Paul L. H. McSweeney
James A. O'Mahony *Editors*

Advanced Dairy Chemistry

Volume 1B: Proteins: Applied Aspects

Fourth Edition

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Preface to the Fourth Edition

Advanced Dairy Chemistry-1B: Proteins: Applied Aspects is the second volume of the fourth edition of the series on advanced topics in dairy chemistry, which started in 1982 with the publication of *Developments in Dairy Chemistry*. The second and third editions of this work were published in 1992 and 2003, respectively, **with the first volume of the fourth edition**, *Advanced Dairy Chemistry-1A: Proteins: Basic Aspects* published in 2013. This series of volumes is an authoritative treatise on dairy chemistry. Like the earlier series, this work is intended for academics, researchers at universities and industry, and senior students; each chapter is referenced extensively.

The chemistry and physico-chemical properties of milk proteins is perhaps the largest and most rapidly evolving area in dairy chemistry and it has proved impossible to cover this topic at the desired depth in one volume. Hence, coverage of dairy proteins in the fourth edition of *Advanced Dairy Chemistry* has been split between basic and applied aspects (this volume).

Most chapters in the third edition on applied aspects of dairy proteins have been retained but have been revised and expanded. The original chapter on production and utilization of functional milk proteins has been split into two new chapters focusing on casein- and whey-based ingredients separately by new authors. The chapters on denaturation, aggregation, and gelation of whey proteins (Chap. 6), heat stability of milk (Chap. 7), and protein stability in sterilized milk (Chap. 10) have been revised and expanded considerably by new authors, and new chapters have been included on rehydration properties of dairy protein powders (Chap. 4) and sensory properties of dairy protein ingredients (Chap. 8). We wish to thank sincerely the 32 contributors (from eight countries) of the 15 chapters of this volume, whose cooperation made our task as editors a pleasure. We wish to acknowledge the assistance given by our editor at Springer Science+Business Media, New York, Ms. Susan Safren and Ms. Sabina Ashbaugh, editorial assistant at Springer, for help in preparing the manuscript.

Cork, Ireland

P.L.H. McSweeney
J.A. O'Mahony

Preface to the Third Edition

Advanced Dairy Chemistry—1: Proteins is the first volume of the third edition of the series on advanced topics in dairy chemistry, which started in 1982 with the publication of *Developments in Dairy Chemistry*. This series of volumes is intended to be a coordinated and authoritative treatise on dairy chemistry. In the decade since the second edition of this volume was published (1992), there have been considerable advances in the study of milk proteins, which are reflected in changes to this book.

All topics included in the second edition are retained in the current edition, which has been updated and considerably expanded from 18 to 29 chapters. Owing to its size, the book is divided into two parts; Part A (Chaps. 1–11) describes the more basic aspects of milk proteins while Part B (Chaps. 12–29) reviews the more applied aspects. Chapter 1, a new chapter, presents an overview of the milk protein system, especially from an historical viewpoint. Chapters 2–5, 7–9, 15, and 16 are revisions of chapters in the second edition and cover analytical aspects, chemical and physiochemical properties, biosynthesis and genetic polymorphism of the principal milk proteins. Nonbovine caseins are reviewed in Chap. 6. Biological properties of milk proteins, which were covered in three chapters in the second edition, are now expanded to five chapters; a separate chapter, Chap. 10, is devoted to lactoferrin and Chap. 11, on indigenous enzymes in milk, has been restructured and expanded. Nutritional aspects, allergenicity of milk proteins, and bioactive peptides are discussed in Chaps. 12, 13, and 14, respectively. Because of significant developments in the area in the last decade, Chap. 17 on genetic engineering of milk proteins has been included. Various aspects of the stability of milk proteins are covered in Chap. 18 (enzymatic coagulation), Chap. 19 (heat-induced coagulation), Chap. 20 (age gelation of sterilized milk), Chap. 21 (ethanol stability), and Chap. 22 (acid coagulation, a new chapter).

The book includes four chapters on the scientific aspects of protein-rich dairy products (milk powders, Chap. 23; ice cream, Chap. 24; cheese, Chap. 25; functional milk proteins, Chap. 26) and three chapters on technologically important properties of milk proteins (surface properties, Chap. 27; thermal denaturation aggregation, Chap. 28; hydration and viscosity, Chap. 29).

Like its predecessors, this book is intended for academics, researchers at universities and industry, and senior students; each chapter is referenced extensively.

We wish to thank sincerely the 60 contributors to the 29 chapters of this volume, whose cooperation made our task as editors a pleasure. The generous assistance of Ms. Anne Cahalane is gratefully acknowledged.

Cork, Ireland

P.F. Fox
Paul L.H. McSweeney

Preface to the Second Edition

Considerable progress has been made on various aspects of milk proteins since *Developments in Dairy Chemistry 1—Proteins* was published in 1982. *Advanced Dairy Chemistry* can be regarded as the second edition of *Development in Dairy Chemistry*, which has been updated and considerably expanded. Many of the original chapters have been revised and updated, e.g. 'Association of Caseins and Casein Micelle Structure', 'Biosynthesis of Milk Proteins', 'Enzymatic Coagulation of Milk', 'Heat Stability of Milk', 'Age Gelation of Sterilized Milks', and 'Nutritional Aspects of Milk Proteins'. Chapter 1 in *Developments*, i.e., 'Chemistry of Milk Proteins', has been subdivided and extended to four chapters: chemistry and physico-chemical properties of the caseins, *b*-lactoglobulin, *a*-lactalbumin, and immunoglobulins. New chapters have been added, including 'Analytical Methods for Milk Proteins', 'Biologically Active Proteins and Peptides', 'Indigenous Enzymes in Milk', 'Genetic Polymorphism of Milk Proteins', 'Genetic Engineering of Milk Proteins', 'Ethanol Stability of Milk', and 'Significance of Proteins in Milk Powders'. A few subjects have been deleted or abbreviated; the three chapters on functional milk proteins in *Developments* have been abbreviated to one in view of the recently published fourth volume of *Developments in Dairy Chemistry—4—Functional Milk Proteins*.

Like its predecessor, the book is intended for lecturers, senior students, and research personnel, and each chapter is extensively referenced.

I would like to thank all the authors who contributed to the book and whose cooperation made my task a pleasure.

Cork, Ireland P.F. Fox

Preface to the First Edition

Because of its commercial and nutritional significance and the ease with which its principal constituents, proteins, lipids, and lactose, can be purified free of each other, milk and dairy products have been the subject of chemical investigation for more than a century. Consequently, milk is the best-described in chemical terms, of the principal food groups. Scientific interest in milk is further stimulated by the great diversity of milks—there are about 4000 mammalian species, each of which secretes milk with specific characteristics. The relative ease with which the intact mammary gland can be isolated in an active state from the body makes milk a very attractive subject for biosynthetic studies. More than any other food commodity, milk is a very versatile raw material and a very wide range of food products are produced from the whole or fractionated system.

This text on proteins is the first volume in an advanced series on selected topics in dairy chemistry. Each chapter is extensively referenced and, it is hoped, should prove a useful reference source for senior students, lecturers, and research personnel. The selection of topics for 'Proteins' has been influenced by a wish to treat the subject in a comprehensive and balanced fashion. Thus, Chaps. 1 and 2 are devoted to an in-depth review of the molecular and colloidal chemistry of the proteins of bovine milk. Although less exhaustively studied than those of bovine milk, considerable knowledge is available on the lactoproteins of a few other species and an inter-species comparison is made in Chap. 3. The biosynthesis of the principal lactoproteins is reviewed in Chap. 4. Chapters 5–8 are devoted to alterations in the colloidal state of milk proteins arising from chemical, physical, or enzymatic modification during processing or storage, viz. enzymatic coagulation, heat-induced coagulation, age gelation of sterilized milks, and chemical and enzymatic changes in cold-stored raw milk. Milk and dairy products provide 20–30 % of protein in 'western' diets and are important worldwide in infant nutrition: lactoproteins in particular are considered in Chap. 9. The increasing significance of 'fabricated' foods has created a demand for 'functional' proteins: Chaps. 10–12 are devoted to the technology, functional properties and food applications of the caseinates and various whey protein products.

Because of space constraints, it was necessary to exclude coverage of the more traditional protein-rich dairy products: milk powders and cheese. It is hoped to devote sections of a future volume to these products.

I wish to thank sincerely the 13 other authors who have contributed to this text and whose cooperation made my task as editor a pleasure.

Cork, Ireland P.F. Fox

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Manufacture and Properties of Dairy Powders

1

Alan L. Kelly and Patrick F. Fox

Abstract

Due to the high intrinsic perishability of milk, strategies for its preservation by partial or almost complete removal of water have been studied and used commercially for over a century. Today, milk-based powders (e.g., whole and skim milk powders) represent a very large sector of dairy commodity production and export trade, and demand for these products, for a range of applications, is increasing. The technology of production of these powders, based around pre-concentration of milk by multi-stage thermal evaporation followed by spray drying in a range of related dryer types, which vary in terms of design, method of atomisation and number of drying stages, is today very sophisticated. Key properties of milk powders, such as their ability to flow, remain stable on long-term storage, and reconstitute on addition to water, are influenced by drying parameters. Understanding these relationships allows processes to be manipulated in order to yield tailored functionality for each application. The technology of drying milk to produce the principal dairy powders is reviewed in this chapter, along with the principal changes to milk constituents induced by such processes, and the potential for use of these powders in applications such as cheese and yoghurt manufacture

Keywords

Milk powder • Spray drying • Properties of milk powders • Dairy ingredients • Powder applications • Speciality milk powders

1.1 Introduction

The first recorded reference to the manufacture of milk powder as a method for preserving milk was by Marco Polo, who observed the use of milk powder by Mongol soldiers in the thirteenth century

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(Hall and Hedrick, 1975). The earliest modern commercial concentrated dairy products were air-dried concentrated milk tablets, developed in 1809 by Nicholas Appert, vacuum-concentrated sweetened condensed milks by Gail Borden in 1856 and sterilized concentrated milk (evaporated milk) by J.B. Meyenberg in 1884. During the period 1850–1900, several 'solidified milk' products were produced, e.g., that patented in 1855 by T.S. Grimwade (UK), by adding sucrose, sodium carbonate and cereal to concentrated milk. Roller-dried milk was first produced by J.R. Hatmaker in 1902 in the UK, and roller-drying was the predominant method for drying milk products, including infant formula, until the 1960s (Pearse, 1998). Spray drying was introduced to the chemical industry by Samuel R. Percy in 1872; the process was improved gradually and used to dry milk by J.C. MacLachlan in 1905 (Hunziger, 1926). Today, spray drying is essentially the only process used to produce milk powders, which have become major, and growing, dairy products. There is an extensive literature on milk powders, including the following textbooks and review articles: Hunziker (1926), Hall and Hedrick (1975), Rothwell (1980), Masters (1991), Caric (1994), Písecký (1997), Westergaard (2003), Tamime (2009), Skanderby (2011) and Schuck (2011a, b) and Schuck *et al*. (2012).

Spray dryers have evolved in design and application, and now a range of dryer types is available, suitable for the production of a wide variety of dairy products. One of the key driving forces in dryer development has been the requirement of consumers for convenient, easily reconstituted ('instant') powders, produced from whole or skimmed milk, and a range of other dairy products, such as whey and buttermilk. Increased understanding of the chemical and physical changes which occur in milk during drying have paralleled developments in drying technology and enable very fine control of the properties, and hence potential applications, of milk powders.

The modern processes used for the production of dairy powders, the effects of these processes on the proteins and other constituents of milk, and the properties of milk powders will be considered in this chapter.

1.2 Types of Milk Powders

A wide range of dry dairy products is produced worldwide, a selection of which are listed in Table 1.1. Milk powder is produced for local consumption, some as reconstituted milk but usually mainly for use as an ingredient in other foods or for export. Developments in end-use applications of milk and milk-based powders, and tailoring functionality for individual applications, was reviewed by Sharma *et al*. (2012).

Much milk powder is traded internationally, from milk-rich to milk-deficient regions. The principal milk powder importing countries are listed in Table 1.2. About 3.8 and 3.6 million tonnes of whole and skimmed milk powder, respectively, were produced in 2009. The principal producers of

Table 1.1 Range of dried dairy products

Skim milk powder	Butter powder
Instant	Cheese powders
Regular	Buttermilk powder
Low-, medium-, high-heat	Whey powders
Whole milk powder	Normal
Instant	Demineralised
Regular	Delactosed
High free fat	Caseinates (sodium, potassium, calcium)
Filled milk powder	Rennet casein
Infant formulae	Acid casein
	Total milk proteinates
	Casein coprecipitates

Table 1.2 Imports of whole milk powder (WMP) and skimmed milk powder (SMP), in thousands of tonnes (IDF, 2010)

Country	WMP	SMP
China	382	128
Japan	0	35
Korea	1	10
Turkey	15	12
EU	1	6
USA	22	0
Russia	20	52
Brazil	52	11
Australia	17	6
Mexico	27	165

WMP in 2009 were China (977), New Zealand (790), EU (739), Brazil (473), Argentina (235) and Australia (126), with all figures being in thousands of tonnes. The principal producers of SMP were EU (1200), USA (778), India (364), New Zealand (360), Australia (190) and Japan (167), in thousands of tonnes. Total exports of WMP in 2009 were 2.13 million tonnes, of which New Zealand supplied 37 %, EU 22 %, Argentina 7 %, Australia 6 %, and the rest of the world 30 %. Total exports of SMP in 2009 were 1.33 million tonnes, of which New Zealand supplied 30 %, USA 19 %, EU 17 %, Australia 13 %, and the rest of the world 21 %. Total exports of whey powders and whey products were 1.31 million tonnes, of which the EU supplied 37 %, USA 30 %, New Zealand 7 %, Switzerland 5 %, Australia 3 %, Canada 3 % and the rest of the world 15 %.

The two principal commercial milk powders are SMP and WMP, which are generally classified as either regular (non-instant) or instant. Food applications of SMP and WMP are summarised in Table 1.3. Commercial SMP is also routinely classified according to the pre-heat treatment applied (heat classification) as low- (or low-low-), medium- or high-heat powders. Fat-filled, or filled, milk powders are products with a fat content close to that of WMP, produced by drying a blend of non-milk fat and skim milk (Kelly *et al*., 2002). Fat-filled milk powder may be produced for nutritional reasons but it is done mainly for economic reasons (Hayman, 1995; Vignolles *et al*., 2010). The fat used is normally cheaper than milk fat, e.g., lard, tallow or vegetable oils, for calf-milk replacers or vegetable oils produced in an importing country blended with reconstituted imported SMP. The influence of oil type (sunflower and palm oil) and spray dryer outlet temperature, in terms of properties of fat-filled milk powders was reported by Kelly *et al*. (2014).

Table 1.3 Principal food applications of skim and whole milk powders

Skim milk powder
Reconstitution
Cheesemaking (low-heat SMP)
Confectionery products
Ice cream and other desserts
Hot and cold beverages
Recombined sweetened condensed milk
Bakery products (high-heat SMP)
Calf milk replacers
Recombined milk production
Chocolate manufacture
Meat products
Whole milk powder
Reconstitution
Convenience soups and sauces
Milk chocolate (high free fat WMP)

Encapsulated milk fat powders (40–60 % fat) may be produced using a blend of emulsifying salts, SMP and flour, starch or sucrose (Holsinger *et al*., 2000). Such powders, which have good flow properties and are resistant to oxidation, compared to other high-fat powders, may be used as substitutes for vegetable shortenings in a range of food products and in applications such as coffee whiteners.

High-fat powders, which may be defined as powders with a fat content in the range 42–65 % (Early, 1990; Munns, 1991), present certain problems in drying due to the crystallisation of fat during handling and storage. Pneumatic transport from dryers may not cool the powder sufficiently to crystallise all the fat therein and some fat may crystallise subsequently on cooling in the package, releasing the latent heat of crystallisation and thus causes an increase in temperature, causing fat to melt and caking of the powder, i.e., the formation of a solid mass of powder. This may be avoided by ensuring complete fat crystallisation using a fluidised bed cooler before packaging the powder (Early, 1998).

In recent years, a number of fractionated or semi-refined milk protein powders have been developed for specific functional applications, with many products arising from the selective use of membrane technologies, such as microfiltration, ultrafiltration or diafiltration (Mistry and Hassan, 1991; Kelly *et al*., 2000). Kelly *et al*. (2000) described a method for the production of native phosphocasein-rich powders using microfiltration and electrodialysis: the latter treatment improved the heat stability of the powder but seriously impaired its rennet coagulation properties. Protein-enriched milk powders may be used as substitutes for SMP and have been shown to have good functional properties for a range of food applications (Mistry and Hassan, 1991). Garem *et al*. (2000) described the manufacture of a milk

powder with improved cheesemaking properties by partial removal of whey proteins by a combination of microfiltration and ultrafiltration. The production of micellar casein powders with significant potential application in cheese manufacture was reviewed by Saboya and Maubois (2000).

Hot tea or coffee is a rather hostile environment (pH < 5, temperature up to 100 °C) for milk and the SMP or WMP products produced in the 1960s were unstable under these conditions. The whey proteins were denatured and precipitated on the surface of powder particles before they could dissolve; the particles flocculated and sedimented. Alternative 'coffee whiteners', made with sodium caseinate, vegetable oil, corn syrup and emulsifiers were developed and became widely used. Due to advances in understanding the factors affecting coffee stability, modern SMP or WMP are suitable as coffee whiteners but casein-based products are still widely used, mainly because they are cheap.

Another highly specialised type of dried milk-based product is infant formula, which has been produced as an alternative source of nutrition for infants to breast milk since 1867, when Justus van Leibig developed a product based on drying a mixture of whole milk, wheat flour, malt flour and potassium carbonate in heated trays. The commercial brand SMA (originally standing for Synthetic Milk Adapted) was first produced in the USA in the early twentieth century.

Today, the manufacture of infant formulae is a highly specialised sector of the dairy industry and these products are more properly referred to as neutraceuticals rather than dairy products (O'Callaghan *et al.*, 2011). The manufacturing protocol varies with the company and type of formula, but a general protocol is shown in Fig. 1.1. The first step is preparation of the blend of ingredients (mainly dry) with the following considerations:

- The base may be SMP or liquid skimmed milk;
- The lactose content is adjusted to that of human milk by addition of lactose or demineralised whey;
- The concentrations and relative proportions of caseins and whey proteins are adjusted, by adding demineralised whey or whey protein concentrate (WPC). Human milk lacks β-lactoglobulin, which is the most allergenic bovine milk protein for human infants. Bovine whey proteins may be fractionated, and the α-lactalbumin (α-la)-rich fraction used for infant formula. The β-lactoglobulin-rich fraction has better gelling properties than regular WPC and is a valuable food ingredient;
- Lipids, e.g., sunflower oil, are added, to reflect the fact that human milk fat is more unsaturated than bovine milk fat;
- The ash (mineral) content must be reduced, by electrodialysis or membrane filtration of the dairy ingredient components, as it is around four times higher than that of human milk and may cause renal overload;
- The blend may be fortified with vitamins and perhaps other important nutrients, e.g., lactoferrin and milk oligosaccharides.

The blend is pasteurised, homogenized and standardized, and then either spray-dried and packaged or UHT-treated and aseptically packaged.

Formulae for healthy infants are normally of two types: 'first-stage' and 'second-stage follow-on' formulae. Speciality formulae are available for infants with special needs, e.g., low birthweight, lactose intolerance, anti-regurgitation, low-caloric, extensively hydrolysed and low phenylalanine content (Maldonado *et al.*, 1998; Thompkinson and Khard, 2007).

1.3 Technology of Milk Powder Manufacture

The production of a number of common milk powders has been described extensively (e.g., Písecký, 1997; Caric, 2003; Kelly, 2006; Schuck, 2011a), and is summarised below.

1.3.1 Milk Pre-Treatment

Milk of high microbiological quality is required for powder manufacture; bactofugation or microfiltration may be used to remove both bacterial cells and spores from milk, and thus ensure a

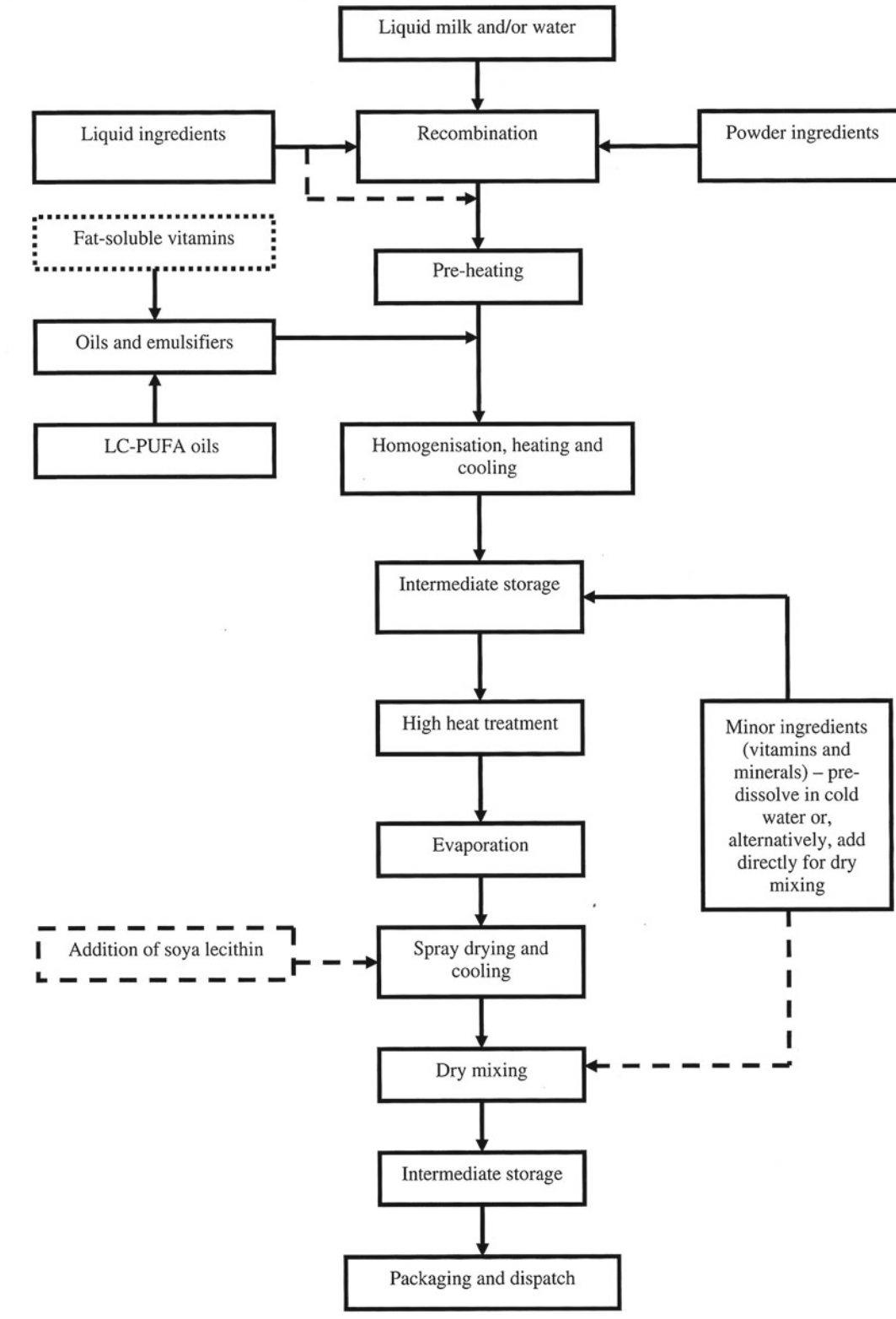

Fig. 1.1 Schematic flowchart for the production of infant formula (from Montagne *et al*., 2009)

milk powder of very high microbiological quality. Whole milk is usually standardised, typically to a fat to solids-non-fat ratio of 1:2.76, to control the fat content of the final powder.

1.3.2 Preheating and Concentration

Heating of milk immediately before evaporation ensures the microbiological quality of the concentrate and of the final powder, but is also a critical step in the control of the functional properties of the powder; the exact conditions vary depending on the product in question, as discussed below. Preheating is usually the highest temperature step applied during manufacture and is therefore the step at which most whey protein denaturation occurs (Singh and Creamer, 1991). Preheating may be performed using any of a range of heat exchangers, including plate heat exchangers, spiral heat exchangers wrapped around the tubes in the evaporator itself or using very short-time steam injection heating systems. Direct heat exchangers are preferred over indirect systems, as biofilms of thermophilic bacteria may develop within indirect heat exchangers (Early, 1998). In recent years, new technological approaches, such as steam-injection processes, have been developed (Murphy *et al.*, 2013).

As discussed in Sect. 1.2, skim milk powder (SMP) is often classified according to the heat treatment applied during preheating. There are three principal heat categories: low heat (typically heated at 75 °C for 15 s), medium heat (typically heated at 75 °C for 1–3 min) and high heat (heated at 80 °C for 30 min or 120 °C for 1 min); some manufacturers may also produce a low-low heat product, with even a milder heat treatment than that for low-heat products (often minimum pasteurisation). Whole milk powder (WMP) is generally not heat-classified, but the concentrate is heated at 85–95 °C for several minutes to ensure inactivation of indigenous lipase and to expose sulphydryl groups with antioxidant activity (Hols and Van Mil, 1991).

After pre-heating, the milk is concentrated to 45–50 % or 42–48 % total solids (TS) for whole or skim milk, respectively. Typically, evaporation is performed in a multiple effect (stage) falling-film evaporator, where thermal efficiency is maximised by maintaining each subsequent effect at progressively lower pressure, and thus reduced boiling point, allowing the vapour from each effect to be used as the heating medium for the

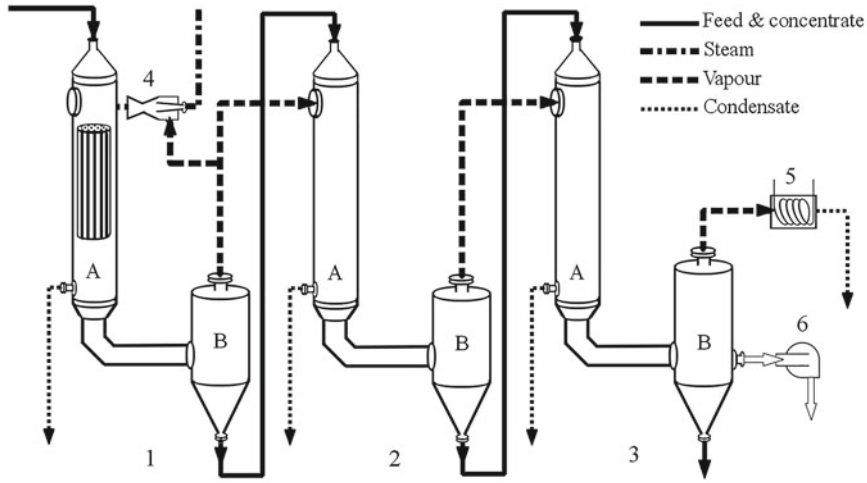

Fig. 1.2 Schematic diagram of a triple effect evaporator showing (1) first effect, (2) second effect, (3) third effect, (4) thermocompressor, (5) vapour condenser and (6) vacuum pump; calandria (**A**) and vapour-liquid separating cyclone (**B**) are shown. The function of the thermocompressor is to mix vapour from the first effect with steam and compress the mixture, thereby increasing its temperature and allowing its use as heating medium for the first effect and reducing the overall consumption of steam

next effect (Fig. 1.2). Steam consumption is reduced further, and the economy of operation increased, by use of thermal or mechanical vapour recompression to increase the temperature of some or all of the vapour and allow it to be used as an additional heating medium. Conventionally, concentration is monitored using an in-line refractometer, although the use of an on-line viscometer has been recommended (O'Donnell *et al*., 1996).

As an alternative to evaporation, membrane processing may be used for specific applications. While processes such as ultrafiltration (UF) fractionate milk constituents, reverse osmosis (RO) or hyperfiltration or nanofiltration (NF) remove essentially only water (and monovalent ions in the case of NF), and thus can serve as a preconcentration step. RO is limited to a low solids level (<20 % TS) and a medium flow rate, but has lower operation costs and is far less thermally intensive than evaporation (Písecký, 1997). The concentration of milk by UF membranes, which are far less susceptible to fouling than RO membranes (El-Gazzar and Marth, 1991), has been suggested also. Concentration by UF offers advantages in that heat treatment during concentration is avoided and the levels of protein and lactose in the powder may be standardised and controlled (Sweetsur and Muir, 1980; Muir and Sweetsur, 1984; Mistry and Pulgar, 1996; Horton, 1997; Sikand *et al*., 2008, 2010). The significance of these alterations in powder composition is discussed in Sect. 1.5.

Crystallisation of lactose prior to drying is desirable for many products, particularly high-lactose products, such as whey powders and, consequently, the concentrate may be held before drying under conditions which promote crystallisation and/or may be seeded with finely-ground lactose crystals, which promote crystallisation by acting as nuclei (Sanderson, 1978).

Milk for WMP or fat-filled milk powder manufacture may also be homogenised, typically after concentration and before drying. Conventionally, two-stage homogenisation is used at a temperature in the range 60–70 °C; typical homogenisation pressures are 15 MPa followed by 5 MPa.

1.3.3 Drying

The first commercial milk powder plants used roller dryers, consisting typically of two co- or counter-rotating steam-heated drums, with or without a preliminary concentration step. Roller drying entails the direct transfer of heat from the drum to a thin film of milk (Fig. 1.3). The water is evaporated off and the dried solids are removed and pulverised, which produces very irregularly shaped powder particles. This method of drying results in severe thermal damage to milk constituents, with adverse effects on flavour and quality. Modifications to the basic roller dryer to reduce damage to the product include drying in a vacuum chamber at a lower drum temperature. Generally, roller drying has been replaced by spray drying, although it is still used for specific applications,

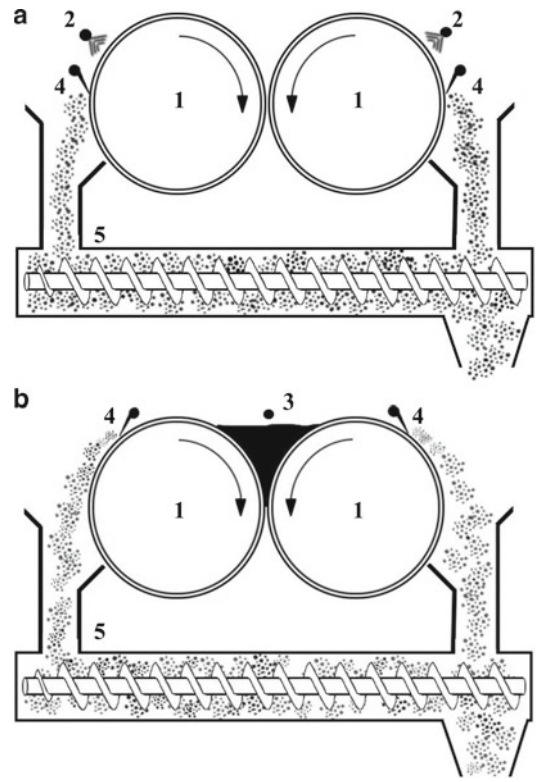

Fig. 1.3 Principle of operation of roller dryers fed either by (**a**) spray applicators or (**b**) feed sump, showing (1) steam-heated drums (may be co- or counter-rotating); (2) spray applicator; (3) sump; (4) knife for removing dry product after completion of one (partial) rotation; (5) conveyor for grinding and transporting dry product

Table 1.4 Characteristics of different types of atomisation

Pressure nozzle atomisation	Centrifugal wheel atomisation
Advantages	*Advantages*
Low occluded air in powder	Good flexibility
High powder bulk density	Handles high feed rates
Good powder flowability	Handles viscous concentrates
Simple and low cost	Wheel speed controls droplet size
Low energy consumption	Steam sweep to control bulk density
Good control over spray flow	Insensitive to concentration variation
Low deposit levels in chamber	Low risk of blocking during run
Can agglomerate with angled nozzles	Can handle crystalline feeds
	Can co-dry separate feeds
Disadvantages	*Disadvantages*
Susceptible to blockage	High energy consumption
	High air incorporation
	High capital costs
	May give deposits in drying chamber

such as the production of WMP with a high free fat content, which is desirable for chocolate manufacture (Clarke and Augustin, 2005).

Today, the majority of milk powder is produced by spray drying, as summarised briefly in the following section. For more detail on technological and engineering aspects of this process, comprehensive descriptions have been given by Hansen (1985), Masters (1991), Westergaard (1994, 2003), Caric (1994), Písecký (1997) and Refstrup and Bourke (2011).

In spray dryers, concentrate is taken from the evaporator, using a positive displacement pump, to an atomiser, usually located at the top of a spray drying chamber, which produces a spray of droplets which contact hot air and are dried to individual powder particles. The concentrate is generally heated to ~72 °C before atomisation, to reduce viscosity and obtain optimal atomisation. The objective of atomisation is to convert the liquid feed to a spray of droplets, 10–400 μm in diameter. Particle size and, in particular, the distribution of particle sizes is very important in determining the properties of milk powder, as will be discussed in Sect. 1.5. Atomisation may be achieved using nozzles under pressure or by centrifugal force using a rotating disk or wheel. Pressure nozzle atomisers have either grooved core inserts or swirl chambers to impart rotary motion to the feed (Masters, 1991). The selection of atomiser type is critical in determining the properties of the powder produced. For maximum flexibility, different types of atomiser can be used interchangeably in many spray dryers. The influence of pressure nozzle and centrifugal atomisers on the properties of milk powder is compared in Table 1.4.

There are three principal classes of spray dryer, based on the number of separate drying stages (one, two or three) used to achieve the final moisture content of the powder (typically 3–5 %; Woodhams and Murray, 1978). These spray dryer types are illustrated in Fig. 1.4.

Fig. 1.4 (continued) dryer (CSD, three stage) with annular integrated and external fluidised beds; (vi) multi-stage spray dryer (MSD, three stage) with circular integrated and external fluidised beds. Dryer components indicated include (1) hot air inlet, (2) atomiser (may be nozzle or rotary), (3) main drying chamber, (4) cyclone, (5) external fluidised bed cooler, (6) external fluidised bed dryer, (7) integrated annular bed dryer and (8) integrated circular bed dryer

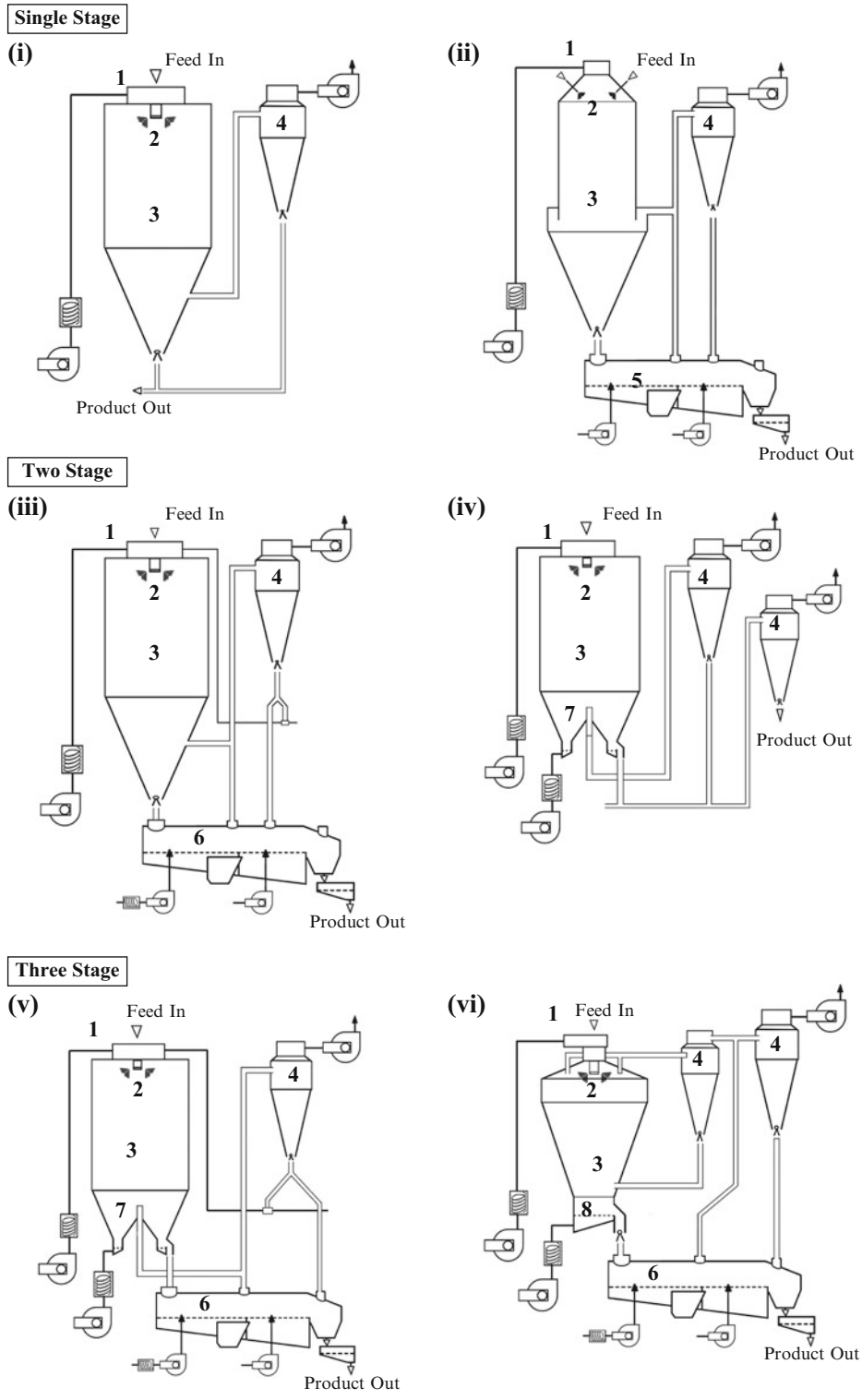

Fig. 1.4 Common spray dryer configurations used for the manufacture of milk powder: (i) single-stage dryer with pneumatic powder conveying; (ii) single stage dryer with fluidised bed cooling and conveying; (iii) two-stage dryer with external fluidised bed dryer; (iv) two-stage dryer with integrated fluidised bed dryer; (v) compact spray

In single-stage dryers, the whole drying process occurs in the main cylindro-conical chamber of the spray dryer itself. Critical parameters which determine both the quality of the final powder and the efficiency of the drying process are the temperature of the drying air on entry to and exit from the main chamber [T_{inlet} (typically 160–220 °C) and T_{outlet} (typically 70–90 °C)]. During drying, milk droplets are cooled continuously by loss of the latent heat of evaporation, and generally never reach a temperature greater than 70 °C during drying. Initially, droplets lose moisture at a constant rate, while saturation conditions exist at the surface, but eventually saturation can no longer be maintained and the rate of drying decreases as a hard dry shell forms at the droplet surface. If this shell becomes too solid or thick, case-hardening occurs, preventing further drying. If exposed to a high air temperature at the end of drying (T_{outlet}), steam and air within powder particles may expand, forming large vacuoles and, potentially, fracturing the powder particles, resulting in an increased level of small, light powder fragments (fines). The rate of drying should be controlled so that the end of the falling rate drying period coincides with the end of heating and drying. The use of advanced process control technologies in spray drying to optimise processes based on understanding the thermodynamic properties of concentrated foods and rheological properties during atomisation, among other factors, was reviewed by O'Callaghan and Cunningham (2005).

Air exiting the chamber carries a significant amount of light powder particles and fines, which are removed from the air by passage through cyclone separators and/or by using bag filters or occasionally wet-scrubbing systems. Recovered powder is added to the main bulk of powder, which is removed from the base of the drying chamber, either by a pneumatic conveying system or a fluidised bed using cold air to cool and transport the powder.

Completion of drying in a single stage necessitates the use of a high air temperature (the air is typically indirectly heated using steam-filled tubular heat exchangers to 200–250 °C), which results in poor-quality non-instant, dusty powder. In two- and three-stage drying the powder exits the main drying chamber at a higher moisture content (~10–15 %) than in a single-stage process, and drying is completed in additional drying stages. Thus, in two-stage drying, the main chamber is followed by a fluidised bed dryer while in three-stage spray dryers, the spray dryer chamber is followed sequentially by an integrated fluidised bed and an external plug-flow fluidised bed (Boersen, 1990). The two main categories of three-stage spray dryers used for milk powders are those with an internal annular fluidised bed surrounding the air outlet (compact spray dryers, CSD) or dryers with an internal circular fluidised bed (multi-stage dryers, MSD).

Separation of drying into two or three stages allows for improved control of powder properties, greater efficiency of the drying process and, because the rate of heat introduction is adjusted to the rate of evaporation, the process is milder than single-stage processes (Písecký, 1997). Another advantage of two- or three-stage spray drying is that such processes are suitable for drying high TS concentrates without deterioration of product solubility. Two- and three-stage drying systems also have reduced stack losses (Písecký, 1997).

Movement of powder within the drying plant may be achieved either by using a fluidised bed or pneumatic system, although the latter method breaks up agglomerates, and is suitable only for powders of high bulk density. Milk powder may be sifted for size classification, and then either packaged directly, filled into intermediate transportation containers, or transferred to silos for storage before packaging. Milk powder may be packaged into wholesale, catering or retail packs (Warren, 1980). Generally, milk powder is packaged in multi-layer paper bags with a polyethylene inner lining. WMP may be packaged in an inert atmosphere, where air is displaced by N_2 or a mixture of N_2 and CO_2, to improve oxidative stability during storage. Occasionally, WMP may be packed in tins or plastic containers, or in bags consisting of multi-layer laminates incorporating metal foil and high density plastic films (Varnam and Sutherland, 1994).

1.3.4 Instantization

Single-stage drying produces dusty, non-agglomerated, non-instant powders of high bulk density. Production of milk powders that reconstitute well when dispersed in cold water (instant powders) has necessitated development of modified spray drying processes.

For SMP, instantization is achieved by agglomeration, i.e., the production of porous clusters of particles, 250–750 μm in diameter, with a high level of entrapped air (Neff and Morris, 1967; Sanderson, 1978; Písecký, 1997). Instantisation reduces the bulk density of milk powder (produces lighter particles) and enhances its wettability, sinkability and solubility (see Sect. 1.5.2).

Agglomeration of SMP may be achieved by (1) returning fines (typically through a separate pipeline) to the drying chamber close to the atomiser, where they act as nuclei for the growth of agglomerates (Refstrup, 1995), (2) by removal of the powder from the spray drying chamber at 8–15 % moisture (and hence use of two- or three-stage drying), which favours agglomerate formation during fluidised bed drying, or (3) by rewetting processes applied at some point after drying (Neff and Morris, 1967). Other methods of agglomeration include the use of multiple atomiser systems where nozzles are arranged so that sprays cross each other (Boersen, 1990), or the use of curved-vane rotary atomisers or gases such as CO_2 or N_2 which are introduced into the feed and expand towards the end of drying, increasing vacuole size. In order to produce agglomerates with optimum reconstitution properties, it has been proposed that low- or medium-heat milk be used and that the initial powder particles should have a high particle density and a diameter in the range 25–50 μm (Sanderson, 1978).

Rewet agglomeration systems involve wetting non-instant powder using either a gaseous phase (humidified air or steam) or a liquid (milk or water) wetting agent, holding for a certain period under conditions which favour the interaction of moist particles to form agglomerates, drying to remove the added moisture, and using size classification to screen agglomerates of the required size. Rewet processes produce better agglomeration and instant properties than straight-through systems, where agglomeration is achieved during the powder manufacturing process itself but the rewet process is more costly and also more prone to microbiological contamination of the product.

To overcome the hydrophobic nature of milk fat, instantization of WMP requires a combination of agglomeration and lecithinisation. Agglomeration of WMP may be achieved by the same procedures as for SMP. Lecithin, typically extracted from soybeans, aids instantization by virtue of its surfactant properties, and is usually added to WMP at a level of ~0.2 % between the spray drying stage and the fluidised bed drying or, alternatively, in a rewet process (Jensen, 1975; Sanderson, 1978). When lecithin is added to WMP, the mixture should be held at ~50 °C for 5 min to ensure complete coating of the particles with lecithin (Jensen, 1975; Sanderson, 1978). Alternative techniques for producing instant WMP were reviewed by Sanderson (1978).

1.3.5 Dust Explosions in Spray Dryers

One of the major risks in spray drying and subsequent handling of products is the possibility of fires and explosions which can be hazardous to personnel and expensive in terms of plant damage and shut-down. Synnott and Duane (1986) listed the following as the principal causes of fires:

- Friction between the rotary valve and cyclone housing;
- External heating, e.g., during welding and duct work;
- Equipment malfunction;
- Self-heating of deposits;
- Nodules of powder forming.

Synnott and Duane (1986) concluded that fires/explosions are not confined to a single type of dryer or powder, although a disproportionate number appear to be associated with fat-filled powders. Increasing the level of fat and the degree of unsaturation of the fat tend to reduce the minimum ignition temperature (MIT) of the powder. The protein fraction seems to be the

component mainly responsible for self-heating. Contamination with NaOH residues left after cleaning reduces MIT. The causes of fires in spray dryers and their prevention are also discussed by Písecký (1997).

The potentially most serious problem with spray drying milk powders is dust explosions, which occur when finely dispersed combustible solids are exposed to an ignition source. They occur under the following conditions (Písecký, 1997):

- Sufficient concentration (usually around 50 g/m^3) of an explosive airborne dust;
- A source of ignition;
- Presence of oxygen in the surrounding atmosphere.

1.4 Physico-Chemical Changes to Milk Constituents During Drying

1.4.1 Proteins

On preheating at a temperature above 75°C at pH ~ 6.5, β-lactoglobulin is denatured, causing significant alterations to its secondary and tertiary structures, and at a temperature above 90°C, extensive whey protein denaturation occurs. Denatured β-lactoglobulin and, to a lesser extent, α-lactalbumin, complex *via* disulphide bond formation with κ-casein (Noh and Richardson, 1989; Corredig and Dalgleish, 1999; Fairise *et al*., 1999). When milk is heated at a pH < ~6.8, the complexes remain attached to the surface of the micelles but, on heating at more alkaline pH values, the complexes are found in the serum phase (Creamer and Matheson, 1978; Mohammed and Fox, 1987; McKenna *et al*., 1999). Oldfield (1998) showed that, as well as interacting with casein micelles, β-lactoglobulin forms disulphide-linked aggregates with bovine serum albumin (BSA) during the manufacture of milk powder, while α-lactalbumin mainly forms hydrophobic-bonded aggregates. The roles of Maillard reactions and isopeptide bond formation in changes in milk proteins during drying and subsequent storage, and implications for nutritional quality, in terms of the level of available lysine, were reviewed by Higgs and Boland (2008), while Le *et al*. (2013) studied the impact of cross-linking due to the Maillard reaction on protein solubility during storage of powder. The Maillard reaction is discussed in more detail in Sect. 1.4.2 below.

The rate of the increase in temperature during preheating may affect whey protein interactions. Indirect heating (with a slower heating rate) favours whey protein-whey protein interactions and higher overall denaturation of whey proteins than more rapid direct heating methods, which favour extensive casein-whey protein interactions (Early, 1998). This may be related to the fact that when milk is heated at a relatively low temperature, formation of disulphide bonds predominates, while a higher temperature is required for the thermal reduction of disulphide bonds and thus sulphydryl-disulphide interchange reactions (de Wit, 1981). Therefore, as caseins do not possess free sulphydryl groups (Swaisgood, 1992), more gentle heating will favour whey protein-whey protein interactions.

Holding concentrate at a temperature above 60 °C for an extended period before spray drying can cause aggregation of casein micelles, increasing the viscosity of the concentrate and affecting the reconstitution properties of SMP (Muir, 1980). During the actual processes of evaporation and spray drying, relatively little denaturation of whey proteins occurs, as the final temperature of milk generally does not exceed 70 °C (Singh and Creamer, 1991). Oldfield (1998) reported only small decreases in the levels of native α-lactalbumin and BSA during evaporation, and no apparent denaturation of β-lactoglobulin. However, association of whey proteins with casein micelles can occur during evaporation, probably because decreasing pH reduces protein charge, facilitating association reactions (Oldfield, 1998). Singh (2007) reviewed the interactions of milk proteins during milk powder manufacture, and related changes during drying to changes in micelle structure during the concentration of milk.

1.4.2 Lactose

Lactose is a reducing disaccharide consisting of galactose and glucose linked by a β1-4 bond (β-*O*-D-galactopyranosyl-1-4-α- or β-D-glucopyranose); it is unique to milk. Lactose is a rather unusual sugar:

- Compared to other mono- and disaccharides, it has low solubility (about 200 g/L in water) but, once dissolved, is difficult to crystallise;
- It occurs as two isomers, α and β, which differ markedly in solubility. β-Lactose is more soluble (around 500 g/L in water) than α-lactose (around 70 g/L in water) at 20 °C but the solubility of the latter is more temperature-dependent and the solubility curves intersect at 93.5 °C; α-lactose is the normal commercial form;
- If α-lactose is dissolved in water, some of it mutarotates to β-lactose, and more α-lactose dissolves. Solubilization and mutarotation continue until equilibrium is established; at 20 °C, the equilibrium ratio is 63 % α and 37 % β;
- α-Lactose crystallises as a monohydrate while crystals of β-lactose are anhydrous.

The structure and properties of lactose are described in McSweeney and Fox (2009) and by Fox (2011).

During spray drying, water is removed rapidly from milk and the lactose present assumes an amorphous glass state which is very hygroscopic and will absorb water readily at moderate or high relative humidities (see Sect. 1.5.7.3). If amorphous lactose absorbs sufficient water during storage, it acquires molecular mobility and it will crystallise in a range of forms with a concomitant release of entrapped water (Roetman, 1979; Saltmarch and Labuza, 1980; Saito, 1985; Jouppila *et al*., 1997). The release of water causes an increase in caking and plasticization, amongst other reactions (Roos, 2002). In milk powder, lactose is the principal constituent and in WMP forms the continuous matrix in which proteins, fat globules and air vacuoles are dispersed. The role of lactose in emulsion stability of milk powders, along with that of proteins and alternative carbohydrates, was reviewed by Vega and Roos (2006).

The concentration of lactose in milk powder affects the microstructure of the powder, with low-lactose powders having a porous structure with large vacuoles and a high level of free fat (Aguilar and Ziegler, 1994a). As discussed in Sect. 1.3.4, small lactose crystals may be added to milk concentrate prior to drying to promote crystallisation under relatively mild conditions (i.e., in the liquid rather than powder state) as the added crystals act as nuclei for crystallisation (Sanderson, 1978). In pre-crystallised products, tomahawk-shaped crystals of α-lactose are formed while, in post-crystallised products, needle-shaped β-lactose crystals predominate (Roetman, 1979).

A further phenomenon of significance to drying, particularly for whey or other high-lactose streams, is the thermoplasticity of lactose, which results in hot, semi-dry powder adhering to dryer walls (Silalai and Roos, 2010). The temperature at which this occurs (the "sticking temperature") depends on the relative levels of amorphous lactose, moisture and lactic acid in the powder. Increasing lactic acid concentration reduces sticking temperature, while increasing the level of pre-crystallised lactose increases this temperature, and this permits a higher feed concentration and drying temperature. The moisture content of the powder is the third major factor, and is dependent on the outlet temperature of the dryer, and drying conditions must be optimised to ensure that the outlet temperature used corresponds to a moisture content which will not result in sticking in the dryer. The sticking of powders during drying, and physico-chemical mechanisms underpinning this, were reviewed by Booyani *et al*. (2004), Hogan and O'Callaghan (2010) and O'Callaghan and Hogan (2013).

The properties of milk powders, including flavour, solubility and colour, deteriorate during storage. Deterioration is due to the Maillard reaction and, in the case of WMP, also to lipid oxidation.

The Maillard reaction, first described by Louis Camile Maillard in 1912, occurs under specific conditions, typically between a carbonyl compound and an amine (see Fig. 1.5 for an overview). In the case of foods, the carbonyl group of a reducing sugar (lactose in the case of milk) and

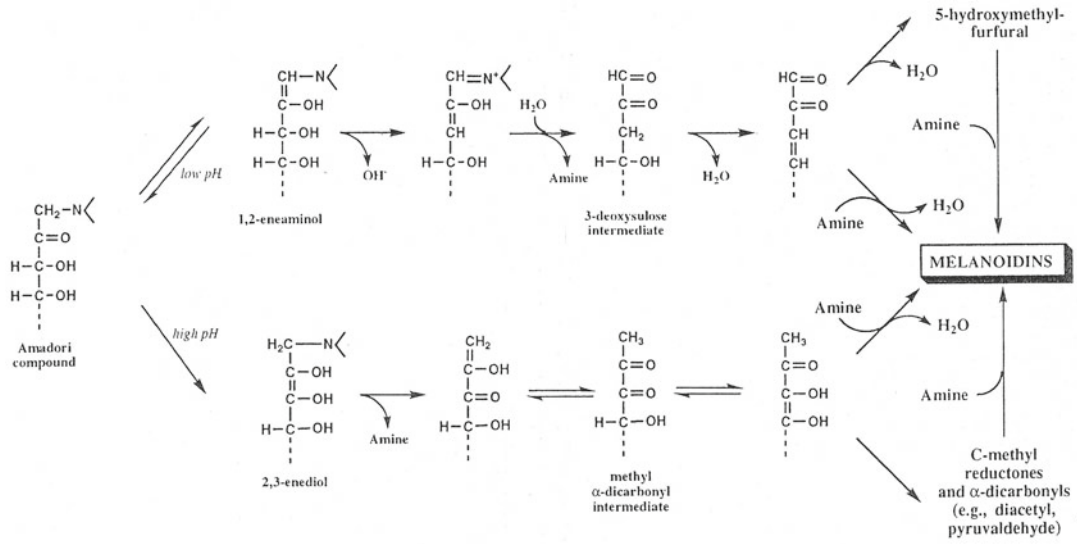

Fig. 1.5 Schematic diagram of the Maillard reaction (Fox and McSweeney, 1998)

the reactive side chains of proteins (principally the ε-amino group of lysine and, to a lesser extent, the indole group of tryptophan, the imidazole group of histidine, the guanidino group of arginine and the α-amino group of N-terminal amino acids) condense to form a glucosamine which, through an Amadori rearrangement, forms an N-substituted-1-amino-1-deoxy-2-ketose (Fig. 1.5). Such compounds may dehydrate to reductones or enolise to either 1-amino-1,2-endiol or 1-amino-2,3-enediol (Nursten, 1981; Walstra and Jenness, 1984). The former compound may be dehydrated to hydroxymethylfurfural (HMF or furfural), while the latter is dealdolized to α-carbonyl compounds which are broken down to fission products which subsequently polymerise to form melanoidins. The literature on the Maillard reaction has been the subject of many reviews, including O'Brien (1997, 2009), Nursten (2011) and Newton *et al.* (2012).

The reaction is base-catalysed (which is not relevant in the case of milk powders) and is influenced by temperature and, in the case of milk powders, by moisture content (the reaction occurs fastest at a_w values around 0.6., i.e., 7 % moisture).

The Maillard reaction affects several attributes of milk, of which the following are the most important:

- Colour: the melanoidins formed in the advanced stages of the Maillard reaction are brown to black in colour; while the formation of a brown colour is desirable in many foods (e.g., the crust of bread, toast, French-fried potatoes), it is undesirable in dairy powders;
- Flavour: many of the intermediate reaction products, e.g., furfural, HMF, or dehydroreductones, are highly flavoured;
- Solubility: the melanoidins are N-containing polymers with low solubility;
- Nutrition: the formation of the initial product, N-substituted glucosamine, is reversible but the further reactions are irreversible and the lysine becomes nutritionally unavailable. Since lysine is an essential amino acid and is limiting in many food products (though not in milk), the Maillard reaction reduces the nutritional value of foods.

Maillard reaction-derived crosslinking of proteins in milk protein systems has been reported (see Friedman, 1977; Pellegrino *et al.*, 1999).

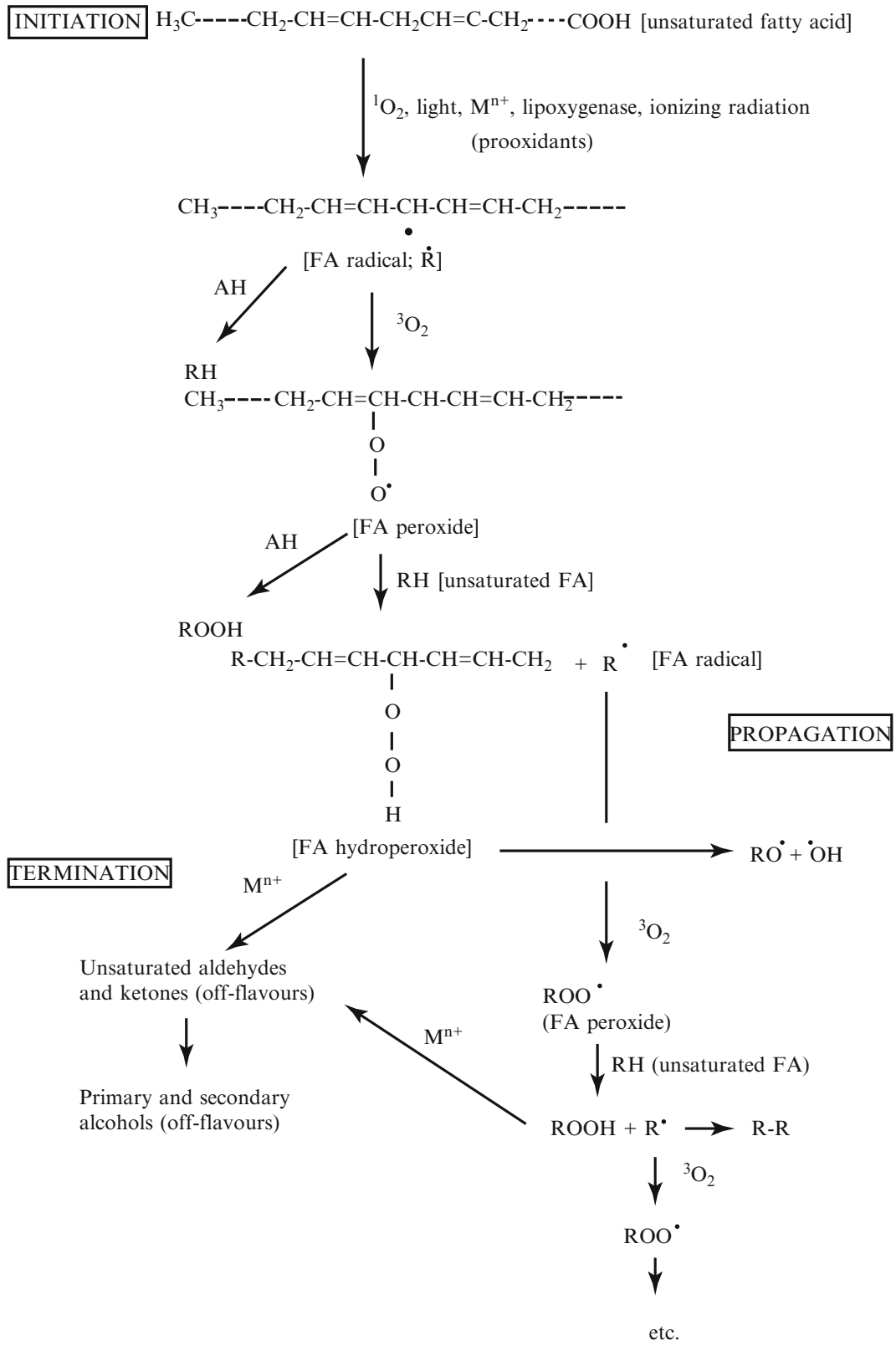

Fig. 1.6 Schematic diagram of lipid oxidation (Fox and McSweeney, 1998)

Jones *et al*. (1998) speculated that Maillard reactions occur during spray drying, although in general these reactions are more prominant in milk powders during storage. In milk powders, Maillard reactions may be favoured by the close proximity of reactive molecules, despite the absence of translational diffusion (Schebor *et al*., 1999). The activation energy of the Maillard reaction in WMP is 203.2 kJ mol^{-1} (Chong *et al*., 1996). Obviously, the reaction is retarded by storage of powder at a low temperature and avoiding the uptake of moisture by storage in moisture-proof bags.

The degradation of lactose during the storage of milk powders has also been reported. Galactose, lactulose and tagatose have been isolated from milk powders and are formed by base-catalysed degradation of lactose and/or the breakdown of the lactose-protein complexes formed during the Maillard reaction (Richards, 1963). Recently, lactose ureide, a urea derivative of lactose, has been reported in milk powders, and was proposed as a potential indicator of the thermal history of dairy products (Suyama *et al*., 2011).

1.4.3 Lipids

Lipids are quite resistant to heating. In the production of WMP, some linoleic acid is converted to conjugated linoleic acid (CLA), which is nutritionally desirable. Non-globular (non-emulsified) fat is formed in roller-dried powders, which is advantageous for WMP used in chocolate manufacture, where a glossy appearance and smooth texture are desirable, as discussed later in this chapter. The significance of free fat in fat-filled powders was reviewed by Vignolles *et al*. (2007).

The most serious fat-related defect in WMP is lipid oxidation, which involves the unsaturated, especially polyunsaturated, fatty acids. The extensive literature on lipid oxidation has been the subject of many reviews (e.g., Richardson and Korycka-Dahl, 1983; O'Connor and O'Brien, 1995, 2006). The reaction, which is autocatalytic, is summarised in Fig. 1.6. The initial step is catalysed by light (especially UV light), metals, especially iron and copper, or enzymes, e.g., lipoxygenases (not relevant in dairy products), with the formation of fatty acid free radicals. These react with O_2 with the formation of peroxy free radicals which abstract a $H^{\bullet}$ from another unsaturated fatty acid, thus continuing the oxidation process. The peroxy free radicals are unstable and break down into two free radicals which continue the reaction, which thus becomes autocatalytic. Some of these radicals are converted to carbonyls which are the cause of the off-flavours, called oxidative rancidity.

The problem is controlled or prevented by using opaque packaging, avoiding contamination with metals (e.g., by using stainless steel), avoiding O_2, e.g., by vacuum packaging and/or flushing with an inert gas, typically N_2, or by using antioxidants (typically natural antioxidants such as tocopherols, e.g., vitamin E) or by using synthetic antioxidants, if permitted. Lloyd *et al*. (2009b) showed that nitrogen flushing significantly extended the shelf-life of WMP, in terms of consumer acceptance and functionality in chocolate, and that the impact of such flushing was greater than the effect of storage temperature.

1.4.4 Minerals

During pre-heat treatment at a medium or high temperature, extensive precipitation of calcium phosphate occurs. Evaporation increases the concentrations of lactose and salts and results in a partially reversible transfer of soluble calcium phosphate to the colloidal form, with a resulting decrease in pH (Le Great and Brule, 1982; Nieuwenhuijse *et al*., 1988; Oldfield, 1998; Lewis, 2011). The extent of transfer of phosphate to the colloidal phase, which is greater than that of calcium, depends on the temperature of preheating. The concentrations of soluble calcium and phosphate in reconstituted SMP generally remain lower than those in the original milk, due to irreversible shifts induced during drying (Le Great and Brule, 1982). Preheating of milk and heating of concentrate reduce calcium ion activity, although these levels increase slowly on reconstitution and storage of milk powder (Oldfield, 1998; Liu *et al*., 2012).

1.5 Functional Properties of Milk Powders

The behaviour of milk powder is determined by the physical and chemical properties of its primary components, namely proteins, lipids and lactose, both individually and in combination. In the following sections, the functional properties of milk powders are described and the effects of process-induced changes during the different stages of manufacture, i.e., milk pre-treatment, drying and storage, on these properties, are discussed. The principal characteristics of milk powder, and the methods used for their measurement, are summarised in Table 1.5 (see Písecký, 1997; Caric and Milanovic, 2003; Schuck, 2011b).

1.5.1 Bulk Density

Bulk density, or packing density, may be defined as the weight of a unit volume of powder (g/mL, g/100 mL or g/L; Písecký, 1997). Bulk density depends on interstitial air (trapped between powder particles) and particle density, the latter being determined by the amount of occluded air (air within powder particles, in cavities called vacuoles) and the actual density of powdered material. Bulk density is closely interrelated with many other properties of milk powder, such as instant properties and flowability.

The bulk density of WMP is generally lower than that of SMP, if dried under similar conditions, due to the lower density of milk fat relative

Table 1.5 Methods for analysis of milk powders

Property	References
Moisture content (oven drying)	IDF No. 26B (1993b)
Protein content (Kjeldahl)	IDF No. 20B (1993a)
Total lactose content	Písecký (1997)
Total fat content (Röse Gottlieb)	IDF No. 123A (1988a)
Free fat content	Písecký (1997)
Sulphydryl content	O'Sullivan *et al*. (1999)
Titratable acidity	IDF (1978)
Oxidation in WMP	Ulberth and Roubicek (1995)
	Doka *et al*. (2015)
Insolubility index (mixing and centrifugation)	IDF (1988b)
Bulk density (tapping test)	IDF (1986)
Scorched particles	ADMI (1971)
Whey protein nitrogen index	ADMI (1971)
Wettability	IDF (1979)
Dispersibility	IDF (1979)
	Chen and Lloyd (1997)
Sludge test	Písecký (1997)
Flowability	Niro Atomiser (1978), Teunnou *et al*. (1999)
Slowly dispersible particles	Písecký (1997)
Particle size distribution	Písecký (1997)
	Aguilar and Ziegler (1994a, b)
Coffee test	Teehan *et al*. (1997)
Hot water sediment	Písecký (1997)
Hygroscopicity	Písecký (1997)
Degree of caking	Písecký (1997)

to protein and lactose. However, this difference is somewhat offset by the fact that the presence of fat in WMP inhibits foaming, which reduces the amount of occluded air (Písecký, 1978). The concentration of lactose in milk affects the bulk density of the powder produced therefrom, with low-lactose powders having a more porous structure, with larger vacuoles than normal (Jimenez-Flores and Kosikowski, 1986; Aguilar and Ziegler, 1994a, b).

Processing steps such as evaporation and pre-heat treatment also influence the bulk density of powders, by determining the extent of denaturation of whey proteins. Denaturation of whey proteins during pre-heat treatment enhances foaming, presumably due to the thermal unfolding of their globular tertiary structure. The bulk density of powder is also markedly affected by processing during drying, particularly atomisation. The bulk density of milk powder produced using a nozzle atomiser is higher than that of powder produced with a centrifugal atomiser (Boersen, 1990). Displacement of air by steam during centrifugal atomisation reduces the amount of occluded air (Woodhams and Murray, 1978).

Increasing the T_{outlet} from 70 to 95 °C improves the efficiency of drying but a further increase in T_{outlet} (95–105 °C) causes over-heating, expansion, cracking, a high vacuole volume and a low powder particle density (Caríc and Kaláb, 1987). In general, the microscopic appearance of milk powder particles is affected directly by the heat treatment applied during manufacture. Increasing T_{inlet} is linked to the formation of wrinkles, or deep folds, on the surface of particles, which are thought to be possibly caused by the presence of casein, as whey powders do not exhibit such wrinkles (Caríc and Kaláb, 1987).

The amount of interstitial air, i.e., air trapped between powder particles, depends mainly on particle size distribution, shape and degree of agglomeration. The low bulk density of roller-dried milk powder and spray-dried powder produced using a centrifugal atomiser compared to powder produced using a nozzle atomiser is due partly to their irregular shape and narrow size distribution (Caríc and Kaláb, 1987).

1.5.2 Reconstitution Properties of Milk Powders

Reconstituted milks, made by dissolution of SMP or WMP, may be distinguished from recombined milks, which are prepared by addition of SMP to water, followed by addition of anhydrous milk fat (AMF) and homogenisation, and filled milks, in which the fat added on recombination is not from milk. Ease of recombination of milk powder is affected primarily by its degree of agglomeration, water temperature and heat classification of the powder, wettability, sinkability and solubility (Table 1.6). The rehydration and solubility characteristics of high-protein dairy powders were reviewed by Crowley et al. (2015). A key variable is the time required for the hydration of proteins and, in the case of recombined products, molten fat should not be added until hydration is complete. Emulsifiers may be added to facilitate and improve emulsification of added fat, which is achieved using high-shear agitators or homogenisation.

Wettability depends on the ability of the powder to overcome the surface tension between it and water; the failure of a powder to wet sufficiently results in the formation of a scum, adherence of undispersed particles to the walls of the container and the gradual appearance of distinct layers in the reconstituted milk (Litman and Ashworth, 1957). The wettability of milk powders

Table 1.6 Parameters involved in the reconstitution of milk powders

Parameter	Depends on
Wettability (ability to absorb water on surface)	Hydrophobicity
	Lecithination
	Agglomeration
	Particle density
Dispersability (disperse without formation of lumps)	Size, agglomeration
Penetrability (ability to penetrate water surface)	Interstitial air
	Particle size distribution
	Liquid viscosity
Sinkability (after being moistened)	Particle density
Ease of solubility (rate of dissolving)	Physical and chemical properties

is markedly affected by the free fat content of the powder (Jensen, 1975; Woodhams and Murray, 1978), which may be a consequence of excessive pumping, homogenisation after concentration or the formation of lactose crystals which penetrate and damage the membranes surrounding the fat globules. Litman and Ashworth (1957) reported that the characteristics of lipids isolated from scum differed markedly from those present in the reconstituted milk; lipids from both sources had a similar saponification number, but the former had a higher melting point and lower iodine number, indicating that they were less susceptible to oxidation (Litman and Ashworth, 1957).

Litman and Ashworth (1957) proposed that the failure of milk powder to wet rapidly and sufficiently may be due to the formation of free fat-protein complexes. Problems associated with the presence of free fat may be alleviated or avoided through the addition of a surfactant such as lecithin, as is generally practised during the manufacture of instant WMP (see Sect. 1.3.4). The wettability of milk powders is also directly affected by the crystallisation of lactose. Lactose present in milk powders which are stored below the glass transition temperature exists in an amorphous state (Jouppila and Roos, 1994b; Teunnou *et al*., 1999). However, if the storage temperature is increased, amorphous lactose crystallises (Jouppila and Roos, 1994a), with subsequent plasticisation and caking of milk powder, which is detrimental to wettability (Mistry and Pulgar, 1996; Fitzpatrick *et al*., 2007). Caking is known to occur above 44 % relative humidity (Teunnou *et al*., 1999). The wettability of milk powder decreases during storage and the extent of the reduction in wettability on storage is reduced if the powders are stored at a low temperature (Litman and Ashworth, 1957). The effect of storage at low temperatures is presumably due to the fact that, under these conditions, most of the lipids in milk have solidified and, consequently, are less mobile and cannot form a film.

The second factor affecting the reconstitutability of milk powder is sinkability. Once the powder has wetted, i.e., the gaseous phase surrounding the particles has been replaced by an aqueous phase, the powder should sink, which in turn facilitates solubilisation. The sinkability of powders is determined largely by particle density (Munns, 1989).

Once the powder particles have been wetted and have sunk, reconstitution depends on solubility, which is a key determinant of overall quality. Standard tests for powder solubility involve mixing powder and water under strictly defined conditions, centrifuging and measuring the volume of insoluble sediment, which is expressed as the insolubility index (sometimes referred to as the solubility index). Typical insolubility indices for standard SMP and WMP are < 2.0 and < 1.5 mL per 100 mL of milk, respectively (Woodhams and Murray, 1978). High-protein milk powders have a high insolubility index (Jimenez-Flores and Kosikowski, 1986).

Seasonal variations in the composition of milk strongly influence the insolubility index of milk powder (Newstead *et al*., 1978; Baldwin and Ackland, 1991), which increases with increasing severity of pre-heat treatment (Baldwin and Ackland, 1991; van Mil and Jans, 1991). The method of atomisation influences powder solubility (Straatsma *et al*., 1999), as does the method of drying, with the insolubility index of roller-dried milk powders being far higher than that of spray-dried powders (Mistry and Hassan, 1991; Mistry and Pulgar, 1996). A mathematical model to predict the insolubility of milk powder based primarily on heat treatment during drying was developed by Straatsma *et al*. (1999).

Baldwin and Ackland (1991) reported that the insolubility index of WMP increased from 2.5 to 3.2 mL over a 12 month storage period. Similar observations were reported by Jimenez-Flores and Kosikowski (1986), Sharma and Tandon (1986) and Celstino *et al*. (1997a).

The mechanism(s) responsible for the increase in the insolubility index on severe heat treatment or during storage have not been elucidated fully, although there is evidence to suggest that Maillard reaction-derived crosslinks (see Sect. 1.4.2 above) play a role (Baldwin and Ackland, 1991; Le *et al*., 2011, 2013).

It is noteworthy that the concentration of HMF in milk powder increases with time and temperature of storage (Al-Talib, 1984; Jimenez-Flores and Kosikowski, 1986; Baldwin and Ackland, 1991; Schebor *et al*., 1999) and with the severity of heat treatment, and that insoluble material in reconstituted milk has been found to contain

Table 1.7 Food applications of skim milk powder (SMP) of different heat classes

Heat classification	Heat treatments typically applied	WPNI	Functional properties	Food applications
Low heat	70 °C for 15 s	>6.0 mg/ml	Solubility, lack of cooked flavour	Recombined milk, milk standardisation, cheese making
Medium heat	85 °C for 1 min	1.5–6.0 mg/ml	Emulsification, foaming, water absorption, viscosity, colour, flavour	Ice cream, chocolate, confectionery
	90 °C for 30 s			
	105 °C for 30 s			
High heat	90 °C for 5 min	<1.5 mg/ml	Heat stability, gelation, water absorption	Recombined evaporated milk
	120 °C for 1 min			
	135 °C for 30 s			
High-high heat	>120 °C for>40 min	<1.5 mg/ml	Flavour, water binding, colour	Bakery, recombined evaporated milk

products of lactose-protein interactions (Parris *et al*., 1990).

A number of alternative mechanisms for the formation of insoluble material in milk powders have been proposed. For instance, it has been suggested that insolubility may be an indirect consequence of lactose crystallisation, as this causes localised increases in calcium ion activity and a concomitant reduction in the stability of casein micelles (Al-Talib, 1984). The possible formation of isopeptide crosslinks (transamination or amidation reactions) or dehydroalanine-derived crosslinks in milk powder and their possible significance for the solubility of milk powders have not been investigated, to the authors' knowledge. Straatsma *et al*. (1999) proposed that insolubility is enhanced by unfolding of β-lactoglobulin and subsequent aggregation with casein, while Singh and Ye (2010) reported that understanding of interactions between proteins, and the MFGM in particular, was key to controlling reconstitution properties of WMP.

1.5.3 Heat Classification of Milk Powders

As mentioned in Sect. 1.3.2, heat classification refers to the extent of heat treatment applied during powder manufacture and generally reflects the severity of treatment during pre-heating before evaporation. The assessment of heat treatments applied to milk and dairy products was comprehensively reviewed by Pellegrino *et al*. (1995). The properties and applications of the major heat classification groups of SMP are outlined in Table 1.7. The majority of SMP produced today is of the medium-heat class (Early, 1998).

The most common analytical parameter used for heat classification of SMP is the whey protein nitrogen index (WPNI), defined as mg undenatured whey proteins/g powder. Typical WPNI values for low-, medium- and high-heat treated milk powders are >6.0, 1.51–5.99 and <1.50 mg/g powder, respectively (Caríc and Kaláb, 1987). The thermal history of milk powders may also be determined by quantifying the free sulphydryl content (cysteine number) or casein number (Staplefeldt *et al*., 1997a). In addition, methods such as SDS-PAGE under reducing and non-reducing conditions, and combinations of these methods into 2-dimensional electrophoretic approaches, can be used to differentiate and characterise the protein-protein interactions in milk powders of different heat classifications (Patel *et al*., 2007; Williams *et al*., 2008).

The validity of heat classification tests may be influenced by variations in the concentration of whey proteins in raw milk, such as caused by seasonal fluctuations in milk composition (Sanderson, 1970), although these are likely to be reasonably minor in extent; such variations may be monitored by powder manufacturers to allow more accurate control of powder properties. Genetic polymorphism of β-lactoglobulin and season, through their influence on level of whey proteins in milk, affect the WPNI and sulphydryl group content of SMP (Sanderson, 1970; O'Sullivan *et al*., 1999).

1.5.4 Flowability of Powder

The flowability of milk powder is a complex phenomenon, dependent on inter- and intra-particle forces, which can be defined loosely as the cohesion, or resistance of the powder to flow, under its own weight. Powder flowability is affected by the shape and size of powder particles, and the moisture and fat content of the powder. Flowability may be assessed by pouring out a heap of powder under standardized conditions and measuring the angle of repose, α, of the resulting heap. The flowability of milk powders is in the order agglomerated SMP > SMP > agglomerated WMP > WMP (Písecký, 1997; Rennie *et al*., 1999). The flowability of milk powder decreases with fat content up to ~20 % fat but from 20 to 45 % fat, flowability is independent of fat content (Woodhams and Murray, 1978). Resistance to flow decreases with decreasing particle size (Woodhams and Murray, 1978; Rennie *et al*., 1999) and increases with the moisture content of the powder (Rennie *et al*., 1999). Flow-conditioning agents, such as silicates, stearates and phosphates, can increase the free-flowing nature of milk powders (see Ontwulata *et al*., 1996).

1.5.5 Organoleptic Properties

Both the natural taste of reconstituted milk powders and the development of undesirable flavours during storage are of critical importance (see Chap. 8). When using milk powder for direct reconstitution, the use of low- or low/medium-heat powder is recommended (Augustin, 1991). However, despite trying to emulate the flavour of fresh milk, reconstituted milk has a distinct type of flavour and there is a system of International Dairy Federation (IDF)-defined standard scales for scoring the flavour of reconstituted milk powders (Hough *et al*., 1992). It is thought that lactones, even-numbered short-chain fatty acids and furanones contribute to the flavour of milk powders (Shiratsuchi *et al*., 1995). The contribution of indigenous enzymes, such as lactoperoxidase, xanthine oxidoreductase, lipase and proteases, as well as enzymes added during dairy processing such as chymosin and lipases, to the flavour of dried dairy products was reviewed by Chen *et al*. (2003) and Campbell and Drake (2013). In addition, the sensory properties of milk protein ingredients was reviewed by Smith et al. (2015).

The volatile flavour compounds in SMP have been identified and shown to originate mainly from breakdown or secondary reactions of residual milk fat, or from feed or forage materials (Shiratsuchi *et al*., 1994a). It has been suggested that specific off-flavours in SMP described as cowhouse-like, hay-like, sulphuric and quinoline-like may be due to the presence of specific compounds such as tetradecanal, β-ionone and benzothiazole (Shiratsuchi *et al*., 1994b).

Preheating is likely to play a major role in the development of specific flavours in WMP, such as the intensity of cooked aroma, cooked taste and creaminess (Baldwin *et al*., 1991). The cooked flavour of milk powder is linked to the production of free sulphydryl groups (Hols and van Mil, 1991) and the Maillard reaction (Shiratsuchi *et al*., 1995). Baldwin *et al*. (1991) proposed that the cooked flavour in WMP stored at 30 °C reaches a maximum after 3 months storage and decreases thereafter and that the intensity of cooked flavour varies with season. Variations in cooked flavour with season may be related to changes in the concentration and profile of whey proteins.

Auldist *et al*. (1996) reported that WMP produced from milk with a high somatic cell count (SCC) did not differ from normal milk powders initially, but that off-flavours developed in the former powders during storage, which was attributed to the high proteolytic and lipolytic activity in milk with a high SCC. The development of off-flavours has been reported in milk powder produced from milk where processing steps included standardisation of the protein content through the addition of cheese whey (Kieseker and Healy, 1996), and which may be due to an altered casein-whey protein ratio (and therefore the sulphydryl content per unit weight).

The most common and serious off-flavour encountered in WMP is oxidative rancidity, as discussed earlier, which is a major determinant of the shelf-life of WMP (Driscoll *et al*., 1985; McGookin and Augustin, 1997; Li *et al*., 2013). Products of oxidative rancidity, particularly unsaturated fatty acids, to peroxides and monocarbonyls, may have

distinct off-flavours (Keen *et al*., 1976). Lloyd *et al*. (2009a) characterised the volatile profile of WMP using solid phase microextraction and correlated sensory characteristics with individual compounds, and described changes in the profile during storage; for example, 'grassy' and 'painty' flavours developed during storage, while the intensity of sweet aromatic flavour decreased. Quantification of free radicals may be used as an early indication of increasing oxidation in WMP (Staplefeldt *et al*., 1997c). Oxidative rancidity in milk powders increases during storage (Mahran *et al*., 1984; Liang, 2000; Whetstine *et al*., 2007) and has been reported to follow zero-order reaction kinetics, with an activation energy of approximately 70 kJ/mol (Chong *et al*., 1996; Liang, 2000).

Lipid oxidation in WMP is greatly affected by storage temperature and heat classification, being highest for low-heat powders at an elevated temperature (Staplefeldt *et al*., 1997b). In general, heat treatment increases the oxidative stability of milk powders (Boon, 1976; Baldwin and Ackland, 1991; van Mil and Jans, 1991; McCluskey *et al*., 1997). Preheat treatment induces the thermal unfolding of β-lactoglobulin, with the concomitant exposure of the buried reducing free sulphydryl group, which has an antioxidant effect (Baldwin and Ackland, 1991). Products of the Maillard reaction between proteins (particularly the caseins) and lactose may also be partially responsible for the increase in oxidative stability of milk powders due to preheating (McGookin and Augustin, 1997).

Many other factors, including season of production, affect the oxidative stability of milk powders (van Mil and Jans, 1991). Celstino *et al*. (1997a) reported that storage of milk for 48 h at 4 °C prior to drying increased the susceptibility of the resultant powder to oxidative rancidity and proposed that an increase in lipolysis may be partly responsible. Reducing the amount of free fat in WMP (as discussed in Sects. 1.4.2 and 1.5.7.1) also affects the oxidative stability of milk powder.

The quality of WMP is also dependent on a_w, with optimum stability being in the range 0.11–0.23 (Staplefeldt *et al*., 1997a, b, c). Storage conditions such as temperature, packaging material and head space in the container also have a marked effect on the oxidative stability of milk powder (Driscoll *et al*., 1985; van Mil and Jans, 1991; McCluskey *et al*., 1997). It has been reported that SMP stored at 32 °C developed an off flavour after 6 months storage and was unacceptable after 24 months, whereas SMP stored at 21 °C was deemed acceptable after 24 months (Driscoll *et al*., 1985). The shelf-life of SMP may also be extended by storing under CO_2 or N_2 (Driscoll *et al*., 1985) or packaging in cans rather than in plastic bags (Driscoll *et al*., 1985).

McCluskey *et al*. (1997) reported that milk produced from cows fed a diet that had been supplemented with vitamin E (22.5 IU vitamin E/cow/day) had an increased vitamin E content and that the powder produced therefrom was less susceptible to oxidative deterioration. The oxidative stability of WMP may also be increased through the direct addition of phenolic compounds as antioxidants to the milk powders (Radayeva *et al*., 1974), or through addition of rice bran oil (Nanua *et al*., 2000). The production of milk rich in phenolic compounds, such as gossypol and genistein, has been advocated, due to their salutary health effects; it is likely that milk powders produced from such milk would also have a high oxidative stability (Parodi, 1996).

During storage, WMP generally becomes darker and more yellow, which is due to lactose crystallisation and the migration of β-carotene and free fat to the surface of powder particles (Nielsen *et al*., 1997a, b). The Maillard reaction is also thought to cause darkening of milk powders during storage.

A defect sometimes associated with milk powders is the presence of scorched particles, or discoloured specks, resulting from deposits of charred powder, which have been discoloured by Maillard reactions at low water activity (a_w) and high temperatures within the drying chamber, entering the bulk dry powder (Early, 1998).

1.5.6 Heat Stability

Most milk powders are reconstituted before use. The ability of reconstituted milk powder to withstand high temperatures is pertinent in the

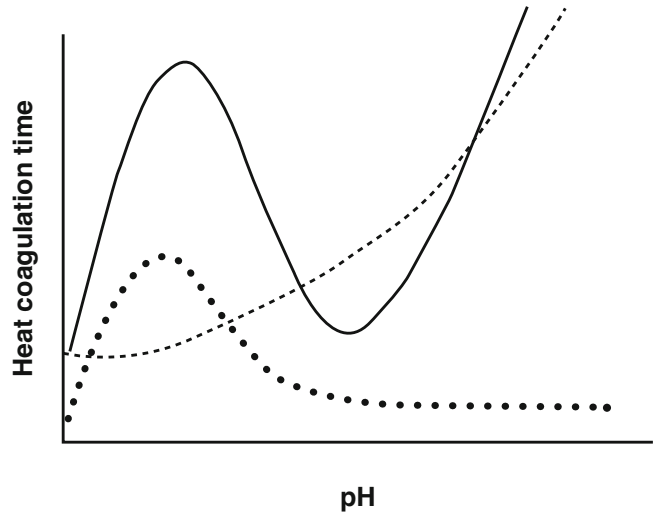

Fig. 1.7 Schematic diagram of the heat stability of milk (from Kelly and Fox, 2012), showing heat coagulation time (HCT)-pH profiles of typical type A bovine milk (*solid line*), type B or serum protein-free milk (*dashed line*), as determined at 140 °C, or concentrated milk (*dotted line*), as determined at 120 °C

production of an array of products such as coffee whiteners, dessert products, bakery products and, perhaps most importantly, sterilised reconstituted whole milk. The ability of reconstituted milk powder to withstand sterilisation may be assessed by the subjective heat stability assay of Davies and White (1966) or by the method of Kieseker and Aitken (1988).

Compared to other biological systems, milk is very stable; good quality fresh milk will withstand heating at 140 °C for at least 15 min but stability is variable. Heat stability is affected by several complicated factors, especially pH, calcium ion activity, and the concentrations of caseins and whey proteins. The effects of free Ca^{2+} and pH on the heat stability of reconstituted low-heat SMP was studied by Faka *et al*. (2009), who reported that reducing Ca^{2+} levels improved the heat stability of such powder on reconstitution. The importance of heat stability for the manufacture of evaporated milk led to early studies on the factors that affect it; the first publication on the subject was that of Sommer and Hart (1919). Since then, there have been many studies on the heat stability of milk, including the mechanism of heat-induced coagulation. These have been reviewed regularly, including Fox and Morrissey (1977), O'Connell and Fox (2003) and Huppertz (2015). The heat stability of unconcentrated milk is usually sufficient to withstand all processing to which it is normally subjected, but the stability of concentrated milk is marginal and must be subjected to certain process modifications. The heat stability of milk and concentrated milk is very dependent on pH and is summarised in Fig. 1.7. The heat stability of reconstituted milk powder is affected by the same factors as fresh milk.

The heat stability of reconstituted milk powder is strongly affected by milk quality (Kelly, 1982). For example, milk powder produced from late lactation milk is inherently unstable (Newstead *et al*., 1978; Auldist *et al*., 1996), which may be related to changes in the ionic calcium content and protein concentration. Auldist *et al*. (1996) also reported that powder produced from milk with a high SCC exhibited low heat stability and attributed this to high proteolytic activity in mastitic milk.

Pre-heat treatment of milk plays a critical role in enhancing the heat stability of powders produced therefrom upon reconstitution. Increasingly severe pre-heat treatments (such as 120–140 °C for 1–15 min in the production of high heat powders) may produce very heat-stable powders (Fox, 1981; Sweetsur and Muir, 1981; Tan-Kintia and Fox, 1999). In New Zealand, such high-temperature pre-heat treatments are widely used in industry (Singh and Newstead, 1992). How pre-heat treatment of milk for powder manufacture enhances the heat stability of reconstituted powder produced from it has not been elucidated fully, although whey protein denaturation and

the precipitation of calcium phosphate under relatively mild conditions and an increase in the stability of caseins to calcium during pre-heat treatment may play a role (Fox, 1981; Tan-Kintia, 1996; O'Connell and Fox, 1999). Sikand *et al.* (2010) showed that the heat stability of reconstituted SMP is affected by heat treatment and method of standardisation (addition of lactose or addition of ultrafiltration permeate). Williams *et al.* (2008) concluded that the key to achieving good heat stability of milk powder was heating to give a low whey protein nitrogen index (WPNI), with a balance of moderately sized soluble protein aggregates and a reduced proportion of smaller micelles, and that aggregate formation, a key factor underpinning heat stability, was influenced by pre-heating conditions and seasonality.

The method used to concentrate milk prior to drying also affects the heat stability of the powder produced therefrom. Sweetsur and Muir (1980) and Muir and Sweetsur (1984) reported that the heat stability of concentrated milk prepared by ultrafiltration was markedly higher than that of concentrates prepared by evaporation. The heat stability of concentrates may be increased by altering the protein profile or mineral content by diafiltration or by the use of different types of membrane (Muir and Sweetsur, 1984; Sikand *et al.*, 2008).

Surprisingly, there is little information on the effect of storage of milk powder on the heat stability of milk reconstituted therefrom. Al-Talib (1984) reported a slight decrease in the heat stability of reconstituted milk powder after storage for 90 days, with the reduction being more pronounced when the powder was stored at a higher temperature (37 °C). The reduction in the heat stability of reconstituted milk powder on storage may be due to an increase in calcium ion activity, as discussed in Sect. 1.4.3, as the ethanol stability of reconstituted milk powder also decreases on storage (Al-Talib, 1984). It has also been shown that the age gelation of ultra-high temperature-treated milk prepared from milk powder is affected by length of time the powder from which the milk was prepared had been stored (Celstino *et al.*, 1997b).

The heat stability of reconstituted WMP is enhanced by the addition of lecithin or buttermilk to milk prior to evaporation and drying (Singh and Tokley, 1990; Singh *et al.*, 1992), with the key consideration being the presence of phospholipid-rich milk fat globule membrane components, which seem to reduce heat-induced interactions between milk proteins (Kasinos *et al.*, 2014). The stabilising effect of lecithin is due partly to its surfactant properties (Singh and Tokley, 1990), while the addition of buttermilk may enhance the stability of milk due to the low calcium and β-lactoglobulin content of buttermilk (O'Connell and Fox, 2000).

1.5.7 Other Functional Properties of Milk Powders

1.5.7.1 Free Fat Content

Fat in WMP is encapsulated within the amorphous matrix of lactose and protein, although some fat is also present in capillary pores and cracks and in pools on the surfaces of powder particles (Buma, 1971). Free fat is generally defined as the fat that can be extracted by organic solvents under defined conditions, and generally originates from non-encapsulated fat, and from fat globules that are accessible to solvent which penetrates through capillaries. The relationships between powder properties such as free fat level, solid fat concentration, particle size and vacuole volume and the rheology of milk chocolate were reported by Twomey *et al.* (2002).

Free fat is considered to be a desirable attribute of WMP used in chocolate manufacture, and such powder is frequently produced by roller-drying (Dewettinck *et al.*, 1996; Keogh *et al.*, 2004; Liang and Hartel, 2004; Clarke and Augustin, 2005), although spray drying processes using simultaneous atomisation of pre-crystallised skim milk concentrate and high-fat cream have also been developed for this application; a processes using high shear and high temperatures in a twin-screw continuous mixer to crystallise lactose and increase free fat levels was described by Koc *et al.* (2003).

1.5.7.2 Cheesemaking Properties

About 40 % of total milk production is used for the manufacture of cheese (about the same percentage as consumed as liquid/beverage milk). About 75 % of all cheese is produced by the rennet-induced coagulation of milk, and most of the remaining 25 % is produced by acid-induced coagulation of the casein at pH around 4.6 at about 30 °C, with a small amount being produced by a combination of acid and heat.

It is generally regarded that premium quality cheese cannot be produced from reconstituted milk powder, which tends to form a soft fragile gel on renneting which is difficult to drain and consequently leads to high moisture curds (Lenoir *et al*., 2000). However, when milk powders are used in the manufacture of cheese it is recommended that low-heat (or low-low-heat) milk powder should be used. The rennetability of milk reconstituted from medium- and high-heat powders is lower than that of normal milk, due to whey protein-κ-casein complex formation leading to the formation of a different type of gel network (Cheftel and Lorient, 1982). The rennetability of reconstituted low-heat SMP decreases slightly during storage of SMP while the firmness of renneted curds produced therefrom decreases markedly (Al-Talib, 1984).

The vast majority of acid- and heat-coagulated cheeses are consumed fresh (unripened). Several varieties of cheese are produced by acid or acid-heat coagulation (Farkye, 2004a, b; Schultz-Collins and Senge, 2004), but the principal varieties are Quarg, Cream cheese, Ricotta (and variants), Bakers' cheese and Cottage cheese. Quarg, Bakers' cheese and Cottage cheese are usually produced from HTST-pasteurized skim milk but may be produced from low-heat SMP. If medium- or high-heat SMP is used, the curds will not synerese efficiently. Obviously, Cream cheese is produced from light cream and Ricotta is produced by acid-heat coagulation but a blend of skim milk and whey is commonly used and, if desired, reconstituted SMP could be used.

Queso Blanco, a cheese of Latin American origin, is produced by acid-heat-induced coagulation of skim milk. Usually, fresh skim milk is used but reconstituted low- or medium-heat SMP could be used.

1.5.7.3 Fermented Milks

According to Surono and Hosono (2011), about 400 names are applied to traditionally and industrially-made fermented milk products throughout the world. Most of these are produced locally on a small scale from fresh untreated milk. They consider that 20 products are 'industrial', i.e., characterized fermented milks which can be classified into three types (for reviews, see Tamime and Robinson, 1999; Robinson, 2011):

1. produced by a lactic acid fermentation, e.g., acidophilus milk and fermented buttermilk;
2. produced by a lactic acid-ethanol fermentation, e.g., kefir;
3. produced as (1) or (2) but in which there is mould growth also.

Many fermented milks are concentrated by draining the fermented product through muslin, e.g., Zadaby, or by centrifugation, or produced from milk with an increased level of total solids, e.g., 13–14 % up to 16–18 %, which may be obtained by adding milk powder to milk, evaporation of milk, or membrane filtration.

Fermented milks could also be produced from reconstituted milk powder, since the acid taste masks the cooked taste of the milk powder, sugar and fruit are added to many fermented milks, and the milk, fortified or not, is subjected to severe heat treatment, e.g., 90–95 °C for 5–10 min for yoghurt. This heat treatment is applied to reduce the tendency of the product to synerese and "whey off"; the objective is the opposite of that in cheese-making, in which syneresis is required. It is not known how widely reconstituted milk powder is used for the production of fermented milk, but it is used for yoghurt. Greek-style yoghurt (around 20 % TS) has become a very popular product in the USA.

1.5.7.4 Water Sorption Properties

Milk powders readily absorb water at moderate to high relative humidities, increasing the rate of many reactions, and greatly influencing the properties of milk powders (Murietta-Pazos *et al*., 2011). Consequently, the water absorption behaviour of milk powder is of critical importance in determining its swelling, gelling, emulsifying,

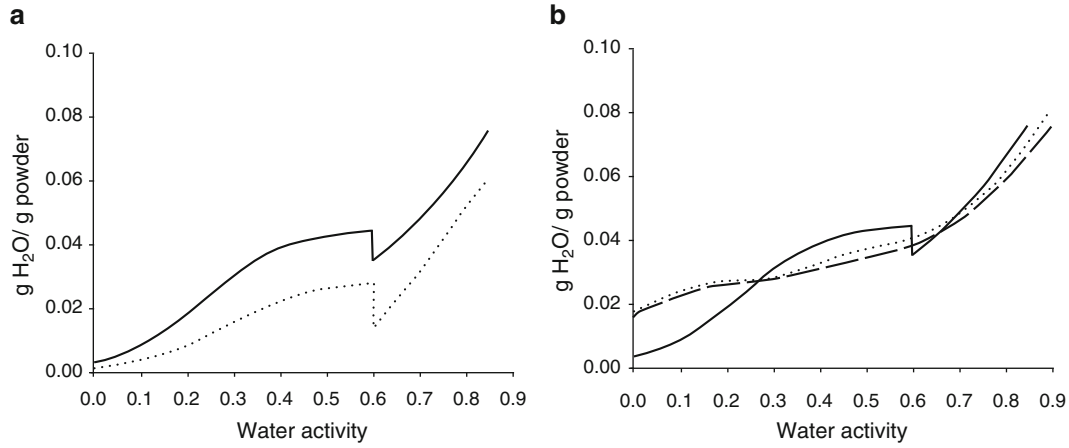

Fig. 1.8 (**a**) Water sorption isotherms of whole milk powder (*dotted line*) and skim milk powder (*solid line*) and (**b**) sorption isotherms of skim milk powder for absorption (*solid line*), desorption (*dotted line*) and reabsorption (*dashed line*) (redrawn from Berlin *et al*., 1968)

foaming and organoleptic properties. The water absorption properties of milk powder are conventionally expressed using water sorption curves, which essentially measure hydration as a function of water activity (Saltmarch and Labuza, 1980). Typical absorption isotherms of WMP and SMP are shown in Fig. 1.8. Differences in the absorption isotherm of the two types of powder are partly related to the fact that lipids are relatively non-hydroscopic. As shown, the isotherms of both WMP and SMP have a distinct sigmoidal shape, with a discontinuity at an a_w of ~0.6 (Berlin *et al*., 1968; Kinsella and Fox, 1985). It has been proposed that this discontinuity is due to lactose crystallisation, which begins at a water activity of 0.33–0.50 (Warburton and Pixton, 1978; Saltmarch and Labuza, 1980). Desorption and resorption isotherms do not have a discontinuity (Fig. 1.8), which shows that the water bound within the discontinuous phase in the absorption isotherm is irreversibly or very strongly bound (i.e., water of crystallisation; Berlin *et al*., 1968).

Berlin *et al*. (1968) proposed that the distinct shape of the sorption isotherm of milk powders can be divided into three specific zones. The initial gradual increase is due to water absorption at bulk phase interaction sites (i.e., external surfaces of particles); this is followed by a rapid increase in absorbed water which is due to absorption by the lactose glass, i.e., lactose crystals are arranged into tightly packed lattices which, compared to amorphous lactose, are non-hygroscopic and consequently release water (see Sect. 1.4.2). The final zone of the sorption isotherms of milk powders (from a_w of 0.6 to 0.8) is due to the diffusion of water into the particles and to protein swelling. Heldman *et al*. (1965) and Al-Talib (1984) showed that the equilibrium moisture content of milk powder decreases during storage and decreases with the severity of pre-heat treatment. Yazdanpanah and Langrish (2011) reported the relationships between crystallisation during manufacture of SMP using multi-stage drying and subsequent moisture sorption properties.

1.5.7.5 Other Functional Properties

There has been little research on the foaming and emulsifying behaviour of milk powders, probably because caseinates are used more widely in applications where such properties are required. In general, milk powders have inferior emulsifying properties to caseinates (Mulvihill and Murphy, 1991; Euston and Hirst, 1999). The reason for this is that, in milk powder systems, caseins are present in micellar form and do not have the same molecular mobility and surface-active properties as sodium caseinate (Mulvihill and Murphy, 1991; Dalgleish, 1996; Euston and Hirst, 1999). However, Euston and Hirst (1999)

reported that the stability of emulsions against creaming is greater when milk powders are used, relative to caseinate systems, and proposed that this may be due either to the former system being less susceptible to depletion flocculation or to the formation of a weak gel in milk powder solutions. It has been reported that milk powder has better emulsifying ability than sodium caseinate at pH 5.2 (Singh and Newstead, 1992), which may be related to the dissociation of protein from micelles in this pH region. High-protein milk powders exhibit better foaming properties than standard milk powders (Jimenez-Flores and Kosikowski, 1986; Mistry and Hassan, 1991), which is presumably related to the high protein content of the former. With the emergence of micellar casein powders (e.g., phosphocaseinate and milk protein concentrate), the use of milk powders as emulsifying and foaming agents may receive more research attention.

A further important property of milk powder is the ability of powders intended for use as coffee whiteners to disperse and remain soluble when added to hot coffee which has a low pH (coffee stability test). Teehan *et al.* (1997) reviewed methods for measuring the coffee stability of milk powder. Oldfield *et al.* (2000) reported that increasing the severity of preheat treatment of milk before evaporation and increasing total solids level in concentrate being fed to the atomiser both negatively affected coffee stability of WMP, while lecithinisation improved coffee stability.

1.6 Conclusion

Drying of milk, while initially a relatively straightforward method of preservation, has developed into a process that can produce a diverse range of products, tailored to the requirements of a wide range of food applications. Such diversification has been made possible by increased understanding of the complex physico-chemical changes that occur during evaporation and drying and, in parallel, understanding of the effects of processing variables on these changes, thus enabling modifications to technology to produce desired functional effects. Regarding future developments in this field, the principal areas of development in the area of drying itself are largely concerned with increasing scale of production, as global utilisation of powders continues to grow. For milk powders, a key ongoing research area is the changes that occur during storage of the powder, and thus enhancement of shelf-life. As regards products, it is likely that significant growth and development will occur in the area of milk powders with altered composition, in particular modified protein content and profile, to provide a new generation of highly functional food ingredients.

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Functional Milk Proteins Production and Utilization: Casein-Based Ingredients

2

Alistair Carr and Matt Golding

Abstract

Casein based ingredients have a long history of use, in both food and non-food applications, due to the ease with which their functional properties can be manipulated. However with the advent of functional and cheaper non-casein alternatives, manufacturers have had to be innovative in both their manufacturing processes and in seeking out new applications. This necessity, through the development of membrane and ion-exchange based ingredients, has led to the demise in the importance of traditional casein products. This chapter reviews the processing and application developments for both traditional and newer ingredients with a focus on how to reduce cost to both the ingredient and application manufacturers while meeting the market demands of ingredient flexibility and quality to enable holistic product design.

Keywords

Casein • Caseinate • Milk protein concentrate • Manufacture • Ingredient • Functional properties

2.1 Introduction

Casein based ingredients are of major importance in both food and to a lesser extent in non-food applications. While caseins have a long tradition of use and hence importance in the global dairy market, the advent of membrane-based separation technologies has resulted in dramatic changes to the casein market product mix. Global membrane-manufactured casein ingredients (i.e., milk protein concentrates (MPC) and micellar casein concentrates (MCC)) production has quadrupled to 1,84,000 tons in the period from 2000 to 2011 (Anonymous, 2012) with the remaining casein product breakdown, for 2010, as follows: rennet casein, 43,000 MT; caseinate, 116,000 MT; acid casein 87,000 MT (Culhane, 2010).

Casein ingredients are added into food systems either primarily for a direct component structuring purpose or for a nutritional purpose

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such as delivery of protein or minerals. There are many different casein based/containing ingredients that are in the market place or are described in the scientific and patent literature. Common to all casein-based ingredients is the need for some form of concentration operation. In the commercial manufacture of casein ingredients concentration is typically a physical separation process and thus relies on differences in particle size and/or density between the caseins and non-desired components of milk. Although casein in milk is present in a micellar form with a relatively large particle size in the range of 80–300 nm, compared to whey protein (2.5–3.7 nm) the technology to exploit this size difference on commercial scale has only been available since the late 1980s.

Historically, the only means available for separating casein from milk was through the use of macro-scale filters. This limitation required the development of methods to destabilise the micro-scale casein micelles and allow the formation of insoluble curd particles with sufficient size and mechanical strength to allow separation from whey *via* the use of screens.

These two approaches form the basis for all the casein-based ingredients that are available commercially and the product of these approaches may be classified by the state of the casein at the point of separation: insoluble or soluble. The sections that follow describe some of the key features for the processes that form the backbone of casein manufacturing.

2.2 Insoluble Casein-Based Ingredients: Acid Casein and Rennet Casein

The separation of casein through the formation of an insoluble product is simplified in this section by focussing on acid casein and rennet casein manufacture. Co-precipitates are essentially produced in the same manner as acid casein apart from some minor variations which are discussed as a special case in Sect. 2.2.6.

To understand the processing steps in casein manufacture it is necessary to describe how casein occurs in its native state. Various models exist for the casein micelle and these are discussed extensively elsewhere. The essential features of the micelle that are relevant to ingredient manufacture are that the micelles are composed of 92 % casein protein, with α_{s1}-, α_{s2}-, β- and κ-casein in an approximate ratio of 3:1:3:1, and 8 % inorganic matter, composed primarily of calcium and phosphate (Swaisgood, 1985). Despite disagreement over the exact structural arrangement of the micellar components, the concept of the casein micelle electrostatically and sterically stabilised by a 'hairy' surface layer of κ-casein appears to be universally accepted. The destabilisation methods rely on reducing the zeta potential of the micelles through charge neutralisation *via* acid addition for the manufacture of acid casein or through a combination of enzymatic removal of the highly electronegative "tail" region of the κ-casein hairs coupled with neutralisation of residual charge *via* the presence of calcium ions in the manufacture of rennet casein. The impact of each method on the zeta potential of the casein micelle is shown in Fig. 2.1.

Although the general processing steps for acid or rennet casein curd are the same, the method employed for zeta potential reduction (acid or enzymatic: cf. Fig. 2.1) does confer unique properties on the resultant curd, subsequent processing and the final casein composition and properties.

Reduction of the zeta potential of the casein micelle does not in itself result in curd formation, it merely decreases electrostatic repulsion. For interaction of casein micelles to occur, and thus curd formation, there also needs to be an attractive force of sufficient strength relative to residual repulsive forces to result in interactions on an industrially useful timescale. The main attractive force acting between micelles is hydrophobic in nature and therefore temperature dependent. Temperature control is an important variable in controlling the rate of curd formation, particle size and the subsequent density and curd strength. Correct management of these factors have downstream effects on the efficiency of curd washing and thus the composition of the final casein produced.

(a) Acid Caseins

Acid addition may be accomplished either through the direct addition of acid in the

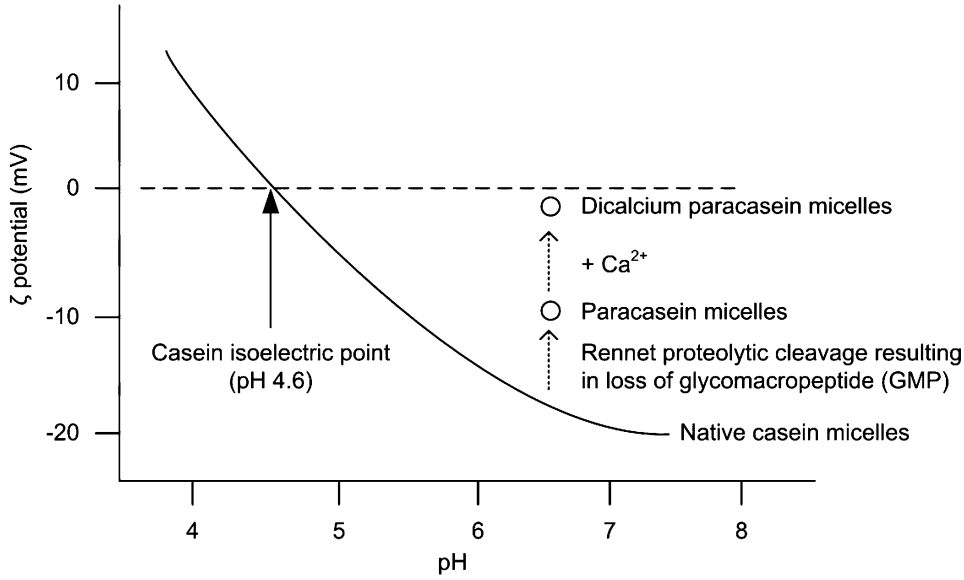

Fig. 2.1 Schematic illustration of the trend in zeta potential of casein micelles in milk as a function of pH and of the effect of ionic calcium on para-κ casein micelles at natural pH of milk

manufacture of mineral acid casein or through a bacterial fermentation process wherein lactose is converted to lactic acid in the manufacture of lactic acid casein.

Precipitation in the production of mineral acid casein is generally achieved through the addition of dilute acid under turbulent conditions to ensure that regions of localised low pH are minimised. Typically the form of mineral acid is hydrochloric acid, although in Australasia, sulphuric acid is more commonly used for reasons of availability and cost. As a general rule of thumb, 60 L of 1.0 N H_2SO_4 or HCl is required to precipitate casein from 1000 L of mid-lactation skim milk. Control of acid addition is a critical step with the most important factor in making good mineral acid casein being the thorough mixing of acid and milk. Inadequate mixing may give rise to micro-domains of low pH, which results in incomplete precipitation; irregular curd; high fines in whey streams; curd breakdown during washing (Teo *et al*., 1997); and sticky curd in the hot wash and during drying. To ensure adequate mixing the recommended acid concentration is 0.25–0.5 M. Ideally mixing should enable the acid to be homogeneously dispersed throughout the milk before it starts to react with the casein. It is also important that mixing is complete before reacted caseins start to precipitate so that fines production is minimised. In reality, acid reacts as soon as it comes in contact with casein and thus gives rise to micro-domains with varying acidity and thus inconsistent curd. However this can be mitigated by diluting the acid which increases both the time for acid to come in contact casein and also the time for reacted casein to come in contact with other reacted casein. However while lower acid concentrations give greater mixing efficiency, they dilute the whey. In contrast, higher acid concentrations are more likely to result in inadequate mixing.

Other acid casein processes have been described in the literature, but to date they have proved uneconomical and, to the authors' knowledge, not implemented on an industrial scale. Tomasula (1995) described a process enabling the continuous processing of acid casein through sparging CO_2 into the

milk stream resulting in the formation of carbonic acid and hence a lowering in pH to the isoelectric point. The use of ion exchange columns has also been described (Rialland and Barbier, 1982) to lower the pH of the skim feed. In this method it is necessary to process the milk through the exchange column, loaded with resin in the hydrogen form, under cold conditions where hydrophobic interactions are at their weakest to prevent curd formation and blocking within the columns. The treated milk is subsequently blended with untreated milk to give a bulk milk with a pH of 4.6.

Lactic casein manufacture involves the addition of specially selected mesophilic strains of starter bacteria to skim milk. The bacteria grow on the lactose in the milk producing lactic acid, which lowers the pH of the milk, eventually causing isoelectric precipitation at a desired final pH of 4.6. The lactic acid casein precipitate forms slowly as a soft gel under quiescent conditions in a large silo, in contrast to mineral acid casein which precipitates rapidly during flow as curd particles. The main requirements for a lactic casein starter are: lactic acid must be the dominant product of metabolism as other metabolic compounds generally lead to poor flavours; bacterial protein requirements must be minimal so that yield losses, and thus contamination of the whey stream, *via* proteolysis are small; the strains must be phage resistant; and preferably stop growing at pH 4.6. Additionally the bacteria must not produce large volumes of gas as this results in a more fragile curd and thus can result in increased fines. A certain level of gas production, however, is required to produce a porous curd that can be cooked and washed easily. The dried casein obtained from gassy curd is structurally weaker and thus can also be ground more easily. From a processing control perspective, it is desirable for the starter strains to have consistent acid production.

After an incubation period of 14–18 h at 22–30 °C (depending on the rate of activity of the starter used) a pH of about 4.6 should be achieved and the lactic casein gel or coagulum is then pumped to the cooker.

(b) Rennet Casein

Destabilisation in rennet casein is achieved through the addition to milk of a preparation containing one or more proteolytic enzymes ("rennet") which causes cleavage of a peptide bond (Phe_{105}–Met_{106}) in κ-casein releasing a peptide commonly referred to as glycomacropeptide (GMP), which due to a terminal sugar moiety possesses a high negative charge. The removal of the negative GMP portion from the surface of the casein micelle results in a decrease in the zeta potential. At pH 6.7 a decrease in zeta potential from about −25 to −11 mV is observed (Dalgleish, 1984). The residual zeta potential on the renneted micelle, however, still has sufficient charge repulsion to stabilise the micelle against aggregation. The residual charge is progressively reduced with increasing calcium ion concentration (Fig. 2.2) until a critical zeta potential is reached when attractive forces dominate and aggregation occurs. The point at which the critical zeta potential occurs varies according to relative ion concentrations, temperature and pH. In unmodified milk there is sufficient calcium present that aggregation will proceed at an industrially useful rate when approximately 80 % of the κ-casein has been hydrolysed, provided that the temperature is high enough (>18 °C).

The renneting of milk, and the production of a clot of aggregated casein micelles (the first stage in making rennet casein), is thus a 2-stage process.

$$\kappa \rightarrow \text{casein} \xrightarrow{\text{chymosin}} \text{para} - \text{k} - \text{casein} + \textit{glycomacropeptide}$$

$$\text{para - k - casein} \xrightarrow{Ca^{2+}} \textit{Dicalcium}\ \text{para - k - casein}$$

Unlike the acid caseins, the pH of the milk does not change significantly during the process and, as no acid is produced or added, the solubility of calcium phosphate does not increase and therefore the calcium phosphate is not lost from the

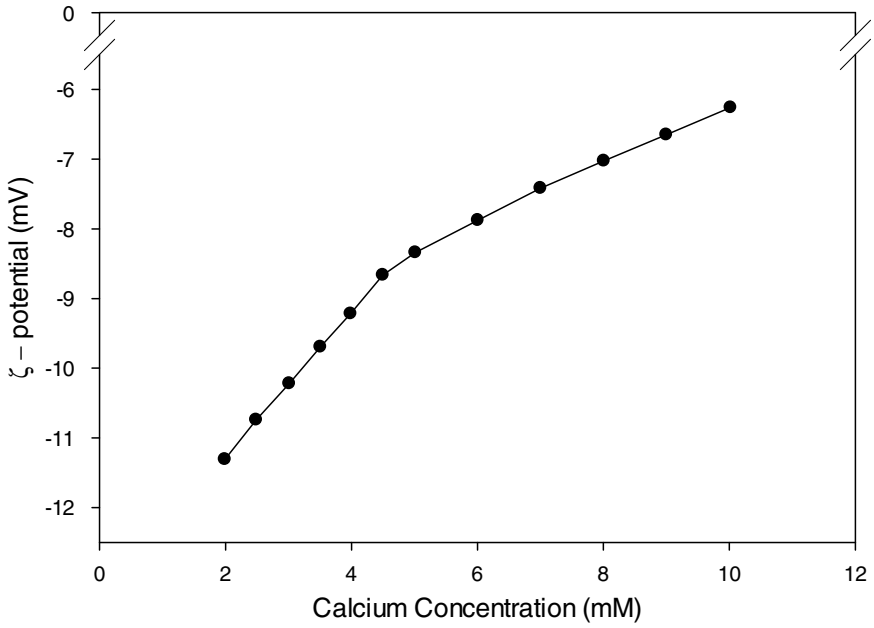

Fig. 2.2 The zeta potential of fully rennetted casein micelles at 25 °C as a function of calcium concentration (graph redrawn from Dalgleish, 1984)

system during dewheying and washing, consequently the ash content of rennet casein is high (Nabenhauer, 1930).

There are two main ways of manufacturing rennet casein: (1) the traditional batch process and (2) the "newer" continuous process.

The traditional batch process, now largely obsolete, is carried out in cheese vats at 30–35 °C. Rennet is mixed in typical proportions of 1:4000–5000 of skim milk. The rennet is typically diluted (approximately 1:20 with water) prior to addition to the vat to ensure that minimal aggregation occurs during mixing. Excessive mixing during aggregation results in small curd granules and thus high fines losses. Increasing the proportion of rennet gives a firm, stringy curd, and increasing the temperature has a similar effect (Bohlin *et al*., 1984). The curd is cut with agitators after 15–20 min. Cutting too early gives a soft curd with high fines losses; cutting too late results in a tough, rubbery curd which is difficult to wash.

The "newer" 'continuous' cook process is described in detail by Weal and Southward (1974). In this process rennet in the ratio 1:7500 or even less is added to skim milk in cylindrical vats at 31 °C. Alternatively cold renneting of the milk at 10–18 °C can also be used but the incubation times required are then much longer. In the cold renneted system, because the strength of hydrophobic attractive forces is reduced, the milk does not clot until the temperature is raised to about 26 °C.

The main variables used for controlling rennet casein precipitation are amount of rennet, incubation temperature and incubation time. 'Warm set' renneting is conceptually similar to lactic casein precipitation in that a casein gel forms in stationary skim milk in a silo, and this gel is then broken up later during cooking to form casein curd particles. 'Cold set' renneting is conceptually similar to mineral acid casein precipitation since no casein gel is formed. Rennet casein curd particles form directly from the cold renneted skim milk as the temperature increases during cooking.

2.2.1 Cooking

To produce an acid curd with uniform mechanical strength, the precipitation is carried out at temperatures wherein aggregation occurs but at a

rate which is slow enough to ensure that a uniform pH throughout the milk is achieved before the point at which curd particle formation begins. The precipitation temperature is therefore necessarily low and therefore the mechanical strength of the curd is also low. Due to the temperature limitations imposed by enzyme activity, rennet casein curd is also formed at temperatures that result in curd with low mechanical strength.

To minimise fines production through the remainder of the process, curd strength is increased through cooking. The cooking process increases the relative strength of hydrophobic bonding and therefore results in aggregation of incipient particles into larger, more readily processed curd with higher density and strength. Concomitantly there is an increase in the degree of whey separation.

During cooking the process stream is typically heated using a combination of indirect heating and multi-nozzle direct steam injection. The direct steam injection serves two purposes: to shatter the curd and effect a rapid increase in temperature. The result of these two processes is that curd entrapped fat is released and that the shattered curd will contract assisting with whey and mineral loss. Following heating the shattered curd enters a low velocity cooker and large diameter cooking pipe where the shear conditions are low enough to allow the curd particles to re-agglomerate. Residence times of 40–60 s are typically used.

The cook temperature is chosen as a balance between being high enough to provide a robust curd that will enable good curd-whey separation while at the same time being low enough to give a friable curd that can be efficiently washed. The maximum cook temperature is also limited by the type of curd and the shear profile within the mechanical dewheying equipment.

(a) Acid casein

When cooking acid curd, it is essential that the correct pH (between 4.6 and 4.7) has been obtained. High pH tends to produce large, rubbery, sticky curd after cooking, partly due to retention of calcium in the curd. In contrast, low pH tends to produce a fine, soft curd, resulting in high losses of casein fines during dewheying and washing (Jablonka and Munro, 1985; Jablonka and Munro, 1986a; Jablonka and Munro, 1986b). This is of particular importance in lactic casein manufacture as the pH of the coagulum varies during cookout of the silo—a 20 m^3 coagulation silo, depending on the through-put capability of downstream equipment, may take more than an hour to empty. pH variation, prior to cooking, may be mitigated by the injection of dilute acid or alkali or through injection of untreated milk (termed pH "trimming"). Adjusting the pH with skim milk has the advantage that protein concentration is not diluted and thus maximum throughput is maintained as downstream processes such as decanter centrifuges are volume constrained. If it is not possible to adjust pH then the impact of pH variations can be minimised by an appropriate cook temperature. As high pH casein is rubbery and more mechanically robust, the cook temperature should be relatively low (about 50 °C) so that the porosity of the curd is maintained to facilitate efficient washing (Jablonka and Munro, 1986a; Fichtali *et al.*, 1990). Low pH curd is not as mechanically robust and therefore is cooked at a higher temperature to increase hydrophobic bond strength and thus increase curd strength.

In addition to the low velocity cooker and large diameter cook pipe post-heating, acid caseins are also passed through an acidulation vat wherein the curd is slowly agitated. Over a residence time of about 10–15 min the casein particles continue to shrink, becoming firmer and tougher, and expelling whey. Due to the low acid conditions the calcium phosphate within the casein micelles dissolves and the acidulation process maximises the amount of calcium which diffuses into the whey (O'Regan and Mulvihill, 2011).

(b) Rennet Casein

The key difference between rennet and acid casein is that rennet casein is not acidified and therefore there is no solubilisation of

calcium phosphate and thus there is no need for an acidulation process as there is for acid casein. Cooked rennet casein is naturally very strong, and sticky tending to form large agglomerates after it is discharged from the cooking pipe. Excessive holding time, prior to dewheying would result in large lumps that would hinder further processing. An acidulation vat is therefore omitted in rennet casein processing and it is very important to separate the cooked rennet curd from the whey as rapidly as possible, and then to cool the curd to soften it and allow the large agglomerates to be broken down so that the curd can be thoroughly washed.

2.2.2 Dewheying

The efficiency of the dewheying step is critical as it determines the volume and purity of the whey that can be processed into valuable co-products. Efficient dewheying also greatly reduces the wash water demand—for example Hobman and Elston (1976) reported a 25 % reduction in wash water requirement when press dewheying is compared to screen dewheying.

The curd/whey slurry ex-cooking consists of approximately 80 % free whey and 20 % curd. Dewheying is typically achieved through a combination of inclined screen and mechanical separation *via* decanter centrifuge or roller press. Screen dewheying produces curd particles with 80–85 % whey retained in the particle pores which is further reduced to 60–65 % by mechanical dewheying. Both devices are important and are typically arranged in series with inclined screen dewheying followed by mechanical dewheying . Mechanical separators are volume-limited and throughput can be increased three-fold by pre-screening and feeding only screened curd to the separator.

The temperature of feed to mechanical separators is an important variable. If the temperature is too high then matting (in the case of roller presses) and plasticization can occur. These phenomena hinder subsequent washing and drying operations. The maximum feed temperature is casein type and equipment-dependent (Munro *et al.*, 1983). Rennet casein is particularly sensitive to plasticization and maximum temperatures range from 40 to 55 °C. If the feed curd is at a less than optimum temperature then a soft and sloppy curd may be produced which, while it will wash well, will result in excessive fines loss during washing.

2.2.3 Washing

To achieve specification limits the dewheyed curd is washed to remove lactose, minerals, whey proteins and residual acid. During washing the soluble constituents in the interstitial spaces between curd particles are readily removed. The removal of soluble constituents within the curd itself, however, is dependent on diffusion. The rate of diffusion depends on the properties of both the liquid phases (wash water and whey) and the curd. The key properties of the liquid phase are the degree of turbulence, viscosity and the relative soluble solids concentration of the wash water to the curd-entrapped whey. The physical features of the curd that impact the rate of diffusion include not only pore size, tortuosity, interconnectivity of channels, and actual curd particle size which dictate the distance that an impurity has to travel to leave a curd particle but also, due to the design of modern wash systems, the texture and compressibility of the curd. Rennet casein curd is more dense than lactic casein curd and, as a result, it is harder to wash. Lactic casein curd is more open and porous or 'spongy', due to the gas-producing bacteria of the starter culture. Mineral acid curd has an intermediate density (Espie *et al.*, 1984; Diamante *et al.*, 1993).

There are two main types of washing systems in use: multi-stage tubular washing and multi-stage wash towers. In the tubular wash system the overall wash water flow is counter current to the curd but within each wash tube the curd and wash water are pumped co-current to each other. Upon exiting each tube the curd and wash water are separated and the exiting wash water becomes the feed wash water to the tube that is upstream, relative to the curd flow. Wash towers, in contrast,

have a true counter-current system where the wash water flow is counter-current between each tower stage and within each tower as the curd falls through an ascending column of water.

The design of wash tubes includes a series of orifice plates that constrict flow with the result that curd is physically squeezed by the increased pressure. The curd compression assists with the removal of impurities from within the curd and as the pressure is relaxed, on exiting the orifice plate, cleaner wash water is absorbed. This process does not occur in wash towers, which are much gentler on the curd and therefore have less tendency to create fines although this is at the expense of wash efficiency. The replacement of two 70 m^3 wash towers, at a New Zealand Dairy factory, with a 5-stage counter-current tubular washing system plant has been reported to result in a 2 h reduction in Cleaning In Place (CIP) turnaround times and a reduction in wash water usage of 50 % Anon (2014).

In a four wash tubular system the washes are all at different temperatures, typical values being 55, 65, 75 and 35 °C. In the first three washes, wash water temperature is progressively increased. Increasing wash water temperature causes the curd to shrink (Teo *et al.*, 1996) so making screen dewatering, between tube washing stages, more efficient, makes the curd particles firmer, and leads to better mechanical dewatering and easier grinding of the dry casein. The increase in temperature is gradual as initial washing is more efficient with a porous, low density curd but as washing progresses it is more efficient to squeeze out residual whey through thermally-induced curd shrinkage. High initial temperatures would decrease pore diameters and thus diffusion rates of whey from the curd. The highest wash tube temperature can also provide a pasteurisation step. The temperature of the final wash, as with dewheying operations, is dictated by the specific equipment utilised for the subsequent dewatering operation. If curd exit temperature is too high the curd becomes sticky and results in matting in a roller press or plasticization in a decanter. Plasticized curd has an impervious surface making both drying and milling operations difficult. A temperature of about 35 °C is normally used, and this is achieved by mixing cold water with the casein curd coming from the hot wash. The composition of the casein wash water itself plays an important role in curd quality: alkaline wash waters in acid casein manufacture, for example, tend to dissolve a portion of the casein surface and make the curd sticky and difficult to dry.

2.2.4 Dewatering and Mincing

The main objective of dewatering is to reduce the thermal load on the drying operation. Removing water by mechanical expression requires only 2–3 % of the energy needed to remove the same water by thermal evaporation. The dewatering step is also the last opportunity to remove residual impurities. Washed casein is initially passed over an inclined screen which reduces the curd moisture in the first screen to about 80–85 %. This is followed by mechanical dewatering, typically using a roller press, decanter centrifuge or screen bowl centrifuge, which further reduces the moisture content to 50–60 %. A secondary reason for mechanical dewatering is to improve the curd firmness for optimum handling in the dryer. On exiting the mechanical dewatering operation, the curd is minced to provide a curd with optimised uniform particle size for drying. In general curd is minced to achieve a particle size that is a trade-off between increasing the surface area for drying and excessive mincing which can result in plasticization and thus decreased drying rates despite an increased surface area.

2.2.5 Drying, Tempering and Grinding

The main aim of drying casein is to remove sufficient moisture from the curd to produce a shelf-stable casein that meets in-pack specification limits (< 12 %) while at the same time minimising the cost associated with unnecessary drying. Due to curd moisture losses of 1.0 % during cooling and tempering, and < 1 % during grinding dry curd processing, curd moisture targets ex-drying

are typically set at 13–14 %. Moisture losses from casein after drying occur during air conveying processes and during grinding operations which are highly energy intensive. Generally dry casein curd is pneumatically conveyed between post drying operations and associated moisture losses are related to the ratio of conveying air to dry curd, air temperature, humidity, temperature of the curd coming off the dryer, and particle size.

Drying may be carried out on vibrating bed, fluidised bed, attrition or pneumatic conveying ring dryers. All of these dryers pass hot air over the curd but vibrating and fluidised bed dryers allow the matching of drying rates to curd moisture contents through the use of multiple beds each with an optimised air temperature. Although caseins *per se* are not prone to denaturation the air temperature during the constant rate drying period should not be too high as the casein surface can dry out, a phenomenon known as "case-hardening", and seal in remaining moisture. Case-hardening can also retard solubilisation processes that may be utilised during end-application use. Attrition and pneumatic conveying ring dryers both incorporate a simultaneous milling operation into the drying process. The milling operation eliminates the problem of case-hardening, and thus the importance of separate temperature control through the constant rate drying period is reduced such that a single temperature air stream may be used throughout the entire drying process.

Following drying, the hot curd particles must be cooled prior to entering tempering bins. Failure to cool the curd may result in browning reactions and in condensation, due to post dryer moisture loss, on bin walls causing patches of high moisture casein which can promote mould growth. Warm curd also has a soft texture that can cause casein particles, particularly those under pressures at the bottom of deep casein bins, to stick together creating handling problems.

Tempering is a holding period of 8–24 h which allows moisture content to equilibrate both within and between curd particles. Tempering is more important following dryer operations that do not incorporate a milling operation and thus produce curd particles with a polydisperse particle size. As the drying time, in vibrating and fluidised bed dryers, is independent of particle size, large casein particles exit with a higher moisture content than small ones. The holding period allows equilibration of moisture contents between curd particles. Casein particles that are not properly tempered (especially the wetter particles) may stick and wrap on to the fluted rolls of a roller mill during grinding (milling) of the casein. When that happens, the mills must be stopped, and the "wrap-on" dug out.

For almost every final use, casein is first dissolved, mostly in alkali for acid caseins, and in the case of processed cheese applications, for rennet casein, through the use of so-called emulsifying salts. As the particle size is reduced (by milling of unground casein), the casein dissolves more quickly and more evenly. This is important to the end-user, as a faster, more uniform dissolving time means lower manufacturing costs and less quality defects.

Roller mills, developed for the flour industry, are most commonly used for grinding, although if a very fine particle size is desired, pin or hammer mills may be used. As with earlier high shear operations, such as mechanical dewatering and dewheying, casein is prone to plasticisation during milling and for this reason should be at a temperature around 20 °C. The milling operation takes the longest time of all the casein manufacturing operations and it is therefore desirable to optimise the processing to maximise milling throughput. The milling time is a function of curd hardness which in part is related to the method of precipitation, with rennet casein being the most difficult to mill followed by mineral acid casein. Lactic acid casein is the easiest to mill and the ease of milling lactic casein improves with gas production by the starter organisms: gas inclusions create discontinuities in the curd structure and thus form areas of weakness. The main opportunity to improve the ease of grinding during processing is in the washing process: in general the higher the casein hot wash temperature the easier the casein is to grind. The milling operation is conducted in conjunction with sifting to ensure that casein particles of the desired particle

size are separated from oversize particles that are recycled through the mill thereby avoiding not only production of below specification fine particles but also reducing the load on the mill. After sifting the casein is generally blended to ensure batch uniformity. The blending operation is necessary as in-process control of casein is difficult due to inherent intra-batch variations resulting from general casein manufacturing variations such as non-uniformity of curd size or casein-type specific variation such as the continuing decrease in pH during the emptying of coagulation silos for lactic casein manufacture.

The dry casein is finally packaged according to particle size. Historically a range of casein specifications was common: 30, 60, 80, and 90 mesh. However due to the high cost associated with milling many manufacturers who use fluid bed dryers, notably those in New Zealand, produce '30 mesh all in', a size fraction that is close to the particle size distribution coming off dryer. Finer casein (90 mesh) is more common in Europe and this is due in part to the more common use of attrition dryers.

2.2.6 Special Case Insoluble Casein Based Ingredients: Co-precipitates

In addition to the manufacture of casein only ingredients, for nutritional, economic and functional reasons, there has been a need to produce high protein ingredients that also incorporate whey proteins. Again, from a historical perspective the inclusion of whey proteins into a high protein ingredient required rendering the whey proteins insoluble so that macro-scale screens could be used to separate them together with the caseins, hence the term co-precipitate, from the remaining undesirable soluble fraction, i.e., lactose and soluble minerals.

Whey proteins are destabilised through thermal denaturation and subsequent interaction with casein proteins. The whey-casein complex is then precipitated by isoelectric precipitation. The pH and calcium concentration during thermal processing can be used to manipulate the properties of the whey-casein co-precipitate (Southward and Aird, 1978; Southward Connolloy, 1983; Connolloy, 1983. In all cases an insoluble curd is formed. However the pH required to precipitate the whey-casein complex increases with increased concentration of calcium: low-calcium (0.5–0.8 % w/w) heat treated milk precipitates at pH 4.6–4.8; medium-calcium (1.0–2.0 % w/w) heat treated milk precipitates at pH 5.3–5.6; high-calcium (2.5–3.0 % w/w) heat treated milk precipitates at pH 5.9. By applying a lower heat treatment to the skim milk under alkali conditions, a whey-casein complex can be formed that will precipitate at pH 4.6 but on neutralisation will become 100 % soluble whereas similarly-treated complexes formed at neutral or acidic pHs are not completely soluble (Connolloy, 1983; Ottenhof and Arnoldus, 1985). More recently, processes for manufacturing co-precipitates and casein-only ingredients have expanded to make use of membrane pre-processing to concentrate either the caseins themselves or total protein prior to precipitation, and thermal denaturation of whey in combination with tranglutaminase treatment (Cuksey *et al.*, 2005).

Once a curd is formed the processing steps follow those for manufacturing acid casein, although co-precipitates are more fragile than casein precipitates and careful handling is required to minimise fines losses.

2.3 Soluble Casein-Based Ingredients: Conversion of Insoluble Casein Ingredients into Soluble Products

The process of casein and co-precipitate manufacturing results in an insoluble product, which limits the functional properties of the ingredient when used as-is or requires extra processing steps by the end-use manufacturer to render the ingredient soluble. It is possible to convert rennet casein into a soluble product (Davis, 1980), however, the most common substrate for conversion is acid casein. The basic process for making a soluble casein through neutralising acid casein in

$$^{+}H_3N\text{-}R\text{-}COO^{-} + OH^{-} \rightarrow H_2N\text{-}R\text{-}COO^{-} + H_2O$$

Acid casein (pH = 4.6)	Caseinate (pH = 6.6)
Exists as insoluble particles	Exists as a solution (monovalent caseinates i.e., Na, K or NH_4); or as a colloidal dispersion (divalent caseinates i.e., Ca, Mg,)

Fig. 2.3 Simple equation showing the neutralisation of acid casein with alkali (where R represents casein protein)

alkali and drying to form a powder (Fig. 2.3), is largely unchanged since it was developed around 1900 (Hall, 1900). Although it is possible to make caseinate from dry casein, in practice this is not desirable as dry casein produces a poorer flavour caseinate and is inherently more expensive to produce due to two drying steps.

During the neutralisation process the manufacturer has the opportunity to manipulate the final intermolecular caseinate structure and hence functional properties through the selection of cations that may be added either as a salt or as a co-ion of the alkali. The balance of monovalent to divalent cations dictates the balance of inter- and intra-molecular interactions between proteins in the caseinate ingredient and allows the manufacturer to customise ingredients for nutritional (i.e., low sodium) or functional reasons (Carr *et al.*, 2002).

At high divalent cation concentrations, for example calcium caseinate, nearly all the divalent cation is tightly bound to the strong anionic sites of the protein, thus allowing inter- and intra-casein molecule hydrophobic bonding to dominate causing rearrangement of the caseins to minimise intermolecular repulsion and so form micelle-like particles with a predominance of charged κ-casein on the surface. Consequently, divalent caseinate particles are poorly hydrated, compact, and have a strong inter-particle repulsion. The relative strength of intermolecular forces seems to be dependent on the species of divalent cation. Carr *et al.* (2002) showed that while the viscosity profiles for sodium caseinate solutions as a function of zinc or calcium concentration were similar, the concentration at which a more compact structure is formed was clearly defined with calcium addition and accompanied with a sharp transition from a translucent solution to an opaque solution. In contrast, the divalent cation concentration at which a rearrangement of casein molecules into a more compact structure occurred was less defined in solutions with added zinc and these solutions did not exhibit a visible change in colour.

In caseinate solutions with low divalent cation concentrations (i.e., monovalent caseinates such as sodium caseinate), the casein–casein interactions are dominated by electrostatic repulsions between the strands of the casein molecules, because the cation–polyanion interactions for the monovalent cations are much weaker (Ho and Waugh, 1965b) than the cation–polyanion interactions for the divalent cations (Ho and Waugh, 1965a). This repulsion overcomes the hydrophobic association energy resulting in loose translucent aggregates that are highly hydrated and thus exhibit high apparent viscosities in solution. As with high divalent cation caseinates, the behaviour of monovalent caseinates is also dependent on the cation species (Carr *et al.*, 2002).

2.3.1 Manufacture of Monovalent Caseinates

One of the main functional properties of monovalent caseinates is the ability of caseinates to impart a high viscosity relative to protein mass. This feature presents several difficulties to the

manufacturer and necessitates a process that is both complex and energy intensive. Due to the particulate and dense nature of casein curd, neutralisation and thus conversion of casein to a soluble form occurs on the surface of curd particles. The highly viscous caseinate surface layer acts as a barrier to alkali diffusion into the unreacted casein interior of the curd particle and additionally acts as a glue and leads to lump formation thus further hindering access of alkali. The caseinate manufacturing process is designed to minimise the negative impact of viscosity on process throughput. The factors that are utilised to minimise the impact of viscosity are:

(a) Particle Size
Particle size reduction of the feed curd so that the surface area available for conversion is maximised and the diffusion distance from unreacted curd in the centre to alkali is minimised;

(b) Temperature
Maximise temperatures throughout processing to reduce viscosity. The correlation between viscosity and the temperature of casein solutions has been well established and decreases logarithmically with increases in temperature (Sergeeva and D'Yachenko, 1973; Towler, 1974; Korolczuk, 1982; Roeper and Winter, 1982; Fichtali and van de Voort, 1993). At the beginning of processing alkali addition occurs at moderate temperatures (approximately 40 °C) to achieve a balance between reducing apparent viscosity and minimising plasticisation of unreacted curd through the mincing processes. After initial mixing, the slurry of reacted and unreacted curd, is heated to 60–75 °C and pumped into a reaction vessel. Higher temperatures coupled with high pH (due to unreacted alkali) can lead to loss of lysine and serine and the production of degradation products such as lysinoalanine. Once the casein has been fully reacted the pH should be around 6.7 and thus there is no longer a temperature constraint due to lysine and serine loss. At this point the temperature is increased to 90 °C prior to the balance tank for the high pressure pump that feeds the atomiser of the spray dryer. During transit, under high pressure, the viscosity can be further reduced by heating to around 130 °C.

(c) pH
To minimise viscosity effects it is desirable to maintain the pH of the aqueous phase at pH 6.7 as caseinate solutions have a broad minimum in apparent viscosity around pH 6.7 with sharp increases in apparent viscosity at lower and higher pHs (Salzberg and Georgevits, 1956; Hayes and Muller, 1961; Puri *et al.*, 1972; Hermansson, 1975). In practice, due to the slow reaction kinetics of caseinating there is initially a higher pH on alkali addition due to unreacted alkali. The pH may be tweaked between reaction vessels *via* additional alkali to ensure that there is sufficient alkali to react all the casein and to ensure the viscosity is at a minimum.

(d) Total solids concentration
The viscosity of caseinate solutions increases logarithmically with protein concentration. The dewatered curd has a moisture content of about 45 % w/w and is diluted with water to 25 % solids to enable the minced curd to be readily handled by the colloid mill and also to minimise viscosity on alkali addition so that adequate mixing can be achieved in reaction vessels. Once the curd has been completely reacted to form caseinate the only viscosity concern is that the particle size of the droplets formed in the atomiser of the spray dryer are small enough to be completely dry before they come in contact with the walls of the dryer and large enough to be readily separated in the main chamber and cyclone from the exiting air phase. Final viscosity adjustments are typically made *via* an inline viscometer that regulates hot water addition. The total solids for optimum drying is dependent on casein manufacturing and is affected by seasonality but is typically in the range of 20–24 % (Westergaard, 2010). Spray drying caseinate from such low total solids is expensive in terms of energy, and also produces a powder with a bulk density as low as 0.40–0.55 g/ml, making it expen-

sive to package and transport. The moisture content of spray dried caseinate must be less than 8 % (Codex Standard 290–1995).

(e) Air incorporation
During the dissolving process, the incorporation of air should be kept to a minimum since air bubbles result in large increases in the viscosity of sodium caseinate solutions. A further problem associated with air inclusion is the formation of stable foams that create handling problems.

A number of approaches have been used in an effort to reduce the main negative factors associated with traditional caseinate manufacture, i.e., the high drying cost, low bulk density, and the poor reconstitution properties due to wettability and dispersibility.

Since its first use in 1900 (Hall, 1900), roller-drying was the main method of drying milk-based products for the first half of the twentieth century and although its use resulted in a powder with good wetting and dispersibility, the product possessed an undesirable flavour profile. Consequently the use of roller drying declined with the advent of spray drying which, despite poorer powder properties, had a blander flavour. Improvements in roller dryer technology, however, have reduced the flavour issue and roller dried caseinate products are commercially available and marketed on the basis of their improved powder handling.

Granular caseinates have been produced by reducing the moisture content of casein curd to such a level (below 40 %) that a free-flowing mass can be maintained by agitation after alkali (carbonate or bicarbonate powder) has been added (Towler, 1978). After allowing sufficient time for conversion, the mixture could be dried and ground in conventional casein equipment. Ammonium caseinate in a granular form has been made simply by passing ammonia gas though low-moisture casein and removing excess ammonia with a stream of air (Girdhar and Hansen, 1974).

A number of processes (Millauer and Wiedmann, 1986; Oelsner, 1990; Asensio *et al.*, 2006) utilising extrusion have been published which have the main advantage of circumventing the high costs of spray drying caseinate by enabling the processing of casein at high total solids (up to 90 % w/w). The processing temperature may be mild (30 °C) to quite high (160 °C). A further advantage is that the conversion of casein to caseinate *via* extrusion is very short (typically < 1 min).

2.3.2 Manufacture of Divalent Caseinates

As with monovalent caseinates, the process for divalent cation caseinates is designed to maximise throughput by minimising viscosity. The general process is the same as for monovalent caseinates, however, processing is complicated by the phenomenon of reversible heat induced gelation and the formation of sedimentable material under certain processing conditions. Roeper (1977) described the manufacturing conditions most suited for the production of high quality spray dried calcium caseinate and recommends:

- The use of a 'soft' moist (65 % moisture) curd rather than 'harsh' dry curd (approx. 50 % moisture) which is more prone to the formation of sedimentable material.
- Relatively low conversion temperatures (< 40 °C) as higher temperatures result in significant levels of sediment.
- Following complete conversion, upon addition of $Ca(OH)_2$, the calcium caseinate dispersion may be heated to reduce the viscosity. The temperature maximum is limited by the reversible gelation temperature which increases with pH from 70 °C at pH 6.65 to 85 °C at pH 7.55. In addition to increasing the reversible gelation temperature, increasing pH also results in less sedimentable material in the final product. The use of ammonia to affect the desired pH increase during processing has the advantage that it flashes off during drying, and thus does not result in an increase in pH of the reconstituted calcium caseinate. As with monovalent caseinates however, increasing the pH above pH 7 has the disadvantage of resulting in an increase in viscosity and thus will negatively impact drying efficiency.

2.4 Soluble Casein-Based Ingredients: Membrane-Concentrated Casein Ingredients

The development of industrial membranes has enabled the physical separation of casein micelles in their native form obviating the need for complex and expensive protein destabilisation operations that result in an insoluble product that then requires further processing to yield a soluble ingredient. Although membrane technology had been applied to milk systems since the late 1960s to concentrate casein proteins (Peri and Pompei, 1973; Maubois *et al*., 1975) for cheese applications, commercial application of membranes to manufacture powdered casein-based ingredients did not begin until the mid to late 1980s (Novak, 1991).

Aside from retaining the casein micelle in its native and thus colloidally stable state, membrane technology gives the manufacturer a greater opportunity to customise the composition of the resulting product with respect to the ratio of relatively large components to those components that are smaller than the molecular weight cut-off of the membrane used in processing. In all uses of membrane technology, for casein ingredient manufacture, soluble salts and lactose are removed. However by choosing a membrane with a larger pore size whey proteins may also be removed. Thus casein based ingredients manufactured *via* membrane technology may be classified as either those which contain both casein and whey proteins in their native ratio (approximately 80:20)—*milk protein concentrates*; or those which contain predominantly casein proteins—*micellar casein products*. There are no regulations defining membrane manufactured powders and consequently there is a great diversity of terms used in the literature and trade dealing with these products. Terms for milk protein concentrates include: "retentate powders", "native milk protein concentrate", "ultrafiltered milk protein concentrate", "milk powder from ultrafiltered skim milk", "skim milk retentate powder", and "high-protein lactose-free milk powder". Micellar casein products may be referred to as "native phosphocaseinate", "micellar casein isolate" or "micellar casein concentrate".

A generalised schematic for manufacturing membrane processed casein ingredients in comparison to traditional isoelectric precipitated casein is shown in Fig. 2.4.

The reader is referred Cheryan (1998) for details on membrane processing in general and to Peinemann *et al*. (2009) and Tamine (2013) for specific information related to membrane processing in the dairy industry. In its simplest form membrane processing consists merely of pumping a feed solution under pressure over the surface of a suitably chosen selectively permeable membrane. Membrane selection depends primarily on the relative difference in size of the species that are to be separated. For micellar casein manufacture a microfiltration membrane with a relatively large pore size of approximately 0.1 μm is chosen to allow the separation of whey proteins from micellar casein whereas for milk protein concentrates an ultrafiltration membrane with a pore size of approximately 10,000 Da is chosen so that only soluble salts and lactose are separated.

Through appropriate selection of membranes and processing conditions the operator is able to produce a range of products with customisable protein compositions. For example a skim milk may be microfiltered at 50 °C to separate micellar casein from whey proteins. This whey depleted retentate could subsequently be cooled to 4 °C, to allow diffusion of β-casein from the casein micelle, and then passed through a second microfiltration membrane to produce a β-casein depleted micellar casein retentate and a β-casein permeate stream. Alternatively a preferential removal of whey proteins can be achieved by appropriate process design: Yukio *et al*. (1994) described the application of heat treatment to the feed skim milk followed by microfiltration to produce a casein- β-lactoglobulin complexed retentate and an α-lactalbumin rich permeate.

The pressure gradient across the membrane forces the solvent and sufficiently small components through the pores of the membrane, while the larger molecules are retained. The retained phase, referred to as the "retentate" or "concentrate"

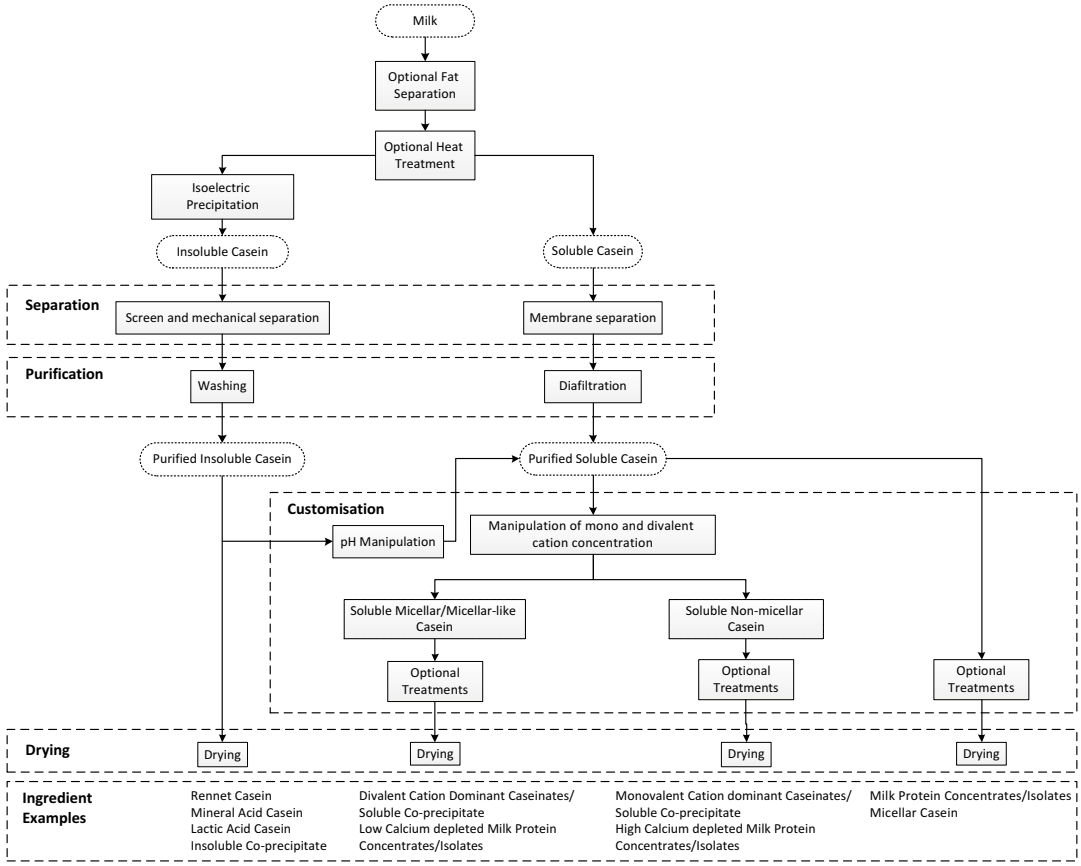

Fig. 2.4 Schematic diagram for the manufacture of casein ingredients

stream, is enriched in the retained macromolecules, while the permeate stream is depleted of the macromolecules. The retentate also contains some of the permeable solutes. The relative concentration of permeable solute to solvent in the retentate compared to the permeate stream may differ, depending on that species' rejection by the membrane. Overall however, since the retentate forms a much smaller volume than the feed, there is a net separation/enrichment of the retained macromolecules. The degree of separation that is possible by direct filtration is limited due to the concomitant increase in viscosity of the retentate as total solids increases. The increase in viscosity results in a decrease in flux to uneconomically low values if the trans-membrane pressure is kept constant. Flux may be improved by increasing trans-membrane pressures, however, there is an upper limit, which is imposed by the strength of the membrane itself. Thus, in order to effect a further purification, it is necessary to resort to "diafiltration". Diafiltration is equivalent to "washing", in casein manufacture, and refers to the process of adding water to the retentate and continuing the elimination of membrane permeating species along with the added water during re-filtering.

The degree of protein purification forms the basis of product nomenclature: for example an MPC56 is milk protein concentrate with a protein content of 56 % (dry basis) while an MPC85 is a milk protein concentrate containing 85 % protein. Milk protein concentrates with higher total protein concentrations (typically >90 %) are referred to as milk protein isolates (Kelly, 2011). In contrast to acid caseins these products have

relatively high ash and calcium contents, since protein bound minerals, such as micellar calcium phosphate, are retained.

2.4.1 Customisation of Membrane Concentrated Casein Ingredients

The primary advantage of manufacturing an ingredient that exists as a fluid is that it offers the opportunity to manipulate the product easily. While the use of membranes *per se* can be used to modify the gross protein composition of the final product, the functional properties of the ingredient are dictated by the state of those proteins. There are many ways to modify the functional properties of protein-based ingredients; however, as with caseinates a key tool for customisation is adjusting the mineral balance and this has formed the basis of much of the early MPC customisation work.

The first membrane produced casein-based products on the market were MPCs, with cheese milk extension as the target market. MPCs remain the primary product. A key hurdle to the uptake of MPC products has been the decrease in solubility that occurs post manufacture, particularly in products with greater than 70 % protein and those that have been subjected to high (>20 °C) storage temperatures (Anema *et al*., 2006). The decrease in solubility seems to be associated with the presence in cheese of small fat-free translucent protein lumps referred to as "nuggets" when MPC was used as a cheese milk extender (Bhaskar *et al*., 2001; Carr *et al*., 2005). The solubility issue has received much attention and is discussed in detail in Chap. 4. Initial solutions to the solubility issue necessitated customer implementation: the powders were recommended to be reconstituted at>50 °C or combined with homogenisation (McKeena, 2000; Mistry, 2002; Gaiani *et al*., 2006, 2007; Mimouni, *et al*., 2009, 2010a; Jeantet *et al*., 2010). The work of Hallström and Dejmek (1988) demonstrated that solubility issues exist prior to actual drying in the case of heat treated ultrafiltered milks and that homogenisation is effective in reducing aggregate size.

The key to market success, however, requires removing barriers to customer uptake and thus an ingredient solution was sought. The approaches to providing an ingredient solution have focussed on manipulating the mineral balance prior to drying. Carr *et al*. (2005) found that after diafiltration, restoration of the monovalent cation to protein ratio for high protein MPCs (>70 % Protein) significantly decreased the subsequent rate of deterioration in solubility on storage. The restoration of the monovalent cation concentration did not, however, alter the solubility/reconstitution profile on the day of manufacture.

Bhaskar *et al*. (2001) developed the first cold-soluble high protein MPC by reducing the calcium concentration of the final product, *via* ion exchange or addition of a chelating agent. This process produced a powder with an altered micellar structure, dependent on the degree of calcium depletion, and a product that possessed both an improved solubility profile and had improved shelf stability . Depending on the level of calcium depletion, an MPC with a continuum of micellar structures can be produced from a native micelle structure to a structure more similar to sodium caseinates. A significant difference, particularly when used in acidified applications, between caseinates and MPCs is that MPCs contain phosphate whereas caseinates possess reduced phosphate levels due to the acidic precipitation and subsequent washing. Micellar phosphate levels increase buffering due to dihydrogenphosphate formation (Ferreira *et al*., 2003) on acid mediated solubilisation of colloidal calcium phosphate.

A recent paper (Amelia and Barbano, 2013) has approached the solubility and ease of use issues of membrane-produced casein ingredients by investigating the possibility of manufacturing a liquid concentrate thus obviating the need for specialist reconstitution operations. They have successfully made an 18 % total solids MCC with a shelf life of 16 weeks when stored at 4 °C. For commercial success it would be desirable to have a product with a higher solids concentration and a longer ambient shelf life. It seems likely that

long life liquid concentrate ingredients will be a fruitful area for ingredient development.

Subsequent to the development of calcium reduction as a tool for manipulating MPC solubility, Bhaskar and co-workers developed a range of MPCs with customised levels of calcium depletion with the means of calcium depletion-focussed ion exchange. The preferred procedure, by this group, for achieving target calcium depletion levels is described by Dybing *et al.* (2002) as applying ion exchange to part of the MPC solution and then blending this with an MPC solution that was not treated by ion exchange. By increasing the level of calcium depletion to greater than 50 %, Kells and Bhaskar (2001) were able to reduce the opacity of a reconstituted MPC sufficiently to enable the utilisation of MPCs not only in milky beverages but also in clear partially acidic beverages (pH > 5.6) such as carbonated soft drinks.

One disadvantage of standard MPC ingredients when used for cheese milk extension is that the whey protein fraction of the MPC is lost to the whey stream and thus does not contribute to cheese yield. Attempts have been made to denature thermally the whey proteins and attach these to the casein micelle so that the whey is incorporated into the cheese matrix; however, the solubility of heat treated MPCs progressively decreases with increases in the whey protein denaturation index (Carr, 1999). The application of calcium depletion technology by Bhaskar *et al.* (2004) to this system resulted in a soluble MPC comprising 50–100 % denatured whey proteins that produced nugget free cheese. Bhaskar *et al.* (2004) also note that the flavour profile of the product can be varied through manipulation of pH and the choice of the cation (sodium or potassium or hydrogen or a mixture) used in the ion exchange resin. A suggested optimum cation combination to minimise off-flavours is a 1:2 M mixture of Na^+ and K^+ resins (Ranjith *et al.*, 1999).

The heat stability of membrane casein ingredients seems to be dependent on mineral balance and on the presence and structuring of whey proteins. Bhaskar *et al.* (2012) demonstrated that 35 % calcium depleted MPC, that had been heat treated at 90 °C for 4 s prior to evaporation and drying, was significantly more stable at 140 °C over a range of pHs than standard MPCs (Fig. 2.5). The effect was most marked at the lower pHs where in the worst case at pH 6.4, the standard MPC showed signs of immediate aggregation whereas the heat-treated 35 % Ca-depleted MPC was stable for 15 min. In marked contrast, Beliciu *et al.* (2012) reported the formation of large, visible agglomerates and coagulation in dried and non-dried MCC solutions heated at 141.7 °C for 3 s, at concentrations of 5–10 % w/w in the pH range of 7.08–7.17, which had similar total Ca:casein ratios as the standard MPC solution of Bhaskar *et al.* (2012). In the same pH range both MPC solutions of Bhaskar *et al.* (2012) were visibly stable for about 18 min. In a complementary study, Sauer and Moraru (2012) reported that the instability of thermally sterilised ($F_o = 6$) MCC solutions could be minimised by increasing the process pH to 7.3 or by decreasing the sterilisation temperature.

There is evidence from early work on calcium caseinate that calcium depletion will be of benefit to the manufacture of MCC. The data of Tessier and Rose (1961) shows that at a Ca:casein ratio of 0.028 solutions were immediately unstable at 140 °C but when the Ca:casein ratio was reduced to 0.011 the caseinate solutions had a similar pH/heat stability profile to standard MPC. Typically micellar casein concentrates have a whey to casein ratio of about 8:92. The impact of heat treatments on MCC prior to drying has not been established and a key question that needs to be answered is how much whey/κ-casein complexation is necessary to afford a commercially significant improvement in heat stability. A window for manufacturing a heat stable MCC may exist through manipulating the fractional whey composition, heat treatment and the calcium to casein ratio.

There is a growing consumer trend for healthier foods, which, in part, manifests itself as a desire for "clean label" or "E-number free" products. Recognition of this trend has resulted in the development of new ingredients for applications that have previously required the use of additives. The use of rennet casein in cheese gels, for example,

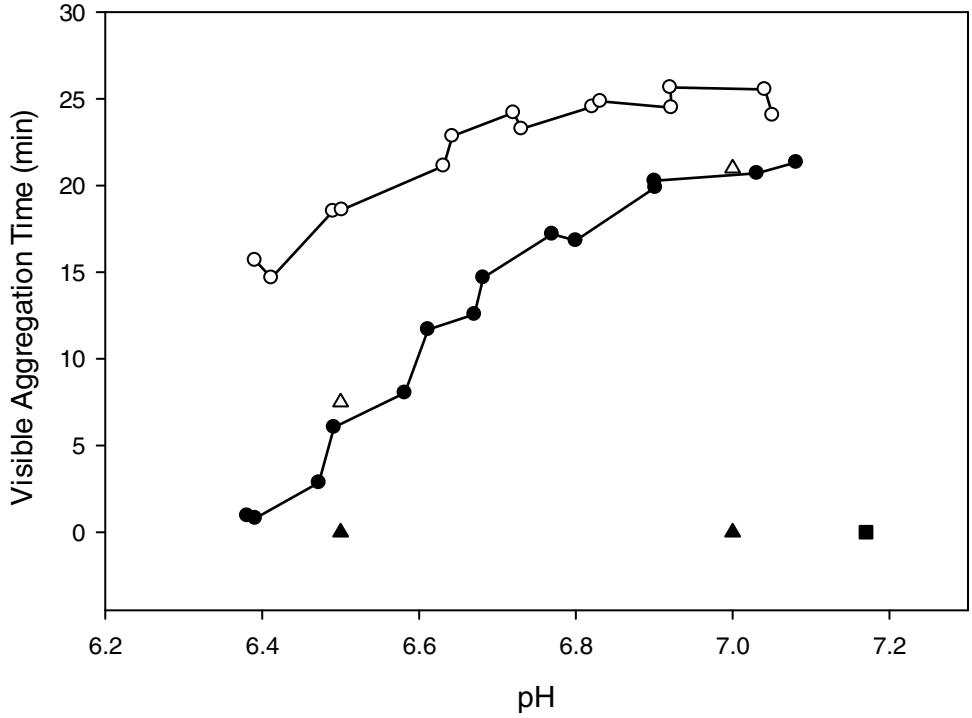

Fig. 2.5 Heat stability curves of 5 % solutions of standard MPC85 with a Ca:casein ratio of 0.033 (*filled circle*) and heat treated 35 % Ca-depleted MPC85 with a Ca:casein ratio of 0.023 (*open circle*) at 140 °C (Redrawn from Bhaskar *et al*., 2012); 5 % MCC with a Ca:casein ratio of 0.038 (*filled square*) (Data from Beliciu *et al*., 2012); 2.8 % calcium caseinate solution with a Ca:casein ratio of 0.011 (*open triangle*) and 2.8 % calcium caseinate solution with a Ca:casein ratio of 0.028 (*filled triangle*) (data calculated from Tessier and Rose 1961). *Note*: All solutions are on an as-is basis

has necessitated the need for calcium sequestering agents, commonly referred to as "melting salts", to solubilise the rennet casein. Through maintaining the zeta potential of the casein micelle *via* calcium depletion (Fig. 2.2), Carr *et al*. (2010) developed a colloidally stable membrane processed rennet casein ingredient that was able to be evaporated and spray dried to produce a fully soluble product thus obviating the necessity to include chelating agents in a formulation.

2.4.2 Fractionated Casein Ingredients

There are numerous methods available for the fractionation of casein into its individual proteins (Ram *et al*., 1995; Huppertz *et al*., 2006; O'Mahony *et al*., 2007; Sinclair *et al*., 2007). These methods generally make use of calcium chloride to precipitate the α_s fraction and cold temperatures to solubilise the β-casein fraction followed by a macro or micro-scale separation technique. With a worldwide trend towards amalgamation of small factories and co-operatives into larger processing facilities it is likely that the sale of casein fractions, that would hitherto have been economically unfeasible, will increase.

2.5 Food Applications of Casein

2.5.1 Application and Function of Casein and Casein Derivatives in Foods

Milk proteins are a well-established component of many food products. This includes not only application in traditional dairy-based foods, such as cheese, yoghurt and ice cream, but also in a

diverse range of other manufactured foods, including processed meats, chilled desserts and flavouring systems.

From a consumer perspective, the inclusion of dairy proteins in foods is associated with high quality nutrition and the delivery of dairy sensory notes to a given product. Whilst this is also true from a manufacturing perspective, the functional role of milk proteins is also recognised as being of critical importance in assembling the requisite product microstructure, which in turn ensures that appropriate material properties, product (physical) stability and texture are likewise achieved.

This is particularly relevant for soft solid and colloidal food systems where milk proteins encompass a range of technical functions, including viscosification, gelation, foaming, emulsification, oil- and water-binding. For such products, the particular functionality is dependent on a number of parameters, such as concentration, effect of processing conditions (notably thermal, but also including the effects of high pressure and shear), the medium of use (ionic strength, pH) and interactions with other components of a food formulation. In considering these various parameters, it is equally important to consider which specific milk protein will be most appropriate in delivering the preferred product structure and attributes. For manufacturers, an attractive aspect of formulating with milk proteins, is that dairy ingredient suppliers are increasingly able to selectively refine protein composition and properties to deliver specific functionalities for most effective use in any given product.

2.5.2 Food Applications and Functions of Soluble Casein Derivatives: Caseinates and MPCs

2.5.2.1 Thermal Properties

The isolation of the casein fraction of the milk protein system is advantageous for the formulation of many food systems. Whilst caseinates can be produced from a number of hydroxides, sodium and calcium derivatives remain the most common. Functional benefits of the caseinates include excellent heat stability, which limits alterations to product properties as a consequence of thermal processing. The caseins, lacking in secondary and tertiary structure, and having relatively few sulphur containing amino acids, do not undergo definitive denaturation and aggregation that is characteristic of many globular proteins. Accordingly, caseinate solutions and emulsions can be processed to pasteurisation temperatures for extended periods with no change in structure-function properties, and can be treated at UHT temperatures for short periods of time, again with minimal alteration to protein properties. More extensive heating of sodium caseinate can result in hydrolysis of the casein proteins (Jahaniaval *et al*., 2000), which can lead to alterations in functionality such as reduced viscosity in solution, improvements in solubility at low pH and the isolation of fractions with modified emulsifying and foaming properties (Guo *et al*., 1996). Heat treatment of calcium caseinate (121 °C, 15 min) was shown to result in both hydrolysis and polymerisation of casein molecules. Both peptide and polymeric fractions were shown to have emulsifying capacity, and enabled the formation of emulsions with excellent stability to thermal processing under retort conditions (Srinivasan *et al*., 2003). Liberation of functional polypeptides with demonstrated benefits through hydrolysis is however limited in application due to the formation of bitter components (Slattery and Fitzgerald, 1998).

2.5.2.2 Colloidal Food Applications

The notable amphiphilicity of the casein proteins imparts excellent emulsifying properties, and accordingly both calcium and sodium caseinates have widespread application as food emulsifiers. Benefits of the caseinates as emulsifiers is a tolerance to heat with no loss of functional properties (as indicated above), as well as maintenance of functional properties in the presence of ethanol. Accordingly, one of the major food application areas of caseinates has been in the production of cream liqueurs (Lynch and Mulvihill, 1997; Singh, 2011). Current formulations tend to comprise a combination of sodium caseinate and

monoglyceride emulsifiers to produce an emulsion with appropriate stability and organoleptic properties.

Long-term shelf stability requires non-interacting droplets (in the presence of ethanol) that are sufficiently small (typically < 0.5 μm) so as to minimise the risk of creaming during the storage life of the product. Casein proteins under relatively neutral conditions provide these requisites when homogenised with cream, typically at temperatures of 45–55 °C, using two stage homogenisation (to minimise the risk of droplet clustering) (Heffernan *et al*., 2009). Tolerance to alcohol of between 30 and 40 % v/v can be achieved, although commercial compositions to not tend to exceed 20 % v/v alcohol. At elevated ethanol levels, loss of solvent quality causes protein precipitation and coagulation of the emulsion (Medina-Torres *et al*., 2009). For cream liqueurs, sodium caseinate rather than calcium caseinate is used due to better ethanol tolerance, typically at concentrations of 1–4 % w/w.

Higher protein concentrations of sodium caseinate can lead to destabilisation of the emulsion system, due to depletion flocculation (Dickinson and Golding, 1997; Dickinson *et al*., 1997; Srinivasan *et al*., 2001). This is a widely reported phenomenon, caused by the presence of non-adsorbed casein in the form of hydrophobically self-associated protein moieties of size ~20 nm (Chu *et al*., 1995; Farrell *et al*., 1996). For approaching fat droplets, non-adsorbed caseinate aggregates are excluded from the intervening space, leading to an osmotic pressure gradient. This produces an attractive depletion potential between the two droplets, resulting in a weak, reversible form of droplet aggregation. Depletion potential scales with protein concentration, size of fat droplets and size of depleting particles–sodium caseinate aggregates have a greater causative effect on depletion interactions compared to calcium caseinate (Srinivasan *et al*., 2001). The relative phase volume of fat also influences emulsion stability. At relatively low phase volumes, sufficient depletion potential results in rapid phase separation due to formation of large flocculate structures that rapidly cream. With increasing phase volume of fat, increasing the depletion potential can result in a partial re-stabilisation of the emulsion due to formation of a percolating fat network, which is also accompanied by a substantial increase in emulsion zero shear viscosity.

A consequence of network formation is that emulsion instability arises from compaction of the network, resulting in syneresis of the continuous phase at the bottom of the sample (rather than formation of a cream layer). Increasing the depletion potential (as achieved by increasing the concentration of non-adsorbed protein) has a reinforcing effect on the droplet network, increasing the lag time prior to onset of emulsion phase separation (Dickinson *et al*., 1997; Manoj *et al*., 1998a, b). At elevated protein concentrations, the continuous phase viscosity provides a contribution to emulsion material properties and stability, reducing droplet motility and slowing the time of droplet network formation (Liang *et al*., 2014; Manoj *et al*., 1998a, b). The consequences of depletion flocculation impart constraints on the relative protein concentration that can be used in liquid emulsion compositions. For appropriate stable liquid emulsion compositions requiring high levels of protein, calcium caseinate may be a preferred option, or alternatively the use of MPC or SMP may be more effective. However, these protein formats are also comprised of polydisperse aggregate structures, and at sufficiently high concentrations can induce depletion effects due to the presence of sufficient volume fractions of protein particles in the size domain associated with depletion interactions (Radford and Dickinson, 2004).

Emulsion compositions comprising a sufficiently high yield stress, or with a highly viscous (e.g., ice cream) or solid continuous phase are not affected by instabilities arising from depletion interactions, and relative protein concentration has less influence on product stability. For emulsification in solid foods, the most significant application area for which caseinates are able to provide superior emulsion stability is in comminuted meat products, such as patés, burger patties, frankfurter type sausages (Petridis *et al*., 2010) and chicken nuggets (Rao *et al*., 1997). For such products the use of both sodium and calcium

caseinates can be a highly effective way of emulsifying fat (either indigenous to the meat source, or as an addition) within a textured protein network. Fat emulsification in comminuted meat products minimises losses during cooking, whilst also providing good long term stability and homogeneity of fat distribution within these products. This approach also allows the replacement or reduction of highly saturated meat fats with healthier unsaturated alternatives (Morales-Irigoyen *et al*., 2012). Increasing consideration is being given to the use of caseinate in combination with the enzyme transglutaminase for such products. This approach allows caseinate to not only impart good emulsifying and water-binding properties to comminuted meat systems, but enzymatic cross-linking of caseinate and muscle proteins allows the milk protein to provide a mechanical and textural contribution to the product, providing firmer textures with reduced losses during cooking (Buchert *et al*., 2010; Lee and Chin, 2011; Martinez *et al*., 2011).

Caseinate-stabilised emulsions are sensitive to other environmental conditions, such as pH, ionic strength and the presence of multivalent cations (Dickinson and Merino, 2002; Schokker and Dalgleish, 2000). Caseinate proteins are negatively charged at neutral pH, providing effective electrostatic stabilisation to emulsions under these conditions. Addition of soluble calcium ions can lead to cross-linking, notably between phosphoserine amino acid groups. This can cause precipitation of caseinate in solution, and cause flocculation of emulsion droplets, leading to instability and phase separation (curiously, calcium concentrations of <10 mM resulted in limited precipitation of caseinate aggregates, subsequently eliminating depletion flocculation in sodium caseinate stabilised emulsions (Dickinson and Golding, 1998)). Likewise, lowering of pH towards the isoelectric point of the protein causes both protein precipitation and flocculation of emulsions stabilised with caseinate. This can be problematic when wishing to formulate stable emulsions under such conditions. However, the structuring of both protein and emulsions can be advantageous for certain products when carried out in an appropriately controlled manner. An example of this includes yogurt manufacture, where it has been demonstrated that fortification of milk with caseinate during manufacture modifies the buffering capacity of the milk system and results in a reinforcement of the protein and protein-emulsion network (Peng, *et al*., 2009). This produces yogurts with higher viscosity, firmness and adhesiveness (Akalin *et al*., 2012). Curiously, it has been observed that aroma release (in 4 % fat flavoured stirred yogurts) decreases as a consequence of caseinate fortification, which is reported as being influenced by changes to the protein network arising from caseinate addition (Saint-Eve *et al*., 2006).

The use of caseinates has also been investigated as part of cheese production where fortification of cheeses using both sodium and calcium caseinates (Dickinson and Merino, 2002) has been investigated.

2.5.2.3 Protection Against Oxidation

Whilst well-recognised as emulsifiers, caseinates have additional technical properties that can provide functional benefits in a range of food systems. In recent years, the antioxidant properties of caseinate protein have been recognised. For lipid emulsion compositions prepared with caseinate, the protein has been shown to improve oxidative stability of fish oil emulsions (Horn *et al*., 2011; O'Dwyer *et al*., 2013). As well as improving oxidative stability in liquid and soft-solid emulsion compositions, sodium caseinate was shown to contribute antioxidant protection to labile components (polyphenols) in film materials prepared using the protein (Helal *et al*., 2012). The protective benefit has been attributed to iron scavenging, notably by the phosphoserine residues prevalent on the α_{s1}-, α_{s2}- and β-caseins. It is argued that even binding of iron to interfacial protein is still inhibitory to oxidation as the phosphoserine residues are resident in the hydrophilic domains of the protein, and therefore are removed from the interface, instead extending into the aqueous phase. Iron is a key catalyst for lipid oxidation, and barrier systems that prevent its approach and interaction at the oil-water interface have been shown to have an inhibitory effect

on onset and rate of oxidation. The antioxidant properties of proteins have also been attributed to free radical scavenging by sulfhydryl-bearing amino acids groups, which helps to terminate the oxidative free radical reaction (Tong *et al*., 2000).

For whey proteins, this is a more likely mechanistic scenario than iron binding, due to a higher prevalence of sulphur bearing peptides, and an absence of phosphoserine groups. For caseinates, this is less likely to be the case, due to lack of sulfhydryl groups. Maillard cross-linking of carbohydrates to casein proteins has been shown to further enhance the antioxidant properties of caseinates (Augustin *et al*., 2006; Hiller and Lorenzen, 2010). The benefits of conjugation include the ability to formulate stable emulsions over a wider range of conditions, such as variable pH and ionic strength. Additionally, conjugates show enhanced ability to quench free radicals relative to non-modified proteins. The application of native and modified caseinates has been explored in a range of products comprising lipids sensitive to oxidation, including breads, beverages and supplements.

2.6 Application and Function of Casein and Casein Derivatives in Non-Food Materials

While the predominant use of casein and casein fractions is as an ingredient in food products, manipulation of the functional properties of the mixed casein system can result in a remarkable array of structured materials such as films and coating, plastics, adhesives and surfactants, which have been shown to have application in a diverse collection of non-food applications (Audic, *et al*., 2003). Production of casein-based non-food materials was most prevalent in the early nineteenth century through to the mid-twentieth century, due to increasingly widespread availability of milk as a commodity. However, the development and increasing use of cheaper synthetic alternatives (and an increase in the value of dairy ingredients in food products) has seen diminished use of so-called technical grade casein as a component in non-food materials, although it can still be found in specific applications based on aspects such as the naturalness and aesthetics of casein manufactured materials.

2.6.1 Casein Derivatives as Adhesives

Arguably, the oldest non-food use of casein is as glue, evidence for which can be traced back to early Egyptian civilisation where curd from milk was recognised for its adhesive properties. Records also exist from medieval Europe of milk-based adhesives, which were prepared by the mixing of casein with lime. The resultant material was used as an adhesive for a number of applications such as furniture assembly and in the binding of paint pigments. The contemporary use of casein as a wood glue shows greater diversity in its preparation, thereby providing a broader range of functional benefits. However, the principals of casein glue preparation are essentially the same as those used centuries ago. Modern casein glues are based on alkalination of insoluble acid casein, usually with a combination of lime and sodium hydroxide, essentially producing a non-edible caseinate derivative. Use of the hydroxide on its own produces a glue with poor water resistance, but with a long working life. Conversely, whilst the use of lime is able to impart water resistance (due to calcium-mediated electrovalent cross-linking of the casein proteins) the working life of such glues is relatively short (NPCS Board of Consultants and Engineers, 2007). Consequently a combination of the two base materials is used in practice, often being combined with acid casein in the dry state to produce a powder which when rehydrates increases in pH, forming the glue. Use of other multivalent cations, such as copper, can improve water resistance.

Glue functionality and properties can be modified by the addition of other materials. For example, metal salts can be added to the composition to improve resistance of the glue to microorganisms, most particularly molds, and the addition of tannates has been shown to improve adhesivity.

Urea can also be added at varying concentrations to modify the viscous properties of the glue, allowing for more or less flowable glues to be produced depending on application. Here, the addition of urea is believed to disrupt the hydrophobic associations that cause the individual caseins to self-assemble into small aggregates structures (as is known to occur for sodium caseinate). At elevated concentrations of protein, close packing of these aggregated moieties generates high viscosities. Disassembly of these aggregates through addition of urea reduces viscosity for solutions at these high protein levels, although there is less effect for dilute protein solutions. Whilst casein glues have mostly been superseded by synthetic alternatives, they are still used for some niche application areas, notably as an adhesive for labels in the bottling industry, for use in woodworking and in bonding paper (Lambuth, 1989).

2.6.2 Casein Derivatives as Plastics

Casein plastics were originally commercially developed in the early twentieth century in Europe, being based on a patent initially granted to Krisch and Spitteler in 1899 (Ralston and Osswald, 2008). Manufacture of these materials was principally based on the high pressure extrusion of rennet casein mixed with water (typically 20–35 %), producing a pliable material. This extruded casein material was not especially effective at being moulded, so shaped products were primarily based on pressing into sheets. This material was then cured in order to produce a rigid, robust plastic material with good water resistance.

Curing was typically carried out using a 4–5 % solution of formaldehyde. Here, the single carbonyl group on formaldehyde reacts with available amine groups on the casein proteins, resulting in the formation of methylene cross-links. This reaction pathway enables rapid and extensive cross-linking of the protein, forming a rigid network that imparts mechanical strength. Compared to modern plastics, the casein material has rather limited mechanical strength, being somewhat brittle in nature. Since the original reaction pathway was determined, numerous studies have sought to investigate how modifications to the microstructural properties of the material (such as through the addition of lipids, plasticizers and a range of different polymers) can be employed to produce casein-based plastics with a broader range of material properties. A particular recent example (Pojanavaraphan, *et al*., 2010) is of a composite of casein protein and sodium montmorrillonite clay which was freeze dried before being cured with glyceraldehyde (considered a more biocompatible cross-linking reagent). The resulting foam-like material (which has been termed a casein aerogel) was reported to give a low density product with mechanical properties similar to expanded polystyrene (Fig. 2.6). A notable feature of the material was its enhanced biodegradation relative to synthetic polymeric systems.

In 1930 the world-wide production of casein-based plastics was estimated at ~10,000 tonnes. However, since the 1960, production has steadily decreased due to the introduction of cheaper synthetic alternatives with a broader range of functionalities. Some limited production of casein plastics does still occur, primarily in the manufacture of buttons, buckles, and other clothing accessories. However, it is clear that there is still considerable scientific interest in the manipulation of plasticised casein structure and function, which may in turn deliver new commercial opportunities for this material.

2.6.3 Caseins as Coating Materials

The structure and amphiphilicity of the caseins produces cohesive, optically transparent film and coating materials on removal of moisture. Casein coatings have been shown to be effective materials in a range of non-food applications, including paper coatings and glazings, protective, pigment binding and anti-static coatings for a range of textiles including leather. Casein films (including composites with other materials) have also shown wide-ranging application as natural packaging films for food and non-food materials.

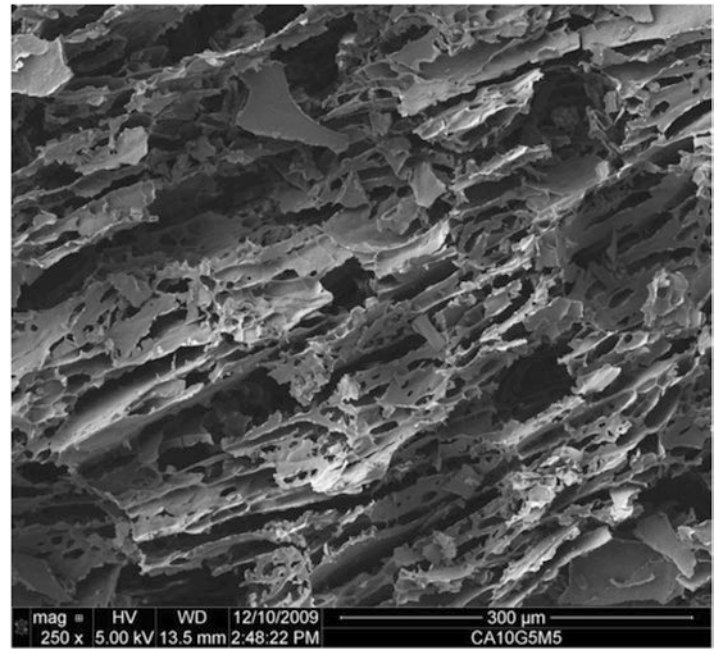

Fig. 2.6 Scanning electron micrograph of casein/clay aerogel composite (taken from Pojanavaraphan, *et al*., 2010)

The role of casein as a coating material for paper dates back to *circa*. 1893. Coating was required to ensure that the pigments used to enhance the texture and appearance of paper were fixed into the material, as well enabling good ink binding to the surface of the paper. Early casein coatings were prepared according to the same principles as for casein-based glues, i.e., the alkalination of acid casein to produce a soluble adhesive. To the solubilised protein solution pigments, usually clays and chalks, were added. Appropriate control over the pigment to coating ratio is required to ensure sufficient delivery of pigment could be achieved without necessarily producing an overly viscous coating solution that would lead to an excessive layering. At high solids, coating viscosities become too high for practical application. As with adhesive applications, the addition of urea provided a means by which the viscosity of the mixture could be reduced. Other approaches included raising pH and the use of proteolytic enzymes.

A particular advantage of the use of casein as a coating was that that treatment with formaldehyde (through dipping or exposure to vapour) produced a material that was insoluble as a consequence of casein cross-linking. For particular applications, washable or stain resistant coatings could be produced using this particular treatment. Coating functionality can be further modified through incorporation of other materials. For example, Khwaldia (2010) investigated the use of waxes as a means of reducing water vapour permeability when sodium caseinate was used as a coating material, thereby reducing the need for treatment with cross-linking reagents. Within the current industry environment, most paper coating is now achieved through the use of starches and their modified derivatives, which provide reduced cost and better functionality. However, a number of application areas still employ the use of casein as a coating material, notably for paper cones and tubes.

For textiles, coating or immersion of fabrics in casein solutions, usually followed by chemical cross-linking (again, most commonly through the use of formaldehyde) can be used to improve aspects of quality, such as prolonging the lifetime of treated materials against abrasion, or providing barrier properties against water absorption.

As with other non-food applications, these particular casein-based coating technologies were originally developed in the early twentieth century. However, novel functionalities for casein materials for treatment of fabrics are continuing to be developed. For example, the combination of caseins with acrylates, coupled with appropriate modification steps, such as grafting, can improve the anti-static properties of certain textile fibres or may prevent greying of cotton from taking place after multiple washes. More recently Shimanovich *et al*. (2012) studied the use of sonochemical processing to produce casein-based microspheres that could be loaded with antibacterial components and deposited onto cotton or polyester fibres.

The ability to retain and release antibacterials post-deposition was considered to have commercial relevance for bandages, sticking plasters and other medical applications, as well as potentially reducing microbial growth post-washing. A further example by Cui *et al*. (2011) investigated the effect of using enzymatic cross-linking transglutaminase on wool fabric that had been treated with casein. The use of the enzyme was considered to improve casein attachment to wool fibres by promoting covalent cross-linking between the casein and keratin proteins. Their findings indicated that treatment with the enzyme reduced the observed shrinkage of the wool, increased the tensile strength and was shown to lead to a smoother surface of the fibres (Fig. 2.7).

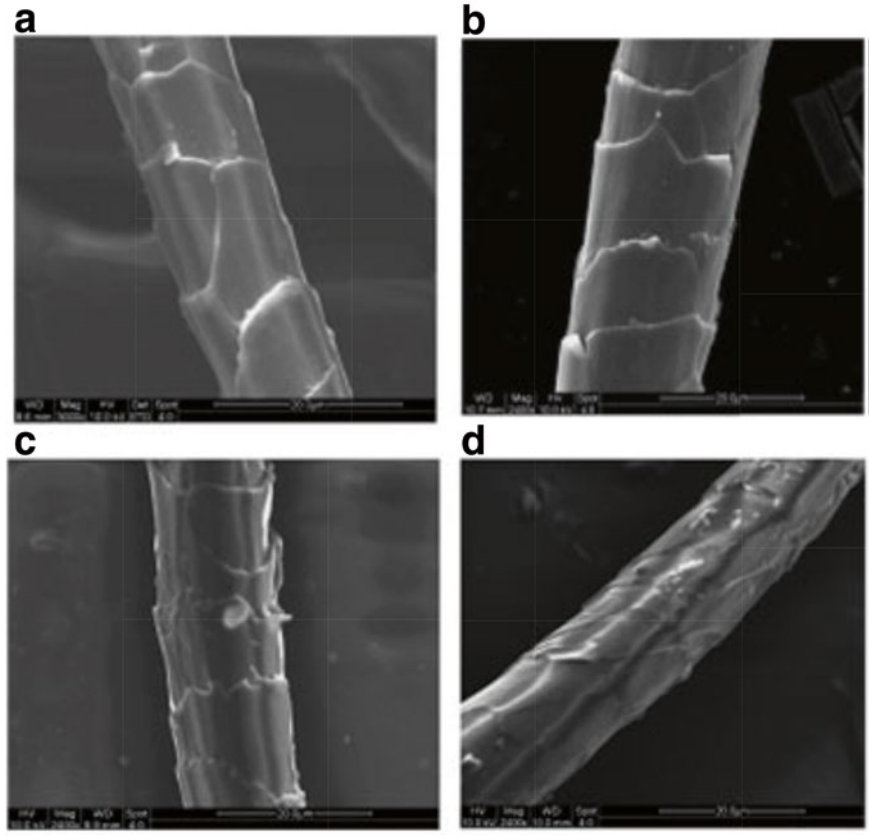

Fig. 2.7 Scanning electron micrographs of surface structure of wool fabrics after different treatments ((**a**) Control, (**b**) KMnO4 pretreatment, (**c**) KMnO4 and casein treatment without TGase, (**d**) KMnO4 and casein treatment with TGase). Taken from Cui *et al*. (2011)

An additional area where casein derivative coatings have application is as packaging films (Khwaldia *et al*., 2004). Commercial advantages to the use of casein-based materials include aspects of biodegradability, through to design of edible packaging films and materials. In the absence of any other additives, alkaline solubilised casein and caseinates can readily form transparent films with good barrier properties for gas permeability. However, compared to starch or synthetic packaging film materials, the mechanical and water barrier properties of casein films are relatively poor. Considerable research has been undertaken in recent years to investigate how adjunct components, notably plasticizers, lipids and cross-linkers can be used to improve both mechanical and water permeability.

As with other coating applications, glues and plastics, water resistance of casein films can be greatly enhanced through the introduction of intermolecular cross-links to the protein network. Chemical cross-linking, such as the use of formaldehyde, is well established as a means for improving water resistance for coatings and plastics, and can be equally applied to packaging films. However, there are limitations in this approach for the preparation of edible packaging materials. Alternative cross-linking mechanisms that have been investigated include the use of γ-irradiation (Brault *et al*., 1997; Lacroix *et al*., 1998), which was used to form permanent cross-links in calcium caseinate films. The resulting film materials were found not only to be more water resistant, but also had improved mechanical properties.

The use of enzymatic protein cross-linkers as a more natural means of altering casein film properties has also been investigated (Heck *et al*., 2013). For example, Juvonen *et al*. (2011) compared transglutaminase to two oxidoreductase enzymes (*Trametes* hirsutalaccase and *Trichoderma reesei* tyrosinase) on caseinate film cross-linking and subsequent properties. They found that both the transglutaminase and *Trichoderma reesei* tyrosinase were effective in increasing the insolubility of the caseinate films.

Inclusion of additive systems to modify the mechanical properties of casein-derived films continues to be an area of considerable research interest, as part of improving the commercial applicability of these systems. Unmodified casein and caseinate films tend to be relatively brittle with low tensile strength and poor extensibility. Composites prepared using caseinate and a number of different plasticising materials can lead to improvements in mechanical properties, notably through the addition of polyol type plasticisers. Arvanitoyannis and Biliaderis (1998) compared a number of polyol systems and showed that the plasticising effect could be ordered accordingly: glycerol < sorbitol < xylose < sucrose. The use of the polyol triethanolamine (which formed part of a study comparing the properties of polyol-caseinate films) was shown to increase film extensibility by 180 % when used in a 1:1 ratio with caseinate (Audic and Chaufer, 2005). Incorporation of lipid components: fats, waxes and emulsifiers, present an additional mechanism by which the material properties of the films can be modified. Inclusion of apolar lipids can also be effective in reducing the water permeability of films (AvenaBustillos *et al*., 1997).

As packaging materials, casein derivatives have been recognised as having particular application in the coating and protection of food products, to improve quality, shelf-life and remove the need for synthetic or non-biodegradable materials (Khwaldia *et al*., 2004). As moisture and gas barriers they have the ability to extend the shelf-life of fruits and vegetable, inhibiting moisture loss from products, reducing ripening rates and retarding browning. Studies have included investigations into the application of modified caseinate films on moisture loss in carrots, zucchini and strawberries. In each case, the applied casein coating was seen to improve moisture retention (Avenabustillos *et al*., 1993, 1994). It has also been demonstrated that caseinate films cross-linked with γ-radiation delayed browning of potatoes and apples after application (Brault *et al*., 1997). Other areas of interest include inhibition of moisture loss in frozen foods and as a vapour barrier for the enhancement of aroma profiles in food systems (Stuchell and Krochta, 1995; Fabra *et al*., 2008).

It is apparent that compared to other non-food application areas, the potential of casein derivatives as packaging materials is receiving renewed commercial interest. Partly this is due to their potential as a natural substrate for innovative active packag-

ing applications, which are increasingly seen as means by which the quality of products (most notably food systems) can be controlled, monitored or enhanced during product lifetime. For example, the incorporation of antimicrobial components into packaging films can provide a means of extending shelf-life and enhancing product safety.

This concept was investigated by Moreira *et al*. (2011), who combined sodium caseinate with the antimicrobial biopolymer chitosan (Rabea *et al*., 2003) to produce composite films which there then coated onto a range of food materials, including cheese, salami and carrot. The resulting material was shown to provide an improvement to the bactericidal properties of the film compared to separate control films based on caseinate and chitosan. The authors purported that the improved antimicrobial performance was derived from an electrostatic complexation between the negatively charged caseinate and the positively charged chitosan. Incorporation of nisin (Cao-Hoang *et al*., 2010) or *Lactobacillus sakei* (Gialamas *et al*., 2010) into caseinate films as means of controlling *Listeria* in food products has also been investigated, with positive results being reported. Additional functionalities include the development of caseinate-based films with enhanced antioxidant properties through the inclusion of alpha-tocopherol (Fabra *et al*., 2011; Jimenez *et al*., 2013).

2.6.4 Other Non-Food Application Areas

The functional properties of casein and its derivatives have been incorporated into an extensive array of additional non-food applications. Benefits in the use of casein materials in such applications have been identified, notably as a readily available, biodegradable raw material. However, commercial uptake of technologies incorporating casein proteins remains limited, primarily due to considerations of cost, not only of the raw materials, but often the requirement to include additional additives to produce the desired technical properties. Additional non-food applications have included surfactancy, texturing and foaming in cosmetic, home personal care applications, and pigment binding, stabilising and emulsifying in a range of water, oil and latex based paints. Other more curious and speculative application areas have included adhesive and binding properties in bitumens and cements, and for the recovery of metal ions from wastes in manufacturing processes (Audic *et al*., 2003).

2.7 Future Perspectives

Increasingly the focus of researchers and manufacturers has been turning towards understanding how the key stakeholders throughout the value chain use casein ingredients and what hurdles exist that are limiting the desirability of these ingredients. Recognising and removing these barriers will assist manufacturers to increase share in existing markets and open access to new markets. The key features that the ingredient designer should be cognisant of will vary from market to market but in general will include the elements shown in Fig. 2.8.

The nutritional profile of a product is key to success in some markets. The textural improvements in yoghurt referred to in the previous section, through the addition of sodium caseinate have been noted as a more general effect of calcium depleted casein systems by Harnett *et al*. (2012). Additionally Harnett *et al*. (2012) noted that systems fortified with calcium-depleted casein ingredients necessarily contain less calcium compared to when non-depleted fortificants are used, thus the reduced calcium level makes it more difficult to make nutrition claims for calcium on the food label—a product that consumers traditionally expect to be rich in calcium. Addition of soluble calcium negates the textural improvements of the Ca-depleted ingredient. Harnett *et al*. (2012) showed that a formulator can obtain both benefits by coupling the Ca-depleted casein ingredient with an insoluble form of calcium.

With the rise of specialised nutrition, from sports recovery beverages to medical foods, formulators are evaluating ingredients not only on total protein or amino acid profiles but on the delivery mechanism to the end consumer. An example of such an approach is in the recent pat-

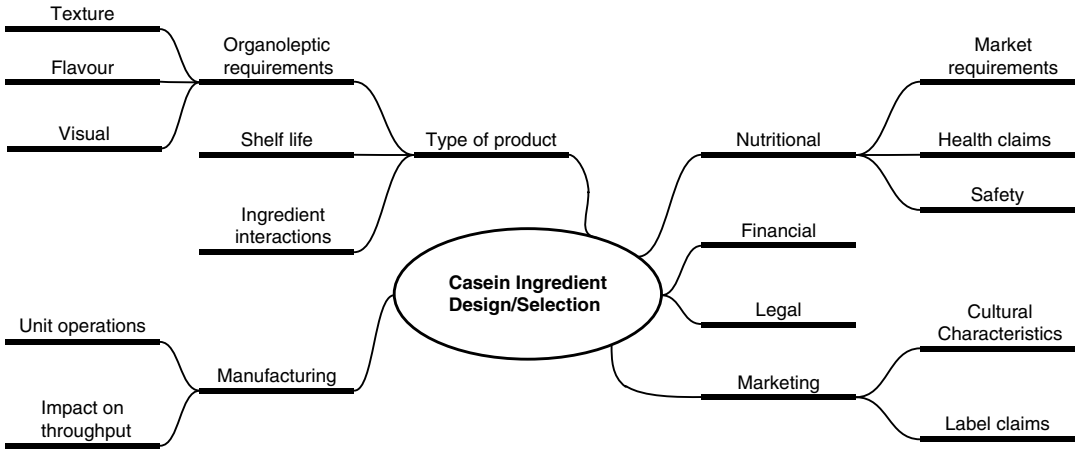

Fig. 2.8 Factors to consider when choosing a casein-based protein ingredient

ent application of Fanning *et al.* (2013) who showed that, on an equivalent protein basis, Ca-depleted MPC produces a larger increase in the blood serum concentration of free leucine compared to either standard MPC or Ca-caseinate.

For other formulators, the choice of ingredients is influenced by the impact of ingredient choice on shelf life. Huppertz *et al.* (2013), for example, have shown that casein manufacturing plays an important role in plasmin activity in the casein ingredient, and thus protein degradation over time in the final consumer application.

The increase in MPC and MCC based products, at the demise in the importance of caseinate ingredients is in part due to cost of manufacture and the high degree of customisation due to the inherent flexibility afforded by membrane processing that enables holistic product design.

The flexibility in process design offers significant opportunities to manufacture customised "black box" casein ingredients that incorporate non-dairy ingredients and so develop specialised ingredients for specific customers. Likely market segments for customised ingredients include complex foods such as nutritional formulations and nutraceuticals where there is a functional need to structure and protect labile bioactives through processing, storage and digestion, such as the casein/starch encapsulated fish oil ingredient described by Trubiano and Makarious (2012).

The increase in specialised casein ingredients in the marketplace is likely to continue and become more important with the rise of cheaper, customised functional proteins from non-dairy sources. To realise the opportunities, researchers will need to move beyond the laboratory and factory and work closely with customers to fully understand the features, from plant design to ingredient interaction and final product attributes, that are important for their business.

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Functional Milk Proteins: Production and Utilization—Whey-Based Ingredients

3

Nidhi Bansal and Bhesh Bhandari

Abstract

Historically, whey was considered to have very little value by the dairy industry. However whey contains about 50 % of the total solids of milk, including almost 100 % of the lactose and about 20 % of the total protein. The excellent nutritional value of whey proteins and the enhanced functional properties of whey-based ingredients have now been widely recognised. Over the years, advancements in science and technology have transformed whey from a troublesome waste product to valuable dairy ingredients. Whey is no longer a 'by-product', but is rather seen as a valuable 'co-product' of cheese making and casein production. This chapter focuses on various unit operations that are commonly utilised for concentration, fractionation and dehydration of whey-based ingredients. The composition and application of whey-based ingredients such as whey powders, whey protein concentrates, whey protein isolate and whey protein fractions are also discussed.

Keywords

Whey • Whey processing • Whey ingredients • Whey powders utilisation • Whey drying

3.1 Introduction

Whey is a pale green coloured translucent liquid phase formed after milk has been coagulated. The colour of the liquid residue depends on milk quality and type of milk used. The United States Code of Federal Regulations (21CFR184.1979) defines whey as "the liquid substance obtained by separating the coagulum from milk, cream, or skim milk in cheesemaking". The milk coagulation pro-

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cess in which casein protein precipitates and whey is formed can be facilitated by growth of microorganisms, acid addition or using enzymes (Killara and Vaghela, 2004). More recently, whey is also produced by separating caseins and whey in skim milk using membrane filtration technology.

Historically, whey was considered a by-product (waste) in cheese making and casein production. It was considered a nuisance and troublesome to dispose of since the amount of whey recovered nearly equalled 90 % of the amount of milk used. At that time, whey was considered to have very little value and dairy companies struggled with its economical disposal (Smithers, 2008). Most commonly, whey was discharged into the sewage systems or water bodies such as lakes, rivers or oceans causing substantial environmental pollution problems due to the high biological oxygen demand (BOD) of whey. The BOD of typical whey is estimated to be >35,000 mg/L (Smithers, 2008). Alternatively, whey was also sprayed onto farmland as fertilizer, but led to contamination of groundwater. The most profitable and innocuous method of whey disposal in the past used to be its utilization in animal feed.

Over the years, through advancements in science and technology, whey has transformed from a troublesome waste product to a valuable dairy material yielding a wealth of quality whey-based ingredients due to its protein, lactose, vitamin and mineral content as well as enzymes, hormones and growth factors (Tunick, 2008). Whey provides quality proteins for good health and is also a good source of essential amino acids. Further to their nutritional contribution, enhancement of functional properties of food due to incorporation of whey-based ingredients is now being increasingly recognized by the food industry and consumers alike. Whey is no longer a 'by-product', but is rather seen as a valuable 'co-product' of cheese making and casein production. Several developments during the recent past have been responsible for transforming the status of whey from a by- to a co-product, including increased cheese production, stricter environmental pollution control measures, advancement in scientific knowledge and technologies, expansion of marketplace and increasing consumer demand for healthy, nutritive and functional foods. Recent growth of the infant formula industry has created a high demand for whey and whey based products. The journey of whey from 'gutter-to-gold' is well narrated by Smithers (2008).

Owing to its exceptional nutritional value and expanding application base, several new commercial processes have been developed to manufacture high quality whey-based ingredients. This chapter will focus on the manufacturing techniques utilised for and applications of several whey-based ingredients, such as whey powders, whey protein concentrates, whey protein isolate and whey protein fractions.

3.2 Sources and Types of Whey

The composition of whey is very similar to milk serum (milk from which casein and fat has been removed). Whey contains about 93 % of the water in milk and 50 % of the total solids of milk. Typical composition of whey is given in Fig. 3.1.

Whey is obtained after removing casein from milk. Casein can be separated from milk by acidification to pH 4.6, by utilising proteolytic enzymes, such as chymosin, or by microfiltration. Acidification of milk can be brought about by microbial fermentation of lactose to lactic acid or by the addition of organic (citric acid or lactic acid) or mineral (sulphuric acid, phosphoric acid or hydrochloric acid) acids. Typically there are two main sources of whey; cheese manufacture and casein/caseinate production. Based on the manufacturing protocol, two major types of whey are produced. Sweet whey is derived from the manufacture of cheese or casein by rennet coagulation of milk at a pH of about 6.0–6.5. Typically sweet whey results from the production of hard and semi-hard cheeses, such as Cheddar cheese. Acid whey results from fermentation during fresh acid-coagulated cheese such as Cottage cheese and direct acidification of milk during casein and caseinate production. The resultant whey has a pH of about 4.6–5.0 (Tunick, 2008; Fox, 2011). The United States Code of Federal Regulations (21CFR184.1979) has defined types of whey as "whey obtained

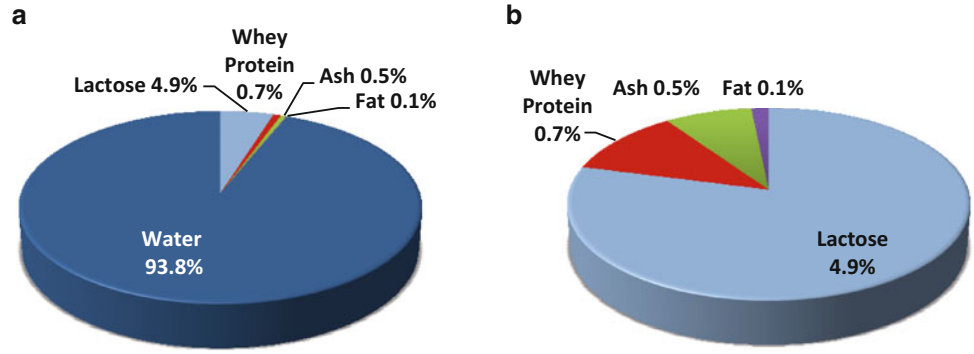


Fig. 3.1 Typical composition (%, w/v) of (**a**) liquid whey and (**b**) solids (assuming 93 % water) in liquid whey on wet (g/100 g liquid whey) basis

from a procedure, in which a significant amount of lactose is converted to lactic acid, or from the curd formation by direct acidification of milk, is known as acid whey. Whey obtained from a procedure in which there is insignificant conversion of lactose to lactic acid is known as sweet whey. Sweet whey has a maximum titratable acidity of not more than 0.16 %, calculated as lactic acid, and an alkalinity of ash of not more than 225 mL of 0.1 N hydrochloric acid per 100 g".

The most widely produced of the two types of whey is sweet whey (Killara and Vaghela, 2004; Tunick, 2008). An increasingly important source of acid whey is Greek-style yogurt. In Greek-style yogurt production, 3 parts of acid whey is produced for every 4 parts of milk used, producing 1 part yogurt. During the last 5 years, the sales and consumption of Greek-style yogurt, and hence acid whey production from it, has grown strongly (Boynton and Novakovic 2013).

The composition of sweet and acid types of whey is quite similar as shown in Table 3.1, the main differences being in the acidity, minerals content and the make-up of the whey protein fraction (Fox, 2011).

Sweet whey produced using rennet to coagulate caseins contains the glycomacropeptide fraction of κ-casein. Acid whey produced by direct acidification of milk to the isoelectric point of the caseins contains higher levels of calcium phosphate solubilised at such low pH. Similarly, fermentation-produced whey may contain polysaccharides, enzymes and flavour compounds contributed by microbial activity. There are batch to batch variations also in the composition of whey and whey-based ingredients. The cause of such variation originates from variations in composition of milk, and the pre-treatments and methods of manufacture utilised for producing whey-based ingredients. The composition of whey resulting from cheesemaking may also be influenced by processing factors such as pasteurization of cheese milk, addition of $CaCl_2$, type of bacterial starter culture used, type and concentration of casein coagulant and pH and temperature at curd cutting, cooking and drainage (Morr and Ha, 1993). The protein composition of whey is depicted in Fig. 3.2 (Hahn *et al.*, 1998).

Table 3.1 Approximate composition and pH of sweet and acid whey (g/L of whey) (de Wit, 2001; Mulvihill and Ennis, 2003; Fox, 2011)

Component	Sweet whey	Acid whey
Total solids (g/L)	63.0–70.0	63.0–70.0
Total protein (g/L)	6.50–6.60	6.10–6.20
Non-protein nitrogen (g/L)	0.27–0.37	0.30–0.40
Lactose (g/L)	46.0–52.0	44.0–47.0
Milk fat (g/L)	0.20–0.50	0.30
Minerals (ash) (g/L)	5.00–5.20	7.50–7.90
Calcium (g/L)	0.40–0.60	1.20–1.60
Phosphate (g/L)	0.50–1.00	2.00
Sodium (g/L)	0.50–0.53	0.50–0.51
Chloride (g/L)	1.10	1.10
Lactate (g/L)	2.00	6.40
pH	5.90–6.40	4.60–4.70

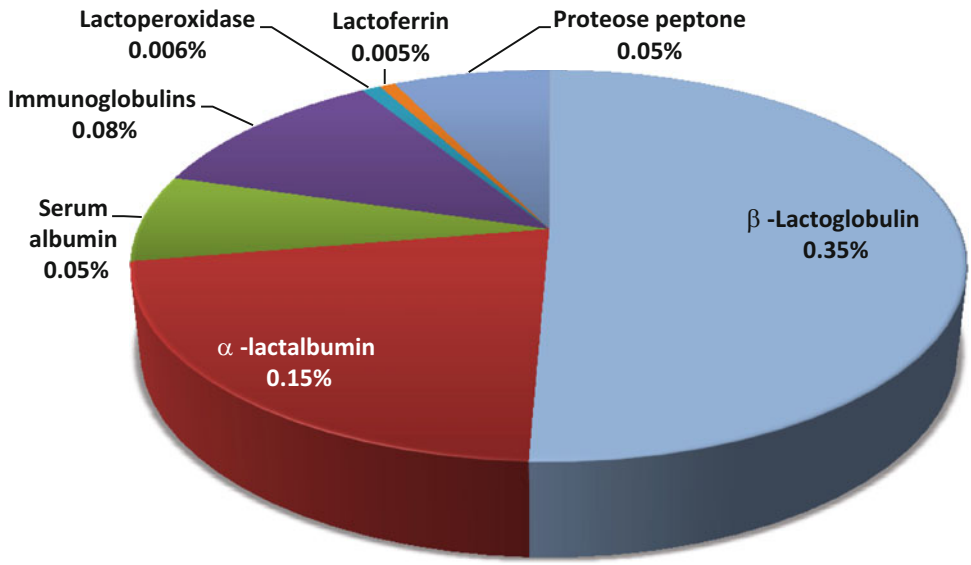


Fig. 3.2 Typical protein composition (%, w/v) of liquid whey

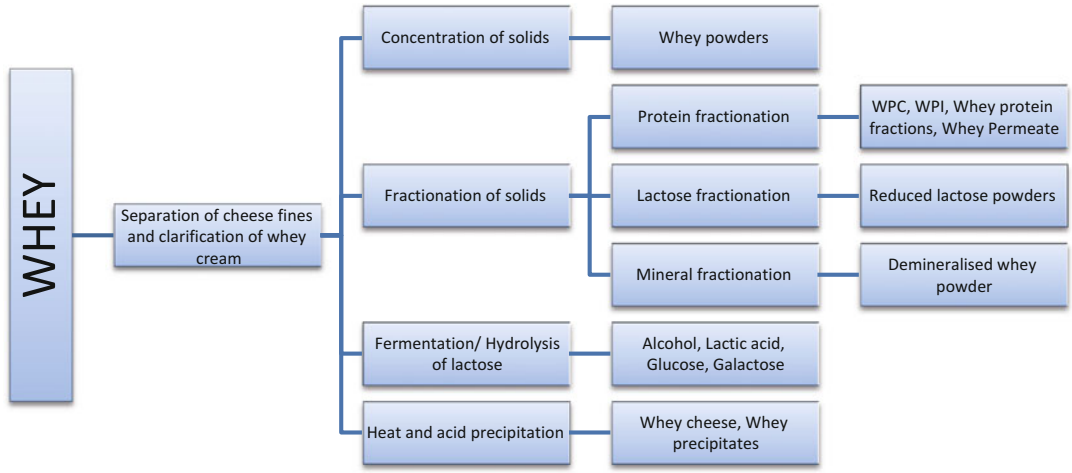


Fig. 3.3 Processes involved in conversion of whey to whey-based ingredients

3.3 Overview of Whey Processing

Whey consists of valuable nutrients and several novel commercial processes have been developed to manufacture high quality whey products. The principal pathways through which liquid whey can be utilized or converted to whey-based ingredients are highlighted in Fig. 3.3. Production of various whey-based ingredients involves combination of the following principal processing steps:

a) *Clarification* for removing cheese fines
b) *Separation* for removing residual milk fat known as whey cream
c) *Concentration of solids* through membrane processing and/ or evaporation and drying yielding whey powders
d) *Fractionation of solids* to
   I. *Recover protein* to produce whey protein concentrates, whey protein isolates and protein fractions such as lactoperoxidase, lactoferrin, α-lactalbumin and β-lactoglobulin

II. *Recover lactose* to yield lactose and lactose fermentation products
III. *De-mineralisation* to produce demineralised whey powders

3.4 Whey Powders

Whey powders are those products that contain less than 15 % protein and are produced simply by concentration and drying of liquid whey. The concentration process may involve only evaporation or a combination of evaporation, nanofiltration and reverse osmosis followed by drying.

3.4.1 Types of Whey Powder

Standard whey powder: Standard whey powder is the simplest whey product produced with little or no major pre-treatments except for clarification for removing cheese fines and separation for residual whey cream. The composition of this powder is relatively similar to that of liquid whey except for the removal of most of the water component. Depending on the source of whey, standard whey powder can be classified as sweet- or acid-whey powder. The composition of acid whey powder is relatively similar to that of sweet whey powder (Table 3.2) except for its slightly lower lactose content. Acid whey powder also has a high titratable acidity which makes the flavour of this powder slightly acidic and may render difficulty on handling due to increased hygroscopicity if the acidity level is >0.2 % lactic acid (Bhandari, 2012).

Demineralized whey powder: This product is also known as reduced-minerals whey powder. It is produced by selective removal of about 70–90 % of minerals from fresh whey through ion-exchange, nanofiltration or electrodialysis and it contains ≥11 % protein (O'Regan *et al.*, 2009).

Delactosed whey powder: This product is also known as reduced lactose whey powder. It is produced by crystallizing out most of the lactose from whey and then recovering the protein-rich stream through ultrafiltration and diafiltration. It may contain about 18–25 % protein and a very high mineral content of about 25 %. It has a proximate composition that is similar to skimmed milk powder and it is possible to use as a skimmed milk powder substitute (Jelen, 2009; Kilara, 2009).

Typical composition of various types of whey powders is given in Table 3.2.

3.4.2 Production of Whey Powders

3.4.2.1 Sweet Whey Powder

The processes used to manufacture whey powders allow the production of non-hygroscopic and non-caking wettable powders. In a normal spray drying process the lactose in the whey powder is in an amorphous and hygroscopic form. Pre-crystallisation of lactose prior to spray drying produces relatively non-hygroscopic whey powder. The key processes that whey powder production will typically follow are illustrated in Fig. 3.4.

Pre-Treatment

After production, whey should be processed as soon as possible; otherwise, it should be cooled quickly to 5 °C to inhibit bacterial growth (de Wit, 2001). If whey is to be stored short-term, then

Table 3.2 Composition of different whey powders (g/100 g) (de Wit, 2001; Mleko *et al.*, 2003; Thomas *et al.*, 2004; Deeth and Hartanto, 2009; Jelen, 2009; Huffman and de Barros Ferreira, 2011)

Product	Water	Fat	Protein	Lactose	Minerals
Sweet whey powder	3–6	0.8–1.5	12–13	70–74	7.5–8.5
Acid whey powder	≤3.5	0.8	9–12	65–69	11–12
Demineralised whey powder	≤3	≤1.5	≥11	75–84	≤4
Delactosed whey powder	2–3	1–4	18–25	40–60	11–27

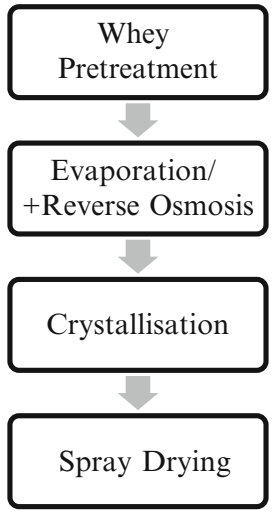


Fig. 3.4 Typical process of manufacturing standard whey powders

cooling is sufficient, otherwise pasteurization is necessary if long-term storage is required prior to further processing (Boland, 2011). The first stage of whey treatment is to remove the fat and casein fines from whey as these interfere with the subsequent unit operations. This can be done by using separation devices such as vibrating sieves, cyclones, centrifugal separators or rotating filters. After the fines and fat are removed, the fines can be returned to cheddaring systems or reused in processed cheese or returned to cheddaring systems while the whey cream can be reused to standardise cheese milk in cheese making or to produce whey butter (de Wit, 2001; Bylund, 2003).

Concentration

After the pre-treatment process, the whey is concentrated under vacuum in a falling-film evaporator with two or more stages. Long-tube, multi-stage evaporators were first utilised for whey concentration in 1933 (Gillies, 1974). Modern evaporators utilise six or seven stages with thermal or mechanical vapour recompressors to reduce the costs of evaporation (Marwaha and Kennedy, 1988).

The evaporators for whey processing are similar to those utilised for skim milk and other dairy fluids. The processing conditions, however, need to be manipulated to suit the properties of whey. The falling film evaporator contains bundles of tubes surrounded by a steam heating jacket and is maintained under vacuum. Whey flows through these tubes as a thin film and is concentrated in a vapour cyclone that is fitted at the evaporator outlet. The vapour is also separated from the partially concentrated whey in the cyclone. This process is repeated through the multiple stages and the vapour produced during the first stage is used as a heating source for the second stage and so on. To increase the thermal efficiency of the evaporator, a thermo-compressor is often used which acts as a heat pump increasing vapour pressure. Using multi-stage evaporators (Fig. 3.5) the whey can be concentrated from ~6.5 to ~45–65 % solids (Bottomley *et al.*, 1990). Reverse osmosis (RO) can also be used to concentrate whey prior to drying; but concentration of whey by RO is limited to around 20 % solids due to the very high osmotic pressure created by concentrated lactose (Pearce, 1992a). Thus further concentration is necessary using evaporators. Multistage recycle RO plants of tubular design are utilised alone or mostly in combination with evaporators to concentrate whey (Kilara, 2009).

Crystallisation

Crystallisation of lactose prior to drying is done to avoid hygroscopicity of lactose in whey powder and hence to prevent stickiness of the powder during drying, storage and handling. After the whey is concentrated to 50–60 % solids, it is cooled to 30 °C. In this concentrated lactose solution, α-lactose and β-lactose are in the supersaturated metastable state. Finely ground α-lactose monohydrate or well-crystallized whey powder is added to the whey to act as nuclei for lactose crystallization. Due to the lower solubility of α-lactose, it crystallises in the α-lactose monohydrate form. It is then cooled to 10 °C at <2 °C/h for 12–16 h while constantly stirring to allow for crystallisation of lactose as α-lactose monohydrate which is less hygroscopic than non-crystalline amorphous lactose glass produced during drying from soluble lactose in whey solution (Jelen, 2009). Flash cooling is avoided to prevent spontaneous nucleation and production of false grain, resulting in exces-

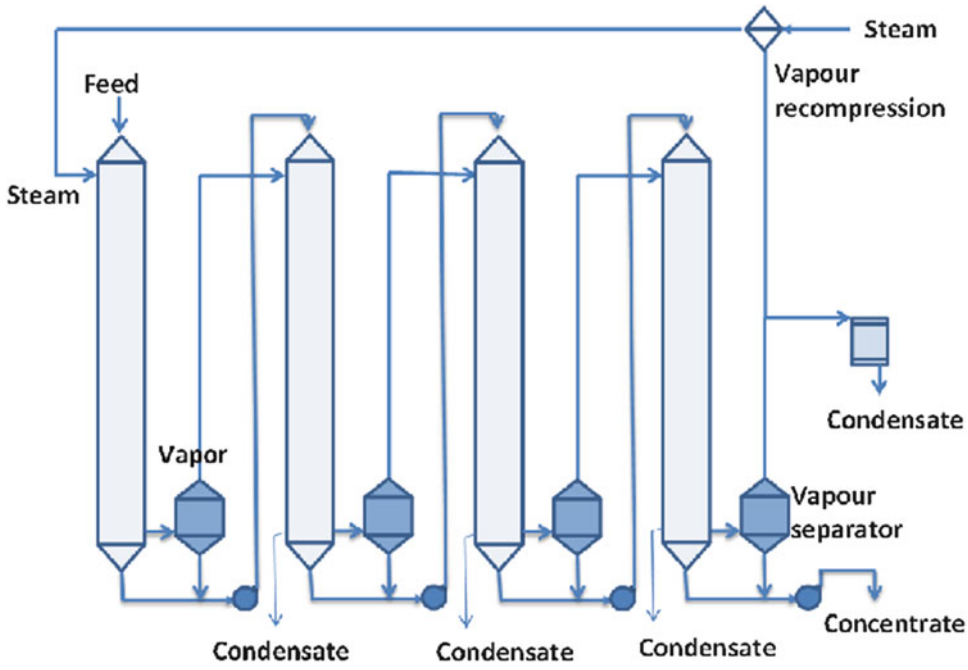


Fig. 3.5 Schematic diagram of a typical 4-effect falling film evaporator

sively large particles. The objective is to produce very small crystals (not exceeding 50–100 μm) that do not impede the subsequent spray drying process. About 75–80 % of lactose in whey will be crystallised during this process.

Drying

The concentrated and pre-crystallised whey is then dried by using either drum/roller dryers or multi-stage spray dryers. Drum/roller drying is much cheaper that spray drying. However, drum drying has been mostly phased out as it causes undesirable heat damage to the whey proteins affecting the functional properties and applications of resultant powders. Also drum dryers are of lower capacity making it difficult to handle large volume of whey. The multi stage spray dryer improves the functionality of the product in which the powder particles are large and have low bulk density and these powders are non-hygroscopic and easily wettable.

A spray drying system consists of at least four main components, which include supply of drying air/gas and heating system, atomization system, drying chamber and powder separator. All spray dryers use some type of atomizer or spray nozzle that disperses the liquid into a controlled drop size spray within the drying chamber (Bhandari *et al.*, 2008). As the concentrated whey, which contains crystallized lactose, is passed through the top of the dryer, as shown in Fig. 3.6, it is atomized producing a range of droplet sizes in the drying chamber. Rotary disc atomizers are commonly used for drying whey products as the crystallized whey contains particles that may block the nozzle-type atomizers (de Wit, 2001). The moisture in the small droplets is driven off quickly as it makes contact with the heated air. Inlet and outlet air temperatures typically used for drying standard whey powders are 160–180 °C and 80–90 °C, respectively (Jelen, 2009). The dried droplets (~6 % moisture) then fall to the bottom of the chamber where they are transported from the drying chamber to either powder handling/packaging system or to an external fluidized bed. Spray dryers used for drying whey products range from the simplest single-stage drier to two-stage and possibly three-stage drying systems and finally to filtermat dryers (Písecký, 2005). The spray dried

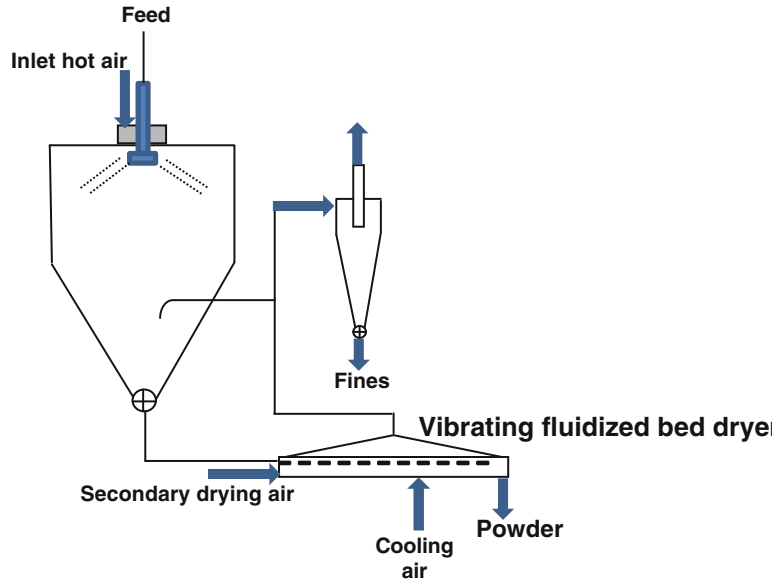


Fig. 3.6 Schematic diagram of a typical two-stage spray dryer

powder exiting the single stage spray dryer will still have around 20–25 % amorphous lactose which is hygroscopic. In a two-stage drying process, post-crystallisation of remaining amorphous lactose and final drying takes place in an external vibrating fluidised bed, reducing the moisture content of the powder further to about 3–4 % (Bhandari *et al.*, 2008). In a three-stage process the second drying stage is on an integral fluid bed within the spray dryer chamber and final drying is in the vibrating fluidized bed (i.e. third stage) located outside the chamber. In an alternative three-stage drying set up, the last two drying stages may occur on a conveyor belt system at the base of the chamber. The choice of the system depends on the physicochemical properties of the product being dried and the required physical stability and quality of the resultant powder.

3.4.2.2 Acid Whey Powder

The process of producing acid whey powder follows the same process outlined above with minor additional pretreatments. Because of the presence of lactic acid which is thermoplastic and also hygroscopic, it is difficult to dry acid whey. Whey with more than 2 % lactic acid (dry basis) poses difficulty on drying due to the depression of the glass transition temperature of lactose by lactic acid (Bhandari, 2012). Additives such as $Mg(OH)_2$ and $Ca(OH)_2$ may be added to neutralise the lactic acid and also during the drying operation, a free-flowing agent, such as sodium silicate or magnesium silicate, can be added to assist with the drying process (Bylund, 2003; Kilara, 2009). Such neutralised and dried whey products are normally used for animal feed.

3.4.2.3 Delactosed Whey Powder

The lactose content of whey can be reduced either by its selective removal or hydolysis. For selective removal, crystallization of lactose in whey concentrated to 65 % solids can be induced by cooling and seeding. Lactose crystals are then removed using a decanter centrifuge (de Wit, 2001). Ultrafiltration and diafiltration can also be employed to manufacture delactosed whey (Kilara, 2009). Alternatively, lactose can be hydrolysed into galactose and glucose using β-galactosidase. This enzyme is inactivated by heating the whey concentrate during subsequent processing. For continuous systems, the enzyme can be immobilised (Bylund, 2003). Typical composition of delactosed whey powder is given in Table 3.2.

3.4.2.4 Demineralized Whey Powder

The mineral content of whey powder (7.5–12 %, Table 3.2) is considered to be high. This high mineral content gives whey powder a salty taste, poses problems during processing of whey and

limits the application of whey powder in the food industry. Partial (~70 %) or extensive (~90 %) removal of these inorganic salts as well as reducing the content of organic ions such as lactates and citrates enables the use of whey powders in a wide range of products. The process of removing these salts is called demineralisation or desalination (Gesan-Guiziou, 2010). The resultant powder is known as demineralised or reduced-minerals whey powder. The unit operations, nanofiltration, ion-exchange, electrodialysis or a combination thereof, are employed in the production of demineralised whey before it is then concentrated and dried are described below.

Nanofiltration

Nanofiltration is a pressure-driven filtration process using a membrane that allows small particles, particularly monovalent ions, to permeate through. This process is common in the production of partially demineralized whey. Nanofiltration utilizes membranes of pore size ~ 1 nm and operates at a transmembrane pressure of about 0.6–4 MPa (Jeantet *et al.*, 2000). The separation of minerals is driven by both steric and electrostatic effects (Kilara, 2009; Gernigon *et al.*, 2011). Due to the differences in electrical potential across the membrane, polyvalent ions are retained in the whey while monovalent ions pass through the membrane into the permeate. Nanofiltration can reduce the mineral content of whey by 40–60 % and concomitantly increase its solids to 15–25 %. This technique can be used as a preliminary step to demineralize whey partially before it goes through the process of electrodialysis or ion exchange for extensive demineralisation, for deacidification of acid whey and for salt reduction in salted cheese whey (Bylund, 2003; Kilara, 2009; Gernigon *et al.*, 2011). Nanofiltration is a cheaper demineralization alternative to install and operate than ion exchange and electrodialysis and generates lesser amount of effluent (van der Horst *et al.*, 1995).

Electrodialysis

Electrodialysis is an electrochemical separation process in which ions are transferred through selective semi-permeable cation or anion ion exchange membranes from one solution to another under the influence of an electric field generated by applying direct current (Bazinet, 2005). An electrodialysis unit is made up of cell pairs consisting of alternating anionic and cationic membranes stacked together. The membranes have a pore size of 10–20 Å and are spaced about 1 mm or less apart. The two electrodes are placed at each end of the cell stack. One elctrodialysis unit can contain up to 500 cell pairs (Huffman and de Barros Ferreira, 2011).

The basic principle involved in electrodialysis is shown in Fig. 3.7. Alternating cell pairs act as concentration and dilution channels (Gernigon *et al.*, 2011). Whey and ion receiver solution (generally acidified 5 % brine solution) are fed in dilution and concentration channels, respectively. When a direct current is passed through the cell stack, ions try to migrate towards the electrode carrying opposite charge. However, the migration of ions is restricted by the membranes acting as barriers to ions of like charge. Anions can pass through an anionic membrane but are stopped by cationic membrane. Similarly, cations pass through a cationic membrane but are stopped by anionic membrane (Houldsworth, 1980). This restricted migration of ions under the influence of direct current leads to migration of ions from whey to a 5 % brine carrier solution thus causing whey demineralization. The degree of demineralization is dependent on factors such as ash content and viscosity of whey, current density and residence time in the electrodialysis unit (Bylund, 2003; Kilara, 2009).

Electrodialysis can operate either in batch mode (commonly used for 70 % or higher demineralization) or in a continuous (demineralization levels between 60 and 70 %) process. The major disadvantage of this process is that it is prone to mineral fouling on cationic membranes and protein fouling on anionic membranes and hence requires frequent cleaning (Gesan-Guiziou, 2010; Ramchandran and Vasiljevic, 2013).

Ion Exchange

Ion exchange involves the adsorption of minerals from a solution through resin in the form of macromolecular porous beads of diameter ~0.3–1.2 mm.

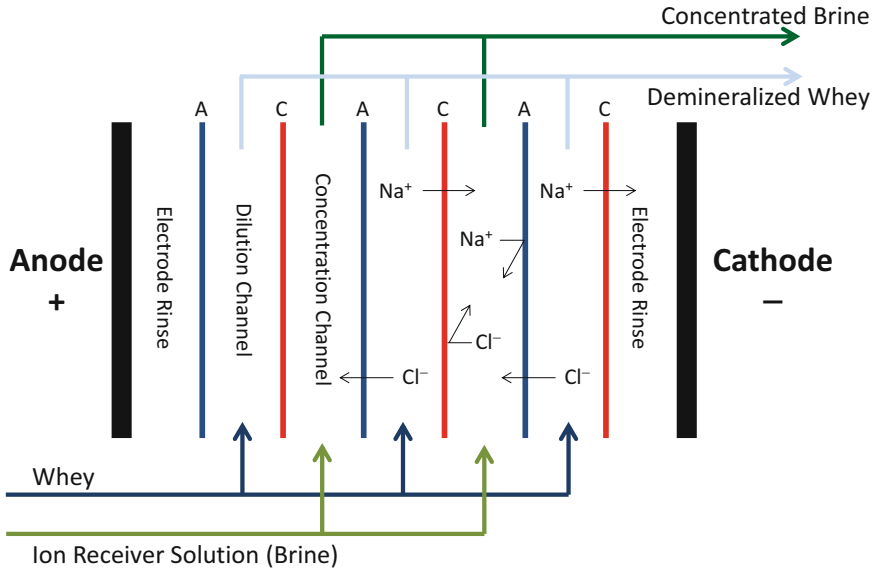


Fig. 3.7 Basic principle of electrodialysis of whey

Ion exchange resins are made up of polymeric materials such as polystyrene/divinyl benzene and polyacrylate. Functional groups such as sulphonic and carboxyl groups (cation exchange resin) and quaternary and tertiary amine groups (anion exchange resins) are chemically bound to these polymeric beads (Hoppe and Higgins, 1992). These functional groups act as insoluble acids or bases, which when converted to salt remain insoluble. These exchange resins are capable of exchanging the mobile ions that they contain for ions of the same charge contained in a solution. This exchange reaction is driven by the concentration of ionic species.

Conventional Ion Exchange Process

Demineralization of whey by ion exchange uses three steps: adsorption, desorption and regeneration. A schematic representation of a conventional ion exchange process is shown in Fig. 3.8. In a conventional demineralization set up, whey is passed through a strong cation exchanger followed by a weak anion exchanger. A cation exchange resin captures positively charged ions and an anion exchange resin negatively charged ions. In fresh resin, the cations attached to the cationic resin are H^+ and anions attached to the anionic resin are OH^-. Demineralization of whey occurs when the mineral ions are replaced with H^+ in the cationic column and these H^+ ions are then replaced by OH^- in the anionic columns. The capacity of the ion exchange process is limited by the amount of ions attached to the resins. Once the resins are saturated, regeneration is done by removing the adsorbed ions from the cationic and anionic exchangers and replacing them with hydrogen and hydroxyl ions, respectively. Hydrochloric acid and sodium hydroxide solutions are generally used to regenerate cationic and anionic resins, respectively. To reduce the consumption of regeneration chemicals, regeneration of resins is carried out using a counter current flow. Before the regeneration is done, the resin beads are back-flushed with water to recover demineralized whey. Ion exchange can yield demineralization up to 92–97 %. Two or three parallel ion exchange systems are used for continuous flow of whey. Several authors have reviewed demineralisation of whey by ion exchange process (Hoppe and Higgins, 1992;

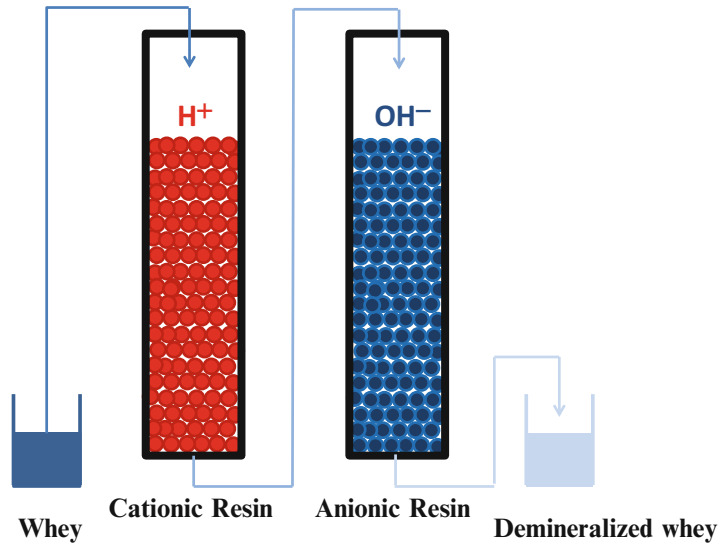


Fig. 3.8 Schematic representation of a conventional ion exchange process for whey demineralization

Table 3.3 Difference in % reduction in mineral content of whey processed by nanfiltration, electrodialysis and ion exchange (Hoppe and Higgins, 1992; van der Horst *et al*., 1995)

	Nanofiltration (45 % demineralization)	Electrodialysis (40 % demineralization)	Electrodialysis (60 % demineralization)	Ion Exchange (95 % demineralization)
Ions	% Reduction			
K^+, Na^+	65	42	64	98
Ca^{2+}	6	24	35	99
Cl^-	54	71	89	95

Greiter *et al*., 2002; Bylund, 2003; Killara and Vaghela, 2004; Kilara, 2009; Gesan-Guiziou, 2010; Huffman and de Barros Ferreira, 2011; Ramchandran and Vasiljevic, 2013).

SMR Ion Exchange Process

To reduce the consumption of regeneration chemicals, the Swedish Dairies Association, SMR, has developed an alternative ion exchange process. In the SMR approach, whey is first passed through a weak anion exchange resin containing HCO_3^- ions and then through a weak cation exchange resin containing NH_4^+ ions. This is in reverse order compared to the conventional ion exchange process. After the process, the salts in whey are thus exchanged for ammonium bicarbonate. Being a thermolytic salt, ammonium bicarbonate is converted into NH_3, CO_2 and H_2O during heating in the subsequent evaporation step. NH_3 and CO_2 are recovered to make new regeneration solution (Durham and Hourigan, 2007). The SMR process is explained in greater detail by Bylund (2003). Some of the advantages of the SMR process over conventional demineralization processes include lower operating costs, higher yield of whey solids and less denaturation of whey proteins during processing.

A comparison of reduction is minerals in whey using nanofiltration, ion exchange or electrodialysis processes is given in Table 3.3.

3.5 Whey Protein Concentrates

The products referred to as whey protein concentrates (WPC) are obtained from whey from which lactose, minerals and water have been removed so that the final dried product has a minimum protein

content of 25 %. Techniques that are commonly used in the process of protein concentration are ultrafiltration and diafiltration. The United States Code of Federal Regulations (21CFR184.1979) has defined WPC as "the substance obtained by the removal of sufficient nonprotein constituents from whey so that the finished dry product contains not less than 25 % protein. Whey protein concentrate is produced by separation techniques such as precipitation, filtration or dialysis. As with whey, whey protein concentrate can be used as a fluid, concentrate or dry powder form. The acidity of whey protein concentrate may be adjusted by the addition of safe and suitable pH-adjusting ingredients".

3.5.1 Types of Whey Protein Concentrates

WPC is usually characterised based on the amount of protein in dry matter it contains. For example WPC-35 contains 34–35 % protein in dry matter. Typically the protein content in WPC powders ranges from 35 to 80 % (de Wit, 2001; Bylund, 2003; Gesan-Guiziou, 2010). A few types of whey protein concentrate that are currently available in the market are briefly explained below.

3.5.1.1 Whey Protein Concentrate 35 (WPC35)

This product contains 35 % of protein in solids and contains some minerals and lactose. The manufacturing process includes clarification, defatting and ultrafiltration before it is concentrated and dried.

3.5.1.2 Whey Protein Concentrate 50 (WPC50)

This product contains 55 % of protein in solids and its manufacturing process is similar to that used for WPC35. However, it is also subjected to an additional process of diafiltration before concentration and drying.

3.5.1.3 Whey Protein Concentrate 80 (WPC80)

This product contains ~80 % of protein in solids and undergoes several diafiltration steps during manufacturing resulting in more lactose and minerals being washed out which increases its protein content.

The composition of the most commonly available whey protein concentrates is outlined in Table 3.4.

3.5.2 Production of Whey Protein Concentrates

WPC are produced utilizing membrane separation techniques including microfiltration, ultrafiltration and sometimes nanofiltration (de Wit, 2001; Bylund, 2003; Ramchandran and Vasiljevic, 2013). The process involving ultrafiltration is commonly employed in the production of whey protein concentrates; however, an additional diafiltration process needs to be carried out when producing WPC with protein content >50 %. This process involves the washing out of low molecular weight components such as lactose and ash using water during the filtration process. A typical process line for the production of whey protein concentrates is shown in Fig. 3.9.

Table 3.4 Typical composition of WPC powders (%) (Grinstead *et al.*, 2000; de Wit, 2001)

	WPC35	WPC50	WPC65	WPC80
Lactose	46.5	30.9	21.1	3.5
Protein (crude)	36.2	52.1	63.0	81.0
Protein (true)	29.7	40.9	59.4	75.0
Moisture	4.6	4.3	4.2	4.0
Ash	7.8	6.4	3.9	3.1
Lactic acid	2.8	2.6	2.2	1.2
Fat	2.1	3.7	5.6	7.2

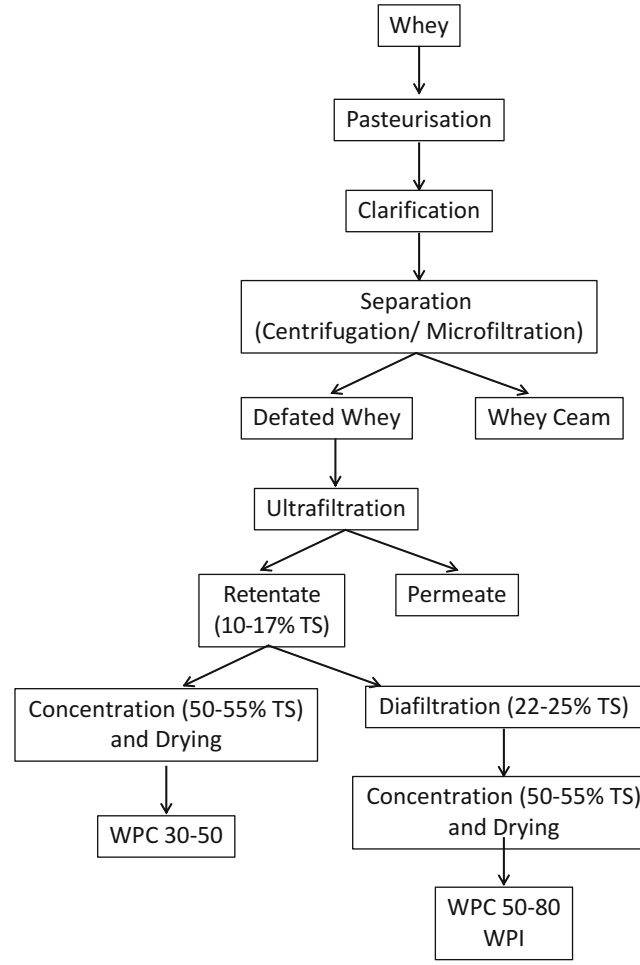


Fig. 3.9 A typical flow chart for producing whey protein concentrates (WPC) and whey protein isolate (WPI)

3.5.2.1 Pre-Treatment

Several pre-treatment processes need to be applied to whey before further processing in order to reduce fouling and improve flux during membrane processing and in some cases manipulate the chemical or functional properties of WPC which results in products with low fat content and improved functionality (Bylund, 2003). Firstly, the whey is clarified and separated using a self-desludging centrifugal clarifier and/or separator primarily to remove residual particles of casein fines, whey cream and the bacterial starter culture cells (in case of whey produced by fermentation). Alternatively, microfiltration may be used to remove fat from the whey (Ramchandran and Vasiljevic, 2013). Microfiltration is a low pressure membrane filtration technique in which ceramic or spiral wound membranes of pore size >0.1 μm are commonly used for whey processing. Fat and casein particles (fines) larger than this pore size are retained. De-fatted whey (i.e., the permeate) is then pasteurized. Precipitation of insoluble calcium phosphate during ultrafiltration is a major cause of membrane fouling (Hobman, 1992). To reduce fouling of membranes, decalcification of whey can be carried out by clarification at pH 7.5 (Musale and Kulkarni, 1998) or heating the whey to 60 °C and holding for 30 min prior to ultrafiltration at 50 °C (Hobman, 1992).

Whey can also be pre-concentrated prior to ultrafiltration and this has several advantages including reduction in costs of whey transportation, volume of storage and energy consumption, possibility of improved separation of fat, increased

retentate total solids in low protein WPC, increased permeate total solids and reduction in the amount of water to be removed during evaporation and drying (Hobman, 1992).

3.5.2.2 Ultrafiltration

Ultrafiltration is a medium pressure physicochemical separation technique in which whey is passed over membranes of pore size 0.001–0.1 μm and molecular mass cut off of 1–200 kDa (García-Garibay *et al*., 2008). The membranes facilitate the separation of small molecules such as lactose, salts and water as permeate from whey proteins, fat globules and suspended solids, which are concentrated relative to other solutes in the retentate. There are four basic membrane designs that are currently used for ultrafiltration: tubular, hollow-fibre, spiral wound and plate and frame. Of these, spiral wound and hollow-fibre designs are more economical (Ramchandran and Vasiljevic, 2013). The optimal temperature at which ultrafiltration is carried out is around 50 °C to a maximum of 55 °C. This is the temperature range in which problems associated with membrane fouling, thermal denaturation of protein and growth of microorganisms can be avoided and acceptable fluxes are achieved (Hobman, 1992; de Wit, 2001; Bylund, 2003). Multiple ultrafiltration units are used in sequence and can increase the total solids of liquid whey up to 10–17 %. As described above, a process of diafiltration is necessary to produce products with protein content (in total solids) greater than 50 %. In diafiltration, the retentate is diluted with water to facilitate increased removal of membrane-permeable molecules. The quantity of water added to retentate ranges from 40 to 95 % of the permeate flow and is dependent on the design of the plant. The number of diafiltration steps used is dictated by the protein content required in the WPC. Diafiltration requires the use of good quality water which is demineralized or softened to avoid problems during processing. The presence of manganese, iron, calcium, silica, aluminium and in some cases chlorine in diafiltration water may cause membrane fouling therefore the absence or appropriate reduction of these minerals in the water to be used in diafiltration is vital (Hobman, 1992).

3.5.2.3 Concentration and Drying

Pasteurization of the retentate may be necessary to reduce microbiological load as the ultrafiltration process for a product containing 85 % protein may have concentrated bacterial cells in the whey (Hobman, 1992; Deeth and Hartanto, 2009).

Concentration, using a falling film evaporator that has a low boiling temperature and high vacuum, is done before drying to reduce the cost of water removal and improve the physical properties of the powder. Drying is then performed using a spray dryer. The inlet and outlet air temperature used are between 160 and 180 °C and less than 80 °C, respectively.

3.6 Whey Protein Isolate

Whey protein ingredients containing ≥90 % protein are known as whey protein isolate (WPI). WPI powder contains 4–6 % moisture, 0.2–2.0 % lactose, 0.2–1.5 % fat and 0.3–4.5 % ash (Morr and Ha, 1993; Foegeding *et al*., 2011). The main difference between WPC and WPI is that WPI contains a higher protein and proportionately lower lactose and minerals content than WPC.

3.6.1 Production of Whey Protein Isolate

To produce WPI, whey is skimmed by centrifugation or microfiltration and demineralized by ion exchange, electrodialysis or nanofiltration. The proteins are concentrated by membrane filtration or ion–exchange chromatographic methods.

3.6.1.1 Ion Exchange Methods

At pH values lower than their isoelectric points (~pH 4.6), amphoteric whey proteins have a net positive charge and behave as cations that can be adsorbed onto ion exchangers. At pH values above their isoelectric points, the proteins have a net negative charge and behave as anions allowing fractionation to be achieved by anion exchangers. In ion exchange methods, pH of the whey is adjusted to provide the required charge on the protein molecules. pH-adjusted whey is passed

through the ion exchanger to adsorb the protein molecules and the deproteinized whey is eluted from the reactor. The pH is readjusted to desorb the proteins. The desorbed proteins are eluted from the ion exchanger, concentrated and spray-dried.

The cellulose-based polyelectrolytes exchanger process was one of the earliest successful methods to recover 90 % of whey proteins. By increasing the charge density in carboxymethyl cellulose (CMC), it is possible to increase yield of protein (Hill and Zadow, 1974). The Vistec Protein Recovery Process, an improved process utilising a CMC derivative, yielded a final dry product containing 97 % protein, 3 % ash, 0.2 % lactose and 0.2 % fat (Palmer, 1977). The process involves acidification of whey to pH < 4.6 and feeding into a continuously stirred tank reactor for adsorption. After changing the pH to moderate alkalinity, protein desorption occurs and proteins are discharged in the eluate. The dilute eluate is concentrated using ultrafiltration and evaporation and is then dried. Drawbacks of the Vistec process include low protein-binding capacity (52 mg/g) and low protein recovery (60 %) (Pearce, 1992b). Using a similar concept but with sulphopropyl cellulose material, gave performance similar to CMC derivative with much lower requirement for regenerative acid/base (Ayers and Petersen, 1985).

The Spherosil (packed column) process uses silica-based fixed-bed ion-exchangers, which do not swell or contract with changes in pH and ionic strength, do not compress under pressure and are stable chemically and physically (Pearce, 1992b). These resins consist of silica beads that are coated with polymeric material containing an appropriate functional exchange group. Spherosil-S is a strongly acidic cation resin and has $–SO_3H$ groups, while Spherosil-QMA can be used as a strongly basic anion resin and has $–N(CH_3)_3^+$ groups (Grandison and Lewis, 1996). Proteins in cationic form in acidic whey are adsorbed on Spherosil-S column and are eluted using ammonium hydroxide. In Spherosil-QMA, anionic proteins from sweet whey are adsorbed and hydrochloric acid is used for elution (Kilara, 2009). Compared to the Vistec process, the Spherosil process suffers from drawbacks such as high costs of exchangers and lower protein binding capacity (79–100 mg/g) compared to CMC (200–500 mg/g) (Kilara, 2009). The anion exchange offers poor fractionation of lactoferrin (LF), lactoperoxidase (LP) and immunoglobulins (Igs), but these could be recovered in a second stage using a weak cationic resin with –COOH groups and eluted with ammonium hydroxide (Pearce, 1992b).

Silica-based ion exchangers have several limitations, including irreversible protein adsorption due to slightly hydrophobic properties, limited flow rates due to slow diffusion of protein into the beads and chemical instability towards strongly alkaline solutions often used for cleaning dairy equipment (Doultani *et al.*, 2003). To overcome these problems, some researchers have utilised agarose-based ion exchange systems for protein recovery from whey (Doultani *et al.*, 2003; Turhan and Etzel, 2004).

The extra steps required to concentrate and purify the dilute protein-containing eluate of protein-rich whey can be time-consuming with additional risk of microbial exposure and growth. Regeneration of the resin can be difficult when the feed is of low quality due to high ion content that binds to the surface of the resin. After several processing cycles, regeneration of exchangers with strong acids and alkalis is required. These complications result in high operating costs. Lan *et al.* (2002) tried to overcome the limitations of ion-exchange systems by using a liquid-solid circulating fluidized bed (LSCFB) ion exchanger that can operate continuously with simultaneous regeneration of resin. Recovery of up to 90 % was achieved by controlling whey feed rate, concentration and solids circulation rate. However, protein binding capacity and performance stability in continuous operation after long term use were not evaluated.

3.6.1.2 Membrane Method

Membrane processing of whey followed by intensive diafiltration is another process to obtain WPI. Lipid removal is essential in this method as it affects functionality and efficiency of downstream

Table 3.5 Protein composition (% w/w) of WPI powders produced by microfiltration (MF) or ion exchange (IE) method

Protein (% w/w)	MF-WPI	IE-WPI
α-Lactalbumin	13	11
β-Lactoglobulin	41	71
Bovine serum albumin	1.2	2.6
IgG	3.5	3.3
Proteose peptone	5.1	0.3
Glycomacropeptide	17	0.4

process (Melachouris, 1984). The membrane method to obtain WPI is similar to that utilised for WPC (Fig. 3.8). Ultrafiltration and extensive diafiltration concentrate the proteins in defatted whey, which is then concentrated and dried.

The differences in protein composition of WPI powders produced by ion exchange or membrane based approaches are given in Table 3.5. WPI produced by an ion exchange method is generally free from glycomacropeptide (GMP). A more thorough recovery of whole protein fraction can be achieved by using two exchangers. Doultani *et al*. (2003) explored the feasibility of using agarose bead cation and anion exchangers in series to extract the principal whey proteins and GMP from whey and achieved ~93 and 98 % recovery of principal proteins and GMP, respectively.

Recently, a process to produce liquid virgin whey protein isolate from skim milk has been described by Marcelo and Rizvi (2008). In their protocol, acidified skim milk (pH 6.0) was first microfiltered using 0.1 μm tubular ceramic membranes to obtain a permeate rich in native whey proteins (i.e. virgin whey). The virgin whey was concentrated and fractioned in a two-step process using a combination of membrane separation techniques. In the first step, the whey was concentrated to ~11 % total solids using spiral wound polysulphone membranes (molecular weight cut off 10 kDa). The whey proteins in the UF retentate were concentrated from ~3.8 to ~6.3 % using diafiltration. In the second step, the protein-rich diafiltered whey was further concentrated by ultrafiltration using polysulphone membranes in hollow fibre configuration. The resulting liquid virgin whey protein isolate had ~24 % whey proteins in 26 % total solids, which is equivalent to >90 % whey proteins on dry matter basis (Marcelo and Rizvi, 2008). The commercial feasibility of this process has not yet been reported.

3.7 Whey Protein Fractions

The major whey proteins include β-lactoglobulin, β-lg (~50 % of total whey protein), α-lactalbumin, α-lac (~25 %), bovine serum albumin, BSA (~6 %) and I_{gs} (~10 %). The other important whey protein fractions are LF, GMP and LP. These fractions differ in their molecular mass, isoelectric points, denaturation properties, chemical reactivity, binding ability, surface charge distribution, polarity, ionic and colloidal behaviour and are fractionated based on these differences (Mulvihill and Ennis, 2003; Bonnaillie and Tomasula, 2009).

3.7.1 Methods of Fractionation

The methods for fractionation of whey proteins can be classified into four different categories: (1) selective precipitation, (2) membrane filtration, (3) selective adsorption and (4) selective elution (Etzel, 2004). In selective precipitation the physical properties of whey protein solutions such as pH and ionic strength are adjusted to promote selective insolubilisation of a particular whey fraction. Heat and manipulation of mineral levels may be used to aid the precipitation process. Membrane filtration processes utilise membranes of specific pore size to fractionate proteins based on the differences in their molecular mass. pH and ionic strength of the solutions may be adjusted to enhance separation where the difference in the molecular mass is not that large. In selective adsorption, the specific target protein fraction is adsorbed using specific immobilised ligands. Whereas, in selective elution, all protein fractions in a mixture are adsorbed on an adsorbent and are then eluted one by one as separate fractions (Etzel, 2004).

3.7.1.1 Fractionation of α-lac and β-lg

Several methods have been reported to fractionate major whey proteins such as heat precipitation (Pearce, 1983), chemical precipitation (Fox *et al.*, 1967), salting-out followed by electrophoresis (Aschaffenburg and Drewry, 1957), chromatography (Chaplin, 1986), membrane separation (Mehra and Donnelly, 1993; Kelly *et al.*, 2000) and aqueous two phase partitioning (Ortin *et al.*, 1992). These techniques either provide exceptional purity with low scale production or low recovery and purity with potential for large scale processing. Some of the recently published novel approaches that can potentially provide scalability while yielding high purity are discussed here.

Membrane Filtration

Membrane filtration processes provide feasibility of large scale production of whey protein fractions and hence, reducing their cost of production. This technique separates proteins based on their molecular mass, but this can be challenging when the differences in molecular masses are not that large. Other commonly confronted problems in filtration technology are membrane damage, fouling, protein or contaminant adsorption, concentration polarization and protein-protein interactions. To resolve some of these issues, high performance tangential flow filtration has been described as a technology that can separate proteins with similar size using semipermeable membranes by exploiting both size and charge properties of the proteins (van Reis *et al.*, 1997; van Reis *et al.*, 1999; van Reis and Zydney, 2001). Optimal separation and high efficiency can be obtained by operating in pressure-dependent flux regime, exploiting effects of concentration polarization, selecting optimal buffer pH and ionic strength and using charged membranes (Saksena and Zydney, 1994; van Reis *et al.*, 1997; van Reis *et al.*, 1999; van Reis and Zydney, 2001).

Selective Elution

Combining anion exchange with gel filtration chromatography can prove to be an important tool in isolating protein fractions by utilising partitioning ability of protein mixtures to mobile or stationary phases. Neyestani *et al.* (2003) were able to fractionate highly purified α-lac, β-lg and BSA with well-preserved antigenicity by using a combination of selective saturation with ammonium sulphate, gel filtration and anion-exchange chromatography. This method of obtaining high purity protein fractions is simple, accessible and less expensive compared to some industrial processes, but is most suitable for small to medium-scale applications.

Immobilized metal ion affinity chromatography (IMAC), a technique which can potentially be scaled up, has been explored for separation of whey protein fractions (Blomkalns and Gomez, 1997). IMAC is based on the binding capacity of proteins to divalent metal ions. IMAC uses a stationary phase with immobilized metal ions. Proteins interact with these metal ions *via* histidine, cysteine and tryptophan residues and are eluted by modifying conditions of the mobile phase (Porath *et al.*, 1975; Porath, 1992; Blomkalns and Gomez, 1997). Blomkalns and Gomez (1997) isolated α-lac from WPC with 90 % purity and 80 % recovery using a Cu(II)-chelating Sepharose FastFlow column. α-lac was selectively eluted by stepwise pH change in the mobile phase from pH 5.5 to pH 3.8.

Chemical and Physical Treatments

Controlled proteolysis by treatment with enzymes such as trypsin and pepsin can change the chemical and physical structure of proteins, thereby altering their solubility and digestibility (Otani, 1981; Schmidt and Poll, 1991; Dalgalarrondo *et al.*, 1995; Guo *et al.*, 1995; Svenning *et al.*, 2000). The differences in resistance to enzymatic digestion has been utilised for fractionation of α-lactalbumin and β-lactoglobulin (Konrad *et al.*, 2000; Konrad and Kleinschmidt, 2008). By utilising a combination of enzymatic treatment and membrane separation, pure β-lactoglobulin with up to 67 % yield and 90–95 % purity and α-lactalbumin with 15 % yield could be obtained (Konrad *et al.*, 2000; Konrad and Kleinschmidt, 2008). Similarly, 81 % of α-lactalbumin present in WPI could be recovered without any traces of β-lactoglobulin by digestion of WPI with α-chymotrypsin (Lisak *et al.*, 2013).

Supercritical carbon dioxide has also been used to precipitate selectively α-lactalbumin from WPI to produce α-lactalbumin- and β-lactoglobulin-enriched protein fractions (Yver *et al*., 2011). Under most economical calculated conditions, 57 % pure α-lactalbumin with 71 % yield and β-lactoglobulin with 89 % yield could be obtained in the solid and soluble fractions, respectively. Recovery and yield of proteins increased with temperature and carbon dioxide pressure (Yver *et al*., 2011).

Tolkach *et al*. (2005) have also obtained native α-lactalbumin with 98 % purity and 75 % recovery by selective thermal denaturation of β-lactoglobulin. The total protein, lactose and calcium content, and pH of starting material (WPC) were optimised to denature β-lactoglobulin selectively by heating. High purity α-lactalbumin was then obtained by removing denatured β-lactoglobulin using microfiltration.

3.7.1.2 Fractionation of Minor Proteins

Several methods that are used for fractionation of lactoferrin and lactoperoxidase are membrane adsorption, size-exclusion, affinity and hydrophobic interaction chromatography and ion-exchange chromatography, with the latter being the most widely used method commercially.

Ion Exchange Chromatography

Lactoferrin and lactoperoxidase can be isolated directly from milk or from whey. Given the cationic nature of these proteins, cation exchange chromatography (CEC) has been extensively researched for their fractionation (Yoshida and Ye, 1991; Ye *et al*., 2000; Fee and Chand, 2006; Lu *et al*., 2007; Du *et al*., 2013).

Yoshida and Ye (1991) fractionated lactoferrin and lactoperoxidase from the albumin fraction of acid whey at lab scale using CEC. Acid whey was first subjected to selective precipitation of globulins using ammonium sulfate, followed by hydrophobic interaction chromatography for adsorption of all the proteins. The adsorbed proteins were eluted using deionized water and subjected to CEC. Lactoperoxidase and lactoferrin were eluted from the CEC column using a linear gradient of NaCl from 0.10 to 0.15 M and 0.4 to 0.55 M, respectively. Similarly, lactoferrin and lactoperoxidase can be recovered from rennet whey using a strong cation exchange resin (Ye *et al*., 2000). Although utilised at lab scale only, Fee and Chand (2006) isolated lactoferrin and lactoperoxidase directly from raw, untreated milk using SP Sepharose Big Beads™. Their method did not require any pre-treatments to separate fat and casein prior to lactoferrin fractionation which has the potential to increase the yield and bioactivity of fractionated proteins. Similarly, lactoferrin could be fractionated from milk or acid whey without pre-treatment using a macroporous cationic cryogel monolithic column with a pore size of 100 μm (Billakanti and Fee, 2009). A yield of over 85 % and a purity of more than 90 % were achieved for lactoferrin. Most recently, Du *et al*. (2013) proposed a single step procedure for purifying lactoferrin from crude sweet whey using an expanded bed adsorption process with cation exchange resin; lactoferrin with ~88 % purity and ~77 % recovery was obtained in a single step.

Andersson and Mattiasson (2006) suggested that compared to conventional chromatographic techniques, simulated moving bed chromatography can enhance the productivity and product concentration and improve the process efficiency of fractionating lactoferrin and lactoperoxidase from WPC.

Lactoferrin has been isolated at production scale from skim colostrum by combining ultrafiltration with CEC (Lu *et al*., 2007). Lactoferrin was first isolated and concentrated from colostrum using two-stage tangential flow ultrafiltration. Crude lactoferrin so obtained was further purified using strong CEC. The resulting product had purity and recovery of ~94 % and ~82 %, respectively (Lu *et al*., 2007).

Hydrophobic Interaction Chromatography

LF can be isolated from WPC by hydrophobic interaction chromatography (HIC) where non-polar regions on protein molecules interact with immobilised hydrophobic ligands (Santos *et al*., 2011). Using similar concept (Alvarez-Guerra and Irabien, 2012), have proposed a novel approach to extract lactoferrin from whey using hydrophobic ionic liquids. It was suggested that

this technique has higher capacity, better selectivity, higher yields and higher purities at a lower cost. Imidazolium-based ionic liquids were used and fractionation was achieved by the interaction between the iron of LF and the cationic imidazole-based moiety of the ionic liquid (Alvarez-Guerra and Irabien, 2012).

3.8 Whey Protein Hydrolysates

Hydrolysis of whey proteins is utilised as a method to change the molecular, nutritional, functional and sensory properties of the proteins. Hydrolysis breaks down proteins into peptides of different sizes and free amino acids, as a result of cleavage of peptide bonds. Proteins can be hydrolysed using enzymes, acids or alkali. Acid and alkaline hydrolysis processes are difficult to control and tend to yield products with reduced nutritional qualities. Compared with acid or alkali hydrolysis, enzymatic hydrolysis of protein using selective proteases can be controlled reasonably well, can be conducted under moderate conditions of pH (6–8) and temperature (40–60 °C) and produce few or no undesirable side reactions or products. Enzymatic modification of proteins to hydrolyse specific peptide bonds is widely used and results in peptides with lower molecular weight, less secondary structure, more ionisable groups and higher exposure of hydrophobic groups than the native proteins (Killara and Vaghela, 2004).

Whey protein hydrolysates have improved absorption in the gut and lower allergenicity compared to intact proteins (Foegeding *et al*., 2011). Hence, the major nutritional application of whey protein hydrolysates is in hypoallergenic infant formulae and nutritional products such as sports nutrition drinks, nutritional bars and enteral formulae (Boland, 2011; Foegeding *et al*., 2011). Enzymatic hydrolysis also improves the functional properties of whey proteins. Compared to native whey proteins, whey protein hydrolysates have increased solubility over a wide range of pH, decreased viscosity, improved water adsorption capacity, improved emulsifying (for hydrolysates with lower degree of hydrolysis) and surface active properties, improved foamability (but lower foam stability) and increased thermal stability (Kuehler and Stine, 1974; Chobert *et al*., 1988; Gauthier *et al*., 1993; Althouse *et al*., 1995; Ju *et al*., 1995; Mutilangi *et al*., 1996; Singh and Dalgleish, 1998; Severin and Xia, 2006; Sinha *et al*., 2007). The surface active properties of commercial whey protein hydrolysates measured in our laboratory (Xu, 2013) are given in Table 3.6. Hydrolysis resulted into increased surface activity (indicated by a reduction of surface tension at the air-water interface) of the partially hydrolysed proteins. Higher degree of hydrolysis also showed higher surface activity of the proteins.

The nutritional and functional properties of whey protein hydrolysates are dependent on the substrate (starting protein material, extent of denaturation), enzyme (enzyme used and its specificity), conditions of hydrolysis (enzyme concentration, pH, temperature, ionic strength, presence or absence of inhibitors) and degree or extent of hydrolysis. Whey protein hydrolysates rich in low molecular weight peptides, especially di- and tripeptides, with as little free amino acids as possible, have high nutritional and therapeutic value (Vijayalakshmi *et al*., 1986). High molecular

Table 3.6 Surface tension (mN/m) of whey protein isolate (WPI) and hydrolysed whey protein (HWP) solutions (2 g/L) at various temperatures

	Surface tension (mN/m)				
Temp	Water	WPI (0 % hydrolysis)	HWP (4 % hydrolysis)	HWP (9.5 % hydrolysis)	HWP (20 % hydrolysis)
24 °C	71.4	62.7	56.1	52.6	52.6
40 °C	69.6	60.9	53.1	51.3	44.4
50 °C	67.9	58.2	51.8	50.4	42.0
60 °C	66.2	54.3	50.1	48.7	39.5

weight peptides (more than 20 amino acid residues) and hence, lower levels of hydrolysis, are associated with the improvement of functionality of hydrolysates. Thus, irrespective of the application of protein hydrolysates, it is always important to characterize them on the basis of their peptide size (i.e. molecular weight profiling) and degree of hydrolysis (Silvestre, 1997).

3.8.1 Method of Manufacture

Several proteolytic enzymes have been utilised to produce whey protein hydrolysates. Some of the common enzymes used are alcalase, neutrase, trypsin, papain, subtilisin carlsberg, chymotrypsin and bromelain (Althouse *et al.*, 1995; Ju *et al.*, 1995; Mutilangi *et al.*, 1996; Sinha *et al.*, 2007).

3.8.1.1 Batch Reactors

The most common method of production of whey protein hydrolysates is batch reaction in which the whey protein substrate is incubated with proteolytic enzymes at elevated temperatures (40–60 °C). Degree of hydrolysis is carefully monitored using pH stat and when the desired degree of hydrolysis is achieved, the reaction is stopped by using appropriate combination of pH, temperature and time (Boland, 2011) or using specific enzyme inhibitors (Kuipers *et al.*, 2007). In industrial production, the mixture of enzymes and processing conditions used vary from manufacturer to manufacturer and is usually proprietary information. The manufacturing process is targeted to avoid bitter flavour and wide spread of molecular weights of peptides.

3.8.1.2 Continuous Reactors

Guadix *et al.* (2006) detailed the production of whey protein hydrolysates using a continuous stirred tank membrane reactor (CSTMR). In this study, whey proteins were hydrolysed at 50 °C and pH 8.5 in a continuous stirred tank membrane reactor including a polyethersulfone plate and frame ultrafiltration module with a molecular weight cut-off of 3 kDa. The CSTMR separated products from the reaction media to increase the yield. The soluble enzyme and the substrate are confined to the retentate side of the membrane, while the products are small enough to permeate through the membrane. CSTMR allows reuse of the enzyme and allows control of the molecular weight of the product by selecting the appropriate membrane pore size (Guadix *et al.*, 2006). CSTMR was found to be a successful technique for the stable, continuous production of whey protein hydrolysate with as high as 80 % substrate conversion.

3.8.1.3 Membrane Technology

Peptides can also be separated from tryptic hydrolysates of whey proteins with charged ultrafiltration/nanofiltration membranes (Pouliot *et al.*, 1999). In this process, commercial WPI was hydrolysed by trypsin, followed by ultrafiltration treatment to remove the enzyme and non-hydrolyzed material from the reaction mixture. It was found that the membrane separation of peptides was mainly based on charge effects and the size of peptides was not the predominating factor. Five polymeric (polyamide and cellulose acetate) charged membranes were investigated and the best results were obtained with a cellulose acetate membrane with a cutoff point between 1000 and 5000 Da. Charged membranes have also been utilised to separate peptides that are naturally present in whey (Recio *et al.*, 2000).

3.9 Microparticulated Whey Protein

The structural and functional properties of whey proteins can be modified by the application of heat. These modifications can improve the functionality of whey protein ingredients in the food industry. Upon heating, denaturation of whey proteins proceeds in three stages (Sanchez *et al.*, 1997). In the first (i.e., denaturation) stage, globular native proteins partially unfold to expose reactive groups that were initially buried inside the macromolecules. In the second (i.e., initiation) stage, partially unfolded molecules interact through noncovalent (e.g., van der Waals and hydrophobic interactions, hydrogen bonds) and covalent (e.g., intermolecular disulfide exchange

reactions) interactions to form aggregates (Sanchez *et al*., 1997). Under quiescent heating conditions of a solution with sufficient protein concentration, denatured protein-aggregate and aggregate-aggregate interactions lead to formation of a continuous three-dimensional gel network in the third (i.e., propagation) stage. If the protein concentrate, however, is sheared simultaneously while heating, the shear forces prevent the formation of a connected gel network and individual protein aggregates are produced. This process is known as microparticulation (Spiegel, 1999). The balance between shear-controlled aggregate growth and shear-induced aggregate break-up defines the aggregate properties during the microparticulation process (Steventon *et al*., 1994).

These engineered protein aggregates impart mouthfeel characteristics similar to fat particles and hence can improve the texture and creaminess of several dairy products such as ice-cream, frozen desserts, yogurt, fermented dairy drinks, flavoured dairy drinks and cheese (Singer and Dunn, 1990; Barrantes *et al*., 1994; Tamime *et al*., 1995; Muir *et al*., 1999; Spiegel, 1999). They are used as fat replacers, texturing or stabilizing agents for ready-to-eat food dispersions or emulsions, and as microcapsules for cosmetic, pharmaceutical or medical purposes (Renard *et al*., 1999).

3.9.1 Methods of Manufacture

Microparticulated whey protein ingredients are produced by simultaneous heat and shear treatment, by high pressure homogenization of a preheated whey protein concentrate or by extrusion cooking in the acid pH range.

3.9.1.1 Heat and Shear Methods

Singer and Dunn (1990) have provided details of the principle and process of manufacturing microparticulated whey proteins (Simplesse®) by simultaneous heating and shearing. The protein solution is first prepared by completely dissolving and dispersing all ingredients. The mix is then heated in a conventional heat exchanger to temperatures just below the coagulation temperature of the proteins. The warm mixture is then simultaneously heated and sheared in a homogenising pasteuriser in less than 10 s. This process leads to formation of protein aggregates in the diameter range of 0.1–3 μm with an average diameter of 1 μm. The product is then cooled to give a creamy, smooth and opaque semisolid dispersion of particles (Singer and Dunn, 1990). The size of the particles will be influenced by conditions of both shearing and heating (Steventon *et al*., 1994; Walkenström *et al*., 1998).

Another device suitable for the particulation process during heating is a scraped surface heat exchanger. Protein aggregates of about 10 μm were produced by heating a whey protein concentrate to 80 °C and by subsequently cooling under a high shearing load (Spiegel and Kessler, 1998). The size of the particles was affected by raw material used and processing parameters such as heating temperature, pH and calcium concentration (Spiegel, 1999; Spiegel and Huss, 2002). Smaller particles (<5 μm) could be obtained at lower pH (Spiegel and Huss, 2002).

3.9.1.2 High Pressure Homogenisation

Alternatively, dynamic high pressure shearing devices, such as a microfluidizer, can be used to create microparticulated whey proteins from completely denatured protein solutions (Paquin *et al*., 1993; Iordache and Jelen, 2003; Dissanayake and Vasiljevic, 2009). Whey protein solution is acidified using HCl or glucono-δ-lactone and heat processed at 80–120 °C for periods varying between 4 s and 10 min to denature the whey proteins. The pH of the cooled suspension is readjusted to 6.7 with NaOH and the suspension is microfluidised at 300–800 bar to obtain microparticulated whey protein (Paquin *et al*., 1993). Particles with mean diameters of 5–10 μm and narrow distributions can be obtained by varying the processing conditions such as pressure, temperature and number of passes.

3.9.1.3 Extrusion Cooking

Microparticulated whey proteins with mean diameters of about 12 μm can also be produced by

extrusion cooking at acidic pH (Queguiner *et al*., 1992; Cheftel and Dumay, 1993). Particle size and properties are affected by barrel heating temperature and pH used.

Several methods for the manufacture of microparticulated whey proteins have been patented. Many of these patented methods have been explained by Cheftel and Dumay (1993). Some of the commercially available microparticulated whey protein products are Simplesse®, Dairy-Lo® and LeanCreme™. Detailed information on commercial methods used for industrial production of microparticulated whey proteins is usually proprietary information and not available in the published literature.

3.10 Utilization and Application of Whey-Based Products

3.10.1 Whey Products as Ingredients in Foods

The uses of whey-based products in the food industry are numerous and continue to grow not only because of their nutritional attributes but also because of the functional properties they impart to the foods. Whey-based ingredients are utilised in a wide range of food categories such as bakery products, meat/fish products, dairy products, confectionary, fruit beverages, medical and nutritional applications, food for catering to special needs (such as infant formula, dietetic foods) and nutraceutical foods (Onwulata and Huth, 2008). The functional properties of whey protein products that are most relevant for their application as food ingredients are solubility in water at a wide range of pH, interaction with water, foaming, emulsification, texture modulation and gelation (Mulvihill and Ennis, 2003; Killara and Vaghela, 2004).

WPC 35 is commonly used as a replacer for skim milk powder as its composition resembles that of skim milk powder. It is also used in bakery mixes, in yoghurt as a stabilizer and fat mimetic, confectionary products, dietetic foods and infant foods. It is quite beneficial in these processed products for its fat-like mouthfeel, water-binding and gelation properties.

WPC 50, 65 and 80 are commonly used in nutritional drinks, protein fortified beverages, bakery products, meat, soups, foods for dietetic purposes and low-fat products. Defatted WPC powder containing 80–85 % protein is used as an egg-white replacer and is commonly used in whipped products such as ice-creams, meringues and toppings. A few examples of utilization of whey and whey-based products as ingredients in different food products and their functional properties relevant to those products are given in Table 3.7.

3.10.2 Nutritional Application of Whey Ingredients

Whey proteins have a high nutritional value. The biological value of whey proteins is much higher than egg proteins and other commonly consumed proteins. Whey protein products are a good source of essential amino acids, a rich source of branched chain amino acids such as leucine, isoleucine and valine and a balanced source of the sulphur-containing amino acids methionine and cysteine (Smithers, 2008). Owing to their superior nutritional value, whey protein-rich ingredients have found niche applications in specialised nutrition, such as infant formulae, sports nutrition, weight control diets and specialised enteral nutrition formulae.

Demineralized whey powders are used extensively in specialized infant and clinical formulations. Milk proteins have been used in infant formulae for a very long time. Recently, a lot more attention is being paid in the infant formula industry to mimic the amino acid composition of human milk. Hence, a mixture of 60 % whey protein with 40 % casein are now being widely used to increase the levels of tryptophan and cysteine in infant formulae (Boland, 2011). β-lactoglobulin is not present naturally in human milk and has been identified as a potential allergen in cows' milk. Hence, whey protein hydrolysates can be utilised to produce hypoallergenic infant formulae. Recent clinical studies of infants fed with mother's milk, or α-lactalbumin enriched modified formula suggest that infants fed with modified formula had weight gain and growth patterns

Table 3.7 Summary of the applications of whey protein ingredients and their functional properties relevant to certain food products (Mulvihill and Ennis, 2003; Killara and Vaghela, 2004)

Food category	Uses	Functionality
Bakery products	Cakes, bread, muffins, croissants	Nutritional, emulsifier and egg replacer
Dairy products	Yoghurt, ricotta cheese, quarg	Nutritional, yield, consistency, curd cohesiveness
	Cream cheeses, cream cheese spreads, sliceable/squeezable cheeses, cheese fillings and dips	Gelling, emulsifier, sensory properties
Beverages	Soft drinks, fruit juices, powdered or frozen beverages	Nutritional
	Milk-based flavored beverages	Colloidal stability, viscosity
Dessert products	Ice-cream, frozen juice bars, frozen dessert coatings	Whipping properties, skim milk solids replacement, emulsifier, body/texture
Confectionary	Aerated candy mixes, sponge cakes, meringues	Emulsifier, whipping properties, egg white replacement, fat binding, foam stability
Pasta products	Macaroni, pasta and imitation pasta	Nutritional, texture, freeze thaw stability, microwaveable
Meat products	Luncheon meats, frankfurters	Gelation, pre-emulsion, water holding capacity, fat binding
	Injection brine for fortification of whole meat products	Yield, gelation
Convenience foods	Gravy mixes, soup mixes, sauces, canned cream soups and sauces, dehydrated cream soups and sauces, salad dressings, microwavable foods, low lipid convenience foods	Flavor enhancer, emulsifier, stabilizer, viscosity controller, freeze thaw stability, egg yolk replacement, water binding capacity, acid solubility
Textured products	Puffed snacks, protein enriched snack-type products, meat extenders	Nutritional, structural, texturing
Infant formula	Pre-term formula, Term formula, follow-on formula	Nutritional
Dietetic foods	Slimming foods, diets for elderly, clinical foods	Nutritional

more similar to those of breastfed infants (Sandström *et al*., 2008; Trabulsi *et al*., 2011).

The whey protein products have very high biological value and are quickly assimilated by the body on ingestion. Also, levels of branched-chain amino acids are high in whey proteins. These qualities make whey protein a suitable product for sports nutrition and body building in products like sports drinks, nutrition bars and blended protein powders (Delgado-Andrade *et al*., 2006). Branched chain amino acids are metabolised in skeletal muscle instead of liver (Etzel, 2004) and enhance synthesis and reduce breakdown of proteins, as well as activate key enzymes for protein synthesis in muscle (Blomstrand *et al*., 2006; Farnfield *et al*., 2009).

Recent research suggests that whey proteins can contribute to regulating body weight by providing satiety signals (Luhovyy *et al*., 2007). The increased sense of satiation can help in managing and preventing obesity. Hence, whey-based products may be suitable for inclusion in the diet of people suffering from obesity. The role of whey proteins as a physiologically functional food ingredient has been recently reviewed (Luhovyy *et al*., 2007). The use of whey protein in products such as protein drink mixes, sport meals, protein bars, high-protein cookies, clear sports drinks, that claim to help with weight management by promoting satiety is increasing (Korhonen and Pihlanto, 2006; Poppitt *et al*., 2011). Although the exact mechanism by which individual whey

proteins and peptides regulate food intake is not fully elucidated yet, it is known that whey proteins reduce short-term food intake, affect satiation and satiety, activate many components of the food intake regulatory system upon ingestion and have insulinotropic effect (Luhovyy *et al*., 2007). Aldrich *et al*. (2011) reported that although a whey protein diet did not significantly improve weight loss, fat loss, or satiety in reduced energy diets among midlife adults, there were significant differences in regional fat loss and in decreased blood pressure.

3.10.3 Encapsulation of Functional Ingredients

Encapsulation is a widely used technique in the food and pharmaceutical industry to protect functional ingredients. Encapsulation can provide additional benefits such as controlled release, flavour masking, improved handling and uniform dispersion in a final product. Major whey proteins, β-lactoglobulin, α-lactalbumin and blood serum albumin, have excellent surface active properties. Hence, whey ingredients can be used as good wall material and emulsifier for encapsulation. Properties of milk protein-stabilised emulsions have been recently reviewed (McClements, 2009; Singh, 2011). The encapsulation of milk fat in WPC and WPI has been studied by Young *et al*. (1993a). Whey ingredients were found to be a suitable material for producing spherical microcapsules for protecting milk fat from the surrounding environment. The encapsulation efficiency of whey ingredients was further enhanced by incorporating carbohydrates in the wall material (Young *et al*., 1993b). Microencapsulation of sunflower oil, lemon and orange oil flavour in coacervates of whey protein and gum Arabic was investigated by Weinbreck *et al*. (2004). Lemon oil encapsulated in these coacervates was incorporated in Gouda cheese and the flavour release of limonene during cheese consumption was studied after 1 month of storage. It was found that the flavour release was correlated to the size of capsules and degree of cross-linking (Weinbreck *et al*., 2004). Encapsulation of β-carotene in WPI-stabilized oil-in-water emulsions has been studied by Xu *et al*. (2013). The loss of β-carotene from the emulsions was positively correlated to the protein oxidation and could be reduced by adding antioxidants such as EDTA and α-tocopherol.

For encapsulation and controlled delivery of bioactive compounds, whey proteins can be used in the form of hydrogels or nanoparticles (Gunasekaran 2009). Heat-induced, pH-sensitive WPC hydrogels have been utilised to study controlled drug release behaviour of caffeine (Gunasekaran *et al*., 2006, 2007). Remondetto *et al*. (2004) studied the influence of microstructure (filamentous or particulate) of cold-set β-lactoglobulin hydrogels on iron delivery under various physiological conditions. It was found that the iron release profiles of filamentous and particulate gels were different from each other and were dependent on pH (Remondetto *et al*., 2004). Under simulated gastrointestinal conditions, the filamentous gel protected iron in the gastric zone and released most of it during the intestinal phase. Hence, filamentous whey protein gels were recommended as feasible matrices for iron transportation (Remondetto *et al*., 2004). WPI micro-beads have also been studied to encapsulate probiotic bacteria such as *Lactobacillus rhamnosus* GG (Doherty *et al*., 2011; Doherty *et al*., 2012). The encapsulated bacteria demonstrated stability under both in vitro simulated and ex vivo porcine gastro-intestinal conditions.

3.10.4 Physiological Applications of Whey Ingredients

Once digested, whey proteins act as a good source of bioactive peptides with strong physiological activity, and offer identified health benefits when consumed. In a murine model study, whey proteins were able to retard colon cancer in young rats efficaciously compared to other sources of protein (McIntosh *et al*., 1998). The study also proposed that sulphur amino acid-rich proteins (especially in WPI) provide biologically available methionine and cysteine that retard the development of colon tumours and tumour precursors. While further substantiation of the benefits ought to be verified by human clinical trials, further

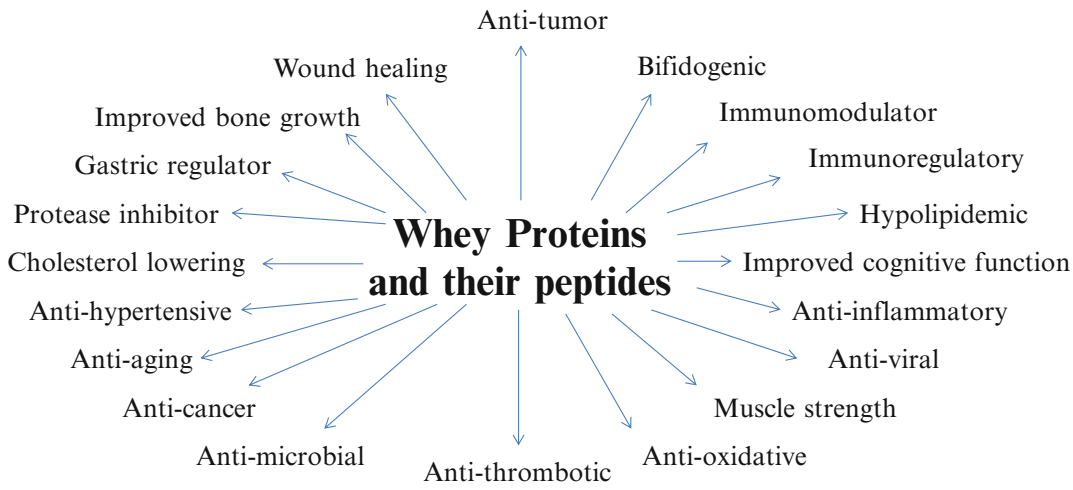


Fig. 3.10 Physiological functions of whey proteins and their peptides

development of whey-protein enhanced food products like ice cream, flavoured milk, muesli bars, dips, puddings and pasta should take precedence to provide a healthy approach to diet. Whey proteins and the bioactive peptides derived from them have several physiological functions and affect several major body systems such as the cardiovascular, digestive, immune and nervous systems (Shah, 2000; Pihlanto and Korhonen, 2003; Manso and López-Fandiño, 2004; Korhonen and Pihlanto, 2006; Krissansen, 2007). A summary of biological functions of whey proteins and their peptides is given in Fig. 3.10.

Research has established that lactoferrin provides tremendous beneficial health effects for humans and animals. It is capable of exerting a diverse range of physiological functions *in vivo* and *in vitro*. *In vivo*, lactoferrin protects the mammary gland and gastro-intestinal tract against infections, provides superior nutrition through increased bioavailability of iron and rich source of amino acids and promotes cell division (mitogenesis), differentiation and cell growth (trophic) of intestinal mucosa (Pihlanto and Korhonen, 2003).

Lactoferrin has been known to participate in iron metabolism, producing antimicrobial, antibacterial, anticancer, antiviral, antiparasitic effects, act like host-defence mechanism, control tumor growth, support cell proliferation and differentiation, help bone formation and hydrolyse RNA (Adlerova *et al*., 2008; Vogel, 2012). *In vitro* inhibition of cytomegalovirus, HIV, herpes, influenza, hepatitis C and poliovirus type I by lactoferrin have also been demonstrated (Vorland, 1999). Infant nutritional formulations containing lactoferrin have been shown to retard coliform bacteria and increase bifidobacteria in infant faeces (Roberts *et al*., 1992).

Lactoferrin is also known to be upregulated in inflammatory disorders, including neurodegenerative disease (Kawamata *et al*., 1993), inflammatory bowel disease (Sugi *et al*., 1996), allergic skin and lung disorders (Zweiman *et al*., 1990; Vandegraaf *et al*., 1991) and arthritis (Decoteau *et al*., 1972).

Lactoferricin, a very strong antimicrobial peptide produced from partial hydrolysis of lactoferrin, has good antitumor activity against fibrosarcoma, melanoma and colon carcinoma (Yoo *et al*., 1997; Eliassen *et al*., 2002).

3.10.5 Whey Protein Films and Coatings

Edible films can help to decrease the amounts of synthetic packaging materials required for food preservation and improve package recyclability. Edible films are generally flavourless, tasteless, flexible and vary in opacity from transparent to

translucent depending on protein source, formulation and composition (Mulvihill and Ennis, 2003). WPC and WPI have been utilised in renewable, biodegradable packaging and plasticised, edible films (McHugh *et al*., 1994; Ramos *et al*., 2012). The water solubility of whey protein edible films can be reduced by acidifying to the isoelectric pH (pI) of proteins or enzymatically cross-linking the proteins (Mulvihill and Ennis, 2003). The edible film provides additional mechanical integrity and support during food handling and functions as a selective permeable barrier for aroma, oxygen, carbon dioxide, moisture and lipids (Miller and Krochta, 1997). In addition to physical (adhesion, cohesion and durability) functionality, infusion of active antioxidants, flavours, vitamins, antimicrobial compounds, fortified nutrients, essential oils, oxygen-scavanging agents in whey protein films is also possible (Cha and Chinnan, 2004; Seydim and Sarikus, 2006; Ramos *et al*., 2012; Fernandez-Pan *et al*., 2013; Janjarasskul *et al*., 2013). Whey protein films have been used as coatings for nuts, confectioneries, eggs, fruits and vegetables and meat products (Dangaran and Krochta, 2009). Whey protein coatings are also applied to traditional packaging materials, such as paper and plastic films to impart new functional properties. Whey protein coating can also improve the wettability and water absorption, grease barrier and oxygen barrier properties of paperboard, plastic laminate and aluminium foil (Dangaran and Krochta, 2009).

3.10.6 Carbon Dioxide Sequestering by Whey Proteins

Carbon dioxide (CO_2) transport in humans is regulated by binding of CO_2 to haemoglobin (protein) forming carbaminohemoglobin (Sharan and Singh, 1985). The physical interaction (adsorption) between protein and CO_2 in packaging of protein foods was established by Mitsuda *et al*. (1975). Carbon capture and sequestration has been proposed as a method to reduce CO_2 emissions in the atmosphere. Recently, a study was conducted to investigate the possibility of using protein ingredients, WPI, to capture CO_2 from the exhaust gases in food-processing industries (Imtiaz-Ul-Islam *et al*., 2011). It was found that the maximum capacity of WPI to capture CO_2 is 0.4 and 0.78 % for commercial WPI and laboratory spray-dried WPI, respectively. Decline in CO_2 capture capacity of WPI was ~1.33 % per cycle, which was much better than ~4.6 % per cycle for CaO (Salvador *et al*., 2003) and ~1.92 % per cycle for Ca_2SiO_4 (Wang *et al*., 2008).

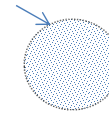
3.10.7 Whey Ingredients as Pharmaceutical Excipients

Pharmaceutical products command high specifications of biodegradability, renewability, absorbability and edibility and such qualities are present in WPI (Caillard *et al*., 2011). In recent studies, succinylated β-lactoglobulin has been shown to be very promising for the controlled release and the protection of active compounds such as riboflavin and probiotic bacteria (Caillard *et al*., 2011; Poulin *et al*., 2011). Caillard *et al*. (2012) studied the tabletability of six commercial WPI samples and found that WPI possesses good flowability and compactability, which are desirable characteristics for tabletability. It was found that the tabletability of WPI was a function of powder particle size and smaller particles have higher compactability (Caillard *et al*., 2012). Also, regular shaped (almost spherical) particles resulted in tablets with highest mechanical strength; powder porosity and moisture content were not correlated to compactability of WPI (Caillard *et al*., 2012). Lactose, derived from whey, is also commonly used an excipient for pharmaceutical products (Siso, 1996).

3.10.8 Whey Ingredients as Drying Aid

Whey protein isolate has been used as drying aid to spray dry various sugar- and acid-rich liquid foods due to its surface active properties. The

Table 3.8 Surface composition of fruit (bayberry) juice powder dried with WPI (from Fang and Bhandari, 2012)

Ratio of fruit juice solid: WPI	Bulk composition in protein (%)	Surface coverage by protein (%)	Powder particle surface structure
99.5:0.5	0.5	58	Particle surface covered with protein 
99.0:1.0	1.0	60	
97.5:2.5	2.5	59	
95.0:5.0	5.0	61	
92.5:7.5	7.5	65	
90.0:10.0	10	63	

surface active property of whey protein isolate and its hydrolysates measured as surface tension of their solutions at the air-water interface is presented earlier in Table 3.6. Fruit juices, honey and acid whey comprise of constituents that depress glass transition temperature and render the product sticky during spray drying and storage conditions. Addition of drying aids that raise the glass transition temperature (such as maltodextrin) is practiced in commercial drying of fruit juices and honey. However a large amount of drying aids (as high as 70 % of total solids) is required to dry these products successfully. This can increase the production cost and also alter the original flavour and taste of the product. In recent years, whey protein has been used to reduce the total amount of drying aids required owing to its surface active property that results in surface coverage of drying particles by proteins making them non-sticky. In Table 3.8 it is shown that addition of as low as 0.5 % (w/w) of WPI solids to bayberry juice solids can result in a coverage of as high as 58 % of powder particle surface by protein. This makes the particle surface non-sticky, improving the powder recovery. Spray drying experiments have shown that a small amount of protein (1 %) was efficient (powder recovery >50 %) to spray dry the bayberry juice, while a large amount of maltodextrin (>30 %) was needed for the same purpose (Fang and Bhandari, 2012). A similar approach can be suggested to assist drying of acid-whey. Due to very low glass transition temperature of lactic acid, spray drying of whey with more than 2 % lactic acid (solid basis) is troublesome. Therefore, an addition of a small amount of whey solids may improve the drying property and reduce the caking behaviour of acid whey.

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Rehydration and Solubility Characteristics of High-Protein Dairy Powders

4

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Abstract

Dairy powders derived from membrane filtration processes, such as milk protein concentrate (MPC) and phosphocaseinate (PC) powders, have considerable potential as functional ingredients due to their high protein content and quality. However, the use of these powders is sometimes limited or impaired by their poor rehydration characteristics in aqueous media, which has been linked with the formation of an inter-linked network of casein micelles at particle surfaces during processing and storage. Analytical tools are now available which can monitor the rehydration of dairy powders dynamically. This is a considerable development, as the rate-limiting stages of rehydration for individual powders (e.g., wetting, dispersion) can now be identified, quantified and targeted in attempts to improve rehydration properties. In addition, these technologies allow the negative effects of sub-optimal processing or storage conditions on powder rehydration and solubility characteristics to be measured, which allows preventative strategies against loss of solubility to be developed. Moreover, it is foreseeable that some of these technologies could be useful for in-line analysis and process control at an industrial scale. This review provides a detailed description of the underlying principles, data outputs and industrial relevance of current methods to monitor dairy powder rehydration. The technologies discussed in this review include viscometry and rheometry, turbidimetry, static light-scattering, focused beam reflectance

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measurement (FBRM), image analysis, nuclear magnetic resonance (NMR) relaxometry, thermochemistry, conductimetry and sound-based technologies. The contribution that these technologies have made to the current understanding of rehydration phenomena, with a particular emphasis on high-protein dairy powders (≥80 % protein), is discussed throughout. In addition, a comprehensive overview of rehydration and solubility characteristics, and the effects of process-, storage-, and additive-induced changes thereon, is given for high-protein dairy powders.

Keywords

Rehydration stages • Solubility • Instant properties • High-protein dairy powders • Rehydration properties • Characterisation of powder rehydration

Abbreviations

ADMI	American Dry Milk Institute
CaCas	Calcium caseinate
CN	Casein
CPMG	Carr-Purcell-Meiboom-Gill
DF	Diafiltration
FBRM	Focused beam reflectance measurement
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
IDF	International dairy federation
MDP	Maltodextrin powder
MF	Microfiltration
MPC	Milk protein concentrate
MR	Maillard reaction
NaCas	Sodium caseinate
NMR	Nuclear magnetic resonance
PC	Phosphocaseinate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLS	Static light-scattering
SMP	Skim milk powder
UF	Ultrafiltration
WMP	Whole milk powder
WP	Whey protein
WPI	Whey protein isolate
XPS	X-ray photoelectron spectroscopy

4.1 Introduction

Drying (e.g., roller-, spray-, freeze-drying) is commonly applied to dairy-based systems to enhance shelf-life, minimise transit costs and increase convenience levels for the end-user (Walstra *et al.*, 1999; Henning *et al.*, 2006; Schuck *et al.*, 2007). The ability of dehydrated ingredients to rehydrate readily in aqueous media is essential if their underlying functionality is to be exploited (Gaiani *et al.*, 2007; Mimouni *et al.*, 2009, 2010a; Fang *et al.*, 2011). Ease of rehydration is not solely an issue of consumer convenience, but is also crucial at an industrial level in relation to unit operations involving powders (Schober and Fitzpatrick, 2005; Hellborg *et al.*, 2010). Powder rehydration properties may be affected by raw material selection, pre-treatments, processing methods and storage conditions (Walstra *et al.*, 1999). In addition, intrinsic powder properties, such as surface and bulk composition, particle structure (e.g., morphology, presence of pores and capillaries) and rehydration conditions (e.g., stirring rate, temperature, solids content), can influence rehydration behaviour (Schuck *et al.*, 2012).

Developments in membrane filtration technologies have facilitated the fractionation of bovine milk to give liquid retentates with high

concentrations of protein (Kelly *et al*., 2000; Mistry, 2002). Retentates derived from membrane processes such as ultrafiltration (UF) and diafiltration (DF) are commonly dried into powders with high proportions of native-state proteins. Milk protein concentrates (MPCs) are produced by heat-treating skim milk and concentrating both whey protein (WP) and casein (CN) fractions in the retentate using UF and sometimes DF (Mulvihill and Ennis, 2003; Udabage *et al*., 2012). Phosphocaseinates (PCs; also termed native phosphocaseinates, native micellar CNs or micellar CN concentrates) are comprised primarily of the CN fraction, and associated minerals, retained following microfiltration (MF) or a combined MF/DF process (Fauquant *et al*., 1988; Pierre *et al*., 1992; Hurt and Barbano, 2010). On the other hand, WP isolates (WPIs) are obtained from UF and perhaps DF of whey or milk microfiltrate (Maubois and Olivier, 1997) or from whey using ion-exchange chromatography (Fox and McSweeney, 1998). After spray drying, which is sometimes preceded by an evaporation step, these dairy products have considerable value due to their innately high protein content and quality (Davenel *et al*., 2002; Mimouni *et al*., 2009); however, high-protein CN-dominant products such as PC powders, and to a lesser extent MPC powders, exhibit poor rehydration characteristics when introduced to aqueous media, due to low concentrations of lactose (Anema *et al*., 2006; Baldwin, 2010; Richard *et al*., 2013) and the poor dispersibility of powder particles with surfaces rich in inter-linked CN micelles (Havea, 2006; Gaiani *et al*., 2007; Mimouni *et al*., 2010a; Haque *et al*., 2012).

Standard methods (e.g., insolubility index) for measuring properties relating to powder solubility have the advantage of being easy to perform and interpret; however, results do not represent the entirety of the rehydration process, can have poor reproducibility and may not be applicable to more recently developed dairy powder ingredients, as discussed by Gaiani *et al*. (2005), Schuck *et al*. (2007) and Fang *et al*. (2008). In addition, these approaches for studying powder solubility do not provide information about the multiple stages of powder rehydration. Furthermore, the poor rehydration characteristics of high-protein dairy powders are probably due to inhibited water transfer during the period of analysis rather than insolubility *per se* (Schuck *et al*., 2007).

Recent research has focused on the dynamic monitoring of powder rehydration *in-situ*, which can allow the identification and quantification of the rate-limiting stage of rehydration for a given powder. Depending on powder type, the rate-limiting step can be wetting, the initial absorption of water by powder particles, or dispersion, the fragmentation of powder particles which have absorbed water. For example, it has been reported that the rate-limiting stage depends on whether the powder is CN- (dispersion-limiting) or WP- (wetting-limiting) dominant (Gaiani *et al*., 2007; Schuck *et al*., 2007; Hussain *et al*., 2011b). This information can be used as a basis to develop processes to improve powder solubility by reducing the duration of the rate-limiting stage of rehydration. In addition, such technologies could be useful for in-line process analysis and control at industrial scale.

This review includes an overview of dairy powder rehydration, the factors (composition-, process-, and storage-related) which influence rehydration behaviour and a discussion of the techniques which have been used to monitor powder rehydration phenomena dynamically. Particular focus has been placed on studies into high-protein dairy powders (≥80 % protein), as this is currently, and will remain for some time, a very active area of research.

4.2 Dairy Powder Rehydration

A general mechanism for the rehydration of an agglomerated, high-protein dairy powder is shown in Fig. 4.1. It is important to note that two or more of these stages may occur concurrently during rehydration. For this reason, stages such as wetting and swelling, or dispersion and dissolution, are often combined and considered as one. However, owing to the complexity of the powder rehydration process, all stages will be considered individually for the purpose of this review. In addition, some stages may not occur for certain

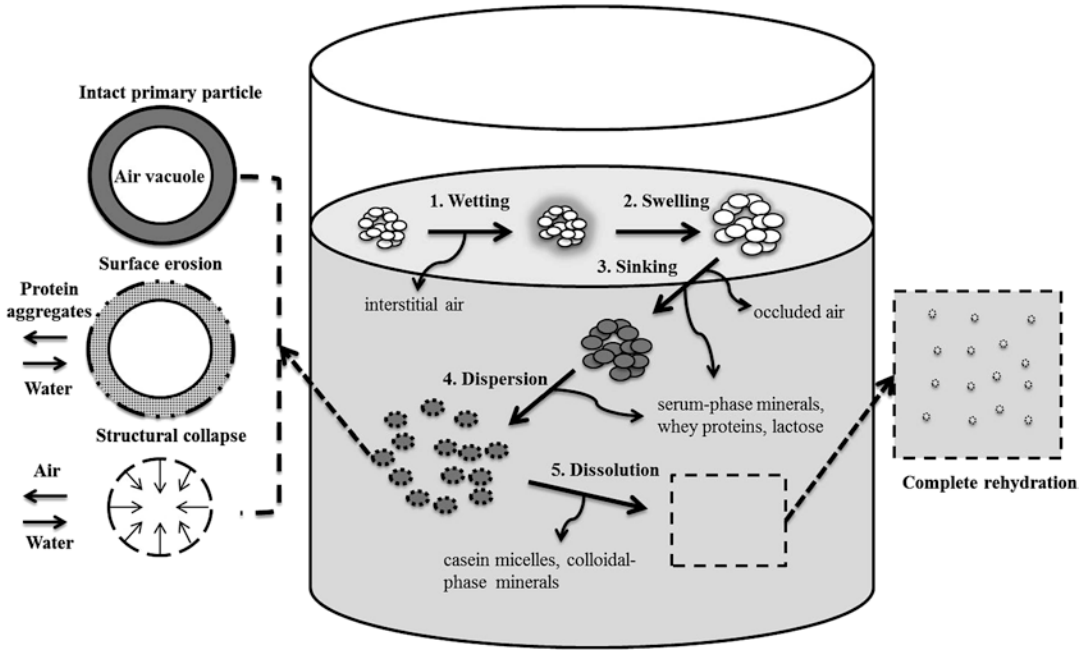

Fig. 4.1 Schematic representation of rehydration profile for an agglomerated high-protein dairy powder, showing *1* Wetting, *2* Swelling, *3* Sinking, *4* Dispersion and *5* Dissolution

powders (e.g., dispersion of agglomerated particles in non-agglomerated powders, swelling of particles and delayed surface erosion in WP-dominant powders).

4.2.1 Wetting

The wetting stage of powder rehydration refers to the initial absorption of water by introduced powder particles, the subsequent immersion of wetted powder particles, and their ultimate disappearance from the free surface of the liquid. Wetting was one of the first powder rehydration stages to be recognised and wettability is considered an important feature of instant powder products (Skanderby *et al*., 2009; Schuck *et al*., 2012). Particles of a skim milk powder (SMP) which has not been agglomerated make contact with the aqueous surface, wet rapidly and create a surface barrier which impedes subsequent wetting of other particles (Masters, 1985). Agglomeration minimises the specific surface area of powder particles and inhibits the formation of a surface film during rehydration, with a concomitant increase in the rate of wetting (Skanderby *et al*., 2009). Wetting of agglomerated powder particles involves the replacement of interstitial air at the solid surface with liquid, followed by inward diffusion of the liquid through the capillary network of the agglomerated powder particle (Galet *et al*., 2010; Forny *et al*., 2011). Wettability of powders may be measured using the International Dairy Federation (IDF) standard test for wettability (IDF, 1979). In this method, a glass plate holding a given quantity of powder is withdrawn over a defined period, the powder falls into the beaker and the time for all particles to become visibly wetted is recorded (Fig. 4.2).

Surface composition and contact angle (θ) have a marked influence on the wettability of powder surfaces (Fig. 4.3). Walstra *et al*. (1999) emphasised the importance of θ in a multi-phase system (solid, liquid and gas), stating that, if θ is <90°, wetting tends to be complete. Values of θ are typically derived from the Young equation (Forny *et al*., 2011):

$$\gamma_{SG=}\gamma_{LS+}\gamma_{LG}.\cos\theta \qquad (4.1)$$

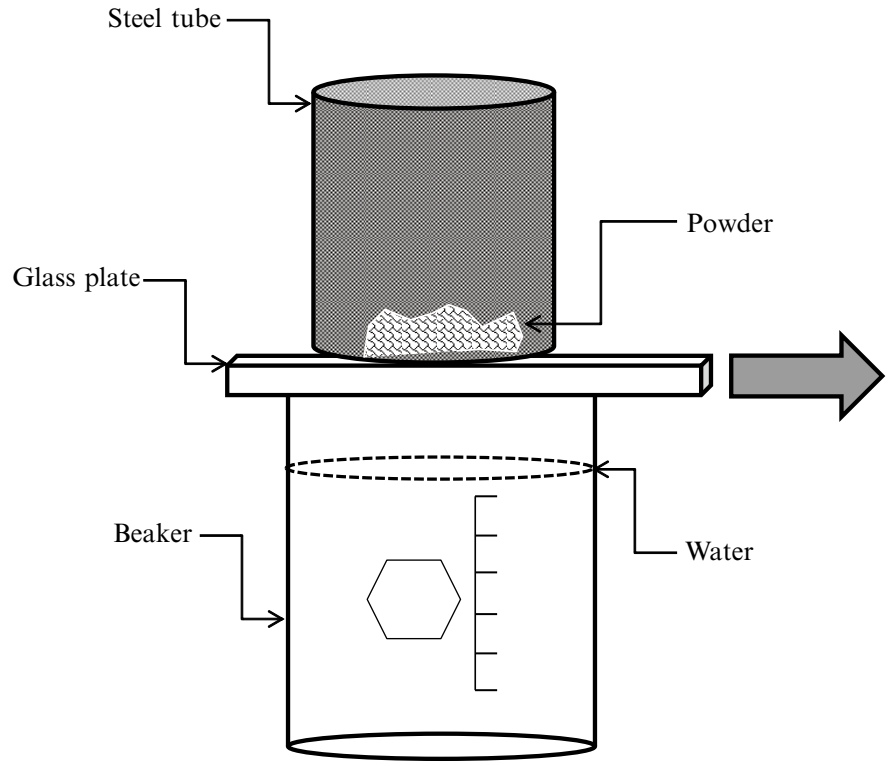

Fig. 4.2 Typical procedure used to determine the wettability of dairy powders

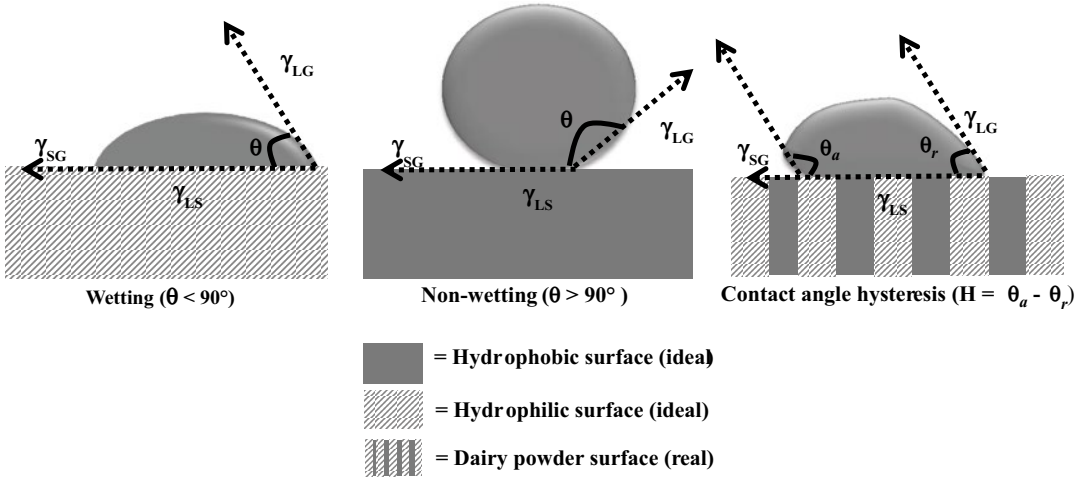

Fig. 4.3 The effect of surface composition on the contact angle (Θ) formed between a liquid drop and a solid surface. Interfacial tensions between solid-gas (γ_{SG}), liquid-solid (γ_{LS}) and liquid-gas (γ_{LG}) phases are illustrated along with Θ, as per Young's equation (4.1). Values of Θ_a and Θ_r represent the advancing and receding Θ, respectively, during contact angle hysteresis

where the interfacial tensions between the solid and gaseous phase, the liquid and solid phase, and the liquid and gaseous phase are represented by γ_{SG}, γ_{LS}, and γ_{LG}, respectively (Fig. 4.3). While it is difficult to obtain a true static Θ value for dairy powders, the change in Θ after a drop of water is deposited on a prepared bed of powder can give a useful indication of wetting properties across different samples. Using dynamic measurement of Θ, Gianfrancesco *et al.* (2011) reported that the presence of heat-denatured β-lactoglobulin or dissociated CN in powders yielded poor wetting properties compared to powders where these proteins were present in unmodified form. In addition, the decrease in Θ over 5 s after a drop of water was deposited on a 35 % protein MPC powder was >40 % compared to <10 % for a 86 % protein MPC powder, indicating that high-protein MPC powders imbibe water less readily (Crowley *et al.*, 2015). The results of Crowley *et al.* (2015) supported those of Fyfe *et al.* (2011), who reported that skins of inter-linked CN micelles at the surface of particles in MPC80 increased both non-polar bonding at dry particle surfaces and the attractive forces between reconstituted MPC80 and a hydrophobic surface, measured using X-ray photoelectron spectroscopy (XPS) and atomic force microscopy, respectively. Methods to measure Θ based on a combination of the Young equation with heat of immersion values, derived from calorimetric measurements, have also been proposed (Marabi *et al.*, 2008).

Young's equation pertains to ideal solid surfaces and yields the intrinsic Θ; however, uneven surface topography and chemical heterogeneity may yield larger apparent Θ values (Kwok and Neumann, 1999; Forny *et al.*, 2011). Deviations from ideality, as in the case of dairy powders, can result in multiple possible values of Θ at a given solid surface (Fig. 4.3). This phenomenon, known as contact angle hysteresis, arises from the difference between Θ values derived from advancing (Θ_a) and receding (Θ_r) liquids and results in discrepancies between experimentally derived and real values for Θ (Kwok and Neumann, 1999). However, despite the inherent difficulty in measuring Θ, the value provides a useful theoretical basis for framing discussions on the effects of surface composition on the wetting of dairy powders.

Hydrophobic materials, such as lipids, increase Θ and affect wetting negatively (Kim *et al.*, 2002) (Fig. 4.3). Conversely, high concentrations of lactose at the surface of dairy powders reduce wetting times, due to the hydrophilic nature of lactose (Gaiani *et al.*, 2006a). However, storage of dairy powders above the glass transition temperature (T_g) for lactose can result in its crystallisation, which has a negative effect on rehydration properties (Vega and Roos, 2006; Marabi *et al.*, 2007).

Gaiani *et al.* (2006a) referred to studies on the surface composition of powders based on scanning electron microscopy and stated that they tended to yield largely qualitative results. In addition, attempts to analyse quantitatively the levels of surface fat using solvent extraction can be compromised by simultaneous extraction of bulk lipids (Vega and Roos, 2006). In recent years, XPS has been a key technique for the characterisation of changes to surface composition in dairy powders. Murrieta-Pazos *et al.* (2012) outlined the principle of XPS in detail: to summarise briefly; irradiation of a sample surface by an X-ray source with known energy results in a total transition from photon energy to atomic electrons; levels of individual components at the powder surface are then calculated based on the elemental composition of the powder surface. Measured ratios of carbon, nitrogen and oxygen are then converted using a matrix formula into surface ratios of protein, lactose and fat.

XPS-based studies have demonstrated how the surface composition of dairy powders can change as a result of processing and storage, with obvious implications for powder wettability. Gaiani *et al.* (2006a) observed an over-representation of fat, relative to bulk composition, on the surfaces of high-protein PC powders. Nijdam and Langrish (2006) reported that higher inlet temperatures during the spray drying of milk concentrates, prepared by mixing reconstituted skim and full cream milk powders to fat in dry-matter contents in the range 1.1–29.8 %, w/w, promoted the transfer of lipid material to

particle surfaces and that lower inlet temperatures favoured the presence of protein at particle surfaces. Gaiani *et al*. (2009b) correlated reduced wettability with the migration of lipids to particle surfaces in a high-protein PC powder over storage. Vignolles *et al*. (2009) reported that, apart from surface fat, wettability of high-fat dairy powders was also affected negatively by factors such as the levels of amorphous lactose at particle surfaces, as well as the size and porosity of particles. Over-representation of fat, relative to bulk powder composition, has also been observed for model infant milk formula powders (McCarthy *et al*., 2013a).

Kim *et al*. (2009) reported that higher feed solids contents reduced surface lipid content while increasing surface protein and lactose content for both SMP and whole milk powder (WMP). Contrary to results reported by Nijdam and Langrish (2006), Kim *et al*. (2009) reported that higher drying temperatures promoted the presence of lactose at powder surfaces, a result which was confirmed by Vignolles *et al*. (2010). Kim *et al*. (2009) also observed that increasing the number of homogenisation passes resulted in lower lipid levels on particle surfaces in WMP samples, which was particularly apparent at low drying temperatures. Hanley *et al*. (2011) reported reduced wettability of infant formula powders with increased air velocity during pneumatic conveying; this coincided with increased bulk density and free fat levels (measured by solvent extraction), due to increased instances of attrition at higher air velocities.

Wetting is often considered to be the rate-limiting step during the rehydration of most dairy powders (Kim *et al*., 2002; Vega and Roos, 2006). Gaiani *et al*. (2007) demonstrated that agglomeration of a WPI powder accelerated wetting; however, the opposite effect was observed for PC powder. WPI powders have been shown to be as poorly-wettable as WMPs, despite the considerably lower fat content of the former (Gaiani *et al*., 2011). Hussain *et al*. (2011b) observed protracted wetting stages for WPI and high-protein PC powders when rehydrated in NaCl or $CaCl_2$ solutions, compared to rehydration in water, with $CaCl_2$ having a more marked influence due to Ca^{2+} having a greater ability than Na^+ to screen charge. Thus, the ionic environment influenced the wettability of both CN- and WP-dominant powders. Numerous studies have demonstrated that wettability is the rate-limiting stage of WP-dominant powders, and that agglomeration is of greater benefit to the rehydration of WP-dominant powders, while being potentially detrimental to the rehydration of CN-based powders (Schuck *et al*., 2007).

4.2.2 Sinking

After a powder has become sufficiently wetted, some occluded air is released from primary particles and replaced by liquid solvent, creating denser particles which descend through the solution (Walstra *et al*., 1999; Kelly *et al*., 2003; Richard *et al*., 2013) (Fig. 4.1). The rate at which this process occurs determines the sinkability of a powder. For sinking to occur, the wetted particle must be denser than the liquid in which it is suspended (Masters, 1985). According to Masters (1985), sinking is promoted by low levels of occluded air and high particle density. Powders with low bulk density will have a greater tendency to float on the surface when added to a liquid.

Determination of the sinkability of dairy powders is traditionally performed in static systems (Bullock and Winder, 1960; Tamsma *et al*., 1967); thus, as most dairy powders are added to liquids under agitation (Schober and Fitzpatrick, 2005; Jeantet *et al*., 2010; Richard *et al*., 2013), sinkability measurements have become largely redundant. Písecký (1997) stated that sinkability was once considered an integral part of the powder rehydration process; nevertheless, its non-decisive role, the difficulty inherent in its measurement and its redundancy as applied to industrial powder rehydration systems have resulted in sinking being regarded increasingly as a minor step during wetting. Hence, few studies have been carried out in relation to sinkability in recent years and it is unlikely to become the focus of studies on dairy powder rehydration in the future.

4.2.3 Swelling

Swelling of powder particles is being recognised increasingly as a distinct stage in the rehydration of CN-dominant powders. In rheological-based studies (Sect. 3.1.1), swelling of particles is observed as a peak in viscosity after initial particle wetting (Gaiani *et al*., 2006b), while turbidimetry (Sect. 3.1.2) and static light-scattering (Sect. 3.1.3) measurements have identified swelling as a minimum in turbidity and a peak in particle size, respectively (Gaiani *et al*., 2006b, 2007). Gaiani *et al*. (2006b) reported increases in PC powder particle size by as much as 35 % due to swelling during rehydration. It is conceivable that swelling may have an impact on subsequent dissolution of solid bridges and dispersion of powder particles; however, this may not be the case for WPI powders, where no clear swelling stage has been observed (Gaiani *et al*., 2007; Hussain *et al*., 2011b). Thus, WP-dominant powders may undergo rehydration as shown in Fig. 4.1, but without a swelling stage. Rehydrating CN-dominant powders at higher temperatures can reduce the duration of the swelling stage (Gaiani *et al*., 2006b), which indicates that the beneficial effect of rehydrating powders at higher temperatures is due in part to a decreased swelling time. Currently, no standard method exists for the measurement of swelling time.

4.2.4 Dispersion

Dispersion involves the fragmentation of wetted powder particles and is closely associated with the instant properties of a powder (Singh and Newstead, 1992). The dispersion of the powder into component agglomerates, fragmentation of agglomerates into primary particles, and erosion of primary particles may all occur during this stage (Mimouni *et al*., 2009; Skanderby *et al*., 2009; Fang *et al*., 2011) (Fig. 4.1).

An IDF standard method is available for measuring powder dispersibility (IDF, 1979). As shown in Fig. 4.4, the method proceeds as per the wettability test (Fig. 4.2), except that the moisture content of the powder is first determined and a spatula is used to prevent adherence of unwetted particles to beaker walls and to promote dispersion. Dispersibility (%) is defined as the ability of the powder to disintegrate into particles small enough to permeate a 150 μm sieve and is determined on a dry solids basis (Schuck *et al*., 2012).

High-protein CN-dominant dairy powders contain particles with surfaces rich in inter-linked CN micelles, which undergo wetting, sinking, and possibly swelling, but do not become sufficiently dispersed to allow complete rehydration after a reasonable period of time (Anema *et al*., 2006; Havea, 2006; Baldwin, 2010; Fang *et al*.,

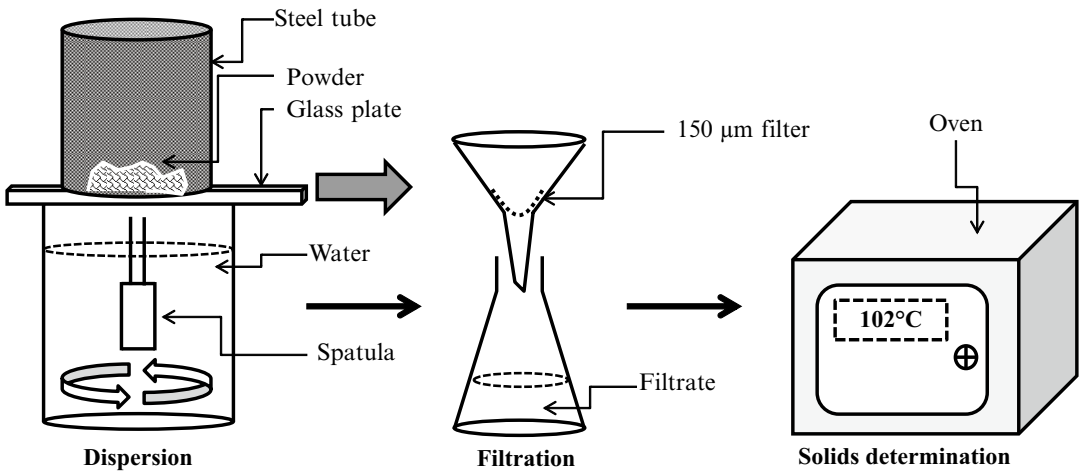

Fig. 4.4 Typical procedure used to determine the dispersibility of dairy powders

2012; Haque *et al*., 2012) results. Mimouni *et al*. (2009) hypothesised that complete rehydration of a high-protein MPC powder does not occur until the CN-rich skins of the primary particles become sufficiently eroded to induce structural collapse and release of all micellar components (such a process is shown during the dispersion stage in Fig. 4.1). This is supported by the continued presence of large primary particle-sized material in MPCs and PCs after extended periods of reconstitution using conventional mixing, even if solubility tests indicate rehydration is complete or near-complete (Chandrapala *et al*., 2014a; Crowley *et al*., 2015).

Mimouni *et al*. (2010a) identified two sets of components in a high-protein MPC powder, fast- and slow-dissolving, and reported that the former group was comprised of lactose, WP and monovalent ions, while the latter group consisted primarily of CN and associated colloidal minerals. As water penetration was sufficient to promote the complete release of fast-dissolving components from powder particles, the authors concluded that wettability could not be the rate-limiting step during rehydration of the MPC powder. Images from scanning electron microscopy seemed to confirm that primary particles of an MPC powder were porous enough to allow rapid inward diffusion of water, with concomitant release of fast-dissolving components, while the surface skin of inter-linked CN micelles prevented effective dispersion of particles, which limited release of micellar material (Mimouni *et al*., 2010b). As will be discussed in Sect. 3.2.5, sound-based technologies have revealed that water penetration can actually be delayed considerably in high-protein powders; moreover, the extrusion-porosification studies of Bouvier *et al*. (2013) demonstrated that increasing the size and number of pores in particles of these powders can markedly improve rehydration properties. Gaiani *et al*. (2011) reported that both PC and WPI powders (although still easy to rehydrate; see Sect. 3.1.2) were poorly-dispersible when compared with skim, semi-skimmed and whole milk powders. The authors attributed poor dispersibility of the powders to high and low concentrations of protein and hygroscopic compounds, respectively.

Augustin *et al*. (2012) produced MPCs through UF, UF and DF, or UF and evaporation, before the MPCs were spray-dried at high and low inlet/outlet temperatures (190/90 or 175/75 °C, respectively) into MPC powders (~82 %, w/w, protein in dry-matter); the authors reported that MPCs which were evaporated and/or spray-dried at high temperatures contained more insoluble material. Fang *et al*. (2012) analysed insoluble material in high-protein MPC powders which were rehydrated and subjected to a second spray drying step using a range of inlet temperatures (77–178 °C); there was an inverse relationship between inlet temperature and the concentration of soluble proteins, as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fang *et al*. (2012) reported that the CNs, in addition to α-lactalbumin, was the fraction most directly affected by higher drying temperatures, while β-lactoglobulin levels remained relatively constant. It is clear that the processing parameters used during the concentration and drying steps in the manufacture of high-protein CN-dominant powder impacts greatly their dispersion properties during subsequent rehydration.

Anema *et al*. (2006) characterised the insoluble material in an MPC powder (84.8 %, w/w, protein) using SDS-PAGE and high-performance liquid chromatography (HPLC); the authors identified the insoluble material as primarily CN-based, with minor WPs also present, and observed that insoluble material increased in quantity with the temperature (20–50 °C) and duration (0–60 days) of powder storage. In addition, Anema *et al*. (2006) demonstrated using mass spectrometry that lactosylation of the MPC powder occurred during storage, which suggested that the development of Maillard reaction (MR) products could contribute to the poor dispersibility of high-protein MPC powders. Le *et al*. (2011a) measured the development of early (lactulosyllysine—measured as furosine, itself a product of lactulosyllysine degradation), intermediate (hydroxymethylfurfural, HMF) and late (melanoidins—measured using colorimetry) MR products in an MPC powder (>80 %, w/w, protein) during storage (1–12 weeks) under a range

of relative humidities (44–84 %) and temperatures (25–40 °C); the authors observed that solubility decreased for the MPC powder with increasing storage time at all conditions studied, while the levels of all MR products increased, and that increased storage temperature and/or relative humidity increased the development of insoluble material and MR products.

Le *et al*. (2011b) compared changes in insolubility and MR product levels in four powders (SMP, WMP, MPC and WPC with 32, 24, 81 and 80 %, w/w, protein, respectively) over storage at 30 °C and a range of relative humidities (44–84 %); the authors reported that the development of furosine, HMF and melanoidins during storage was lower in MPC or WPC powders compared to WMP or SMP, which was attributed to the higher lactose:protein ratios in the latter powders, and that WPC was the only powder which did not become more insoluble over storage, presumably due to its lack of micellar CN. Indeed, Udabage *et al*. (2012) reported that a 95 %, w/w, protein CN-dominant powder without micellar casein (manufactured by spray drying of recombined concentrates of sodium caseinate (NaCas) and WPI to casein:whey ratios of 10:1 or 1:10) maintained 100 % solubility after high pressure treatment and/or 1 month storage at 40 °C; thus, the presence of micellar CN has a significant influence on the initial presence of insoluble material after manufacture and its development over storage. Le *et al*. (2011b) also reported that increased relative humidity during storage affected the solubility of SMP and MPC powders negatively but had no effect on WMP and WPC powder solubility; higher relative humidities promoted the development of furosine, HMF and melanoidins in SMP and WMP, while only furosine and melanoidin levels were affected in WPC and MPC powders. Elsewhere, Semagotoa *et al*. (2014) exposed an MPC70 powder to UV light during storage, which resulted in photoxidation-induced changes including lactosylation of casein and the formation of covalently-linked high molecular weight aggregates, with concomitant loss of solubility; conversely, MPC70 not exposed to UV light retained its solubility, unless the powder had been exposed to high temperature and relative humidity.

The studies of Anema *et al*. (2006) and Le *et al*. (2011a, b) revealed a possible relationship between the development of MR products and the poor dispersion properties of high-protein CN-dominant powders. Le *et al*. (2013) found that the insoluble material which developed over storage of an MPC powder (81 %, w/w protein) was primarily composed of α_{S1}-CN and subsequently investigated the effects of dephosphorylation, addition of lactose or addition of methylglyoxal (an advanced MR product) on the cross-linking behaviour of pure α_{S1}-CN on heating at 90 °C; the authors reported that the addition of lactose or methylglyoxal increased the development of cross-links during heating of α_{S1}-CN, while dephosphorylation had no effect. The results of Le *et al*. (2013) suggested that higher contents of lactose may increase the rate of development of MR products (e.g., methylglyoxal), with a concomitant increase in protein-protein cross-linking *via* amino acid residues such as lysine; conversely, a non-MR product, dehydroalanine (produced by heat- or alkaline-induced β-elimination of cysteine or phosphoserine residues), could not have participated in these protein-protein interactions as removal of phosphate from the phosphoserine residues of α_{S1}-CN did not affect subsequent cross-linking reactions on heating. Thus, it is possible that the development of MR products contributes to the poor dispersion characteristics of high-protein CN-dominant powders; indeed, Ennis and Mulvihill (1999b) also reported impaired solubility of rennet CN powders with increased furosine levels.

Anema *et al*. (2006) suggested that lower levels of "spacer" material, such as lactose, in high-protein MPC powders reduces the proximal distance between proteins, thereby promoting associations between CN micelles. Baldwin (2010) and Richard *et al*. (2013) suggested that lactose improves water penetration into powder particles and that a low level of lactose in high-protein dairy powders has a negative effect on solubility. Dissociation of CNs from the micellar phase of retentates during the manufacture of MPCs, is believed to be due to depletion of serum-phase minerals during UF and DF, which

creates a driving force for re-equilibration of calcium and inorganic phosphate with concomitant loss of micellar calcium phosphate. These effects have been attributed with an increased potential for hydrophobic interactions between CN micelles during processing, which may be responsible for the development of poorlydispersible inter-linked CN micelles in resultant MPC powders (Mimouni *et al.*, 2010b; Udabage *et al.*, 2012).

Udabage *et al.* (2012) applied high pressure treatment to MPCs before spray drying in the production of an 85 %, w/w, protein MPC powder and reported marked reductions in the insolubility of MPC powders pre-treated with high pressures. The application of 200 MPa of pressure at 40 °C resulted in a 19 % increase in solubility; the authors attributed the increased solubility of high pressure-treated MPC powders to increases in serum-based CN which reduced the proximity between CN micelles and thereby prevented their aggregation at particle surfaces during drying. Indeed, high pressure treatment of >100 MPa can cause extensive dissociation of CNs from micelles due to solubilisation of calcium phosphate from the micellar phase and increased ionisation under pressurised conditions (Huppertz *et al.*, 2006). Udabage *et al.* (2012) attributed increased solubility of powders manufactured from high pressure-treated MPCs to reduced levels of micellar CN; however, it is unclear whether increases in non-sedimentable CN caused by high pressure treatment are due to fragmentation of CN micelles into smaller micellar structures or the release of individual CNs from the colloidal phase (Huppertz *et al.*, 2006).

A number of studies on high-protein dairy powders using Fourier transform infrared spectroscopy have found only weak correlations between protein conformational changes and solubility in water (Kher *et al.*, 2007; Haque *et al.*, 2010; Sikand *et al.*, 2011). However, Hussain *et al.* (2011b) reported modifications to the secondary structure of proteins in PC and WPI powders when dissolved in NaCl or $CaCl_2$ solutions. Haque *et al.* (2012) reported enthalpy relaxations and thermal annealing in MPC powders during storage; the authors concluded that structural alterations over time created an increased potential for protein-protein interactions and the formation of a network of interlinked CN micelles at the powder surface. It was proposed that this network impaired the dispersion stage of MPC powder rehydration.

In recent years, numerous attempts have been made to improve the rehydration properties of high-protein CN-dominant powders through modifications to processing and rehydration conditions. Energy (thermal and/or kinetic) may be introduced to enhance powder dispersion (Jeantet *et al.*, 2010; Mimouni *et al.*, 2010a). Heating and stirring enhance dispersion by increasing the breakdown of solid bridges between particles but the energy inputs required depend on the physical structures and state of inter-particle bridges in the powder (Forny *et al.*, 2011). Increasing the stirring rate (Jeantet *et al.*, 2010) or using impeller designs that promote turbulence (Richard *et al.*, 2013) accelerates the rehydration of CN-dominant powders due to disruption of poorly-dispersible particles with surfaces rich in inter-linked CN micelles (Mimouni *et al.*, 2010b). The application of ultrasonication during the rehydration of a high-protein MPC powder was shown to improve its solubility dramatically compared to conventional mixing with an overhead stirrer at 50 °C (McCarthy *et al.*, 2013b); however, ultrasonication-induced cavitation resulted in increases in temperature to >70 °C, which, if not controlled by thermal dissipation, reduced the solubility of the MPC powder due to denaturation of WP. Chandrapala *et al.* (2014a) reported that ultrasonication, rotor-stator mixing or high-pressure homegenisation were all effective technologies for increasing the fragmentation of poorly-dispersible particles in high-protein MPC and PC powders.

Higher temperatures are commonly used to dissolve poorly-soluble powders but, in the case of dairy powders, care must be taken to avoid protein denaturation and aggregation, which could affect solubility negatively (El-Samragy *et al.*, 1993; Gianfrancesco *et al.*, 2011). Indeed, Fang *et al.* (2010) demonstrated that high temperatures could have a positive or negative effect on the rehydration of MPC powders

depending on powder composition, as well as their thermal and storage histories. Furthermore, high-protein dairy powders which develop considerable insolubility (i.e., after prolonged storage) may not undergo effective rehydration even if higher temperatures are used (Udabage *et al*., 2012). Richard *et al*. (2013) reported that temperatures of ≥37 °C were sufficient to affect negatively the rehydration of a PC/WPI powder, a result attributed to aggregation, the opposite of effective dispersion.

An MPC produced by the ion-exchange method of Bhaskar *et al*. (2001), in which ~30 % of Ca^{2+} was replaced by Na^{+}, yielded low levels of insoluble material in the resultant MPC powder, presumably due to the removal of Ca^{2+} as an electrostatic bridging medium between CN micelles (Havea, 2006). Crowley *et al*. (2014) reported that reconstituted high-protein MPCs had a markedly higher Ca-ion activity than lower protein MPCs, due to greater loss of serum-phase minerals during UF/DF, with associated decreases in ionic strength and possibly a reduction in the influence of calcium binding salts (e.g., citrates and phosphates). Indeed, rehydration in phosphate or citrate solutions has been shown to improve the solubility of high-protein PC powders (Davenel *et al*., 2002; Schuck *et al*., 2002), indicating that disruption of inter-linked CN-micelles can be achieved using calcium binding agents.

The mineral environment into which a powder is added greatly impacts the rehydration process. Using milk as a medium for the rehydration of an MPC powder (85 %, w/w, protein), Udabage *et al*. (2012) reported marked increases in solubility compared to powders rehydrated in water; this effect was attributed to the higher total mineral content of milk, which may have induced greater changes to mineral equilbria and promoted the release of CN micelles from powder particle surfaces. Mao *et al*. (2012) manufactured MPCs with a diafiltration step that introduced a range of concentrations of NaCl; increasing the level of NaCl was found to increase MPC powder solubility, presumably due to ion-exchange effects and disruption of micellar integrity (Bhaskar *et al*., 2001; Havea, 2006); in addition, β-mercaptoethanol and SDS also reduced aggregate formation, indicating that electrostatic, hydrophobic and disulphide linkages between proteins may all contribute to the poor dispersion properties of high-protein MPC powders. It has also been observed that the positive influence of rehydrating high-protein MPCs in high-ionic strength media (e.g., 80 mM KCl or milk permeate) is more pronounced at elevated temperatures (Crowley *et al*., 2015).

Augustin *et al*. (2012) used ultrasonication (24 kHz, 600 W), two-stage homogenisation (350/100 bar) or microfluidisation (800 bar) of MPCs at ~50 °C before drying to investigate the effects of retentate pre-treatments with high shear technologies on the solubility of MPC powders (~82 %, w/w, protein in dry-matter) manufactured at pilot-scale; all high shear treatments increased powder solubility but microfluidised samples showed the most marked improvement in solubility after manufacture and after 8 months storage at ~22 °C. When microfluidisation was applied to commercial MPCs before drying into powders (~91 %, w/w, protein in dry-matter), Augustin *et al*. (2012) reported little improvement in powder solubility after manufacture compared to non-microfluidised samples; however, microfluidised samples displayed improved solubility compared to the control after both were stored for 6 months. Yanjun *et al*. (2014) pre-treated MPCs at ≤50 °C with ultrasonication (20 kHz, 600 W) before spray drying into an MPC powder (80 %, w/w, protein); these researchers reported reduced particle size and increased surface hydrophobicity in ultrasonicated samples, effects which were attributed to cavitation, turbulence and microstreaming induced by ultrasonication, with concomitant improvements in functional properties (e.g., solubility, emulsification, and gelation). Chandrapala *et al*. (2014b) ultrasonicated (20 kHz, 450 W) reconstituted MPC, WPC and CaCas powders before subjecting them to a second spray drying step; the sonicated MPC retained its solubility better over storage (60 days) than an unsonicated MPC at low or high relative humidity, with no differences observed for WPC and CaCas powders.

Gaiani *et al.* (2005) reported increased rehydration times for high-protein PC powders following agglomeration, which were correlated with reduced dispersibility values; hence, it is apparent that, where optimum solubility is required, agglomeration should be avoided during the manufacture of spray-dried CN-dominant powders, as it exacerbates their poor dispersion characteristics. Bouvier *et al.* (2013) reported that extrusion-porosification yielded MPC powders with considerably enhanced rehydration properties compared to spray-dried MPC powders due to the presence of micron-sized pores and nano-sized capillaries in the particles of the extrusion-porosified powders.

From the above studies, it is evident that dispersion is the rate-limiting stage during the rehydration of high-protein CN-dominant powders. This is due to the presence of poorly-dispersible particles with surfaces rich in inter-linked CN micelles, which can predominate because of multiple factors including an increased proximity between CN micelles due to high protein concentration factors during membrane processing (Anema *et al.*, 2006; Udabage *et al.*, 2012), changes to micellar structure caused by alterations to the concentration of minerals and mineral:protein ratios during membrane processing (Mimouni *et al.*, 2010b), increased denaturation and aggregation during evaporation and/or spray drying at high temperatures (Augustin *et al.*, 2012; Fang *et al.*, 2012), creation of additional poorly-dispersible material through agglomeration of CN-dominant powders (Gaiani *et al.*, 2005; Schuck *et al.*, 2007), and the development of MR products during prolonged storage, particularly at high temperature and/or relative humidity (Anema *et al.*, 2006; Le *et al.*, 2011a, b, 2013).

A number of strategies are available to reduce levels of poorly-dispersible material in high-protein CN-dominant powders. Introduction of monovalent ions using NaCl or KCl, either in the concentrate before drying (Bhaskar *et al.*, 2001; Mao *et al.*, 2012) or the dispersant before rehydration (Schuck *et al.*, 2002; Gaiani *et al.*, 2007; Crowley *et al.*, 2015) can improve dissociation of poorly-dispersible particles and improve solubility. In addition, Davenel *et al.* (2002) and Schuck *et al.* (2002) showed that rehydration in phosphate or citrate solutions improved the solubility of high-protein PC powders, while Udabage *et al.* (2012) demonstrated that milk had better dispersing characteristics than water for high-protein MPC powders. It should be noted, however, that strategies which involve modifying the mineral environment are often undesirable as mineral content and protein structure can be altered in the final product. Thus, physical interventions which improve powder solubility may be more appropriate in a broader range of applications, examples of which include increased stirring rate or temperature during rehydration (Jeantet *et al.*, 2010), use of turbulence-promoting impeller designs during powder rehydration (Richard *et al.*, 2013), application of ultrasonication during powder rehydration (McCarthy *et al.*, 2013b; Chandrapala *et al.*, 2014a), pre-treatment of concentrates before drying with ultrasonication (Augustin *et al.*, 2012; Chandrapala *et al.*, 2014b; Yanjun *et al.*, 2014), microfluidisation (Augustin *et al.*, 2012) or high pressure processing (Udabage *et al.*, 2012), and the use of alternative manufacturing processes to conventional spray drying, such as extrusion-porosification (Bouvier *et al.*, 2013). However, the benefits of some of these additive-free technologies may be off-set by the higher capital and running (e.g., energy) costs associated with their application.

4.2.5 Dissolution

Dissolution is the final stage of rehydration of dairy powders and coincides with the complete disappearance of granular structures (i.e., agglomerates, primary particles) and the release of constituent molecules (i.e., proteins, carbohydrates, minerals etc.) (Gaiani *et al.*, 2007) (Fig. 4.1). Purported functional properties of a powder will be affected negatively if dissolution is inhibited (Kher *et al.*, 2007). If a powder has successfully wetted, sunk, swelled and dispersed, the powder is said to have dissolved; thus, dissolution could be considered the end-point of rehydration rather than a stage in itself. Mimouni *et al.* (2010a) demonstrated that WP, monovalent

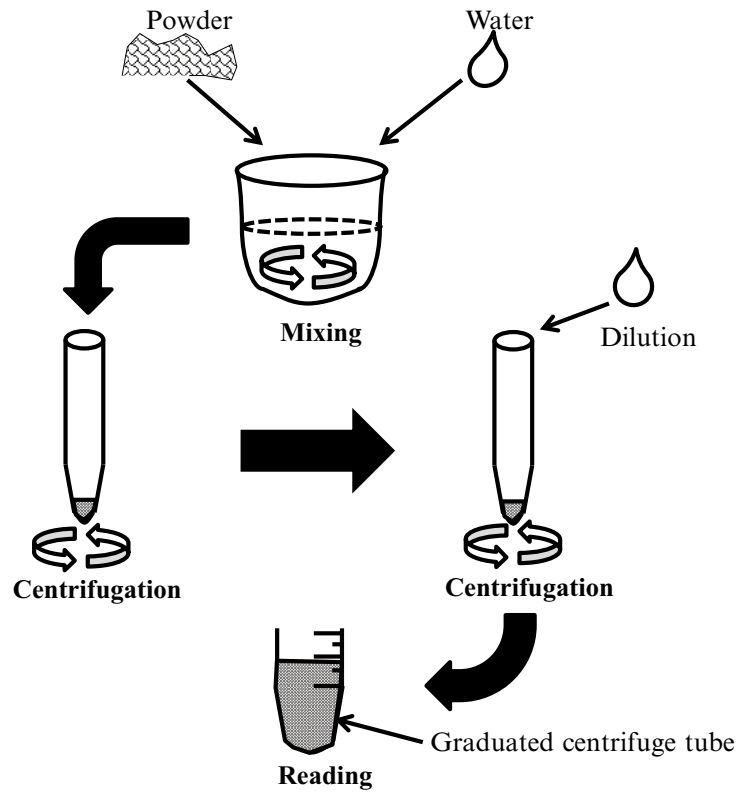

Fig. 4.5 Typical procedure used to determine the insolubility index of dairy powders (redrawn from Skanderby *et al*., 2009)

ions and lactose dissolve rapidly during rehydration and that CN micelles and associated minerals dissolve slowly. Thus, dissolution occurs throughout powder rehydration but is delayed by the poor dispersion of particles containing interlinked CN micelles and inhibited water transfer evident in high-protein CN-dominant powders.

Standard methods for the determination of powder solubility typically involve rehydration of a powder in water under controlled conditions, followed by centrifugation of the resultant mixture, and measurement of the amount of sedimentable or "insoluble" material (Kelly *et al*., 2003). Baldwin and Truong (2007) described the method outlined by the American Dry Milk Institute (ADMI, 1971) which was known as the *solubility index*. The ADMI method was updated in 1988 by the IDF, who entitled the resultant value the *insolubility index* (IDF, 1988; Písecký, 1997). The sediment can be quantified using centrifuge tubes with conically graduated bases or on a dry solids basis (Walstra *et al*., 1999; Skanderby *et al*., 2009). A typical experimental procedure for the measurement of insolubility index is outlined in Fig. 4.5. The insolubility index (ISI, in %) described by the IDF standard (IDF, 1988; Schuck *et al*., 2012) for skim milk is the volume of sediment (for 50 mL) after rehydration (10 g of powder in 100 mL of distilled water, at 25 °C), mixing (at 4000 rev min^{-1} for 90 s) and centrifugation (for 300 s at $160 \times g$). With this method, the quantity of insoluble material can be determined. However, it must be noted that the presence of sedimentable material may not be due to true insolubility, as in the case of denatured protein, but may instead be due to low water transfer during rehydration (Schuck *et al*., 2002).

Once granulate particles have disappeared after dispersion, rehydration is said to be complete; subsequently, numerous intrinsic and extrinsic factors influence the extent to which water becomes associated with proteins, with the extent of these protein-water interactions

influencing the functional properties of the dried ingredient (Kinsella and Fox, 1987). de la Fuente and Alais (1975) reported that solvation of CN micelles in milk decreased with acidification and increased with alkalisation. The authors also reported increasing solvation with heating (72–90 °C). Conversely, Snoeren *et al.* (1984) observed reduced micellar solvation with increasing temperature between 4 and 30 °C. The presence of charged and polar groups plays a central role in protein-water interactions (Hardy *et al.*, 2002). The extent to which bulk solvent can access these groups without steric hindrance is also important (Kinsella and Fox, 1987). The poor rehydration properties of high-moisture powders have been linked with depletion of potential hydrogen-bonding sites due to the presence of bound water (Marabi *et al.*, 2007; Syll *et al.*, 2012).

Heat-induced denaturation and aggregation of WP has been shown to have a negative effect on the solubility of skim milk retentate powders (El-Samragy *et al.*, 1993). However, the presence of denatured WP can have a positive impact on the functional properties of powders, relating to water-holding, viscosity and foaming (Henning *et al.*, 2006). Calcium binding agents are used during the rehydration of rennet CN powders to disrupt protein-protein cross-links and improve solvent access to proteins (Ennis *et al.*, 1998; Ennis and Mulvihill, 1999a, b, 2001). Addition of phosphates and citrates has been shown to increase protein-water interactions and reduce rehydration times for high-protein PC powders (Davenel *et al.*, 2002; Schuck *et al.*, 2002).

Shearing conditions may influence protein hydration positively by disrupting particle structure and making proteins more accessible to solvent, and/or by exposing hydrophilic groups and increasing the affinity of solvent for the proteins; however, shearing has potentially negative consequences if hydrophobic groups become exposed, with concomitant loss of solubility and/or aggregation (Ennis *et al.*, 1998). Ultrasonication of WPI or WP hydrolysate solutions (10 %, w/w, protein) caused an increase in conductivity, which was likely due to exposure of charged groups from within the globular structure of WPs, which resulted in greater solvent access to WPs with a concomitant improvement in solubility (Jambrak *et al.*, 2008); however, ultrasonication had little effect on the solubility of WPC solutions due to their higher content of lactose, which, it was proposed, had a protective effect against structural changes to proteins induced by ultrasonication.

de la Fuente and Alais (1975) reported decreased solvation of CN micelles on addition of $CaCl_2$, which was attributed to the associated pH decrease and clustering of the CN micelles; conversely, the researchers reported increased solvation of CN micelles, coinciding with increased pH, in calcium oxide-supplemented samples. Snoeren *et al.* (1984) also reported reduced solvation of CN micelles on supplementation of milk with $CaCl_2$. Exchange of H^+ for Ca^{2+} at the hydrophobic core of CN micelles has been linked with decreased micellar solvation on addition of $CaCl_2$ (Canabady-Rochelle *et al.*, 2009). Conversely, the introduction of Na^+ has been shown to increase micellar solvation (Le Ray *et al.*, 1998), although the addition of NaCl has a less pronounced effect on protein-water interactions than $CaCl_2$ (Davenel *et al.*, 2002; Schuck *et al.*, 2002), due to the ability of divalent cations to contribute to cross bridging, as well as screening of charge (Hussain *et al.*, 2012).

The rehydration profile of NaCas powder, monitored by turbidimetry (see Sect. 3.1.2), has been shown to be more similar to that of WPI powder than PC powder (Gaiani *et al.*, 2009a), presumably due to the absence of micellar structures in both NaCas and WPI powders. Indeed, protein-water interactions tend to be more pronounced for random-coil (e.g., CN) than globular (e.g., WP) proteins (Kinsella and Fox, 1987; Hussain *et al.*, 2011b). Hussain *et al.* (2011b) reported that higher quantities of NaCl than $CaCl_2$ were required to induce changes in the rehydration profiles of both PC and WPI powders. Structure loss in CN micelles due to Na^+/Ca^{2+} exchange has been linked with improved water transfer in PC powders (Hussain *et al.*, 2011a). Moreover, the rehydration properties of PC powders are affected differently by added salts, depending on the powder production mode

and the co-localisation of both protein and salts in the particle structure: Schuck *et al*. (2002) and Gaiani *et al*. (2007) showed that co-drying of PC and NaCl (i.e., mixing salt into the concentrate prior to spray drying) significantly improved solubility, while bi-drying (simultaneous drying of PC and NaCl through different nozzles) and dry-mixing (mixing of PC and NaCl powders) resulted in no or limited improvement.

4.3 Monitoring Powder Rehydration

Methods available to monitor the rehydration of powders can be divided into those which (1) discriminate between multiple rehydration stages (Sect. 3.1) and (2) solely measure total rehydration time and/or yield information on specific phenomena related to powder rehydration, e.g., mineral release, heat release, water penetration (Sect. 3.2). These differences in rehydration monitoring capability are illustrated in Fig. 4.6, along with information pertaining to any in-line capabilities for industrial powder rehydration processes. In addition, schematic representations of data outputs for high-protein CN-dominant powders using these techniques are shown in Fig. 4.7.

4.3.1 Multi-Stage Characterisation of Powder Rehydration

4.3.1.1 Rheology-Based Approaches

Rheology has been used to monitor dairy powder rehydration dynamically *in situ* and to identify individual stages of rehydration based on changes in viscosity. Ennis *et al*. (1998) monitored the rehydration of rennet CN powders in solutions of disodium orthophosphate, a calcium binding agent, at 55 °C under constant shear conditions (600 rpm). Peaks in the viscosity index were related to changes in particle size, inter-particle interactions and protein-water interactions, with specific stages in the rehydration process being identified by the researchers: (a) an initial increase in viscosity associated with wetting and swelling of particles, (b) a minor viscosity increase due to inter-particle interactions with the creation of small clusters, (c) network formation due to particle clumping and swelling, leading to a further viscosity increase, (d) a maximum viscosity index value related to absorption of solvent by particles, and (e) progressive viscosity decreases with shear, culminating in (f) a steady low viscosity reading indicative of complete rehydration.

Ennis *et al*. (1998) reported that concentrations of disodium orthophosphate up to 0.5 %,

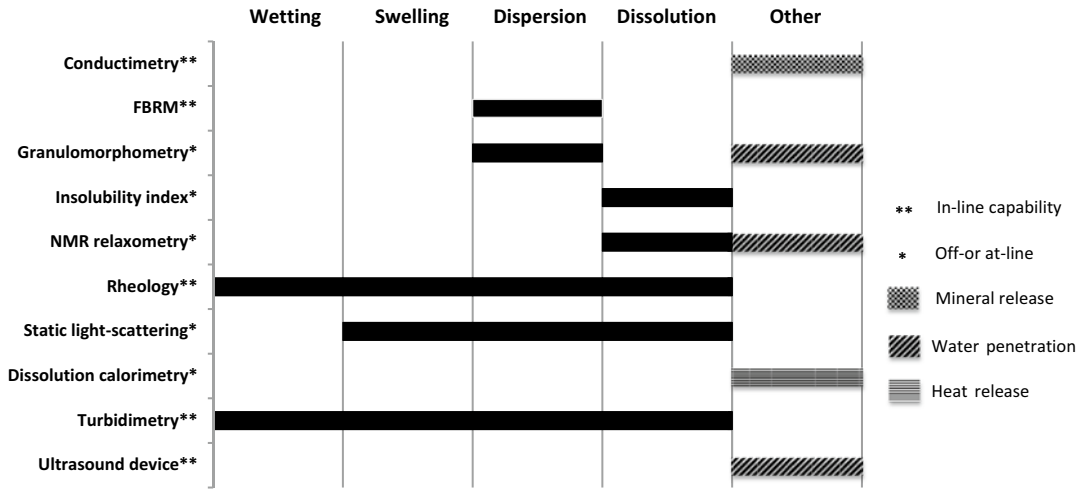

Fig. 4.6 Quantifiable rehydration stages, other data outputs and in- or off-line nature of methods for measuring the rehydration of dairy powders. *FBRM* focused beam reflectance measurement, *NMR* nuclear magnetic resonance

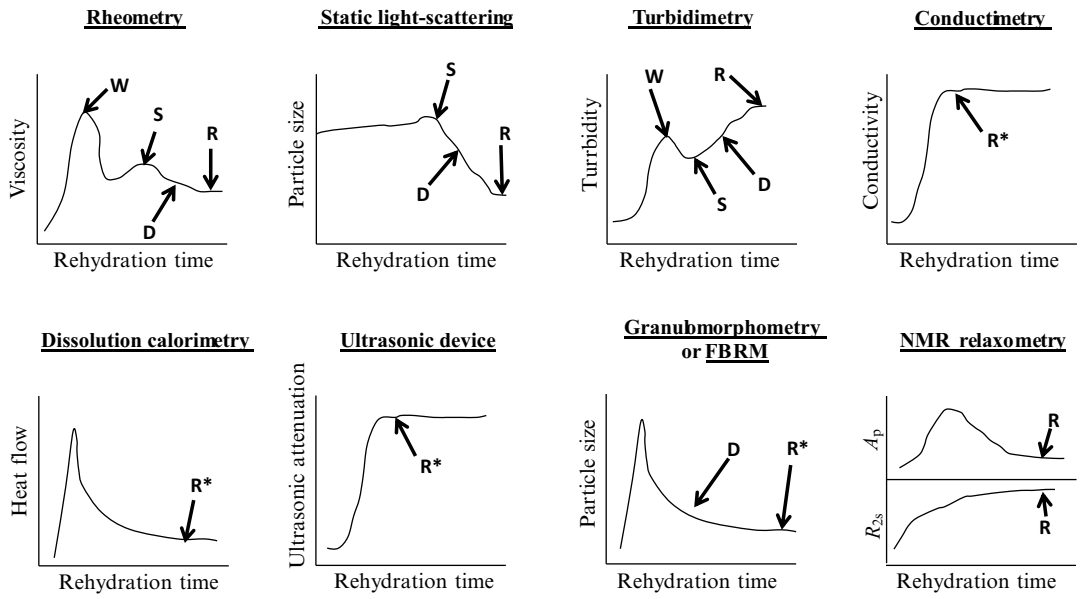

Fig. 4.7 Summary of typical rehydration profiles obtained for dairy powders using different measurement techniques. *W* wetting, *S* swelling, *D* dispersion, *R* time required for total rehydration, *FBRM* focused beam reflectance measurement, *NMR* nuclear magnetic resonance, *R** rehydration time may not indicate complete rehydration of powder in all cases

w/w, disrupted Ca^{2+}-mediated inter-particle cross bridging sufficiently to increase protein hydration, with a concomitant increase in the viscosity index; the authors also reported an increase in both the time required for particles to swell and for maximum viscosity index to be reached. At intermediate addition levels (0.35–0.7 %, w/w, disodium orthophosphate), viscosity decreased on continuing shear after reaching the maximum value, a phenomenon attributed to inhibited protein mobility, with resultant loss of water-holding capacity and reduced inter-protein interactions.

Ennis and Mulvihill (1999a) reported that monitoring of rehydration using viscometry was useful as a predictor of the performance of rennet CN powder during pilot-scale analogue cheese manufacture. After measuring rehydration using the viscometer method and furosine levels by HPLC, Ennis and Mulvihill (1999b) reported that differences in rehydration profiles of rennet CN powders were due, at least in part, to differences in levels of MR products in the rennet CN powders. Ennis and Mulvihill (2001) also observed reduced maximum viscosity index and time taken to reach maximum viscosity index in rennet CN powders manufactured from early- and late-lactation milk, which suggested that milk production season can influence the rehydration properties of rennet CN powders.

Gaiani *et al.* (2006b) used a rheometer to study the rehydration of a high-protein PC powder in water under constant shear rate (100 s^{-1}) at different concentrations, temperatures and rehydration times. This approach, when combined with particle size analysis, allowed the identification and quantification of the individual stages of the rehydration process (Fig. 4.7). Gaiani *et al.* (2006b) reported reduced swelling and rehydration times with increasing temperature from 5 to 45 °C. In efforts to improve rehydration properties, enhancement of water transfer into powder particles is thought to be crucial (Murrieta-Pazos *et al.*, 2011) and increased temperatures during rehydration are known to achieve this (Davenel *et al.*, 1997). The results of Gaiani *et al.* (2006b) suggested that the positive effect of high temperatures (40–50 °C) on the rehydration of CN-dominant powders may be linked to a reduced duration of the swelling stage.

Both Ennis *et al.* (1998) and Gaiani *et al.* (2006b) reported using "custom-built" paddle geometries for their viscometer- and rheometer-based studies, respectively. Ennis *et al.* (1998) used a paddle stirrer constructed from two flat stainless steel plates, both perforated twice, which were arranged at 90° to each other and positioned vertically in series along the rotating shaft, while the geometry used by Gaiani *et al.* (2006b) comprised four unperforated blades, also arranged at right angles relative to each other. The most noticeable geometrical variation between the paddle stirrers used by the two research groups was the presence or absence of plate perforations. The presence of narrow orifices in the design adopted by Ennis *et al.* (1998) would have increased mechanical agitation through the promotion of more turbulent mixing and thereby aided the dispersion of aggregated material; conversely, the configuration of Gaiani *et al.* (2006b), operated in the laminar regime, would have had a reduced energy input due to its lack of plate perforations. Indeed, Richard *et al.* (2013) demonstrated, using dynamic image analysis, that differences in impeller design can influence particle fragmentation and powder rehydration in the turbulent regime, which the authors attributed to associated variations in energy dissipation, suction phenomena and particle circulation.

The studies by Ennis *et al.* (1998) and Gaiani *et al.* (2006b) involved the analysis of different sample materials, thus negating any potential assessment of the influence of paddle geometry, stirring rate and flow regime; however, in future studies, with common sample bases, the effect of these factors on rehydration profiles should be taken into consideration. Indeed, it is often favourable to operate in the turbulent regime due to increased energy dissipation and promotion of powder rehydration. Thus, increased focus should be placed on the rehydration performance of dairy powders during turbulent flow, as it is probably of more direct relevance to industrial mixing operations, where shear rates in the range 10–500 s^{-1} and 10^2–10^4 s^{-1} are common during mixing and dispersion processes, respectively. According to Schuck *et al.* (2007), constant stirring rates are commonly used in industrial processes. Despite this, Gaiani *et al.* (2009a) considered that the constant speed stirring used in the studies of Ennis *et al.* (1998) and Gaiani *et al.* (2006b) deviated considerably from industrial stirring rates; if this is so, then adjusting the rate of shear to replicate these stirring rates more closely would seem a logical progression. It is also important to note that as Ennis *et al.* (1998) measured the rehydration of a CN-dominant powder in a solution of a calcium binding agent, which would cause a certain degree of *para*-caseinate dissociation with concomitant thickening, it is difficult to compare their results with those of Gaiani *et al.* (2006b); in addition, it is unclear if the viscometer method would be as sensitive to the comparatively lower viscosity changes measured by the latter researchers using rheometry for PC powders dispersed in water.

Rheological devices are available for direct in-line process stream analysis (Cullen *et al.*, 2000) (Fig. 4.6), making rheology an industrially relevant technique for monitoring dairy powder rehydration without requiring sampling for off-line analysis. In addition, indirect methods of monitoring viscosity based on following the powder consumption (amperage) of the stirrer could be useful for in-line measurements of powder rehydration during processing; indeed, Schuck *et al.* (2005) found a strong correlation between the amperage of a concentrate vacuum pump and the viscosity of dairy concentrates.

4.3.1.2 Turbidimetry

The optical phenomenon of turbidity may be visualised as a haze or cloudiness in an otherwise transparent sample. Herri *et al.* (1999) outlined the physical principle of turbidity as follows: on passage through a fluid medium, a light beam of defined wavelength (λ) may encounter suspended particles with concomitant scattering of the incident beam. The resultant global extinction phenomenon yields an extinction coefficient of light-scattering, defined as turbidity (τ_λ) (see 4.2):

$$\tau_\lambda = \frac{\frac{1}{L}\log I_0}{I_L} \tag{4.2}$$

where I_0 equates to the intensity of the incident beam and I_L represents the intensity of the transmitted beam following passage through an optical path of length L. Thus, as the intensity of the transmitted beam decreases, turbidity is observed to increase.

Utilising light in the near-infrared region (860 nm), turbidity probes direct an incident beam into a sample of interest; any suspended particles which pass the incident beam reflect it back at a 180° angle into an electronic receptor located within the instrument. Data is reported in nephelometric turbidity units as a function of time, allowing continuous monitoring of dynamic processes such as dairy powder rehydration (Gaiani *et al*., 2005, 2009a).

Using turbidimetry and particle size analysis, Gaiani *et al*. (2005) studied the rehydration properties of both agglomerated and non-agglomerated high-protein PC powders, along with PC/ultrafiltrate powders. Four stages of rehydration for PC powder were observed: wetting, swelling, dispersion, and dissolution (Figs. 4.6, 4.7). Agglomeration had a negative effect on the rehydration properties of PC powders, which was correlated with reduced dispersibility values in agglomerated powders. It was also demonstrated that mixing with ultrafiltrate by co-drying yielded rehydration times 14 and 20 times faster for agglomerated and non-agglomerated PC powders, respectively, when compared to PC powders which were not co-dried with ultrafiltrate. Gaiani *et al*. (2007) demonstrated that the rate-limiting stage of PC powder rehydration was dispersion, that agglomeration had a positive impact on the rehydration of WP-dominant powders, and that adding WP to CN-dominant powders before spray drying can improve their rehydration properties.

Gaiani *et al*. (2009a) also used turbidimetry in a study of the rehydration of high-protein PC, NaCas, and WPI powders. During rehydration, turbidity increased steadily for PC powder but remained relatively low for WPI powder; as NaCas displayed a similar rehydration profile to WPI, the lack of micellar structures in both powders presumably caused reduced light-scattering effects. PC powders underwent rapid wetting compared to WPI powders; however, the total rehydration time for PC was 48,000 s compared to 239 s for WPI, due to a protracted dispersion stage for the former. A swelling stage was not observed for WPI, possibly due to a noisy turbidity signal. Hussain *et al*. (2011b) attributed the noisy turbidity signal of WPI powders during rehydration to the formation of lumps. Hussain *et al*. (2011b) speculated that reduced water binding by globular WPs, compared to random-coil CNs, explained the lack of a swelling stage during rehydration of the WPI powders.

Hussain *et al*. (2011b) reported that rehydration in NaCl solutions (0.75–3 %, w/v) instead of water delayed the stabilisation of turbidity for a high-protein PC powder; conversely, higher NaCl concentrations (6–12 %, w/v) eliminated the swelling stage and caused more rapid stabilisation of turbidity. In agreement with Gaiani *et al*. (2009a), these authors observed no distinct swelling stage, a longer wetting time and rapid turbidity stabilisation for a WPI powder rehydrated in water compared to a PC powder. Rehydration in 9–12 %, w/v, NaCl or 2.25–12 %, w/v, $CaCl_2$ solutions caused marked elongation of total rehydration time for WPI powder.

Turbidimetry is a useful tool to characterise dairy powder rehydration *in-situ*. In combination with particle size analysis, it gives particularly detailed information about the rehydration of CN-dominant powders (Gaiani *et al*., 2005; Schuck *et al*., 2007). Available as probes which can be inserted directly into a sample or process stream, turbidimetry has good potential as an in-line method for process analysis (Fig. 4.6). In addition, it is a versatile technique, which has been used to study both CN- and WP-dominant powders, rehydrated in water (Gaiani *et al*., 2009a) and in variable ionic environments (Hussain *et al*., 2011a, b).

4.3.1.3 Static Light-Scattering

Techniques based on light-scattering are commonly used to determine particle size and particle size distributions in both wet and dry samples. Scattering is a composite of light-matter interactions (reflection, refraction, diffraction) and as such is not a singular phenomenon (Webb, 2000).

Extinction of incident light is associated with the combined effects of both light scattering and absorption (Mori, 2007). Static light-scattering (SLS), also known as laser diffractometry or small-angle SLS, is an analytical technique which estimates particle size based on the specific angle at which particles scatter light; put simply, small particles scatter at large angles and large particles scatter at small angles (Keck and Müller, 2008; Mimouni *et al*., 2009).

In SLS, patterns of scattering are derived from intensity characteristics of the entire population of particles, with a particle size distribution and a mean particle diameter being determined (Moughal *et al.,* 2000; Gaiani *et al*., 2006b). The scattering pattern depends on the ratio between the particle size and the wavelength of incident light; thus, if the value for either parameter is kept constant whilst the other is modified, the resultant scattering pattern will be altered. Depending on the ratio between particle size and incident wavelength, Fraunhofer, Rayleigh or Mie scattering patterns may be observed (Keck and Müller, 2008). Fraunhofer patterns occur in systems wherein the particle size is much greater than the incident wavelength, whilst the inverse is true for Rayleigh scattering; Mie scattering occupies the intermediate region between these two patterns (Mori, 2007).

Particle size data from SLS instruments are typically derived from calculations based on Mie theory (also known as Lorenz-Mie theory; see Mie, 1908), which requires that some theoretical assumptions are made regarding the particles themselves (Webb, 2000; Mori, 2007; Keck and Müller, 2008). In essence, Mie theory considers the scattering and absorption of an incident beam of monochromatic light composed of plane waves by spherical particles which are isotropic in nature (Webb, 2000). As such, the data output of SLS relates to the equivalent spherical diameter of particles (Moughal *et al.*, 2000).

Moughal *et al*. (2000) used SLS technology to study particle size changes during the dissolution of calcium caseinate (CaCas) powders. Following an initial 180 min period of rehydration in water, the sample was introduced to the dispersing unit of the SLS system and analysed for changes in volume distribution and obscuration over 100 min. During analysis, the peak representing particles of 0.1–2.0 μm in size was observed to increase as the 2–80 μm peak decreased; this coincided with a steady decrease in obscuration (optical concentration) values, indicating the dissolution of particles which would otherwise contribute to light-scattering phenomena. The authors reported that rehydration times of up to 6 h were required for some CaCas powders to become rehydrated and that, as such, standard tests using rehydration times of ~1 h were inadequate.

In a study on the rehydration of high-protein PC powder, Gaiani *et al*. (2005) also used SLS technology, observing that, as rehydration time elapsed, particle size initially increased, before a substantial reduction in particle size, followed by a continuous reading for small particles (Fig. 4.7). Mimouni *et al*. (2009) studied the rehydration of a high-protein MPC powder at different temperatures using SLS. Aliquots (4 mL) of solution were taken and analysed for the change in size and volume concentration of particles. The rate at which the relative volume concentration of agglomerated particles decreased was more rapid than that of primary particles. The latter was thus identified as the rate-limiting step during the rehydration of MPC powder; the researchers found that the duration of this stage was reduced on increasing temperature (24–35 °C). Based on their results, Mimouni *et al*. (2009) developed a model for the rehydration of an agglomerated high-protein MPC powder which encompassed disruption of CN agglomerates, dispersion of primary particles, hydration of air vacuoles and erosion of the proteinaceous outer surface skin of particles, with concomitant release of CN micelles and associated minerals into solution.

Harper *et al*. (1963) hypothesised that high stirring rates increased powder solubilisation by reducing the concentration of solids in regions where particles are dissolving; to investigate this, the authors increased the spatial distance between powder particles by mixing them with sand before rehydration and found that minimising the

local concentration of solids around dissolving particles improved solubility. Indeed, mechanical agitation is commonly used to increase the rate of rehydration (Schober and Fitzpatrick, 2005; Jeantet *et al*., 2010). Jeantet *et al*. (2010) monitored the rehydration of a high-protein PC powder under different hydrodynamic conditions based on changes in particle size over time, as derived from SLS measurements. Increasing temperature from 26–30 °C had the same impact as a two-fold increase in stirring rate. The number of impeller rotations required for complete rehydration increased at higher solids content, but was independent of stirring rate. Thus, it was proposed that strategies to improve PC rehydration should proceed in order of most to least effective, i.e., temperature > stirring rate > solids content. Richard *et al*. (2013) showed that these findings could be extended to a range of other dairy powders (PC, mixes of PC and WPI, mixes of PC and lactose) and impeller designs.

SLS has been used to validate and supplement data from more recently developed methods of measuring dairy powder rehydration. Gaiani *et al*. (2005, 2006b) used SLS as a reference method for identifying different rehydration stages when studying the rehydration of a high-protein PC powder with turbidimetry and rheometry, respectively (see Sects. 3.1.1 and 3.1.2). However, SLS does not provide data pertaining to powder wetting (Fig. 4.6), which is a disadvantage when studying powders where it is the rate-limiting stage, such as WP-dominant powders (Gaiani *et al*., 2007; Schuck *et al*., 2007; Hussain *et al*., 2011b). In addition, it has also been noted that the SLS methods reported in the literature involved off-line sampling and dilution of samples prior to analysis (Fang *et al*., 2010), calling into question the extent to which the SLS-derived data represents the true nature of the powder rehydration process. It should be noted, however, that in-line SLS technology has recently been developed. If such technology can be verified for use in the monitoring of powder rehydration processes, the aforementioned issues associated with SLS measurements in these systems could be resolved.

4.3.1.4 Image Analysis

Gaiani *et al*. (2009a) used a phase contrast microscope with video camera attachment to study the rehydration of a high-protein PC powder; the microscopy images clearly displayed swelling of a PC powder particle from approximately 250–400 μm, with dispersion and disintegration of the particle as time elapsed. Mimouni *et al*. (2009) used a light microscope with video camera attachment to study morphological changes to MPC powder particles after exposure to a drop of water; this allowed the identification of air vacuoles inside primary particles (also observed as indentations using scanning electron microscopy) caused by spray drying. The authors hypothesised that erosion of the surrounding proteinaceous skin of these vacuoles would be required for structural collapse and concomitant solubilisation. Also using light microscopy, Fang *et al*. (2010) captured images of a poorly-soluble MPC powder, as well as a highly-soluble MPC powder, after rehydration; the images showed that larger particles persist in the former. Scanning electron microscopy images of an MPC powder after rehydration seemed to show that particles in a poorly-soluble MPC powder, which exhibited a surface skin consisting primarily of inter-linked CN micelles, was porous enough to imbibe water readily but that release of CN micelles was restricted (Mimouni *et al*., 2010b).

A granulomorphometer was used by Richard *et al*. (2013) to capture images of dairy powders during rehydration, and to measure particle counts, size distributions, inner diameter and mean particle diameter of powder particles during dissolution (Fig. 4.8). As the method required dilution of samples prior to analysis, it was not sensitive to the early stages of powder rehydration; in addition, due to limitations in the optics of the instrument, it did not allow a value for total rehydration time to be obtained (Fig. 4.6); however, the technique was applied successfully to visualise the impact of different impeller designs on fragmentation of particles and imbibing of liquid into particles. In addition, Richard *et al*. (2013) used granulomorphometry to investigate the interaction of water or ethanol with powder

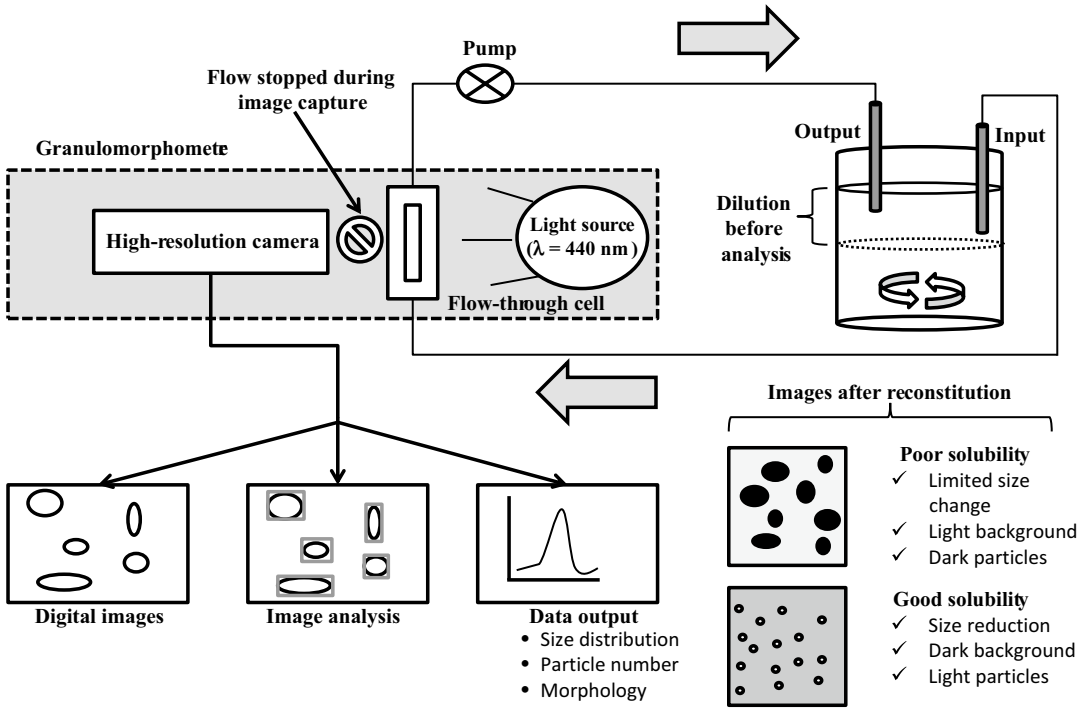

Fig. 4.8 Schematic representation of the principle of operation of the granulomorphometer (redrawn from Richard *et al.*, 2013)

particles; lightening of particle cores and darkening of image background were observed with water as the dispersant but not with ethanol; thus, these effects were reported as being due to water penetration into air vacuoles within primary particles and the advanced stages of powder dissolution, respectively.

As the above examples demonstrate, visual assessment of powder particles in solubilisation studies are commonly performed using off-line microscopy; these methods, however, require intensive sample preparation, are time-consuming, and are prone to yielding results with a high degree of error, as discussed by Gaiani *et al.* (2011). Thus, it is beneficial to use methods, such as granulomorphometry, which can monitor the rehydration of powders dynamically. However, there are a number of inherent limitations to granulomorphometry, as applied to studies into powder rehydration, namely, its requirement for sample dilution, its poor discrimination of fine particles and its off-line nature.

4.3.2 Single-Stage Characterisation of Powder Rehydration

4.3.2.1 Focused Beam Reflectance Measurement

Techniques based on laser light back-scattering, such as focused beam reflectance measurement (FBRM), are used in the analysis of particle size, particularly in the study of active pharmaceutical ingredients, where it is used to track dynamic phenomena such as crystallisation (Kail *et al.*, 2009).

The instrument (Fig. 4.9) houses its optics in a probe which can be immersed into a reaction vessel or process stream of interest. From within the probe, a laser ($\lambda = 780$ nm) is directed through an optics module, which focuses the beam at a narrow point near a sapphire window at the probe's base (Fang *et al.*, 2010). In a unique configuration, controlled speed rotation (2 m s^{-1}) of the optics directs the laser away from the middle axis of the probe, causing the beam to scan passing

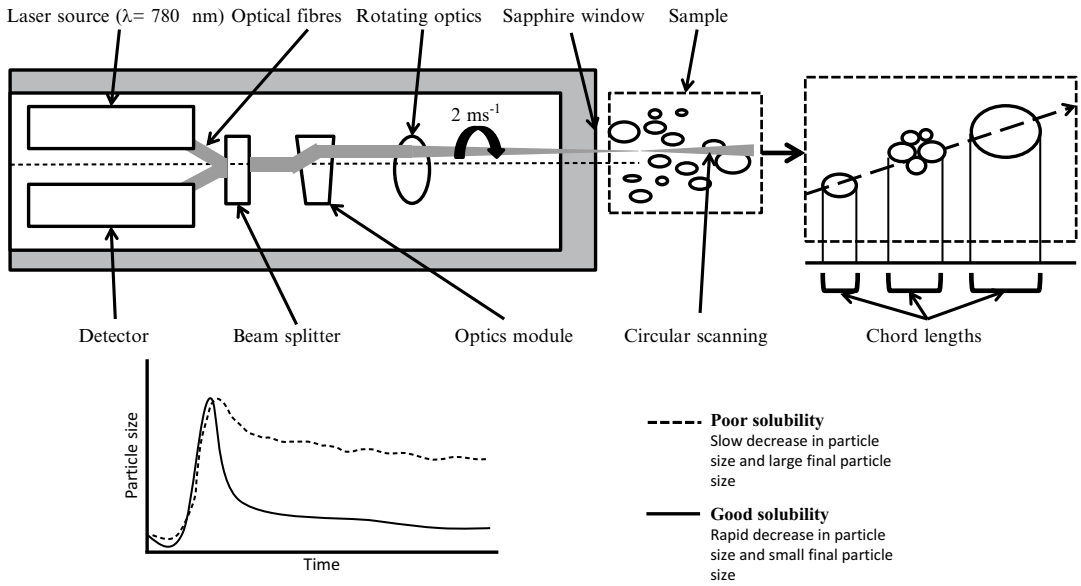

Fig. 4.9 Schematic representation of the principle of focused beam reflectance measurement (FBRM) (redrawn from Fang *et al.*, 2010)

particles in a circular manner (Dowding *et al.*, 2001). The rotating mechanism increases the spatial expanse covered by the laser and provides a more accurate representation of the particle size distribution (Singh, 2009). During analysis, laser light is first back-scattered (scattering angle of >90°) from particles; this light is then coupled by a beam splitter to an optical fiber and finally conducted onto a detector (Kail *et al.*, 2009). The duration of the resultant pulse depends on the time taken to scan completely a particle from one edge to another, which is computed by a special discrimination circuit (Dowding *et al.*, 2001). The pulse duration value is multiplied by the scan speed to yield the chord length. Thousands of chord lengths are typically recorded, which results in a chord length distribution, which can be re-calculated as either a number- or volume-weighted particle size distribution (Dowding *et al.*, 2001; Fang *et al.*, 2011). A chord length can be defined as a straight line stretching between two edges at opposite sides of a particle; in this sense, aggregates and agglomerates are analysed as single particles (Fig. 4.9).

Fang *et al.* (2010) used FBRM in their analysis of the rehydration properties of six MPC powders with varying protein contents, thermal and storage histories. For the six powders tested, a variable and rapid increase in chord length, coinciding with a low particle count, was attributed to wetting and sinking of particles. During rehydration, some MPC powders displayed reduced solubility with increasing temperature and others increased solubility with increasing temperature; these opposing effects were attributed to the differing compositions as well as storage and thermal histories of the samples. Thus, the most appropriate temperature for powder rehydration may be influenced considerably by the extent of storage- and process-induced changes.

Using FBRM, Fang *et al.* (2011) observed that the rehydration of agglomerated MPC powders was affected negatively by long storage durations (2 months compared to 2 weeks). Chord length decreased more rapidly for samples rehydrated at 50 °C (equilibrium particle size reading after 200 s) compared to 20 °C (equilibrium particle size reading after 30 min). The authors proposed a model of the rehydration of agglomerated MPC powders based on two stages: (1) dispersion of agglomerates (initial dissolution) and (2) dispersion of primary particles (equilibrium

dissolution); of course, the application of this model is restricted to agglomerated powders. Counts for different particle populations by FBRM showed that the number of large particles (150–300 μm) decreased rapidly over a period of ~300 s, while the number of small particles (1–10 μm) increased consistently during the 1800 s of rehydration. These data suggested that stages (1) and (2) initially occur in parallel but, as the former concludes, the latter continues for a protracted period; as such, stage (2) was considered to be the rate-limiting stage of rehydration for the agglomerated MPC powders studied.

Fang *et al.* (2012) rehydrated MPC powders and spray-dried them again at a range of inlet temperatures (77–178 °C), before analysing them for rehydration performance using FBRM. MPC powders subjected to a second spray drying step at low inlet temperatures (77 or 107 °C) maintained the same level of solubility as the original powder (made with one spray drying step), with both plateauing at a particle size of ~80 μm after 200 s. This effect was not observed for MPC powders dried at higher inlet temperatures (155 or 178 °C), which did not fully disperse after 30 min. The comparatively slow decrease in particle size for the control (one drying step), was attributed to the presence of greater amounts of agglomerated material, which necessitated additional dispersion; indeed, the presence of agglomerated material has been shown to affect negatively the rehydration of CN-dominant powders (Schuck *et al.*, 2007).

FBRM is a technique which allows the monitoring of dynamic processes *in situ* without the need for sample preparation or dilution (Dowding *et al.*, 2001) and it has been demonstrated to be a useful technique for studying the rehydration of dairy powders, particularly the dispersion stage of agglomerated CN-dominant powders (Fang *et al.*, 2011; see Figs. 4.6, 4.7). As such, FBRM has significant potential for use in the study of dairy powder rehydration in-line in a processing environment. However, in FBRM studies on the rehydration of dairy powders, powders were considered fully rehydrated when relatively large particle sizes (~50–100 μm) were still being measured; although particles of this size can remain in suspension in MPCs after prolonged rehydration (Mimouni *et al.*, 2009; Chandrapala *et al.*, 2014a; Crowley *et al.*, 2015), there is a question as to the sensitivity of FBRM to smaller particle sizes (e.g., CN micelles) and the time taken to reach total rehydration of powders (Fig. 4.7). In addition, the technology has yet to be evaluated for its suitability in the analysis of WP-dominant powders, or, indeed, dairy powders which are known to exhibit good rehydration properties.

4.3.2.2 Nuclear Magnetic Resonance Relaxometry

A nuclear magnetic resonance (NMR) relaxometry method, designed by Davenel *et al.* (1997), can be used to study dairy powder rehydration and has been shown to be highly sensitive to protein-water interactions. Davenel *et al.* (2002) and Schuck *et al.* (2002) both used a Minispec Bruker PC 10 NMR spectrometer (Bruker, Wissembourg, France) operated at a 10 MHz resonance frequency. During rehydration, decay curves were retrieved by recording a maximum of 845 spin echoes every 20 s from a Carr-Purcell-Meiboom-Gill (CPMG) sequence. Two exponential curves were summed in order to attain the CPMG curve, yielding the following equation:

$$S(t) = A_p \exp\left(-t.R_{2p}\right) + A_s \exp\left(-t.R_{2s}\right) \quad (4.3)$$

A_p and R_{2p} comprised the fast decay component, representing the amount of protons and relaxation rate, respectively, derived from exchangeable protons and water protons in the non-rehydrated solution. A_s and R_{2s} comprised the slow decay component, representing the amount of protons and relaxation rate, respectively, derived from exchangeable protons and water protons in the fully rehydrated solution. The relaxation rate value attributed to the slow component, R_{2s}, increased steadily as mixing progressed, whilst the number of protons associated with the fast component, A_p, decreased steadily as rehydration time elapsed; complete rehydration was observed as a constant reading for R_{2s} and a reduction to zero of protons associated with the fast component, A_p (Fig. 4.7).

Using the NMR method, Schuck *et al*. (2002), monitored the rehydration of a high-protein PC powder with minerals incorporated through different technological approaches (co-drying, bi-drying, dry-mixing; see Sect. 2.5), and observed absorption of water by particles (increased A_p) and solubilisation of particles (evolution of R_{2s}), in agreement with Davenel *et al*. (2002) (Fig. 4.7); thus, the NMR method was particularly sensitive to protein-water interactions associated with the advanced stages of rehydration (Fig. 4.6). The authors concluded that no loss of CN micelle integrity occurred when mineral salts were added by dry-mixing or bi-drying, but that structural alteration may occur if minerals are co-dried with PC. NaCl (for all methods of incorporation) elevated the concentration of free Ca^{2+} ions by 8 % compared with the control, reducing both the insolubility index and rehydration time for the PC, with little change in R_{2s} values. Davenel *et al*. (2002) also reported reduced rehydration times for PC, with no apparent change in R_{2s}, when NaCl was added before drying. These results indicated that minimal alterations to micellar properties occurred with NaCl addition and that improved rehydration was probably due to the innate hygroscopicity of the salt.

Addition of $CaCl_2$ resulted in a more pronounced impact on micellar integrity, with a large decrease in R_{2s} due to inhibited water transfer as a result of protein precipitation, with concomitant increase in insolubility and time to rehydration (Davenel *et al*., 2002; Schuck *et al*., 2002). Two calcium binding agents, sodium phosphate and citrate, were also tested by Schuck *et al*. (2002), with the greater reduction in R_{2s} values and rehydration times induced by the latter suggested a more marked effect on micellar integrity. Davenel *et al*. (2002) also observed greater reductions in rehydration time and relaxation rates for high-protein PC powders with the addition of citrates compared to phosphates. PC powder which had been freeze-dried showed delayed rehydration times compared to spray-dried PC powder; in addition, enrichment with WP or carbohydrate before drying improved rehydration of PC powder, as monitored by NMR relaxometry (Davenel *et al*., 2002). This was in agreement with the results of Mimouni *et al*. (2010a), who demonstrated that WP and carbohydrates readily dissolve during the rehydration of a poorly-soluble MPC powder.

NMR relaxometry is a highly sensitive method for studying changes to protein structure, relaxation rates and proton populations, making it a valuable tool for dairy powder rehydration studies (Schuck *et al*., 2007). Gaiani *et al*. (2009a) noted that, although the NMR method facilitated the study of certain aspects of powder rehydration, it did not allow characterisation of the wetting stage; moreover, it has little potential as an in-line method of analysis. However, the NMR technique facilitated the determination of rehydration rate and rehydration time; incomplete rehydration, due to the presence of insoluble material, could also be detected using the NMR method. Thus, NMR relaxometry is a highly sensitive method for studying the more advanced stages of powder rehydration (Fig. 4.6).

4.3.2.3 Thermochemistry

Marabi *et al*. (2007) used dissolution calorimetry to monitor thermodynamically the dissolution of SMP and maltodextrin powder (MDP). The microcalorimeter used (Calvet calorimeter, C80 Setaram, Caluire, France) compartmentalised the powder and the liquid prior to mixing. Following thermal equilibration, the powder was brought into contact with the dispersing medium to initiate wetting. Exothermic (negative enthalpy) and endothermic (positive enthalpy) responses during powder rehydration were then measured against a reference sample (water with no powder). High moisture contents and the presence of crystallised lactose were observed to yield reduced exothermic responses, which was in agreement with the negative effects of these compounds on powder solubility observed using image analysis. It was speculated that high-moisture powders had fewer hydrogen bonding sites due to the presence of bound water, and that the dissolution of crystallised material may have produced a positive enthalpic response which could have dampened the overall exothermic effect. Syll *et al*. (2012) reported an approximately linear correlation

between the water activity of powders and dissolution enthalpy values; this reduced exothermic response was again speculated to be a result of decreased availability of hydrogen bonding sites.

Also using dissolution calorimetry, Marabi *et al*. (2008) observed decreased heat release from lyophilised skim milk- or maltodextrin-based powders as fat content was increased. In addition, the authors investigated the mixing of pure fat with water, which yielded an endothermic response. It was concluded that increasing the fat level in powder systems has the effect of producing a higher endothermic response and that this would have a negative effect on powder rehydration by lowering the dissolution of enthalpy. The negative effect of a reduced heat of dissolution was correlated with longer rehydration times (determined by conductimetry; see Sect. 3.2.4). These data highlighted the fact that the negative effect of fat on powder solubilisation, widely attributed to increased hydrophobicity, may also be a result of reduced enthalpy of dissolution due to an increased endothermic response. However, the increase in rehydration time (113 s) for a powder with 45.0 % fat compared to 35.7 % fat coincided with only a minor decrease in the exothermic response; this led the authors to propose that the heat of dissolution was not capable of overcoming the rate-limiting step (wetting) for the 45 % fat powder.

Forny *et al*. (2011) highlighted the crucial role that thermodynamic approaches, such as dissolution calorimetry, could play in future studies of powder rehydration. The authors postulated that poorly-soluble powders could be characterised by their low exothermic response or an endothermic reading; furthermore, the authors proposed that powders composed of both amorphous and crystalline material could constitute a "thermodynamic microenvironment", in which poor solubility could be amended by a localised supply of heat flow from the amorphous to the crystalline phase during dissolution.

Citing the example of materials such as sodium chlorite, which is highly-soluble despite yielding an endothermic response of dissolution, Syll *et al*. (2012) stated that localised heat release as a method of improving powder rehydration was not feasible. In contrast to reports by Marabi *et al*. (2007, 2008) and Forny *et al*. (2011), these authors could find no reliable correlation between calorimetric response and dissolution time, determined by isothermal calorimetry and SLS, respectively, for various dairy powders. Moreover, Syll *et al*. (2012) reported rapid rehydration (measured using SLS) in powders which exhibited low exothermic responses. These results did not support the conclusions of Forny *et al*. (2011); thus, Syll *et al*. (2012) cautioned against using dissolution enthalpy values to investigate powder rehydration phenomena. They proposed that, used in conjunction with relaxation enthalpy measurements from differential scanning calorimetry, heat release data from isothermal calorimetry were most suitable for studying process- and storage-induced changes in amorphous powders, such as those occurring during ageing.

4.3.2.4 Conductimetry

Conductimetry measures the ability of a given solution to conduct an electric current (St-Gelais *et al*., 1995). The primary contributors to conductivity in milk systems are ions, amino acids (basic and acidic) and proteins; conversely, lactose and lipids are non-conductors (Therdthai and Zhou, 2001). Zhuang *et al.* (1997) discussed how the presence of lipids can reduce conductivity in milk by inhibiting the movement of ions. Mucchetti *et al*. (1995) demonstrated that skim milk ultrafiltrate had a similar conductivity to milk, while the retentate from diafiltration of skim milk had a significantly lower conductivity. Thus, it is minerals in the serum phase and not the colloidal phase which have the dominant influence on conductivity readings in milk (Zhuang *et al*., 1997). St-Gelais *et al*. (1995) reported that increasing temperature or acidification increased the conductivity of milk, presumably due to modifications in mineral equilibria between the serum and colloidal phases (Mucchetti *et al*., 1995).

Using a conductivity probe, inserted into a jacketed vessel, Marabi *et al*. (2008) monitored the dissolution of model food powders (SMP and MDP with different fat contents). Conductivity

values increased initially as powder was rehydrated (Fig. 4.7), presumably due to the release of charged species. An equilibrium conductivity reading was interpreted by the researchers as indicating complete rehydration. Increased fat content was reported to delay equilibrium conductivity readings (reduce solubility), with 45 % fat resulting in the longest dissolution time (~120 s). However, this effect could have been due to the inhibiting role of fat on any change in conductivity (Zhuang *et al*., 1997), rather than fat affecting directly the rehydration of other constituents in the powder.

Marabi *et al*. (2008) demonstrated that conductimetry was capable of monitoring the rehydration of both highly and poorly-soluble powders over time. Available as probes which can be inserted into samples or process streams, conductimetry also has significant potential as a method for in-line analysis. However, Marabi *et al*. (2008) conceded that conductivity data are not sufficient for physical modelling of the dissolution process, while Syll *et al*. (2012) questioned whether conductivity probes were capable of accurately identifying the end of the rehydration process. Indeed, in powders where particles containing poorly-dispersible surface skins of CN micelles remain after a reasonable period of rehydration (Mimouni *et al*., 2010a), an equilibrium conductivity reading may still be observed; thus, it would only indicate the rehydration of those constituents which are innately soluble within the time-course of the analysis.

Yanjun *et al*. (2014) reported reduced levels of insoluble material when MPC powder (80 %, w/w, protein) was ultrasonicated; despite this, conductivity levels did not change, indicating that conductimetry may not be able to detect changes in solubility. However, Gianfrancesco *et al*. (2011) used conductimetry to measure differences in the rehydration profiles of protein-based powders resulting from changes in the physicochemical state of the proteins in the powders; these researchers observed that heat-denatured β-lactoglobulin powder or NaCas powder, with or without lactose, exhibited considerably slower rehydration times than native β-lactoglobulin powder or PC powder, respectively; these results suggested that conductimetry was sufficiently sensitive to detect changes to rehydration behaviour induced by structural modifications to proteins. Indeed, Jambrak *et al*. (2008) reported increases in conductivity and solubility in ultrasonicated WPI and WP hydrolysate solutions, which was likely due to exposure of charged groups from within WPs with a concomitant increase in solvent access to proteins. Conductimetry is a useful method to measure the ionic strength of dairy systems (Crowley *et al*., 2014); thus, in studies on the rehydration properties of high-protein CN-dominant powders, it may be best applied to measure ionic strength for the determination of appropriate addition levels of salts such as NaCl, which are known to improve their solubility (Schuck *et al*., 2002).

4.3.2.5 Sound-Based Technologies

Ultrasonic spectroscopy has been used to characterise the rehydration of NaCas powder (Povey *et al*., 1999) and instant milk powder (Meyer *et al*., 2006); however, these studies were based on the properties of powders which had already been fully rehydrated. Richard *et al*. (2012) were the first to use ultrasonic tests *in situ* to monitor the rehydration of dairy powders dynamically. A schematic representation of the device is shown in Fig. 4.10. Instant extinction of the acoustic signal was observed as a high-protein PC powder was added to the stirred vessel (Fig. 4.7). As the powder dissolved and attenuation decreased, the acoustic signal recovered to an equilibrium value after 42 min; however, relaxation of the ultrasound signal did not coincide with complete dissolution, with particle sizes of between 20 and 30 μm measured by SLS after an equilibrium ultrasound reading; thus, ultrasonic relaxation measurements may not be suitable for assessing the total rehydration times of powders (Fig. 4.6). Crucially, the results of the study suggested that the release of occluded air is not as rapid as has been suggested previously by Mimouni *et al*. (2010a, b) for high-protein MPC powders and that, in fact, water penetration may be inhibited considerably by the surface skin of inter-linked CN micelles present at the surfaces of particles in high-protein CN-dominant powders.

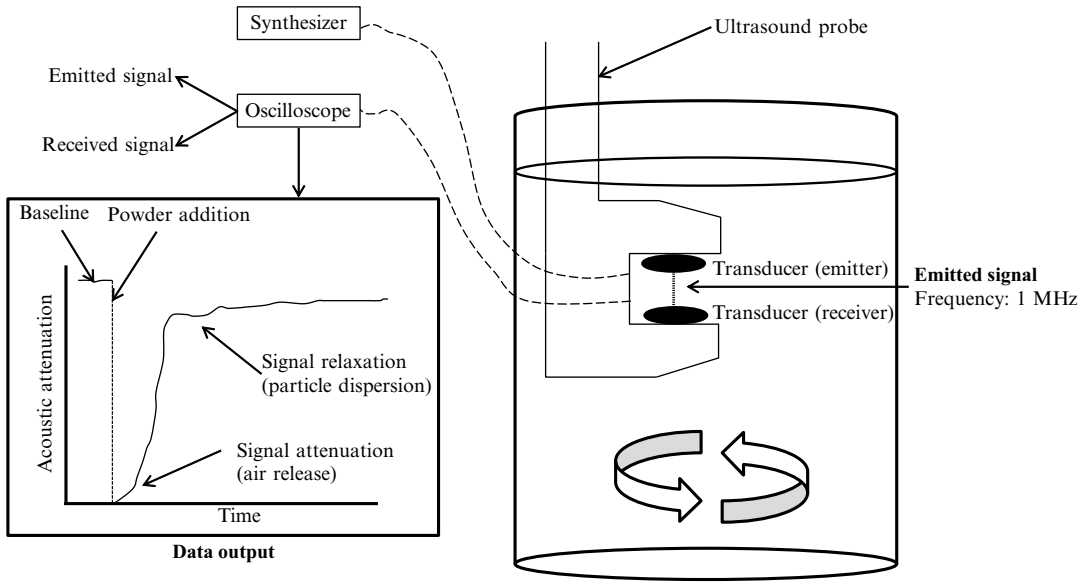

Fig. 4.10 Schematic representation of the principle and data outputs for an ultrasound device used for measuring the rehydration of dairy powders (redrawn from Richard *et al.*, 2012)

Release of occluded air from primary particles and its replacement with water is known to occur during the rehydration of dairy powders (Walstra *et al.*, 1999; Kelly *et al.*, 2003). Richard *et al.* (2012) determined that air release, as a result of solvent penetration with concomitant release of air occluded in the primary particles, was primarily responsible for ultrasound signal attenuation. The observation that attenuation levels did not recover completely following ultrasound relaxation suggested that air release did not occur during the later stages of rehydration. Thus, the technique was shown to be useful as a method to monitor the early to intermediate stages of powder rehydration. Moreover, it was shown that the ultrasound relaxation times were highly correlated with the overall rehydration times for different dairy powders (WPI, mix of PC and WPI, mix of PC and lactose). These results suggested that penetration of water into powder particles, with concomitant release of occluded air, is a key step during the rehydration of powders, as it precedes the fragmentation of primary particles and thus markedly impacts the time required to achieve complete rehydration. However, it is possible that primary particles of CN-dominant powders may imbibe water readily while retaining a surface rich in inter-linked CN micelles (Mimouni *et al.*, 2010a, b); in this case, air release would not necessarily precede fragmentation of the primary particles, but rather their sedimentation due to increased particle density (Masters, 1985).

It is clear from the aforementioned studies that further research is required to confirm whether delayed water penetration inhibits the effective dispersion of particles in high-protein dairy powders. Sound-based technologies will be important in this respect, as they are sensitive to the release of air, which can be used to account for changes in the penetration of water into particles between different powders. Other sound-based technologies also have potential applications in studies of dairy powder rehydration. For example, broadband acoustic resonance spectroscopy (BARDS), developed by Fitzpatrick (2011), has been used to study the rehydration of chemical compounds and mixtures but, until recently, had not been used in the analysis of complex, multi-component systems such as dairy powders. As the acoustic resonance measured by BARDS is associated with the release of gas from dissolving compounds, it is a useful method for monitoring the

stages of powder rehydration associated with water penetration and air release. Indeed, results from BARDS analysis indicated that air release took ~10 min for an MPC35 powder compared to ~50 min for an MPC90 powder (Vos *et al*., 2015); both powders were rehydrated to 0.2 %, w/w, in water, with significant increases in air release times expected at the higher target concentrations that these powders would typically be rehydrated to.

4.4 Conclusions

Rehydration should be considered the first and most essential attribute of high-protein dairy powders, as their use as ingredients in the food industry and other industries commonly requires them to rehydrate easily in aqueous media. A wide range of technologies are now available to study powder rehydration. Certain technologies, such as turbidimetry, conductimetry and FBRM, could find applications in industrial processes due to their in-line capabilities. Others, such as NMR-, thermochemical-, and sound-based technologies, will continue to yield new insights into the complex interactions which occur between powder components and water during rehydration.

Schuck *et al*. (2007) compared results from NMR, turbidimetry and rheometry studies into the rehydration of dairy powders. There were wide variations between the rehydration times obtained from the different methods and little agreement with insolubility index results in certain cases. The mechanisms behind rehydration, particularly for novel high-protein powders, remains poorly understood. There is a multitude of technologies available, but each varies considerably in terms of their underlying principles and data-outputs, and no method, used in isolation, would appear to be capable of fully characterising the rehydration process. Thus, future studies would benefit from an integrated approach, where two or more of these technologies are combined, to study high-protein CN-dominant powders such as PC, MPC and milk protein isolate powders. Moreover, measuring the rehydration properties of dairy powders is one of the best methods of determining the degree to which ageing-related changes have occurred during storage (Anema *et al*., 2006; Mimouni *et al*., 2009). Thus, the evolution of structure in dairy powders during storage should be a central focus in future research, as it will help explain resultant rehydration behaviour and make it possible to control this important property better.

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Emulsions and Foams Stabilised by Milk Proteins

5

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Abstract

Milk proteins are known to possess a wide range of functional properties, such as emulsification, thickening, gelling and foaming. Milk proteins facilitate the formation and stabilisation of oil droplets in emulsions or of air bubbles in foams in formulated foods. These functional properties of milk proteins are exploited in the manufacture of dairy and other products, such as recombined milk, cream, butter, yoghurt, ice cream, cream liqueurs, dressings, mayonnaise, sauces and desserts. This chapter provides an overview of the emulsifying and foaming properties of milk proteins, focusing on the adsorption of milk proteins at oil–water and air–water interfaces with emphasis on the preferential adsorption among milk proteins and the stability of milk-protein-based emulsions and foams. Highlights on the behaviour of milk-protein-stabilised emulsions after consumption that have recently attracted a great deal of research interest are discussed briefly.

Keywords

Milk-protein-stabilised emulsions • Foam stability • Emulsion stability • Emulsion digestion • Gastrointestinal tract

5.1 Introduction

Milk proteins are generally classified into caseins and whey proteins. Caseins are flexible proteins that have no rigid α-helix and β-pleated sheet structure and comprise four distinct proteins, α_{s1}-, α_{s2}-, β- and κ-casein, all of which are phosphoproteins (Fox, 2009; Singh, 2011). In contrast, whey proteins are globular in nature and

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possess high levels of secondary, tertiary and, in most cases, quaternary structure. Whey proteins can be fractionated into β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin, lactoferrin, immunoglobulins and several minor proteins. Most commercially available milk protein ingredients are mixtures of various caseins or whey proteins, e.g., caseinates, whey protein concentrates (WPCs), whey protein isolates (WPIs) and milk protein concentrates (MPCs). These ingredients are widely used in the preparation of a broad range of food emulsions and foams.

Emulsions (milk, cream, butter, mayonnaise, coffee whiteners, whipped toppings, cream liqueurs and low fat spreads) and foams (whipped cream and ice cream) are dispersed oil–water and air–water systems, respectively, and represent a major proportion of processed food formulations. During the emulsification or foaming process, both caseins and whey proteins adsorb rapidly at oil–water or air–water interfaces, forming a film around the oil droplets or air bubbles (Damodaran, 1997; Dickinson and Patino, 1999). This adsorbed layer or film protects the oil droplets or air bubbles against various physicochemical processes of instability. Knowledge of protein structures at the interfaces and their mechanical and rheological properties is essential for controlling the stability of these dispersed systems. Protein interfacial structures and properties are affected by changes in pH, ionic strength, temperature, shear and pressure, which in turn alter the stability of these dispersed systems. The aim of this chapter is to provide readers with an overview of the formation and properties of emulsions and foams stabilised by various forms of milk proteins, focusing mainly on recent studies.

In the case of emulsions, major advances have been made in understanding the adsorption process, the composition and structure of adsorbed layers of proteins and how these proteins influence their physical and chemical properties (Dickinson and Stainsby, 1988; Dickinson, 1998, 1999a; Damodaran, 2005; McClements, 2005). Interestingly, in recent years, the physical and biochemical stability of emulsions after consumption has generated a great deal of research interest (McClements *et al*., 2009; Singh *et al*., 2009; Golding and Wooster, 2010; Le Révérend *et al*., 2010; Singh, 2011; Singh and Sarkar, 2011; Singh and Ye, 2013). Some progress has been made on understanding how the adsorbed layers and the physical structures of food emulsions influence the rates of lipid digestion. Knowledge of these complex interactions between the emulsion droplets and the physiological components, such as mucin, gastric and intestinal enzymes (e.g., pepsin, trypsin and lipases) and bile salts, is key to understanding the physiological behaviour of emulsions during their transit through the gastrointestinal tract. Hence, in this chapter, we discuss current advances in our understanding of the physiological behaviour of emulsions, particularly those stabilised by milk proteins, from a physicochemical viewpoint.

5.2 Formation and Stability of Protein-Stabilised Emulsions

Generally, emulsions can be prepared using a wide range of high shear apparatus, such as colloid mills, high speed blenders, high pressure valve homogenisers and ultrasonic equipment, that mix an oil phase and an aqueous phase together in the presence of a surfactant (McClements, 2005). During high pressure valve homogenisation a coarse mixture of the oil and aqueous phases is forced through a narrow slit under the action of high pressure, resulting in cavitation, intense laminar shear flow and turbulence. Consequently, the structurally amphiphilic emulsifier molecules, such as proteins, are adsorbed at the interface, creating a stabilising interfacial layer at the droplet surface and leading to the generation of fine, uniformly dispersed droplets (Dickinson, 2003).

The physicochemical properties and the stability of emulsions depend on a number of factors such as the types and concentrations of the dispersed phase and the continuous phase, the nature of the stabilising layer, temperature, pH, the viscosity of both phases, the homogenisation conditions and other processing parameters employed, such as heat treatment, high pressure

processing and enzymatic hydrolysis (McClements, 2005). The stability of an emulsion therefore refers to its ability to resist any alteration in its properties and structure over the time scale of observation. Interfacial layers of different structures, compositions and charges can be carefully designed using specific proteins to meet the physicochemical demands and the required stability of food emulsions. Interestingly, as long as sufficient surfactant to cover the newly-created interface is present during homogenisation, emulsions are generally very stable to coalescence over prolonged storage periods. However, these emulsions are susceptible to different types of instability as a result of various types of physical and chemical processes, which in turn lead to enhanced creaming or serum separation. Generally, physical instability refers to modifications in the spatial arrangement or size distribution of the emulsion droplets, such as creaming, flocculation and coalescence, whereas chemical instability includes changes in the composition of the emulsion droplets themselves, such as oxidation and hydrolysis (McClements, 2005).

Stokes' Law can be used to describe creaming, which involves the movement of oil droplets under gravity or an applied centrifugal force to form a concentrated cream layer at the top of the emulsion without any change in the droplet size distribution. The rate of creaming can be calculated using the following mathematical expression (Hunter, 1989; McClements, 2005; Singh *et al.*, 2009):

$$\nu_{stokes} = \frac{2r^2\left(\rho_1 - \rho_2\right)}{9\eta} \tag{5.1}$$

where ν_{stokes} = velocity of creaming, r = radius of the emulsion droplets, ρ_1 and ρ_2 = densities of the continuous and dispersed phases, respectively, and η = shear viscosity of the continuous phase.

Hence, the kinetic stability of an emulsion can be increased or the creaming rate can be decreased by lowering the radius of the droplets, by increasing the viscosity of the continuous phase or by decreasing the difference in density between the two phases.

However, instabilities other than creaming, such as flocculation or coalescence, cannot be described by this law. Emulsion flocculation is an aggregation process that arises when droplets associate because of unbalanced inter-atomic attractive and repulsive forces (Dalgleish, 1997). Commonly, there are two types of droplet–droplet interaction, i.e., depletion flocculation and bridging flocculation. Generally, depletion flocculation occurs because of the presence of a non-adsorbing biopolymer in the continuous phase of the emulsion, which can promote the association of emulsion droplets by inducing an osmotic pressure gradient within the continuous phase surrounding the droplets. In contrast, bridging flocculation occurs when a high molecular weight biopolymer at a sufficiently low concentration adsorbs on to two or more emulsion droplets, resulting in bridges (McClements, 2005).

In contrast to flocculation, coalescence refers to a completely irreversible increase in droplet size by the accretion of two or more primary emulsion droplets, gradually leading to the separation of the oil phase and the aqueous phase. Coalescence generally occurs when the stabilising film surrounding the emulsion droplets is thinned to a certain critical thickness, resulting in film breakage, thus joining emulsion droplets (van Aken *et al.*, 2003; van Aken, 2004). Generally, emulsions are stable to coalescence as the proteins or other biopolymer molecules adsorb at the droplet surfaces, forming a dense viscoelastic interfacial layer (Dickinson and Stainsby, 1988). However, any extreme processing conditions, such as high shear or enzymatic hydrolysis, that lead to significant attrition of the interfacial film can give rise to gradual agglomeration of bare emulsion droplets, resulting in coalescence and oiling-off. For instance, coalescence has been widely reported in emulsions stabilised by whey protein hydrolysates because of the formation of a thinner interfacial film and the reduced surface viscosity of an interface formed with predominantly short peptides as opposed to intact proteins (Agboola *et al.*, 1998 Singh and Dalgleish, 1998).

5.2.1 Aspects of Emulsions Stabilised by Milk Proteins

Milk proteins such as caseins, caseinates, WPIs, β-lg and bovine serum albumins are known to be excellent emulsifiers because of their amphiphilic nature (Morr, 1982; Mulvihill and Fox, 1989). In most food emulsions, the oil droplets are coated by a continuous film of adsorbed material, such as caseins and/or whey proteins. They reduce the interfacial tension between the oil and aqueous phases, form films with different rheological properties and, thus, stabilise the emulsion droplets. The structure and the composition of the adsorbed layers can be quite complicated because foods in general contain a variety of surface-active agents; all are possibly adsorbed at the interface either individually as monolayers or aggregates or in combination, resulting in complex multi-layered interfacial layers (Singh *et al.*, 2009), as illustrated in Fig. 5.1. The nature of the interfacial layers formed depends largely on the type, concentration, charge and conformation of the adsorbed milk protein, and on the types of interaction and competition that occur between the adsorbed species (Dickinson, 2003; McClements, 2005).

The role of the caseins and whey proteins in stabilising emulsions has been thoroughly investigated (see reviews by Kinsella, 1984; Morr and Ha, 1993; Dalgleish, 1995, 2006; Wong *et al.*, 1996; Dickinson, 1999b, 2001; Singh, 2005). The most commonly used forms of milk protein in food emulsions are sodium caseinate and whey proteins (WPIs or WPCs). Because of their highly surface-active properties, it is possible to make stable emulsions at a relatively low ratio of milk protein to oil (about 1:60). In these emulsions, the surface protein coverage is a function of increasing protein concentration until it reaches a plateau value of about 2.0–3.0 mg/m^2 (Euston and Hirst, 1999; Srinivasan *et al.*, 2001). Because of the flexible structures of caseins, they adsorb rapidly at the interface, forming extended adsorbed layers up to about 10 nm thick (Holt and Sawyer, 1988; Dalgleish, 1990, 1995, 1996a; Mackie *et al.*, 1993; Dickinson and McClements, 1995; Fang and Dalgleish, 1998). In contrast, globular

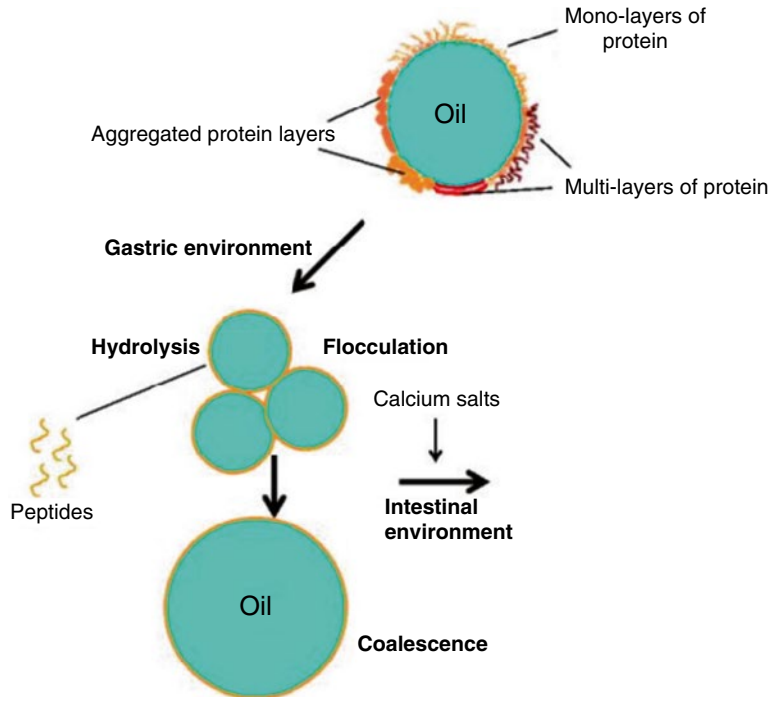

Fig. 5.1 Schematic illustration of the possible changes in milk protein-stabilised emulsions as they pass through the *in vitro* physiological model (Singh and Ye, 2013: reproduced with the permission of Elsevier Inc.)

whey proteins such as β-lg unfold partially, somewhere intermediate between the native state and the fully denatured conformation, resulting in compact adsorbed layers that are only about 2 nm thick (Dickinson, 1998). The sequence of surface activity reported for milk proteins is β-casein > monodispersed casein micelle > serum albumin > α-la > α_s-casein = κ-casein > β-lg > euglobulins (Ennis and Mulvihill, 2000).

An overview of milk protein layers adsorbed at oil–water interfaces and their relationship to the physicochemical stabilisation of emulsions is given in the following subsections.

5.2.1.1 Emulsions Stabilised by Caseins and Caseinates

Caseins, because of their surface activity, are known to adsorb strongly at an oil–water interface during emulsification, thus protecting the emulsion droplets against physicochemical instability (Dickinson, 1999b). The long-term stability of emulsions against coalescence can be attributed to both electrostatic and steric stabilisation effects (Dickinson, 2006). The relative absence of tertiary and secondary structure (Holt and Sawyer, 1988) and the presence of distinct hydrophobic and hydrophilic domains in the primary structure (Swaisgood, 1992) contribute to the relatively high surface activity of the caseins. However, as there is a lack of clarity of the native structure of caseins, the conformational changes upon adsorption at the oil droplet surface are not completely understood (Dalgleish, 2004).

Generally, α_{s1}-casein and β-casein, which contribute almost 75 % of the total casein of milk, provide similar emulsifying properties based on their amino acid sequences (Swaisgood, 1982). Both α_{s1}-casein and β-casein carry a net negative charge at neutral pH, are distinctly amphiphilic, have similarities in terms of linear disordered chains of around 200 residues with phosphoserine chains and have strong abilities to adsorb at oil–water interfaces. However, β-casein is reported to have higher surface activity and is more flexible in nature, because of its numerous proline residues, little ordered structure and negligible intermolecular cross-links, than α_{s1}-casein (Swaisgood, 1982; Dickinson, 1994). Experimental analysis has shown that β-casein adsorbs to the droplet surface with its hydrophobic region strongly anchored to the oil phase and its hydrophilic region (4–50 residues at the *N*-terminal) protruding into the aqueous phase (Dalgleish, 1996a; Dickinson, 1999b). In contrast to the tail-like anchoring phenomenon of β-casein, α_{s1}-casein has a loop-like conformation that binds to the droplet surface *via* peptides towards the middle of the sequence (compared with the end of the sequence in the case of β-casein).

Both β-casein and α_{s1}-casein have been used to prepare stable oil-in-water emulsions (Dickinson, 1989, 1999b; Swaisgood, 1992). Both caseins lower interfacial tension at the oil–water interface but the rate of lowering of the interfacial tension is greater for β-casein than for α_{s1}-casein. Moreover, α_{s1}-casein-stabilised emulsion droplets have higher negative charge at neutral pH and are relatively more susceptible to flocculation at high ionic strengths than β-casein-stabilised emulsion droplets (Dickinson *et al.*, 1988). Based on a few experimental studies, it can be inferred that α_{s1}-casein accounts for less surface coverage, resulting in thinner interfacial films, than β-casein (Brooksbank *et al.*, 1993; Dalgleish, 1993, 1996b). β-Casein, because of its relatively higher surface activity, also adsorbs preferentially at the oil–water interface compared to α_{s1}-casein, and appears to displace α_{s1}-casein from the droplet surface (Dickinson and Stainsby, 1988).

In the food industry, individual caseins are generally not used to prepare emulsions because of the cost of pure fractions and their sparse availability. Instead, various types of caseinate, such as sodium caseinate, are widely used in the preparation of emulsion-type products. Caseinates are produced from skim milk by lowering the pH to 4.6, by adding either lactic or hydrochloric acid or microbial cultures to precipitate the casein, then resolubilising it with alkali or alkaline salts of sodium, potassium or calcium at neutral pH followed by spray drying (Mulvihill, 1989). Sodium caseinate comprises not only α_{s1}- and β-caseins but also κ- and α_{s2}-caseins and small quantities of lipids and

inorganic salts. The stability of an oil-in-water emulsion made with sodium caseinate largely depends on the composition of the adsorbed layer, the quantities of proteins and the conformation of the casein in the continuous phase. In sodium caseinate-stabilised oil-in-water emulsions, all forms of caseins (α_{s1}-, α_{s2}-, β- and κ-caseins) are adsorbed at the emulsion droplet surface (Robson and Dalgleish, 1987; Hunt and Dalgleish, 1994a; Srinivasan *et al.*, 1996), providing stability against coalescence and flocculation.

In contrast to pure caseins, the competitive adsorption of β-casein rather than the other casein fractions in sodium caseinate appears to be driven by the total protein content or the volume ratio of caseinate to oil in the emulsion. It has been shown that β-casein adsorbs preferentially at the interface only at lower caseinate concentrations (<2.0 %) and/or when the ratio of caseinate to oil is low, i.e., when the caseins are predicted to exist as monomers (Srinivasan *et al.*, 1996, 1999). However, at higher total caseinate concentrations (caseinate:oil ratio of > 1:60), α_{s1}-casein adsorbs preferentially and β-casein loses its competitive adsorption ability; this has been attributed to the self-aggregating tendency of β-casein to form micelles or to complex with other casein fractions, such as α_{s1}-casein, *via* hydrophobic interactions (Lucey *et al.*, 2000). Furthermore, these aggregated complexes appear to have less emulsifying capability, as the hydrophobic areas are mutually blocked in the process of complex formation (Lorient *et al.*, 1989). Irrespective of the caseinate concentration, κ-casein from sodium caseinate has been found to be least adsorbed at droplet surfaces.

The creaming stability of sodium caseinate emulsions (20–30 % w/w oil) shows a complex dependence on the caseinate content. At lower caseinate concentrations, the emulsion is destabilised by bridging flocculation because there is insufficient protein to fully cover all the droplets in the emulsion. At an intermediate caseinate concentration of about 2.0 % w/w, the emulsion is stabilised against flocculation, coalescence and creaming for several weeks as the protein content is sufficient to cover the droplet surface. However, when the caseinate concentration is increased to above 3.0 % w/w, unadsorbed caseinate gives rise to depletion flocculation (Dickinson and Golding, 1997; Srinivasan *et al.*, 2001). Further increasing the protein concentration above 6.0 % w/w results in a very high degree of depletion flocculation, leading to a strong emulsion droplet network, which is stable to creaming.

Interestingly, concentration-dependent depletion flocculation is not common in whey-protein-stabilised emulsions. It appears that depletion flocculation in sodium caseinate-stabilised emulsions is caused by the presence of casein aggregates (sub-micelles) formed from the self-assembly of casein molecules in the aqueous phase of the emulsion at concentrations above 2 % w/w. The addition of moderate amounts of calcium chloride to emulsions containing excess sodium caseinate has been shown to eliminate depletion flocculation and to improve the creaming stability (Ye and Singh, 2001). This effect can be attributed to an increase in the average size of the casein aggregates in the aqueous phase, resulting in a large increase in the molecular mass of the caseins (Dickinson *et al.*, 2001). In addition, there is a reduction in the concentration of unadsorbed caseinate.

5.2.1.2 Emulsions Stabilised by Whey Proteins

Whey proteins (β-lg, α-la, bovine serum albumin, lactoferrin and immunoglobulins) are characterised by three-dimensional structures that are held together by disulphide bridges (Kinsella, 1984). They are soluble over a wide pH range. Whey proteins in general are highly susceptible to thermal denaturation above 70 °C because of their globular nature (Kinsella and Whitehead, 1989; Hunt and Dalgleish, 1995; Singh, 2005). The most important whey protein fractions include β-*lg* and α-la, which account for ~70–80 % of the total whey protein and possess excellent emulsifying properties. These proteins adsorb on to oil–water interfaces and form stable emulsions, although the emulsions formed are slightly less stable than casein-stabilised emulsions under the same conditions (Hunt and Dalgleish, 1994a; Dalgleish, 1995).

Structurally, β-lg is a compact, folded, globular protein, containing 162 amino acids along with two disulphide bonds and one free thiol group (Swaisgood, 1982). The three-dimensional structure of β-lg comprises nine strands of anti-parallel β-sheets, joined together into a conical β-hydrophobic barrel unit, and a flanking three-turn α-helix (Sawyer *et al.*, 1985; Papiz *et al.*, 1986; Oliveira *et al.*, 2001). Under ambient temperatures (~25 °C) and at neutral pH, β-lg exists mostly as a non-covalently linked dimer with a molecular weight of ~36 kDa (McKenzie and Sawyer, 1967; Ziegler and Foegeding, 1990). Because of its amphiphilic nature, β-lg shows good emulsifying properties by adsorbing at the interfacial layer, where it partially unfolds and forms a continuous interfacial film through intermolecular β-pleated sheet interactions. The exposed reactive free thiol groups at the interface lead to slow polymerisation of the adsorbed protein *via* sulphydryl–disulphide interchange mechanisms (Dickinson and Matsumura, 1991; McClements *et al.*, 1993; Lefèvre and Subirade, 2003). β-Lg has been the most extensively studied of all food proteins for its role in stabilising oil-in-water emulsions, because of its well-defined structure and properties (McKenzie, 1971; Kinsella and Whitehead, 1989).

α-La, another major whey protein, is a globular, calcium metallo-protein, which is stabilised by four intra-chain disulphide bonds (Swaisgood, 1982). In contrast to β-lg, α-la does not contain a free thiol group. These two whey proteins also differ in their amino acid composition, with β-lg having more proline residues than α-la (eight and two, respectively), resulting in higher hydrophobicity, and α-la having more cysteine residues than β-lg (eight and five, respectively), resulting in more internal disulphide bridges (Ng-Kwai-Hang, 2003). α-La denatures at a relatively low temperature (~66 °C) compared with β-lg (~73 °C) but does not aggregate because of the absence of a free thiol group (Dalgleish *et al.*, 1997; Schokker *et al.*, 2000; Considine *et al.*, 2007). Native α-la has good emulsifying capabilities, but has poor gelation properties.

Dickinson *et al.* (1989) studied competitive adsorption at the oil–water interface in emulsions stabilised by β-lg and α-la model systems using classical exchange measurements. They showed that competitive displacement between β-lg and α-la was rather slow and very limited, in contrast to that between caseins, which was shown to be much faster and essentially reversible in character (Dickinson *et al.*, 1988). For instance, β-lg was not displaced from the interface in β-lg-stabilised emulsions when α-la was added at a level of 1:1 % w/w until a prolonged time period of 32 h. On increasing the proportion of α-la to β-lg (to 10:1), only 15 % of the β-lg was displaced to the serum phase. In contrast, on increasing the proportion of β-lg in an α-la-stabilised emulsion (to 10:1), nearly 30 % of the α-la was displaced. This suggests that, for β-lg and α-la model systems, the interfacial adsorption is relatively irreversible (in comparison with casein systems) and the protein that is initially introduced to the interface will probably dominate at the interface, irrespective of its relative surface activity (Dickinson *et al.*, 1988, 1989). Both these whey proteins are highly structured globular proteins and undergo conformational changes upon adsorption at an interface (Fang and Dalgleish, 1997, 1998). However, of the two globular proteins, β-lg is even more difficult to displace than α-la. This can be attributed to the sulphydryl–disulphide interchange reactions that occur in β-lg-stabilised emulsions, but not in pure α-la-stabilised emulsions because of the absence of a free thiol group in α-la (Dickinson and Matsumura, 1991; Monahan *et al.*, 1995; Damodaran and Anand, 1997). Upon adsorption, β-lg undergoes partial unfolding, stretches and becomes densely packed at the interface, enabling the free thiol group on each molecule of β-lg to link *via* intermolecular covalent disulphide bridges at the droplet interface. As the extent of polymerisation increases during storage, the interfacial film continues to strengthen irreversibly with time, resulting in high surface rheology (Dickinson, 1989; Damodaran and Anand, 1997; Dalgleish, 2004). Thus, its displacement from the interface by α-la becomes highly unlikely.

Whey proteins also contain low levels of lactoferrin, a glycoprotein of molecular weight ~80 kDa, which has about 700 amino acid

residues and is well known for its iron-binding capacity (Baker and Baker, 2005). Unlike most milk proteins, which have isoelectric points (p*I*s) ranging from 4.5 to 5.5, lactoferrin has a relatively high p*I* of ~8.0 and thus has the unique property of possessing a high positive surface charge at neutral pH (almost +50 mV) (Ye and Singh, 2006a). This high positive charge density of lactoferrin has been predicted to allow the formation of cationic emulsion droplets over wide pH ranges. The adsorption behaviour of lactoferrin in oil-in-water emulsions was explored by Ye and Singh (2006a). Similar to other milk proteins such as caseinates and β-lg, lactoferrin adsorbed to the oil–water interface, producing stable emulsion droplets with a net positive charge.

In contrast to caseinates and β-lg, emulsions stabilised by lactoferrin were stable over a wide range of pH from 7.0 to 3.0. The droplet sizes of lactoferrin emulsions were reported to be very similar to those of β-lg emulsions prepared under the same conditions of pH, oil:protein ratio and homogenisation pressure. However, lactoferrin-stabilised emulsions had a comparatively higher surface coverage because of the higher molecular weight of lactoferrin. As lactoferrin in solution is highly positively charged, lactoferrin has been shown to exhibit electrostatic complexation with anionic β-lg at neutral pH (Wahlgren *et al*., 1993). Using this theory, multi-layered oil-in-water emulsions were produced from the electrostatic interactions of oppositely charged milk proteins, i.e., lactoferrin and β-lg, at neutral pH at the droplet surface, resulting in stable emulsion droplets with thick multi-layered interfacial layers and greater amounts of protein adsorbed at the oil–water interface (Ye and Singh, 2007). The primary emulsion, containing either cationic (lactoferrin-coated) or anionic (β-lg-coated) droplets, was produced initially. The secondary emulsion was then formed by adding either lactoferrin or β-lg solution to the primary emulsion based on opposite charges. Interestingly, the overall charge of emulsion droplets stabilised using the binary protein mixtures was close to zero at some concentrations. However, the multi-layered emulsions were protected against flocculation because of the strong steric effects of the dense interfacial film at the droplet surface.

In addition to pure proteins, the commercially available forms of whey protein that are widely used as emulsifiers in food industries are WPCs (comprising 25–80 % protein) and WPIs (comprising >90 % protein). These concentrated forms of whey protein are produced by ultrafiltration, diafiltration or ion exchange followed by drying steps to obtain protein levels of ~80–95 % (Morr and Ha, 1993; Mulvihill and Ennis, 2003). Both WPC and WPI are widely used in processed food applications because of their water-binding, gelling, foaming and surface-active properties (Mulvihill and Ennis, 2003; Singh, 2005). Processing treatments during the manufacture of WPC and WPI tend to denature some of the whey proteins because globular proteins are highly susceptible to conformational changes (denaturation) and aggregation when the pH, ionic strength or temperature is changed and this generally affects their functional properties.

It is interesting to note here that, when WPC or WPI is used to stabilise emulsions, similar in quantities to pure fractions of the individual proteins, there is little preferential adsorption of β-lg over α-la or *vice versa* at the droplet surface regardless of the proportion of protein to oil (Euston *et al*., 1996; Ye and Singh, 2000, 2006b). Ye and Singh (2000) showed that in WPC-stabilised emulsions (30 % w/w oil, 0.5 % w/w WPC), the proportions of adsorbed α-la and β-lg were ~18 % or ~82 %, respectively, compared with those in the original WPC solution (α-la ~ 25 %; β-lg ~ 75 %), suggesting that β-lg was adsorbed slightly in preference to α-la under these conditions.

Preferential adsorption of α-la and β-lg was more clearly demonstrated when WPI-stabilised emulsions were subjected to pH changes. Shimizu *et al*. (1981) showed that the total protein adsorption was highest at pH 5 in whey-protein-stabilised emulsions, possibly because of the dense network formed at a pH close to the p*I*. Interestingly, they also observed that the adsorption of β-lg decreased as a function of decreasing pH from 9 to 3, whereas the adsorption of α-la

increased in the same pH range. This decrease in the adsorption of β-lg was attributed to pH-dependent conformational changes of tertiary and quaternary structures (Shimizu *et al.*, 1985; Hunt and Dalgleish, 1994b).

In emulsions formed with both caseinate and whey protein, Hunt and Dalgleish (1994b) reported that the preferential adsorption depended on the protein concentration. This was further validated by a study by Ye (2008), which suggested that in emulsions made with mixtures of sodium caseinate and WPC, caseins adsorb preferentially at the oil–water interface at high protein concentrations whereas whey proteins adsorb preferentially at low protein concentrations (<3 %).

5.3 Formation and Stability of Protein-Stabilised Foams

Because of their surface-active properties, proteins are known to contribute to the formation of foams and to the physical stability of foam-based food formulations such as whipped cream, mousse and ice cream. Proteins are less effective than low molecular weight surfactants in reducing the air–water interfacial tension but they form an interfacial film that exhibits viscoelastic properties and that enables the foam to resist destabilisation (Murray and Ettelaie, 2004).

In general, foams can be generated by two mechanical methods, i.e., bubbling and stirring (Bikerman, 1973; Prud'homme and Khan, 1996; Exerowa and Kruglyakov, 1998; Weaire and Hutzler, 1999). In the bubbling method, the foam is produced by bubbling gas or air through the aqueous phase containing the foaming agent (protein, surfactants, etc.) using a single capillary, a set of capillaries or a porous plate. The size of the foam bubbles thus generated depends on the pore size of the capillaries or the porous plate, the properties of the surfactant solution, such as dynamic surface tension, surface elasticity and bulk viscosity, and the conditions of foam formation, i.e., rate of gas flow, temperature, pressure, etc. In contrast, the stirring method involves mixing the gaseous phase and the aqueous phase, which contains the foaming agent, mechanically using a stirrer or shaker or allowing simultaneous flow of the gas and the liquid in a tube.

The foaming capacity (or foamability) is defined either as the volume of foam generated under fixed conditions of temperature and intensity of mechanical agitation or by the time needed to generate a certain volume of foam. Both the foam formation process and foamability depend largely on the physicochemical properties of the stabilising substances. From a protein perspective, foam formation depends on the rate at which the proteins can transfer to the air–liquid interface and the stability of the foam generated depends on the ability of the adsorbed proteins to form a cohesive viscoelastic film *via* intermolecular bonds (Damodaran, 1997).

Similar to emulsions, foams also require high energy and subsequent thermodynamic instability makes them liable to separate into their two original phases over time. Thus, foams are also kinetically stable colloidal dispersions and undergo destabilisation over different time scales mainly by three mechanisms, i.e., liquid drainage, bubble coalescence and disproportionation of individual bubbles (Ivanov, 1988; Prud'homme and Khan, 1996; Exerowa and Kruglyakov, 1998; Weaire and Hutzler, 1999; Pereira *et al.*, 2003; Denkov, 2004; Murray and Ettelaie, 2004; Saint-Jalmes *et al.*, 2005; Denkov and Marinova, 2006). Drainage is driven mainly by gravity and involves a gradual rise of bubbles through the foam mass, while the aqueous phase drains through the lamellae and the plateau borders between the foam bubbles. In contrast, bubble coalescence involves thinning and rupturing of the isolated liquid interfacial films separating two neighbouring bubbles. Foam bubbles are stabilised against coalescence by the generation of strong colloidal forces that act between the film surfaces and the adsorption of surface-active molecules such as proteins to form a dense film. The third type of foam destabilisation is disproportionation, which involves bubble coarsening because of the diffusion of gas through the foam films, from the smaller bubbles to the larger bubbles.

5.3.1 Aspects of Foams Stabilised by Milk Proteins

Milk proteins are widely known for their foam-forming and foam-stabilising properties (Anderson and Brooker, 1988). Despite their high molecular weight and (in the case of whey proteins) their complex secondary and tertiary structures, milk proteins are able to diffuse from the aqueous phase and adsorb at the air–water interface during foam formation because of the compatibility of the hydrophobic regions of their structure with the hydrophobic nature of the gaseous phase. The different molecular structures of flexible and globular milk proteins generally lead to different structures of the adsorbed layers at the air–water interface. Milk proteins are well known to alter their charge and surface activity with pH and accordingly their foamability is also affected.

Marinova *et al*. (2009) compared the foaming behaviours of caseins and WPC as a function of pH and over a range of ionic strengths. As expected, both caseins and whey proteins showed a rapid increase in foam volume as a function of protein concentration until a plateau value was reached; there was a corresponding increase in protein adsorption at the air–water interface and a decrease in dynamic surface tension with increasing protein concentration. Both types of protein led to the stabilisation of foams against bubble coalescence. However, there were significant differences between foams stabilised with WPC and foams stabilised with caseins. For example, a relatively lower concentration of sodium caseinate (>0.3 % w/w) than of WPC (>1 % w/w) was needed to generate stable foam. Also, the volume of foam generated by WPC (10–11 mL) was nearly half of that generated by sodium caseinate (22–23 mL). When the pH was varied, WPC had maximum foamability near the p*I* whereas sodium caseinate had minimum foamability near the p*I*.

Marinova *et al*. (2009) explained these differences based on the molecular structures of the adsorbed milk protein layers and the different aggregation behaviours, as schematically presented in Fig. 5.2. At the natural pH (pH 6.5–6.8), i.e., far from the p*I* (pH 4.6), flexible casein molecules allowed the formation of denser adsorbed layers comprising hydrophobic amino acid residues in a "loop"-like configuration and the hydrophilic chain extending farther away as a "tail" within the serum phase, thus ensuring better foam stabilisation (Dickinson *et al*., 1993) (Fig. 5.2). In contrast, WPC could not anchor strongly at the surface of the foam bubbles because of its intact globular conformation, at least initially (Gurkov *et al*., 2003; Freer *et al*., 2004). The minimum foamability of sodium caseinate near the p*I* can be explained on the basis of the unavailability of sufficient quantities of casein for adsorption at the surfaces because of the precipitation of casein at the p*I*. However, in the case of whey proteins at pH 4.5, slightly positively charged *β*-lg (p*I* 5.1), and slightly

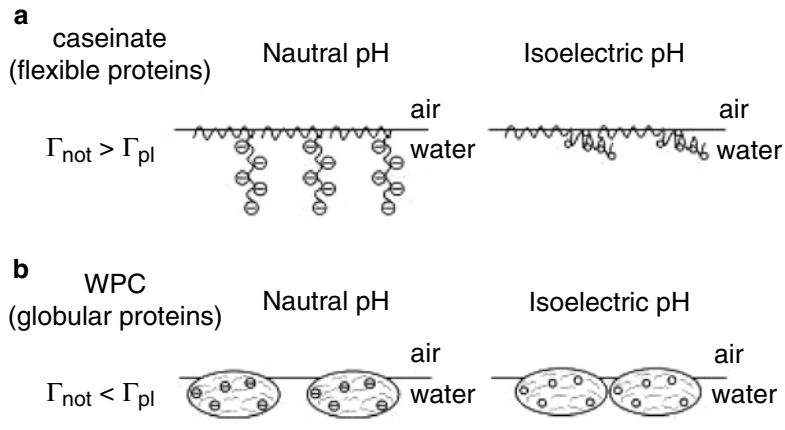

Fig. 5.2 Schematic presentation of sodium caseinate (**a**) and WPC (**b**) molecules adsorbed at the water–air interface (Marinova *et al*., 2009: reproduced with the permission of Elsevier Inc.)

negatively charged α-la (p*I* 4.5), may have interacted and strengthened the compaction of the molecules at the air–water interface *via* electrostatic interactions and thus allowed stronger interfacial films, ensuring better bubble coverage and thus high foamability. This is in line with the results obtained by other authors; it was found that, even in mixed protein systems, globular whey proteins, such as β-lg, and flexible random coil caseins, such as β-caseins, typically have different adsorption rates and also different foaming abilities because of their different abilities to adapt their conformation at the air–serum interface (Martin *et al*., 2002; Ridout *et al*., 2004).

Interestingly, the foaming properties of β-lg and WPI can be improved by increasing the ionic strength in the range 0–0.1 M NaCl at pH levels above or below their p*I*, as evidenced by dynamic surface tension measurements (Davis *et al*., 2004; Zhang *et al*., 2004). This is because of the charge screening effect of the salt, which allows the proteins to be compacted at the interface, resulting in increased protein adsorption, and the formation of a strong viscoelastic network, which provides stabilisation of the foam against bubble coalescence.

Furthermore, certain chemical (Enomoto *et al*., 2007; Wooster and Augustin, 2007), physical (Phillips *et al*., 1990; Yang *et al*., 2001) or enzymatic (Davis *et al*., 2005) treatments are also known to improve the foaming properties of whey proteins. These treatments generally influence the conformation of whey proteins, especially β-lg, by modifying the protein structure, and thus alter the kinetics of protein adsorption at the interface, the time needed for the whey protein to rearrange upon adsorption at the interface and the ability to interact with other interfacial proteins, in the case of mixed systems.

The effects of heat treatment on the foaming properties of β-lg have been studied because heat treatment is commonly used in food processing to make many food foams. When native β-lg was compared with thermally treated β-lg, it was demonstrated that heated β-lg had higher surface hydrophobicity, adsorbed at a much faster rate at the air–water interface and had better foaming properties with respect to the initial rheology of the interfacial film (Phillips *et al*., 1995; Kim *et al*., 2005; Croguennec *et al*., 2006). It has been shown that β-lg aggregates produced by heat treatment of β-lg solution at 85 °C for 15 min can significantly improve the foaming properties (Schmitt *et al*., 2007; Unterhaslberger *et al*., 2007; Rullier *et al*., 2008). Moro *et al*., (2011) showed that preheating a 5.5 % (w/v) β-lg solution at 85 °C for 3 min generated a considerable change in its aggregation profile, producing non-native monomers (51 %) and dimers (33 %) and trimers (16 %). Because of the formation of these polymeric aggregates, the surface hydrophobicity was increased dramatically, which in turn improved the foamability; the foamability was of the order of ~800 % higher than that of the corresponding foam formed with unheated β-lg. This greater foam stability against disproportionation or collapse was attributed to an increase in the viscosity of the protein solution because of the presence of aggregates, which slowed the rate of liquid drainage because of compaction of the interfacial film.

As well as the individual proteins, the interactions between β-lg and α-la during the thermal treatment of WPI have been found to play a significant role in the foaming properties and the stability of foams. Zhu and Damodaran (1994) showed that the heat treatment of WPI at neutral pH generated aggregates that improved foamability (at a monomeric:polymeric ratio of 60:40) or foam stability (at a monomeric:polymeric ratio of 40:60). Davis and Foegeding (2004) further confirmed these findings by showing similar foam stability improvements even when native whey protein was mixed with whey protein polymers generated by heat treatment at a similar monomeric:polymeric ratio of 40:60. This can be explained on the basis of rapid movement of monomeric whey protein at the interface upon foaming to decrease the surface tension followed by the formation of a viscoelastic interfacial network (mainly driven by disulphide bond formation and hydrophobic interactions) at the interface by the soluble aggregates. Nicorescu *et al*. (2009) showed that an optimal heat treatment of 2 % w/v WPI at 80 °C at ionic strength of 50 mM NaCl and at neutral pH was effective in obtaining an

improved firmness of the interfacial films because of the simultaneous formation of a cohesive network of protein aggregates (the generation of approximately 10 % soluble whey protein aggregates) at the interface and throughout the foam lamellae, which enhanced stability against liquid drainage. However, at temperatures of 80–100 °C, the generation of more than 50 % soluble aggregates caused weakening of the interfacial network because the presence of a large number of heavy clusters of aggregates, which behaved as a "solid", led to more rapid drainage.

Our understanding of the role of the molecular structure and the processing of milk proteins on the formation and stability of foams has advanced significantly over recent years. For the future, it is crucial to understand how factors such as protein–protein interactions, interactions with other ingredients such as sugar, the emulsifiers in food foams and the interfacial composition of mixed interfaces contribute to foam stability holistically and the generation of "novel" foam properties.

5.4 Interactions of Milk-Protein-Stabilised Emulsions Under Physiological Conditions

For the last decade or so, the effects of processing (e.g., heat, high pressure and shear) on the properties of food emulsions (e.g., viscosity, droplet size distribution and phase stability) have been studied extensively. Interestingly, much of this work has involved the use of milk proteins and has focused on understanding the functionality of milk proteins in stabilising emulsions and exploiting their unique properties to produce novel structures and sensory perceptions. In contrast, efforts to elucidate the fate of milk-protein-stabilised emulsions following consumption during *in vitro* gastrointestinal digestion are relatively recent and are now generating a great deal of interest.

When a complex food emulsion is consumed, the properties of each of its components, together with its interactions with physiological factors, including mucin, pepsin, lipase, gastric mucins and phospholipids, should be considered. These biochemical agents may interact with the emulsion and result in modification of the adsorbed protein layers and the droplet characteristics, affecting the stability of the emulsion and the digestibility of its components (Fig. 5.1). Although emulsions can be carefully manipulated using various physical or chemical processes before they are consumed, understanding of the interactions during transit through the gastrointestinal tract is of great importance to gain insights into the post-consumption structural and physicochemical changes in these emulsions. As this chapter is concerned with milk proteins, we briefly describe the behaviour of milk-protein-stabilised emulsions in physiological environments and discuss how the milk protein-based interfacial layer influences the various steps involved in the digestibility of emulsified lipids.

When a milk-protein-stabilised food emulsion is consumed, it resides for a short period in the mouth and is exposed to a wide range of biochemical conditions, such as dilution effects, because of mixing with saliva, and access to salivary enzymes such as amylases, biopolymers such as mucins and different electrolytes in the saliva, as well as physicochemical conditions, such as moderate changes in pH and temperature (to around 37 °C) and shear forces between the tongue and the oral palate (Malone *et al*., 2003a, b; de Wijk *et al*., 2004; de Wijk and Prinz, 2005; Vingerhoeds *et al*., 2005). Interestingly, there is some evidence to show that the behaviour of milk-protein-stabilised emulsions in the mouth is largely driven by the non-covalent interactions of salivary components with the adsorbed milk protein layer at the oil droplet surface (van Aken *et al*., 2005; Sarkar *et al*., 2009a; Vingerhoeds *et al*., 2009). Emulsions formed with WPI, sodium caseinate and lactoferrin showed flocculation of the droplets when mixed with human saliva. This flocculation was predominantly driven by the highly glycosylated negatively-charged mucin present in human saliva. The emulsion flocculation in the presence of saliva was considered to be regulated by depletion forces, van der Waals' forces and/or electrostatic interactions between emulsion droplets and salivary proteins, and was largely dependent on the

initial charge of the milk-protein-stabilised emulsion droplets (Silletti *et al*., 2007; Sarkar *et al*., 2009a).

Sarkar *et al*. (2009a) investigated the behaviour of negatively and positively charged oil-in-water emulsions stabilised by milk proteins in the presence of artificial human saliva. At neutral pH, negatively-charged β-lg-stabilised emulsions underwent some degree of depletion flocculation because of strong repulsive forces with anionic mucin. In contrast, positively charged lactoferrin-stabilised emulsions interacted with mucin *via* electrostatic interactions, which led to bridging-type flocculation under certain conditions. These kinds of emulsion–saliva electrostatic interactions might occur when emulsions are consumed in real situations and could result in different sensory and textural perceptions *in vivo*.

After oral transit, emulsions are swallowed, are subjected to shear effects in the oesophagus and are finally exposed to a highly acidic pH (typically between 1 and 3) and shear forces because of peristaltic movements of the stomach (Weisbrodt, 2001; Kalantzi *et al*., 2006). During the gastric phase, emulsions are exposed to digestive juices, containing proteolytic (pepsin) and lipolytic (gastric lipase) enzymes, mucins and salts. It is obvious that milk-protein-stabilised emulsions would undergo major changes in the stomach because of the possible action of pepsin on the protein layer at the interface, the effects of low pH and ionic strength on the droplet charge and the interactions of gastric mucin with interfacial protein. For example, flexible caseins are highly susceptible to hydrolysis by pepsin in aqueous solutions (Guo *et al*., 1995). However, globular whey proteins, particularly β-lg, are known to be largely resistant to peptic hydrolysis in their native state (Schmidt and Poll, 1991).

Studies by Macierzanka *et al*. (2009) and Sarkar *et al*. (2009b), which are in agreement with other reports, suggest that β-lg becomes highly prone to hydrolysis by pepsin when present as the interfacial layer in an emulsion. This is due to a conformational change of the β-lg molecules upon adsorption at the droplet surface, which opens up the peptic cleavage sites for enzymatic attack by pepsin. Interestingly, in milk-protein-stabilised emulsions, generally not all the protein is present at the droplet surface, i.e., in the adsorbed state; a considerable proportion remains in the aqueous phase. As the adsorbed and unadsorbed proteins are likely to exist in different conformational states, their susceptibilities to pepsin could be different under gastric conditions; the rate of hydrolysis of β-lg, from sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels, is shown in Fig. 5.3. Sarkar *et al*. (2009b) showed that adsorbed β-lg was more susceptible to pepsin hydrolysis (85 % decrease in the intact protein) than unadsorbed protein (about 50 % decrease in the intact protein). In contrast, β-lg in solution in its native state was largely resistant to pepsin digestion (about 20 % decrease in the intact protein) under the same simulated gastric conditions. This suggested that the conformation of unadsorbed β-lg is significantly altered during emulsion formation, possibly as a result of the high turbulence during homogenisation, as mentioned in the previous section.

The hydrolysis of the adsorbed layers by pepsin also results in a loss of positive charge on the droplet surface as well as a reduction in the thickness of the adsorbed layer (Sarkar *et al*., 2009b). The peptides that remain at the interface are unable to provide sufficient electrostatic and steric stabilisation effects. As a result, these emulsions with hydrolysed interfaces are highly susceptible to flocculation and coalescence (Fig. 5.1). Such a phenomenon has been demonstrated for lactoferrin- and β-lg-stabilised emulsions, which undergo flocculation followed by some degree of coalescence on exposure to simulated gastric conditions (Sarkar *et al*., 2009b; Sarkar, 2010).

In addition to the important effect of gastric enzymes, highly glycosylated mucin, which forms a self-associated gel-like structure at gastric pH and protects the stomach from digesting itself, also has an important role in its interaction with milk-protein-stabilised emulsions (Bansil and Turner, 2006). The work of Sarkar *et al*. (2010a) suggests that the addition of a low level of soluble mucin promotes the flocculation of

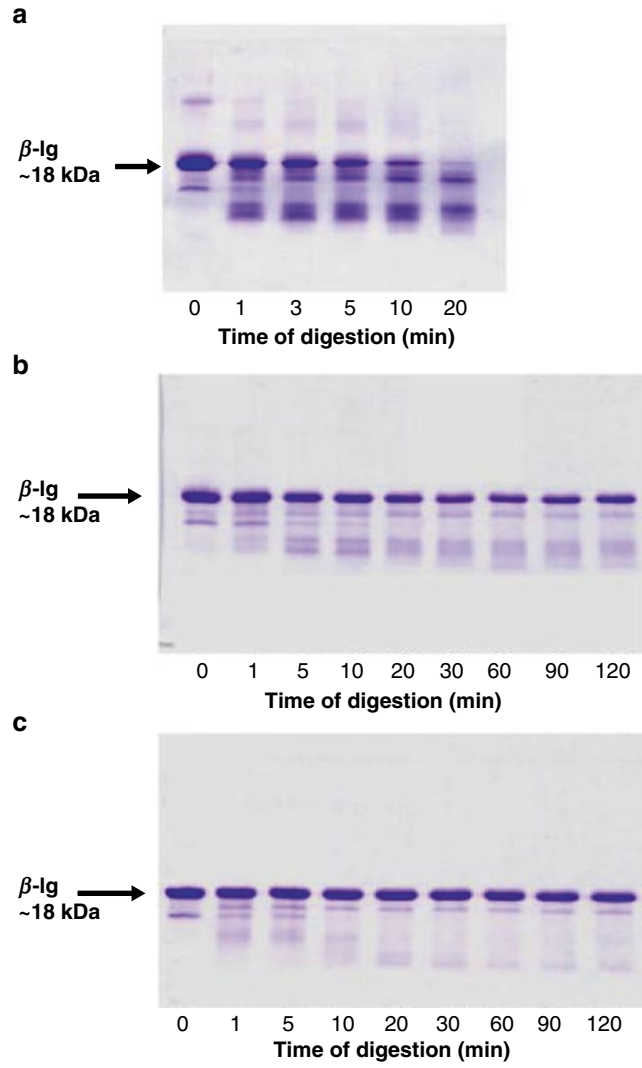

Fig. 5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoretograms of β-lg emulsions: (**a**) cream phase; (**b**) continuous phase; (**c**) native β-lg solutions [containing 0.36 % β-lg (the same as the concentration of β-lg in the continuous phase of the emulsion)] after mixing with simulated gastric fluid as a function of incubation time (Sarkar *et al*., 2009b: reproduced with the permission of Elsevier Inc.)

β-lg-stabilised emulsions, possibly through a bridging mechanism, but does not significantly affect the action of pepsin on the adsorbed β-lg layer. Overall, when they reach the gastric tract, emulsions stabilised by milk proteins show a significant level of instability, which is predominantly driven by the proteolytic effect of pepsin.

Following transit through the gastric tract, emulsions enter the human intestinal tract, which is a very complex environment, as it contains various salts, pancreatic enzymes, coenzymes, bile salts, phospholipids, remnants of oral and gastric digestion and various microbial species at neutral to alkaline pH (6.0–7.5) (McClements *et al*., 2009; Singh *et al*., 2009; Singh and Ye, 2013). As milk protein might be susceptible to the actions of pancreatic enzymes such as trypsin and chymotrypsin, both the adsorbed proteins/peptides and the unadsorbed proteins/peptides might be further hydrolysed into smaller peptides and amino acids. Over the last few decades, several studies have reported the effects of the hydrolysis of milk-protein-stabilised oil-in-water emulsions by trypsin on their physical stability (Kaminogawa *et al*., 1987; Leaver and Dalgleish, 1990). As expected, extensive hydrolysis of adsorbed protein layers results in rupturing of the interfacial layer, leading to the coalescence of

emulsion droplets and subsequent oiling-off (Agboola and Dalgleish, 1996). However, the behaviour of milk protein-based emulsions in a more complex intestinal environment, simulating intestinal conditions, has not been reported until very recently, by Sarkar *et al*. (2010b, c).

In addition to intestinal enzymes, the interaction of surface-active bile salts with the adsorbed protein layer are important. Bile salts, which originate from the liver *via* the gall bladder, consist mainly of sodium salts of taurocholic, taurodeoxycholic, taurochenodeoxycholic, glycocholic and glycodeoxycholic acids. These surface-active compounds displace the adsorbed proteins/peptides from the surface of emulsion droplets because of their relatively higher surface activity, thus promoting the accessibility of the active site of lipase to the hydrophobic lipid core (Wickham *et al*., 1998; Fave *et al*., 2004; Mun *et al*., 2007; Sarkar *et al*., 2010b). The nature and, in particular, the charge of the adsorbed milk protein layer seem to drive the preferential adsorption of bile salts and the subsequent displacement of the protein layer (Singh *et al*., 2009). For instance, bile salts have been shown to displace whey proteins more readily than caseinates from the interface of emulsion droplets during storage (Mun *et al*., 2007). In emulsions stabilised by negatively charged β-lg, displacement of protein was observed even at the lowest concentration of bile salts (Sarkar *et al*., 2010b), but the bile salts did not displace positively-charged lactoferrin from the emulsion droplets. The bile salts appeared to bind to the adsorbed lactoferrin layer *via* an electrostatic mechanism.

Upon entering the intestine, pancreatic lipase adsorbs to the droplet interface, usually *via* complexation with colipase and/or bile salts (Bauer *et al*., 2005). Colipase is a short polypeptide with a molecular weight of 10 kDa, which forms a stoichiometric complex with lipase in a 1:1 w/w ratio, enabling the water-soluble pancreatic lipase to attach firmly to the hydrophobic lipid core at the oil droplet surface (Erlanson-Albertsson, 1992). Bile salts may either facilitate or inhibit the activity of pancreatic lipase depending on their concentration (Lowe, 2002; Bauer *et al*., 2005). At low concentrations, bile salts promote pancreatic lipase activity, mainly by allowing the adsorption of lipase to the oil–water interface (Gargouri *et al*., 1983; Mun *et al*., 2007) as well as solubilising and removing the inhibitory reaction products from the oil–water interface. However, at high concentrations, bile salts generally compete with lipases for adherence to the droplet surface, thus inhibiting the point of contact between the hydrophobic lipid core and the lipase (Gargouri *et al*., 1983) and retarding lipase activity. Pancreatic lipase cleaves triacylglycerols to form 2-monoacylglycerols and fatty acids; some of these digestion products are surface active and could potentially displace the initial adsorbed material from the droplet surface (McClements *et al*., 2009; Singh *et al*., 2009; Sarkar *et al*., 2010c).

Most studies of lipid digestion in milk protein-based emulsions have used *in vitro* intestinal models containing pancreatic lipase and bile salts. The extent of lipid hydrolysis was found to be similar in caseinate- and whey protein-stabilised emulsions, although the oil droplets in the whey protein-stabilised emulsions were less stable (Mun *et al*., 2007). Our study showed that lactoferrin- and β-lg-stabilised emulsions underwent a significant degree of coalescence on the addition of physiological concentrations of pancreatin and bile salts (Sarkar *et al*., 2010c). For both emulsions, destabilisation in simulated intestinal fluid was largely attributed to the lipolysis of the hydrophobic lipid core by the lipase fractions of the pancreatin as well as the proteolysis of the adsorbed protein layer by the trypsin or other proteolytic fractions present in pancreatin.

In addition to pure whey protein systems, studies on the *in vitro* digestion of WPI emulsions showed that they did not undergo pronounced structural changes during simulated gastric digestion although the α-la and a portion of the β-lg adsorbed at the interface were hydrolysed by pepsin. However, during the subsequent intestinal phase of digestion, the partially digested WPI-stabilised emulsion droplets underwent coalescence (Li *et al*., 2013). In contrast, in the case of sodium caseinate-stabilised emulsions, the droplets underwent droplet flocculation

with some degree of coalescence during the gastric phase itself (Li *et al.*, 2012). Because of its open flexible structure, casein was easily hydrolysed by pepsin, which in turn led to coalescence of droplets under gastric conditions. Overall, at both sodium caseinate- and whey-protein-stabilised interfaces, digestion in the gastric fluid containing pepsin apparently accelerated the coalescence of the emulsion droplets during subsequent exposure to intestinal fluid containing pancreatic lipase. However, for both milk-protein-stabilised emulsions, the rate and the extent of lipid digestion in the intestinal environment were found not to be influenced by the previous structural changes that may have occurred during the gastric phase.

Recently, a number of researchers (Hur *et al.*, 2009; Li *et al.*, 2012; Kenmogne-Domguia *et al.*, 2013) have studied the effect of proteolysis of the adsorbed milk protein layer and subsequent physicochemical changes of the emulsion droplets during the entire physiological transit. Emulsions stabilised by casein and bovine serum albumin were treated under *in vitro* gastric conditions at various pH values and at various concentrations of pepsin, as a function of incubation time. The adsorbed protein was hydrolysed to different degrees by pepsin, which resulted in droplet flocculation and coalescence in the emulsions. When these gastric-treated samples were exposed to *in vitro* intestinal digestion, the results showed that gastric conditions could modify the kinetics of lipolysis, but had limited impact on the final extent of lipolysis in the intestinal step of digestion.

Studies in our laboratory on lipid droplets initially coated with lactoferrin (cationic) and β-lg (anionic) and sequentially treated with simulated oral, gastric and intestinal fluids in an *in vitro* physiological model further validated that milk-protein-stabilised interfaces, irrespective of their high original electrostatic charges, offer little protection to the droplets against pepsin- and pancreatin-induced destabilisation and thus cannot help, individually, in controlling the rate and the extent of lipid digestion (Sarkar, 2010; Singh and Sarkar, 2011). The mechanism of destabilisation and re-stabilisation in intestinal fluid following pre-processing in oral and gastric fluids could not be interpreted reliably because of interference from one or more of the factors in the chemically complex, simulated physiological media used. There is a clear need for further research in this area to have a better understanding of the different competitive displacement mechanisms and hydrolytic reactions occurring in the intestine and to characterise the final state of the droplets and the products of lipid hydrolysis. More complete *in vitro* digestion models to simulate various physiological processes occurring in the mouth, stomach and small intestine need to be developed and then validated by *in vivo* and clinical studies. Further research in this area is likely to lead to new knowledge that can be used in designing food matrices by manipulating milk proteins effectively at the oil–water interface during physiological transit for controlled lipid delivery applications.

5.5 Conclusions

Milk proteins in both soluble and dispersed forms have excellent surface-active, foaming and emulsion-stabilising properties. Differences in structure, flexibility and state of aggregation of the different milk proteins give rise to differences in their emulsion- and foam-stabilising properties. These attributes of milk proteins have been exploited to manufacture various prepared foods. For decades, research has been performed on oil-in-water emulsions and foams using purified or simple mixtures of caseins and whey proteins to manufacture a wide range of products and there is now a great deal of understanding on the conformation of proteins at oil–water interfaces, competitive exchange reactions and factors controlling the rheology and stability of emulsions under different environmental conditions (temperature, pH and ionic conditions). However, much less is known about the further processing of emulsions after they have been consumed, i.e., during oral processing in the mouth and during the digestion processes. This area of research

needs to be developed further before the interactions between milk-protein-stabilised emulsions and physiological factors can be carefully utilised to develop novel products with sensory and/or health benefits.

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Heat-Induced Denaturation, Aggregation and Gelation of Whey Proteins

6

André Brodkorb, Thomas Croguennec, Said Bouhallab, and Joseph J. Kehoe

Abstract

Whey proteins, a group of acid-soluble proteins, represent approximately 20 % of the total protein in bovine milk. The two main proteins, β-lactoglobulin (18.3 kDa) and α-lactalbumin (14.2 kDa) have been the subject of numerous studies. The purpose has mostly been to elucidate and exploit potential structure/function relationships. Both proteins provide high nutritional value and are utilised in the production of nutritional beverages such as infant formula and energy drinks. In addition, β-lactoglobulin offers a range of useful techno-functional properties, such as thickening, emulsification, gelation or foaming. Exposure of whey proteins to heat is a common industrial processing step that causes structural changes in proteins and that can lead to increases in viscosity and/or formation of potentially extensive gel networks above a critical protein concentration. This chapter describes such heat-induced unfolding, denaturation and aggregation processes, and their kinetics, as well as cases of ordered protein structures such as fibrils or nanoparticles. Heat-induced and cold gelation of whey proteins is also described. Gel formation is brought about by the assembly of soluble aggregates formed during the initial stages of heating. Such gel networks develop through electrostatic, hydrophobic and covalent interactions between denatured whey proteins. The micro- and

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macro-structure of whey protein gels vary widely and are dependent on the nature of the aggregation processes involved. The influence of protein type and concentration, salt type and ionic strength, pH and heating conditions on the above processes are reviewed.

Keywords
Dairy proteins • Whey proteins • Denaturation • Aggregation • Gelation

6.1 Introduction

Whey proteins are widely used in the food industry and can be used to provide a variety of functionalities. Whey proteins are used for nutritional purposes, as thickening agents, emulsifiers, gelling and foaming agents. For some of these functionalities thermal treatment is necessary or can be used to improve functionality. During thermal treatment above a certain temperature, whey proteins unfold, denature and aggregate. A good understanding of these processes can help to maximise the desired functionalities tailored for a particular use and to control/reduce issues associated with thermal processing of whey protein solutions. The whey protein content of bovine milk and liquid whey is quite low and further processing of whey normally occurs to make whey protein ingredients. The most common whey protein products are whey protein concentrates (WPC) and whey protein isolates (WPI); additionally the individual whey proteins such as β-lactoglobulin, α-lactalbumin and lactoferrin can be fractionated from whey.

6.2 Heat-Induced Denaturation of Whey Proteins

For over 60 years the denaturation kinetics of whey proteins, in particular of β-lactoglobulin, has been studied. Tables 6.1 and 6.2 summarise denaturation details and an overview of the studies conducted to date of whey protein denaturation kinetics. One of the earliest papers on the subject (Briggs and Hull, 1945) divided denaturation/aggregation into two processes. The first process occurred only at temperatures over 65 °C. The authors applied moving boundary electrophoresis using a Tiselius apparatus to native and heated β-lactoglobulin. There was no change in the electrophoretic mobility ("slow fraction") of β-lactoglobulin heated above 65 °C but they observed an increase in "particle weight". This first process followed first order kinetics. The second stage could only proceed once the first stage had occurred, but could proceed at lower temperatures. The second process followed second order kinetics and caused an increase in electrophoretic mobility ("fast fraction"). It is likely that the first process was protein unfolding and the second process was protein aggregation. It was noted that increasing the pH or reducing the ionic strength decreased the rate of the second process (aggregation). In β-lactoglobulin the two processes have been identified by differential scanning calorimetry (DSC) (Fitzsimons *et al.*, 2007), where the denaturation process is endothermic and the aggregation process is exothermic. Previous

Table 6.1 Thermal denaturation temperature and enthalpy of whey proteins

Protein	T_D (°C)	T_{tr} (°C)	ΔH (kJ mol^{-1})
β-Lactoglobulin	78	83	311
α-Lactalbumin	62	68	253
Bovine serum albumin (BSA)	64	70	803
Immunoglobulin (Ig)	72	89	500

T_D is the initial denaturation temperature
T_{tr} is the temperature at the DSC peak maximum
ΔH is the enthalpy of denaturation
Adapted from de Wit (1984) and Kinsella and Whitehead (1989)

Table 6.2 Summary of studies of β-lactoglobulin denaturation and their kinetic orders

Author	Order	Comments
Briggs and Hull (1945)	Two processes: 1.0 and 2.0	pH 6.9, 0.1 M ionic strength
		pH and ionic strength affect rate of second process Used moving boundary electrophoresis
Gough and Jenness (1962)	1.0 (assumed)	In skim milk and in buffer at pH 6.9
Lyster (1970)	2.0	Skim milk
		First order with respect to β-lactoglobulin concentration.
Hillier and Lyster (1979)	2.0	Skim milk
		Used polyacrylamide gel electrophoresis (PAGE) for analysis
Dannenberg and Kessler (1988)	1.5	Skim milk
		Used ultra thin-layer isoelectric focusing for protein quantification
Roefs and de Kruif (1994)	1.5	Pure β-lactoglobulin solutions
		Proposed mechanism
Anema and McKenna (1996)	1.5	Reconstituted whole milk
		Native-PAGE for protein quantification
Verheul *et al.,* (1998)	1.5	Pure β-lactoglobulin solutions
Oldfield *et al.,* (1998)	β-lg A 1.0±0.3	Skim milk
	β-lg B 1.4±0.4	Heating carried out in UHT plant
		Higher orders obtained above 95 °C
Hoffmann and van Mil (1999)	1.5	Pure β-lactoglobulin in 20 mM Tris–HCl buffer
Oldfield *et al.,* (2000)	0.6±0.7 to 1.4±0.4	Skim milk
		Studied the effect of pH
		Values are pH dependent
Croguennec *et al.,* (2004b)	β-lg A 1.96	Pure β-lactoglobulin solutions
	β-lg B 1.98	
Hinrichs and Rademacher (2005)	2.0	Skim milk
		Combination of pressure and heat treatment
Sava *et al.,* (2005)	Pseudo 1.0	Pure β-lactoglobulin solutions
		Measured surface hydrophobicity and exposed sulfhydryl groups
Oldfield *et al.,* (2005)	1.3±0.7 to 1.7±0.2	Whey protein enriched/depleted skim milk
Mounsey and O'Kennedy (2007)	1.97	Pure β-lactoglobulin solutions
Kehoe *et al.,* (2007)	1.4±0.1 to 1.7±0.1	Mixed β-lactoglobulin/BSA solutions
Kehoe *et al.,* (2011)	1.6±0.4 to 1.0±0	β-Lactoglobulin and WPI solutions at a range of concentrations

studies had only observed the endothermic unfolding process as the aggregation step rapidly follows unfolding and the exothermic peak produced is relatively small compared to the endothermic peak. The individual whey proteins present in WPC and WPI all unfold at different temperatures ranging from 62 to 78 °C (de Wit, 1984; Kinsella and Whitehead, 1989). Table 6.1 gives an overview of the denaturation parameters of the main whey proteins.

The kinetics of denaturation of β-lactoglobulin has been widely studied and is highly dependent on the environment as well as genetic variants. Gough and Jenness (1962) studied the denaturation kinetics of β-lactoglobulin A and B. They concluded that β-lactoglobulin B denatured at a faster rate than β-lactoglobulin A in skim milk and in buffer. Lyster (1970) found that the denaturation of β-lactoglobulin in skim milk followed second order kinetics when milk samples were heated at

85 °C for 0–3 min. It was found that the reaction order for β-lactoglobulin denaturation with respect to concentration was 1.0. This unusual behaviour of the protein in having one reaction order with respect to temperature and another with respect to concentration had already been noted for ovalbumin (Haurowitz *et al.*, 1952). However, it should be noted that the denaturation of β-lactoglobulin was carried out at 100 °C, which is well in excess of the denaturation temperature (Table 6.1). Denaturation at a lower temperature might have yielded different results, especially as the authors had already noted a change in temperature dependence in the Arrhenius plot at around 90 °C. It was also noted that the rate of α-lactalbumin denaturation increased when β-lactoglobulin was added. The addition of a sulfhydryl blocking agent to the milk had no effect on the denaturation rates at temperatures below 78 °C; at temperatures above 78 °C there was a large decrease in the rate of β-lactoglobulin denaturation. This result was important as it supported earlier findings as to the importance of sulfhydryl groups (Chaudry and Humbert, 1968). The two-step process involving the denaturation of whey proteins through an initial unfolding step followed by an irreversible aggregation step was accepted for many years; more recently, it has become clear that as the protein unfolds during thermal treatment, several rearrangements of the protein structure occur. These "activated" intermediate states then undergo irreversible aggregation.

During the initial unfolding step the hydrophobic groups at the core of the native protein molecule become exposed on the surface, increasing the hydrophobic interactions between molecules. The unfolding process also exposes some of the disulphide bonds and free sulfhydryl groups (in the case of β-lactoglobulin) making them available to participate in intramolecular disulphide bonds. These bonds may form *via* oxidation of the free sulfhydryl groups or by rearrangement of existing disulphide bonds.

The most widely accepted model for β-lactoglobulin denaturation and aggregation kinetics was proposed by Roefs and de Kruif (1994). The mechanism was based on a polymerisation reaction involving three steps: activation, propagation and termination, with aggregation occurring *via* disulphide linkages, as follows:

$$B_2 \leftrightarrows 2B \rightarrow B^* \text{ (activation)}$$

where B_2 is the non-covalent dimer of β-lactoglobulin, B the monomer and B^* the activated monomer. Upon heating the protein initially undergoes some unfolding, which leaves a sulfhydryl group exposed on the surface of β-lactoglobulin, available for reaction.

$$B + B^*_i \rightarrow B^*_{i+1} \text{ (propagation)}$$

The activated β-lactoglobulin (B^*) can react with a native β-lactoglobulin molecule to form an activated dimer (B^*_2). The aggregation occurs *via* a disulphide interchange reaction whereby a disulphide bond in the native β-lactoglobulin breaks and one of the sulfhydryl groups forms a disulphide bond with the sulfhydryl group on the surface of the activated β-lactoglobulin. The remaining sulfhydryl group, which had been part of a disulphide bridge in the native β-lactoglobulin (B) is now exposed on the surface of the aggregate and available for further propagation reactions.

$$B^*_i + B^*_j \rightarrow B_{i+j} \text{ (termination)}$$

Termination reactions occur when two activated β-lactoglobulin monomers or aggregates (B^*_i and B^*_j) form a disulphide linkage between their exposed sulfhydryls. This means that the newly formed aggregate no longer has any exposed sulfhydryl groups, so it is unable to react further.

Using this model it was predicted that the reaction order would be 1.5; this value had been previously determined as the order of β-lactoglobulin denaturation (Dannenberg and Kessler, 1988). Roefs and de Kruif (1994) found that experimental data also fitted the model. The denaturation process was slowed by using low concentrations of β-lactoglobulin and a relatively low temperature (65 °C); this allowed measurements to be made over a long period of time (48 h). This led to more accurate measurement of denaturation kinetics. In a follow-up study, the same group looked at the effect of pH,

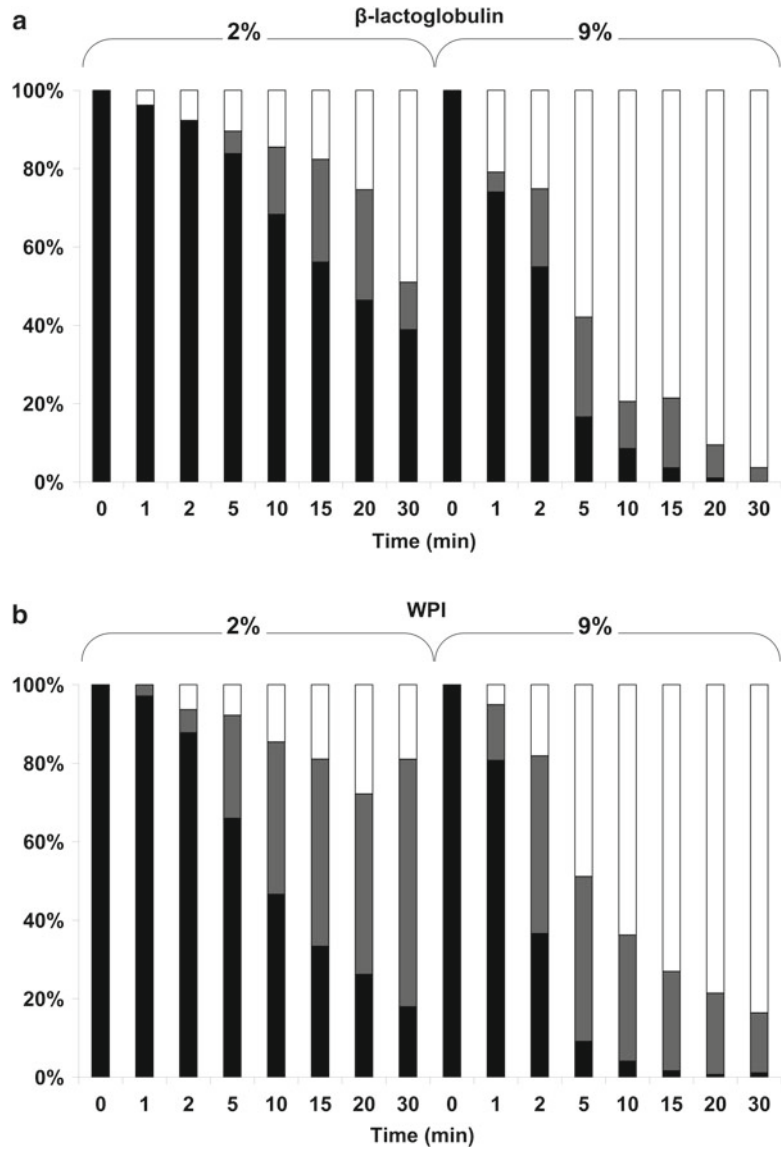

Fig. 6.1 Distribution of protein in (**a**) 20 and 90 g L^{-1} β-lactoglobulin and (**b**) 20 and 90 g L^{-1} WPI solutions. *Black bars* represent the proportion of native β-lactoglobulin as measured by reversed phase-HPLC (RP-HPLC). *Grey bars* represent non-native monomeric β-lactoglobulin (the difference in monomeric by gel permeation—HPLC (GPC) and native by RP-HPLC). *Clear bars* represent the quantity of aggregated β-lactoglobulin (measured by GPC). Reprinted from Kehoe *et al.*, 2011. Kehoe, J. J., Wang, L., Morris, E. R., & Brodkorb, A. (2011). Formation of non-native β-lactoglobulin during heat-induced denaturation *Food Biophysics*, *6*(4), 487–496. Copyright (2013), with permission from Springer

heating temperature and NaCl on the denaturation kinetics (Verheul *et al.*, 1998). The reaction order decreased with increased NaCl concentrations, and increasing pH caused an increase in reaction order. Based on the mechanism above, if the unfolding step becomes rate limiting, the overall reaction order will be close to 1.0. There are three possible ways to alter the relative rate of unfolding to aggregation, making unfolding the rate limiting step: lower the heating temperature, bring the pH close to the pI or increase the salt concentration. Conversely, to bring the reaction order closer to 2 the aggregation step has to become rate limiting. This will occur at pH values away from the pI, at low ionic strength and high heating temperatures.

A recent study by the authors quantified the β-lactoglobulin present as stable intermediates (Fig. 6.1) as a function of concentration and heating time at 78 °C (Kehoe *et al.*, 2011). The concentration of non-native monomers increased initially with heating time but then began to decrease as the concentration of aggregates increased.

Measuring the denaturation of pure whey proteins is interesting in itself, but it is rare to have pure or fully native whey proteins in commercial products, except raw milk. The kinetics of denaturation of the whey proteins in milk and enriched milks has also been studied. The presence of casein, salts and lactose in milk alter the reaction rates and orders for whey protein denaturation. Denatured whey proteins form aggregates with the caseins in milk. β-Lactoglobulin aggregates with the casein micelle *via* disulphide linkages formed with κ-casein at the micelle surface, however this process is highly dependent on the pH of the serum. The amount of β-lactoglobulin associated with the casein micelle also varies greatly with heating conditions. On heating milk between 75 and 90 °C for 80 min nearly all the β-lactoglobulin associates with the micelle (Corredig and Dalgleish, 1996b). However, when a pilot scale UHT plant was used to heat the milk at temperatures up to 130 °C for a shorter period of time, 100 s, only ~50 % of the β-lactoglobulin associated with the micelles (Oldfield *et al.*, 2005). Under these heating conditions all the β-lactoglobulin is denatured but it does not all form aggregates with the micelle. Comparison of a direct heating system, direct steam injection (DSI), with an indirect heating system, UHT, showed that a greater quantity of β-lactoglobulin was associated with the micelles in UHT than in DSI treatments (Corredig and Dalgleish, 1996a). The rate and order of denaturation of whey protein depend on the concentration relative to the other components in the milk. Oldfield *et al.,* (2005) studied the kinetics of whey protein denaturation in whey protein depleted and whey protein enriched milks. The samples were heated at temperatures between 90 and 120 °C and the protein was quantified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The reaction order and rate for β-lactoglobulin denaturation both decreased with increasing concentration of whey proteins. Two other whey proteins, α-lactalbumin and BSA, were also studied; in both cases the reaction order was not affected by whey protein concentration, but the reaction rate increased with whey protein concentration.

Sava *et al.,* (2005) studied the rate of increase in surface hydrophobicity and surface sulfhydryl groups in β-lactoglobulin as a function of heating temperature and time. The rate of increase in surface hydrophobicity increased dramatically over the temperature range studied on heating at pH 7.5. Using a first order kinetics model, the rate was approximately seven times faster at 82.5 °C than at 67.5 °C. The number of sulfhydryl groups exposed on the surface of β-lactoglobulin increased with heating time. The rate of the increase was temperature-dependent. As with the denaturation studies mentioned above there was a change in temperature dependence in the Arrhenius plot. In this case the change in activation energy occurred at around 78 °C. The authors suggest that below 78 °C the protein is only unfolding and above this temperature aggregation starts. The activation energy for sulfhydryl exposure can be up to six times greater for temperatures above 78 °C.

6.3 Heat-Induced Aggregation of Whey Proteins

The mechanisms by which whey proteins aggregate have been widely studied. The main irreversible linkages occur through covalent bonds formed between thiol groups of cysteine residues. There are, however, other bonds and interactions that arise during heat denaturation; hydrophobic, van der Waals and electrostatic interactions are also important. All these interactions are affected by salt concentration, pH, heating time and temperature.

After denaturation, β-lactoglobulin aggregates initially into oligomers and subsequently into soluble aggregates (Baussay *et al.*, 2004; Pouzot *et al.*, 2005). Upon further heating the soluble aggregates form larger colloidal particles, which can be detected by gel permeation chromatography coupled with multi-angle laser light scattering (MALLS, Fig. 6.2). Depending on the ionic conditions these particles may be unstable in solution and aggregate further.

One of the earliest papers that studied bond formation in heated β-lactoglobulin found that heating temperature played a key role in aggregate formation (Sawyer, 1968). Using starch gel electrophoresis, Sawyer (1968) established that larger aggregates

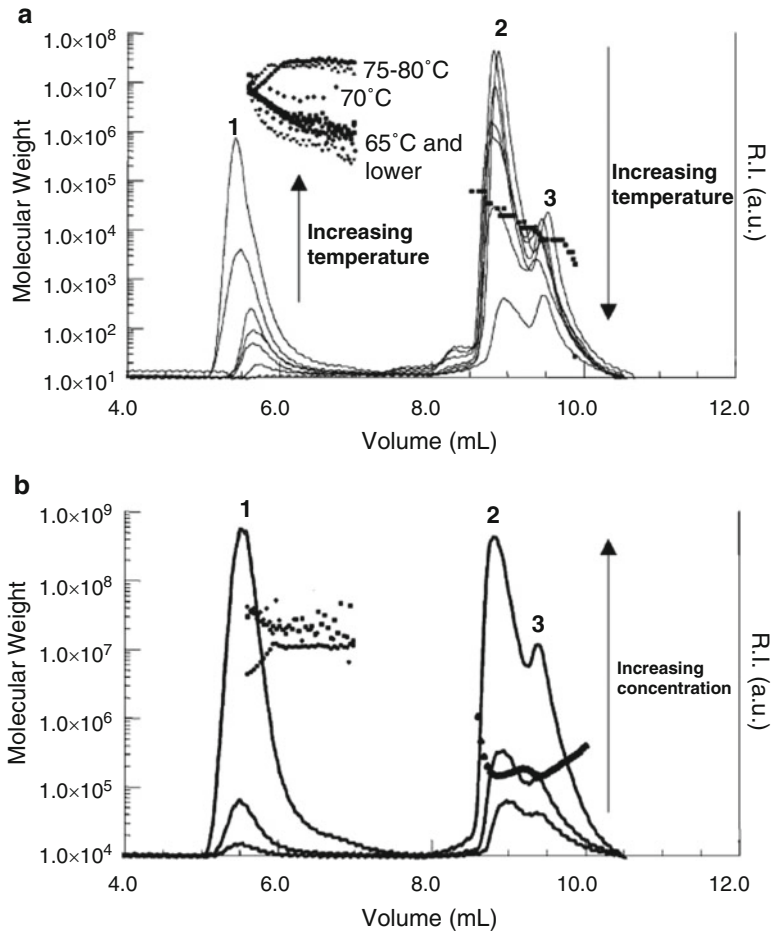

Fig. 6.2 Molecular weight distributions as determined by gel permeation—HPLC (GPC) coupled with multi-angle laser light scattering (MALLS, dotted line) and refractive index (*full line*) of whey proteins isolate: (**a**) fixed protein concentration (5 %, w/w) with increasing heating temperatures from 50 to 80 °C for 10 min, and (**b**) fixed heating conditions (75 °C for 10 min) with increasing concentrations (1, 2, 5, and 8 %, w/w). Reprinted from Beaulieu *et al.*, 2005. Beaulieu, M., Corredig, M., Turgeon, S. L., Wicker, L., & Doublier, J.-L. (2005). The formation of heat-induced protein aggregates in whey protein/pectin mixtures studied by size exclusion chromatography coupled with multi-angle laser light scattering detection. *Food Hydrocolloids*, *19*(5), 803–812. Copyright (2013), with permission from Elsevier

were formed at 75 °C than at 97.5 °C. The protein concentration used was quite low, 4.8 g L^{-1}, but the protein was heated for a long period of time (150 min). When the protein was heated in the presence of the sulfhydryl blocking agent N-ethylmaleimide (NEM), aggregate formation was inhibited. These results were related to earlier findings of Briggs and Hull (1945); Sawyer (1968) concluded that the primary reaction at 97.5 °C involved the formation of disulphide linkages, while the secondary reaction was due to non-specific aggregation. This was confirmed when a protein solution was heated at the higher temperature for 10 min and cooled; this allowed the formation of the primary aggregates. NEM was added and the solution was heated again at 75 °C. The secondary reactions still occurred in the presence of NEM. The mechanism proposed by Sawyer (1968) is shown in Fig. 6.3. Native β-lactoglobulin (N) is in a monomer/dimer equilibrium at pH 7.0. Upon heating, β-lactoglobulin unfolds and increases the reactivity of the sulfhydryl group in the species (2D). The unfolded species then aggregates (D_n') *via* sulfhydryl oxidation or sulfhydryl interchange reactions. These aggregates then associate further by non-specific interactions (D_n'' and D_n''').

A later study looked at the effect of pH, temperature and oxygen on the sulfhydryl groups in β-lactoglobulin (Watanabe and Klostermeyer, 1976). The authors found that in the presence of oxygen, the concentration of sulfhydryls decreased with heating time and the quantity of disulphides increased, reaching a constant concentration, while the quantity of free sulfhydryl groups was still decreasing. In anaerobic conditions this decrease was much smaller. When the

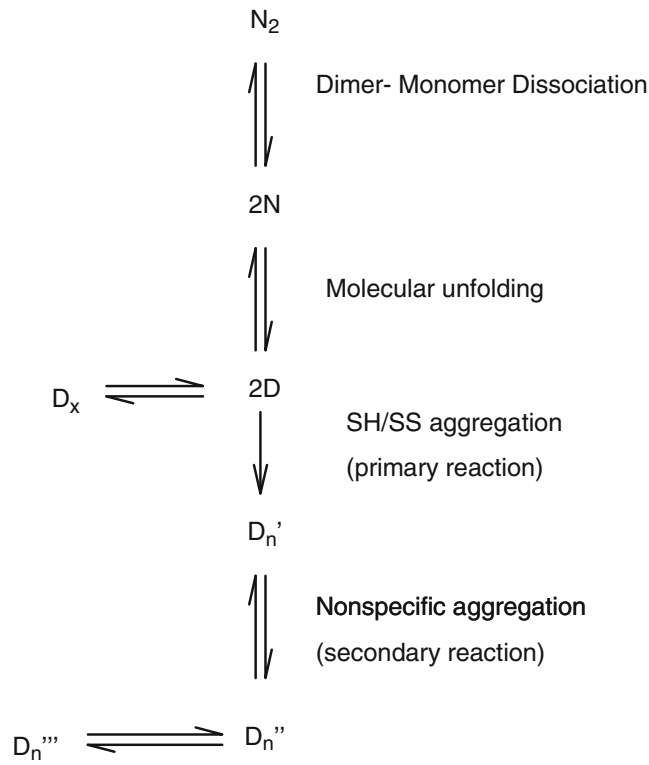

Fig. 6.3 Denaturation/aggregation mechanism of β-lactoglobulin, adapted from Sawyer (1968); N—native β-lactoglobulin, D- denatured β-lactoglobulin, D_x and D_n—various aggregated forms of β-lactoglobulin. Sawyer, W. H. (1968). Heat denaturation of bovine β-lactoglobulin and relevance of disulfide aggregation. *J. Dairy Sci.*, **51**, 323–329

temperature effect was studied it was found that the decrease in sulfhydryl groups did not match the increase in disulphide bonds. The rate of sulfhydryl group decrease was also found to increase with increasing pH. Gel permeation chromatography showed that aggregates formed in samples heated under aerobic or anaerobic conditions. These results supported the theory that aggregates can form *via* mechanisms other than disulphide linkages, although no aggregates formed when β-lactoglobulin was heated in the presence of NEM.

While the model put forward by Sawyer (1968) (Fig. 6.3) showed that covalent and non-covalent interactions occurred sequentially, it was also possible that they occurred simultaneously. In an investigation aimed at establishing, which interactions occurred during the early stages of aggregate formation, Hoffmann and Van Mil (1997) found that at pH values >7.0 and low ionic strength, the free sulfhydryl group played an important role in the early stages of aggregation of β-lactoglobulin. The authors used relatively low temperatures and concentrations, 65 °C and 10 g L^{-1}, which slowed down the reactions and made it easier to study aggregation. Using native, reducing SDS and non-reducing SDS–PAGE, they found that β-lactoglobulin formed covalently linked aggregates when heated. However, when β-lactoglobulin was heated in the presence of NEM, aggregates also formed, although they were not covalently linked. In fact, during the first 1.5 h of heating, the concentration of native β-lactoglobulin decreased faster in the presence of NEM than in its absence. The authors attributed this to pH changes in the solution. The effect of NEM was studied at pH 6.0, 7.0 and 8.0. Thiol groups still played an important role at pH 7.0 and 8.0. However, at pH 6.0 there was actually more aggregation in the sample containing NEM than in the one without. At pH 8.0 all interactions were *via* sulfhydryl interchange, as no aggregation occurred in the presence of NEM at this pH.

Shimada and Cheftel (1989) determined whether the covalent linkages were due to the

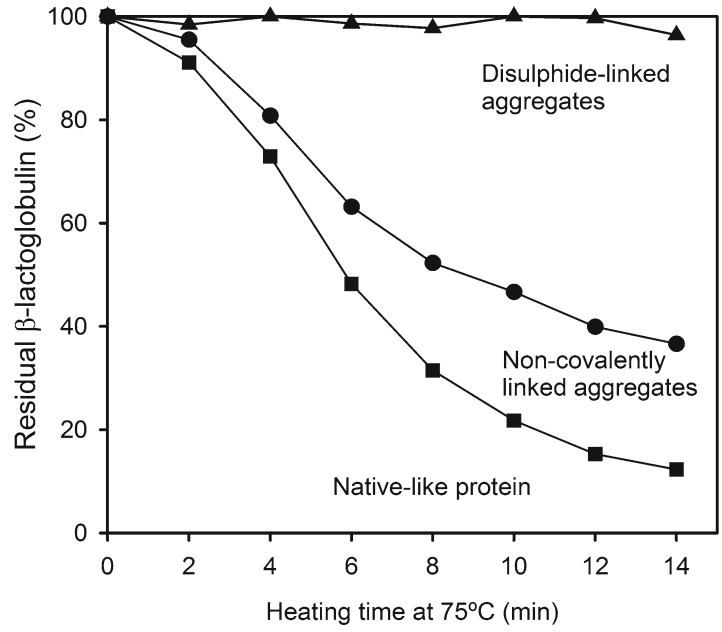

Fig. 6.4 Loss in native-like (*squares*), "SDS-monomeric" (*circles*) and "total-reducible" (*triangles*) β-lactoglobulin on heating WPC solution (12 % (w/w), pH 6.9) at 75 °C for various times. Obtained using native-PAGE, SDS-PAGE and SDS-reducing PAGE (from Singh *et al.*, 2000, reproduced with permission). Singh, H., Ye, A. & Havea, P. (2000) Milk Protein Interactions and Functionality of Dairy Ingredients. *Aust. J. Dairy Technol.* 55, 71–77

oxidation of sulfhydryls to disulphides or due to a sulfhydryl/disulphide interchange reaction. It was found that the cysteines involved in the Cys_{60}-Cys_{160} disulphide bond were involved in disulphide interchange reactions. Intramolecular disulphide bonds had a negligible contribution to gel network formation at pH 2.5; the study concluded that van der Waal forces are most important at this pH.

McSwiney *et al.,* (1994) studied the early stages of β-lactoglobulin denaturation and concluded that the initial step in the aggregation process was the rearrangement of the protein to form non-native monomers. Around the same time, Roefs and de Kruif (1994) proposed their model, outlined in Sect. 6.2 above, which had the exposure of a free sulfhydryl on the surface of the β-lactoglobulin molecule as the initiation step in the aggregation process. McSwiney *et al.,* (1994) noted that disulphide rearrangements were found to occur even after all the β-lactoglobulin had formed covalently linked aggregates, as the gel strength continued to increase after this time.

Hydrophobic interactions are also important in the formation of aggregates. A combination of native-PAGE and SDS-PAGE has been used (Havea *et al.*, 1998) to separate the role of covalent and non-covalent bonds during heat-induced aggregation in WPC (see Fig. 6.4). The amount of β-lactoglobulin involved in hydrophobic interactions increased with increasing protein concentration. The same occurred for α-lactalbumin, but to a lesser extent. A more recent study by Sava *et al.* (2005) found that when β-lactoglobulin was heated at temperatures above 78 °C, the surface hydrophobicity of the protein initially increased and then decreased. This decrease in surface hydrophobicity was attributed to β-lactoglobulin aggregation. Heat treatment of β-lactoglobulin B at temperatures between 72 and 93 °C caused the formation of disulphide linked dimers (Considine *et al.*, 2007). When the same samples were analysed by native-PAGE, there were trimers and larger aggregates present in the samples. These protein aggregates would have been formed by hydrophobic interactions.

While native β-lactoglobulin has a free sulfhydryl group on Cys_{121}, it has been shown that disulphide interchange reactions occur during the early stages of heat denaturation (Croguennec *et al.*, 2003). This suggested that intermolecular disulphide linkages other than Cys_{121}-Cys_{121} were responsible for the formation of covalent dimers and larger covalent aggregates of β-lactoglobulin. A mass spectrometric study of dimers and trimers formed in β-lactoglobulin, heat denatured at

68.5 °C, was carried out by performing an in-gel digest on the dimer and trimer bands from a non-reducing SDS-PAGE gel (Surroca *et al.*, 2002). It was found that Cys_{121}, Cys_{66} and Cys_{160} were the residues mainly involved in inter-molecular disulphide linkages. Another study (Livney *et al.*, 2003) found that peptides containing Cys_{66} were most commonly involved in inter-molecular disulphide linkages between β-lactoglobulin and α-lactalbumin. The peptides containing Cys_{106}, Cys_{119}, Cys_{121} were next most predominant. The results obtained by Surroca *et al.,* (2002) suggested that Cys_{121} was involved in the inter-molecular linkage and Cys_{106} and Cys_{119} formed intra-molecular disulphide bonds. They also found some linkages between Cys_{160} and peptides derived from α-lactalbumin.

Croguennec *et al.,* (2003) studied the early stages in the denaturation/aggregation process of β-lactoglobulin. By adding NEM to β-lactoglobulin prior to heating, a monomer containing Cys_{119} blocked with NEM could be isolated. Mass spectrometry was used to confirm that the non-native species contained a new disulphide bond, Cys_{121}-Cys_{106} as Cys_{119} was blocked with NEM. These two non-native monomers blocked by NEM, $MCys_{121}$-NEM and $MCys_{119}$-NEM, were studied spectroscopically to determine structural differences (Croguennec *et al.*, 2004a). Circular Dichroism (CD) results showed that both species contained 21 % α-helix, 40 % β-structure and 39 % coil. However, they both differed from the native β-lactoglobulin species. This showed that even though the $MCys_{121}$-NEM did refold upon cooling and remained soluble at pH 4.6, it had been altered by the binding of NEM. The intrinsic fluorescence also showed slight differences between native β-lactoglobulin and $MCys_{121}$-NEM. It was suggested that this could be brought about by a reduction in quenching of the Trp_{61} because $MCys_{121}$-NEM does not dimerise, and also the binding of NEM may move the Arg_{124} away from Trp_{19}, also reducing quenching. The emission spectrum of $MCys_{119}$-NEM showed both a red-shift and an increase in fluorescence intensity. This implied that the Trp residues were in a more polar environment and that the quenching of Trp_{61} by the Cys_{66}-Cys_{160} bond had been reduced.

A recent improvement in microscopic techniques, atomic force microscopy (AFM) in particular, has made it possible to visualise small nanometre-sized aggregates of β-lactoglobulin with high enough resolution to distinguish between individual protein monomers (Fig. 6.5). Under specific conditions, more ordered structures can be formed during the heat denaturation and aggregation of whey proteins, the formation of these structures are postulated to be possible with all proteins. The conditions giving rise to these highly ordered structures vary from one protein to another; whey proteins will form fibrils, ribbons, spherulites and nanotubes under appropriate conditions of concentration, ionic strength and temperature.

Amyloid-like fibrils have been widely studied because of their role in diseases such as Parkinson's and Alzheimer's disease. These fibrils are ordered repeating aggregates of protein or peptides 3–10 nm in width. They can range from hundreds of nanometres up to micrometres in length (Trexler and Nilsoon, 2007; Lara *et al.*, 2011).

The three main whey proteins β-lactoglobulin (Langton and Hermansson, 1992; Tobitani and Ross-Murphy, 1997; Kavanagh *et al.*, 2000; Schokker *et al.*, 2000; Arnaudov and de Vries, 2006), α-lactalbumin (Goers *et al.*, 2002) and BSA (Veerman *et al.*, 2003b) have all been reported to form fibrils; as with other areas of whey protein functionality, β-lactoglobulin has been studied to the greatest extent. For the formation of fibrils, hydrophobic patches on the protein need to be exposed to the solvent while some net charge is retained on the surface; to induce fibril formation from globular whey proteins, a combination of elevated temperature, low pH and low ionic strength are typically used (Akkermans *et al.*, 2008; Lara *et al.*, 2011). Urea has also been used to induce fibril formation in β-lactoglobulin (Rasmussen *et al.*, 2007; Hamada *et al.*, 2009) and it is also possible to form fibrils using high levels of alcohol (Gosal *et al.*, 2004). The fibril formation process in β-lactoglobulin seems to require acid-induced hydrolysis into large peptide fractions (Akkermans *et al.*, 2008) which associate and form extended β-sheets, following a nucleation and growth pathway (Arnaudov and de Vries,

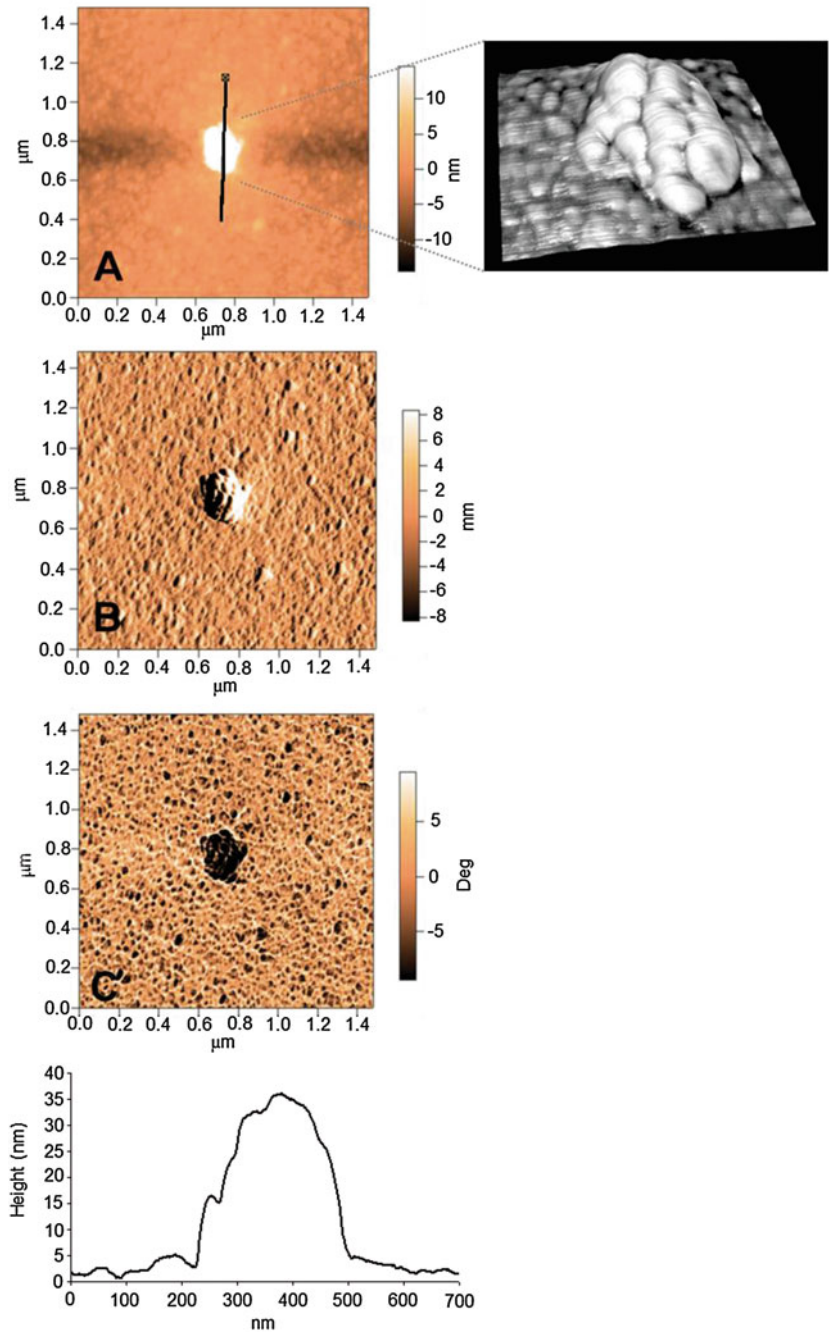

Fig. 6.5 Atomic force microscopy images of nanometer size whey protein aggregates (recorded in air) showing height (**A**), amplitude (**B**) and phase (**C**) data for a WPI solution 50 g L^{-1} heated for 30 min at 78 °C. A three dimensional representation (with an overlaid phase image) of the larger aggregate is shown in the image to the right of panel A. The graph at the bottom shows the height profile across the cross-section marked in panel A. Reprinted from Kehoe *et al.*, 2011. Kehoe, J. J., Wang, L., Morris, E. R., & Brodkorb, A. (2011). Formation of non-native β-lactoglobulin during heat-induced denaturation *Food Biophysics*, *6*(4), 487–496. Copyright (2013), with permission from Springer

2007); the initial nucleation process can be accelerated by shearing, seeding or increased temperature (Bolder *et al.*, 2007; Loveday *et al.*, 2012).

Fibrils can form larger ordered structures under certain conditions; stacking of fibrils gives rise to helical ribbons (Adamcik *et al.*, 2010). These spherulites can be formed by whey proteins during extended heating at acidic pH (Bolder *et al.*, 2006). Spherulites have a semi-crystalline structure (Bromley *et al.*, 2005) and exhibit a Maltese cross pattern when viewed with polarised light under the microscope (Domike *et al.*, 2009). The mechanisms of spherulite formation are not fully understood, as with fibril formation, pH, temperature and the balance between electrostatic, hydrophobic and hydrophilic interactions all play a role in spherulite formation (Domike and Donald, 2007).

Another example of altering β-lactoglobulin aggregation by carefully controlling the environmental conditions was presented by Schmitt *et al.,* (2009). It was possible to form relatively monodisperse nanoparticles, stable to sedimentation, with a size of around 160 nm. The authors attributed this phenomenon to a fine balance of attractive and repulsive interactions occurring between unfolded β-lactoglobulin molecules, when heated at 85 °C for 15 min at pH 5.8. α-Lactalbumin has been shown to form nano-tubes under certain conditions. Ipsen *et al.,* (2001) found that these nano-tubes formed in partially hydrolysed α-lactalbumin; the hollow straight strands were 20 nm in diameter. However, these nano tubes only form under very specific conditions of hydrolysis, protein concentration and ionic conditions (Graveland-Bikker *et al.*, 2004; Graveland-Bikker and de Kruif, 2006). The formation of nano-tubes is inhibited by the presence of other proteins and certain ions.

6.4 Whey Protein Gelation

6.4.1 Heat-Induced Whey Protein Gels

The ability of whey proteins to form heat-induced gels has been known for many years. Gels are three-dimensional, self-supporting, networks entrapping within their scaffolding the aqueous solution and/or dispersed elements. Gel properties are dependent on the balance between protein-protein and protein-solvent interactions in the system. Protein-protein interactions form the network, whereas protein-solvent interactions allow the aqueous phase to be retained in the system; an excess of protein-protein interactions result in the collapse of the network and the exudation of the aqueous phase (syneresis). In contrast to this, a soft gel (or no gel) is obtained if protein-solvent interactions are in excess compared to protein-protein interactions. Hence, the maximum gel strength is obtained by an optimum balance between protein-protein and protein-solvent interactions. Mulvihill and Kinsella (1987) suggested that factors such as protein concentration, heating temperature, heating time, pH, salt concentration, salt type, cysteine and sucrose affected gel strength by acting on the protein-protein and protein-solvent interactions. However, the authors also pointed out that the interrelationship of these factors and the mechanism whereby they affect structure, gel strength and gel texture were still poorly understood at the time. Commercial whey protein products exhibit considerable variation in protein content, extent of protein denaturation, the presence of other components including lactose, minerals, caseins fines and lipids. This diversity explains why the gelation properties of whey protein products are rather unpredictable based on the commercial information delivered by the manufacturers (Ramos *et al.*, 2012). For this reason, numerous studies on whey protein gelation have been conducted on purified proteins.

6.4.1.1 Effect of Protein Concentration

Gelation is observed subsequent to protein denaturation when protein concentration is higher than the critical concentration for gelation and the amount of denatured protein is sufficient. Below this critical concentration, soluble aggregates or precipitates are obtained. The minimum concentration for gelation depends upon several factors like pH, ionic strength and degree of denaturation (Sullivan *et al.*, 2008). The mechanism of gelation is usually divided into two phases: the formation of primary

aggregates from denatured proteins, which then aggregate further (secondary aggregation) until a network is obtained (Nicolai and Durand, 2013). Not surprisingly protein concentration is a key factor influencing gel strength. For β-lactoglobulin and BSA there is an exponential relationship between protein concentration and gel hardness (Matsudomi *et al.*, 1991). A later study, modelling the factors affecting gel strength, found that protein concentration was the most significant factor affecting the deformation properties of gels at 20 and 70 % compression (Twomey *et al.*, 1997).

6.4.1.2 Effect of Protein Composition

The denaturation temperature of the proteins involved in gel formation is important for the formation of whey protein gels and determines some of the characteristics of the gel. A study by Matsudomi *et al.,* (1991) found that at pH 8.0, BSA formed softer gels than β-lactoglobulin below 80 °C. However, the strength of BSA gels increased more rapidly as a function of temperature; at temperatures above 80 °C, BSA gels were harder than those of β-lactoglobulin. For mixtures of proteins, the denaturation temperature of the individual proteins becomes more important in determining the structures that are formed. The gelation behaviour of mixtures of BSA and β-lactoglobulin have been studied (Considine *et al.*, 2007). Like β-lactoglobulin, BSA has a free sulfhydryl group, thus a likely candidate to participate in inter- and intra-molecular disulphide rearrangements. BSA will form a heat-induced gel in the absence of other whey proteins. BSA has been shown to enhance the strength of whey protein gels (Hines and Foegeding, 1993; Matsudomi *et al.*, 1994). Even at concentrations where the proteins were incapable of forming gels individually, a mixture of β-lactoglobulin and BSA formed gels (Matsudomi *et al.*, 1994). A later paper reported a difference in the way the proteins interacted within the gel, depending on the temperature at which the gel was formed (Gezimati *et al.*, 1996). If the gel was formed at 70 °C, then BSA formed a gel network first followed by gelation of β-lactoglobulin to form interpenetrating protein strands. However, if the denaturation was carried out at a higher temperature (75 °C) then both proteins formed linked interpenetrating strands. If the proteins were heated separately, β-lactoglobulin formed a much stronger gel than BSA. It was proposed that the protein first aggregated by non-covalent means and then covalent linkages occurred.

The addition of α-lactalbumin to β-lactoglobulin also resulted in the formation of stronger gels (Matsudomi *et al.*, 1992; Hines and Foegeding, 1993). α-Lactalbumin does not usually gel under conditions where β-lactoglobulin gels are formed. Gels containing a mixture of β-lactoglobulin and α-lactalbumin were harder than gels containing only β-lactoglobulin formed at the same concentration. Furthermore, increasing the concentration of α-lactalbumin caused the gel hardness to increase. β-Lactoglobulin and α-lactalbumin formed disulphide-linked soluble aggregates during gel formation even though α-lactalbumin does not have a free sulfhydryl group. Hines and Foegeding (1993) showed that when 7 % α-lactalbumin was heated at 80 °C for 3 h gelation occurred.

In the presence of caseinomacropeptide (CMP), the C-terminal fragment of κ-casein, the strength of the whey protein gels decreased (Outinen and Rantamäki, 2008) and the gel had a coarser structure with larger pores (Meng *et al.*, 2012). Martinez *et al.,* (2010) showed that the heat-set gels formed at pH 7.0 with a CMP:β-lactoglobulin ratio of 1:4 exhibited higher elasticity than gels formed form β-lactoglobulin alone. With a higher proportion of CMP, the gels become softer. The strength and the water holding capacity of whey protein gels formed upon heating at 70 °C for 30 min at pH 6.7 increased in the presence of a small amount of sodium caseinate. The sodium caseinate fills the pores of the whey protein network leading to a less porous and more uniform structure (Picone *et al.*, 2011).

6.4.1.3 Effect of pH

The pH of the protein solution has been shown to affect several aspects of whey protein gelation. Altering the pH of the solution alters the charge on the protein and thus the electrostatic interactions that take place during gel formation.

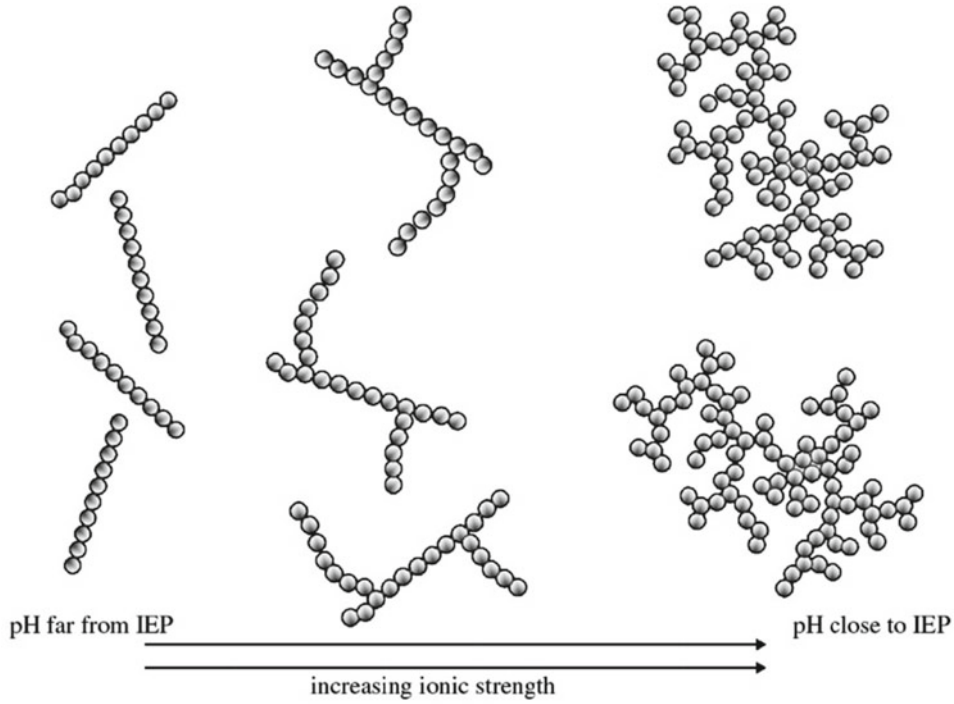

Fig. 6.6 Effect of pH and ionic strength on the aggregation of whey proteins. Depending on protein concentration, different supra-molecular structures (below critical concentration for gelation) or gels (above critical concentration for gelation) are obtained. Reprinted from van der Linden and Venema, 2007. van der Linden, E. and Venema, P. (2007). Self-assembly and aggregation of proteins. *Current Opinion in Colloid & Interface Science*, 12, 158–165. Copyright (2013), with permission from Elsevier

The change in electrostatic interactions altered the strength and appearance of whey protein gels; BSA and β-lactoglobulin gels reached a maximum hardness when heated at pH 6.5 (Matsudomi *et al.*, 1991). Above or below this pH, gel hardness decreased. Gels that were formed at pH values below 6.0 were white and spongy.

The pH of the solution also determined the denaturation temperature of the proteins (Twomey *et al.*, 1997). The denaturation temperature of whey proteins increases by decreasing the pH from pH 6.7 to 5.8. By altering the denaturation temperature the gelation time can be changed. Tobitani and Ross-Murphy (1997) found that decreasing the pH of the protein solution also caused a decrease in gelation time. Secondary aggregation is facilitated when the pH is closer to the pI of the whey protein due to lower electrostatic repulsions (Nicolai and Durand, 2013). The structure of the gel formed varied with pH. At pH values away from the pI of the protein, electrostatic repulsive forces are dominant. Under these conditions a fine stranded gel network is formed; the strands formed were of the nanometer scale. If the pH of the protein solution was closer to the pI then the repulsive forces between the proteins were reduced and a particulate gel network formed (Fig. 6.6). The particulates were quite coarse, up to micrometer in size (Clark *et al.*, 1981; Ikeda *et al.*, 1999; Ikeda and Li-Chan, 2004). The effect of pH on the structures formed during the gelation of β-lactoglobulin was studied using TEM (Kavanagh *et al.*, 2000). The authors found that the pH at which the protein was heated affected the length of the fibrils formed. As the pH moved away from the pI of the proteins, the fibrils became longer. While pH is clearly important in determining the characteristics of whey protein gels, the agent used to adjust the pH is also

important. β-Lactoglobulin gels formed at pH 3.5 showed large differences in structure depending on the acidulant (Resch *et al.*, 2005). Acidification using phosphoric acid increased the denaturation temperature of β-lactoglobulin and led to the formation of very soft pudding-like gels. Gels acidified using lactic acid or hydrochloric acid (HCl) were very ordered in structure, containing long filaments of protein and small pores. These very ordered gels took longer to form than those acidified with other acids. Adjusting the pH with citric acid resulted in the formation of milky white gels, which were of similar strength to gels acidified with HCl. The micro-structure of the gels containing citric acid were less homogeneous and the gels contained large pores.

6.4.1.4 Effect of Salts

Depending on the pH value, the amount of salts required for obtaining a whey protein gel varies. At pH 8.0, β-lactoglobulin did not form a gel in the absence of salts even after heating at 90 °C for 30 min (Mulvihill and Kinsella, 1988). If NaCl or $CaCl_2$ were added to the solutions, gels formed upon heating under the same conditions. The maximum gel strength was at 100 mM NaCl or 20 mM $CaCl_2$ (10 % β-lg, 200 mM Tris-HCl, pH 8.0). The addition of NaCl had the same effect as reducing the pH, as some of the charge on the surface of the protein was neutralised, reducing electrostatic interactions. The repulsive forces between the proteins were reduced, allowing other interactions such as covalent bonding to occur. In the case of BSA gels, the addition of NaCl had no effect on the hardness of the gels (Matsudomi *et al.*, 1991). In β-lactoglobulin gels (10 % β-lg, 100 mM Tris–HCl, pH 8.0), a NaCl concentration of 20 mM produced the hardest gels; above or below this concentration the gels became softer. High concentrations (up to 400 mM) of NaCl produced opaque spongy gels similar to those formed at low pH. An optimum level of added NaCl in WPI dispersions is also necessary to get maximum gel strength (Hussain *et al.*, 2012). As for pH, NaCl addition affects the structure of the gels, changing it from fine stranded at low NaCl content to particulate at higher NaCl content (Fig. 6.6). The change in the structure of the gels is accompanied by a transition in its appearance, from translucent to opaque (Ako *et al.*, 2009). The addition of NaCl alters not only the physical characteristics of the gels but also the temperature and length of time necessary to form a gel. The addition of a small amount of NaCl altered the gelation temperature and gelation time of BSA (Tobitani and Ross-Murphy, 1997). Increasing the concentration of NaCl was found to have no further effect on the gelation time or temperature. The addition of NaCl increases the gelation temperature of WPI dispersions (5 % protein, pH 6.5) (Hussain *et al.*, 2012).

Given that pH and salt concentration both altered the electrostatic protein-protein interactions, there was a very significant interaction between pH and NaCl concentration in determining the onset of gelation of β-lactoglobulin (Twomey *et al.*, 1997). At a pH of 6.0 the addition of salt (25–400 mM) had no effect on the gel point.

$CaCl_2$ is another salt that is often added to whey protein solutions to aid gelation as calcium causes bridging between proteins. At optimal concentrations this leads to stronger gels, but excessive bridging can also lead to matrix collapse (Matsudomi *et al.*, 1991). Like NaCl, $CaCl_2$ had an influence both on gelation time and temperature as well as the physical characteristics of the gel. In the presence of low concentrations of Ca^{2+} (2–5 mM) BSA formed clear, elastic gels (Matsudomi *et al.*, 1991). Higher concentrations (>10 mM) gave gels that were turbid, possibly because the Ca^{2+} bridging led to larger aggregates. What is your thinking? gels did not recover after deformation. β-Lactoglobulin gels were affected to a greater extent by $CaCl_2$ concentration; weaker gels were formed from solutions containing $CaCl_2$ concentrations of 5 mM or greater. Twomey *et al.,* (1997) showed that $CaCl_2$ decreased the temperature at which β-lactoglobulin gelled and increased the turbidity of the gels. Depending on the initial composition of powders, the gels of WPI and WPCs exhibit maximum firmness for different levels of added calcium (Riou *et al.*, 2011).

The effect of a variety of anions on whey protein gelation has also been studied. Anion

concentration of 100 mM resulted in weaker β-lactoglobulin gels and the gel hardness decreased in the order $SO_4^{2-} > Cl^- > Br^- > I^- > SCN^-$ (Matsudomi *et al.*, 1991). In the case of BSA gels, the addition of SO_4^{2-} produced the hardest gels. There were only small differences between the strength of the gels formed when the other anions were added.

Veerman *et al.,* (2003b) studied the effect of ionic strength on the formation of BSA gels at pH 2.0, and found that gels could be formed at lower protein concentrations under conditions of higher ionic strength. Both increased heating time and higher ionic strength was found to lead to longer protein fibrils. Not surprisingly, the study also found that the rate of conversion of BSA monomers to aggregates was higher under conditions of higher ionic strength. The strength of the gels also increased with increasing ionic strength.

Adjusting the pH or salt content of whey protein solutions is a means of controlling the electrostatic interactions between the proteins during gelation. However, there are other interactions that occur during gelation, which are more difficult to control. As discussed above, the formation of new or additional disulphide bonds is an important reaction in the aggregation of whey proteins. The process of disulphide rearrangements and formation of intermolecular disulphide bonds continues during the gelation process.

6.4.1.5 Influence of Disulphide Bonds

As discussed above, disulphide bonds are important in the aggregation of β-lactoglobulin and the same can be said of their role in gelation. Porcine β-lactoglobulin shows a large degree of homology with bovine β-lactoglobulin. However, the porcine protein does not contain a free sulfhydryl. Gallagher *et al.,* (1996) showed that porcine β-lactoglobulin did not form heat-set gels at pH 7. By adding a sulfhydryl blocking agent (NEM) it was found that the strength of bovine β-lactoglobulin and BSA gels was reduced (Matsudomi *et al.*, 1991; Mulvihill *et al.*, 1991). The addition of 2 mM NEM was sufficient to cause a decrease in the strength of the gels made from BSA, while 10 mM NEM was required for β-lactoglobulin gels to have the same effect. These concentrations of NEM were in molar excess of the free sulfhydryls present. This would suggest that it was necessary to block all free sulfhydryls to prevent disulphide rearrangements. Havea *et al.,* (2009) observed that adding NEM (NEM:protein molar ratio of 1:1) to WPC dispersion (15 % (w/w) powder, 85 % (w/w) protein content, pH 6.5) increased the stiffness of the heat-set gel and it required less deformation to break.

The addition of up to 5 mM dithiothreitol (DTT) to reduce the disulphide bonds in BSA caused an increase in gel hardness (Matsudomi *et al.*, 1991). Breaking the disulphide bonds may lead to an increase in the intermolecular disulphide bonds or, alternatively, unfolding the protein may lead to increased hydrophobic interactions. The addition of DTT concentrations >10 mM resulted in the formation of softer gels. Higher concentrations of DTT increased attractive interactions and caused the proteins to coagulate rather than to gel. The addition of >20 mM DTT caused the proteins to gel without heating. Similar results were observed with β-lactoglobulin, except that lower concentrations of DTT were required to bring about a reduction in gel strength. In the presence of DTT, the gelation temperature of WPC solution was reduced and the stiffness of the gel increased (Havea *et al.*, 2009).

The pH also influenced the extent of disulphide bond formation in whey protein gels. Gels formed at pH 7.0 were formed by covalently linked aggregates, which subsequently formed a gel network by non-covalent interactions (Otte *et al.*, 2000). At pH 5.0, the gel structure was stabilised mainly by disulphide bonds. At pH 3.0 non-covalent interactions were predominant in stabilising the gel.

6.4.2 Cold Whey Protein Gelation

Cold gelation is a two-step process. Initially the protein solution is heated under conditions where denaturation is encouraged but aggregation is limited (low ionic strength and pH away from pI). The heating step causes the formation of

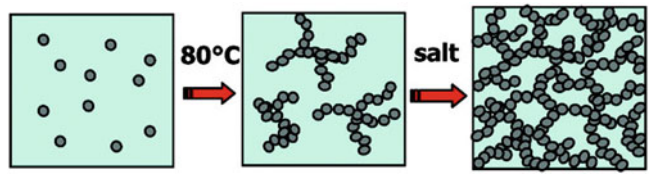

Fig. 6.7 Schematic of salt-induced cold gelation of whey proteins. Step 1: formation of soluble aggregates of whey proteins by heating the solution at low ionic strength and away from pI. Step 2: cold-gelation induced by addition of salt. Reprinted with permission from Ako *et al.*, 2010. Ako, K., Nicolai, T. and Durand, D. (2010). Salt-induced gelation of globular protein aggregates: Structure and kinetics. *Biomacromolecules*, **11**, 864–871. Copyright (2013) American Chemical Society

soluble aggregates. Gelation of the soluble aggregates is induced by a pH change, or by the addition of a salt to the solution. The structure and the properties of the gels depend on the choice of agent used to induce cold-gelation (pH or salt).

Note that an early cold gelation study involving whey proteins was actually only a one step process and involved no heating stage (Xiong and Kinsella, 1990). In this study WPI was denatured by adding urea to a concentration of 6 M. It was found that at pH values between 7.0 and 10.0 gels were formed. The concentration of free sulfhydryl groups decreased, particularly at higher pH values. This agreed with studies previously carried out in heat-set gels, showing disulphide linkages occurring during gel formation (Schmidt *et al.*, 1978; Hillier *et al.*, 1980; Zirbel and Kinsella, 1988). When NEM was added to block sulfhydryl groups, gelation was inhibited. The alkali cold gelation of whey proteins (pH > 10) is also achieved after heating whey proteins at pH 6.9 (Mercadé-Prieto and Gunasekaran, 2009). The authors suggested that the gel network was formed by the aggregates produced at pH 6.9 that associate *via* non-covalent interactions during pH increase. As the extreme pH conditions used for the gel formation are not relevant to the food industry, alkali-induced cold gelation was not studied further for food applications.

6.4.2.1 Salt-Induced Cold Gelation

Salt-induced cold gelation is usually induced by the addition of $CaCl_2$ (Barbut and Foegeding, 1993; Hongsprabhas and Barbut, 1998; Ju and Kilara, 1998b; Khun *et al.*, 2010), NaCl (Ako *et al.*, 2010; Khun *et al.*, 2010) and $MgCl_2$ (da Silva and Delgado, 2011) but also $ZnCl_2$ or $CuCl_2$ (Navarra *et al.*, 2009) to a solution of denatured whey proteins (Fig. 6.7). The properties and the gel structure changed depending on the kind of salt used to induce gelation (Ju and Kilara, 1998b; Khun *et al.*, 2010). Basically, salt-induced cold gelation results from the screening of the electrostatic repulsions between negatively charged aggregates. However, divalent cations are also able to form bridges between negative groups on the surface of the aggregates (Bryant and McClements, 2000).

Barbut and Foegeding (1993) formed cold-set WPI gels by inducing gelation of pre-heated solution using $CaCl_2$. They found that solutions heated below 70 °C (up to 10 min) did not form gels when $CaCl_2$ was added. The temperature at which the soluble aggregates are formed affects the characteristics of the gel. Hongsprabhas and Barbut (1996) found that both heating temperature and the Ca^{2+} concentration used for gelation could affect the type of gel formed. Increasing the heating temperature and the $CaCl_2$ levels both led to more translucent gels. Higher heating temperatures also led to harder gels as did the addition of higher concentrations of $CaCl_2$. Gels formed from aggregates heated at temperatures greater than 80 °C had a significantly higher water holding capacity than those heated at lower temperatures.

It was found that there was no difference in the number of accessible sulfhydryl groups (Ellman's assay) between protein solutions heated at 70 and 90 °C (Hongsprabhas and Barbut, 1997b). Gel electrophoresis showed that

the quantity of covalently-linked proteins increased with heating temperature. The viscosity of the solutions heated at 90 °C was much greater than solutions heated at 70 °C. The authors attributed this to the formation of larger aggregates, rather than formation of a greater number of aggregates. While aggregate size was not measured, they concluded that aggregate size rather than the level of exposed sulfhydryls influenced gel strength. Using scanning and transmission electron microscopy it was determined that gels produced from aggregates formed at 70 °C were more porous than those produced from aggregates formed at 90 °C. This was found to be independent of the $CaCl_2$ concentration used to induce gel formation. Larger and smoother aggregates were observed in the gels that had been pre-heated at 70 °C. They attributed the increased gel opacity to the presence of these larger aggregates. The $CaCl_2$ concentration used to induce gelation also affected the size of the aggregates formed within the gels. Higher concentrations of $CaCl_2$ (120 mM) produced larger aggregates than lower concentrations (10 mM). It was concluded that the interactions occurring during gel formation induced by $CaCl_2$ were mainly electrostatic. The same authors showed that when some of the sulfhydryl groups were blocked during the formation of heat-induced aggregates, a reduction in hardness of the gels was observed (Hongsprabhas and Barbut, 1997a). The gels also became less cohesive when the aggregates were formed in the presence of NEM. However, the authors maintained that gelation was primarily due to electrostatic interactions, as gels were formed in the presence of $CaCl_2$.

The kinetics of whey protein denaturation is concentration dependent (Roefs and de Kruif, 1994) and the protein concentration during heating should affect the properties of soluble aggregates. When WPI solutions were heated at concentrations between 4 and 9 % protein and then diluted to form gels containing 3 % protein, a linear relationship was observed between the protein concentration during heating and the hardness of the gels subsequently formed (Ju and Kilara, 1998a). An increase in size and number of protein aggregates was observed with increasing protein concentration. When the protein concentration was kept constant (3 % protein) the hardness of the gels increased linearly with the heating time (0–12 min). For heating times between 12 and 30 min, the rate of increase of gel hardness slowed. The heating time where the rapid increase in hardness occurred corresponds to the rapid increase in the size of the soluble aggregates. This study suggested there was a relationship between protein aggregates (size and number) and gel hardness.

Gel hardness was found to increase linearly with $CaCl_2$ concentration when cold gelation was induced using $CaCl_2$ concentrations between 10 and 20 mM (Ju and Kilara, 1998b). There was only a small increase in gel hardness between 20 and 40 mM. NaCl concentrations between 50 and 200 mM also caused a linear increase in hardness of cold gels, while increasing the concentration from 200 to 400 mM resulted in a slow decrease in gel hardness.

$CaCl_2$ induced harder and more elastic gels than gels induced by NaCl, but the water holding capacity of the former gels was lower. In addition, the gels induced by $CaCl_2$ exhibited more open structure and had more opaque appearance than the gels obtained with added NaCl (Khun *et al.*, 2010). Usually the salt-induced (NaCl and $CaCl_2$) cold-set gels of whey proteins are harder and have more translucent appearance than the heat-induced gels with the same amount of salt (McClements and Keogh, 1995; Hongsprabhas and Barbut, 1998). This is related to the microstructure of the gels, which is more particulate in the heat-set gels and more fine-stranded in cold-set gels (Barbut and Foegeding, 1993).

6.4.2.2 Acid-Induced Cold Gelation

Bringing the pH of the pre-heated solution of whey proteins close to the pI of the aggregates is another way to obtain cold set gels. In this case, the gel network is formed following reduction of the charge density of the aggregates. Rabiey and Britten (2009) indicated that the first sign of aggregation occurred when the zeta potential of the aggregates reached −18.2 mV regardless of the composition of whey protein aggregates.

glucono-δ-lactone (GDL) is classically used to induce the gelation of the aggregates. In aqueous solution, GDL undergoes hydrolysis to form gluconic acid and causes a progressive decrease in pH. The kinetics of acidification is an important parameter to get gels with good properties (Cavallieri and da Cunha, 2008). When GDL was used to induce gelation, a concentration of 0.8 % was found to induce the hardest gels (8 % WPI). The final pH of the gel under these conditions was 4.7. Either increasing (lower final pH) or decreasing (higher final pH) the GDL concentration caused the gels to be softer. β-Lactoglobulin was mainly responsible for the hardness of the acid-induced whey protein gels. Increasing the proportion of α-lactalbumin and CMP in the whey protein mixture resulted in softer gels (Rabiey and Britten, 2009; de Faria *et al.*, 2013). Ju and Kilara (1998b) found that the effect of protein concentration on gel hardness was dependent on how gelation was induced. Previously a power-law relationship had been established between protein concentration and gel hardness in heat-set gels (Mleko *et al.*, 1994; Ju *et al.*, 1995). This relationship applied to cold-set gels formed using $CaCl_2$ and GDL. Gels formed by the addition of NaCl followed an exponential relationship between concentration and gel hardness. Gels formed using GDL were harder than gels formed using NaCl or $CaCl_2$, while the gels formed using a protease were weakest. It has been suggested that the mechanism of cold gel formation is different in the presence of NaCl than in the presence $CaCl_2$ (Marangoni *et al.*, 2000). Using size-exclusion chromatography, it was determined that all aggregates (three different sizes) formed during the heating of WPI decreased at the same rate during gelation induced by NaCl. The authors suggested that this was a one-step process involving the slow and simultaneous aggregation of the protein aggregates. On the other hand, when $CaCl_2$ was added, a two-step process occurred, involving the rapid aggregation of the larger aggregates followed by a slower aggregation of the smaller ones.

An advantage of forming cold-set, rather than heat-set gels, is that the process is slower, allowing the different reactions occurring during gelation to be studied more easily. The gelation process is often dealt with strictly in terms of the formation of disulphide linkages. However, it is clear that other interactions can be important in gel formation (Cavallieri *et al.*, 2008). Alting *et al.,* (2002) modified the charge on the whey proteins by succinylation of the lysine residues on the protein in β-lactoglobulin or WPI solutions, which caused conversion of the positively charged amino group to a negatively charged carboxylic group. This alteration in the overall charge of the protein led to a shift in the pH range where an acid-induced cold gel was formed. The size of the covalently linked aggregates formed during gelation also decreased with increased succinylation. The results suggested that the "driving force" of cold-gel formation was the decrease in electrostatic repulsion between the aggregates. The reduction in repulsive forces allows secondary aggregation to occur aggregation to occur.

An earlier paper by the same authors (Alting *et al.*, 2000) had shown that inter-molecular disulphide linkages occurred between protein both during the heating stage, where soluble aggregates were formed, and during the acidification stage, when the gel is formed. If a sulfhydryl blocking agent is added to the soluble aggregates before acidification, there is a decrease in the size of disulphide linked aggregates present after gelation has occurred. In fact, no increase in the size of the aggregates was observed when the blocking agents NEM and *p*-chloromercuribenzoic acid (PCMB) were used. The rate of acidification also determined the degree of covalent linkage during the acidification step. A more rapid acidification did not allow time for covalent linkages to occur. At high GDL concentrations, the size of the covalently linked aggregates present decreased during acidification. The same group showed that it was not the size of the aggregates present but rather the quantity of exposed sulfhydryl groups that determined the hardness of the gel formed by the soluble aggregates (Alting *et al.*, 2003a). Further studies showed that blocking the formation of disulphide bonds during the acidification step led to increased permeability, turbidity and syneresis, and spontaneous rupture of the gels formed (Alting *et al.*, 2003a, b). However, these studies only blocked the formation of disulphide bonds

during the acidification step of the cold gelation process, allowing disulphide linkages to occur freely during the initial heating step. A later study took the process a step further and blocked the sulfhydryl groups during the heat denaturation step (pH 7.0) and the acidification step (Mounsey and O'Kennedy, 2007). Surprisingly, considering the prominence given to the role of disulphides, blocking the sulfhydryls had little effect on the storage modulus of the gels. However, when a strain sweep was applied to the gels, it was found that the gels, which had been formed in the presence of NEM, were weaker.

Britten and Giroux (2001) investigated the effect of pH and calcium on the cold-gelation process. It was found that pH and Ca^{2+} could affect the size and shape of the aggregates formed during heat denaturation of WPI. At pH 6.5 in the presence of 4 mM Ca^{2+}, large aggregates formed, giving the solution a milky white colour. It was thought that at pH values >7.0, Ca^{2+} controls the rate of disulphide interchange reactions. When the solutions were acidified to form gels, the aggregates formed at pH 6.5 gave weaker gels in the presence of increased Ca^{2+} concentrations, while at pH 8.5 Ca^{2+} had the opposite effect.

While it is normal to form protein aggregates by heating at pH values ~7.0 and at low ionic strength, to prevent gelation occurring during the heating process, gels have also been formed by aggregates formed at extreme pH values. If β-lactoglobulin is heated at 80 °C for 10 h at pH 2.0, protein fibrils are formed. Gelation of these fibrils can be induced by adjusting the pH to 7.0 and adding $CaCl_2$ (Veerman *et al.*, 2003a). The fibrils formed at pH 2.0 were compared, using TEM, to those formed during "conventional" cold gelation, i.e., heated for 30 min at pH 7.0. The fibrils formed at pH 2.0 were longer and stiffer than those formed at the higher pH. The fibrils formed at pH 2.0 remained stable when the pH was adjusted to 7.0. However, only 60 % of the protein was converted into aggregates at pH 2.0 compared to 94 % at pH 7.0. The gels formed using the aggregates formed at pH 2.0 were stronger than those formed using the conventional method.

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Heat Stability of Milk

7

Thom Huppertz

Abstract

Fresh milk at its natural pH can be heated at 140 °C for more than 10 min before coagulation occurs, but when the pH is changed or milk is concentrated, heat stability can be reduced. Heat-induced coagulation of milk is the result of aggregation of the milk proteins. Heat-induced dissociation of κ-casein from the micelles and the acid-induced collapse of the κ-casein brush on the micelle surface, as a result of heat-induced acidification, destabilize the micelles, making them susceptible to aggregation. Heat-induced denaturation of whey proteins and association with the casein micelles can stabilize against heat-induced coagulation. However, in concentrated milk, heat-induced whey protein aggregation can be a strong destabilizing factor, particularly at high pH. Controlled pre-denaturation of whey proteins prior to sterilization is an adequate manner of improving heat stability. Addition or removal of minerals like calcium and phosphate strongly influences the heat stability of milk, as does the amount or urea naturally present in milk and the heat-induced degradation of lactose. Improving and controlling the heat stability of milk therefore requires control of a wide variety of intricate relationships between constituents and physicochemical properties of milk.

Keywords

Heat stability • Milk • Whey protein denaturation • Casein dissociation • Lactose hydrolysis • Calcium

7.1 Introduction

Throughout the dairy industry and related industries, such as those producing clinical and infant foods, a wide variety of heat treatments are applied to a range of milk products, differing widely in

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composition and physicochemical properties. Heat treatments are mostly applied to extend the shelf-life of the product and the heat-load applied to products varies widely. Comparatively mild heat treatments include thermization and pasteurization, either low-temperature long-time (LTLT) or high-temperature short-time (HTST), which cause little or no noticeable instability to the product. At the other end of the spectrum, several, often concentrated, products are subjected to intense sterilization treatments. It is in this latter category that physical instability of the product may occur as a result of heat treatment. Instability of milk products to heat treatment may manifest itself in several forms, e.g., as flocculation or gelation during heat treatment, but also in the form of (protein) aggregates formed during heat treatment which, although not directly visible to the naked eye, sediment during storage. The ability of milk to withstand heat treatment, and thus not result in aforementioned physical instabilities, is commonly referred to as the heat stability of milk.

Over the past century, the heat stability of milk has intrigued dairy scientists from both an academic and industrial perspective. From an industrial perspective, the heat treatment of concentrated milk systems subjected to sterilization treatments has been of primary concern, e.g., in the manufacture of evaporated milk products. In addition, heat stability is also important when concentrates of milk, infant formula and clinical formula are heated prior to spray-drying. Likewise, current developments of liquid milk protein-based high-protein drinks, often containing >10 % protein, also warrant careful consideration of heat stability, as retention of low viscosity and the absence of sediment formation during storage are crucial in these products.

At its natural pH, unconcentrated milk is extremely stable to heat treatment and heating for >15 min at 140 °C, i.e., conditions far in excess of those commercially applied, is possible without visible signs of instability. However, even small changes in pH, i.e., by 0.1 pH unit, can result in big changes in heat stability. This strong pH-dependence of the heat stability of unconcentrated milk has also been the subject of much academic research and has often been used to provide further mechanistic understanding of the heat stability of milk systems.

While directly applicable to a wide variety of different dairy products, mechanisms underlying the (in)stability of milk systems to heat-induced coagulation are similar and translatable between product categories. The heat stability of milk systems is influenced by a myriad of compositional and physicochemical properties of the products, as well as the various processing steps used. Such factors were reviewed in detail in previous chapters in this series (Fox, 1982; Singh and Creamer, 1992; O'Connell and Fox, 2003) and since the work of O'Connell and Fox (2003), comparatively little new information has been published on the heat stability of milk. However, considerable progress has been made in understanding heat-induced changes in milk systems, which can be utilized to understand further the mechanistics of heat stability. Therefore, rather than covering all aspects of the influence of composition and processing on heat stability, the aim of this chapter is to discuss the heat stability of unconcentrated and concentrated milk systems from a mechanistic perspective; i.e., what are the crucial factors determining the (in)stability of dairy products to heat-induced coagulation and how they can be controlled. For excellent reviews on the influence of compositional and processing factors on the heat stability of milk, the reader is referred to Rose (1963), Fox and Morrissey (1977), Fox (1982), Singh (1995, 2004) and O'Connell and Fox (2003).

7.2 Assessment of the Heat Stability of Milk

Ever since the first reports on the heat-induced coagulation of milk, a number of methods and indicators have been used to quantify the stability of milk to heat treatment. Both subjective and objective methods have been proposed to determine the heat stability of milk. In addition, some in-line methods have also been proposed. The most widely-used method for determining heat

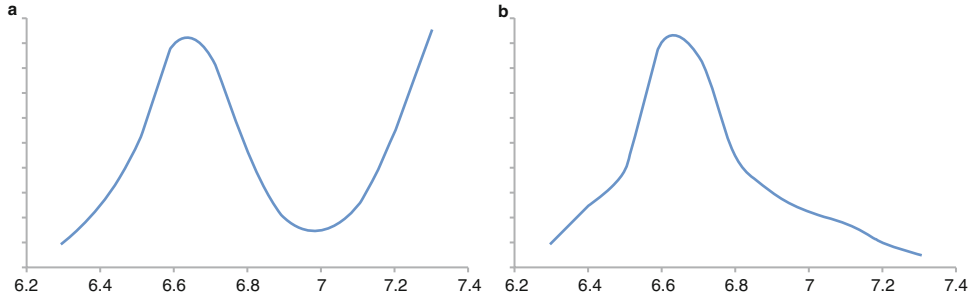

Fig. 7.1 Typical pH-heat coagulation time (*HCT*) profile of unconcentrated milk at 140 °C (**a**) or concentrated milk at 120 °C (**b**)

stability of milk is the subjective heat stability assay originally developed by Miller and Sommer (1940) and later refined by Davies and White (1966). In this assay, the heat coagulation time (HCT) of milk samples is defined as the time elapsing between putting samples held in a small tube in a thermostatically controlled oil bath and the onset of visible coagulation of the samples. The assay temperature used depends on the type of milk used, and is typically 140 °C for unconcentrated milk and 110–120 °C for concentrated milk samples. Detection of the onset of coagulation is aided by gently rocking the samples in the oil bath. To obtain mechanistic insights, the HCT of samples is generally determined as a function of pH and yield typical profiles as outlined in Fig. 7.1.

Unconcentrated milk samples, at 140 °C (Fig. 7.1a), show a pH-HCT profile in which HCT increases with increasing pH up to a maximum, which commonly coincides with the natural pH of the milk, i.e., pH 6.6–6.7. At higher pH values, HCT decreases with increasing pH to a minimum around pH 6.9–7.0, while HCT progressively increases with pH on the alkaline side of the minimum. For concentrated milk, the pH-HCT profile at 120 °C also shows a maximum in HCT at pH values close to the natural pH of the milk (pH 6.5–6.6), but no minimum in HCT is observed at the alkaline side of the maximum (Fig. 7.1b).

A much less frequently used parameter is the so-called heat coagulation temperature (HCTemp) of milk. In this, also subjective, assay, HCT is determined at a range of temperatures and HCTemp is determined by interpolation as the temperature at which the sample has a certain HCT; in many cases, a HCT of 2 min has been used in determining HCTemp of samples. While HCTemp is a measure of the susceptibility of milk to spontaneous coagulation and is thus a measure of the intrinsic heat stability of milk, and arguably a better indicator of the susceptibility of products to heat treatment in industrial settings, HCTemp is rarely measured. The limited application of HCTemp is primarily due to the fact that measurements at various temperatures are time-consuming.

An objective assay for determining the heat stability of milk was determined by White and Davies (1966). In this assay, the amount of total nitrogen or protein sedimentable at low centrifugal force (e.g., 400 g) after heat treatment is expressed as a function of heating time at a certain temperature. The so-called nitrogen- or protein-depletion curves obtained by this assay show a strong increase in sedimentable nitrogen/protein at the point at which HCT is visually observed in the subjective heat stability assay described above, indicating a good correlation between the subjective and objective methods for determining heat stability. The fact that the objective method is more labor-intensive and time-consuming has meant that this method, like that for determining HCTemp, is rarely employed.

In addition, a number of other methods have been proposed for determining heat stability, based on viscosity measurements (De Wit *et al.*, 1986; Kieseker and Aitken, 1988), an electromagnetic device (Foissy and Kneifel, 1984), or

ultrasound spectroscopy (Lehmann and Buckin, 2005) at high temperature. These methods all can provide valuable information of not only the time point at which coagulation occurs, but also of changes in the samples occurring during the time leading up to the point of coagulation. However, the need for specialized equipment has thus far limited application of these methods.

7.3 Colloidal Stability of Milk Systems

In essence, heat-induced coagulation of milk occurs as a result of a reduced colloidal stability of the casein system due to changes occurring during heat treatment. The heat stability of milk is often described as the stability of the casein micelles in milk to heat-induced coagulation. Coagulation is based on colloidal instability and not polymerization of proteins, which has been postulated in some works as to be responsible for the heat-induced coagulation of milk under certain conditions (Darling, 1980; Van Boekel *et al.*, 1989a). Polymerization is, most likely, merely a concomitant intra-micellar reaction that occurs up to the point of coagulation. After the coagulation point has been reached, polymerization reactions can also be intramicellar. In this respect, it is important to realize that at the HCT, which is determined subjectively, aggregate sizes have to be in excess of ~0.1 mm to be visible; at this point, depending on their size and fractal nature, particles are likely to consist of at least tens of thousands of casein micelles. Hence, extensive flocculation of the micelles has already occurred and some inter-micellar polymerization can already be present at the point of coagulation. This explains why in some cases heat-induced coagula, even taken immediately after HCT was reached, were not fully dispersible in solutions containing urea and a calcium chelator (Dalgleish *et al.*, 1987; Van Boekel *et al.*, 1989a). Particularly for samples far outside the HCT minimum, the long lead-up to eventual flocculation provides ample opportunity for inter-micellar polymerization to occur before the HCT.

Given the aforementioned conjecture that the heat-induced coagulation of milk is colloidal in nature, a brief overview of the colloidal stability of milk is presented in this section for mechanistic understanding. For more complete overviews on this topic, the reader is referred to previous reviews on the topic (Holt and Horne, 1996; De Kruif, 1999; De Kruif and Holt, 2003; Dalgleish, 2011). In essence, casein micelles can be described as hydrated association colloids stabilized by a polyelectrolyte brush. The core of the micelles consists of an amorphous protein matrix and amorphous nanoclusters of calcium phosphate, generally referred to as colloidal calcium phosphate (CCP). Various models have been proposed for the substructure of the casein micelles, containing notable structural features such as submicelles (Walstra, 1990), nanoclusters (Holt, 1992; De Kruif and Holt, 2003; De Kruif *et al.*, 2012; Holt *et al.*, 2013), tubular structures (Dalgleish *et al.*, 2004), water channels (Dalgleish, 2011) and a dual-binding model (Horne, 1998). While there is thus far no consensus on a defined substructure of the casein micelles, all aforementioned publications generally agree on several aspects; i.e.:

1. The presence of considerable amounts of water in the core of the micelles
2. The presence of calcium phosphate nanoclusters in the core of the micelles
3. The necessity for both the calcium phosphate nanoclusters and interactions between the proteins to maintain structural integrity.
4. The presence of κ-casein on the surface of the casein micelles

It is particularly the last aspect, i.e., the interaction between the caseins, which remains a topic of debate. Horne (1998) described these interactions as hydrophobic interactions and further specified that these interactions occur when the interaction energy arising from hydrophobic interactions is greater than that of electrostatic repulsion. De Kruif *et al.,* (2012), however, reasoned that it is not only hydrophobic and electrostatic interactions that determine the interaction

potential, but also hydrogen bonding, van de Waals' interactions, and described these together under the collective name 'cohesive weak interactions' based on the fact that quantification of contribution of each individual interaction is not possible. These 'cohesive weak interactions' specifically exclude the much stronger interactions of the phosphoserine residues with the calcium phosphate nanoclusters as well as disulphide bridging between proteins. More recently, Holt *et al.,* (2013) argued that casein association is better termed as 'entropic interactions' for what can be described to be an endothermic protein association process wherein the distinction cannot be made between polypeptide chain and side chain interactions. Overall, while the exact nature of casein interaction remains poorly quantifiable; one can state with certainty that the interaction is a balance between attractive and repulsive forces.

Compared to the ongoing debate on the substructure of the casein micelles, a general consensus has long existed on its colloidal stability. It is widely recognized that the colloidal stability of casein micelles is governed primarily by the C-terminal end of κ-casein, which protrudes from the micelle surface (Holt and Horne, 1996; De Kruif and Holt, 2003). Such stabilization has been described in the form of a grafted polyelectrolyte brush, wherein the N-terminus of κ-casein is grafted to the micelle and the C-terminus protrudes into the solvent. This C-terminus of κ-casein carries significant net-negative charges due to the presence of a large number of Glu residues, as well as the sialic acid termini on the carbohydrate moieties of glycosylated Thr residues (Holt and Horne, 1996; De Kruif and Holt, 2003). The stabilization provided by this brush has been described both as electrostatic and steric stabilization. However, high levels of NaCl, i.e., >0.5 M, can be added to milk without causing any notable micellar instability. Hence, the stabilization provided by the C-terminus of κ-casein appears to be predominantly of steric nature.

Colloidal instability of micelles can be reduced through either collapse or removal of the κ-casein brush. Brush removal is the basis of the conversion of milk into a coagulum during cheesemaking, whereby the C-terminus is removed from the micelle by enzymatic hydrolysis (Dalgleish, 1993; Holt and Horne, 1996). As outlined in more detail in Sect. 7.4.2, heat treatment of milk can also result in partial removal of the κ-casein brush. In this case, however, it is the entire κ-casein molecule, rather than only the C-terminus, that is removed from the micelle. In both cases, the removal of the steric stabilization provided by the C-terminus of κ-casein yield a residual micellar structure which has significantly reduced colloidal stability and is extremely prone to aggregation in a medium of sufficiently high calcium ion activity.

In addition to removal of the κ-casein brush, colloidal instability can also be induced by collapse of the κ-casein brush. The most notable examples where this occurs is on acidification of milk and on the addition of ethanol, or other solvents, to milk. In the case of acidification, collapse of the brush is a result of a reduction in solvency of the brush as a result of the protonation of the carboxylate groups of Glu and Asp residues of the protein, as well as of sialic acid residues of the carbohydrate moieties (De Kruif and Zhulina, 1996). As outlined in Sect. 7.4.1, heating induces considerable acidification of milk; hence, the stability of casein micelles to acidification is of primary importance in governing heat stability. The collapse of the κ-casein brush as a result of the addition of ethanol is the result of a loss of solvency of the brush because of a reduced solvent quality (Horne, 2003).

Overall, when considering the colloidal stability of casein micelles in respect to heat stability of milk, two aspects appear to be most crucial, i.e., removal of the κ-casein brush as a result of heat-induced dissociation of κ-casein from the surface, yielding a residual micelle extremely prone to calcium-induced aggregation. In addition, collapse of the κ-casein brush can also occur as a result of heat-induced acidification of milk. Heat-induced acidification of milk and heat-induced dissociation of κ-casein are dealt with in further detail in the next section.

7.4 Heat-Induced Changes in Milk

The physicochemical properties of milk are essentially the result of a large number of equilibria in milk; most notably, these equilibria include the solubility and ionization of salts, the interactions of salts with proteins and the association of proteins. These equilibria are all strongly affected by temperature. As a result, properties of milk change as a result of heating. Unfortunately, many factors that are widely believed to contribute strongly to the heat stability of milk cannot be measured at high temperature, particularly not at typical assay temperatures for heat stability of milk. Some notable exceptions to data are turbidity measurements, which show that light scattering intensity is retained at high temperature (Van Boekel *et al.*, 1989a, b; Nieuwenhuijse *et al.*, 1991), and ^{1}H-NMR measurements on casein micelles, which show considerable increases in relaxation with the protein matrix at increasing temperature (see results by Rollema presented by De Kruif and Holt, 2003).

However, for most cases, mechanistic insights of the heat stability of milk have to be derived from extrapolation of changes occurring at lower temperature to assay temperatures, or the comparison of samples before and after heat treatment. In this section, the predominant heat-induced changes in milk believed to contribute to heat-induced coagulation are described; i.e., heat-induced changes in milk pH, heat-induced changes in the mineral balance, heat-induced dissociation of caseins and heat-induced denaturation of whey proteins and their interaction with caseins.

7.4.1 Heat-Induced Changes in Milk pH and the Mineral Balance in Milk

It is widely recognised that the pH of milk decreases considerably with increasing temperature. In addition, the importance of heat-induced acidification of milk on the heat stability of milk is strongly exemplified by the fact that if periodically readjusted to natural pH, milk may be heated for up to 3 h at 140 °C before coagulation occurs (Pyne, 1958; Fox, 1981). *In situ* measurements carried out at temperatures up to 80 °C indicate a near-linear reduction in milk pH, from 6.7–6.9 at 5 °C to 6.2–6.3 at 80 °C (Chaplin and Lyster, 1988; Ma and Barbano, 2003). pH values of milk at 25 and 90 °C were studied by Chandrapala *et al.,* (2010), who noted that the magnitude of the decrease in pH on heating increased by ~0.1 pH unit with increasing solids content from 9 to 21 % of the milk, but increased by ~0.2 pH units with increasing the original pH of the milk from 6.2 to 7.2, irrespective of milk solids content. *In situ* pH measurements of milk at higher temperatures have thus far not been reported.

Hence, for insights in changes in milk pH occurring at these temperatures, researchers have thus far had to rely on determining changes in milk pH after subsequent cooling to temperatures at which pH measurements could be carried out. Extrapolations by Fox (1981) indicate that milk pH at the point of coagulation at 140 °C is <5.0, suggesting a strong role of acid-induced destabilization of casein micelles in heat-induced coagulation of milk. During the initial stages of heating, e.g., the first few minutes, rapid reductions in milk pH are observed on cooling, whereas subsequent prolonged heating produces a further, slower decrease in pH with increasing heating time. At heating times >5 min, near-linear reductions in pH with increasing heating time are observed. Three factors have been identified as major contributors to the heat-induced acidification of milk (Van Boekel *et al.*, 1989a): (1) the thermal degradation of lactose to organic acids, of which formic acid is the most important, (2) the heat-induced precipitation of tertiary calcium phosphate, with a concomitant release of H^+, and (3) the heat-induced dephosphorylation of casein, with the subsequent precipitation of the released phosphate as tertiary calcium phosphate.

The rapid reduction in milk pH during the first few minutes of heating is attributed primarily to reaction (2), i.e., the heat-induced precipitation of tertiary calcium phosphate. With increasing temperature, the solubility of calcium phosphate

decreases. *In situ* separation of the serum phase of milk at different temperatures by Pouliot *et al.,* (1989) show reductions in the concentration of serum calcium and phosphate from ~9 to ~3 and from ~12 mmol L^{-1} to ~8 mmol L^{-1}, respectively, on increasing temperature from 5 °C to 90 °C; similar trends were reported by Rose and Tessier (1959), On-Nom *et al.,* (2010) and Kaombe *et al.* (2012). In contrast, levels of serum magnesium and citrate remained relatively constant in this temperature range (Rose and Tessier, 1959; Pouliot *et al.*, 1989). In addition, considerable reductions in calcium ion activity have been observed on heating milk from 25 to 60 °C (Chandrapala *et al.*, 2010). Indications of mineral equilibria in milk at higher temperatures are currently not available.

The more gradual reduction in milk pH on subsequent prolonged heating is due primarily to the formation of organic acids, arising from the heat-induced degradation of lactose. Heat-induced degradation of lactose occurs through two routes, i.e., through isomerization/degradation and through the Maillard reaction, which account for ~80 % and ~20 %, respectively, of lactose degraded during heat treatment of milk at temperatures in the region 110–150 °C (Berg and van Boekel, 1994). The isomerization/degradation route involves the isomerization of lactose to lactulose through the Lobry de Bruin-Alberda van Eckenstein transformation, followed by the subsequent degradation into galactose, lactose and other C5 and C6 compounds. The Maillard reaction involves the interaction of lactose with the ε-amino group of lysine to form an Amadori product, lactulosyllysine, which can subsequently be degraded, to galactose and formic acid as the main products. Formic acid accounts for ~80 % of total acids formed, and is formed at a rate of ~0.4 mmol L^{-1} min^{-1} at 140 °C, resulting in reduction in pH of ~0.03 pH units min^{-1} (Berg and Van Boekel, 1994). Other acids formed on heating include lactic acid, acetic acid, pyruvic acid and propionic acid (Morr *et al.*, 1957; Berg and Van Boekel, 1994).

The extent of the contribution of heat-induced dephosphorylation of casein to the reduction in milk pH during heating is still not fully established. For sodium caseinate, heat-induced dephosphorylation has been reported to be complete after 5 h at 120 °C, with 50 % dephosphorylation occurring within the first hour; however, for calcium caseinate, heat-induced dephosphorylation proceeds more slowly, with only 80 % dephosphorylation reached after 5 h at 120 °C (Belec and Jenness, 1962a). Such differences are probably due to the association of calcium with phosphoserine residues. In milk, heat-induced dephosphorylation is also considerably slower than for sodium caseinate, with the rate constant for heat-induced dephosphorylation of casein in unconcentrated skim milk at 140 °C being almost 50 % lower than that for sodium caseinate. Likewise, at 120 °C, the rate constant for heat-induced dephosphorylation of casein in twofold concentrated pre-heated milk was ~25 % lower than that in sodium caseinate. For unconcentrated milk, data by Belec and Jenness (1962b) indicate ~55, 70 and 90 % dephosphorylation after heat treatment at 140 °C for 20 min, 30 min or 60 min, respectively. In twofold concentrated pre-heated milk, such values were ~15, 20 and 35 % after heat treatment at 120 °C for 20, 30 and 60 min, and ~25, 40 and 60 % after heat treatment for the same duration at 130 °C.

7.4.2 Heat-Induced Dissociation of Caseins

As mentioned in previous sections, heat-induced dissociation of κ-casein from the casein micelle is a prime contributor to the heat-induced coagulation of milk. First reported in the studies of Kudo (1980a) and subsequently in the works by Singh and Fox (1985a, 1986, 1987a, b, c), its role in the heat stability phenomenon has now been firmly established. As quantification of the degree of dissociated caseins at high temperature is unfortunately not possible, most work on the topic has focused on the differences in non-sedimentable caseins before and after heat treatment. As such, one should always keep in mind that the effects observed are the result of the combination of the dissociation of caseins during heat treatment, and the subsequent (partial) reversal of the effects,

through reassociation, on cooling, and subsequent storage. In addition to κ-casein, heat-induced dissociation of other caseins has also been studied. Heat-induced dissociation of α_s-caseins and β-casein increases with increasing temperature in whey protein-free systems; however, in milk, the extent of dissociation, which increases with increasing pH, shows a maximum at ~70 °C, after which levels of non-sedimentable α_s- and β-casein readily decrease with further increases in temperature (Anema and Klostermeyer, 1997; Anema, 1998). Hence, in relation to the heat stability of milk at temperatures far in excess of 100 °C, heat-induced dissociation of κ-casein is believed to be considerably more important than that of the other caseins.

One of the most important aspects of the heat-induced dissociation of κ-casein is its pH dependence. In unconcentrated milk, up to pH ~6.6–6.7, little or no heat-induced dissociation of κ-casein is observed after heat treatment; however, the degree of heat-induced dissociation increases strongly with increasing pH >6.7 (Singh and Fox, 1985, 1986, 1987a, b, c; Anema and Klostermeyer, 1997; Anema *et al.*, 1993, 2004). This heat-induced dissociation of κ-casein from the micelles leaves κ-casein-depleted micelles which are much more susceptible to calcium-induced coagulation, perhaps in a manner similar to *para-casein* micelles after renneting of milk.

The strong effects of κ-casein dissociation on the heat stability of milk were particularly exemplified in experiments by Singh and Fox (1985), in which milk was pre-heated at a range of pH values and the pH-HCT profiles of the micellar phase of such milks were determined. For the micellar phase of milk pre-heated at high pH, i.e., where considerable heat-induced dissociation of κ-casein had occurred, very low heat stability was observed at low pH, probably as a result of the high calcium activity and the absence of stabilizing κ-casein or κ-casein-whey protein complexes (Singh and Fox, 1985). These findings highlight the strong destabilizing role of heat-induced κ-casein dissociation on the stability of milk to heat treatment.

As outlined above, pH is a dominant factor determining the extent of heat-induced dissociation of κ-casein, but other factors also contribute, most notably the presence of whey proteins, the solids content of the milk and the composition of the milk serum and of the casein micelle. With respect to the role of whey proteins, two different aspects can be distinguished. At pH <6.7, the addition of β-lactoglobulin to a whey protein-free casein micelle suspension reduces the extent of heat-induced dissociation of κ-casein, whereas at pH >6.7, the extent of heat-induced dissociation is actually higher in micelle suspensions supplemented with β-lg than in their whey protein-free counterparts (Singh and Fox, 1987b). In skim milk heated at 90 °C for 20–30 min at various pH values (6.5–7.1), a linear relation is found between the level of non-sedimentable κ-casein and the level of non-sedimentable denatured whey protein (Anema, 2007), suggesting a close relationship between heat-induced whey protein denaturation and aggregation and heat-induced dissociation of κ-casein, or at least its inability to reassociate with the micelle on subsequent cooling.

In concentrated milk prepared by evaporation or by reconstitution of milk powder, higher levels of non-sedimentable κ-casein are observed after heat treatment than in unconcentrated milk, and the pH above which heat-induced dissociation of κ-casein is observed decreases with increasing concentration (Singh and Creamer, 1991a, b, c). However, when milk is concentrated by ultrafiltration, the amount of heat-dissociated κ-casein, as well as the influence of pH thereon, was found to be independent of protein concentration (Anema *et al.*, 1993). Hence, such results strongly indicate an important role of the serum phase constituents in governing heat-induced dissociation of κ-casein. Accordingly, more extensive heat-induced dissociation was observed in milk samples prepared by reconstituting milk powder in milk permeate than in powder samples reconstituted in water (Anema *et al.*, 1993). With respect to the serum phase composition of milk, it has been reported that increasing the serum calcium content in milk reduced the extent of heat-induced dissociation of κ-casein, whereas increasing soluble phosphate content in milk had comparatively little effect on the heat-induced dissociation of κ-casein.

With respect to the influence of casein micelle composition, it has been shown that changing the CCP content of the micelles to values ranging from ~40 to 120 % of that naturally present has little effect on heat-induced dissociation of κ-casein (Singh and Fox, 1987c). Furthermore, heat-induced dissociation of κ-casein has been shown to be more extensive from smaller casein micelles and it has been suggested that the susceptibility of κ-casein to heat-induced dissociation increases with the degree of glycosylation (Aoki and Kako, 1984).

7.4.3 Heat-Induced Denaturation of Whey Proteins and Casein-Whey Protein Interactions

As outlined before, heat-induced denaturation of whey proteins and their interactions with caseins are believed to affect significantly the heat stability of milk, as is exemplified, for example, by the very different pH-HCT profiles of normal milk and whey protein-free milk, and the effect of pre-heating on the heat stability of unconcentrated and concentrated milk. During heat treatment of milk, a number of sequential changes can happen to whey proteins; i.e., the dissociation of non-covalently linked oligomers, the (partial) unfolding of the native structure and the concomitant exposure of reactive amino acids. This can result in the aggregation of whey proteins, either through non-covalent interactions or through thiol/disulphide interchange reactions, either with other whey proteins or with caseins (Anema, 2009).

In cows' milk, β-lactoglobulin is the primary whey protein and has a free sulfhydryl group. Hence, it is the behavior of this whey protein that largely determines the heat-induced denaturation and aggregation of whey proteins in milk. Kinetic evaluation of the heat-induced denaturation of β-lactoglobulin and α-lactalbumin in milk indicated high values for the activation temperatures and enthalpy at temperatures up to 90 and 80 °C for these respective proteins, indicating that a large number of bonds were disrupted. However, at higher temperatures, considerably lower inactivation energies and enthalpies were observed, typical of chemical reactions. This suggests that at temperatures >90 °C, i.e., temperatures relevant for heat stability of milk, chemical (aggregation) reactions are rate limiting (Dannenberg and Kessler, 1988; Anema and McKenna, 1996; Oldfield *et al.*, 1998a).

The extent of heat-induced denaturation of whey proteins is enhanced with increasing milk pH (Law and Leaver, 2000). Furthermore, heat-induced denaturation of β-lactoglobulin is less extensive in concentrated milk than in unconcentrated milk, whereas the denaturation of α-lactalbumin is hardly affected by concentration (Anema, 2000, 2001). However, important to realize in many cases is that under conditions typically used in assaying the heat stability of milk, the degree of heat-induced denaturation will be very high. For β-lactoglobulin in milk, ~90 % denaturation is observed after heating for ~30 s, ~20 s or ~15 s at 120 °C, 130 °C or 140 °C, respectively, with ~99 % denaturation observed after heat treatment at 80 s, 40 s or 30 s at these respective temperatures; ~90 % denaturation of α-lactalbumin requires heating at 120 °C, 130 °C or 140 °C for ~150 s, 80 s or 50 s, respectively (Dannenberg and Kessler, 1988). Hence, one should consider that, even given aforementioned influences of pH and milk concentration on whey protein denaturation, near-complete denaturation of whey proteins is observed in the early stages of determining HCT and the degree of whey protein denaturation can thus probably be ruled out as a major factor explaining the pH-dependence of HCT.

However, a further factor that should be considered in this respect is the distribution of denatured whey proteins between the casein micelles and the serum phase of milk. The extent of association of denatured whey proteins with the casein micelles increases with increasing heating temperature and time. During the initial stages of heating at 80–130 °C, primarily denatured β-lactoglobulin is found associated with the casein micelles, whereas association of denatured α-lactalbumin with casein micelles is only observed after prolonged heating (Oldfield *et al.*, 1998b).

Like the heat-induced dissociation of κ-casein, the distribution of denatured whey proteins in heated milk also shows marked pH dependence. In milk heated at low pH, the majority of denatured whey protein is found associated with the casein micelles, whereas with increasing pH, a larger proportion of denatured whey protein is found in the serum phase of milk (Vasbinder *and De Kruif*, 2003; Anema *et al.*, 2004). Whereas, the ratio of denatured whey protein:κ-casein in the serum phase is rather constant as a function of pH, (~2.5 whey protein molecules per κ-casein molecule), the ratio of denatured whey protein:κ-casein shows a marked pH dependence; i.e., it decreases from ~1:1 in milk heated at pH 6.5 to ~0.5:1 in milk heated at pH 7.1 (Anema, 2007).

It should be noted that the association and distribution of whey proteins between the micellar and serum phase has a marked effect on the functional properties of milk, most notably the acid-induced coagulation of milk. Due to the heat-induced denaturation of whey proteins and their association with casein micelles, acid-induced gelation of milk is typically enhanced, and the pH at which acid gelation commences increases, as well as improved firmness of acid-induced milk gels (McKenna and Anema, 1993; Horne and Davidson, 1993; Lucey *et al.*, 1997). In addition, the pH at which acid-induced flocculation commences (Vasbinder and De Kruif, 2003), as well as the firmness of acid-induced milk gels increase with increasing proportion of denatured whey protein found in the serum phase of milk (Anema *et al.*, 2004). This altered acid-gelation behavior of milk can also be relevant for the stability of milk to heat-induced acidification.

7.5 Heat-Induced Coagulation of Milk

Van Boekel *et al.,* (1989b), in a series of kinetic studies on the heat-induced coagulation of milk, outlined that two main aspects should be considered when considering the heat-induced coagulation of milk; i.e., the rate of encounter of particles and their colloidal stability and interactions. With respect to the first aspect, i.e., the rate of encounter, primary determinants in this respect are the temperature (the rate of encounters increases approximately tenfold with increasing temperature from 20 to 140 °C), the volume fraction of particles, which is primarily determined by milk concentration, but also, as outlined later, by the degree of homogenization if fat is present. In addition, serum phase viscosity will hinder particle mobility and can hence influence the rate of particle encounter.

Whether or not particle encounter leads to particle interactions is largely determined by the colloidal interactions between the particles. As outlined in Sect. 7.3, the colloidal stability of casein micelles can be largely described as that of colloidal particles sterically stabilized by a polyelectrolyte brush. This stabilization, however, is prone to many changes during heating of milk, depending on particularly pH, mineral content and composition and the presence or absence of whey proteins. As outlined in Sect. 7.4, the heat-induced coagulation of milk is the result of a large number of changes happening during heat treatment of milk. The most important of these are (1) heat-induced acidification of milk, (2) heat-induced precipitation of calcium phosphate, (3) heat-induced dissociation of κ-casein and (4) heat-induced denaturation of whey proteins and their interaction with κ-casein. When taking these factors, and particularly their pH-dependence, into account, and combining them with the required dependencies of particle encounter and interactions, one can mechanistically understand the pH-HCT profiles observed for milk.

For unconcentrated milk, the most striking features in the pH-HCT profile are the maximum and minimum observed at pH ~6.6–6.7 and ~6.9–7.0, respectively. At pH values below the maximum, heat-induced dissociation of κ-casein does not occur; hence, micelles remain sterically stabilized by κ-casein, as well as by denatured whey protein, which associates with κ-casein on the surface of the micelles. Although calcium ion activity is high at these pH values, the micelles are expected to be sufficiently stable to calcium-induced coagulation. Most likely, heat-induced acidification plays the crucial role here in

determining the stability of milk to heat-induced coagulation. In addition, at low pH, rather large appendages of denatured whey proteins protruding from the micelle surface have been observed, which can contribute to more rapid coagulation. It is important to take into account in these reactions that heat-induced denaturation of whey proteins is a rapid process at the standard assay temperature of HCT measurements. Hence, whey proteins can be a prime determinant in tailoring the micelle surface, and hence its susceptibility to acid-induced coagulation. Hence, heat stability increases with increasing pH in this pH range, which is related to a lower calcium ion activity and a longer time required to reach a sufficiently low pH. The increased amount of denatured whey protein and the reduced association thereof with casein micelles would be expected to increase the susceptibility of milk to acid-induced coagulation, but this effect is obviously of lesser importance in this pH range.

At pH values above that of the HCT max, a reduction in heat stability of milk is observed, down to a minimum in HCT at pH 6.9–7.0. The decrease in heat stability in this pH range can be attributed to the fact that heat-induced dissociation of κ-casein becomes a significant contributor. The dissociation of κ-casein from the micelle surface leaves a rather bare micelle surface, which, in addition to acid-induced coagulation, is also very prone to calcium-induced coagulation. Hence, the HCT in this pH range is determined primarily by a balance between the extent of heat-induced dissociation of κ-casein, which increases with increasing pH, and calcium ion activity, which decreases with pH, in addition to, of course, the influence of heat-induced acidification of milk on micelle stability. In the region of the minimum, HCT is extremely low due to the fact that heat-induced dissociation is extensive, whereas calcium ion activity is still sufficiently high, leading to coagulation of the milk within minutes. When pH is further increased, heat-induced dissociation of κ-casein increases further, but calcium ion activity is further reduced, ultimately leading to strong increases in HCT.

For concentrated milk, similar factors to those outlined above contribute to the stability (or instability) of the milk to heat-induced coagulation, but the extent to which they contribute differs. In addition, a further factor needs to be taken into account, i.e., the fact that the concentration of whey proteins in the concentrated milk is sufficiently high to form networks, resulting in the heat-induced coagulation of milk. This appears to be the case for concentrated milk at pH values <6.5. At these conditions, electron micrographs after the occurrence of heat-induced coagulated milk show little or no aggregation of casein micelles (Nieuwenhuijse *et al.*, 1991). Hence, the network formed on heat-induced gelation of milk under these conditions appears to derive from the formation of a network of denatured whey proteins, possibly augmented by the participation of whey protein-covered casein micelles in the network. At higher pH values, i.e., in the range 6.5–7.0, HCT is, like for unconcentrated milk, a balance of the extent of heat-induced association of whey proteins with the micelles and the extent of heat-induced dissociation of κ-casein. At pH >7.0, where HCT of unconcentrated milk increases with pH, HCT for concentrated milk remains low, which may be attributed to the formation of a network of complexes of denatured whey proteins and κ-casein in the serum phase of the milk.

7.6 Compositional and Processing Factors Affecting the Heat Stability of Milk

7.6.1 Influence of Urea on the Heat Stability of Milk

Urea has long been known to have a notable beneficial effect on the heat stability of unconcentrated milk. In fact, natural variations in urea levels in milk have been reported to be highly correlated with the heat stability of bulk milk, and have been estimated to be responsible for up to 90 % of natural variability in the HCT of bulk milk (Holt *et al.*, 1978a; Kelly *et al.*, 1982). When considering the pH-HCT profile of milk, it is apparent that at urea concentrations <20 mM,

HCT of milk is increased with increasing urea level, except for around the HCT minimum (Muir and Sweetsur, 1977; Fox *et al.*, 1980; Metwalli and Van Boekel, 1996; Shalabi and Fox, 1982a). For concentrated milk, urea generally has little effect on heat stability. The limited effect of urea on the heat stability of unconcentrated milk in the pH-region of the minimum, as well as in the entire pH range of concentrated milk, can be related to the comparatively limited heat-induced degradation of urea as a result of the relatively short coagulation time or the low assay temperature, respectively. As a result, the extent of heat-induced degradation of urea is too limited to have a notable effect on HCT. Only at very high levels of added urea, i.e., >20 mM, notable increases in HCT of unconcentrated milk in the region of the HCT minimum are observed (Metwalli and Van Boekel, 1996).

For unconcentrated milk in regions outside the minimum, however, considerable increases in HCT are observed with increasing urea concentration. On heating, urea decomposes gradually, according to first order reaction kinetics, into ammonia and carbon dioxide (Metwalli *et al.*, 1996). The ammonia formed stabilizes the milk against heat-induced acidification through the neutralization of acid. As a result of this neutralisation, a higher extent of heat-induced dissociation of κ-casein and a reduced calcium ion activity are found in heated urea-fortified milk, compared to their non-fortified counterparts (Metwalli and Van Boekel, 1996). In addition, to the buffering effect, further urea-induced stabilization against heat-induced coagulation of milk is derived from the decomposition of urea into isothiocyanate. This isothiocyanate can react with Lys residues to form homocitrulline (Metwalli and Van Boekel, 1996). It is not clear yet whether it is because this compound is a structural analogue to Arg, or because of further reaction of homocitrulline with lactose that creates the actual stabilization, possibly analogous to the increases in heat stability observed on modification of arginine with dicarbonyls (Shalabi and Fox, 1982b). The further reaction is apparent from the fact that homocitrulline concentrations initially increase with increasing heating time, but subsequently decrease on prolonged heating (Metwalli *et al.*, 1996). Furthermore, such findings are also in agreement with the fact that low levels of lactose can stabilize milk against heat-induced coagulation, but do so more efficiently in the presence of urea (Kudo, 1980b; Shalabi and Fox, 1982a).

7.6.2 Influence of Lactose and Other Carbohydrates on the Heat Stability of Milk

As outlined above, lactose plays an important role in the heat stability of milk. It is a primary contributor to the heat-induced acidification of milk, and can thereby reduce heat stability. However, heat stability of lactose-free milk is lower than that of milk containing low levels of lactose (e.g., ~1 % lactose), suggesting involvement of lactose in stabilizing mechanisms as well. This stabilization could be related, as outlined in Sect. 7.6.1, to the reaction of lactose with homocitrulline, which was formed as a result of the reaction of isothiocyanate, a degradation product of urea, with lysine.

In addition to lactose, a number of other carbohydrates also affect heat stability. The non-reducing carbohydrates sucrose and trehalose, as well as the sugar alcohol lactitol have little effect on the heat stability of lactose-free milk (Holt *et al.*, 1978b; Tan-Kintia, 1996). However, reducing sugars, such as glucose, galactose, lactulose, maltose, mannose and fructose can stabilize lactose-free milk against heat-induced coagulation (Holt *et al.*, 1978b; Tan-Kintia, 1996). In general, monosaccharides are more efficient in stabilizing milk against heat-induced coagulation than di- or trisaccharides, and pentoses are more efficient than hexoses (Tan-Kintia, 1996). In addition, 2-deoxyribose is also highly efficient in stabilizing milk against heat-induced coagulation, probably due to its open structure (Holt *et al.*, 1978b).

7.6.3 Influence of Protein Cross-Linking on the Heat Stability of Milk

As outlined, it is envisioned that the heat-induced cross-linking of caseins is a concomitant effect rather than a major cause of heat-induced coagulation of milk. However, cross-linking of proteins prior to heating, or during the initial stages of heating, can affect heat stability. The two most notable examples of this are the chemical cross-linking with formaldehyde and the enzymatic cross-linking with transglutaminase (TGase).

The ability of formaldehyde to stabilize milk against heat-induced coagulation has been known since the 1950s (Nelson, 1954) and has intermittently been the subject of study ever since. The most striking feature of formaldehyde on the heat stability of unconcentrated milk is the elimination of the minimum in the pH-HCT profile, transferring it to one where HCT increases with pH (Holt *et al.*, 1978b; Singh and Fox, 1985b; Metwalli and Van Boekel, 1995). Likewise, in the pH-HCT profile of concentrated milk, the maximum is also eliminated from the pH-HCT profile, and HCT strongly increases with pH in the presence of formaldehyde (Nieuwenhuijse *et al.*, 1991). Given that similar observations were made for whey protein-free systems, it appears that the stabilizing reaction is primarily related to the caseins. The specific involvement of lysine residues was suggested by Singh and Fox (1985b) and later confirmed by Metwalli and Van Boekel (1995). Most likely, the lysine residues interaction with formaldehyde to form a methylol residue, which can subsequently interact with glutamine, asparagine or arginine residues, thus forming intra- or inter-molecular cross-links. As a result of these cross-links, heat-induced dissociation of κ-casein is prevented and its destabilizing effect on the heat stability of milk is nullified.

However, while interesting from an academic perspective, the addition of formaldehyde to dairy products is not permitted. A potential alternative, however, may be presented in the form of TGase, which enzymatically cross-links protein through the formation of an isopeptide bond between lysine and glutamine residues. Enzymatic cross-linking of milk proteins with TGase also prevents heat-induced dissociation of κ-casein (O'Sullivan *et al.*, 2002) and strongly affects the pH-HCT profile of unconcentrated (O'Sullivan *et al.*, 2001, 2002), pre-heated (O'Sullivan *et al.*, 2002), serum protein-free (O'Sullivan *et al.*, 2002; Mounsey *et al.*, 2005) or concentrated (O'Sullivan *et al.*, 2001) milk.

7.6.4 Influence of pre-Heating on the Heat Stability of Milk

The effects of pre-heating on the heat stability of both unconcentrated and concentrated milk have long been known and studied. While such studies have been primarily of academic interest for unconcentrated milk, pre-heating has long been a crucial step in providing concentrated milk systems with sufficient heat stability to withstand sterilization. In these cases, the milk is typically pre-heated prior to the concentration step. In the unconcentrated phase, pre-heating results in the denaturation of whey proteins, which either associate with the casein micelles or form aggregates in the serum phase. However, concentrations of whey proteins in unconcentrated milk are too low to result in gelation. By denaturing whey proteins prior to concentration, the heat coagulation due to gelation of whey proteins in concentrated milk on the acidic side of the maximum can be prevented and considerable increases in heat stability are noted. In contrast, pre-heating generally has little effect on the heat stability of concentrated milk on the alkaline side of the maximum (Nieuwenhuijse *et al.*, 1991).

When unconcentrated milk at its natural pH is pre-heated and subsequently, without readjustment of pH, its HCT is measured, little effect of pre-heating is observed (Darling, 1980; Tan-Kintia and Fox, 1999). However, pre-heating does induce a reduction in milk pH, and when the pH-HCT profile for pre-heated unconcentrated milk is determined, a shift in the pH-HCT profile to more acidic values, without affecting the HCT at the maximum, is typically observed (Rose, 1962). The pH of milk at the point of

pre-heating is also crucial. When pH is on the acidic side of the maximum, pre-heating generally increases heat stability, whereas reductions in heat stability are observed when pre-heating is performed on the alkaline side of the minimum (Newstead, 1977).

7.6.5 Influence of Phosphates on the Heat Stability of Milk

In addition to pre-heating, a further step commonly employed to improve the heat stability of milk, particularly concentrated milk, is the addition of phosphates, typically in the order of 10–50 mM phosphate per kg concentrate. The addition of phosphates results in a shift in the pH-HCT profile to more acidic values, as well as a higher HCT at the maximum in the profile (Sweetsur and Muir, 1980; Augustin and Clarke, 1990; Nieuwenhuijse *et al.*, 1991). According to Nieuwenhuijse *et al.,* (1991), a primary contributor to the phosphate-induced increases in the heat stability of milk is a reduction in calcium ion activity. Furthermore, effects on heat-induced dissociation of κ-casein and changes in the association of denatured whey proteins with casein micelles will contribute to the effects observed.

7.6.6 Influence of Fat on the Heat Stability of Milk

As outlined in previous sections, heat-induced coagulation of milk is primarily a result of the coagulation of the proteinaceous fraction of milk. As such, the presence of fat in milk *per se* does not influence the heat stability of milk. This is clearly illustrated by the similarity in heat stability of whole milk and skim milk prepared therefrom (Sweetsur and Muir, 1983a; McCrae, 1999). However, in virtually all cases in industry relating to heat stability, milk is homogenized prior to heat treatment and a reduced stability of milk to heat-induced coagulation is observed (Sweetsur and Muir, 1983a, b; McCrae, 1999). The extent of destabilization is strongly influenced by the homogenization conditions used, most notably the pressure and temperature. In general, a reduction in heat stability is observed with a reduction in particle size (Sweetsur and Muir, 1983a, b; McCrae, 1999). Such observations can be related to the fact that the membrane of fat globules in homogenized milk is comprised primarily of milk proteins, of which caseins again represent the predominant fraction. As a result, the homogenization of milk effectively increases the volume fraction of casein micelles, thus increasing the rate of encounter of casein particles during heating and thereby increasing susceptibility to heat-induced coagulation. In addition, it should also be considered that, when casein micelles are absorbed on the fat globule surface, they tend to show some spreading (Oortwijn and Walstra, 1979). Hence, not the entire casein-covered surface is stabilized by κ-casein, further increasing susceptibility to calcium-induced destabilization on heating. The inclusion of emulsifiers, such as lecithin, has been proposed as a measure of controlling heat-induced coagulation of homogenized milk concentrates (McCrae, 1999), in addition to aforementioned options to increase heat stability such as pre-heating and the addition of phosphates.

7.7 Conclusions and Future Perspectives

After a century of active research, the heat-induced coagulation of milk still remains a topic of interest and concern to not only the dairy industry, but also to those producing infant formula and nutritional beverages. Considerable research effort has been dedicated to understanding the heat stability of both unconcentrated milk and milk concentrates. Whereas the former is primarily of academic interest, it has provided the basis of understanding the heat stability of the latter in considerable detail. The heat stability of concentrated milk systems will undoubtedly long remain an area of focus for the dairy industry, not only in traditional areas such as evaporated milk and recombined milk, but also in other areas. For instance, mechanistic understanding of the heat stability of concentrated milk can be used to underpin heat-induced coagulation phenomena observed in other systems, such as structure

formation during the heating step applied in the manufacture of for example, caramels and toffee, as well as the development of insolubility during the drying of milk-based powders, e.g., infant formula. Development of insolubility in these systems tends to occur primarily at the final stages of drying, where a highly concentrated system is still at sufficiently high temperature to induce coagulation phenomena.

A further topic of interest for the future is the range of new milk protein-based ingredients that have made their appearance in recent years. In particular, the heat stabilities of milk protein concentrate (MPC), milk protein isolate (MPI) and micellar casein isolate (MCI) should be considered. All are prepared by membrane filtration and result in ingredients with strongly differing casein:lactose and casein:serum mineral ratios, and, in the case of MCI, also in differing casein:whey protein ratios. With these ingredients becoming more popular, and being applied in, e.g., high protein drinks, their heat stability needs to be considered and placed in the context of mechanistic understanding outlined throughout this chapter.

Finally, a decade ago, O'Connell and Fox (2003) concluded that further development of techniques to monitor changes in milk *in situ* during heating should significantly advance our understanding of the heat-induced coagulation of milk. Unfortunately, one can only conclude that that particular issue still remains. Over the past decade, comparatively little progress has been made in this particular area. Diffusing wave spectroscopy (DWS) could provide a valuable tool in monitoring heat-induced coagulation of milk *in situ*, whereas NMR techniques can also provide considerable further insights. Only by incorporating these, and other techniques into the area, can heat-induced coagulation of milk be fully understood.

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Sensory Properties of Milk Protein Ingredients

8

T.J. Smith, R.E. Campbell, and M.A. Drake

Abstract

The importance of dairy protein ingredients to the domestic and global dairy industry is increasing. These ingredients possess an abundance of functional and nutritional properties, but flavor of these ingredients also plays an important role. Sensory characteristics of dairy protein ingredients are widely variable. There are a concurrent variety of flavor sources including raw product stream, processing and storage. The dairy protein ingredient category encompasses a wide variety of products, including dried milk, milk protein concentrates and isolates, whey protein concentrates, isolates, hydrolysates, caseinates, and serum protein concentrates. This chapter addresses the current body of work and continued research on the sensory properties and profiles of dairy proteins.

Keywords

Dairy • Protein • Whey • Milk • Sensory • Flavor

8.1 Introduction

Dairy proteins provide significant value to the food industry and have an abundance of functional and nutritional properties (Foegeding *et al*., 2002; Miller, 2005; O'Connell and Flynn, 2007). The term "dairy protein" encompasses an increasingly large variety of ingredients, including whey, serum, and milk concentrated and unconcentrated products. Included in this category also are caseins and caseinates. Dairy proteins have many valuable functional properties, such as gelation, thermal stability, foam formation and emulsification properties (Foegeding *et al*., 2002). Consumption of whey proteins, specifically, provides many health benefits, including exercise recovery, weight management, cardiovascular health, anti-cancer effects, anti-infection activity, wound repair and infant nutrition (Smithers, 2008). Due to their ability to enhance functional characteristics of foods, dairy

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proteins are included in many different types of foods. Foods which use dairy proteins for their functional properties include fish products (used as binding agents), cheese and yogurt (to improve yields), cakes (as a replacement for egg whites), minced meats, coffee whiteners, salad dressings, frozen desserts (as an emulsifier) and many others (Jayaprakasha and Brueckner, 1999).

The varied useful functional characteristics of dairy proteins would not benefit the food industry, however, if their flavor profile was such that use as an ingredient detrimentally affected the purchase habits of consumers. Flavor plays a large role in acceptance and product success in all foods, and dried ingredients impact the flavor of the final product in which they are included (Caudle *et al*., 2005; Wright *et al*., 2009). As such, the sensory profiles of dairy proteins and ingredients which contain them cannot be overlooked. Due to their primary use as functional or nutritional enhancers, dairy proteins typically have a bland and mild flavor.

8.2 Sensory Analysis

Sensory science has been around for less than 70 years, making it a fairly young discipline. Sensory science analyzes all properties of food or other materials which are perceived by the human senses including, but not limited to, sight, taste, touch, and smell. Sensory properties are critical for a product to enjoy success in the marketplace. There are two main types of sensory testing: analytical tests and affective tests (Drake, 2007). Several types of different tests exist for each of these two main categories giving scientists a variety of tests from which to select.

Analytical sensory tests use trained or screened panelists and are objective in nature. Some examples of these tests include descriptive analysis, discrimination tests and threshold tests. Affective sensory tests use consumers, who are untrained, and are subjective in nature. These tests often measure degree of liking using a 9-point hedonic scale or measure preference among products (Meilgaard *et al*., 2007). Selection of the appropriate sensory test before data collection is imperative in order to fulfill the specific objective. Should an inappropriate test be selected, or if sensory testing is left as an afterthought, meaningful results and conclusions cannot be made (Drake *et al*., 2007a).

Dried dairy ingredients should ideally be bland or very mild in flavor so that ingredient flavor does not carry through into the finished product and influence consumer liking. Dairy foods can possess two types of off-flavors: those that are already present in the milk and those that develop later as a consequence of actions, such as processing or storage (Bodyfelt *et al*., 1988). Since off flavors are designated by consumers and not by trained experts, flavors are more appropriately classified as dairy and nondairy flavors (Drake, 2004). A sensory language has been developed for whey and dried whey ingredients, milk powders, and milk proteins, which includes both dairy and nondairy flavors (Tables 8.1 and 8.2).

Undesirable flavors that carry through to the finished product limit the utilization of dairy ingredients. In general, dairy products display a wholesome and flavorful image making them well accepted with consumers. In order for dairy products to continue a positive perception by consumers, understanding flavor, flavor variability, sources of off-flavors and consumer perception is imperative (Drake *et al*., 2009a, b).

8.3 Off Flavor Formation in Milk Products

One would expect dairy proteins to inherit flavors primarily from the milk, but secondary flavors are introduced by processing. Figure 8.1 elucidates some of the common processes through which dairy proteins and ingredients are produced, any of which may introduce flavors.

While unprocessed dairy ingredients tend to have a very bland flavor, once processed and stored, dried dairy protein ingredients can develop undesirable flavors, such as cardboard, cabbage, animal, tortilla, fatty, brothy and soapy (Carunchia Whetstine *et al*., 2005; Drake *et al*., 2009a; Wright *et al*., 2009). Undesirable ingredient flavors are often detectable in finished products and are a limiting factor in foods which lack

Table 8.1 Language and preparation of reference materials for dried skim and whole milk powders and milk proteins (Adapted from Drake *et al*., 2003; Lloyd *et al*., 2009a, b; Drake *et al*., 2009a)

Descriptor	Reference	Preparation
Cooked/sulfurous	Heated milk	Heat pasteurized skim milk to 85 °C for 45 min
Caramelized/butterscotch	Autoclaved milk	Autoclave whole milk at 121 °C for 30 min
	Caramel syrup	Dilute a tablespoon of caramel syrup in 400 mL skim milk
Sweet aromatic/cake mix	Pillsbury-white cake mix	
	Vanillin	Dilute 5 mg of vanillin in skim milk
Cereal/grass-like	Breakfast cereals (corn flakes, oat and wheaties)	Soak one cup cereal into three cups milk for 30 min and filter to remove cereals
Grassy/Hay	Timothy Hay	
Barny	p-Cresol	20 ppm in skim milk
Brothy/potato-like	Canned white potato slices	Remove the sliced potatoes from the broth
	Methional	20 ppm on filter paper in sniff jar
Animal/gelatin-like/wet dog	Knox-unflavored gelatin	Dissolve one bag of gelatin (28 g) in two cups of distilled water
Milkfat/lactone	Heavy cream	
	Delta dodecalactone	40 ppm on filter paper in sniff jar
Fried fatty/frier oil/painty	(E,E,)-2,4-decadienal or nonanal	2 ppb on filter paper in sniff jar
Fishy	Fresh fish with skin	
	Canned tuna juice	
Mushroom/metallic	Fresh mushroom	Slice fresh mushroom in skim milk for 30 min and filter to remove mushroom slices
Papery/cardboard	Cardboard paper	Soak pieces of cardboard paper in skim milk or water overnight
Tortilla	Packaged corn tortilla, or *o*-aminoacetophenone	Packaged corn tortillas or 100 ppm *o*-aminoacetophenone on filter paper in sniff jar
Musty/earthy	Aroma of damp basement or potting soil	One part per trillion trichloroanisole on filter paper in sniff jar, potting soil
Burnt feathers/glue	Stale, degraded proteinaceous aroma	Elmers glue stored at 21 °C for more than 12 months
Burnt/charcoal	Over toasted bread slice	
Vitamin/rubber	Enfamil Liquid polyvisol vitamins	
Diacetyl/buttery	Diacetyl	Diacetyl, 20 ppm on filter paper
Sweet taste	Sucrose	5 % sucrose solution
Salty taste	NaCl	2 % NaCl solution
Sour taste	Citric acid	1 % citric acid solution
Bitter taste	Caffeine	0.5 % caffeine solution
Umami	Monosodium glutamate	1 % monosodium glutamate in water
Astringent	Tea	Soak six tea bags in water for 10 min

flavor strong enough to overpower or mask the flavor of the dried dairy protein ingredient (Caudle *et al*., 2005; Drake, 2006; Drake *et al*., 2009a, b; Wright *et al*., 2009; Evans *et al*., 2010). Lipid oxidation and Maillard reactions have a significant effect on off flavor formation.

8.3.1 Lipid Oxidation

Dairy protein ingredients contain varying amounts of fat, but flavor volatiles caused by lipid oxidation play a major role in the flavor of all dried dairy ingredients. The two types of lipid

Table 8.2 Sensory language for descriptive analysis of fluid whey and whey proteins (Adapted from Carunchia Whetstine *et al*., 2003; Russell *et al*., 2006; Liaw *et al*., 2011; Drake *et al*., 2009a, b; Wright *et al*., 2009)

Term	Definition	Reference	Example/preparation
Overall aroma intensity	The overall orthonasal aroma impact		Evaluated as the lid is removed from the cupped sample
Sweet aromatic	Sweet aromatics associated with dairy products		Vanilla cake mix or 20 ppm vanillin in milk
Cooked/milky	Aromatics associated with cooked milk	Cooked milk	Heating skim milk to 85 °C for 30 min
Doughy	Aromatics associated with canned biscuit dough	(*Z*)-4-heptenal	1 ppm (*Z*)-4-heptenal in water from canned biscuit dough, or cooked pasta water
Fatty/frying oil	Aromatics associated with old frying oil and lipid oxidation products	2,4-decadienal	Old (stored) vegetable oil
Metallic/meat serum	Aromatic associated with metals or with juices of raw or rare beef	Aroma of fresh raw beef steak or juices from seared beef steak	
Cucumber	Aromatics associated with freshly sliced cucumber	(*E*)-2-nonenal	1 ppm (*E*)-2-nonenal or freshly sliced cucumbers
Grassy/hay	Aromatics associated with dried grasses	Alfalfa or grass hay	
Cardboard/wet paper	Aromatics associated with wet cardboard and brown paper	Cardboard paper	Brown cardboard or brown paper bag cut into strips and soaked in water
Potato brothy	Aromatics associated with boiled potatoes	Boiled potatoes	Drained broth from canned potatoes
Cabbage brothy	Aromatics associated with boiled cabbage	Boiled cabbage	Cabbage leaf boiled in 500 mL water for 5 min
Raisin/Spicy	Aromatic associated with stewed raisins	Boiled raisins	Boil 50 g dark raisins in 500 mL water
Animal/wet dog	Aromatics associated with wet dog hair	Knox gelatin	One bag of gelatin (28 g) dissolved in two cups of distilled water
Pasta water/cereal	Aromatics associated with water after pasta has been boiled in it or oatmeal	Boiled pasta or plain boiled oats	Pasta boiled in water for 30 min
Soapy	Aromatics associated with soap	Lauric acid	1 ppm lauric acid or shaved bar soap
Bitter	Basic taste associated with bitterness	Caffeine	Caffeine, 0.5 % in water
Astringency	Chemical feeling factor characterized by a drying or puckering of the oral tissues	Alum	Alum, 1 % in water

degradation reactions in dairy protein ingredients are hydrolytic rancidity and autoxidation. Hydrolytic rancidity is the release of fatty acids from the glycerol backbone of a glyceride molecule (McClements and Decker, 2008). Flavors associated with free fatty acids are the result of hydrolytic rancidity. In the case of certain cheeses, this reaction is enzyme-mediated (lipase) and is a source of desirable flavor. In dried dairy ingredients, free fatty acid flavors (rancid, butyric, sweaty, soapy flavors) are not desirable. Autoxidation refers to the production of radicals caused by the reaction of lipids with oxygen and is a much more frequent source of off-flavors in dried dairy ingredients (Frankel, 2005). Lipid oxidation occurs in the presence of

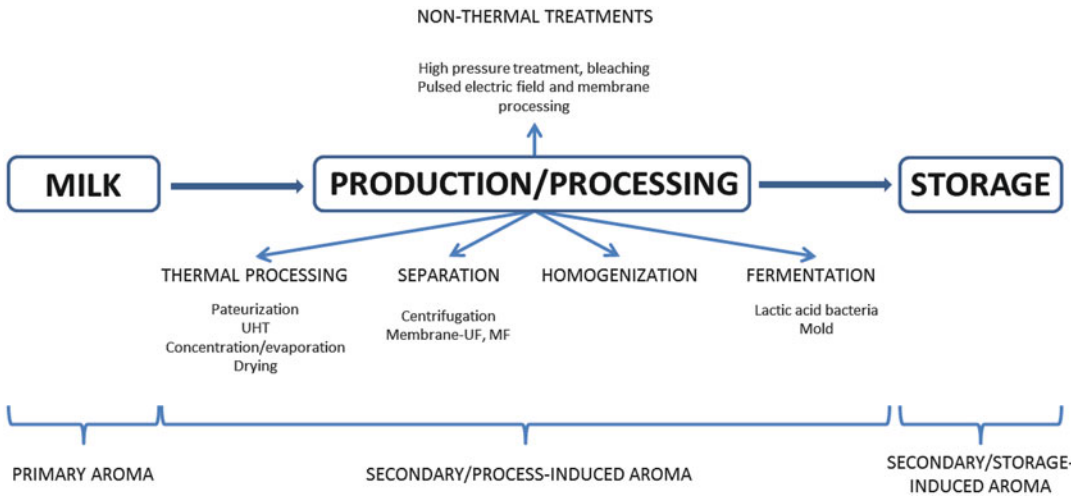


Fig. 8.1 Various typical steps in a milk processing application. *UHT* Ultra-high temperature processing, *UF* ultrafiltration, *MF* microfiltration. (Adapted from Cadwallader and Singh, 2009)

initiators (oxidizers) when unsaturated lipids lose a hydrogen radical and form lipid free radicals. The alkyl radical of the unsaturated lipids containing a labile hydrogen reacts with molecular oxygen to form peroxyl radicals. This is followed by a hydrogen transfer reaction with unsaturated lipids to form hydroperoxides (Frankel, 2005). Once the peroxyl radicals accumulate, they begin to interact with each other to form non-radical products, terminating the reaction. Lipid oxidation begins in the earliest stages of dairy protein ingredient processing and can decrease sensory quality. Oleic, linoleic, and linolenic acids are unsaturated fatty acids commonly found in milk which, when auto-oxidized cause volatile flavor compound formation. Specifically, auto oxidation of oleic acid produces octanal, nonanal, decanal, 2-decenal, 2-undecenal, while auto-oxidation of linoleic acid produces hexanal, 2-octenal, 3-nonenal, and 2,4-decadienal, and auto-oxidation of linolenic acid produces propanal, 2-hexenal, 2,4-heptadienal, 3,6-nonadienal, and 2,4,7-decatrienal (Cadwallader and Singh, 2009). These compounds, at the appropriate concentrations, cause a host of undesirable flavors including cardboard, painty, fatty and cucumber (Drake *et al*., 2009a, b; Lloyd *et al*., 2009b; Whitson *et al*., 2010).

8.3.2 Maillard Reactions

Maillard reaction products are also a major component of off flavors in milk and milk protein ingredients. Maillard reactions typically occur during processing and storage (Sikorski *et al*., 2008). Maillard reactions require a reducing sugar and an amine-containing compound such as a protein, peptide, or amino acid. Lactose is a reducing sugar and is a component of virtually all dairy protein ingredients, making the Maillard reaction a predominant interaction in dairy protein ingredients. Maillard reactions have three main stages as follows:

1. Reaction of an amine with a reducing sugar to form a glycosyl amine, which is then followed by the Amadori rearrangement;
2. Dehydration of intermediate products, fragmentation of the saccharidic moiety, and Strecker degradation of the products;
3. Reactions of intermediate products to produce heterocyclic flavor compounds (Sikorski *et al*., 2008)

Figure 8.2 is a representation of many of the off flavor compounds produced during the different steps of the Maillard reaction.

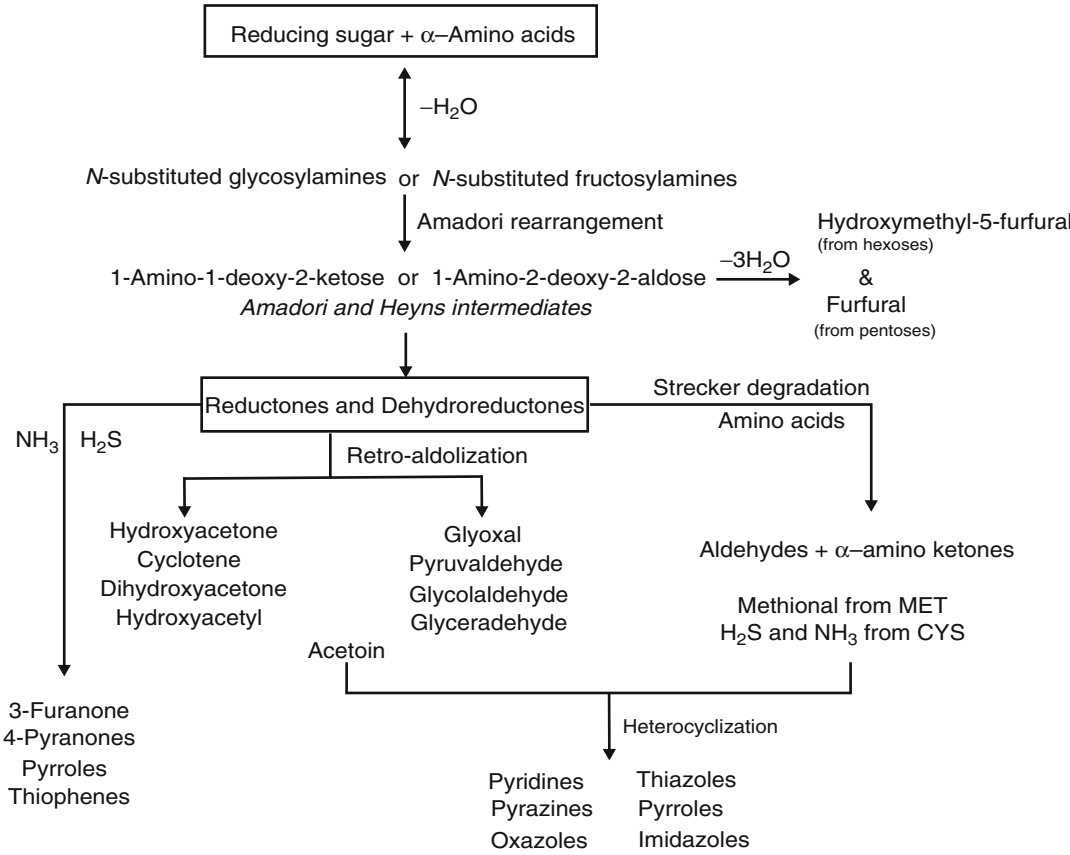


Fig. 8.2 Formation of aroma compounds *via* Maillard reaction/non-enzymatic browning (Cadwallader and Singh, 2009; used with permission)

The moisture content of a dry dairy protein ingredient must fall below 3 % before Maillard reactions cease, which is below the target moisture content for most dried dairy ingredients (Sienkiewicz and Riedel, 1990). As such, it is rare for Maillard reactions not to affect the flavor of a dairy protein ingredient. Maillard reactions are sourced to sweet aromatic and caramelized flavors in freshly manufactured milk and whey ingredients and brothy, vitamin and stale flavors in stored milk and whey ingredients (Karagul-Yuceer *et al.*, 2001; Karagul-Yuceer *et al.*, 2002; Drake, 2006; Drake *et al.*, 2007b; Drake *et al.*, 2009b; Wright *et al.*, 2009).

8.4 Fluid Milk

As the precursor of all dairy protein ingredients, fluid milk flavor is an important factor. Fluid milk is a complicated system and many compositional factors affect flavor formation. Calvo and de la Hoz (1992) stated that the origins of flavor-contributing compounds are typically due to the feed, microbial metabolism before dairy processing, or are generated by heat treatment or caused by storage of the fluid milk. Fat content, fatty acid composition, vitamin and mineral content, lactose, and salts all have an effect on the flavor of fluid

milk (Clark *et al*., 2009). Seasonal variability, primarily of milk fat levels, is seen due to differences in feed and stage of lactation (Varnam and Sutherland, 1994). The age and genetics of the cow, and the feed also have an effect on milk composition and flavor (Varnam and Sutherland, 1994; Croissant *et al*., 2007) as does processing. (Z)-4 Heptenal (doughy/fatty), 1-octen-3-one (metallic/mushroom), hexanal (grassy), 12:2 lactone (baby powder) and dimethyl disulfide (onion) are significant flavor components of fluid milk (Chandan, 1997). Bendall (2001) identified 66 different aroma-active compounds in fluid milk, and classified them as nitrogen heterocycles, linolenic acid oxidation products, γ-lactones, phenolics, phytol derivatives, fatty acids, strecker esters, sulfur compounds, δ-lactones, terpenes, diacetyl and related compounds, and Strecker degradation products. Croissant *et al.,*. (2007) studied the sensory attributes of fluid milk from both Jersey and Holstein cows on a total mixed ration (TMR) and a pasture based (PB) diet. Sensory profiles of the milks from different feeding methods were different, with pasture-fed cows producing milk with distinct grassy, mothball flavors, and salty taste profiles, while TMR fed cows produced milk characterized by a sweet feed/malty flavor (Table 8.3).

Table 8.3 Sensory profiles of 1.5 % pasteurized milk from pasture-based and total mixed ration feeding system for each treatment and breed group

	Jersey		Holstein	
Sensory attribute	TMR	Pasture	TMR	Pasture
Aroma intensity	2.0[a]	2.0[a]	2.0[a]	2.0[a]
Sweet aromatic	2.5[a]	2.0[a]	2.4[a]	1.9[b]
Cooked	2.8[a]	2.8[a]	2.8[a]	2.7[a]
Milk fat	2.1[a]	2.1[a]	2.1[a]	2.1[a]
Grassy	ND	1.3[a]	ND	1.5[a]
Mothball	ND	1.1[a]	ND	1.2[a]
Sweet feed/malty	1.9[a]	ND	1.9[a]	ND
Sweet	2.4[a]	2.1[b]	2.4[a]	2.0[c]
Salty	ND	0.9[a]	ND	1.0[a]
Astringency	1.1[a]	1.1[a]	1.0[a]	1.1[a]

[a,b,c]Means within rows with different superscripts are different ($P < 0.05$)
Scores based on a universal 0–15 point intensity scale
Means are from duplicate analyses by ten trained panelists
ND not detected
Adapted from Croissant *et al.,* (2007)

Figure 8.3 is a biplot from principal component (PCA) of these descriptive analysis results and shows that the variation in flavor of fluid milks was due to feed (pasture versus TMR) more so than breed. Dimick *et al,*. (1969) also found that pasture-fed cows had fewer lactones in their milk than silage-fed cows. Badings (1980) and Bendall (2001) reported that pasture feeding of cows caused an increase in indole, skatole, sufides, mercaptans, nitriles, and thiocyanates, compounds that cause common off-flavors and potential sources of grassy and mothball flavors.

It is also well established that processing has an effect on fluid milk flavor. To ensure microbial safety, the vast majority of commercial milk is thermally processed. High-temperature short-time (HTST, typically 72 °C for 15 s) ultra-pasteurized (UP, typically 138 °C for 2 s), and ultra high-temperature (UHT, 135–150 °C for 3–5 s) processing are typical thermal treatments for fluid milk. Heat treatment increases lipid oxidation products and causes thermal degradation of milk proteins (Scanlan *et al*., 1969; Badings and Neeter, 1980; Moio *et al*., 1994). Impactful sulfur compounds, methyl ketones and lactones are all formed during heat treatment and as severity of heat treatment increases, levels of volatile flavor compounds increase (Scanlan *et al*., 1969; Badings and Neeter, 1980; de la Hoz, 1992) and cooked, sulfurous, cabbage, and caramelized flavors also are increased with heat treatment.

8.5 Milk Powders

Milk powders are another widely used raw material in the formulation of many different foods. They have many uses including recombination back into milk, in the baking industry to increase volume of bread and improve water binding capacity, as a substitute for eggs in baked goods, as the milk solids in milk chocolate, in sausages and ready-cooked meals, baby food, ice cream, and as animal feed (USDEC, 2013a, b).

Milk powders in the US are separated into three categories: dry whole milk/whole milk powder (WMP), nonfat dry milk (NFDM) and skim milk powder (SMP). WMP in the US is

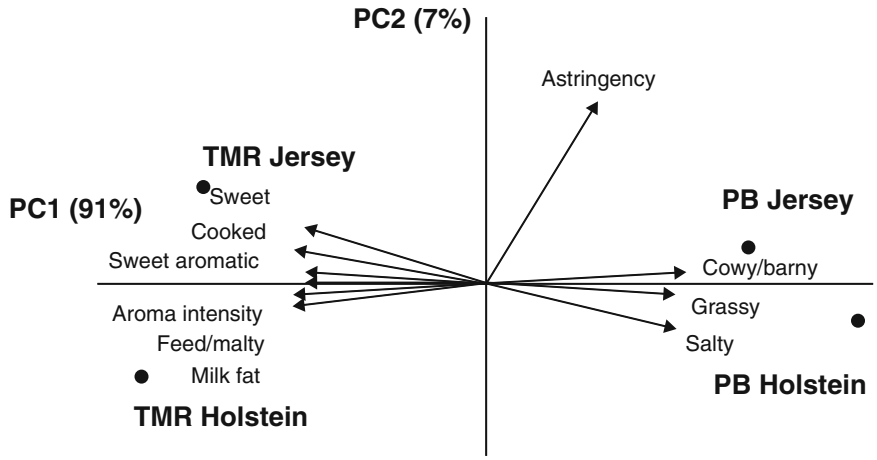


Fig. 8.3 Principal component anaysis biplot of sensory analysis of fluid milk derived from TMR and PB Jersey and Holstein cows. Croissant *et al.,* (2007). Used with permission

24.5–27 % protein, 26–40 % fat, 2–4.5 % moisture, 36–38.5 % lactose, and 5.5–6.5 % ash while NFDM and SMP are 34–37 % protein, 49.5–52 % lactose, 0.6–1.25 % fat, 8.2–8.6 % ash, and 3–4.5 % moisture (USDEC, 2013a, b). WMP is usually obtained by removing water from whole milk although it may also be a blending of fluid, condensed or dry SMP with liquid or dry cream or with fluid, condensed or dry milk. NFDM and SMP are very similar to each other and are both made by removing water from pasteurized skim milk. The difference between the two powders is that SMP has a minimum milk protein content of 34 % while NFDM has no standardized protein level (USDEC, 2013a, b). The protein content of SMP can also be adjusted by adding either ultrafiltered milk retentate or permeate while NFDM cannot (ADPI, 2013).

8.5.1 Skimmed Milk Powder/ Non-fat Dry Milk

SMP and NFDM can be designated low, medium or high heat based on heat treatment of milk prior to spray drying and subsequent whey protein nitrogen index (WPNI). WPNI is a method for the determination of soluble whey proteins in NFDM and gives a good indication of the heat treatment used on the milk before spray drying (USDEC, 2013b). Low heat NFDM has a WPNI of over 6.00 mg soluble whey protein per g powder, while medium heat NFDM has a WPNI between 1.51 and 5.99 mg/g, and high heat has WPNI under 1.50 mg/g (USDEC, 2013b). Sensory profiles of SMP/NFDM vary with heat treatment, age, and country of origin (Table 8.4).

Freshly manufactured low heat skim milk powders are characterized by cooked/milky and sweet aromatics. Like fluid milk from pasture feeding, skim milk powders from countries where pasture-feeding is common, may have grassy/hay flavors. These sweet fresh milk flavors decrease with storage and nondairy flavors (cardboard, animal, fatty) increase in intensity (Caudle *et al*., 2005; Drake *et al*., 2007b). Karagul-Yuceer *et al,*. (2001) also conducted a study on NFDM with low, medium and high heat treatments which differed in their sensory characteristics (Table 8.5).

By using direct solvent extraction, gas chromatography-olfactometry and gas chromatograpy-mass spectrometry, Karagul-Yuceer *et al,*. (2001) found that free fatty acids, lactones and browning/Maillard reaction products including maltol, Furaneol and aldehydes were the primary contributors to the aroma of NFDM while ketones and skatole played a smaller, but significant part. Heat-induced compounds such as Furaneol, maltol, sotolon, vanillin, butanoic acid, o-aminoacetophenone,

Table 8.4 Descriptive analysis profiles of various SMP (Adapted in part from Caudle *et al.*, 2005)

Attribute	SMP1	SMP2	SMP3	SMP4	SMP5
Cooked	3.3[a]	3.1[a]	2.0[b]	3.5[a]	3.8[a]
Sweet aromatic	3.0[a]	3.5[a]	1.5[b]	1.5[b]	1.0[c]
Grassy	ND	ND	ND	ND	1.5[a]
Animal	ND	ND	ND	2.5[a]	ND
Fatty/frier oil	ND	ND	2.0[a]	ND	ND
Sweet taste	2.5[a,b]	3.0[a]	2.3[b]	1.5[c]	2.3[b]
Astringency	1.5[a,b]	1.0[b]	2.0[a]	1.5[a,b]	1.8[a]

[a,b,c]Means within rows with different superscripts are different ($P < 0.05$)
Scores based on a universal 0–15 point intensity scale
Means are from duplicate analyses by ten trained panelists
ND not detected

Table 8.5 Descriptive analysis profiles of nonfat dry milk treated with low, medium or high heat adapted from Karagul-Yuceer *et al.,* (2001)

Attributes	Low	Medium	High
Cooked/sulfurous	3.0[c]	3.6[b]	4.3[a]
Sweet aromatic/cake mix	1.0[a]	0.5[b]	ND
Cardboard	1.0[b]	1.5[a]	1.5[a]
Sweet taste	1.9[a]	1.5[b]	1.0[c]
Astringent	0.5[b]	0.7[b]	1.2[a]

[a,b,c]Means within rows with different superscripts are different ($P < 0.05$)
Scores based on a universal 0–15 point intensity scale
Means are from duplicate analyses by ten trained panelists
ND not detected

(*E*)-4,5-epoxy-(*E*)-2-decenal, nonanal, and 1-octen-3-one were detected at higher levels in high-heat-treated NFDM.

8.5.2 Whole Milk Powder

Studies have also evaluated the flavor of WMP (Biolatto *et al.*, 2007; Lloyd *et al.*, 2009a, b). WMP has a flavor distinct from SMP and undergoes seasonal shifts in dimethyl sulfide, *n*-pentanal, *n*-hexanal and butyric acid concentrations (Biolatto *et al.*, 2007). An increase in *n*-hexanal, *n*-pentanal and dimethyl sulfide concentration occurs in the summer season due to changes in the feed availability (Biolatto *et al.*, 2007). Lloyd *et al.,* (2009a) performed a study on a range of WMP produced in the US which had been stored for up to a year. Figure 8.4 is a PCA biplot of the initial descriptive analysis of US WMP from four different facilities.

Flavor variability was evident amongst freshly manufactured WMP. Lactones play a large role in flavor of WMP and contribute a distinct caramelized flavor (Carunchia *et al.*, 2007a). Flavor changes with storage, and lipid oxidation flavors (fatty, grassy and painty) were quickly evident due to the high fat content of WMP (Table 8.6).

8.5.3 Milk Protein Concentrates

Milk protein concentrates (MPCs) and isolates (MPIs) are a relatively new category of dairy ingredients with USDA statistics of US production only going back to 2008 (USDA, 2013). Production of MPC is on the rise in the US with an increase from 34,000 tonnes in 2008 to almost 47,000 tonnes in 2012 (USDA, 2013). Milk protein concentrates are manufactured by removing most of the lactose and minerals from skim milk using ultrafiltration (O'Donnell and Butler, 1996).

Like whey proteins, MPC are separated by protein content into different categories and have different sensory properties. MPC with lower protein levels have fluid milk-type flavors including cooked/milky, sweet aromatic, cereal and sweet taste (Drake *et al.*, 2009a). As protein content increases the flavor profile changes. MPC (70 and 80 % protein) and MPI (>90 % protein) are characterized by tortilla, brothy, cardboard and animal flavors in addition to higher astringency, and a decrease in sweet aromatic and milky flavors. Sensory profiles of MPC at 45 and 85 % protein directly after production (0 month),

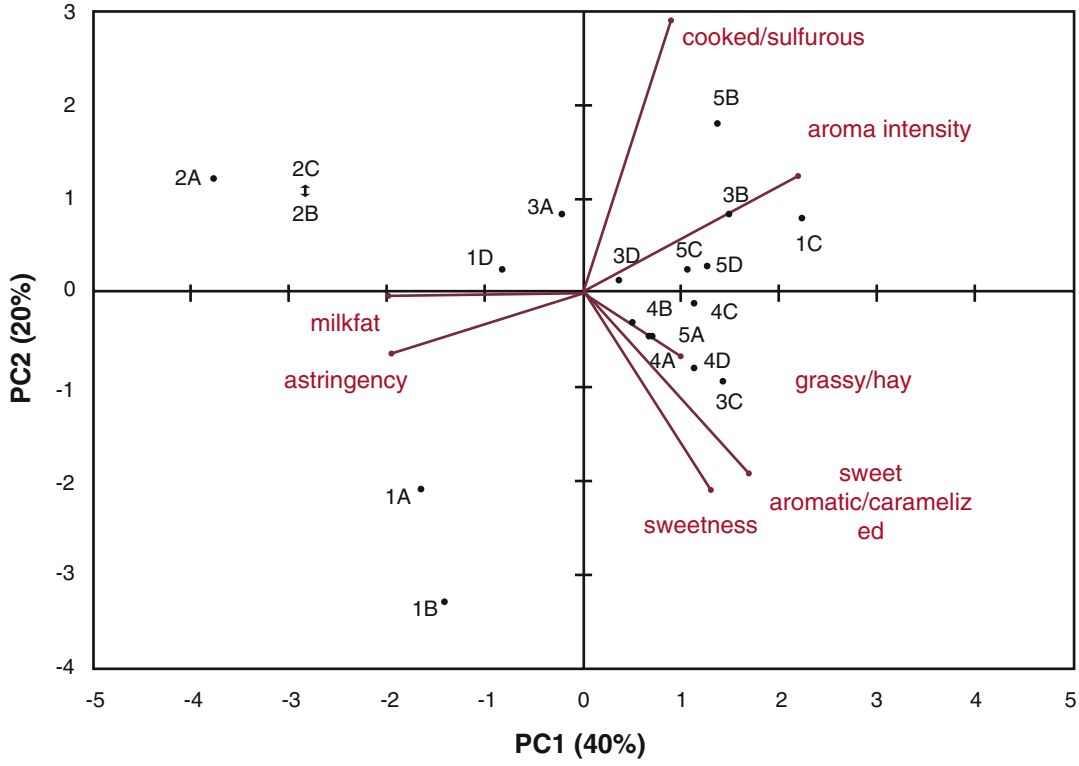


Fig. 8.4 Principal component analysis biplot of initial descriptive analysis of whole milk powder from four facilities. Numbers represent shipments (1–5) and letters represent facilities (A to D). Each point represents duplicate analyses from two bags produced on different days. Reproduced with permission from Lloyd *et al.,* (2009a)

Table 8.6 Mean flavor attributes in US whole milk powders through 1 year storage

Storage time (month)	0	2	4	6	8	10	12
Aroma intensity	2.0^{b}	2.0^{b}	2.0^{b}	2.1^{b}	2.2^{b}	2.4^{a}	2.5^{a}
Cooked/sulfurous	2.5^{a}	2.3^{b}	2.2^{c}	$2.1^{c,d}$	2.0^{d}	2.0^{d}	2.0^{d}
Milk fat	2.6^{a}	2.4^{b}	2.2^{c}	2.1^{d}	1.9^{d}	2.0^{d}	1.9^{d}
Sweet aromatic/caramelized	2.5^{a}	2.2^{b}	2.1^{c}	$2.0^{c,d}$	1.8^{d}	1.8^{d}	1.8^{d}
Grassy/hay	ND	1.1^{d}	1.4^{c}	$1.6b^{c}$	1.8^{b}	1.9^{b}	2.4^{a}
Fatty/fryer oil/painty	ND	ND	0.5^{e}	1.0^{d}	1.4^{c}	1.6^{b}	2.0^{a}
Sweet taste	2.1^{a}	2.1^{a}	2.1^{a}	2.0^{a}	2.0^{a}	1.9^{a}	1.9^{a}
Astringency	1.3^{b}	1.3^{b}	1.3^{b}	1.4^{b}	1.4^{b}	$1.5^{a,b}$	1.7^{a}

Values represent pooled means from duplicate panel measurements from five shipments from four facilities. Different letter with a row indicate significant difference ($P<0.05$)

ND not detected

Intensities were scored on a 0–15 point universal Spectrum™ intensity scale where 0 = absence of the attribute and 15 = very high intensity of the attribute

Adapted from Lloyd *et al.,* (2009a)

and after 1 and 3 months of storage at 3, 25, and 40 °C demonstrate the impact of protein, storage temperature and storage time on the sensory characteristics (Tables 8.7 and 8.8). Overall, higher protein MPC had lower cooked/milky and sweet aromatic flavors. The MPC85 developed higher cardboard flavor during storage while sweet aromatic flavor decreased. Tortilla flavor

Table 8.7 Sensory profiles of milk protein concentrates containing 45 % and 85 % protein (MPC 45 and MPC 85 samples stored for 0, 1 and 3 months at 3, 25 or 40 °C)

	45	85	45	45	45	45	45	45	85	85	85	85	85	85
	0 month	0 month	3 °C 1 month	3 °C 3 months	25 °C 1 month	25 °C 3 months	40 °C 1 month	40 °C 3 months	3 °C 1 month	3 °C 3 months	25 °C 1 month	25 °C 3 months	40 °C 1 month	40 °C 3 months
Aroma intensity	2.5	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1.5	1.5	2.0	2.0	1.5	2.8
Cooked/milky	3.5	1.8	3.1	3.0	3.0	2.8	2.4	2.3	1.5	1.5	1.0	1.0	1.0	0.5
Sweet aromatic	3.0	1.0	2.2	2.0	2.5	2.3	2.0	2.0	1.0	ND	0.5	ND	ND	ND
Cardboard	1.5	1.5	1.0	1.3	1.5	1.5	1.3	1.5	1.5	2.0	1.5	2.2	1.5	2.7
Cereal	ND	ND	ND	ND	ND	ND	ND	1.0	ND	ND	ND	ND	1.3	2.4
Tortilla	ND	1.5	ND	ND	ND	ND	ND	ND	1.5	1.8	1.5	2.0	2.3	2.5
Sweet taste	1.8	ND	1.8	2.0	1.7	1.7	1.4	1.3	ND	ND	ND	ND	ND	ND
Astringency	1.8	2.5	2.5	2.0	2.5	2.5	2.8	2.8	3.0	3.0	3.5	3.7	3.5	3.5

Values represent pooled means from duplicate panel measurements
ND not detected
Intensities were scored on a 0–15 point universal Spectrum™ intensity scale where 0 = absence of the attribute and 15 = very high intensity of the attribute

Table 8.8 Sensory profiles of pilot plant and commercial milk protein concentrates 45, 70, 80, 85 and milk protein isolate within 4 weeks of manufacture

	MPC45 (PP)	MPC85 (PP)	MPC70 (A)	MPC85 (A)	MPI90 (A)	MPC80 (B)	MPC80 (C)	MPC85 (C)
Aroma intensity	2.5	1.5	2.0	2.5	2.5	1.0	2.0	1.2
Sweet aromatic	3.2	1.2	1.8	1.5	ND	1.0	0.5	1.5
Cardboard	1.4	2.0	1.6	2.3	2.5	ND	1.5	2.0
Potato brothy	ND	ND	ND	1.5	1.5	ND	ND	ND
Tortilla	0.5	1.4	1.0	1.0	2.2	1.8	2.2	1.0
Soapy	ND	ND	ND	ND	ND	1.0	1.0	1.3
Fatty	ND	ND	ND	ND	2.0	ND	1.0	ND
Sweet taste	2.3	ND	1.0	ND	ND	ND	ND	ND
Astringency	1.5	2.5	2.0	2.5	2.3	2.3	2.2	2.5

Values represent pooled means from duplicate panel measurements
ND not detected, *PP* pilot plant
A, B, and C denote products from three different commercial suppliers
Intensities were scored on a 0–15 point universal Spectrum™ intensity scale where 0 = absence of the attribute and 15 = very high intensity of the attribute

was only detected in MPC85 and increased with storage time and storage temperature. Both time and temperature variables decreased cooked milky and sweet aromatic flavors of low and high protein MPC (Table 8.7). High protein MPC were characterized by tortilla flavors with some exhibiting potato brothy and soapy characteristics.

8.6 Caseins

The primary protein fraction in bovine milk is casein. Caseins are extremely heat stable and have been isolated from milk and produced commercially for over 80 years (Mulvihill and Ennis, 2003). Originally caseins were used as glues or synthetic fibers and caseins did not gain popularity as a functional food ingredient until about 50 years ago (Mulvihill and Ennis, 2003). To remove caseins from milk, the milk is first skimmed to remove fat and then the caseins are destabilized so that the casein proteins will coagulate/precipitate. Destabilization can be achieved by lowering the pH to the isoelectric point (pH 4.6) and achieving isoelectric precipitation (acid casein) or by enzymatic destabilization (rennet casein) (Rollema and Muir, 2009).

8.6.1 Acid Casein

Changing the pH of milk from about 6.7 (its natural pH) to 4.6 causes caseins to precipitate. This decrease in pH can be achieved two ways: mineral acids (e.g., HCl) or through fermentation of the lactose in the skim milk which produces lactic acid (O'Kennedy, 2011). The functionality of acid casein is severely limited due to its insolubility and thus is often pH neutralized through alkali addition to form a caseinate (O'Kennedy, 2009). Some alkalis commonly used include sodium hydroxide, calcium hydroxide or sodium carbonate (O'Kennedy, 2009, 2011; Drake *et al.*, 2009b).

Caseins and caseinates have distinct and generally non-dairy-like flavors and as such, are generally selected/used for their functionality rather than pleasant or dairy-like flavor (Table 8.9).

Both acid and rennet caseins are high in aroma intensity and tortilla flavor. Acid and rennet casein are also characterized by a dirty brothy/animal flavor which has been documented in the literature (Karagul-Yuceer *et al.*, 2003; Isleten and Karagul-Yuceer, 2006). Since caseins are functional proteins and can be used to alter viscosity and other texture attributes, they have been studied recently in yogurt (Guzman-Gonzalez *et al.*, 2000; Guzman and Saint-Eve *et al.*, 2006;

Table 8.9 Sensory profile of commercial milk protein concentrates containing 80 or 85 % protein (C80 and 85, respectively), acid casein (AC), and rennet casein (RC)

	C80	C85	AC	RC
Aroma intensity	2.0^{c}	1.2^{d}	4.5^{a}	3.7^{b}
Sweet aromatic	0.5^{b}	1.5^{a}	ND	ND
Sour aromatic	ND	ND	1.8^{a}	ND
Cardboard	1.5^{c}	2.0^{b}	ND	2.8^{a}
Dirty brothy/animal	ND	ND	3.3^{a}	2.0^{b}
Tortilla	2.2^{b}	1.0^{c}	4.5^{a}	2.5^{b}
Soapy	1.0^{a}	1.3^{a}	ND	ND
Fatty	1.0^{a}	ND	ND	ND
Sour taste	ND	ND	1.0^{a}	ND
Astringency	2.2^{a}	2.5^{a}	ND	ND

Values represent pooled means from duplicate panel measurements

ND not detected

Intensities were scored on a 0–15 point universal Spectrum™ intensity scale where 0 = absence of the attribute and 15 = very high intensity of the attribute. Means in a row followed by different letters are different ($p < 0.05$)

C80 MPC80, *C85* MPC85, *AC* Acid Casein, *RC* Rennet Casein

Isleten and Karagul-Yuceer, 2006; Damin *et al.*, 2009; Routray and Mishra, 2011; Akalin *et al.*, 2012). To increase total solids in low and non-fat yogurt, dried dairy ingredients such as sodium caseinate are added to prevent textural defects such as poor gel firmness and syneresis (Isleten and Karagul-Yuceer, 2006; Routray and Mishra, 2011). Istelen and Karagul-Yuceer (2006) compared physical and sensory properties of fat-free yogurts made with SMP, whey protein isolate (WPI), sodium caseinate, or a yogurt texture improver, during storage. Yogurts which used sodium caseinate displayed a distinct animal-like off flavor which had been previously identified as a key sensory attribute in dried rennet caseins (Karagul-Yuceer *et al.*, 2003; Isleten and Karagul-Yuceer, 2006). Cardboard flavor and astringency were also detected in yogurts which used dried dairy ingredients but were highest in those fortified with sodium caseinate. Although these off-flavors were present, yogurts enriched with sodium caseinate had higher viscosities and less syneresis than the control yogurt. In addition, consumers preferred products fortified with sodium caseinate to all other treatments in this study (Istelen and Karagul-Yuceer, 2006).

Saint-Eve *et al,.* (2006) investigated the role of protein composition at a constant protein level on 4 % fat, strawberry flavored, stirred yogurt. Yogurts enriched with caseinates had a higher viscosity than those enriched with whey proteins. In addition, release of major aroma compounds were lower in caseinate-enriched yogurts leading to lower flavor intensity and fruity notes which was in agreement with physicochemical measurements as yogurts with a high caseinate level had higher retention of aroma compounds suggesting protein/flavor binding (Hansen and Booker, 1996; Saint-Eve *et al.*, 2006). The inclusion of dirty brothy/animal and tortilla flavors present in acid caseins and caseinates must be carefully weighed against their many functional benefits.

8.6.2 Rennet Casein

The production of rennet casein is nearly identical to the production of cheese curd and depends on the enzyme (rennet) cleaving the crucial peptide bond (Phe_{105}-Met_{106}) in κ-casein (O'Kennedy, 2009). Like acid casein, in order to render rennet casein soluble, polyphosphates or citrates must be used to produce caseinates (Wallington, 1997). Rennet casein is used in the food industry not nearly as much as caseinates due to its insolubility (Wallington, 1997). Rennet caseins are used in cheese analog production and to make non-food items such as plastics (Wallington, 1997). Like caseinates, rennet caseins exhibit a distinct odor described as that of animal or wet dog. Figure 8.5 documents the aroma profiles of two rehydrated rennet caseins.

Karagul-Yuceer *et al,.* (2003) identified 20 neutral/basic and 14 acidic odorants from rennet caseins. While *o*-aminoacetophenone was detected at the highest flavor dilution factor, sensory studies of model mixtures demonstrated that this compound may play only a minor role in the flavor of rennet casein with hexanoic acid, indole, guiacol, and *p*-cresol being the major contributors to the previously described animal/wet dog-like odor (Karagul-Yuceer *et al.*, 2003).

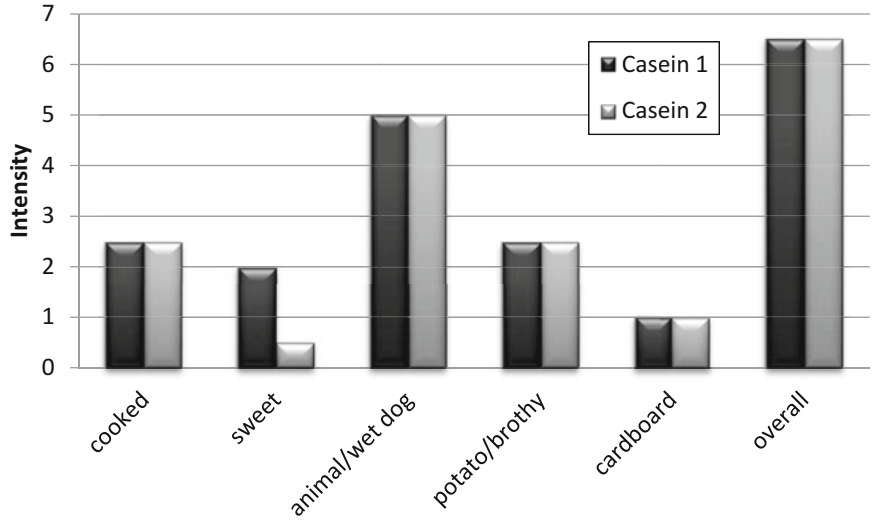


Fig. 8.5 Sensory profiles of rehydrated rennet casein (adapted from Karagul-Yuceer *et al*., 2003)

A relatively recent review of cheese analogs detailed the texture properties of various proteins, such as caseinates and rennet casein, but only mentioned one study which incorporated sensory properties (Bachmann, 2001).

8.6.3 Whey Proteins

The United States is the largest cheese producer in the world, at almost 4.9 million tonnes in 2012 (USDA, 2012). Whey proteins are the byproduct of cheesemaking and as such, the US is the largest whey producer in the world. The US has seen growth in the production of both whey protein concentrate (WPC, 34–89 % protein) and whey protein isolate (WPI, >90 % protein) over the past 10 years. WPC production has increased from 142,000 tonnes in 2002 to 200,000 tonnes in 2012. WPI production in 2003 was about 10,000 tonnes while 2012 production was almost 30,000 tonnes (USDA, 2013). Whey protein concentrates are typically membrane processed and concentrated to reach the desired protein levels. Different whey products are used for a variety of food products. Whey concentrates and protein concentrates are used in breads and other baked goods, pre-cooked meats, confections, dry beverage mixes, beverages, seafood products, ice cream mixes, mayonnaise-type dressings, dairy products such as yogurt and processed cheese, snacks, animal feed, baby food, diet foods and soups (USDEC, 2013d).

To understand better the introduction of flavor and off-flavor development, it is important to understand the processing steps that whey proteins are subject to before a final product is reached. During Cheddar cheese manufacture, for instance, the milk is received, pretreated, standardized, and heat treated/pasteurized. Calcium chloride and color (typically annatto) are also often added. The cheese milk is acidified through the addition of a lactic acid-producing starter culture or by direct addition of lactic acid and then a coagulant (rennet) is added. After curd formation and initial whey drainage, the curds are cut and cooked to help expel the whey from the coagulated casein (curd). These steps influence the flavor and flavor chemistry of the fluid whey.

8.6.4 Liquid Whey

Liquid whey is the unconcentrated byproduct of Cheddar, Swiss, Mozzarella, Monterey Jack and similar cheeses (Mahajan *et al*., 2004). The dried powder is typically composed of about 70 %

Table 8.10 Sensory flavor attributes of liquid wheys from Cheddar and Mozzarella manufacture initially and after 3 days at 3 °C

	Cheddar				Mozzarella			
	No fat separation		Fat separation		No fat separation		Fat Separation	
Sensory attribute	0 day	3 days	0 day	3 days	0 day	3 days	0 day	3 days
Aroma intensity	2.4^{a}	2.1^{b}	2.4^{a}	2.0^{b}	$2.2^{a,b}$	2.0^{b}	$2.0^{b,c}$	1.6^{c}
Sweet aromatic	2.0^{a}	1.1^{b}	2.0^{a}	1.0^{b}	1.1^{b}	0.9^{b}	1.1^{b}	0.8^{b}
Sour aromatic	ND	ND	ND	ND	1.1^{a}	0.7^{b}	0.6^{b}	0.6^{b}
Cardboard	ND	1.4^{a}	ND	0.6^{c}	ND	1.0^{b}	ND	0.5^{c}
Cooked/milky	$3.2^{a,b}$	$2.7^{c,d}$	3.2^{a}	$2.8^{b,c,d}$	$3.2^{a,b}$	$2.4^{d,e}$	$2.9^{a,b,c}$	2.2^{e}
Sweet aromatic	2.0^{a}	2.0^{a}	2.0^{a}	2.0^{a}	1.7^{b}	1.6^{b}	1.6^{b}	1.5^{b}
Astringent	1.5^{a}	1.5^{a}	1.5^{a}	1.5^{a}	1.5^{a}	1.5^{a}	1.5^{a}	1.5

Means in a row followed by different superscript letters signify differences ($P<0.05$). *ND* not detected. Attributes were scored using a 0–15 point universal Spectrum™ scale where 0 = absence of the attribute and 15 = extremely high intensity of attribute

Used with permission from Liaw *et al.,* (2011)

lactose, 1.5 % fat, 12 % protein, 4 % moisture and 8.5 % other solids (USDEC, 2013a, b). Many studies have evaluated the flavor attributes of liquid whey (Carunchia Whetstine *et al*., 2003; Karagul-Yuceer *et al*., 2003; Tomaino *et al*., 2004; Drake *et al*., 2009a, b; Wright *et al*., 2009; Liaw *et al*., 2010, 2011; Campbell *et al*., 2011b). Starter culture and type of set (rennet or acid) both have significant effects on the flavor of the liquid whey. Gallardo-Escamilla *et al,*. (2005) reported that whey from rennet-set cheese was bland, sweet, and milky, while acid casein wheys were described as bitter, stale, rancid and chemical (Table 8.10).

Campbell *et al*. (2011a, b) also reported bland, milky flavor and lower oxidation products in rennet-set whey without the addition of starter culture compared to that from rennet-set whey with starter culture. The type of cheese is also a source of variability (Tomaino *et al*., 2004; Gallardo-Escamilla *et al*., 2005; Drake *et al*., 2009a, b; Campbell *et al*., 2011b). Liaw *et al,*. (2011) studied the differences between liquid wheys from Mozzarella and Cheddar manufacture. Mozzarella and Cheddar wheys were distinct in flavor profile following 3 days of storage at 3 °C. Lipid oxidation continues in fluid whey despite pasteurization, fat separation and cold storage (Tomaino *et al*., 2004; Liaw *et al*., 2011; Campbell *et al*., 2011a, b). This increase in lipid oxidation products results in increases in cardboard and serumy flavors in liquid wheys (Liaw *et al*., 2011; Campbell *et al*., 2011a, b).

Cheddar whey is more prone to lipid oxidation than Mozzarella whey, and further differences exist within different strains of starter cultures (Carunchia Whetstine *et al*., 2003; Campbell *et al*., 2011a; Liaw *et al*., 2011).

8.6.5 Sweet Whey Powder

Sweet whey powder (SWP) is the spray dried product of liquid whey manufactured from rennet coagulation of casein in milk. SWP goes through several steps before drying, including separations, pasteurization, concentration and crystallization (USDEC, 2013c; Tunick, 2008). SWPs are characterized by cooked, carmelized, cardboard, oxidized, and barny flavors (Sithole *et al*., 2005, 2006a, b). Many factors affect the stability of SWPs including variability in liquid whey, raw milk composition and processing (Sithole *et al*., 2006a). Different suppliers of SWP produce distinct products with unique flavor profiles (Table 8.11).

Mahajan *et al,*. (2004) found that the important aroma volatiles in sweet whey powder were the short-chain fatty acids, aldehydes and ketones, lactones, sulfur compounds, phenols, indoles, pyrazines, furans and pyrroles and that autooxidation of lipids, caramelization of sugar,

Table 8.11 Sensory profiles of commercial sweet whey powders

	SW1	SW2	SW3
Sweet aromatic	1.4	1.2	1.1
Caramelized	2.4	2.7	2.9
Cooked	3.9	3.8	4.0
Cardboard	1.8	1.9	2.0
Oxidized	1.2	1.7	1.6
Barny	0.8	1.6	1.6
Sweet taste	4.1	4.8	3.5
Sour taste	1.6	1.2	1.4
Salty taste	2.6	2.2	2.6

Rating scale was a 15-point scale, where 3 = slight, and 7 = moderate
Adapted from Sithole *et al.,* (2005)

and Maillard reactions could explain the generation of many aroma compounds. Sithole *et al.* (2005) concluded that the shelf life of SWP is longer than 12 months; however, the deterioration rates of SWP from different commercial suppliers vary and should be taken into account. SWP contains a high concentration of lactose and is susceptible to Maillard reactions, which play a role in the rate of deterioration of flavor profiles of whey powders.

8.6.6 Whey Protein Concentrates

Processing variables have perhaps the most significant effect on the flavor of whey protein ingredients. A typical whey protein concentrate process includes holding, clarification/fat separation, pasteurization, ultrafiltration, diafiltration, evaporation and spray drying (Varnam and Sutherland, 1994; Huffman, 1996). Additional steps are necessary depending on the specific whey protein product. Production of some lower protein whey concentrates do not require diafiltration, while whey protein isolates (WPI) often require an extra fat removal step, such as microfiltration, in order to reach desired protein levels (Table 8.12).

Tremendous variability in sensory profile is documented among WPC80 (80 % protein) from different suppliers (Carunchia Whetstine *et al.*, 2005; Wright *et al.*, 2009; Evans *et al.*, 2010). Differences are due to the source of whey as well as differences in processing procedures. Differences in fluid whey from different cheese manufacturers translate to differences in whey protein flavor. WPC80 produced from Mozzarella whey had higher sweet aromatic and cereal flavors compared to WPC80 from Cheddar whey (Wright *et al.*, 2009; Drake *et al.*, 2009b). WPC66 (66 % protein) from rennet-set whey with no starter culture was characterized by milky flavor and low cardboard flavor (Campbell *et al.*, 2011a).

Storage of fluid product is an important factor in whey protein ingredient flavor. Tables 8.13 and 8.14 show a comparison of cardboard flavor intensity to aldehyde relative abundance (μg/kg) in WPC spray dried from Mozzarella whey and Cheddar WPI produced from liquid retentates stored for 0, 6, 12, 24, and 48 h before processing. Similar increases in lipid oxidation and cardboard flavor were demonstrated by Liaw *et al,.* (2011) in WPC produced from fresh and stored fluid Cheddar and Mozzarella wheys. Fluid storage of whey or retentate should be minimized (<6 h) to minimize lipid oxidation and associated off-flavors.

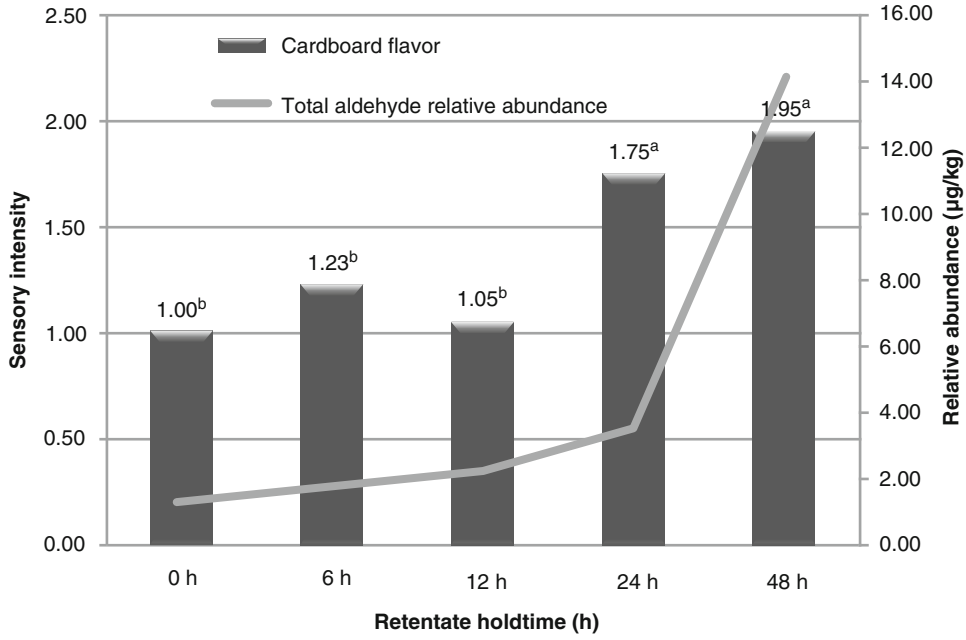
Bleaching is another common liquid whey processing step for colored Cheddar whey which increases off flavor formation. Annatto, a pigment extract from the *Bixa orellana* shrub, is often added to milk intended for Cheddar cheese manufacture to provide desired orange color many consumers expect. Chemical bleaching is the most common method applied to remove the color despite the oxidative load this introduces to the system. Numerous publications have connected the bleaching of liquid whey with off-flavor formation in dried whey protein products (Croissant *et al.*, 2009; Listiyani *et al.*, 2011; Campbell *et al.*, 2011a, 2012; Kang *et al.*, 2012; Jervis *et al.*, 2012). Jervis *et al.* (2012) studied volatile components produced by lipid oxidation and sensory attributes of WPC80 made from fluid whey bleached by benzoyl peroxide (BP) or hydrogen peroxide (HP). Figure 8.6 is a (PCA) biplot of sensory attributes and lipid oxidation volatile components in those WPC80 ingredients. The HP-bleached WPC80 were character-

Table 8.12 Descriptive sensory analysis of whey protein concentrates (WPC80; 80 % protein) from different manufacturers

Column1	WPC 1	WPC 2	WPC 3	WPC 4	WPC 5	WPC 6	WPC 7	WPC 8	WPC 9	WPC 10
Aroma intensity	1.6^{c}	3.0^{a}	2.0^{b}	3.0^{a}	1.8b^{c}	3.5^{a}	3.5^{a}	3.3^{a}	2.0^{b}	1.4^{c}
Sweet aromatic	0.8^{c}	ND	ND	2.9^{a}	2.0^{b}	1.5^{c}	ND	ND	1.3^{c}	2.1^{b}
Cooked milky	1.1^{b}	ND	ND	ND	ND	ND	2.0^{a}	1.5^{a,b}	ND	ND
Cardboard	1.3^{c}	3.3^{a}	2.0^{b}	ND	1.5^{b}	ND	ND	3.3^{a}	1.6^{b}	0.6^{c}
Pasta water	ND	ND	1.5^{d}	1.5^{d}	2.5^{c}	3.3^{b}	4.0^{a}	2.5^{c}	ND	ND
Brothy	ND	ND	ND	ND	ND	ND	1.5	ND	ND	ND
Doughy fatty	ND	ND	ND	1.5	ND	ND	ND	ND	ND	ND
Cereal	1.9	ND	ND	ND	ND	ND	ND	ND	ND	ND
Astringent	2.3^{b}	3.4^{a}	3.0^{a,b}	2.8^{b}	2.5^{b}	3.0^{a,b}	2.5^{b}	2.4^{b}	2.3^{b}	2.3^{b}

^{a,b,c}Means in a row followed by different letters are different ($p<0.05$)
WPC from different manufacturing sites
Intensities were scored on a 0–15-point universal scale where 0 = none and 15 = very high (Meilgaard *et al*., 1999). *ND* Not detected
Adapted from Carunchia Whetstine *et al.,* (2005), Wright *et al.,* (2009) and Evans *et al.,* (2010)

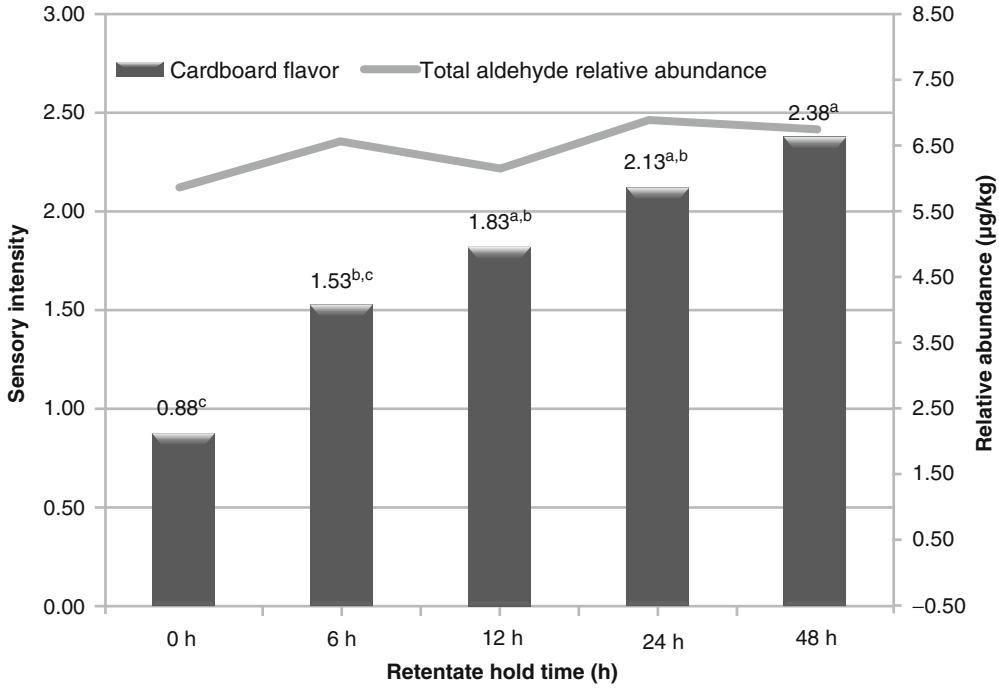
Table 8.13 Comparison of cardboard flavor intensity to total aldehyde relative abundance (μg/kg) in Mozzarella whey protein concentrate from liquid retentate stored for 0, 6, 12, 24 or 48 h at 3 °C. Means followed by different superscripts indicate differences ($p<0.05$) among hold times. Used with permission from Whitson *et al*. (2011)



ized by higher concentrations of hexanal, heptanal, octanal, nonanal, decanal, dimethyl disulfide and 1-octen-3-one when compared with unbleached and BP-bleached WPC80. HP-bleached WPC80 were also characterized by higher intensities of cardboard and fatty flavors (Table 8.15).

Hydrogen peroxide and benzoyl peroxide are the two chemical bleaching agents currently allowed for liquid whey, but the effect of

Table 8.14 Comparison of cardboard flavor intensity to total aldehyde relative abundance (μg/kg) in Cheddar whey protein isolate from liquid retentate stored for 0, 6, 12, 24 or 48 h. Means followed by different superscripts indicate differences ($p<0.05$) among hold times. Used with permission from Whitson *et al.,* (2011)



alternative bleaching agents has also been studied (Kang *et al*., 2012; Campbell *et al*., 2012).

Alternative bleaching agents, including ultraviolet light, acid-activated bentonite, ozone, and lactoperoxidase compared to hydrogen peroxide and benzoyl peroxide, have variable effects on cardboard flavor intensity and total aldehyde levels in WPC80. Every bleaching agent increased aldehyde levels and cardboard flavor intensity when compared to an unbleached control (Campbell *et al*., 2012; Jervis *et al*., 2012; Kang *et al*., 2012). Of the bleaching agents evaluated, only bentonite did not use oxidation as the primary method of norbixin destruction, which explains the low aldehyde increase compared to other bleaching agents studied. Bleaching of colored whey is a major factor in off-flavor formation.

Storage and agglomeration of the dried powder also have significant effects on flavor. Agglomeration is a process which produces small clumps of several whey powder particles, and is a common process because it increases dispersability and decreases dispersion time due to an increase in the particle size and porosity of the powder (Pietsch 2005; Turchiuli *et al*., 2005). A wetting agent may also be applied to increase solubility further, and soy lecithin is the most commonly used agent. Wright *et al,*. (2009) studied the effect of agglomeration and storage on the flavor of WPC80. Both agglomeration and instantization decreased storage stability of WPC80 and WPI (Figs. 8.7 and 8.8). The addition of lecithin also can contribute grassy and cucumber flavors and bitter taste to WPC80 and WPI.

8.7 Whey Protein Isolates

Many of the processing steps for WPI are the same as those used for high protein content WPC (>80 % protein) production, although a considerable reduction in fat content is necessary to reach >90 % protein levels. As such, the flavor profiles for the two are similar, although differences do exist. Table 8.16 displays sensory profiles of

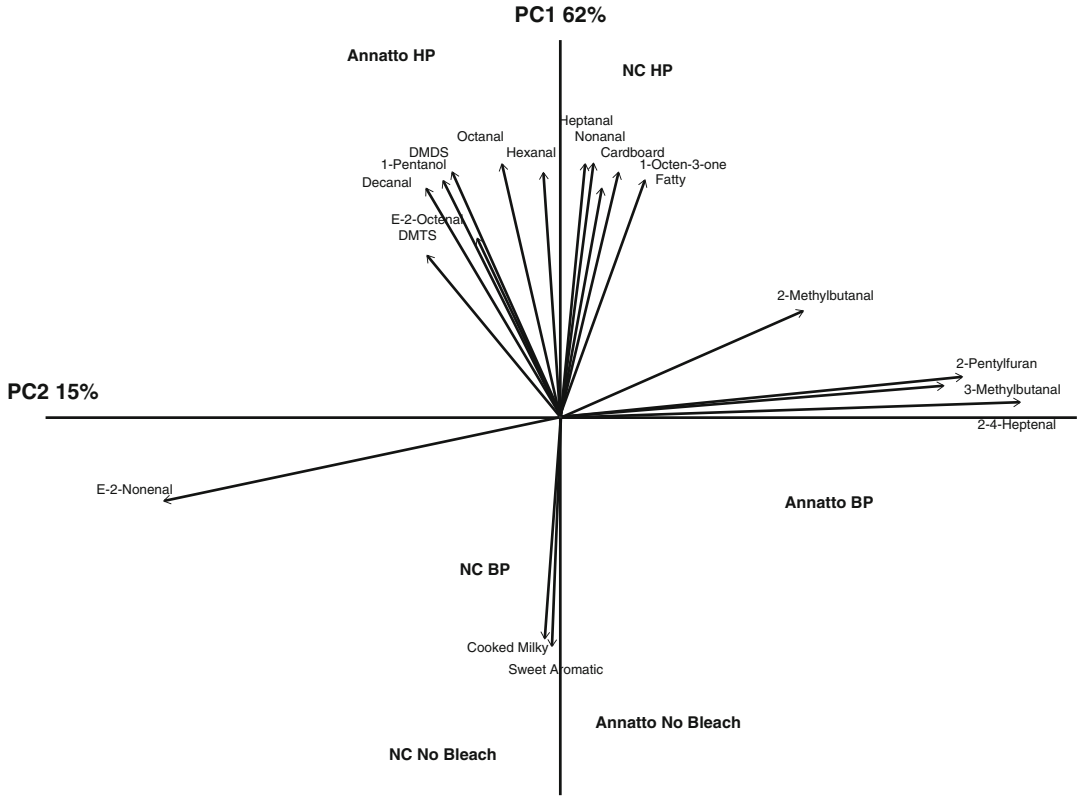
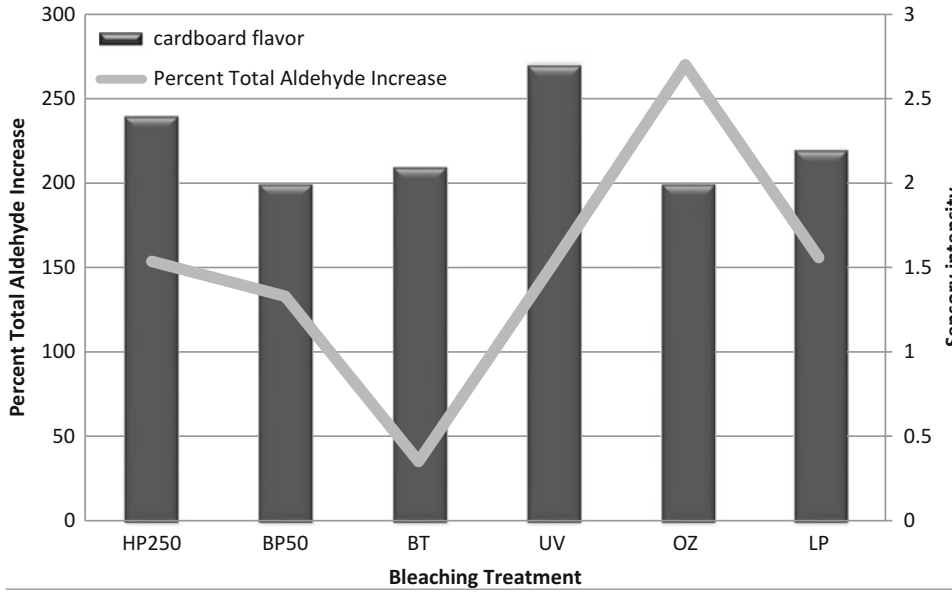


Fig. 8.6 Principal component analysis biplot of sensory attributes and selected lipid oxidation volatile components of WPC80 with and without annatto color added to the milk followed by no bleaching, bleaching with benzoyl peroxide (BP; 50 mg/kg) or bleaching with hydrogen peroxide (HP; 500 mg/kg). Used with permission (Jervis *et al.*, 2012)

Table 8.15 Comparison of descriptive analysis cardboard flavor intensity to percent total aldehyde increase in Cheddar whey protein concentrates bleached with 250 ppm hydrogen peroxide (HP250), 50 ppm benzoyl peroxide (BP50), acid activated bentonite (BT), ultraviolet light (UV), ozone (OZ), and the lactoperoxidase system using 20 ppm hydrogen peroxide (LP). Adapted from Kang *et al.,* (2012), Jervis *et al.,* (2012) and Campbell *et al.,* (2012)



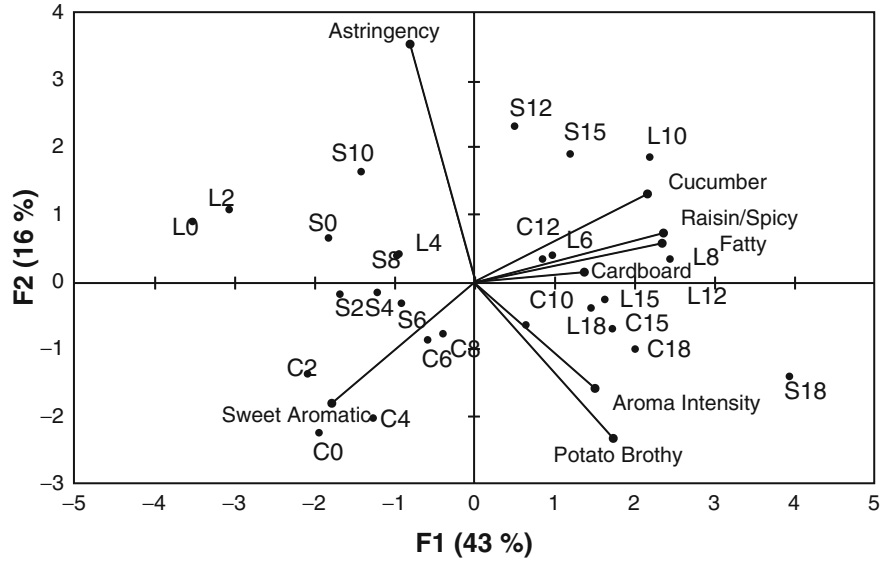


Fig. 8.7 Principal component analysis biplot of sensory changes in nonagglomerated and agglomerated WPC80 from Plant 1 over time. Numbers indicate treatment type and storage time. The letter "C" represents the nonagglomerated control. "S" represents the steam-agglomerated samples. "L" represents the lecithin-agglomerated samples. The number next to each letter represents the storage in months at the time of analysis. Used with permission (Wright *et al.*, 2009)

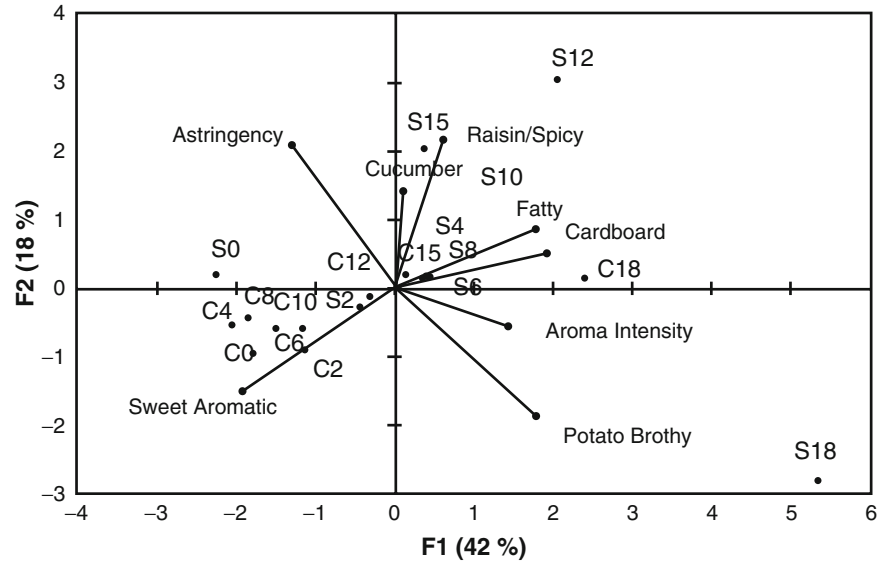


Fig. 8.8 Principal component analysis biplot of sensory changes in nonagglomerated and agglomerated WPC80 from Plant 2 over time. Numbers indicate treatment type and storage time. The letter "C" represents the nonagglomerated control. "S" represents the steam-agglomerated samples. The number next to each letter represents the storage in months at the time of analysis. Used with permission (Wright *et al.*, 2009)

rehydrated unbleached and hydrogen peroxide- and benzoyl peroxide-bleached WPI. As with WPC80, chemical bleaching alters flavor profiles of WPI. WPIs from bleached whey have lower sweet aromatic flavor and higher cardboard flavor than WPI from unbleached whey.

Flavor variability within commercial WPI has been studied, especially the occurrence of

Table 8.16 Descriptive sensory profiles of unbleached WPI (Control) and WPI bleached using 250 ppm hydrogen peroxide (HP) or 50 ppm benzoyl peroxide (BP)

	Control	HP	BP
Aroma intensity	2.0[b]	2.5[a]	2.0[b]
Sweet aromatic	2.0[a]	ND	0.5[b]
Cardboard	1.5[c]	2.5[a]	2.0[b]
Cabbage	ND	1.0	ND
Fatty	ND	ND	1.2
Bitter	ND	1.1[a]	ND

Means in a row followed by different lowercase letters signify differences ($P<0.05$). *ND* not detected. Attributes were scored using a 0–15 point universal Spectrum™ scale where 0 = absence of the attribute and 15 = extremely high intensity of attribute

cabbage off-flavor. Wright *et al,*. (2009) studied the volatile origin of this off-flavor. WPI with cabbage off-flavor had higher concentrations of dimethyl trisulfide, a sulfurous compound with a threshold in water of 0.07 parts per trillion (Wright *et al*., 2006). Dimethyltrisulfide, along with dimethyldisulfide and dimethyl sulfide, are all caused by degradation of methionine which can occur from oxidative processes during whey processing or storage.

8.8 Whey Protein Hydrolysates

Whey protein hydrolysates (WPH) are another category within whey protein concentrates and isolates that are commonly used as food ingredients. Whey proteins are first ultrafiltered/diafiltered to desired protein levels. Enzymatic hydrolysis using a variety of proteolytic enzymes is used to reach the desired level of proteolysis after which they are heated to inactivate the added enzymes (Havea *et al*., 2009). Whey protein hydrolysates are often touted for their bioactive properties stemming from specific protein fragments (i.e., peptides) that may improve health (Nnanna and Wu, 2007). WPH have been suggested to have a protective effect against cardiovascular disease in addition to ion binding, antioxidant, immunomodulatory, satiety, and antiallergenicity effects (Nnanna and Wu, 2007).

Despite the health effects of WPHs, their general use is hindered due to strong, and often objectionable, sensory attributes. WPHs differ from whey protein concentrates and isolates in enzymatic treatments, additional thermal treatments and clarification steps. Reducing peptide chain length through enzymatic hydrolysis can also significantly increase bitter taste, further differentiating WPH from typical WPC and WPI (Spellman *et al*., 2009). Bitter taste of whey protein hydrolysates is variable, and dependent on the specific enzyme used for hydrolysis, the degree to which the protein is hydrolyzed and processing (Spellman *et al*., 2009; Drake *et al*., 2009a, b). Leksrisompong *et al*. (2010) reported that high bitter taste was correlated with low concentrations of large and medium chain peptides (2 to >10 kDa) and higher concentrations of low molecular weight peptides (<0.5 to 1 kDa). These findings are consistent with previous studies linking low molecular weight peptides with bitter taste (Cheison *et al*., 2007; Spellman *et al*., 2009). Leksrisompong *et al*. (2012) conducted work on masking the bitter taste inherent in whey protein hydrolysates, and suggested that fructose, sucrose, sucralose, monosodium glutamate, sodium acetate, sodium gluconate, 5′adenosine monophosphate (AMP), and 5′AMP disodium were all effective to some degree, with sweeteners showing the most promise.

Bitter taste is not the only sensory challenge of WPH. Many of the flavors in WPH have been documented in WPC and WPI, but the bitterness can range anywhere from 2.4 to 13.0 on a 15 point universal scale, far higher than what is detected in WPC or WPI. Other flavors documented in WPH were cooked/sulfur, potato/brothy, cheesy, tortilla/animal, and malty flavors. It is likely that the unique flavor profile of WPH is due to protein degradation compounds in addition to lipid oxidation products found in WPI and WPC. Leksrisompong *et al,*. (2010) reported that 2-methyl butanal (malty/chocolate) and methional (potato brothy), protein degradation products, were key volatile compounds in all WPH. Protein degradation products were key volatile compounds in all WPH. Sulfur compounds commonly found in

WPH were dimethyl sulfide, 2-methyl-3-furanthiol, methyl 2-methyl-furyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide. Other Strecker degradation compounds detected in some of the WPH samples were phenylacetaldehyde, 2-phenethanal, *p*-cresol, guaiacol, 2-acetyl pyrroline and sotolon. Lipid oxidation compounds were also evident (Table 8.17).

8.9 Serum Protein

Whey proteins that have been removed prior to the cheese making process have been referred to as "native" whey proteins or milk serum proteins (Drake *et al*., 2009a, b; Evans *et al*., 2009, 2010). Milk serum proteins are a valuable milk fraction and are not exposed to cheese making, thus leaving them free of any functional or sensory effects from this process. It may be advantageous to remove the milk serum proteins prior to cheese making for several reasons, particularly to produce more mild-tasting and consistent value-added whey protein products. When milk serum proteins are removed before the manufacture of cheese, the subsequent composition of the cheese is the same because the majority of these proteins are not retained in the cheese (Nelson and Barbano, 2005a). Serum proteins are prepared by microfiltering and diafiltering skimmed milk (Nelson and Barbano, 2005a).

Lipid oxidation is initiated during the cheese making process (Campbell *et al*., 2011b) and subsequent steps in whey processing increase levels of volatile oxidation products (Croissant *et al*., 2009; Campbell *et al*., 2011a; Whitson *et al*., 2011). Serum protein concentrates are lower in fat (0.53 % vs. 4.67 % on a dry weight basis) than WPC (Evans *et al*., 2009, 2010) because the fat remaining in skim milk is retained in the retentate of the MF process, while the serum proteins pass into the permeate. During production of WPC, the fat not removed from whey is concentrated by UF and DF in the WPC retentate. The high content of fat in WPC has a negative effect on flavor (and functionality) and provides a source for increased levels of lipid oxidation compounds, such as aldehydes, compared to SPC (Fig. 8.9) (Evans *et al*., 2010; Liaw *et al*., 2010; Jervis *et al*., 2012; Campbell *et al*., 2013). Serum proteins contain lower amounts of volatile compounds and thus lower intensities of flavors commonly found in WPC due not only to the decreased fat content, but also to the absence of starter culture and cheese making residuals. In addition, WPC often has to be bleached as some of the annatto added to the cheese milk to impart desired color, remains in the fluid whey. Previous studies have demonstrated that bleaching significantly impacts flavor in both WPC and SPC but by removing proteins prior to cheese making, bleaching can be avoided (Jervis *et al*., 2012; Campbell *et al*., 2013). Bleaching using hydrogen peroxide, the most commonly used bleaching agent in US industry, increases total aldehydes (hexanal, heptanal, octanal, nonanal, decanal) nearly 20-fold in WPC and SPC (Fig. 8.9).

Evans *et al.* (2009) compared the flavor profile of serum protein concentrates at 34 % protein to whey protein concentrates at 34 % protein. Spray dried SPC lacked both diacetyl and cardboard flavors (Table 8.18). Evans *et al.* (2010) subsequently compared the flavor profile of SPC80 (80 % protein) to that of WPC80 (80 % protein) as well as to several commercial WPC80s. The WPC80 had higher overall aroma intensity and also had cardboard and diacetyl flavors that were not present in the SPC80 (Tables 8.18 and 8.19).

8.10 Conclusions

Milk protein ingredient products and applications continue to increase in number as technology and processing methodologies continue to adapt to the demand for functional and nutritious ingredients. An understanding of the sensory properties of milk proteins and milk protein ingredients is essential to ensure the healthy growth and development of the dairy industry. While the nutritional and functional characteristics of milk protein ingredients are a marketing boon, disregarding the sensory aspects of these products would most certainly lead to missed opportunities and underutilization. A significant amount of

Table 8.17 Descriptive sensory profiles of selected whey protein hydrolysates using a 0–15 point Spectrum™ scale where 0 = absence of attribute and 15 = extremely high intensity

Sample	Aroma intensity	Cooked/milky	Cooked/sulfur	Malty	Cardboard	Potato brothy	Cheesy brothy	Grassy/herbal	Tortilla/animal	Cucumber	Scorched	Sour	Bitter	Astringency
1	$2.9^{c,d,e}$	ND	$1.7^{a,b}$	ND	0.8^{a}	1.1^{c}	1.3^{a}	1.0^{a}	ND	ND	2.0^{b}	2.3^{a}	$12.4^{a,b}$	$3.0^{b,c}$
2	$2.9^{c,d,e}$	2.0^{a}	$1.0^{a,b}$	ND	ND	0.5^{d}	ND	0.5^{b}	0.6^{d}	ND	ND	2.5^{a}	10.1^{c}	$3.1^{b,c}$
3	$2.1^{e,f}$	$1.1^{a,b}$	$1.4^{a,b}$	ND	ND	1.5^{c}	0.5^{b}	1.5^{a}	1.1^{c}	ND	0.9^{c}	ND	2.4^{h}	$2.5^{c,d,e}$
4	1.9^{f}	ND	ND	$0.9^{b,c}$	ND	1.3^{c}	1.0^{a}	ND	ND	ND	0.8^{c}	ND	5.6^{e}	2.1^{e}
5	$3.5^{b,c}$	ND	$0.9^{a,b}$	3.0^{a}	1.0^{a}	2.7^{b}	1.4^{a}	ND	1.1^{c}	ND	0.9^{c}	ND	$3.6^{f,g}$	$2.4^{d,e}$
6	$2.6^{d,e,f}$	ND	ND	$0.6^{b,c}$	0.5^{b}	1.6^{c}	$0.9^{a,b}$	ND	0.6^{d}	ND	3.1^{a}	2.6^{a}	11.7^{b}	$2.3^{d,e}$
7	$2.5^{d,e,f}$	ND	ND	$0.5^{b,c}$	1.0^{a}	1.6^{c}	1.5^{a}	ND	2.6^{b}	ND	ND	0.8^{b}	13.0^{a}	$3.8^{a,b}$
8	3.7^{b}	ND	$2.0^{a,b}$	ND	ND	2.3^{b}	ND	ND	2.4^{b}	0.9^{b}	ND	ND	$2.8^{g,h}$	$3.0^{b,c}$
9	3.7^{b}	ND	$1.2^{a,b}$	ND	ND	1.5^{c}	ND	1.3^{a}	2.3^{b}	ND	ND	ND	4.0^{f}	$3.1^{b,c}$
10	4.6^{a}	ND	ND	ND	ND	3.6^{a}	ND	ND	4.4^{a}	ND	2.8^{a}	2.8^{a}	$9.2^{c,d}$	4.1^{a}
11	3.7^{b}	ND	3.4^{a}	$1.8^{a,b}$	ND	1.5^{c}	ND	ND	2.0^{b}	2.4^{a}	$2.4^{a,b}$	ND	$4.7^{e,f}$	$2.4^{d,e}$

Most aromatics in dried ingredients fall between 0 and 4 on this scale
Means in a column not followed by a common letter are statistically different ($p < 0.05$)
ND not detected
Used with permission (Leksrisompong *et al.*, 2010)

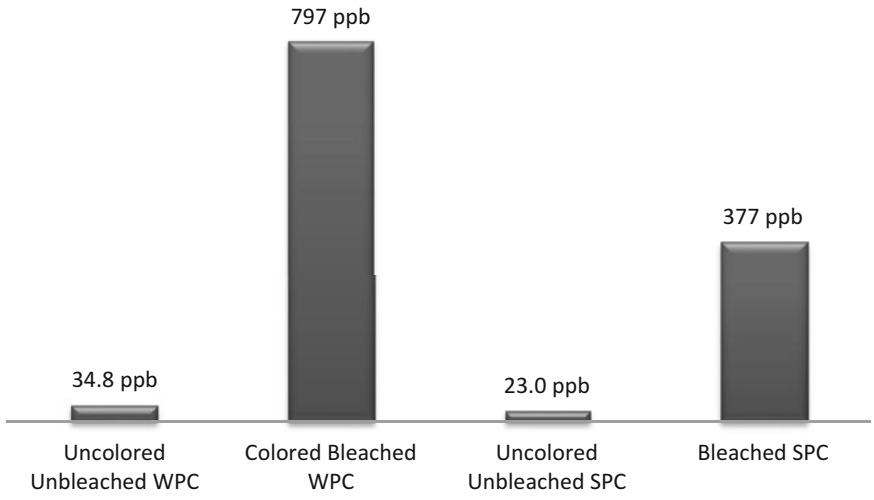


Fig. 8.9 Total aldehydes (hexanal, heptanal, octanal, nonanal, decanal) in bleached (500 ppm hydrogen peroxide) and unbleached whey protein concentrate (WPC) and serum protein concentrate (SPC). Adapted from Jervis *et al.,* (2012) and Campbell *et al.,* (2013)

Table 8.18 Sensory attributes of spray-dried (SD) serum protein concentrate (SPC) and whey protein concentrate (WPC)

Product	Aroma intensity	Sweet aromatic	Diacetyl	Cardboard	Cereal	Cooked/milky	Sweet taste	Astringent
SD WPC	1.8^{b}	1.1^{b}	0.5^{a}	0.6^{a}	0.7^{b}	1.4^{a}	2.0^{a}	1.7^{a}
SD SPC	$1.7^{b,c}$	0.7^{c}	ND	ND	1.0^{a}	1.5^{a}	2.0^{a}	1.7^{a}

Adapted from Evans *et al.,* (2009)
Intensities were scored on a 0–15-point universal scale where 0 = none and 15 = very high intensity (Meilgaard *et al.*, 1999)
ND not detected
^{a,b,c}Means in the same column not sharing a common superscript are different ($p < 0.05$)

Table 8.19 Mean sensory attributes of the experimental 80 % serum protein concentrate (SPC) and whey protein concentrate (WPC) and five commercial 80 % WPC products

Source	Product	Aroma intensity	Sweet aromatic	Diacetyl	Cardboard	Cereal	Cooked/milky	Potato	Astringent
Commercial WPC	1	$1.8^{b,c}$	ND	ND	1.6^{b}	1.1^{b}	ND	ND	$2.3^{a,b}$
	2	1.2^{d}	0.8^{b}	ND	$1.1^{c,d}$	ND	ND	ND	2.1^{a}
	3	$1.4^{c,d}$	1.5^{a}	ND	$1.0^{c,d}$	1.1^{b}	ND	ND	$2.3^{a,b}$
	4	2.3^{a}	ND	ND	2.2^{a}	ND	ND	1.4	$2.2^{a,b}$
	5	1.0^{d}	0.8^{b}	1.1	$1.3^{b,c,d}$	0.8^{c}	ND	ND	2.5^{a}
Experimental	SPC	1.2^{d}	ND	ND	0.8^{d}	1.2^{b}	1.1^{a}	ND	1.6^{d}
Experimental	WPC	$1.6^{b,c}$	0.7^{b}	ND	$1.3^{b,c,d}$	1.9^{a}	1.1^{a}	ND	1.9^{c}

Adapted from Evans *et al.,* (2010)
Intensities were scored on a 0–15-point universal scale where 0 = none and 15 = very high intensity (Meilgaard *et al.*, 1999)
ND not detected
^{a,b,c}Means in the same column not sharing a common superscript are different ($p < 0.05$)

information on the flavor characterization of different milk protein products exists in literature; however, continued research must be done on flavor reduction and improvement to realize fully the potential of milk protein products.

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Ethanol Stability and Milk Composition

9

David S. Horne

Abstract

This chapter reviews the history of the use of the ethanol stability test and surveys the milk compositional factors influencing its result. Developed originally as an indicator of fresh milk quality, it now finds use in some countries as a warning of the lack of suitability for high temperature heat treatment. Relevant factors influencing the heat stability of milk are introduced and mechanisms are proposed and compared for both instability reactions. It is concluded that a fairer option would be to develop a direct rapid heat stability method for use by field operatives.

Keywords

Ethanol stability of milk • Heat stability of milk • Casein micelle stability • Dual-binding model of casein micelle • Casein interactions

9.1 Introduction and History

Milks are secreted by mammals for the primary function of providing the complete nutritional requirements (protein, fat, carbohydrates, minerals and sometimes water) for the suckling young of the species. Consumption directly from the mammary gland by the neonate was Nature's design but recognition of the benefits of including milk in the diet of man has led to the growth of the dairy industry we see today.

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The gap in both time and distance between production and consumption of liquid milk lends itself to opportunities for contamination and spoilage. To ensure product quality and to avoid unscrupulous traders passing aged milk as fresh, public health regulations or city statutes laid down tests to assess the quality of milk offered for retail sale. One can imagine that sniffing or tasting would feature in the earliest tests but a more objective test assessment was provided by the alcohol test, accounts of which first appeared in the fourth quarter of the nineteenth century.

The simple unsophisticated single volume test demanded that if a precipitate formed when an equal volume of ethanol solution, usually 70 % v/v, was added to a milk sample, the batch

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of milk was rejected. It was believed that the alcohol test responded to acidity developed in the milk as it spoiled. But it was soon recognised that the alcohol test was neither selective nor specific. Positive tests were obtained with fresh milks from individual cows but were taken to indicate abnormalities due to pathological or physiological conditions in the cow. Colostral milks and late lactation milks also failed in this test. Ayers and Johnson (1915) found that artificially acidifying milks brought about failure but that some milks where acidity occurred due to bacterial growth did not fail the test until excessive growth was present. Moreover, it was not possible to correlate bacterial counts with failure in the alcohol test. They observed that many milks with low counts of colony-forming-units (CFU) failed and, equally unsatisfactorily, many milks with high counts passed the alcohol test. It was concluded at that time that the alcohol test was not of any particular value in the control of market milk supply. Interestingly, the same conclusion was reached in a recent publication from Iran (Shekarforoush *et al*., 2003), with almost the same percentage of high CFU milks passing and low CFU milks failing, and again no correlation between alcohol stability and titratable acidity.

Despite this lack of confidence in the efficacy of the alcohol test, Dahlberg and Garner (1921) concluded that "the alcohol test shows good possibilities as a practical and reliable test for determining the quality of milk for condenseries making evaporated milk". In other words, milks which were likely to have heat stability problems would also fail the ethanol stability test. In a series of laboratory trials with 90 samples from a local creamery (Dahlberg and Garner, 1921), 45 coagulated when mixed with an equal volume of 75 % v/v alcohol. Of these 45 samples, 43 showed evidence of coagulation when the milks were evaporated and sterilised at 112.8 °C (235 °F). Of the 45 samples that passed the alcohol stability test, only 3 of them showed coagulation when evaporated and sterilised. Factory trials were less successful. All samples which failed the alcohol stability test gave an unsatisfactory product, but of 22 samples showing no reaction to alcohol, only 8 gave a completely satisfactory evaporated and sterilised product in one factory. In a second factory with a smaller group of eight trials, four milks which coagulated with alcohol resulted in poor product quality on heating, whilst four milks which passed the alcohol test gave condensed and evaporated products satisfactory in all respects.

Notwithstanding this recommendation, the alcohol test was not widely adopted, and following a report from Ramsdell *et al.,* (1931), who concluded that no available test, including the alcohol test, was sufficiently "definite for advantageous use in grading milk to sterilization purpose", the alcohol test fell into disfavour and its usage declined in the USA and Europe. It is still employed, however, in south east Asia and throughout South and Central America as a means of rapidly defining the capability of milk to withstand heat treatment, the milks being tested with 72 % v/v, ethanol on arrival at the dairy plant. Many of the milks failing the test are of low titratable acidity, giving rise to a particular class of milks, designated LINA (Leite instavel ao alcool e nao acido). This has resulted in a number of recent papers, many from South American countries, dealing with this phenomenon and its causes, but with no definitive solution to this issue suggested. The problem is not one of ensuring that fresh, wholesome milk is rejected, but of making sure that accepted milk does not cause problems downstream when subjected to heat treatment for sterilization purposes. This review summarizes our knowledge and understanding of ethanol stability of milk, and through that, its relationship to heat stability.

9.2 Ethanol Stability/pH Profile

When the alcohol test first came into use, it was based on a single point assay at the pH of milk at the time of measurement. It was regarded as a measure of acidity, particularly of acidity produced by bacterial fermentation, but few studies investigated the role of pH in defining the ethanol stability. By modifying the alcohol test to an

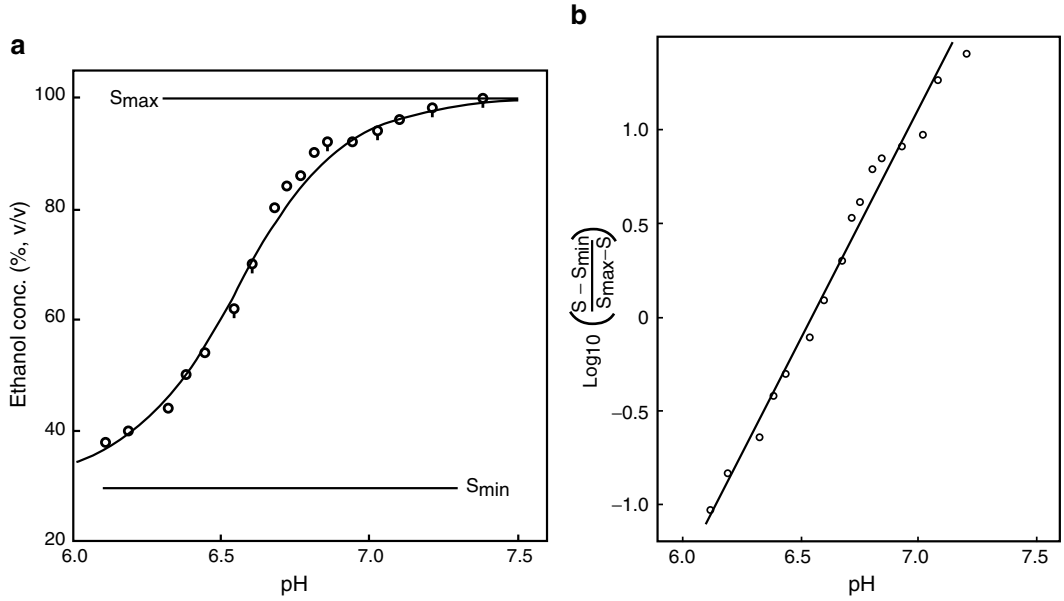

Fig. 9.1 (**a**) Effect of adjusting milk pH on the minimum concentration of ethanol solution required to coagulate the milk. *Vertical dashes* below some of points indicate coagulation occurred on standing at the next lowest ethanol concentration. The *solid curve* was calculated using Eq. 1 with parameters, $S_{min}=31$, $S_{max}=100$, $pK=6.552$ and slope = 0.416 (see Fig. 9.1b). From Horne and Parker (1980), copyright Elsevier. Reprinted with permission. (**b**) The fit of the experimental ethanol stability measurements from Fig. 9.1a to the straight line transform of Eq. 9.1b. A maximum correlation coefficient of 0.993 was obtained using asymptotic values $S_{min}=31$ and $S_{max}=100$. From Horne and Parker (1980), copyright Elsevier. Reprinted with permission

addition of two volumes of ethanol solution to one volume of milk, Horne and Parker (1980) were able to induce coagulation in milks whose pH had been adjusted to cover the range 6.0–7.5 by adding appropriate amounts of acid or alkali, always balancing the amounts with water to maintain a constant final concentration of milk. They found that on artificial adjustment of the pH, the ethanol stability was a sigmoidal function of pH, and, rather than follow a single universal curve for all milks, each individual milk had its own characteristic profile, defined by four parameters. These were the asymptotic values (S_{min} and S_{max}) of the two arms of the sigmoid at low and high pH respectively; an inflexion point or pK value, denoting the position of the profile along the pH-axis; and a gradient or slope parameter, through the inflexion point. Such a profile can be fitted empirically to the functional form given by the equation

$$\frac{S_{max}-S}{S-S_{min}} = 10^{b(pH-pK)} \quad (9.1a)$$

Or its logarithmic transform

$$pH = pK + b\log_{10}\left[\left(S-S_{min}\right)/\left(S_{max}-S\right)\right] \quad (9.1b)$$

Figure 9.1a shows the fit of the stability data to this function and Fig. 9.1b to the linear transform.

9.2.1 Role of Serum Phase Components

Individual milks from animals in mid-lactation could give rise to distinctly different profiles, mainly showing lateral shifts of their pK values along the pH-axis. Examples are shown in Fig. 9.2. Also shown are the profiles of these milks after the milk sera have been interchanged

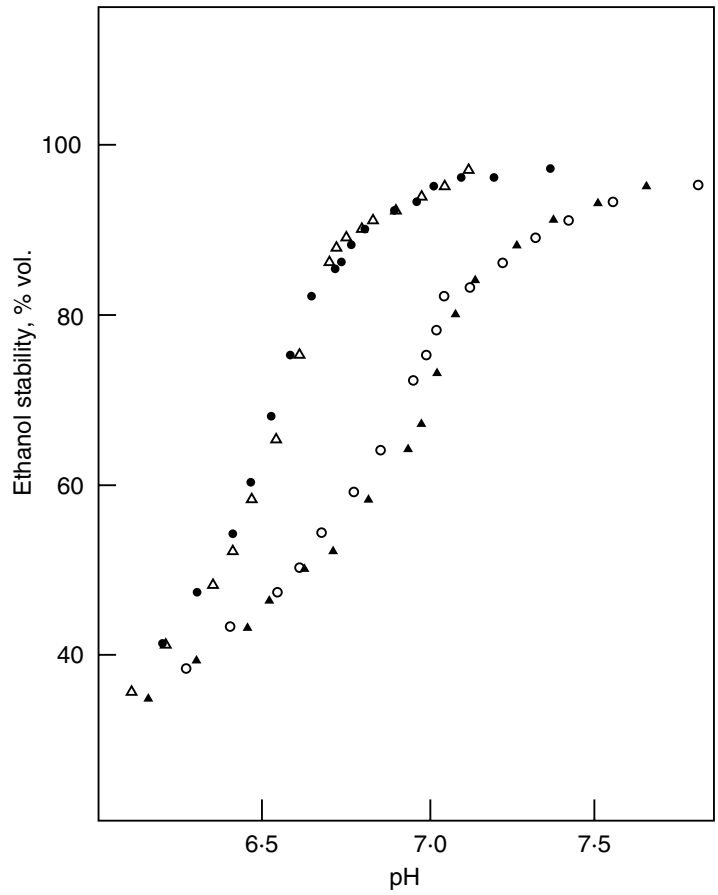

Fig. 9.2 Examples of ethanol stability/pH profiles and the effect on those profiles of interchanging milk sera by dialysis. *Filled circle*, Milk A; *open circle*, milk A, dialysed against milk B; *filled triangle*, milk B; *open triangle*, milk B, dialysed against milk A. From Horne and Parker (1981a). Copyright Cambridge University Press. Reprinted with permission

by dialysing a small volume of one milk against a large volume of the other. Milk A dialysed against an excess of milk B acquired the ethanol stability/pH profile of milk B. Conversely milk B dialysed against milk A acquired the profile of milk A. The shift is complete and the profiles are virtually superimposable. The shape and position of the ethanol stability/pH profile is determined purely by dialyzable components in the serum phase. Colloidal or protein components, particularly whey proteins, play no major role when comparing profiles of milks from the same species (Horne and Parker, 1981a). Further confirmation of the lack of involvement of whey proteins comes from an experiment where the ethanol/pH stability profile of casein micelles re-suspended in milk dialysate, then dialysed against the original milk was found to be identical to the profile of the original milk (Fig. 9.3). It is therefore not surprising that Botaro *et al.,* (2007) were unable to find any influence of β-lactoglobulin polymorphism on the ethanol stability of milk; if the protein itself has no role, genetic variants will have even less effect. The experiment of Horne and Parker (1981a) verifies, however, that the coagulating species are the casein micelles and confirms the dominating role of soluble, diffusible components in defining their stability.

In order to determine which components play an active role in ethanol-induced coagulation, Horne and Parker (1981a) observed the effects of controlled additions of selected components in turn. Lactose and urea additions had no effect. The addition of ionic calcium (as calcium chloride) to milk displaced the profile to higher values, but did not alter its sigmoidal character, nor

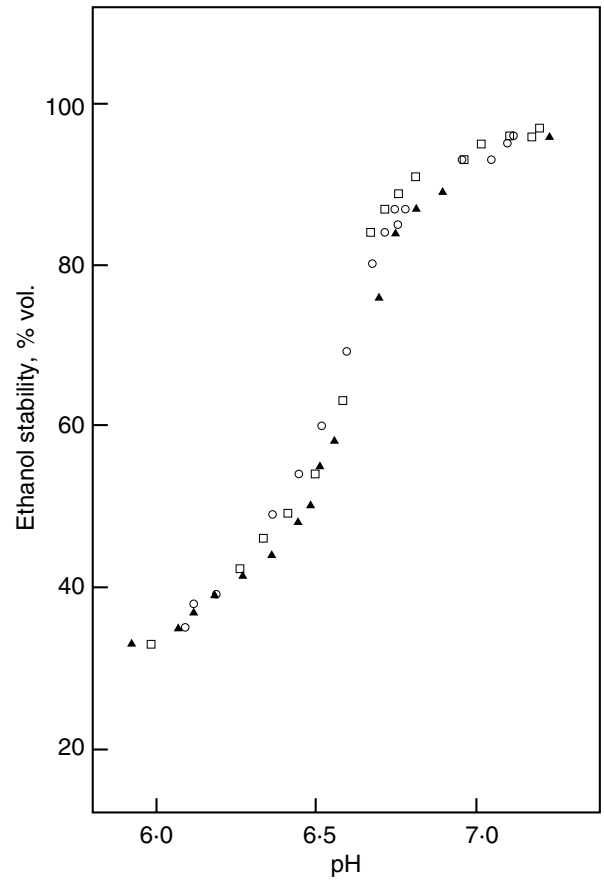

Fig. 9.3 Comparison of the ethanol stability profile of original skim milk, *open circle*; casein micelles from that milk, resuspended in dialysate, *open square*; casein micelles resuspended in whey, *filled triangle*. Both resuspended systems were dialysed overnight at 4 °C against the original milk before determination of their stability profiles. From Horne and Parker (1981a). Copyright Cambridge University Press. Reprinted with permission

did it significantly change the values of S_{min} or S_{max}. Removal of Ca^{2+} by addition of the sequestrant, ethylene diamine tetracetic acid (EDTA), had the reverse effect of shifting the entire profile to lower pH. Contrary to the effect with added cation, enrichment of milk with up to 5 mM phosphate anion had no effect on the ethanol/pH stability profile. However, additions of 5 mM citrate resulted in a slight shift to lower pH. Slight shifts can be important, however, in defining the measured stability around the natural pH of the milk, as demonstrated in Fig. 9.4, taken from Horne and Parker (1981b), where the effect of adding Ca^{2+} or EDTA on the ethanol stability of milk maintained at pH 6.5 is shown. Small additions of either species can produce large shifts in measured ethanol stability. The importance of ionic calcium level for ethanol stability was thus verified.

The role of phosphate in determining the sigmoidal shape of the profile was demonstrated in a series of experiments (Horne and Parker, 1981b), first comparing the stability profile of milk dialysed against a synthetic serum devoid of phosphate with the profile of the original milk, showing removal of the transition (Fig. 9.5). In a separate experiment, the sigmoidal transition was created by introducing phosphate into calcium caseinate solutions. Phosphate is known to become a more effective sequestrant of calcium as pH is increased and competes with the casein in this regard in the latter series of experiments, decreasing the amount of calcium bound to casein, increasing its negative charge and increas-

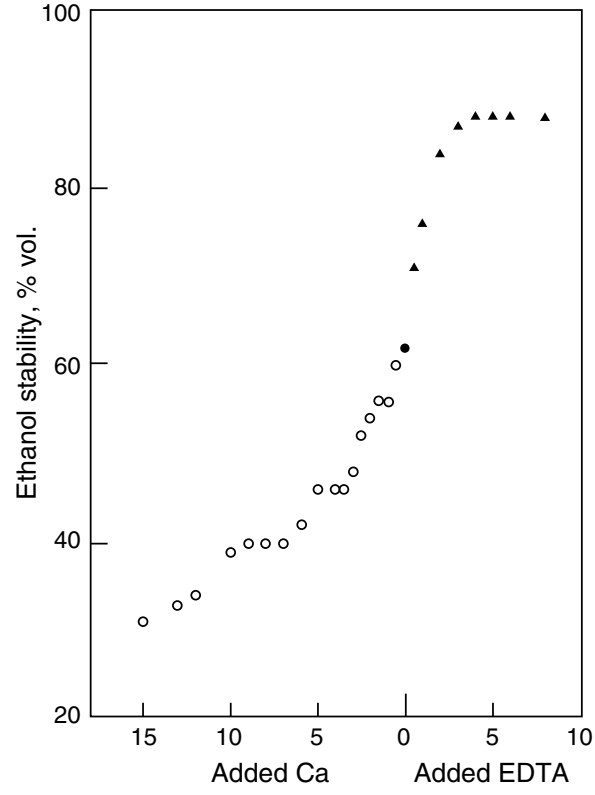

Fig. 9.4 Effect of adding Ca^{2+} (as $CaCl_2$) or EDTA on the ethanol stability of skim milk maintaining pH at 6.5. *Filled circle*, Control sample at pH 6.5; *open circle*, Ca^{2+} enriched milks, concentrations in mM added; *filled triangle*, samples to which EDTA was added. From Horne and Parker (1981b). Copyright Cambridge University Press. Reprinted with permission

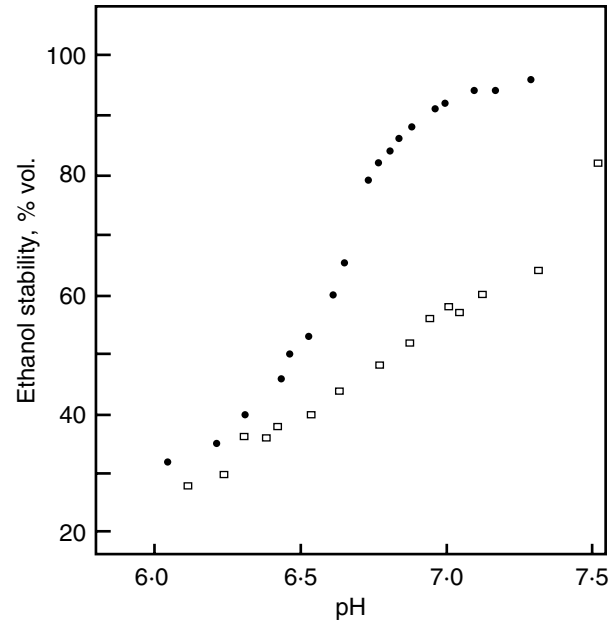

Fig. 9.5 Effect of dialysing skimmilk (16 h, 4 °C) against a synthetic serum devoid of phosphate. Original bulk milk, not dialysed *filled circle*, milk dialysed against Jenness and Koops (1962) type serum, with phosphate omitted *open triangle*. From Horne and Parker (1981b). Copyright Cambridge University Press. Reprinted with permission

ing its ethanol stability. This is completely the reverse of the role of calcium phosphate suggested by Pierre (1985); this author envisaged the ethanol causing calcium phosphate to precipitate onto the micelle and destabilise it.

9.2.2 Role of Protein Charge

Another series of experiments emphasized the role of protein charge in controlling the ethanol stability of the casein micelle system (Horne and Parker, 1982). The casein micelles are negatively charged in the entire pH range of interest. When positively charged lysine residues were acylated by reaction with acetic anhydride, ethanol stability was increased and the ethanol stability/pH profiles were shifted to lower pH. Conversely, the amidation of carboxyl groups and the consequent removal of their negative charge reduced ethanol stability and shifted the profile to higher pH.

9.3 Theoretical Considerations in Ethanol Stability

9.3.1 Steric Stabilization

Casein micelles are charged colloidal species and to some extent their stability is determined by the electrostatic repulsion between them, but it has long been recognised that this repulsion alone is insufficient and that the inter-micellar interaction potential has a steric stabilisation component also contributing repulsion. This steric stabilisation is provided by a diffuse layer of hydrophilic, polypeptide chains, mainly the C-terminal portion of κ-casein, the so-called 'hairy layer' model. Convincing verification of this model was provided by Walstra *et al.,* (1981), who used dynamic light scattering on dilute suspensions of casein micelles to measure directly changes in hydrodynamic diameter. On addition of rennet to a diluted solution of milk in calcium buffer, a reduction in diameter of about 5 nm was observed, consistent with the removal of a macropeptide hairy layer. At longer times they observed an increase in apparent size as the depilated, destabilized micelles aggregated. Horne (1984) used the same light scattering technique to measure the hydrodynamic radius of casein micelles in the presence of ethanol up to alcohol concentrations inducing aggregation. At these sub-critical concentrations of ethanol, the radius of the micelles, diluted from a resuspended micellar fraction of narrow size distribution, exhibited a maximum decrease of approximately 10 nm from the size measured in the absence of ethanol. The hydrodynamic radius (Fig. 9.6) passed through this minimum and increased dramatically as the concentration of ethanol was increased through the critical level, the time-dependent increase in radius here indicating aggregation of micelles. Further experiments and observations were consistent with the hairy layer being collapsed onto the micellar surface on addition of the poor solvent, ethanol. Subsequent aggregation beyond the critical ethanol concentration clearly demonstrated the role of a steric stabilising component in micellar interactions and stability.

All theories of steric stabilisation highlight the essential role of the stabilising polymer and the thickness of the stabilising layer. Solvent quality, which dictates the conformation and thickness, is a function also of the ionic constituents of the buffer system. This is of particular importance when the polymeric stabiliser is a polyelectrolyte or protein, as in the case of the casein micelle. Extensive studies, carried out by Horne (1986) and Horne and Davidson (1986), confirmed the role of pH, ionic strength and Ca^{2+} in influencing barrier thickness when included in the ethanolic buffer system. Such results are primarily of academic interest when discussing the stability and interactions of the casein micelle, and are only of secondary interest in the discussion of the ethanol stability of milk. They are included for completeness and because the steric component has to be removed or counteracted to allow micellar aggregation to proceed, whether in a dilute suspension or in a milk. In this context, Huppertz and DeKruif (2007), in milks previously treated with transglutaminase, found that cross-linking the caseins within the micelle increased the ethanol stability across the usual pH range but prevented the ethanol induced collapse of the hairy

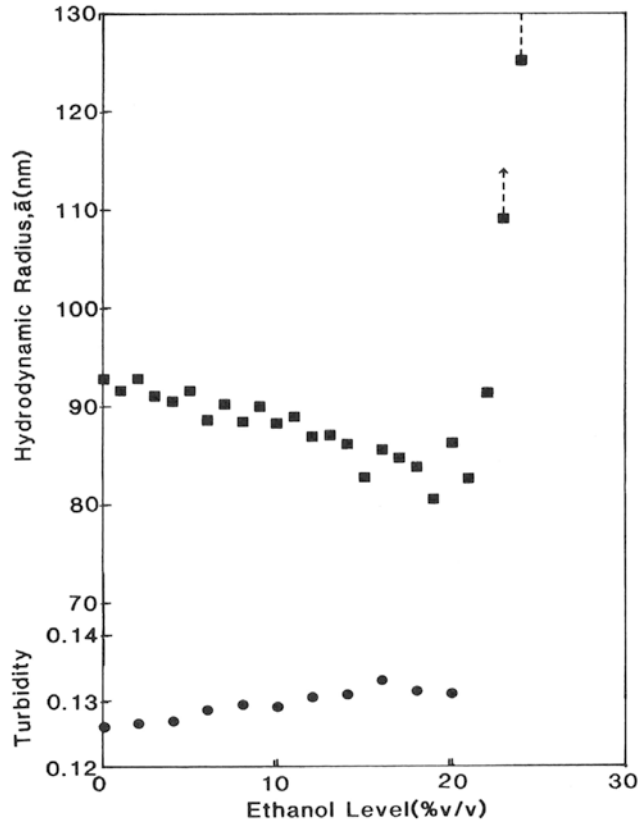

Fig. 9.6 Apparent hydrodynamic radius (upper data set, *filled square*) and total scattering (turbidity) (lower data set, *filled circle*) of casein micelles as a function of ethanol content of diluting buffer system. Buffer was 20 mM imidazole, 50 mM NaCl, 5 mM $CaCl_2$, adjusted to pH 7.0. Radius values are the mean of four consecutive measurements. *Dashed lines* at higher ethanol levels indicate successively increasing values and aggregation. From Horne (1984), copyright John Wiley and Sons Inc. Reprinted with permission

layer. Very small decreases in radius of gyration were observed for the enzyme-treated milks. Huppertz and DeKruif (2007) suggest that the increase in stability is due to the inability of the cross-linked hairs to collapse on addition of ethanol. But this is contrary to the theories of steric stabilisation where the polymer contribution is conceived as an entropic term relying on mobility and multiple conformations in the hairs, contributions which would be lost on creating a network of cross-links. It is also contrary to the observations of Horne and Davidson (1986) who found that thin layers provided less stability than thick, as in accord with theory. In this case of transglutaminase-treated milks, it is more likely that the increase in ethanol stability is due to an increase in the negative charge carried by the micelles, since the enzyme cross-links proteins between uncharged glutamine residues and positively charged lysine residues, removing the contribution of the latter to the net sum of charges. This must also be done in sufficient amounts to compensate for the loss of the steric stabilising component. This influence of chemical modification is akin to that reported previously by Horne and Parker (1982).

9.3.2 Role of Alcohol and Dielectric Constant

The final factor we have to consider in our examination of the alcohol stability test is the alcohol and its function. In a study involving the use of a series of alcohols (methanol, ethanol and 2-propanol) Horne and Parker (1981c) showed that the different stability/pH profiles for each alcohol reduced to a single curve when plotted as

a function of solvent dielectric constant versus pH. The interactions of organic solvents, including alcohols, with protein systems as complex as the casein micelle and milk, are many and various. Mezdour *et al.,* (2006) investigated the solubility of caseinate as a function of pH in ethanolic solutions and put our earlier empirical equations on a sounder physicochemical foundation. They found that the pK values of the various ionization equilibria in the casein shifted linearly with reciprocal dielectric constant but in different directions for carboxyl and amino groups—to higher pH for carboxyl and to lower pH for amino, but the latter shift never reaching as low as the pH range of interest (pH 5.0–8.0). These movements would imply that the isoelectric point of the casein would shift to higher pH as ethanol concentration was increased. In milk, the solubility and calcium binding equilibria must also be considered and one would anticipate them moving in a similar direction to the carboxyl group.

Mezdour *et al.,* (2006) carried out all their studies at ambient temperature and, indeed, all studies of alcohol stability of milk appear to have been done at prevailing ambient temperature. O'Connell *et al.,* (2001a, b) observed that when heated in the range 40–60 °C in the presence of ethanol, far from inducing coagulation, the micelles dissociated and translucent solutions were obtained, the ease of dissociation increasing with alcohol concentration. A similar pattern of behaviour was reported by Horne and Davidson (1987) who found that low levels of the hydrophobic alcohol, trifluoroethanol, induced precipitation but high concentrations brought about dispersion of the micellar casein. Within the casein micelle it is believed that hydrophobic bonding is one form of interaction holding the casein micelle together, once the charge repulsion between the proteins is neutralised or overcome. It is possible that at higher temperatures or with a high level of a hydrophobic alcohol, the alcohol competes for the binding sites between proteins or otherwise induces conformational change in the caseins, disrupting such bonds and thereby bringing about dispersion.

9.3.3 Mechanism for Ethanol-Induced Coagulation

We are now in a position to make a plausible attempt to describe a mechanism for the alcohol-induced destabilization of skim milk. Much of this appeared in Horne (1987) but parts have been confirmed by more recent studies (Mezdour *et al.*, 2006). Essentially the alcohol-induced reaction is an isoelectric precipitation process, as confirmed by Mezdour *et al.,* (2006), the point of zero charge being raised in pH as alcohol concentration is increased. For both the caseinate solution and the micellar system, the addition of calcium, which binds to the protein, further reduces the protein charge, in effect further raising the isoelectric point. In the case of the micellar system, the calcium-binding and neutralization contributes to the thinning of the steric stabilizing layer and the removal of the steric component in alcohol solution (Horne, 1986; Horne and Davidson, 1986). Through the changes made in dielectric constant, the mineral equilibria are also shifted. In particular, the solubility of calcium phosphate is decreased. Pierre (1985) suggested that this precipitation onto the micelles induced instability in the micellar system. Far from being a destabilizing influence, it appears that the precipitation of calcium phosphate is in fact a stabilizing factor and is the origin of the sigmoidal profile. Driving the calcium phosphate equilibrium to precipitate decreases the free Ca^{2+} level, drawing caseinate-bound calcium, and increasing protein charge, with a consequent weakening of internal micellar structure, but, by doing so gradually, all contributing to increased system stability. Countering these effects are the shifts in isoelectric point and the collapse of the steric stabilizing layer. A catch-up situation therefore exists, but at any pH, there is a particular alcohol concentration when the system reaches balance and precipitation ensues. The sigmoidal profile is developed because higher pH promotes calcium phosphate precipitation driving the system towards stability and requiring a higher alcohol content to produce the shift in isoelectric point to overcome that.

But pulling calcium and calcium phosphate from the casein micelle can drive micellar disintegration. We do not visually see that with experiments conducted at room temperature, but at higher temperatures, as used by O'Connell *et al.,* (2001a, b) or with trifluoroethanol, the enhanced precipitation of calcium phosphate, aided perhaps by competition for hydrophobic binding sites results in dispersion of the micelle and no precipitation. Through the involvement of calcium phosphate in this mechanism, we have explained the controlling influence of components in the soluble phase on the ethanol stability test in a purely physico-chemical fashion.

9.4 Lactational and Seasonal Effects

How the seasonal and lactational variation of milk composition, particularly milk minerals, correlates with the ethanol stability of milks, has been the subject of several investigations. Measuring ethanol stability at natural pH, Mitamura (1937), Davies and White (1958) and Horne *et al.,* (1986) all found milk to be very unstable to ethanol in early lactation. This was confirmed most recently by Tsioulpas *et al.,* (2007), who also measured levels of free ionic calcium in milk, sampling for the first 5 days post partum, then at 15, 30, 60 and 90 days into lactation. With samples from eight cows, milks were high in free Ca^{2+}, low in pH and low in ethanol stability on day 1 post partum, but decreased in Ca^{2+}, increasing in pH and ethanol stability thereafter. Figure 9.7, drawn from their data, shows that free Ca^{2+} concentration correlated highly with their measure of ethanol stability ($r^2 = 0.9721$).

Unlike the other groups, Horne *et al.,* (1986) measured entire ethanol/pH stability profiles over the lactations of eight individual cows, examining trends in profile parameters. They observed a general movement of the profiles to more alkaline values as lactation progressed (Fig. 9.8), together with a decrease in the gradient at the profile pK value. If measured at a fixed pH, this behaviour would produce a decline in ethanol stability through lactation. Unfortunately Horne *et al.,* (1986) collected no compositional data to assist with interpreting these data, but this failing was rectified to some extent in the paper of Donnelly and Horne (1986). They observed highly significant correlations ($P < 0.001$) between profile pK and soluble phosphate ($r = -0.841$), with soluble phosphate + citrate ($r = -0.801$) and with soluble salt balance ratio (SBR) ($r = 0.804$) (Fig. 9.9). This molar ratio is defined as the ratio of the soluble calcium plus magnesium concentrations to the sum of concentrations of soluble anionic species, phosphate plus citrate. SBR values showed a

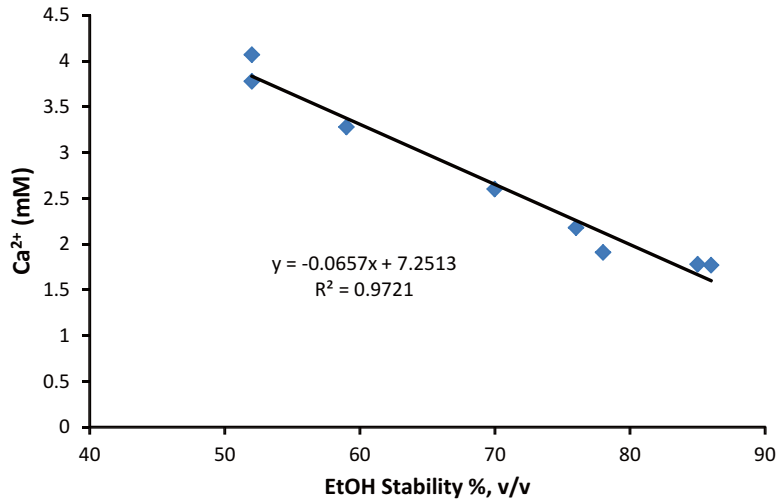

Fig. 9.7 Correlation of free ionic calcium levels with ethanol stability of milk measured at natural pH. Milk samples were taken in early lactation as detailed in the text. Data, taken from Tsioulpas *et al.,* (2007), is averaged over measurements from eight cows

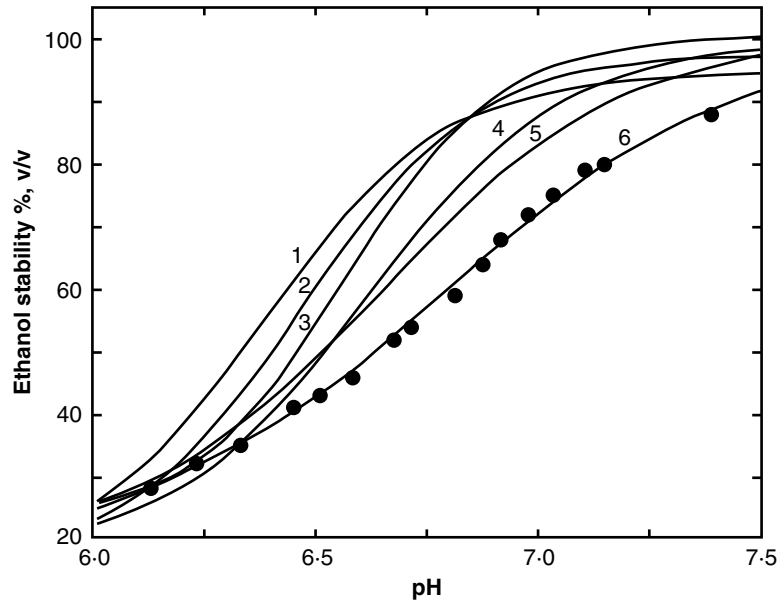

Fig. 9.8 Ethanol stability/pH profiles of milks drawn at various times during lactation from an individual cow (spring calver). *Curves* 1–6 were calculated from measurements made on milks obtained on day 6, 45, 116, 172, 242 and 316 of lactation, respectively. For clarity data points are shown for profile 6 only. From Horne *et al.,* (1986). Copyright Cambridge University Press. Reprinted with permission

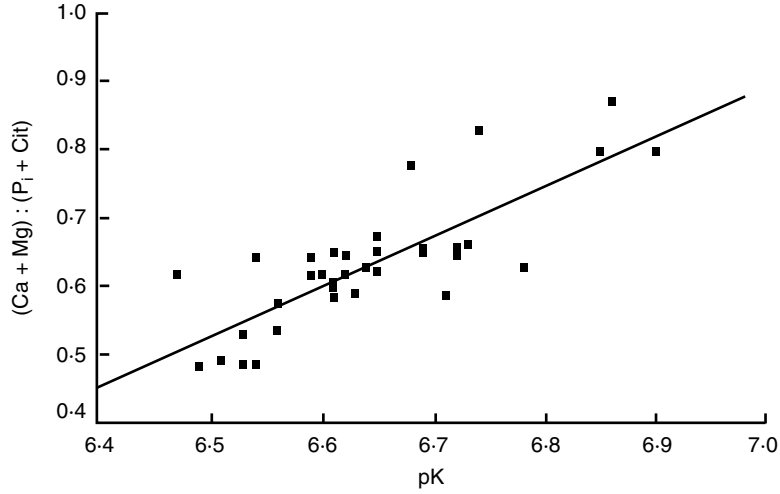

Fig. 9.9 Correlation of ethanol stability profile pK with the molar soluble salt balance ratio, (Ca+Mg):(P_i+Cit). Linear regression line included, r=0.804. From Donnelly and Horne (1986). Copyright Cambridge University Press. Reprinted with permission

gradual increase with advancing lactation, coincident with the rise in profile pK, this rise in pK agreeing with the trend observed by Horne *et al.,* (1986). Increase in SBR was predominantly the result of the decline in soluble phosphate, as also observed previously by White and Davies (1958). This rise in profile pK would again be consistent with a decline in ethanol stability at a fixed pH through to the end of lactation, as observed also by Horne *et al.,* (1986).

As far as the more recent studies originating from South America on the occurrence of LINA milks (Barchiesi-Ferrari *et al.,* (2007); Botaro *et al.,* (2007); Chavez *et al.,* (2004);

Marques *et al.,* (2007) and Zanela *et al.,* (2006)), the majority of these appear not to consider the role of soluble salts, so it is not possible to carry this analysis further. Nor do they appear concerned with lactational effects, unless seasonality and calving patterns are tightly linked. Rather there seems to be a tendency to suggest that instability is linked with forage availability but the evidence does not appear overwhelming.

9.5 Relationship Between Heat Stability and Ethanol Stability

Currently, the ethanol stability test is employed in many instances as a predictor of the suitability of milks for UHT sterilization. In a short review of heat stability of milk, it is impossible to do justice to all the research that has been published on the subject over the last century or so. For more detailed information, the reader is referred to the excellent reviews of Fox and Morrissey (1977), Singh and Creamer (1992), O'Connell and Fox (2003) and Chap. 7 of this volume. In comparing ethanol and heat stability of milk, it is immediately apparent from the test protocols that each test could be viewing different aspects of micellar stability.

Ethanol stability tests are subjective and instantaneous. Milk is mixed with an alcohol solution. If immediate precipitation occurs, the sample has failed the test. If no precipitate, then the sample has passed. Over the years, however, a number of heat stability tests have been developed. Miller and Sommer (1940) defined heat stability as the *temperature* required to coagulate milk protein instantaneously (effectively within two minutes). In practice, the time to induce coagulation is measured at a range of temperatures, and heat coagulation temperature is intrapolated to give a value at 2 min. This makes the test time-consuming, and it became customary only to measure the heat coagulation *time* at a definite temperature, usually 140 °C for unconcentrated milk, or 120 °C for concentrated milk. The milk sample (1–2 mL) is sealed in a glass tube, clipped/clamped onto a platform which is rocked in the oil bath until particles of coagulated protein can be seen in flowing milk. This is referred to as the 'subjective' heat stability test, since the observation of coagulation depends on the operator. An 'objective' test for heat stability was developed by White and Davies (1966). This involves measuring the percentage of the total protein sedimentable at low centrifugal force, after various heating intervals. The resulting protein depletion curve shows a sudden break at the onset of coagulation and provides an objective assessment of heat stability. But again, the method is very time-consuming and is unsuitable for routine use.

9.6 Heat Stability/pH Profiles

Since most published research relates to the heat coagulation time behaviour, either using 'subjective' or 'objective' methods, these results will be considered first. Just as Horne and Parker (1980) drew up an ethanol stability/pH profile, so previously had Rose (1961a, b) reported the effect of adjusting pH on the heat stability of milk. Unlike ethanol stability/pH profiles, heat coagulation time/pH curves fall into two classes. As shown in Fig. 9.10, Type A profiles or milks show a pronounced maximum and minimum as pH is increased, whereas the heat stability of Type B milks increases gradually throughout the pH range. Interestingly, the heat coagulation time/pH profile of a Type A milk, determined by the objective protein depletion methodology, also has a maximum and minimum. Outwith the region of the minimum, coagulation proceeds in a single stage depletion curve. Within the region of the minimum, however, a two-stage depletion curve is observed. The first drop in the depletion curve is the observed visual coagulation time, while the second occurs at a time, visually undetectable, which would be expected if the minimum did not exist (Fig. 9.11) (Sweetsur and White, 1974; O'Connell and Fox, 2000). Parker *et al.,* (1979) showed that such curves could be

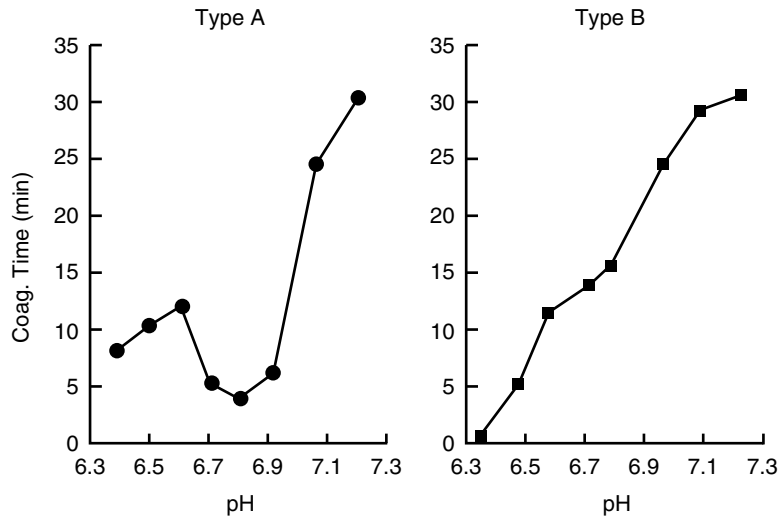

Fig. 9.10 Typical Type A and Type B heat coagulation time/pH profiles. From Horne and Muir (1990). Copyright Elsevier, Reprinted with permission

modelled by treating the aggregating micelles as tri-functional units and applying the theory of branching processes, originally derived to describe polymer condensation and gelation. To give the two-stage depletion behaviour, two distinct types of active particles were assumed to exist, each of which could exclusively react only with its own type, with few or no cross-reaction products.

Further evidence of the occurrence of two reactions (at least) is the observation that the coagulum first formed in the minimum is soluble in 6 M urea, whereas that formed outside or in a Type B milk is insoluble in this dissociating medium. This highlights a major difference between alcohol-induced and heat-induced coagulation. Alcohol coagulation is a physically induced phenomenon. The inclusion of the ethanol modifies the dielectric constant of the system and it is as a consequence of this change on the physical interactions and equilibria that protein coagulation is induced. Heat-induced coagulation, on the other hand, is the result of a number of chemical reactions brought about by heating of the sample, at a suitable temperature until coagulation is observed. Before we discuss these reactions, it is pertinent to insert a short digression into micellar interactions and internal structure. For a full description, see Horne (1998) or Horne (2006).

9.7 Micelle Interactions and Internal Structure

Colloids (and casein micelles are colloidal particles) are stable because a sufficiently large repulsive energy barrier exists between particles and prevents close approach, aggregation and ultimately coagulation. All other things remaining equal and unchanged, inputting enough thermal energy would be sufficient to overcome the barrier and initiate aggregation. The simplest approximation to the energy barrier in the case of casein micelles views it as a summation of steric and electrostatic components. The consensus is that the steric component is composed of contributions from the outermost layer of κ-casein on the surface of the micelle. The electrostatic repulsive component comes from the net negative charges on the micelle, arising from dissociated acid and basic protein groups. The number of such charges is thus pH dependent and also depends on the level of binding of anionic ions (calcium and magnesium) from the serum phase, as well as the overall ionic strength of the system.

There has been a tendency to develop, on the basis of this theory, a picture of the casein micelle as an immutable hard sphere particle with an adsorbed layer of κ-casein forming the steric sta-

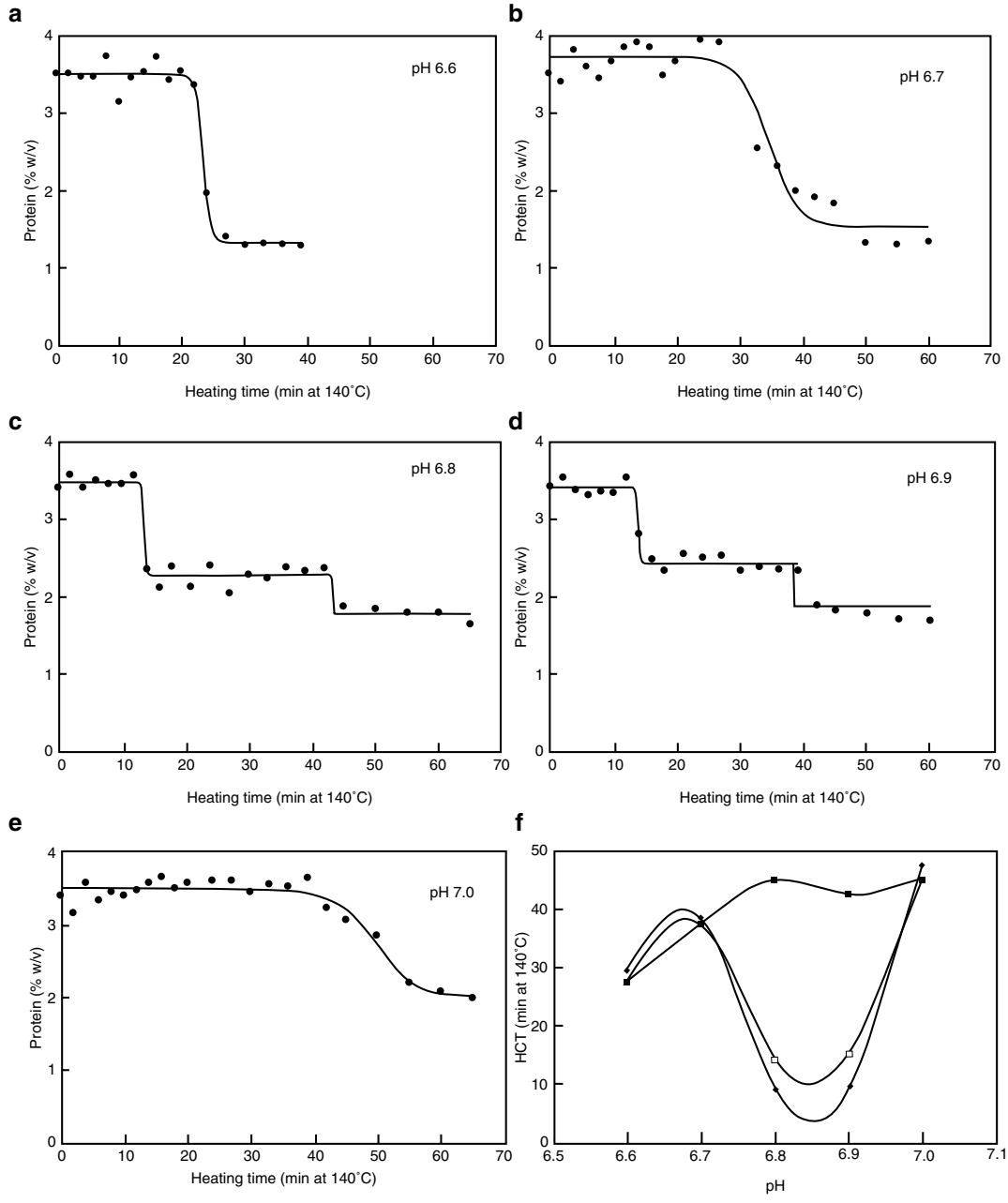

Fig. 9.11 (**a–e**), Protein depletion curves for skim milk at pH values 6.6–7.0 on heating at 140 °C and (**f**) the heat coagulation time-pH profile of skim milk determined by the subjective heat assay (*filled diamond*), the objective heat stability assay taking the first inflexion points within the minimum (*open square*), the objective heat stability assay taking the second inflexion points within the minimum (*filled square*). From O'Connell and Fox (2000). Copyright Elsevier. Reprinted with permission

bilizing hairy layer. However, in reality, each micelle is an open, gel-like aggregate, consisting of many thousand protein molecules. Several models have been developed to describe the assembly and structure of the micelle, but amongst these, the dual-binding model (Horne, 1998; Horne, 2006) appears most successful in reproducing micellar behaviour. The dual-binding model emphasises the role of the properties of the individual caseins in its approach to micellar assembly, with those members of the casein family possessing clusters of phosphoserine residues utilizing these in cross-linking across calcium phosphate nanoclusters. Multiple groupings of phosphoserines on α_{S1}- and α_{S2}-caseins allow such binding pathways to form a three-dimensional network but the neutralization of protein negative charge, which this linking brings about, also facilitates and enhances the second binding pathway through hydrophobic interactions between hydrophobic regions on all of the caseins. This forms a second intermingled network with the nanocluster crosslinks, both contributing to micellar integrity at natural pH. Hydrophobic interactions are important and essential because they are the only way by which κ-casein is linked onto the micelle – κ-casein does not possess a phosphoserine cluster. κ-Casein is also amphiphilic, one end of the molecule is hydrophobic but the other end is hydrophilic, the macropeptide end. κ-Casein is therefore the chain terminator for the growth of the network and naturally ends up on the external surface of the micelle.

This dual-binding model reproduces much of the technological behaviour of the casein micelle. It does not conflict in any way with the mechanism of ethanol-induced coagulation described earlier in this chapter (see also Horne, 2003) and has proved useful in rationalizing acid gelation and renneting at both natural pH and under cheesemaking conditions. When milk is heated, a host of competitive but sometimes inter-dependent reactions occur. It could prove instructive to consider these reactions and their consequences in the light of this model of the internal structure of the micelle. Before considering those reactions, it is pertinent to make several points. Because of the open structure of the micelle, small molecules to be found in milk are as equally at home within the micelle as outside it. Differences in concentration of these components within and outside of the micelle are likely to be slight. The same cannot be said regarding the casein. Casein concentration overall may be around 2.5 % or 25 g per litre, in bovine milk. But this protein is packaged into micelles where the effective protein concentration could be a factor of 10, or more, higher. This could speed up reactions significantly. Conversely if the reaction involves two different protein molecules or different parts of the same molecule, it should be recalled that many of the proteins are tethered into a network, albeit a dynamic one, restricting their freedom of movement.

9.8 Heat-Induced Reactions in Milk

Fox (1981a) reviewed the heat-induced changes in milk and classified them into five groups: (i) acid development, (ii) precipitation of calcium phosphate, (iii) Maillard reactions, (iv) casein modification and (v) interaction of sulphydryl groups, the latter including whey protein denaturation. More detailed information on these reactions can be found also in O'Connell and Fox (2003) and Chap. 7 of this volume. The reagents for all of these are, of course, already present in milk and some of these reagents may take part in more than one of the groupings proposed by Fox (1981a).

Acid development can be brought about by the thermal degradation of lactose to organic acids but this is thought to account for only up to 50 % of heat-induced acidification. The second reaction responsible for acidification is the precipitation of primary and secondary calcium phosphate as tertiary calcium phosphate with concomitant release of hydrogen ions (H^+). Calcium phosphate is released from the micelle on acidification anyway, through the titration of phosphoserines, freeing up caseins bound through nanocluster links and making internal bonding within the micelle

more labile and dynamic. The role of acid development cannot be underestimated, as it has been shown that heat stability at 140 °C can be prolonged for at least 3 h if pH is adjusted occasionally back to its original value (Fox, 1981b). Such reactions therefore contribute to instability and eventual precipitation.

The second major reaction of lactose brought about by heating is the wide-ranging group of reactions included under the umbrella of Maillard reaction. In its first stage this is a reaction of lactose and protein, predominantly with lysine residues but to a lesser extent with tryptophan, histidine and arginine residues. But these represent only the first stages in a cascade of parallel and competing reactions, some of which can lead to cross-linking of protein molecules. From the point of view of the micelle model, most of the residues involved are positively charged and their loss makes the protein more negatively charged and therefore more inherently stable. This can be counterbalanced, however, if some of the cross-linking reactions occur with negatively charged carboxyl groups, again illustrating the complexity of the problem. Maillard reactions can, of course, also occur between whey proteins and lactose.

Other heat-induced modifications of the caseins are dephosphorylation and proteolysis. Dephosphorylation removes the sites for nanocluster cross-linking, weakening internal micellar structure but a reaction product of this is dehydroalanine, which may then cross-link with nucleophilic amino acids (lysine, cysteine, arginine, histidine) (Manson and Carolan, 1980). Heat-induced proteolysis has been detected in the formation of soluble peptides and non-protein nitrogen but the latter may also arise from the deamidation of glutamine and asparagine residues. If this is the case, then the negative charge on the protein is increased and the micelle is possibly stabilised or even moved towards disintegration, detectable as an increase in level of serum casein. Cleavage of peptide bonds in κ-casein is perhaps the most important proteolysis result which has been detected, as this could influence the steric stabilization of the micelle. Indeed Fox and Hearn (1978a) found that 20 % hydrolysis of κ-casein by chymosin prior to heating caused instantaneous coagulation at 140 °C.

Yet more casein modifications can be brought about by reaction with the thermal degradation product of urea, cyanate, which can react with lysine or cysteine residues. Loss of positively charged lysine residues from the caseins, makes the micelle more negative and, if occurring in moderation, would tend to enhance heat stability, but, if excessive modification occurs, then some degradation of the micelle or release of caseins to serum might be the outcome. However, it may be in reaction with cysteine that urea degradation products play their most important role in their contribution to heat stability. Free, or readily available, cysteines in the caseins are found only in κ-casein and there they provide a reaction partner on heating with such residues in the whey proteins. An extensive literature has built up on these complex-forming reactions since they are of considerable importance not only in heat stability but also in the manufacture of fermented milk products. The majority of these are beyond the scope of this article and the reader is referred to the reviews of O'Connell and Fox (2003), Donato and Guyomarc'h (2009) and Chap. 7 of this volume. We return to relevant portions of this behaviour during the discussion of the mechanisms giving rise to the Type A and Type B heat coagulation/ pH curves that now follows.

9.9 Interchanging Heat Stability Profiles and Mechanism of Heat-Induced Coagulation

It is obvious from the above that determining a heat coagulation time allows a vast range of reaction possibilities between and involving milk components, some competing, some destabilising, some stabilising. The occurrence of the Type A profile with its minimum in coagulation time and the ease with which shifts from Type A to Type B behaviour, and *vice versa*, can be achieved further attest to the complexity of heat coagulation behaviour. Table 9.1 lists some of the meth-

Table 9.1 Methods of interchanging form of heat stability/pH curves

Conversion	Reference
Type A is converted to Type B when	
Assay temperature is decreased from 150 to 120 °C	Sweetsur and White (1974)
κ-casein is added	Tessier and Rose (1964)
Colloidal calcium phosphate is removed	Rose (1962)
Phosphate is replaced by another anion	Morrissey (1969)
Type A milk is dialysed against excess Type B milk	Fox and Morrissey (1977), Morrissey (1969)
Type B is converted to Type A when	
Assay temperature is raised from 130 to 150 °C	Sweetsur and White (1974)
The milk is forewarmed at 80 °C for 30 min	Sweetsur and White (1974)
β-lactoglobulin is added	Tessier and Rose (1964)
Divalent cations (Ca^{2+}, Mg^{2+} or both) are added	Morrissey (1969)
Type B milk is dialysed against excess Type A	Fox and Morrissey (1977)
	Morrissey (1969)

ods of interchanging the types of heat stability profile. Some of these are controversial in that not every group has found them to be operative in every milk on every occasion. In this category is the effect of raising assay temperature. Sweetsur and White (1974) found that most milks were Type B at 130 °C but showed Type A behaviour with increasing temperature, whereas Fox and Hearn (1978b) were unable to confirm that assay temperature had any major influence on the shape of the heat stability/pH curve.

Conversion from Type B to Type A on raising temperature is easily explainable as a consequence of the occurrence of two reactions with different activation energies. The first reaction in the minimum has to have the higher activation energy, and it is, in fact, observed to have an activation energy 30–40 kJ/mol higher than that for the coagulation of micelles at the pH of maximum stability (Muir and Sweetsur, 1977). However, reaction rates are also concentration dependent, and failure to observe the temperature effect on every occasion is probably associated with compositional differences affecting the availability of reactants for the two pathways. Generally, researchers agree that the presence of the minimum in a Type A curve is associated with the formation of a complex between β-lactoglobulin and κ-casein. Many Type B milks are low in β-lactoglobulin and can be converted to Type A by the addition of this whey protein (Sweetsur and White, 1974). The κ-casein partner, however, must be micellar. Addition of free κ-casein to the milk allows the complex formation to take place in the serum phase, and a Type B curve results (Tessier and Rose, 1964). Similarly, forcing the dissociation of κ-casein from the micelle into the serum phase by the addition of NaCl produces a Type B profile (Tessier and Rose, 1964). Although β-lactoglobulin is the principal whey protein, other whey proteins such as α-lactalbumin and bovine serum albumin can destabilize milk subjected to heating. The effect of α-lactalbumin is similar to that of β-lactoglobulin, whereas addition of bovine serum albumin results in a more general destabilization (Fox and Hearn, 1978b).

Many of the observations in Table 9.1 can be rationalized by postulating an equilibrium between serum and micellar κ-casein mediated by calcium ion activity. This is not necessarily the calcium binding to the κ-casein, but rather to the micelle and reducing its negative charge, thereby favouring the bonding of the (also negatively) charged κ-casein, all within the spirit of the dual-binding model. Equally, the calcium could influence the binding of the κ-casein/β-lactoglobulin complex to the micelle, especially since the location of the complex and its ease of dissociation form the micelle is pH dependent, or, perhaps the calcium ion can exert some influence on the actual formation reaction of the protein complex.

The evidence seems incontrovertible, however, that the formation of the whey protein/κ-casein complex is the switch which triggers entry into the minimum of the Type A curve. It has also been demonstrated that this complex dissociates from the micelle at pH>6.7, leaving the denuded micelle inherently unstable. Why the complex dissociates remains unclear, but it has been suggested that the conformational change in the

β-lactoglobulin around pH 6.8 may be involved in influencing the reactivity of the thiol group of β-lactoglobulin (O'Connell and Fox, 2003).

More controversy surrounds the explanations for the influence of urea on milk heat stability and the form of the heat coagulation profile. O'Connell and Fox (2003) express difficulties in understanding why urea, which promotes extensive dissociation of caseins from the micelle (Dalgleish *et al.*, 1987; Metwalli *et al.*, 1996), stabilizes milk. But they are forgetting that if cyanate, the thermal degradation product, reacts with lysine residues, this increases net negative charge of the parent protein. If that protein is κ-casein, it reduces the interaction energy binding that protein, promoting its dissociation. Whereas, if the protein is micellar, then net micelle charge is increased and this promotes stability through increased electrostatic repulsion.

Urea increases heat stability when single stage coagulation occurs, as in Type B milk across the pH range, or in a Type A milk outside the minimum, but is less effective when two-stage coagulation is evident. In other words, urea in this pH range is losing out to another reagent for its reaction partners. This led Horne and Muir (1990) to propose that urea and β-lactoglobulin compete for reaction with the –S–S– bonds of κ-casein. Outside the minimum, urea/κ-casein interactions are favored, while within the pH range of the minimum, β-lactoglobulin is more reactive, possibly due to the exposure of a buried thiol group at pH values >6.8. Horne and Muir (1990) postulated that the formation of β-lactoglobulin/κ-casein complexes on the surface reduced the effectiveness of steric stabilization and decreased micellar stability but equally the dissociation of the complex would be as effective in stripping out the steric stabilization component, leaving the micelle open to calcium-mediated, charge-controlled coagulation.

O'Connell and Fox (2003) have criticized this hypothesis on the grounds that it fails to explain the following phenomena:

- Urea stabilizes serum protein free micelle dispersions (Metwalli and van Boekel, 1996)

  Rebuttal: Urea would act in this situation just as it does in milk with its degradation product reacting with lysine and increasing protein negative charge.
- Whey proteins stabilize serum protein free casein micelle dispersions at pH 6.7 (Rose, 1963).

  Rebuttal: This is more difficult to explain in terms of the original Horne and Muir (1990) hypothesis of the complex reinforcing the κ-casein hairs but is less so if the complex dissociating hypothesis is favored, and in no way conflicts with the idea of competition between urea and whey proteins for κ-casein S–S bonds.
- Degradation of indigenous urea in a Type A milk does not alter the pH-dependence of the heat stability (Muir and Sweetsur, 1977).

  Rebuttal: Urea is envisaged as a competitor with β-lactoglobulin. The fact that a Type A profile is observed shows that the urea is already losing out to the whey protein. Thus removal of urea will not alter this state, though it might produce changes in values.
- Cyanate interacts with only a fraction of the –SH groups in milk (Manson *et al.*, 1985).

  Rebuttal: But reaction of cyanate with cysteines is only one possible pathway for this reagent. Cyanate also reacts with lysine groups and this promotes stability.
- Inhibition of sulphydryl interactions does not eliminate the stabilizing effect of urea (Muir and Sweetsur, 1977).

  Rebuttal: But yet again reaction of cyanate with lysine residues, unaffected by such inhibitions, would be the main stabilizing effect of urea.

Overall, we might envisage the heat-induced coagulation of skim-milk to be one of isoelectric precipitation, mediated by binding of calcium-ions. If we pour in enough energy by heating at a high temperature, we may bring about instantaneous coagulation if the temperature is high enough. Prolonged heating drives down milk pH and lowers micellar charge by lactose degradation and calcium phosphate precipitation. If we start from a higher pH, we have to heat longer to reach the precipitation point. Extra calcium promotes coagulation by neutralizing charge; reagents such as urea or lactose remove positively charged groups, effectively increasing micellar negative

charge and promoting micellar stability, until we reach a pH where extensive formation/dissociation of the β-lactoglobulin/κ-casein complex occurs and the micelle is dramatically destabilized. Increase in pH beyond the minimum, then brings about increased micellar charge and increased stability.

This provides a mechanism for heat-induced coagulation at a selected assay temperature, but, in a measurement of heat coagulation time, it is heating prolonged until coagulation occurs and one could question its relevance to the instantaneous coagulation occurring on ethanol addition in the alcohol stability test, or even to the manufacture of a UHT milk, where the milk has to withstand heating at 140 °C for only a few seconds. The latter conditions are more closely related to the situation existing in the heat stability test where coagulation temperature is the parameter of interest.

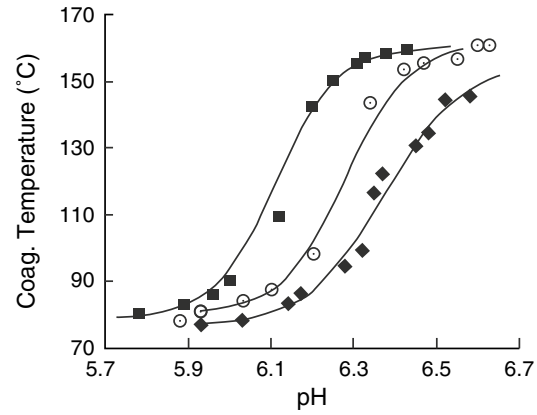

Fig. 9.12 Reworked heat stability data of Miller and Sommer (1940) demonstrating the fit of the sigmoidal profile equation, previously used in the treatment of ethanol stability data, to their coagulation temperature versus pH data. Curves and data points are drawn for control milk (*open circle*), milk with added calcium (*filled diamond*) and milk with added phosphate (*filled square*). Reproduced from Horne and Muir (1990) Copyright Elsevier. Reprinted with permission

9.10 Heat Coagulation Temperature and its Response to pH, Calcium and Phosphate

Miller and Sommer (1940) developed the heat coagulation temperature assay and used it to examine the effect of acidifying milk on its heat stability. The coagulation temperature versus pH profile shows a remarkable similarity to the ethanol stability/pH profile with heat stability increasing sigmoidally with pH (Fig. 9.12). Indeed the data sets can be fitted with the same analytical form employed to fit ethanol/pH profiles (Horne and Muir, 1990). We only have the reworked data of Miller and Sommer (1940) to examine and it would be unwise to take too much from such meager information, but the pK value for their milk seems lower than a typical value for an ethanol stability profile.

What we do know from their published data is the effect of added calcium or phosphate on the position of the heat stability/pH profile. Again, exemplary data have been extracted from their paper and plotted in Fig. 9.12. The response to the addition or removal of calcium is similar to that shown by the ethanol stability profile, namely, a decrease in stability and a shift of the profile to higher pH when calcium level is increased. Conversely, an increase in stability and a shift in profile to lower pH occur when the effective calcium content of the milk is reduced by the addition of a calcium sequestrant. In this instance, the data of Miller and Sommer (1940) show phosphate to be very effective in shifting the profile. Addition of citrate (5 mM) was equally effective. Nevertheless, in some of their other examples, phosphate addition had no detectable effect on the calculated profile pK value. They give at least four examples with calcium addition in their paper, all of them show the shift to higher pH demonstrated in the example shown in Fig. 9.12.

9.11 Conclusions and Suggestions

Marked similarities are thus detected between ethanol stability of milk and the coagulation temperature assay for heat stability. They demonstrate a similar response to pH adjustment but we note that the heat stability data do not extend

above pH 6.6, that is, they fail to include the pH range where minima are observed in heat stability in other test protocols. In the coagulation temperature tests, milks respond at least qualitatively in the same manner as ethanol stability to increases or decreases in available calcium. These similarities suggest that in both tests, the reaction pathway might be identical. If the destabilization reactions are simply overcoming a reaction energy barrier, in both instances the magnitude of the electrostatic component is controlled to some extent by calcium binding and hence similarly influenced by available calcium levels and pH. Beyond that, however, there are a number of milk components, lactose, urea and whey proteins, which have been shown to have no influence on ethanol stability (Horne and Parker, 1981a, b) but whose heat-induced reactions have considerable influence on heat stability, as discussed above. Indeed, the destabilizing complex formation between κ-casein and whey proteins, is enhanced at higher temperatures and dominates heat stability in the pH range of the minimum in the type A heat stability-pH profile. This makes it unlikely that an ethanol stability measurement made at room temperature would directly correlate with heat stability at 140 °C, as evidenced by the measurement of a heat coagulation time. On the other hand, it might be argued that ethanol stability gives a measure of intermicellar energy barrier height and heating to a coagulation temperature is overcoming and modifying this barrier in a prescribed manner from the same starting point, albeit that the rate of decline is dependent on the concentrations of the afore-mentioned reagents. If we ignore this last point and if the heat coagulation temperature test is adjudged an indicator of suitability for UHT processing (e.g., heat coagulation temperature >140 °C say), then the ethanol stability test could be an equally good indicator, the proviso being that in a single point test, we have not determined the ethanol concentration that would provide the required measure of stability. The chosen concentration of 72 % v/v ethanol may be setting the bar too high or too low. This concern needs to be addressed or at least the presently selected concentration justified. A fairer alternative might be to devise a rapid test of heat stability which could be carried out in the field by the tanker operative or at reception of the milk at the processing plant.

Bearing in mind the suggested mechanism for ethanol-induced coagulation and the role of the soluble salts in the serum phase, the high correlation with the salt balance ratio, is there any way in which instability can be 'cured'? For example, blending 'unstable' with 'stable' milks? What proportions would be required? If instability was due to early or late lactation, would mixing with other milks from herds with different calving patterns overcome the problem? Even when bulking milks from the same herds, would it be beneficial to have different groups of cows calving at different times of the year? If the instability is due to too high a salt balance ratio (SBR), could this SBR be modified directly by addition of sodium citrate, say? Or if SBR is high because citrate is low, could the citrate level be manipulated by modifying the animal's diet, as has been shown by Holt *et al.,* (1979)? More data is required on SBR in milks showing LINA failures to eliminate this as a possibility for these.

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Protein Stability in Sterilised Milk and Milk Products

10

Hilton Deeth and Mike Lewis

Abstract

Sterilised milk and milk products are produced by intense heat treatments and are stored at ambient temperature for several months. These conditions present a considerable challenge to the stability of their protein components. Several changes occur during the heat treatment, usually more during in-container sterilisation than UHT treatment, and some changes continue during storage. Most are time- and temperature-dependent and some are pH-dependent. The major chemical changes are denaturation of whey proteins, covalent cross-linking of proteins, redistribution of proteins between the colloidal and serum phases, lactosylation and subsequent Maillard reactions, degradation of individual amino acids *via* deamidation and dephosphorylation as well as generation of volatile sulfur compounds, and proteolysis by indigenous and bacterial proteinases. Physical changes also occur, including deposit formation during heating, and further sediment formation and gelation during storage. The mineral fraction plays an important role in some of these reactions. The changes to proteins have implications for the nutritional status of the product. There is a reduction in bioavailability of essential amino acids, particularly lysine, and formation of potentially toxic compounds resulting from Maillard reactions and protein cross-linking. However, the proteins in some sterilised products may be more digestible due to physical changes causing increased accessibility to digestive proteinases.

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Keywords

Heat stability • Gelation • Maillard reactions • Protein cross-linking • Storage stability

10.1 Introduction

10.1.1 Definition of Sterilisation

Heat treatment is widely practised in the dairy industry, whether the product is destined for consumption as liquid milk or for conversion into an increasingly large range of dairy products. The focus of this chapter is on sterilisation of milk and milk products and specifically the effects of heat treatment on the protein fraction and how this relates to both milk quality and safety.

Milk and milk products can either be sterilised in bottles or other sealed containers, or by a continuous process known as ultra high temperature (UHT) processing followed by aseptic packaging. Comprehensive accounts of the procedures for producing in-container sterilised milk and problems associated with it were provided by Cronshaw (1947) and Davis (1955). In-container sterilised milk is still produced throughout the world and the range of products that are sterilised in this way has increased, examples being custards, milk desserts with rice, tapioca and semolina, and flavoured milk. UHT procedures were developed more recently and are not featured in work of Cronshaw (1947) and Davis (1955). UHT processing involves the use of higher temperatures and shorter times, which reduce the extent of chemical changes as well as providing opportunities for larger throughputs and greater energy efficiency. Sterilised products are ambient-stable and do not require refrigeration throughout distribution and storage. However, ambient temperature in some parts of the world is often over 30 °C and occasionally above 50 °C. Thus, under these conditions, changes in the protein fraction will be accelerated and will influence how long the product will remain acceptable.

The aim of sterilisation is to produce products which are commercially sterile, i.e., containing no microorganisms likely to grow in the product under normal conditions of storage, with target spoilage rates of less than one in 10,000 containers. Milk is a low-acid product (pH>4.5), so a major safety issue is to inactivate *Clostridium botulinum*, which is the most heat-resistant pathogenic spore. This is facilitated by heating the product at 121 °C for 3 min (total process lethality, $F_0=3$), resulting in 12 decimal reductions (12D) in numbers of *C. botulinum*. (IFST, 1991). Higher temperatures and much shorter times are used in UHT processes. However *Cl. botulinum* is rarely found in raw milk, but other *Clostridium* species and more heat-resistant bacterial spores of organisms such as *Geobacillus stearothermophilus* and *Bacillus sporothermodurans* (Hammer *et al.*, 1996) may be found. Thus a minimum "botulinum cook" produces a product which is safe, but not necessarily sterile. For milk and milk products it is recommended to achieve at least two decimal reductions of *G. stearothermophilus*, which corresponds to an F_0 value of 8. In general, the use of higher temperatures and shorter times employed in UHT processes to achieve this results in less chemical change taking place than in in-container sterilisation (Burton, 1988). In most cases this is desirable, but in the case of heat-resistant proteases and lipases, this may not be the case.

There is now a bewildering choice of milk products available, both sterilised and pasteurised. Milk is standardized for both protein and fat: semi-skim milk at 1.5–1.8 % fat is now popular and skim milk at 0.1 % fat provides an even lower-fat version. For those who prefer a richer, fuller flavour, milk from Jersey and Guernsey cows and other rare breeds is an option. Flavoured milk products have become more numerous and cream products ranging from 12 to 55 % fat, with a wide range of viscosities are available. In addition to cow's milk are milks from goats, sheep

and buffalo, as well as specialty milks for pets, such as cats and dogs. Evaporated milk is also available in many countries. Milk products are now being consumed in parts of the world where there has previously been no culture of doing so. In many of these locations, the amount of fresh milk is not sufficient to meet the demand, so many milk products are made from reconstituted milk powders.

10.1.2 Overview of Sterilisation Methods

10.1.2.1 In-Container Sterilisation

Foods have been sterilised in sealed containers, such as cans, for over 200 years. Milk was originally sterilised in glass bottles sealed with a crown cork but more recently plastic bottles have been used. In general the basic principles have remained the same since the 1930s, with the advent of the crown cork, which helped with the mechanization of the bottle filling process and the reuse of bottles. Practical drawbacks of in-container sterilisation processes are that the product heats and cools relatively slowly and the maximum temperature that can be achieved is limited by the internal pressure generated.

Milk is clarified using a centrifuge with claimed bacterial spore removal of greater than 99 %. It is homogenised at 63–82 °C, for example at a single stage pressure of about 20 MPa or double stage at about 17 and 3.5 MPa. It is then filled into glass bottles between 74 and 80 °C under conditions which give minimal frothing, and sealed. Plastic bottles are sealed at a lower temperature of 54–55 °C. Care should be taken to avoid conditions in balance tanks which may be conducive to growth of thermophilic bacteria. Ashton and Romney (1981) cite sterilisation processing conditions of 110–116 °C for 20–30 min, depending upon the extent of cooked flavour and colour preferred by the consumer. Batch or continuous retorting processes may be used (Davis, 1955). Other processing details are outlined by Ashton and Romney (1981); these include more detail on continuous retorts such as hydrostatic or rotary valve sealed sterilisers which are capable of higher temperatures and shorter times (132–140 °C for 12 min) and the use of steam for glass bottles or steam/air mixtures for plastic bottles. A more recent innovation is a combined process which involves first heating milk at UHT conditions (e.g., 137 °C for 4 s) and filling into bottles which are then sealed and passed through a conventional retorting process, but for a reduced time. Milk products such as custards, canned desserts and rice puddings are also produced by in-container sterilisation, with some agitation to promote heat transfer and ensure good mixing. Sterilised milk has a rich creamy appearance, perhaps helped by coloured Maillard reaction products, a distinct cooked flavour (rich, nutty, caramelised), which, once acquired, makes other heat-treated products taste bland. The turbidity test developed by Aschaffenburg in (1950) is a well established test for ensuring adequate sterilisation. Regulations in some countries require that sterilised milk should produce a negative turbidity result, ensuring that it has been heated sufficiently to denature fully the whey proteins.

10.1.2.2 UHT Processing

UHT processing of milk combined with aseptic packaging was introduced to produce a shelf-stable product with minimal chemical damage compared with in-container sterilised milk. UHT milk may have a shelf life of up to 12 months although in practice it is usually consumed much earlier. UHT treatment is normally in the range 135–145 °C in combination with appropriate holding times (1–10 s) necessary to achieve commercial sterility (Burton, 1988; Lewis and Heppell, 2000). In practice, the products are checked for sterility by incubating at 55 °C for 7 days and at 30 °C for 15 days and testing for bacterial growth (Codex Alimentarius, 2003). Some useful bacteriological and chemical indices have been developed to describe the effects of a particular heating regime on the bacterial and chemical components of milk, respectively. The major indices of heat treatment are B* (bacterial) and C* (chemical) (Kessler, 1981). A B* value of 1 corresponds to a 9D reduction of thermophilic spores ($z = 10.5$ °C) and is achieved at 135 °C for

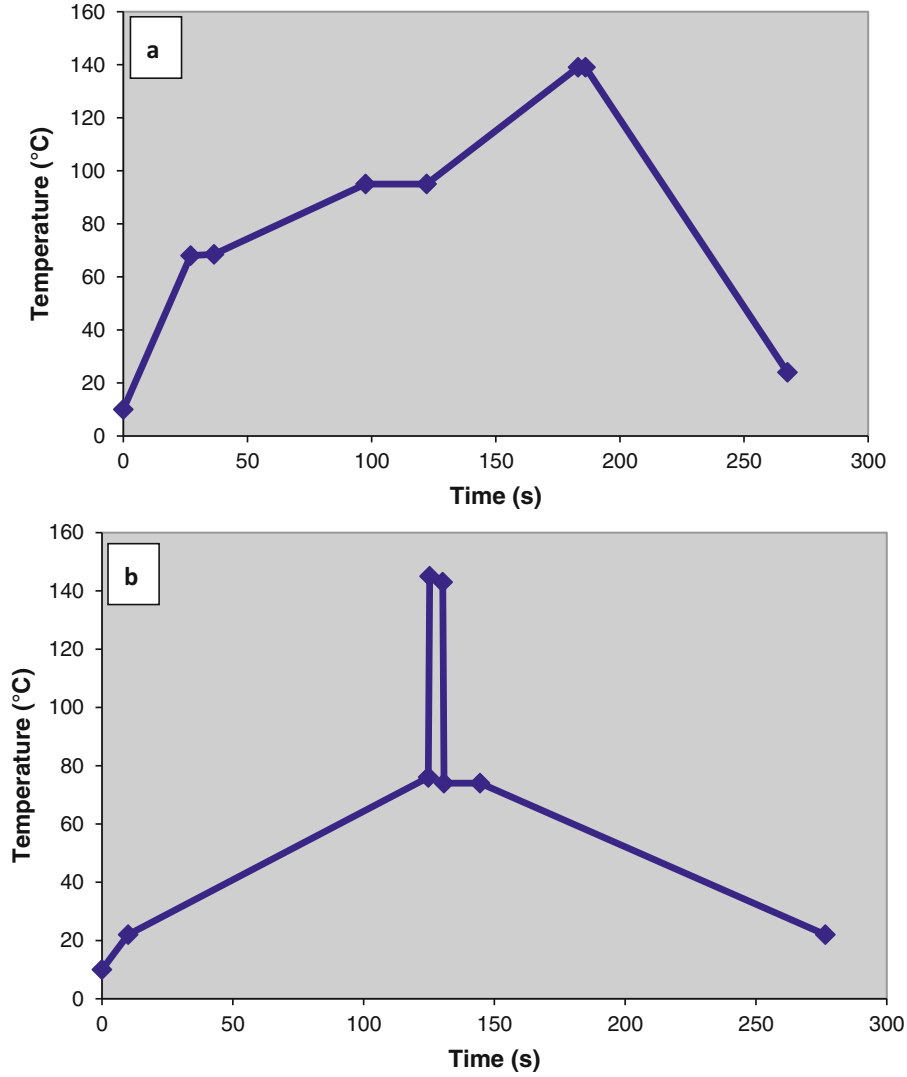

Fig. 10.1 Temperature–time profiles of (**a**) an indirect UHT plant and (**b**) a direct UHT plant. The B* for both plants is 2.9

10.1 s while a C* of 1 corresponds to 3 % loss of thiamine (z = 31.4 °C) and is achieved at 135 °C for 30.5 s (Kessler, 1981). B* and C* values for milk processed on different commercial UHT plants in Australia have been reported by Tran *et al.,* (2008).

UHT heating can be either 'direct' or 'indirect'. Indirect processes are more common with both plate and tubular heat exchangers being widely used. For direct heating, saturated steam (free of entrained water droplets) is mixed with milk (Lewis and Heppell, 2000). The main differences between the two processes which are pertinent to this chapter are the temperature–time profiles (Fig. 10.1). The different heating and cooling profiles of direct and indirect systems have been compared by Tran *et al.,* (2008). Direct heating results in very rapid heating. Equally rapid cooling is achieved in a vacuum flash cooling process. This removes excess water and volatiles in the milk, which include dissolved oxygen, heat-induced sulfur volatiles and added volatiles

in flavoured milk drinks. Indirect heating involves slower heating and cooling. Other key differences have been listed by Lewis and Deeth (2009).

10.2 Stability During Heating

10.2.1 Heat Stability

10.2.1.1 Overview

Heat stability can be defined as the ability of milk to withstand high processing temperatures without visible coagulation or gelation (Singh, 2004). Arguably it is the most important property of milk destined to be sterilised. Inherently, cow's milk has a high heat stability, which is due to the loose, ill-defined three dimensional structure of its principal proteins, the caseins. This is despite the fact that the whey protein fraction is heat labile and starts to denature at about 70 °C. In fact, one relevant area relates to the interactions that take place between denatured whey protein and casein during the heating process (see Sect. 10.2.2.1).

The most-used method for estimating heat stability is measurement of the heat coagulation time (HCT). HCT is defined as the time that elapses between placing a sample of milk in an oil bath at a definite temperature, usually 140 °C for unconcentrated milk or 120 °C for concentrated milk, and the onset of visible coagulation (O'Connell and Fox, 2003). Rose (1961) performed experiments where the pH of milk samples was adjusted to a range of pH values (typically between 6.4 and 7.0), prior to determination of their HCT. In this way, the relevance of starting pH to heat stability could be observed. It was found that for the majority of milk samples (Type A), the heat stability passes through a maximum and a minimum as the pH is increased from 6.4 to 7.0; for a minority of samples (Type B), there is a steady increase in heat stability as pH increases. Type A milks predominate in most countries (with the exception of Japan), accounting for 70 % of Scottish, 80 % of Canadian and ~100 % of Irish and Australian milks of individual cows (Horne and Muir, 1990). At a pH well below the maximum, milk coagulates rapidly because the low pH and high calcium ion activity decreases electrostatic repulsions between the proteins. In addition, relatively high amounts of whey proteins associated with the micelles at low pH may promote aggregation of casein particles through cross-linking of whey proteins bound to different micelles. The occurrence of a maximum (at ~pH 6.7) in the HCT–pH profile of normal milk is essentially due to greater stability of whey protein-coated micelles, as the formation of a complex between κ-casein and β-lactoglobulin on the surface of the casein micelles alters the steric and electrostatic interactions and prevents the dissociation of micellar κ-casein. At pH values above 6.7, the stability decreases due to the dissociation of micellar κ-casein, thus reducing the stabilizing effect of this protein. The κ-casein-depleted micelles are sensitive to calcium ions. Therefore, the minimum in the HCT–pH profile is a result of coagulation of κ-casein-depleted micelles; coagulation is essentially due to reduced pH during heating caused by calcium ions. At higher pH, although dissociation of micellar κ-casein increases, the HCT increases due to the increase in protein charge and low calcium ion activity. It is also possible that the dissociated κ-casein may reassociate during extended heating because of a heat-induced decrease in pH (Singh, 2004).

Many studies have investigated how the HCT–pH coagulation profile is influenced by factors such as milk composition (such as proteins, lactose, salts, urea), preheating conditions, concentration and other parameters; these have been reviewed by Singh (2004). Good earlier reviews are those of Rose (1962), Fox and Morrissey (1977) and Horne and Muir (1990). Sievanen *et al.,* (2008) have compared such profiles for normal milk and concentrated milk with added calcium chloride. There is an element of subjectivity in determining the point at which coagulation first takes place. However, it is claimed by regular users that it is possible to obtain reproducible results with practice. A variant on the HCT procedure is to determine the heat coagulation temperature, which is the temperature required to coagulate milk in a given time period (Rose, 1962; Horne and Muir, 1990).

Other methods for determining heat stability include the ethanol test, a whitening test, a protein sedimentation test and viscosity determination (Singh, 2004).

However, despite all the research work, problems related to poor heat stability of milk are still encountered in commercial processes. Some possible reasons for this are discussed below. Singh (2004) stated that the heat coagulation time (heat stability) often correlates very poorly with the stability of milk on commercial sterilization. He also pointed out that from an industry point of view, the use of a pilot scale or laboratory scale sterilizer which simulates sterilization conditions used in practice provides more reliable results and prediction of behaviour of milk in commercial plants, compared with laboratory-based subjective HCT tests.

Milk can show very good heat stability and can take over 20 min to coagulate at 140 °C in HCT studies. However, during UHT processing, milk only reaches a temperature of ~140 °C for a few seconds. Also, there are differences in the heating and cooling rates for different UHT plants, as discussed by Tran *et al.,* (2008), and most of these will be different to the rates of heating experienced in the HCT test. During in-container sterilisation, temperatures rarely exceed 120 °C and relatively few HCT studies have been done on unconcentrated milk at this temperature. Another difference is that in-container sterilisation is essentially a static process (although containers may be agitated), which will encourage coagulation and the formation of a gel. In contrast, during UHT processing, milk is subjected to more shear, which might disrupt gelation and result in sediment formation. It has also been reported that the rate of fouling is lower in a plate heat exchanger compared to a tubular heat exchanger because of the higher shear rates in the former (Bansal and Chen, 2006).

Most investigators interpret the mechanisms of heat stability from measurements of the properties of milk at ambient temperature. Thus, important determinants such as pH, ionic calcium and conditions on the surface of the micelle at sterilisation temperatures are little considered. It was recognised some time ago that pH decreases as temperature increases. For example, Pyne and McHenry (1955) found that at the point of coagulation, the pH of milk (heated at 130 °C) decreased by approximately 0.7 units. Fox (1981) reported that pH decrease is the most important single factor which leads to the coagulation of milk during heating. Indeed, if the pH of milk is readjusted periodically to its original value, it may be heated at 140 °C for at least 3 h without coagulating (Fox, 1981). It was also concluded that different factors control the heat coagulation process at different initial pH values. Therefore heat stability will be better understood when important variables can be measured at high temperatures and a better understanding is gained about how pH and ionic calcium change with temperature and how this affects conditions on the surface of the micelle, and the dissociation of soluble caseins from the micelle (see Sect. 10.2.2.3). It can be argued that factors that minimize the pH changes caused when the temperature of milk increases will have a beneficial effect (a temperature buffering effect) on heat stability. Salaun *et al.,* (2005) wrote an excellent review on the buffering capacity of dairy products and states that addition of citrate and phosphate salts to milk induces quantitative changes in the buffering capacity.

Another compounding influence is biological variability, so those factors influencing heat stability will vary in different milk samples. In an ideal world, it would be desirable to be able to determine whether milk is likely to be stable to any particular thermal process from a knowledge of its composition. Some studies which report or discuss differences in heat stability of milk samples from individual cows and bulk milk samples are those of White and Davies (1958b), Burton (1988), Chavez *et al.,* (2004), Grandison (1988) and Grimley *et al.,* (2009). White and Davies (1958b) reported that HCT for bulk milks ranged from 17.2 to 59 min at 130 °C, whereas that from individual cows ranged from 0.6 to 86.2 min. They also pointed out that, in general, HCT was not related to the concentration of ionic calcium, except when the calcium phosphate content of the caseinate complex was very low, in which

case it was inversely related. It has been suggested that the pH maximum often corresponds to the natural pH of the milk (Horne and Muir, 1990), although this has not been specifically investigated.

In UHT treatment, milk with poor heat stability can give rise to fouling of heat exchangers and sediment formation and it is best to avoid UHT treating such milk. Some suggestions for avoiding milk which may be unsuitable for sterilisation are given in Sects. 10.2.3.1 and 10.2.3.2. Of the vast literature covering heat stability, only a small fraction deals specifically with UHT processes, making it less simple to suggest practical solutions to improve performance.

10.2.1.2 In-Container Sterilisation and Comparisons with UHT Processing

Heat stability problems related to in-container sterilisation of unconcentrated milk are rarely reported, but for milk concentrates this is a major issue. Tsioulpas *et al.,* (2010) sterilised milk containing trisodium citrate (TSC), disodium hydrogen phosphate (DSHP), sodium hexametaphosphate (SHMP), dihydrogen sodium phosphate (DHSP) and $CaCl_2$ at 121 °C for 15 min. These had variable effects on ionic calcium, Ca^{2+}; for DSHP and DHSP, Ca^{2+} decreased during sterilisation, but for SHMP it remained little changed or increased. Milk containing 3.2 mM SHMP and more than 4.5 mM $CaCl_2$ coagulated on sterilisation. It was interesting that DHSP reduced pH slightly but had no adverse effect on sediment and resulted in less browning during storage.

Grimley *et al.,* (2009) examined changes in heat stability of milk from five farms over the Spring flush period, when cows move from indoor to pasture feeding. Sediment formation ranged from 0.41 to 0.77 % when milk was sterilised at 120 °C for 15 min and from 21 to 26 min when measured by the HCT method at 140 °C. No correlation between the methods was reported. Although there were clear variations in milk composition over this period, there was no evidence that heat stability changed as a result of these. On-Nom *et al.,* (2012) heated reconstituted skim milk powder (9 % total solids) containing up to 25 mM $CaCl_2$ from 60 to 120 °C for different times. At 60 °C, milk could accommodate 16.2 mM $CaCl_2$ without coagulating whereas at 120 °C, milk containing only 3.6 mM of calcium chloride coagulated. One interesting observation was that at all temperatures, poor heat stability was manifested first by sediment formation, which was then followed by coagulation at higher calcium chloride additions. Milk samples were also dialysed during their respective heat treatments to recover the soluble phase and to measure pH and ionic calcium. No coagulation was observed if Ca^{2+} concentrations were <0.5 mM and pH was >6.3, measured at their respective coagulation temperatures.

Milk was found to become unstable to UHT processing at lower calcium additions compared to in-container sterilisation (Omoarukhe *et al.*, 2010). The range of addition, as calcium gluconate and calcium lactate, used was 0–10 mM. Nian *et al.,* (2012) evaluated the heat stability of milk from individual cows and found that milk with the highest Ca^{2+} produced the least sediment on in-container sterilisation ($R^2=0.41$). Ezeh and Lewis (2011) compared the heat stability of milk which had been reduced to different pH values and then restored. Sediment ranged from 0.1 up to 0.19 % for UHT treatment and from 0.1 to 0.23 % for in-container sterilisation. In summary, milk could be adjusted to a pH as low as 5.45 and then restored without adversely affecting its heat stability. During this pH reduction and restoration, considerable amounts of calcium and phosphorus move out of and back into the micelle without adversely affecting heat stability. In contrast, rennet coagulation time was considerably reduced by such readjustments.

Two further studies highlight differences in heat stability when the same milk samples were subjected to UHT and in-container sterilisation. Chen *et al.,* (2012) showed that goat's milk produced significantly more sediment following UHT processing compared to in-container sterilisation. When ionic calcium was increased slightly by adding 2 mM $CaCl_2$, sediment increased

drastically after UHT treatment, but by much less following in-container sterilisation (see also Sect. 10.2.2.4).

Addition of up to 12.8 mM DSHP or TSC resulted in a progressive increase in sediment for in-container sterilization. In contrast for UHT milk, adding 6.4 mM of these salts reduced sediment formation but sediment increased on addition of 12.8 mM of these salts. Thus it is also possible to add too much DSHP and TSC, as sediment formation went through a minimum with increasing additions of these compounds. Results for casein micelle size showed that an increased amount of sediment was accompanied by an increased casein micelle size.

Similar observations were found for cow's milk (Chen, 2013). In a comparison study, 25 batches of cow's milk showed good heat stability when subjected to UHT treatment and in-container sterilisation. The average sediment level was 0.19 % for UHT treatment (range 0.1–0.29 %) and 0.24 % for in-container sterilisation (range 0.02–0.56 %). Adding 10 mM DSHP or TSC increased sediment much more for in-container sterilisation than for UHT treatment. Thus, trends were similar to those found for goat's milk, although sediment levels were lower in the control cow's milk. Adding only 2 mM calcium chloride increased sediment significantly for UHT treatment but not for in-container sterilisation. Such differences have only recently been reported. However, although variations were found in amounts of sediment, the overall heat stability of these milk samples was consistently high and none of them produced sediment levels that would be detectable to consumers. It is not clear why there are these differences and why relatively small reductions in ionic calcium make milk more susceptible to sediment formation during in-container sterilisation compared to UHT treatment. Some of these findings for in-container sterilisation are in agreement with HCT–pH relationships for Type A milk samples in that narrow pH range between their minimum and maximum values.

There is a strong argument for avoiding using milk for commercial processes which shows poor heat stability, but for the reasons discussed, avoiding the use of such is not straightforward in practice. Since measuring HCT is not simple, one suggestion is to monitor the ethanol stability of the milk. Horne and co-workers have investi gated the factors affecting ethanol stability, starting in the 1980s and summarised by Horne (2003). Several workers have observed that ethanol stability is influenced by ionic calcium, as reviewed by Lewis (2010). Shew (1981) recommended that milk should be stable in 74 % ethanol to be suitable for UHT processing. As far as the authors are aware, there are no similar recommendations for in-container sterilisation. A key question is whether ethanol stability is a reliable indicator of stability to UHT processing conditions. The authors' experience with pilot plant and laboratory experiments on cow's milk and goat's milk suggests that reducing ionic calcium is beneficial in terms of reducing fouling of heat exchangers and sediment formation (Boumpa *et al.*, 2008; Prakash *et al.*, 2007). Also, reducing ionic calcium was found to increase ethanol stability and good correlations have been found between ethanol stability and Ca^{2+}. Therefore, in situations where sediment formation or fouling is a problem, the following suggestions were offered by Lewis and Deeth (2009). The pH, ethanol stability and ionic calcium should be routinely monitored in raw milk to establish their effects on sediment and fouling-related problems. Over time, this should provide data to assess, understand and eventually reduce problems arising from poor heat stability. Chavez *et al.,* (2004) grouped bulk milk samples into those having alcohol stability values above and below 72 %. Over 30 % of bulk milk samples had ethanol stabilities less than 72 %; these had Ca^{2+} values between 1.84 and 2.59 mM and pH values in the narrow range of 6.67–6.69. Those showing ethanol stability greater than 72 % had Ca^{2+} from 1.66 to 2.04 mM and pH from 6.70 to 6.72. The mean heat coagulation times were 23.8 and 19.9 min for samples with ethanol stabilities above and below 72 %, respectively.

There are two main reasons why milk may have a low ethanol stability (<74 %). The first is poor microbial quality which is accompanied by a fall in pH and the second is a salt imbalance

(Horne, 2003). The former situation is likely to arise with milk of poor hygienic quality or stored with poor refrigeration. Note, that as raw milk bacteriological quality deteriorates, its pH is reduced, which in turn increases ionic calcium and reduces ethanol stability. It is important to establish whether the microbial count is high and if so to solve the problem by improving raw milk quality. However, as Horne (2003) cautioned, milk with a low ethanol stability may just have a salt imbalance. For example, any factors which reduce the negative charge, such as concentrations of H^+, Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions, as well as the proportions of different casein fractions in the micelle, may reduce ethanol stability.

For formulated products, such as flavoured milk, reconstituted milk or ice cream mix, there is also the opportunity to manipulate composition to improve heat stability. Also, with reconstituted products, water quality may play an important role, especially with regard to the amount of calcium and magnesium which may be present.

10.2.1.3 Concentrated Milk

In principle, the procedures for producing evaporated or condensed milk are straightforward. The aim is to produce a product which is pourable and which does not thicken or coagulate during the final sterilisation procedure. The target for viscosity is rarely recorded, but it should be in the range of 20–40 cp at 20 °C. Viscosity measurement is the preferred method for assessing heat stability. Tarassuk and Tamsma (1956) proposed that the critical zone of viscosity that coincided with the first and partial appearance of gel formation was 90–100 cp (measured at 25 °C). The key steps are forewarming prior to evaporation and ensuring the correct amount of stabiliser is added prior to sterilisation. It is then important to prevent further thickening and gelation and crystal formation during storage. Sediment formation is not reported to be a major issue, although a small amount of sediment is also formed.

Cronshaw (1947) reviewed studies on factors affecting the heat stability of evaporated milk, especially the role of forewarming. It was suggested that using high-temperature, short-time conditions results in good heat stability and that this could also be improved by further heat treatment of the concentrate prior to sterilisation. There is also a suggestion that applying correct forewarming conditions would eliminate the need for the addition of stabilisers. This is an interesting proposal that is worthy of further investigation. Benton and Aldbery (1926) measured heat stability of milk which had been concentrated to 18 % solid-not-fat, by measuring the time required to curdle the product at 120 °C. They reported considerable variations in heat stability of milk from individual cows (4–24 min). They also observed that addition of citrate or borate to improve heat stability was only effective up to a point, beyond which the stability decreased. It was observed that adding these stabilisers increased alcohol stability. In the majority of the samples studied the optimum heat stability lay between alcohol stabilities of 70 and 75 %. They considered that the alcohol test provided a better means of avoiding overstabilisation than pH adjustment, as there was no particular pH to which milk could be adjusted to provide uniform results. However, it was observed that samples which were naturally or adjusted above or below the pH range 6.55–6.65, prior to evaporation, were never heat stable.

Forewarming conditions initially involved 80–95 °C for 10–30 min, although more recently temperatures over 100 °C have been used for shorter time periods. Augustin and Clark (1991) observed that 120 °C for 2 min (both direct and indirect) resulted in lower viscosities than 85 °C for 30 min. Tarassuk and Tamsma (1956) reported that optimum conditions of preheating vary seasonally and with milk from different cows.

The concentrate is homogenised prior to stabiliser addition and sterilisation. The stabilisers most investigated are TSC and DSHP. Most of the early literature makes reference to TSC, but the stabiliser of choice in the UK is now DSHP. As discussed, the optimum amount to be added will be different for every batch as levels of phosphate and citrate naturally present in the milk are variable. Traditionally, the amount to be added is determined experimentally, by sterilising a few cans with different amounts of salts under the conditions employed and determining which

level does not cause coagulation (Cronshaw, 1947). This procedure is still widely practiced today. Thus recommended levels of stabiliser addition are rarely reported. Cronshaw (1947) gives a range of 2–10 oz/1000 lb (0.013–0.063 %) of TSC or DSHP: these values seem low.

Thus, although procedures seem relatively straightforward, ensuring a product of consistent viscosity is difficult because of the variability in heat stability. It is also important to ensure that total solids content is controlled tightly as heat stability is influenced by small changes in total solids. Viscosity data for concentrated milk of 26–34 % TS during storage was provided by Tarassuk and Tamsma (1956). The time to reach an unacceptable viscosity increased as total solids was reduced. Marked retardation of gelation during storage was also achieved by a process that involved over-concentrating the milk (28–40 % TS), preheating the concentrate and then diluting back to 26 % TS. At higher concentrations, preheating conditions of less severity are required for optimum retardation of gelation in storage.

TSC is effective in preventing thickening and coagulation during sterilisation. Deysher and Webb (1952) reported that the crystals which formed during storage of evaporated milk were calcium citrate and that this could be retarded by addition of DSHP and by storage at low temperature. Thus, one drawback of TSC might be that it promotes crystallisation of calcium citrate which adversely affects the texture of the product. This was found to be the case when citric acid was added to evaporated milk (Deysher and Webb, 1952).

Sterilisation was found to result in a decrease in calcium ion activity and a decrease in pH. The decreases in calcium ion concentration caused by sterilisation were not influenced by the preheating treatment. Nieuwenhuijse *et al.,* (1988) reported that calcium ion concentration appeared to be an essential factor for the heat stability of concentrated milk (TS = 31.3 %) at pH values below the HCT maximum. Generally, March milk and October milk were less stable than June and September milks (note this was in the Northern Hemisphere).

Where fresh milk is not available, concentrated milk is made by reconstituting milk powder. High-heat powders are normally recommended for this purpose. Augustin and Clark (1991) produced recombined milks from powders which had been subjected to different preheat treatments. They were sterilised at 120 °C for 13 min and heat stability was measured by viscosity. The ranges found for two experiments were 11.5–49 cp and 13 to >80 cp. Faka *et al.,* (2009) found that low-heat skim milk powder reconstituted to 25 % TS had poor heat stability. This could be improved by removal of some calcium by ion exchange or by addition of TSC prior to drying. Tsikritzi (2011) compared the heat stability of medium-heat SMP reconstituted to 20 and 25 % TS. When sterilised at 115 °C for 15 min, the less concentrated sample was heat-stable whereas the more concentrated one coagulated. Improved heat stability was best achieved by addition of DSHP (0.1–0.5 %) or TSC (0.1 %). Higher concentrations of TSC resulted in a higher viscosity but no coagulation. The disodium salt of EDTA and DHSP caused coagulation, whereas the tetrasodium salt of EDTA did not cause coagulation and viscosity decreased as its concentration increased from 0.1 to 0.4 %. SHMP caused coagulation at about 6.6 mM (0.4 %) and was the only salt to increase viscosity prior to sterilisation.

10.2.2 Chemical Changes

10.2.2.1 Whey Protein Denaturation and Disulfide Protein Cross-Linking

Extensive denaturation of the whey proteins occurs during sterilisation, reaching close to 100 % in in-container sterilised milk. The degree of denaturation is less in UHT milk and dependent on the type of processing (i.e. direct or indirect) and the temperature–time profile of the heating process. Denaturation is dominated by the two major whey proteins, β-lactoglobulin and α-lactalbumin, the former being denatured more during UHT treatment than the latter. Of the other whey proteins, the immunoglobulins are the most heat-sensitive, followed by bovine

serum albumin, both of which are completely denatured by sterilisation processes. Proteose peptones are not denatured by heat (Donovan and Mulvihill, 1987).

The extent of whey protein denaturation is greater for indirect UHT plants than for direct plants. Tran *et al.,* (2008) determined the temperature–time profiles of several commercial direct and indirect UHT plants and calculated the extent of denaturation for each, based on the kinetics of denaturation reported by Dannenberg and Kessler (1988) and Lyster (1970). This showed that β-lactoglobulin is almost completely denatured by commercial indirect plants but direct plants cause only 74–92 % denaturation. However, the percentage denaturation of α-lactalbumin was 25–90 % for indirect and 27–58 % for direct plants (Tran *et al.*, 2008). Since the range of the residual levels of α-lactalbumin in UHT milks is large and seldom, if ever, reaches zero, the concentration of α-lactalbumin in UHT milk has been suggested as an index of heat treatment (Kondal Reddy *et al.*, 1999; Dupont *et al.*, 2004, Tran *et al.*, 2008).

Table 10.1 shows the calculated denaturation percentages of β-lactoglobulin and α-lactalbumin in milk processed on an indirect and a direct UHT plant after the preheat section, the high-heat holding tube and after cooling. The temperature–time profiles of the two plants are shown in Fig. 10.1. The bactericidal effect (B*) (Kessler, 1981) of the two plants is the same, 2.9 (see Sect. 10.1.2.2). Table 10.1 demonstrates that in the indirect plant, most of the denaturation occurs prior to the sterilisation holding tube whereas in the direct plant, most of the denaturation occurs in the holding tube. A similar situation is obtained for α-lactalbumin, albeit at much lower denaturation percentages.

Table 10.1 Denaturation (%)[a] of major whey proteins during UHT processing on indirect and direct UHT plants[b]

Stage in UHT plant	β-Lactoglobulin		α-Lactalbumin	
	Indirect	Direct	Indirect	Direct
At end of preheating including preheat hold	92	7	50	<1
At end of high-temperature holding tube	92	68	53	15
At end of cooling	94	69	66	21

[a]Based on kinetics of Lyster (1970)
[b]The temperature–time profiles are shown in Fig. 10.1; both plants had a B* of 2.9

The data in Table 10.1 also illustrate why the amount of residual undenatured β-lactoglobulin in UHT milk is not a good indicator of the severity of heat treatment. It is also the reason why many UHT milks show a negative turbidity in the Aschaffenburg test (Lewis and Heppell, 2000). This demonstrates why residual α-lactalbumin is a better index of heat treatment than β-lactoglobulin.

Concentration of milk affects the rate of heat-denaturation of whey proteins. In concentrated milk, the denaturation rate of β-lactoglobulin is reduced while denaturation of α-lactalbumin is similar to that in unconcentrated milk (McKenna and O'Sullivan, 1971). According to Anema (2009), for both β-lactoglobulin and α-lactalbumin, increasing the non-protein solids level decreases the denaturation rate but increasing the protein content increases the denaturation rate; these opposite effects cancel out for α-lactalbumin but for β-lactoglobulin, the retarding effect of the non-protein components exceeds the enhancing effect of the proteins.

Denaturation of β-lactoglobulin and α-lactalbumin has been studied in detail, both in pure forms and in mixtures with other milk proteins. When β-lactoglobulin is heated alone, it firstly undergoes a transformation from its natural dimeric form to a monomeric form, or forms, which then aggregate into oligomers or polymers depending on the severity of the heating conditions. Denaturation commences at temperatures as low as 40 °C but at temperatures below about 70 °C it is mostly reversible (Iametti and Bonomi, 1996). Heating above about 70 °C causes irreversible unfolding of the globular monomer exposing hydrophobic amino acids and a free sulfhydryl group, at Cys121, which is normally buried inside the native globular structure. The native monomer undergoes intramolecular sulfhydryl–disulfide reactions which result in the formation of non-native monomers with other

sulfhydryl groups exposed, e.g., Cys119 and Cys160 (Creamer *et al.*, 2004). Aggregation of the monomers occurs through both hydrophobic interactions and disulfide bonding, caused by intermolecular sulfhydryl–disulfide reactions.

In mixtures of β-lactoglobulin and α-lactalbumin, the reactive β-lactoglobulin monomer reacts with other molecules of β-lactoglobulin as well as α-lactalbumin, presumably *via* a sulfhydryl–disulfide reaction with the very reactive Cys6–Cys120 S–S bond of α-lactalbumin (Kuwajima *et al.*, 1990). Melo and Hansen (1978) purported to show that a β-lactoglobulin–α-lactalbumin adduct was formed. However, Noh *et al.,* (1989), using column chromatography, found no evidence for such a species while Havea *et al.,* (1998), using 2D electrophoresis identified a faint spot which they attributed to a disulfide-linked dimer of α-lactalbumin and/or a β-lactoglobulin–α-lactalbumin complex. α-Lactalbumin is very resistant to heat denaturation when heated alone or with micellar casein but is much less stable when heated in the presence of β-lactoglobulin (Calvo *et al.*, 1993). Bovine serum albumin has a free sulfhydryl group and in mixtures with β-lactoglobulin and α-lactalbumin, such as in whey protein isolate or concentrate, it also interacts with both β-lactoglobulin and α-lactalbumin (Havea *et al.*, 2001). However, bovine serum albumin only constitutes about 5 % of the whey proteins and hence makes a minor contribution to the denaturation process.

When a milk system containing both whey proteins and casein micelles is heated, the whey proteins not only interact with each other but also with the casein, in particular κ-casein which is located on the surface of the micelle. The reactive monomer of β-lactoglobulin primarily reacts with κ-casein but may do so after reacting with α-lactalbumin. Thus at the normal initial pH of milk, ~6.7, heat treatment such as UHT results in formation of casein micelles with whey proteins attached. However, during heating some κ-casein is released from the micelle and appears in the serum in association with denatured whey proteins. The relative amounts of denatured whey proteins associated with the casein micelle and associated with κ-casein in the serum phase is dependent on several factors such as the nature of the heat treatment and pH (Corredig and Dalgleish, 1996a, b). In indirect UHT systems in which heating is relatively slow, most of the denatured β-lactoglobulin and α-lactalbumin associate with the casein micelle while in direct UHT systems in which heating is rapid, about half of the denatured whey proteins occur in the milk serum and half on the casein micelle (Corredig and Dalgleish, 1996a). Mottar *et al.,* (1989) showed that the intensity of heat treatment also affected the ratio of β-lactoglobulin to α-lactalbumin attached to the micelle; at low heat intensities, such as in direct UHT heating, the ratio was high and at higher intensities, such as in indirect UHT treatments, it was lower as more α-lactalbumin became attached. This has significant practical implications because the hydrophobicity increases with increased α-lactalbumin attached to the micelle and this also increases water-holding capacity, an important consideration for some products. The pH of heating also significantly affects the distribution of these proteins; at pH ~6.5, most of the denatured whey proteins attach to the casein micelle while at higher pH, >6.8, most appears in the serum with κ-casein (Kudo, 1980). Probably not coincidentally, an increasing amount of κ-casein is released into the serum at the higher pH levels. There has been a lively debate about whether the whey protein complexes associate with the κ-casein before or after they dissociate from the casein micelle (Oldfield *et al.*, 1998; Corredig and Dalgleish, 1999; Anema, 2009). How this might affect heat stability is further discussed in Sect. 10.2.2.3.

One of the important practical implications of whey protein denaturation is fouling of heat exchangers during heat treatment (see Sect. 10.2.3.1). Fouling in the early stages is largely due to denatured β-lactoglobulin and hence the kinetics of deposit formation are related to the kinetics of denaturation (de Jong *et al.*, 1992). It has been shown that the denatured/unfolded monomer of β-lactoglobulin is largely responsible for the deposit formation (Jeurnink *et al.*, 1996; Mounsey and O'Kennedy, 2007) and the longer it is present in the UHT plant, the

greater the deposit formation or, conversely, the shorter the unfolded form is present, the longer the run-time of the plant (Grijspeerdt *et al.*, 2004). This suggests that the unfolded form is "sticky" and readily attaches to the walls of the stainless steel tube or plate and forms a base for further deposit formation.

There has been considerable interest in improving the heat stability of whey proteins because of the adverse effects of their denaturation during heat treatment. Several approaches have been investigated with some success. These include enzymatic cross-linking, mineral chelation, ultrasonication, enzymatic hydrolysis and addition of chaperone proteins (Burrington, 2012). The last approach uses caseins as "chaperone" proteins (Morgan *et al.*, 2005). α_{s1}-casein (Bhattacharyya and Das, 1999), α_{s1}/β-casein and micellar casein (O'Kennedy and Mounsey, 2006) and sodium caseinate have been shown to be effective. The protective effect of α_{s1}-casein has been attributed to its high hydrophobicity and highly flexible nature. The protective behaviour is manifested in reduced aggregation of the denatured whey proteins which results in greater solubility and smaller particles of the denatured whey protein. Another research approach has been to add sulfhydryl-blocking agents such as N-ethylmaleimide (NEM) (Sawyer, 1968) or dithio(bis)-*p*-nitrobenzoate (DTNB) (Iametti and Bonomi, 1996) and oxidising agents such as iodate or hydrogen peroxide (Marshall, 1986). These reagents, which react with the free sulfhydryl group of the denatured β-lactoglobulin monomer, prevent aggregation of β-lactoglobulin and interaction of β-lactoglobulin with κ-casein and α-lactalbumin, and decrease fouling of heat exchangers during UHT processing (Skudder *et al.*, 1981). To date, this has not been a practical approach as the reagents used are not permitted food additives in most countries. One food-grade compound, dihydrolipoic acid, was recently shown to decrease aggregation of β-lactoglobulin on heating (Wijayanti *et al.*, 2013); however, it was not useful in heated whey protein isolate as it enhanced aggregation of α-lactalbumin (Wijayanti et al., 2014).

A further approach which allows the use of whey proteins in heated products is microparticulation. This involves thermal denaturation, aggregation and precipitation of the whey proteins as microparticles (Steventon *et al.*, 1994). It is not clear whether microparticulated whey protein would withstand commercial sterilisation conditions.

10.2.2.2 Non-disulfide Protein Cross-Linking

Protein cross-links other than through disulfide bonds can be formed *via* dehydroalanine or Maillard reaction products. Proteins cross-linked *via* dehydroalanine cannot be dissociated with reducing agents such as mercaptoethanol as can the disulfide-linked protein complexes. Proteins cross-linked *via* dehydroalanine, on hydrolysis, yield isodipeptides such as lysinoalanine (LAL), histidinoalanine (HAL) and lanthionine (Friedman, 1999). The first step in the cross-linking process is production of dehydroalanine from *O*-phosphorylserine, *O*-glycosylserine or cysteine by elimination of phosphate, a sugar or H_2S, respectively. The dehydroalanine then reacts with an amino group of another amino acid, such as the ε-amino group of lysine (Friedman, 1999) forming a cross-linked protein. The reaction is favoured at high pH and hence is relevant during the manufacture of products such as sodium caseinate and calcium caseinate which involves addition of alkalis. pH here typically does not exceed pH 7.0 during manufacture.

Dephosphorylation of the phosphoserine residues in milk occurs during sterilisation (Meisel and Schlimme, 1995). It can occur by hydrolysis with release of phosphate and serine or by β-elimination with formation of dehydroalanine and phosphate. van Boekel (1999) found that, in heated caseinate solutions, more phosphate and serine than dehydroalanine was formed. When a caseinate solution was heated at 140 °C, very little dephosphorylation occurred in the short term but increased with time to more than 50 % after 30 min (van Boekel, 1999) and 100 % after 1 h (Singh, 1995). Dephosphorylation

does not appear to be an important factor in the heat stability of concentrated milk (Nieuwenhuijse *et al.*, 1988).

Non-disulfide covalently cross-linked proteins are present in raw milk and increase during heat treatment and during storage. Zin El-Din *et al.,* (1991) found that 7.5 % of the casein in UHT milk was polymerised with 5 % being formed by the UHT process (indirect plate heat exchanger at 138 °C for 2 min) while Andrews (1975) and Lauber *et al.,* (2001) reported 14 % non-reducible polymerised casein in freshly processed UHT milk. However, other authors using electrophoretic methods found negligible cross-linked proteins in fresh UHT milk (Al-Saadi and Deeth, 2008; Holland *et al.*, 2011). Lorient (1979) reported formation of lysinoalanine cross-links at pH 7.0 but not at pH 6.0 when α_{S1}-casein was heated at 120 °C for 30 min. Much more cross-linking occurred at pH 11.9. Fritsch *et al.,* (1983) found that UHT milk contained very little lysinoalanine (from 0 to 50 μg/g protein) but autoclaved milk had considerably more (from 110 to 710 μg/g protein) when heated at 110–129 °C for 10–25 min. Higher temperatures, higher pH and longer heating times increased lysinoalanine formation.

It has been suggested that some cross-linking in UHT milk is related to Maillard reaction products (Andrews and Cheeseman, 1971; Andrews, 1975) but definitive proof of the cross-links has not been reported. However, heating milk with and without lactose at 100 °C for 5 h provided indirect evidence as considerably more cross-linking occurred when lactose was present (Andrews and Cheeseman, 1972). Al-Saadi *et al.,* (2013) heated milk with and without lactose at 95 °C for up to 8 h and concluded that the cross-linking which occurred with lactose present mostly occurred *via* Maillard reaction products while the cross-linking in the absence of lactose occurs mainly *via* isodipeptides such as lysinoalanine (LAL). The Maillard reaction products which could act as cross-linking agents include glyoxal and methylglyoxal. Le *et al.,* (2013) have recently shown that incubating milk protein concentrate with methylglyoxal produced similar polymerised proteins to those which form in this product during storage.

Protein cross-linking, whether *via* dehydroalanine or Maillard reaction products, has practical significance since it reduces the nutritional availability of amino acids such as lysine and reduces the digestibility of the proteins (Friedman *et al.*, 1981). It has also been shown to increase the viscosity of milk for yogurt manufacture in a similar manner to disulfide cross-linking in conventional yogurt manufacture (Lauber *et al.*, 2001). Protein cross-linking has also been investigated in relation to age gelation of UHT milk. Venkatachalam *et al.,* (1993) concluded that the Maillard reaction neither promoted nor deferred age gelation. McMahon (1996) suggested that the reduced susceptibility of UHT milks to gelation when stored at 35 °C may be due to non-disulfide protein cross-linking.

10.2.2.3 Protein Dissociation from the Casein Micelle

De la Fuente (1998) reported the following concentrations (mg/L) of caseins in the micellar and soluble phases of unheated milk, respectively: α_{s1}—(10,900 and 700); α_{s2}—(3000 and 100); β—(9000 and 1300) and κ—(2900 and 500). There is considerable evidence that casein dissociation from the micelle takes place during heating of milk. Most investigations have focussed on the dissociation of κ-casein from the micelle and its interactions with whey proteins, especially β-lactoglobulin. One argument is that dissociation of κ-casein from the surface of the micelle will make it more susceptible to calcium-ion-mediated heat-induced aggregation, so this has a marked effect on the heat stability of the milk. An important feature of all these studies is the pH dependence of these interactions. This topic has been reviewed by Anema (2009). Many of the studies focus on the effects of pH on dissociation of caseins and their subsequent interaction with denatured whey proteins. At pH values >6.7 the amount of non-sedimented protein increases in heat-treated milk. Kudo (1980) showed that at pH 6.5, the amount of non-sedimented protein in heated milk was less than

in non-heated milk. As pH was increased, the amount was also found to increase in heated milk, until at pH 6.7 it had exceeded that in non-heated milk. Anema and Li (2000) reported that when milk at pH >6.7 was heated, the quantity of α_S- and β-caseins dissociated increased with increasing temperature to a maximum at about 60 °C, decreased at temperatures between 60 and 100 °C and then increased again at temperatures above 100 °C. This produced a local minimum in the dissociated casein–temperature curve at about 100 °C. The dissociation of κ-casein increased essentially linearly with increasing temperature up to 100 °C (Anema and Li, 2000). Singh and Latham (1993) studied the aggregation and dissociation of protein in milk heated at 140 °C and found that initial heating gave rise to the formation of high molecular weight complexes of whey proteins and κ-casein. With continued heating, the quantities of these complexes remained more or less constant but the amounts of intermediate-sized protein material cross-linked through covalent (non-disulfide) bonds increased gradually. Increasing the pH at heating resulted in increased quantities of whey protein–κ-casein complexes and monomeric protein in the ultracentrifugation supernatant. Anema (2009) concluded that further detailed investigations are required to clarify whether dissociation of κ-casein occurs before or after interaction with the denatured whey protein.

Raw milk is usually held chilled prior to heat treatment. When milk is kept at 4 °C, there is considerable dissociation of β-casein from the micelle. However, at 30 °C only a small amount of β-casein dissociates, even when pH is reduced. The dissociation of β-casein is reported to be reversible but there is no evidence that this movement of β-casein affects heat stability. It has been reported that urea addition increases soluble casein (Dalgleish *et al.*, 1987). Addition of 10 mM urea was found to increase protein solubilisation in milk heated at 130 °C. Although this might result in a reduction in heat stability, it has been postulated that urea more likely acts to prevent crosslink formation and aggregation and to also diminish pH drift. Udabage *et al.,* (2000) reported that adding mixtures of DSHP and DHSP, and $CaCl_2$ increased sedimentable casein (and calcium phosphate), whereas adding TSC or EDTA had the opposite effect.

In slower heating processes and at higher initial pH, more κ-casein dissociation is likely to occur. This might provide an explanation for the observed decrease in heat stability found for in-container sterilised milk when pH is increased by addition of stabilisers and in evaporated milk when stabiliser addition exceeds the optimum, which is the situation found when DSHP and TSC are added to milk which is then in-container sterilised (see Sect. 10.2.1.2). This is in agreement with the findings of On-Nom (2012). When milk is heated more rapidly (as in UHT treatment), milk pH will fall much more quickly and less κ-casein dissociation is likely to occur. This might explain why milk with small amounts of added phosphate and citrate are more stable to UHT conditions than to in-container sterilisation.

There are relatively few studies on the extent of protein dissociation at high temperature. Ideally it would be informative to ultracentrifuge or otherwise partition milk at high temperature. On-Nom (2012) used ultrafiltration (MWCO of 200,000 Da) and dialysis (MWCO of 300,000 and one million Da) to partition milk at different pH and temperatures. $CaCl_2$, DSHP and TSC were added to milk and pH, Ca^{2+}, total calcium, magnesium (Mg), phosphorus (P) and protein concentration were investigated using different partitioning methods in the temperature range 20–120 °C. It was found that pH, mineral content and soluble protein decreased as temperature increased for the control milk. The addition of TSC and DSHP to milk increased pH, phosphorus and soluble protein but decreased Ca^{2+} whereas $CaCl_2$ had the opposite effect. TSC addition resulted in a higher amount of soluble protein than DSHP. When comparing these four separation techniques, dialysis was considered to be the best method for investigating these properties at high temperature. Further work needs to be done in this area.

10.2.2.4 Role of Minerals and pH

Ionic Calcium and pH

One crucial requirement for milk destined for sterilisation is that it has good heat stability. Two important parameters affecting heat stability are H^+ concentration (pH) and ionic calcium (Ca^{2+}). Both are positively charged and will tend to neutralise the negative charge on the casein micelle. Pyne and McHenry (1955) pointed out that heat coagulation was slow in milk which was low in calcium ion concentration and colloidal phosphate. There are considerable variations in both pH and Ca^{2+} (White and Davies, 1958a; Lin, 2002; Tsioulpas *et al.*, 2007) in milk from individual cows. Bulking of milk reduces these variations but does not eliminate them (Chavez *et al.*, 2004, Tsioulpas *et al.*, 2007, Grimley *et al.*, 2009). Much of the earlier literature focused on the influence of pH on heat stability but paid much less attention to that of Ca^{2+}. Tsioulpas *et al.,* (2007) found that Ca^{2+} for 234 samples from individual cows was between 1.05 and 5.29 mM; the average was 1.88 mM. Chen (2013) found for 25 bulk milk samples collected over a 1 year period from the same farm had an average Ca^{2+} concentration of 2.05 mM and a range of 1.68–2.55 mM. White and Davies (1958a) reported an inverse relationship between pH and ionic calcium and others have observed the same, although none has reported a strong correlation. Thus, pH and ionic calcium are independent parameters; both individual and bulk milk samples with a particular pH can have a wide range of Ca^{2+} concentrations and *vice versa* (Lin, 2002; Nian *et al.*, 2012).

However, reduction in pH of any milk sample, by whatever means, increases the Ca^{2+} concentration. This was investigated by Zadow *et al.,* (1983) for goat's milk and for cow's milk by Geerts *et al.,* (1983) and Tsioulpas *et al.,* (2007). The last authors found a linear relationship between pH and log (Ca^{2+}), with a slope of –0.62. Holt (2004) showed how Ca^{2+} concentration could be predicted to change in milk over the pH range 5.0–7.0. Ca^{2+} is influenced by the amount and type of casein and by phosphates, citrates and by the pH of the milk. Therefore, milk will have a Ca^{2+} concentration and pH, which are determined by its own unique composition and which can be predicted by models developed by Holt *et al.,* (1981) and Holt (2004). Ionic calcium in milk from other species has been reviewed by Lewis (2010).

One important fact which is less often discussed is that the pH of milk decreases as its temperature increases. When pH was measured directly in the holding tube of a heat exchanger it decreased from 6.57 at 40 °C to 6.26 at 80 °C (Ma and Barbano, 2003). Walstra and Jenness (1984) illustrated that milk pH decreases to just below 6.0 when milk is heated to 100 °C. More recent data suggest that it may be as low as 5.6 at 140 °C (On-Nom, 2012). Thus, increasing the temperature of milk induces two changes which have competing effects. One might expect that this reduction in pH would increase calcium phosphate solubility, but calcium phosphate becomes less soluble at high temperature. This latter factor dominates and soluble calcium has been found to decrease as temperature increases (Pouliot *et al.*, 1989). Rose (1962) proposed that Ca^{2+} would increase with increasing temperature, due mainly to the fall in pH, despite some contradictory evidence reported by Tessier and Rose (1958). Only recently have measurements of Ca^{2+} at high temperatures been reported; directly using electrodes up to 60 °C (Chandrapala *et al.*, 2010) and by UF up to 80 °C and dialysis up to 120 °C (On-Nom *et al.*, 2010). The dialysis is performed by placing dialysis tubing containing a small amount of water into the milk, bringing the milk to the desired temperature for a predetermined time, quickly removing the dialysis bag and analysing its contents. More recently ultrafiltration (UF) has been performed up to 140 °C, by placing the UF module in the holding tube of a UHT plant (On-Nom, 2012).

These different approaches show that Ca^{2+} concentration decreases as temperature increases. Thus, it is now known that both pH and Ca^{2+} concentration of milk decrease as temperature increases. It is our opinion that heat stability will be better understood by further studies related to measurement of pH and Ca^{2+} (and other components of interest) at the heating temperature.

Rose (1963) concluded that a definite correlation between calcium ion concentration at ambient temperature and heat stability at high temperature could not be established. This opinion was also expressed recently by McKinnon *et al.,* (2009), where pH was monitored when milk was heated to 90 °C.

When milk is subjected to heat treatments, it is important to distinguish between changes in Ca^{2+} concentration that result from different heat treatments and the effect of temperature on Ca^{2+}. It is also prudent to measure the resulting pH changes, because of the interrelationship between pH and Ca^{2+} concentration. UHT treatment has little effect on pH of milk, whereas sterilisation reduces pH by about 0.2–0.3 units. Bringing milk to the boil and then immediately cooling decreased Ca^{2+} from 2.78 to 2.09 mM (Demott, 1968). UHT treatment shows a larger reduction in soluble Ca $^{2+}$ concentration than does pasteurisation (Geerts *et al.*, 1983; Ranjith, 1995). However, Ca^{2+} quickly recovers, but not to its original value. A linear relationship was found between the logarithm of time and the recovery of the calcium ion activity following heat treatment (Geerts *et al.*, 1983). In-container sterilisation may result in either an increase or decrease in Ca^{2+}, but there is always a considerable drop in milk pH (Tsioulpas *et al.*, 2010). Nieuwenhuijse *et al.,* (1988) reported that forewarming slightly reduced Ca^{2+} but had little effect on pH. Pyne and McHenry (1955) estimated that half the acidity developed on heating in the temperature range of 100–130 °C was due to lactose decomposition, a third due to liberation of phosphate from the casein micelle and a sixth due to displacement of the calcium phosphate equilibrium.

Addition of Phosphates, Citrate, EDTA and Calcium Salts

Salts of phosphoric acid and citric acid (both tribasic) are frequently added to prevent coagulation during the production of evaporated milk. Christiannson *et al.,* (1954) drew attention to the role of TSC in reducing Ca^{2+}. Tessier and Rose (1958) reported that addition of phosphate and citrate both decreased Ca^{2+}. However phosphate addition precipitated calcium, whereas citrate dissolved colloidal calcium. Singh and Fox (1987) and Udabage *et al.,* (2000) reported that the amount of soluble casein increased with the addition of TSC, whereas the addition of calcium and mixtures of DSHP and DHSP had the opposite effect.

These stabilisers are rarely used for commercial sterilisation of cow's milk but they may be required for formulated or fortified milk drinks and are definitely required for UHT treatment of goat's milk. Tsioulpas *et al.,* (2010) reported that addition of TSC and DSHP to milk reduced Ca^{2+}, increased pH and increased ethanol stability in a concentration-dependent fashion. SHMP also reduced soluble Ca^{2+} concentration considerably, but its effect on pH was less noticeable. In contrast, DHSP reduced pH but had little effect on Ca^{2+}. In-container sterilisation had variable effects on Ca^{2+}; for DSHP and DHSP, Ca^{2+} decreased after sterilisation, but for SHMP it remained little changed or increased. Milk containing 3.2 mM SHMP and more than 4.5 mM $CaCl_2$ coagulated on sterilisation.

EDTA is a calcium-chelating agent, which has been much investigated, although rarely added to milk. More often, the disodium salt has been investigated, which decreases pH, whereas the tetrasodium salt increases pH. Thus, addition of both these salts will reduce soluble Ca^{2+} concentration provided that the pH is adjusted to its original value. It could be argued that it is immaterial which form is added, provided that the pH is adjusted, although this has not been established experimentally. For both salts, excessive additions (>20 mM) will cause destabilisation of the micelle; the milk becomes whey-like in appearance. Udabage *et al.,* (2000) investigated EDTA, mixtures of DSHP and DHSP, and TSC. They measured Ca^{2+} in milk adjusted to pH 6.65 and found significant reductions in Ca^{2+}.

Calcium supplementation of milk is challenging, because it is already saturated with calcium and because adding most soluble calcium salts generates hydrogen ions, reduces pH and increases Ca^{2+} (Philippe *et al.*, 2003; Tsioulpas *et al.*, 2010). Another approach is addition of calcium salts of weaker acids such as calcium gluconate and calcium lactate. In a study comparing six calcium

salts, calcium chloride showed the largest destabilizing effect, followed by calcium lactate and calcium gluconate. Milk became unstable to UHT processing at lower calcium additions compared to in-container sterilisation (Omoarukhe *et al.*, 2010). However, addition of three insoluble calcium salts (30 mM) did not change any of the properties influencing heat stability, such as pH and Ca^{2+}. There were no major signs of instability associated with coagulation, sediment formation or fouling when subjected to UHT and in-container sterilization. The buffering capacity was also unaltered. On-Nom *et al.,* (2012) studied the combined effects of calcium chloride addition (up to 25 mM) and heat treatment (60–120 °C) on heat stability and found that the amount of calcium chloride required to induce poor heat stability decreased as temperature increased.

Calcium removal from milk can be achieved by ion exchange resins (Ranjith *et al.*, 1999). Changes in Ca^{2+} and pH depend upon the exchanging counter ions; effects are different for Na^+ or K^+, compared to H^+. Although it may seem counterintuitive from a nutritional viewpoint to remove calcium, it has been found to improve heat stability, presumably by reduction in Ca^{2+}. This has been reported by Ranjith *et al.,* (1999), Jeurnink and DeKruif (1995), Prakash *et al.,* (2007) and Grimley *et al.,* (2010). Demott (1968) reported that treatment with anionic exchanger increased Ca^{2+}, because of removal of phosphates. An alternative procedure which may be less disruptive to the micelle is to separate milk by UF and to then remove calcium from the permeate, rather than from milk (Ranjith, 1995).

10.2.2.5 Protein-Derived Flavours

The flavour of sterilised milk is distinctive and different from that of raw or pasteurised milk. For many consumers, particularly those accustomed to pasteurised milk, this is not a positive attribute of UHT milk (Perkins and Deeth, 2001). Typical consumer descriptions given to the flavour of UHT milk are "cooked" or "heated". In-container sterilised milks have a more intense cooked flavour than UHT milks because of the higher intensity of the heat treatment.

Sterilisation heat treatments cause denaturation of whey proteins (see Sect. 10.2.2.1) and some degradation of the milk proteins. One consequence is the release of low molecular-weight sulfur compounds. Thus, immediately after manufacture, UHT milk has a sulfurous flavour sometimes likened to boiled egg or cooked cabbage. This defect is commonly referred to as "cooked". The volatile sulfur compounds which are largely responsible for this flavour were reviewed by Al-Attabi *et al.,* (2009). The major ones in UHT milk are hydrogen sulfide, carbonyl sulfide, methanethiol, dimethyl sulphide, carbon disulfide, dimethyl disulfide, dimethyl sulfoxide, dimethyl sulfone and dimethyl trisulfide. From calculated odour activity values, Vazquez-Landaverde *et al.,* (2006) considered methanethiol, dimethyl sulfide and dimethyl trisulfide to be the most important contributors to the sulfurous flavour while Al-Attabi (2009) concluded from aqueous flavour threshold values that hydrogen sulphide also contributed to this flavour. The high concentrations of volatile sulfur compounds in UHT milk immediately after manufacture decrease during storage at a temperature-dependent rate. Figure 10.2 shows the decrease in hydrogen sulfide concentration in a commercial indirectly processed UHT milk during storage at ~22 °C immediately after manufacture; a similar decline was observed for methanethiol (Al-Attabi *et al.*, 2014).

In skim milk, the volatile sulfur compounds are generated from the thermal degradation of whey proteins, principally β-lactoglobulin (Hutton and Patton, 1952) while in whole milk they are also produced from milk fat globule membrane proteins (Mulder and Walstra, 1974). Thus as the fat percentage increases, the levels of hydrogen sulfide, dimethyl sulfide, methanethiol and dimethyl trisulfide in the sterilised milk increase (Gaafar, 1987; Vazquez-Landaverde *et al.*, 2006). This is illustrated for hydrogen sulfide in Fig. 10.2 for skim and whole milks produced commercially under the same conditions (Al-Attabi *et al.*, 2014). Thermal degradation of cysteine and methionine is largely responsible for production of these compounds.

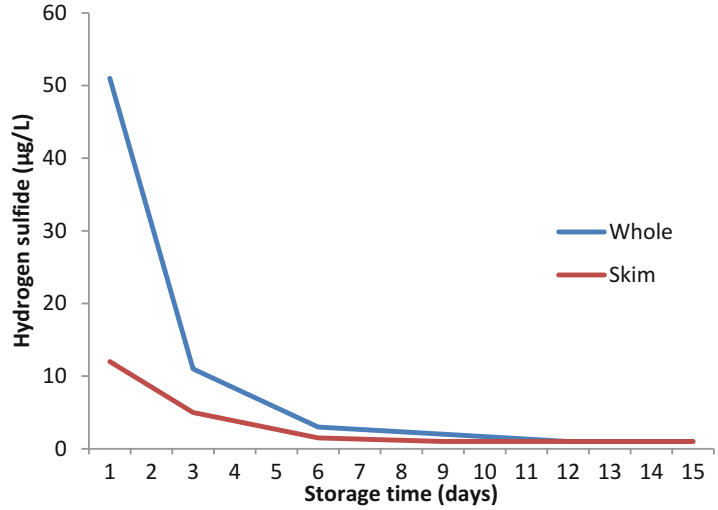

Fig. 10.2 Decrease in hydrogen sulfide concentration in UHT whole and skim milk during storage (data from Al-Attabi, 2009)

For example, hydrogen sulfide is formed from cysteine *via* a Strecker degradation in the presence of dicarbonyls, such as diacetyl, while methanethiol is formed from a Strecker degradation of methionine *via* methional (Schutte, 1976).

Another flavour in UHT milk has been designated as "heated" and is considered to be the major flavour of UHT milk after the initial sulfurous flavour has disappeared and before stale flavours caused by fat oxidation appear. Presumably, this flavour exists immediately after manufacture but is masked by the dominant sulfurous notes. Shipe *et al.,* (1978) identified lactones, methyl ketones, maltol, vanillin, benzaldehyde, acetophenone and diacetyl as components of this flavour. These compounds are mainly Maillard reaction products and/or lipid oxidation products. Colahan-Sederstrom and Peterson (2005) found that compounds generated by the Maillard reaction were present at much lower concentrations when the polyphenol epicatechin was added to milk before UHT treatment. The Maillard reaction initiated during heating continues during storage of the product and it appears that the more intense the heat treatment the more Maillard browning occurs during storage. Furthermore, the Maillard reaction rate is temperature-dependent and long-term storage of milk at temperatures above ~30 °C is inadvisable.

10.2.3 Physical Changes

10.2.3.1 Fouling of Heat Exchangers

When milk is heated, aggregation reactions may take place which give rise to deposit formation on heat exchanger surfaces, which is termed fouling. Fouling limits run times of UHT plants, particularly indirect heating plants and hence has significant economic effects. If the aggregates or deposits do not attach or become dislodged, they may cause problems downstream of the holding tube (Bansal and Chen, 2006) or in the final product and be perceived as sediment. Swartzel (1983) linked sediment formation during storage in aseptic packages to the severity of heat treatment and the extent of fouling.

Fouling of heat exchangers has been widely studied but the mechanisms are still not fully understood. Burton's review articles (Burton, 1968, 1988) provide a good summary of the state of knowledge at those times, and an update was provided by Bansal and Chen (2006). Methods for measuring fouling have been reviewed by Lewis and Heppell (2000) and Prakash *et al.,* (2005). Early investigations on fouling of UHT milk led to the recognition of two distinct types of deposit (Lyster, 1965; Burton, 1968). The major area where deposits occur is the preheating section. Maximum deposit formation is in the

temperature range 95–110 °C (Burton, 1988) and at 110 °C according to Skudder *et al.,* (1986). The deposit forming between 80 and 105 °C is a white voluminous deposit, which has a high protein content (50–70 %) and a significant mineral content (30–40 %) and tends to block the flow passages. This is known as Type A deposit. At the lower end of the temperature spectrum, the protein is predominantly denatured β-lactoglobulin but toward the top end of the range it is predominantly casein. Tissier *et al.,* (1984) also found two major temperature zones for deposit formation; the first at ~90 °C (predominantly protein (50 %) and the second at ~130 °C, which was predominantly mineral (75 %)). The major protein contributing to the lower temperature peak was β-lactoglobulin (62 %) while in the second peak, β-casein (50 %) and α_{s1}-casein (27 %) were predominant. Work by Lalande *et al.,* (1984) was in general agreement with this. Type B deposits, which form at higher temperatures, are finer, more granular and predominantly mineral in origin (70–80 %), with only small amounts of protein (10–20 %). The mineral component is probably β-tricalcium phosphate. Fouling deposits reduce the efficiency of the heat transfer process and make it difficult to reach the product outlet temperature without raising the temperature of the heating medium to an undesirable extent.

It is interesting that fat does not feature significantly in any of the deposits, usually less than 5 %, despite it being present in equal concentrations to protein and in greater concentration than minerals in whole milk. However Newstead *et al.,* (1998) reported that fouling deposits from recombined milk had higher levels of fat (up to 60 %) compared to fresh milk (10 % or less). Fouling in creams, where the fat might represent up to 75 % of the dry matter is also reported to be predominantly protein and mineral in character, with the fat playing an insignificant role.

More fouling has been reported in tubular heat exchangers than plate heat exchangers, attributed to the higher shear rates found in plate heat exchangers (Bansal and Chen, 2006). However the total cross sectional area for flow is also much higher in tubular heat exchangers and this may be the overall controlling factor. It is interesting that direct UHT plants are considered to be capable of dealing with poorer quality raw milk much better than indirect plant, as longer run times can be achieved on a direct plant. Fouling in direct plants is much reduced, due to the reduced heat transfer surface at high temperatures and the very quick rise in temperature. However this may lead to more sediment in the final product compared to indirect plants (Perkin *et al.*, 1973; Ramsey and Swartzel, 1984). Deposits may still form in the holding tube, the back-pressure valve and the beginning of the cooling section. Higher proportions of fat (0–35 %) are found in deposits from direct steam injection plants, attributed to fat destabilisation during the injection process; post-sterilisation homogenisation at high temperature (138 °C) also produce deposits with a higher proportion of fat.

The role of pH in deposit formation was found not to be straightforward, most probably because it was not considered together with ionic calcium. Fresh milks with the same pH showed considerable variations in their susceptibility to fouling (Burton, 1988; Grandison, 1988). However, for any individual batch of milk, in general, a reduction in pH of about 0.15 units during storage, from 6.67 to 6.52 would significantly increase fouling. Reducing pH was also found to increase the amount of fat within the deposit. The addition of sodium hydroxide to increase the pH by about 0.1 unit prior to processing had little effect, whereas addition of TSC (4 mM) increased the pH by 0.7 and increased both processing times and product quality, and slightly increased product viscosity. Burton (1968) showed that for the same milk sample, the amount of deposit formed increased as the pH was reduced, using hydrochloric acid, with significant changes taking place below pH 6.5. Kastanas *et al.,* (1995) showed that fouling increased as pH was reduced in milk heated at 140 °C. This was measured by a decrease in overall heat transfer coefficient (OHTC) and an increase in pressure drop in a miniature tubular UHT plant. Skudder *et al.,* (1986) observed that it was extremely useful to measure pH as a slight increase in pH improves processing times. Similar results were obtained for reconstituted milks (Zadow and Hardham,

1978). Other additives have been used to reduce fouling, but they may not be legally acceptable in all countries. Sodium and potassium pyrophosphates, added at 100 ppm, were both effective and doubled the running time in a plate heat exchanger (Burdett, 1974). Skudder *et al.,* (1981) found that the addition of iodate extended running time considerably by interfering with the formation of type A deposit, largely polymerised β-lactoglobulin. Addition of only 10 ppm doubled the running time before requiring cleaning but 20 ppm caused bitterness due to plasmin-induced proteolysis during subsequent storage (Grufferty and Fox, 1986) (see Sect. 10.3.2.1.4).

Grandison (1988) also suggested that pH was not a reliable indicator by itself, concluding that there were other factors in milk which would make a significant contribution towards fouling. Experiments designed to investigate seasonal variations in milk composition showed that there were significant differences in fouling behaviour throughout a complete year. However it was not possible to correlate fouling with any physical or chemical parameters, so the reasons for this observation were not clearly established. Milks with high levels of κ-casein were more prone to deposit formation. Note that ionic calcium was not measured by Grandison (1988). Jeurnink and DeKruif (1995) produced low- and high-calcium milk and found that both had reduced HCT. In terms of deposit formation, the high-calcium milk produced significantly more than the control whereas the low-calcium milk produced only slightly more. Ionic calcium was also measured but only three samples were investigated. It is recommended that both pH and ionic calcium should be monitored to gain a better understanding of the fouling process.

Lalande and Corrieu (1981) showed that there was a strong positive correlation between the rate of deposit formation and the concentration of ammonia; an increase in ammonia concentration from 3.7 to 7.2 ppm was associated with a doubling of the fouling rate constant. One suggestion is that ammonia concentration is related to the urea content of milk; natural urease may break down the urea producing ammonia, thereby increasing its susceptibility to fouling.

Aging raw milk at 4 °C for 12–24 h, without change of pH, was found to reduce its susceptibility to fouling. This was thought to be due to lipolysis and the production of fatty acids (Burton and Burdett, 1974). Addition of capric acid reduced fouling whereas addition of stearic acid increased fouling (Al-Roubaie and Burton, 1979). Capric acid was thought to associate with the casein micelle and prevent interactions which would lead to the build-up of the deposit. Kastanas *et al.,* (1996) found that raw milk could be stored chilled for a considerable time without change in its susceptibility to fouling.

One approach to reducing fouling in UHT plants is to carry out a heat treatment prior to high-temperature sterilisation. This has been termed forewarming, preholding and preheating. While some authors have used these terms interchangeably, forewarming and preholding were used in early literature to refer mainly to a procedure performed before introduction of the milk to a UHT plant while preheating is commonly used today to refer to a step within the UHT plant. In general, such heat treatment, which denatures β-lactoglobulin to some degree, decreases fouling and increases run time (Burton, 1968; Mottar and Moermans, 1988; Foster and Green, 1990), with higher temperatures being more effective than lower temperatures. Mottar and Moermans (1988), using response surface methodology, found that the optimum preheating/forewarming conditions to reduce fouling were 70–90 °C for 40–80 s. However, because the more intense treatments can lead to off-flavour production, they concluded that the optimum preheating conditions are 70–80 °C for 40–70 s. In contrast to the above, Srichantra *et al.,* (2006) reported the opposite effect of preheating, that is, the more severe the preheating the greater the rate of fouling. They suggested the reason for their different results could be related to the different processing procedures, including the stage of homogenisation; they preheated milk which had been previously homogenised (and pasteurised).

Gynnig *et al.,* (1958) showed that removal of air from milk reduced the total amount of deposit produced by between 50 and 75 % in a laboratory pasteuriser operating at 85 °C. Fouling is believed

to result from the presence of bubbles as the air becomes less soluble at increased temperature and provides nucleation sites for deposit formation. On commercial UHT plants this can be achieved by using a deaerator, but there is no reported evidence that this will reduce fouling. Burton (1968, 1988) suggested that the pressure under which UHT processing is performed (~0.5 MPa) prevents air bubbles escaping from the product and facilitating fouling. He recommended operating pressures in UHT plants of at least 0.1 MPa higher than that corresponding to the highest product temperature in the plant to minimise fouling (Burton, 1988). However, Prakash (2007) altered the dissolved oxygen content of milk from 1.15 to 7.15 ppm and found no difference in fouling in a UHT pilot plant.

There is still uncertainty whether protein denaturation or protein aggregation are the key reactions in fouling and whether it is denatured whey protein or minerals that deposit first on the heat exchange surface. UHT equipment must be sterilised downstream of the holding tube prior to processing milk. This is most often done by circulating hot water through the cooling section at 130 °C for 30 min. In hard water areas, it is important that this water should be softened. In over 200 UHT pilot plant trials with good quality cow's milk, excessive fouling was observed to occur quickly on only one occasion. It was subsequently discovered that the water softener was not working and hard water had been used for plant sterilisation. This most probably resulted in calcium being deposited, which then accelerated further accumulation of proteins at the surface. This is an argument for mineral deposits initiating the fouling process, but in other circumstances it may be initiated by protein deposition (Lewis, M. J., unpublished data). Lewis and Deeth (2009) provide an overview whereby fouling is initiated by denatured whey protein adsorbing onto the heated surface (see also Sect. 10.2.2.1).

From personal experience, goat's milk is difficult to UHT process. It is both very susceptible to fouling and also to sediment formation. Its alcohol stability is also well below that of cow's milk, at between 40 and 60 %. Kastanas (1996) found that goat's milk fouling led to a rapid decrease in OHTC, with no initiation period. Reconstituted goat's milk, made from freeze-dried powder, which had a lower ionic calcium level was less susceptible to fouling than fresh goat's milk. Kastanas *et al.,* (1996) showed that a number of treatments, such as citrate addition, forewarming and pH adjustment, all reduced fouling in goat's milk. Prakash *et al.,* (2007) showed that SHMP, TSC and some calcium reduction by ion-exchange resin treatment were also effective. The findings of Boumpa *et al.,* (2008) on sediment in UHT goat's milk are in agreement with these findings. On the other hand, buffalo milk is widely available in some countries (e.g., Egypt and India), some of which is UHT processed, often mixed with cow's milk. There are no reports that this provides a severe fouling problem, despite its higher total solids of 16–18 %. In fact the alcohol stability and heat stability of buffalo milk compare favourably with cow's milk (Laxminarayana and Dastur, 1968).

10.2.3.2 Sediment Formation

It has been suggested that mechanisms that give rise to sediment formation also are responsible for fouling (Burton, 1988). Most heat treated milk contains a slight amount of sediment, which is not usually sufficient to be a problem for the consumer (Burton, 1988). This appears to become clearly noticeable by taste above about 1 % (dwb). Good quality raw cow's milk usually shows high heat stability. For example, Chen (2013) found that the average sediment level was 0.19 % for UHT treatment of 25 samples of bulk cow's milk, collected at 2 weekly intervals (range 0.1–0.29 %). However, if heat stability is poor, then problems will arise and such milk should be avoided (see Sect. 10.2.1.2). It has been found that sediment increases with severity of the heat treatment of a particular heat process (Swartzel, 1983; Boumpa *et al.*, 2008) and is present in greater quantities following direct processes than indirect processes (Perkin *et al.*, 1973; Ramsey and Swartzel, 1984). Zadow (1978) found that little sediment was formed in UHT cow's milk if the pH was kept above 6.62; below this value sedimentation increased rapidly. In contrast,

sedimentation was severe in goat's milk when the pH was below 6.9. Similar trends were observed for concentrated skim milk, which was found to be stable above pH of 6.55 but below this value, severe sedimentation occurred (Zadow and Hardham, 1981). Lewis *et al.,* (2011) reported that addition of only 4.5 mM calcium chloride produced a voluminous sediment following UHT treatment. This could be reduced by addition of DSHP and TSC. Sediment formation was only slight for milk samples whose ethanol stability was greater than 80 %. Experiments adding calcium salts and reducing pH need to be done with care, so as to avoid blocking a UHT plant, as may easily happen.

Sediment formation is also much more of a problem in UHT goat's milk than cow's milk (Zadow *et al.*, 1983; Montilla and Calvo, 1997). In fact goat's milk behaves like cow's milk with added calcium chloride. Zadow *et al.,* (1983) found that adding DSHP reduced sediment in UHT goat's milk; adding 0.2 % DSHP reduced soluble Ca^{2+} concentrations from 3.2 to 1.4 mM, with pH adjustment. They also looked at variations of Ca^{2+} with pH, comparing goat's milk with cow's milk. They had no success with calcium fortification (using calcium chloride) of goat's milk. Montilla and Calvo (1997) reported that goat's milk heated at 135–150 °C showed a higher stability with added phosphate than those adjusted to alkaline pH. They speculated that phosphates improved stability by changes in Ca^{2+}, but they did not measure this. Boumpa *et al.,* (2008) investigated sediment formation during UHT treatment of goat's milk. Without stabiliser, UHT goat's milk produced considerable sediment. Addition of SHMP, DSHP and TSC each reduced Ca^{2+}, increased ethanol stability and reduced sediment. Chen *et al.,* (2012) found that when ionic calcium was increased in goat's milk by addition of calcium chloride, sediment increased drastically after UHT treatment. They also showed that DSHP and TSC addition decreased sediment formation but if too much was added, sediment would increase; in other words sediment formation went through a minimum with increasing additions of these compounds.

10.3 Stability During Storage

10.3.1 Chemical Changes

The proteins of sterilised milks undergo considerable change during storage which is dependent on both the temperature and time of storage. The extensive changes which occur in the proteins in UHT milk during storage are demonstrated by reversed-phase HPLC (see Fig. 10.3) (Al-Saadi and Deeth, 2008) and by 2-D electrophoresis (Holland *et al.*, 2011). The changes in the proteins have been assessed by various other chemical methods such as stability to added ethanol, ionic calcium or phosphate (Gaucher *et al.*, 2008, 2011), changes in non-protein nitrogen (NPN) and non-casein nitrogen (NCN), reduction in casein percentage and increases in content of high-molecular weight protein material. Changes in casein micelle size, hydration and zeta potential have also been measured (Gaucher *et al.*, 2011; Baglinière *et al.*, 2012). While the exact nature of all the changes has not been determined, several changes have been characterised. These include cross-linking, deamidation, glycosylation and proteolysis.

10.3.1.1 Protein Cross-Linking

In addition to the cross-linking which can occur during heat treatment (see Sect. 10.2.2.2), considerable cross-linking occurs in UHT milk during storage. The extent of cross-linking increases at elevated storage temperatures (Andrews, 1975; Al-Saadi and Deeth, 2008). The cross-linking has been observed using size-exclusion chromatography (Andrews and Cheeseman, 1972; Zin El-Din *et al.*, 1991) and one- (Al-Saadi and Deeth, 2008) and two-dimensional electrophoresis (Holland *et al.*, 2011). Cross-linking occurs between caseins with little involvement of whey proteins. α_{S1}-Casein is the major protein involved (Andrews, 1975; Holland *et al.*, 2011).

Lauber *et al.,* (2001) found that cross-linking during storage of UHT milk for 30 days at 37 and 50 °C increased from 14 % to 25 % and 63 %, respectively, while Andrews (1975) reported 21 and 50 % of the proteins were polymerised in UHT milk stored for 6 months at 4 °C and 37 °C,

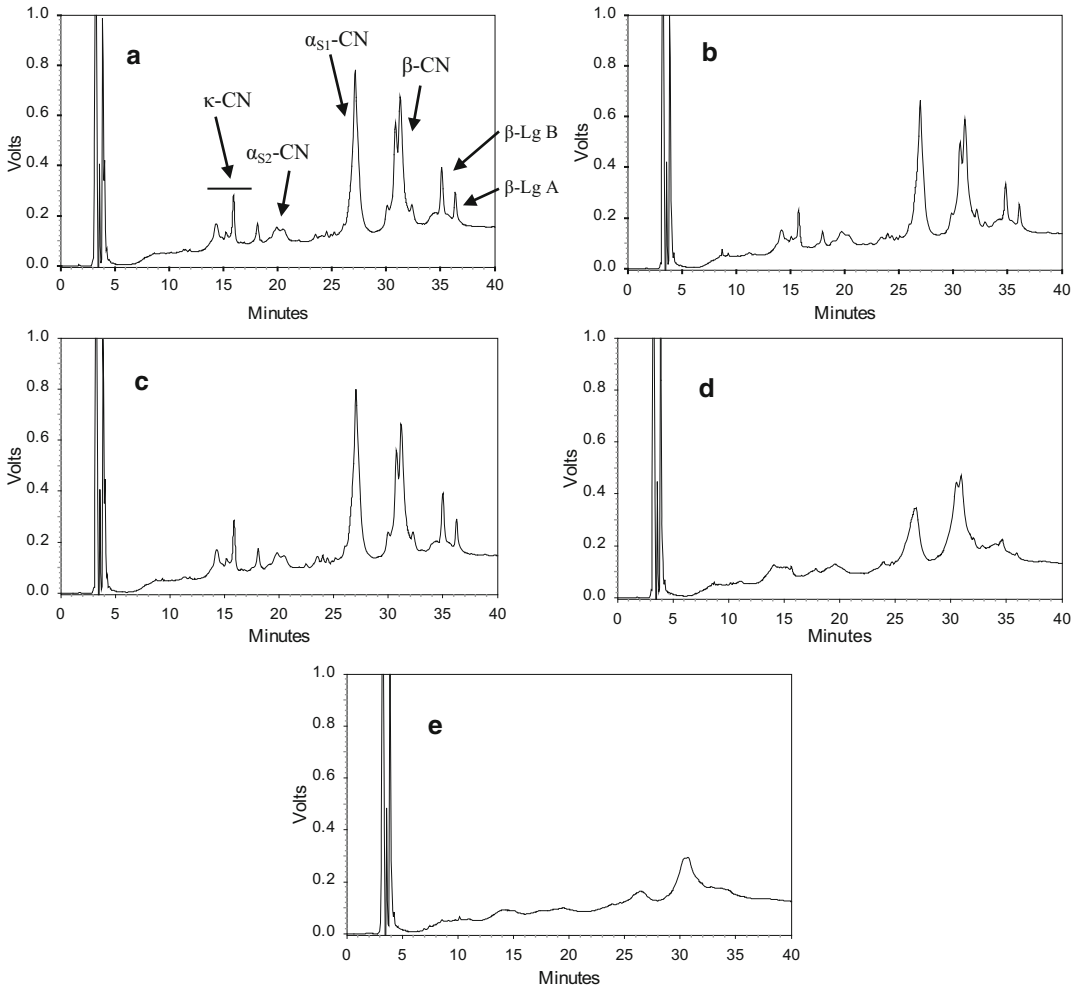

Fig. 10.3 Reversed-phase HPLC profiles of freshly processed UHT milk (*a*) and of UHT milks stored at 5 °C (*b*), 20 °C (*c*), 37 °C (*d*) and 45 °C (*e*) for 12 weeks (From Al-Saadi and Deeth, 2008; reproduced with permission)

respectively. Henle *et al.,* (1996) reported similar increases for UHT skim milk stored for 6 months at 4 and 27 °C, from 8.2 % to 11.7 % and 53.9 %, respectively. Al-Saadi and Deeth (2008) found about 37 % of the proteins in UHT milk were cross-linked after storage at 45 °C for 12 weeks. Protein cross-linking during storage has been associated with sedimentation of UHT milk (Andrews and Cheeseman, 1972) and has been implicated in reduced solubility of some milk powders (Le *et al.*, 2013).

Cattaneo *et al.,* (2008) suggested that lysinoalanine, a measure of cross-linking, was a sensitive index of the storage conditions of UHT milk. Henle *et al.,* (1996) measured lysinoalanine and histidinoalanine in stored UHT milks and calculated that they could account for only 14–50 % of the polymerised casein formed during storage. They hypothesised that other forms of cross-linking, probably related to the Maillard reaction, also occurred. While this has not been proven for sterilised milk, it has been shown to be significant in milk powders (Le *et al.*, 2013).

10.3.1.2 Deamidation

Non-enzymatic deamidation (amide hydrolysis) of the asparagine and glutamine residues of proteins to aspartic acid and glutamic acid,

respectively, and ammonia occurs during intense heating of milk (e.g., 140 °C for several minutes) and in sterilised milks during storage. It occurs to only a small extent during sterilisation, either in-container (van Boekel, 1999) or UHT (Holland *et al.*, 2011); however, significant deamidation occurs during storage of UHT-sterilised milk (Holland *et al.*, 2011). The highest rates of deamidation are observed for asparagine residues followed by glycines although significant rates also occur with asparagines next to serine and histidine residues. The reaction occurs *via* formation of a cyclic imide (succinimide or glutarimide for asparagine and glutamine, respectively). The extent of deamidation can be measured by the increase in non-protein nitrogen (NPN) (due to ammonia release) (Metwalli and van Boekel, 1998) or the appearance of new protein species with higher negative charge using isoelectric focussing as used in 2-D electrophoresis (Holland *et al.*, 2011). The 2-D gels of stored UHT whole milk clearly show new spots for deamidated proteins, particularly α_{S1}-casein, α-lactalbumin and β-lactoglobulin (Holland *et al.*, 2011, 2012). The extent of deamidation increased with temperature and time of storage; considerable deamidation occurred at 28 and 40 °C over 2 months.

There is commercial interest in deamidation as it has been reported to increase the heat stability of milk proteins, reduce fouling, improve some physical functional properties and improve the nutritional value of proteins, due to reduction of blocked lysines (Miwa *et al.*, 2010, Timmer-Keetels *et al.*, 2011). Targeted deamidation can be achieved by enzymatic treatment (Miwa *et al.*, 2010).

10.3.1.3 Lactosylation and Maillard Reactions

Lactosylation of lysine residues in milk proteins is the first step in the Maillard series of reactions which leads to brown discoloration, off-flavours, reduction in pH through production of acids such as formic acid, and, as discussed above, protein cross-linking. It is readily seen on 2-D electrophoresis gels of stored UHT milk in the spots for α-lactalbumin and β-lactoglobulin which appear as stacked spots with up to five spots per stack corresponding to proteins with molecular weights differing by the molecular weight of lactose, i.e., the proteins contain multiple molecules of lactose (Holland *et al.*, 2011). Lactosylation probably occurs on the caseins also but that is not obvious on 2-D gels. The lactosylation occurs during heating and continues during storage. It is often measured in the form of furosine, an acid degradation product, of the Amadori product lactulosyllysine. Furosine is used as an index of the severity of heat treatment but as lactosylation continues during storage, its value in stored UHT milk is limited. Recently lactosylated whey proteins have been quantified by mass spectrometric methods and proposed as a measure of the thermal history of UHT milk (Losito *et al.*, 2007); these methods would also have limited value for stored milk.

It has recently been shown that the Maillard reaction in UHT milk can be inhibited by adding polyphenols before UHT processing. Schamberger and Labuza (2007) found that the green tea flavonoids, epicatechin and epigallocatechin gallate, reduced the production of Maillard-associated fluorescence and colour changes (see Sect. 10.2.2.5).

10.3.1.4 Proteinases and Proteolysis

Proteolysis is a major destabilising process of milk proteins in sterilised milks. Its main effects are flavour change with production of bitter flavours, due to hydrophobic peptides, and physical destabilisation particularly gelation during storage. It is caused by two major types of proteinase, the indigenous milk proteinases of which plasmin (milk alkaline proteinase) is the most important, and bacterial proteinases produced by bacterial contaminants, mainly pseudomonads, in the raw milk prior to heat treatment. Both enzyme types are heat-resistant and under certain processing conditions can remain active in sterilised milks and cause proteolysis during storage. While plasmin is by far the most important milk proteinase, Gaucher *et al.,* (2008) reported that some of the 181 peptides they identified in stored UHT milk resulted from the action of elastin and cathespins G, B and D; the others were produced by plasmin and bacterial enzymes.

Plasmin exists in milk in a complex system containing its inactive precursor, plasminogen, together with activators and inhibitors which ultimately determine its activity in the product. In raw milk most exists as plasminogen which is converted to the active plasmin by plasminogen activators. Reported ratios of plasminogen to plasmin vary; for example, Richardson (1983) concluded the ratio was ~9 while Auldist *et al.,* (1996a) reported a range of ratios from ~5 to ~2. Auldist *et al.,* (1996a) examined the plasmin/plasminogen levels in milk with low and high somatic cell counts and from cows in early and late lactation and found that milk from cows in late lactation with high somatic cell counts had the highest levels of plasmin and also the highest plasmin/plasminogen ratio. Furthermore, the proteolysis levels, measured as non-casein nitrogen, in UHT milks prepared from the four types of raw milk increased during storage for 6 months to much higher levels in the high somatic cell count milk than in the low somatic cell count milk. For high somatic cell count milk, the late lactation milk exhibited more proteolysis than the early lactation milk but in the low somatic cell count milks there was no difference in the extent of proteolysis (Auldist *et al.*, 1996b). Somatic cells contain a plasminogen activator which enhances the conversion of inactive plasminogen to plasmin (Kelly and Foley, 1997).

The heat inactivation of plasmin is quite complex. At low levels of heat treatment such as pasteurisation the plasmin activity increases due to inactivation of inhibitors of the plasminogen activator which converts plasminogen to plasmin (Richardson, 1983). At higher temperatures, there is an increased inactivation such that in some UHT milk little or no plasmin remains. In general, indirect UHT processes inactivate more plasmin than direct processes. For example, Manji *et al.,* (1986) reported that indirectly processed UHT milk immediately after processing had no detectable plasmin activity but had 19 % of its original plasminogen level while directly processed milks had 19 and 37 % of the original plasmin and plasminogen, respectively. Plasminogen activators have high heat stability (Lu and Nielsen, 1993) and remain active in UHT milk and convert plasminogen to plasmin during storage (Manji *et al.*, 1986). It has also been shown that bacterial proteinases can activate the plasminogen to plasmin in milk and enhance plasmin activity (Nielsen, 2002).

The inactivation of plasmin is closely associated with denaturation of β-lactoglobulin through sulfhydryl–disulfide interactions (Kelly and Foley, 1997). This is indicated by the protection of plasmin activity by potassium iodate which oxidises the free sulfhydryl of denatured β-lactoglobulin and prevents interaction with plasmin (Kelly and Foley, 1997). This interaction with β-lactoglobulin during heating was used as the basis of a method of inactivating plasmin during the preheating stages of UHT processing by van Asselt *et al.,* (2008). They devised a pretreatment of 80 °C for 300 s which inactivated plasmin and prevented proteolysis in milk treated by an innovative steam injection method (150–180 °C for 0.2 s). Previously this very high temperature treatment had been shown to inactivate effectively highly heat-resistant bacterial spores but led to bitter flavours in stored UHT milk due to plasmin action (Huijs *et al.*, 2004). An alternative preheat treatment of at least 90 °C for 30 s was found to inactivate plasmin and prevent proteolysis of directly processed UHT reconstituted skim milk during storage (Newstead *et al.*, 2006).

The sterilisation temperature conditions are also important for controlling plasmin-induced proteolysis in UHT milk. This was demonstrated by Topçu *et al.,* (2006) who, using an indirect heating process, found that UHT milk processed at 150 °C rather than 140 °C showed less proteolysis during storage.

Bacterial proteinases are produced in raw milk at the end of the log phase of growth of psychrotrophic bacteria. This is when the count exceeds ~10^6 cfu/mL. However, the count at which proteinases are produced and can cause proteolysis depends on the nature of the bacteria present; milk can have 10^7 cfu/mL of psychrotrophs but contain no proteinase (Haryani *et al.*, 2003). Similar results were reported by Baglinière *et al.,* (2012) for nine strains of *P. fluorescens* grown to ~2.5×10^6 cfu/mL; three produced no proteinase, one a small amount and six consider-

able amounts. The significance of these bacterial proteinases in sterilised milks is their extreme heat resistance. For example, Griffiths *et al.,* (1981) showed that *Pseudomonas* proteinases retained 20–40 % of their activity after exposure to 140 °C for 5 s while Mitchell and Ewings (1985) reported decimal reduction times at 140 °C ($D_{140\,°C}$) ranging from 2 to 300 s.

More bacterial enzyme mediated proteolysis occurs in directly heated UHT milk than in indirectly heated milk. This is due to the greater inactivation of the proteinases by the higher overall heat load of the indirect treatment (Corradini and Pecchini, 1981). As an alternative to using a high heat intensity to inactivate the bacterial proteinases, a low temperature inactivation (LTI) treatment has been shown to be effective. It involves treatment at ~55 °C for 30–60 min (Barach *et al.*, 1976) although the exact effective conditions may vary for different proteinases. It can be applied either before or after UHT treatment and is most effective when applied 1 day after UHT processing. Explanations for the effectiveness of the unusual process are that the enzyme autodigests at ~55 °C or that it undergoes a conformation change and interaction with casein to form an enzymatically inactive proteinase–casein complex. The use of LTI with UHT sterilization has been shown to reduce proteolysis and increase the shelf-life of UHT milk containing psychrotrophic bacterial proteinases (West *et al.*, 1978; Kocak and Zadow, 1985c).

The fat content of milk affects the level of proteolysis in UHT milk. López Fandiño *et al.,* (1993a) found greater proteolysis during storage in UHT skim milks than in UHT whole milks subjected to the same UHT treatments. The finding applied to proteolysis by both plasmin and bacterial proteinases. This suggests that UHT heating conditions should be more severe for skim milk than for whole milk, a practice followed by some processors.

Plasmin and bacterial proteinases have different specificities for individual caseins. The preferred substrates for plasmin are β- and α_{S2}-casein; α_{S1}-casein is a poor substrate (de Rham and Andrews, 1982; Richardson, 1983, McSweeney *et al.*, 1993, 1994). κ-Casein is generally considered to be resistant (Eigel, 1977). Plasmin does not hydrolyse native whey proteins, in fact, whey proteins have some inhibitory effects on plasmin activity (Politis *et al.*, 1993). While an array of peptides are produced from plasmin action in milk, γ-casein and proteose peptones are the major ones.

In contrast to plasmin, many bacterial proteinases have a preference for κ-casein, in much the same way as does chymosin in rennet, but can also hydrolyse other caseins (Law *et al.*, 1977). Miralles *et al.,* (2003) showed that the peptides released from κ-casein by *Pseudomonas fluorescens* B52 were *para*-κ-casein, fragment 1-105, and related peptides 1-103, 1-104, 1-106 and 1-107. Therefore the *Pseudomonas* proteinase is less specific than chymosin. However, not all *Pseudomonas* proteinases have the same specificity. For example, Carini and Todesco (1977) and Deepa and Mathur (1994) reported that β-casein was degraded most, although the possibility of plasmin causing the degradation in these cases cannot be excluded. Recently Baglinière *et al.,* (2012) reported that the specificity of proteinases from five strains of *P. fluorescens* was in the order β- > α_{S1}- > κ- > α_{S2}-casein. These differences in reported casein preferences can be attributed to the use of different bacterial species and strains and/or different experimental conditions (Nicodème *et al.*, 2005).

The difference in the peptides released by plasmin and bacterial proteinases forms the basis of analytical methods for distinguishing between the two types of proteolysis. The peptides released by plasmin are larger and more hydrophobic than those released by bacterial proteinases. Hence the former precipitate in 4 % trichloroacetic acid (TCA) and are soluble at pH 4.6 while the latter are soluble in both 4 % TCA and at pH 4.6. Furthermore, the plasmin peptides elute from reversed phase HPLC much later than the peptides produced from bacterial proteinases (López Fandiño *et al.*, 1993b; Datta and Deeth, 2003).

Measuring the low levels of bacterial proteinases that could cause proteolysis in UHT milk during storage is quite difficult. If this could be achieved, suspect milk could be diverted to other uses. Several methods have been proposed (Cliffe

and Law, 1982; Kwan *et al.*, 1983; Christen and Senica, 1987; Fairbairn, 1989; Chen *et al.*, 2003) but to date none has been universally accepted. The ideal method should be specific for the bacterial enzymes and relate well to the action of the enzyme in milk. Thus it should involve the use of the natural substrate, i.e., casein or casein derivative, rather than an alternative substrate. Button *et al.,* (2011) developed such a method using fluorogenic FITC-casein which was able to detect very low levels of proteinase activity using long assay incubation times. It could detect proteinase in UHT milk containing 0.0003 % of a cell-free culture of *P. fluorescens* (grown to ~10^6 cells/mL) using a 10-day incubation, and in milk with 0.003 % addition in an 8-h incubation. The 0.0003 % addition was the lowest addition level to cause measurable proteolysis in UHT milk during room temperature storage.

Proteolysis by bacterial proteinases is easier to measure than trace levels of bacterial proteinase activity. Several methods have been developed to study this phenomenon. Commonly used methods include measurement of NPN (12 % TCA-soluble N) and non-casein nitrogen (NCN) (pH 4.6-soluble N), measurement of free amino groups using fluorescamine, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and *O*-phthalaldehyde (OPA), as well as HPLC and electrophoretic methods (Recio *et al.*, 1996; Al Kanhal, 2000; Datta and Deeth, 2003; Le *et al.*, 2006).

10.3.2 Physical Changes

10.3.2.1 Age Gelation

Gelation during storage (age gelation) is a major change in sterilised milks which limits their shelf-lives. This is particularly an issue in countries where the expected shelf-life is greater than 6 months and in some cases up to 12 months. It is not so much an issue where the expected shelf life is much less, for example, 12 weeks (McKellar *et al.*, 1984). Erasmo *et al.,* (1981) even suggested that the recommended storage time for UHT milk should be reduced from 4 to 2 months because of the problem of gelation.

Typically in milks in which gelation occurs, the viscosity of the milk increases gradually during storage and then sharply immediately before gelation, when the viscosity reaches ~10 mPa s. Some authors have observed a decrease in viscosity, i.e., a thinning effect, before the viscosity increase (e.g., de Koning *et al.*, 1985). The gelation is irreversible and generally renders the product unacceptable. Some authors (e.g., Snoeren *et al.*, 1979) refer to the gelation as coagulation and liken it to rennet coagulation. Several authors (e.g., Visser, 1981) also refer to sedimentation in sterilised milks and it is not always clear whether this is the same as or different from gelation. This variation in nomenclature causes some difficulty in interpreting the mechanism of so-called gelation. It is highly probable that different mechanisms apply in different situations.

In the previous edition of this book, Nieuwenhuijse and van Boekel (2003) presented an excellent summary of the literature in a figure entitled "Simplified scheme for the various pathways for destabilisation of the protein in milk and milk products". In it they identified four types of gel: particle gel, fine-stranded gel, gel by bridging flocculation and gelled sediment. The associated causes of the gelation were identified as: bacterial proteolysis, plasmin proteolysis, low-temperature storage and high-temperature storage, respectively.

Age gelation has been extensively researched and reviewed (e.g., Harwalkar, 1992; Datta and Deeth, 2001; Nieuwenhuijse and van Boekel, 2003, Chavan *et al.*, 2011). Several different factors which promote gelation, and some which delay or inhibit gelation, have been identified and mechanisms of the gelation process have been proposed. However, the precise biochemical and/or physical changes which cause the gelation have not been elucidated. It is possible that the gelation occurs *via* different mechanisms in different products; this is certainly the case for unconcentrated milks and concentrated milks.

The factors which have been shown to affect the rate, and possibly the nature, of gelation

include: proteolysis by native plasmin or bacterial proteinases, severity and nature of the sterilisation heating, temperature of storage, and various additives.

Proteolysis

Proteolysis is the factor which has attracted most attention. It has been shown by many, but not all, researchers to be highly significant in age gelation of unconcentrated milk. It is widely considered not to be a factor in age gelation of concentrated milk; however, McKenna and Singh (1991) concluded that proteolytic processes may be involved in gelation of UHT reconstituted concentrated skim milk. The onset of gelation in unconcentrated milk is often, but not always, associated with bitterness caused by the released peptides (McKellar *et al.*, 1984; Mitchell and Ewings, 1985). Conversely bitterness may develop in a milk without gelation occurring.

Proteolysis caused by plasmin has been identified as a definite cause of gelation in milks known to be free of bacterial enzymes. However, plasmin may be present together with bacterial proteinases and in such cases it is difficult to identify which enzyme is responsible for the gelation. As discussed in Sect. 10.3.1.1, directly processed UHT sterilised milk contains more plasminogen/plasmin than indirectly processed milk. As a result, gelation occurs earlier in directly processed milk than in indirectly processed milk (McKellar *et al.*, 1984). However, the time to gelation does not always correlate with the extent of proteolysis (Manji *et al.*, 1986).

Bacterial proteolysis and its relationship to age gelation has attracted a great deal of attention. Many authors have shown that either addition of bacterial proteinases to, or growth of proteolytic bacteria in, milk before sterilisation or bacterial proteinases added aseptically after sterilisation can result in gelation during storage. Very low concentrations of proteinase can cause sufficient proteolysis to cause gelation during storage at room temperature. For example, Button *et al.,* (2011) reported that 0.0003 % (v/v) of a cell-free milk culture of *P. fluorescens* (grown to ~10^6 cells/mL) aseptically added to UHT milk caused sufficient proteolysis, measured as 12 μM free amino acids (Leu-Gly equivalents), in 5 months to cause gelation. Proteolysis was measured by the fluorescamine method (Chism *et al.*, 1979). Several authors have attempted to correlate levels of bacterial proteinase activity with shelf-life as determined by the onset of bitterness or gelation/coagulation. For example, Mitchell and Ewings (1985) determined a level of proteinase of ~0.3 ng/mL for a shelf-life of at least 4 months while Richardson and Newstead (1979) suggested a level of 1–2 ng/mL for a storage life of at least 3 months. While these levels are a useful guide, it must be stressed that the threshold limits will vary with the proteinase as these enzymes differ in specific activity and in substrate specificity and hence in the peptides they produce in UHT milk.

It has been reported that the gels produced in UHT whole milks as a result of proteolysis by plasmin and bacterial proteinase differ in appearance. Bacterial proteinases lead to formation of a custard-like gel (Harwalkar, 1992) whereas plasmin proteolysis causes a creamy surface layer which thickens into a curd-like layer (Hardham, 1998). The gels have also been examined microscopically. Gels caused by bacterial proteinases have a tighter protein network with thicker strands and contain more intact casein micelles and micelle aggregates than plasmin-initiated gels. de Koning *et al.,* (1985) showed that a UHT skim milk gel had a thread-like structure containing casein micelles which were almost completely disintegrated. Corresponding concentrated milk gels showed a network containing aggregated, partly-deformed casein micelles which had not been degraded by proteolysis.

Severity and Nature of the Sterilisation Heating

It is well documented that, for unconcentrated milk, the more severe the sterilisation heating, i.e., the higher the C* (Tran *et al.*, 2008), the less the risk of gelation or the longer time required for gelation to occur (see Sect. 10.1.2.2). As discussed above, milks subjected to direct UHT treatment were more susceptible to gelation than milks processed by indirect methods because the severity of heating for the same bactericidal

effect is greater, i.e., have higher C* (Tran *et al.*, 2008). Within each of these modes of UHT heating, the processes with the highest C* lead to the lowest incidence of gelation (e.g., Topçu *et al.*, 2006). In milks subjected to the most severe sterilisation heating (*i.e.* in-container sterilisation) gelation seldom occurs (Samel *et al.*, 1971). However, Harwalkar *et al.,* (1983) reported that in-container sterilised evaporated milk occasionally exhibits gelation on storage; they found that cold storage of the concentrate before sterilisation promoted thickening and gelation of the product during storage and samples sterilised without cold storage were stable.

The major reason advanced for the improved stability of milk subjected to the more intense treatments is inactivation of proteinases. While this is obviously a major factor, it is possible there are other physico-chemical factors also involved.

Temperature of Storage

The reports are quite consistent that storage at low temperature, i.e., ~4 °C, and high-temperature (>~30 °C) retard gelation compared with the intermediate temperatures (Kocak and Zadow, 1985a; Manji and Kakuda, 1986; Gaafar and El Sayed, 1991). Unfortunately, the temperatures favourable to gelation development are the normal room-temperature storage conditions. The mechanism for the retardation of gel formation at the higher temperatures has not been definitively elucidated but it has been suggested that at these temperatures proteolysis is too fast and gel development is prevented (Manji *et al.*, 1986). It has also been suggested that the protein cross-linking which is known to occur at these temperatures could stabilise the micelles and prevent release from casein micelles into the milk serum of proteins or protein complexes such as the β-lactoglobulin–κ-casein-complex (see Sect. 10.3.2.1.5) which could form a gel (McMahon, 1996). As noted above, the cross-linking could be *via* dehydroalanine forming linkages between lysine and alanine or histidine on adjacent molecules, or *via* advanced Maillard reaction products such as methylglyoxal. This is consistent with the suggestion of Samel *et al.,* (1971) that gelation may be inhibited if regions of proteins that would otherwise be involved in protein-protein interactions were blocked by interactions with lysine, such as with lactose in the first step of the Maillard reaction.

Additives

Various additives, both food-compatible and food-incompatible, have been investigated for their effect on age gelation. Some have been investigated for practical use while others have been investigated in relation to determining the mechanism of gelation. The most common additive is SHMP commonly known as polyphosphate although this is a non-specific term which can also apply to other phosphates. SHMP has been shown to have a beneficial effect by considerably extending the time to gelation although, as Kocak and Zadow (1985c) warned, it varies in its effectiveness depending on the supplier. It is added routinely by some UHT milk processors at a rate of about 0.1 % w/w. Interestingly, it does not influence proteolysis and hence milk containing SHMP can be considerably proteolysed but still not gelled. According to a proposed mechanism, proteinase-induced gelation is a two-stage process where the first stage is proteolysis of a casein or caseins which releases proteins into the serum and the second stage involves storage-induced physico-chemical changes which result in aggregation of destabilised casein micelles (Kocak and Zadow, 1985a). Therefore if SHMP does not affect proteolysis it must affect the second stage, possibly by interacting with caseins and hindering aggregation or altering the charge on the micelle. SHMP certainly stabilises the casein because milk containing it maintains a high ethanol stability throughout storage while milks which gel show decreased ethanol stability. SHMP also decreases ionic calcium (Mittal *et al.*, 1990) which may be related to the high ethanol stability, often used as an indicator heat stability. Curiously, Mittal *et al.,* (1990) found that the heat stability of UHT recombined milk containing SHMP declined more during storage than control milk without SHMP.

In contrast to polyphosphate, addition of monophosphates accelerates gelation. In an electron

microscopic study of UHT concentrated skim milk, Harwalkar and Vreeman (1978) showed that the structure of the casein micelles in milk with orthophosphate was similar to that of micelles in milk with no additive, that is, the micelles were slightly distorted, had thread-like tails on their perimeters and aggregated into interconnected chains at gelation. The micelles in corresponding milk containing SHMP did not change during storage and remained separated. It is interesting that the ability of UHT milk to withstand increasing amounts of added phosphate (0.5 M KH_2PO_4) with heating (100 °C for 10 min), without coagulating is used as a measure of its stability (Gaucher *et al.*, 2008). Unstable milks have very low phosphate test values, i.e., very little added phosphate causes instability.

The addition of potassium iodate to milk before UHT treatment results in increased plasmin activity in the UHT milk (Grufferty and Fox, 1986; Kelly and Foley, 1997). The mode of action of iodate appears to be in preventing thiol–disulfide interchange between denatured β-lactoglobulin and plasmin (see Sect. 2.2.2) and hence reducing the level of inactivation of plasmin (Skudder 1981; Grufferty and Fox, 1986). Inhibition of thiol–disulfide interchange reactions and subsequent aggregation of β-lactoglobulin was also used to explain the reduction by iodate of fouling during UHT heating (Skudder *et al.*, 1981).

Mechanisms

There have been several attempts at elucidating the mechanism of age gelation in sterilised milk. These have been mostly based around a central role of proteolysis together with physicochemical effects (Nieuwenhuijse and van Boekel, 2003). Any overarching mechanism must take into account the facts that: (1) proteolysis by plasmin and bacterial proteinases enhance gelation in unconcentrated milk but not concentrated milk; (2) in unconcentrated milk, the degree of proteolysis often does not correlate well with gelation and (3) gelation is retarded when the milk is stored at >~30 °C. A variation of the two-stage process mentioned in the previous section in which a proteolysis stage is followed by aggregation of destabilised casein micelles (Kocak and Zadow, 1985a) is that proposed by McMahon (1996) in which the proteolysis stage is followed by formation of a gel network by the β-lactoglobulin–κ-casein-complex released from the casein micelle, after it reaches a certain critical concentration in the serum; the network incorporates casein micelles (McMahon, 1996). McMahon (1996) suggested the reason why gelation was retarded at higher storage temperatures was that intramicellar covalent cross-linking occurred which prevented release of the β-lactoglobulin–κ-casein complex.

A three-stage mechanism was proposed by Deepa and Mathur (1994) in which the first was proteolysis, as in the two-stage models above, the second stage was the enzymatic formation of plastein products by synthesis from smaller released peptides and the third was the aggregation of the plastein peptides by non-covalent bonds and possibly hydrophobic interaction of the plastein peptides with hydrophobic regions of casein micelles. Plastein formation from caseins, particularly β-casein was reported by Sukan and Andrews (1982). Andrews and Alichanidis (1990) confirmed the formation of plastein from caseins and that under some circumstances the plastein can form a gel; however, they concluded that the plasteins were formed by non-covalent bonding and that plastein gels were reversible. This does not accord with gels in sterilised milk formed during storage which are not reversible and casts doubt on this proposed mechanism

10.3.2.2 Sedimentation

Sediment formation during storage can be a major problem for UHT milk (Vesconsi *et al.*, 2012), particularly concentrated milk (Dalgleish, 1992). It is initiated during sterilisation of milk and is influenced by the extent and nature of the heat treatment as well as milk compositional factors (see Sect. 10.2.3.2). However, it increases with both storage time and temperature (Ramsey and Swartzel, 1984). The level of fat in the milk has been found to not affect sediment formation (Hawran *et al.*, 1985).

Dalgleish (1992) concluded that in undisturbed UHT milk even native casein micelles

tend to sediment by gravity during storage but the rate of sedimentation increases significantly as the molecular weight of the micelles increases. Regular disturbance of UHT milk packages decreases sediment formation.

10.4 Nutritional Implications

Much of the interest in the nutritional implications of the proteins in sterilised milks has focussed on the reduced availability of essential amino acids, particularly lysine. As discussed in previous sections, lysine is the primary target of the Maillard reaction and also cross-linking reactions involving dehydroalanine. Both series of reactions are initiated during heating and continue during storage in a temperature- and time-dependent manner. Andersson and Öste (1995) concluded that the loss of lysine in UHT milk (up to about 5 %) has little nutritional significance. Langhendries *et al.,* (1992) reported a loss of 6.2 % in UHT milk but a much greater loss, 15.6 %, in in-can sterilised milk. Higher lysine losses occur in UHT lactose-hydrolysed milks because of the higher Maillard reactivity of the monosaccharides over lactose (Renzschauen, 1983). As mentioned in Sect. 10.2, some cystine and methionine residues are degraded to volatile sulfur compounds during sterilisation but again the extent of the destruction of these amino acids is minimal and considered to be of little nutritional significance (Hurrell *et al.*, 1980).

The formation of Maillard reaction products and lysinoalanine cross-links during heating and storage has potential toxicological consequences. Animal experiments have shown that both products can cause histological changes in the kidney although adverse effects in humans is considered unlikely (Fritsch *et al.*, 1983). Langhendries *et al.,* (1992) used UHT milk and in-can sterilised milk in feeding trials with healthy preterm babies and found no evidence of changes in their kidney function. Rerat *et al.,* (2002) concluded from pig feeding trials with skim milk intensely heated (to cause 50 % reduction in available lysine) that the levels of Maillard reaction products normally encountered in heated milks would have negligible nutritional consequences.

Several authors have reported that UHT processing does not adversely affect the digestibility of milk proteins (e.g., Povoa and Moraes-Santos, 1997). Lonnerdal and Hernell (1998) found that infants fed a UHT-treated formula had similar metabolic functions to those of breast-fed infants. Lacroix *et al.,* (2008) used a sensitive method based on milk labelled with ^{15}N to assess the effects of pasteurised, microfiltered and UHT milk on postprandial kinetics in humans and found a higher anabolic use of the proteins from the UHT milk than from the pasteurised and microfiltered milks. They attributed the difference to the consequences of the UHT heat treatment, in particular to the greater accessibility of the proteins to enzymatic digestion due to loosening of the casein micellar structure and to the presence of small soluble aggregates of proteins dissociated from the casein micelle. However, Carbonaro *et al.,* (1998) found that the *in vitro* digestibility of whey proteins from UHT milk and in-container sterilised milk was reduced by the heat treatments and suggested this was due to aggregation of the proteins which reduced their accessibility to proteolytic enzymes. The reduction in digestibility increased with the severity of the heat treatment.

Al Kanhal *et al.,* (2001) examined the nutritional quality of the protein in fresh and reconstituted UHT milk at manufacture and after storage for up to 6 months. They found no difference between the fresh UHT milk at manufacture and pasteurised milk but the reconstituted UHT milk had lower protein quality at manufacture than the pasteurised milk. The nutritional quality of both UHT milks decreased during storage; the net protein utilisation values of the protein in the fresh and reconstituted UHT milks after storage for 6 months were 5 % and 12 % lower than at manufacture, respectively.

In summary, UHT treatment has little effect on the biological value of milk proteins and may even increase their utilisation in humans. In-container sterilisation causes substantially more change in the proteins than UHT treatment

and may significantly reduce their biological value. The nutritional quality of proteins in sterilised lactose-hydrolysed and reconstituted milks is lower than that of the corresponding unmodified and fresh products. Storage of all sterilised milks decreases the nutritional quality of their proteins in a temperature-dependent manner.

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Enzymatic Coagulation of Milk

11

M. Corredig and E. Salvatore

Abstract

The enzymatic gelation of milk is one of the most studied topics in food colloids, as the reaction is based on the gradual hydrolysis of a polyelectrolyte layer on the surface of the casein micelles. The loss of the polyelectrolyte layer causes the destabilization of these protein particles in milk, and results in aggregates and a gel network. This chapter discusses the mechanisms of rennet-induced gelation of milk, with particular attention to the release of caseinomacropeptide from κ-casein and the rennet-induced aggregation of casein micelles. These two phases are strictly dependent on temperature, pH and the presence of calcium and other components in milk. The recent findings on the effects of heating, high pressure and concentration on enzymatic coagulation are also described.

11.1 Introduction

The process of cheese making is based on a simple, specific, proteolytic reaction that causes the destabilization of protein particles in milk, which aggregate and form a gel network. The final structure of the gel will be a function of other factors, including, but not limited to, pH, calcium concentration and milk processing history. By modulating the early stages of structure formation or manipulating the cheese curd it is possible to obtain the large variety of cheese products available today.

Cheese curd is a protein-based gel in which the hydrolyzed casein micelles form a continuous network containing fat globules. The reaction is initiated by the enzyme chymosin (in most rennets), which hydrolyzes specifically κ-casein, a protein present on the surface of the casein micelles. Upon hydrolysis of this protein, the overall free energy of association between the micelles decreases and the casein particles stick to one another forming a three-dimensional gel.

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The present chapter will outline the current understanding of the mechanism of rennet-induced gelation of the proteins in milk. The reader is also encouraged to consult previous reviews for fundamental aspects related to the kinetics of the enzymatic reaction as well as the models of rennet coagulation, as they have been well described (Hyslop, 2003; Horne and Banks, 2004). This chapter will focus on the preliminary stages of aggregation, as well as the recent findings on the effect of environmental and processing conditions on the enzymatic gelation of casein micelles. The effect of extrinsic factors on the aggregation of caseins in milk continues to be an important topic, as manufacturing practices evolve, and products are no longer obtained only using traditional methods, but other ingredients and more advanced processing technologies are put in place for increasing yields, modifying texture, creating novel products, responding to the demands of a very competitive market.

A number of experimental approaches have been developed over the years to follow the primary stages of rennet-induced aggregation of the casein micelles, as these initial coagulation steps are thought to be critical to the development of the final gel network. However, much less is understood, from a fundamental standpoint, about the rearrangements of the protein network after gelation, as studies at the molecular level become really challenging at this point. This phase is critical and affected by intrinsic and extrinsic factors, such as for example, temperature, salt concentration, time of cutting, or manipulations of the gel. These manipulations after gelation as well as ripening will not be discussed in this chapter, as they are beyond the scope of this review; however, they ultimately affect the final properties of the cheese (Fox *et al*., 2000, 2004).

11.2 Rennets

Rennet coagulation of milk is a key step in the preparation of several dairy products. Traditionally, the separation of milk into curds and whey is caused by the addition of an extract from the abomasum (the fourth and last compartment of the stomach of ruminants). The most common rennet extract derives from unweaned calves; however, in the Mediterranean areas, rennet paste from small ruminants (lambs or kids) is also used to produce cheeses. In this case, the final products are characterized by complex flavor profiles due to the presence of other enzymes, for example, pre-gastric and gastric lipases in the rennet extracts. These lipolytic enzymes are absent from calf rennet or are present at much lower concentrations.

Animal rennet extracts are mainly comprised of three enzymes, belonging to the pepsin-like family of aspartic proteinases: chymosin (EC 3.4.23.4), pepsin A (EC 3.4.23.1), and gastricsin (EC 3.4.23.3). The proportion of these enzymes depends on the feeding regime and the age of the animal at slaughter. In particular, the proportion of pepsin increases in adult cattle, whilst chymosin is reduced. Chymosin is the specific milk-clotting enzyme contained in rennet, and it cleaves the Phe_{105}-Met_{106} bond of κ-casein, while pepsin A and gastricsin are generic proteases, which exhibit less specific activity.

For cheese production, it is critical to have a weak general proteolytic activity and narrow specificity. High proteolytic activity will result in the production of small and medium-sized peptides, which will impart bitter or unpleasant notes to the cheese and reduce yield. The clotting activity, expressed as International Milk-Clotting Units (IMCU), refers to the specific ability of the enzyme to cleave the Phe_{105}-Met_{106} bond of κ-casein, starting the coagulation process, while the general proteolytic activity is linked to the hydrolysis of peptide bonds elsewhere in the casein system. A low ratio of clotting (C)/proteolytic (P) activity may cause a reduction of cheese yield, losses of fat and protein in the whey and the onset of defects, such as bitterness, during cheese ripening. The residues of the enzyme in cheese curd can lead to the formation of peptides responsible for bitter taste during ripening, and also have a role in cheese texture development.

There are other sources of coagulant enzymes generally indicated as rennet substitutes, and may have different origins (recombinant, animal,

microbial and vegetable). The performances of calf rennet and fermentation-produced chymosins in terms of yield and C/P ratio are higher than for other enzymes (Barbano and Rasmussen, 1992; Crabbe, 2004).

Nowadays, most chymosin is produced by fermentation. Calf chymosin (genetic variant B) is widely produced through recombinant DNA technology by microorganisms such as *Aspergillus niger* and *Kluyveromyces lactis* (Jaros *et al*., 2008). Camel chymosin is also commercially available, produced by *Aspergillus niger*. This enzyme has 85 % sequence identity with bovine chymosin and when added to cow's milk, it has a reduced generic proteolytic ability with 70 % higher specificity compared to bovine chymosin (Kappeler *et al*., 2006). Bovine chymosin, on the other hand, cannot cause gelation of camel milk (Kappeler *et al*., 2006). These differences are attributed to the differences in the amino acid residues of the substrate binding site, causing a better association between camel chymosin and κ-casein in bovine milk (Jensen *et al*., 2013). Other recombinant chymosins have been obtained from goat and buffalo. In particular, goat chymosin seems to have the highest catalytic specificity compared to the other enzymes (Vallejo *et al*., 2012).

Rennet substitutes from other sources, such as animals, and plants or microorganisms have been used for cheese production. The substitutes of animal origin are extracts of pepsin A from pig or chicken stomachs. Microbial coagulants, containing proteinases (EC 3.4.23.6) are commercially extracted from three microorganisms: *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica*. Plant proteinases are extensively used in the production of raw sheep and goat milk cheeses from Spain and Portugal. These coagulants (mainly two aspartic proteinases such as cardosin A and B) are extracted from the flowers of *Cynara cardunculus*, and are characterized by a high specific milk-clotting activity, but also by a higher proteolytic activity throughout cheese ripening compared to chymosin or calf rennet (Galán *et al*., 2008, 2012; Pino *et al*., 2009). The higher proteolytic activity of plant enzymes compared to bovine chymosin allows optimal sensory characteristics for cheeses to be obtained in shorter times, causing an acceleration of the ripening process. Mathematical models have been proposed to relate the concentration of these enzymes with milk clotting time, and to evaluate their proteolytic activity on different milk samples (Silva and Malcata, 2005).

11.2.1 Chymosin

Chymosin belongs to the family of aspartic proteinases, which are widely distributed in many organisms and tissues. The structure-function relationships of various aspartic proteinases have been extensively reviewed (Chitpinityol and Crabbe;, 1998; Crabbe, 2004). Bovine chymosin is a globular protein consisting of 323 residues and presenting a 55 % sequence homology with pepsin. Natural calf chymosin is found in three isoforms: A, B and C. The difference between A and B forms is related to a different amino acid residue in the position 243 of the molecular chain, which is an Asp in the A form and a Gly in the B form (Chitpinityol and Crabbe, 1998). The isoform B is the most abundant form and most stable, while chymosin C is a degradation product of the A isoform (Danley and Geoghegan, 1988).

The three-dimensional structure and active site geometry of aspartic proteinases have been widely studied using X-ray crystallography (Gilliland *et al*., 1990; Sielecki *et al*., 1990; Newman *et al*., 1991, 1993). A high resolution (1.8 Å) study has been recently published using X-ray synchrotron data (Jensen *et al*., 2013). The major differences among aspartic proteinases are related to a basic region for the substrate binding specificity, the so-called flap region, the movement of which is essential for enzyme-substrate binding. In bovine chymosin, this flap corresponds to amino acid residues 73–85.

The substrate-binding pockets of chymosin for κ-casein have been previously described (Gilliland *et al*., 1990; Newman *et al*., 1991; Chitpinityol and Crabbe, 1998). Both bovine and camel chymosins have positively charged patches on their surface, which are responsible for interactions with the negatively charged

κ-casein (Jensen *et al*., 2013). Bovine chymosin has two similar β-barrel domains, with an active site consisting of Asp-Thr-Gly amino acid residues in N- and C-terminal lobes of chymosin (Newman *et al*., 1991). An interdomain region held together by hydrogen bridges is present between the two domains, often referred to as "fireman's grip". This area consists of two catalytic aspartic residues containing an activated water molecule (Gilliland *et al*., 1990; Sielecki *et al*., 1990; Newman *et al*., 1991). The specificity of chymosin for κ-casein arises from the amino acid sequence in the region 98–102 of the substrate molecule. This sequence consists of alternating His and Pro residues, acting as an allosteric regulator, converting the self-inhibited form of chymosin to the active form (Visser *et al*., 1987; Gutchina *et al*., 1996). This amino acid cluster binds to the C-terminal domain of chymosin (Palmer *et al*., 2010). The two lysine residues at positions 111 and 112 contribute to form positively charged clusters on the surface of κ-casein, that facilitate the docking of the chymosin enzyme at the cleavage site (Farrell *et al*., 1999). Camel chymosin differs from bovine chymosin by two amino acid residues: Lys_{221} and Val_{223} in bovine chymosin are substituted with Val_{221} and Phe_{223} in the camel enzyme. This difference, as already mentioned, affects the geometric and charge characteristics of the substrate binding site, improving its specificity for κ-casein (Palmer *et al*., 2010; Sørensen *et al*., 2011; Jensen *et al*., 2013).

11.3 The Initial Stages of Milk Renneting

κ-casein molecules are not free in solution, but are part of a supramolecular structure, the casein micelle. The location of κ-casein on the casein micelles is of particular relevance to rennet-induced gelation of milk. The cleavage of κ-casein causes destabilization of the micelles, and precipitation of the proteins. Casein micelles are highly hydrated particles (about 3.5 g of water per g of protein) which are composed of four different caseins (α_{s1}-,α_{s2}-, β-and κ-casein), with a diameter ranging between 80 and 400 nm. More details on the structure of these particles can be found elsewhere (see for example, Dalgleish and Corredig, 2012; de Kruif *et al*., 2012). The caseins are held together by non-covalent forces; hydrogen bonding, electrostatic interactions, van der Waals, hydrophobic interactions, and salt bridges between phosphoserine residues and colloidal calcium phosphate, are all present in the micelles to form small clusters (about 800 of them) of 2 nm in size (de Kruif *et al*., 2012). Figure 11.1 shows the microstructure of casein micelles as analyzed using cryo-transmission electron microscopy (cryo-TEM). The micelles appear spherical in nature with a diameter around 140 nm, and with a rugged surface. The darker areas in the images have been attributed to the calcium phosphate nanoclusters (Marchin *et al*., 2007). κ-Casein lacks phosphate centers and cannot participate in the formation of the nanoclusters; hence, while β-casein and α_s-caseins are predominantly found in the interior, κ-casein is present on the surface of the casein micelles (Dalgleish *et al*., 1989). The protein extends in solution forming a polyelectrolyte layer ("the hairy layer"). This layer creates steric and charge repulsion between the protein particles, providing stability (de Kruif and Zhulina, 1996). The gelation of casein micelles by renneting is then the result of two important reactions: the hydrolysis of the polyelectrolyte layer of the casein micelles and the destabilization and aggregation of the casein micelles.

During the enzymatic reaction, the hydrodynamic diameter of the casein micelles, measured by dynamic light scattering, decreases by about 10 nm (Walstra *et al*., 1981; Horne and Davidson, 1993). The viscosity of the milk also shows a decrease, which has been attributed to the change in the hydrodynamic radius of the micelles (Scott Blair and Oosthuizen, 1961). However, the decrease in viscosity is not apparent when renneting occurs at a lower pH than the natural pH of milk, for example pH 6.2 (van Hooydonk *et al*., 1986). At a lower pH, there is a partial collapse of the hydrated κ-casein layer. It is then clear that the rennet reaction on κ-casein is complex, as the hydrolysis occurs on the surface of a much larger

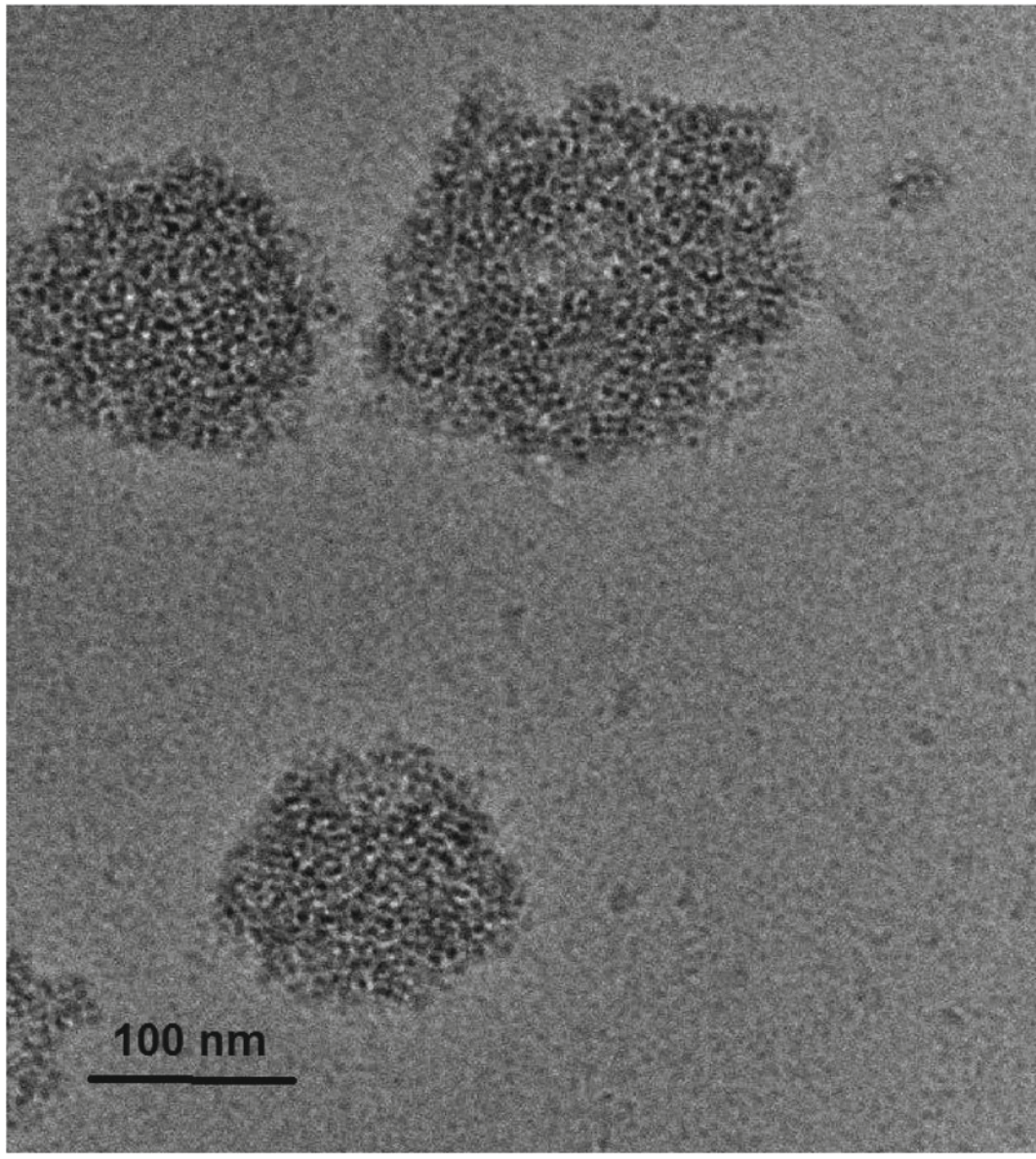

Fig. 11.1 Cryo-transmission electron microscope image of casein micelles from native milk, dispersed in their own milk serum. Samples were prepared in liquid ethane

colloidal structure, and it is not a simple hydrolysis reaction on protein molecules in solution.

Rennet causes the release of the C-terminal portion of κ-casein, often referred to as the caseinomacropeptide (CMP), which diffuses away from the casein surface. A frequently used method to follow the extent of the reaction in milk is the determination of non-protein nitrogen (NPN) in the soluble phase; however, this method may not be sensitive enough to measure initial proteolysis rates. The release of CMP is better quantified using HPLC, after acid precipitation of the proteins, using trichloroacetic acid or perchloric acid (see for example, van Hooydonk and Olieman, 1982; López-Fandiño *et al*., 1993).

In unheated milk, all the κ-casein is accessed by the enzyme. The optimum pH for the enzymatic activity is 5.5–5.3, where the rate of cleavage is maximal (Visser *et al.*, 1980; van Hooydonk *et al.*, 1984). At lower pH it has been reported that general proteolysis on the other caseins (α_{s1}-, α_{s2}-, β-caseins) occurs (Lindqvist and Storgards, 1960), with important consequences for cheese ripening.

Several models have been proposed to describe the kinetics of gelation of the casein micelles; these aspects have been thoroughly examined in previous editions of this book (see for example, Hyslop, 2003). Most authors suggest that the reaction is a first order reaction (van Hooydonk *et al.*, 1984). There are differences in the kinetics of the reaction between proteolysis of κ-casein in solution or in native casein micelles, as chymosin has strong affinity for *para*-κ-casein (Dalgleish, 1979; Dunnewind *et al.*, 1996; de Roos *et al.*, 2000).

The rate of the reaction is proportional to enzyme or protein concentration, and the relationship is linear for protein concentrations up to 8 % using skim milk concentrated by ultrafiltration (van Hooydonk *et al.*, 1984). The initial velocity of the reaction reaches a plateau at higher milk protein concentrations (Garnot and Corre, 1980). It has been suggested that the reduction in the rate of the reaction with concentration may be due to reduction of the effective diffusion rate of the enzyme (van Hooydonk *et al.*, 1984); however, it is important to note that processing history or serum composition may also have an effect. The reaction is strongly dependent on temperature, pH, calcium concentration, or micellar structure, and the effect of concentration on the rate of the enzymatic reaction, as well as the coagulation has yet to be fully elucidated.

There seems to be general agreement that the chymosin reaction is diffusion controlled, with a low energy of activation (van Hooydonk *et al.*, 1984) and a constant reaction rate, which is affected by the volume fraction of the casein micelles. The enzyme is active on a very heterogeneous system; it is known that κ-casein can form disulphide-linked oligomeric aggregates (Groves *et al.*, 1998) and it is probably present in this form in native casein micelles. The protein is not homogeneously present on the surface of the particles, as there is not enough κ-casein to cover the entire surface area (Dalgleish, 1998; Dalgleish and Corredig, 2012). The rate depends on the number of effective collisions, whereby the micelles diffuse very slowly compared to the rate of enzyme diffusion (van Hooydonk *et al.*, 1984).

Chymosin activity is affected by ionic strength and type of ions present. The casein micelles are also strongly affected by ionic strength and in general, serum composition and it is challenging to distinguish between the effects of ionic strength on the reaction and that on the casein micelles structure. However, it has been demonstrated that the rate of hydrolysis is diminished by increasing the ionic strength to 0.1, for example using NaCl (Visser *et al.*, 1980). In addition, a maximum rate of proteolysis has been reported for 8 mM calcium chloride (Bringe and Kinsella, 1986).

11.4 Colloidal Aspects of Rennet-Induced Aggregation of Casein Micelles

The clotting process triggered by chymosin has been studied for decades, and the aggregation is explained adopting models from colloidal theories. The mechanism of aggregation needs to take into account the relationship between the extent of κ-casein proteolysis and the probability of the casein micelles to aggregate when in close proximity with one another. As previously mentioned, κ-casein acts as a charged-polymer brush, hence its solvation and stability depends on solvent quality and charge density. The removal of the CMP from the surface results in a reduction of the overall charge of the particles, measured as a reduction in ζ-potential (Green, 1973; Anema and Klostermeyer, 1996); however, electrostatic repulsion is not the prime factor stabilizing casein micelles in skim milk. At large distances, the behaviour of casein micelles can be approximated to that of hard spheres. The presence of κ-casein on the surface causes steric repulsion forces to dominate over short-range van der

Waal's and electrostatic interactions, and the steric influence of this polyelectrolyte layer is strong enough to keep the casein micelles far apart (de Kruif and Zhulina, 1996; de Kruif, 1998), even at high concentrations (Bouchoux *et al*., 2010; Krishnankutty Nair *et al*., 2014).

In milk, at its natural pH, extensive (80–90 %) hydrolysis of κ-casein needs to occur before the casein micelles show visible aggregation (Dalgleish, 1979). Aggregation occurs only in the presence of calcium ions (Bringe and Kinsella, 1986; Martin *et al*., 2010). Temperature, pH and concentration of enzyme affect the kinetics of aggregation (Okigbo *et al*., 1985a, b; Kawaguchi *et al*., 1987; Crabbe, 2004). Aggregation of casein micelles is inhibited at refrigeration temperatures, even after a nearly complete release of the CMP. This behaviour has developed the general consensus that hydrophobic forces play a major role in casein aggregation; however, this may also be due to rearrangements in the structure of the casein micelles at low temperature.

The clotting reaction can be modeled after von Smoluchowski's rate theory for bi-molecular reactions, and it is assumed to be diffusion-controlled and independent of the size of the reacting particles (Payens, 1979; Dalgleish, 1980). This model well explains the increase in the turbidity of casein micelle suspensions during renneting (Payens, 1979; Dalgleish, 1980). The collisions between the casein micelles will not be effective (will not lead to aggregates) until a sufficient portion of the surface can come into direct contact with another surface. The elimination of patches of depleted CMP is critical. A simple geometrical model of steric stabilization has been employed to describe this behaviour (Dalgleish and Holt, 1988). Once the protein particles are sufficiently modified, the collisions between the particles will lead to adhesion and the formation of clusters (Dalgleish, 1980). There is then a change from a stable colloidal system, with a high occurrence of ineffective particle encounters, to an unstable system, in a very short time scale, that is nearly instantaneous when a sufficient amount of κ-casein has been hydrolyzed. The apparent lag phase observed in milk from the addition of rennet to the visible milk gelation is caused by the time necessary for κ-casein to be hydrolyzed. This model is well supported by research that demonstrates that the ability of the casein particles to aggregate when partly treated with chymosin does not increase linearly with the extent of proteolysis (Dalgleish, 1979, 1980).

As the casein micelles come closer to each other, they are subjected to long range forces that rule how close they can approach. If closer approach can be achieved, then the interactions are short ranged, and may depend on the local composition and conformation of the κ-casein in the portion of the surfaces that could interact. This will not happen with intact layers. It has also been demonstrated that close approach by osmotic stressing cannot cause aggregation of the casein micelles (Krishnankutty Nair *et al*., 2013). The time necessary to observe visible milk gelation decreases with increasing enzyme concentration or protein concentration; however, literature results are contradictory in this regard, and may result from differences in the methods used to measure gelation and differences in the processing history of the sample (Dalgleish, 1980; Waungana *et al*., 1998; Karlsson *et al*., 2007; Salvatore *et al*., 2011; Sandra *et al*., 2011). The gelation point is more accurately identified using the extent of CMP release and a summary of the dependence of the gelation point on the amount of CMP released is shown in Fig. 11.2, for milk containing different concentrations of protein or treated at different pH values.

Several methods were developed over the years for monitoring the coagulation of milk, and a chronological list of them has been reported by Lucey (2002). Most of the available methods can accurately measure the milk coagulation time, and rheological measurements together with microstructural analysis can follow structure formation and the properties of the final gel; however, more analytical approaches are needed to follow structural rearrangements and structural heterogeneities once the gel has formed.

The early stages of gelation can be followed using light scattering. The changes in the diffusivity of the casein micelles allow measuring with accuracy the point of gelation, often expressed by a rapid increase in the radius of the casein micelles, or by a change in the turbidity of the

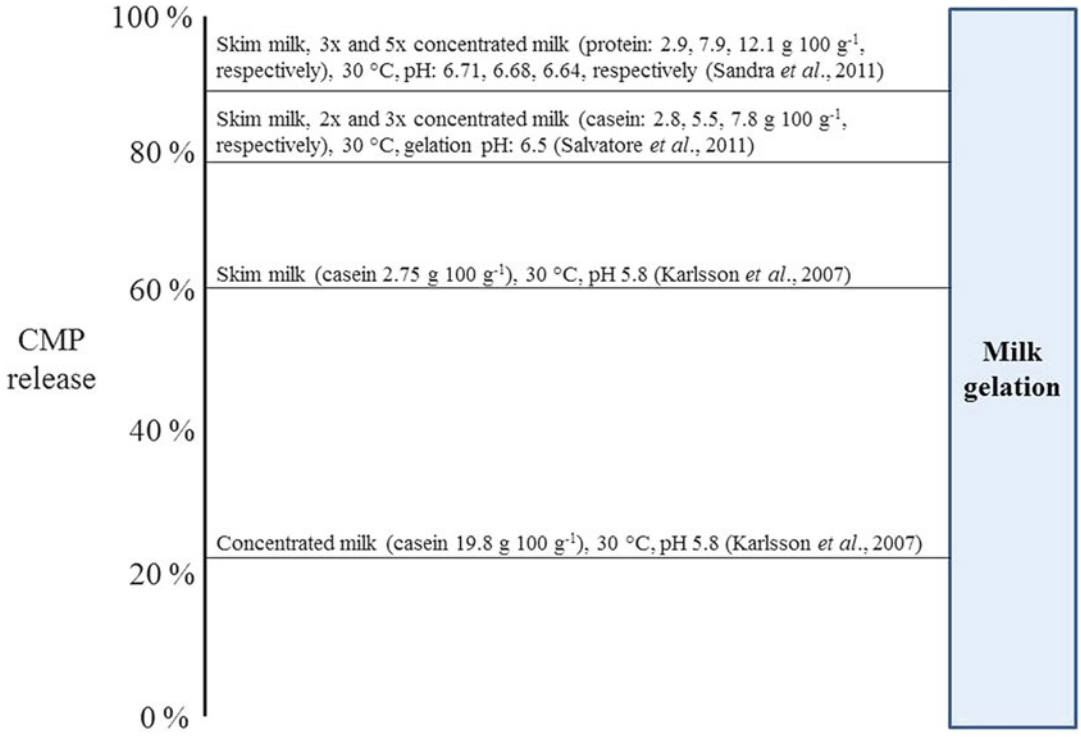

Fig. 11.2 Summary of reports on the effect of protein concentration (by ultrafiltration) and pH on the gelation point (recent studies)

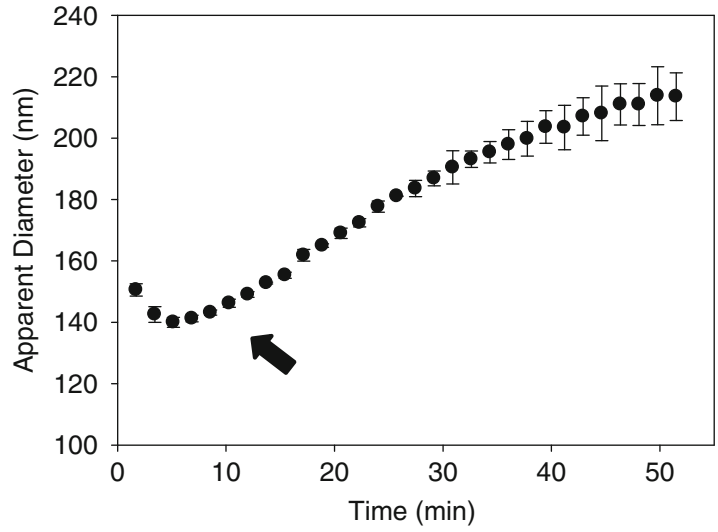

Fig. 11.3 Change in apparent diameter as a function of time from rennet addition, measured by dynamic light scattering. The casein micelles from untreated skim milk are extensively diluted in milk permeate, and rennet is added. The phase corresponding to the removal of the hairy layer of κ-casein is indicated by the *arrow*. For experimental details see Gaygadzhiev *et al.*, (2009)

milk. Before the point of gelation, there is a reduction of the casein micelle size (Walstra *et al.*, 1981; Horne and Davidson, 1993), as shown in Fig. 11.3. After the addition of rennet, it is possible to follow a reduction in the viscosity of the milk, (which has been attributed to the release of CMP from the casein micelles), before the increase due to coagulation (de Kruif, 1999).

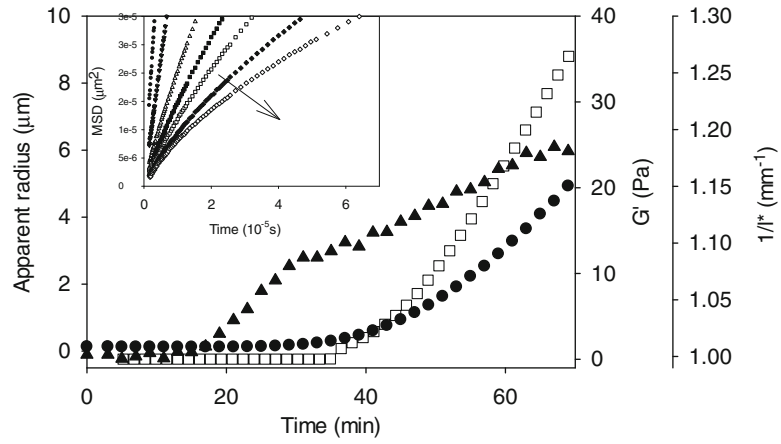

Fig. 11.4 Representative data on a rennet induced aggregation experiment using skim milk, indicating the changes in radius (circles), 1/l* (turbidity, *triangles*) as measured by diffusing wave spectroscopy, compared to changes in the elastic modulus (G', *squares*) measured by rheology. Time indicates the time after addition of rennet. Insert shows mean square displacement as a function of time (not change in time scale); arrow indicates different time points during renneting

The diffusivity of the casein micelles sharply decreases (as indicated by the sharp increase in the radius measured by light scattering) and the casein particles show arrested motion. This can be clearly measured by following the slope of the mean square displacement, measured using Diffusing Wave Spectroscopy (DWS), as shown in the insert of Fig. 11.4 (Alexander and Dalgleish, 2004; Gaygadzhiev *et al*., 2009). Figure 11.4 summarizes the development of light scattering and rheological parameters during a rennet gelation experiment. The changes in the turbidity parameter (1/l*) obtained by light scattering (transmission DWS) are related to the beginning of inter-particle interactions, and indicate that with the release of CMP the spatial correlation of the casein micelles begins to be affected at an earlier point than the complete release of CMP (Alexander and Dalgleish, 2004; Sandra *et al*., 2007).

Dynamic oscillatory testing is often employed to follow the development of the gel structure in rennet-treated milk. The elastic modulus (G') shows a rapid increase at the point of gelation, in full agreement with the decrease in the diffusivity of the casein micelles, indicated by the increase in the apparent radius measured by light scattering. This rapid increase in the apparent size of the micelles or the elastic modulus is referred to as gelation point, and in milk at natural pH this point corresponds to about 90 % of CMP released from the micelles (Sandra *et al*., 2007). Using oscillatory rheology it is not only possible to measure the development of the gel structure, by following the changes in the elastic modulus (G'), but it is also possible to measure the transition point from a sol to a gel, often defined as a change of phase (tan $\delta = 1$, where δ is the phase angle). Tan δ decreases rapidly through the gelation point, indicating that the increase in the stiffness is due to an increase in the number of linkages in the protein network, but not to the nature or strength of the bonds.

It is possible then to conclude that the kinetics of rennet-induced gelation strongly depend on the quality of the solvated κ-casein layer. At the natural pH of milk, gelation occurs only when nearly all the CMP has been removed from the surface of the casein micelles. Under these conditions, upon physical contact, the casein micelles no longer behave as rigid spheres, and can deform or even coalesce, leading to a loss of particle identity, showing "fusion" between the protein particles, similar to what has been described for model rubbery particles (Gauer *et al*., 2009).

At a lower pH, gelation occurs at a lower degree of hydrolysis (van Hooydonk *et al*., 1986) because of the decrease in steric and electrostatic repulsion. Similarly, the addition of ethanol can cause partial collapse of the κ-casein layer and a decrease in steric repulsion (Bringe and Kinsella, 1986; de Kruif, 1999; O'Connell *et al*., 2006). The internal organization of the proteins in the casein micelles and the amount of colloidal calcium phosphate present are not important to the initial stages of aggregation, and the behaviour of the casein micelles can be approximated to that of colloidal hard spheres (de Kruif, 1998). However, the internal characteristics and the extent of colloidal calcium phosphate present in the micelles become of importance after aggregation, as they affect the structural rearrangements of the network. It has been reported that a partial loss of colloidal calcium may affect charge interactions, and the formation of a weaker, more flexible protein gel (Choi *et al*., 2007). The composition of the serum phase and the presence of other molecules interacting with the casein micelles also can strongly affect the renneting properties of milk, at the early stages of aggregation as well as during structure formation. In particular, heat denatured whey proteins, soluble caseins and small molecular mass emulsifiers can affect the interactions of the casein micelles and the efficiency of the collisions (Gaygadzhiev *et al*., 2009; Ion Titapiccolo *et al*., 2010a; Kethiredipalli *et al*., 2010). For example, it has been shown that by addition of polysorbate, the casein micelles have a higher propensity to aggregate, and even at the natural pH of milk, the gelation time occurs at an earlier extent of CMP release than for control milk samples. On the other hand, recent work has demonstrated that, in the absence of added calcium, aggregation of casein micelles is inhibited by the presence of soluble caseins (Gaygadzhiev *et al*., 2009). The soluble proteins in milk at its natural pH may inhibit close range attachment of the micelles by shielding the hydrophobic or calcium-sensitive patches on the surface of the protein particles. This effect is demonstrated using dynamic light scattering under diluted conditions: while after addition of rennet there is still a decrease in the hydrodynamic diameter of the casein micelles, in the presence of sodium caseinate, there is no aggregation of the protein particles. The presence of soluble casein, and the re-arrangements of the casein micelles during cooling have also been considered as an important factor for the inhibition of rennet-induced aggregation at temperatures below 18 °C (Bansal *et al*., 2007).

11.5 Effect of Calcium Chloride on Rennet Induced Gelation of Casein Micelles

It is well recognized that the addition of calcium chloride to milk has positive effects on texture and cheese curd yield (Udabage *et al*., 2001). Calcium ions are essential for aggregation and gelation of casein micelles, and the reason behind this mechanism has yet to be fully established. *Para*-κ-casein binds to calcium ions more strongly than κ-casein (Bringe and Kinsella, 1986), and it has been recently hypothesized using molecular simulations that the charge asymmetry on the *para*-κ-casein may play a key role in affecting CMP conformation on the micelle surface and, as a consequence, its stabilizing properties (Ettelaie *et al*., 2014). Indeed, although free calcium ions shield negatively-charged amino acid residues, reducing the overall charge of the casein micelles, this cannot be the only reason behind its essential role in rennet-induced aggregation of casein micelles.

In milk, addition of 1 mM calcium chloride does not increase the enzymatic activity, as shown by kinetics of CMP release (Sandra *et al*., 2012), but it shortens coagulation time and forms stiffer gels (van Hooydonk *et al*., 1986; Sandra *et al*., 2012). The aggregation of the casein micelles occurs at a lower degree of κ-casein hydrolysis, by decreasing the extent of solvation of the polyelectrolyte brush around the micelles (van Hooydonk *et al*., 1986; de Kruif, 1999). The mode of addition of calcium (fast addition as $CaCl_2$, or slowly by dialysis) to the milk does not seem to have an impact on the aggregation behaviour (Sandra *et al*., 2012). A recent study followed the increase in turbidity of milk after

addition of rennet using diffusing wave spectroscopy and showed no difference in the turbidity behaviour as a function of CMP release with 1 mM calcium added, in spite of the earlier onset of coagulation of the micelles (Sandra *et al.*, 2012). These results may suggest that calcium ions play a major role in strengthening short range interactions. Evidence of calcium binding to the micelles after rennet coagulation was also shown in a study on mixed acid and rennet coagulation, whereby soluble calcium was shown to be reduced during early acidification after complete CMP release (Salvatore *et al.*, 2011). It is also important to note that although additional calcium increases stiffness of the gels, too much calcium added to milk can form weaker gels (Fox *et al.*, 2000; Udabage *et al.*, 2001).

While the amount of soluble calcium is of great significance in the early stages of rennet gelation, differences in the concentration of colloidal calcium affect the structural rearrangements of the gel network (Choi *et al.*, 2007). Loss of colloidal calcium phosphate will weaken the internal structure of the micelles once the gel has formed, and will affect the forces stabilizing the gel, namely, hydrogen bonds, van der Waals interactions and calcium bridges. Hence, the presence of colloidal calcium is an important factor in determining the properties of the gel network. This can be clearly noted in the value of tan δ of rennet gels measured by rheology (Choi *et al.*, 2007).

11.6 Effect of Acidification on Rennet Induced Gelation

Many cheese making processes involve an acidification step, usually occurring simultaneously to chymosin activity. A mild acidification can improve rennet coagulation properties, by shortening the coagulation time and strengthening the gel (Renault *et al.*, 2000). As the pH decreases, the surface of the casein micelles changes, micellar calcium is released in the serum phase, but, at temperatures above 25 °C, this pH change does not cause a substantial release of protein in the soluble phase (Dalgleish and Law, 1988). At a more acidic pH there is also a faster release of CMP, as shown in Fig. 11.5.

With decreasing pH, the steric repulsion between casein micelles decreases and the attractive forces, as described quantitatively using an adhesive hard sphere model, increase (de Kruif, 1997). At about pH 5.6, there is a change in the apparent diameter of the micelles (Alexander and Dalgleish, 2005). Hence, the extent of CMP release necessary to cause the micelles to aggregate becomes smaller as the pH is decreased (van Hooydonk *et al.*, 1986).

The mechanism of gel formation by acidification is quite different from that caused by renneting, as well as the properties of the final gel. In contrast with rennet-induced gelation, in the case of acid gelation, CMP is not released, but the micelles loose

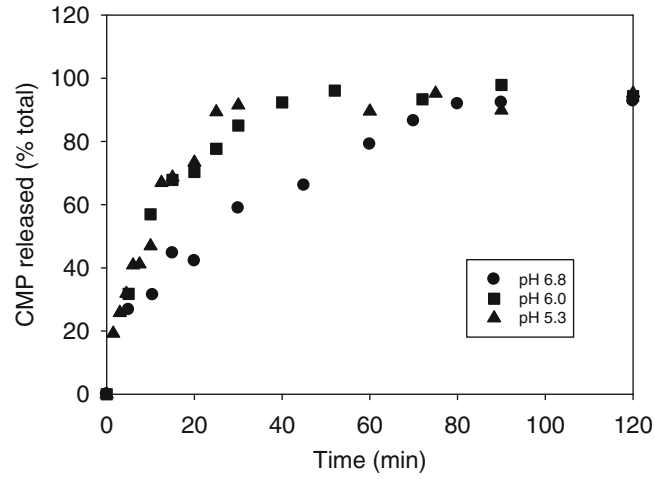

Fig. 11.5 Release of caseino macropeptide during renneting of milk at different pH values. Samples were analyzed using HPLC after precipitation with 3 % perchloric acid

electrostatic repulsion because of the decrease in the overall charge on the casein micelles. This causes the collapse of the hairy layer and the decrease in steric stabilization, leading to aggregation near the isoelectric point of the proteins.

The rheological properties of milk gels obtained by a combination of acid and enzymatic gelation are less understood, as the two mechanisms occur simultaneously and the properties of the gel network formed are different than in the case of rennet alone, or acid alone (Lucey *et al*., 2000; Gastaldi *et al*., 2003). The order and the extent of the modification of the casein micelles will strongly affect the structure and properties of the final gel (Li and Dalgleish, 2006). In the case of low addition of rennet, whereby there is an insufficient release of CMP for aggregation to occur, by increasing the extent of proteolysis of κ-casein the pH of gelation increases (Gastaldi *et al*., 2003; Li and Dalgleish, 2006). On the other hand, in the case where a sufficient amount of CMP is released to cause rennet-induced aggregation, the release of calcium from the casein micelles during further acidification changes the properties of the gel (Li and Dalgleish, 2006; Salvatore *et al*., 2011).

In gels prepared with a combination of rennet and acid, the storage modulus is larger than that of either acid or rennet gelation alone. In addition, these gels show a very distinct development of the storage modulus, as exemplified in Fig. 11.6. When a rennet gel is formed at a pH where the calcium phosphate is still mostly present in the micelles, the gel modulus shows first a marked increase and a subsequent decrease caused by the release of colloidal calcium phosphate from the micelles. The development of the value of tan δ demonstrates the weakening of the gel network, explained by a weakening internal structure of casein micelles already part of a network, because of increased charge repulsion between the protein chains. A further decrease in pH then causes an increase in the storage modulus, indicating a further rearrangement of the structure (Fig. 11.6).

In summary, gels formed during partial enzymatic gelation and acidification will share both properties, but their characteristics will depend on the interactions between the particles, the presence of an inter-particle layer of CMP and the presence of colloidal calcium phosphate. This will lead to a different extent of breakdown of the internal micellar structure and formation of inter-particle interactions in the network, and may promote fusion of the casein micelles (Aichinger *et al*., 2003).

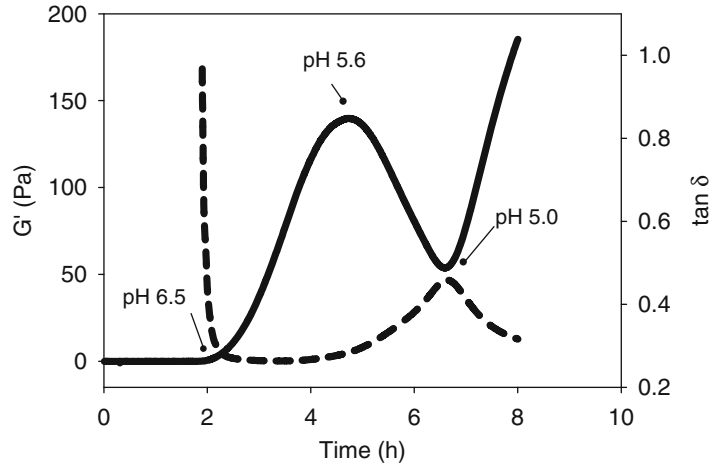

Fig. 11.6 Rheological pattern of rennet gelation during fermentation with mesophilic bacteria. Storage modulus (G', *solid line*), and loss tangent (tan δ, *broken line*) as a function of time. The pH during fermentation at various points of the curve are indicated. Redrawn from Salvatore *et al.,* (2011)

11.7 Effect of Temperature on Enzymatic Coagulation

It is common practice to cool the milk immediately after milking, to improve its microbiological quality. However, it has been demonstrated that cold refrigeration affects clotting time and reduces curd firmness (Walstra and van Vliet, 1986; Raynal and Remeuf, 2000). This effect has been attributed to the solubilization of β-casein from the micelles, resulting in a slight increase in the micellar stability. The cold-induced solubilisation of β-casein and calcium are partly reversed with re-warming of milk (Raynal and Remeuf, 2000).

When milk is incubated with rennet at refrigeration temperatures, the enzymatic reaction occurs slowly but no visible gelation is observed (Berridge, 1942). Recent work measured the rheological properties of milk during renneting at 10 °C and demonstrated that aggregation may occur at this temperature, but at a very slow rate. This effect has been recently attributed to the presence of β-casein, which may be playing a protective role being present in the serum phase and causing rearrangements to the casein micelle structure (Bansal *et al*., 2007).

11.8 Enzymatic Coagulation of Heated Milk

Heating of milk at temperatures above 60 °C causes structural changes to the whey proteins. These proteins unfold, expose their reactive sites and form aggregates, either in solution or on the surface of the casein micelles. The presence of heat-induced whey protein complexes in milk has important consequences for the technological properties of milk. The aggregates present in the non-sedimentable fraction of heated milk contain β-lactoglobulin, α-lactalbumin and κ-casein, and are <100 nm in diameter (Jean *et al*., 2006).

The presence of heat-induced aggregates can cause significant changes to the rate of rennet-induced aggregation and the properties of the curd. The effect has been attributed in the past to the reduction of the accessibility of chymosin to κ-casein, because of the linkage of denatured whey protein molecules to Cys_{11} and Cys_{88} of κ-casein, the loss of soluble calcium and the steric hindrance caused by the aggregates (van Hooydonk *et al*., 1987).

It has been recently demonstrated that the rate of CMP release is very similar in heated and unheated milk (Vasbinder *et al*., 2003; Kethireddipalli *et al*., 2010) and, regardless of where the κ-casein is located (i.e., in the serum complexes or on the casein micelles) the accessibility of the enzyme is similar (Mollé *et al*., 2006). The formation of heat-induced whey protein aggregates hinders interactions of casein micelles, decreasing the rate of effective collisions (Vasbinder *et al*., 2003). The aggregation is limited regardless of the distribution of the complexes on the micelles or in the serum phase. The whey protein aggregates present in the unsedimentable fraction prior to renneting bind to the casein micelles as renneting proceeds, affecting the potentially interacting sites of the micelles (Kethireddipalli *et al*., 2010).

Rennet curds prepared from heated milk have poor texture, poor stretching and melting properties (Singh and Waungana, 2001). Strategies have been reported to restore, at least partially, the ability of skim milk to gel with rennet addition. The rennet coagulation properties of milk can be partially recovered after heating by acidifying milk to pH below 6.2, or by acidifying and then readjusting to pH 6.7, or by calcium addition (Banks *et al*., 1987; Singh and Waungana, 2001). However, the curds obtained with milk treated this way differ from traditional cheese curds. It is also important to note that by careful control of heating and modification to the processes, it is possible to transfer the whey proteins from milk to cheese curds, improving the yield. In this case also the properties of the cheese may be substantially different from the control cheese made from unheated milk (see for example, Chromik *et al*., 2010).

11.9 Renneting of High Pressure-Treated Milk

High pressure treatment induces changes in the milk constituents that affect the cheese making properties, in particular the rennet clotting time, the rate of gel formation, the stiffness and texture of the gel, as well as the water holding capacity of the curd (López-Fandiño *et al*., 1997; Needs *et al*., 2000). The distribution between soluble and colloidal calcium and phosphate is affected, and the increase in soluble calcium and phosphate reaches a plateau at about 300 MPa (López-Fandiño *et al*., 1997). This is caused by a change in the non-micellar casein fraction, with the extent of dissociation in the order β->κ->α_s-caseins (López-Fandiño *et al*., 1996). The dissociation of casein micelles during high pressure processing is partly reversible, but depending on the pressure, can either induce aggregation or disruption of the casein micelles, with changes to the turbidity of the milk (Huppertz *et al*., 2006). Although the enzymatic hydrolysis (as measured by the release of CMP) seems to be little affected by high pressure, the changes in the size of the micelles reduce the clotting time (López-Fandiño *et al*., 1997). Indeed the presence of partially-disrupted casein micelles will cause an increase in the surface area for inter-micellar interactions, and will promote aggregation. At higher pressures (i.e., >300 MPa) whey protein denaturation occurs (López-Fandiño *et al*., 1996), and the formation of complexes with the casein micelles has a negative impact on casein aggregation. It has been reported that about 90 % of the β-lactoglobulin is no longer native at 400 MPa (López-Fandiño *et al*., 1996; Needs *et al*., 2000). Although the association of whey proteins with the casein micelles may cause hindrance to the secondary phase of gelation, curd yields increase, because of the incorporation of whey proteins and the increase in moisture content (Needs *et al*., 2000; Huppertz *et al*., 2006).

In addition to static high pressure, some work has also been reported on the effect of dynamic high pressure homogenization on rennet-induced aggregation of skim milk. The surface of the casein micelles is slightly altered by high pressure homogenization, causing a shorter coagulation time, as some κ-casein is removed (Sandra and Dalgleish, 2007).

11.10 Enzymatic Coagulation in Milk Concentrated by Membrane Filtration

In recent years, there has been an increased interest in understanding the effect of the volume fraction of casein micelles on the renneting properties of milk concentrates, as processes such as ultrafiltration and microfiltration are increasingly common unit operations in the dairy industry. The higher extent of linkages formed and the decreased mobility of the micelles may require changes in the cheese manufacturing processes, as they will affect yield, structure and texture of the final product.

Rennet aggregation is a diffusion controlled reaction (Dalgleish, 1980) and it may be affected by casein volume fraction. Earlier studies reported either a decreased or a constant reaction rate for milk concentrated by ultrafiltration (Garnot and Corre, 1980; van Hooydonk *et al*., 1984). The rate also depends on pH (with a lower increase at pH 6.2) (Garnot and Corre, 1980). Visible gelation of milk concentrated by ultrafiltration occurs earlier than in normal milk, with a higher firmness at higher protein concentration (Garnot *et al*., 1982).

Recent work reported the gelation of casein micelles as a function of concentration of milk by ultrafiltration, following the extent of CMP release (Salvatore *et al*., 2011; Sandra *et al*., 2011). These studies clearly demonstrated that in all cases, at the natural pH of milk, casein aggregation occurs at about 90 % CMP release (Sandra *et al*., 2011). The concentration also seems to affect the association of chymosin with the casein micelles. In a study with artificial micelles it was shown that in a more diluted system, chymosin dissociates faster than in concentrated systems (de Roos *et al*., 2000). This has important consequences to the recovery of chymosin in the cheese curd and cheese ripening (de Roos *et al*., 2000).

When treating concentrated milk, it is important to take into account the processing history of milk as well as the composition of the serum phase, as the amount of soluble calcium will affect the coagulation behaviour of milk (Ferrer *et al*., 2011). This is critical in the case of reconstitution of milk protein concentrates (Martin *et al*., 2010). The rennetability of these micellar concentrates depends on the ionic composition of the serum phase (Ferrer *et al*., 2008; Martin *et al*., 2010). Milk protein concentrates reconstituted in water do not gel upon rennet treatment, unless calcium is added to the serum phase. The amount of soluble casein present in the milk has to be carefully controlled as it may hinder the aggregation of the micellar fraction (Martin *et al*., 2010; Ferrer *et al*., 2011; Sandra *et al*., 2012).

11.11 Presence of Other Components in Milk

Although most mechanistic studies on the enzymatic gelation of casein micelles have been carried out in skim milk, in dairy products the aggregation occurs in the presence of other components, which may or may not affect the behaviour of the micelles.

In most milk products, fat globules play an important role in imparting structure and texture to the matrix, and contribute to creamy mouthfeel and flavour. The renneting behaviour of milk is affected by the size and the interfacial composition of the fat globules. In untreated whole milk, fat globules are polydisperse in size from less than 100 nm to about 10 μm in diameter (Michalski *et al*., 2002). Homogenization of milk modifies the size of the globules, resulting in an increase of their surface area. The original fat globule membrane material is no longer sufficient to stabilize the newly formed interfaces, and casein micelles are found preferentially adsorbed onto the interface, either intact or as fragments of the original micelle (Sharma and Dalgleish, 1993). The properties of rennet gels containing fat globules, prepared by homogenizing milk are quite different than those of unhomogenized milk, and have been widely studied, in particular for the effect on moisture retention, the whitening of cheese and increases in yield (Peters, 1956).

Recent data suggest that the release of CMP is faster in homogenized milk compared to skim milk, and all the κ-casein is available to chymosin hydrolysis (Ion Titapiccolo *et al*., 2010b). Homogenized milk coagulates faster than non-homogenized milk (Ion Titapiccolo *et al*., 2010b). The spreading of the caseins at the interface will increase the susceptibility of κ-casein to rennet, as previously hypothesized (Robson and Dalgleish, 1984). The micelles present at the surface of homogenized fat globules interact with the gel and aggregate with each other. The presence of these micelles does not modify their aggregation behaviour, but the system aggregates earlier than skim milk as a consequence of the earlier CMP release kinetics (Ion Titapiccolo *et al*., 2010b). However, milk homogenization may impair the quality of cheese obtained by rennet-induced gelation of milk, leading to soft curds containing high moisture levels.

Research has also been carried out using model systems consisting of milk gels filled with oil droplets. The rheological behaviour of these emulsion-filled milk gels depends on the structural properties of the filler particles, the viscoelasticity of the matrix as well as the physical properties of the droplets, the concentration of the components, and most importantly, the interactions between the interface of the droplet and the protein network (Sala *et al*., 2007).

In recombined systems, with a careful design of the interfacial composition of the fat globules and control of their particle size and colloidal state, it is possible to affect the structure of rennet gels, as the ability of the droplets to interact with the gel and participate in the formation of the casein network will result in very different structures. While interacting droplets will be part of the gel network, as in the case of homogenized fat globules containing casein micelles, oil droplets with interfacial material not subjected to destabilization with rennet will not become part of the network gel. Small non-interacting droplets will reside within the pores of the network,

and will have little effect on the viscoelasticity of the system. Larger droplets or flocculated droplets will cause increases in the stiffness of the gel, even when not interacting. As the casein gel develops, the droplets are now entrapped inside the pores of the protein network, and the droplets, as well as the caseins have much less available space to interact, causing stiffening of the gel while the mobility of the droplets continue to decrease within the pores (Corredig *et al*., 2011). During cutting of the curd, these oil droplets may be lost in the serum phase, but have contributed to the structuring of the rennet gels.

Enzymatic cross-linking of caseins, which results in interesting properties for milk gels (increased gel strength and reduced syneresis), also affects the coagulation properties of milk. If rennet is added to casein micelles enzymatically cross-linked with transglutaminase (TGase; EC 2.3.2.13), the aggregation of *para*-κ-casein is reduced, due to a further level of steric stabilisation created by cross-linked micelles (Huppertz and de Kruif, 2007). Conversely, other authors (O'Sullivan *et al*., 2002; Özer *et al*., 2012) also demonstrated that the primary stage of milk coagulation (the release of CMP) is influenced by cross-linking. The coagulation times of milk with added TGase are strictly dependent on the amount of enzyme used, the coagulation temperature and the initial pH of the milk (Özer *et al*., 2012).

The drive to healthier products in the market place is also leading to the study of enzymatic coagulation in mixed systems containing polysaccharides, in quantities that could be either nutritionally significant, as dietary fiber, or as functional ingredients in low fat products. Polysaccharides are high molecular weight molecules that cause structuring in milk, and above a critical concentration can cause phase separation. The careful control of microphase separated domains during renneting is of increasing interest as it has the potential to create novel structures in dairy matrices. By controlling the kinetics and the extent of phase separation it is possible to form different gel microstructures (Acero-Lopez *et al*., 2010).

Recent work has been published on the effect of addition of polysaccharide molecules on the renneting properties and coagulation kinetics of skim milk (Fagan *et al*., 2006; Arango *et al*., 2013). The addition of fiber can change the time of rennet gelation as well as the stiffness and the microstructure of the gels. For example, in the case of skim milk containing κ-carrageenan and xanthan gum, increasing polysaccharide concentration causes inhibition of the aggregation phase, while adding high methoxyl pectin enhances aggregation. These changes have been attributed to differences in the structuring of these polysaccharide molecules (Tan *et al*., 2007).

The structuring behaviour of polysaccharide molecules depends on biopolymer concentration, milk volume fraction, environmental conditions, processing history and all these factors need to be carefully controlled to fine tune the final texture of the rennet gel. For example, above a certain concentration, phase separation may occur, and even before macroscopic phase separation, localized casein micelle clusters will form, limiting the formation of a self-supporting gel.

11.12 Conclusion and Outlook

The enzymatic gelation of milk is one of the most studied topics in food colloids, as the reaction is based on the gradual hydrolysis of a protein polyelectrolyte layer on the surface of the casein micelles. A better understanding of the renneting reaction has also great applied importance to dairy processors. By modulating environmental conditions, casein volume fraction and processing history of the milk, it is possible to control the preliminary stages of gelation, and form cheese curds with different properties, and as a consequence, influence the final cheese. Different conditions of enzymatic coagulation will also have important consequences to ripening, bacterial distribution and their lysis in the matrix.

The mode of action of the enzyme is well understood, and the details of the reaction well described, at least in the case of unprocessed milk at its natural pH. More knowledge is needed to link the changes occurring in the preliminary stages of gelation to the final gel, to be able to

ultimately control the structure and form particular types of networks. Better experimental approaches are needed to be able to study the interactions occurring in the complex cheese curd matrix, to follow rearrangements of structure and how these are affected by the initial rennet coagulation and the destabilization of the casein micelles. Little is also known of the effects caused by partial enzymatic modifications to the casein micelles. Indeed, a multitude of processes are known where aggregation of *para*-κ-casein is one of the competing mechanisms occurring during milk gelation, and it is important to understand better how to fine tune the conditions to obtain a particular curd structure. Such processing conditions are often ruled by practical knowledge as it is still necessary to tease out the various mechanisms at play. This is obvious in rennet curds obtained in less traditional processes, whereby components, other than fat globules and native whey proteins may be present, or the processing history of the milk is complex, as for example, in reconstituted milk powders. This chapter highlights the current knowledge on the fundamental aspects of enzymatic induced coagulation of milk, but it clearly points out the need for more studies to bridge the gaps between model systems and practical application. In addition, it is clear that more needs to be understood on how to control the colloidal destabilization reaction to reach a particular structure and texture. Cheese is a nutritionally valued product widely consumed in various forms; a better knowledge of the complexity of the gelling process will lead to improved or novel products and processes.

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Acid Coagulation of Milk

12

John A. Lucey

Abstract

The acid coagulation of milk is the basis for a wide diversity of cultured dairy products. Acidification directly impacts the stability of casein micelles, reducing their charge, dissolving some of the insoluble calcium phosphate crosslinks and modifying internal bonding between proteins. The formation of aggregates and ultimately gels occurs at some critical point when electrostatic repulsion is reduced and is not sufficient to overcome attractive forces, like hydrophobic interactions. Acid-induced milk gels increase in stiffness with time due to on-going bond formation between casein particles within the network. In gels made from heated milk, an increase in the loss tangent parameter is observed for a short period after gelation; this phenomenon is due to the loss of insoluble calcium phosphate crosslinks within the casein particles that are already forming the gel matrix. The texture and physical properties of acid-induced gels are dependent on the specific conditions used for gel formation including: the rate of acidification, temperature, extent of whey protein denaturation, protein content, and presence of polysaccharide stabilizers. On-going studies are still investigating the exact physicochemical mechanisms involved in the acid coagulation of milk including trying to gain a better understanding of how exopolysaccharides modify yoghurt properties.

Keywords

Yoghurt • Fermented milk • Acid gels • Whey protein denaturation • Exopolysaccharides • Wheying-off

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12.1 Introduction

Fermented milks and yoghurts are one of the oldest and most popular foodstuffs that are produced throughout the world. Worldwide production of fermented milk products (such as yoghurt) probably exceeds 25 million tonnes (IDF, 2012). In 2011, per capita consumption of yoghurt in the US was 6.2 kg (IDFA, 2013) but this is still only a fraction of the consumption levels in many parts of the world.

Historically, the souring of milk was unpredictable and the exact processes involved were not known until well into the twentieth century. For example, Revis and Payne (1907) in an article entitled "*The Acid Coagulation of Milk*" investigated why during milk souring the initial increase in bacterial numbers was not directly proportional to the increase in acidity, as well as the rate of calcium solubilisation from the caseins as a function of acid development. It was just over one hundred years ago that Metchnikoff first proposed that consumption of fermented milk could help to prolong the life of man, igniting interest in health benefits. Over the past 100 years much has been discovered about the microbiology/biochemistry of the various cultures used for these fermentations, the therapeutic benefits of certain probiotic bacteria, as well as the formation and physicochemical properties of acid coagulated products. The formation and physical properties of acidified milk gels have been reviewed (Lucey and Singh, 1997, 2003; Horne, 1999).

A wide variety of fermented milk products are produced; some of the main types are briefly described here. Yoghurt is formed by the slow fermentation of lactose to lactic acid by the thermophilic starter bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. In set-style yoghurt, gels are formed (undisturbed) in the retail pot. Stirred-type yoghurt is made by breaking a set gel before mixing with fruit or other inclusions and filling into retail containers. Concentrated yoghurt (called Greek or Greek-style yoghurt in the US) is made by removing some acid whey after fermentation using straining with cloth bags, mechanical separation or membrane filtration. The microbiology/biochemistry of the starter cultures used, as well as the manufacturing technologies involved in yoghurt and fermented milks, have been extensively reviewed (e.g., Chandan, 2006; Tamime, 2006; Tamime and Robinson, 2007).

The effects of compositional and processing parameters on the textural properties of acid milk gels and fresh acid cheeses have been reviewed (Lucey and Singh, 1997, 2003; Lucey, 2011). In fresh acid cheeses, the coagulation of milk, cream or whey is achieved *via* a combination of acid and heat. The production of fresh acid cheeses generally involves pre-treatments (which may include heat treatment and/or homogenisation of the milk), slow acidification (usually with mesophilic starter cultures) and gelation, whey separation and/or curd treatment (Guinee *et al.*, 1993). A small amount of rennet may be used in the production of Quarg, Cottage cheese and *Fromage frais* to promote greater syneresis. Compositional and processing parameters, such as fat content of the milk, milk heat treatment, method of whey separation, heat treatment of the curd and addition of hydrocolloids to the curd, are varied to produce different types of fresh cheese. The use of rennet in acid gels has a dramatic influence on the rheological properties of these "combined" gels (Lucey *et al.*, 2000; Tranchant *et al.*, 2001). Acid casein is produced by the acidification of milk by starter (lactic) or mineral acids (e.g., HCl), followed by heating, whey separation, washing with water, mechanical dewatering and drying of the resultant precipitate (Chap. 2). A common factor in all of these different acidified milk products is that the initial step involves the formation of an acid-induced gel, which is then further processed. This chapter focuses primarily on the formation of acid milk gels and their physical, rheological and microstructural properties.

12.2 Acidification of Milk

12.2.1 Method of Acidification of Milk

Milk can be acidified by bacterial cultures, which ferment lactose to lactic acid, the direct addition of mineral acids, such as hydrochloric acid (HCl), the use of glucono-δ-lactone (GDL) which

hydrolyses to gluconic acid, injection of carbon dioxide, or by a combination of these methods. Many studies on the formation of acid gels have involved the use of GDL (Lucey and Singh, 1997). An extensive study on the formation and rheological properties of acid gels formed by cold acidification of milk with HCl and subsequent heating to the gelation temperature has been reported (Roefs *et al.*, 1990; Roefs and van Vliet, 1990). The cold acidification method allowed the acidification process to be completed separately from gelation, which only occurred during the warming step. The rate of acidification is different between milk acidified with GDL and bacterial cultures; GDL is hydrolysed rapidly to gluconic acid (especially at high temperatures) whereas after the addition of starter bacteria, the pH changes little initially, but then decreases gradually with time. The final pH attained in GDL-induced gels is a function of the amount of GDL added to the milk whereas starter bacteria can continue to produce acid until a very low pH (e.g., <4.1) is attained when the bacteria are inhibited by the low pH; in practice, bacterially acidified gels are cooled when sufficient acidity has been attained (Tamime and Robinson, 2007) or the product will become too acidic.

In the manufacture of casein, skim milk is mixed with mineral acid at room temperature and the decrease in pH is very rapid. The rate of pH change during fermentation or addition of acid is controlled by the buffering properties of milk (Lucey *et al.*, 1993). The rheological and physical properties of gels made with GDL differ from those of fermentation-produced gels (Lucey *et al.*, 1998d), probably due to the different rates of acidification during the critical stage of aggregation of the casein particles, different degrees of particle/cluster rearrangements, as well as concomitant physico-chemical changes in casein micelles.

Preacidification of milk (addition of acid prior to addition of culture) has been explored as a means of accelerating the fermentation process and facilitating a more continuous manufacturing process (Driessen *et al.*, 1977; Peng *et al.*, 2009a). Yoghurt gels made with lower preacidification pH and longer fermentation time had lower storage modulus and yield stress values, higher whey separation and permeability and formed coarse networks (Peng *et al.*, 2009a). Preacidification removed colloidal calcium phosphate (CCP) crosslinks within casein micelles resulting in weaker gels. Fermentation time significantly affects the rate of solubilization of CCP. Long fermentation times (slow acidification) allows more time for the solubilization of CCP (higher soluble Ca content in gels), whereas short fermentation times (fast acidification) allows less time for this process to occur (lower soluble Ca content in gels) (Peng *et al.*, 2009a).

12.2.2 Effect of Acidification on the Properties of Casein Micelles

Caseins constitute approximately 80 % of the protein in bovine milk, present as four types (α_{s1}-, α_{s2}-, β-, and κ-casein) in combination with appreciable quantities of CCP in the form of aggregates called casein micelles. The structure and formation of casein micelles has been intensively investigated in the past with many different models proposed; but now most researchers are using the dual-binding model (Horne, 1998) as the most realistic approach. The dual binding model emphasizes that the assembly of the micelle occurs due to the combined impact of both hydrophobic and electrostatic interactions. The hydrophobic interactions occur between hydrophobic segments on the casein molecules. Phosphoserine clusters on casein molecules provide the location for the formation of CCP nanoclusters, but they also help to prevent excessive growth of these nanoclusters by capping these growing structures, limiting their growth. Recently, the molecular weight of CCP was experimentally estimated to be around 7500 g/mol (Choi *et al.*, 2011), which was within the range (4900–9800 g/mol) predicted theoretically by Horne *et al.* (2007).

Originally, it was assumed that micelles were held together only by bridges of CCP (Schmidt, 1982) but it is now clear that a number of factors are responsible for maintaining the integrity of

the micelle. The formation of CCP is an effective means of "burying" a considerable amount of Ca and phosphate within casein micelles (which is important for milk to achieve its primary role of nutrition of the young calf) but CCP can be removed without completely disrupting the micelle if milk is acidified at temperatures >20 °C (Dalgleish and Law, 1988; Lucey *et al.*, 1997a); casein dissociation is limited at >20 °C. The CCP-depleted casein particles dissociate from the micelle if the pH is adjusted back to 6.6, which indicates that electrostatic interactions are important for micellar stability (Lucey *et al.*, 1997a). Hydrophobic and hydrogen bonding are important for micelle integrity since the addition of urea disrupts the micelle structure (McGann and Fox, 1974); calcium ions (Ca^{2+}) also play a role in the integrity of the micelle (Holt *et al.*, 1986).

During acidification of milk, many of the physicochemical properties of the casein micelles undergo considerable change, especially in the pH range 5.5–5.0, including increased voluminosity and solvation of the caseins (Roefs *et al.*, 1985; Walstra, 1990). The decrease in pH also results in a reduction in the charge on the κ-casein hairs and they eventually collapse on the micellar surface (de Kruif, 1999). During acidification there is an initial (small) decrease in average micellar mass and radius and also a redistribution of mass (rearrangements) within the micelles (Moitzi *et al.*, 2011). These complex changes occurring during acidification are dependent on environmental conditions including concentration/dilution of the system, rate of acidification, and temperature (Moitzi *et al.*, 2011).

As the pH of unheated milk is reduced, CCP is dissolved (Pyne and McGann, 1960) and the caseins are liberated into the serum phase (Roefs *et al.*, 1985; Dalgleish and Law, 1988). The extent of liberation of caseins depends on the temperature at acidification; at 30 °C, a decrease in pH causes virtually no liberation; at 4 °C about 40 % of the caseins are liberated into the serum at pH~5.5 (Dalgleish and Law, 1988). The temperature of acidification has little (direct) effect on the solubilization of CCP. Apparently, little change in the average hydrodynamic diameter of casein micelles occurs during acidification of (unheated) milk to pH~5.0 (Roefs *et al.*, 1985). Aggregation of casein occurs as the isoelectric point (pH 4.6) is approached.

Milk used for yoghurt manufacture is subjected to an extensive heat treatment. Heat treatment of milk at a temperature above 70 °C causes denaturation of whey proteins, some of which associate with casein micelles, involving κ-casein, *via* hydrophobic interactions and intermolecular disulphide bonds (Haque and Kinsella, 1988; Singh, 1995). Moderate heating (<90 °C) does not appear to affect the size of casein micelles although these treatments cause the whey proteins to denature and bind to micellar κ-casein; more extensive heat treatment (>100 °C) causes some degree of micellar aggregation and an increase in particle size (Dalgleish *et al.*, 1987). Heat treatment of milk markedly effects the formation and properties of acid gels, as described in detail in Sect. 12.3.1.2.

The effect of heat treatment on the solubilization of CCP and the release of caseins during acidification of milk has been studied (Law, 1996; Singh *et al.*, 1996). Heat treatment (in the range 70–90 °C) prior to acidification has little effect on the extent of solubilization of calcium and P_i from the micelles (Law, 1996; Singh *et al.*, 1996).

From this discussion, it appears that during acidification of unheated or heated milk to pH values ~5.1 at temperatures above 22 °C, most of the CCP in the micelles is solubilized, the charge on individual caseins is altered and the ionic strength of the solution increases. As a result, the forces responsible for the integrity of these "micelle-like" CCP-depleted casein particles are considerably different from those in native micelles even if their average hydrodynamic diameter appears largely unchanged. The balance between the (intermolecular) attractive and repulsive forces, which is important for gelation properties, is also modified (Horne, 1999).

12.3 Formation and Properties of Acid-Induced Gels

12.3.1 Mechanisms Involved in the Formation of Acid-Induced Gels

12.3.1.1 Theoretical Models

Acid milk gels (like yoghurt) are examples of particle gels (Horne, 1999). At least three theoretical models, namely fractal, adhesive hard spheres and percolation models have been used to model the formation of acidified milk gels.

Fractal aggregation theory has been applied to the formation of various casein gels (Bremer *et al.*, 1989, 1990, 1993; Vertier *et al.*, 1997; Chardot *et al.*, 2002) and is described in this section. In particle gels, a fractal scaling regime may occur only over small length scales, which are of the order of the aggregating clusters. At longer length scales, the microstructure appears homogeneous. Fractal behaviour is not expected in gels made from high volume fraction systems (Dickinson, 1997; Horne, 1999). Fractal aggregation assumes that (hard) spherical particles of radius a can move by Brownian motion and that they can aggregate when they encounter each other. The aggregates formed then also aggregate with each other. If no further changes occur among the particles in an aggregate, once they are incorporated, this cluster-cluster aggregation process leads to aggregates obeying the scaling relation:

$$\frac{N_p}{N_0} = \left(\frac{R}{a_{eff}}\right)^{D-3} \quad (1)$$

where N_p is the number of particles in an aggregate of radius R, N_0 is the total number of primary particles that could form such a floc, D is a constant called the fractal dimensionality ($D<3$) and a_{eff} is the radius of the effective building blocks forming the fractal cluster.

For casein gels (both rennet- and acid-induced), $D \approx 2.3$ has been observed generally (Bremer *et al.*, 1989, 1993), although the rheological properties of these gels differ markedly. This simple fractal approach, although it has successfully described semi-quantitative features in irreversibly aggregating systems, appears to have some deficiencies, including the lack of any allowance for aggregate rearrangement (before, during and after gelation) and interpenetration, and the assumption that all aggregates have the same size at the gel point (Dickinson, 1997). Horne (1999) concluded that although casein gels can exhibit some scaling behaviour, the fractal approach does not provide insights into the dynamics of gel development.

The aggregation of casein particles during the acidification of milk has also been modelled using the adhesive hard sphere theory (de Kruif *et al.*, 1995; de Kruif and Roefs, 1996; de Kruif, 1997, 1999). In this model, it is proposed that the glycomacropeptide (GMP) part of κ-casein sterically stabilizes casein micelles and the GMP is considered as a polyelectrolyte brush which collapses on the surface of the micelle as the pH of the system approaches the pK_a of the charged groups on the brush (reduced charge density).

Horne (1999, 2003) pointed out that the adhesive hard sphere model could be applied only to situations where there is weak attraction between particles (i.e., onset of instability) and it has no inherent time scale. Acidification of milk causes significant changes in the internal structure and integrity of casein micelles which greatly impacts milk gelation properties (Choi *et al.*, 2007; Ozcan Yilsay *et al.*, 2007). These results indicate that simple hard sphere models are inadequate for the characterization of milk gel networks.

Horne (1999) reviewed the suitability of percolation models for acid milk gels. In this theory, it is assumed that percolation clusters form bonds between close neighbours on a lattice. Bond formation is assumed to be random and as the number of bonds between neighbours increases, the clusters increase in size. In computer simulations, these functions can be calculated (de Kruif *et al.*, 1995). Above a certain threshold, a large cluster occurs which extends through the lattice. Analogies between percolation and gelation can be drawn with particles establishing an increasing number of links as they aggregate, until, at a certain threshold, a cluster is created which spans the system. At this stage, only a fraction of bonds

have been joined and, even more importantly, not all individual fractions have been incorporated into the system-spanning cluster and this mechanism would explain the continued increase in G′ after gelation (Horne, 1999); however, the author suggests that the percolation model may be suitable only at the gel point and suggests that it is difficult to use this theory to model the mechanical properties of acid gels.

12.3.1.2 Possible Physico-Chemical Mechanisms Involved in the Formation of Gels from Unheated and Heated Milks

Native casein micelles (in milk at normal pH) are stabilized by a negative charge and steric repulsion (Walstra, 1990; Mulvihill and Grufferty, 1995). On acidification, casein particles aggregate as a result of (mainly) charge neutralization, leading to the formation of chains and clusters that are linked together to form a three-dimensional network (Mulvihill and Grufferty, 1995). Acid casein gels can be formed from sodium caseinate and these gels can have generally similar properties to acid gels made from milk (Lucey *et al.*, 1997c, d), which suggests that CCP is not essential for acid gelation. Hydrophobic interactions are unlikely to play a direct role in the strength of acid gels as the elastic or storage modulus (G′) of acid gels increases with decreasing assay temperature (Roefs and van Vliet, 1990; Lucey *et al.*, 1997b, c). Cooling gels to a low temperature results in an increase in the G′ probably due to swelling of casein particles and an increase in the contact area between particles (Lucey *et al.*, 1997c). Roefs and van Vliet (1990) reported that increasing the concentration of NaCl added to cold-acidified skim milk samples resulted in a decrease in the dynamic moduli of gels formed on subsequent heating, confirming that electrostatic interactions are important for particle interactions. With increasing ionic strength, charged groups on casein would be screened, thereby weakening interactions between particles (Roefs and van Vliet, 1990; Lucey *et al.*, 1997c); calcium binding by caseins also decreases with increasing ionic strength (Dalgleish and Parker, 1980).

Lucey *et al.* (1997b) suggested that gels made from unheated milks undergo extensive particle rearrangements during the gel formation, resulting in the formation of dense clusters of aggregated casein particles, which aggregate to form a gel. From these dense clusters, it would be expected that many particles would hardly contribute to cross linking of the network; thus, unheated milk gels would probably have a low G′ value, which has indeed been reported (Lucey *et al.*, 1997b).

Acid gels made from heated milk have a higher pH at gelation (Heertje et al., 1985; Horne and Davidson, 1993) and produce considerably firmer gels than unheated milk (Lucey *et al.*, 1997b, 1998c). High heat treatment of milk causes denaturation of whey proteins and subsequently a proportion of denatured whey protein associates with the casein micelles, involving κ-casein. These whey proteins appear as appendages or filaments on the casein micellar surface in electron micrographs (Davies *et al.*, 1978; Kalab *et al.*, 1983; Mottar *et al.*, 1989). When heated milk is acidified, the denatured whey proteins associated with the casein micelles become susceptible to aggregation, as the repulsive charge on the proteins is reduced. The isoelectric pH of the major whey protein, β-lactoglobulin, is ~5.3 which is higher than that of the caseins (Kinsella and Whitehead, 1989). This would explain the high pH of gelation of heated milk. Denatured whey proteins associated with casein micelles ("bound") act as bridging material by interacting with other denatured whey proteins associated with micelles (Lucey *et al.*, 1998c). This increases the number and strength of bonds between protein particles. Altering the pH of heating impacts the proportion of denatured whey protein associated with the micelle or in the form of soluble complexes ("soluble"). There has been considerable interest in understanding the impact of different proportions of "bound" and "soluble" denatured whey proteins on acid gels (Lucey *et al.*, 1998c; Donato *et al.*, 2007; Guyomarc'h *et al.*, 2009).

The different physico-chemical mechanisms involved in the formation of acid gels made from heated milk result in the formation of gels with

different microstructure from those formed from unheated milk. There is more branching or interconnectivity of the gel network in heated milk gels than in unheated milk gels (Lucey *et al.*, 1998e). The presence of denatured whey proteins on the surface of casein particles may hinder the close approach of other casein particles and lessen the likelihood that dense clusters of casein particles could be formed. Cross-linking of aggregating particles may occur *via* the denatured whey proteins associated with the casein micelle surface (Lucey *et al.*, 1998c) instead of between charged residues or hydrophobic groups on casein molecules. Thus, "bound" denatured whey proteins play a vital role in strengthening acid gels made from heated milk.

12.3.2 Physical Properties of Acid-Induced Gels

12.3.2.1 Rheological Properties of Acid Milk Gels

In many studies, the textural properties of acid gels have been measured empirically as firmness or viscosity (e.g., Parnell-Clunies *et al.*, 1986; Dannenberg and Kessler, 1988b). Empirical methods do not help in understanding the interactions or mechanisms involved in the formation of acid gels and are more suited for purposes of quality control. The relevance of viscosity measurements in a set gel is unclear, although it is important in stirred-style yoghurts. Fundamental small and large deformation studies of acid gels are much more powerful and useful tools in understanding the formation and physicochemical properties of acid gels.

Most rheological parameters characterizing casein gels depend on the number and strength of bonds between the casein particles, on the structure of the latter and the spatial distribution of the strands making up these particles (Roefs *et al.*, 1990; Lucey and Singh, 1997).

Dynamic non-destructive testing, which involves an oscillatory applied strain or stress, can provide very useful information on the gel formation process (Lopes da Silva and Rao, 1999). Some of the main parameters that are usually determined from these tests include the G′, which is a measure of the energy stored per oscillation cycle, the viscous or loss modulus (G″), which is a measure of the energy dissipated as heat per cycle, and the loss tangent (tan δ), which is the ratio of the viscous to elastic properties (Lopes da Silva and Rao, 1999). These parameters are defined as follows:

$$G' = \left(\frac{\tau_0}{\gamma_0}\right)\cos\delta \tag{2}$$

$$G'' = \left(\frac{\tau_0}{\gamma_0}\right)\sin\delta \tag{3}$$

$$\tan\delta = \frac{G''}{G'} \tag{4}$$

where τ_0 is the amplitude of the shear stress, γ_0 is the amplitude of the strain and δ is the phase angle.

The rheological properties of yoghurt gels made from unheated and heated milk at 40 °C are shown in Fig. 12.1. Unheated milk forms a weak gel and the pH at the onset of gelation is generally ~4.9–4.8. After gelation, G′ initially increases rapidly but starts to plateau (<30 Pa) during ageing of the gel, tan δ decreased to <0.4 soon after gelation and decreased to ~0.25 during ageing of the gel. Roefs (1986) demonstrated that for acid gels made by cold acidification and quiescent heating, G′ continues to increase for a period of up to several days, due presumably to slow ongoing fusion of casein particles.

There have been a number of reports on the effects of heat treatment on the rheological properties of acid gels determined by dynamic low amplitude (strain) oscillation (van Vliet and Keetels, 1995; Lucey *et al.*, 1997b, 1998c). van Vliet and Keetels (1995) reported that acid skim milk gels made from reconstituted low-heat skim milk powder (SMP) had much lower dynamic moduli than gels made from high-heat SMP. Lucey *et al.,* (1997b) reported that heating milk at a temperature ≥78 °C greatly increased the G′ of GDL-induced acid milk gels compared to unheated milk. Increased cross-linking or bridging, by denatured whey proteins, within

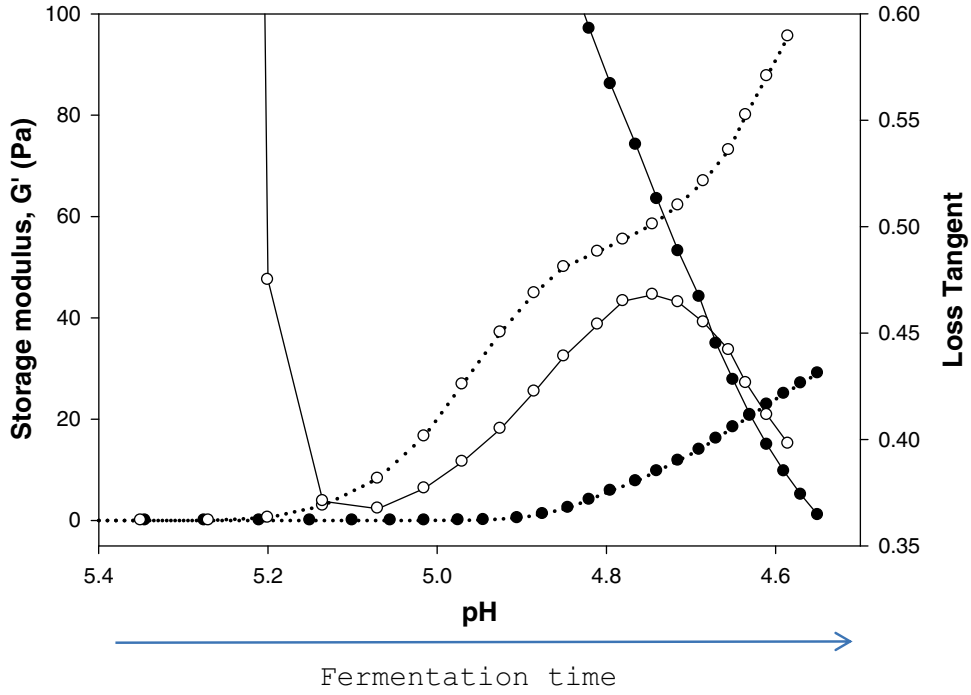

Fig. 12.1 Rheological properties of acid milk gel made by yoghurt fermentation at 40 °C from unheated (*filled circle*) or heated (85 °C for 30 min) (*unfilled circle*) milk. *Dotted lines* for storage modulus (G′) and *solid lines* for loss tangent profiles (Pachekrepapol and Lucey, unpublished results)

gels made from heated milk may be responsible for the increased rigidity and G′ of the network (Lucey *et al.*, 1997b, 1998c).

An unusual rheological phenomenon is observed soon after the formation of an acid gel from heated milk; tan δ decreases initially but then increases to a maximum value before decreasing again (Fig. 12.1) (Biliaderis *et al.*, 1992; Rönnegård and Dejmek, 1993; van Marle and Zoon, 1995; Lucey *et al.*, 1998c). A high tan δ indicates an increased susceptibility of bonds and strands in the gel to break or relax, thus facilitating more rearrangements of the gel (van Vliet *et al.*, 1991). The solubilization of CCP also results in a change in the light scattering properties of the milk system, as can be observed from the second peak in first derivative (R′) profile of yoghurt gels (Castillo *et al.*, 2006) (Fig. 12.2). The maximum in tan δ is a consequence of a partial loosening of the weak initial gel network due to the solubilization of CCP (Fig. 12.3), while at lower pH values there would be increased protein-protein attractions between casein particles as the net charge decreases on approaching of the isoelectric point (Lucey *et al.*, 1998c). The maximum in tan δ does not occur in acid gels made from unheated milk or in milk heated in the presence of a sulfhydryl blocking agent because gelation in these systems occurs at low pH values (<5.2), which is below the pH range where most of the dramatic changes in the physicochemical properties of casein micelles (e.g., solubilization of CCP) occur (Lucey *et al.*, 1998c).

Horne (1999) reported that the rheological properties of acidified milk gels exhibit a form of scaling behaviour. In this procedure, the complex shear modulus (G*) for individual milk samples are replotted as a function of reduced time, defined as the reaction time, *t*, divided by the gelation time for that profile. Then, each of these individual reduced time plots is normalized

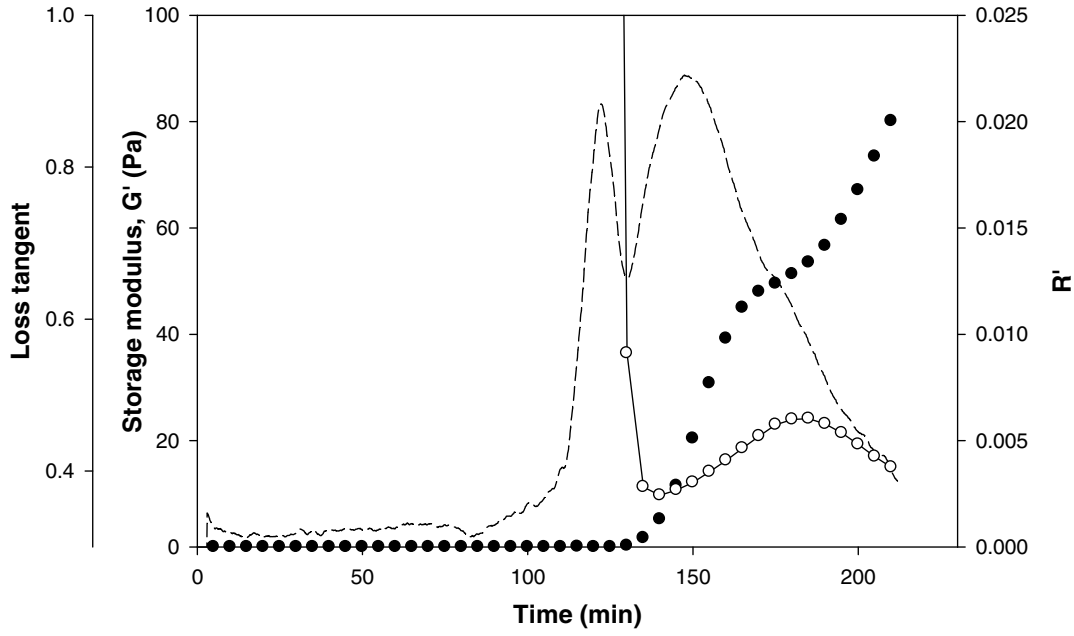

Fig. 12.2 Comparison of light scattering and rheological properties during yoghurt gelation. *Dashed line* light is R′, the first derivative of the light backscatter ratio (R), which is calculated by dividing the sensor output voltage by the initial/starting sample voltage. Method basically as described by Castillo *et al.,* (2006) using a CoAguLab instrument (Reflectronics Inc., Lexington, KY, USA). Storage modulus (G′) (*filled circle*) and loss tangent (*unfilled circle*) values (Pachekrepapol and Lucey, unpublished results)

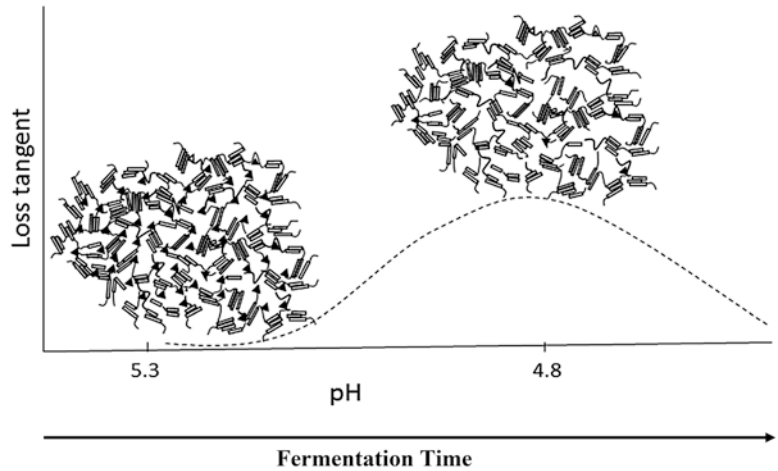

Fig. 12.3 Schematic (simplistic) representation of the possible internal structural changes in casein particles due to the loss of colloidal calcium phosphate during yoghurt gelation, which are responsible for the initial increase in the loss tangent values

against the value of its own shear modulus at three times the gelation time. For acid gels made from unheated or heated (90 °C for 10 min) milk, there are two distinct "master curves". Horne (1999) suggested that since heated milk and unheated milk had different "master curves" this implies that there are fundamental differences in the kinetics and dynamics of the gel formation

process in these two types of gels. It would appear that the rate at which bonds form between protein particles and the mechanism by which clusters grow into a network are altered by heat treatment.

The effects of fat on the fundamental rheological properties of acid milk gels have been reported (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988; Lucey *et al.*, 1998b; Cho *et al.*, 1999). The nature of the fat globule membrane determines the types of interaction that can occur between fat globules and the protein matrix. Fat globules act as an inert filler if the native fat globule membrane is intact since this membrane does not interact with casein particles (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988). The G′ value of acid milk gels decreases with an increasing volume fraction of fat, which has an intact native fat globule membrane (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988). In homogenized or recombined milk, the native membrane is replaced largely with casein and some whey protein so that the surfaces of fat particles can interact with the protein matrix (largely casein but some denatured whey proteins when the gel is made from heated milks) of acid milk gels (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988). In acid gels made from recombined milk, G′ increases with an increasing volume fraction of fat (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988; Lucey *et al.*, 1998b). Cho *et al.,* (1999) showed that the G′ of acid gels made from either heated or unheated milk was influenced by the nature of fat globule membrane; gels containing fat globules stabilized by sodium caseinate or denatured whey proteins had very high G′ values compared with those stabilised with SMP (containing casein micelles and whey proteins) or native (undenatured) whey proteins.

In experiments where the time-scale of the applied deformation was varied (frequency sweeps), log G′ versus log angular frequency gave linear lines with a slope of ~0.15 for various types of acid casein gels (Roefs and van Vliet, 1990; Lucey *et al.*, 1997b). This suggests that similar structural components are present in all types of acid casein gels.

Large deformation studies provide information on properties that may be related to the consistency of the gel during consumption or shearing (which is used in the production of stirred-style yoghurt). There is little information on the large scale deformation properties of yoghurts (Rönnegård and Dejmek, 1993). Mixing and stirring of set gels prior to rheological testing means that many reported (e.g., Dannenberg and Kessler, 1988b) yield properties are not those of the original 'set' gel. Fundamental large deformation rheological properties of acid casein gels have been reported (Bremer *et al.*, 1990; van Vliet *et al.*, 1991; van Vliet and Keetels, 1995; Lucey *et al.*, 1997b, c). Gross fracture of acid casein gels made with GDL was observed at a strain of 0.5–0.6 (Roefs, 1986; van Vliet *et al.*, 1991; van Marle and Zoon, 1995). Lucey *et al.,* (1997c), using a low constant shear rate technique for acid casein gels made *in situ*, found that the apparent shear stress at fracture increased with decreasing gelation temperature. The apparent shear stress at fracture of acid casein gels increased with ageing while the strain at fracture decreased (Lucey *et al.*, 1997c). Lucey *et al.,* (1997b) reported that heat treatment of milk prior to acidification resulted in a large reduction in the strain at fracture, from ~1.5 for gels made from unheated milk to 0.5–0.8 for gels made from milks heated at temperatures ≥80 °C.

12.3.2.2 Microstructure of Acid Gels

Electron microscopy (EM) studies on acid gels, such as yoghurt, have shown that these gels consist of a coarse particulate network of casein particles linked together in clusters, chains and strands (Kalab *et al.*, 1983). The network has pores or void spaces in which the aqueous phase is confined. The diameter of these pores varies considerably, with larger pores in gels made at a high gelation temperature or from milk with a low protein content. There have been several EM studies on the microstructure of gels formed by acidification of heated milk (Davies *et al.*, 1978; Parnell-Clunies *et al.*, 1987; Mottar *et al.*, 1989). Harwalkar and Kalab (1980) proposed, based on the examination of electron micrographs, that

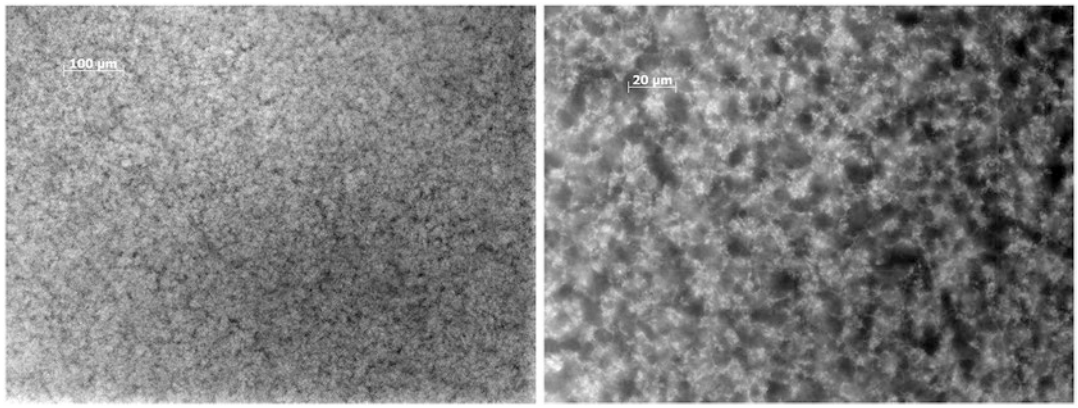

Fig. 12.4 Fluorescence microscopy of yoghurt gels made by fermentation at 40 °C from heated (85 °C for 30 min) milk. The protein matrix appears white (Pachekrepapol and Lucey, unpublished results)

yoghurt gels made from unheated milk had larger protein clusters than gels made from heated milk, which they described as highly branched.

Unless great care is taken, many of the preparation steps required in EM, including dehydration, fixation, embedding, sectioning and staining, can disrupt the native structure of milk products and lead to the creation of artefacts. Confocal scanning laser microscopy (CSLM) is a technique which enables samples to be observed with minimal preparation due to its unique optical sectioning capabilities and high spatial resolution (Brooker, 1995) and is very suitable for observing the overall microstructure of milk gels (Hassan *et al.*, 1995; Lucey *et al.*, 1997d, 1998b, e). Confocal images are very amenable to image analysis since the images are already in a digital form and, for example, they can be used to calculate the *D* of acid casein gels (Bremer *et al.*, 1993).

Many of the preparation steps used in EM of whole milk yoghurt can result in partial extraction of fat globules (Allan-Wojtas and Kalab, 1984). Barrantes *et al.* (1996) reported that in yoghurt made from recombined milk, the fat globules were not noticeable using scanning EM but could be observed using transmission EM. The microstructure of acid gels with different fat contents was investigated using CSLM by Lucey *et al.,* (1998b). In acid-induced gels made from recombined milk, fat globules appeared to be dispersed throughout the gel and the microstructure was quite different from that of skim milk gels (Lucey *et al.*, 1998b). No large pores were visible in acid gels made from recombined milk; probably fat globules obscured the finer details of pores and strands. Fluorescence micrographs of a yoghurt gel made from heated milk are shown in Fig. 12.4 (at two different magnifications). The effects of heat treatment on the microstructure of GDL-induced milk gels have been reported (Lucey *et al.*, 1998e).

12.3.2.3 Permeability of Acid Milk Gels

Permeability measurements give information about inhomogeneities at the level of the gel network (van Dijk and Walstra, 1986; Roefs *et al.*, 1990; Lucey *et al.*, 1998e) and the permeability coefficient of acid gels can be calculated as follows:

$$B = -\left[\ln\frac{\left(h_\infty - h_{t_2}\right)}{\left(h_\infty - h_{t_1}\right)}\eta\ H/\left[\rho\ g\left(t_2 - t_1\right)\right]\right] \quad (5)$$

where B is the permeability coefficient (m^2), h_∞ is the height of the whey in the reference tube (m), h_{t1} is the height of the whey in the gel tube at t_1 (m), h_{t2} is the height of the whey in the gel tube at t_2, η is the viscosity of the whey, H is the height of the gel (m), ρ is the density of the whey and g is acceleration due to gravity. In acid gels made at 30 °C with GDL, the value of B is usually in the range $1–2\times10^{-13}$ m^2 (Roefs *et al.* 1990; Lucey *et al.*, 1998e).

In rennet-induced milk gels, B increases with time, which has been taken as evidence of "microsyneresis" or breakage of strands in the network resulting in the formation of larger pores (Walstra, 1993). Studies on the permeability of acid-induced gels have shown that the value of B does not change with time (Roefs *et al.*, 1990; Lucey *et al.*, 1997d).

12.3.2.4 Appearance

Set-style yoghurt gels should have a smooth, semi-solid consistency with no surface whey (Lucey and Singh, 1997). The appearance of a set gel should be smooth with no cracks or 'blemishes'. Acid gels made from severely heated milks with GDL had a "rough" surface with visible cracks and some whey separation (Lucey *et al.*, 1998a, e). It was suggested that spontaneous rearrangement of the network just after gel formation might be responsible for these defects. Gels made from severely heated milk had a low strain at fracture compared to gels made from unheated milk and this may make heated gels more susceptible to localized fracturing of strands in the network (Lucey, 2001). The "transition" in the rheological properties (as indicated by the maximum in loss tangent) may increase the susceptibility of protein-protein bonds to relax and if these bonds have a relatively short lifetime, this may lead to yielding or breaking of strands (van Vliet *et al.*, 1991).

12.3.2.5 Whey Separation

Whey separation refers to the appearance of liquid (whey) on the surface of a milk gel and is a common defect in fermented milk products. Syneresis is defined as shrinkage of a gel and this occurs concomitantly with expulsion of whey. It is useful to define spontaneous syneresis as the contraction of a gel without the application of any external force (e.g., centrifugation) and this is related to instability of the gel network (i.e., due to large scale rearrangements) (Walstra, 1993). In practice, yoghurt manufacturers try to prevent whey separation by adding stabilizers (e.g., pectin or gelatin) or whey protein concentrate (WPC).

A simple method for quantifying spontaneous whey separation in acid gels is often used (Lucey *et al.*, 1998a). Older studies had determined the quantity of whey expelled from yoghurt as a result of high speed centrifugation or drainage through a screen (Harwalkar and Kalab, 1983, 1986; Dannenberg and Kessler, 1988a). The drainage of whey from a broken gel distributed over a screen measures whey separation when a very large surface area is available and is more relevant to products such as Cottage cheese or casein, than to set gels like yoghurt (Lucey *et al.*, 1998a). High speed centrifugation measures the water holding capacity of the gels under relatively high forces. Therefore, both of these methods for measuring whey expulsion are not relevant to the spontaneous separation of whey defect from set-style gels. The approach of Lucey *et al.* (1998a) was to make gels in containers and determine the amount of surface whey that was expelled during gelation. Whey separation (in volumetric flasks) was increased significantly by heat treatment and gelation temperature (Lucey *et al.*, 1998a); coincidentally a high heat treatment and high gelation temperature are commonly used in the manufacture of yoghurt.

A high incubation temperature is one of the main causes of whey separation in acid gels like yoghurt (Lucey and Singh, 1997). Acid-induced milk gels formed by slow acidification of milk at a low temperature and quiescent heating exhibit little wheying-off or spontaneous syneresis (Roefs, 1986).

It has been shown (van Dijk and Walstra, 1986) that the one dimensional syneresis of milk gels is related to the flow of liquid (whey) through the network and is governed by the equation of Darcy:

$$v = \frac{B}{\eta}\frac{p}{x} \tag{6}$$

where v is the superficial flow velocity of the syneresing liquid, B is the permeability coefficient, η is the viscosity of the liquid, p is the pressure acting on the liquid and x the distance over which the liquid must flow. In milk gels, an endogenous syneresis pressure can occur if there is a tendency of the casein network to rearrange after its formation (Lucey *et al.*, 1997d). It has been shown that endogenous syneresis pressure is mostly small in acid sodium caseinate gels and

this results in lesser tendency for shrinkage of these gels compared to rennet-induced gels (Lucey *et al.*, 1997d). One-dimensional syneresis of acid milk gels made with GDL increased at high gelation temperatures and high pH values (van Vliet *et al.*, 1997).

Differences in the ease of water loss from different casein gels have been related to the susceptibility of the network to rearrangements just after gel formation (van Vliet and Walstra, 1994). Parameters that affect the time-scale for rearrangements of bonds in a gel include the dynamic moduli, which indicates the strength and number of bonds in the network, the yield stress and shear deformation at yielding, which determine the susceptibility of the strands to breakage, and tan δ, with higher values favouring the relaxation of bonds (van Vliet *et al.*, 1991; Lucey *et al.*, 1997b, d). In freshly made gels, the number of bonds between each junction is not yet very high, as indicated by the low dynamic moduli and tan δ is higher than in aged gels; these factors might explain why wheying–off occurs sometimes in young but not as much in aged gels. The "maximum in tan δ" which has been observed in acid gels made from heated milk (Fig. 12.1) would indicate an even greater likelihood of relaxation of bonds during the initial period after gel formation.

Acid-induced milk gels that were first cooled to a low temperature (e.g., 5 °C) before wetting their surface actually increased slightly in height, possibly due to the absorption of water by casein particles, which swell at low temperatures (Lucey *et al.*, 1997d). Surface whey that was expelled during gelation is sometimes reabsorbed by the gel on cooling and storage at low temperature (Lucey and Singh, 1997).

12.3.2.6 Textural Defects

A wide range of cultured milk products is now on the market and each has very different textures or consistencies. Many processing parameters influence the properties of yoghurt gels (Table 12.1). The textural properties of acid milk gels can be assessed by a range of fundamental and empirical (instrumental) methods such as dynamic low amplitude oscillation, large amplitude oscillatory shear, creep or stress relaxation, penetration, rotational viscometry and flow through an orifice such as a Posthumus funnel. An excessively firm texture can be caused by factors such as a very high total solids content of the mix or an excessive amount of added stabilizers (this is exploited in the manufacture of "custard-style" yoghurt). A weak body can be caused by factors such as a low solids (fat) content of the mix, insufficient heat treatment of the milk, low acidity (high pH) and a high gelation temperature (>40 °C). Textural defects described as 'lumpiness' or 'granular' are objectionable as consumers usually expect a smooth, fine-bodied product (Tamime and Robinson, 2007; Clark *et al.*, 2009) Lumpiness usually refers to the presence of large protein aggregates in yoghurt that can often range in size from 1 to 5 mm (Lucey and Singh, 1997). Factors such as excessive production of acid at a high incubation temperature, poorly rehydrated powders, and the use of rennet have been associated with these defects (Lucey and Singh, 1997). Some of these defects may be caused by conditions that favour the formation of dense protein clusters during gelation. Excessive heat treatment of milk and the addition of a high level of WPC have also been associated with other textural defects.

Table 12.1 Some factors influencing gel strength/viscosity and whey separation of cultured milk products (general trends)

With an increase in this condition	Firmness/viscosity	Wheying-off
Protein content	↑	↓
Fat content	↑	↓
Homogenization pressure	↑	↓
Heat treatment	↑	↓ or ↑
Incubation temperature	↓	↑
pH value	↓	↑
Rennet level	↑	↑

12.3.3 Addition of Dairy Proteins

In many acid gel systems additional dairy proteins are added to improve textural attributes, reduce whey separation, or for nutritional fortification.

There are many options for adjusting the dairy protein levels of cultured products including the addition of skim milk powder, WPC, membrane concentrates, and milk protein concentrates.

There has been considerable interest in replacing skim milk powder with whey powders. The decision on possible substitution depends on factors such as their relative prices and the type of whey product. Bland whey products should be used to avoid off-flavours. Whey proteins have been used to substitute for up to 35–40 % of skim milk protein in yoghurt formulations. High concentration can result in defects and quality depends on processing conditions (i.e., heat treatment and incubation temperature). High protein whey products contain little lactose and have high gelling protein content. In yoghurt samples in which ≥20 % of milk solids-non-fat (SNF) were replaced by WPC, a 'grainy' texture was observed (Greig and Van Kan, 1984). Substituting WPC for SMP to elevate the total solids content of yoghurt mixes increased the 'lumpy' or 'granular' defect (Guirguis *et al.*, 1988), while replacement of casein by WPC resulted in a yoghurt with a 'less smooth and clumpy' appearance (Jelen *et al.*, 1987). Addition of WPC to milk, followed by heat treatment, resulted in acid gels becoming more brittle (Lucey *et al.*, 1999). With the addition of high levels (4 %) of WPC, there is an increased risk of coagulation during heat treatment (Jelen *et al.*, 1987). Possible benefits of using whey powders include: cost reduction compared with skim milk powder, improved texture, reduction in wheying-off, possible replacement of non-dairy ingredients ("cleaner label"), as well as the addition of nutritionally beneficial whey proteins, minerals and other bioactive compounds.

Peng *et al.,* (2009b) prepared yoghurt bases from reconstituted SMP with 2.5 % protein and fortified with additional 1 % protein (wt/wt) from four different milk protein sources: SMP, milk protein isolate (MPI), micellar casein (MC) and sodium caseinate. Heat-treated, yoghurt mixes were fermented at 40 °C with a commercial yoghurt culture until pH 4.6. They reported that yoghurt firmness increased in the order: skim milk powder = micellar casein < milk protein isolate < sodium caseinate. Various other studies have also suggested that the use of sodium caseinate to fortify milk was the most effective means of increasing yoghurt firmness and reducing syneresis compared to other types of milk powders (Modler *et al.*, 1983; Tamime *et al.*, 1984; Sodini *et al.*, 2004).

12.4 Interactions Between Acidified Casein Systems and Polysaccharides

12.4.1 Possible Interactions Between Caseins and Polysaccharides

In many dairy systems polysaccharides are added to increase/control viscosity or to stabilize the system, e.g., from sedimentation. Many different types of polysaccharides are used including pectins, starches, gelatin and gums.

In stirred-type yoghurt, stabilizers are added to control textural defects and prevent whey separation but stabilizers are not normally added to plain, set-style yoghurt (Lucey and Singh, 1997) although pectin is sometimes added in the US. Generally, usage levels for stabilizers in stirred yoghurt are <0.5 %. The most commonly added stabilizers in cream cheeses are xanthan gum and galactomannans (locust bean gum and guar gum), which act synergistically to increase viscosity or form a weak gel.

The nature of the interaction between milk proteins and polysaccharides is dependent on many factors including the specific types of intermolecular forces between the two biopolymers, concentrations of the two biopolymers and environmental factors (pH, ionic strength and calcium content) (Dickinson, 1998; Syrbe *et al.*, 1998).

Some possible interactions between caseins and polysaccharides are shown in Fig. 12.5. In the presence of a nonadsorbing biopolymer there can be an attractive force between casein particles due to an osmotic effect associated with the exclusion of polysaccharides from the narrow region around

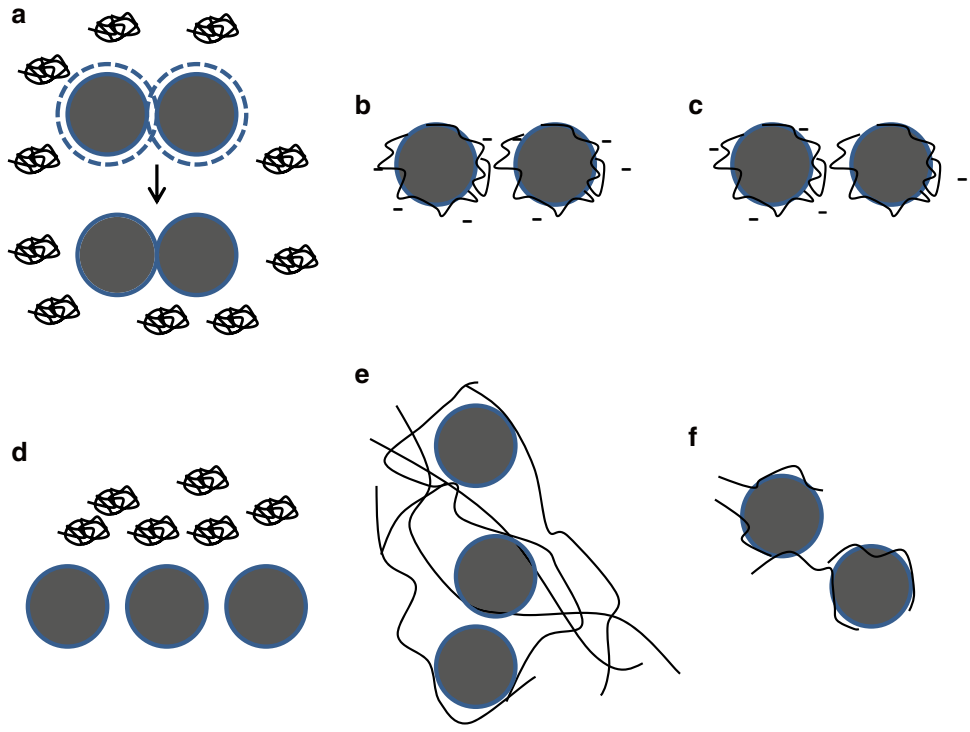

Fig. 12.5 Schematic representation of possible interactions between casein particles and polysaccharides. (**a**) depletion flocculation, (**b**) electrostatic repulsion, (**c**) electrostatic attraction, (**d**) phase separation, (**e**) high viscosity or gelling polysaccharides, and (**f**) bridging flocculation

each casein particle (Fig. 12.5a). The polysaccharides are "depleted" in the region around, and between, casein particles if they are too large to enter the depletion region. Flocculation of casein particles causes the depletion layers to overlap, thereby increasing the volume of solvent available for the polymer. Some polysaccharides are able to complex with proteins at specific pH values, for example high methoxy pectin can adsorb on caseins at low pH and provide electrostatic repulsion for acidified beverages (Fig. 12.5b). At neutral pH an electrostatic complex can be formed between oppositely charged biopolymers (Fig. 12.5c), e.g., κ-casein and κ-carrrageenan. Phase separation (Fig. 12.5d) results from incompatibility between the protein and polysaccharide molecules. Addition of high methoxy pectin to milk (at neutral pH values) can cause phase separation. Some polysaccharides have the ability to form weak gels (or systems with high zero shear viscosity) and thereby stabilize the sedimentation of casein particles (e.g., xanthan is able to form weak gels, especially in the presence of galactomannans) (Fig. 12.5e). Other biopolymers can form stiff gel networks, e.g., gelatin. Bridging flocculation occurs where there is a mixture of an adsorbing biopolymer and protein molecules and if there is insufficient polymer to cover all the particle surface completely and some polymer becomes attached simultaneously to more than one particle; this is called bridging flocculation (Fig. 12.5f). Bridging flocculation has been reported as possible between caseins and biopolymers like pectins or carrageenans (De Kruif and Holt, 2003). With a change in biopolymer concentration, there can be change in the nature of the interaction with caseins, e.g., the system can change from a depletion type interaction to a biopolymer gel network with an increase in the biopolymer concentration.

Low methoxy pectins are widely used as gelling/thickening agents in yoghurts. Pectin is an

anionic carboxylated polysaccharide and in yoghurt its gelation occurs due to the release of calcium ions from micelles during acidification, which then initiates interactions between blocks of galacturonic acid on the pectin molecules (Matia-Merino and Singh, 2007).

12.4.2 Impact of Exopolysaccharides in Acid-Induced Gels

Some yoghurt starter cultures produce exopolysaccharides (EPS) during the fermentation process and are considered to help increase the viscosity of yoghurt and reduce whey separation. They can be viewed as a naturally produced thickener. This EPS can be produced as a capsular layer around the bacterial cell or excreted into the medium to produce an effect sometimes called "ropy" or "stringy" (Hassan, 2008); popular examples of ropy yoghurt are Viili and Långfil from Scandinavia. Capsular EPS has little impact on yoghurt gelation or texture. Ropy EPS can be either charged or uncharged. Some bacterial strains produce EPS which are considered to help increase the viscosity of yoghurt and reduce whey separation. The reported EPS concentrations produced in yoghurt fermentations is relatively small <150 mg/L. It is not clear how EPS modify yoghurt texture but various mechanisms have been suggested including: EPS in the serum phase increasing product viscosity, EPS forming bridges with the casein matrix and incompatibility between the EPS and casein modifying gel structure. EPS-producing starter cultures produce a number of specific types of EPS that differ in chemical structure/sugar composition, molar mass, concentration, charge, location and structural characteristics. It is possible that charged EPS may associate electrostatically with the caseins, depending on the pH of the milk, whereas uncharged EPS may influence gelation *via* a depletion flocculation type mechanism (Girard and Schaffer-Lequart, 2007). The exact period during fermentation (before, during or after gelation) when EPS is produced may play a role in determining the impact of EPS on yoghurt gels.

12.5 Concluding Remarks

Although acidified milk gels have been made for thousands of years only the technological and microbiological aspects of these products were studied in any detail until relatively recently. Considerable progress has been made during the past 20 years on the formation and rheological properties of acidified milk gels. Recent developments were reviewed but it is believed that further work is needed in the following areas:

- The mechanism by which EPS impact the formation of acid gels, as well as, their influence on the detailed textural and sensory attributes of yoghurt. More knowledge is needed about precise physicochemical properties (molar mass, charge, branching, etc) of EPS and their importance for texture modifications of yoghurt.
- Studies that probe possible linkages between fundamental rheological properties and empirical tests and their correlation with sensory perception of texture and taste.
- Developing or modifying existing theoretical models for the formation of acid milk gels; such theories should be able to describe the kinetics of gelation and the dynamics of the rheological properties, help explain the different gelation behaviour of heated milk, be consistent with rearrangements of the aggregating particles before, during and after gelation and explain the continued increase in G′ after visual gelation.
- Concentrated yoghurts are becoming more popular but little is known about the impact of processing on their textural or physical properties, e.g., impact of heating, shearing or pumping of the fermented product. Different approaches to concentration (fortification, mechanical separation or membrane filtration) are used industrially and they produce different products but little is known about the reasons for these differences.

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Milk Proteins in Ice Cream

13

H. Douglas Goff

Abstract

Milk proteins contribute three important structural functions to ice cream. They emulsify the fat phase during homogenization to produce a stable emulsion in the mix state. Their subsequent interaction with emulsifiers during the ageing process reduces the adsorbed protein level, thus producing a fat emulsion that is able to partially coalesce in the whipped and frozen ice cream and produce desirable fat structure. Proteins present in the serum phase of the mix during whipping contribute to the development of an air bubble interface that is capable of maintaining small and stable air bubbles. Unadsorbed proteins also increase mix viscosity, particularly in the unfrozen serum phase after cryo-concentration, which leads to enhanced body and texture and reduced rates of ice recrystallization. There are many protein ingredients available, from the traditional sources of milk solids-not-fat to the isolated and modified casein-based or whey-protein based ingredients, and it is essential for product developers to utilize the appropriate sources of proteins to deliver the functional attributes needed for their specific products. This chapter, therefore, outlines the structure of ice cream and the functional contributions of proteins to deliver that structure and also reviews the understanding to-date of protein modifications that are available to dairy and ice cream manufacturers for optimizing structure and quality.

Keywords

Ice cream • Frozen dessert • Fat partial coalescence • Adsorbed protein • Protein functionality in ice cream

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13.1 Introduction

The term "ice cream" is used in the context of this chapter to represent a family of whipped dairy products that are manufactured by freezing and are consumed in the frozen state. These include: ice cream, which consists of either dairy or non-dairy fats; premium, higher fat versions; "light", lower fat versions, and ice milk; sherbet; frozen yogurt; and other related products. The structure of ice cream can be described as a complex colloid consisting of three discrete phases: (i) fat globules (some partially coalesced) and their adsorbed interfacial material, (ii) air bubbles and their adsorbed interfacial material, and (iii) ice crystals, surrounded by a freeze-concentrated aqueous serum or matrix phase that contains the sugars, proteins, polysaccharides and salts (Goff, 1997, 2002). Milk proteins are added as part of the milk solids-not-fat component; the protein content of a mix is usually about 4 %. Proteins contribute three very important functional roles to the development of structure in ice cream: emulsification and resulting contribution to partial coalescence and fat structure formation; aeration and foam stability; and solution properties (Schmidt, 1994; Goff, 1997, 2002).

Proteins exist in part at the fat-serum phase interface, together with the added surfactants. Proteins adsorb to the fat globules at the time of homogenization. They are then partially displaced by surfactants during cold aging of the mix emulsion (Goff, 2002, 2006). The resulting composition and structure of the fat globule membrane partly determines the susceptibility of the mix emulsion to partial coalescence, which in turn forms the fat structure that is responsible for many of the structural and textural attributes of ice cream. Proteins also exist at the air-mix interface. Ice cream is a frozen foam, and proteins contribute both to formation of the foam during aeration and to stabilization of the foam, together with added surfactants and partially-coalesced fat (Pelan *et al.*, 1997; Zhang and Goff, 2004, 2005; Xinyi *et al.*, 2010). Those proteins not at an interface exist in the aqueous, unfrozen phase. The water-holding capacity of proteins contributes to viscosity in the mix, especially due to freeze-concentration, which imparts a beneficial body and texture to the ice cream, increases the meltdown time of ice cream and contributes to reduced icyness (Schmidt *et al.*, 1993; Alvarez *et al.*, 2005; Patel *et al.*, 2006). Milk proteins may also be partially incompatible with the added polysaccharides in ice cream, both in the mix but especially during freeze-concentration, and thus the mix may separate into phases (Syrbe *et al.*, 1998; Vega *et al.*, 2004). The resulting networks of polysaccharide and aggregated protein may be partially responsible for controlling recrystallization of the ice phase during storage and temperature fluctuations.

Traditional sources of milk solids-not-fat (e.g., condensed skim or skim milk powder) provide micellar casein and serum proteins in the same ratio as found in milk. Modern technology can provide any blend of micellar casein, soluble caseins, and serum proteins, either in their native state or modified by various means. As ingredient manufactured isolated milk proteins, blends and fractions become increasingly commercially available, it is important to understand their functional contribution to the structure and texture of ice cream and to ensure that these functional roles are being met. Thus, the objective of this chapter is to overview briefly the ingredients and processes used for ice cream manufacture, focusing on the protein sources, and then to examine in detail the functional contributions of proteins to ice cream. This chapter is updated from the previous edition (Goff, 2003) with research from the last decade. Readers are referred to the earlier edition for a review of the older literature. Other pertinent reviews include the ice cream chapters in the lipids (Goff, 2006) and lactose (Goff, 2009) volumes of the third edition of this series, and the recently-updated *Ice Cream* text (Goff and Hartel, 2013).

13.2 Ice Cream Manufacture

13.2.1 Ingredients

13.2.1.1 Fat

The ingredients normally found in ice cream formulations are presented in Table 13.1. The fat content is an indicator of the perceived quality and/or value of the ice cream. The fat component

Table 13.1 Components of typical ice cream mix formulations

Component	Range (%)
Fat, dairy or non-dairy	10–16
Milk solids-not-fat	9–12
Sucrose	9–12
Corn syrup solids	4–6
Stabilizers/emulsifiers	0–0.5
Total solids	36–45
Water	55–64

of the mix contributes to richness of flavour of ice cream, produces a characteristic smooth texture by lubricating the palate, helps to give body, and aids in producing desirable melting properties (Goff, 2006; Goff and Hartel, 2013). Milkfat, usually supplied by cream, sweet (unsalted) butter or anhydrous milkfat ("butteroil"), is the principal, or perhaps only, fat source for dairy ice cream formulations. Vegetable fats can also be used as fat sources in non-dairy ice cream, depending on regulations. Blends of oils are often used in ice cream manufacture, selected to take into account physical characteristics, flavor, availability and cost. The naturally-saturated fats—palm kernel oil, coconut oil or palm oil—are most typically used (Goff and Hartel, 2013). During freezing of ice cream, the fat emulsion that exists in the mix will partially coalesce or destabilize as a result of emulsifier action, air incorporation, ice crystallization and high shear in the freezer (Kokubo *et al.*, 1998). This partial coalescence of the fat is necessary to set up the structure and texture in ice cream, which is very similar to the structure in whipped cream (Stanley *et al.*, 1996), and crystalline fat is necessary to establish the correct structure, hence the use of saturated sources of fat. The process of partial coalescence and the role of the milk protein in this process will be discussed in Sect. 13.5.1.

13.2.1.2 Milk Solids-Not-Fat

The milk solids-not-fat (SNF), or serum solids, contain the lactose, caseins, whey proteins, minerals (ash), vitamins, acids, enzymes and gases of the milk or milk products from which they were derived. Limitations on their use include off-flavours from certain products and an excess of lactose, which may lead to problems due to excessive freezing point depression or lactose crystallization. Traditionally, the best sources of milk SNF for high quality ice cream products have been fresh concentrated skimmed milk or spray dried low-heat skim milk powder. Others include those containing whole milk protein (e.g., condensed or sweetened condensed whole milk, dry or condensed buttermilk or milk protein concentrates), those containing casein (e.g., microfiltered micellar casein (phosphocaseinate), sodium caseinate), or those containing serum or whey proteins (e.g., dried or condensed whey, whey or serum protein concentrates, whey or serum protein isolates) (Goff and Hartel, 2013), as will be discussed in Sect. 13.3. The specific functionality of proteins in ice cream will be discussed in Sect. 13.5.

13.2.1.3 Sweeteners

Sweeteners improve the texture and palatability of ice cream and enhance flavours. Their ability to lower the freezing point of a solution imparts a measure of control over the temperature-hardness relationship (Goff and Hartel, 2013). The most common sweetening agent is sucrose, alone or in combination with other sugars. Sucrose and lactose are most commonly present in ice cream in the supersaturated, or glassy, state, with ideally no crystals present (Caldwell *et al.*, 1992; Goff, 2009). In many ice cream formulations, sweeteners derived from corn or other starch-based syrups are substituted for all or a portion of the sucrose. The use of starch hydrolysis products (also referred to as "glucose solids", although they contain only a small quantity of the monosaccharide, glucose) in ice cream is generally perceived to provide greater smoothness by contributing to a firmer and more chewy body, to provide better meltdown characteristics, to reduce heat shock potential, which improves the shelf life of the finished product, and to provide an economical source of solids.

13.2.1.4 Stabilizers

Ice cream stabilizers are a group of hydrocolloid ingredients (usually polysaccharides, although gelatin was one of the first stabilizers used) used in ice cream formulations to produce smoothness

in body and texture, retard or reduce the growth of ice and lactose crystals during storage, especially during periods of temperature fluctuation known as heat shock, and to provide uniformity to the product and resistance to melting. They also increase the viscosity of the mix, stabilize the mix (e.g., carrageenan), to prevent serum separation aid in suspension of flavouring particles, produce a stable foam with easy cut-off and stiffness at the barrel freezer for packaging, slow down moisture migration from the product to the package or the air, and help to prevent shrinkage of the product volume during storage (Goff and Hartel, 2013). Stabilizers commonly used include locust bean (carob) gum, guar gum, carboxymethyl cellulose and carrageenan. Others that may be used include sodium alginate, xanthan, gelatin or other indigenous gum sources (BahramParvar and MazaheriTehrani, 2011). Each stabilizer has its own characteristics and often two or more of these stabilizers are used in combination to lend synergistic properties to each other and improve their overall effectiveness. Carrageenan is a secondary colloid used to prevent serum separation in the mix, which is usually promoted by one of the other stabilizers (Thaiudom and Goff, 2003; Vega *et al.*, 2005). This polysaccharide-protein interaction is discussed more fully in Sect. 13.5.3.

13.2.1.5 Emulsifiers

Emulsifiers have been used in ice cream mix manufacture for many years, starting with egg yolk in traditional recipes. They are usually integrated with the stabilizers in proprietary commercial blends but their function and action is very different from that of the stabilizers. They are used to: (i) improve the whipping quality of the mix; (ii) produce a drier ice cream to facilitate moulding, fancy extrusion and novelty product manufacture; (iii) produce a smoother body and texture in the finished product; and (iv) promote superior drawing qualities at the freezer to produce a product with good stand-up properties and melt resistance (Barfod *et al.*, 1991; Goff, 1997, 2002; Goff and Hartel, 2013). Their mechanism of action can be summarized as follows: they lower the fat/aqueous phase interfacial tension in the mix, resulting in protein displacement from the fat globule surface, which in turn reduces the stability of the fat globule to partial coalescence that occurs during the whipping and freezing process, leading to the formation of an aggregated fat structure in the frozen product which contributes greatly to texture and melt-down properties. The extent of protein displacement from the membrane, and hence the extent of fat destabilization achieved, is a function of the concentration of emulsifier (Bolliger *et al.*, 2000). The interaction of emulsifiers with proteins and role in structure formation will be described in Sect. 13.5.1. Emulsifiers used in ice cream manufacture today are of two main types: mono- and di-glycerides and sorbitan esters. Of the latter, Polysorbate 80 is a very strong promoter of fat destabilization in ice cream (Goff *et al.*, 1987) and is used in many commercial stabilizer/emulsifier blends.

13.2.2 Processes

13.2.2.1 Mix Manufacture

Ice cream processing operations can be divided into two distinct stages, mix manufacture and freezing operations (Fig. 13.1). The manufacture of ice cream mix involves the following unit operations: combination and blending of ingredients, batch or continuous pasteurization, homogenization and aging (Goff and Hartel, 2013). Pasteurization is designed to kill pathogenic bacteria. In addition, it serves a useful role in reducing the total bacterial load and in solubilization of some of the components (proteins and stabilizers). Both batch (>~69 °C for ~30 min) and continuous (high temperature-short time, HTST, >~80 °C for ~15–25 s) systems are in common use. Homogenization is responsible for the formation of the fat emulsion by forcing the hot mix through a small orifice under pressures of 15.5–18.9 MPa (2000–3000 psig), depending on the composition of the mix. A large increase in the surface area of the fat globules is responsible in part for the formation of the fat globule membrane, comprised of adsorbed materials that lower the interfacial free energy of the fat globules. With single stage homogenizers, fat globules tend to cluster as

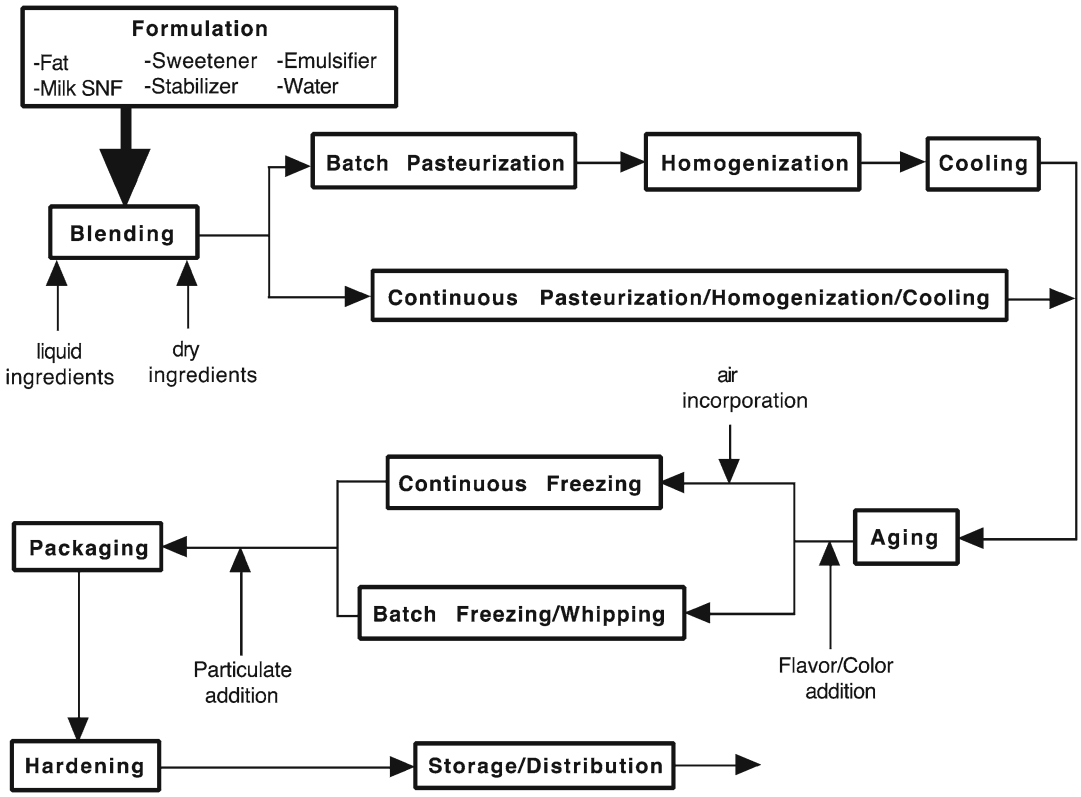

Fig. 13.1 Schematic illustration of the processing steps in ice cream manufacture (SNF, solid-non-fat)

newly created fat surfaces not coated with emulsifier come together or adsorbed emulsifier molecules are shared. Therefore, a second homogenizing valve is frequently placed immediately after the first with applied back-pressures of 3.4 MPa (500 psig), allowing more time for surface adsorption of emulsifier molecules to occur. An aging time of 4 h or greater is recommended following processing of the mix and prior to freezing to produce a smoother texture and better quality product. The temperature of the mix should be maintained as low as possible without freezing (≤4 °C). Aging allows for hydration of milk proteins and stabilizers (some increase in viscosity occurs during the aging period), crystallization of fat and rearrangement of the fat globule membrane (protein displacement by surfactant). The appropriate ratio of solid:liquid fat must be attained at this stage, a function of temperature and the triglyceride composition of the fat used, as a partially crystalline emulsion is needed for partial coalescence during the whipping and freezing steps (Barfod *et al.*, 1991; Boode and Walstra, 1993; Boode *et al.*, 1993; Mendez-Velasco and Goff, 2012a). Emulsifiers generally displace milk proteins from the fat surface during the aging period (Goff *et al.*, 1987; Euston *et al.*, 1995, 1996); this is discussed in Sect. 13.5.1.

13.2.2.2 Freezing

Ice cream freezing also consists of two distinct stages: passing the mix after aeration through a swept-surface heat exchanger, jacketed with a liquid, evaporating refrigerant, under high shear conditions to promote extensive ice crystal nucleation and air bubble formation (dynamic freezing); and freezing (hardening) the ice cream quiescently, after addition of particulate ingredients and packaging, under conditions that promote rapid freezing and small ice crystal sizes

(−30 °C or colder, either forced convection or plate-type conduction freezers) (Adapa *et al.*, 2000b; Cook and Hartel, 2010). The dynamic freezing and whipping process is one of the most important unit operations for the development of quality and yield of finished product, due to the incorporation of air creating the foam, the formation of the ice phase, and the destabilization of the fat emulsion due to freeze-concentration of the emulsion, air incorporation and high shear (Goff, 2002, 2006). One objective in ice cream manufacture is to produce ice crystals that are below, or at least not significantly above, the threshold of sensory detection at the time of consumption. This threshold has been suggested by Cook and Hartel (2010) to be between 40 and 50 μm. Consequently, the freezing steps of the manufacturing process and the temperature profile throughout the distribution system are critical factors in meeting this objective (Adapa *et al.*, 2000b; Cook and Hartel, 2010). Following rapid hardening, ice cream storage should occur at a low, constant temperature, usually −25 °C.

13.3 Sources of Milk Proteins in Ice Cream

13.3.1 Whole Milk Protein Products

There are a large variety of potential sources of milk proteins for use in ice cream, either in a milk SNF mixture that also includes variable lactose and ash components, or in an enriched form. Whole milk protein blends contain both caseins and whey proteins, and this category includes most of the traditional sources of milk SNF, typically condensed or dry skim milk, as discussed in Sect. 13.2.1.2. However, most ice cream formulations now use another source or sources of milk SNF or milk protein to replace all or a portion of skim milk solids, for both functional and economical reasons (Goff and Hartel, 2013). When assessing replacements for skim milk solids, an important consideration is the concentration of protein, lactose and ash in the ingredient preparation. Lactose is not very sweet and not very soluble, and therefore during freezing of ice cream, it is freeze-concentrated beyond maximum solubility and thus potentially prone to crystallization. Lactose crystals are very undesirable in ice cream, causing the defect known as sandiness. Lactose, being a disaccharide, also contributes to freezing point depression in the mix, so its concentration must be controlled closely (Goff, 2009). The milk salts also affect both the flavour and texture of ice cream (Goff and Hartel, 2013). Also, when replacing skim milk solids, sufficient total solids must be added to limit the water content of the mix and meet legal minimum total solids requirements. For these reasons, it is often desirable to find a skim milk replacement with similar concentrations of lactose and protein. Lactose can be reduced through ultrafiltration or modified by limited hydrolysis to its constituent monosaccharides; either change will affect the concentration of the ingredient that can be used and the subsequent protein level achieved in the ice cream.

Buttermilk solids have often been cited as a useful substitute for skim milk solids. Buttermilk contains a higher concentration of fat globule membrane phospholipids than skim milk. Thus, it can be used for its emulsifying properties to reduce the need for emulsifiers, or in formulations where it is undesirable to add emulsifiers (Goff and Hartel, 2013). The use of milk protein concentrates, produced by ultrafiltration and diafiltration of skim milk, has also been explored in ice cream applications, as a means of enhancing protein levels and reducing lactose (Alvarez *et al.*, 2005; Patel *et al.*, 2006).

13.3.2 Casein Products

It is possible to produce concentrated protein products from the casein fraction of milk proteins, the most common for use as a food ingredient being sodium caseinate. The use of sodium caseinate in ice cream has been investigated, and a small percentage may be useful in contributing to functional properties, particularly aeration and emulsification (Goff *et al.*, 1989; van Camp *et al.*, 1996). However, the functionality of sodium

caseinate is different than that of micellar casein, which needs to be considered when proposing its use. Sodium caseinate can contribute positively to aeration, but may lead to an emulsion that is too stable to undergo the required degree of partial coalescence. It is therefore most desirable in the serum phase rather than at the fat interface.

Recently, microfiltration has been used to separate native serum proteins from micellar casein (Nelson and Barbano, 2005; Hurt and Barbano, 2010). Although the focus was on the casein for cheesemaking and the functionality of the serum proteins, it demonstrates the potential to develop native streams of casein devoid of serum proteins. This product is also referred to as phosphocaseinate, to distinguish it from the caseinates devoid of phosphate, although it should be noted that there is much more native micellar structure retained in casein preparations derived from microfiltration than fromacid precipitation and neutralization to form caseinates

Recent research has also focused on modifications of casein micelles by altering the balance of micellar to soluble casein. High hydrostatic pressure processing of either concentrated skim milk for ice cream manufacture (Lim *et al.*, 2008) or of ice cream mix (Huppertz *et al.*, 2011) has demonstrated enhanced functional properties of the proteins and improved development of structure in ice cream. This has largely been attributed to alterations in casein micelle structure to enhance soluble caseins, and suggests that alternative technologies to accomplish the same goal may also enhance ice cream structure: the use of various mineral salts or mineral salt chelators, for example. It was demonstrated that EDTA could enhance soluble protein and improve ice cream structure, by chelating serum calcium, which reduces micellar calcium and causes an increase in soluble casein (Zhang and Goff, 2004). The same concept has been demonstrated with the use of citrate salts, although not specifically for ice cream applications (Augustin and Clarke, 2008). On the other hand, the addition of calcium salts to ice cream mix causes a reduction in soluble casein, which negatively impacts on ice cream structure (Costa *et al.*, 2008). Another recent modification of casein micelle functionality discussed in the literature is through its interaction with κ-carrageenan, to produce an aggregated particle that has been shown to enhance viscosity and creaminess in dairy products (Ji *et al.*, 2008a, b; Flett *et al.*, 2010a, b). Modifications of caseins and milk fat globules through microfluidization has also been demonstrated to enhance sensory properties (Ciron *et al.*, 2011, 2012). They demonstrated that the microstructure of low-fat yoghurts prepared with microfluidized milk consisted of smaller and more uniform fat globules, well incorporated into more interconnected fat-protein gel networks, compared with those of control yoghurts. This modification in microstructure increased the gel particle size, gel strength and viscosity; and significantly improved creaminess and desirable texture characteristics such as smoothness, cohesiveness, thickness, and oral and spoon viscosity. The same improvements might also be seen in low-fat ice cream mixes.

13.3.3 Whey Protein Products

There has been a great deal of attention to the use of whey products in ice cream. Whey contains fat, lactose, whey proteins and water but no casein. While skim milk powder contains 54.5 % lactose and 36 % protein, whey powder contains 72–73 % lactose and only about 10–12 % protein. Thus, it can aggravate some of the problems associated with high lactose (Smith *et al.*, 1984). However, an increasing number of whey products are available that have higher protein and lower lactose contents, mostly processed by membrane technology (Lee and White, 1991). Use of these ingredients can provide much higher quality than the traditional whey ingredients (Goff and Hartel, 2013). Whey protein concentrates with similar protein and lactose contents to skim milk solids can be produced by ultrafiltration. Protein content can vary from 20 to 35 %. The use of diafiltration can increase protein content to 75 %. If desired, the remaining lactose can be modified by hydrolysis, although the freezing point depression effect of the higher monosaccharide content

must be considered. Ash content can be reduced by demineralization. Whey protein isolates are produced either by a combination of ultrafiltration and microfiltration or by ion exchange processing, which contain no lactose. These are also available for blending with other ingredients to increase the SNF content of ice cream formulations and to modify the protein: lactose ratio or the whey protein:casein ratio. Recent work has also demonstrated the potential to develop native serum proteins, separated from casein by microfiltration (Nelson and Barbano, 2005; Hurt and Barbano, 2010; Adams and Barbano, 2013). These are extracted directly from skim milk rater than from whey, thus not exposed to the processing steps used in cheesemaking thus keeping a high level of functionality and flavor.

There has been considerable recent research looking at the potential to develop whey protein-based products with enhanced functionality for ice cream applications through heat-aggregation of the proteins (Sourdet *et al*., 2002; 2003; Granger *et al.*, 2005a; Udabage *et al.*, 2005; Relkin *et al.*, 2006). Casein micelles play an important role in ice cream functionality, as described in Sect. 13.5. So the concept is that a heat-aggregated particle of whey protein may play a role closer to that of the casein micelle than a soluble protein. Indeed, limited heat aggregation has been shown to enhance functionality, but the balance of heated whey protein to unheated whey protein to casein is critical for optimal functionality.

One area where aggregated whey proteins have been successful is in the development of fat replacers for low fat applications. These proteins are usually processed by thermally aggregating under shear to produce minute particles (0.5–2 μm diameter) that can promote a creamy textural sensation. This process is called microparticulation. Size of the particulates is an important determinant of mouthfeel. The particles, being hydrophobic on their surfaces due to their content of non-polar amino acids, resist interactions with each other. They can be spray dried and reconstituted without affecting particle integrity. They hold water and disperse well in aqueous systems. The application of microparticulated whey proteins into frozen dessert products has been studied by Schmidt *et al.,* (1993), Ohmes *et al.,* (1998), Adapa *et al.,* (2000a), Liou and Grün (2007) and Aykan *et al.,* (2008). An improvement in texture from the use of whey protein-based fat replacers in light and low fat formulations has been demonstrated in this work, although it can also lead to higher mix viscosity.

13.4 Ice Cream Structure

The texture of ice cream is perhaps one of its most important quality attributes. It is the sensory manifestation of structure; thus, establishment of optimal ice cream structure is critical to maximal textural quality. It is also critical to processing parameters (e.g., dryness on extrusion), shelf-life, and quality parameters during consumption (e.g., shape retention during melting) (Muse and Hartel, 2004). Structurally, ice cream is comprised of thee discrete phases—fat globules, some of which may be partially coalesced, air bubbles, and ice crystals—these are embedded into a freeze-concentrated unfrozen matrix of soluble sugars, proteins, minerals salts, stabilizers and water (Fig. 13.2). Both the fat phase and the air phase also have interfacial layers associated with them. An understanding of the functional role of proteins in ice cream depends on conceptualization of this structure. The structure of ice cream begins with the mix as a simple emulsion, with a discrete phase of partially crystalline fat globules surrounded by an interfacial layer comprised of proteins and surfactants (Fig. 13.3). The continuous, serum phase consists of the unadsorbed casein micelles in suspension in a solution of sugars, unadsorbed whey proteins, salts and high molecular weight polysaccharides. Ice cream is a complex food colloid in that the mix emulsion is subsequently foamed, creating a dispersed phase of air bubbles, and is frozen, forming another dispersed phase of ice crystals (Fig. 13.4). Air bubbles and ice crystals are usually in the size range of 20–50 μm (Caldwell *et al.*, 1992). The serum phase is freeze-concentrated. In addition, the partially-crystalline fat phase, at refrigerated temperatures,

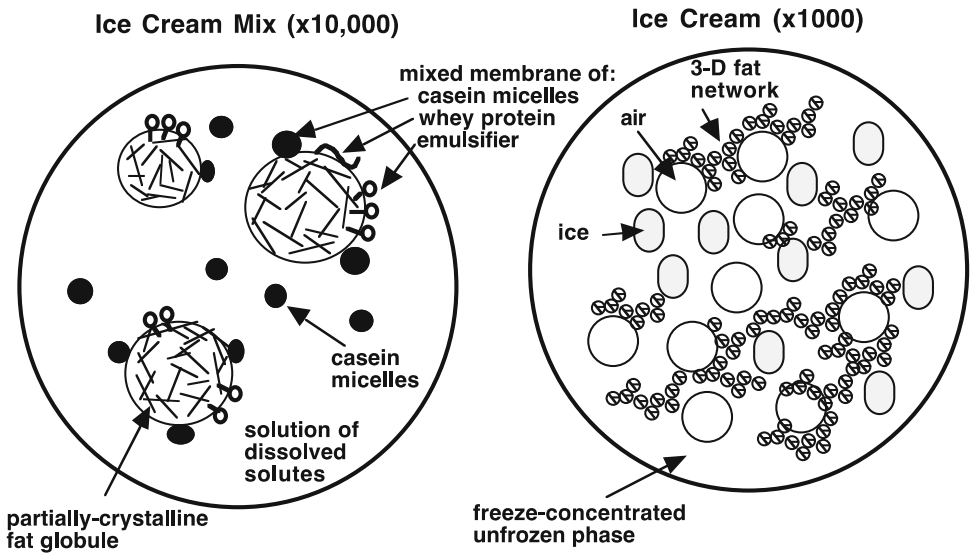

Fig. 13.2 Highly schematic illustration of the structure of ice cream mix and ice cream. Ice cream mix contains the partially crystalline fat globules and casein micelles as discrete particles in a solution of sugars (including lactose), salts, dispersed whey protein and stabilizers, etc. The surface of the fat globule demonstrates the competitive adsorption of casein micelles, globular, partially denatured whey proteins, β-casein and added emulsifiers. Ice cream contains the ice crystals, air bubbles and partially-coalesced fat globules as discrete phases within an unfrozen serum containing the dissolved material (including lactose). The partially-coalesced fat agglomerates adsorb to the surface of the air bubbles, which are also surrounded by protein and emulsifier, and link the bubbles through the lamellae between them

undergoes partial coalescence during the concomitant whipping and freezing process, resulting in a network of agglomerated fat, which partially surrounds the air bubbles and gives rise to a solid-like structure (Fig. 13.5) (Goff, 2002, 2006). Given this context, the functional role of protein can be examined, considering their behaviour at the fat interface, the air interface and in the serum phase.

13.5 Functional Roles of Milk Proteins in Ice Cream

13.5.1 Emulsification

The interfacial behaviour of milk protein in emulsions is well documented, as is the competitive displacement of proteins by low molecular weight surfactants (Chen and Dickinson, 1993; Euston *et al.*, 1995, 1996; Granger *et al.*, 2005a, b). In ice cream, the emulsion must be stable to withstand mechanical action in the mix state, but must undergo sufficient partial coalescence to establish desirable structural attributes when frozen. These include dryness at extrusion for fancy molding, slowness of melting, some degree of shape retention during melting and smoothness during consumption. This implies the use of small molecule surfactants (emulsifiers) to reduce protein adsorption and produce a weak fat membrane that is sensitive to shear action (Goff *et al.*, 1987, 1989; Gelin *et al.*, 1994, 1996a, b; Goff, 2002). Figure 13.3 demonstrates the action of emulsifiers in ice cream by showing protein adsorption to fat globules in the absence of emulsifier (Fig. 13.3a), in which considerable casein micelle adsorption can be seen, and in the presence of emulsifier (Fig. 13.3b), which shows little or no casein adsorption. The loss of steric stability from the globule, which was contributed by micellar adsorption, accounts for its greater propensity for partial coalescence during shearing. Partial coalescence is responsible for establishing a three-dimensional aggregation of fat globules that provide structural integrity (Fig. 13.5).

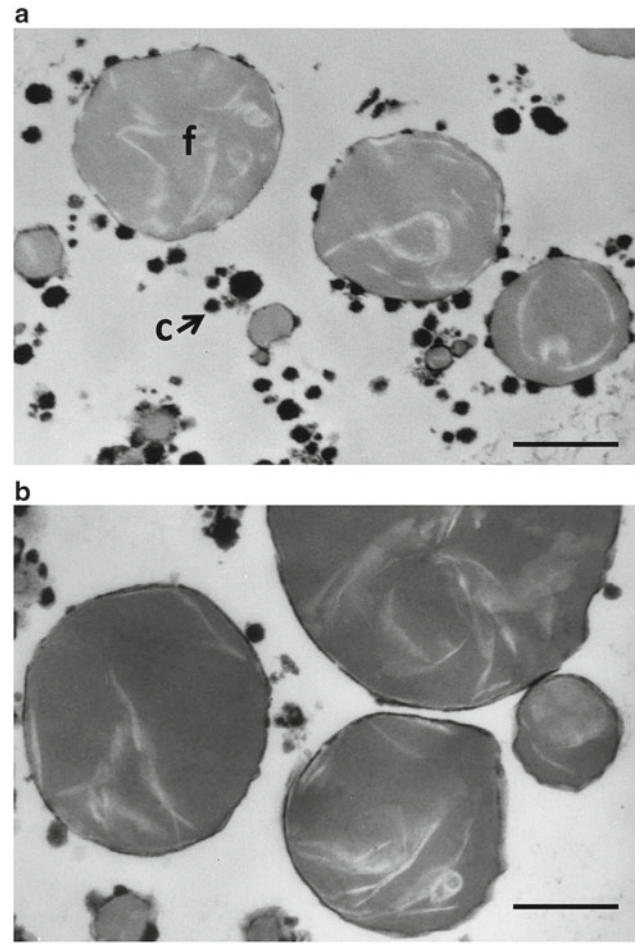

Fig. 13.3 Transmission electron micrographs showing the structure of fat and protein in ice cream mix in the absence (**a**) or presence (**b**) of emulsifier. f = fat, c = casein micelle, bar = 1 μm. For preparation method, see Goff *et al.,* (1987).

This is especially important if such integrity is needed when the structural contribution from ice is weaker (i.e., before hardening or during melting). Variables that affect the destabilization of fat in ice cream have been well studied (Kokubo *et al.*, 1998; Sourdet *et al.*, 2003; Mendez-Velasco and Goff, 2012a, b).

With respect to protein contribution to fat globule integrity, it is obvious from the studies to date that a weak surface layer is most desirable. Segall and Goff (1999) examined the susceptibility of ice cream emulsions to partial coalescence during shear when the emulsion was prepared with varying concentration and type of protein, while still retaining sufficient quiescent emulsion stability. The membranes of fat globules stabilized by an excess of whey protein (from whey protein isolate) are generally too stable to undergo partial coalescence (Sourdet *et al.*, 2002; Zhang and Goff, 2005). However, the membranes of fat globules stabilized by limited whey protein isolate were more susceptible than those made from sodium caseinate or casein micelles, while those made from partially hydrolyzed whey proteins did not show sufficient quiescent emulsion stability (Segall and Goff, 1999). The problem with this approach, though, is that when casein was added after homogenization to the limited whey protein-stabilized emulsion, further casein adsorption to the whey protein stabilized membrane was rapid, unless suitably controlled (Segall and Goff, 2002a, b).

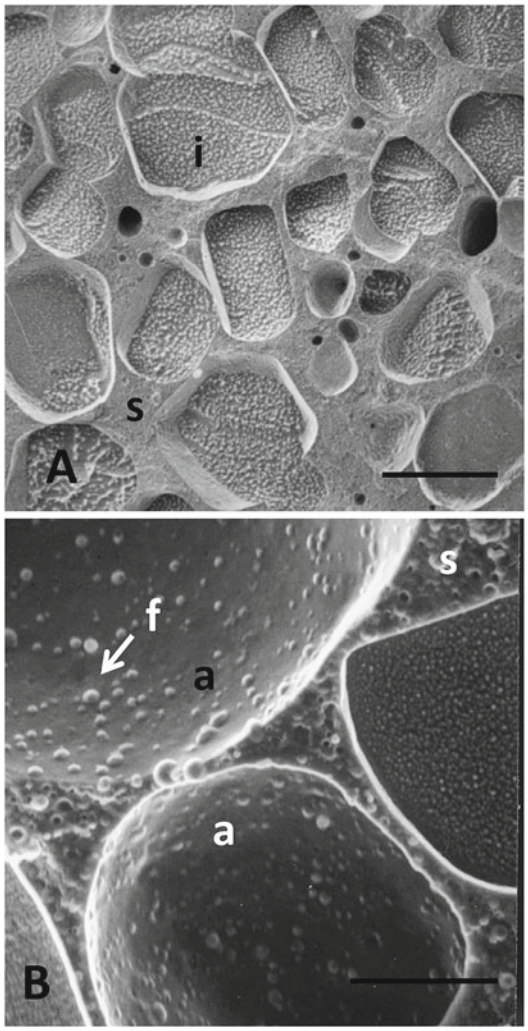

Fig. 13.4 Cryo-scanning electron micrographs of ice cream structure. (**A**) low magnification showing region dominated by ice crystals. Bar = 30 μm. (**B**) higher magnification showing close-up of air bubble interior and fat globules. Bar = 10 μm. a = air bubble, f = fat globule, i = ice crystal, s = unfrozen serum phase, For preparation method, see Caldwell *et al.,* (1992)

Heat aggregation of whey proteins plays an important role in producing a fat globule interface that is more susceptible to partial coalescence (Sourdet *et al.*, 2002; 2003; Granger *et al.*, 2005a; Relkin *et al.*, 2006). However, the heat-aggregated proteins by themselves may not be sufficient to establish a stabilizing membrane to get good homogenizing effects (Granger *et al.*, 2005a). Thus, an understanding of protein structures and protein-surfactant interactions at the fat interface is critical to develop the optimal level of partial coalescence in the finished product.

13.5.2 Aeration

Milk proteins are well known for their foaming properties (Xinyi *et al.*, 2010) and during the manufacture of ice cream, air is incorporated to about 50 % phase volume. Thus, it should be unsurprising that milk proteins contribute to stabilizing the air interface in ice cream. Foam formation and stabilization in ice cream has recently been reviewed (Xinyi *et al.*, 2010). Continuous air interfaces in ice cream can always been seen by scanning electron microscopy (Fig. 13.4b). During sublimation, these interfaces remain intact, suggesting that a combination of protein, emulsifier and fat forms a continuous layer separating the air bubble from direct contact with ice. This air interface is very important for overall structure and structural stability (Turan *et al.*, 1999; Sofjan and Hartel, 2004). Loss of air, usually due to a lack of functional protein and the development of air channels, can lead to a defect known as shrinkage, the occurrence of which is fairly common and very significant for quality loss and unacceptability of the product (Dubey and White, 1997; Turan *et al.*, 1999). The process of whipping heavy cream includes an initial protein adsorption to the air interface and a subsequent adsorption of fat globules and their associated membrane to the existing protein membrane of the air bubble (Stanley *et al.*, 1996; van Camp *et al.*, 1996). Globular fat adsorption to air interfaces is known to stabilize air bubbles from rapid collapse (Stanley *et al.*, 1996; Chang and Hartel, 2002a, b). Proteins at the fat interface have also been shown to play an important role during the aeration of emulsions (van Camp *et al.*, 1996; Zhang and Goff, 2005). Incorporation of air into ice cream in commercial whipping/freezing equipment is rapid, occurring within seconds, and at the same time, viscosity of the surrounding matrix increases exponentially due

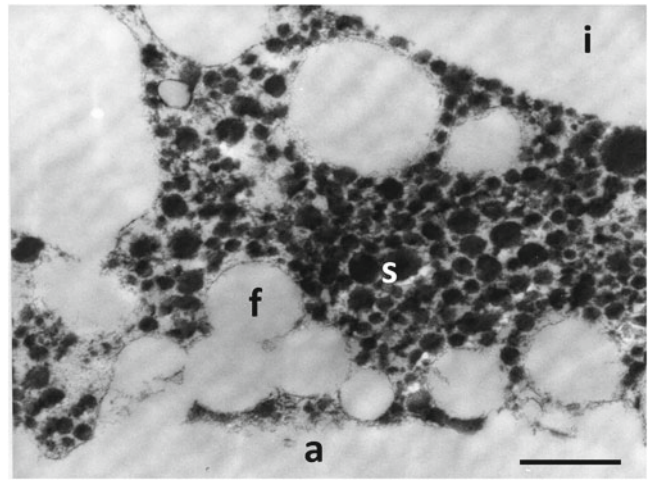

Fig. 13.5 A transmission electron micrograph prepared by freeze substitution showing the structure of partially coalesced fat, the freeze-concentration of casein micelles in the unfrozen phase and the interaction of fat at the air interface. a = air bubble, f = fat globule, i = ice crystal, s = serum phase packed with casein micelles, bar = 1 μm. For preparation method, see Goff *et al.,* (1999a)

to freezing, such that air bubbles after formation become physically trapped in a semi-solid matrix, making their collapse quite difficult.

Goff *et al.,* (1999a) examined air interfaces in ice cream and fat-air interactions using transmission electron microscopy with freeze-substitution. The structures created by increasing levels of fat destabilization in ice cream (achieved through increased emulsifier concentration in the mix and batch versus continuous freezing) were observed as an increasing concentration of discrete fat globules at the air interface (as in Fig. 13.4b) and increasing coalescence and clustering of fat globules both at the air interface and within the serum phase (Fig. 13.5). Air interfaces at the highest levels of fat destabilization were not completely covered by fat globules. There was no evidence of a surface layer of free fat in the work of Goff *et al.,* (1999a). Further, air interfaces in ice creams showing low level of fat adsorption to the air interface, due to very stable fat globule membranes Fig. 13.6 shows a very similar, continuous membrane to those from a formulation containing adsorbed fat, offering further suggestion that the air bubble membrane itself is comprised of protein, with discrete and partially-coalesced fat globules subsequently adsorbed.

Proteins adsorbed to the air interfaces in ice cream are difficult to quantify. Zhang and Goff (2004) used immune-gold labeled β-casein and β-lactoglobulin in ice cream and visualized protein adsorption to the air interfaces by transmission electron microscopy. They showed that the air interfaces were stabilized to a great extent by soluble casein and whey proteins, when available, compared to casein micelles. Modifications of micellar casein to enhance soluble casein, for example by the addition EDTA, also enhanced protein adsorption to the air interface (Zhang and Goff, 2004).

13.5.3 Solution Behaviour

Milk proteins interact with water and the subsequent hydration is responsible for a variety of functional properties, including rheological behaviour. Thus, freeze-concentration of proteins in ice cream must lead to a sufficient concentration to have a large impact on the viscosity of the unfrozen phase and its subsequent effect on ice crystallization, ice crystal stability and solute mobility (Flores and Goff, 1999; Regand and Goff, 2002). The cryo-concentration of casein micelles in the unfrozen serum phase in ice cream can easily be seen in Fig. 13.5. This has a large impact on all the other structural elements in ice cream (Sofjan and Hartel, 2004). Jonkman *et al.,* (1999), who studied the effect of ice cream manufacture on the structure of casein micelles, found that the micelles *per se* were not affected by the process. Although the stability of the

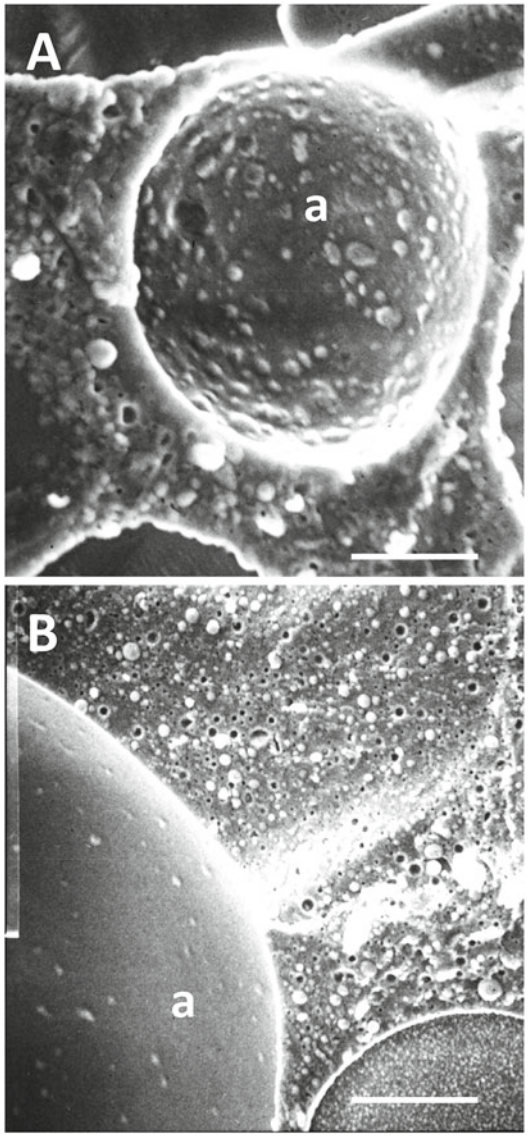

Fig. 13.6 Cryo-scanning electron micrographs of the air interface in ice cream showing a high level of fat adsorption (**A**) or a low level of fat adsorption (**B**). In both cases, the air interface surrounding the fat is comprised of protein. a = air bubble, bar in (**A**) = 30 μm, bar in (**B**) = 10 μm. For preparation method, see Caldwell *et al.,* (1992)

micelle was expected to be affected by low temperature, this was offset by an increasing concentration of milk salts in solution during freeze-concentration, such that the micelle remained intact in a similar state to that found in the mix (Fig. 13.5).

Polysaccharides are also added to ice cream mix to enhance solution viscosity and to impact on ice crystallization behaviour. Commonly used polysaccharides can be incompatible in solution with milk proteins leading to a microscopic or macroscopic phase separation (Syrbe *et al.*, 1998; Vega *et al.*, 2005), a phenomenon that has been studied in milk and ice cream mixes (Garnier *et al.*, 1995; Bourriot *et al.*, 1999; Schorsch *et al.*, 1999a, b, 2000). Goff *et al.,* (1999b) examined the interaction between milk proteins and polysaccharides in frozen systems using labelled polysaccharides and fluorescence microscopy, and demonstrated a clear phase separation between the two, leading to discernable networks created by freezing from both locust bean gum and milk proteins (Fig. 13.7). The same phenomenon can be seen by transmission electron microscopy of ice cream, where it can be seen that when in solution with polysaccharides, the casein aggregates into distinct networks (Fig. 13.8). Flores and Goff (1999) demonstrated that milk proteins had a large impact on ice crystal size and stability. It thus appears that microscopic phase separation of the milk protein induced by polysaccharides, and "aggregation" of casein into a weak gel-like network, promoted also by freeze-concentration, may be at least partly responsible for ice crystal stability and for body and texture of the ice cream during consumption. It is well known that κ-carrageenan controls macroscopic phase separation between casein micelles and polysaccharide stabilizers in ice cream or ice cream mixes, although it has recently been shown that they are still phase separated at a microscopic scale (Vega and Goff, 2005; Vega *et al.*, 2004, 2005). Spagnuolo *et al.,* (2005) demonstrated that κ-carrageenan interacts directly with casein micelles and also κ-carrageenan helices interact with each other, to form a weak structural network that holds the individual protein-rich or polysaccharide-rich phases intact in a water-in-water type "emulsion", so they do not lead to macroscopic phase separation.

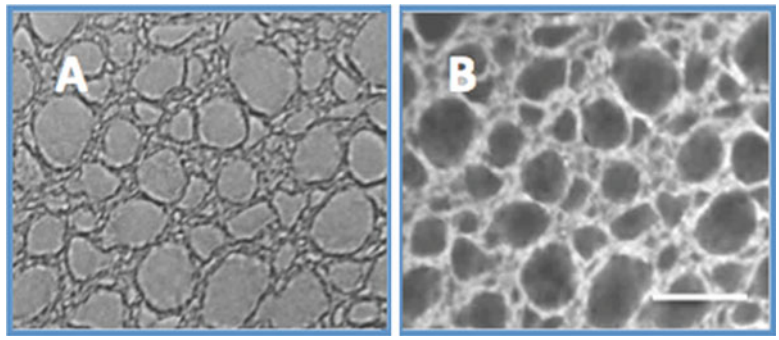

Fig. 13.7 Confocal scanning laser micrographs of frozen and melted non-fat ice cream mix, showing phase separation of protein and locust bean gum (tagged with a fluorescent marker). The structure resulted from the formation of ice; however, melting has caused the ice to disappear but the protein and polysaccharide structure to remain. (**A**) phase contrast image, showing the structure that results from cryo-aggregated protein. (**B**) Fluorescent image from the same field as (**A**), showing the structure that results from cryo-gelled, fluorescent-labeled locust bean gum. Bar = 50 μm. For preparation method, see Goff *et al.,* (1999b)

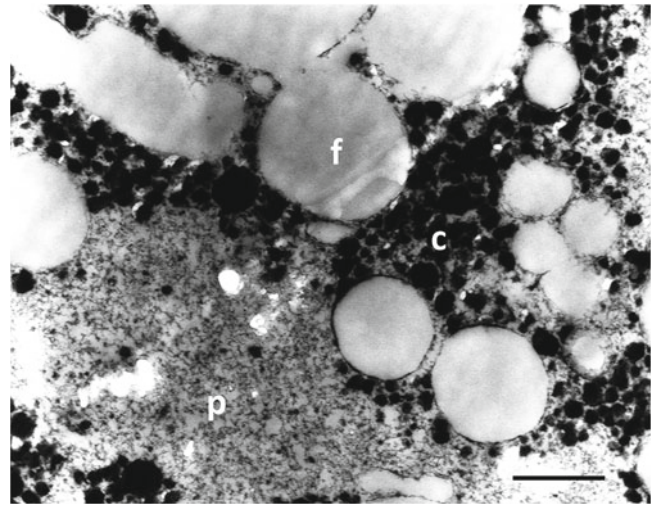

Fig. 13.8 A transmission electron micrograph prepared by freeze substitution showing the phase separation and aggregation of casein micelles resulting from the combination of partial coalescence of the fat and addition of polysaccharide. f = fat globule, c = casein micelle, p = polysaccharide network devoid of casein micelles, bar = 1 μm. For preparation method, see Goff *et al.,* (1999a)

13.6 Conclusions

Ice cream is a complex food colloid, with at least three discrete phases (ice, air and fat) and a continuous unfrozen phase. Proteins contribute several important functional roles to ice cream that can be divided into three categories: emulsification, aeration and solution behaviour. They also contribute to sensory properties (flavour and body) of the product in ways that are exclusive of any of these physical contributions. Several choices are available to manufacturers for their source of milk proteins, including caseins, whey proteins, either of these as modified proteins, fractions and blends of each, with or without lactose and minerals. It should be obvious that the selection of an ingredient to supply milk protein should be made on the basis of the extent to which that ingredient is able to meet all of the expectations (functional roles) placed on it. Much recent research has focused on modifications of proteins—enhanced soluble caseins or heat-aggregated whey proteins as examples—to deliver optimal functionality in ice cream formulations. In the next few years, these research findings

will undoubtedly be translated into new protein-based products for ice cream formulations to a greater extent than they are now. New manufacturing techniques that can modify existing protein sources better to deliver the functional demands placed on them, such as high pressure processing, will continue to evolve.

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Protein in Cheese and Cheese Products: Structure-Function Relationships

14

Timothy P. Guinee

Abstract

Cheese manufacture involves the controlled destabilization of the casein micelle in milk by enzymatic hydrolysis of the surface κ-casein layer, acidification to the isoelectric pH of the casein, or a combination of pH reduction to ~5.6 and high temperature (~90 °C) in rennet-curd, acid-curd and acid heat-curd cheeses, respectively. Under suitable conditions, the destabilized micelles undergo limited aggregation to form a gel, which is dehydrated to a curd with the desired moisture content by a series of unit operations including cutting the gel into pieces (curd particles), *in situ* acidification, heating and stirring the curd particle/whey mixture, removal of the expressed whey, pressing and/or salting of the curd. Microstructurally, rennet-curd cheese is a matrix comprised of a hydrated calcium phosphate *para*-casein network that occludes the fat phase which occurs as discrete and coalesced globules or pools. The microstructure is influenced by the concentration of *para*-casein and the degree to which the component *para-casein* micelles are aggregated and fused, as affected by manufacturing operations. Macrostructurally, rennet-curd cheese is an assembly of curd particles or pieces (e.g., chips) that fuse to varying degrees according to their microstructure, which affects their potential to deform, and curd handling treatments (e.g., pressing) which effect the level of stress applied to the amalgam of curd particles/pieces. The matrix of acid- or acid heat-curd cheese is similar to that of rennet-curd cheese, but the network is formed from casein (with little, or no, bound calcium) or casein complexed with whey protein, denatured by high heat treatment of the milk prior to acidification and gelation. Most acid-curd and acid heat-curd cheeses have a very uniform texture and lack a macrostructure as the curd particles, low in calcium and high in moisture, coalesce easily to form a structural

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continuum. Heating of rennet-curd cheese to 90–100 °C in culinary applications leads to contraction and shrinkage of the *para*-casein network and liquefaction and coalescence of fat. These microstructural changes are the basis of the melt properties, including softening, flow and stretchability. Owing to their low pH, acid-curd cheeses generally tend to be unstable during heating, as reflected by protein precipitation and the release of excess free moisture. The micro- and macro-structure of cheese has a major influence on various aspects of quality including composition, rheology, texture, cooking properties, opacity/translucence, and behaviour during curd processing operations such as portioning, shredding, slicing, and processed cheese manufacture.

Keywords
Cheese • Protein • Structure • Structure-function relationships • Rheology • Functionality

Abbreviations

ACP	Analogue cheese product
CB	Cheese base
CCP	Colloidal calcium phosphate
CN	Casein
ES	Emulsifying salt
EU	European Union
G′	Storage or elastic shear modulus
G″	Viscous modulus
HHT	High heat treatment
LMMC	Low moisture Mozzarella cheese
LT	Loss tangent
LT_{max}	Loss tangent max
M-HCFUF	Medium-to-high concentration factor ultrafiltration
MNFS	Moisture-in-non-fat substances
PCF	Pasteurized processed cheese food
PCP	Pasteurized processed cheese product
PDWPC	Partially denatured whey protein concentrate
PIM	Concentration of protein in the moisture phase of cheese (g protein/100 g protein + moisture)
UF	Ultrafiltration

14.1 Introduction

Global cheese production is about 21.3×10^6 tonnes per annum (OECD/FAO, 2014) and accounts for ~25 % of total milk usage (IDF, 2012). While cheese-like products are produced in most parts of the world, the principal cheese-producing regions are Europe, North America and Oceania. Within these regions, the production and consumption of cheese varies widely with country, as does the proportion of milk used for cheese which ranges from ~20 % in New Zealand, Greece or Romania to ~90 % in Italy. Approximately 10 % of total cheese production is traded on the global market, the major suppliers being the EU (~31 %), New Zealand (~11 %), USA (10 %) and Australia (~9 %), and the major importers being Russia (~19 %), Japan (~10 %), USA (~6.5 %) and Saudi Arabia (5 %).

Overall, cheese consumption has increased continuously worldwide at a rate of ~4.5 % per annum between 2009 and 2011 (IDF, 2012) and is projected to grow on average by 1.7 % per annum from 21.4×10^6 tonnes in 2014 to 23.6×10^6 tonnes in 2020 (OECD/FAO, 2014).

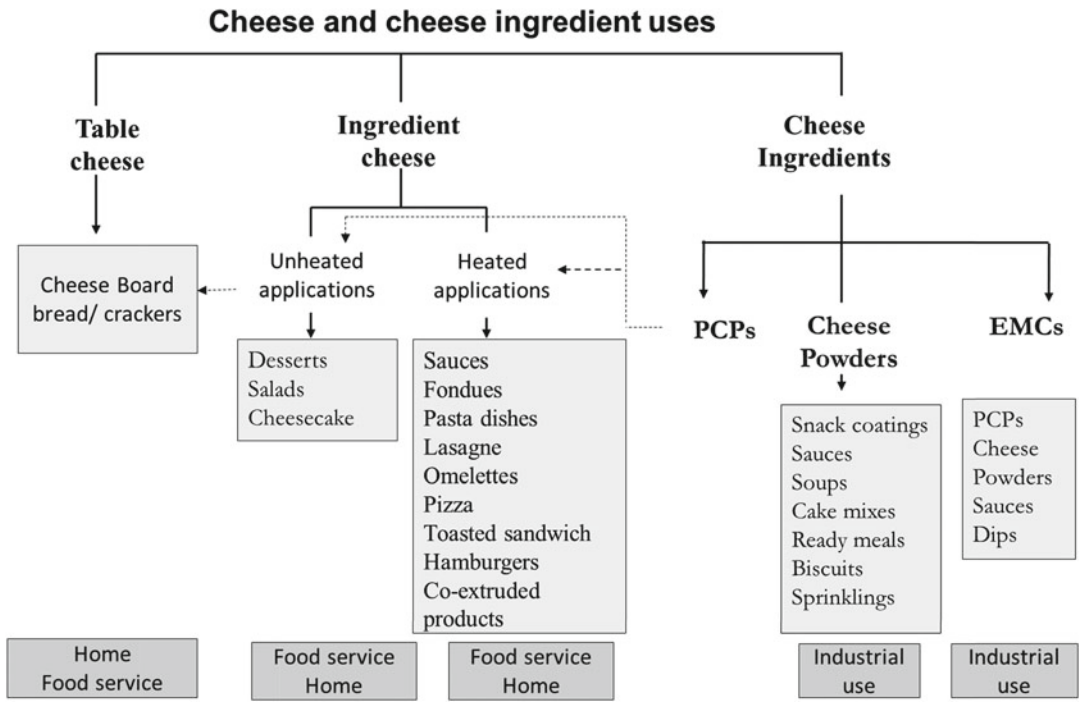

Fig. 14.1 Uses of cheese as an ingredient and food sectors where it is used. *PCP* processed cheese product, *EMC* enzyme-modified cheese

The accelerated demand is being driven by a number of factors including: (a) increases in global population and per capita income, (b) changes in consumer life-style (e.g., eating out), and (c) the expansion of food service and snack food sectors allied with the versatile functionality of cheese, which enables it to be used as an ingredient in, and enhance the quality of, prepared foods/meals and snack foods. Simultaneously, there has been an increase in demand for more consistent quality, with respect to sensory properties (e.g., taste, tactile texture, aesthetic), usage characteristics (e.g., convenience, shreddability, melt, flowability) and nutrient profiling (e.g., ratio of saturated-to-unsaturated fatty acids and levels of calcium). This is motivated by higher consumer expectations, health agencies, legislators, suppliers and retailers trying to gain more market share by differentiation of branded products.

Cheese is an extremely versatile product, which may be consumed directly as table cheese (e.g., as an accompaniment to crackers and bread) or indirectly as an ingredient in other foods (Fig. 14.1). It is a major ingredient in the prepared foods and food service sectors, where it is used in an extensive array of products including sandwiches, pizza, pasta dishes, meat dishes, omelettes, quiches, sauces and soups (Guinee, 2011a). Cheese is also used extensively in the industrial food sector for the preparation of ready-to-use grated/shredded cheeses and cheese blends and for the mass production of cheese-based ingredients such as pasteurized processed cheese products (PCPs), cheese powders and enzyme-modified cheeses (EMCs).

When used as an ingredient in foods, cheese is required to perform one or more functions (cf. Sects. 14.7 and 14.8). In the unheated state, the cheese may be required to exhibit a number of rheological properties to facilitate its size reduction and use in the preparation of various dishes, e.g., the ability to crumble easily, to slice or to shred cleanly, or to bend when in slice form. The rheological properties also determine the textural properties of the cheese during mastication. Generally, cheese is required to contribute to the

organoleptic characteristics (i.e., taste, aroma and texture) of the food in which it is an ingredient. On grilling or baking, the cheese may be required to melt, flow, brown, oil-off and/or stretch to varying degrees. The functional properties may be defined as those rheological, physicochemical, micro-structural and organoleptic (i.e., flavour and texture) characteristics that affect the behaviour of the cheese when consumed or used as an ingredient in culinary applications, or in the preparation of cheese based ingredients. These functions ultimately determine the acceptability of cheese or cheese-containing products to the consumer.

Protein, as a major component of most cheese varieties, has a marked influence on its functional properties (Guinee, 2003; Lucey *et al.*, 2003). Similarly, the proteins of cheese, along with optionally added protein from dairy ingredients (e.g., skim milk powders, caseins), affect the physico-chemical, rheological, stability and usage appeal characteristics of pasteurized processed cheese products (PCPs) and analogue cheese products (ACPs) significantly (Guinee, 2009, 2011b; O'Riordan *et al.*, 2011). Hence, protein has a major influence on cheese quality and its industrial applications.

In this chapter, some industrial aspects of protein in cheese products, including its contribution to acid- and rennet-induced gel formation, curd formation and texturization, cheese structure, cheese rheology and functionality, and the formation and stabilization of processed cheese products, are discussed. Protein, of course also contributes to other aspects of cheese products which have been studied extensively and reviewed, including yield and manufacturing efficiency (Fox *et al.*, 2000; Guinee, 2003), cheese flavour (McSweeney and Sousa, 2000; Singh *et al.*, 2003; Upadhyay *et al.*, 2004; Hassan *et al.*, 2013), aeration and foaming characteristics of fresh cheese-based desserts. While little information is available on the foaming properties of cheese-based products, the foaming properties of milk proteins have been reviewed extensively (Anderson and Brooker, 1988; Brooker, 1993; Jana *et al.*, 1994; Dickinson, 2003).

14.2 Contribution of Protein to Gelation of Milk and Curd Formation

Cheese is a concentrated protein gel, which occludes fat and moisture. Manufacture involves: gelation of cheese milk, dehydration of the gel to form a curd, and treatment of the curd (e.g., dry stirring, cheddaring, texturization, salting, moulding, pressing). The moulded curd may be consumed fresh (shortly after manufacture, for example, within 1 week) or matured for periods of ~2 weeks to 2 years to form a ripened cheese.

A central step in the manufacture of cheeses is the gelation of milk, which is achieved by increasing the surface hydrophobicity of casein micelles either by:

- selective hydrolysis of the κ-casein at the Phe_{105}-Met_{106} peptide bond by the addition of acid proteinases, referred to generically as rennets (chymosin, pepsin), in the manufacture of rennet-curd cheeses such as Cheddar, Gouda and Mozzarella;
- acidification (using starter cultures or food-grade acids and/or acidogen) at a temperature of 20–40 °C to a pH value close to the isoelectric pH of casein, i.e., ~4.6, in the manufacture of fresh acid-curd cheeses such as Quark, Cream cheese and Fromage frais;
- a combination of acid and heat, for example, heating milk at ~pH 5.6 to ~90 °C, in the manufacture of acid-heat coagulated cheeses such as Mascarpone and Ricotta (Farkye, 2004b; Lucey, 2011a).

14.2.1 Rennet-Induced Gelation of Milk

On treatment of milk with chymosin (rennet), the κ-casein is hydrolysed, with the primary cleavage point being the peptide bond Phe_{105}-Met_{106}, resulting in the liberation of the highly-charged, hydrophilic Met_{106}-Val_{169} caseino-macropeptide

into the milk serum (whey). This results in an effective 'shaving' of the hairy layer from the micelle surface, a marked reduction in the negative surface charge to ~−10 mV, and an increase in the attractive forces between, or 'stickiness' of, the *para*-casein micelle surfaces. Consequently, the latter begin to aggregate when sufficient κ-casein is hydrolysed (~80–90 % of total), resulting in the formation of clusters/aggregates of *para*-casein micelles that fuse into strands that 'knit' gradually into a continuous network, that leads to gelation when the milk is quiescent (Fig. 14.2). The strength of the gel then increases with time, as reflected by the sigmoidal-shaped increases in storage modulus, G′, and loss modulus, G″ (Fig. 14.3). A controlled, moderate degree of contact between the surfaces of touching *para*-casein micelles promotes the formation of a gel which may be described as a protein network that occupies the full volume of the milk and encloses the fat globules and serum, analogous to the way that a sponge holds water (Fig. 14.2). Surface contact and knitting of the *para*-casein micelles is mediated by inter-and intra-protein linkages as affected by the cross-linking effect of calcium ions (attaching to acidic amino acid residues, such as glutamate and aspartate) and colloidal calcium phosphate (attached to serine phosphate groups), and hydrophobic interactions between uncharged amino acid residues (Swaisgood, 2003; De Kruif and Holt, 2003).

The strength of a gel is dependent on the volume fraction and homogeneity of the calcium phosphate *para*-casein network, which determines the number of stress-bearing strands per unit area of the gel. Considering a gel to which a relatively small stress (i.e., much less than the yield stress) is applied in the direction x, the storage modulus (G′, i.e., ratio of shear stress to shear strain, σ/γ), which is an index of elasticity or strength of the gel, can be related to the number of strands per unit area according to the equation (Walstra and van Vliet, 1986):

$$G = CN\,d^2A / dx^2 \qquad \text{(Equation 14.1)}$$

where: N = number of strands per unit area of the gel in a cross section perpendicular to x, bearing the stress; C = is a coefficient related to the characteristic length determining the geometry of the network; dA = change in elastic energy when the aggregates in the strands are moved apart over a distance, dx, on the application of the stress. The number of strands per unit area of a gel is determined by:

- the concentration of gel-forming protein, and
- the fineness or coarseness of the gel network, with a fine gel network having a greater number of stress bearing-strands than a coarse gel.

G′ increases more than proportionally with protein concentration (P; Fig. 14.4), with $G' \propto P^n$, where the value of n is typically within the range 2.0–2.6 (Culioli and Sherman, 1978; Tokita *et al.*, 1983; Guinee *et al.*, 1996).

The factors affecting rennet-induced gelation of milk have been reviewed extensively (Horne and Banks, 2004; Lucey, 2011b); key parameters include milk composition, treatments of the cheese milk (e.g., cooling, heat treatments), and gelation conditions (rennet type and level, pH, temperature).

14.2.2 Acid-Induced Gelation of Milk

The caseins in milk are insoluble at their isoelectric points (~pH 4.6) at temperatures >8 °C. This property is exploited in the formation of acid curd cheeses, such as Cottage cheese, Quark and Cream cheese, the manufacture of which involves slow quiescent acidification of the cheesemilk to ~pH 4.6–4.8 by starter culture, organic acid or acidogen (e.g., glucono-δ-lactone) at temperatures of 20–30 °C. Acidification results in a number of physicochemical changes promoting hydration/dispersion or dehydration/aggregation effects on the casein micelle, with the ratio of these effects changing as the pH declines during the acidification (fermentation) process. The reduction in pH from 6.6 to ~5.2–5.4 results in a decrease in the negative charge of the micelles due to titration of negative charges with H^+ ions. Nevertheless, this is not generally accompanied by the onset of

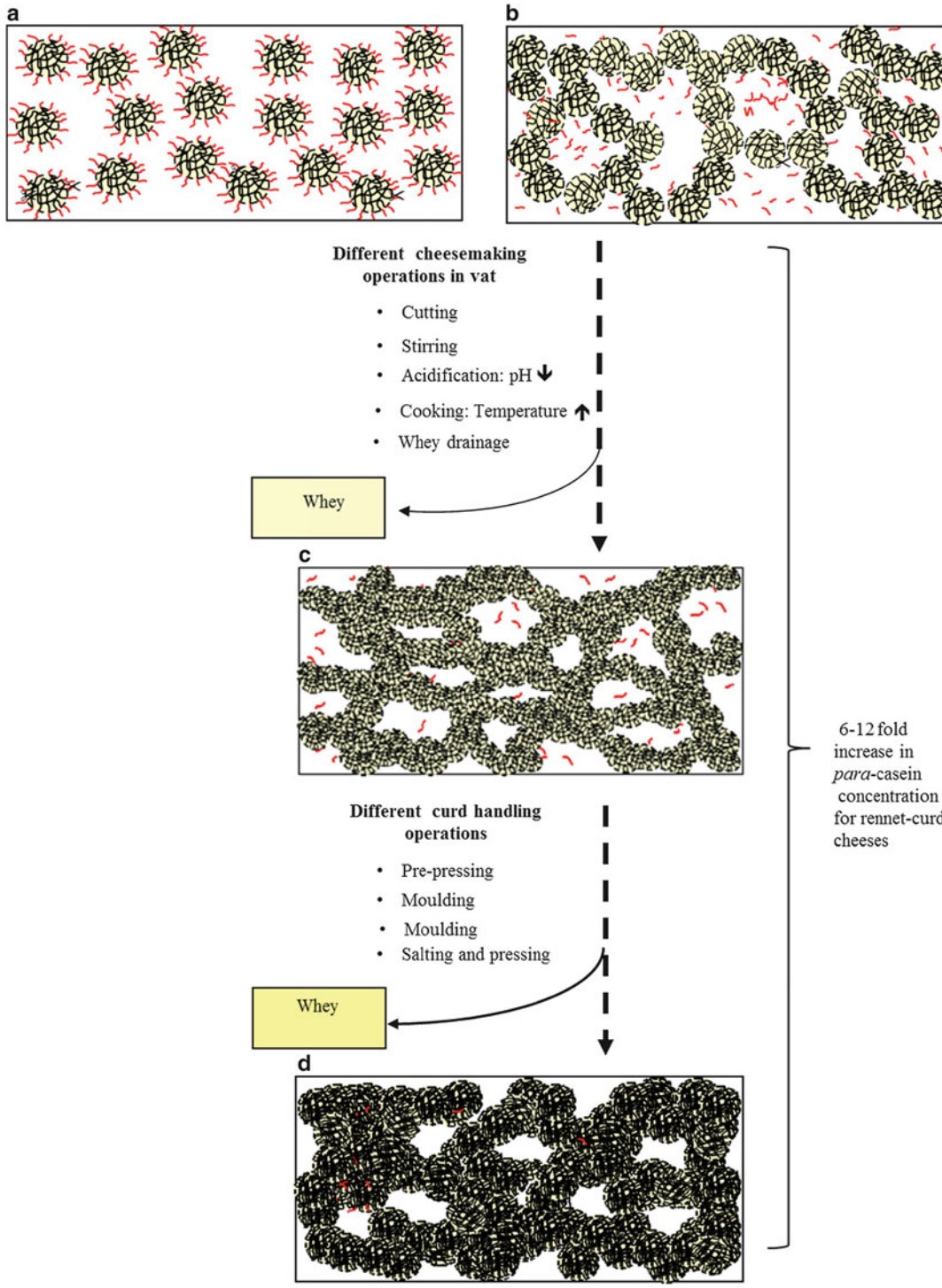

Fig. 14.2 Schematic representation of the various stages involved in the formation of cheese curd from milk: (**a**) Milk with starter culture at rennet addition, showing intact casein micelles dispersed in bulk phase serum (*white*). Each micelle consists of (i) a core, comprised of internal serum (*yellow*) and proteins (mainly α_{s1}-, α_{s2}- and β-caseins; *black lines*) self-assembled into an internal network by calcium-, calcium-phosphate and hydrophobic mediated interactions, and (ii) a surface layer of mainly of κ-casein with the with its c-terminal region (macropeptide, *red*) protruding from the surface. Fat globules in the milk are not shown; (**b**) milk gel formed following rennet-induced hydrolysis of the κ-casein macropeptide and aggregation of the resultant *para*-casein micelles into a

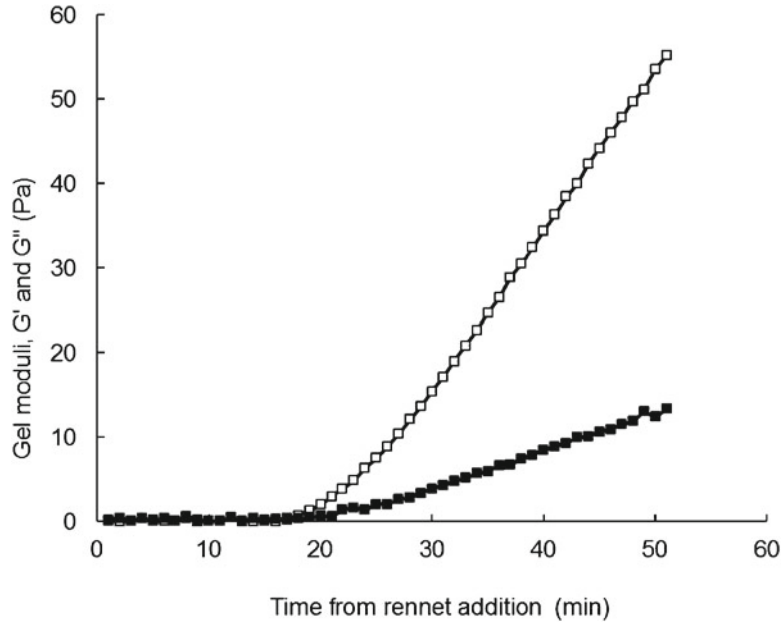

Fig. 14.3 Development of storage modulus (G′; *open square*) and loss modulus (G″; *filled square*) during the rennet-induced gelation of milk

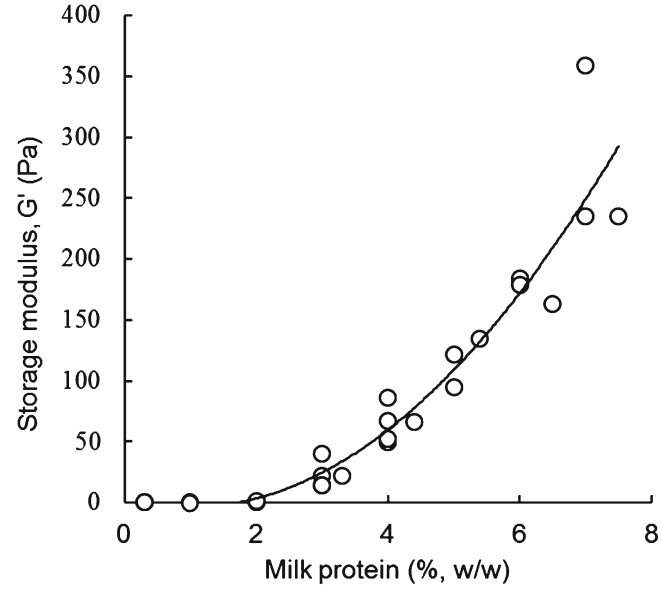

Fig. 14.4 Effect of protein content of skim milk on the strength (storage modulus) of rennet gels. Milks of different protein content were prepared by ultrafiltration of skim milk, and blending the resultant retentate, permeate, and non-ultrafiltered skim milk in appropriate quantities (modified from Guinee *et al.*, 1997)

Fig. 14.2 (continued) three-dimensional network, the pores of which are filled with fat globules and bulk phase serum (*white*) including the released macropeptide (*red*); (**c**) dehydration and concentration of the *para*-casein network into curd, as affected by various operations in the cheese vat including cutting the gel, stirring and cooking of curd particle-whey mixture and drainage of the whey; and (**d**) further dehydration of the *para*-casein network into the finished cheese by subjecting the curd to various operations which depending on the cheese variety may include cheddaring, moulding, salting, and/or pressing. The network is highly constrained and presses in on the enclosed fat phase (*white*); all the bulk-phase solvent and much of the internal serum within the *para*-casein micelles has been removed

gelation mainly because of the solubilization of colloidal calcium phosphate (CCP) (fully soluble at ~pH 5.2 at 20 °C) and the attendant diffusion of all caseins from the micelle to the serum and the increase in ionic strength of the serum phase. However, further reduction in pH in the range ~5.2–4.6 results in aggregation of casein and gel formation as the forces promoting dispersion of the casein micelle are overtaken by the sharp reductions in the negative charge and dehydration of the casein, the collapse in the steric effect associated with the κ-casein C-terminal 'hairs', and the increase in hydrophobic interactions. The onset of gelation occurs typically at pH ~5.1 and further reduction in pH toward 4.8–4.6 promotes further aggregation of casein, further development of the gel structure and rigidity, enabling separation of whey from the curd by physical means (e.g., breakage, stirring, and whey drainage, or centrifugation). Gel rigidity increases sigmoidally as the pH of the gelling cheesemilk continues to decrease towards 4.6 during incubation with the fermentation of lactose to lactic acid (Fig. 14.5). The principal interactions between the casein molecules are likely electrostatic and hydrophobic, with the strength or contribution of each type governed by the residual charge on the CN molecule (which is influenced directly by pH, ionic strength and Ca binding) and the temperature of the gel. However, covalent (disulphide) bonding between κ-CN and β-lactoglobulin are considered to contribute significantly to gel formation and elasticity of acid-induced milk gels formed from high-heat treated milk and to acid-heat-induced milk gels (Fig. 14.6; Lucey *et al.*, 1999; Lucey and Singh, 2003; Vasbinder *et al.*, 2003).

Similar to rennet-induced milk gels, the rheological properties of acid-induced milk gels are affected strongly by protein level. Hence, the G′ of acid casein gels prepared from model milks which were subjected to a standard pasteurization treatment (95 °C for 5 min) increased progressively as the total protein content was increased in the range 3.0–5.5 % (Fig. 14.7). The model milks, which were prepared by blending phosphocasein, whey protein isolate and ultrafiltered milk permeate in de-ionized water, had a fixed level of denatured whey protein (38 % of total whey protein) after pasteurization. Similarly, the G′ of acid casein gels prepared from model milks with equal concentrations of total protein (4.8 %) and subjected to a similar heat treatment (95 °C for 5 min)

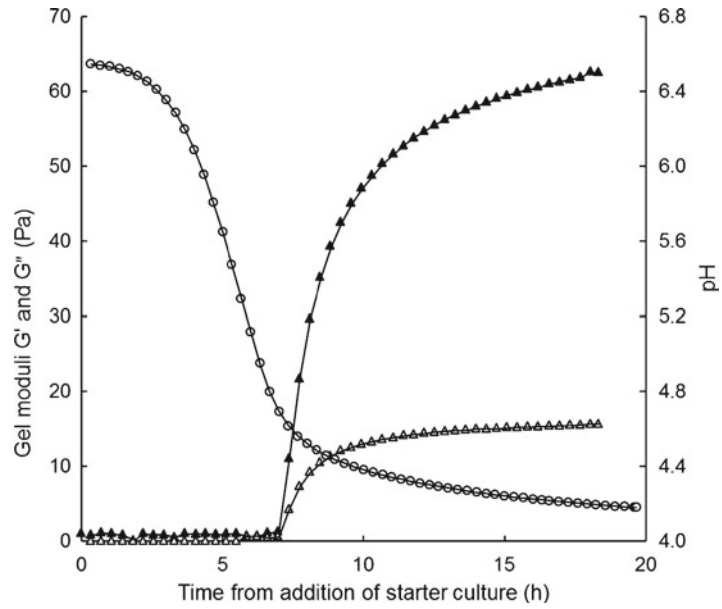

Fig. 14.5 Dynamic changes in pH (*open circle*), storage modulus, G′ (*filled triangle*) and loss modulus, G″ (*open triangle*) during the formation of an acid-induced skim milk gel. The skim milk (3.1 % protein) was pasteurized at 72 °C, cooled to 30 °C, inoculated with 2 % (w/w) *Lactococcus lactis* subsp. *cremoris* and incubated for 18 h (modified from Guinee and Hickey, 2009)

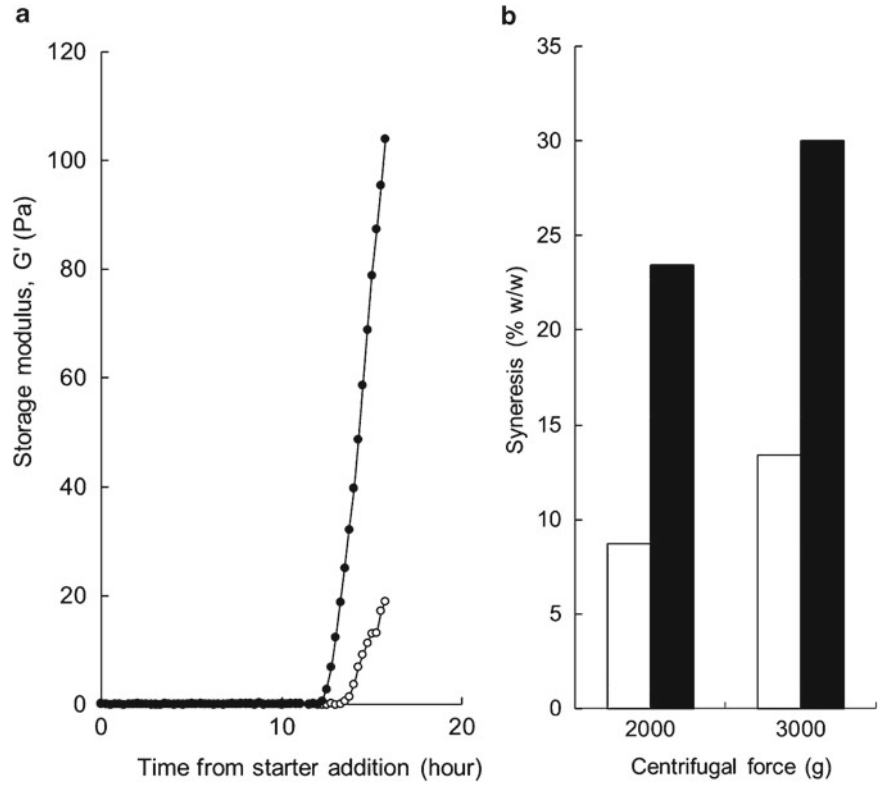

Fig. 14.6 Development of storage modulus during fermentation of skim milk pasteurized at 72 °C × 15 s (*open circle*) or 90 °C × 300 s (*filled circle*) (**a**) and the level of syneresis of Quark cheeses made from the resultant acid-gels (**b**). The skim milk was inoculated with starter culture and incubated at 22 °C; following fermentation, the gels (pH 4.6) were stirred gently and samples were weighed in centrifuge tubes and allowed to stand at 8 °C for 36–48 h prior to centrifugation. Percentage syneresis was defined as the weight of whey expelled on centrifugation as a percentage of the original sample weight (from Guinee, 2003)

increased as the level of *in situ* whey protein denaturation was increased (Fig. 14.8). The latter trend is expected, as increasing the concentration of denatured whey protein as a proportion of the total gel-forming protein results in a finer gel network (Guinee *et al.*, 1993; Mulvihill and Grufferty, 1995).

14.3 Contribution of Protein to Syneresis of Milk Gels

Following gelation of the milk, further casein aggregation is promoted to expel whey and reduce the moisture content to that of the final cheese. The expulsion of whey, referred to as syneresis, is a consequence of rearrangement of the casein network due mainly to increases in hydrophobic and electrostatic interactions between the casein molecules, as promoted by heating, pH reduction, and pressure application (during stirring and pressing). In rennet curd cheeses, syneresis is achieved by:

- Cutting the gel into pieces (cubes, particles), which leads to a large increase in surface area, allowing the enclosed serum (whey) to escape through the freshly cut surfaces;
- Stirring the curd particles in the whey, which leads to local deformations at the surfaces

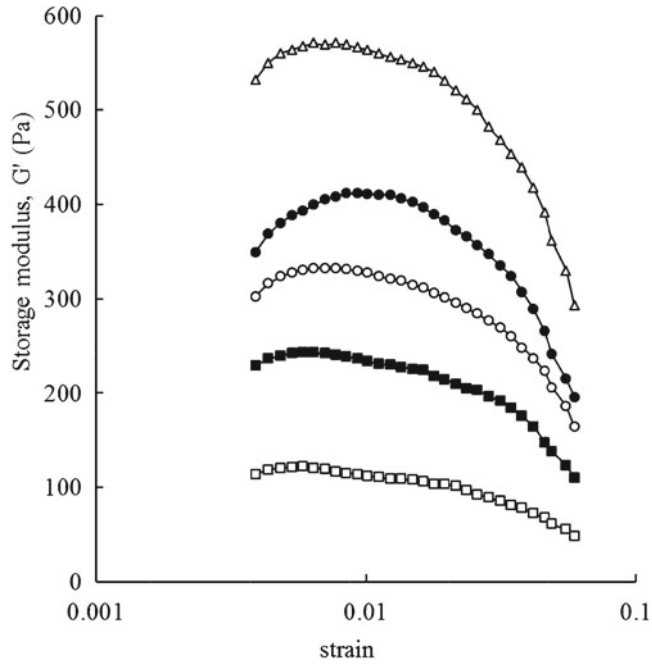

Fig. 14.7 Effect of protein on storage modulus of low-fat (0.4 %, w/w) acid-induced gels from model milks with casein and whey protein contents of 55 and 45 % of total protein, respectively. The model milks were prepared by blending micellar casein powder, whey protein isolate and ultrafiltered milk permeate in appropriate quantities in de-ionized water. The model milks were made to different protein levels while maintaining the casein-whey protein ratio constant at 1.2, adjusted to pH 6.65 and heat-treated (95 °C for 5 min). Denatured whey protein was ~83 % of total whey protein and ~38 % of total protein. The concentrations (%, w/w) of total protein and gel-forming protein (casein plus denatured whey protein) in the milks were 3.9 and 3.6 (*open square*), 4.4 and 4.1 (*filled square*), 4.9 and 4.5 and (*open circle*); 5.4 and 5.0 (*filled circle*); 5.9 and 5.5 (*open triangle*) (redrawn from Guinee, 2003)

of the curd particles, which in turn cause internal structural re-arrangements of the casein gel network, and ensuing moisture expulsion;

- Cooking the curd particle-whey mixture (from ~31 to 35–55 °C), which increases the degree of casein aggregation of the casein network within the curd particles, resulting in shrinkage and moisture expulsion;
- Gradual pH reduction in tandem with the development of lactic acid following fermentation of lactose by the starter culture (from ~pH 6.5 in the set gel to ~5.5–5.0 in the fresh curd);
- Removal of the expelled whey from the curd particles using curd screens;
- Physical pressing of the recovered curd particles, which forces the surfaces of the curd particles together into a cohesive mass and eliminates most of the interstitial whey pockets and surface whey;
- Salting of the curd to ~1.0–2.5 %, which removes whey from the curd by diffusion.

The moisture content decreases from ~88 % in the gel prior to cutting to values of ~35–55 % in the curd after pressing depending on the variety; moreover, the moisture content may decrease further during ripening depending on the presence/absence of packaging, the type of packaging, and the relative humidity of the environment. Simultaneously, the casein is concentrated from ~2.5 % (w/w) in the milk to 16–20 % in semi-hard chesses such as Feta, Camembert, and to 25–32 % in hard-cheeses like Cheddar, Emmental and Parmesan. The concentration of protein in the moisture phase (PIM)

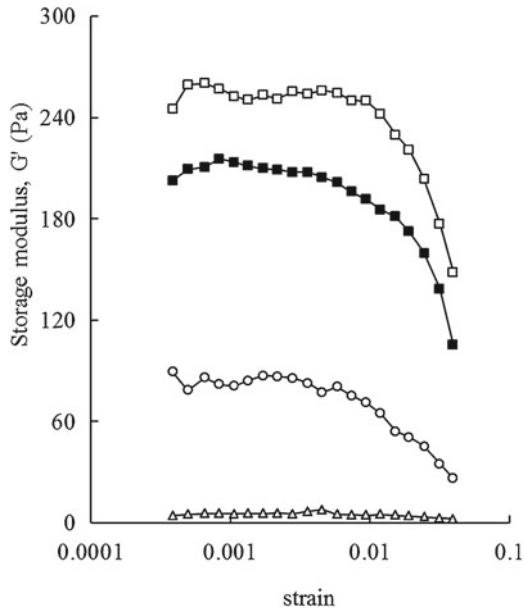

Fig. 14.8 Effect of whey protein denaturation on the storage modulus of low-fat (0.4 %, w/w) acid-induced gels from model milks with ~4.8 % total protein and whey protein ranging from ~5 to 40 % of total protein. The model milks were prepared by blending micellar casein, whey protein isolate and ultrafiltered milk permeate in different proportions in de-ionized water, adjusted to pH 6.55 and heat-treated at 95 °C for 5 min; denatured whey protein was ~80 % of total whey protein The concentrations of gel-forming protein (i.e., casein and denatured whey protein, g/100 g) and denatured whey protein (% of total gel-forming protein) protein were: 4.3 and 34.4 (*open square*); 4.3 and 26.1 (*filled square*); 4.3 and 17.3 (*open circle*); and 4.3 and 4.1 (*open triangle*), respectively (redrawn from Guinee, 2003)

increases from ~20 % in the native casein micelle in the milk to 22 % in Feta-type cheese to ~53 % in Parmesan (Table 14.1).

Consequently, the dehydration process in rennet-curd cheeses results in the removal of essentially all the bulk phase serum (moisture and dissolved solutes including lactose, soluble salts and non-protein nitrogen) and part of the moisture entrapped within the native casein micelle, as whey. The moisture content remaining in the cheese curd following pressing corresponds to moisture that has not been removed from the *para*-casein micelles during the cheesemaking process. The originally-dispersed casein micelles fuse increasingly with time and transform into a concentrated structural continuum (calcium phosphate *para*-casein network) where no bulk free (non-immobilised) solvent is available to confer liquidity to the cheese; the network may be described as being very constrained or "jammed" (Fig. 14.2; Guinee and O'Kennedy, 2012). It is noteworthy that ultracentrifugation of milk, e.g., at 100,000 × *g* for 60 min at ~20 °C, results in concentration of the casein micelles in the form of a pellet with ~20 % protein and 80 % moisture, and which at 5–10 °C behaves as a viscoelastic solid, the elasticity (G′, storage modulus) of which is independent of shear rate (Horne, 1998). This behaviour illustrates the tendency of casein to form constrained polymeric-type structures when concentrated. In this context, the experiments of Bouchoux *et al.* (2009) involving the concentration of casein micelle dispersions in milk ultrafiltrate by equilibrium dialysis against solvents of different osmotic pressure, are of interest. Increasing the concentrations of dispersions progressively to >20–25 % (w/w) resulted in their transformation from opaque liquids to translucent coherent solids as the micelles became increasingly dehydrated, concentrated and interacted irreversibly to form a structural continuum. The extent of casein dehydration, and hence, concentration and aggregation determines the number and balance of attractions (hydrophobic and electrostatic) between the *para*-casein molecules, and thereby affects the microstructure of the cheese. Hence, controlling the degree of dehydration, through manipulation of the different manufacturing steps and their sequence, is critical in defining the various aspects of cheese quality such as the ratio of viscous to elastic characteristics, texture, physical properties and opacity (see. Sect. 14.4.2). The voluminosity of the *para*-casein network is temperature dependant, decreasing with increase in temperature (Pastorino *et al.*, 2002), and this principle is exploited during cheesemaking (e.g., by altering the scald/cook temperatures) to change the properties of the final cheese.

Table 14.1 Approximate composition of different cheese varieties[a]

Cheese	Moisture (%, w/w)	Protein	Protein-in-moisture	Salt-in-moisture	Calcium (mg/g protein)
Quark	82.0	13.0	13.7	0.2	7.7
Cottage cheese	79	13.8	15.0	1.3	6.8
Feta	56.5	15.6	22.0	5.3	23.7
Camembert	50.7	20.9	29.0	4.1	18.6
Danish blue	43.0	18.4	30.0	7.7	25.3
LMM[b]	49.8	25.1	34.0	3.0	28.4
Stilton	40.5	21.6	35.0	8.5	19.4
Edam	43.8	26.0	37.2	4.2	28.1
Gouda	40.1	24.0	37.4	5.2	30.0
Cheddar	37.2	25.0	40.0	4.8	29.2
Half-fat Cheddar	42.9	33.4	43.8	4.4	28.0
Emmental	35.7	28.7	45.0	1.8	32.6
Gruyere	33.6	27.3	45.0	3.6	24.4
Skim milk cheese	50.5	42.0	45.4		25.5
Parmesan	30.6	34.9	53.0	8.8	31.4

[a]Cheeses ranked in order of increasing concentration (%) of protein in the moisture phase (g protein/100 g protein + moisture)
[b]*LMM* low moisture Mozzarella cheese

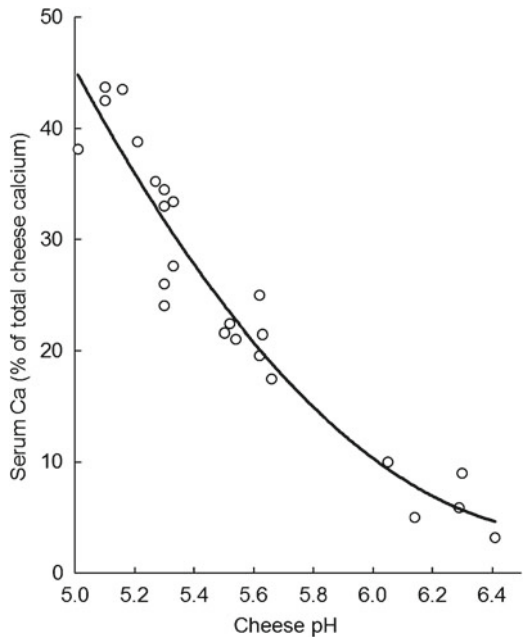

Fig. 14.9 Effect of the pH of cheese on the calcium content (*open circle*, % of total cheese Ca), in cheese serum expressed on hydraulic pressing of various commercial cheeses, including Cheddar, Mozzarella, Dubliner, Kashkaval, Provolone and analogue pizza cheese (redrawn from Guinee *et al*., 2000b)

For a given composition of the final cheese, on conclusion of syneresis, the volume fraction of the casein network in the formed cheese is determined by the casein concentration, and the degree of hydration of the protein network is determined primarily by the salt content of the aqueous phase and the pH which affects the ratio of insoluble calcium (contributing to calcium bridges between the casein molecules) to soluble calcium in the aqueous phase (Fig. 14.9). However, increasing the temperature of the final cheese during storage (e.g., to temperatures of 25–35 °C) appears to reduce the voluminosity of the casein network, as evidenced by the leakage of serum from the cheese and its accumulation in the vacuum wrap, especially when the cheese is held for a long period, e.g., 6 months (Guinee, unpublished results). Increasing the calcium content of the final cheese through high-pressure injection with calcium chloride solutions (40 %, w/w) has been found to reduce the voluminosity of the *para*-casein network and promote whey expulsion (Pastorino *et al.*, 2003b). The latter observations suggest that syneresis in the formed cheese is

promoted by increased intermolecular attractions between the caseins by hydrophobic interactions or calcium crosslinking.

Similarly, in the formation of acid-curd cheeses, the acid-induced milk gel is dehydrated and concentrated to the desired dry matter of the final fresh cheese using various operations including: breaking/stirring the gel; heating; and straining, filtration or centrifugation of the broken curd. Compared to rennet-curd cheeses, fresh acid-curd cheeses generally have a high moisture content (~60–80 %) and low contents of protein (5–14 %) and calcium (<~0.18 %); the fat content varies from ~0.5 % in Quark to ~35 % in double Cream cheese and ~50 % in Mascarpone. The PIM is much lower than that of rennet-curd chesses and even lower than that of the native casein micelle, e.g., ~5.5 in double cream cheese, 6.3 in Quark to ~8.5 in Labne (Table 14.1). This is because the calcium-to-casein ratio of acid-curd cheeses (e.g., 6-8 mg/g casein in Cottage cheese) is lower than that of the casein micelle (e.g., 28–30 mg/g protein) owing to the low pH and concomitant solubilization of all the CCP and much of the calcium bound directly to the casein prior to cutting/stirring the gel and whey drainage. Moreover, most or all of the calcium present is soluble at the low pH of acid-curd cheeses (~4.4–4.9) and contributes little to intermolecular electrostatic interactions between the casein molecules. Consequently, the balance of electrostatic-to-hydrophobic interactions is likely to be lower in acid curd cheese compared to rennet-curd cheese (Lucey *et al.*, 2003). Owing to their low PIM, the casein network of acid-curd cheeses are less constrained than the corresponding calcium phosphate *para*-casein network of rennet-curd cheeses. Consequently, the casein networks of acid-curd cheeses are more susceptible to rearrangement and shrinkage, and the development of defects (e.g., syneresis, granular, sandy or grainy texture) during storage. These defects are accelerated by slow cooling of the concentrated curd after separation, and by temperature fluctuations during storage.

14.3.1 Relationship Between Milk Gel Characteristics and Extent of Syneresis

Syneresis and gel shrinkage are accelerated on cutting the coagulum into particles (e.g., typically 1.0–0.5 cm cube) due to the shorter distance that whey has to travel through the gel matrix before reaching a surface where it is released. The result is a two-phase mixture of curd and whey. The consequences of curd shrinkage are increases in whey volume and in the ratio of whey to curd, and a reduction in the moisture content of the curd particles (Guinee and O'Callaghan, 2010, Fig. 14.10a). In rennet-curd cheeses, the curd particles are stirred in the increasing volume of expelled whey for a predetermined length of time during which the majority of syneresis takes place, even though the rate of syneresis decreases with time (Fig. 14.10b). Hence, in commercial cheese manufacture, the whey is drained off (pumped out) after a given time, e.g., typically at 90 min after cutting in the case of Cheddar cheese. The drained whey accounts for ~80–90 % of the milk volume in hard rennet-coagulated cheeses such as Cheddar, Gouda and Emmental, with further whey being removed during moulding, pressing and/or dry-stirring operations.

The relationship between degree of syneresis and the curd particle characteristics of rennet- and acid-induced milk gels and syneretic pressure can be expressed by Darcy's equation, in the following form for one-dimensional flow:

$$v = \left(\frac{B}{\eta}\right) \times \left(\frac{p}{l}\right) \qquad \text{(Equation 14.2)}$$

where, v is the velocity of the whey, B is the permeability coefficient of the gel (index of porosity), η is the viscosity of the whey, p is the pressure exerted by the contracting network on the enclosed whey and l is the distance through which the whey has to travel to the surface (index of curd particle size).

Cutting the gel disrupts the gel structure creating cracks in the gel, which initiate syneresis by creating new interactions between

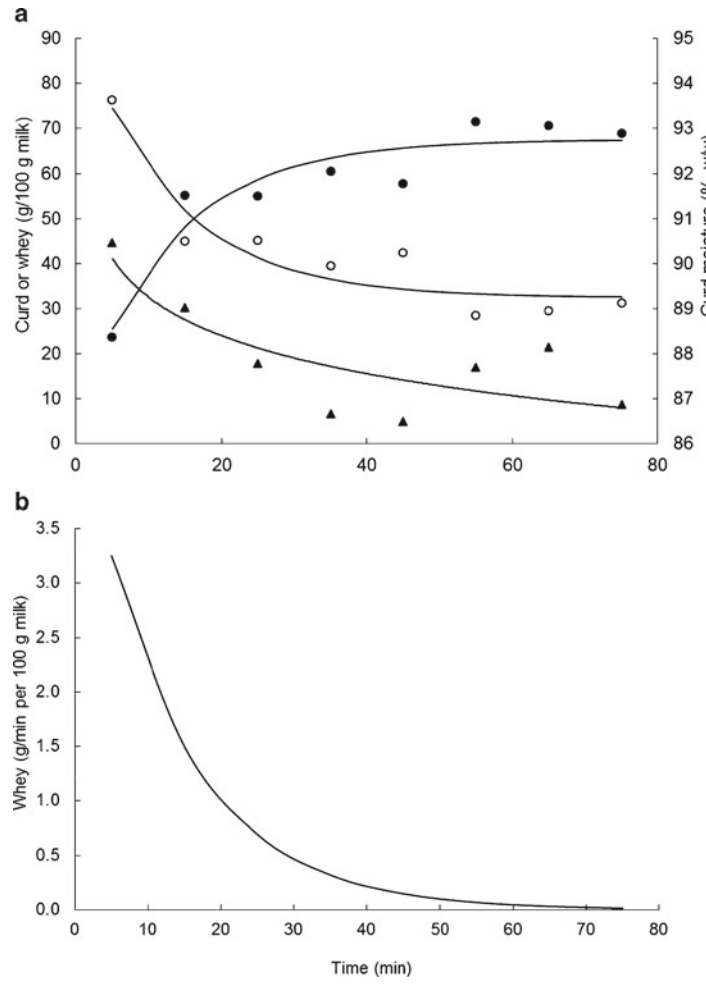

Fig. 14.10 Effect of stirring time on (**a**) weights of whey (*filled circle*) and curd (*open circle*) and curd moisture (*filled triangle*), and (**b**) rate of syneresis. The curd was made in an experimental vat with controlled cutting and stirring from milk with protein and fat levels of 3.2 and 1.4 % (w/w), respectively (redrawn from Guinee and O'Callaghan, 2010)

para-casein molecules (Dejmek and Walstra, 2004). The extent of cutting of rennet-induced milk gels determines the size of curd particles, which is related inversely to the velocity of whey exudation (Everard *et al.*, 2007) (See Equation 14.2), and directly related to moisture content of the final curd (Whitehead and Harkness, 1954; Czulak *et al.*, 1969). Additionally, smaller curd particles provide more surface area for syneresis, which together with the increased velocity of whey release, increases the rate of syneresis. Consequentially, Grundelius *et al.* (2000) reported that curd particle size was a key factor influencing shrinkage of the curd particles *per se* during the initial stage of syneresis, and that smaller curd particles shrink more rapidly than larger ones. Additionally, shrinkage in rennet-curd cheeses is accelerated by pH reduction and by increasing the temperature of the curd-whey mixture (Walstra *et al.*, 1985; Lodaite *et al.*, 2000; Dejmek and Walstra, 2004); both factors promote hydrophobic interactions between the *para*-casein molecules and increase the pressure

exerted by the curd particle casein network on occluded moisture, which is related directly to the velocity of whey exudation (See Equation 14.2). Reviews on the factors affecting the syneresis of rennet-induced milk gels include Patel *et al.* (1972), Pearse and Mackinlay (1989), Walstra *et al.* (1985), and Dejmek and Walstra (2004).

Likewise, gel shrinkage and syneresis of acid-induced milk gels is influenced by many factors including temperature of coagulation and separation, method of acidification (e.g., starter culture, GDL, and rate of acidification, ionic strength, set pH, addition of rennet and rheological properties of the gel (Lucey *et al.*, 1998; Schkoda *et al.*, 1999; Lucey, 2001; Gastaldi *et al.*, 2003; Castillo *et al.*, 2006).

14.4 Contribution of Protein to Cheese Structure

Cheese is essentially a concentrated protein gel, which occludes fat and moisture. *Para*-κ-casein is the principal structural component of the gel in rennet-curd cheeses. At the pasteurization conditions (72 °C × 15 s) generally applied to milk for rennet-curd cheese, ≤5 % of total whey proteins are denatured and complex with κ-casein (Fenelon and Guinee, 1999) and are retained in the cheese curd. Casein is the major component of acid-coagulated cheeses. However, high heat treatment (e.g., 95 °C for 2 min) is frequently applied in the manufacture of acid-coagulated cheeses (e.g., Quark and Cream cheese) and acid-heat coagulated cheeses (e.g., Ricotta, Paneer, Mascarpone, some Quesoblanco types) and results in substantial denaturation of whey proteins. The denatured whey proteins interact and complex with the casein micelles and become part of the gel formed during subsequent acidification (van Hooydonk *et al.*, 1987). This interaction has a marked influence on the gel structure and properties of acid- and rennet-curd cheese varieties (Harwalkar and Kaláb, 1980, 1988; Guinee *et al.*, 1993; Farkye, 2004b).

The concentration and type of protein have a major influence on the micro-structure of acid- and rennet-coagulated milk protein gels, which on dehydration and concentration, form the structural fabric of the cheese. The micro-structure of the gel markedly affects its rheological and syneretic properties, the recovery of fat, protein and moisture from milk to cheese, and the yield of cheese (Kaláb and Harwalkar, 1974; Schafer and Olson, 1975; Harwalkar and Kaláb, 1980, 1981; Green *et al.*, 1981a, b; 1983; Green, 1990a, b; Marshall, 1986; Banks et al., 1987; Banks *et al.*, 1994a, b; McMahon *et al.*, 1993; Guinee *et al.*, 1995, 1998). The structure of the final cheese influences its functional properties, e.g., rheology, behaviour on heating, and texture (Emmons *et al.*, 1980; Green *et al.*, 1981a, b; Green, 1990a; b; Guinee *et al.*, 1995, 1999, 2000a; McMahon *et al.*, 1996; Fenelon *et al.*, 1999).

14.4.1 Structure of Rennet-Curd Cheeses

Protein is a major determinant of cheese structure, which may be considered at two levels, namely the microstructure and the macrostructure. The former may be arbitrarily defined as the structure in the smallest piece of the cheese (e.g., curd particles), and the latter as the structure of the assembly of curd particles (in the case of brine salted cheeses), or curd chips or pieces (in the case of dry-salted cheeses such as Cheddar and Stilton), that fuse into a whole (moulded cheese) during pressing and ripening (Fig. 14.11).

14.4.1.1 Microstructure

The microstructure of cheese represents the spatial distribution of the compositional components (casein, minerals, fat, moisture and dissolved solutes such as lactose, lactic acid, soluble salts and peptides) at the micro-scale level (e.g., > 25× magnification) and the level of intra- and intermolecular attractions between the components. It has been studied extensively (Hall and Creamer, 1972; Kaláb and Harwalkar, 1974; Kimber *et al.*, 1974; Kalab, 1977; de Jong,

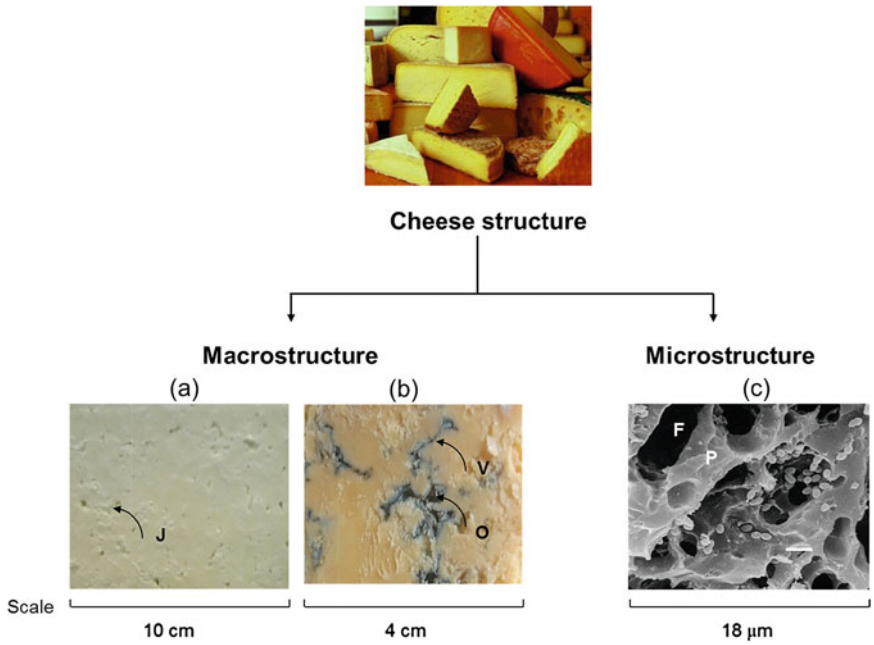

Fig. 14.11 Structural resolution of cheese showing the macrostructure of 1 day-old Cheddar after removal from the cheese press (*a*) and retail Shropshire blue (*b*) and the microstructure of 1 month-old Cheddar cheese (*c*). The macrostructure consists of an assembly of curd particles or curd pieces (chips) fused through interaction of surfaces, and includes discontinuities in the form of curd particle/curd chip junctions (J), veins of blue mould (V) or openings (O) between curd chips. The microstructure within curd particles or curd chips consists of a calcium phosphate *para*-casein network (P) which immobilizes the serum phase (moisture and dissolved salts) within its fused para-casein micelles and encases the fat (F) which occurs as globules in varying degrees of coalescence. The degree of fusion at curd particle/curd chip junctions (J) depends on potential of their microstructure to deform and flow under stress as applied during moulding and pressing

1978a; Green *et al.*, 1981a, b, 1983; Green, 1990a, b; Kiely *et al.*, 1992, 1993; Mistry and Anderson, 1993; Desai and Nolting, 1995; Everett *et al.*, 1995; Bryant *et al.*, 1995; Fenelon *et al.*, 1999; Guinee *et al.*, 1999, 2000a; Ong *et al.*, 2013).

The microstructure of rennet-curd cheese may be defined as a highly concentrated matrix (Fig. 14.12), consisting of:

- a calcium phosphate *para*-casein network of extensively dehydrated, fused *para*-casein micelles;
- a fat phase (in the form of globules, coalesced globules and/or pools) that is encased within the casein network;
- a solvent phase consisting of water and dissolved solutes (e.g., lactose, lactic acid, soluble salts, water soluble peptides, enzymes) entrapped within the *para*-casein micelles, and that corresponds largely to residual solvent not removed from the micelle during manufacture;
- micro-organisms, mainly starter and non-starter lactic acid bacteria, mainly located at the fat-protein interface

The fusion of casein molecules within the network is mediated by various interactions, including calcium bridges, formed by divalent calcium ions which bind to dissociated carboxyl groups of acidic amino acid residues and to phosphate (attached to serine groups), and hydrophobic interactions between uncharged amino acid residues on individual casein molecules.

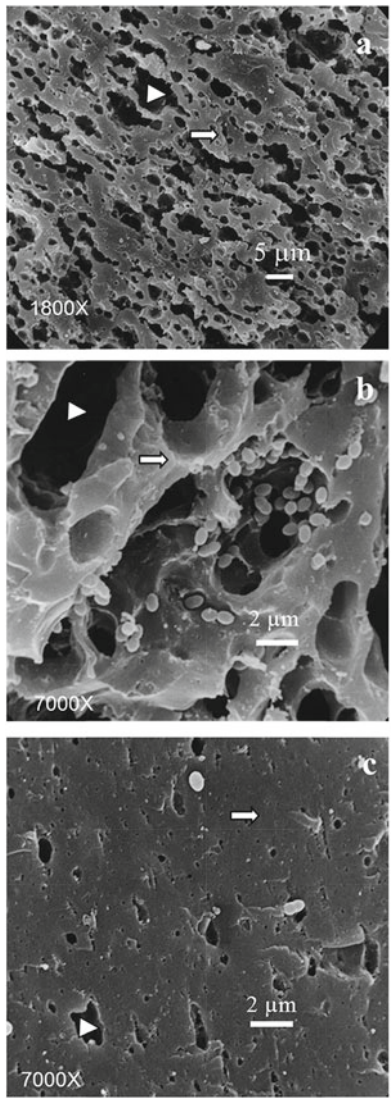

Fig. 14.12 Scanning electron micrographs of full (33 %, w/w, **a**, **b**) and low (0.4 %, w/w, **c**) fat Cheddar cheeses at low (1800, **a**) or high (7000, **b** and **c**) magnifications. The *arrows* correspond to the *para*-casein matrix and the *arrowheads* to the areas occupied by fat and free serum prior to their removal during sample preparation; bacteria (most likely starter lactococci) are visible in (**b**), being concentrated mainly at the fat-*para*-casein interface (Plate **a**, modified from Guinee *et al*., 1998; plates **b** and **c**, modified from Fenelon *et al*., 1999)

Overall, the microstructure of the *para*-casein network may be envisaged as a polymer network, comprised of polymer chains of fused *para-casein* micelles that are interconnected by numerous calcium- and hydrophobic-cross links to form a single macroscopic entity. The volume fraction of the *para*-casein network and degree of fusion (aggregation) of the *para*-casein particles making up that network increase as the contents of fat and moisture are reduced. Conversely, the volume fraction of the casein network decreases as the levels of the latter components, which may be considered as diluents, increase. This is clearly evident on comparing the structures of Cheddar cheese of varying fat content (Fig. 14.12; Fenelon and Guinee, 1999). Hence, controlling the degree of *para*-casein concentration and fusion (aggregation), through manipulation of the different manufacturing steps and their sequence, is critical in defining the properties of the final cheese, including rheological properties (e.g., ratio of viscous to elastic characteristics), physical properties (e.g., deformability, firmness, chewiness, ability to be sliced, shredded or grated) and opacity/translucence.

The fat in cheese occurs as globules in varying degrees of coalescence, as confirmed by the presence of fissures, or irregularly-shaped openings in the *para*-casein matrix, which remain after removal of fat during sample preparation for scanning electron microscopy (Fig. 14.12; Mistry and Anderson, 1993; Bryant *et al.*, 1995; Guinee *et al.*, 1998; Fenelon *et al.*, 1999; Rowney *et al.*, 2003; Lopez *et al.*, 2006; Richoux *et al.*, 2008). The frequency of these fissures decreases as the fat content is reduced, e.g., from 33.2 to 8.2 % (w/w) fat. Clumping and coalescence are the result of shear stresses imposed on the fat globule membrane by various manufacturing steps (e.g., cutting, stirring, pressing, plasticization) and the shrinkage of the surrounding *para*-casein network, which forces the occluded globules into close contact. Shrinkage of the *para*-casein network coincides with its dehydration during the various stages of cheesemaking, e.g., acidification, heating and pressing. In the temperature range used for cheesemaking (~30–55 °C) most, or all, of the milk fat is liquid (Norris *et al.*, 1973; Lopez *et al.*, 2006) and therefore flows on the application of stress.

The cheese matrix also contains starter and non-starter bacteria, which attach themselves, *via* filaments from their cell walls, to the casein net-

work (Kimber *et al.*, 1974) and are concentrated at the fat-casein interface (Laloy *et al.*, 1996). Bacterial proteinases and intracellular peptidases, released on autolysis, contribute to the hydrolysis of casein (Beresford and Williams, 2004; McSweeney, 2004). In addition, the surface layer of the cheese may be colonized by various microorganisms, including bacteria, moulds and yeasts, which release enzymes (e.g., proteinases, peptidases, lipases) into the outer layers of the cheese matrix at various rates during maturation (Spinnler and Gripon, 2004; Brennan *et al.*, 2004). The enzymes released into the surface layers of the cheese diffuse into the cheese a short distance only owing to their high molecular weight (Lee *et al.*, 1980; Noomen, 1983). However, it is likely that water-soluble products of enzyme hydrolysis (water-soluble fatty acids, low molecular mass peptides and amino acids) and other compounds (e.g., lactic acid and soluble calcium) diffuse through the cheese mass (Lee *et al.*, 1980) and affect zonal variations in pH, *para*-casein hydration, rheology, functionality and flavour (Noomen, 1983; Karahadian and Lindsay, 1987).

Various physicochemical changes occur in the structural components of the *para*-casein matrix during maturation; these changes are mediated by the residual rennet, microorganisms and their enzymes, and changes in mineral equilibrium between the serum and *para*-casein network. The type and level of physicochemical changes depend on the cheese variety, cheese composition and ripening conditions. They include:

- hydrolysis of the *para*-casein into peptides, of varying molecular mass, and amino acids (FAA); degradation of the FAA into compounds such as amines, aldehydes, alcohols and ammonia (Upadhyay *et al.*, 2004);
- changes in the equilibrium concentrations of calcium and inorganic phosphate between the para-casein network and the enclosed serum, with the equilibrium being influenced by maturation time, pH and other factors such as the concentration of sodium (Na) in the moisture phase and soluble Ca (Le Graet *et al.*, 1983; Karahadian and Lindsay, 1987; Guo and Kindstedt, 1995; Guo *et al.*, 1997; Paulson *et al.*, 1998; Lee *et al.*, 2005, 2010);
- increase in hydration of the *para*-casein, as reflected by the decrease in the level of serum expressed by centrifugation or by hydraulic pressing of the cheese during maturation (Kindstedt, 1995; Guo and Kindstedt, 1995; Guo *et al.*, 1997; Thierry *et al.*, 1998; Boutrou *et al.*, 1999; Guinee *et al.*, 2000a; Hassan *et al.*, 2004). Hydration is mediated by factors such as proteolysis, an increase in pH, and the solubilization of casein-bound calcium;
- physical expansion or swelling of the *para-casein* network, at least in Mozzarella cheese, as a result of the increase in casein hydration. The age-related increase in the degree of swelling of the casein network of Mozzarella cheese is clearly observed on examination of the cheese by confocal laser scanning microscopy during maturation (Fig. 14.13);
- coalescence of fat globules, resulting in the formation of fat pools. This appears to occur in all cheeses, as demonstrated by various types of microscopy (Kimber *et al.*, 1974; Laloy *et al.*, 1996; Lopez *et al.*, 2006). The occurrence of fat coalescence is also supported by the increases in the level of fat that can be expressed from the cheese when subjected to hydraulic pressure or centrifugation, or exudes from the cheese on baking (Kindstedt, 1995; Thierry *et al.*, 1998; Guinee *et al.*, 2000a, c). Fat coalescence may be mediated by an increase in free fat due to degradation of the fat globule membrane and/or by hydration and swelling of the *para*-casein, which in effect forces the partially denuded fat globules into closer proximity (Kindstedt and Guo, 1997). It is noteworthy that ~30–60 % of the total milk fat is liquid (Norris *et al.*, 1973; Lopez *et al.*, 2006) in the temperature range used for the ripening of different cheese varieties (e.g., 8–22 °C) and flows under pressure.

Hence, cheese is a biologically, enzymatically and biochemically dynamic system in which the protein and other structural components undergo

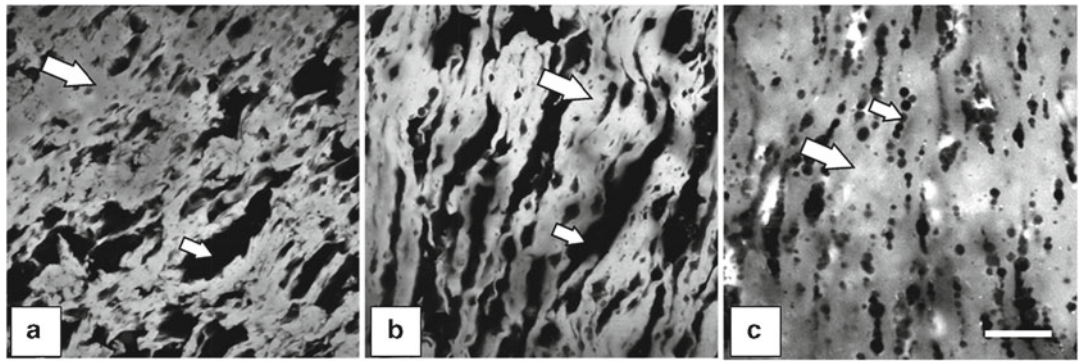

Fig. 14.13 Confocal laser scanning micrographs of low-moisture Mozzarella cheese at different stages of production and storage at 4 °C: (**a**) before plasticization; salted and milled curd, showing *para*-casein matrix strands (*long arrows*) and void spaces containing fat globules (*short arrows*); (**b**) post plasticization (24 h storage at 4 °C), stretched curd showing extensive linearization of *para*-casein into fibres (*long arrows*) and globules/pools of fat (*short arrows*); and (**c**) after 43 days storage at 4 °C; aged cheese showing hydrated swollen *para*-casein fibres forming a continuous protein phase with occluded fat mainly as pools. Bar = 25 μm (Modified from Guinee *et al*., 2002)

physicochemical and micro-structural changes during ripening. These changes assist in the conversion of fresh 'green' curd to a mature cheese and influence its rheological, textural, functional and flavour characteristics markedly (see Sects. 14.7 and 14.8). Thus, a storage period is generally required for most rennet-coagulated cheeses before they attain the desired attributes (e.g., flavour, aroma, or the ability to melt, flow and stretch on cooking) associated with the particular variety.

14.4.1.2 Macrostructure

The macrostructure, representing the gross aspects of structure within the moulded cheese, such as curd granule junctions, eyes, and slits/cracks, is observed visually by the unaided eye or at very low levels of magnification (<10× magnification) with the aid of light microscopy. The knitting of individual curd particles or chips/pieces into a macrostructure (intact moulded cheese) is influenced by many factors including:

- the microstructural-related properties of the particles or chips *per se*, which determines their potential to deform and flow into, and fuse with, other curd particles when subjected to moulding and pressing; critical factors include *inter alia* the degree of *para*-casein hydration (g water/g protein), level of calcium bound by *para*-casein, protein-to-fat ratio of matrix, salt content and pH of the moisture (solvent) phase which influences protein hydration;
- the curd particles surface properties such as composition (protein-to-fat ratio, salt content, moisture content), degree of protein hydration (as affected by temperatures of curd particle/whey mixture during scalding, moulding, and pressing), and aspect (e.g., shape, size, aspect ratio, uniformity);
- the size distribution and surface area of curd particles or pieces, which affects the packing arrangement, i.e. the neatness of fit, into a continuum;
- pressing conditions including temperature and pressure which affect factors such as the strength of hydrophobic interactions, the extent of protein solubilization and fat crystallization, and the ability of curd particles to flow and knit into a seamless whole, without notable inter-particle microstructural discontinuities and junctions;
- the presence of interstitial air;
- storage conditions (temperature, time) which affect the extent of age-related changes in pH, equilibrium between soluble and *para*-casein bound calcium, proteolysis, fat coalescence, and hence, the ability of curd particles to merge and lose their identity.

In young cheese, the macrostructure consists of an assembly of curd particles that are fused with neighbouring particles to varying degrees. Discontinuities at the macro-structural level exist in the form of curd granule junctions or curd chip junctions (in Cheddar and related dry-salted varieties) (Kaláb, 1979; Lowrie *et al.*, 1982; Paquet and Kaláb, 1988). Curd granule junctions in low-moisture Mozzarella are well defined, ~3–5 μm wide, and appear as veins running along the perimeters of neighbouring curd particles (Kalab, 1977). Unlike the interior of the curd particles, the junctions are comprised mainly of casein, being almost devoid of fat. Factors which contribute to the formation of these junctions include leaching of the fat from the surface of the curd particles and dehydration of surface protein during the cutting, acidification, cooking and pressing stages of curd manufacture. Chip junctions in Cheddar and related dry-salted varieties are clearly discernible on examination of the cheese by light microscopy and, like curd granule junctions, have a higher casein-to-fat ratio than the interior of the chips. The difference in cheese composition at junctions, compared to the interior of the curd particles, is likely to cause differences in the molecular attractions between adjacent *para*-casein layers in the interior and exterior of curd particles, and thus to differences in structure-function relationships.

On-going fusion of particles during maturation leads to a more coherent structural continuum as reflected by the disappearance of inter-particle boundaries and the formation of a more homogeneous mass (Kimber *et al.*, 1974; de Jong, 1978a).

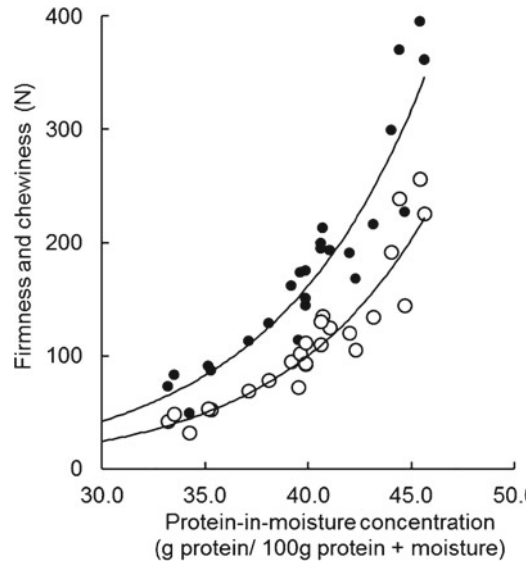

Fig. 14.14 Firmness (*filled circle*) and chewiness (*open circle*) of model skim-milk cheeses decay exponentially as the protein-in-moisture (PIM) is reduced, by increasing the moisture of the cheese through manipulation of cheese-making protocol. The range of PIM values (30–46 %) represents the range found across a spectrum of different semi-hard/hard cheeses from ~30 % in Camembert to ~46 % in Gruyére and Emmental (see Table 14.1)

14.4.2 Structure-Function Relationships of Rennet-Curd Cheeses

14.4.2.1 Unheated Cheese

As the concentration of *para*-casein (network) increases, its volume fraction increases, and its microstructure becomes less particulate and more of a single continuum (rather than assembly of individual sub-structures). Simultaneously, the mobility of the system becomes increasingly restricted as the lubricating contribution of moisture diminishes, where mobility may be defined as the potential of contiguous planes of the cheese matrix to move, or undergo displacement, when cheeses are subjected to stresses or strains. However, for a given casein concentration, the level of calcium, which may be envisaged as the binding 'glue' between the caseins, is a critical factor affecting the potential of the casein network to undergo displacement. Consequently, cheeses tend to become more elastic, firm and chewy as the levels of protein (para-casein), calcium and PIM increase (Fig. 14.14; Fenelon and Guinee, 2000; Pastorino *et al.*, 2003b; O'Mahony *et al.*, 2006). Simultaneously, the cheese becomes less opaque (less white) and more translucent as the volume fraction of the casein matrix increases, owing to the increasing degree of structural continuity and the reduction in extent of structural interfaces (e.g., moisture/network interface) where light scattering occurs. However, the

degree of opacity increases as the fat content increases, owing to the increase of light scattering interfaces between the fat globules/pools and the casein and the moisture phase.

The impact of macrostructure-functionality relationship is readily evident when comparing some key functional attributes (e.g., sliceability, shreddability, grating ability) across different cheese types, for example brine-salted and dry-salted hard cheeses. Brine-salted cheeses such as Gouda, Mozzarella and Emmental usually display excellent sliceability and shreddability, owing to the high degree of curd particle knitting into a seamless whole moulded structural continuum. The slices are flexible, bendable and when stacked slice-on-slice (without separating foils) and can peel easily without breaking. On the other hand, dry-salted cheese such as Cheddar do not perform as well functionally, with the slices being generally more brittle and prone to breaking along curd-chip junctions that may be considered as 'fault lines' where chip-to-chip surface knitting is poor, owing to a number of factors such as a relatively high degree of dehydration of the *para*-casein network (commensurate with a salting-out effect at the high salt-in-moisture concentrations in the surface layer of the chips during the dry salting), high concentrations of calcium phosphate and calcium phosphate deposits (which contribute to seaminess, the occurrence of white crystalline deposits in the area between curd chips, in dry-salted cheeses), low contents of moisture and fat and relatively low temperatures during pressing. Nevertheless, the adverse effects of 'discontinuous' macrostructure on sliceability and shreddability of dry salted cheeses are likely to be overcome to greater or lesser degrees by optimizing the microstructure-macrostructure interaction. This involves altering the microstructure and rheology of the curd chips which make them more amenable to flowing and knitting together (e.g., alteration of pH, calcium level, ratio of soluble-to-insoluble calcium, and modifying the conditions of salting).

Similarly, a very high degree of curd-particle surface dehydration may impair the fusion of curd particles to knit into a cohesive, structural continuum, and thereby the ability of the cheese to slice. This is most readily evident in Parmesan-type cheeses, where conditions of manufacture (e.g., small cut particle size, high scald temperature, and long brining time) and curd composition (e.g., high protein-to-fat ratio and low moisture content) are conducive to a very high degree of *para*-casein dehydration and aggregation. Consequently, the cheese is very dry with a hard texture that fractures easily along its curd granule junctions (on application of stress or strain) to yield a 'mealy-like', granular/grainy texture on mastication, as the poorly knitted curd particles (granules) come apart and are perceived separately. Hence while the cheese is very hard, it is very brittle and unsuited to slicing and shredding, but is ideally suited to grating and for use as a sprinkling onto dishes such as spaghetti Bolognese.

14.4.2.2 Heated Cheese

Heating cheese to temperatures encountered during baking and grilling (90–98 °C), results in two major microstructural changes:

- Contraction and shrinkage of the *para*-casein network (owing to a temperature-induced increase in the extent of hydrophobic interactions between the casein molecules) and a simultaneous expulsion of moisture from the casein network, and
- Liquefaction and coalescence of fat globules, resulting in formation of free fat, which is readily observed as an oily layer at the surface of the melting cheese mass.

Consequently, the melting cheese mass becomes more fluid, as both the oil and water act as lubricants between contiguous layers of the cheese network. These heat-induced changes in microstructure form the basis of what are generally known as melt properties (see Sects. 14.8, Table 14.4), including softening of the melting cheese, flow and spread of the melting cheese mass under its own weight during grilling or baking, stretchability and stringiness of the molten cheese mass when extended, oil exudation and formation of free oil on the surface of molten cheese mass.

Table 14.4 Functional properties of grilled/baked cheese which influence its functionality as an ingredient

Property	Definition	Cheeses which generally display this property	Property related to physico-chemical state
Meltability	The ability of cheese to soften to a molten cohesive mass on heating	Most cheeses after a given storage period, dependent on the variety, PCPs, APCs, cream cheese	Fat liquifaction, fat coalescence
Flowability	The ability of the melted cheese to flow	Most cheeses after a given storage period, dependent on the variety, PCPs, OACs, cream cheese	Fat liquifaction, heat-induced casein dehydration, high degree of fat coalescence, limited oiling-off
Stretchability	The ability of the melted cheese to form cohesive fibres, strings or sheets when extended	Low-moisture Mozzarella, Kashkaval, young Cheddar (i.e., 15 days)	Moderate degree of heat-induced casein dehydration and casein aggregation, level and type of molecular attractions between *para*-casein molecules
Flow resistance (often referred to as melt-resistance)	The resistance to flow of melted cheese	Paneer, PCPs, OACs, natural cheeses from high heat-treated milk	Absence of fat coalescence, heat-induced aggregation or gelation of a particular component(s) (e.g., whey proteins), thermo-irreversibility of protein aggregation or gel system in the uncooked product upon heating
Chewiness (rubbery, tough, elastic)	High resistance to breakdown on mastication	Low-moisture Mozzarella, Kashkaval, young Cheddar (i.e., 15 days)	As for stretchability
Viscous (soupy)	Low resistance of melted cheese to breakdown on mastication	Mature Cheddar, aged Mozzarella, cream cheese, PCPs, some ACs	Relatively high level of casein hydration
Limited cooling-off	Ability of cheese to express a little free-oil on heating, so as to reduce cheese dehydration. Maintain succulence of, and impart surface sheen to, melted cheese	Most natural cheeses (if not very mature or very young), PCPs, APCs	Limited degree of fat coalescence
Desirable surface appearance	Desired degree of surface sheen with few, if any, dry, scorched black or brown particles	Mature Cheddar, aged Mozzarella, Cream cheese, PCPs, OACs (depending on formulation)	Adequate degree of casein hydration and of oiling off during baking; low level of residual reducing sugars (e.g. lactose, galactose) and Maillard browning

Key: *PCP* processed cheese products, *APC* analogue pizza cheese, *OAC* analogue cheeses

14.4.3 Structure of Acid-Curd Cheeses

While the structure of acid-curd cheeses is generically similar to rennet curd cheese, in that it is essentially a polymer network of casein, it differs in the following respects:

- the polymer network is comprised of casein instead of calcium phosphate *para*-casein;
- the degree of calcium bridging contributing to casein interconnectivity and casein network formation is significantly lower. Owing to the low pH (~4.5–4.8), all the colloidal calcium phosphate has been solubilised during gel formation and removed in the cheese whey during subsequent concentration. Only calcium attached directly to acidic amino acids remains with the casein, and this decreases as the pH is reduced. Despite the overall low level, the quantity of calcium is, nevertheless, a critical determinant of texture (chewy or mushy) in the case of Cottage cheese, which may be described as a 'mainly acid-coagulated cheese' even though a small quantity of rennet is used in manufacture (Farkye, 2004a);
- the volume fraction of the casein network is much lower and the degree of fusion between the casein particles making up the network is generally much lower (Kaláb, 1979) because of the lower casein concentration and higher moisture content.

14.4.4 Structure-Function Relationships of Acid-Curd Cheese

Owing to the low volume fraction of the casein network and degree of fusion between the casein particles, acid curd cheeses generally have a soft, smooth consistency and mouthfeel. Compared to rennet-curd cheeses, they typically have high moisture content, low protein and high moisture-to-protein ratios (e.g., ~5.7 g water/g protein for Quark compared to ~1.5 and 0.9 for Cheddar and Parmesan cheeses, respectively). Indeed some acid-curd cheeses, especially high moisture products such as Quark, Labneh and *Fromage frais* may be considered as viscoelastic liquids, rather than viscoelastic solids as in the case for rennet-curd cheeses in general (Metzger, 2011). Owing to these properties, acid curd cheeses tend to be susceptible to uncontrolled post-manufacture casein aggregation and dehydration *via* mobility and re-arrangement of their casein networks caused by external stresses on the product (for example during transport/distribution and retailing) or internal stresses induced by changes in temperature during storage. In extreme cases, this can lead to protein precipitation and extensive wheying-off. This tendency is likely to be more pronounced when the moisture content of the product is high, as this facilitates the sedimentation of protein aggregates; however, this defect can be alleviated by inclusion of stabilisers (e.g., neutral hydrocolloids), which increase the viscosity of the aqueous phase thereby restricting mobility of the casein network.

Protein aggregation is the basis of major sensory defects including excessive wheying-off and the development of sandy/grainy textures during storage, especially where the concentrated milk gel is heated and hot-filled post fermentation (e.g., as in Cream cheese) and where the product is required to have a long shelf-life (e.g., up to 6 month for hot-packed Cream cheese and some fresh cheese preparations). Avoidance of such defects requires optimization of the degree of casein aggregation at the different stages of manufacture through the control of appropriate tools such as milk composition (e.g., casein-to-whey protein ratio), heat treatment of the milk and levels of whey protein denaturation, optimization of product pH, treatment of gel post whey separation (homogenization, shearing, rate and extent of cooling) and the use of suitable stabilisers (e.g., hydrocolloids such as gums). Where permitted, suitable stabilisers (e.g., guar gum, locust bean gum) can increase the viscosity of the aqueous phase, and thereby, minimise casein aggregation *via* mobility and re-arrangement of the gel structure. The options available depend on the product, which determines the steps involved in manufacture (including the heat load and degree

of shear applied to the concentrated acidified milk gel), the sequence of steps, product composition and shelf life.

14.5 Role of Protein in the Curd Texturization and Plasticization Processes in the Manufacture of Cheddar and Pasta-Filata Varieties

In the manufacture of Cheddar, and *pasta-filata* (stretched-curd) cheeses such as Mozzarella and Kashkaval, the curd is subjected to texturization processes, which involve fusion of *para*-casein strands into fibres which have a linear orientation.

14.5.1 Cheddaring

Texturization in the manufacture of Cheddar involves cheddaring, whereby drained curd is piled and pressed under its own weight while the pH decreases from ~6.15 to 5.2 as a result of growth of the starter culture. In traditional manufacture, piling was achieved by cutting the bed of drained curds into slabs which were piled higher as the pH decreased. In modern cheesemaking, the drained curds are discharged continuously onto moving perforated belts with side plates (e.g., Tetra Tebel Alfomatic®) or into towers (e.g., APV CheddarMaster®-tower system) where the respective height and degree of flow of the curd layer or column are controlled by altering belt speed/dimensions or residence time in the tower (Bennett and Johnston, 2004). The cheddaring process creates conditions conducive to curd flow and texturization (i.e., development of a fibrous or chicken breast-like texture) (King and Czulak, 1958). These conditions include:

- holding the curd at ~34–35 °C for ~45–60 min
  - to allow continued growth of the starter culture and acid development and hence a decrease in pH from ~6.15 to 5.2;
  - to ensure that the milk fat remains essentially in the liquid state and thereby contributes to the viscous, rather than elastic, character of the curd;
- piling the curd and allowing it to press under its own weight in equipment (e.g., finishing vat, cheddaring tower or cheddaring belts) designed to accommodate a certain degree of curd flow.

Cheddaring promotes a number of physicochemical conditions that are conducive to curd flow and texturization. These include:

- a decrease in pH and solubilization of micellar calcium phosphate which is bound to the casein and acts as a cementing agent between the casein micelles/sub-micelles (van Hooydonk *et al.*, 1986; Swaisgood, 2003);
- an increase in the ratio of soluble-to-casein bound Ca; soluble Ca increases from ~5 to 40 % of total Ca in the curd as the pH decreases from 6.15 to 5.2 (Guinee *et al.*, 2000b; Fig. 14.9);
- an increase in *para*-casein hydration which increases with decreasing pH in the range 6.6 to ~5.15 (Creamer, 1985; Fig. 14.15), and
- an expected increase in the ratio of viscous-to-elastic character of the curd.

The increase in casein hydration with decreasing pH is probably in part a consequence of the increase in the ratio of soluble-to-micellar Ca. Studies in model (dilute) casein systems have shown that casein hydration is inversely related to the concentration of casein-bound Ca (Sood *et al.*, 1979). As a consequence of the reduction in casein-bound Ca and the increase in casein hydration, the viscoelastic casein network undergoes limited flow if physically unrestricted, especially when piled and pressed under its own weight. Curd flow gives the desired planar orientation of the strands of the *para*-casein network (Kalab, 1977, 1979; Lowrie *et al.*, 1982; Figs. 14.12a and 14.13). The physicochemical changes in curd during cheddaring are summarised in Fig. 14.16.

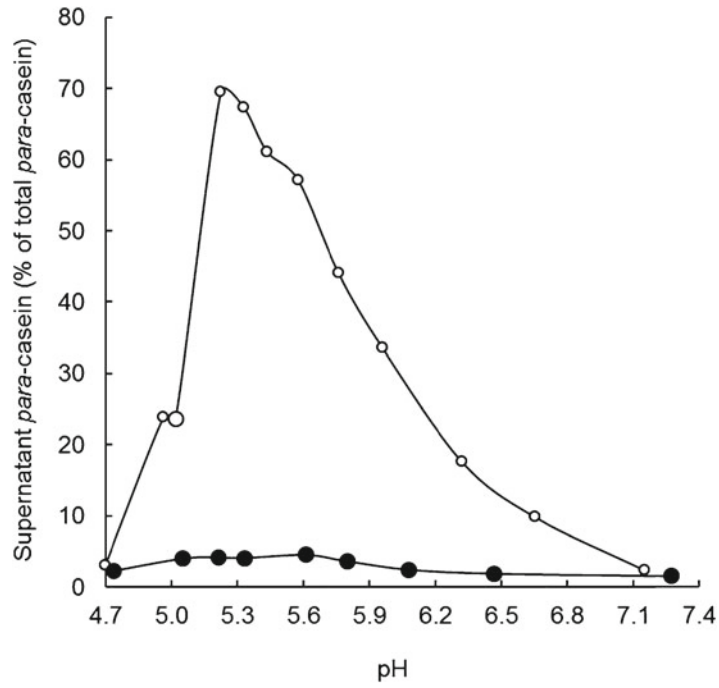

Fig. 14.15 Effect of NaCl, added at 5 % (w/w) on the solubility of a 5 % (w/w) dispersion of rennet casein (80 %, w/w, protein; 7.1 %, w/w, ash). The rennet casein was thoroughly mixed with water (*filled circle*) or 5 %, w/w, NaCl (*open circle*) and the pH adjusted to different values using 20 % (w/v) lactic acid. The dispersions were held at 15 °C for 22 h and then centrifuged at 3000×*g* for 30 min at 15 °C. The protein content of the supernatant was measured and expressed as a percentage of the total protein (redrawn from Guinee and Fox, 2004)

Following cheddaring, the curd is milled into chips which are dry-salted, moulded and pressed. The latter processes result in the random orientation of salted curd chips, and relatively little evidence of distinct casein fibres with a preponderant orientation in the pressed Cheddar cheese, at least compared to freshly prepared Mozzarella. Hence, it is difficult to ascertain the specific contribution of the cheddaring process *per se* to the structure and characteristics of Cheddar cheese. This would necessitate comparison of the structures of control Cheddar cheese and experimental Cheddar cheese manufactured under conditions whereby curd flow during acidification and cheddaring were restricted, for example by moulding the curd following whey drainage prior to subsequent milling and dry-salting.

14.5.2 Curd Plasticization

The manufacture of Mozzarella and other *pasta-filata* cheeses, e.g., Kashkaval and Provolone, involves a plasticization stage towards the end of manufacture, whereby the milled cheese curd is heated and subjected to shear and extension stress that transform it to a uniform molten, glossy, fibrous mass that forms fine strings when peeled.

The manufacture of Mozzarella and other *pasta-filata* cheeses, e.g. is similar to that of Cheddar up to the completion of the cheddaring process and subsequent milling of the curd into chips but differs markedly in subsequent treatments. The curd chips (pH ~5.2–5.3) are slivered, plasticized, moulded, cooled and salted. During plasticization, the shredded curds are typically heated to ~57–60 °C and stretched by kneading in hot water or dilute brine (e.g., 5 % NaCl) at ~78 °C. Alternatively, the curds may be subjected to extrusion as in the formation of string cheese, whereby the curds are heated by direct steam injection, worked by rotating screws into a hot molten mass which is extruded under pressure through die plates (Fritchen *et al.*, 1990; Mulvaney *et al.*, 1997). During plasticization, the relatively low curd pH and the high temperature are conducive to limited aggregation of the casein and the formation of *para*-casein fibres of relatively high tensile strength (Taneya *et al.*, 1992; Oberg *et al.*, 1993; Apostolopoulos, 1994; Pagliarini and

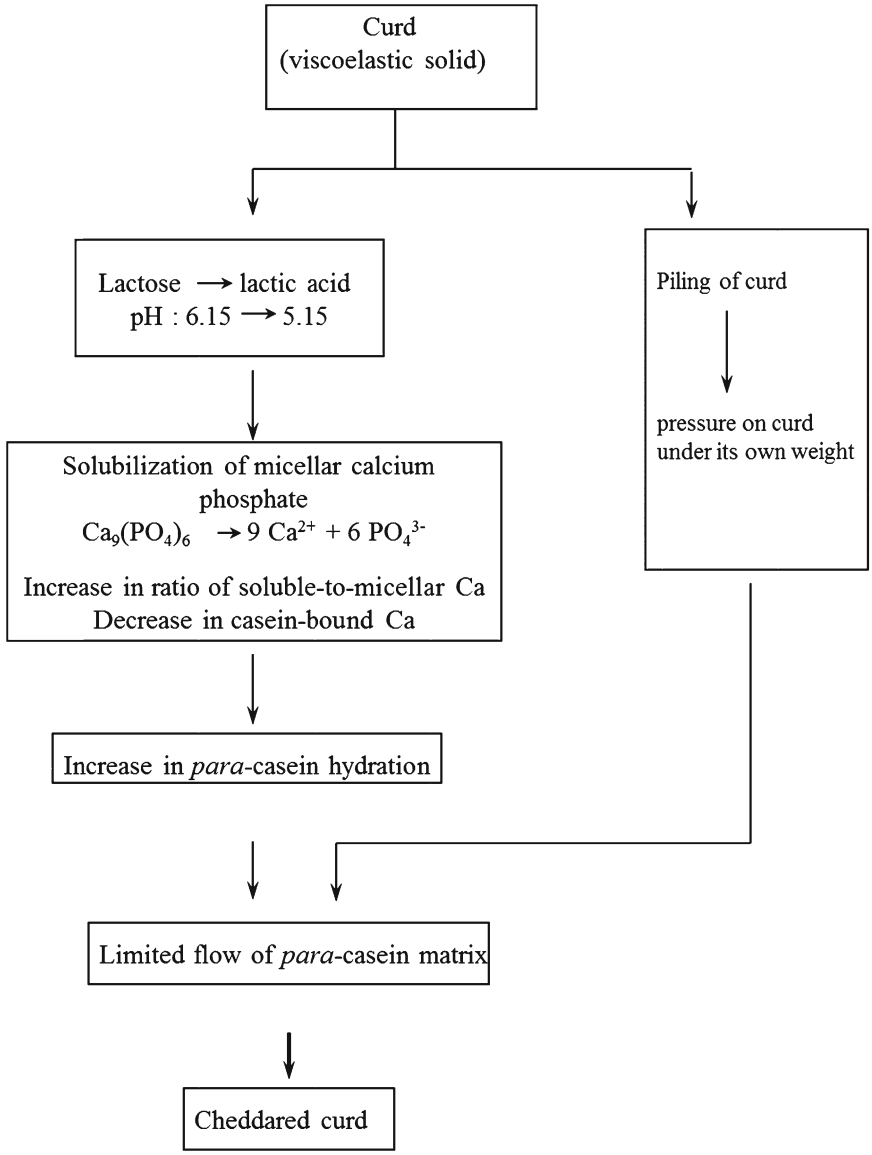

Fig. 14.16 Summary of physico-chemical changes in cheese during cheddaring (redrawn from Fox *et al*., 2000)

Beatrice, 1994; Guinee and O'Callaghan, 1997; McMahon *et al.*, 1999). At a micro-structural level, plasticization results in linearization of the *para*-casein network into fibres and clumping of fat into pools that are trapped between, and show the same orientation as, the fibres (Kalab, 1977; Kiely *et al.*, 1992; Taneya *et al.*, 1992; Oberg *et al.*, 1993; Fig. 14.13). When *pasta filata* cheeses are subsequently cooked on pizza, the fibres endow the cheese topping with the desired levels of stretch or stringiness and chewiness (Guinee and O'Callaghan, 1997).

The physicochemical changes responsible for plasticization include:

- a reduction in the extent of crosslinking of *para*-casein molecules constituting the network, as facilitated by pH reduction and commensurate solubilization of colloidal calcium phosphate;

- an increase in the degree of casein hydration in the cheddared curd, as affected by a reduction in curd pH and an increase in the ratio of soluble-to-micellar Ca;
- heat-induced coalescence of free fat which lubricates the flow of the *para*-casein network (Paquet and Kaláb, 1988; Guinee *et al.*, 2000c; Lefevre *et al.*, 2000);
- extension and shear stresses applied to the curds which assist in the displacement of adjoining planes of the *para*-casein matrix.

The relationship between *para*-casein hydration and pH may be explained by the dominance of two opposing forces over the pH region 6.0–5.0:

- those that promote charge neutralization, which lead to dehydration and aggregation of the *para*-casein which in turn is expected to impede shear and extension displacement (flow) of the casein network at the high temperature, and
- those that promote solubilization of colloidal calcium which is conducive to casein hydration and flow of the network.

At pH values in the region 6.0–5.2, solubilization of CCP appears to be dominant since reducing pH results in an increase in the hydration of *para*-casein (Fig. 14.15; Creamer, 1985). In contrast, charge neutralization appears to dominate in the pH range 5.2–4.6, as reduction in pH leads to a marked decrease in *para*-casein hydration.

The total concentration of calcium in the curd, which is controlled mainly by the pH of the milk at setting and that of the curd at whey drainage (Lawrence *et al.*, 1984), determines the pH at which plasticization of the curd is possible. In the conventional manufacture of Mozzarella, the milk is, typically, set at pH 6.55, the whey is drained off at pH 6.15 and the cheddared curd is, ideally, plasticized at pH ~5.2–5.3. At this pH, the concentration of calcium in the curd (~28 mg/g protein) and the ratio of soluble-to-colloidal calcium (~30:70) permit adequate hydration of the *para*-casein for proper plasticization. At higher pH values, the curd does not plasticize properly: the individual slivers do not cohere well, and the heated and kneaded curd mass remains 'lumpy' and 'bitty', has a short consistency, and a rough (knobby), dull surface appearance. The latter defects, which become more pronounced as the pH is increased in the region 5.3–5.8, reflects the decrease in *para-casein* hydration due to the concomitant reduction in the ratio of soluble-to-colloidal Ca. For similar concentrations of total calcium in cheese, soluble calcium as a percentage of total Ca decreases from ~40 % at pH 5.0 to ~15 % at pH 5.8 (Guinee *et al.*, 2000b; Fig. 14.9).

However, successful plasticization can be achieved at a high curd pH, e.g., 5.6–5.8, if the concentration of total Ca in the curd is sufficiently low (e.g., <18 mg/g protein), as in the case of directly-acidified Mozzarella (DAM). In this case, acidification is achieved by the addition of food-grade organic acid(s), rather than the conversion of lactose to lactic acid by the starter culture (Shehata *et al.*, 1967; Kindstedt and Guo, 1997; Guinee *et al.*, 2002). Typically, the milk pH is adjusted to ~5.6 prior to rennet addition and no further change in pH occurs during curd manufacture which is otherwise similar to that for conventional Mozzarella made using a starter culture. Following whey drainage, the curd, typically with a pH of ~5.6, plasticizes quite well at 57 °C. The ability of curd made using direct acidification to plasticize at the relatively high pH can be explained on the basis of its lower total calcium content, which gives a level of casein-bound calcium that is comparable to that found in conventionally-manufactured curd at pH ~5.2. While soluble Ca decreases from ~40 to 20 % of total Ca as the pH is raised from ~5.2 to 5.6, the overall concentration of calcium in DAM is markedly lower (e.g., 18 *vs.* 28 mg/g protein). Hence, the estimated concentration of micellar casein-bound Ca in DAM (~14.5 mg/g protein) is similar to, or lower than that (~16.5 mg/g protein) of conventional Mozzarella which is plasticized at pH 5.2. Owing to the inverse relationship between casein-bound Ca and casein hydration (Sood *et al.*, 1979), the degree of *para*-casein hydration in DAM curd at pH 5.6 is, therefore, similar to, or somewhat greater than, that in conventionally-produced Mozzarella curd at pH 5.2 (Kindstedt and Guo, 1997; Guinee *et al.*, 2002).

14.6 Effect of Protein on the Rheological Properties of Unheated Cheese

The ability of cheese to fulfil its requirements as a food or as an ingredient depends on its functional properties, which may be defined as those physico-chemical, micro-structural, rheological and organoleptic characteristics which affect the behaviour of cheese in food systems during food preparation, processing, cooking and consumption. Protein, a major component in most cheeses, has a major influence on the functional properties of both unheated and heated cheese.

When used as an ingredient in food applications, cheese is required to perform one or more functions. In the unheated state, cheese is generally required to exhibit a number of rheological-based properties so as to facilitate its size reduction in the primary stages of preparation of various dishes, e.g., the ability to crumble easily, to slice or to shred cleanly and to bend when in sliced form (Table 14.2). During size reduction operations, cheese is generally subjected to relatively high stresses (e.g., >600 kPa) and strains (e.g., ≫0.02) which result in fracture, e.g., portioning of cheese into retail sizes, shredding into thin narrow cylindrical pieces (e.g., 2.5 cm long and 0.4 cm diameter), dicing into very small cubes (0.4 cm) and comminution by forcing pre-cut cheese through die plates with narrow apertures. Similarly when eaten, cheese is subjected to a number of strains which reduce it to a paste capable of being swallowed; first, the cheese is bitten (cut by the incisors), compressed (by the molars) on chewing, and sheared (between the palate and the tongue, and between the teeth).

Cheese rheology and the factors that affect it have been reviewed extensively (van Vliet, 1991; Visser, 1991; Prentice *et al.*, 1993; Lucey *et al.*, 2003; O'Callaghan and Guinee, 2004; Guinee, 2011c). The concentration, type, and degree of hydrolysis of protein have a major influence on cheese rheology. The effects of protein on rheology and texture are confirmed by the positive correlation between the volume fraction of the casein network and cheese firmness and fracture stress (de Jong, 1977; Guinee *et al.*, 2000a), and by the effects of gel fineness or coarseness on the rheological characteristics of the acid-curd cheeses (Green *et al.*, 1983; Green, 1990a; Guinee *et al.*, 1993; Lucey, 2002).

14.6.1 Effect of Protein Concentration on Rheology

It is difficult to elucidate the direct effects of altering the concentration of any one compositional component, including protein, on the rheology of cheese since the levels of the different components tend to vary simultaneously, e.g., fat reduction is accompanied by increases in the levels of protein and moisture and decreases in the levels of moisture-in-non-fat substance and fat-in-dry matter. Nevertheless, results on the functionality of reduced-fat cheese do provide insights on the effects of protein on cheese rheology.

Increasing the protein content of cheese results in significant increases in storage modulus, firmness (force required to attain a given deformation) and fracture stress of the unheated cheese (Fenelon and Guinee, 2000). This is confirmed by the positive correlations between the content of intact casein and fracture stress and firmness of Cheddar cheeses with fat and protein contents ranging from 7 to 33 % (w/w) and from 26 to 40 % (w/w), respectively (Fig. 14.17) (Emmons *et al.*, 1980; Bryant *et al.*, 1995; Mackey and Desai, 1995; Drake *et al.*, 1997). Such a trend is expected based on the:

- increase in the concentration and volume fraction of the *para*-casein network, the number of stress-bearing protein network strands, and the number of intra- and inter-strand linkages (Fig. 14.12) (see Sects. 14.2 and 14.3);
- increase in PIM concentration, from 40 to 46 % on reducing the fat content of Cheddar-type cheese from ~32 to 1 % (w/w) and simultaneously increasing the protein from ~25 to 42 % (w/w);
- reduction in the lubricating effects of moisture and fat on the fracture surfaces of the *para-casein* network during deformation.

Table 14.2 Functional properties of raw cheese which influence its functionality as an ingredient

Property	Definition	Cheeses which generally display the property	Positively associated rheological parameters	Some applications where property is important
Shreddability	The ability of a cheese block to:	Low moisture Mozzarella, Swiss-type cheese, medium-aged Cheddar, Gouda, Provolone, APC	Elasticity, springiness, firm, long	Cheese on pizza
	• Shred into thin strips of uniform dimensions			
	• Resist fracture during shredding			Retail shredded cheese packs
	• Resist clumping/balling during shredding			
Sliceability	Ability of cheese to:	Low-moisture Mozzarella	Elasticity, springiness, firm, long	Sliced cheese for salads, crackers, sandwiches, burgers, continental breakfast slices
	• Cut cleanly into thin slices without fracturing or crumbling or sticking to cutting implement	Swiss-type cheese		
	• Retain peelability (without curling up at ends or drying out) on exposure under ambient conditions for moderate time (e.g., 1–2 h)	Provolone		
	• Bend without visually fracturing	APC, PCP's (some)		
Gratability	The ability of the cheese to fracture (elastically) into small particles, with a low tendency to stick, on shearing and crushing	Parmesan	Elastic fracturability	Grated cheese
		Romano		Cheese sprinklings
Spreadability	• The ability to spread easily when subjected to a shear stress	Mature Camembert, Cream cheese, mature Blue cheese	Plastic fracturability	Fondues
			Soft, adhesiveness	Cheese sauces
		PCPs and some AC (depending on formulations)	Short	Cheese cakes
				Spreads on crackers
Crumbliness	• The ability to break down into small irregular shaped pieces when rubbed (at low deformation)	Blue cheese	Elastic fracture at low deformation	Salads
		Cheshire	Low cohesion	Home-made soups

Key: *PCPs* processed cheese products, *APC* analogue pizza cheese, *AC* analogue cheeses

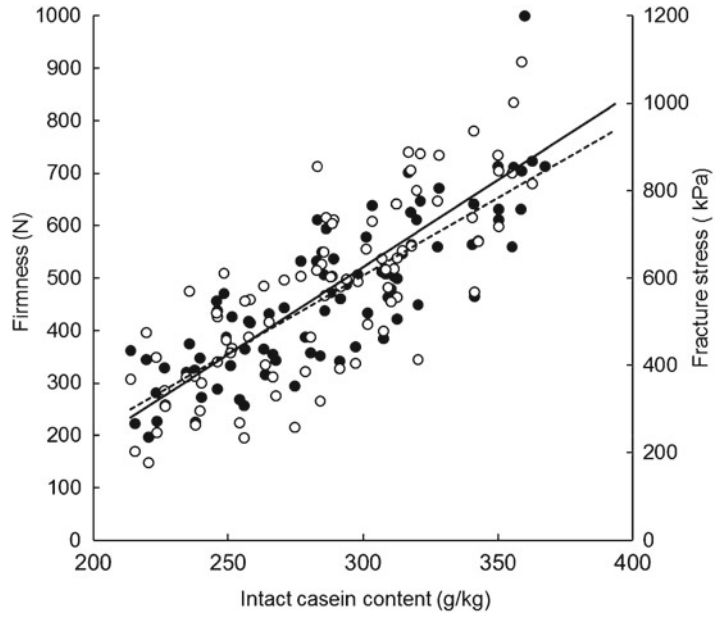

Fig. 14.17 Relationship between the content of intact casein and firmness (*open circle*) and fracture stress (*filled circle*) in Cheddar cheeses of varying fat content in the range 6–33 % (w/w) (Redrawn from Guinee *et al*., 2000a)

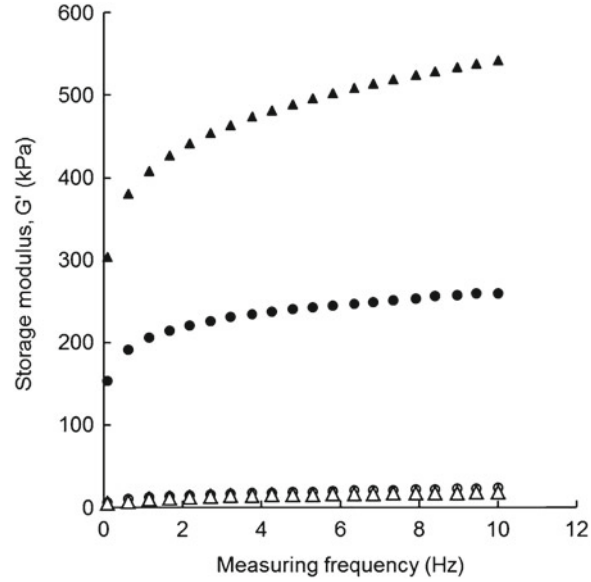

Fig. 14.18 Elastic shear modulus as a function of frequency for 120 day-old full-fat (*filled triangle*, *open triangle*) and half-fat (*filled circle*, *open circle*) Cheddar cheeses at 4 (*filled triangle*, *filled circle*) or 40 (*open triangle*, *open circle*) °C. Samples were tempered at the assay temperature for 20 min and subjected to a low amplitude oscillating strain of 0.06 (Redrawn from Guinee, 2003)

On the application of a stress to a cheese, the volume fraction of the *para*-casein network determines the extent of deformation. As the concentration of casein increases, the intra- and inter-strand linkages become more numerous and the network becomes more elastic and more difficult to deform at room temperature (de Jong, 1976, 1977, 1978a, b; Visser, 1991; Ma *et al.*, 1997; Fenelon and Guinee, 2000; Guinee *et al.*, 2000a).

At low temperatures (<5 °C), milk fat is predominantly solid and adds to the elasticity of the casein network (Fig. 14.18). The solid fat globules/pools limit the deformation of the casein network, as deformation of the latter would also require deformation of the fat globules enmeshed

within its pores. However, the contribution of fat to the elasticity of cheese decreases rapidly as the ratio of solid-to-liquid fat decreases with increasing temperature and is very low at 40 °C, where all the milk fat is liquid. Hence, the G′ for half-fat Cheddar is lower than that of full-fat Cheddar at 4 °C, where the fat is solid but similar where fat is liquid, even though the dry matter content of the latter is higher than that of the former (Fig. 14.18). Temperature has comparatively little effect of the elasticity of 'skim' cheese (fat ~ ≤1.4 %).

14.6.2 Effect of Denatured Whey Proteins

The ratio of casein-to-whey protein in cheese can be altered by *in situ* denaturation of whey proteins in the milk during pasteurization, by milk ultrafiltration (UF), or by the addition of denatured whey proteins.

14.6.2.1 High Heat Treatment of Milk

The effects of high heat treatment (HHT) of milk on the properties of acid-induced milk gels have been studied extensively (Lucey *et al.*, 1998a; Lucey, 2001; Anema *et al.*, 2004; Guyomarc'h *et al.*, 2006). In general, these have found that gelation occurs at a higher pH, and that the resultant gels have a lower porosity and are more rigid (higher G′) compared to gels from unheated milk. Nevertheless, the effects of heat treatment and its severity on the tendency of acid-induced milk gels to undergo syneresis are less clear. It is likely that the effect of heat treatment on syneresis is influenced by numerous factors such as milk composition, extent of heat treatment, incubation temperature, method of acidification (starter culture or GDL), rennet addition, rate of gelation, rheology of the gel, protein concentration and extent of post-gelation network rearrangement and method of measurement (Lucey *et al.*, 1998a, b; Lucey, 2001, 2002; Castillo *et al.*, 2006).

In contrast, much less is known about the effect of milk heat treatment on the properties of acid-curd cheeses, which are produced by concentration of these gels using various techniques such as centrifugal separation or UF. In the manufacture of fresh acid-curd cheeses, such as Quark, HHT of the milk (e.g., 95 °C × 5 min) prior to culturing tends to give a smoother and firmer consistency (Fig. 14.6) (Mahaut and Korolczuk, 1992; Mulvihill and Grufferty, 1995). This effect is due to the increase in the level of gel-forming protein and the finer gel structure. HHT of milk causes extensive denaturation of the whey proteins (e.g., denaturation levels >70 % of total whey protein) and their binding (especially β-lactoglobulin), *via* disulphide interaction, to κ-CN; the denatured whey proteins subsequently become part of the gel, which may be considered as a complex gel. Protein gels formed from two-protein systems have been categorized as filled gels or complex gels (Lanier, 1991). In complex gels, the minor protein interacts and complexes with the main gel-forming protein, whereas in filled gels the minor protein behaves as a non-interactive filler within the gel pores (either in solution or as dispersed particles). Electron microscopic analysis of HHT treated milk shows that these complexes result in the formation of filamentous appendages which protrude from the micelle surface (Harwalkar and Kaláb, 1981, 1988; Heertje *et al.*, 1985) and prevent the close approach, and hence extensive fusion, of micelles on subsequent acidification. Hence, the micelles with attached appendages form into thin strands (chains) giving a highly branched, continuous gel network with smaller interstitial spaces or pores. The more finely structured gel has a higher volume fraction, a lower permeability coefficient (and hence lower porosity) and a reduced propensity to spontaneous wheying-off. The relatively low porosity of the gel is conducive to a relatively high water-holding capacity and a low tendency towards syneresis of the resultant fresh cheese, e.g., Quark (Fig. 14.6b). Conversely, when micelle fusion is more extensive, the resultant gel has thicker strands, is less continuous, more porous and more susceptible to syneresis on storage. The rheological and syneretic properties of the curd (i.e., Quark) formed after breaking/stirring and concentration of the gel reflect those of the gel.

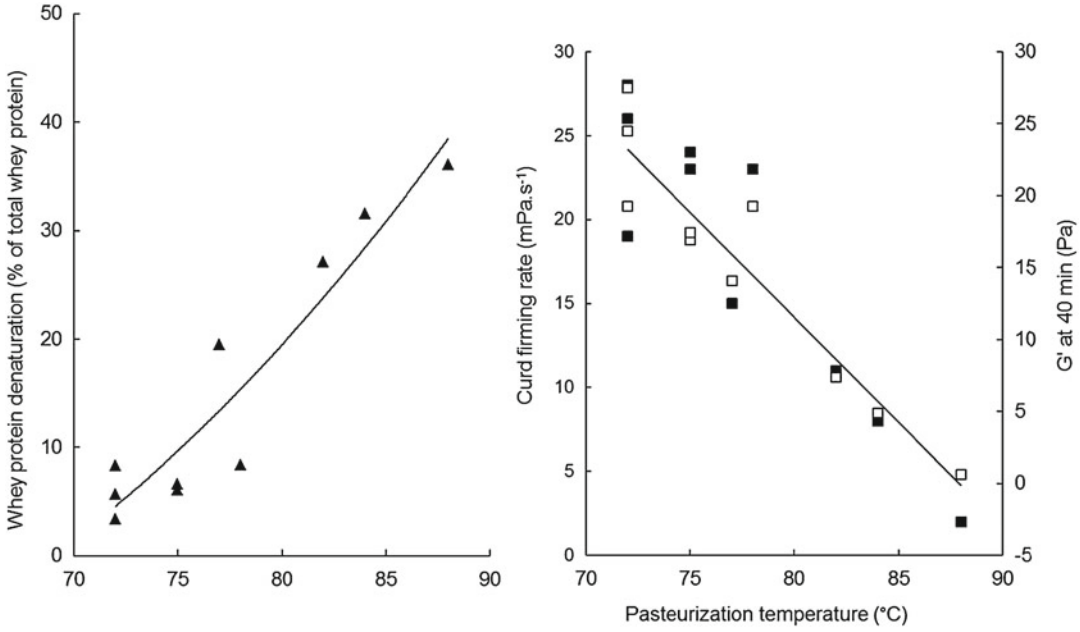

Fig. 14.19 Effect of pasteurization temperature (°C) (for 15 s) of milk on (**a**) the level of whey protein denaturation (closed triangle) and (**b**) rennet coagulation properties of milk, curd firming rate (*filled square*) and elastic shear modulus at 40 min (*open square*) (redrawn from Guinee *et al.*, 1997)

Similar to acid milk gels, *in situ* denaturation of whey proteins by HHT of milk limits the degree of aggregation of the *para*-casein micelles in rennet-induced milk gels. However, in contrast to acid-induced gels, the resultant incorporation of whey proteins into the rennet-induced milk gels affects their rheological properties and those of resultant cheese adversely, as reflected by longer gelation times, lower curd firming rates, lower gel strength, lower curd firmness and fracture stress (Singh *et al.*, 1988; Green, 1990b; McMahon *et al.*, 1993; Guinee *et al.*, 1997, 1998). Indeed, increasing the level of whey protein denaturation to >30 % of total, by HHT of milk (e.g., >80 °C × 15 s), impairs rennet coagulability to such an extent that the milk is unsuitable for commercial cheese manufacture (Fig. 14.19). The negative effect of HHT on the rennet coagulation properties of rennet gels has been attributed to:

- the complexing of denatured whey proteins with κ-CN, leading to the formation of appendages which protrude from the micelle surfaces and render the Phe_{105}-Met_{106} bond of κ-CN less susceptible to hydrolysis by rennet;
- a reduction in the concentration of native micellar calcium phosphate and ionic calcium, and
- the steric hindrance caused by the filamentous appendages (i.e., the denatured whey protein) protruding from the surface of casein micelles (van Hooydonk *et al.*, 1987; Lucey, 1995).

These complexes impede casein aggregation/fusion during gel formation and post-cutting operations, shrinkage of the gel network and, hence, syneresis (Pearse *et al.*, 1985; Green, 1990a, b; Guinee *et al.*, 1995, 1996). The relatively fine structure of the rennet-induced gel from HHT milk increases its water holding capacity through increased capillary action. Consequently, cheese prepared from HHT milk (e.g., 82 °C for 15 s) has a higher moisture content, a lower fracture stress and a lower firmness than cheese made from milk pasteurized at normal temperature (e.g., 72 °C for 15 s) (El-Koussy *et al.*, 1977; Marshall, 1986; Guinee *et al.*, 1998).

Owing to its effect on cheese rheology, high levels of denatured whey proteins in cheese milk may be exploited as a means of improving the texture (reducing the firmness and elasticity) of low-fat cheeses which tend to be excessively firm and rubbery (Guinee *et al.*, 1998).

The rennet coagulability of HHT milk can be improved by increasing coagulation temperature, acidification to a lower than normal pH at setting or addition of $CaCl_2$ (Banks et al., 1987; Singh *et al.*, 1988; van Hooydonk and van den Berg, 1988; Lucey, 1995). The beneficial effects of adding $CaCl_2$ include increases in calcium ion concentration [Ca^{2+}] and [CCP] and a concomitant decrease in pH (the addition of $CaCl_2$ to 0.02 %, i.e., 1.8 mM Ca, reduces the pH by ~0.05–0.1 units, depending on the concentration of protein). The compositional changes induced by $CaCl_2$ addition contribute to an increase in the extent of aggregation of rennet-treated *para-casein* micelles, an effect likely due to the combined effects of protonation of, and additional calcium-induced cross-linking of, negatively charged groups on the casein (e.g., carboxyl groups of glutamate and aspartic acid residues). The rennet coagulation properties of severely heat-treated milk are also improved by acidification (e.g., to pH < 6.4), holding for ~2 h at 20 °C, and re-neutralization to the natural pH of milk; the level of improvement increases with the degree of acidification in the pH range 6.4–5.8 (Singh *et al.*, 1988; Lucey, 1995; Fig. 14.20). The improved rennet coagulation properties appear to result from the solubilization of indigenous CCP and the subsequent increase in the [Ca^{2+}]; HHT milk acidified to pH 5.8 and re-neutralized to pH 6.6 has a higher [Ca^{2+}] than non-acidified milk (Singh *et al.*, 1988). Nevertheless, despite the improvement in rennet coagulation obtained with acidification/re-neutralization, the curds from such milks (e.g., with whey protein denaturation ≈50 % of total whey protein) are susceptible to shattering on cutting and exhibit impaired syneresis, as reflected by a high level of curd fines, low recovery of fat and high moisture content of the cheese (Table 14.3). The impaired syneresis may be associated with an alteration in the ratio of soluble-to-colloidal calcium required for optimum casein aggregation and syneresis. Nevertheless, Banks *et al.* (1994a, b) reported the manufacture of good quality Cheddar cheese with acceptable moisture content from HHT milk by pre-acidification of milk pH to 5.8 prior to renneting and adjustment of cheesemaking parameters.

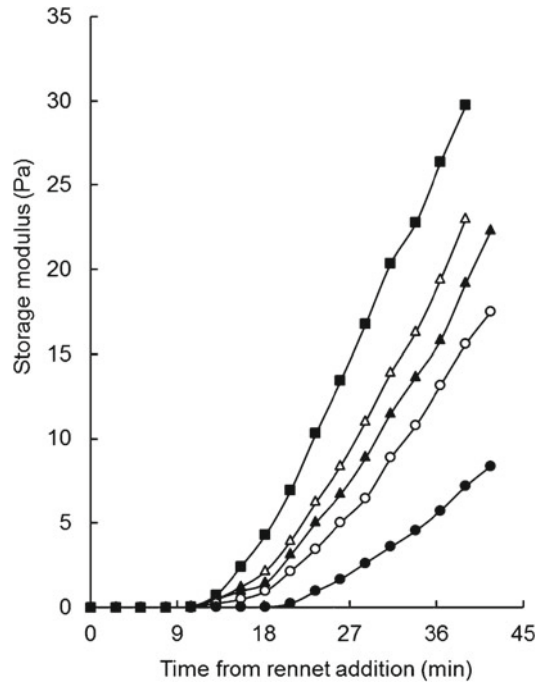

Fig. 14.20 Development of elastic shear modulus in rennet-treated milk blends prepared by mixing equal weights of unheated skim milk (protein, 3 %, w/w) and low heat skim milk powder reconstituted in de-ionized water (protein, 3 %, w/w). The skim milk blends were adjusted to pH 6.6 (*open circle*), 6.3 (*filled triangle*), 6.0 (*open triangle*) or 5.8 (*filled square*) at 21 °C, allowed to stand for 2 h at 21 °C, and re-neutralized to pH 6.6; G′ for reconstituted low-heat skim milk powder in de-ionized water (protein, 3 %, w/w) (*filled circle*) (Redrawn from Guinee, 2003)

14.6.2.2 Addition of Denatured Whey Proteins

Whey protein preparations with varying degrees of denaturation may be in different formats, including micro-particulates such as Simplesse® or Dairy-Lo® containing whey protein particles/aggregates with different size (e.g., typically 1–5 μm), and whey protein concentrates prepared

Table 14.3 Cheddar cheesemaking characteristics of milks prepared by blending fresh skim milk and reconstituted low-heat skim milk powder

	Ratio of fresh skim milk to reconstituted skim milk powder in the cheesemilk			
	100:00	50:50[a]	50:50[b]	SED[c]
Cheese composition				
Moisture (g/kg)	379.4[d]	383.8[d]	396.4[d]	10.6
Fat (g/kg)	327.5[d]	322.5[d]	316.9[d]	7.3
Protein (g/kg)	254.9[d]	253.7[d]	250.3[d]	3.9
Whey composition				
Fat (g/kg)	3.6[e]	4.4[d,e]	4.8[d]	0.53
Protein (g/kg)	9.7[d]	9.5[d]	9.6[d]	0.36
Curd fines (mg/kg)	178.0[d]	288.0[d]	279.0[d]	81.9
Yield				
Actual (kg/100 g)	9.67[d]	9.59[d]	9.58[d]	0.16
MACY (kg/100 g)[f]	9.81[d]	9.70[d]	9.66[d]	0.11

Values presented are the means of four replicate trials

[a]Low-heat skim milk powder was reconstituted to a protein level similar to that in the fresh skim milk and the blend was then standardized to a protein-to-fat ratio as for the control Cheddar cheese

[b]Similar to 1, except that following pasteurization and cooling to 31 °C, the standardized milk was acidified to pH 6.3, using dilute (5 g/100 mL) lactic acid, allowed to stand for 2 h and then neutralized to pH 6.55, using dilute sodium hydroxide (5 g/100 mL) prior to rennet addition

[c]*SED* standard error of difference

[d,e]Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

[f]*MACY* moisture-adjusted (to 370 g/kg) yield per 100 kg milk

by the Centriwhey, Lactal or UF processes. These are generally referred to as partially denatured whey protein concentrates (PDWPCs).

Microparticulation typically involves acidification to the isoelectric pH, HHT, and homogenization, the size of the aggregates being controlled by concentration, pH, calcium level, ionic strength, heat treatment and homogenization pressures (Singer, 1996; Spiegel, 1999). Such particles are reported to behave as inert fillers in cheese matrices and to impart a creamy mouthfeel, as their size (~1–5 μm) is close to that of native milk fat globules (CP Kelco, 2014). In contrast, concentrated whey protein solutions do not contain aggregates >0.05–0.1 μm.

Varying results have been obtained with the addition of PDWPC to milk for the manufacture of hard or semi-hard cheese, such as Cheddar and Gouda. There is general agreement that the addition of PDWPC increases the moisture content, actual yield and moisture-adjusted yield, with the extent of the increase being correlated positively with the degree of denaturation of the added PDWPC (van den Berg, 1979; Brown and Ernstrom, 1982; Banks and Muir, 1985; Baldwin *et al.*, 1986). However, the addition of PDWPC has, generally, been found to cause defective body (greasy, soft) and flavour (unclean, astringent) characteristics in Gouda and Cheddar cheese (van den Berg, 1979), with the intensity of the defects becoming more pronounced with increasing level of the PDWPC added. It has been suggested that these defects may be a consequence of the large size of whey protein particles (aggregates), which do not fit compactly within the pores of, and thereby impede shrinkage of the *para*-casein matrix (van den Berg, 1979) and therefore give relatively large increases in moisture content.

The addition of Simplesse® to cheese milk has been found to improve the texture of reduced-fat hard to semi-hard cheeses such as Cheddar, Gouda, Mozzarella and Kashar (as reflected by the reduced fracture stress, fracture strain or hardness) and has little influence on flavour (Lucey and Gorry, 1994; Fenelon and Guinee, 1997; Koca and Metin, 2004; Sahan *et al.*, 2008). Mackey and Desai (1995), who studied the micro-structure of reduced-fat Cheddar containing Simplesse® 100 using transmission electron microscopy, concluded that the microparticulated whey protein acts as a non-interacting filler in the cheese matrix, in the formation of a filled gel, and mimics the structural properties of fat. A similar observation was made by McMahon *et al.* (1996) in reduced-fat Mozzarella, who concluded that Simplesse® was present as spherical particles of a relatively narrow size distribution (0.5–1.0 μm diameter) in the serum channels between the protein fibres. The improvement in cheese texture in

these studies coincided with an increase in moisture content of Simplesse®-containing cheese, likely to be a result of the combined effects of increased water binding by the whey proteins *per se* and the impedance to shrinkage of the *para-casein* network by the occluded whey protein particles. Nevertheless, a recent study (Schenkel *et al.*, 2013) found that Simplesse® significantly reduced the hardness, gumminess, cohesiveness and springiness of reduced- and low-fat Gouda cheeses where the moisture contents of the corresponding pairs of control- and Simplesse®-containing cheeses were equal.

Dairy-Lo® or Dairy-Lo™ has also been found to increase moisture content and reduce hardness (Fenelon and Guinee, 1997), but the extent of softening is less than that reported for Simplese® (Koca and Metin, 2004).

14.6.3 Effect of Casein Hydrolysis on Rheology

Factors that promote weakening of the casein matrix reduce the stress required to achieve a given deformation. Numerous studies have reported reductions in fracture stress, fracture strain and firmness of different cheeses during ripening (de Jong, 1976, 1977, 1978a, b; Creamer and Olson, 1982; Guinee *et al.*, 2000a; Rynne *et al.*, 2004, Hou *et al.*, 2014). These changes are inversely correlated with the extent of primary proteolysis, as measured by the increase in pH 4.6 soluble nitrogen as % of total nitrogen (pH 4.6 SN/TN) or by reduction in levels of intact casein (Guinee *et al.*, 2000a, 2001; Feeney *et al.*, 2001).

Creamer and Olson (1982) reported that the early hydrolysis of α_{s1}-CN at the Phe_{23}-Phe_{24} peptide bond, to α_{S1}-casein (f1-23) and α_{S1}-CN (f24-199), by residual chymosin is responsible for the marked weakening of the *para*-casein matrix and reductions in fracture stress and firmness/hardness of Cheddar cheese during the first few weeks of ripening. The hardness of the cheese decreases rapidly when only ~20 % of the α_{s1}-casein is hydrolyzed at Phe_{23}-Phe_{24}, and then more slowly as the remaining α_{s1}- and other caseins are hydrolysed further during ripening. The sequence of residues 14–24 of α_{s1}-CN is strongly hydrophobic and confers intact α_{s1}-CN with strong self-association and aggregation tendencies in the cheese environment, and thereby contributes to the integrity and elasticity of the calcium phosphate *para*-casein network (Creamer *et al.*, 1982). Nevertheless, O'Mahony *et al.* (2005) concluded that the softening of Cheddar cheese during the early stages of ripening was essentially independent of the hydrolysis of α_{S1}-CN at Phe_{23}-Phe_{24} and was instead correlated more closely to the partial solubilization of CCP associated with the *para*-casein network of the curd. These authors reported that the addition of pepstatin (a potent competitive inhibitor of chymosin) at a level of 10 μmol/L to the curds/whey mixture at the start of cooking inhibited the hydrolysis of α_{s1}-casein significantly, completely inhibited of formation of α_{S1}-CN (f24-199), but had little effect on the hydrolysis of β-casein during the first 21 days of ripening. Yet, the hardness of all cheeses decreased significantly during this period, albeit at a much lower rate than in the pepstatin-containing cheeses.

In contrast to the above, the firmness of some cheeses (e.g., brine-salted and/or surface dry-salted varieties that are not packaged for part of their ripening period) may increase initially even though proteolysis occurs during this period. The increase in firmness is a consequence of the loss of moisture and the concomitant increase in the protein concentration (de Jong, 1978b; Visser, 1991). Other factors such as changes in pH and the increase in the salt content in the inner regions (Visser, 1991) as a result of inward diffusion from the surface rind zone, may also contribute to the initial increase in firmness. However, when the composition has stabilised, the softening associated with proteolysis becomes dominant, and the firmness and fracture stress decrease (de Jong, 1978b; Visser, 1991).

Few studies have measured the rheological characteristics of cheeses with similar levels of α_{s1}-CN degradation but with markedly different degrees of β-CN hydrolysis. This is probably because of the fact that β-CN generally undergoes markedly less breakdown than α_{s1}-CN

during the ripening of most cheeses such as Cheddar, Gouda and Mozzarella (Visser and de Groot-Mostert, 1977; de Jong, 1977; Fox, 1989; Yun *et al.*, 1993a; Fox and McSweeney, 1996). The slow breakdown appears to be related to:

- intermolecular hydrophobic interactions between the hydrophobic C-terminal region of β-CN which contains the chymosin-sensitive bonds, and/or
- the selective inhibition of chymosin/pepsin hydrolysis of β-CN at the relatively low water activity in cheese, e.g., ~0.91–0.96 for hard/semi-hard varieties (Fox, 1989).

Hence, it is difficult to alter the degree of degradation of β-casein, especially while maintaining the degree of α_{s1}-CN degradation constant. However, differences in the level of β-CN degradation between cheeses have been achieved by the use of either calf rennet or *Cryphonectria parasitica* proteinase as coagulant, with the higher level of degradation being obtained with the latter. A higher degree of β-CN breakdown in Cheddar cheese with a similar level of α_{s1}-CN degradation resulted in a significantly lower fracture strain at all times (up to 270 days of ripening), a numerically lower fracture stress at <100 days, a numerically lower stress at 80 % compression and a higher stress at 20 % compression at <200 days (Bogenrief and Olson, 1995). Yun *et al.* (1993a, b) reported that higher levels of β-CN degradation (e.g., 30 *vs.* 18 % of total) in Mozzarella cheese, with similar levels of α_{s1}–CN degradation, coincided with numerically lower firmness values at ≤30 days.

14.7 Effect of Protein on the Functional Properties of Unheated Cheese

The functional properties of unheated cheese are determined largely by its rheological and flavour/aroma characteristics. While the rheological properties do not affect the flavour and aroma directly, their influence on the rate and extent of breakdown of the cheese mass during mastication may alter the threshold concentrations at which flavour compounds are perceived, and hence, the cheese flavour (Delahunty and Drake, 2004).

The primary stage of preparation of any food containing cheese requires that the cheese mass be reduced in size to facilitate dispersion, mixing and/or layering onto the food. Size reduction is usually achieved by cutting into relatively large pieces, crumbling, slicing, shredding, dicing, grating and/or shearing; these actions usually involve a combination of compressive and shear stresses. The behaviour of the cheese when subjected to the different size-reduction methods constitutes a group of important functional properties, including sliceability, shreddability, and gratability (Table 14.2). These properties are determined by the rheological characteristics, which determine the displacement and force at which cheese fractures (fracture strain and stress) and the type of fracture (elastic or plastic) (O'Callaghan and Guinee, 2004). Hence, the rheology-related functional properties of the unheated cheese have a major impact on its suitability for a particular application (Table 14.2).

Little or no published information is available on the direct effect of protein on the rheology-related functional properties (e.g., sliceability, crumbliness) of unheated cheese. However, the effect of protein on these properties may be deduced from its effect on rheological parameters such as fracture stress, fracture strain and firmness (see Sect. 14.6). The dependency of functionality on rheology is supported by observations on the uses of cheese in practice. Mature Camembert or Chaumes, which are soft, "short" and adhesive, are not used in shredded/diced cheese applications, such as pizza, because of their tendency to ball and clump. However, the ability of these cheeses to undergo plastic fracture and flow under shear (i.e., spread) makes them ideal for blending with other materials such as butter, milk or flour in the preparation of fondues and sauces. The brittleness and tendency of hard cheeses such as Parmesan and Romano, with low levels of moisture and fat-in-dry matter, to undergo elastic fracturability endows them with excellent gratability (when crushed between rollers) and makes them suitable for sprinkling onto pasta dishes. However, these properties render the latter cheeses unsuitable for applications that require slices (e.g., sandwiches and cheeseburgers) or shredded cheese. Conversely, other

hard cheeses, such as Cheddar and Gouda-type, are unsuitable for grating owing to their lack of brittleness and to their elasticity and relatively high fracture stress and strain, which enables a relatively high degree of recovery to their original shape and dimensions following crushing. Moreover, the relatively high levels of moisture and fat-in-dry matter in the latter cheeses are conducive to a higher degree of flow following fracture, compared to Romano or Parmesan, and hence to the development of tackiness following crushing. Owing to its springiness, elasticity and long body, Swiss-type cheese is ideal for slicing very thinly and therefore is particularly well suited for applications such as sandwiches and stuffed cheese slices. Similarly, the springiness of low-moisture Mozzarella cheese (LMMC) endows it with good shreddability (a low tendency to fracture and form fines or curd dust) and non-stick properties and facilitates uniform distribution on the surface of pizzas (Table 14.2). Owing to their crumbliness, cheeses such as Feta, Cheshire or Caerphilly are particularly well suited for easy inclusion into mixed salads.

14.8 Effect of Protein on the Functional Properties of Heated Cheese

Cheese is used extensively in cooking applications owing to its heat-induced functionality, which is a composite of different attributes or functionalities, including softening (melting), stretchability, flowability, apparent viscosity and tendency to brown. These functionalities have a major impact on the quality of products in which cheese is used, e.g., grilled cheese sandwiches, pizza, cheeseburgers, pasta dishes and sauces. Depending on the application, one or more functional properties may be required (Table 14.4). The heated cheese, following grilling or baking, may be required to melt, flow, brown, blister, oil-off and/or stretch to varying degrees. It may also be expected to be chewy (as in pizza) and contribute to certain mouth-coating characteristics (as in sauces and pasta dishes). In many dishes, e.g., sauces, the cheese is required to have the ability to interact with other food components such as water, carbohydrates, proteins and fats during food preparation. In food service, the cooked cheese may be expected to remain smooth and moist, without congealing, developing a 'skin' or becoming 'stodgy' over time or during cooling (during service and consum ption).

The functionality of heated cheese is influenced by many factors.

14.8.1 Impact of Structure: Structure-Function Relationships

Heating cheese to temperatures encountered during baking and grilling (90–98 °C), results in two major microstructural changes:

- Contraction and shrinkage of the *para*-casein matrix (owing to a temperature-induced increase in the extent of hydrophobic interactions between the casein molecules) and a simultaneous expulsion of moisture from the casein network, and
- Liquefaction and coalescence of fat globules, resulting in formation of free fat, which is readily observed as an oily layer at the surface of the melting cheese mass.

Clear evidence for heat-induced coalescence of fat globules in natural cheese is provided by microscopy of cheese during heating (Paquet and Kaláb, 1988; Auty *et al.*, 1999; Guinee *et al.*, 2000c; Lefevre *et al.*, 2000; Fig. 14.21) and by the release of oil on baking (Rudan and Barbano, 1998). In the uncooked state, most of the cheese fat is crystallised (>50 % of total fat temperatures <10 °C) and augments the rigidity of the cheese matrix. In effect the fat globules can be envisaged as solid inclusions encased within the *para-casein* network, which contribute to the resistance of the network during deformation. However, on heating the cheese to temperatures >40 °C, the fat melts completely and the fat globules collapse, creating 'soft spots' within the

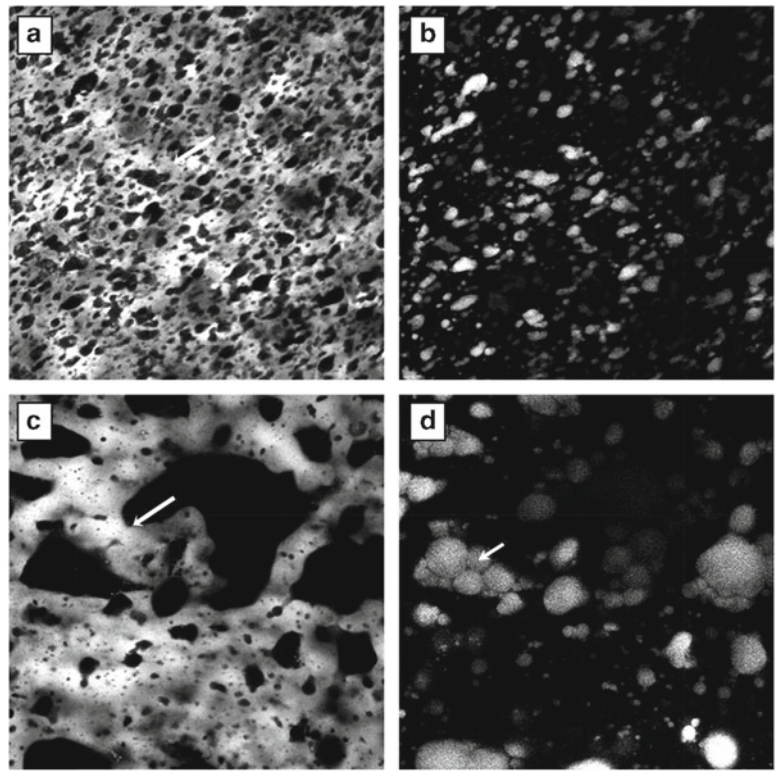

Fig. 14.21 Confocal laser scanning micrographs of unheated full-fat Cheddar cheese (**a**, **b**) and the same cheese after heating to 90 °C at a rate of 5 °C/min and cooling to room temperature (**c**, **d**). The micrographs show protein (**a**, **c**; *long arrows*) and fat (**b**, **d**; *short arrows*) as light areas against a dark background. The effect of heating was studied using a warm-stage attachment to the Zeiss LSM 310 confocal scanning laser microscope. Samples were labelled with Nile Blue and images acquired at 488 nm excitation for fat and 633 nm for protein. Bar corresponds to 25 µm (modified from Guinee *et al.*, 2000c)

matrix. Thus, heating leads to localised internal stresses within the melting cheese matrix that are conducive to flow.

This decrease in the voluminosity (shrinkage) of the particulate *para*-casein network, on heating cheese (e.g., to 60–90 °C), results in expression of free serum, which confers fluidity/liquidity (Dave *et al.*, 2001; Pastorino *et al.*, 2002) and thereby increases the mobility of the melting cheese mass; simultaneously, the cheese becomes more opaque as the casein particles shrink. The extent of microphase separation of serum depends on various factors such as the protein-in-moisture concentration, the protein-to-fat ratio, and the strength of the links between the *para*-casein polymers as affected by pH and calcium level. Conversely, on cooling the heated cheese, the network re-adsorbs some of the expressed serum, the voluminosity increases, and the cheese becomes more translucent and less fluid (congeals).

14.8.2 Functionality of Different Cheese Varieties

Studies on the functional properties of different cheese types (natural cheese, processed cheese and analogue cheese) on heating indicate that there are considerable intra- and inter-variety differences in melt time, flowability, stretchability and apparent viscosity (Park *et al.*, 1984; Guinee *et al.*, 2000b; Table 14.5). The inter-varietal differences in functional properties probably reflect differences in conditions of manufacture (e.g., pH at whey drainage, texturization), composition (e.g., pH, calcium-to-casein ratio, protein content, PIM ratio, protein-to-fat ratio), degree of maturity (e.g., pH 4.6 soluble N) and/or formulation (e.g., level and types of added ingredients and processing conditions) in the case of the PCPs and analogue pizza cheese. These in turn influence the volume fraction of the *para*-casein network, its water-binding capacity and rheologi-

Table 14.5 Effect of variety on the functional attributes of heated cheese

Cheese type	Sample size	Melt time (s)	Flowability (%)	Stretchability (cm)	Apparent viscosity (Pa s)
Pasta filata-type					
– Low-moisture Mozzarella	8	108 (6)	53 (8)	83 (21)	623 (303)
– Kashkaval	2	96 (11)	67 (4)	87 (13)	522 (330)
– Provolone dolce	3	86 (6)	64 (21)	80 (13)	950 (–)
– Provala fumica	1	99	67	77	–
– Provala	1	92	71	76	–
Cheese with eyes					
– Gruyère	1	105	78	67	391
– Jamsberg	1	82	52	35	371
– Emmental	1	81	74	35	269
Cheddar	8	100 (7)	69 (9)	23 (10)	349 (129)
Analogue pizza cheese	8	105 (13)	42 (19)	27 (8)	668 (307)

Compiled from Guinee *et al.* (2000a, b, c), Guinee (unpublished results)
Note: Where the sample size ≥2, mean values are presented; values in parentheses are standard deviations
Melt time measured at 280 °C; flowability using the Schreiber test at 240 °C for 4 min; stretchability using uniaxial extension following heating at 280 °C or 4 min and viscosity using heliopath viscometry (Guinee and O'Callaghan, 1997)

cal response of the natural cheese when subjected to heating.

Compared to other varieties, *pasta filata* cheeses (e.g., Mozzarella, Provolone and Kashkaval) are differentiated by their superior stretchability, relatively high apparent viscosity and moderate flowability and melt times (Table 14.5). These functional attributes endow the *pasta filata* cheeses with the characteristics that are typically associated with the melted cheese on pizza, i.e., sufficiently rapid melt and desirable levels of stringiness, chewiness and flow. The superior stringiness of the *pasta filata* cheeses on baking, compared to other natural cheeses, may be attributed primarily to curd plasticization and the formation of the tensile casein fibres (see Sect. 14.5). In contrast to the *pasta filata* cheeses, other types of cheese, including analogue pizza cheese and retail Cheddar and Emmental, have relatively low stretchability, low apparent viscosity and high or impaired flowability characteristics. Pizza with such cheeses on their own would lack the desired stringiness and chewiness, and flow excessively. Conversely, stringiness is an undesirable attribute for applications such as sauces, gratins, *cordon-bleu* applications or fondues. Cheeses such as mature Cheddar, Emmental, Raclette and Gouda are more satisfactory for the latter applications because of their higher degree of proteolysis, excellent flowability and flavour and absence of stringiness on grilling.

Many parameters contribute to the functional characteristics of heated cheese products (Rayan *et al.*, 1980; Harvey *et al.*, 1982; Rudan and Barbano, 1998; Paulson *et al.*, 1998; Rowney *et al.*, 1999). The concentration and type of protein (i.e., casein or whey proteins), degree of calcium-induced crosslinking (Guinee *et al.*, 2002; Metzger *et al.*, 2001; Pastorino *et al.*, 2003b), the pH, which affects the ratio of casein-bound calcium-to-soluble calcium (Pastorino *et al.*, 2003a), and the extent of protein hydrolysis are major determinants of functionality (Arnott *et al.*, 1957; Schulz, 1976; Lazaridis *et al.*, 1981; Mahoney *et al.*, 1982; Lawrence, 1989; Rüegg *et al.*, 1991; Yun *et al.*, 1993a, b; Kindstedt, 1993, 1995; Bogenrief and Olson, 1995; Guinee *et al.*, 2000a, b; Kaláb *et al.*, 1991; Rynne *et al.*, 2004).

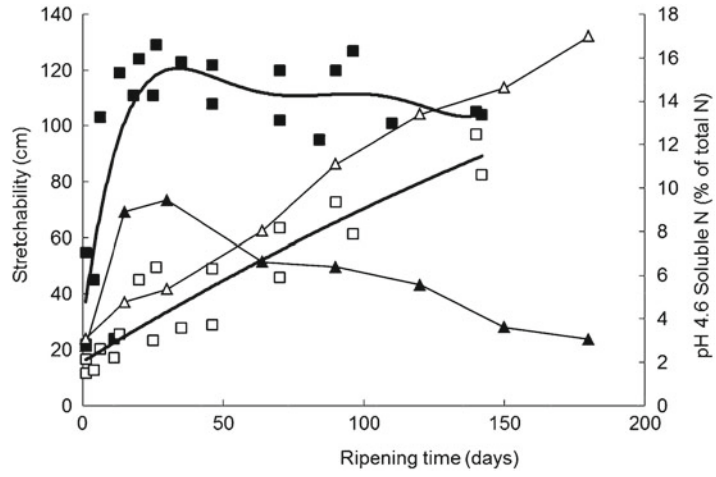

Fig. 14.22 Changes in the stretchability (solid symbols) and content of pH 4.6-soluble nitrogen (open symbols) in low- moisture Mozzarella (*filled square*, *open square*) and full-fat Cheddar (*filled triangle*, *open triangle*) during ripening at 4 °C (Mozzarella) or 8 °C (Cheddar). Data presented are the means of three replicate trials for Cheddar, and from two typical Mozzarella cheeses (From Guinee, 2003)

14.8.3 Relationship Between Cheese Proteolysis and Heat-Induced Stringiness (Stretchability)

Compared to other varieties, e.g., Cheddar, the level of proteolysis in commercial LMMC, as measured by pH 4.6 soluble N as a percentage of total N (pH 4.6 SN/TN) is low. A survey of commercial cheeses indicated that the mean concentration of pH 4.6 SN/TN in Cheddar and LMMC was 20.3 and 4.7, respectively (Guinee *et al.*, 2000b). The low level of proteolysis in LMMC is a consequence of heat inactivation of residual rennet in the curd during the plasticization process (Matheson, 1981; Singh and Creamer, 1990) and storage conditions (low temperature, ≤4 °C, short time).

Nevertheless, the rate of formation of pH 4.6 SN/TN in experimental LMMC (~50 % moisture) stored at 4 °C was found to be only slightly lower than that of Cheddar (Fig. 14.22). Proteolysis in LMMC is probably mainly a consequence of plasmin activity (Richardson and Pearce, 1981; Grufferty and Fox, 1988; Fox, 1989; Farkye and Fox, 1990, 1992). Undoubtedly, the higher moisture and lower salt content, compared to Cheddar, are more favourable for proteolysis by residual rennet activity in LMMC than in Cheddar (Creamer, 1976). The stretchability of experimental LMMC stored at 4 °C remained essentially constant from day 15 to day 140, though proteolysis increased significantly over the same period, as manifested by the increase in pH 4.6 SN/TN from ~5 to 12 % (Fig. 14.22). This trend was confirmed by subsequent investigations (Feeney *et al.*, 2001; Guinee *et al.*, 2001) that showed that the stretchability of LMMC was not significantly impaired until the concentration of pH 4.6 SN/TN exceeded ~15 %. In contrast, the stretchability of young Cheddar cheese is acceptable up to ~30 days but then deteriorates rapidly on further ageing as the level of pH 4.6 SN/TN increases to values ~8 % (Guinee *et al.*, 2000a; Rynne *et al.*, 2004). The different stretchability/pH 4.6 SN profiles of Cheddar and LMMC probably reflects differences in the state of aggregation of *para*-casein (as affected by the occurrence/absence of a plasticization process), the ratio of soluble-to-colloidal Ca, pH and type of proteolysis (i.e., hydrolysis of α_{s1}- *vs*. β-CN). Compared to Cheddar, LMMC is plasticized and has a higher pH (~5.53 *vs*. 5.15), a similar Ca content (~27.5 mg/g casein), a lower ratio of soluble-to-total Ca (~22 *vs*. 37 % total Ca; Fig. 14.9), and more extensive degradation of β-CN (Yun *et al.*, 1993b; Fox *et al.*, 1996; Upadhyay *et al.*, 2004). A comparative study of 12-week old Cheddar, Gouda and Mozzarella cheeses showed that the latter had the highest levels of intact α_{s1}-CN, α_{S1}-CN (f24-199) and intermediate levels of β- and γ-CNs (Creamer, 1976).

14.8.4 Effect of Casein Concentration, Hydrolysis and Hydration on Flowability and Apparent Viscosity

Flowability may be defined as displacement of connecting planes of the *para*-casein matrix as a result of heat-induced stress and concomitant events: shrinkage of the *para*-casein network and fat liquefaction and coalescence (Fig. 14.21). The impact of the latter is very significant, as is evident from the significant reduction in fluidity (loss tangent, LT) of heated Cheddar cheese (at ~80–90 °C) on reducing the fat content from ~30 to 1 %, and on high pressure homogenization of the cheese milk (Fig. 14.23). Once fat coalescence is initiated, the *para*-casein matrix flows to a degree determined by the concentration of casein and the level of *para*-casein hydration, which is controlled by the levels of moisture in non-fat substances (MNFS) and total calcium, pH and the ratio of soluble-to-micellar Ca.

Marked inter- and intra-varietal differences occur in the flowability of different cheese types (Park *et al.*, 1984; Rüegg *et al.*, 1991; Kindstedt, 1993; Guinee *et al.*, 2000b; Guinee and Kilcawley, 2004), reflecting differences in make procedure, composition and maturity. Guinee *et al.* (2000a) showed that the level of intact casein in Cheddar cheese, with a fat content in the range 7–30 %, was correlated positively ($P<0.05$) with apparent viscosity and negatively with flowability (Fig. 14.24). The latter changes in functionality are attributable to the increase in volume fraction of the calcium phosphate *para*-casein network (Fig. 14.12), and reductions in moisture-in-non-fat substances and the level of free oil released on cooking. Hence, less moisture and free fat are available to confer lubricity and liquidity to the melting cheese mass.

For a given level of protein, flowability increases significantly with the degree of proteolysis. Hence, a strong positive correlation between the flowability and concentration of non-protein nitrogen (in the range 0–38 % total

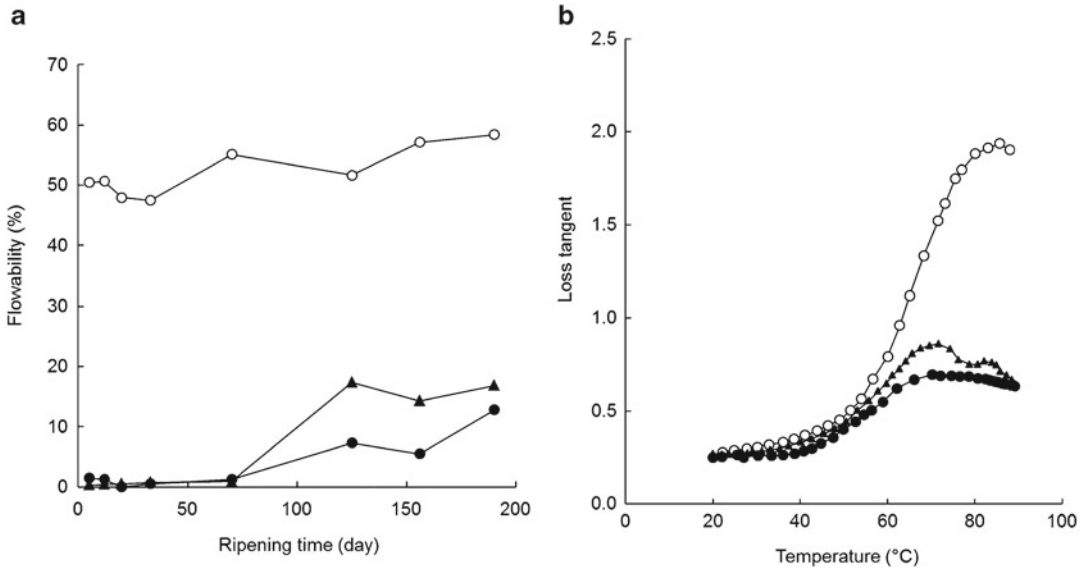

Fig. 14.23 Flowability (**a**) and loss tangent (**b**) of melted full-fat Cheddar (30 %, w/w) cheese prepared from control (non-homogenized) (*open circle*) or homogenized (20 kPa-stage 1, 5 kPa-stage 2) (*filled circle*) milk, and low-fat Cheddar-type cheese (1.3 % fat) prepared from non-homogenised skimmilk (filled triangle). The loss tangent was measured using rheometry; the samples were subjected to a low amplitude strain of 0.06 and an oscillation frequency of 1 Hz. Flowability represents the percentage increase in diameter of a cheese disc after heating at 280 °C for 4 min (Modified from Guinee *et al.*, 2000c).

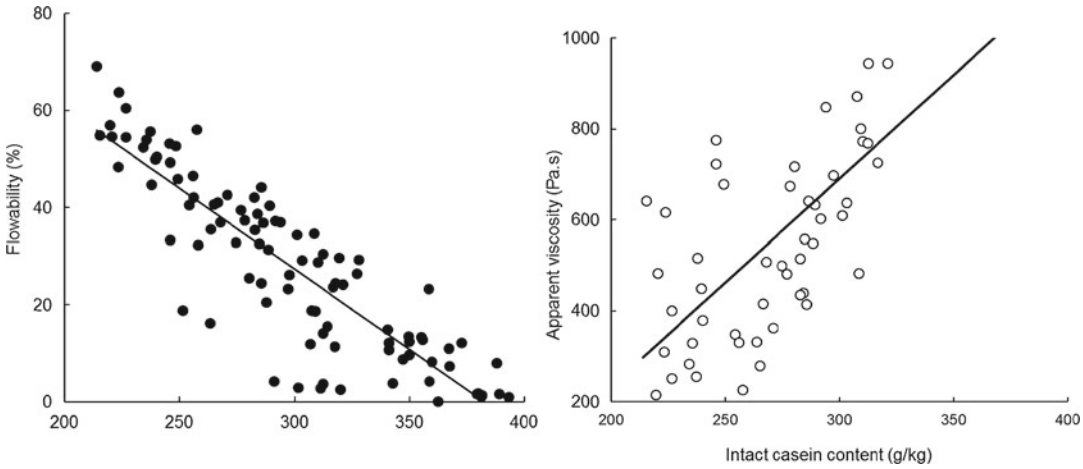

Fig. 14.24 Relationship between the level of intact casein and flowability (*filled circle*) and apparent viscosity (at 70 °C) (*open circle*) of melted Cheddar cheese with fat content in the range 6–33 % (w/w) (Redrawn from Guinee *et al*., 2000a)

N) was reported in model acid-type cheese, treated with varying levels of a proteinase derived from *Aspergillus oryzae* (Lazaridis *et al.*, 1981). The model cheeses had optimum flowability when the molecular mass of the casein peptides (measured using SDS polyacrylamide gel electrophoresis) was in the range 10–24 kDa (Mahoney *et al.*, 1982); at higher concentrations of peptides of molecular mass <10 kDa, the cheese became too flowable.

The flowability of Cheddar cheeses with similar levels of α_{s1}-CN degradation was correlated closely with the extent of β-CN hydrolysis (Bogenrief and Olson, 1995). Yun *et al.* (1993a) reported that Mozzarella cheese made with *C. parasitica* proteinase as coagulant had a higher level of pH 4.6 SN throughout ripening than the corresponding cheeses made using either chymosin or *Rhizomucor miehei* proteinase (e.g., 14 % vs. 8 % pH 4.6 SN at 50 days) and had a higher level of β-CN degradation at times ≤30 days. The higher level of primary proteolysis in cheese made using *C. parasitica* proteinase coincided with higher flowability and free oil release (at ≥25 days) and lower apparent viscosity on melting (Yun *et al.*, 1993b).

14.8.5 Effect of Whey Protein and Casein-Whey Protein Interaction on Flowability

In some cheese applications, softening or melt is essential but very limited flow, or a high degree of flow resistance, is required so as to preserve the shape and identity of the cheese. Examples of the latter include fried Paneer, fried Haloumi (Fig. 14.25), grilled, deep-fried breaded cheese sticks, and casseroles in which the identity of cheese pieces following cooking is desirable (Chandan *et al.*, 1979). Most cheeses, especially natural rennet-curd cheeses when mature, are unsuitable for these applications owing to their excessive flow and oiling-off on cooking. Instead, specially formulated, flow-resistant PCPs or ACPs are frequently used in such applications. However, the inclusion of whey proteins in PCPs (Schulz, 1976) and natural rennet-curd cheese (as discussed below) has been generally found to retard the flowability and alter the cooking properties. Nevertheless, varying results have been reported for the effect of whey proteins on the flowability of natural rennet-curd cheeses, deepening on the level, type and method of inclu-

Fig. 14.25 Grill-frying Halloumi cheese pieces. Unlike most other cheeses, the Halloumi cheese pieces retain their shape, with little or no flow principally because of (i) the relatively high pH (~5.86), which results in a high ratio of casein-bound calcium to soluble calcium, thereby impeding the ability of the casein network to undergo-heat-induced displacement, (ii) the manufacturing procedure which involves cooking curd blocks in the heated cheese whey (~90 °C) for ~40–50 min resulting in denaturation of whey proteins and their precipitation on to the surface of the cheese blocks where they form a surface film, and (iii) the low level of casein hydrolysis due to the inactivation of residual rennet activity during heating of the curd

sion of the whey protein, and other factors such as the addition of exogenous proteinases and the make procedure of the cheese.

The whey protein content of natural cheese can be increased by *in situ* denaturation of the native whey proteins in the cheese-milk by HHT, by the manufacture of cheese from high concentration factor ultrafiltered milk retentates, or by the addition of whey protein powders or concentrates to the cheese milk (see Sect. 14.6.2).

14.8.5.1 High Heat Treatment of Milk and In Situ Denaturation of Whey Proteins

HHT of milk (e.g., 95 °C × 5 min) and the resultant incorporation of a high level of denatured whey protein is a feature of the manufacturing process of acid-heat coagulated cheese types, e.g., Queso-blanco types and Paneer (Chandan *et al.*, 1979; Torres and Chandan, 1981; Kaláb *et al.*, 1988; Farkye, 2004a). The former cheeses exhibit excellent flow resistance and for this reason are used in applications where maintenance of visual identity and shape of the cheese on cooking is desirable, e.g., fried cheese pieces, curries, casseroles and deep fried-cheese sticks. In contrast, HHT of milk is normally not practiced in the manufacture of rennet-curd cheese varieties, because of the impaired curd forming properties, higher moisture content, and, generally, higher fat losses, and texture defects (Guinee *et al.*, 1998; Punidadas *et al.*, 1999; Mead and Roupas, 2001; Menard *et al.*, 2005; Guyomarc'h, 2006). Nevertheless, interest in HHT for the manufacture of rennet-curd cheeses continues owing to its potential to increase the recovery of

protein from milk to cheese, or as means of increasing the moisture content and improving the quality of reduced-fat cheese.

Rynne *et al.* (2004) found that the heat-induced flowability and stretchability of half-fat Cheddar cheese decreased significantly as the degree of whey protein denaturation in the cheese-milk was increased from ~3 % (in control milk pasteurized at 72 °C × 26 s) to values ≥20 % of total whey protein (at pasteurization temperatures ≥82 °C for 26 s), despite the fact that the moisture content of the cheese increased simultaneously; the corresponding levels of denatured whey protein in the control and experimental cheeses were 0.21 % (w/w) and ≥1.35 % (w/w), respectively. A lower level of whey protein denaturation in the cheese-milk (8.4 % of total on pasteurization at 77 °C × 26 s), corresponding to ~0.6 % (w/w) whey protein in the cheese, had little effect on meltability or stretchability. Guinee (2002) found that the degree of flowability and maximum LT (LT_{max}) on heating half-fat Cheddar cheese from 25 to 88 °C decreased significantly (from ~1.75 to 0.8) as the level of denatured whey protein in the cheese was increased from 0.33 % (w/w) to values ≥1.05 % (w/w) by HHT of the cheese-milk. A similar trend was reported by Horne *et al.* (1994) for full-fat Cheddar cheese made from milk heated at 110 °C for 60s. The latter results suggest some form of thermal aggregation, or gelation, of denatured whey proteins and/or complexes of denatured whey proteins and *para*-κ-CN that reduce the flow when cheese is heated to temperatures >65 °C. The formation of such aggregates, or pseudo-gels, is likely to be enhanced by the high contents of protein and soluble calcium in the concentrated cheese environment (Doi *et al.*, 1983a; Jelen and Rattray, 1995).

In contrast to the above, Schafer and Olson (1975) reported that increasing the level of whey protein denaturation in milk from ~3 to 35 % of total whey protein, by increasing the pasteurization temperature from 80 to 130 °C for a 2 s holding time, did not significantly affect the heat-induced meltability (flowability) of Mozzarella cheese prepared by chemical acidification, even though the moisture content of the cheese decreased with increasing heat treatment. The discrepancy between the previous studies may relate to the additional steps of re-pasteurization (63 °C × 30 min) and high pressure homogenization (13.3 MPa) of the HHT milk prior to cheese manufacture in the study of Schafer and Olson (1975). Such conditions may favour enhanced interaction of denatured proteins into aggregates, which would be size-reduced by homogenization into micro-whey protein particles; such particles, similar to commercial microparticulated whey proteins, could behave as inert fillers in the rennet-induced milk gel, and thereby reduce the degree of *para*-casein aggregation during cheese manufacture, and enhance the flowability of the lower-moisture cheese from the HHT homogenized milks. In contrast, homogenization of the control milk (80 °C × 2 s) would impede the flowability of the control cheeses significantly. High pressure homogenization of cheese milk transforms the native milk fat globule into fat-filled casein particles and significantly reduces the flowability and LT_{max} of cheese on heating (Guinee *et al.*, 2000c; Fig. 14.23). The latter particles are incorporated into the *para*-casein network during cheese manufacture and are stable to thermal-induced coalescence when the cheese is heated.

14.8.5.2 High Concentration Factor Ultrafiltration of Cheese Milk

The use of medium-to-high concentration factor ultrafiltration (M-HCFUF, e.g., 6–9×) has been investigated as a means of manufacture of semi-hard/hard rennet-curd cheeses (Covacevich, 1981; Green, 1990a, b; Mistry and Maubois, 2004). The resultant M-HCFUF retentates (also referred to as liquid pre-cheese) can be converted directly into cheese by gelation and acidification (Madsen and Qvist, 1998) or indirectly by gelation and acidification to form a curd that is exposed to further syneresis by additional operations such as cutting and cooking (Garrett, 1987; Guinee *et al.*, 1995). In either situation, the whey protein content of the cheese made by M-HCFUF is high compared to that of cheeses made from standard milk or low concentration factor ultrafil-

tration (LCFUF) milk retentates (~ 4.0 – 4.5% protein) using conventional technology, where most of the whey proteins are lost in the cheese whey, apart from procedures applying HHT tenement of the milk or LCFUF retentate, as discussed above.

Madsen and Qvist (1998) reported that the meltability (flow) of Mozzarella cheese made from M-HCFUF retentates and containing ~29–38 % of the total native whey proteins in the milk, was markedly lower than that of conventionally made commercial Mozzarella of comparable composition. However, the meltability of the M-HCFUF cheese was improved significantly on increasing the extent of casein hydrolysis by treatment of the retentate with *Bacillus licheniformis* proteinase. In corollary, the flow of processed cheese made with a high level of added UF retentate (Sood and Kosikowski, 1979) improved significantly on increasing the extent of casein hydrolysis by treatment of the retentate with *Bacillus subtilis* proteinase. The latter results concur with observations indicating a direct correlation between flowability and the concentration of non-protein nitrogen (as % total N) in model chemically acidified curds treated with different levels of *A. oryzae* proteinase (Lazaridis *et al.*, 1981; Mahoney *et al.*, 1982). These findings indicate that the impairment of the heat-induced melt properties of cheese and processed cheeses due to the thermal denaturation and aggregation of included whey proteins may be compensated for by increasing the degree of hydrolysis and flow of the calcium phosphate *para*-casein network that forms the skeletal structure. An alternative approach to improving the flow of cheese products containing a high level of whey protein is structural attenuation of the *para-casein* network through significant reduction in degree of calcium-induced crosslinking between the *para*-casein polymers. This is confirmed by the findings of Anis and Ernstrom (1984) who found that the flowability of PCPs made from milk M-HCFUF ultrafiltrates was significantly improved on reducing the calcium content of the retentate *via* acidification of the milk prior to UF and diafiltration.

14.8.5.3 Addition of Whey Protein Concentrates or Powders

The whey protein content of cheese may also be increased by the addition of addition of whey protein concentrates or powders, in which proteins are subject to varying treatments during preparation, including UF, diafiltration, heat treatment, pH adjustment, aggregation and/or microparticulation (see Sect. 14.6.2).

McMahon *et al.* (1996) evaluated the effect of different fat replacers, including the whey protein-based fat mimetics, Simplesse® D100 (53 %, w/w, protein) and Dairy-Lo® (35 % protein), added at levels of 0.06 and 0.23 % (w/w) based on manufacturer's recommendations, on the micro-structure and heat-induced functionality of low-fat (4–5 %, w/w) Mozzarella cheese. The estimated level of whey protein added to the cheese-milk from these mimetics was 0.03 and 0.09 % (w/w), respectively. The fat mimetics resulted in higher moisture content than the control low fat-cheese (e.g., 55.3 % vs. 53.0 %, w/w) but did not significantly affect the flowability or apparent viscosity of the melted cheese over a 28 day ripening period. Similarly, a subsequent study (Koca and Metin, 2004) found that the addition of 1 % (w/w) Simplesse® D100 or Dairy-Lo™ to the cheese milk did not influence the mean value of heat-induced flowability of reduced-fat (~7 %, w/w) Kashar, a semi-hard Turkish cheese, over a 90 day ripening period, despite the fact that the cheese containing Simplesse® D100 or Dairy-Lo™ had higher moisture (2–4 %) than the control reduced-fat cheese; the flow of all reduced-fat cheeses, as measured using modifications of the Schreiber (230 °C × 5 min) and Price-Olson (104 °C × 60 min) methods, was markedly inferior to that of the full-fat cheese. Nevertheless, a subsequent study on Kashar (Sahan *et al.*, 2008) found that the addition of Simplesse® D100 to the milk (1 %, w/w) significantly increased the heat-induced flowability of reduced-fat Kashar cheese (13.5 % fat in dry matter, FDM) compared to the control low-fat cheese (17.5 % FDM), where the moisture contents of both the Simplesse®-containing and control cheeses were similar (~55.5 %). However, the flow of both reduced-fat cheeses was significantly

lower than the control full-fat cheese (43 % FDM). Schenkel *et al.* (2013) investigated the effects of adding Simplesse® 100 (53 %, w/w, protein) at a rate of 1 % (w/w) to the milk on the properties of full-fat- (FFC), reduced-fat- (RFC) and low-fat- (LFC) Gouda-type cheeses, where the gross compositions and levels of primary proteolysis of the corresponding pairs of cheeses, with Simplesse® 100 and without (controls), were similar. Simplesse® addition did not significantly affect LT_{max} (fluidity) on heating the cheeses between 20 and 80 °C, but significantly reduced both the gel-to-sol transition temperature (i.e., where $G' = G''$) and the temperature of LT_{max} in all three cheeses. These results suggest that even though the Simplesse® 100-containing cheeses did not become any more fluid than the corresponding control cheeses on heating to 80 °C, they acquired their fluidity at lower temperatures. This trend was further corroborated by the greater reduction in the height of cheese cylinders containing added Simplesse®100 compared to the control cheeses, when subjected to a normal compression force of 0.3 N at 60 °C. The authors concluded that whey protein micro-particles act as inert fillers in the cheese matrix and behave as spherical barriers between the casein strands. Consequently, they reduce the number of interactions between the caseins and, thereby, facilitate easier thermal-induced displacement of the *para-casein* network. Hence, Punidadas *et al.* (1999) found that high pressure homogenization (and microparticulation) of a denatured whey protein dispersion (obtained by pH adjustment of whey to 4.6, high heat treatment, and sedimentation) prior to addition to cheese milk (at a level of ~0.01 %, w/w protein) significantly improved the meltability of reduced-fat Mozzarella-style cheese.

In conclusion, the addition of whey protein concentrates has been investigated mainly as a potential means of improving the texture and cooking properties of reduced-fat cheeses. However, inconsistent results have been found for their effects on various aspects of cooking properties, such as flowability as measured by modifications of the Schreiber and Price-Olson methods, apparent viscosity, or temperature related changes in LT_{max} or sol-to-gel transition temperature. Some studies have shown little of no effect (McMahon *et al.*, 1996; Mead and Roupas, 2001; Zalazar *et al.*, 2002; Koca and Metin, 2004), others an improvement (Punidadas *et al.*, 1999; Sahan *et al.*, 2008; Schenkel *et al.*, 2013) or impairment (Mead and Roupas, 2001). Inter-study discrepancies may relate to the level of whey protein added, the type of whey protein treatment (e.g., microparticulated or not), point at where the whey proteins are added (before or after pasteurization), degree of heat treatment of the milk, differences between control and treatment cheeses (in moisture, fat, protein and pH), method of evaluation of cheese properties, and other factors. Few of the studies provide data on calcium content of the cheeses, despite extending the set-to-cut times and curd-treatment times prior to whey separation; variation in the calcium-to-casein ratio, which has been shown to have major impact on cooking properties (Guinee *et al.*, 2002; Metzger *et al.*, 2001; Pastorino *et al.*, 2003b), is likely to confound further the effects obtained with added whey proteins.

14.8.6 Effect of Protein on Age-Related Changes in Functionality: Extent of Protein Hydration and Hydrolysis

Age-related changes in the functionality of cheese have been studied extensively, especially for LMMC (Kindstedt, 1993, 1995; Yun *et al.*, 1993a, b; Paulson *et al.*, 1998; Guinee *et al.*, 1998; Guinee *et al.*, 2001, 2002; McMahon *et al.*, 1996; Rudan *et al.*, 1998; Metzger *et al.*, 2001; Sheehan *et al.*, 2004; Govindasamy-Lucey *et al.*, 2007; Wadhwani *et al.*, 2011) and Cheddar (Bogenrief and Olson, 1995; Guinee *et al.*, 2000a, b; Rynne *et al.*, 2004), but also for other cheeses including Gouda (Schenkel *et al.*, 2013), Kashar (Koca and Metin, 2004, Sahan *et al.*, 2008), Swiss-type cheeses (Richoux *et al.*, 2009) and Colby (Lee *et al.*, 2010). All the above studies have shown that functionality is dynamic, with the various attributes undergoing age-related changes to a degree

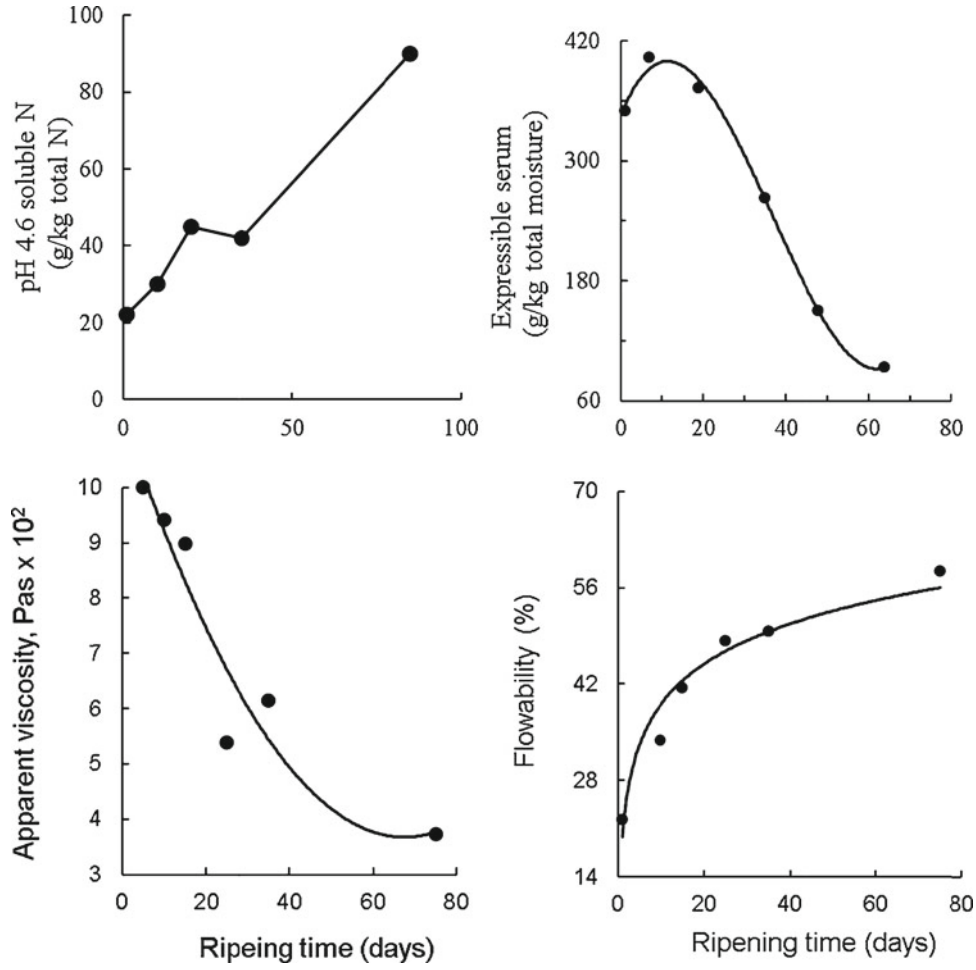

Fig. 14.26 Typical changes in the levels of pH 4.6-soluble nitrogen (N), expressible serum (on hydraulic pressing at 3 MPa for 3 h at 21 °C), flowability after heating to 280 °C for 4 min and apparent viscosity (at 70 °C) of melted low-moisture Mozzarella cheese as a function of storage time at 4 °C (redrawn from Guinee, 2003)

depending on the composition and functional attribute of the cheese, e.g., whether stretch or flow (Fig. 14.26). Changes in the extent of protein hydrolysis and hydration (Fig. 14.26) are major factors contributing to the age-related changes in functionality, as discussed below.

LMMC made by conventional procedures is generally non-functional on cooking during the first 5–10 days of storage at 4 °C after manufacture, with drying-out, crusting and skin formation being common defects in the baked cheese. The lack of functionality is due to the low water-binding capacity of the *para*-casein in the unheated cheese and the low propensity to the heated cheese to express free oil on baking. Both factors are conducive to excessive evaporation of moisture from the cheese during baking because of the high cheese temperature (typically 90–100 °C in the mass of the melting cheese when heated in a convection oven at 280 °C for 4 min; Guinee and O'Callaghan, 1997). The dried-out, crusted cheese lacks succulence and fails to flow or stretch. Moreover, on heating to 70 °C, the cheese is extremely tough and chewy, as reflected by the high apparent viscosity (Fig. 14.26). Freshly made Cheddar behaves in a similar manner (Guinee *et al.*, 2000a).

However, the functionality of LMMC and Cheddar improves markedly during the first 2 weeks of ripening at 4 °C, as reflected by reductions in melt time and apparent viscosity and increases in flowability and stretchability, and maintains this status until ~40–50 days (Fig. 14.26). The improved functionality may be attributed to increases in protein hydration and free fat during ageing of the cheese as reflected by the reduction in the quantity of serum and increase in the quantity of free fat expressed from the cheese when subjected to hydraulic pressing or ultracentrifugation, i.e., for Mozzarella (Guo and Kindstedt, 1995; Kindstedt and Guo, 1997; Guinee *et al.*, 2001; Fig. 14.26), Cheddar (Guinee *et al.*, 2000a; Hassan *et al.*, 2004), Emmental (Thierry *et al.*, 1998) or Camembert (Boutrou *et al.*, 1999). The vapour pressure of water bound by the *para*-casein is lower than that of free water and thus has a lower propensity to evaporate during baking (Geurts *et al.*, 1974; Masters, 1976). The exudation of free oil from the shredded cheese during baking also restricts dehydration (Rudan and Barbano, 1998); the free oil forms an apolar surface coat, which impedes the escape of water vapour. The changes in protein hydration appear to be the result of a number of factors, including small increases in pH (from ~5.15 to ~5.35–5.40 at 5 days) and primary proteolysis and the solubilization of casein-bound calcium (Guo and Kindstedt, 1995; Hassan *et al.*, 2004). The physical swelling of the casein associated with its increased hydration is likely to contribute to the coalescence of non-globular liquid fat.

The contribution of casein hydrolysis to the improvement in functionality of the heated cheese is verified by the increases in flowability and stretchability, and reduction in apparent viscosity as the level of pH 4.6-soluble N increases with ageing (Figs. 14.22 and 14.26) (Arnott *et al.*, 1957; Yun *et al.*, 1993a, b; Bogenrief and Olson, 1995; Guinee *et al.*, 2000a; Rynne *et al.*, 2004). Solubilization of casein-bound Ca appears to occur during the ripening of Mozzarella cheeses, as reflected by the age-related increase in the level of soluble Ca in the cheese serum (Guo and Kindstedt, 1995). Calcium in Mozzarella is solubilized when the calcium attached to the casein matrix is partially replaced by sodium (Guo and Kindstedt, 1995; Guo *et al.*, 1997). The *para-casein* in salted Mozzarella binds more water than that in unsalted Mozzarella, as reflected by the higher level of serum expressed from the unsalted cheese on centrifugation, at the various stages during ripening (Guo *et al.*, 1997). Conditions in the cheese that favour the increased water binding are the simultaneous occurrence of low concentrations of Na and Ca in the serum phase (i.e., 2.0 % NaCl and 0.4 % Ca) (Geurts *et al.*, 1972; Guinee and Fox, 1986; Kindstedt and Guo, 1997). Hence, Paulson *et al.,* (1998) found that the flowability of directly acidified non-fat Mozzarella increased with increasing salt content to ~1 % salt-in-moisture (S/M) and remained relatively constant thereafter to ~3.55 % S/M.

The increased propensity of the cheese to oil-off on cooking may be associated with age-related degradation of the fat globule membrane and/or the continued coalescence of the partially denuded fat globules. Coalescence is likely to be accelerated by the swelling of the casein network following hydration, an occurrence that would promote shearing of any remaining fat globule membrane and forcing of the partially denuded fat globules into close proximity (Fig. 14.12; Kindstedt and Guo, 1997).

On prolonged storage, e.g., to 75 days, unbaked LMMC tends to become too soft and sticky while the baked cheese is excessively flowable, has a 'soupy' consistency and lacks the desired chewiness (as reflected by the relatively low apparent viscosity). These changes in functionality are attributable to excessive proteolysis (Arnott *et al.*, 1957; Bogenrief and Olson, 1995; Guinee *et al.*, 2001). However, the stretchability remains relatively constant even when the product is stored for a prolonged period (up to 4 months at 4 °C; Guinee and O'Callaghan, 1997). The constant stretchability of LMMC indicates that the level of primary proteolysis in the cheese at this time (i.e., pH 4.6-soluble N ~14–16 % of total N) is insufficient to cause a significant impairment of stretchability (Fig. 14.22).

14.9 Contribution of Protein to Formation and Stabilization of Pasteurized Processed Cheese Products (PCPs) and Analogue Cheese Products (ACPs)

Pasteurised processed cheese products are produced by comminuting and melting into a smooth homogeneous blend, one or more natural cheeses and optional ingredients using heat, mechanical shear and (usually) emulsifying salts (ES). The type and level of optional ingredients permitted are determined by the product type, and include dairy ingredients, vegetables, meats, stabilisers, ES, flavours, colours, preservatives and water. There are various types of PCPs, the type/standard of which depends on national legislation. They generally differ with respect to specifications on composition (e.g., contents of fat and dry matter), minimum content of natural cheese, and permitted ingredients (e.g., cheese, dairy ingredients and stabilisers) (Hickey, 2011). The categories and related specifications vary between countries. Hence, in the UK, two categories of PCPs namely Processed Cheese and Cheese Spread (or Cheese Food) were specified in the Cream Regulations 1995 (HMSO, 1995). Cheese was the only dairy product that could be used in Processed Cheese, while other milk products could be used in Cheese Spread. In Germany four categories of PCP are defined in the Deutsche Käseverordnung (Bundesministeriums der Justiz in Zusammenarbeit mit der juris GmbH (2010)): Schmelzkäse (processed cheese), Schmelzkäsezubereitung (processed cheese preparation), Käsezubereitung (cheese preparation) and Kasekomposition (cheese composition). In the USA, the Food and Drug Administration, FDA (2012a) identifies three main categories, namely: pasteurized process cheese, pasteurized process cheese food (PCF), and pasteurized process cheese spread (PCS). In process cheeses, permitted ingredients include natural cheese, water, anhydrous milk fat at a level ≤5 % (w/w) of finished product, ES, flavouring, colouring agent and condiments. Based on the levels of non-cheese ingredients, the level of natural cheese can be as high as 85 % (w/w) of the final product but varies depending on the processed cheese product being manufactured (e.g., processed Cheddar cheese, processed Edam cheese, processed cheese), compositions of the cheeses used, and the moisture content of the PCP. A minimum cheese content of ≥51 % (w/w) of the final product is required in pasteurised process cheese foods and spreads, in which non-cheese ingredients (e.g., dairy ingredients) can be used at levels up to ~15 % depending on composition of the PCP.

The manufacture of PCPs essentially involves the following major steps: shredding of cheese, blending with ES, water and optional ingredients, processing of the blend, and hot packing and cooling (Dixon, 2011). Processing refers to the heat treatment of the blend, by direct or indirect steam, with constant agitation. In batch processing, the temperature-time combination varies (70–95 °C for 4–15 min) depending on the formulation, extent of agitation, and the desired product texture, body and shelf-life characteristics. In continuous cooking, the blend is mixed and heated to 80–90 °C in a vacuum mixer from where it is pumped through a battery of tubular heat exchangers and heated to 130–145 °C for a few seconds and then flash-cooled to 90 °C. In continuous cookers, the blend is continuously pumped through a steam-injection nozzle which affects instant agitation and heating to 85–95 °C. The heated product then continues to a holding tube, with dimensions and operating back pressures that ensure the desired residence time at high temperature. The cooked product is then flash cooled to ~74–75 °C and hot-filled.

In contrast, ACPs generally contain no added cheese, except where a small amount is added to impart a cheese flavour or as required by customer specifications. Similar to PCPs, ACPs contain added stabilisers, ES, flavours, colours, preservatives and water. ACPs may be categorized arbitrarily as dairy, partial dairy or non-dairy, depending on whether the fat and/or protein components are from dairy or vegetable sources (Shaw, 1984). Partial dairy analogues, in which the fat is mainly vegetable oil (e.g., soy oil, palm

oil, rapeseed and their hydrogenated equivalents) and the protein is dairy-based (usually rennet casein and caseinate), are the most common. Similar to PCPs, the other main ingredients are ES and water. The manufacturing procedures of ACP are similar to those for PCP, and involve formulation, processing and hot packing of the hot molten product (O'Riordan *et al.*, 2011). While production method varies somewhat, a typical manufacturing procedure involves the following sequence of steps:

- simultaneous addition of the required amounts of water and dry ingredients (e.g., casein, ES),
- addition of oil,
- cooking to ~85 °C (using direct steam injection) while continuously shearing until a uniform homogeneous molten mass is obtained (typically 5–8 min).
- addition of flavouring materials (e.g., enzyme-modified cheese, starter distillate) and acid(s) (e.g., citric acid) to the molten mass, followed by blending for a further 1–2 min, and
- hot packing and cooling.

14.9.1 Role of Protein in Manufacture and Structure Formation

The principles of the manufacture of processed cheese have been reviewed extensively (Caríc and Kaláb, 1993; Fox *et al.*, 2000; Guinee *et al.*, 2004; Glass and Doyle, 2005; Kapoor and Metzger, 2008). Application of heat (70–90 °C) and mechanical shear to natural cheese in the absence of stabilisers usually results in the formation of a heterogeneous, gummy, pudding-like mass which undergoes extensive oiling-off and moisture exudation during manufacture and, especially, on cooling. These defects arise from:

- liquefaction of fat, and the coalescence of free fat into pools as a result of shearing and removal of the MFGM surrounding the fat, and
- dehydration, aggregation and shrinkage of the *para*-casein network as a result of
  - an increase in hydrophobic interactions between the casein molecules, as a result of the combined effects of high temperature and low pH (pH 4.6–5.6),
  - precipitation of soluble (serum) calcium and phosphate, leading to further calcium phosphate mediated interactions between the *para-casein* molecules (especially, in rennet-curd cheeses).

The addition of ES (1–3 %), such as the sodium salts of citric acid and/or phosphoric acid, during processing promotes emulsification of free fat and rehydration of the *para*-casein and thus contributes to the formation of a smooth, homogeneous, stable product. These salts, referred to as emulsifying or melting salts generally consist of a monovalent cation (e.g., sodium) and a polyvalent anion (e.g., phosphate or citrate). While these salts are not emulsifiers *per se*, they promote, with the aid of heat and shear, a series of concerted physico-chemical changes in the cheese blend which result in rehydration of the aggregated *para*-casein and its partial conversion into an active emulsifying agent, sodium phosphate *para*-caseinate, which emulsifies the fat released on heating and shearing. These changes include upward adjustment of pH and stabilization (buffering), calcium sequestration, *para-casein* hydration and dispersal, emulsification and structure formation (Fig. 14.27).

14.9.1.1 Physico-chemical Changes During Processing

The use of the correct blend of ES usually shifts the pH of cheese upwards (typically from ~5.0–5.5 in the natural cheese to 5.6–5.9 in the PCP) and stabilises it by virtue of their high buffering capacity (Gupta *et al.*, 1984; Caríc and Kaláb, 1987; Lucey *et al.*, 2011). This change contributes to an increased negative charge on the *para-casein* and enhanced calcium-sequestering ability of the ES (Irani and Callis, 1962).

Calcium sequestration involves the exchange of the Ca^{2+} (attached to casein *via* phosphoserine residues and/or the carboxyl groups of acidic amino acids) of the *para*-casein network for the monovalent Na^{+} of the ES. Recent studies indicate that most of the Ca (≥75 % of total) and P

A. Overall Reaction

Rennet-curd cheese (calcium *para*-casein network with entrapped moisture and globular fat) + water + emulsifying salts and energy

↓

Processed cheese

B. Physico-chemical changes during processing

Change	Main causative agent
• Ion exchange (Ca sequestration)	- Emulsifying salt
• pH buffering	- Emulsifying salt
• Protein dispersion	- Emulsifying salt - Thermal and mechanical energy
• Protein hydration	- Emulsifying salt - Thermal energy
• Emulsification	- Dispersed hydrated protein - Mechanical energy
• Structure formation	- Protein-protein interactions - Emulsified fat globules - Fat crystallization

Fig. 14.27 Summary of physico-chemical changes that occur during manufacture of processed cheese products (Modified from Fox *et al*., 2000)

(≥66 % of total) in PCPs is water-insoluble (Guinee and O'Kennedy, 2009, 2012; Shirashoji *et al.*, 2010), suggesting that the formed calcium-ES complex (e.g., calcium phosphate and/or calcium citrate) occurs as finely dispersed inclusions (Caríc and Kaláb, 1993). The partial demineralization of the *para*-casein at the elevated pH results in degradation of most of the intra- and inter-casein calcium crosslinks, and partial transformation from a tightly aggregated calcium phosphate *para-casein* network in natural cheese to a water-dispersible sodium *para*-caseinate (Nakajima *et al.*, 1975; Sood *et al.*, 1979; Matheis and Whitaker, 1984; Southward, 1985; Swaisgood, 2003; Lee *et al.*, 1986; Marchesseau *et al.*, 1997; Mulvihill and Ennis, 2003). These changes are confirmed by the large increase in the level of water-soluble protein following processing (e.g., from ~5–20 % of total protein in the natural cheese to ~60–90 % in processed cheese depending on the type and level of ES), and by the low calcium-to-protein ratio of the solubilised protein (~5–10 mg/g soluble protein in processed Cheddar cheese versus ~21 mg/g protein in natural Cheddar).

The degree of calcium sequestration and casein hydration (as determined by the proportion of total N that is non-sedimentable on centrifugation of a homogenate prepared by blending grated processed cheese and distilled water) are dependent on processing conditions and the type and level of ES (calcium chelating

strength, pH and buffering capacity) (Irani and Callis, 1962; van Wazer, 1971; Cavalier-Salou and Cheftel, 1991; Guinee *et al.*, 2004; Lucey *et al.*, 2011; Guinee, 2011b). Hence, the buffering capacity of the ES is a critical factor controlling the textural and melting attributes of PCPs and ACPs (Rayan *et al.*, 1980; Thomas *et al.*, 1980; Gupta *et al.*, 1984; Cavalier-Salou and Cheftel, 1991; Marchesseau *et al.*, 1997; Lee and Klostermeyer, 2001; Guinee, 2009, 2011b; Lucey *et al.*, 2011).

Under the conditions of cheese processing, the dispersed hydrated *para*-caseinate contributes to:

- emulsification—by coating the surfaces of dispersed free fat droplets, and
- emulsion stability by immobilization of a large amount of free water.

14.9.1.2 Structure Formation During Cooling

On cooling PCPs, the homogeneous molten viscous mass sets to form a characteristic body, which, depending on blend formulation, processing conditions and cooling rate, may vary from firm and sliceable to soft and spreadable (Piska and Štětina, 2004). Micro-structural studies on PCPs or ACPs indicate that the structure is an emulsion of discrete, rounded fat droplets of varying size in a continuous protein matrix (Kimura *et al.*, 1978; Taneya *et al.*, 1980; Rayan *et al.*, 1980; Heertje *et al.*, 1981; Lee *et al.*, 1981; Tamime *et al.*, 1990, 2011; Savello *et al.*, 1989; Marchesseau and Cuq, 1995; Marchesseau *et al.*, 1997; Guinee *et al.*, 1999; O'Riordan *et al.*, 2011). Compared to natural cheese, there is less clumping or coalescence of fat globules and the mean fat globule size is generally smaller but depends on formulation and processing conditions (e.g., ES, milk protein additions, processing time and extent of shear). High resolution transmission electron microscopy (60,000×) with negative staining of the protein reveals that the protein occurs as a network of strands that are finer than those of natural cheese and appear to be composed of *para*-caseinate sub-particles (20–30 nm diameter) joined end-to-end. It has been suggested that these particles may correspond to casein sub-micelles released from the calcium phosphate *para*-casein network of the natural cheese as a result of calcium chelation by the ES. The thickness of the strands decreases as the pH is raised from 5.2 to 6.1, an effect attributed to a change in the proportions of different types of protein interactions: hydrophobic, electrostatic, hydrogen bonds and residual calcium crosslinks (Marchesseau *et al.*, 1997).

The surfaces of emulsified sodium *para-caseinate-coated* fat globules, which can be considered as fat-filled pseudo-protein particles, appear connected to protein network, and may be considered to increase its effective protein concentration (see van Vliet and Dentener-Kikkert, 1982; Marchesseau *et al.*, 1997). The positive correlations between the degree of emulsification and firmness or elasticity, and the inverse relationship between the degree of emulsification and flowability of PCPs support this suggestion (Rayan *et al.*, 1980; Caríc *et al.*, 1985; Savello *et al.*, 1989).

14.9.2 Effect of Protein on the Properties of PCPs and ACPs

14.9.2.1 Differences in Protein Hydration/Aggregation in PCP Owing to Variations in Manufacturing and Storage Conditions

In 'hard' PCPs, the strands of the protein network are thicker, longer, and more connected into a structural continuum than in 'soft' PCPs (Taneya *et al.*, 1980; Heertje *et al.*, 1981; Caríc *et al.*, 1985; Tamime *et al.*, 1990). Minute electron-dense areas in the strands of the protein phase may correspond to regions of strand overlap and/or reflect areas with a relatively high degree of aggregation and fusion of the *para*-caseinate particles. Hence, the number and area of electron-dense zones in very firm PCF that had been cooked to 85 °C and held for 5 h were markedly higher than in control PCF that had been cooled after 3 min at 85 °C (Kaláb *et al.*, 1987).

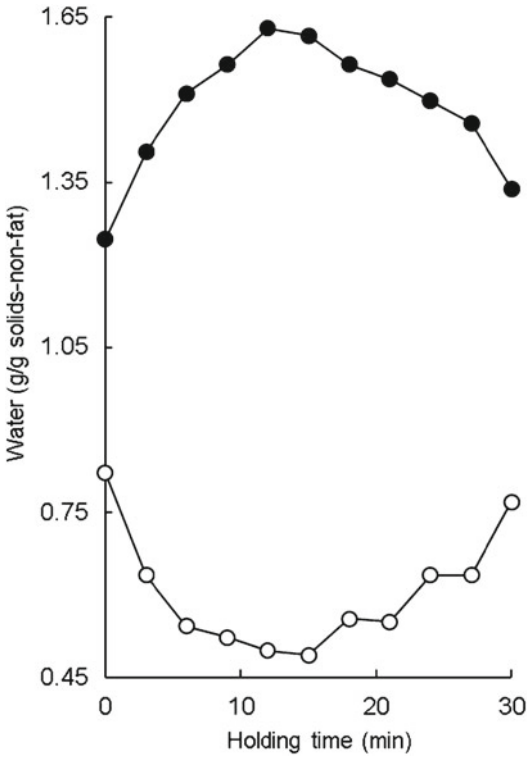

Fig. 14.28 Changes in the level of free water (*open circle*) and total bound water (*filled circle*) in pasteurized processed cheese as a function of processing time at 95 °C (redrawn from Csøk, 1982)

Prolonged holding (e.g., 5 h) at a high temperature is conducive to aggregation and dehydration of *para*-casein. Hence, Csøk (1982) reported that on holding a cooked processed cheese at 95 °C, the bound water increased to a maximum (e.g., at ~15 min) and decreased thereafter (Fig. 14.28). The initial increase may be attributed to increased solution of the ES (not fully solubilized at the end of the 'normal heating step', e.g., ~2–5 min) and calcium chelation, while the eventual decrease reflects aggregation of the *para*-caseinate on prolonged heating. In commercial practice, it is well known that prolonged holding of formed PCPs at a high temperature (e.g., due to delay or stoppage of packaging lines) can lead to a process known as 'over-creaming' whereby the product acquires an appearance resembling that of an 'orange-peel' surface, develops an over-firm and heavy pudding-like (coarse) structure which leaks free moisture and exudes beads of free oil (through the 'surface dimples'). The release of moisture and free oil during 'over-creaming' suggests that the process coincides with the onset of protein dehydration, emulsion destabilization and phase inversion; the increased degree of protein aggregation is consistent with the increase in firmness and elasticity. The reduction in casein hydration is supported by the changes reported on the characteristics of PCP on increasing the holding time from 1 to 32 min at 80 °C (Guinee, 2011b):

- a reduction in the level of water soluble nitrogen (WSN) from ~70 to 35 % of total N;
- increases the firmness and elastic shear modulus, G′ at 25 °C (by ~12.6 Pa/min in the range 1–32 min) of the unheated PCP, and reductions in the flow and maximum loss tangent (index of fluidity of the melted PCP, by ~0.03 min^{-1}) of the heated/melted PCP (Fig. 14.29).

The extent of these changes, which are consistent with those reported elsewhere, depends on other processing conditions (e.g., shear, temperature), formulation (e.g., degree of proteolysis, calcium level), and composition (e.g., moisture) of the product (Rayan *et al.*, 1980; Harvey *et al.*, 1982; Glenn *et al.*, 2003; Shirashoji *et al.*, 2006).

The micro-structure of the protein matrix also appears to change during storage, especially at a high temperature. A study on the age-related changes on processed cheese indicated that firmness and elasticity (i.e., force required to push a wire through a cheese) increased during storage over 3 months, with the extent of the increase being higher as the storage temperature was raised from 10 to 30 °C (Tamime *et al.*, 1990). The latter effect may be attributed to an increase in protein aggregation by hydrophobic interaction (Bryant and McClements, 1998), and is consistent with the decrease in moisture content of, and expression of moisture from, PCPs stored at 30 °C for 3 months, compared to control PCP stored at 4 °C (Guinee, unpublished results).

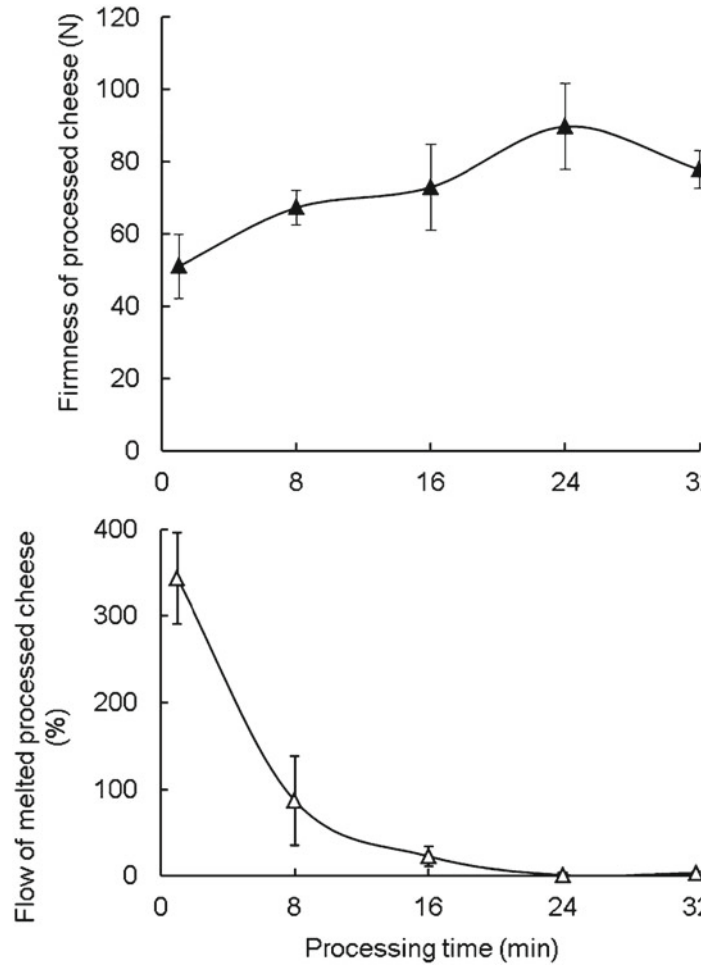

Fig. 14.29 Effect of processing time on the properties of processed cheese. The intact casein content of the Cheddar cheese was ~81 % of total casein. Data are the means of three replicate trials, and error bars show standard deviations (redrawn from Guinee, 2011b)

14.9.2.2 Protein Content of PCP

The concentration and type of protein has a major impact on the properties of PCPs and ACPs (Guinee, 2009; O'Riordan *et al.*, 2011).

Increasing the protein content of PCPs (by reducing the fat content while maintaining the moisture level and ES-to-protein ratio constant) increased the firmness of the unheated PCP and reduced the flowability and maximum loss tangent (fluidity) of the melted PCP (Guinee and O'Callaghan, 2013). These changes coincided with reductions in the levels of water-soluble protein and Ca (as percentages of total protein and total Ca).

14.9.2.3 Maturity and Level of Proteolysis of Natural Cheese Used in Manufacture of PCP

The *para*-casein in rennet-curd cheeses, such as Cheddar or Gouda, is enzymatically hydrolysed to peptides, of varying molecular mass, and free amino acids during maturation; simultaneously, the level of intact casein, as measured by the levels of total protein that is insoluble in water at pH 4.6, decreases. For a given cheese type, increasing the extent of proteolysis generally coincides with a reduction in the storage modulus (G'), fracture stress and firmness of the unheated

PCP, and an increase in the flowability of the melted PCP and its fluidity (as indicated by higher loss tangent) (Brickley *et al.*, 2007; Guinee, 2011b). Arnott *et al.,* (1957) reported a linear increase in the meltability of processed Cheddar cheese with increasing tyrosine content of Cheddar cheese, when the calculated concentration of free tyrosine level exceeded a critical level (~17 mg/g under the test conditions) after ~100 days maturation; no relationship between tyrosine content and flow of the melted PCP was evident at lower levels of tyrosine. Moreover, the extent of increase in meltability of PCPs with degree of maturity and proteolysis of the natural cheese used in the formulation is influenced by a number of factors such as pH (Vakaleris *et al.*, 1962) and calcium content of the natural cheese used (Guinee and O'Kennedy, 2009; Guinee, , 2011b) and the degree of shear during processing (Garimella Purna *et al.*, 2006). Likewise, the meltability of PCPs made using acid-curd cheeses increased linearly as the extent of proteolysis in the cheese was increased, by treatment with fungal proteinase, from 16 to 38 % soluble N (% of total N soluble in a mixed solvent comprised of 12 % trichloroacetic acid and 0.2 % phosphotungstic acid) (Lazaridis *et al.*, 1981). Nevertheless, excessive proteolysis (>20 % total soluble N) in the cheese was associated with increasingly shorter texture, faulty body, grainy mouthfeel, and bitterness in the resultant PCPs. The above effects of increasing proteolysis of cheese on PCPs may be envisaged as an attenuation of the PCP network, as water-soluble peptides are unlikely to become part of the casein/*para*-caseinate network of the PCP.

The effects of the degree of cheese maturity, and hence intact protein, has long been recognised and reflected in industrial practices. Thus, the use of cheese age as a major selection criterion for blend formulation at commercial level is common practice. Block processed cheeses with good sliceability and elasticity are generally formulated with a high proportion of young cheese (85–95 % intact protein) in the blend whereas predominantly medium ripe cheese (60–85 % intact casein) is used for cheese spreads.

14.9.2.4 Type of Milk Protein Ingredients Used in PCP

Milk protein ingredients (e.g., rennet casein, acid casein, caseinates, whey proteins) and cheese base are used widely in ACPs and PCPS, at levels determined by customer and legislation specifications (Hickey, 2011), product composition, and the composition of the ingredient *per se*. Their use is motivated by the desire to reduce formulation cost, improve consistency, and/or to innovate and differentiate PCP characteristics. Depending on their solubility, structure and chemistry, added proteins have a marked influence on the physicochemical, rheological, stability and usage appeal characteristics of pasteurized processed cheese products (PCPs) and analogue cheese products (ACPs) (Savello *et al.*, 1989; Abou El-Nour *et al.*, 1996; Guinee, 2009; O'Riordan *et al.*, 2011).

14.9.2.5 Caseins and Caseinates

Rennet casein is the preferred protein type for use in block processed cheese or analogue/substitute pizza cheese. In both products, it behaves like fresh natural rennet-curd cheese curd conferring a high degree of elasticity and firmness to the unheated processed cheese, and moderate meltability. Partial replacement of young Cheddar cheese (intact casein content, ~93 % of total protein) with commercial rennet casein (intact casein, ~99 % of total protein) results in firmer cheese that flows to a lower degree on melting. This effect is expected owing to the higher levels of intact casein and calcium phosphate in rennet casein (~33 mg/casein compared to ~27 mg/casein in Cheddar cheese).

While rennet casein also contributes to the desired stringiness and stretchability of the hot molten analogue pizza cheese (APC) at the end of manufacture prior to cooling, the finished APC products nevertheless have inferior stringiness compared to natural Mozzarella cheese when subsequently melted on pizza (Guinee *et al.*, 2000b). This is expected because of a lower degree of *para*-casein aggregation in the APC commensurate with the sequestration of calcium phosphate from the casein by the ES during processing, and the difference in product structure

(concentrated oil-in-water emulsion in APC compared to concentrated rennet-induced gel in Mozzarella cheese). Moreover, microstructural studies have shown that the aligned calcium phosphate *para*-casein fibres that confer natural molten Mozzarella with its stringiness are absent from APC, which consists of discrete rounded fat droplets of varying size in a continuous protein phase (Guinee *et al.*, 2000b; Kindstedt *et al.*, 2004).

Acid casein is generally not used, or used only at very low levels (e.g. 1–3 %) in ACPs and PCPs because of its insolubility at low pH, and its depressing effect on the pH, which in turn reduces the degree of dissociation of the ES and their ability to sequester calcium from other proteins (e.g., cheese protein or rennet casein). Consequently, the use of acid casein at quantities of 1–3 % (w/w) of the processed cheese formulation can markedly extend the product make time, unless the pH of the blend is increased to its normal value (e.g., ~5.8–6.0) *via* addition of alkali and/or an ES blend with the desired pH-buffering effect. A comparison of the functionality of acid casein and rennet casein on the melt properties of model processed cheeses (made from casein powder, vegetable fat, water and ES) was made by Savello *et al.,* (1989). The response of meltability to casein type depended on type of ES and the pH to which the acid casein was adjusted (upwards) during processing. Acid casein (pH adjusted to ~7.0–7.7) gave model processed cheeses with higher meltability than those made with rennet casein on using disodium hydrogen orthophosphate or tetrasodium pyrophosphate, and an opposite effect when using sodium aluminum phosphate or trisodium citrate.

Caseinates (especially sodium) find most application in spreadable processed cheese spreads where their high water-binding capacity and good emulsifying properties promote a desired creaming effect. Gouda *et al.,* (1985) reported that full replacement of cheese solids-non-fat by calcium caseinate caused deterioration in spreadability of Cheddar PCS, probably due an excessive creaming effect. However, partial replacement in a formulation (with skim milk powder, calcium caseinate, ripe Cheddar, butter oil and ES at respective levels of 6–8, 5–7, 15, 14, and 3 %, w/w) improved the meltability of the PCS, suggesting a desirable level of creaming.

14.9.2.6 Whey Protein Ingredients

In practice, whey powders (e.g., sweet whey powder with ~12–15 % protein) are used widely in PCPs as a cost-effective filler to impart a mild sweet taste and a smooth consistency, especially desirable in highly processed cheese spreads and dips. Whey protein concentrates (WPCs) or whey protein isolates (WPIs), because of their relatively high cost are used less frequently, to convey specific functions such as (i) to give low-flow or flow-resistance in "controlled–melt" PCPs targeted to specific cooking applications (e.g., cheese insets/pieces in meat-based products, fried cheese), and (ii) to confer "body" (stiffness and viscosity) to high moisture spreadable PCPs. An early patent (Schulz, 1976) describes a process for the manufacture of PCP that is resistant to flow on cooking, based on the addition of a heat-coagulable protein (3–7 %, w/w, lactalbumin or egg albumen), at a temperature <70 °C, to the PCP formulation near the completion of processing. Numerous studies have investigated the effects of added whey proteins on the texture and cooking characteristics of PCPs (Savello *et al.*, 1989; Hill and Smith, 1992; Gupta and Reuter, 1993; Kaminarides and Stachtiaris, 2000; Mleko and Foegeding, 2000, 2001; French *et al.*, 2002; Mounsey *et al.*, 2007). These studies have generally shown that the loss of flowability depended on concentration of whey proteins but was essentially independent of whether the whey protein was denatured or undenatured or the pH (in the pH range 7–10) at which the whey protein was denatured. Mleko and Foegeding (2001) postulated that processed cheese with added whey proteins behaved like a two-component system that on heating, comprised of a melting casein network and a non-melting whey protein network. Such a network may be comprised of whey protein particles/aggregates that interact to form larger particles, or a network, in the processed cheese environment at the high temperatures (80–100 °C) reached during manufacture and subsequent cooking of the product in the

particular food application (e.g., in sandwich or pizza). The high calcium content and relatively low pH (~≤6.0) of the processed cheese environment would be conducive to a high degree of interaction of heat-denatured whey proteins by covalent (disulphide), hydrophobic and electrostatic interactions. Model studies have shown that the heat-induced aggregation of β-lactoglobulin is enhanced by reducing pH in the range 7.0–5.0 and increasing the concentration of ionic calcium (Law and Leaver, 2000; Spiegel and Huss, 2002; O'Kennedy and Mounsey, 2009; Petit *et al.*, 2011; Ryan *et al.*, 2013) The use WPCs as a replacement of cheese solids has also been found to accelerate storage-related flavour deterioration, which increased with the level of WPC added in the range 0–20 %, w/w (Thapa and Gupta, 1992).

14.9.2.7 Casein-Whey Protein Co-precipitates (CWPCPs)

Conventionally, CWPCPs, also referred to as total milk proteinates, are protein products containing casein and whey proteins and are formed by heat treatment of the milk and subsequent precipitation of the protein complex by acidification to pH 4.6 and calcium addition (Mulvihill and Ennis, 2003). Depending on the level of $CaCl_2$ added, three types of CWPCPs may be obtained, namely, high, medium and low calcium containing 2.5–3.0, 1.0–2.0 and 0.5–0.8 %, w/w, calcium, respectively. Their addition to PCP formulations has been generally found to increase the firmness and sliceability, but similar to whey proteins, to reduce the flowability on heating to an extent that increases with level of addition (Thomas, 1970; Abou El-Nour *et al.*, 1996; French *et al.*, 2002; Mounsey *et al.*, 2007). The adverse effect of CWPCPs on meltability may be associated with a number of factors including the formation of large whey-protein casein aggregates (O'Kennedy and Mounsey, 2009) which on inclusion in processed cheese impede slippage of the protein layers and thereby restrict heat-induced flow, similar to the melt-inhibitory effect of some fat replacers in reduced-fat natural cheese (McMahon *et al.*, 1996), and/or the formation of a non-melting whey protein network (Mleko *et al.*, 2003).

However, new approaches aimed at controlling heat-induced denaturation and aggregation of whey protein, and the size/gelation capacity of the resultant reaction products may improve the functionality of CWPCPs (Mleko and Foegeding, 1999; Donato and Guyomarc'h, 2009). Mounsey *et al.,* (2007) reported the effects of varying the pH of skim milk (9.5, 7.5, 3.5) at heating (90 °C × 20 min) prior to re-acidification to pH 4.6 on the performance of the resultant liquid CWPCPs on the cooking behaviour of model PCPs. Used as a substitute for acid casein powder, the CWPCPs were added to the PCPs at a level of ~8 %, resulting in concentrations of ~1.0–1.4 % whey protein. The meltability and fluidity (maximum value of loss tangent on heating from 20 to 90 °C) of PCPs improved significantly as the pH of the skim milk at heating was increased, while the meltability of the PCP made using the pH 9.5 CWPCP was similar to the control processed cheese. Conversely, reducing the pH at heating to 3.5 had the opposite effect.

14.9.2.8 Cheese Base (CB)

CB is manufactured by UF and diafiltration of skim milk (typically to 20–25 % dry matter), inoculation of the retentate with lactic culture, incubation to a set pH (5.2–5.8), pasteurization and scraped-surface evaporation to ~60 % dry matter (Ernstrom *et al.*, 1980; Sutherland, 1991; Ganguli, 1991). However, rennet may be added to the retentate to form a curd from which a small quantity of whey is removed (compared to that in natural cheese manufacture) and which is dry-salted and pressed, and stored as natural cheese. It is frequently used as a substitute for cheese in the manufacture of processed cheese, the main advantages being its lower cost and more consistent quality (i.e., intact casein content and calcium-to-casein ratio). Increasing the level of substitution of natural cheese by CB generally results in a "longer-bodied," firmer PCP which is less flowable on heating (Collinge and Ernstrom, 1988; Collinge *et al.*, 1988; Tamime *et al.*, 1990; Younis *et al.*, 1991). The latter effects probably reside in the characteristics of the CB, compared to cheese:

- a higher degree of intact casein and calcium-to-casein ratio (Brickley *et al.*, 2007; Guinee and O'Kennedy, 2009; Guinee and O'Kennedy, 2012);
- the presence of whey proteins (~8.7 %, w/w) which are likely to denature and complex with *para*-κ-casein to form a pseudo-gel at the high temperatures used during processing (85–90 °C for 3 min) (see Doi *et al.*, 1983a, b, 1985).

The relatively high levels of protein and soluble calcium, and low pH in the CB (e.g., relative to milk) enhance the tendency of whey proteins to aggregate and gel (Doi *et al.*, 1983a, b; O'Kennedy and Mounsey, 2009; Donato and Guyomarc'h, 2009). Hence, the flowability of PCPs made using CB is significantly improved by reducing the calcium content of the CB by pre-acidification of the milk prior to UF, and reducing the intact casein in the CB by the addition of exogenous proteinases (Sood and Kosikowski, 1979; Anis and Ernstrom, 1984; Tamime *et al.*, 1990, 1991).

Analogous to the effect of CB, substitution of Cheddar cheese (to a level of 33 % w/w) with a freshly made curd, prepared by acid-heat coagulation (pH 5.5, 90 °C for 10 min) of milk significantly increased the firmness of the resultant processed cheese (Kaláb *et al.*, 1991). Similarly, increasing the level of substitution of rennet casein (to levels of 0–50 %) by total milk protein resulted in a progressive increase in firmness, and a decrease in flowability, of an ACP (Abou El-Nour *et al.*, 1996).

14.10 Conclusions

Protein is the major structural component in most cheese varieties, apart from some acid curd varieties (e.g., Mascarpone), brown whey cheeses (e.g., Mysost) and some cheese-like imitation products where it amounts to <20 % of dry matter. Consequently, the concentration and structure of the protein phase, which occurs in the form of a hydrated network filled with fat globules, has a major influence on cheese texture, size reduction properties, cooking properties, and hence, the acceptability of the cheese to the consumer, food service provider and manufacturer of formulated foods containing cheese.

There are two main categories of proteins in bovine milk, namely caseins (~2.6 %) and whey proteins (~0.65 %). Whey proteins occur as macromolecular solutes that are soluble in the serum or aqueous phase (solvent) of the milk, while caseins are in the form of spherical-shaped colloidal particles (~200 nm), micelles, dispersed in the serum phase. The micelles comprise casein (20 %), insoluble calcium and phosphate (~1.8 %) and internal solvent (~78.2 %), which can be assumed to be compositionally similar to the bulk phase serum in which they are dispersed. The stability of micelles is extremely sensitive to factors that alter the balance between hydrophobic attractive forces and electrostatic repulsive forces at their surfaces. The balance affects the micelle surface in terms of its attraction to, or repulsion of, neighbouring micelles. Destabilization and aggregation of casein micelles is promoted by increasing the surface hydrophobicity either through enzymatic hydrolysis of the surface κ-casein layer by rennet, charge neutralization by acidification to the isoelectric pH, or a combination of charge reduction and increased hydrophobic interaction by pH reduction to ~5.6 and high heat treatment to ~90 °C. The sensitised micelles then begin to aggregate. A controlled, moderate degree of contact between the surfaces of touching micelles promotes the formation of a gel, which may be described as a *para*-casein (rennet-curd cheeses) or casein (acid-curd cheeses) network that occupies the full volume of the milk and encloses the fat globules and serum, analogous to the way that a sponge holds water. Following gelation of the milk, further casein aggregation is promoted to reduce the moisture content of the gel (~88 % moisture) to the final moisture content of the cheese (~33–55 % depending on the variety). This is achieved by subjecting the gel to the interactive effects of different operations which vary depending on the type of cheese: cutting into pieces/particles, stirring, cooking and acidification of gel particles in whey; physical removal of whey (drainage); and acidification, heating, salting, and/or pressing of the resultant curd mass.

Rennet-curd cheeses may be defined as viscoelastic solid structures, comprised of a highly concentrated, constrained network of dehydrated *para*-casein micelles that encase the milk fat in the form of globules and/or pools. Controlling the concentration of *para*-casein and its degree of aggregation, through manipulation of the different manufacturing steps and their sequence, is critical in defining the various aspects of cheese quality: ratio of viscous to elastic characteristics, texture, functionality (e.g., shreddability) and opacity/translucence. High levels of aggregation promote cheeses that are quintessentially elastic, hard and chewy, have good shreddability and sliceability, and melt/flow moderately on heating. On the other hand, moderate degrees of aggregation favour higher moisture cheeses that are relatively soft, adhesive, spreadable, easily mixed with other ingredients and flow extensively on heating. Other factors, however, influence the attributes of rennet-curd cheese, e.g., milk pretreatments, starter cultures, added ingredients, maturation conditions, macrostructure as affected by curd handling treatments, and storage/maturation conditions. Maturation results in several biochemical changes (e.g., proteolysis, pH, protein hydration) and affects the partition of calcium between the serum phase and the *para*-casein network. Consequently, cheese rheology and heat-induced functionality exhibit dynamic behaviour, undergoing marked changes during ripening. Protein and its degradation products also contribute to cheese flavour.

Limited heat-induced destabilization of rennet-curd cheeses, as manifested by a reduction in *para*-casein voluminosity and fat coalescence, is generally a prerequisite for correct functionality in heated cheeses, where some oiling-off and protein aggregation are important for properties such as surface sheen, succulence, stringiness and chewiness of the melted cheese. The decrease in the voluminosity of, and attendant expulsion of solvent from, the *para*-casein network on heating cheese to temperatures in the range 60–90 °C (e.g., during baking and grilling) confers fluidity/liquidity and mobility to the melting cheese mass. This heat-induced microphase separation of moisture within the cheese, which may be considered as the basis of the heat-induced melting and/or stretching of cheese, occurs to a degree dependent on various factors such as protein-in-moisture concentration, protein-to-fat ratio, degree of fat emulsification, presence of whey proteins, and the strength of the links between the *para-casein* molecules as affected by pH, calcium level, and extent of protein hydrolysis.

Acid-curd cheeses such as Quark, Labneh and reduced-fat cream cheeses generally have a soft, uniform consistency, a smooth mouth-feel free of any sandiness or graininess; in contrast to rennet-curd cheeses, chewiness is generally an undesirable attribute, except in Cottage cheese where the individual curd granules are ideally somewhat chewy. However, owing to their relatively high moisture level (~60–80 %) and low content of protein (5–14 %), acid-curd cheeses are susceptible to uncontrolled post-manufacture casein aggregation and dehydration *via* mobility and re-arrangement of their casein matrices when subject to stresses, for example during transport/distribution and retailing. This is the basis of major sensory defects including excessive wheying-off and the development of sandy/grainy textures during storage. Avoidance of such defects requires optimization of the degree of casein aggregation at the different stages of cheese manufacture through the use of appropriate tools such as temperature, pH, ionic strength, whey protein denaturation and their complexation with the casein micelles prior to fermentation.

Heating of cheese to a high temperature (e.g., 90 °C) while shearing generally results in extensive protein (*para*-casein or casein) dehydration and the formation of free fat, resulting in moisture leakage and fat exudation. Re-hydration of the protein by the addition of emulsifying salts, such as sodium phosphates or citrates, assists in a structural transition from a concentrated gel to a concentrated *para*-caseinate-stabilised oil-in-water emulsion, which is the basic structure of PCPs and ACPs. The degree of *para*-caseinate aggregation or hydration and size distribution of emulsified oil droplets are major determinants of the rheology and heat-induced functionality of the resultant PCPs. However, similar to rennet-curd chesses, the

functionality of unheated and heated PCPs is influenced by many factors such as protein concentration, protein-to-fat ratio, calcium-to-casein ratio, and the concentration of whey proteins.

14.11 Projection of Future Work

Much experimental knowledge is now available on the general contribution of milk proteins to the structure-function relationships of cheese and cheese products. However, little is known about the direct effects of protein (volume fraction and type) on rheology and heat-induced viscoelastic changes in cheese. For a given variety of natural cheese, it is difficult to quantify the effect of any one particular component (e.g., protein) since the concentrations of different components generally vary simultaneously. A more systematic understanding of the role of protein could be gleaned from analysis of model systems (e.g., liquid precheeses of varying solids content based on micellar casein with different calcium-phosphate levels and milk ultrafiltrate) in which the concentration and type of protein, solvent properties (e.g., ionic strength, NaCl content, pH), and other factors influencing protein aggregation and structure (temperature, time) are more easily controlled. The use of model systems, together with non-invasive measuring techniques with resolution at the molecular level (e.g., atomic force microscopy and ultrasound) may provide a better understanding of the contribution of protein, under different conditions, to the physical properties of cheese.

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Milk Protein Hydrolysates and Bioactive Peptides

15

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Abstract

Milk proteins and milk protein-derived peptides have been widely studied for their health enhancing properties. This chapter presents the updated scientific knowledge on the bioactive properties of milk protein-derived peptides. The different bioactive properties which have been attributed to milk protein-derived peptides are discussed. These include mineral binding, cardioprotective, antidiabetic, satiating, opioid, antimicrobial, immunomodulatory, anticancer and antioxidant activities. The structure-function relationship is presented for the aforementioned bioactive properties based on current scientific knowledge. For each bioactive property, the data obtained *in vitro* is discussed, followed by an analysis of the information obtained from animal and human intervention studies. To date, most studies have been conducted *in vitro*. However, an increasing number of *in vivo* studies testing the efficacy of milk protein-derived peptides are being conducted. In certain instances, the *in vivo* studies have confirmed the bioactivity of specific milk protein-derived peptides or milk protein hydrolysates. However, conflicting data still exist in the scientific literature, which demonstrates that the bioactive properties observed *in vitro* do not always translate *in vivo*. Detailed knowledge of the peptide sequences responsible for the bioactive properties, together with a better understanding of the

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bioavailability and stability of these peptides *in vivo* may help to enhance the development of milk protein hydrolysates with health promoting capabilities in humans. Ultimately, this may lead to the approval of health claims by the relevant regulatory agencies.

Keywords
Bioactive peptides • Hydrolysates • Milk • Bioavailability • Stability • Peptide discovery

15.1 Introduction

During the past 20–30 years, there has been a growing interest in utilising compounds from natural sources as a means to prevent/modulate various health conditions in humans. More particularly, extensive research has been carried out on the health benefits of milk proteins and peptides with a particular emphasis on the identification of bioactive peptide (BAP) sequences. It is well documented that intact milk proteins possess many potential nutritional and health benefits in general which can help prevent certain disease conditions (Ricci-Cabello *et al.*, 2012). For example, mineral binding and delivery properties have been described for the caseins (CNs) and a broad spectrum of antibacterial activity has been linked with the whey protein, lactoferrin (LF). In addition, numerous BAP sequences which are encrypted within the primary structures of milk proteins have been identified (FitzGerald and Meisel, 2003b). Bovine milk is the most studied source for BAPs, and for this reason, the majority of the BAPs identified to date originate from milk proteins (Panchaud *et al.*, 2012). Milk protein-derived BAP sequences have been shown to modulate key biomarkers associated with the cardiovascular, nervous, gastrointestinal and immune systems. These BAPs may be involved in specific interactions with cellular receptors or key metabolic enzymes, resulting in the modulation of specific physiological responses. Therefore, the targeted utilisation of these peptides may beneficially modulate physiological systems within the human body. Using *in vitro* assays, a diverse range of potential physiological targets for milk protein-derived BAPs have been identified. Bioactive properties including immunomodulatory, opioid, mineral binding/bone formation, hypotensive, antithrombic, antimicrobial, anticancer, antidiabetic and anti-obesity activities have been reported for milk protein-derived BAPs (Meisel, 1997; Clare and Swaisgood, 2000; FitzGerald and Meisel, 2003b; Korhonen and Pilhanto, 2006; Korhonen and Pihlanto, 2007; Pihlanto, 2011). In addition, an increasing number of studies now exist which have demonstrated the bioactive potential of milk protein-derived peptides *in vivo* using animal models and human trials. To date, the most frequently performed small animal and human intervention studies have been conducted with BAPs targeting antihypertensive (van der Zander *et al.*, 2008; Xu *et al.*, 2008; Jauhiainen *et al.*, 2010a, b; Miguel *et al.*, 2010; Norris and FitzGerald, 2013), mineral binding (Nongonierma and FitzGerald, 2012a) and antidiabetic (Power *et al.*, 2009; Akhavan *et al.*, 2010; Morifuji *et al.*, 2010; Geerts *et al.*, 2011; Gaudel *et al.*, 2013) properties. An increasing number of ingredients, foods and oral hygiene products containing BAPs are now available on the marketplace.

Milk proteins are naturally hydrolysed into peptides and amino acids following the combined action of gastric and pancreatic hydrolases during gastrointestinal transit in addition to degradation by microbial enzymes in the gut microflora (Nehir El and Simsek, 2012). This natural process increases the digestibility of food proteins and generates bioavailable nutrients to assist the body in its various functions. Many bioactive sequences have therefore been found in the digestate during gastrointestinal transit.

In addition to the *in vivo* formation, BAPs can also be generated using:

- physical and/or chemical processes including ultrasonic, microwave and chemical treatments;
- microbial fermentation involving mostly bacterial proteolytic/peptidolytic activities during the manufacture of fermented dairy products;
- and enzymatic hydrolysis using proteolytic activities from mammalian, microbial and plant sources (Nongonierma and FitzGerald, 2011).

Various methodologies are now being employed to identify milk protein-derived BAPs. Bioinformatic, quantitative structure activity relationship (QSAR) and artificial neural network (ANN) approaches, for instance, have been utilised in order to understand better the interactions between BAPs and specific biological receptors and/or to predict bioactive sequences which may efficiently bind to the active site of certain key metabolic (biomarker) enzymes (Pripp, 2007; Norris *et al.*, 2012; Nongonierma *et al.*, 2013b). These strategies have led to a better understanding and prediction of the behaviour of BAPs in regard to their potential physiological effects. Mass spectrometry (MS) has been utilised as a tool to identify new BAP sequences in various milk protein-based hydrolysates/fermentates and in the digestate (Hernández-Ledesma *et al.*, 2004; Tsopmo *et al.*, 2011; Kunda *et al.*, 2012; Boutrou *et al.*, 2013). The objective of this chapter is to review recent developments in milk protein-derived BAPs. The current methodologies used to enrich, fractionate, identify and discover new BAP sequences will also be described and particular attention will be given to the impact of food formulation on the stability and bioavailability of milk protein-derived BAPs.

15.2 Bioactive Properties of Milk Protein Hydrolysates

Most work on milk-derived BAPs has been conducted with bovine milk; however, milk from other species including goat, sheep, buffalo, camel, mare, jenny, donkey and yak have been identified as a source of BAPs (Pandya and Haenlein, 2009; Hernández-Ledesma *et al.*, 2011; Mao *et al.*, 2011; Nikkhah, 2011; Bidasolo *et al.*, 2012). BAPs can target different organ sites within the human body. Some peptides, for example, can display their bioactive properties directly in the oral cavity. Other peptides affect specific targets located within the gastrointestinal system. BAPs modulating systemic targets must be bioavailable, i.e., have the ability to cross the gut and/or brain barrier and be stable to epithelial and serum peptidases. To date, the most studied milk protein-derived BAPs are mineral binding and angiotensin-converting enzyme (ACE) inhibitory peptides.

15.2.1 Mineral Binding Peptides and Hydrolysates

Within the human body, calcium (Ca) is mainly localised in the bones and the teeth with 99 % of the total Ca in the adult body being in the form of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Bonjour *et al.*, 2009). Due to health concerns related to risks of osteoporosis in different population groups, including teenage girls, post-menopausal women and the elderly, supplementation of Ca has been proposed as a means to meet daily Ca intake requirements (Gueguen and Pointillart, 2000; Bonjour *et al.*, 2009). Decreased ileal absorption of dietary Ca following the formation of insoluble calcium phosphate ($Ca_3(PO_4)_2$) complexes with foods has been reported (Gueguen and Pointillart, 2000). In this context, the use of caseinophosphopeptides (CPPs) has been suggested as a means to improve Ca bioavailability while increasing its intestinal absorption. A deficit in other minerals such as iron is responsible for the development of anemia. An increase in iron bioavailability in the presence of CPPs has therefore been suggested (García-Nebot *et al.*, 2010). CPPs have also been described as anticariogenic compounds owing to their role in the promotion of dental enamel remineralisation (Reynolds, 1993; Nongonierma and FitzGerald, 2012a). Dental caries and periodontal diseases

have been described as the most prevalent or widespread conditions within humans (Petersen and Kwan, 2009); it is thought that 90 % of the world's population will experience caries at least once in their life (Marchisio *et al.*, 2010). Carious lesions can be repaired by promoting remineralisation and reducing demineralisation of dental enamel (Chen and Wang, 2010; Peters, 2010). Unhydrolysed CNs have been reported to have anticariogenic properties (Guggenheim *et al.*, 1999; Rodrigues *et al.*, 2011). However, this effect has been observed with high dosage of CNs (Aimutis, 2004; Cross *et al.*, 2006, 2007). CPPs have been identified as more potent anticariogenic CN-derived peptides compared to unhydrolysed CNs (Reynolds, 1998; Moezizadeh and Moayedi, 2009; Gupta and Prakash, 2011; Nongonierma and FitzGerald, 2012a).

15.2.1.1 Structure Function

Binding of minerals to milk-protein-derived BAPs has mainly been described for CPPs. However, a few studies have demonstrated that whey protein-derived peptides could also bind minerals (Vegarud *et al.*, 2000; Rui, 2009; Pan *et al.*, 2013). The α-lactalbumin (α-La)-derived peptide Ser-Thr-Glu-Tyr-Gly has been identified for its Ca^{2+} binding properties (Pan *et al.*, 2013). Setarehnejad *et al.* (2010) have shown the protective effect of caseinomacropeptide (CMP, κ-CN (f 106–169)) against hydroxyapatite dissolution *in vitro*, which was attributed to its Ca^{2+} binding properties. To date, most milk-derived mineral binding BAPs have been shown with CPPs; for this reason these will be further discussed within this section.

CPPs may be released following microbial fermentation, physical processing or enzymatic hydrolysis of CNs. They are rich in clusters of phosphorylated seryl (and occasionally threonine) residues. CPPs have been linked with the binding of divalent ions such as Ca^{2+}, magnesium and iron (Fe^{2+}) and trace elements including zinc (Zn^{2+}), barium, selenium, nickel, cobalt and chromium (FitzGerald, 1998). They are reported to improve mineral bioavailability by acting as a mineral carrier and/or by enhancing mineral solubility. The two most frequently studied CPPs are α_{s1}-CN (f 59–79)5P and β-CN (f 1–25)4P. These CPPs contain a specific sequence known as the "acidic motif" which consists of three serine phosphate groups followed by two glutamic acid residues, i.e., Ser(P)-Ser(P)-Ser(P)-Glu-Glu (Bouhallab and Bouglé, 2004). The enhancement of Ca uptake by CPPs in intestinal cells depends on the amino acid residues upstream and downstream from the "acidic motif". Removal of the four C-terminus amino acids from β-CN (f 1–25)4P resulted in a reduction of Ca uptake by HT29 cells (Fig. 15.1). Removal of a phosphorus residue or removal of the N terminal region of β-CN (f 1–25)4P both resulted in a complete loss of Ca uptake by HT29 cells. These results show the crucial role of the phosphorylated acidic domain and the N-terminal region of β-CN (f 1–25)4P on Ca^{2+} bioavailability (Ferrarretto *et al.*, 2003).

In the oral cavity, the mechanism of action for CPPs involves an increase in $Ca_3(PO_4)_2$ solubility, which results in a supersaturated state of Ca^{2+} at the tooth surface acting as a driving force for dental enamel remineralisation of carious lesions (Cochrane *et al.*, 2010). Another mechanism involves the buffering capacity of CPPs in the oral cavity, counteracting the pH decrease generally observed in carious lesions (Rahiotis *et al.*, 2008; Kitasako *et al.*, 2010). Amorphous calcium phosphate (ACP-$Ca_3(PO_4)\bullet 3H_2O$) can be converted into hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) in an aqueous medium (Boskey and Posner, 1973). Combinations of CPP and ACP have been utilised for their remineralising properties (Walsh, 2009). CPPs from tryptic CN digests when combined with ACP have been shown to possess anticariogenic properties (Adamson and Reynolds, 1996; Kitts, 2006). The bioactive properties of CPP-ACPs have been classified into four categories, i.e., remineralisation, prevention of demineralisation, anti-plaque and other bioactivities (Fig. 15.2). It has been shown that enamel remineralised with CPP-ACP was more acid resistant than normal enamel (Iijima *et al.*, 2004). A relatively long half-life of 124.8 min has been reported for CPPs in the plaque (Cochrane *et al.*, 2010), indicating a significant residence time in the plaque.

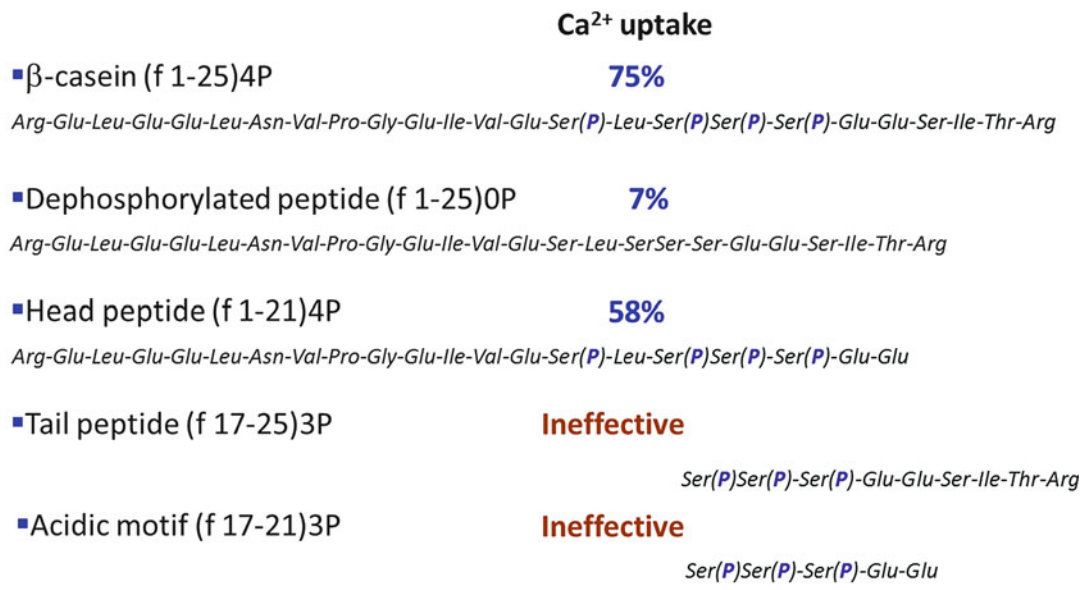
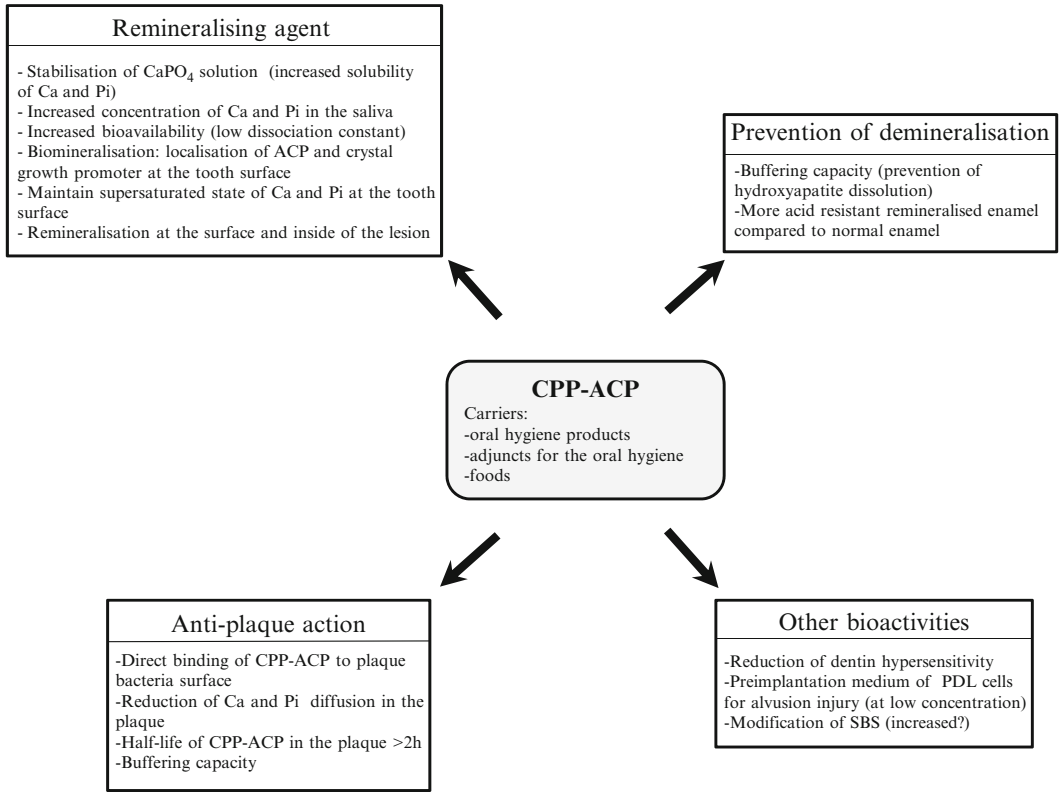


Fig. 15.1 Schematic representation of the role of upstream and downstream amino acid residues on calcium uptake by HT29 cells (adapted from Ferrarretto *et al.*, 2003)

15.2.1.2 *In Vitro* and *In Situ* Assessment/Screening of Mineral Binding

Various *in vitro* and *in situ* protocols have been described to assess the mineral binding properties of CPPs. These protocols differ depending on whether it is for evaluation of the remineralising properties in the oral cavity or at the gut and bone level. In the oral cavity, *in vitro* protocols include investigation of the protective effect of CPPs against demineralisation of hydroxyapatite on animal tooth enamel. *In situ* protocols involve removable intra-oral appliances holding enamel slabs which had undergone a demineralisation protocol prior to CPP application (Nongonierma and FitzGerald, 2012a). Cell cultures have been used for the evaluation of CPPs at the intestinal and bone level. *In vitro* and *in situ* protocols are generally utilised as a means of screening various samples for their mineral binding properties.

Remineralisation of dentin and enamel has been demonstrated *in vitro* and *in situ* with CPP-ACP (Reynolds *et al.*, 1999; Manton *et al.*, 2010; Walker *et al.*, 2010; Zhang *et al.*, 2011). Increased remineralisation of the enamel subsurface has been observed when CPP-ACP was combined with fluoride following the formation of fluoroapatite (ten Cate, 1999; Kumar *et al.*, 2008; Reynolds, 2008; Walsh, 2009; Jayarajan *et al.*, 2011; Shen *et al.*, 2011). However, some studies did not demonstrate any positive effect of CPP-ACP on tooth remineralisation (Lata *et al.*, 2010; Wegehaupt *et al.*, 2012) or did not find any differences with fluoride (Tantbirojn *et al.*, 2008; Uysal *et al.*, 2010; Wang *et al.*, 2011a). In intestinal cell culture models, differences in mineral binding properties have been shown depending on the nature of the CPP preparation. A higher number of HT29 epithelial cells were responsive to Ca uptake with β-CN (f 1–25)4P compared to α_{s1}-CN (f 59–79)5P (Ferrarretto *et al.*, 2003). This may be linked to differences in the binding affinity for Ca between the two CPPs, with β-CN (f 1–25)4P having a lower Ca binding affinity and higher stoichiometry (0.63 mM^{-1} and 4 Ca^{2+}:peptide, respectively) than α_{s1}-CN (f 59–79)5P (0.84 mM^{-1} and 1 Ca^{2+}:peptide, respectively) (Meisel and Olieman, 1998). A dose-response relationship was reported between the concentration of β-CN (f 1–25)4P (from 50 to 200 μmol L^{-1}) and Ca uptake by HT29 cells in contrast with α_{s1}-CN (f 59–79)5P. Differences in



CPP: caseinophosphopeptide
ACP: amorphous calcium phosphate
PDL: periodontal ligament
SBS: shear bond strength

Fig. 15.2 Summary of the bioactive properties of caseinophosphopeptide-amorphous calcium phosphate (CPP-ACP) in the oral cavity (taken from Nongonierma and FitzGerald, 2012a)

the aggregation behaviour between β-CN (f 1–25)4P and α_{s1}-CN (f 59–79)5P in the presence of Ca were seen. It has been suggested that the bioactive form of β-CN (f 1–25)4P may be aggregated (Gravaghi *et al.*, 2007).

Fe uptake by Caco-2 intestinal cell cultures and rat duodenal loop has been shown to depend on the CPP preparation. Fe uptake was about twofold higher with β-CN (f 1–25)4P than with α_{s1}-CN (f 59–79)5P (Kibangou *et al.*, 2005). It has been reported that the CPP fragment β-CN (f 1–25)4P can bind 4–5 Fe (Bouhallab and Bouglé, 2004). Furthermore, Fe was not released from Fe-β-CN (f 1–25)4P complexes during *in vitro* simulated gastrointestinal digestion (SGID). This was attributed to the negatively charged phosphate groups having a protective effect against CPP degradation by gastrointestinal enzymes (Ait Oukhatar *et al.*, 2000).

The the influence of CPP preparation on the bioavailability of Fe and Zn has been studied (García-Nebot *et al.*, 2013). It was demonstrated for the first time that CPPs (β-CN (f 1–25)4P, α_{s1}-CN (f 64–74)4P and α_{s2}-CN (f 1–19)4P) induced an increase in ferritin (a surrogate biomarker for Fe^{2+} storage) synthesis in Caco-2 cells. The highest ferritin synthesis being obtained with β-CN (f 1–25)4P. When Fe^{2+} was added to the CPPs, a further increase in ferritin synthesis was seen as compared to the CPPs free from Fe. Different CPPs were shown to increase Zn uptake in Caco-2 cells with different

mechanisms being involved depending on the type of CPP. For the SGID of α_{s1}-and α_{s2}-CN, an increase in Zn retention in Caco-2 cells was seen whereas an increase in Zn permeation was involved for the SGID of β-CN and α_{s1}-CN (f 64–74)4P (García-Nebot *et al.*, 2013).

It has been demonstrated that a commercial CPP preparation (CE90 CPP III, DMV International, Veghel, The Netherlands) could modulate Ca uptake by human osteoblasts with a dose-response relationship (Donida *et al.*, 2009). Tulipano *et al.* (2010) showed that only short CPP sequences (ten amino acid residues) had a biological activity (i.e., cell growth, Ca uptake and deposition on the extracellular matrix) in mouse osteoblast like-cells. In contrast, no, or very little biological effect was seen for these markers with the 13 amino acid residue CPPs studied. Besides peptide length, it has been shown that slight modifications in CPP sequence can affect their biological activity in osteoblast cells. Two CPP sequences derived from α_{s2}-CN differing in one amino acid, which was due to genetic variant differences (variant C vs. variants A, B and D), displayed different effects regarding cell viability. The CPP containing a Thr residue could bind more Ca than the peptide with an Ile residue. This was related to the ability of Glu-Gln-Leu-Ser-Thr-Ser-Glu-Glu-Asn-Ser to form more stable complexes with Ca. The negative role of these two peptides on extracellular remineralisation might be due to the fact that they prevent Ca precipitation with inorganic phosphate (Pi), therefore, hindering the deposition of hydroxyapatite crystals in osteoblast cells (Tulipano *et al.*, 2010).

Picariello *et al.* (2010) subjected milk proteins to SGID. CPPs were found amongst the major CN-derived peptides which survived SGID. CPPs such as α_{s1}-CN (f 104–119)1P and β-CN (f 26–41)1P were degraded when CN was dephosphorylated, suggesting that phosphorylation may play a role in the stability of these CPPs to SGID (Ait Oukhatar *et al.*, 2000; Picariello *et al.*, 2010). Similarly, after SGID of an adapted and a follow-up milk-based infant formula, CPPs were detected with 50 % of the fragments containing the acidic motif. In addition, the CPP sequences initially present in the infant formulas were still found after SGID, indicating that they were not degraded during digestion and would have the potential to play a physiological role in the organism (Miquel *et al.*, 2005). β-CN (f 1–25) binding to different minerals (Ca, Mg, Zn and Cu) has been studied in conditions mimicking the ileum (pH 8 and 37 °C) using isothermal titration calorimetry. It was shown that 1 mol of β-CN (f 1–25)4P could bind 2 mol of Ca, Mg or Zn. However, only a weak or no binding to Cu could be determined (Zidane *et al.*, 2012). Low binding affinity between 4900 and 11,200 M^{-1} was observed, suggesting that the minerals may be released *in vivo*. Some studies suggest that certain CPP sequences have a small enough size to be able to cross the gut barrier. Following SGID of two different infant formulae, CPPs with a molecular mass ranging from 1.4 to 9.6 kDa were generated (Miquel *et al.*, 2006). However, it is not clear if CPP complexes are able to cross the gut and reach the circulatory system. To our knowledge, there is no report in the literature demonstrating the presence of CPPs in the blood of mammals.

Due to the susceptibility of BAPs to enzymatic degradation in the gastrointestinal tract and in the serum, and the need to cross the gut barrier for BAPs with a systemic target, it has been suggested that the limiting factor in the *in vivo* bioactivity of BAPs may be their bioavailability (Gardner, 1983; Panchaud *et al.*, 2012). For this reason, short peptides may be more bioavailable BAP candidates which may survive enzymatic degradation *in vivo* (Udenigwe and Aluko, 2012). Foltz *et al.* (2009) studied the intestinal stability of dipeptides and showed that half of the 228 dipeptides studied were highly stable to hydrolysis by pancreatic enzymes. Nasogastric intubation methodology has been employed to study the peptides released in humans following the ingestion of CNs. It was shown that BAPs could be detected in the jejunum in amounts which were sufficient for a bioactivity to be seen (Boutrou *et al.*, 2013). Peptides are absorbed through a transcellular (endocytosis/transcytosis) or paracellular route in the intestine (Jahan-Mihan *et al.*, 2011; Panchaud *et al.*, 2012; Fernández-Musoles *et al.*, 2013a). It is thought that hydrophilic peptides

are mainly absorbed through the paracellular route whereas hydrophobic peptides are absorbed through the transcellular route (Panchaud *et al.*, 2012). During permeation through intestinal cells, a high proportion of peptides (>90 %) may be further degraded to amino acids (Jahan-Mihan *et al.*, 2011). The totality of parameters which dictate the stability of BAPs in the gastrointestinal tract are still poorly understood (Jahan-Mihan *et al.*, 2011).

15.2.1.3 *Ex Vivo* and *In Vivo* Evaluation

Despite the fact that CPPs are present in the gastrointestinal system following dairy protein or CPP ingestion, conflicting evidence exists in the literature regarding the relationship between Ca absorption and CPP intake (Meisel and Frister, 1988; FitzGerald, 1998; FitzGerald and Meisel, 2003a; Meisel and FitzGerald, 2003). To date, a limited number of *in vivo* studies have been carried out in respect of the biological effects of CPP mineralising properties. CPPs have been detected in the intestinal fluid of humans with an ileostomy. Higher levels were detected after ingestion of milk while none or very low CPP levels were detectable following direct ingestion of CPPs (Meisel *et al.*, 2003). CPPs have been found in the distal small intestine of the rat, and in the stomach, duodenum and distal ileum of human subjects (Hartmann and Meisel, 2007) following the consumption of dairy ingredients and products. The presence of CPPs in the gastrointestinal tract and in the faeces of rats fed with CNs has also been reported, which suggested their resistance to gastrointestinal digestion (Kasai *et al.*, 1995). The resistance of CPPs to gastrointestinal digestion has been attributed to their anionic characteristic (Kitts, 2005).

Different animal studies have shown an increase in mineral bioavailability in the presence of CPPs. The mineral binding properties of CPPs have been linked with an increase in Ca absorption in rats by 40 and 60 % at a Ca:Pi of 1:1 and 1:2, respectively (Erba *et al.*, 2002). Addition of CPPs in the diet of growing male rats stimulated the availability of Ca in the femur (Mora-Gutierrez *et al.*, 2007). In contrast, other studies have demonstrated no role or a negative role of the ingestion of CPPs on bone tissue remineralisation (Tulipano *et al.*, 2010) or bone mineral content and density (McKinnon *et al.*, 2010).

Because of the relatively low bioavailability of many BAPs, it has been suggested that their biological effect may be related to an accumulation of the BAPs in target tissues (Jauhiainen *et al.*, 2007) or to a direct activity of such peptides in the gut (van der Pijl *et al.*, 2008). Increased Fe absorption has been shown *ex vivo* using rat duodenum perfused with β-CN (f 1–25)-Fe as compared to iron gluconate (Pérès *et al.*, 1999). It was demonstrated that the Fe originating from β-CN (f 1–25)-Fe could be absorbed by passive diffusion and endocytosis when bound to an amino acid or a peptide. It was also demonstrated in rats that intestinal perfusion with β-CN (f 1–25)-Fe resulted in an increased net Fe absorption (Pérès *et al.*, 1999). In the presence of β-CN (f 1–25) and Ca^{2+}, a significant increase in Fe bioavailability was seen in rats (Peres *et al.*, 1997). MS studies of degradation products obtained after β-CN (f 1–25) infusion in rat duodenum revealed the presence of various peptide fragments. However, the phosphorylated fragment of β-CN (f 15–24) could not be detected in the lumen. Therefore, it has been proposed that this fragment may be able to act as an iron vector at the brush border level (Bouhallab *et al.*, 1999). The impact of membrane alkaline phosphatase activity on the degradation of β-CN (f 1–25) and β-CN (f 1–25)-Fe was studied in perfused rat intestinal loops (Ani-Kibangou *et al.*, 2005). In the presence of an alkaline phosphatase inhibitor (Na_2WO_4), the concentration of β-CN (f 1–25)-Fe in the lumen decreased, suggesting that the complex could be absorbed in the intestine. The amounts of Fe absorbed were lower without phosphatase inhibitor, which may suggest that a certain proportion of the Fe may be released from β-CN (f 1–25) following the hydrolytic action of phosphatases. α_{s1}-CN derived CPPs may have an inhibitory effect on Fe^{2+} absorption due to their high levels of phosphorylation which negatively impact on Fe^{2+} bioavailability (Bouhallab *et al.*, 2002).

Addition of CPPs to the diet of rats fed with phytates, which are inhibitors of mineral uptake,

led to a better absorption for Ca and Zn in a rat pup model. Other inhibitors of Zn absorption include trace elements such as Fe. It has been shown in a rat duodenal loop that the utilisation of β-CN (f 1–25) could have a protective effect against the inhibition of Zn absorption in the presence of Fe (Pérès *et al.*, 1998). The nature of the CPP salt has been shown to have different effects on the absorption of Zn with a better absorption being seen with CPP-Ca complexes compared to CPP-Na complexes (Hansen *et al.*, 1996).

Nongonierma and FitzGerald (2012a) examined the scientific evidence supporting the bioactive potential of CPPs as anticariogenic agents in humans. Out of 16 human intervention studies conducted with CPP-ACP, 13 (80 %) of the studies concluded in a beneficial role of CPP-ACP on teeth remineralisation (Table 15.1). The contradictory outcomes from *in vivo* clinical trials on dental enamel remineralisation with CPP-ACP may be related to the setup of the trial. Parameters such as the vehicle, residence time and contact of CPP-ACP with the teeth are crucial for bioactivity to be observed.

It was shown that consumption of milk supplemented with β-CN (f 1–25)-Fe could increase Fe tissue uptake but no significant differences were found in terms of its absorption compared to ferrous sulfate in young females (Ait-Oukhatar *et al.*, 2002). A randomised human trial was carried out with 15 human subjects consuming one control drink (Ca lactate drink) and 2 test drinks (Ca lactate drink with CPP1 or CPP2 obtained following the hydrolysis of milk proteins with two different enzyme activities). Differences in the true fractional Ca absorption (TFCA), which was calculated by dividing the proportion of Ca in the urine by the proportion of Ca ingested, were seen. TFCA was reduced from 26.0 for the control to 22.7 and 23.5 for CPP1 and CPP2, respectively (Teucher *et al.*, 2006). The conflicting bioavailability results in the literature may be due to differences in the methodology utilised for mineral quantification, the presence of chelating agents (such as phytates, oxalates and tannins), the food matrix, CPP preparation, CPP dose and CPP:mineral ratio (Gueguen and Pointillart, 2000; Nongonierma and FitzGerald, 2010).

15.2.2 ACE and Renin Inhibitory Peptides and Hydrolysates

High blood pressure, or hypertension, is a risk factor for the development of cardiovascular disease (CVD) including stroke, myocardial infraction and heart failure. CVD claimed 17.3 million lives worldwide in 2008 and this figure is expected to rise to 23.3 million by 2030 according to the latest World Health Organisation (WHO) data (WHO, 2013b). The lowering of blood pressure is therefore a common target for the prevention and treatment of CVDs. Renin is part of the renin-angiotensin system (RAS) where it is responsible for the conversion of angiotensinogen to angiotensin I. ACE further hydrolyses angiotensin I to angiotensin II, leading to downstream vasoconstriction. ACE is also part of the kinin-nitric oxide system (KNOS) where it converts the highly potent vasodilator, bradykinin, into inactive fragments (FitzGerald *et al.*, 2004). Dysfunctions in the RAS and/or KNOS systems lead to hypertension and resultant CVD. Inhibition of both renin and ACE therefore, present targets for the control of hypertension.

ACE inhibitors, by blocking the action of ACE, are responsible for the systemic lowering of blood pressure thus acting as antihypertensive agents. Pharmacological inhibitors of ACE include Captopril, Enalapril, Ramipril and Lisinopril. However, these drugs may result in many undesired side-effects. This has led to the search for ACE inhibitors derived from natural sources which generally tend to be less potent but do not have such undesirable side-effects. ACE inhibitory peptides have been released from many food protein sources as reviewed elsewhere (Hartmann and Meisel, 2007; Erdmann *et al.*, 2008; Korhonen, 2009; Martinez-Maqueda *et al.*, 2012; Norris and FitzGerald, 2013). Milk proteins (Korhonen, 2009), including CN (Phelan *et al.*, 2009b) and whey (FitzGerald and Meisel, 1999; Pihlanto-Leppälä, 2000; Madureira *et al.*, 2010; Hernandez-Ledesma *et al.*, 2011) are rich sources of ACE inhibitory peptides.

15.2.2.1 Structure Function

There are many bovine milk protein-derived ACE inhibitory peptides reported in the literature with varying potencies (see Table 15.2). In gen-

Table 15.1 Summary of the outcomes of human trials evaluating the mineralising role of caseinophosphopeptide-amorphous calcium phosphate (CPP-ACP) complexes in the oral cavity (taken from Nongonierma and FitzGerald, 2012a)

Product tested	Number of participants	Duration	Results	Reference
Test group: Mouth rinse with (1) 2 % CPP-ACP Recaldent™ (2) 6 % CPP-ACP Recaldent™ (3) unstabilised slurry of calcium and sodium phosphate	30 adults	5 days	Increase in Ca and Pi level in the plaque in a dose-dependent manner. CPP-ACP localised at the bacteria surface and in the intercellular plaque matrix	Reynolds *et al.* (2003)
Control group: Deionised water				
Test group: Sugar-free chewing gum Recaldent™ containing 9.5 mg of CPP-ACP	30 adults	4 days	Increase in CPP level in the plaque. 132 ng of CPP/mg plaque (25 % of this amount still present in the plaque 3 h after gum chewing)	
Control group: None				
Test group: 3 months treatment with CPP-ACP paste without fluoride (Topacal C5) + 3 months fluorinated toothpaste	26 adolescents	6 months (12 months follow-up)	63 % WSL sites totally disappear with the CPP-ACP treatment vs. 25 % for the control after 12 months (55 and 18 %, respectively, after 6 months)	Andersson *et al.* (2007)
Control group: 6 months 0.05 % NaF mouth wash + fluoride toothpaste (1000–1100 ppm)				
Test group: Microabrasion of WSL followed by CPP-ACP paste (Tooth Mousse) application (15 min, twice daily)	Not disclosed	Up to several months (not specified)	Natural tooth appearance recovered with elimination of superficial WSL	Ardu *et al.* (2007)
Control group: None				
Test group: (1) mouth rinse (Recaldent™) with 2 % CPP-ACP + 450 ppm fluoride and (2) mouth rinse with 450 ppm fluoride	14 adults	5 days	Plaque fluoride level doubled with the fluoride mouth rinse as compared to the control	Reynolds *et al.* (2008)
Control group: Deionised water			Plaque fluoride level with the 2 % CPP-ACP + fluoride mouth rinse more than two times that obtained with the fluoride mouth rinse	
Test group: Sugar free gum containing (54 mg CPP-ACP)	2720 adolescents	24 months	18 % reduction in approximal caries and 53 % greater regression with the CPP-ACP group as compared to the control group	Morgan *et al.* (2008)
Control group: Sorbitol-based sugar-free gum				
Test group: Tooth cream with 10 % CPP-ACP (Tooth Mousse/ MI Paste) + fluoride toothpaste (1100 ppm) and mouth rinse (900 ppm)	45 adolescents (post orthodontic population)	12 weeks	31 % more regression of the WSL as compared to the control	Bailey *et al.* (2009)
Control group: Placebo cream + fluoride toothpaste (1100 ppm) and mouth rinse (900 ppm)				

(continued)

Table 15.1 (continued)

Product tested	Number of participants	Duration	Results	Reference
Test groups: Tooth Mousse topical application after tooth brushing	10 orthodontic patients (average age 17.7 years)	2 months follow-up	Reduction of enamel demineralisation of WSEL	Zhou *et al.* (2009)
Control group: None				
Test groups: (1) Toothpaste with 2 % CPP and (2) Toothpaste with 1190 ppm Fluoride and 0.76 % sodium monofluorophosphate (SMFP)	150 adolescents	24 months	Regression of caries as compared to the control group	Rao *et al.* (2009)
Control group: Placebo toothpaste without CPP			No significant differences of decayed surface of the carious lesions in the CPP group and the SMFP group	
Test group: Tooth Mousse in combination with fluoride toothpaste	60 adolescents	4 weeks	No significant difference in the regression of WSL between the test and the control groups	Brochner *et al.* (2011)
Control group: Fluoride toothpaste				
Test group: CPP-ACP (10 % (w/w) – MI paste) + non-fluoride toothpaste	8 adults	24 months	Increase of the pH of WSEL during 24 months (from 5.94 to 6.70) toward that of sound enamel	Kitasako *et al.* (2010)
Control group: None			Visual improvement of the appearance of WSEL	
Test group: CPP-ACP (0.2 % (w/w)) paste (Recaldent™ GC Tooth Mousse) applied with fluoride trays without rinsing	30 children (6 to 9 years) with molar incisor hypomineralisation (MIH)	3 years 4 months follow-up	More geometric, mature and mineralised MIH	Baroni and Marchionni (2011)
Control group: Healthy premolar of the same subjects studied			Increase in the potential MIH enamel structure	
Test group: CPP-ACP (Tooth Mousse) + sodium fluoride (1450 ppm F) toothpaste	26 adults (22–31 years)	3 weeks	Significant reduction of laser fluorescence in the CPP-ACP group after 15 days, compared to the control group. Reduction of the enamel surface porosity in the CPP-ACP group	Altenburger *et al.* (2010)
Control group: sodium fluoride (1450 ppm F) toothpaste			No difference between the 2 groups while performing a visual analysis of the fissures	
Test group: CPP-ACP (Tooth Mousse) + sodium fluoride topical gel	21 adolescents (13–17 years)	60 days	Prevention of demineralisation of the enamel around orthodontic brackets with CPP-ACP and fluoride. No significant difference between CPP-ACP and fluoride	Uysal *et al.* (2010)
Control group: No agent applied on the tooth surface				

(continued)

Table 15.1 (continued)

Product tested	Number of participants	Duration	Results	Reference
Test group: CPP-ACP (0.2 % (w/w)) + sodium fluoride (900 ppm) paste (MI Paste plus) in combination with fluoride toothpaste	54 orthodontic adolescents after debonding	12 weeks (follow-up 3 months)	No significant change in the size of the WSL between the two groups	Beerens *et al.* (2010)
			Decrease in the percentage of aciduric bacteria from 47.4 to 38.1 % and *S. mutans* from 9.6 to 6.6 %	
Control group: Fluoride-free control paste (Ultradent) in combination with fluoride toothpaste			No advantage of CPP-ACFP paste in addition to normal oral hygiene	
Test group: CPP-ACP (MI Paste) applied after teeth brushing with a fluoride paste	60 orthodontic adolescents	3 months	Reduction of enamel decalcification index score by 53.5 % with MI Paste	Robertson *et al.* (2011)
Control group: Placebo fluoride paste			Reduction of the number of WSL and protective effect of CPP-ACFP	
Test group: CPP-ACP (Tooth Mousse) on demineralised teeth	40 adolescents (10–16 years)	1 month	Formation of an amorphous layer on demineralised teeth following CPP-ACP treatment	Ferrazzano *et al.* (2011)
Control group: placebo gel on demineralised teeth/sound and demineralised teeth with no topical treatment				

Table 15.2 Some bovine milk protein-derived angiotensin-I-converting enzyme (ACE) inhibitory peptides (adapted from Meisel *et al.*, 2006; Saito, 2008)

Protein source[a]	Fragment	Peptide sequence	IC_{50} (μM)[b]
α_{s1}-CN	(f 25–27)	VAP	2
	(f 23–27)	FFVAP	6
	(f 24–27)	FVAP	10
	(f 90–91)	RY[c]	10.5
	(f 1–9)	RPKHPIKHQ	13.4
	(f 194–199)	TTMPLW	16
	(f 104–109)	YKVPQL	22
	(f 197–199)	PLW	36
	(f 198–199)	LW	50
	(f 142–147)	LAYFYP	65
	(f 23–34)	FFVAPFPEVFGK	77
	(f 157–164)	DAYPSGAW	98
	(f 91–92), (f 94–95)	YL[d]	122
	(f 28–34)	FPEVFGK	140
	(f 32–34)	FGK	160
	(f 27–30)	PFPE	>1000
	(f 143–147)	AYFYP	>1000
	(f 136–139)	LFRQ	17[e]
	(f 143–148)	AYFYPE	106[e]

(continued)

Table 15.2 (continued)

Protein source[a]	Fragment	Peptide sequence	IC_{50} (μM)[b]
α_{s2}-CN	(f 174–179)	FALPQY	4.3
	(f 174–181)	FALPQYLK	4.3
	(f 92–98)	FPQYLQY	14
	(f 182–184)	TVY	15
	(f 204–207)	VRYL	24.1
	(f 25–32)	NMAINPSK	60
	(f 201–202)	IPY	206
	(f 81–89)	ALNEINQFY	219
	(f 81–91)	ALNEINQFYQK	264
	(f 190–197)	MKPWIQPK	300
	(f 198–202)	TKVIP	400
	(f 189–192)	AMKPW	580
	(f 189–197)	AMKPWIQPK	600
β-CN	(f 74–76)	IPP[f]	5
	(f 169–174)	KVLPVP	5
	(f 84–86)	VPP	9
	(f 177–183)	AVPYPQR	15
	(f 49–61)	IHPFAQTQSLVYP	19
	(f 52–61)	FAQTQSLVYP	25
	(f 50–61)	HPFAQTQSLVYP	26
	(f 48–61)	KIHPFAQTQSLVYP	39
	(f 57–61)	SLVYP	40
	(f 56–61)	QSLVYP	41
	(f 59–61)	VYP	44
	(f 55–61)	TQSLVYP	64
	(f 54–61)	QTQSLVYP	73
	(f 53–61)	AQTQSLVYP	76
	(f 177–181)	AVPYP	80
	(f 58–610)	LVYP	170
	(f 183–190)	RDMPIQAF	209
	(f 179–181)	PYP[g]	220
	(f 59–64)	VYPFPG	221
	(f 177–183)	AVPYPQR	274
	(f 193–198)	YQEPVL	280
	(f 59–61)	VYP	299
	(f 193–202)	YQEPVLQPVR	300
	(f 62–63), (f 111–112), (f 157–158), (f 205–206)	FP[h]	315
	(f 177–179)	AVP	340
	(f 108–113)	EMPFPK	423
	(f 140–143)	LQSW	500
	(f 60–66)	YPFPGPI	500
	(f 80–90)	TPVVVPPFFQP	749
	(f 191–197)	LLYQQPV	>1000
	(f 133–138)	LHLPLP	7[e]
	(f 58–76)	LVYPFPGPIPNSLPQNIPP	19[e]

(continued)

Table 15.2 (continued)

Protein source[a]	Fragment	Peptide sequence	IC_{50} (μM)[b]
κ-CN	(f 185–190)	VTSTAV	30[e]
	(f 25–34)	YIPIQYVLSR	100
	(f 58–59)	YP[i]	720
α-La	(f 104–108)	WLAHK	77
	(f 99–108)	VGINYWLAHK	327
	(f 50–52)	YGL	409
	(f 105–110)	LAHKAL	621
	(f 50–53)	YGLF	733.3
	(f 50–51)	YG	1522.6
	(f 52–53)	LF[j]	349
β-Lg	(f 142–148)	ALPMHIR	42.6
	(f 102–103)	YL	122.1
	(f 78–80)	IPA	141
	(f 102–105)	YLLF	171.8
	(f 142–146)	ALPMH	521
	(f 15–19)	VAGTW	534
	(f 9–14)	GLDIQK	580
	(f 34–40)	LDAQSAPLR	635
	(f 148–149)	RL	2438.9
	(f 106–111)	CMENSA	788
	(f 146–148)	HIR	953
	(f 94–100)	VLDTDYK	946
	(f 81–83)	VFK	1049
	(f 22–25)	LAMA	1062
	(f 146–149)	HIRL	1153.2
	(f 147–148)	IR	695.5
	(f 15–20)	VAGTWY	1682
	(f 10–14)	LDIQK	17[e]
	(f 1–5)	LIVTQ	17[e]
	(f 81–82)	VF	19[e]
	(f 7–9)	MKG	24[e]
BSA	(f 215-223)	ALKAWSVAR	3
	(f 228-228)	FP	315

[a]*CN* casein, *α-La* α-lactalbumin, *β-Lg* β-lactoglobulin, *BSA* bovine serum albumin

[b]IC_{50}, concentration of peptide resulting in a 50 % inhibition of ACE activity

[c]This sequence is also found in α_{s2}-casein (f 170–171) and (f 205–206), κ-casein (f 34–35) and serum albumin (f 409–410)

[d]This sequence is also found in α_{s2}-casein (f 95–96), (f 98–99), (f 179–180) and (f 206–207), serum albumin (f 30–31), (f 137–138) and (f 451–452) and in β-lactoglobulin (f 102–103)

[e]IC_{50} values expressed as mg L^{-1}

[f]This sequence is also found in κ-casein (f 108–110)

[g]This sequence is also found in κ-casein (f 57–59)

[h]This sequence also occurs in α_{s1}-casein (f 28–29), α_{s2}-casein (f 92–93) and serum albumin (f 222–223)

[i]This sequence is also found in α_{s1}-casein (f 146–147) and (f 159–160) and in β-casein (f 114–115)

[j]This sequence also occurs in α_{s1}-casein (f 149–150), serum albumin (f 69–70) and (f 505–506) and in β-lactoglobulin (f 104–105)

eral, peptides derived from CNs are more potent than those derived from whey proteins. As can be seen from Table 15.2, most of the ACE inhibitory peptides derived from bovine milk proteins are relatively short (2–12 amino acids). Although a detailed structure activity relationship has yet to be confirmed for ACE inhibitory peptides it is generally accepted that the three C-terminal peptides are relevant (Murray and FitzGerald, 2007). The presence of one or more Pro residues in these three terminal positions has been shown to be of importance. Moreover, the amino acids Tyr, Phe, Trp or Leu are notably present in the C-terminus of many potent ACE inhibitory peptides (Ruiz *et al.*, 2004; Meisel *et al.*, 2006). Furthermore, the presence of a positively charged Lys or Arg at the C-terminus seems to contribute to the potency of ACE inhibitory peptides (López-Fandiño *et al.*, 2006).

15.2.2.2 *In Vitro* Assessment/ Screening

Traditionally, two main strategies have been employed in the research of milk protein-derived ACE inhibitory peptides: (i) enrichment and isolation of BAPs from hydrolysates and fermentates and (ii) the chemical synthesis of potential ACE inhibitory peptides and their analogues. More recently, *in silico* approaches have been used for the discovery of ACE inhibitory peptides as discussed in Sect. 15.4.2. Among the milk protein-derived peptides, those derived from CNs have shown the greatest potency with Val-Ala-Pro from α_{s1}-CN (IC_{50} 2.0 μM), Phe-Ala-Leu-Pro-Gln-Tyr and Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys from α_{s2}-CN (IC_{50} of both 4.3 μM) and Ile-Pro-Pro from β-CN (IC_{50} 5.0 μM), being the most potent to date, see Table 15.2.

Research to date has, in general, found that there is a poor correlation between ACE inhibition *in vitro* and hypotensive effects *in vivo*. This is probably due to peptide bioavailability issues, leading to the need to review the *in vitro* methods currently used to assess ACE inhibition (López-Fandiño *et al.*, 2006). Furthermore, there may be other hypotensive effects involved such as inhibition of endothelin converting enzyme, opioid-induced blood pressure regulation, renin inhibition, nitric oxide, bradykinin and calcium channel blockers (Norris and FitzGerald, 2013).

Much research has been conducted into investigating the ability of ACE inhibitory peptides to cross the intestine wall into the bloodstream and thus to become bioavailable. Transepithelial transport of ACE inhibitory peptides through Caco-2 cells has been demonstrated with Ala-Leu-Pro-Met-His-Ile-Arg (Vermeirssen *et al.*, 2002), His-Leu-Pro-Leu-Pro (Quirós *et al.*, 2008), Val-Pro-Pro (Satake *et al.*, 2002), Gly-Gly-Tyr-Arg (Shimizu *et al.*, 1997), and Arg-Trp-Gln (LF, B(f 21–23)) and Trp-Gln (LF B (f 23–24)) (Fernández-Musoles *et al.*, 2013a). Hydrophobic peptides have been associated with a high transepithelial permeation through a Caco-2 cell monolayer (Shimizu *et al.*, 1997). Di and tri-peptide uptake in the intestine occurs through a proton coupled transporter PepT1, resulting in a greater extent of uptake than that of free amino acids (Adibi and Morse, 1971; Panchaud *et al.*, 2012). The level of BAPs which have been found to cross the Caco-2 cells in their intact form is, however, quite low. A proportion <2 % of Val-Pro-Pro was shown to permeate through Caco-2 cell monolayers in 60 min (Satake *et al.*, 2002). It was shown that dipeptides (Leu-Trp, Phe-Tyr and Ile-Tyr) and the ACE inhibitory tripeptide Ile-Pro-Pro could reach the circulation without being degraded following ingestion of a lacto-tripeptide enriched yogurt beverage (Foltz *et al.*, 2007). Similarly in rats, it was found that Ile-Pro-Ile was rapidly absorbed intact after oral administration and was slowly excreted in faeces and urine. Ile-Pro-Pro was distributed in different tissues, when administered at 7 mg kg^{-1} (Jauhiainen *et al.*, 2007). The pharmacokinetics of Ile-Pro-Pro, Leu-Pro-Pro and Val-Pro-Pro, which were administered intragastrically *via* a saline solution, was studied in piglets. Bioavailability of the three peptides was found to be as low as 0.1 %. The half-life of absorption and elimination for the tripeptides were 3 and 15 min, respectively, suggesting an acute response (van der Pijl *et al.*, 2008). Studies have shown that many peptides retain their ACE inhibitory properties following SGID even in situations where there was further breakdown of peptides by the gastrointestinal

enzymes (Tavares *et al.*, 2011a; Ruiz-Giménez *et al.*, 2012).

Discovery of renin inhibitory peptides is still in its infancy. A number of studies have reported renin inhibitory peptides from different protein sources including hydrolysates of the macroalga, *Palmaria palmata* (Harnedy and FitzGerald, 2013), chicken skin protein hydrolysates (Onuh *et al.*, 2013), rapeseed protein hydrolysate (He *et al.*, 2013) and pea protein hydrolysates (Li and Aluko, 2010). Although there are, to our knowledge, no reported studies on milk protein-derived renin inhibitory peptides to date, it should be noted that, for example, two of the renin inhibitory di-peptide sequences discovered in rapeseed, namely Leu-Tyr and Thr-Phe, are also found within the sequences of bovine milk proteins.

15.2.2.3 *In Vivo* Studies (Small Animal and Human)

Animal studies on ACE inhibitory peptides have, in the main, been carried out on spontaneously hypertensive rats (SHR), a commonly accepted model of human hypertension. The CN-derived tripeptides, Ile-Pro-Pro and Val-Pro-Pro with IC_{50} values of 5 and 9 μM, respectively (Nakamura *et al.*, 1995a) were among the first food protein-derived peptides shown to have a blood pressure lowering effect on SHRs following a single oral dose of peptide (Nakamura *et al.*, 1995b). ACE inhibitory peptides from a bovine CN hydrolysate resulted in a reduction in systolic blood pressure (SBP) and a marked reduction in diastolic blood pressure (DBP) as measured by the rat tail cuff method following a single oral dose of the pepsin-hydrolysed CN and its 3 kDa permeate (Miguel *et al.*, 2009). Furthermore, specific CN-derived peptide sequences resulted in reductions in SBP and DBP, including the β-CN derived peptides, Leu-Val-Tyr-Pro-Phe-Thr-Gly-Pro-Ile-Pro-Asp (−28.0 mmHg) and His-Leu-Pro-Leu-Pro (−23.5 mmHg) (Miguel *et al.*, 2010). Bovine LF derived peptides Leu-Ile-Trp-Lys-Leu, Arg-Pro-Tyr-Leu and Leu-Asn-Asn-Ser-Arg-Pro with IC_{50} values of 0.47, 56.5 and 105.3 μM, respectively, reduced SBP by −25.3, −18.9 and −15.3 mmHg, respectively. Furthermore, Leu-Ile-Trp-Lys-Leu and Arg-Pro-Tyr-Leu induced contraction in vascular tissue in an *ex vivo* assay on the rabbit carotid artery (Ruiz-Giménez *et al.*, 2012). The <3 kDa fraction of the pepsin hydrolysate from which these peptides arose also showed inhibitory effects on vasoconstriction mediated by endothelin converting enzyme (Fernández-Musoles *et al.*, 2013b). Peptides arising from hydrolysis of whey proteins have been shown to have antihypertensive effects in SHRs in many studies (Abubakar *et al.*, 1998; FitzGerald and Meisel, 2000; Pihlanto-Leppälä, 2000) that resulted in lowering of SBP in SHRs. A summary of the antihypertensive effects of some bovine milk protein-derived peptides on SHRs can be found in Table 15.3.

Some studies have investigated the antihypertensive potential of ACE inhibitory peptides when consumed/administered to humans. The *in vivo* antihypertensive effects of the most studied milk protein-derived ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, have been shown in several human studies as reviewed by Korhonen (2009), where it was noted that reductions in SBP and DBP of 1.5–14.0 and 0.5–6.8 mmHg, respectively, have been achieved. These peptides are found in the commercially available milk products, Ameal (Calpis Co. Ltd, Tokyo, Japan) and Evolus® (Valio Ltd, Helsinki, Finland). Recent studies by Goudarzi and co-workers found that a whey protein hydrolysate whose production had been optimised for the generation of peptides with high *in vitro* ACE inhibition and antioxidant activity (Goudarzi *et al.*, 2012) did not reduce blood pressure in pre-hypertensive subjects following a 1 week feeding period (Goudarzi and Madadlou, 2013). This highlights the importance of *in vivo* testing of ACE inhibitory BAPs.

A number of meta-analyses of antihypertensive peptides derived from milk proteins have been performed. Xu *et al.* (2008), in a meta-analysis (total 623 participants) of randomised controlled trials of the effect of Ile-Pro-Pro and Val-Pro-Pro on blood pressure, found that these CN-derived tripeptides had a beneficial effect on the blood pressure of both hypertensive and pre-hypertensive subjects with decreases of 4.8 and 2.2 mmHg in SBP and DBP, respectively. Pripp

Table 15.3 Antihypertensive activity in spontaneously hypertensive rats of some bovine milk protein-derived peptides (adapted from FitzGerald *et al.*, 2004; Saito, 2008; Martinez-Maqueda *et al.*, 2012)

Source protein[a]	Preparation	Peptide sequence	Dose (mg kg^{-1})	SBP (mmHg)[b]
α_{s1}-CN	Trypsin hydrolysate	FFVAPFPGVFGK	100	−34.0
	L. helviticus CPN4 fermentation	YP	2	−32.1
	Pepsin hydrolysate	RYLGY	5	−25.0
	Pepsin hydrolysate	AYFYPEL	5	−20.0
	Trypsin hydrolysate	TTMPLW	100	−13.6
	Proteinase of *L. helveticus* CP 790	YKVPQL	2	−12.5
	Cheese	RPKHPIKHQ	6.1–7.5	−9.3
α_{s2}-CN	Pepsin hydrolysate	PYVRYL	3	−23.4
	Pepsin hydrolysate	YQKFPQY	5	−15.0
	Proteinase of *L. helveticus* CP 790	TKVIP		−9.0
	Proteinase of *L. helveticus* CP 790	AMPKPW	2	−5.0
	Proteinase of *L. helveticus* CP 790	MKPWIQPK	2	−3.0
β-CN	Proteinase of *L. helveticus* CP 790	KVLPVP	1	−32.2
	Proteinase of *L. helveticus* CP 790	KVLPVPQ	2	−31.5
	L. helviticus and *S. cerevisiae* fermentation	IPP	1	−28.3
	Caprine kefir	LVYPPFTGPIPN	10	−28.0
	Proteinase K	FP	8	−27.0
	E. faecalis fermentation	LHLPLP	3	−25.0
	E. faecalis fermentation	HLPLP	7	−23.5
	Proteinase K hydrolysate	VYPFPG	8	−22.0
	Proteinase K hydrolysate	VYP	8	−21.0
	Enzyme modified cheese	MAP	3	−17.0
	E. faecalis fermentation	VLGPVRGPFP	10	−16.2
	E. faecalis fermentation	VRGPFPIIV	10	−16.1
	E. faecalis fermentation	LVYPFPGPIPNSLPQNIPP	6	−14.9
	Trypsin hydrolysate	AVPYPQR	100	−10.0
	Proteinase K hydrolysate	TPVVVPPFLQP	8	−8.0
	E. faecalis fermentation	LHLPLPL	10	−7.7
	Cheese	YPFPGPIPN	6.1–7.5	−7.0
	Proteinase of *L. helveticus* CP 790	LQSW	2	−2.0
κ-CN	Pepsin, chymotrypsin and trypsin hydrolysate	YAKPVA	6	−23.1
	Pepsin, chymotrypsin and trypsin hydrolysate	IASGQP	6.7–7.1	−22.5
	Pepsin, chymotrypsin and trypsin hydrolysate	IAK	4	−20.7
	Pepsin, chymotrypsin and trypsin hydrolysate	WQVLPNAVPAK	7	−18.4
	Flavourzyme and *S. thermophilus* and *L. bulgaricus*	YPYY	3.4	−15.9
	Pepsin, chymotrypsin and trypsin hydrolysate	HPHPHLSF	10	−15.7
CMP	Trypsin hydrolysate	MAIPPKK	10	−28.0
α-La	Pepsin, chymotrypsin and trypsin hydrolysate	YGLF	0.1	−23.4

(continued)

Table 15.3 (continued)

Source protein[a]	Preparation	Peptide sequence	Dose (mg kg^{-1})	SBP (mmHg)[b]
β-Lg	Proteinase K hydrolysate	IPA	8	−31.0
	Thermolysin hydrolysate	LLF	10	−29.0
	Thermolysin hydrolysate	LQKW	10	−18.1
LF	Pepsin hydrolysate	RRWQWR	10	−16.7
		WQ	10	−11.4

[a]*CN* casein, *CMP* caseinomacropeptide, *α-La* α-lactalbumin, *β-Lg* β-lactoglobulin, *LF* lactoferrin

[b]Systolic blood pressure

(2008) conducted a meta-analysis of placebo-controlled trials of food peptides (a high proportion of which were the CN-derived Val-Pro-Pro and Ile-Pro-Pro) and found that there was a pooled effect of −5.13 and −2.42 mmHg on SBP and DBP, respectively. Moreover, a meta-analysis of the blood pressure lowering effects of lactotripeptides (LTPs—including Val-Pro-Pro and Ile-Pro-Pro) on a total of 1919 subjects found a statistically significant, if modest, effect on blood pressure (Qin *et al.*, 2013). Interestingly, a population specific effect of the efficacy of Val-Pro-Pro and Ile-Pro-Pro has been reported. In a meta-analysis of placebo-controlled clinical trials a more marked effect of LTPs on Asians (SBP −6.93 mmHg, DBP −3.98 mmHg) was reported compared to Caucasians (SBP −1.17 mmHg and DBP −2.82 mmHg) (Cicero *et al.*, 2011). These authors further investigated the effect of LTPs on European populations in another meta-analysis where they found a small but significant decrease in SBP (−1.28 mmHg) and DBP (−0.59 mmHg). Furthermore, this study found a significant effect of age on the reduction in BP with each additional year resulting in a further decrease of SBP by 0.09 mmHg, an effect which may be due to isolated systolic hypertension (Cicero *et al.*, 2013). Contradicting data has, however, been presented in a recent meta-analysis by Usinger *et al.* (2012) where it is reported that milk fermented with various lactobacilli had a modest overall effect on SBP (−2.45 mmHg) and no effect on DBP. The authors conclude that there is no supporting evidence of a blood pressure lowering effect of fermented milk as the studies included were heterogeneous with weak methodology.

15.2.3 Antithrombic Peptides

Thrombosis is a pathological condition that occurs when a blood clot forms in a blood vessel, thereby interrupting the flow of blood through the vessel. There are marked similarities between milk clotting and the blood clotting process as reviewed by Rutherfurd and Gill (2000) leading to the suggestion that milk proteins may be a good source of antithrombotic peptides.

15.2.3.1 Structure Function

Inhibition of platelet aggregation is a critical element of antithrombotic therapies. Fibrinogen plays an important role in platelet aggregation and fibrin formation. Structural similarities have been noted between bovine κ-CN, or its glycomacropeptide (GMP), and human fibrinogen γ-chain. Jollès and Henschen (1982) observed the structural and functional similarities between the C-terminal dodecapeptide of the human fibrinogen γ-chain His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val and various casoplatelins (i.e., peptides from the (f 106–116) region of bovine κ-CN corresponding to Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys). Residues Ile_{108}, Lys_{112} and Asp_{115} of casoplatelin have been shown to be important for antithrombotic activity (Fiat *et al.*, 1989).

15.2.3.2 *In Vitro* Assessment/ Screening

Peptides from sheep milk have been shown to have antithrombotic activities as reviewed by Hernández-Ledesma *et al.* (2011). Casoplatelin, liberated by trypsin and chymosin hydrolysis of

bovine κ-CN, is an antithrombotic peptide that inhibits ADP-induced platelet aggregation and binding of the γ-chain of fibrinogen in a dose-dependent manner (Jolles *et al.*, 1986). The κ-CN (f 112–116) peptide was found to be 30-fold more potent than the (f 106–116) with IC_{50}s of 2 and 60 μM, respectively. The C-terminal fragment of β-CN, (f 193–209) corresponding to the amino acid sequence Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val, was shown to inhibit the proteolytic activity of thrombin on fibrinogen. This peptide, which was released by fermentation of CN with *Lactobacillus casei* Shirota, had a thrombin inhibition efficacy ratio of 4.6 % per μg peptide mL^{-1} and also showed an ACE inhibition efficacy ratio of 0.14 % per μg peptide mL^{-1} (Rojas-Ronquillo *et al.*, 2012). Two antithrombotic peptides, from bovine and human κ-caseinoglycopeptides, were found in the plasma of 5-day-old babies following the ingestion of bovine milk-based formula or human breast milk, showing that such peptides can be absorbed into the bloodstream (Chabance *et al.*, 1995). There is also much evidence to suggest that high levels of oxidative stress leads to a dysfunctional vasculature including thrombus formation as reviewed in Leopold and Loscalzo (2009). It is therefore conceivable to expect that milk proteins, being a rich source of antioxidant peptides (Power *et al.*, 2013), may also play an important role in the control of thrombosis. Table 15.4 summarises some of the antithrombotic peptides derived from milk.

15.2.3.3 *In Vivo* Studies (Small Animal and Human)

GMP, the undecapeptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys (κ-CN f 106–116) and Lys-Asn-Gln-Asp-Lys (κ-CN f 112–116) were shown to have a significant antithrombotic activity for arterial thrombosis in guinea-pigs (Bal dit Sollier *et al.*, 1996). Lys-Arg-Asp-Ser corresponding to (f 38–41) of human LF was shown to have an antithrombotic effect in guinea pig and rat in experimental models of arteriolar thrombosis (Drouet *et al.*, 1990).

15.2.4 Antidiabetic Peptides and Hydrolysates

Diabetes can be defined as an inability of the body to produce or optimally respond to insulin. It has been classified into two types which differ in their clinical presentation. Type 1 diabetes, an autoimmune condition, is characterised by the destruction of pancreatic β cells which results in the inability of the organism to secrete insulin. Type 2 diabetes (T2D) corresponds to an insulin resistance in peripheral tissues (skeletal muscle, liver and fat tissue), which can evolve into deficiencies of insulin secretion and β cell dys-

Table 15.4 Some milk protein-derived antithrombotic peptides (table adapted from Hernández-Ledesma *et al.*, 2011)

Species	Protein source and fragment[a]	Peptide sequence	IC_{50} (μM)	Produced by	Reference
Sheep	CMP (f 112–116)	KDQDK	C. I.[b]	Tryptic hydrolysis	Qian *et al.* (1995)
	CMP (f 163–171)	TAQVTSTEV	C. I.[b]	Tryptic hydrolysis	Qian *et al.* (1995)
	CMP (f 165–171)	QVTSTEV	C. I.[b]	Tryptic hydrolysis	Qian *et al.* (1995)
Cow	β-CN (f 193–209)	YQEPVLGPVRGPFPIIV	4.6 %[c]	*Lactobacillus casei* Shirota fermentation	Rojas-Ronquillo *et al.* (2012)
	κ-CN (f 106–116)	MAIPPKKNQDK	60	Tryptic hydrolysis and synthetic	Jolles *et al.* (1986)
	κ-CN (f 112–116)	KNQDK	2	Tryptic hydrolysis	Maubois *et al.* (1991)
	κ-CN (f 113–116)	NQDK	400	Tryptic hydrolysis	Maubois *et al.* (1991)
Human	LF (f 38–41)	KRDS	350	Synthetic	Mazoyer *et al.* (1990)

[a]*CN* casein, *CMP* caseinomacropeptide, *LF* lactoferrin
[b]Complete inhibition
[c]Expressed as thrombin efficacy ratio % per μg mL^{-1} of peptide

function in more severe cases (Schrezenmeir and Jagla, 2000). The figures for diabetes worldwide are alarming; according to the WHO, T2D incidence is estimated to increase to 366 million sufferers in 2030 (WHO, 2006). Other illnesses have been associated with the development of insulin resistance syndrome (IRS), including impaired glucose metabolism and hyperinsulinemia together with dyslipidemia, central obesity and hypertension. The management of T2D and IRS involve strategies including changes in lifestyle, physical exercise and a balanced diet (Schrezenmeir and Jagla, 2000). The consumption of dairy products, particularly low-fat dairy products, has been linked with a reduction in the risk of T2D (Choi *et al.*, 2005; Tremblay and Gilbert, 2009; Ricci-Cabello *et al.*, 2012). Different strategies have been developed to help in the management of T2D. These comprise the stimulation of insulin secretion by pancreatic β cells (insulinotropic activity) and the regulation of key enzyme activities involved in the regulation of blood glucose such as α-glucosidase, α-amylase and dipeptidyl peptidase IV (DPP-IV).

15.2.4.1 Structure Function

While the insulinotropic action of CN-derived hydrolysates has been reported (Manders *et al.*, 2006a, b; Geerts *et al.*, 2011), most studies in the area of antidiabetic peptides to date have been performed with whey proteins and whey protein hydrolysates. The mechanisms governing the insulinotropic properties of milk protein hydrolysates are not completely understood. It has been proposed that the enhanced insulinotropic activity of hydrolysates may be correlated with higher rates of intestinal absorption of amino acids and short peptides (Geerts *et al.*, 2011). The consumption of milk protein hydrolysates by humans has been associated with an increase in plasma concentration of specific insulinotropic amino acids such as Leu, Phe, Arg and Tyr (van Loon *et al.*, 2000), and branched chain amino acid (BCAA)-containing dipeptides such as Ile-Leu and Val-Leu (Morifuji *et al.*, 2010). It has been proposed that the combined activity of free BCAA and whey protein-derived peptides was responsible for the insulinotropic activity of milk protein hydrolysates (Luhovyy *et al.*, 2007).

The regulation of plasma glucose can also be achieved through another mechanism involving incretin hormones. Incretins comprising glucose dependent insulinotropic polypeptide (GIP) and more particularly glucagon-like peptide-1 (GLP-1) have been shown to enhance insulin secretion from pancreatic β cells in the presence of nutrients *in vivo* (Drucker, 2006). The half-life of GLP-1 and GIP can be significantly reduced following degradation by DPP-IV (Guasch *et al.*, 2012). DPP-IV inhibitors are reported to protect GLP-1 and GIP from enzymatic degradation, resulting in a prolonged insulinotropic action and normalisation of blood glucose *in vivo* (Nauck and El-Ouaghlidi, 2005; Drucker, 2006). Various studies have demonstrated the potential of milk protein-derived peptides to act as a natural source of DPP-IV inhibitory peptides. An, an *in silico* approach was used in order to identify potential dietary protein precursors of DPP-IV inhibitory peptides (Lacroix and Li-Chan, 2012b). It was reported that CN hydrolysates were more potent DPP-IV inhibitors than whey protein hydrolysates since β-CN is the milk protein substrate displaying the highest proportion of DPP-IV inhibitory peptide sequences (Lacroix and Li-Chan, 2012b). An hydrophobic pocket at the active site of DPP-IV, composed of Tyr_{666}, Tyr_{662}, Val_{711}, Val_{656}, and Trp_{659}, has previously been described (Engel *et al.*, 2003). DPP-IV inhibitors include peptides having sequences such as Xaa-Pro, Pro-Xaa or Xaa-Ala (where Xaa is an amino acid residue) (Yan *et al.*, 1992). It has been reported that peptides with 2–8 amino acids containing hydrophobic amino acids including Pro residues can inhibit DPP-IV (Hatanaka *et al.*, 2012; Lacroix and Li-Chan, 2012b). However, peptides without Pro residues have also been identified as potent DPP-IV inhibitors. For example, Trp-Val and Val-Ala, have been identified as DPP-IV inhibitors (Nongonierma and FitzGerald, 2013a).

Lineweaver and Burk kinetic analysis has been used to determine the mode of DPP-IV inhibition of peptide inhibitors or hydrolysates and to determine the type of inhibition by amino acids, peptides and milk protein hydrolysates. Figure 15.3 illustrates the Lineweaver and Burk double reciprocal plots obtained with three

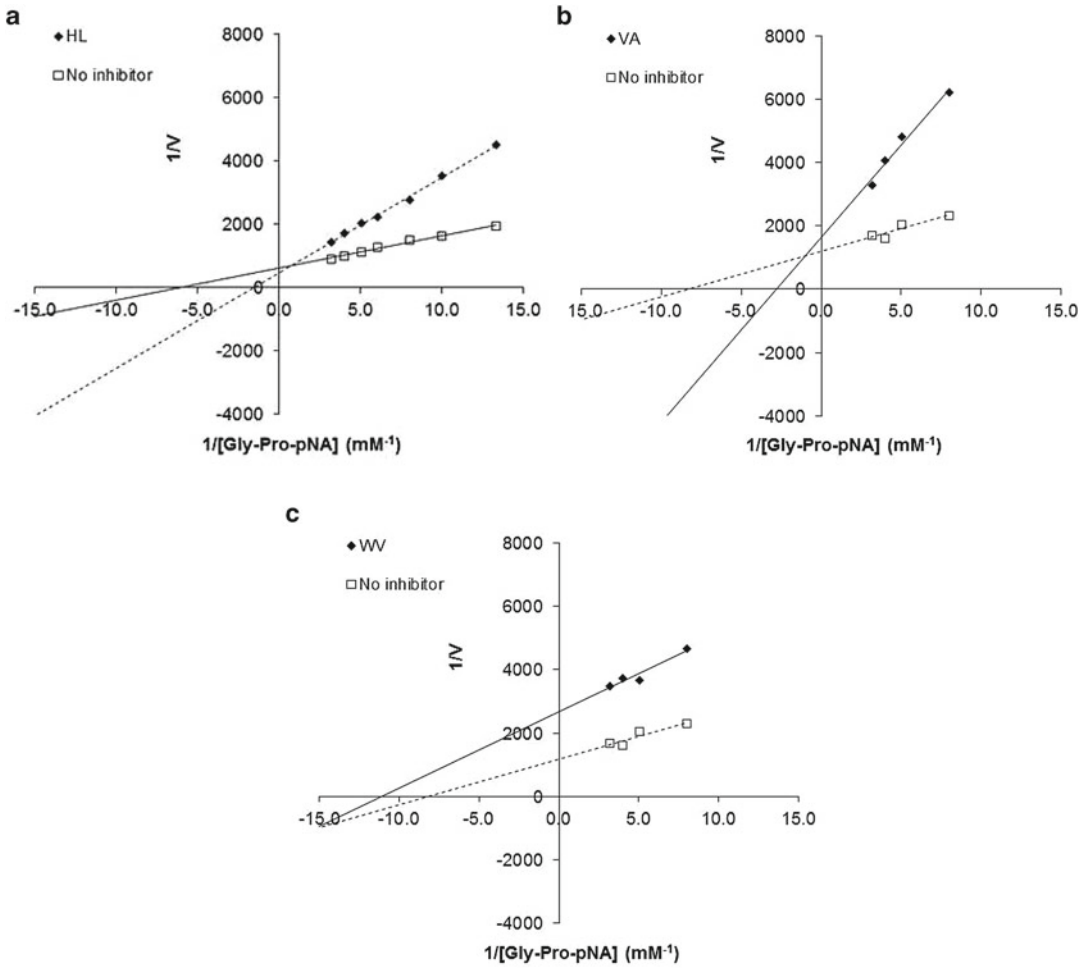


Fig. 15.3 Lineweaver and Burk plots for dipeptidyl peptidase (DPP-IV) with synthetic peptides. (**a**) His-Leu, (**b**) Val-Ala and (**c**) Trp-Val (adapted from Nongonierma and FitzGerald, 2013a)

dipeptides (His-Leu, Val-Ala and Trp-Val). Analysis of these plots reveal that His-Leu and Val-Ala where competitive inhibitors of DPP-IV while Trp-Val was a non-competitive inhibitor (Nongonierma and FitzGerald, 2013a). Milk protein hydrolysates have been shown to behave as competitive inhibitors of DPP-IV, suggesting a direct interaction between the hydrolysates and the active site of DPP-IV (Nongonierma and FitzGerald, 2013a, b). However, it is likely that some milk protein-derived peptides may also behave as DPP-IV substrates (Nongonierma and FitzGerald, 2014). DPP-IV peptide inhibitors such as Diprotin A (Ile-Pro-Ile) and Diprotin B (Val-Pro-Leu), two bacterially-derived DPP-IV inhibitors, have also been shown to behave as DPP-IV substrates (Rahfeld *et al.*, 1991). The dipeptides Ile-Pro and Val-Pro released by the hydrolytic activity of DPP-IV on Diprotin A and B, respectively, are also inhibitors of DPP-IV (Hatanaka *et al.*, 2012). Most peptide inhibitors described in the literature are competitive inhibitors of DPP-IV. However, linear mixed- or parabolic mixed-type inhibition of DPP-IV with peptides derived from the human immunodeficiency virus-1 (HIV) transactivator Tat (Lorey *et al.*, 2003) and Trp-containing dipeptides (Nongonierma and FitzGerald, 2013a, c) have been reported. It has been suggested that Trp-Val may bind to a secondary binding site near the

active site of DPP-IV (Nongonierma *et al.*, 2013b). Furthermore, it has been suggested that the N-terminal amino acid residue plays an important role in DPP-IV inhibition. In this regard, a few examples are available in the literature showing DPP-IV inhibitory activity of dipeptides whereas the reverse peptides could not inhibit DPP-IV (Hatanaka *et al.*, 2012; Nongonierma and FitzGerald, 2013a, c).

15.2.4.2 *In Vitro* Assessment/ Screening

Inhibition of α-glucosidase can be used to reduce post-prandial hyperglycemia following the ingestion of disaccharide rich foods. To our knowledge, no report of milk protein hydrolysate with α-amylase inhibitory activity has been made in the literature. Lacroix and Li-Chan (2013) have shown that peptic whey protein hydrolysates (WPH) could inhibit α-glucosidase, with IC_{50} values for α-glucosidase of 3.5 and 4.5 mg mL^{-1} for β-lactoglobulin (β-Lg) and whey protein isolate hydrolysates, respectively. Val-Trp, which is also present in α-La has been described for its α-glucosidase inhibitory properties when isolated from sardine muscle protein hydrolysates (Matsui *et al.*, 1999; Oki *et al.*, 1999).

The insulinotropic properties of milk-derived components have been well documented. *In vitro* studies using pancreatic β cell lines or primary islet cells have highlighted acute insulinotropic effects of various amino acids including Ala, Arg, Leu and Gln (Dixon *et al.*, 2003; Newsholme *et al.*, 2006, 2007). A WPH has been identified for its insulinotropic activity in pancreatic β cells *in vitro*. A dose-dependent relationship between the concentration of WPH and insulin secretion was demonstrated. In addition, a two tiered model consisting of Caco-2 and pancreatic β cell monolayers was used to study permeation of WPH peptides *in vitro*. It was shown that the bioactive components of WPH could cross the Caco-2 cell monolayer and stimulate pancreatic β cells (Gaudel *et al.*, 2013). The insulinotropic activity of WPH was associated with the hydrophilic fraction (amino acids and peptides) of WPH as highlighted when different fractions were tested *in vitro* using pancreatic β cells (Nongonierma *et al.*, 2013a).

Peptides derived from various milk protein substrates have been described as DPP-IV inhibitors (Tulipano *et al.*, 2011; Uchida *et al.*, 2011; Lacroix and Li-Chan, 2012a; Uenishi *et al.*, 2012; Nongonierma and FitzGerald, 2013a). For instance, β-Lg, α-La, LF and bovine serum albumin (BSA) hydrolysates have been shown to possess DPP-IV inhibitory activity (Tulipano *et al.*, 2011; Uchida *et al.*, 2011; Lacroix and Li-Chan, 2013; Nongonierma and FitzGerald, 2013a). Nongonierma and FitzGerald (2013a) have identified an LF-derived hydrolysate and various milk-derived dipeptides with DPP-IV inhibitory properties. Dipeptide sequences present in milk proteins with DPP-IV inhibitory activities have been identified including Trp-Val, Ala-Leu, Glu-Lys, Gly-Leu, Ser-Leu, Phe-Leu, His-Leu. The IC_{50} values for DPP-IV inhibition reported in the literature (Table 15.5) range from μM to mM. In addition, it has been shown that some amino acids can also inhibit DPP-IV (Nongonierma *et al.*, 2013b). Three amino acids out of the 20 studied were shown to have DPP-IV inhibitory properties (Table 15.5). However, their IC_{50} values were relatively high indicating a low potency compared to DPP-IV inhibitory peptides. The inhibitory potency of certain peptides naturally found in milk is generally higher than that of milk protein hydrolysates with typical IC_{50} values of 0.0015 and 0.0360 mg mL^{-1} for peptides and hydrolysates, respectively (Hatanaka *et al.*, 2012; Lacroix and Li-Chan, 2012a, 2013; Nongonierma and FitzGerald, 2013a). This may be explained by the fact that the BAPs within milk protein hydrolysates may be present at relatively low concentrations (Lacroix and Li-Chan, 2012a).

WPH was subjected to SGID in order to study the stability of DPP-IV inhibitory peptides to further hydrolysis by digestive enzymes. Following SGID of WPH, an increase in the DPP-IV inhibitory potential was observed. This suggested that the peptides involved in DPP-IV inhibition were short peptides, which was further reinforced by the fact that processing WPH through ultrafiltration membranes with a 5 and 2 kDa molecular weight cut off (MWCO) allowed further enrichment in the BAPs within the WPH. It has also been shown that mixtures of Sitagliptin, a pharmaceutical drug inhibitor of DPP-IV, and milk-

Table 15.5 Amino acids and milk protein-derived peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory properties and their associated half maximal inhibitory concentration (IC_{50}) value

Amino acid sequence	Protein fragment[a]	IC_{50} (μM)	Reference
IPAVF	β-Lg (f 78–82)	44.7	Silveira *et al.* (2013)
TPEVDDEALEK	β-Lg (f 125–135)	319.5	
IPAVFK	β-Lg (f 78–83)	143.0	
VLVLDTDYK	β-Lg (f 98–106)	424.4	
W	Various sources	4280.40	Nongonierma *et al.* (2013b)
M	Various sources	2381.51	
L	Various sources	3419.25	
FL	Various sources	399.58	Nongonierma and FitzGerald (2013a)
WV	α-La (f 26–27)	65.69	
EK	Various sources	3216.75	
AL	Various sources	882.13	
VA	Various sources	168.24	
SL	Various sources	2517.08	
GL	Various sources	2615.03	
HL	Various sources	143.19	
VAGTWY	β-Lg (f 15–20)	174	Uchida *et al.* (2011)
VPITPT	α_{s2}-CN (f 117–122)	130	Uenishi *et al.* (2012)
LPQNIPP	β-CN (f 70–76)	160	
PQNIPPL	β-CN (f 71–77)	1500	
VPITPTL	α_{s2}-CN (f 117–123)	110	
FPGPIPN	β-CN (f 62–68)	260	
PGPIHNS	β-CN (f 63–69)	1000	
IPPLTQTPV	β-CN (f 74–82)	1300	
VPPFIQPE	β-CN (f 84–91)	2500	
YPFPGPIPN	β-CN (f 60–68)	670	
LPQNIPPL	β-CN (f 70–77)	46	
LPQ	β-CN (f 70–72), α_{s1}-CN (f 11–13)	82	

[a]*CN* casein, *α-La* α-lactalbumin, *β-Lg* β-lactoglobulin

derived peptides were able to inhibit DPP-IV in an additive manner. This indicates that milk-derived peptides may augment the DPP-IV inhibitory activity of synthetic drugs (Nongonierma and FitzGerald, 2013b).

15.2.4.3 *In Vivo* Evaluation

The antidiabetic properties of milk proteins and milk protein hydrolysates have been demonstrated in small animal models. Supplementation of whey proteins for 11 weeks in mice receiving a high fat diet improved glucose tolerance and insulin sensitivity (Shertzer *et al.*, 2011). Similarly, oral gavage of intact (WP) and WPH in Wistar rats in a post-absorptive phase (3 h after food removal) for 30 days was conducted. This resulted in higher serum Leu and insulin levels 60 min after gavage with WPH compared to WP (Toedebusch *et al.*, 2012). Gaudel *et al*, (2013) also demonstrated that administration of a WPH by oral gavage for 8 weeks could restore the capacity of pancreatic islets to secrete insulin in obese and diabetic (*ob/ob*) mice, improve blood glucose homeostasis and reduce hyperinsulinemia. The DPP-IV inhibitory properties observed *in vitro* were shown to translate to antidiabetic activities *in vivo*. A tryptic digest of β-Lg with DPP-IV inhibitory activity was able to induce a decrease in blood glucose level in mice following an oral glucose tolerance test when administered orally at 300 mg kg^{-1} body weight (Uchida *et al.*, 2011). Similarly, the DPP-IV

inhibitory peptide Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu (β-CN (f 70–77)) isolated from Gouda-type cheese induced a significant reduction of blood glucose in rats following a glucose challenge (Uenishi *et al.*, 2012).

Amino acids (Leu, Arg, Ile, Phe and Ala) or milk-derived peptides ingested in combination with carbohydrates have been shown to increase the secretion of insulin in humans (van Loon *et al.*, 2000; Luhovyy *et al.*, 2007; Geerts *et al.*, 2011). The insulinotropic action of intact and hydrolysed whey proteins has been demonstrated in studies with normoglycaemic (Power *et al.*, 2009; Akhavan *et al.*, 2010) and diabetic subjects (van Loon *et al.*, 2000, 2003; Frid *et al.*, 2005; Manders *et al.*, 2006a, b; Geerts *et al.*, 2011). Furthermore, human studies have shown that whey protein hydrolysates had a higher insulinotropic effect than the unhydrolysed proteins (Power *et al.*, 2009; Morifuji *et al.*, 2010). Protein breakdown prior to food intake has been shown to yield a greater increase in plasma amino acids and di-peptides compared to unhydrolysed proteins (van Loon *et al.*, 2000; Koopman *et al.*, 2009; Morifuji *et al.*, 2010).

15.2.5 Satiety Peptides and Hydrolysates

Worldwide, the incidence of obesity is increasing (Nguyen *et al.*, 2012). In 2008, 1.5 billion adults were overweight, of which 500 million were obese (WHO, 2013c). Excess body weight can result in negative health consequences such as impaired glucose metabolism, dyslipidemia and hypertension (Schrezenmeir and Jagla, 2000; Gustafson *et al.*, 2001; Nguyen *et al.*, 2012). Satiety is defined as the hunger moderation which prevails after food intake and is responsible for the delay in food intake between meals. Satiety is controlled by different hormones in the gut and in the brain. A link between the gut and brain signals exists, which is termed "gut-brain axis". Various gut hormones have been identified for their satiating properties including cholecystokinin (CCK), peptide YY (PYY) and GLP1 (Sam *et al.*, 2012; McGregor and Poppitt, 2013). Modulation of appetite and satiety can also be achieved directly in the gastro-intestinal tract or the central nervous system (CNS) involving increased serotonin (5-hydroxytryptamine, 5HT) neurotransmission (Schellekens *et al.*, 2012). The modulation of appetite through regulation of the serotonergic system involves systemic targets. Central regulation of appetite can be achieved through 5-HT_{2C} serotonin receptor agonism (Miller, 2005; Jensen, 2006; Schellekens *et al.*, 2012). Modifications of the diet combined with exercise and bariatric surgery or anti-obesity pharmaceutical drugs are currently being employed to tackle obesity (Miller, 2005; Redman and Ravussin, 2010; Martin *et al.*, 2011; Nguyen *et al.*, 2012). However, many safety concerns and side-effects have been associated with anti-obesity pharmaceutical drugs (Connolly *et al.*, 1997; Powell *et al.*, 2011; Kang and Park, 2012; Schellekens *et al.*, 2012). Furthermore, non-compliance of overweight people to diet and exercise changes can be the cause for inefficient weight loss (Nguyen *et al.*, 2012). Therefore, there has been a focus on the inclusion of natural compounds with appetite lowering properties in the diet to help people to lose weight (Zemel, 2005; Luhovyy *et al.*, 2007; van Meijl *et al.*, 2008; Dougkas *et al.*, 2011; Nehir El and Simsek, 2012). Alteration of food structure has also been suggested as a means to improve the formulation of low energy foods. In this regard, different strategies have been suggested including higher incorporation of water and air or a decrease in the particle size of fat droplets (Nehir El and Simsek, 2012).

15.2.5.1 Structure Function

Milk proteins have been described as being more satiating than milk fats or carbohydrates (Anderson and Moore, 2004; Luhovyy *et al.*, 2007; Dougkas *et al.*, 2011). In addition, whey proteins are generally regarded as more satiating than CNs because they are digested faster and induce a faster appearance of amino acids and peptides in the plasma. The slower digestion of CN has been linked to its ability to coagulate at the acidic gut pH (Turgeon and Rioux, 2011). Protein intake can induce satiety signals in the gastrointestinal tract, inducing slower gastric

emptying, stimulation of gut hormone receptors and possibly opioid receptors (Anderson and Moore, 2004; Luhovyy *et al.*, 2007; Turgeon and Rioux, 2011).

CMP and other CN-derived peptides have been shown to play a role in satiety. It has been suggested that the mechanisms behind gastric secretion regulation might involve gut regulatory peptides including gastrin, somatostatin and vasointestinal polypeptide (VIP). CPPs and CMP have been proposed as ingredients for the reduction of gastric secretions such as HCl. This would additionally help to combat growth of bacteria such as *Helicobacter pylori* which have been associated with the development of gastric ulcers (Guilloteau *et al.*, 2010). GMP, the glycosylated fraction from CMP, has also been shown to regulate satiety and several studies have investigated the link between GMP and satiety. The satiety-inducing mechanism of GMP is thought to involve increased release of CCK and inhibition of gastric secretion (Yvon *et al.*, 1994; Gustafson *et al.*, 2001; Thomä-Worringer *et al.*, 2006).

Different studies have shown the indirect effect of milk proteins on serotonin activation. This has been linked with Trp, which is the precursor of serotonin (Turgeon and Rioux, 2011). Furthermore, 5-HT_2-serotonin receptor antagonism by human and bovine β-casomorphin-7 (Tyr-Pro-Phe-Val-Glu-Pro-Ile) has been reported (Sokolov *et al.*, 2005). Nongonierma *et al.* (2013c) have shown that milk protein hydrolysates can activate serotonin 5-HT_{2C} receptor in foetal cell lines (HEK 293A) engineered to express the 5-HT_{2C}-serotonin receptor. Fractionation of a sodium caseinate (NaCN) hydrolysate (NaCNH) using approaches including ultrafiltration (UF), reverse-phase high performance liquid chromatography (RP-HPLC), solid-phase extraction (SPE) and isoelectric focusing (IEF) allowed determination of the physicochemical characteristics of the BAPs. Low molecular mass (<1 kDa) hydrophobic peptides with a basic isoelectric point (>8.6) were able to stimulate the 5-HT_{2C} serotonin receptor (Nongonierma *et al.*, 2013c). However, the sequence of the serotonin agonist peptides have not yet been identified.

15.2.5.2 *In Vitro* Assessment/ Screening

In vitro assays targeting specific biomarkers of satiety in cell cultures have been used to evaluate the potential satiating properties of milk protein-derived peptides. It was shown that Leu, Ile, skim milk, and CN could stimulate GLP-1 release from NCI-H716, a human intestinal cell line, in a dose-dependent manner. However, whey proteins did not show any bioactivity (Chen and Reimer, 2009). A link with the satiating properties of milk protein-derived peptides has been proposed due to the increased half-life of GLP-1 and the DPP-IV inhibitory properties of milk protein hydrolysates (Lugari *et al.*, 2004).

It has been shown that serotonergic peptides could be released by *in vitro* gastric digestion from a wide range of CN substrates including skim milk powder, acid CN, NaCN and GMP. The unhydrolysed proteins could not substantially activate the 5-HT_{2C} serotonin receptors. However, unhydrolysed GMP was able to activate the 5-HT_{2C} serotonin receptor. The NaCN hydrolysate was subjected to *in vitro* intestinal digestion to further study the stability of the BAPs therein. Serotonin agonism was unaffected by the intestinal digestion suggesting that the BAPs within the hydrolysate may be stable to intestinal digestion (Nongonierma *et al.*, 2013c).

15.2.5.3 *In Vivo* Evaluation

Because of the poor correlation between *in vitro* biomarkers of satiety and food intake in animals and in humans, most studies have directly evaluated the satiating properties of milk protein-derived peptides *in vivo*. An additive effect involving CCK receptors and peripheral opioid receptors has been demonstrated with rats receiving a premeal load of CN hydrolysate. Utilisation of CCK antagonists and opioid antagonists suppressed the reduction of food intake observed when the rats were pre-fed with the CN hydrolysate (Pupovac and Anderson, 2002). It has been shown in addition that the interaction of the milk-derived peptides with opioid and CCK receptors was independent. The effect observed with CN hydrolysates on the reduction of food intake has been attributed to the opioid agonist

properties of β-casomorphins (Froetschel *et al.*, 2001). It has been shown that the agonist activity of β-casomorphins may result in a delaying of gastric emptying and intestinal transit in rats (Pupovac and Anderson, 2002), thereby affecting satiety (Ricci-Cabello *et al.*, 2012).

CPPs alone or in association with CMP have been described in the regulation of gastric secretions of calves (Guilloteau *et al.*, 2010). CPPs were shown to inhibit gastric secretions (gastric juice, HCl and pepsin) and to decrease electrolyte concentration in the gastric juice. This effect was more marked when CPPs were added with CMP. However, despite its satiating properties, most dietary interventions with GMP have failed to result in a reduction of food intake in humans (Chung Chun Lam *et al.*, 2009; Cliftont *et al.*, 2010). In addition, it has been shown that following ingestion of a preload of 50 g of two GMP preparations with different levels of glycosylation or a GMP-depleted whey, no significant difference could be found compared to a glucose control in terms of plasma CCK levels, food intake and subjective satiety in obese subjects (Keogh *et al.*, 2010). It appears that more complex mechanisms than satiety regulation by the secretion of CCK alone play a role in food-intake in humans (Keogh *et al.*, 2010; Ricci-Cabello *et al.*, 2012).

To our knowledge, no study has yet specifically demonstrated the role of BAPs in serotonin agonist activity *in vivo*. The only link between the consumption of milk and increased serotonin *in vivo* has been proposed through an increase in dietary free Trp. Increased hypothalamic serotonin release has been reported with rats (Orosco *et al.*, 2004) and in humans (Markus *et al.*, 2000) fed with α-La. This has been linked with the high Trp content of α-La.

15.2.6 Opioid Peptides

Opiates are compounds which behave like morphine in their physiological action. Opioid peptides bind to opioid receptors and exert effects similar to the opiates. Opioid peptides can be divided into two groups; (a) endogenous or typical opioids produced by the body such as the endorphins, enkephalins and dynorphins, and (b) exogenous or atypical opioid peptides that can be ingested in food.

15.2.6.1 Structure Function

The typical or endogenous opioid peptides all have a common N-terminal amino acid sequence, Tyr-Gly-Gly-Phe, while in the atypical or exogenous opioids only the N-terminal Tyr residue is conserved (Teschemacher *et al.*, 1997). The N-terminal sequence of the atypical opioid receptors is Tyr-Xaa-Phe (where Xaa is an amino acid residue) or Tyr-Xaa_1-Xaa_2-Phe. These structures form a structural motif that support binding of the peptide to the opioid receptors (Pihlanto-Leppälä, 2000). Opioid peptides may bind to three different types of opioid receptors in the cell, μ receptors which are associated with the control of intestinal motility and emotional behaviour, δ receptors which are associated with the control of emotional behaviour and κ receptors which are associated with analgesia and satiety (Höllt, 1983). Opioid peptides exert their activities by binding to such receptors.

15.2.6.2 *In Vitro* Assessment/ Screening

The first milk protein-derived opioid receptor ligands were discovered by Schulz *et al.* (1977) when they observed an opioid peptide in the blood of mammals that appeared to permeate into their milk. This peptide was later identified by Brantl *et al.* (1979) and was termed β-casomorphin. Milk protein-derived opioid peptides include casomorphins and lactorphins that are derived from CN and whey, respectively (Hartmann and Meisel, 2007). Opioid peptides derived from bovine whey proteins (Pihlanto-Leppälä, 2000; Madureira *et al.*, 2010) and milk (Meisel, 1998; Korhonen and Pilhanto, 2006; Nagpal *et al.*, 2011) are well documented. Milk protein-derived opioid peptides can exert agonistic or antagonistic effects as was found in a study on opioid peptides released from different cheese varieties where the agonistic β-casomorphins and the antagonistic

Table 15.6 Some bovine milk protein-derived opioid peptides (adapted from Pihlanto-Leppälä, 2000; FitzGerald and Meisel, 2003b)

Opioid peptide	Source protein and fragment[a]	Peptide sequence	IC_{50}[b] (μM)	Produced by
Opioid agonists				
β-casomorphin-11	β-CN (f 60–70)	YPFPGPIPNSL	10	Enzymatic hydrolysis[c]
β-casomorphin-7	β-CN (f 60–66)	YPFPGPI	14	Enzymatic hydrolysis and synthetic peptide
β-casomorphin-6	β-CN (f 60–65)	YPFPGP	3.2	Synthetic peptide
β-casomorphin-5	β-CN (f 60–64)	YPFPG	1.1	Enzymatic hydrolysis and synthetic peptide
β-casomorphin-4	β-CN (f 60–63)	YPFP	2.7	Synthetic peptide
Morphiceptin	β-CN (f 60–63)	YPFP.NH_2	3	Enzymatic hydrolysis and synthetic peptide
α_{s1}-CN exorphin	α_{s1}-CN (f 90–96)	RYLGYLE	1.2	Enzymatic hydrolysis and synthetic peptide
α_{s1}-CN exorphin	α_{s1}-CN (f 90–95)	RYLGYL	12	Enzymatic hydrolysis and synthetic peptide
α_{s1}-CN exorphin	α_{s1}-CN (f 91–96)	YLGYLE	45	Synthetic peptide
α-lactorphin	α-La (f 50–53)	YGLF	67	Pepsin hydrolysis and synthetic peptide
β-lactorphin	β-Lg (f 102–105)	YLFF	38	Pepsin/trypsin hydrolysis and synthetic peptide
β-lactotensin	β-Lg (f 146–149)	HIRL	N.D.[d]	Chymotrypsin hydrolysis
Serorphin	BSA (f 35–42)	YPSYGLNY	85	Enzymatic hydrolysis
Opioid antagonists				
Casoxin A	κ-CN (f 35–42)	YPSYGLNY	400	Enzymatic hydrolysis and synthetic peptide
Casoxin B	κ-CN (f 58–61)	YPYY	100	Synthetic peptide
Casoxin C	κ-CN (f 25–34)	YIPIQYVLSR	50	Enzymatic hydrolysis and synthetic peptide

[a]*CN* casein, *α-La* α-lactalbumin, *β-Lg* β-lactoglobulin, *BSA* bovine serum albumin
[b]Peptide concentration required to inhibit [^{3}H]-ligand binding by 50 %
[c]Isolated from the *in vivo* hydrolysis product of the duodenum of the Goettingen mini pig
[d]*N.D.* not determined

casoxin-6 and -C, and lactoferroxin A were found to be released from semi-hard and ripened mould cheeses (Sienkiewicz-Szłapka *et al.*, 2009b). Table 15.6 details the structure of some opioid peptides derived from bovine milk. Research has shown the ability of the opioid peptides β-casomorphin-5 and -7, casoxin-6 (Sienkiewicz-Szłapka *et al.*, 2009a) and lactoferroxin A (Iwan *et al.*, 2008) to cross Caco-2 cell monolayer in both the apical-to-basolateral and the basolateral-to-apical directions suggesting that these peptides may cross from the human intestine into the bloodstream.

15.2.6.3 *In Vivo* Studies (Small Animal and Human)

The *in vivo* opioid effects of milk protein-derived peptides have been studied. Meisel and co-workers reported the *in vivo* formation of β-casomorphins following digestion of β-CN with the discovery of these peptides in the duodenal chyme of minipigs (Meisel, 1986; Meisel and Frister, 1989). Moreover, following ingestion of 1 L of bovine milk by human volunteers, considerable amounts of β-casomorphin-7, smaller amounts of β-casomorphin-4 and -6 but no β-casomorphin-5 were found in the intestinal

contents showing the ability to generate these opioid peptides *in vivo* and the suggestion that such peptides may act as regulators of intestinal function (Svedberg *et al.*, 1985). Elevated levels of bovine β-casomorphin-7 and depressed levels of the enzyme that hydrolyses it, DPP-IV, were found in the sera of infants with 'near miss' sudden infant death syndrome events, leading to the suggestion that this opioid peptide may play a role in inhibiting the respiratory centre in the brainstem and may explain the occurrence of apnoea following exposure to cow's milk (Wasilewska *et al.*, 2011). β-Casomorphin-7 can be produced by typical digestive processes of the A1 and B variants of β-CN, but not the A2 variant. This has led to the suggestion by some that consumption of milk from the A2 variant may lead to a reduction in the incidence of CVD and type 1 diabetes (Kamiński *et al.*, 2007). However, the true effect of the A1/A2 variant remains controversial with other reports concluding that there is no evidence of an adverse effect on human health from the consumption of A1 variant milk (Truswell, 2005; De Noni *et al.*, 2009). The tetrapeptide, Tyr-Gly-Leu-Phe, also known as α-lactorphin, was shown to have both opioid-like and antihypertensive activities without displaying the negative effects on nociception, locomotor activity, motor co-ordination, rectal temperature or the length of pentobarbital anaesthesia in mice, which occur for example with morphine (Ijäs *et al.*, 2004). Multifunctional BAPs have been identified such as α-lactorphin, whose antihypertensive activity is thought to be achieved through the vasodilatory action of binding to opioid receptors (Nurminen *et al.*, 2000). In a study where 5- to 18-day-old rat pups were removed from their mothers it was seen that separation anxiety was significantly reduced through pre-injection of the pups with bovine milk protein-derived LF through an opioid-mediated mechanism (Takeuchi *et al.*, 2003). In a review of the effects of opioid peptides on the control of human ingestive behaviour Yeomans and Gray (2002) suggest that an opioid reward process may be involved in the short-term mediation of food and alcohol consumption, thus opioid antagonists may be used as a preventative measure for alcohol abuse and obesity. It is therefore plausible to suggest that milk protein-derived opioid antagonist peptides may present a dietary option in preventing and controlling alcohol and food abuse.

15.2.7 Antimicrobial Peptides

The antimicrobial properties of intact LF and various milk protein-derived peptides have been identified. Therefore, the utilisation of these proteins and peptides as food biopreservatives or health supplements with antibiotic-type effects has been proposed (Benkerroum, 2010; Agyei and Danquah, 2012; Wilson *et al.*, 2012). Peptide sequences originating from all the major milk proteins have been identified as antimicrobial agents (Benkerroum, 2010). Examples of different milk protein-derived antimicrobial peptide sequences are given in Table 15.7. Peptides derived from LF, α_{s1}- and α_{s2}-CN have been most studied (Hartmann and Meisel, 2007). However, other whey-derived peptides have also been described for their antimicrobial properties (Morris and FitzGerald, 2009). Milk protein-derived antimicrobial peptides display their bioactive properties against a wide range of microbial strains including Gram (+) and Gram (−) bacteria (*Escherichia, Helicobacter, Listeria, Salmonella, Staphylococcus*), yeast and filamentous fungi (Clare and Swaisgood, 2000; Chatterton *et al.*, 2006; Atanasova and Ivanova, 2010; Benkerroum, 2010; Danquah and Agyei, 2012; Sinha *et al.*, 2013).

15.2.7.1 Structure Function

Different mechanisms of action for the antimicrobial effects of milk proteins and peptides have been described. Direct binding to the lipopolysaccharide membrane of bacteria has been reported for LF, resulting in increased membrane permeability. The bacteriostatic activity of LF has been attributed to the sequestration of iron from the growth medium, which in turn impairs bacterial growth (Sanchez *et al.*, 1992; Morris and FitzGerald, 2009). Milk protein-derived antimicrobial peptides generally share common physicochemical characteristics. Antimicrobial

Table 15.7 Examples of antimicrobial milk protein-derived peptides (adapted from Panyam and Kilara, 1996; Clare and Swaisgood, 2000; FitzGerald and Meisel, 2003b; Chan and Li-Chan, 2005)

Starting protein	Enzyme	Bioactive peptide sequence[a]	Antimicrobial activity
Whole CN	Chymosin	Casecidins (β-CN (f 193–209), β-CN (f 193–207))	*Staphylococcus*, *Sarcina*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*
	Papain	Iracidin (α_{s1}-CN (f 1–23))	antimicrobial, antiviral and antipathogen adhesion
α_{s1}- CN	Trypsin	–	
	Chymosin	Iracidin (α_{s1}-CN (f 1–23))	*Candida albicans*, Gram (+) and Gram (−) bacteria
α_{s2}-CN	Chymosin	Casocidin	Growth inhibition
	Acid treated	Casocidin I (α_{s2}-CN (f 165–203))	*Escherichia coli*, *Streptococcus carnosus*
κ-CN	Chymosin	Kappacin	Dental pathogens
	Pepsin	(κ-CN (f 63–117))	Gram (+) and Gram (−) bacteria, yeast
	Chymosin	GMP residues (κ-CN (f 106–109))	*Porphyromonas gingivalis*, *Streptococcus mutans*, *Escherichia coli*
	Trypsin	GMP (κ-CN (f 106–169))	
κ-CN (kappacin- derivative)	Glu-C	κ-CN (f 138–158)	*Streptococcus mutans*
β-Lg	Trypsin	Four antimicrobial peptides negatively charged	Gram (+) bacteria
	Trypsin	β-Lg (f 15–20), β-Lg (f 25–40), β-Lg (f 78–83), β-Lg (f 92–100)	Gram (+) bacteria
	Trypsin	Two antimicrobial peptides	Gram (−) and *Bacillus subtilis*
	Chymotrypsin	One antimicrobial peptide	
β-Lg and α-La	Gastrointestinal proteinases	–	Growth inhibition
α-La	Trypsin	α-La (f 1–5), α-La (f 17–31)	Gram (+) bacteria
	Chymotrypsin	α-La (f 61–68) alpha chain, α-La (f 75–80) beta chain	Gram (+) bacteria
LF	Pepsin	LF-cin B (LF (f 17–41))	Gram (+) and Gram (−), fungi, protozoa
	Pepsin	LF-cin (LF (f 17–41))	Growth inhibition
	Trypsin	LF (f 345–689), LF (f 1–280), LF (f 222–230) and LF (f 264–269)	Growth inhibition
LF B	Pepsin	LF (f 17–41), LF (f 20–25)	Gram (+) and Gram (−) bacteria, yeast, fungi, parasites, viruses

[a]*CN* casein, *GMP* glycomacropeptide, *α-La* α-lactalbumin, *β-Lg* β-lactoglobulin, *LF* lactoferrin, *LF-cin* lactoferricin

peptides have been described as relatively short with cationic and hydrophobic characteristics (Hancock and Sahl, 2006; Cederlund *et al.*, 2011; Agyei and Danquah, 2012). Their mechanism of action is thought to involve disruption of the cell membrane, leading to increased permeability (Hartmann and Meisel, 2007; Cederlund *et al.*, 2011). Structure-activity relationship studies with α_{s2}-CN (f 183–207) revealed that the antimicrobial activity could be altered by modifying peptide sequence. It was shown that C-terminal sequence, the positive charge, and hydrophobicity of the peptide positively impact on antimicrobial activity (Alvarez-Ordóñez *et al.*, 2013).

15.2.7.2 *In Vitro* Assessment/ Screening

To date, many milk protein-derived antimicrobial peptides have been identified *in vitro*. Numerous sequences of antimicrobial peptides derived from CNs have been reported (Table 15.7). For example, peptide sequences displaying antimicrobial activity against *Listeria monocytogenes* and *Cronobacter sakazakii* have been identified and the structure-activity relationship of α_{s2}-CN (f 183–207) was studied (Alvarez-Ordóñez *et al.*, 2013). It was shown that maternal milk contains >300 milk peptides with a majority being derived from β-CN. These milk peptides displayed antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus* (Dallas *et al.*, 2013). Three peptides isolated from a CN hydrolysate (α_{s1}-CN (f 16–15), α_{s2}-CN (f 165–188) and κ-CN (f 136–146)) were able to inhibit the growth of *Escherichia coli* and *Bacillus subtilis* (Elbarbary *et al.*, 2012).

CPPs may also display anticariogenic activities. Bacteria can colonize the tooth surface and adhere to the pellicle through adhesion-receptor interactions, resulting in the formation of biofilms at the tooth surface known as dental plaque (Hannig and Hannig, 2010; Marsch *et al.*, 2011). Direct binding of CPP-ACP to dental plaque has been shown (Reynolds *et al.*, 2003; Cross *et al.*, 2006). A binding capacity of 0.16 and 0.11 g CPP-ACP/g wet weight cells at pH 7.0 and 5.0, respectively, has been reported (Rose, 2000). Inhibition of bacterial enzymes by CPP-ACP has also been reported (Rose, 2000; Azarpazhooh and Limeback, 2008; Rahiotis *et al.*, 2008). Another milk protein-derived peptide, CMP, has been identified for its antimicrobial properties in the oral cavity. Its mode of action involves inhibition of adhesion and growth of bacteria involved in the formation of bacterial plaque (Korhonen and Pilhanto, 2006; Thomä-Worringer *et al.*, 2006). Intact CMP has been proposed as a preventative agent against dental caries. It has been shown to inhibit adhesion of cariogenic bacteria such as *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus sobrinus* in the oral cavity and to modulate the composition of dental plaque microbiota. Hydrolysed CMP was also described as an anticariogenic agent. The monophosphorylated sequence Ser(P)149 κ-CN A (f 138–158), obtained after hydrolysis of CMP by Glu-C endopeptidase was shown to possess antibacterial properties against *Streptococcus mutans*, *Porphyromonas gingivalis* and *Escherichia coli* (Thomä-Worringer *et al.*, 2006). Two other κ-CN peptides (Lys-Cys-Gly-Pro and Lys-Cys-Pro) have been reported for their antimicrobial properties against *Enterococcus faecalis* (Liu *et al.*, 2012).

In general, intact milk proteins do not have any antimicrobial activity, with the exception of the lactoperoxidase system (O'Mahony *et al.*, 2013) and LF (Clare and Swaisgood, 2000). In the context of gastroenteric infections, LF has been described for its antimicrobial (bacteriostatic, bactericidal and anti-adhesive) and prebiotic properties (Morris and FitzGerald, 2009; Yen *et al.*, 2011). Enzymatic hydrolysis of LF results in the release of various antimicrobial peptides which have been shown, in certain instances, to be more potent than intact LF (Yen *et al.*, 2011). Potent peptide inhibitors of *Candida* growth were obtained after peptic digestion of LF (Ueta *et al.*, 2001). Different peptides derived from LF have been identified for their antimicrobial activity, including lactoferricin (LF-cin, LF (f 17–41)) and lactoferrampin (LF-ampin, LF (f 268–284)) (Bellamy *et al.*, 1992; van der Kraan *et al.*, 2004). Production of antimicrobial LF hydrolysates using the microbial coagulant from *Rhizomucor miehei* has been described as a means to generate hydrolysates which would be suitable for Halal food formulations. Two potent antimicrobial peptides (the 11-residue LF-cin B, Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly, and Lys-Leu-Leu-Ser-Lys-Ala-Gln-Glu-Lys-Phe-Gly-Lys-Asn-Ser-Arg-Ser-Phe-Gln-Leu) could inhibit the growth of *Escherichia coli* and *Bacillus subtilis* (Elbarbary *et al.*, 2010). The antiadhesive activity of intact β-Lg and α-La have also been described (Pellegrini *et al.*, 2001). β-Lg was shown to play a role in the prevention of pathogen colonisation by decreasing pathogen (*Escherichia coli* and *Klebsiella oxytaco*) adhesion to human ileostomy glycoproteins. This was achieved by direct

binding of β-Lg to the ileostomy glycoproteins. Binding of β-Lg to *Escherichia coli* appeared to involve the same glycoproteins (Ouwehand *et al.*, 1997). New antimicrobial peptide sequences such as β-Lg (f 50–54), β-Lg (f 123–125), β-Lg (f 134–136), β-Lg (f 143–146), β-Lg (f 147–149) and α-La (f 117–121) have been identified from whey proteins (Théolier *et al.*, 2013).

15.2.7.3 *In Vivo* Evaluation

Despite the numerous reports on the antimicrobial properties of milk protein-derived peptides, very few studies appear to have been conducted *in vivo* reporting a biological effect of these peptides. This may be due to the modest potency of these peptides or to the fact that their bioactivity can be impaired or masked in the presence of other food constituents. However, the antimicrobial role of CPPs *in situ* with humans wearing dental appliances has been demonstrated (Reynolds, 2008). The buffering capacity of CPP-ACP following a carbohydrate challenge has been shown in humans using a miniature pH electrode approach (Caruana *et al.*, 2009). However, contradictory results have been found *in situ* showing no conclusive effect of CPP-ACP in humans wearing fixed orthodontic appliances (Marchisio *et al.*, 2010). To date, very few studies have clearly demonstrated the antimicrobial role of CPPs in humans. One human intervention study showed that CPP-amorphous phosphate calcium fluoride induced a reduction in the percentage of aciduric bacteria from 47.4 to 38.1 % and *Streptococcus mutans* from 9.6 to 6.6 % after 4 weeks treatment (Beerens *et al.*, 2010).

15.2.8 Immunomodulatory Peptides and Hydrolysates

Low-grade inflammation has been linked with the development of a range of metabolic conditions including obesity, insulin resistance, metabolic syndrome and coronary heart disease (Danesh *et al.*, 2000; Rosa *et al.*, 2012). Immunomodulatory peptides are described as peptides which can enhance immune function *in vivo* through stimulation or suppression of the immune system (Gauthier *et al.*, 2006; Nagpal *et al.*, 2011; Agyei and Danquah, 2012). The immunostimulatory properties of milk peptides have been described in several reviews (Gauthier *et al.*, 2006; Thomä-Worringer *et al.*, 2006; Nagpal *et al.*, 2011; Agyei and Danquah, 2012; Udenigwe and Aluko, 2012). Chatterton *et al.* (2013) have reviewed the anti-inflammatory properties of milk proteins in the intestinal tract of newborns, infants and children.

15.2.8.1 Structure Function

Different mechanisms have been identified involving regulation of cytokine expression and antibody production by milk protein-derived peptides (Udenigwe and Aluko, 2012). Various milk protein-derived peptides have also been shown to stimulate proliferation of lymphocytes, enhance the phagocytic activity of macrophages and to increase bacterial resistance (Nagpal *et al.*, 2011). The sequences of immunomodulatory milk protein-derived peptides have not yet been elucidated, possibly due to the nonspecific activity of such peptides (Agyei and Danquah, 2012). However, it is generally thought that there is a positive relationship between immunostimulation and the presence of an Arg residue at the N or C terminus of the peptide (Nagpal *et al.*, 2011).

15.2.8.2 *In Vitro* Assessment/ Screening

The effect of milk protein-derived peptides on cytokine production has been demonstrated in different studies. Phelan *et al.* (2009a) showed that CN-derived peptides could induce an increase in the production of concanavalin A (ConA)-stimulated interleukin-2 (IL-2), whereas no effect on the production of ConA-stimulated interleukin-10 (IL-10) could be seen in cell cultures of human Jurkat cells. The opposite results were found with other CN-derived hydrolysates which induced an increase in ConA-stimulated IL-10 but had no effect on ConA-stimulated IL-2. These results suggest that CN-derived peptides played a role in T cell-mediated immune response in cell culture models. O'Sullivan *et al.* (2013) studied the effect of

enzymatic cross-linking on the anti-inflammatory response of Jurkat cells. Cross linking of NaCN before enzymatic hydrolysis was shown to impact positively on anti-inflammatory reactions in Jurkat cells. They found that the production of Con-A stimulated IL-2 and IL-10 significantly decreased (≥50 %) following incubation of Jurkat T cells with a cross-linked NaCN sample which was subsequently hydrolysed with Prolyve 1000. Therefore, it was concluded that peptide structure/sequence had a significant effect on the immunostimulatory outcome observed *in vitro*.

The immunostimulatory properties of CPPs have been demonstrated in mouse spleen and rabbit Peyer's patch cell cultures. The CPPs, α_{s1}-CN (f 59–79) and β-CN (f 1–25), were shown to affect the humoral response and to impact positively on proliferation of mouse spleen and rabbit Peyer's patch cell cultures owing to their mitogenic activity (Hata *et al.*, 1998). β-CN (f 1–28) has also been shown to induce stimulation of IgA production and to have a mitogenic effect on human T, B, and monocyte cells *in vitro* (Kawahara *et al.*, 2004).

15.2.8.3 *In Vivo* Evaluation

A link between antimicrobial and immunomodulatory peptides has been established. It has been shown that certain milk BAPs behave as immunomodulatory peptides and thereby increase resistance to pathogens in the gastrointestinal tract (Gauthier *et al.*, 2006). It was shown in mice that intravenous administration of Val-Glu-Pro-Ile-Pro-Tyr, human β-CN (f 54–59), enhanced resistance to infection with *Klebsiella pneumoniae* (Parker *et al.*, 1984). In a mouse model of Crohn's disease induced with trinitrobenzene sulfonic acid (TNBS), it was shown that a β-CN fermentate produced with *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 reduced mortality and inflammation (Espeche Turbay *et al.*, 2012). The induction of oral tolerance by β-Lg hydrolysates has been studied in a mouse oral tolerance model (Adel-Patient *et al.*, 2011; Adel-Patient *et al.*, 2012). The role of intact β-Lg (administered intravenously) in the induction of oral tolerance in mice has been demonstrated. The induction of oral tolerance was seen following *de novo* generation of T*reg* cells which were responsible for inhibition of sensitisation and elicitation of the allergic reaction. In contrast, the tolerogenic properties of β-Lg were decreased following tryptic hydrolysis, suggesting that extensive hydrolysis may be detrimental to the induction of oral tolerance (Adel-Patient *et al.*, 2011). It was also shown that the larger β-Lg derived peptides, β-Lg (f 25–107) and β-Lg (f 84–106), did not induce oral tolerance, suggesting that a mixture of peptides covering the whole sequence of β-Lg may be required. In addition, large peptides comprising a disulphide bond were reported to be responsible for the oral tolerance induced by β-Lg hydrolysates (Adel-Patient *et al.*, 2012). Tryptic and chymotryptic β-Lg hydrolysate acidic fractions (p*I* 2–5), which were further fermented with *Lactobacillus paracasei* NCC2461, were shown to induce oral tolerance in mice. The mechanism of action of the tolerogenic peptides involved stimulation of Type 1-regulatory T (Tr-1) cells, which was shown to suppress immune response through the secretion of IL-10 (Prioult *et al.*, 2004).

There appear to be very few human studies in the literature reporting the immunostimulatory properties of milk peptides. The ATTICA study (18 months, 3042 subjects) has demonstrated that the intake of dairy products was linked to a significant reduction of low-grade inflammation in healthy adults consuming dairy products (Panagiotakos *et al.*, 2010). It is not clear however if the origin of this effect was the protein components in milk. As already mentioned, the use of milk protein-derived BAPs to induce oral tolerance in humans has been proposed. Induction of oral tolerance may be employed as a means to prevent the immune system from over-acting on exposure to common antigens and microbiota. It has been reported that partially hydrolysed milk proteins ingested in small amounts may display tolerogenic properties (Gauthier *et al.*, 2006).

15.2.9 Anticancer Peptides and Hydrolysates

Cancer is currently the leading cause of death worldwide, with 7.6 million cancer-related deaths in 2008. This figure is continuing to rise with an estimated 13.1 million cancer-related deaths predicted for 2030 (WHO, 2013a). Cancer treating therapies continue to have many undesirable side-effects; therefore, the search for more tolerable treatments continues, making a functional food/nutraceutical approach attractive. A number of milk components are of potential relevance to tumour development; these include milk fat, protein, Ca, vitamin B12, riboflavin and retinoids (Tsuda *et al.*, 2000). Data from epidemiological studies on the influence of the consumption of milk on the development of cancers are ambiguous. Consumption of milk has been shown to help to prevent colorectal cancer (Cho *et al.*, 2004) and various other cancers as reviewed by Melnik (2009). Conversely, a meta-analysis showed a correlation between milk consumption and the development of prostate cancer (Qin *et al.*, 2004). Furthermore, little or no association was found between consumption of milk and the development of cancers of the bladder (Li *et al.*, 2011), breast, ovaries and endometrium (Parodi, 2012) and prostate (Parodi, 2009). Bovine LF has been shown to inhibit tumour cell growth in a number of studies (Duarte *et al.*, 2011; Tung *et al.*, 2013) and this has been reviewed by Tomita *et al.* (2009) and Tsuda *et al.* (2010).

15.2.9.1 *In Vitro* Assessment/ Screening

There is growing and substantial evidence to show the anticancer effects of peptides arising from hydrolysis of milk proteins, some of which can be seen in Table 15.8. The cytomodulatory and cancer cell growth inhibitory effects of milk protein-derived peptides have been reviewed (Meisel and FitzGerald, 2003). Five different casomorphins; α_{s1}-CN (f 90–95), α_{s1}-CN (f 90–96), β-casomorphin-7, β-casomorphin-7 (f 1-5) and morphiceptin inhibited cell proliferation in the T47D human breast cancer cell line (Hatzoglou *et al.*, 1996). Various NaCN hydrolysates resulted in an inhibitory effect on viability and growth of the Jurkat T and Caco-2 human cancer cell lines (Phelan *et al.*, 2010). Moreover, these hydrolysates were shown to increase the antioxidant controlling activities, glutathione (GSH, Cys-Glu-Gly) and catalase, in Jurkat T cells (Lahart *et al.*, 2011). The presence of persistent or chronic oxidative stress is a common feature of many human malignancies (Kryston *et al.*, 2011); therefore, increases in antioxidant

Table 15.8 Some milk protein-derived anticancer peptides

Animal	Source protein and fragment[a]	Peptide sequence	Cell type	Reference
Cow	α_{s1}-CN (f 90–95)	RYLGYL	TD47 (breast cancer)	Hatzoglou *et al.* (1996)
Cow	α_{s1}-CN (f 90–96)	RYLGYLE	TD47 (breast cancer)	Hatzoglou *et al.* (1996)
Cow	β-CN (f 60–66)	YPFPGPI	TD47 (breast cancer)	Hatzoglou *et al.* (1996)
Cow	β-CN (f 60–65)	YPFPG	TD47 (breast cancer)	Hatzoglou *et al.* (1996)
Cow	LF-B (f 17–41)	FKCRRWQWRMKKLGAPSITCVRRAF	Leukemia, fibrosarcoma various carcinoma, neuroblastoma	Yoo *et al.* (1997); Eliassen *et al.* (2002); Mader *et al.* (2005); Eliassen *et al.* (2006)
Human	α_{s1}-CN (f 158–162)	YVPFP	TD47 (breast cancer)	Kampa *et al.* (1996)

[a]*CN* casein, *LF* lactoferrin

enzymes may help prevent such cancers. A CPP preparation (CE 90 CPP III, DMV International, Veghel, The Netherlands), derived from a CN hydrolysate, prevented the apoptosis of normal, differentiated HT-29 colon cells while it promoted apoptosis of the undifferentiated HT-29 cell line by stimulating Ca uptake by the cell (Perego *et al.*, 2012). Bovine LF-cin is an antimicrobial peptide which exhibits cytotoxic effects against many cancer cell lines *in vitro* and is non-toxic to the tested normal cell lines (Hoskin and Ramamoorthy, 2008). Furthermore, a shorter form of bovine LF-cin, LF-cin (f 4–14) was found to inhibit growth of the human colon cancer cell line, Caco-2, by means of prolonging the S-phase of growth and possibly increasing deoxyribonucleic acid (DNA) repair in ultraviolet (UV)-induced DNA damaged cells (Freiburghaus *et al.*, 2012). Ubiquitin was identified in bovine milk using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and was shown to inhibit the growth of a Caco-2 cell line (Freiburghaus *et al.*, 2010). A proprietary whey peptide product (AminoAct®, OncoNutrition, Riverside, CA, USA) was reported to cause apoptosis in HT-29 colon cancer cells through inhibition of the tyrosine kinase activity of epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR2) and insulin receptor having IC_{50} values of 9.85, 7.7 and 6.18 μM, respectively (Kreider *et al.*, 2011). Furthermore, AminoAct® was found to increase the lifespan of the nematode *Caenorhabditis elegans* and to be clinically safe in healthy human volunteers.

However, there is also some conflicting evidence which shows proliferative effects of CN-derived peptides. Tryptic fragments of β-CN, (f 1–18) and (f 105–117) stimulated DNA synthesis in the mouse fibroblast BALB/c3T3 cells (Azuma *et al.*, 1989). Furthermore, a bacterial fermentate of CN resulted in a reduction in the rate of cell division in ileum epithelial cells (IEC) but led to an increase in cell division in Caco-2 cells (MacDonald *et al.*, 1994).

15.2.9.2 *In Vivo* Studies (Small Animal and Human)

Peptides released through the fermentation of milk by *Lactobacillus helviticus* resulted in a decrease in the size of fibrosarcomas in mice fed the fermented product for 7 days (LeBlanc *et al.*, 2002). The anticancer effects associated with increases in cellular GSH following the consumption of whey proteins have been documented (Bounous *et al.*, 1988, 1991; McIntosh *et al.*, 1995; Bounous, 2000). The increase in cellular GSH level has been attributed to the high levels of Cys found in whey proteins. These anticancer effects may be related to the production of peptides on digestion of the proteins in the gastrointestinal tract. Ingestion of 3 g/day of bovine LF for 12 months resulted in a significant decrease in adenomatous colorectal polyp growth in patients aged 63 or younger and it was proposed as an alternative to the surgical removal of such polyps (Kozu *et al.*, 2009). Furthermore, repeat injections of LF-cin resulted in inhibition of neuroblastoma xenograft growth *in vivo* (Eliassen *et al.*, 2006). Moreover, intra-tumoural injection of a shorter, 9-mer form of LF-cin, (Trp-Lys-Lys-Trp-Dip-Lys-Lys-Trp-Lys-NH_2, where Dip represents the non-coded residue β-diphenylalanine) resulted in necrosis, infiltration of inflammatory cells and resultant regression of lymphomas as well as inducing long-term cellular immunity against the specific lymphoma, leading to the suggestion that this may provide a strategy for vaccination against cancer (Berge *et al.*, 2010). Bovine LF-cin was also shown to inhibit basic fibroblast growth factor and vascular endothelial growth factor-induced angiogenesis in a murine model of angiogenesis (C57BL/6 mice with Matrigel plug inserted subcutaneously) (Mader *et al.*, 2006).

15.2.10 Antioxidant Peptides and Hydrolysates

The human body naturally produces free radicals and reactive oxygen species (ROS) which are processed/inactivated by the body's natural

antioxidant defences (superoxide dismutase, catalase and peroxidase). However, in certain conditions, the antioxidant system cannot cope with the excess ROS and free radicals generated in the body. This may happen when the body is subjected to stress-related factors such as those occurring in certain diseases (e.g., Alzheimer's disease, diabetes, artherosclerosis, obesity, etc.), physical exercise or ageing. The ultimate consequence of an excess of oxidants is cellular death. Dietary supplementation with antioxidants has been proposed to assist/strengthen the natural antioxidant system. Dietary compounds present certain advantages over synthetic antioxidants as they can be part of food intake and not have the associated adverse side-effects which may occur with synthetic antioxidants (Pihlanto, 2006; Udenigwe and Aluko, 2012). Various studies have demonstrated the antioxidative properties of milk proteins and milk protein-derived hydrolysates *in vitro* (Suetsuna *et al.*, 2000; Hernandez-Ledesma *et al.*, 2005; Liu *et al.*, 2005; Pihlanto, 2006; Elias *et al.*, 2008; Shahidi and Zhong, 2010; Contreras *et al.*, 2011; Nongonierma and FitzGerald, 2012b; Sousa *et al.*, 2012). Milk protein hydrolysates may contain both pro- and antioxidant peptides (Pihlanto, 2006).

15.2.10.1 Structure Function

It has been suggested that antioxidative peptides from milk are generally short (<11 amino acids) and contain hydrophobic residues (Pro, His, Try and Trp) (Pihlanto, 2006). The antioxidant properties of milk protein-derived peptides and hydrolysates have been reviewed. Different mechanisms have been described for the antioxidative properties of milk proteins and peptides including the scavenging of ROS and the inhibition/activation of key metabolic enzymes involved in oxidative processes *in vivo*. In addition, it has been reported that low molecular mass peptides, such as carnosine (Ala-His) and GSH, can act as antioxidants within cells whereas larger molecular mass peptides may display their antioxidative activity directly in the plasma (Power *et al.*, 2013).

The radical scavenging activity of milk protein-derived peptides has been described in several studies *in vitro* (Rival *et al.*, 2000; Pihlanto, 2006; Nongonierma and FitzGerald, 2013a). It has been suggested that both the composition and sequence of milk peptides play a role in their antioxidant activity (Pihlanto, 2006). Specific amino acid residues have been associated with high antioxidant properties. These include amino acids with sulphur residues (Cys and Met) and aromatic structures (Trp, Tyr, His and Phe) (Meucci and Mele, 1997; Saito *et al.*, 2003; Elias *et al.*, 2008; Komagoe *et al.*, 2010; Shahidi and Zhong, 2010; Udenigwe and Aluko, 2012). The antioxidant properties of Cys may also be attributed to the fact that it is a precursor of GSH (Meisel, 2005; Udenigwe and Aluko, 2012). The antioxidant properties of peptides containing phenol and indole amino acids has been linked to the fact that these can act as potential hydrogen donors (Pihlanto, 2006). Radicals formed with phenol and indole amino acids are relatively stable, which can result in slowing down of oxidation propagation, thereby increasing antioxidant capacity (Saito *et al.*, 2003). Amino acids have been reported to display a lower antioxidant activity compared to peptides as they have a lower radical scavenging activity (Elias *et al.*, 2008; Nongonierma and FitzGerald, 2012b). The role of peptide sequences in antioxidant properties has also been demonstrated in several studies. Suetsuna and Chen (2002) found that the antioxidant activity of dipeptides depended on the C or N terminal position of certain amino acid residues. They found that Trp-Lys had a stronger antioxidant activity compared to its reverse peptide (Lys-Trp). Similarly, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging properties of Trp-Val and its reverse peptide (Val-Trp) were also found to differ. It was shown that Trp-Val had an half maximal effective concentration (EC_{50}) value which was half that of Val-Trp (Nongonierma and FitzGerald, 2012b).

15.2.10.2 *In Vitro* Assessment/ Screening

The fermented milk, kefir, was found to scavenge superoxide (SO) radicals (Liu *et al.*, 2005). CN-derived-peptides isolated from a peptic hydrolysate of CN have also been identified for their

SO scavenging activity (Suetsuna *et al.*, 2000). A potent SO radical scavenger (Tyr-Phe-Tyr-Pro-Glu-Leu, α_{s1}-CN (f 144–149)) was isolated from this peptic hydrolysate (Suetsuna *et al.*, 2000). Structural modifications of this peptide affected its bioactive properties. A reduction in the SO scavenging activity was seen following removal of N terminal fragments (Tyr, Tyr-Pro or Tyr-Pro-Tyr residues) from the parent peptide. In addition, it was shown that Glu-Leu was responsible for the SO radical scavenging activity (Suetsuna *et al.*, 2000). Other milk protein-derived dipeptides have been identified for their SO scavenging activity (Table 15.9). It was found that milk protein hydrolysates had a higher SO scavenging activity than dipeptides. This was evident from the fact that larger peptides had a higher SO scavenging activity than lower molecular mass peptides as shown by the lower EC_{50} value for SO scavenging obtained with the 5 kDa retentate of a whey protein hydrolysate (0.14 mg mL^{-1}) compared to the 5 kDa permeate (0.37 mg mL^{-1}) of the same hydrolysate (Nongonierma and FitzGerald, 2013a). Similar results were found by Suetsuna *et al.* (2000). Upon fractionation of a peptic CN hydrolysate, three fractions with a wide range of EC_{50} values from less than 0.1 mg mL^{-1} to greater than 10 mg mL^{-1} were identified (Suetsuna *et al.*, 2000). This suggests that potent SO scavengers can be found within milk protein hydrolysates. In addition, enrichment of BAPs following fractionation could result in an increased potency for SO scavenging.

The antioxidant properties of CPPs have been reviewed by Kitts (2005). Milk protein-derived CPPs can act both as primary non-site specific (direct scavenging of free radicals) and secondary site specific (metal pro-oxidant sequestering) antioxidants. Their antioxidant properties have been linked with the phosphoserine residues in the CPP sequence. This specific feature of CPPs results in anionic and polar domains that have the ability to sequester cationic metal ions. CPPs can also interact with iron and reduce its prooxidative action on deoxyribose (Kitts, 2005).

Milk protein-derived hydrolysates have been shown to affect a wide range of metabolic enzymes that are involved in the antioxidant system *in vivo*. It has been shown that CN hydrolysates could induce an increase in cellular catalase activity and GSH level in human lymphocyte (Jurkat) cells (Phelan *et al.*, 2009a). Furthermore, WPC hydrolysates were shown to beneficially modulate genes involved in the antioxidant process, as well as catalase and GSH activities, in human umbilical vein endothelial cells in culture (O'Keeffe and FitzGerald, 2014). Inhibition of xanthine oxidase (XO), a key enzyme involved in the generation of oxidative species *in vivo*, has also been described. Free Trp, Trp containing dipeptides (Val-Trp, Val-Trp, Ile-Trp, Arg-Tp and Lys-Trp), and different LF hydrolysates have been identified as XO inhibitors *in vitro* (Nongonierma and FitzGerald, 2012b; Nongonierma *et al.*, 2013b). The XO inhibition was attributed to the

Table 15.9 Half maximal effective concentration (EC_{50}) of milk protein-derived peptides with superoxide scavenging activity

Peptide sequence	Protein fragment[a]	SO EC_{50} (mM)[*]	Reference
EK	Various sources	45.16	Nongonierma and FitzGerald (2013a)
GL	Various sources	20.73	
AL	Various sources	8.60	
VA	Various sources	>370	
WV	α-La (f 26–27)	131.02	
VW	LF (f 346–347), LF (f 548–549)	114.52	
SF	Various sources	163.54	
FL	Various sources	13.81	
HL	Various sources	>370	
SL	Various sources	24.92	
YFYPEL	α_{s1}-CN (f 144–149)	79.2	Suetsuna *et al.* (2000)
FYPEL	α_{s1}-CN (f 145–149)	127.5	
YPEL	α_{s1}-CN (f 146–149)	189.3	
PEL	α_{s1}-CN (f 147–149), BSA (f 151–153)	306.0	
EL	α_{s1}-CN (f 148–149), various sources	63.1	

[a]*CN* casein, *α-La* α-lactalbumin, *LF* lactoferrin, *BSA* bovine serum albumin

[*]*SO EC50: superoxide half maximal effective concentration*

Trp residue, which has structural similarities with xanthine and Allopurinol, a potent drug inhibitor of XO. Trp-containing peptides in the LF hydrolysates were shown to be responsible for the inhibition of XO as demonstrated by the enrichment in BAPs upon solid-phase extraction (SPE) fractionation using activated carbon.

15.2.10.3 *In Vivo* Evaluation

The exact mechanisms by which milk protein-derived BAPs display their anti- or pro-oxidant activity is still unclear (Pihlanto, 2006) and very few studies have demonstrated the role of these antioxidants *in vivo*. To date most studies reporting on the antioxidant properties of milk peptides have been focused in the area of sport science and exercise physiology. The increase in ROS following increased respiratory metabolism can result in an inflammatory reaction in the muscles which is generally manifested by an increase in phagocytes in muscle following physical exercise (Aoi *et al.*, 2004). This increase in antioxidative and inflammatory status can have adverse effects as it may lead to increased fatigue during exercise and ultimately results in lower performance. It has been shown that feeding mice for 4 weeks with antioxidant whey-derived peptides could retard fatigue during a swimming exercise. The effect of the antioxidant whey peptides on fatigue was seen by an increase in swimming time by more than 50 % compared to the control group consisting of mice which did not receive the whey peptides (Liu *et al.*, 2011). Similarly, consumption of milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* with antioxidant properties was linked to decreased muscle damage caused by prolonged exercise in Wistar rats (Aoi *et al.*, 2007). It was shown that the decrease in muscle damage corresponded with an increase in antioxidant activity (thiobarbituric-acid-reactive substances, TBARS) in muscle tissues, which in turn may reduce the inflammation caused by ROS. In addition to the direct antioxidant activity of milk peptides, it was also shown that milk peptides could increase the expression of endogenous antioxidant enzymes including superoxide dismutase (SOD) and catalase (Aoi *et al.*, 2007). A study conducted in humans has shown similar outcomes where consumption of milk fermented by *Lactobacillus helveticus* induced an improvement in glucose metabolism and reduced muscle soreness after high-intensity exercise. This was linked to the fact that ROS can decrease insulin-dependent glucose uptake in damaged muscles following impairment of insulin signalling pathways in muscle cells (reduction of glucose transporter protein 4 (GLUT4) translocation). A reduction in muscle soreness was linked with the reduction in muscle damage (reduction in inflammation post exercise) observed following consumption of the fermented milk (Iwasa *et al.*, 2013). To our understanding, the specific peptide sequences involved in these processes have not yet been identified.

A wide range of milk protein-derived BAPs have been identified using *in vitro* assays and, in certain instances, have been confirmed *in vivo*. BAPs with similar sequences may target different organs/systems; these peptides are generally termed as multifunctional BAPs. For example, multifunctional BAPs with antioxidant and antidiabetic (Nongonierma and FitzGerald, 2013a), immunomodulatory and antimicrobial (Gauthier *et al.*, 2006) and opioid and antihypertensive (Nurminen *et al.*, 2000) activities have been reported. Interestingly, some regions of the CNs known as "strategic zones" are rich in BAP sequences. These "strategic zones" appear to be relatively resistant to the action of proteases (Fiat and Jollès, 1989; Meisel, 1998; Schanbacher *et al.*, 1998; Meisel and FitzGerald, 2003).

15.3 Hydrolysate Formulations for Populations with Specific Needs

Specialised food products based on milk protein hydrolysates have been developed to address the nutritional needs of various specialist population groups. These include infants, infants with cow's milk protein allergies (CMPA), the elderly and people suffering from phenylketonuria (PKU). Milk protein hydrolysates targeting these population groups generally have different compositional

and physicochemical characteristics. The objectives of milk protein hydrolysis/fermentation differ depending on the final application. For infants and the elderly, the targeted release of specific amino acids or peptides is carried out with the view of releasing specific nutritional and/or biofunctional compounds. For CMPA and PKU, the aim during protein breakdown is to cleave and remove specific peptides (allergenic epitopes) or amino acids (Phe). In the case of PKU, removal of Phe involves further processing of the hydrolysate whereby Phe is fractionated from the hydrolysate. It is generally accepted that hypoallergenic formulae require extensively hydrolysed proteins, while protein hydrolysates used in nutritional supplements are generally moderately hydrolysed. Different enzymes are used to generate hypoallergenic formulae, however, this process currently mainly involves gastrointestinal enzyme activities (Nongonierma and FitzGerald, 2011).

15.3.1 CMPA

CMPA, which is described as the most common food allergy in children, has been linked to the early introduction of cow's milk to the infant's diet, with the CNs and β-Lg being described as major allergens (Restani *et al.*, 2009; Brożek *et al.*, 2012). In particular, β-Lg, which is absent from breast milk, has a high allergenic potential (van Esch *et al.*, 2011). CMPA can be IgE or non-IgE mediated, involving different clinical reactions in the skin, and in the gastrointestinal and respiratory tracts (Bossios *et al.*, 2011; De Greef *et al.*, 2012). It is often recommended in cases of CMPA to exclusively breast feed infants and/or to follow a cow's milk protein elimination diet until 9–12 months of age (De Greef *et al.*, 2012). In severe cases of CMPA, complete removal of the allergenic epitopes is achieved through the utilisation of elemental (amino acid) formulae which have been shown to possess no antigenic potential as these are free from proteins and peptides (De Greef *et al.*, 2012). The prevention and treatment of CMPA through induction of oral tolerance to the allergens, which consists of a progressive introduction of the allergens to the infant diet, has also been suggested (Pecquet *et al.*, 2000; Vandenplas, 2010). The induction of oral tolerance *in vivo* has been described in several studies, see Sect. 15.2.8 (Adel-Patient *et al.*, 2011; Adel-Patient *et al.*, 2012).

Degradation of the allergenic epitopes can be achieved by enzymatic hydrolysis of milk proteins yielding partially and extensively hydrolysed protein ingredients (Elsayed *et al.*, 2004; Zheng *et al.*, 2008). Various allergenic epitopes of milk proteins, such as β-CN (f 60–69), β-CN (f 122–135), α_{s1}-CN (f 16–35) α_{s1}-CN (f 136–155), α_{s2}-CN (f 98–115), α_{s2}-CN (f 86–103), κ-CN (f 42–65), κ-CN (f 68–89), β-Lg (f 41–60), β-Lg (f 102–124), β-Lg (f 149–162), β-Lg (f 31–48), β-Lg (f 67–84), β-Lg (f 75–86), β-Lg (f 77–107), β-Lg (f 97–117), β-Lg (f 119–128), α-La (f 1–16), α-La (f 47–58) and α-La (f 93–102), have been identified (Sélo *et al.*, 1999; Elsayed *et al.*, 2004; Monaci *et al.*, 2006; Minkiewicz *et al.*, 2011; Caira *et al.*, 2012). Additional allergenic epitopes may also be found in the BIOPEP database (Dziuba *et al.*, 2003). Cleavage of these epitopes can decrease the allergenic potential of milk proteins. Therefore, hydrolysis of milk proteins with the view of developing hypoallergenic formulae has been conducted with a wide range of enzyme preparations (Zheng *et al.*, 2008; Nilantha Lakshman *et al.*, 2011). A gastric and pancreatic digest of β-Lg, conducted in the presence and absence of phosphatidylcholine (PC), was tested for allergenic potential *in vitro* and with CMPA sufferers. In the presence of PC, the extent of β-Lg hydrolysis was lower, resulting in a higher IgE binding with sera from CMPA infants and higher skin prick test reactivity (Bossios *et al.*, 2011). Pre- or post-hydrolysis treatments including heat treatment and UF fractionation have been employed as a means to reduce the allergenicity of milk protein hydrolysates (Clemente, 2000). Cross-linking of milk protein-derived peptides has also been reported to decrease the antigenic potential of milk protein hydrolysates. However, in certain instances, cross-linking with transglutaminase was shown to have no impact on the antigenicity of CN hydrolysates (O'Sullivan and FitzGerald, 2012; O'Sullivan *et al.*, 2013). Several studies have

evaluated the hypoallergenic potential of milk protein hydrolysates using *in vitro* tests. However, a poor correlation between *in vitro* antigenicity and the hypoallergenic potential of compounds *in vivo* has been reported (Vandenplas, 2010). Therefore, there is a requirement for *in vivo* testing in order to validate the hypoallergenicity of milk protein hydrolysates (Vandenplas, 2010; van Esch *et al.*, 2011).

15.3.2 PKU

PKU is a genetic metabolic disorder characterised by a non-functional or deficient phenylalanine hydrolase, which converts Phe to Tyr. Malfunction of this enzyme impairs Phe metabolism and causes Phe accumulation in the body. This can lead to serious medical complications notably the onset of mental disorders (Clemente, 2000). Therefore, PKU sufferers require a low Phe diet throughout their life. The protein source most readily utilised for PKU nutrition consists of milk protein hydrolysates which have been depleted of their Phe content. Various studies have utilised enzymatic hydrolysis of milk protein substrates to specifically release Phe residues. The milk protein hydrolysates are subsequently processed with different adsorbents such as activated carbon or synthetic resins in order to remove the Phe residues (Cogan *et al.*, 1981; Lopez-Bajonero *et al.*, 1991; Lopes *et al.*, 2005; Silva *et al.*, 2007; Silvestre *et al.*, 2011, 2013). Removal efficiencies of Phe ranging from 69 to 99 % have been achieved. Different enzyme activities have been used including animal and plant-derived enzymes for hydrolysis. Hydrolysis of whey protein concentrate (WPC) with Pancreatin™ to a degree of hydrolysis (DH) > 30 % allowed production of an hydrolysate which was then further fractionated with activated carbon in order to remove Phe. This procedure allowed removal of up to 80 % of the Phe from the hydrolysate (Silvestre *et al.*, 2011). The production of hydrolysates with microbial proteases was described for the cleavage of Phe from milk proteins (Silvestre *et al.*, 2013). GMP has also been identified as a potential nitrogen source for PKU sufferers. The interest in utilising GMP comes from the fact that it is free from Phe residues (Ney *et al.*, 2009).

15.3.3 Elderly Populations

The ageing of the world population has been linked to various health risks related to malnutrition/undernutrition in the elderly leading to issues such as the loss of muscle mass. The loss of muscle results in a reduction in mobility, which may in turn lead to an increased risk of fractures. The loss in skeletal muscle mass, known as sarcopenia, has been linked to "anabolic resistance", a situation whereby protein ingestion results in less efficient muscle synthesis (Breen and Phillips, 2011). Milk appears to be the protein of choice for a balanced nutritional composition notably in terms of essential amino acid supply for population groups including the elderly. Increased protein intake in elderly populations has been proposed as a means to improve the bioavailability of proteinaceous material. In addition, combination of nutritional intervention with an exercise regimen has been proposed as a means of increasing muscle mass gain in the elderly. Exercising before CN intake resulted in an increase in muscle protein synthesis in elderly men (Pennings *et al.*, 2011b). Whey proteins have been shown to be more efficient for muscle synthesis in elderly subjects as compared to CNs. Myofibrillar proteins are responsible for muscle contractions and may therefore play a crucial role in improving mobility in the elderly population. Burd *et al.* (2012) studied the effect of whey and CN ingestion on myofibrillar protein synthesis in elderly people at rest and after resistance exercise. During post-exercise recovery, a 60 % greater myofibrillar protein synthesis was found with whey compared to CN ingestion. This has been linked with the higher extent of circulating amino acid levels following ingestion of whey compared to CN, possibly due to the faster rate of digestion of whey proteins. Ingestion of 20 g of whey protein isolate resulted in an increase in myofibrillar muscle protein synthesis (MPS) above fasting levels in non-frail elderly subjects.

However, no further increase in MPS could be seen with the ingestion of 40 g protein. Differences in MPS response post-exercise were seen, showing a dose response behaviour between whey protein intake and MPS up to 40 g protein. In addition, it has been shown that ingestion of ≥2 g Leu was required in order to overcome the 'Leu threshold' which may be the rate limiting step for increasing myofibrillar MPS (Yang *et al.*, 2012b). Co-ingestion of CNs and carbohydrate did not result in a higher post-prandial muscle protein synthesis compared to the ingestion of CN alone. This demonstrated that a higher post-prandial insulin level was not required to increase muscle protein accretion (Hamer *et al.*, 2013). In addition to the increase in MPS, a reduction in fat mass is often a target in the elderly population. Ingestion of a mixture of whey proteins and essential amino acids used as a meal replacement in an hypocaloric diet (800 kcal/day) for 8 weeks, resulted in a 7 % weight loss in elderly obese subjects. The meal replacement formula affected body weight and also body composition as the overall fat mass was shown to decrease (Coker *et al.*, 2012). Most studies to date describe the positive role of intact milk proteins on MPS. There appear to be very few studies which have investigated increased MPS following consumption of milk protein hydrolysates by elderly people. The influence of a CN hydrolysate on the fractional synthesis rate (FSR) of muscle protein in the elderly has been studied. However, it was shown that the CN hydrolysate yielded lower FSR of muscle protein than intact whey proteins. This was linked to the fact that ingestion of the CN hydrolysate yielded a lower plasma Leu concentration than whey proteins (Pennings *et al.*, 2011a).

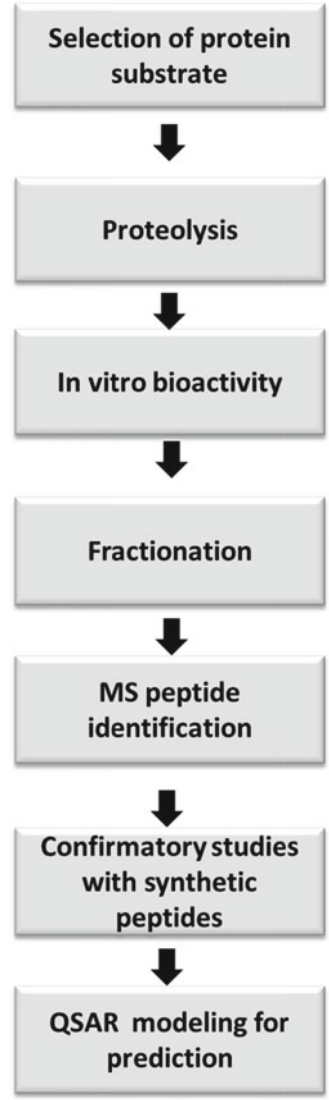


Fig. 15.4 General work flow for the discovery of bioactive peptides (adapted from Carrasco-Castilla *et al.*, 2012; Panchaud *et al.*, 2012). *MS* mass spectrometry, *QSAR* quantitative structure activity relationship

15.4 Enrichment, Fractionation, Identification and Discovery of BAPs

The work flow for the discovery of BAPs has to date been based on an empirical approach (Carrasco-Castilla *et al.*, 2012). It generally involves the enzymatic release of BAPs from an isolated individual protein or from a mixture of different milk proteins (Fig. 15.4). Bioactivity testing of the enzymatic hydrolysates or microbial fermentates is carried out *in vitro* using specific bioassays (Panchaud *et al.*, 2012). Promising samples are generally fractionated in order to enrich for the BAPs and to reduce the complexity of the sample. This is usually achieved using a bioactivity-driven fractionation strategy. Selected fractions are analysed by MS in order to identify

the peptide sequence(s) of the BAPs therein. Confirmatory studies are then generally conducted *in vitro* and *in vivo* with the potential BAP candidate(s). Subsequently, quantitative structure activity relationship (QSAR) approaches may be conducted in order to predict the structure of other related BAPs with similar or enhanced bioactivity (Carrasco-Castilla *et al.*, 2012). Identification of BAPs may be conducted using several approaches. MS is generally used for peptide identification (Panchaud *et al.*, 2012). However, other methods have been described for the targeted detection of specific BAP sequences. For instance enzyme linked immunosorbent assay (ELISA) has been used to detect low levels of peptides within samples, which is useful for instance in the detection of allergenic epitopes (Carrasco-Castilla *et al.*, 2012). An ELISA-based assay has been developed specifically to detect β-CN (f 1–25) in CPP preparations (Kume *et al.*, 2013). However, to date, the identification of short peptides ≤ 4 amino acids still remains a challenge. This is due to the difficulty of obtaining high quality spectra in MS and to the general lack of databases and software for MS analysis for these short peptides (Carrasco-Castilla *et al.*, 2012; Panchaud *et al.*, 2012).

15.4.1 Enrichment and Fractionation of BAPs

Various methods may be used to enrich and fractionate BAPs from complex mixtures of peptides found within milk protein hydrolysates. It has been shown that most BAPs are relatively short peptides (Panchaud *et al.*, 2012). Of the peptides reported within the BIOPEP database, 42 % were shown to contain 2–6 amino acid residues, 46 % had 7–25 amino acids and 12 % were >25 amino acids in length (Panchaud *et al.*, 2012). Lab-scale fractionation of BAPs, allows better characterisation of the bioactivity *in vitro* and *in vivo* and subsequent industrial preparation of fractions enriched in BAPs. Fractionation of BAPs at an industrial scale can be relatively expensive and it has been reported that it can account for 70–80 % of the total production cost (Carrasco-Castilla *et al.*, 2012). Fractionation and enrichment techniques are generally based on physicochemical differences between inactive peptides and BAPs. Different physicochemical characteristics have been exploited during fractionation including molecular mass, solubility, hydrophobicity and charge (Panchaud *et al.*, 2012).

Differential solubility of BAPs has been exploited for their fractionation from complex peptide mixtures. Particular examples may be found in the extraction of ACE inhibitory, antioxidant and antimicrobial BAPs from cheese in water soluble and ethanolic extracts (Pritchard *et al.*, 2010; Meira *et al.*, 2012; Sagardia *et al.*, 2013a). The utilisation of food-grade solvents such as ethanol has also been described for the selective precipitation of CPPs. Ca^{2+} and ethanol at pH 3.5 has been used to selectively precipitate CPPs containing the acidic motif (Reynolds *et al.*, 1994). Deproteinisation methods have also been described to separate selectively large peptides and proteins from lower molecular mass peptides using different solvents, acids or salts (Sforza *et al.*, 2003).

The separation of BAPs by membrane processing has been reviewed (Bazinet and Firdaous, 2009, 2013). Membrane processing techniques which have been applied to the separation of BAPs have been classified in three main categories where the driving force for separation utilises pressure, electrophoretic mobility and a combination of pressure and electrical field. Pressure driven methods, including UF, represent one the most common techniques described in the literature for the fractionation of BAPs whereby BAPs are generally fractionated on the basis of differences in their molecular mass (Muro *et al.*, 2013). UF fractionation of milk protein hydrolysates has been utilised to enrich BAPs with ACE inhibitory and antioxidant (Hernández-Ledesma *et al.*, 2007; Pan *et al.*, 2012; Holder *et al.*, 2013), immunomodulatory (Rodríguez-Carrio *et al.*, 2014), serotonergic (Nongonierma *et al.*, 2013c), insulinotropic (Nongonierma *et al.*, 2013a) and DPP-IV inhibitory activities (Lacroix and Li-Chan, 2012a; Nongonierma and FitzGerald, 2013b). However, UF can be a relatively time consuming procedure which involves different

filtration stages and cleaning steps, due to the possibility of membrane fouling (Bazinet and Firdaous, 2013). UF processing, however, generally displays a lack in selectivity for peptide fractionation (Poliwoda and Wieczorek, 2009; Bazinet and Firdaous, 2013). Because most BAPs described to date have a relatively low molecular mass, their fractionation may be a challenge due to the unavailability of commercially available large-scale membranes with a relevant MWCO. In addition, when the BAPs have low molecular masses, they can passively diffuse through the membranes. Diafiltration may be used to improve UF yield for low molecular mass peptides. Nevertheless, this results in longer duration UF procedures and in the utilisation of relatively high volumes of water. Although UF allows the processing of relatively large amounts of hydrolysate, it does not always allow a targeted fractionation of BAPs from milk protein hydrolysates. It has been observed that UF fractionation can yield fractions which still have a very complex peptide profile, making it difficult to identify the BAPs responsible for a specific bioactivity (Nongonierma *et al.*, 2013a, c). UF appears, however, to be a suitable technique for enrichment of BAPs for subsequent use in human or animal nutrition as it is up-scalable and does not require the use of harmful solvents. A specific application of membrane filtration is electromembrane filtration whereby the separation of peptides is based on both their molecular mass and also on electrophoretic mobility (Carrasco-Castilla *et al.*, 2012). Electromembrane filtration allows for reduced fouling and increased selectivity during peptide fractionation; however, some undesirable oxido-reduction reactions in the vicinity of the electrodes have been reported (Bazinet and Firdaous, 2013).

Fractionation techniques based on differences in isoelectric point include isoelectric focusing (IEF). This technique has been utilised to fractionate milk protein-derived BAPs. Immunostimulatory peptides have been fractionated with IEF from whey protein hydrolysates (Mercier *et al.*, 2004; Saint-Sauveur *et al.*, 2008). The fractions were more potent than the parent hydrolysate, inducing a higher lymphocyte secretion (Mercier *et al.*, 2004). The higher immunostimulatory properties of the IEF fractions were explained by the possible occurrence of peptide-peptide interactions (hydrophobic and electrostatic interactions) in the unfractionated sample. In certain instances, these physicochemical interactions may hinder the bioactive properties of the peptides within milk protein hydrolysates, reducing their bioactive potential (Groleau *et al.*, 2003; Mercier *et al.*, 2004). Serotonergic peptides were concentrated within fractions in the pH range 8.6 and 13.2 in contrast with fractions arising from the same milk protein hydrolysate within the acidic pH range (0.9 and 2.9). However, significant losses of peptides may occur during IEF, where up to 38 % of the peptides were lost during IEF of a sodium caseinate hydrolysate (Nongonierma *et al.*, 2013c). IEF generally employs ampholytes, which need to be removed subsequently. However, IEF has also been used to fractionate proteins and peptides in the absence of ampholytes (Yata *et al.*, 1996; Groleau *et al.*, 2001; Hashimoto *et al.*, 2006; Elbarbary *et al.*, 2012). IEF may be scaled up and utilised as a low cost effective means to isolate BAPs using a food-grade technique (Akahoshi *et al.*, 2000; Hashimoto *et al.*, 2006).

Chromatographic techniques including RP-HPLC have been described as a means to fractionate peptides based on differences in their hydrophobicity. Indeed, RP-HPLC separation has been described as the most effective method for peptide separation (Akahoshi *et al.*, 2000). Semi-preparative RP-HPLC may be utilised to fractionate larger amounts of BAPs. It also allows real-time isolation of BAPs as UV detection occurs simultaneously as RP fractionation. Semi-preparative RP-HPLC can be relatively time consuming depending on the size and characteristics of the RP-HPLC system used, including the volume of the injection loop and the capacity of the RP-HPLC column. Semi-preparative RP-HPLC may involve pooling fractions collected during multiple runs in order to collect sufficient material. In addition, the relatively high flow rate of mobile phase used can result in large fraction volumes for subsequent drying, which extends the duration and cost of the overall extraction.

In order to get a better separation of peptide peaks, relatively long shallow gradients may also be required. Nevertheless, a good knowledge of the physicochemical properties of BAPs allows a more targeted approach for selective isolation of the active fractions using an RP approach.

Fractionation of peptides by SPE has been successfully carried out (Voirin *et al.*, 1991; Herraiz and Casal, 1995). Fractionation using matrices with different hydrophobicities is achieved *via* elution with increasing apolar solvent concentrations. SPE has been applied for the isolation of BAPs from milk protein hydrolysates. This has allowed enrichment of BAPs with XO inhibitory (Nongonierma and FitzGerald, 2012b), insulinotropic (Nongonierma *et al.*, 2013a) and serotonergic activity (Nongonierma *et al.*, 2013c). Recoveries ranging from 15 to 101 % for peptides and from 6 to 100 % for milk protein-derived peptides have been reported with various SPE cartridges (Herraiz and Casal, 1995; Nongonierma and FitzGerald, 2012b). These differences in peptide recovery may come from the fact that irreversible adsorption of milk peptides can occur on various SPE adsorbents (Voirin *et al.*, 1991; Herraiz and Casal, 1995). The polarity of the SPE sorbent can significantly affect the peptides which elute from the SPE cartridges. In general, the higher the hydrophobicity of the sorbent, the stronger the retention of peptides leading to the requirement for higher concentrations of hydrophobic solvent to elute such peptides (Herraiz and Casal, 1995; Nongonierma *et al.*, 2013a, c). Application of a custom made SPE cartridge containing activated carbon has been described for the specific binding of Trp and Trp-containing peptides from a LF hydrolysate (Nongonierma and FitzGerald, 2012b). Potent insulinotropic activity was identified in the unbound fraction which was eluted in water from the Strata-X cartridges loaded with a whey protein hydrolysate. Amino acid analysis showed a significant enrichment in BCAA in this fraction, which is in agreement with its bioactive properties as BCAA have been associated with insulinotropic properties (Nongonierma *et al.*, 2013a). One advantage of SPE over RP-HPLC fractionation is that it is generally less time consuming and may allow fractionation of larger quantities (>6 times more) than semi-preparative RP-HPLC (Nongonierma *et al.*, 2013c). SPE has been described as being low in selectivity as it only allows separation of the peptides on the basis of their hydrophobicity or charge in certain instances (Poliwoda and Wieczorek, 2009). The utilisation of appropriate concentrations of hydrophobic solvent to desorb peptides bound to the SPE cartridge may help to overcome this issue. In addition, simultaneous extractions may be carried out in parallel, using a manifold where different SPE cartridges are attached. Concentration of the fractions can be achieved by optimising the amount of solvents used for elution of the SPE cartridges, which can reduce the drying times for the fractions. The major drawbacks of SPE are in relation to possible losses of BAPs onto the adsorbent following irreversible adsorption (Voirin *et al.*, 1991; Herraiz and Casal, 1995; Nongonierma and FitzGerald, 2012b). Therefore, the choice of adsorbent is crucial when using SPE as a fractionation technique for BAP separation. Nevertheless, preconditioning of the SPE may help to avoid or reduce irreversible binding onto the adsorbent (Voirin *et al.*, 1991).

The application of different fractionation techniques may not in some instances allow identification of BAPs. This may arise from the fact that several components (peptides/amino acids) may be needed for the activity. Therefore, fractionation to a single component may prove futile (Panchaud *et al.*, 2012).

Recombinant DNA technologies have been identified as a new means to enhance the production of specific BAPs and improve their yield compared to conventional techniques involving the isolation of BAPs from food protein-derived hydrolysates (Losacco *et al.*, 2007; Carrasco-Castilla *et al.*, 2012; Zambrowicz *et al.*, 2013). These may be utilised for the production of specific peptide sequences which, for example, have been predicted to be bioactive by *in silico* approaches. These techniques may also serve as a new avenue to produce high levels of BAPs at an industrial scale (Losacco *et al.*, 2007). These approaches generally utilise food-grade bacteria (probiotic strains) as a cell factory producing high purity BAPs. They have been successfully developed for

the production of ACE inhibitory BAPs (Losacco *et al.*, 2007; Carrasco-Castilla *et al.*, 2012; Losurdo *et al.*, 2013). Three ACE inhibitory peptides (bovine β-CN variant A2 (f 47–52), (f 57–66) and (f 73–82)) were produced with a recombinant DNA approach using *Lactobacillus helveticus* (Losacco *et al.*, 2007). The peptides were further purified by RP-fast protein liquid chromatography (FPLC) and it was shown that 1 g of purified recombinant fusion protein yielded ~50 mg of peptide. In another study, the same ACE inhibitory peptides (β-CN variant A2 (f 47–52), (f 57–66) and (f 73–82)) and their precursors produced using an *Escherichia coli-Bifidobacterium pseudocatenulatum* M115 shuttle vector were collected in cell lysates (Losurdo *et al.*, 2013). The *Bifidobacterium pseudocatenulatum* M115 strain is naturally found in the human microbiota and, it has therefore been suggested that transfected strains may be used as a means to release ACE inhibitory peptides directly *in vivo* (Losurdo *et al.*, 2013).

15.4.2 Discovery of New BAP Sequences Using *In Silico* Approaches

Food protein hydrolysates are generally relatively complex in terms of their peptide composition because the food-grade enzyme preparations used contain a mixture of different enzyme activities. This complexity makes the identification of BAPs relatively challenging. The utilisation of *in silico* approaches can therefore help improve the discovery of BAPs in a time and cost effective fashion (Carrasco-Castilla *et al.*, 2012; Panchaud *et al.*, 2012). Various databases, capturing information on BAP sequences together with their biological activity, are available online (Minkiewicz *et al.*, 2009). It has been pointed out that most of the BAP sequences found to date within the BIOPEP database, for example, are ACE inhibitory peptides. These are followed by antimicrobial, antioxidative and other BAP sequences (Panchaud *et al.*, 2012).

In silico approaches have been widely used for the identification and discovery of pharmaceutical drugs. However, they have more recently been applied to the discovery of food-derived BAPs (Pripp, 2007). *In silico* approaches have been used in different ways in order to:

- predict the release of BAP sequences from specific protein substrates with selected proteolytic/peptidolytic enzymes;
- identify precursor proteins for BAPs based on known BAP sequences which are generally readily available in databases or in the scientific literature;
- perform QSAR analysis whereby the peptide structure is utilised to predict its biological activity;
- virtually screen a large amount of peptides using molecular docking approaches which allow determination of the interactions between peptides and receptors.

Protein hydrolysis (peptide cutter) programmes may be used to digest food protein sequences *in silico* employing a wide range of enzymes. Based on the structural similarity of Trp with drug inhibitors of XO which have xanthine-like structures (e.g., Allopurinol), an *in silico* approach was recently used to predict the release of milk protein-derived dipeptides on digestion with gastrointestinal enzymes. This approach led to the discovery of new Trp containing dipeptides with XO inhibitory properties (Nongonierma and FitzGerald, 2012b).

In silico approaches can assist in the selection of appropriate strategies for a more targeted release of BAPs, allowing a better prediction for the selection of the starting substrate and the enzyme (Gu *et al.*, 2011). *In silico* approaches have also been utilised to determine food protein-derived starting substrates potentially enriched in BAPs with ACE inhibitory (Gu *et al.*, 2011) or DPP-IV inhibitory properties (Lacroix and Li-Chan, 2012b). It was shown that within the milk proteins, β-CN was a good source of DPP-IV inhibitory peptides (Lacroix and Li-Chan, 2012b) and that LF was enriched in potent ACE inhibitory peptides (Gu *et al.*, 2011). While these *in silico* approaches utilise known peptide sequences available in the literature, they are restricted by the current level of knowledge on the characteristics of peptide sequences displaying specific bioactivities. The

utilisation of response surface approaches has allowed determination of the optimum degree of hydrolysis (DH) for WPC and α-La hydrolysates (Tavares *et al.*, 2011b) and the optimum processing conditions (van der Ven *et al.*, 2002) for enzymatic release of ACE inhibitory peptides. The peptide mapping tool, EnzymePredictor, has been developed to further interpret MS data. This software allows identification of enzyme activities which have been used to generate an hydrolysate with unknown or poorly characterised enzyme activities. This tool may be used to substitute one enzyme with another or to identify enzymes which could mimic microbial cleavages in fermentates (Vijayakumar *et al.*, 2012). ANN approaches have been used to locate BAP sequences within a parent protein. PeptideLocator has been developed to detect BAPs within a given protein sequence. It also provides a probability value for a given peptide to be bioactive. This may aid in the selection of protein substrates and enzyme activities which may allow the release of selected BAPs (Mooney *et al.*, 2013).

A QSAR approach has been utilised to assess how the peptide composition of a milk protein hydrolysate may influence the generation of ACE inhibitory BAPs following gastrointestinal digestion. It was shown that the release of peptides with a Pro or Ile at the C-terminus should yield potent (low IC_{50} values) ACE inhibitors when subjected to gastrointestinal digestion *in silico* (Pripp, 2005). A chemical feature-pharmacophore approach, utilising 28 peptides to train a model, was described for the prediction of peptide ACE inhibitory potency (IC_{50} value). There was a good agreement between experimental and predicted ACE IC_{50} values (Wang *et al.*, 2011b). A QSAR study was conducted to identify ACE inhibitory peptides from cheese extracts (Sagardia *et al.*, 2013a) using a previously developed QSAR model for determination of the ACE IC_{50} values of pentapeptides (Sagardia *et al.*, 2013b). When applied to selected peptides identified by MS in cheese, the QSAR model overestimated ACE inhibitory potency compared to the experimentally determined IC_{50}. However, the QSAR model allowed correct prediction of peptide ranking according to their ACE inhibitory potency. Subsequently, the model was used to predict the most potent ACE inhibitory peptides within the cheese extracts. This strategy highlighted the fact that MS combined with QSAR allowed identification of novel ACE inhibitory peptides which may not be identified using a bioactivity guided fractionation approach. This was attributed to the fact that fractionation may not allow sufficient enrichment of peptides present at low concentrations in complex food matrices (Sagardia *et al.*, 2013a). Therefore, coupling MS and QSAR allowed a more comprehensive profile of the BAPs present within a specific cheese matrix.

Testing of a wide range of compounds can be relatively time consuming and expensive. For this reason, virtual screening has been proposed as an efficient approach to discover new peptide sequences with specific bioactive properties. Virtual screening is carried out with *in silico* methodologies, including molecular docking, which allow prediction of peptide binding to proteins (Pripp, 2007). To date, there are a limited number of studies describing the utilisation of molecular docking to predict binding of specific milk protein-derived peptides to protein receptors. Most molecular docking studies have been described for peptide sequences with ACE inhibitory properties (Pripp, 2007; Norris *et al.*, 2012; Pan *et al.*, 2012). Molecular docking showed good agreement between the Vina scores, the predicted binding affinity of the peptide to the enzyme, and ACE inhibitory IC_{50} values and allowed the discovery of new ACE inhibitory dipeptide sequences (Asp-Trp and Trp-Pro) (Norris *et al.*, 2012). Molecular docking has been applied to study binding of amino acids and milk protein-derived dipeptides to the active site of XO and DPP-IV (Nongonierma *et al.*, 2013b). Intestinal stability of the dipeptides was also predicted using the model developed by Foltz *et al.* (2009). It was shown that this strategy could not be applied to peptides with a non-competitive mode of inhibition as they do not bind to the active site of the enzyme. In this study, no direct correlation between the Vina scores for XO and DPP-IV and the IC_{50} values of milk dipeptides could be found. This highlighted the necessity to understand the mode of inhibition of the peptides

prior to molecular docking analyses. Utilisation of an *in silico* approach (Pepsite2 tool) allowed determination of the possible site of interaction of Trp-Val on DPP-IV (Nongonierma *et al.*, 2013b). Similarly, linear mixed- or parabolic mixed-type DPP-IV inhibitors (peptides derived from the N terminus of the human immunodeficiency virus-1 (HIV) transactivator Tat) were also reported to bind to a secondary site on DPP-IV (Lorey *et al.*, 2003). Because secondary binding sites on XO and DPP-IV have not been well characterized, there is a lack of knowledge on the structure of these specific sites. Therefore, this is currently a limitation for molecular docking strategies where prior knowledge of the interaction sites on a specific enzyme or receptor is required to conduct virtual screening of peptides. In addition, the results of *in silico* approaches need to be validated by experimental testing of the BAPs in order to confirm their outcome (Carrasco-Castilla *et al.*, 2012).

15.5 Overview of Foods Formulated with BAPs

BAPs may be found naturally in dairy products. This is the case for instance in dairy foods which were generated with a fermentation step. These include cheese, yoghurt and other fermented milks (FitzGerald and Murray, 2006; Korhonen and Pilhanto, 2006; Carrasco-Castilla *et al.*, 2012; Pihlanto, 2013). Various ingredients enriched with respect to BAPs together with food and oral hygiene products which have been formulated with BAPs are available on the marketplace (Table 15.10). To date, most foods formulated with BAPs are composed of hydrolysates or fermentates and not of purified peptides because of the high cost and the low yield generally associated with peptide purification (Zambrowicz *et al.*, 2013). Many of the products currently found on the market contain either CPPs or hypotensive BAPs. These food products are generally termed as functional foods since in addition to their basic nutritional properties, they display health promoting properties (Joana Gil-Chávez *et al.*, 2013).

Functional foods represent a growing market worldwide. Japan is the largest market for functional foods followed by the USA and Europe. The popularity of functional foods is thought to be driven by the increased cost of healthcare, ageing of the population and the desire for a good quality of life by consumers. The perception of consumers that such foods are linked with a healthy lifestyle, improved intellectual and physical performance and a reduction in the incidence of various nutrition related chronic diseases has also been identified as a key driver for the purchase of functional foods (Bigliardi and Galati, 2013).

15.6 Effect of Food Processing and the Food Matrix on the Stability of BAPs

BAPs are often produced from by-products of the dairy industry in order to add value and to reduce production costs (Carrasco-Castilla *et al.*, 2012). The processing conditions during the generation of milk BAPs, their incorporation into food matrices and their storage may affect their stability and bioactive properties. Microencapsulation techniques may be used to increase the stability of BAPs in food matrices (de Vos *et al.*, 2010; Onwulata, 2012; Udenigwe and Aluko, 2012). Another application has been described as a strategy to mask the bitterness and reduce the hygroscopy of a whey protein hydrolysate, which was encapsulated in spray-dried capsules of maltodextrin or a mixture of maltodextrin and β-cyclodextrin (Yang *et al.*, 2012a). Spray-drying of a CN hydrolysate in gum arabic was conducted, resulting in a reduction in the CN hydrolysate bitterness and improve its predicted stability (increase in glass transition temperature and reduction in water activity and hygroscopy) during storage (Subtil *et al.*, 2014). Similar results were found with a CN hydrolysate encapsulated in maltodextrin (Rocha *et al.*, 2009) or mixtures of gelatin and soy protein isolate (Favaro-Trindade *et al.*, 2010) by spray-drying. The CN hydrolysate encapsulated in maltodextrin was subsequently formulated in

Table 15.10 Examples of commercially available products containing milk protein-derived biofunctional peptide ingredients (adapted from Phelan *et al.*, 2009b; Carrasco-Castilla *et al.*, 2012; Nongonierma and FitzGerald, 2012a)

Product type	Manufacturer	Format	Health claim
Ingredients			
C12 Peptide	DMV, Netherlands	Ingredient	Hypotensive
Cardi-04	Chr. Hansen A/S, Denmark	Ingredient	Hypotensive
Biozate 1	Davisco Foods International	Ingredient	Hypotensive
Ameal BP, Ameal peptide	Calpis Co.	Ingredient	Hypotensive
PeptoPro	DSM Food Specialists, Netherlands	Ingredient	Improves athletic performance
Alphalactalbumin	Davisco Foods International	Ingredient	Helps sleep and memory
Lactium	Ingredia Nutritional	Ingredient	Reduction of stress effect
CE90CPP	DMV, International	Ingredient	Mineral binding
Lacprodan D1-2021	Arla Foods	Ingredient	Improves oral hygiene and uptake of vitamins and minerals
Glycomacropeptide	Davisco Foods International	Ingredient	Anticariogenic, antimicrobial and antithrombic
Peptigen 110	MD Foods	Ingredient	Mineral binding
Capolac MM0525	Arla Foods	Ingredient	Mineral binding
CPPB and CPPC	Armor Proteines	Ingredient	Anticariogenic
CPP-I, II & III	Meiji Seika	Ingredient	Anticariogenic
Recaldent	Cadbury Adams	Ingredient	Anticariogenic
Food products			
ProDiet F200	Ingredia, France	Milk Drink Confectionary	Reduces stress
Calpico (Europe) or Calpis AMEAL S (Japan)	Calpis Co., Japan	Fermented milk	Hypotensive
Casein DP Peptio Drink	Kanebo, Japan	Soft drink	Hypotensive
Evolus	Valio, Finland	Fermented milk, calcium enriched	Hypotensive
Tekkotsu Inryou	Suntory	Soft drink	Mineral binding
Kotsu Calcium	Asahi	Soft drink	Mineral binding
Meiji Milk Recaldent	Meiji	Milk beverage	Maintains strong teeth
Oral hygiene products			
Trident white sugar gum	Cadbury	Gum (with Recaldent)	Actively strengthens and rebuilds teeth with Recaldent
Recaldent Mints	Cadbury	Mints	–
MI Paste (GC Tooth Mousse[a])	GC	Tooth paste (with Recaldent)	Help remineralize and rejuvenate teeth
MI Paste Plus (GC Tooth Mousse Plus[a])	GC		
Prospec™ MI Paste	GC	Topical paste (with Recaldent)	Reduces dental erosion
Topacal C-5[b]	Nulite Systems International	Dental cream	–
Phoscal[b]	Nulite Systems International	–	–

sports bars. It was shown that the sports bar with the encapsulated CN hydrolysate were significantly less bitter than the bars with the unencapsulated CN hydrolysate (Rocha *et al.*, 2009). Other encapsulation methods based on liposome/liposphere technologies have also been described for the encapsulation of milk protein hydrolysates (Barbosa *et al.*, 2004; Morais *et al.*, 2004).

Heat-treatment can cause protein denaturation, allowing access of the enzyme to cleaving sites which were previously hidden within the folded protein structure (Mullally *et al.*, 1998; Hernández-Ledesma *et al.*, 2006; O'Loughlin *et al.*, 2012). This in turn may influence the type and extent of BAP release (Lourenço da Costa *et al.*, 2007; Adjonu *et al.*, 2013). Regulation of pH (7.0) during enzymatic hydrolysis of whey proteins was shown to affect the release of insulinotropic BAPs. In the absence of pH regulation, a lower protein breakdown was seen as compared to the hydrolysate generated with pH control. This resulted in a higher insulinotropic activity of the pH regulated hydrolysate as compared to the non-pH regulated hydrolysate (Nongonierma *et al.*, 2013a). Response surface methodologies have been utilised to determine the influence of different processing parameters (pH, temperature, hydrolysis time, enzyme:substrate ratio and pretreatment temperature) during whey protein enzymatic hydrolysis on ACE inhibitory activity. Consequently, a model was developed to predict ACE IC_{50} values for whey protein hydrolysates generated using different conditions (van der Ven *et al.*, 2002).

BAPs present in cheese or other fermented dairy products may be further degraded by microorganisms during storage, resulting in an increase or a decrease in their bioactive potency (Pihlanto, 2013). Milk has been shown to have a protective effect against CPP degradation in the gastrointestinal tract (Meisel *et al.*, 2003). Direct ingestion of CPPs resulted in their extensive degradation as they were barely detected in the ileum fluid. Better absorption of divalent cations bound to CPPs has been reported for liquid than solid foods. This might involve transfer limitations prevailing in solid foods, due to physical entrapment of the CPPs within the food matrix. Solid food matrices may slow down the release of CPPs in the intestinal lumen, which may adversely affect the bioavailability of the divalent cations (Miquel and Farré, 2007). A fruit beverage was reported to have the ability to protect CPPs against degradation by gastrointestinal enzymes. A fruit beverage supplemented with milk or CPPs was subjected to SGID (García-Nebot *et al.*, 2009). Following SGID, 10 different CPPs, with one presenting the acidic motif, were identified in the fruit beverage sample supplemented with milk. However, when CPPs were added to the fruit beverage, 16 different CPPs were identified following SGID with more than one peptide containing the cluster sequence (Fig. 15.5).

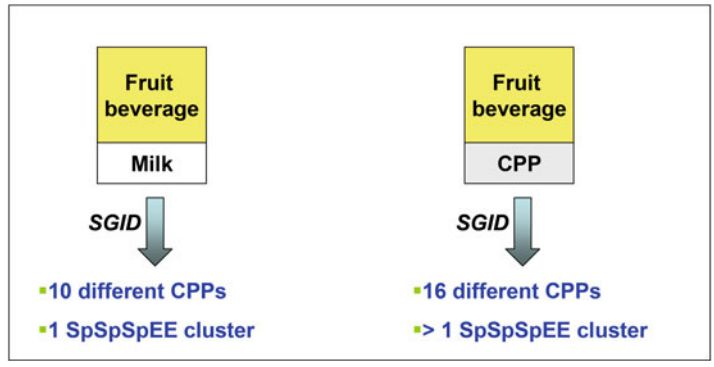


Fig. 15.5 Release/retention of caseinophosphopeptides (CPPs) following simulated gastrointestinal digestion (SGID) of fruit beverage supplemented with milk or CPPs (adapted from García-Nebot *et al.*, 2009)

15.7 Regulatory Aspects

Functional foods are a strictly regulated area. Health claims are granted by different agencies such as the European Food Safety Authority (EFSA) in Europe, the Ministry of Health, Labour and Welfare (MHLW) in Japan and the Food and Drug Administration (FDA) in the US (Phelan *et al.*, 2009b). Japan is the leader in terms of functional foods as it was the first country to put in place a licencing system known as Food for Specified Health Uses (FOSHU) to define a regulatory framework. Differences have been highlighted for health/functional food regulations depending on the geographical area, highlighting that various approaches are being taken to establish regulations depending on the country (Lalor and Wall, 2013). In Japan, the FOSHU license is attributed to the food containing bioactive ingredients and not to the ingredient itself. The physiological effects and safety of the food must be demonstrated in humans. Three types of FOSHU approvals are granted by the MHLW. These include qualified, standardised and reduction of disease risk FOSHU claims. The qualified FOSHU claim is attributed to foods where the bioactive properties are not supported by scientific evidence or where the mechanism of action is not known. The standardised FOSHU claim is attributed to foods where the health benefits are substantiated by scientific evidence. The reduction of risk disease is attributed to foods where there is clinical or nutritional evidence of a reduction of a risk disease by a specific bioactive compound (Shimizu, 2003). A large number of products (almost 1000 were FOSHU approved Shimizu, 2012) have been granted FOSHU approval with a few milk protein-derived BAPs having mineral binding and blood pressure reducing properties (MHLW, 2013).

In the USA, the FDA has defined three types of claims: health claims, nutrient content claims, and structure function claims. Health claims are attributed to bioactives which have been shown to reduce a risk of a specific disease following extensive review of the scientific literature. Nutrient content claims describe the level of a specific nutrient, for which a recommended daily intake has generally been established, in a food product. Structure function claims are not evaluated by the FDA. They apply to dietary compounds which play a role in the normal function in the human body (FDA, 2013).

In the EU, the Regulation on Nutrition and Health Claims has been enforced since July 2007. It comprises three articles. Article 14 (disease risk reduction and child development) is aimed at bioactive components with risk reducing disease properties or child development health promoting characteristics. Article 13 ("general function" health claims) are not applicable to areas covered by article 14, they apply to components targeting growth, development and body functions; psychological and behavioural functions; slimming and weight control along with satiety or reduction of available energy from the diet. Article 13.5 ("new function" health claims) relates to newly developed scientific evidence and proprietary bioactives (EFSA, 2013).

Evaluation of bioactive constituents by regulatory agencies is based on the scientific evidence supporting the health claim. In particular with EFSA, the outcomes of human intervention studies appear to be crucial for a positive opinion to be granted to a specific bioactive component (Jäkälä and Vapaatalo, 2010; Lalor and Wall, 2013). In this context, characterisation of the bioactive within functional foods and structure activity relationship is a prerequisite for acceptance of health claims. To date, no BAPs have been granted a health claim by EFSA even though they are naturally released *in vivo*, and therefore considered as safe for human consumption (Carrasco-Castilla *et al.*, 2012; Udenigwe and Aluko, 2012; Zambrowicz *et al.*, 2013). A few studies have looked at the safety of milk protein-derived ACE inhibitory peptides (Ile-Pro-Pro and Val-Pro-Pro) and these have reported no cytotoxicity *in vitro* and *in vivo* (Bernard *et al.*, 2005; Maeno *et al.*, 2005; Ponstein-Simarro Doorten *et al.*, 2009; Anadón *et al.*, 2010). EFSA has facilitated the submission process for health dossiers through the publication of guidance notes to assist future applicants in this complicated process (Lalor and Wall, 2013).

15.8 Conclusions

Numerous studies have shown that milk protein-derived BAPs have the potential to modulate different biomarkers of health *in vitro*. It is well established that milk protein-derived peptides can positively impact on human health, modulating different systems including the gastrointestinal, cardiovascular, endocrine, pancreatic and muscular systems. However, there are still only a limited number of studies which have clearly demonstrated the mechanisms involved in the bioactive properties of milk protein-derived BAPs *in vivo*. To date, the detailed role of milk BAPs on human health is not clearly understood. Intensive research has been conducted in the area of milk protein-derived peptides for the past 30 years. State-of-the-art techniques, mainly coming from the pharmaceutical sector, including proteomics/peptidomics, *in silico* analyses and intervention studies are being applied to the discovery and validation of milk protein-derived BAPs. These new techniques have made it possible to process a large amount of data, to detect specific peptides with an increased accuracy and to speed up the discovery rate of BAP sequences. The generation of BAP databases allows a more rapid identification of new BAPs while using *in silico* approaches. The utilisation of computer based programs has increased scientific outputs in the area of food protein-derived BAPs. However there is still a need to develop methodologies for the identification of low molecular mass BAPs. Numerous ingredients containing BAPs are available in the marketplace. Different studies have shown the effect of food formulation and processing on the biological activity of the BAPs. This suggests that not all food matrices are suitable carriers for BAPs and that there is a requirement to validate retention of the biological activity following modifications in the formulation or processing conditions of foods. Various intervention studies have been conducted with BAPs in humans, particularly with ACE inhibitory and mineral binding milk protein-derived peptides. However, these human studies do not always provide conclusive evidence for the bioactivity of milk protein-derived BAPs. This may be due to the potency of BAPs, which is generally low compared to pharmaceutical drugs. In addition, it is likely that the biological activity of BAPs may only be seen over the long term if there is a need for accumulation of BAPs in target tissues. This may indicate that human intervention studies with BAPs may need to be standardised and conducted over a longer period of time. Because of a lack of clear scientific evidence on the biological activity of BAPs in humans, regulatory agencies are slow to deliver health claims for BAPs. However, scientific advances made over the past few years in the area of milk protein-derived BAPs, may help to establish clearly the role of BAPs in human health. Combined with a healthy lifestyle, functional foods may help to prevent and/or alleviate major health issues associated with the metabolic syndrome. Functional foods may also improve the quality of life of individuals suffering from various health conditions or improve physical and mental health performances of specific population groups.

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