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Acceleration of cheese ripening

P.F. Fox, J.M. Wallace, S. Morgan, C.M. Lynch, E.J. Niland & J. Tobin
Department of Food Chemistry, National Food Biotechnology Centre, University College, Cork, Ireland

Abstract

The characteristic aroma, flavour and texture of cheese develop during ripening of the cheese curd through the action of numerous enzymes derived from the cheese milk, the coagulant, starter and non- starter bacteria. Ripening is a slow and consequently an expensive process that is not fully predictable or controllable. Consequently, there are economic and possibly technological incentives to accelerate ripening. The principal methods by which this may be achieved are: an elevated ripening temperature, modified starters, exogenous enzymes and cheese slurries. The advantages, limitations, technical feasibility and commercial potential of these methods are discussed and compared.

Introduction

The original objective of cheese manufacture was to conserve the principal nutrients in milk, i.e., lipids and proteins. This was achieved by a combination of acidification, dehydration, low redox potential and salting. Although a few minor cheese varieties are dehydrated sufficiently, or contain a sufficiently high level of NaCl, to prevent microbiological and/or enzymatic changes during storage, the composition of most varieties permits biological and enzymatic activity, i.e., ripening (maturation), during storage. The characteristics of the individual cheese varieties develop as a result of the biochemical changes that occur during ripening, as determined by curd composition, microflora, residual coagulant and residual milk enzymes.

Although cheese ripening is a very complex biochemical process (for reviews, see Fox et al., 1993, 1995, 1996), it primarily involves glycolysis, lipolysis and proteolysis, together with numerous secondary changes that are responsible for the characteristic flavour and texture of each cheese variety. These changes are catalysed by: (1) residual rennet, (2) starter bacteria and their enzymes, (3) secondary cultures and their enzymes, (4) non-starter adventitious microflora and their enzymes, and (5) indigenous milk enzymes.

Most (~ 98%) of the lactose in milk is removed in the whey but fresh cheese curd contains 0.7 to 1.5% lactose which is fermented, mainly to L-lactic acid, in all cheese varieties to give a pH of ~ 5.0. Lactose is usually completely fermented within, at most, a few weeks; however, a high level of salt may cause its incomplete fermentation (Thomas & Pearce, 1981). Lactic acid may be metabolized to propionic acid, acetic acid and CO_2, as in Swiss- type cheeses, to H_2O and CO_2, eg, in Camembert, or to D-lactate and some acetate as in Cheddar, Dutch and Italian varieties.

Only limited lipolysis occurs in most cheese varieties, notable exceptions being Blue cheeses and some Italian varieties. Lipases secreted by *P. roqueforti* are the principal lipolytic agents in blue cheeses, the characteristic peppery flavour of which is due to methyl ketones produced by partial β-oxidation of free fatty acids. An exogenous lipase, pregastric esterase, is the principal lipolytic agent in Italian cheeses.

Proteolysis occurs in all cheese varieties, ranging from limited, e.g., Mozzarella, to very extensive, e.g., Blue, Parmesan and extra-mature Cheddar. Proteolysis is largely responsible for the textural changes in most varieties, makes a direct contribution to flavour, e.g., peptides and amino acids (and perhaps off-flavour, e.g., bitterness), produces substrates (amino acids) for the generation of sapid compounds, e.g., amines, acids, thiols and thioesters, and facilitates the release of sapid compounds from the cheese mass during mastication. Proteolysis is perhaps the most important reaction during cheese ripening, with the exception of blue and Ital-

ian varieties, in which lipolysis and fatty acid oxidation dominate, although proteolysis is also very important.

When the objective of cheese production was primarily the conservation of milk constituents, then the more stable the product, i.e., the less change, the better. While storage stability is still important, it is no longer the primary objective of cheese manufacture, a consistently high quality being the target. Since ripening is expensive, acceleration of ripening, especially of low-moisture, slow-ripening varieties, is desirable, provided that the proper balance can be maintained.

Objective of accelerating ripening

Proteolysis appears to be rate-limiting in the maturation of most cheese varieties and hence has been the focus of most research on the acceleration of ripening. Acceleration of ripening is most pertinent for low-moisture, slow-ripening varieties and most published work has been on Cheddar. Techniques for the acceleration of ripening are also applicable to low-fat cheeses which ripen more slowly than their full-fat counterparts.

The extensive literature on the acceleration of cheese ripening has been reviewed by Law (1978, 1980, 1982, 1984, 1987), Moskowitz & Noelck (1987), Fox (1988/89), El-Soda and Pandian (1991), El-Soda (1993) and Wilkinson (1993). This article will concentrate on recent work by our group rather than attempt to review again the whole subject.

Proteolysis in naturally ripened cheese

Since accelerating proteolysis is the usual objective of accelerated ripening of cheese, elucidation of the extent and type of proteolysis in naturally-ripened cheese would appear to be a desirable prerequisite. Considerable progress has been made on this subject during the past 10 years or so.

The development and standardization of methods for the quantitation and characterization of proteolysis in cheese is essential for studies on cheese ripening. The subject has been reviewed by Grappin et al. (1985), Rank et al. (1985), Fox (1989), IDF (1991), McSweeney & Fox (1993) and Fox et al. (1995) and will not be discussed further here; suffice it to say that the methods used fall into 3 principal categories:

1. Quantitation of nitrogen soluble in various extractants/precipitants [water, pH 4.6 buffers, 2–12% TCA, 30–70% ethanol, phosphotungstic acid (PTA)], usually by Kjeldahl but less frequently by the method of Lowry or dye-binding methods, absorbance of 280 nm or amino group-reactive agents, eg, TNBS, ninhydrin, fluorescamine or o-phataldialdehyde. The suitability of these methods has been compared by Wallace and Fox (1994); the Lowry modification of the biuret method appears to give best results.
2. Release of amino groups, as quantified by reaction with TNBS, ninhydrin, fluorescamine or o-phataldialdehyde.
3. Electrophoresis, usually in urea-containing polyacrylamide gels, or chromatography, usually RP-HPLC of small water-soluble peptides or IE-HPLC of larger, water-insoluble peptides.

Proteolysis in mature cheeses, especially in Cheddar, Blue and Parmesan, is so complex that fractionation of the cheese is necessary to fully appreciate its extent. Various fractionation methods were compared by Kuchroo & Fox (1983); some of these have been developed further and combined into a protocol, Figure 1 (O'Sullivan & Fox, 1990; Singh et al., 1994; Fox et al., 1994) which is used by many other investigators, sometimes in modified form.

Contribution of individual agents to proteolysis

The proteolytic enzymes involved in the ripening of cheese originate from 4, and in some varieties 5, sources: (1) Milk, (2) Coagulant, (3) Starter bacteria, (4) Non-starter lactic acid bacteria (NSLAB), (5) Secondary/adjunct microorganisms, e.g., *P. roqueforti* (Blue cheeses), *P. camemberti* (Camembert and Brie), *Br. linens*, yeasts and *Micrococcus* (surface smear cheeses), *P. freudenreichii* subsp *shermanii* (Swiss varieties); these organisms dominate the ripening of cheeses in which they are used through their proteolytic and/or lipolytic activity and secondary metabolism, e.g., β-oxidation of fatty acids (Blue cheeses), amino acid catabolism (smear ripened cheeses) and/or lactate metabolism (Swiss varieties and Camembert). *L. lactis* ssp *lactis* var. *diacetylactis* and *Leuconostoc* spp, components of the starter for Dutch-type cheeses, metabolise citrate to diacetyl and CO_2, which are important for flavour and eye development, respectively; traditionally, secondary/adjunct starters are not used in Cheddar-type cheeses but, as discussed in Section 4.4, the development and application of such cultures are among the promising approaches toward accelerating ripening.

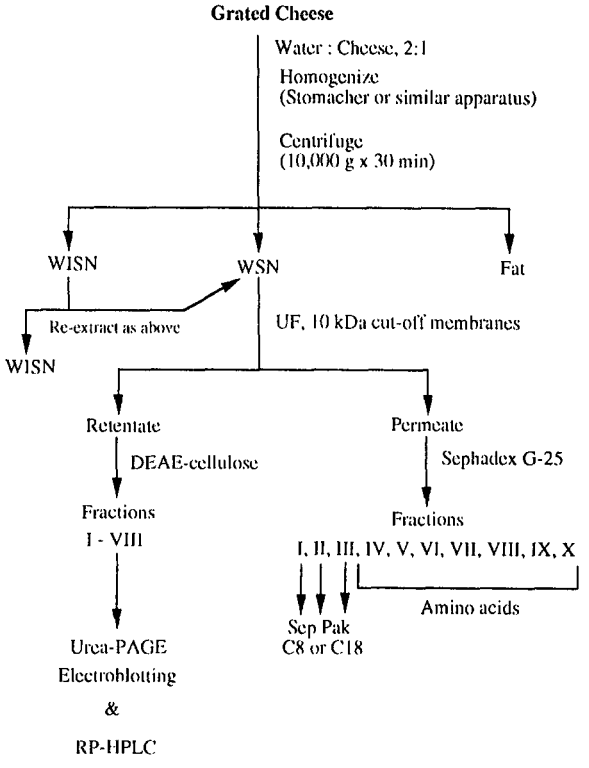

Figure 1. Scheme for the fractionation of cheese nitrogen (Fox et al., 1994).

Starting with the pioneering work of Mabbitt et al. (1955), techniques have been developed which permit the elimination of one or more of the 5 ripening agents, permitting assessment of their contribution to ripening. These methods have been reviewed by Fox et al. (1993). Essentially, they involve:

1. Selection of milk with a very low microbiological count, followed by pasteurization (or perhaps microfiltration) to give essentially sterile milk.
2. Manufacture of cheese in a sterile environment (enclosed vats, sterile room or laminar airflow unit) to eliminate adventitious contaminants (NSLAB).
3. Use of an acidogen, usually glucono-δ-lactone, to replace the acidifying function of the starter.
4. Inactivation of the coagulant, after it has hydrolysed κ- casein, by heating and/or pH adjustment.
5. Inhibition of plasmin (the principal milk proteinase) by 6- aminohexanoic acid.

Studies on such model cheeses have shown (Mabbitt et al., 1959; Visser, 1977a, b, c; Visser & de Groot-Mostert, 1977; O'Keeffe et al., 1976a, b, 1978; Le Bars et al., 1975; Lynch et al., 1996a, b) that:

1. The coagulant is principally responsible for the formation of water- or pH 4.6-soluble N and the changes indicated by PAGE but makes little contribution to the formation of small peptides or free amino acids (TCA- or PTA-soluble N).
2. Although plasmin contributes relatively little to the formation of water- or pH 4.6-soluble N, and even less to the formation of TCA- or PTA-soluble N, it does hydrolyse some β-casein with the formation of γ- caseins and proteose peptones. The contribution of plasmin is more important in high-cooked cheeses, e.g., Swiss and Parmesan, than in Cheddar or Dutch types due to the extensive inactivation of the coagulant and to more extensive activation of plasminogen, possibly due to the inactivation of inhibitors of plasminogen activators at high cook temperatures (Farkye & Fox, 1991).
   The contribution of the indigenous acid proteinase (cathepsin D) has not been established; its specificity is similar to that of chymosin (McSweeney et al., 1995) and is probably overshadowed by chymosin in normal cheese.
3. Although lactic acid bacteria are weakly proteolytic, they possess a very comprehensive proteolytic system which is necessary for their extensive growth in milk which contains very little free amino acids and small peptides. The proteolytic system of *Lactococcus* and, to a lesser extent, *Lactobacillus* has been studied extensively at the molecular, biochemical and genetic levels; the extensive literature has been reviewed by Thomas & Pritchard (1987), Tan et al. (1993), Visser (1993), Monnet et al. (1993), Pritchard and Coolbear (1993), Law & Haandrikman (1996) and summarized by Fox & McSweeney (1996a, b).
   The proteolytic system consists of a cell wall-associated proteinase, 3 or 4 intracellular proteinases (Stepaniak et al., 1995), 2 intracellular endopeptidases (PepO, PepF), at least 3 aminopeptidases (PepN, PepA and PepC), X-prolyl dipeptidyl aminopeptidase (PepX), iminopeptidase, at least one tripeptidase, at least one general dipeptidase and a number of proline-specific peptidases; a carboxypeptidase has not been reported in *Lactococcus* and in only a few species of *Lactobacillus*. Through their concerted action, the proteolytic system of LAB can hydrolyse casein to amino acids (Figure 2).
   Studies on controlled microflora cheeses have shown that the proteolytic system of starter LAB contributes little to primary proteolysis in cheese,

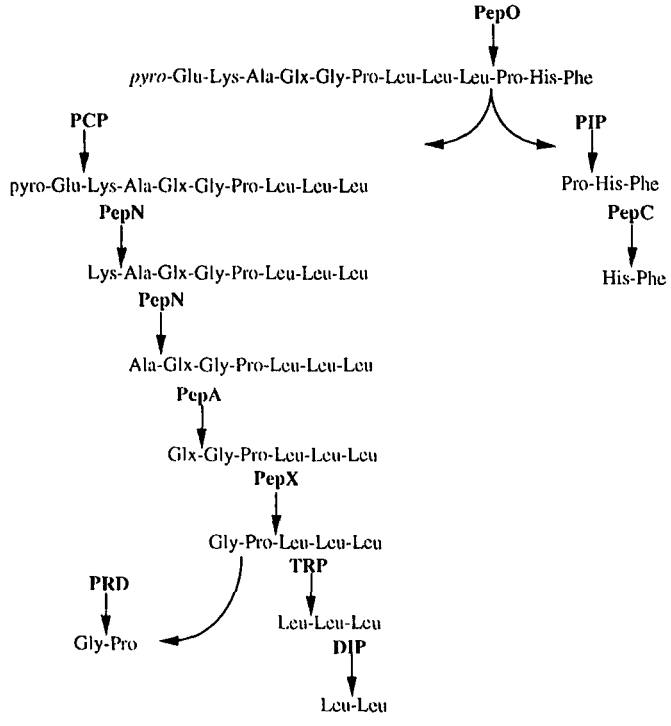

Figure 2. Schematic representation of the hydrolysis of a hypothetical dodecapeptide by the combined action of endo- and exopeptidases of *Lactococcus* spp.

as detected by PAGE or the formation of water- or pH 4.6-soluble peptides, but is mainly responsible for the formation of small peptides and amino acids (i.e., TCA- or PTA-soluble N).

4. Although cheese made from pasteurized milk in modern mechanized plants contains very few NSLAB immediately after manufacture (typically < 50 cfu/g), these multiply rapidly to reach, typically, 10^7 cfu/g within about 2 months. During this period, the starter cells which are present initially at, typically, 10^9 cfu/g, decrease to 10^7 cfu/g; hence, in long-ripened cheeses, e.g., Cheddar, NSLAB dominate the viable microflora throughout most of the ripening period.

The cheeses investigated in all the above-mentioned studies on controlled microflora cheeses were intended to be free of NSLAB and hence their contribution to proteolysis, or ripening in general, was not studied. Recent studies in our laboratory (Lane & Fox, 1996; Lynch et al., 1996a, b) using chemically acidified (GDL) or biologically acidified (starter) cheese, with or without an adjunct culture of non-starter lactobacilli, showed that lactobacilli appeared to produce many of the same peptides as the starter, although at a slower rate, accelerated the production of free amino acids and certain volatile compounds and influenced the flavour of the cheeses.

The specificity of chymosin, plasmin, cathepsin D and lactococcal cell wall-associated proteinases on individual caseins in solution are known and have been summarized by Fox et al. (1994, 1995, 1996) and Fox & McSweeney (1996a, b).

Proteolysis in cheese

The principal water-insoluble peptides are produced either from α_{s1}-casein by chymosin or from β-casein by plasmin; several minor water-insoluble peptides remain to be identified (McSweeney et al., 1994a; Mooney & Fox, unpublished). In mature (> 6 mo) Cheddar, all α_{s1}-CN is hydrolyzed at Phe_{23}-Phe_{24} to yield α_{s1}-CN f1–23 and α_{s1}-CN f24–199 (α_{s1}-I). About 50% of the latter is hydrolysed at Leu_{101}-Tyr_{102} to yield α_{s1}-CN f24–101 and α_{s1}-CN f102–199. The bonds Phe_{32}-Gly_{33} and Leu_{109}-Glu_{110} are also hydrolysed, probably in α_{s1}-CN f24–199. The bond Leu_{109}-Glu_{110} was not found by McSweeney et al. (1993b) to be hydrolysed in α_{s1}-CN in solution. Although the bond Trp_{164}-Tyr_{165} in α_{s1}-CN in solution is hydrolysed rapidly (McSweeney et al., 1993b; Exterkate et al., 1995), it does not appear to be hydrolysed in cheese - at least no peptide commencing with Tyr_{165} has yet been identified. Although α_{s1}-CN is readily hydrolysed by plasmin (McSweeney et al., 1993c; Le Bars & Gripon, 1993), it does not appear to be hydrolysed to a significant extent by plasmin in cheese.

β-Casein in solution is readily hydrolysed by chymosin, especially at Leu_{192}-Tyr_{193} and also at Ala_{189}-Phe_{190}, Leu_{165}-Ser_{166}, Gln_{167}-Ser_{168}, Leu_{163}-Ser_{164}, Leu_{139}-Leu_{140}, Leu_{127}-Thr_{128}. However, very little of the primary product, β-CN f1–192, is normally produced in cheese. Instead, ~ 50% of the β-casein is hydrolysed by plasmin at Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}- Glu_{108}, yielding β-CN f1–28 (PP8f), f1–105/107 (PP5), f28–105/107 (PP8s), f29–209 (γ^1-CN), f106–209 (γ^2-CN) and f108–209 (γ^3-CN).

The principal water-insoluble peptides are common to several internal-bacterially ripened cheeses, i.e., Cheddar, Cheddar types (English territorials), Edam, Gouda, Maasdammer, Emmental and Parmesan. At least the bonds Phe_{23}-Phe_{24} in α_{s1}-casein and Lys_{28}-Lys_{29}, Lys_{105}- His_{106} Lys_{107}-Glu_{108} in β-casein are cleaved in all these cheeses. The bond Leu_{101}-Glu_{102} of α_{s1}-CN is hydrolysed in all except Emmental and Parmesan, possibly because chymosin is extensively

inactivated in these cheeses; the bond Phe_{23}-Phe_{24} in these cheeses may be hydrolysed by cathepsin D rather than by chymosin.

Many of the water-soluble peptides in Cheddar have been isolated and identified (Singh et al., 1994, 1995, 1996; Fox et al., 1994); these are summarized in Figures 3 and 4. The N-terminal of many of these peptides corresponds to a chymosin (α_{s1}-CN) or a plasmin (β-CN) cleavage site while that of many others corresponds to a known lactococcal CEP cleavage site. Few of the isolated peptides contain a primary chymosin (α_{s1}- CN) or plasmin (β-CN) cleavage site, suggesting that lactococcal CEP cleaves polypeptides produced from α_{s1}-CN by chymosin or from β-CN by plasmin rather than the intact proteins. The C-terminal of many of the peptides does not correspond to a known chymosin, plasmin or lactococcal CEP cleavage site, suggesting: (1) carboxypeptidase activity, which has not been identified in *Lactococcus* and in only a few *Lactobacillus* strains, (2) unreported lactococcal CEP cleavage sites, or (3) activity of other proteinases, eg, from NSLAB or intracellular proteinases from *Lactococcus* or NSLAB or endopeptidases (PepO, PepF).

Significantly, the vast majority of water-soluble peptides are produced from the N-terminal half of α_{s1}- or β-casein; only 4 peptides from α_{s2}-CN and none from κ- CN have been identified so far.

The small peptides in other varieties have not been studied as extensively as those in Cheddar; although some peptides are common to several varieties, RP-HPLC indicates varietal-specific profiles (see Fox & McSweeney, 1996a, b).

There appears to be general agreement that the intensity of cheese flavour correlates with the concentration of free amino acids (Aston et al., 1983). In mature Cheddar and Parmesan, ~ 3 and 20% of total amino acids are free. The concentrations of individual amino acids in a selection of cheeses are summarized in Table 1. Free amino acids contribute directly to cheese flavour and serve as substrates for other flavour-generating reactions, eg, deamination, decarboxylation, desulfuration, Strecker reaction, Maillard reaction.

It is clear from the discussion in this section that considerable information is now available on the extent and nature of proteolysis in Cheddar cheese and to a lesser extent in a number of other varieties. Attempts to accelerate ripening must aim to accelerate in a balanced way the key flavour-generating proteolytic reactions. Unfortunately, the key reactions have not yet been identified - a detailed comparison of the peptide profiles in Cheddar cheeses (or other varieties) varying in quality from poor to excellent would appear to be a desirable objective.

Table 1. Free Amino Acid Composition of Selected Cheeses

Amino Acid	Cheddar mmol/g[a]	Swiss mmol/g[b]	Gouda mg/g[c]	Blue[d]
Ala	4.04	5.66	0.50	1729
Arg	6.37	nd	1.18	445
Asn	-	-	-	-
Asp	11.65	5.40	0.45	860
Cys	0.37	nd	-	-
Gln	-	-	-	-
Glu	21.62	9.79	3.38	4775
Gly	4.14	1.74	0.37	466
His	-	-	0.22	449
Ile	3.58	-	0.65	1929
Leu	21.19	15.72	3.34	3015
Lys	7.80	1.12	2.02	2231
Met	2.95	0.42	0.66	1111
Phe	9.02	6.40	1.67	1785
Pro	2.95	2.41	-	3076
Ser	4.00	2.65	0.54	1045
Thr	3.78	2.80	1.55	1123
Trp	-	-	-	552
Tyr	3.75	1.03	1.00	1154
Val	9.47	8.36	1.13	2408
Orn	nd	nd	-	77
Asn,Gln	-	-	-	876
Citrulline	-	-	-	423
γ-ABA	-	-	-	221

nd = not detected
- = not analysed
γ-ABA = g-amino butyric acid
[a] Wood et al. (1985). 8 m old cheese analyzed by capillary GC.
[b] Wood et al. (1985). 2 m old Swiss-type cheese analyzed by capillary GC.
[c] Visser, F.M.W. (1977c). 6 m old cheese made with starter *Lactococcus lactis* ssp *cremoris*.
[d] Ismail & Hansen (1972). 248 d old Danablue cheese analyzed by amino acid analyzer, results expressed as mg amino acid residue/15.7 g total N.

In the following sections, published studies on accelerated ripening of cheese will be discussed in the context of the preceding discussion on proteolysis during normal ripening.

Methods for accelerating cheese ripening

The methods used to accelerate ripening can be categorised into 6 groups: (1) elevated ripening tempera-

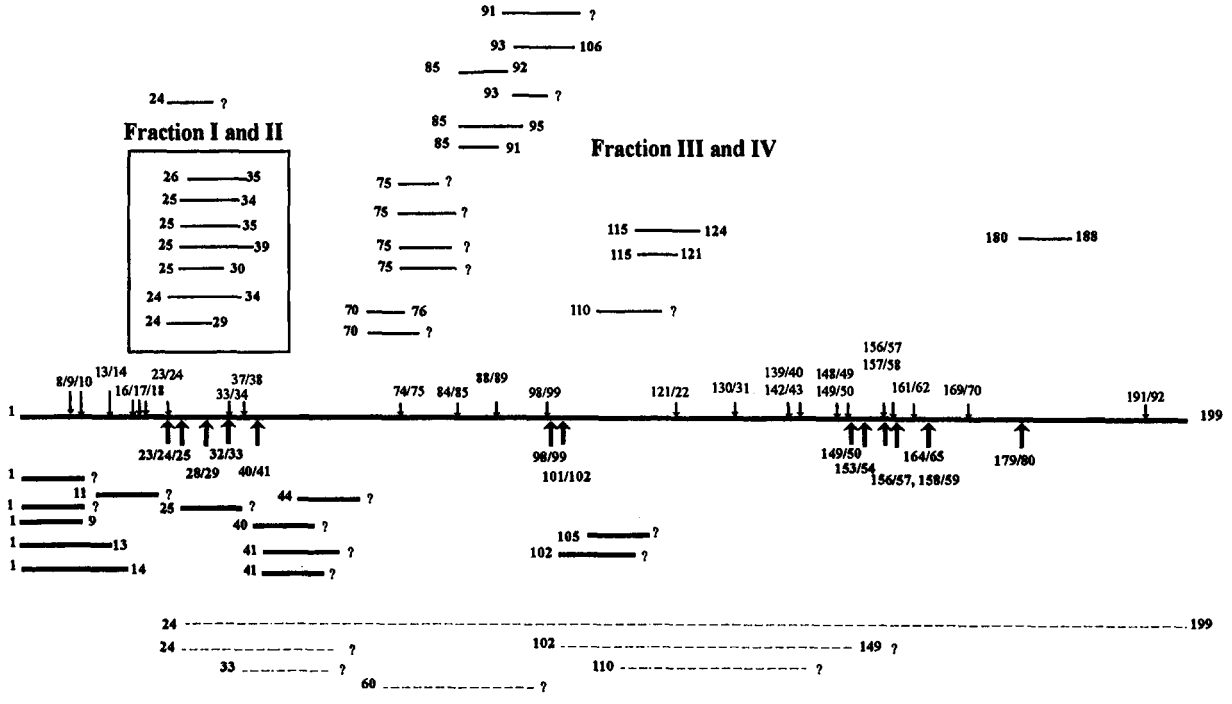

Figure 3. α_{s1}-Casein-derived peptides isolated from the water-insoluble (- - -) or from the diafiltration permeate (____) or retentate (▬▬) of the water-soluble fraction of Cheddar cheese. ? Incomplete sequence (from Singh et al., 1996).

tures, (2) exogenous enzymes, (3) chemically or physically modified cells, (4) genetically modified starters, (5) adjunct cultures, (6) cheese slurries (Table 2). These methods aim to accelerate cheese ripening either by increasing the level(s) of putative key enzymes or by making the conditions under which the 'endigenous' enzymes in cheese operate more favourably for their activity.

Elevated temperature

Traditionally, cheese was ripened in caves or cellars, probably at 15–20 °C for much of the year. Since the introduction of mechanical refrigeration for cheese ripening rooms in the 1940s, the use of controlled ripening temperatures has become normal practice in modern factories. These range from 22–24 °C for Parmesan and Emmental, 12–20 °C for mould and smear-ripened cheeses, 12–14 °C for Dutch varieties to 6–8 °C for Cheddar; thus, the ripening temperature for Cheddar is exceptionally low. The ripening temperature for most varieties is profiled - the above temperatures are the 'maximum' in the profiles and are usually maintained for 4–6 weeks, usually to induce the growth of a desired microflora, after which the cheese is transferred to a much lower temperature, e.g., 4 °C, e.g., Emmental or mould-ripened cheeses. Again, Cheddar is an exception since it is normally ripened at 6–8 °C throughout.

The scope for accelerating the ripening of most cheese varieties by increasing the ripening temperature is quite limited since fat will exude from the cheese > 20 °C. However, this approach has potential for Cheddar and offers the simplest method for accelerating ripening: no additional costs are involved (indeed savings may accrue from reduced refrigeration costs) and there are no legal barriers. However, considering the numerous complex biochemical reactions that occur during ripening, it is unlikely that all reactions will be accelerated equally at elevated temperatures and unbalanced flavour or off-flavours may result.

Based on the results of a study on the influence of starter type, number of NSLAB and ripening temperature (6 or 13 °C) on the flavour of Cheddar cheese, Law et al. (1979) concluded that ripening temperature was the most important single factor in determining flavour intensity, irrespective of the type of starter and number of NSLAB. The time required to reach maturity was at least 50% less at 13 than at 6 °C. Bitterness was more marked at the lower temperature, possibly due to the

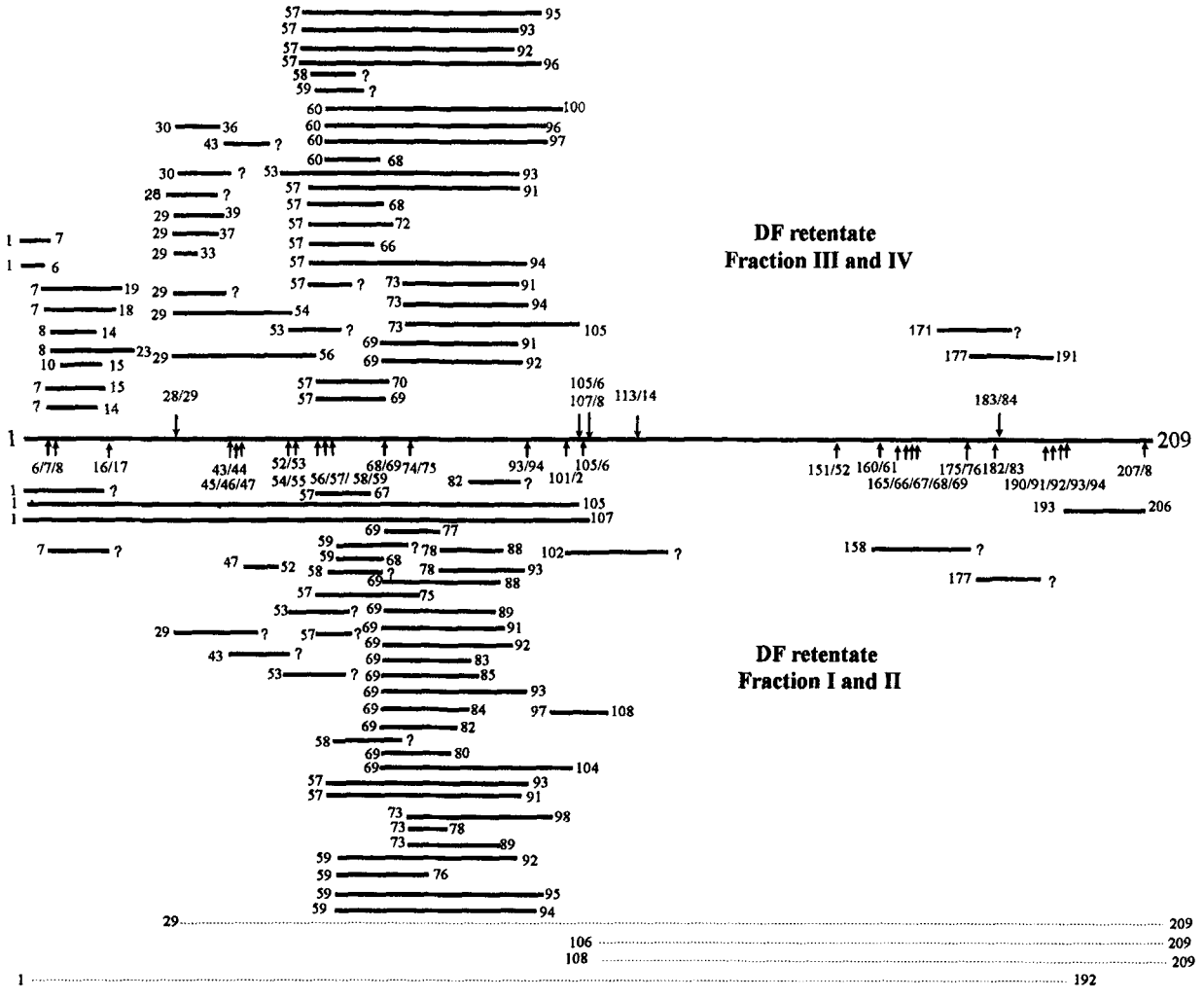

Figure 4. β-Casein-derived peptides isolated from the water-insoluble (- - -) or from the diafiltration retentate (▬) of the water-soluble fraction of Cheddar cheese. ? Incomplete sequence (from Singh et al., 1996).

lower intensity of Cheddar flavour, or perhaps because the peptidases needed to hydrolyse the bitter peptides required the higher temperature for adequate activity.

A very comprehensive study on the effect of temperature (8–20 °C, applied consistently or profiled), alone or in combination with lactose-negative starter or exogenous proteinase (Neutrase), was conducted in Australia in the 1980s by Aston, Dulley, Fedrick and collaborators (Aston et al., 1983; Fedrick et al., 1983; Fedrick & Dulley, 1984; Fedrick, 1987). Ripening was monitored by various chemical and rheological criteria and compositional parameters specified. The essential conclusions of this study, which was reviewed in detail by Fox (1988/89), were that it is possible to reduce the maturation time by ~ 50% by ripening at 13–15 °C but it was emphasised that cheese should be of good compositional and microbiological quality.

High populations of NSLAB present a risk of off-flavour development in cheese ripened at elevated temperatures. Fryer (1982) recommended that Cheddar cheese should contain $< 10^3$ NSLAB/g at hooping and that their numbers should be controlled by rapid cooling to < 10°C. If the cooled cheeses are held for 14 days, NSLAB grow slowly, producing only lactic acid, and should not exceed 10^6/g at 14 days. After this initial period at a low temperature, balanced ripening could be accelerated at a relatively high temperature without a risk of off-flavour development (Fryer, 1982).

The potential of elevated temperatures to accelerate the ripening of Cheddar was also studied by

Table 2. Methods for accelerating cheese ripening

Method	Advantage	Limitations/Problems
Elevated temperature	Effective; no legal barriers; technically simple; no cost, perhaps saving	Non-specific; increased risk of microbial spoilage; applicable to relatively few varieties, e.g. Cheddar
Exogenous enzymes		
rennet	Natural additive; cheap	not effective
plasmin	Indigenous milk enzyme; effective	expensive
Other proteinases/ peptidases	Low cost; specific action; choice of flavour options	Limited choice of useful enzymes; possible legal barriers; difficult to incorporate uniformly; risk of over-ripening; limited commercial use to date
Lipases	Traditional for certain cheese varieties	Risk of rancidity; very limited general use
Selected/activated/modified starters		
Selected starters Enzyme profile Rapid lysis	Normal additives;	None
Attenuated starters Lysozyme treatment Heat or freeze shocked Solvent treated Neutralized starters Mutant cultures, e.g. Lac$^-$	Easily incorporated; natural enzyme profile	May be expensive
Other types of bacterial cells	Easily incorporated; range of enzyme options?	Perhaps legal problems in some cases
Genetically engineered starters	Easily incorporated; desirable enzyme profiles	Possible legal barriers; key enzymes not yet identified
Adjunct starters	Natural microflora; appear to be effective; flavour options; commercially available	Careful selection required
Cheese slurries/ high moisture cheese	Very rapid flavour development; commercially used	High risk of microbial spoilage; suitable only as a food ingredient
Addition of free amino acids to cheese curd	choice of flavour	May be too expensive; limited work to date

Folkertsma et al. (1996). Commercially-made Cheddar cheeses were cooled either rapidly or slowly and ripened for various time/temperature combinations at 8, 12 or 16 °C. NSLAB grew very slowly in the cheese which was cooled rapidly and ripened at 8 °C, although ripening temperature had little influence on

the final numbers of NSLAB (10^7–10^8 cfu/g). Proteolysis (monitored by the formation of water- and PTA-soluble N and total free amino acids, urea-PAGE and RP-HPLC) and lipolysis were accelerated by increasing the ripening temperature. Cheeses ripened at 16 °C received good flavour scores early during ripening but their texture deteriorated after prolonged storage at 16 °C. Ripening at 12 °C was considered to be optimal and ripening could be accelerated or decelerated by increasing or lowering the temperature at any stage of the process.

According to El Soda & Pandian (1991), the use of an elevated temperature to accelerate the ripening of Cheddar cheese is likely to be limited to those made under very hygienic conditions in commercial factories. However, since most Cheddar is now made in highly automated plants from pasteurized milk with initial low counts, elevated ripening temperatures appear to be feasible; at least, ripening at temperatures as low as 6°C is unnecessary unless a very slow rate of ripening is desired, for whatever reasons.

The ripening of Manchego cheese can be accelerated and flavour intensified, especially in cheese made from pasteurized milk, by ripening at an elevated temperature (16 °C); an earlier study had shown that ripening at 20 °C had a negative effect on cheese quality although proteolysis and lipolysis were accelerated compared with cheese ripened at 10 °C (Gaya et al., 1990).

Exogenous enzymes

A number of options are available, ranging from the quite conservative to the more exotic.

Coagulant

Since the coagulant is principally responsible for primary proteolysis in most cheese varieties (see Section 3.1), it might be expected that ripening could be accelerated by increasing the level or activity of rennet in the cheese curd. Although, Exterkate and Alting (1995) suggested that chymosin is the limiting proteolytic agent in the initial production of amino N in cheese, several studies (Stadhouders, 1960; Creamer et al., 1987; Guinee et al., 1991; Johnston et al., 1994) have shown that increasing the level of rennet in cheese curd (achieved by various means) does not accelerate ripening and in fact probably causes bitterness. However, as far as we are aware, the combined effect of increasing rennet level and starter and/or NSLAB population has not been investigated.

The natural function of chymosin is to coagulate milk in the stomach, thereby increasing the efficiency of digestion. It is fortuitous that chymosin is not only the most efficient milk coagulant but also gives best results in cheese ripening. However, it seems reasonable to suggest that the efficiency of chymosin in cheese ripening could be improved by protein engineering. The chymosin gene has been cloned and expressed in several microorganisms (*Kluyveromyces marxianus* var. *lactis*, *E. coli* and *Aspergillus niger* var. *awamori*) and chymosin from such sources is now used widely, but not universally, in commercial cheese manufacture, with excellent results (see Teuber, 1990; IDF, 1992). The gene for the acid proteinase of *R. miehei* has also been cloned and expressed in *A. oryzae*, and the product is commercially available (Marzyme GM; Texel, Cheshire, UK). In all these cases, the parent gene has not been modified but a number of studies on the genetic engineering of chymosin have been published (see Fox & McSweeney, 1996b). As far as we know, the cheesemaking properties of such mutants have not been assessed.

As discussed in Section 3.1, chymosin has very little activity on β-casein in cheese, probably because the principal chymosin- susceptible bond in β-casein, Leu_{192}-Tyr_{193}, is in the hydrophobic C-terminal region of the molecule which appears to interact hydrophobically in cheese, rendering this bond inaccessible. However, *C. parasitica* proteinase preferentially hydrolyses β- casein in cheese (possibly because its preferred cleavage sites are in the hydrophilic N-terminal region) without causing flavour defects (Rea & Fox, unpublished). A rennet containing chymosin and *C. parasitica* proteinase might be useful for accelerating ripening.

Plasmin

Plasmin contributes to proteolysis in cheese, especially of high- cooked varieties in which chymosin is extensively or totally inactivated (see Section 3.1). Plasmin is associated with the casein micelles in milk, which can bind at least 10 times the amount of plasmin normally present (Farkye & Fox, 1992) and is totally and uniformly incorporated into cheese curd, thus overcoming one of the major problems encountered with the use of exogenous proteinase to accelerate cheese ripening.

Addition of exogenous plasmin to cheesemilk accelerated the ripening of cheese made from that of

milk without off-flavour development (Farkye & Fox, 1992; Farkye & Landkammer, 1992; Kelly, 1995). At present, plasmin is too expensive for use in cheese on a commercial scale. Perhaps the gene for plasmin can be cloned in a suitable bacterial host which could be engineered to excrete the enzyme. Since milk normally contains 4 times as much plasminogen as plasmin, an alternative strategy might be to activate indigenous plasminogen by adding a plasminogen activator, eg, urokinase, which also associates with the casein micelles. However, the cost of this approach may also be excessive.

Since plasmin is a trypsin-like enzyme, trypsin, which is relatively cheap and readily available commercially, may also be suitable for accelerating ripening. Careful use of trypsin has been reported (Madkor & Fox, 1994) to accelerate ripening but these findings must be confirmed. Since trypsin is more proteolytic than plasmin, greater care is required in its use.

Exogenous proteinases

The possibility of accelerating ripening through the use of exogenous (non-rennet) proteinases has attracted considerable attention over the past 20 years. The principal problems associated with this approach, which has been reviewed by Law (1984, 1987) and Fox (1988/89), are ensuring uniform distribution of the enzyme in the curd and the prohibition of exogenous enzymes in many countries.

The earliest reports on the use of exogenous enzymes to accelerate the ripening of Cheddar cheese appear to be those of Kosikowski and collaborators who investigated various combinations of commercially available acid and neutral proteinases, lipases, decarboxylases and lactases (see Fox, 1988/89 for references). Acid proteinases produced pronounced bitterness but the addition of certain neutral proteinases and peptidases with the salt gave a marked increase in flavour after 1 month at 20 °C but an overripe, burnt flavour and free fluid were evident after 1 month at 32 °C. Incorporation of the enzyme- treated cheese in processed cheese gave a marked increase in Cheddar flavour at 10% addition and a very sharp flavour at 20%. Good quality medium-sharp Cheddar could be produced in 3 months at 10 °C through the addition of combinations of selected proteinases and lipases. Up to 60% enzyme-treated (fungal lipases and proteinases) UF retentate could be successfully incorporated into processed cheese.

On the assumption that a mixture of enzymes is likely to be more effective at accelerating ripening than a single enzyme, Law (1980) described the results of Cheddar cheesemaking trials in which a proteinase-peptidase preparation from a *Pseudomonas* culture was incorporated into the curd at salting (the organism secreted an extracellular proteinase and released intracellular peptidases when grown in media containing surfactants). A low level of enzyme addition accelerated flavour development, especially during the early stages of ripening, but larger amounts of enzyme caused bitterness and other off- flavours.

Law and Wigmore (1982a, b, 1983) compared the influence of acid, neutral and alkaline proteinases on proteolysis and flavour development in Cheddar cheese. Neutrase (*B. subtilis*), which enhanced flavour development at a low level of enzyme addition but caused bitterness at higher levels, was considered to be the most promising of the enzymes tested, possible because it is unstable in cheese (and hence its activity is somewhat limited), whereas acid proteinases are more stable. Use of an optimum level of Neutrase reduced the ripening time by ~ 50% but enzyme-treated cheese had a softer body and was more brittle than control cheeses of the same age. A combination of Neutrase and streptococcal cell-free extract (CFE) gave better results than Neutrase alone. Although increasing the level of CFE progressively increased proteolysis, flavour intensity did not increase *pro rata*, suggesting that subsequent amino acid transformations to sapid compounds were rate-limiting and were not catalysed by the enzymes in the CFE. This combined enzyme preparation was commercialized by Imperial Biotechnology, London, and marketed as 'Accelase'. Its use in several large-scale commercial cheesemaking trials was described by Fullbrook (1987). However, in spite of the claimed success of the Accelase in pilot-scale and commercial-scale studies, it has not been commercially successful and, as far as we are aware, is not currently available; its commercial failure may be due to the prohibition on the use of exogenous enzymes (other than rennet) in cheesemaking in the UK.

Frick et al. (1984) reported that proteinase 11 (a neutral proteinase from *A. oryzae*; Miles Marshall) added to Colby cheese curd at salting accelerated ripening without bitter flavour development. However, Fedrick et al. (1986a) could not confirm this; the lowest level of this enzyme that gave detectable flavour enhancement also resulted in bitterness that intensified with increasing level of added enzyme; proteinase P11 produced a slightly higher level of bitterness for com-

parable levels of proteolysis than Neutrase. [Law & Wigmore (1982a, b) had found that the acid proteinase of *A. oryzae* was unsuitable for cheese ripening.]

The results of a comparative study on proteolysis and textural changes in granular Cheddar cheeses supplemented with Neutrase, calf lipase, Neutrase plus calf lipase or NaturAge (a culture-enzyme mixture; Miles Marshall) was reported by Lin et al. (1987). TCA- soluble N increased rapidly in all proteinase-supplemented cheeses but free amino acid levels increased more slowly. Textural changes reflected gross proteolysis better than the formation of free amino acids. Unfortunately, the flavours of the cheeses were not reported.

The combined influence of Neutrase, a lac^- prt^- starter and ripening temperature (8 or 15 °C) was studied by Fedrick et al. (1986b). All treatments accelerated ripening compared to the control at 8 °C. Storage at 15 °C was the most effective single treatment, reducing ripening time by > 50%. Neutrase alone gave ~ 25% reduction. A slightly bitter flavour was noted in the Neutrase-treated cheeses but did not significantly affect panel preferences or grade until late in ripening.

A proteinase (*P. candidum*) - peptidase (*Lc. lactis* or *Lb. casei*) preparation for accelerating the ripening of Dutch, Tilsit or Lowicki-type cheese was described by Kalinowski et al. (1979, 1982). The enzyme, added to the cheesemilk, accelerated proteolysis and approximately halved the ripening time. Addition of a CFE from *Lb. casei, Lb. helveticus* or *Lb. bulgaricus* to the curd accelerated proteolysis and lipolysis in Cheddar cheese but the cheeses were bitter after 2 months (El Soda et al., 1981, 1982).

Guinee et al. (1991) reported that Neutrase, FlavourAge FR (a lipase-proteinase preparation from *A. oryzae*) or extra rennet added to Cheddar curd at salting accelerated flavour development when the cheese was ripened at 5°C for a relatively short period (4–5 months) but excessive proteolysis and associated flavour and body defects occurred on further storage, especially at a higher temperature. According to Wilkinson et al. (1992), neither FlavourAge FR nor DCA 50 (a proteinase-peptidase blend; Imperial Biotechnology, London) caused substantial acceleration of flavour development and in some cases led to off-flavours and textural defects.

Addition of exogenous proteinases to curd. With the exception of rennet and plasmin (which adsorbs on casein micelles), the incorporation and uniform distribution of exogenous proteinases throughout the cheese matrix poses several problems: (1) proteinases are usually water-soluble, and hence when added to cheesemilk, most of the added enzyme is lost in the whey, which increases cost, (2) enzyme-contaminated whey must be heat-treated if the whey proteins are recovered for use as functional proteins; the choice of enzyme is limited to those that are inactivated at temperatures below those that cause thermal denaturation of whey proteins, (3) according to Law & King (1985), the amount of Neutrase that should be added to milk to ensure a sufficient level of enzyme in the curd (Law & Wigmore, 1982a) caused a 25–80% decrease in rennet coagulation time, yielded a soft curd and at least 20% of the β- casein was hydrolysed at pressing; presumably, this would reduce cheese yield, which was not measured.

Consequently, most investigators have added enzyme, usually diluted with salt to facilitate mixing, to the curd at salting. Since the diffusion coefficient of large molecules, like proteinases and lipases, is very low, this method is applicable only to Cheddar-type cheeses, which are salted as chips at the end of manufacture, and not to surface- salted (brine or dry) cheeses which include most varieties. Even with Cheddar-type cheeses, the enzyme will be concentrated at the surface of chips, which may be quite large. Uneven mixing of the salt-enzyme mixture with the curd may lead to 'hot spots' where excessive proteolysis and lipolysis, with concomitant off-flavours, may occur.

Enzyme encapsulation offers the possibility of overcoming the above problems. The microcapsules, being sufficiently large, are occluded in the curd; the main problem is to achieve the release of the enzymes after curd formation. Several studies on the microencapsulation of enzymes for incorporation into cheese have been reported (see Fox, 1988/89; Pandian & El-Soda, 1991; Wilkinson, 1993; Skeie, 1994). Although microcapsules added to milk are incorporated efficiently into cheese curd, the efficiency of enzyme encapsulation is low, thus increasing cost. As far as we know, encapsulated enzymes are not being used commercially in cheese production.

Exogenous lipases

Lipolysis is a major contributor, directly or indirectly, in flavour development in strong-flavoured cheeses, eg, hard Italian, Blue varieties, Feta. Rennet paste or crude preparations of pre-gastric esterase (PGE) are normally used in the production of Italian cheeses (see Nelson et al., 1977; Fox, 1988/89; Kilara, 1985; Fox

& Stepaniak, 1993). *M. miehei* lipase may also be used for Italian cheeses, although it is less effective than PGE; lipases from *P. roqueforti* and *P. candidum* may also be satisfactory.

The ripening of blue cheese may be accelerated and quality improved by added lipases (see Fox, 1988/89; Kilara, 1985; Fox & Stepaniak, 1993). A Blue cheese substitute for use as an ingredient for salad dressings and cheese dips can be produced from fat-curd blends by treatment with fungal lipases and *P. roqueforti* spores (see Fox, 1988/89; Kilara, 1985; Fox & Stepaniak, 1993, for references).

Although Cheddar-type and Dutch-type cheeses undergo little lipolysis during ripening, it has been claimed that addition of rennet paste or gastric lipase improves the flavour of Cheddar cheese, especially that made from pasteurized milk; several patents have been issued for the use of lipases to improve the flavour of 'American' or 'processed American' cheeses (see Nelson et al., 1977; Kilara, 1985). The enzyme mixtures used by Kosikowski and collaborators (see Fox, 1988/89) to accelerate Cheddar cheese ripening contained lipases. Law & Wigmore (1985) reported that the addition of PGE or *M. miehei* lipase, with or without Neutrase, to Cheddar cheese curd had a negative effect on flavour quality.

FlavorAge contains a unique lipase from a strain of *A. oryzae* which has an exceptionally high specificity for C_6-C_8 acids and forms micelles, $\sim$ 0.2 μm in diameter, in aqueous media as a result of which $\sim$ 94% of the enzyme added to milk is recovered in the cheese curds (Arbige et al., 1986). According to these authors, FlavourAge accelerated the ripening of Cheddar cheese; the formation of short-chain fatty acids paralleled flavour intensity in Cheddar cheese. In contrast to the FFA profile caused by PGE, which liberated high concentrations of butanoic acid, the FFA profile in cheese treated with FlavourAge was similar to that in the control cheese except that the level of FFA was much higher (Arbige et al., 1986).

Frick et al. (1984) compared the fatty acid profiles in Colby cheese to which FlavourAge or Miles 600 lipase plus proteinase was added. The latter produced a Romano-type flavour while FlavourAge produced a flavour more typical of an aged Cheddar at similar enzyme activities. Addition of an unspecified lipase to Samsoe yielded a cheese with a flavour closely resembling that of Greek Kasseri cheese (Jensen, 1970). Feta cheese produced from cow's milk with a blend of *Lc. lactis* and *Lb. casei* as starter and a blend of kid and lamb PGEs developed the body, flavour and texture of authentic Feta cheese (Efthymiou & Mattick, 1964). The flavour of Egyptian Ras cheese was improved by addition of PGE or lipases from *M. miehei* or *M. pusillus* (El Shibiny et al., 1978). Low levels of PGE improved and accelerated flavour development in Domiati cheese but prolonged ripening led to rancid off-flavours in enzyme-treated cheeses (El Neshawy et al., 1982). The flavour of Latin America White cheese was improved by low levels of pre-gastric esterase (Torres & Chandan, 1981).

Selected, activated or modified starters

Since the proteolytic system of the starter bacteria is responsible for the formation of small peptides and amino acids and probably for flavour development in cheese (Section 3.1), it seems obvious to exploit these enzymes to accelerate ripening; at least 4 approaches to do so have been employed.

Selected starters

The primary function of starters is to produce acid at a reliable and predictable rate. Traditionally, cheesemakers relied on the indigenous microflora of milk or on 'slop-back' natural starters for acid production. Such methods are still used for artisinal cheeses and even for such famous varieties as Parmesan. However, selected, undefined starters have been used for Cheddar, Dutch and Swiss cheeses since the beginning of this century and have been refined and improved progressively over the years. In the case of Cheddar, cocktails of phage-unrelated, single-strain starters were introduced in New Zealand by Whitehead in the 1930s, and are now widely used in New Zealand, Australia, Ireland, USA and probably elsewhere.

The principal criterion applied in the selection of single-strain starters is phage-unrelatedness; other important criteria include the ability to grow well and produce acid at the temperature profile used in cheesemaking and inter-strain compatibility (Martley & Lawrence, 1972; Crow et al., 1993); selection is usually made by the protocol of Heap and Lawrence (1976). Bitterness is a common problem with fast acid-producing strains, apparently because these strains have high heat tolerance and usually reach high numbers in the cheese curd (Lemieux & Simard, 1991, 1992). Fast acid-producing strains are usually *Lc. lactis* ssp *lactis*; consequently, strains of *Lc. lactis* ssp *cremoris* are now usually used as starters for Cheddar cheese.

Although the selection protocol of Heap and Lawrence (1976) does not include specific criteria for the selection of starter strains with the ability to produce high quality cheese, commercial experience has provided evidence for the exclusion of strains with undesirable cheesemaking properties, eg, bitterness, and the use of strains that more or less consistently produce high quality cheeses. The scientific selection of starter strains with desirable cheesemaking properties is hampered by the lack of precise knowledge as to which enzymes are most important.

Selection based on enzyme profiles. Lactococcal strains differ considerably with respect to total and cell wall-associated proteinase activity (Coolbear et al., 1994; Crow et al., 1994); however, no information is available on the comparative cheesemaking properties of these strains. Breen and Fox (unpublished) studied the cheesemaking properties of 19 single-strain starters in Cheddar cheese manufactured on a small (20 L) scale; results indicated considerable inter-strain variations in proteolysis, lipolysis and sensory quality (Figure 5). Unfortunately, information is not available at present on the enzyme complement of these strains. The influence of starter strain on the sensory properties of Cheddar cheese was also demonstrated by Muir et al. (1996). Further studies on the cheesemaking properties, preferably on a large scale, and the enzyme complement of single-strain *Lactococcus* starters is warranted.

The only extracellular enzyme in *Lactococcus* is the cell wall- associated proteinase. The peptidases are intracellular, although some may have a peripheral location (Tan et al., 1992). The esterase(s) and phosphatase(s) are also intracellular. The significance of lactococcal exopeptidases in cheese quality is unclear but they are responsible for the production of free amino acids and probably thus influence flavour development (see Section 3.1). Dephosphorylation of casein-derived peptides occurs during ripening (Singh et al., 1995, 1996). The significance of dephosphorylation is not known although Martley & Lawrence (1972) suggested that phosphatase activity was an important attribute of starters.

Selection based on starter cell lysis. Since the growth of lactococci ceases at or shortly after the end of curd manufacture (Martley & Lawrence, 1972; Visser, 1977b), their intracellular enzymes are ineffective until the cells die and lyse. Generally, *Lc. cremoris* cultures die faster than *Lc. lactis* ssp *lactis* strains although there is considerable interstrain variation within each subspecies (Martley & Lawrence, 1972; Visser, 1977b; Chapot-Chartier et al., 1994; Wilkinson et al., 1994b; O'Donovan, 1994). Information on the rate of lysis of *Lactococcus* species in cheese is rather limited but available evidence indicates substantial inter-strain differences (Wilkinson et al., 1994b; Chapot-Chartier et al., 1994).

It would be expected that the sooner starter peptidases are released through lysis, the sooner they can participate in proteolysis and hence the faster the rate of ripening. However, the stability of lactococcal exopeptidases in cheese is unknown. If they are unstable, it is possible that enzymes released early during ripening through accelerated lysis may contribute little to flavour development since the concentration of suitable peptides is low at this time. The stability of some intracellular marker enzymes was studied by Wilkinson et al. (1994a) who found that PepX activity was quite unstable (15% of initial activity remained after 24 h) in a cheese slurry system (pH 5.17). The other enzyme activities studied (glucose–6-phosphate hydrogenase and lactate dehydrogenase) were also relatively unstable. In contrast, Chapot- Chartier et al. (1994) found that PepX and PepC/N activities were stable in an extract of St. Paulin cheese (pH 5.8). Further research in this area appears warranted.

The release of intracellular peptidases into the matrix of St. Paulin cheese as a consequence of lysis was confirmed by Chapot-Chartier et al. (1994). Cheese made with fast-lysing *Lc. lactis* subsp *cremoris* AM2 developed higher levels of amino nitrogen than that made with slow-lysing *Lc. lactis* subsp *lactis* NCDO 763; lower levels of bitterness were reported in the cheese made with the fast-lysing starter. Wilkinson et al. (1994b) reported that the production of free amino acids was 5 times faster in Cheddar cheese made using a fast-lysing strain (AM2) than in cheese made using a slow-lysing strain (HP); the latter cheese was bitter.

Considering the presumed importance of cell lysis, a number of authors have attempted to accelerate ripening by increasing the rate of starter lysis. Four principal approaches have been investigated:

Selection of naturally fast-lysing strains. There have been few systematic studies on rate of lysis of *Lactococcus* but many known fast-lysing strains have undesirable cheesemaking properties, eg, slow acid production or phage sensitivity. Further studies in this area are warranted.

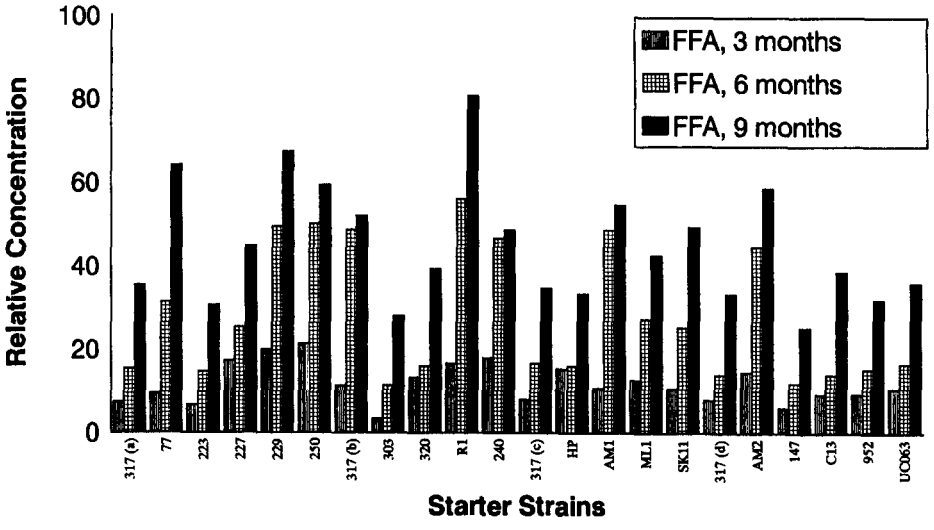

Figure 5. Relative concentration of free amino acids in Cheddar cheeses manufactured using single strain *Lactococcus* starters (Breen D & Fox PF, unpublished).

Thermoinducible lysis. Feirtag & McKay (1987a, b) isolated a *Lactococcus* mutant which underwent lysis during the cooking of Cheddar cheese (38 to 40 °C) because it harboured a thermoinducible prophage. The authors speculated that such strains would be useful for accelerating cheese ripening through the early release of intracellular enzymes; however, extensive loss of these enzymes in the whey may occur. No further reports on this or similar strains appear to have been published. Should this approach prove effective in practice, it should be possible to construct thermoinducible mutants of any desired starter by genetic techniques.

Bacteriophage-assisted lysis. Crow et al. (1996) accelerated the lysis of *Lc. lactis* subsp *lactis* ML8 using its homologous phage, ml_8. Although the phage was added to the milk at the start of cheese- making, it did not adversely affect acid production during manufacture. Phage treatment accelerated the decline in viable starter numbers during ripening, accelerated the release of free amino acids and ammonia and reduced bitterness. Phage-induced lysis may have potential for accelerating ripening but the technique may be unacceptable to cheese manufacturers due to fears of unpredictable acid production.

Bacteriocin-induced lysis. In recent years, there has been a surge of interest in broad- spectrum bacteriocins, mostly due to their potential to preserve foods against spoilage and pathogenic organisms. The classical example of a commercially successful bacteriocin is nisin (Daeschel, 1993). The potential applications of narrow-spectrum bacteriocins have not been investigated but they have been studied extensively at the molecular level.

Morgan et al. (1995) identified a narrow-spectrum bacteriocin producer, *L. lactis* subsp *lactis* biovar. *diacetylactis* DPC3286, which differed from other lactococcal bacteriocin producers in that it exhibited a bacteriolytic effect on sensitive lactococci; generally, lactococcal bacteriocins exhibit either a bacteriostatic or bactericidal effect. Analysis of strain DPC3286 revealed that it is both proteinase- and lactose-negative and that bacteriocin production is encoded on a 78-kb plasmid, pSM78. DPC3286 produces three bacteriocins, lactococcins A, B and M, all of which have been studied in detail (van Belkum et al., 1989, 1991a; Venema et al., 1993, 1994). The genetic organization of the genes encoding the lactococcins was found to be highly conserved between DPC3286 and *Lc. lactis* subsp. *cremoris* 9B4 (the strain investigated by van Belkum et al., 1989). The mechanism of action of lactococcins A and B has been identified (van Belkum et al., 1991b; Venema et al., 1993). Since neither A nor B is capable of lysing cells and although the mechanism of action of lactococcin M has not been reported, it is thought that a combination of the three lactococcins may initiate lysis.

All Cheddar cheese starter cultures tested were found to be sensitive to the bacteriolytic activity of DPC3286, although to different extents. The potential of DPC3286, used as an adjunct culture to accelerate the lysis of starter lactococci and consequently the

maturation of Cheddar cheese made using *Lc. lactis* subsp. *cremoris* HP, a strain which exhibits a low level of autolysis and produces bitter cheese (O'Donovan, 1994; Wilkinson et al., 1994b), was investigated.

Laboratory-scale (3 L) cheesemaking trials were carried out to determine a suitable ratio of adjunct to starter culture. Since DPC3286 is Lac^- and Prt^-, it does not contribute to acid production during manufacture but its lytic effect on the starter reduced the rate of acid production by the starter culture. Since acid production is critical in cheese manufacture, a level of adjunct that increased the manufacturing time by not more than 30 min was established.

Two pilot-scale trials (500 L) were then conducted using HP with differing levels (0.0–0.225%) of bacteriocin-producing adjunct. In the second trial, a bacteriocin-negative adjunct was included as a control (this strain differs from the bacteriocin-positive strain only in that it lacks the plasmid pSM78, responsible bacteriocin production). The cheeses, which were within the compositional range for Cheddar, were ripened at 8 °C. Lysis was monitored by the release of intracellular enzymes assayed in 'cheese-juice' expressed from cheese under hydraulic pressure. Greater release of intracellular LDH was observed in cheeses containing the bacteriocin-producing adjunct than in the control cheeses, indicating that the adjunct promoted lysis of the HP starter. In trial 1, 0.03 or 0.125% adjunct resulted in average increases of 26 and 66%, respectively, in LDH activity over a 6-month ripening period, relative to the control; corresponding values for intracellular PepX were 60 and 180%. In trial 2, cheese with a 0.225% inoculum of the bacteriocin-producing adjunct exhibited average increases of 62 and 33% LDH and PepX activity over the control while cheeses containing the bacteriocin-negative adjunct had similar LDH activity to control cheese but the level of PepX was increased.

In trial 1, the total concentration of free amino acids in the cheeses containing 0.03 or 0.125% bacteriocin-producing adjunct was 26 and 47% higher, respectively, than in the control. In trial 2, the bacteriocin-producing strain increased the level of free amino acids by 22%. RP- HPLC of cheese juice showed distinctly different peptide profiles for the experimental and control cheeses; the former contained an increased level of hydrophilic peptides, which may indicate reduced bitterness. The experimental cheeses received higher grades than controls for both flavour/aroma and body/texture.

This study revealed that Cheddar manufactured with the bacteriocin-producing strain, DPC3286, as a starter adjunct exhibited increased levels of starter cell lysis, higher concentrations of free amino acids, a reduction in bitterness and higher grading scores; the adjunct did not inhibit the growth of non-starter lactic acid bacteria (since it is a narrow spectrum bacteriocin producer). This novel method for increasing starter cell lysis in Cheddar cheese has many advantages over more conventional methods for accelerating cheese ripening: it requires no special legal approval, avoids the occurrence of hot spots since the bacteriocin-producing cells are distributed throughout the cheese curd and involves no extra costs for specialized equipment. However, extension of the cheese make-time may be a cause for concern.

Attenuated starters

Since the starter plays a key role in cheese ripening it might be expected that increasing cell numbers would accelerate ripening. However, Lowrie & Lawrence (1972) reported that, at least in the case of Cheddar, high numbers of starter cells are associated with bitterness. Not all authors (e.g., Stadhouders et al., 1983) agree that bitterness is related simply to starter cell numbers and suggest that too much or the wrong type of proteolytic activity is responsible, e.g., too little peptidase activity relative to proteinase activity. In fact, a number of authors (see Fox, 1988/89 for references) reported that stimulating starter growth, eg, by adding starter autolysate, protein hydrolysate or trace metals accelerated ripening; this approach appears to run contra to the view that high starter cell numbers cause bitterness. Perhaps the significance of starter cell numbers on cheese ripening should be reinvestigated.

An alternative to the use of high starter cell numbers is the addition of attenuated starter cells to the cheese milk, the rational being to destroy the acid-producing ability of the starter (since excessively rapid acid development is undesirable), but causing as little denaturation of the cell's enzymes as possible. The discussion in the preceding paragraph suggests that adding attenuated cells might cause bitterness but this has not been reported to be a problem, the opposite usually being reported. However, most or all of the studies on the use of attenuated starters have been on varieties other than Cheddar.

Five alternative treatments/approaches have been investigated for the production of attenuated starters.

Lysozyme treatment. Law et al. (1976) report that the addition of lysozyme- treated cells to a level equivalent to 10^{10} cells/g cheese had little influence on the rate of flavour development in Cheddar cheese although the level of free amino acids was increased up to 3 fold compared with controls. Law (1980) considered that while the procedure is suitable for laboratory-scale studies, lysozyme is too expensive for commercial, large-scale cheesemaking; a cheaper supply of lysozyme may render this approach viable.

Heat- or freeze-shocked cells. The lactic acid-producing ability of lactic acid bacteria can be markedly reduced by a sub-lethal heat treatment while only slightly reducing proteinase and peptidase activities; heating at 59 or 69 °C for 15 sec was optimal for mixed mesophilic and lactobacilli cultures, respectively (Pettersson & Sjostrom, 1975). When concentrates of heat-shocked cultures were added to cheese milk at a level of 2% (v/v), ~ 90% of the added cells were entrapped in the curd but entrapment efficiency decreased at higher levels of addition. Proteolysis in Swedish household cheese was increased and quality improved by addition of the heat-shocked cells to the cheesemilk, *Lb. helveticus* being the most effective. The extent of proteolysis increased *pro rata* with the level of heat-shocked *Lb. helveticus* culture added but not for a mesophilic culture, suggesting some limiting factor in the latter. Bitterness was not observed in any of the cheeses.

Essentially similar results were reported by Bartels et al. (1987a) for Gouda cheese. Heat shocking at 70 °C for 18 sec was found to be optimal and 2% addition was almost as effective as 4%. Of several thermophiles investigated, *Lb. helveticus* gave best results; *Lb. bulgaricus* and one strain of *Str. thermophilus* had negative effects on flavour quality due mainly to bitterness. An acetaldehyde-like or yoghurt flavour was noted in most of the cheeses containing heat-shocked lactobacilli.

Heat-shocked (67 °C × 10 s) *Lb. helveticus* cells accelerated amino nitrogen formation and enhanced flavour development in Swedish hard cheese; although Neutrase when added alone accelerated proteolysis, it caused bitterness which was eliminated when both heat-shocked *Lb. helveticus* cells and Neutrase were added to the curd (Ardö & Pettersson, 1988). The effect of heat treatment on the proteolytic system of *Lb. delbrueckii* ssp *bulgaricus* was studied by López-Fandiño & Ardö (1991).

Freeze-thawing also kills bacteria without inactivating their enzymes. Addition of freeze-shocked *Lb. helveticus* CNRZ 32 cells to cheesemilk markedly accelerated proteolysis and flavour development in Gouda cheese without adverse effects (Bartels et al., 1987b). The greatest flavour difference between the control and experimental cheeses was observed after 5 weeks of ripening. Addition of untreated *Lb. helveticus* cells also accelerated proteolysis but caused off-flavours. *Lb. helveticus* peptidases appeared to be capable of degrading and debitterizing bitter peptides.

Solvent-treated cells. Exterkate (1979, 1984) and Exterkate & de Veer (1987) reported that treatment of starter cells with n-butanol activated some membrane-bound proteinases and peptidases, presumably by increasing accessibility for substrate. Addition of a suspension of butanol-treated cells to cheese milk accelerated ripening slightly and, perhaps more importantly, reduced the intensity of bitter flavour compared to control cheeses (Stadhouders et al., 1983). This approach is probably impractical for use in cheesemaking at present because of its complexity and possible legal barriers.

Neutralized inactivated cultures. Shchedushnov and D'Yachenko (1974) described a method for the preparation of inactive starter (*Lactobacillus* spp) by continuous neutralization of the growth medium (whey or skim milk) using marble chips. After 3 days, most of the cells had died but their proteolytic enzymes remained active. Addition of the inactivated starter (1–1.5%), together with the regular starter (1–1.5%), to milk for Cheddar cheese intensified proteolysis and accelerated ripening.

Mutant starters. Because the rate of acid development is a critical factor in cheese manufacture, the amount of normal starter cannot be increased without producing an atypical cheese. This has led to consideration of the use of Lac^- mutants, incorporation of which does not affect the rate of acid development but provides additional proteinases and peptidases.

The use of a Lac^-, Prt^- mutant, *Lc. lactis* C2, to accelerate cheese ripening was described by Grieve & Dulley (1983). Mutant concentrates containing ~ 10^{11} cfu/ml were added to the cheese milk to give levels of starter cells in the curd at milling 10–60 times higher than in the control cheese. Proteolysis was accelerated in the experimental cheeses, flavour quality was improved and flavour development was advanced by up

to 12 weeks over controls. Exposure of some control and experimental cheeses to elevated ripening temperatures (20 °C) for one month further increased proteolysis and advanced flavour development. This work was extended by Aston et al. (1983) and Fedrick et al. (1986b) who studied the combined effects of Lac^- starter, exogenous proteinase and elevated temperatures on cheese ripening; regardless of the other treatments employed, supplementation with Lac^- starter accelerated ripening.

Richardson et al. (1983) recommended the use of Prt^- starters to reduce bitterness in cheese. It was claimed (Oberg et al., 1986) that the rate of proteolysis in Cheddar cheese made using Prt^- starters was similar to that in control cheese, but this was not confirmed by Law et al. (1992) who found considerably higher levels of small peptides and free amino acids in cheese made using Prt^+ starter than in those made with a Prt^- mutant.

Lac^- *Lactococcus* strains with high exopeptidase activity are commercially available as cheese additives. A selection of such cultures obtained from Chr. Hansen's Laboratories (Reading, UK) was assessed by Tobin and Fox (unpublished) in Cheddar cheese with a controlled microflora. The cheeses containing individual Lac^- *Lactococcus* mutants consistently received higher scores for flavour and body than the controls. Proteolysis and lipolysis in these cheeses are being studied.

The current active programmes on the genetics of lactic acid bacteria will probably lead to the development of Lac^- starters with superior cheese ripening properties, e.g., with increased proteinase and/or peptidase or perhaps other activities that may be important in the rate of cheese ripening and/or quality.

Other types of bacterial cells as additives. *Pseudomonas* spp are extremely proteolytic bacteria. They produce very active, heat-stable extracellular proteinases and lipases which have been studied extensively (see McKellar, 1987), owing to their spoilage potential in dairy products, meat and fish. *Pseudomonas* spp also possess a range of intracellular peptidases which have been the subject of relatively little research: an aminopeptidase (Shamsuzzaman & McKellar, 1987; Gobbetti et al., 1995) and a dipeptidase (Gobbetti & Fox, 1996) from *Ps. fluorescens* and an iminopeptidase and a dipeptidase from *Ps. tolaasii* (Baral, 1995) have been studied. *Ps. tolaasii* also possesses a carboxypeptidase, which has not been isolated (Baral, 1995). Since *Pseudomonas* spp are strict aerobes, they will not grow in or on vacuum-packed cheese. Hence, washed *Pseudomonas* cells (i.e., washed free of extracellular proteinase and lipase) should serve as a useful source of peptidases. Niland & Fox (1996) reported a preliminary study on the use of washed *Ps. tolaasii* cells to accelerate the ripening of Cheddar. Washed cells added to cheesemilk at 10^5–10^8 cfu/ml were entrapped in the curd to give approx 10^6–10^9 cfu/g of fresh curd. The cells died very quickly (to $\sim 10^4$ cfu/g after 4 weeks). Even at 10^9 cfu/g, the *Pseudomonas* enzymes did not affect proteolysis as detected by PAGE but did increase the concentration of WSN and amino acids and accelerated textural and flavour development without the occurrence of off-flavours. Inoculation of cheesemilk with 10^7–10^8 cfu/ml was necessary to have a significant effect. Such a large inoculum may be uneconomic although the ability of *Pseudomonas* to grow on cheap minimal media would reduce production costs. It may also be possible to select or genetically engineer strains with very high peptidase activity; unfortunately, as for genetically engineering starter strains, the key peptidase(s), or other key enzymes, required for accelerated ripening are not yet known.

Genetically engineered starters

The considerable knowledge now available on the genetics of cell wall-associated proteinase and many of the intracellular peptidases makes it possible to specifically modify the proteolytic system of starter *Lactococcus*.

The gene for the neutral proteinase (Neutrase) of *B. subtilis* was cloned in *L. lactis* UC317 by McGarry et al. (1995). Cheddar cheese manufactured with this engineered culture as the sole starter underwent very extensive proteolysis and the texture became very soft within 2 weeks at 8 °C. The cheese was not tasted but its aroma was satisfactory. By using a blend of unmodified and Neutrase-producing cells as starter, a more controlled rate of proteolysis was obtained and ripening was accelerated (McGarry et al., 1994). An 80:20 blend of unmodified:modified cells gave best results. Since the genetically-modified cells were not food grade, the cheese was not tasted but the results appear sufficiently interesting to warrant further investigation when a food-grade modified mutant becomes available.

Since free amino acids are widely believed to make a major contribution, directly or indirectly, to flavour development in cheese, the use of a starter with increased aminopeptidase activity would appear to be

attractive. Two studies have been reported (McGarry et al., 1995; Christensen et al., 1995) on the use of a starter genetically engineered to super-produce aminopeptidase N; although the release of amino acids was accelerated, the rate of flavour development and its intensity were not, suggesting that the release of amino acids is not rate limiting. The availability of *Lactococcus* mutants lacking up to 5 peptidases (Mierau et al., 1996) should facilitate identification of key peptidases and hence the engineering of mutants that superproduce these peptidases.

Adjunct starters

The fourth group of contributors to the ripening of cheese are non- starter lactic acid bacteria (NSLAB) which may originate in the milk, especially if raw milk is used, or the cheesemaking environment (equipment, air, personnel). Cheese is quite a hostile environment (low pH, low E_h, lack of fermentable carbohydrate, probiotics produced by the starter) and consequently very few genera of bacteria can grow or even survive in properly made cheese. Apart from *Clostridium* spp, which can grow in the interior of most cheeses (Cheddar types are the major exceptions) unless adequate precautions are taken, NSLAB are the principal bacteria capable of growth in the interior of cheese.

Although NSLAB have been reported to include *Micrococcus, Pediococcus* and *Enterococcus* (in special cases), the predominant species are mesophilic lactobacilli, which may be the only non-starter bacteria present (Jordan & Cogan, 1993). In Cheddar and Dutch-type cheeses made from high-quality pasteurized milk in modern enclosed automated plants, the number of NSLAB is < 50 cfu/g in 1 day-old cheese. These grow at a temperature-dependent rate to $\sim 10^7$, typically within about 2 months in the case of Cheddar. As discussed in Section 3.1, the significance of NSLAB to cheese ripening and quality is unclear; experiments on cheese with a controlled microflora suggest that they perform a similar proteolytic function to starter *Lactococcus* but are less effective.

There is a widely held view that cheese made from raw milk ripens faster and develops a more intense flavour than cheese made from pasteurized milk, suggesting that the indigenous microflora may be responsible. However, pasteurization causes other changes in addition to killing the indigenous microorganisms, e.g., inactivation of indigenous enzymes, denaturation of whey proteins, minor shifts in milk salts. The development of microfiltration permits the removal of indigenous microorganisms without other concomitant changes.

The ripening of Cheddar cheese made from raw, pasteurized or microfiltered milk was compared by McSweeney et al. (1993a). The cheeses made from pasteurized or microfiltered milk were essentially similar with respect to proteolysis, lipolysis, microflora and quality but were considerably different from the cheeses made from raw milk. Flavour developed faster and more intensely in the raw milk cheeses than in the other two cheeses, although it was considered atypical of modern Cheddar. The number of NSLAB was about 10-fold higher in the raw milk cheese than in the others (10^8 compared with 10^7 cfu/g) and were more heterogeneous. Essentially similar results were reported by Bouton & Grappin (1995) for Gruyère Comte cheese made from raw or microfiltered milk. It is concluded from these studies that the microflora of raw milk cheese makes a significant and perhaps a positive contribution to cheese quality.

The development of a more intense flavour in raw milk cheese has stimulated interest in *Lactobacillus* cultures for addition to pasteurized milk to simulate the quality of raw milk cheese. Such cultures are now available from commercial starter suppliers but little scientific information is available on their performance. Published studies include: Puchades et al. (1989), Broome et al. (1990), Lee et al. (1990) and McSweeney et al. (1994b). In all of these studies, low numbers of selected mesophilic lactobacilli were added to the cheesemilk; there is general agreement that the lactobacilli modified proteolysis: in particular, they resulted in the formation of a higher concentration of free amino acids than in the control cheese and improved sensoric quality.

Two further studies on the use of mesophilic lactobacilli as adjunct starters have been completed in our laboratory using cheese made with a controlled microflora (Lynch et al., 1996a, b). In one study, adjunct cultures of 4 species of mesophilic lactobacilli (*Lb. plantarum, Lb. casei* ssp *pseudoplantarum, Lb. casei* ssp *casei* and *Lb. curvatus*) were added individually to the cheese milks at a level of $\sim 10^3$ cfu/ml; a fifth uninoculated vat served as control. Numbers of lactobacilli in the experimental cheeses ex-press were 10^4 to 10^5 cfu/g and increased rapidly during the first month of ripening, reaching a maximum in all cases of 10^7 to 10^8 cfu/g after ~ 3 months. Numbers of *Lb. casei* ssp *casei* and *Lb. curvatus* showed no decline to the end of ripening but numbers of *Lb. plantarum* and *Lb. casei* ssp *pseudoplantarum* decreased by ~ 1 log

cycle between 3 and 6 months. The control cheeses remained free of 'wild' NSLAB for 34 (trial 1) and 97 (trial 2) days and their numbers were always at least 2 log cycles lower than the number of lactobacilli in the experimental cheeses.

The 4 month-old control and experimental cheeses received similar scores for flavour intensity and flavour acceptability. After 6 months, the control cheeses received the highest scores for flavour intensity but the lowest scores for flavour acceptability; slight bitterness in the control cheeses detected by some graders may have accounted for this. *Lb. plantarum* and *Lb. casei* ssp *pseudoplantarum* improved flavour acceptability to a greater extent than *Lb. casei* or *casei* and *Lb. curvatus*.

Urea-PAGE showed essentially no differences in proteolysis between the cheeses and only minor quantitative differences between the water-soluble extracts. However, the level of total free amino acids (FAA) was higher in the experimental cheeses than in the controls towards the end of ripening, in agreement with earlier studies. The effectiveness of *Lb. plantarum* and *Lb. casei* ssp *pseudoplantarum* as adjuncts has not been reported previously; they appear to warrant further investigation.

The second study was designed to assess the influence of a mixed *Lactobacillus* adjunct culture (comprising of strains of *Lb. casei* ssp *casei, Lb. casei* ssp *pseudoplantarum*, *Lb. plantarum* and *Lb. curvatus*) on the ripening of Cheddar cheese acidified by starter or with lactic acid and glucono-δ-lactone (GDL) (O'Keeffe et al., 1976a). Numbers of lactobacilli in the adjunct-containing cheeses were 10^6 to 10^7 cfu/g of curd ex-press and $\sim 10^8$ cfu/g after 1 month; numbers decreased slightly thereafter. The control starter cheese remained free of 'wild' NSLAB for $\sim$ 1 month while the chemically-acidified control cheeses remained free for only 1 or 2 weeks; in both control cheeses, the number of NSLAB remained at least 2 log cycles lower than in the experimental cheeses throughout ripening.

Adjunct lactobacilli considerably intensified the flavour of the GDL/NSLAB cheese in comparison to the GDL control; however, the flavour was considered unacceptable by many members of the panel and was downgraded for flavour acceptability. The starter-acidified cheeses (with or without adjunct lactobacilli) received considerably higher grades for flavour intensity and flavour acceptability than GDL cheeses. The starter/NSLAB cheese received slightly higher scores for flavour intensity but slightly lower scores for flavour acceptability than the starter control cheese. The sensory data suggest that the adjunct was capable of intensifying but not necessarily improving cheese flavour.

Urea-PAGE showed essentially no differences in primary proteolysis between the cheeses. Levels of WSN were higher in the starter-acidified than in the chemically-acidified cheeses and NSLAB influenced WSN development to only a very minor extent. Both starter-acidified cheeses had considerably higher levels of FAA than the chemically-acidified cheeses, highlighting the importance of the starter in FAA formation. Both the GDL/NSLAB and starter/NSLAB cheeses had higher levels of FAA than their corresponding controls throughout ripening, probably due to increased peptidase activity contributed by the adjunct lactobacilli.

RP-HPLC of 70% ethanol-soluble fractions of the cheeses showed few differences in peptide profiles between the starter and starter/NSLAB cheeses but there were major differences between the chemically and starter-acidified cheeses. Quantitative and qualitative differences were also apparent between the chromatograms of GDL and GDL/NSLAB cheeses.

The results of these studies show that adjunct cultures of mesophilic lactobacilli do influence proteolysis in Cheddar cheese during ripening (to a greater extent in the absence of a starter than in its presence), mainly at the level of FAA formation. While the adjunct used did not accelerate ripening to a significant extent, some modification of cheese flavour was achieved. The use of a single species as adjunct appears to be a more promising than a 'cocktail' of species in enhancing the sensory properties of Cheddar. Further research is being undertaken to confirm this.

Although the *Lactobacillus* strains used in the studies by McSweeney et al. (1994b) and Lynch et al. (1996a, b) were isolated from the highly flavoured raw milk cheese studied by McSweeney et al. (1993a), the impact of these strains when used either individually or as cocktails was very much less than the more heterogeneous indigenous microflora of raw milk. Although the flavour of the raw milk cheese studied by McSweeney et al. (1993a) was atypical of 'modern' Cheddar, it was very much more intense than that of the cheeses made from pasteurized or microfiltered milk. Lactobacilli clearly have the potential to modify cheese flavour and accelerate flavour development but further research is required to select the best strains.

Secondary cultures

The final agent involved in the ripening of many cheese varieties are the secondary starters, especially *Propionibacterium, Brevibacterium, Penicillium* and some yeasts. As discussed in Section 3.1, these cultures play key and characterizing roles in the ripening of cheeses in which they are used. With the exception of some Swiss varieties, cheeses in which secondary starters are used have relatively short ripening times, due to a relatively high moisture content and the very high activity of the secondary starter.

Apart from the production of blue cheese substitutes (as discussed in Section 4.2.4), we are not aware of work on the accelerated ripening of cheeses with a secondary starter and they will not be discussed in this review.

Cheese 'slurries'

The greatest acceleration of ripening has been achieved using the slurry system introduced by Kristoffersen et al. (1967) and refined by Singh & Kristoffersen (1970, 1971b, 1972). Cheddar cheese curd slurried in ~ 3% NaCl to ~ 40% cheese solids developed full flavour in 4–5 days at 30–35 °C when reduced glutathione (100 mg/kg) was included. A relationship was shown between flavour development and the formation of active SH groups and free fatty acids. Ripening of slurries made from chemically acidified curd showed the importance of rennet, lactic acid starter, glutathione and pH (~ 5.3, to retard the growth of undesirable bacteria). Addition of cheese slurries to cheese milk or cheese curd was reported (Abdel Baky et al., 1982a, b) to accelerate the ripening of Cephalotyre 'Ras' cheese. Inclusion of proteinases, lipases or trace elements in the slurries improved their effectiveness.

Ripened Cheddar cheese slurries have been successfully incorporated into processed cheese up to ~ 20% of the blend (Sutherland, 1975). Dulley (1976) reported that addition of a slurry of ripened cheese, which was considered to serve as a source of lactobacilli, to cheese milk reduced the ripening time of the resultant cheese by ~ 25%. A similar principle was described by von Bockelmann and Lodkin (1974): mature cheese was homogenized in Na_3 citrate and added to cheese milk before manufacture; ripening of the resultant cheese was accelerated, apparently due to an increased population of lactobacilli in the cheese.

'Cheese slurries' have been adopted for the rapid ripening of Brick (Kristoffersen et al., 1967), Feta (Zerfiridis & Kristoffersen, 1970) & Swiss (Singh and Kristoffersen, 1971a). A fast-ripening procedure for the preparation of particulate Blue cheese of normal composition was described by Harte & Stine (1972).

Cheese slurries have been used as models in which to study the biochemistry of cheese ripening, to screen the suitability of proteinases and lipases for use as cheese additives and of bacterial cultures as starter or adjuncts. The principal attractions of cheese slurries for such purposes are the short ripening time, the low cost and the possibility of including numerous parameters in a single study which is not possible with cheesemaking, even on a pilot scale. While acknowledging these important advantages, we believe that slurries do not approximate the composition of cheese sufficiently closely and are suitable only for screening cultures or enzymes.

A variant of the slurry system is used in the Novo process for the production of 'Enzyme Modified Cheese' (EMC). Medium-aged cheese is mixed with water, 'emulsified', homogenized and pasteurized to control the indigenous microflora. After cooling, exogenous enzymes, e.g., 'Palatase' (a *M. miehei* lipase) and/or proteinase, are added at the required level and the mixture incubated at 40 °C for 12–96 h. The mixture is then repasteurized (66–72 °C for 4–8 min) to yield an EMC paste that may be used in processed cheese, soups, dips, dressings, snack foods, etc. EMC has 5–20 times the flavour impact of the mild Cheddar from which it is made and may be used at 2–3% of a processed cheese blend.

A similar approach is used by the Miles Marshall Company in the preparation of Marstar enzyme-modified cheese products for use in processed cheeses or in recipes containing cheese (Talbott & McCord, 1981). A range of enzyme preparations is available for Cheddar, Romano and Swiss cheese. The enzyme preparations are added to cheese pastes 40–55% (of total solids) at a level of ~ 1.0%. An essentially similar protocol was described by Lee et al. (1986): mild cheese or fresh cheese curd was mixed with cream, treated with a combination of neutral proteinase (Neutrase) and a lipase (preferably a mixture of gastric and pregastric lipases or *M. miehei* lipase) and incubated at 35 °C for ~ 48 h. Several other methods have been described for the preparation of enzyme-modified cheese (see Lee et al., 1986; Kilara, 1985).

Enzyme-modified cheeses, produced by propriety technology, are used commercially by processed cheese manufacturers. While such products may be very bitter and do not resemble cheese flavour, they

apparently do intensify the flavour of processed cheese products and cheese ingredients.

Effect of adding free amino acids to cheddar cheese curd in flavour development

The most abundant amino acids in Cheddar cheese are glutamic acid, leucine, valine, isoleucine, lysine and phenylalanine (Law et al., 1976; Hickey et al., 1983; Puchades et al., 1989; Wilkinson, 1993); histidine and alanine are also present at high concentrations (Broome et al., 1990). The concentration of total amino acids is considered not to be directly responsible for Cheddar flavour but the release of certain amino acids, particularly glutamic acid, methionine and leucine, coincides with flavour development (Broome et al., 1990). Leucine and methionine are considered to be the main contributors to cheesy flavour in the water-soluble extract of Cheddar (Kowalewska et al., 1985; Marsili, 1985; Aston & Creamer, 1986).

Amino acids undergo various catabolic reactions, such as deamination, decarboxylation, transamination and side chain modification, yielding α-keto acids, NH_3, amines, aldehydes, acids or alcohols (Gripon et al., 1991). These degradation products are thought to play a significant role in the formation of specific cheese flavours (Hemme et al., 1982; Fox et al., 1993, 1995). Amino acid catabolism is less intense in Cheddar than in varieties in which moulds or non-lactic bacteria are present. Sulphur compounds, e.g., methanethiol, are major flavour components in washed-rind or mould-ripened cheeses. Methanethiol can be produced from methionine by strains of *Brevibacterium linens* (Law & Sharpe, 1978; Hemme et al., 1982; Ferchichi et al., 1985). It has been reported (Manning, 1979; Law, 1981) that the concentration of methanethiol in Cheddar cheese correlates closely with flavour intensity and its absence from headspace volatiles coincides with the lack of typical Cheddar flavour and aroma (Manning & Price, 1977). Production of methanethiol in Cheddar, which does not contain *Br. linens*, is thought to arise from non-enzymatic decomposition of methionine (Wainwright et al., 1972; Law & Sharpe, 1978) or by the combination of H_2S, produced from cysteine, with methionine (Hemme et al., 1982). A cystathionine β-lyase, capable of producing methanethiol, dimethyldisulphide dimethyltrisulphide from methionine, has been isolated by Alting et al. (1995) from *Lc. lactis* ssp *cremoris* B78. The enzyme was active under conditions equivalent to those in cheese and may be responsible for the biosynthesis of sulfur- containing compounds in cheese, although methionine was a poor substrate for the isolated enzyme. Methanethiol is a precursor of other sulphur compounds, e.g., hydrogen sulphide, carbonyl sulphide and dimethyl sulphide, which can be produced either by the cheese microflora and their enzymes or non-enzymatically (Adda et al., 1982; Hemme et al., 1982). Hydrogen sulphide is desirable in Cheddar but it is not essential for balanced flavour development and may even cause a sulphur flavour defect in ripened cheese if its concentration is too high (Law, 1981; Hemme et al., 1982).

Since free amino acids are released rather slowly during cheese ripening, a study was undertaken by Wallace & Fox (1996) to assess the possibility of accelerating flavour development in Cheddar cheese by adding free amino acids to the curd at salting.

According to Wood et al. (1985), the total concentration of free amino acids in Cheddar is $\sim$ 84 mmol/kg ($\sim$ 11 g/kg). In this experiment, cas-amino acids were added to milled Cheddar curd with the salt at concentrations of 0, 1.4, 2.8, 5.7 and 8.5 g/kg (cheeses A, B, C, D and E, respectively). Proteolysis was monitored by measuring water and PTA-soluble N, RP-HPLC and amino acid analysis on the water-soluble extract (WSE), and urea-PAGE of cheese and WSE. Cheeses were graded after 1, 3 and 6 months on the basis of flavour and texture.

The composition of the experimental and control cheeses were similar and within the expected ranges for Cheddar. Very small differences in the concentration of WSN were observed between the control and experimental cheeses throughout ripening. Increases in PTA-SN were more pronounced in the experimental cheeses and were directly proportional to the level of cas-amino acids added to the curd. Urea-PAGE showed no differences, either quantitative or qualitative, between the control and experimental cheeses or their WSE's at any stage of ripening. RP-HPLC indicated that the control and experimental cheese E contained considerably lower levels of all major peptides than cheeses B, C and D, suggesting that low concentrations of amino acids activated proteolysis but a very high concentration appeared to be inhibitory.

Free amino acid levels, which were proportional to the amount added, remained static or decreased slightly during the first 5 weeks of ripening; however, concentrations increased substantially (1–2 g/kg cheese) in all cheeses between 1 and 3 months and especially between 3 and 6 months, particularly in those supplemented with intermediate levels of cas-

amino acids. Cheeses C and D showed increases of 2.5 and 3 g of total amino acids per kg cheese between 3 and 6 months of ripening. Although cheese E had the highest concentration of total amino acids throughout ripening, substantially greater increases in the concentration of amino acids, particularly serine, isoleucine, leucine and phenylalanine, occurred in all other experimental cheeses and the control during ripening (e.g., the increase in free amino acids was 0.8 g/kg higher in cheese C than in cheese E), suggesting that while intermediate levels of free amino acids enhanced peptidolytic activity, higher levels tended to be inhibitory.

The principal amino acids in all 1 day old cheeses were glutamic acid, proline, arginine and leucine; high concentrations of NH_3 were also present in all cheeses. The concentrations of NH_3, lysine and proline decreased during the first 5 weeks of ripening (particularly in the experimental cheeses, the lost being proportional to the level of amino acids added), suggesting that these amino acids were catabolised by the cheese microflora. In agreement with previous workers (Law et al., 1976; Hickey et al., 1983; Puchades et al., 1989; Wilkinson, 1993), there was a substantial increase in the concentrations of leucine, glutamic acid and phenylalanine during ripening, with leucine being the dominant amino acid after 6 months. There was also a substantial increase in the concentration of ammonia in all cheeses, which has not been reported previously. Arginine appeared to be catabolised rapidly in all cheeses during the latter half of ripening, perhaps by intracellular lactococcal enzymes or by NSLAB which are capable of utilising Arg, perhaps producing ornithine (which was not monitored in this study).

Off-flavours were detected in all experimental cheeses (but not in the control) when they were first graded after 5 weeks. Cheese E was described as having a very advanced flavour at 3 months but was 'overripe', with an 'unclean' flavour and a weak, pasty texture, at 6 months. Cheese D had a slight burnt off-flavour up to 3 months but after 6 months it had the best flavour and texture. The quality of cheese C was also very high (although it received a lower flavour intensity score than cheese E at 6 months, its flavour was described as superior). Cheese B was downgraded due to a bitter, over-acid flavour; the control was also bitter at 6 months.

It is concluded that addition of intermediate levels of free amino acids to Cheddar cheese curd during manufacture has a beneficial effect on the development of cheese flavour. Amino acids appear to stimulate proteolysis, particularly secondary proteolysis involving the breakdown of small peptides to free amino acids, either due to the activation of peptidases, increased cell lysis or increased growth of NSLAB (which was not studied). The products of amino acid catabolism were not studied; perhaps this would merit further study as these products are thought to be major contributors to cheese flavour. While cas-amino acids are expensive and would not be practical for industrial-scale use, it may be possible to economically manufacture a protein hydrolysate by acid hydrolysis for use in accelerated cheese ripening.

Prospects for accelerated ripening

There is undoubtedly an economic incentive to accelerate the ripening of low-moisture, highly-flavoured, long-ripened cheeses. Although consumer preferences are tending towards more mild- flavoured cheeses, there is a considerable niche market for more highly flavoured products. While the ideal might be to have cheese ready for consumption within a few days, this is unlikely to be attained and in any case it would be necessary to stabilize the product after reaching optimum quality, eg, by heat treatment, as is used in the production of enzyme-modified cheeses.

Although the possibility of using exogenous (non-rennet) proteinases, and in some cases peptidases, attracted considerable attention for a period, this approach has not been commercially successful for which a number of factors may be responsible: (1) primary proteolysis is probably not the rate-limiting reaction in flavour development, (2) the use of exogenous enzymes in cheese is prohibited in several countries, and (3) uniform incorporation of enzymes is still problematic and the use of encapsulated enzymes is not viable at present. Because it can be easily incorporated into cheese curd, is an indigenous enzyme active in natural cheese and has narrow specificity, producing non-bitter peptides, plasmin may have potential as a cheese ripening aid; however, at present it is too expensive but its cost may be reduced via genetic engineering.

Attenuated cells appear to have given useful results in pilot-scale experiments but, considering the mass of cells required, the cost of such cells would appear to be prohibitive for commercial use, except perhaps in special circumstances. Selected peptidase-rich, Lac^-/Prt^- *Lactococcus* cells added as adjuncts have given promising results but further work is required and they may not be cost-effective.

We believe that the selection of starter strains according to scientific principles holds considerable potential. Such selection procedures are hampered by the lack of information on the key enzymes involved in ripening. Preliminary studies on the significance of early cell lysis have given promising results and further studies are warranted; bacteriocin-induced lysis appears to be particularly attractive.

The ability to genetically engineer starters holds enormous potential but results to date using genetically engineered starters have been disappointing. Again, identifying the key enzymes in ripening is essential for the success of this approach. It is hoped that current research on cheese ripening will identify the key sapid compounds in cheese and hence the critical, rate-limiting enzymes. Genetic manipulation of Lac^-/Prt^- adjunct *Lactococcus* will also be possible when key limiting enzymes have been identified. We believe that adjunct starters, especially lactobacilli, hold considerable potential. It appears to be possible to produce cheese of acceptable quality without lactobacilli but they appear to intensify (Cheddar) cheese flavour and offer flavour options. The volume of literature published on starter adjuncts has been rather limited to date; further work will almost certainly lead to the development of superior adjuncts. There is the obvious possibility of transferring desirable enzymes from lactobacilli to starter lactococci.

At present, elevated ripening temperatures ($\sim$ 15 °C) offer the most effective, and certainly the simplest and cheapest, method for the accelerating ripening of Cheddar, which is usually ripened at unnecessarily low temperatures; however, this approach is less applicable to many other varieties for which relatively high ripening temperatures are used at present.

The key to accelerating ripening ultimately depends on identifying the key sapid compounds in cheese. This has been a rather intractable problem; work on the subject commenced nearly 100 years ago and has been quite intense since about 1960, i.e. since the development of gas chromatography. Although as many as 400 compounds which might be expected to influence cheese taste and aroma have been identified, it is not possible to describe cheese flavour precisely. Until such information is available, attempts to accelerate ripening will be speculative and empirical.

References

Abdel Baky AA, El Fak AM, Rabia AM & El Neshewy AA (1982a) Cheese slurry in the acceleration of Cephalotyre 'Ras' cheese ripening. J. Food Prot. 45: 894–897

Abdel Baky AA, El Neshewy AA, Rabia AHM & Farahat SM (1982b) Ripening changes in Cephalotyre 'Ras' cheese slurries. J. Dairy Res. 49: 337–341

Adda J, Gripon JC & Vassal L (1982) The chemistry of flavour and texture generation in cheese. Food Chem. 9: 115–129

Alting AC, Engels WJM, van Schalkwijk S & Exterkate FA (1995) Purification and characterization of cystathionine β-lyase from *Lactococcus lactis* subsp. *cremoris* B78 and its possible role in flavor development in cheese. Appl. Environ. Microbiol. 61: 4037–4042

Arbige MV, Freund PR, Silver SC & Zelro JT (1986) Novel lipase for Cheddar cheese flavor development. Food Technol. 40(4): 91–98

Ardö Y & Pettersson H-E (1988) Accelerated cheese ripening with heat treated cells of *Lactobacillus helveticus* and a commercial proteolytic enzyme. J. Dairy Res. 55: 239–245

Aston JW & Creamer LK (1986) Contribution of the components of the water-soluble fraction to the flavour of Cheddar cheese. N.Z. J. Dairy Sci. Technol. 21: 229–248

Aston JW, Durward IG & Dulley JR (1983) Proteolysis and flavour development in Cheddar cheese. Aust. J. Dairy Technol. 38: 55–65

Aston JW, Grieve PA, Durward IG & Dulley JR (1983) Proteolysis and flavour development in Cheddar cheeses subjected to accelerated ripening treatments. Aust. J. Dairy Technol. 38: 59–65

Baral A (1995) Isolation and Characterization of Enzymes from *Pseudomonas tolaasii*. PhD Thesis, National University of Ireland, Cork

Bartels HJ, Johnson ME & Olson NF (1987a) Accelerated ripening of Gouda cheese. 1. Effect of heat-shocked thermophilic lactobacilli and streptococci on proteolysis and flavor development. Milchwissenschaft 42: 83–88

Bartels HJ, Johnson ME & Olson NF (1987b) Accelerated ripening of Gouda cheese. 1. Effect of freeze-shocked *Lactobacillus helveticus* on proteolysis and flavor development. Milchwissenschaft 42: 139–144

Bouton Y & Grappin R (1995) Comparison de la qualite de fromages a pate pressee cuite fabriques a partir de lait cru on microfiltre. Le Lait 75: 31–44

Broome MC, Krause DA & Hickey MW (1990) The use of non-starter lactobacilli in Cheddar cheese manufacture. Aust. J. Dairy Technol. 45: 67–73

Chapot-Chartier M-P, Deniel C, Rousseau M, Vassal L & Gripon J-C (1994) Autolysis of two strains of *Lactococcus lactis* during cheese ripening. Int. Dairy J. 4: 251–269

Christensen JE, Johnson ME & Steele JC (1995) Production of Cheddar cheese using a *Lactococcus lactis* ssp *cremoris* SK11 derivative with enhanced aminopeptidase activity. Int. Dairy J. 5: 367–379

Coolbear T, Pillidge CJ & Crow VL (1994) The diversity of potential cheese ripening characteristics of lactic acid starter bacteria. 1. Resistance to cell lysis and levels and cellular distribution of proteinase activities. Int. Dairy J. 4: 697–721

Creamer LK, Iyer M & Lelievre J (1987) Effect of various levels of rennet addition on characteristics of Cheddar cheese made from ultrafiltered milk. N.Z. J. Dairy Sci. Technol. 22: 205–214

Crow VL, Holland R, Pritchard GG & Coolbear T (1994) The diversity of potential cheese ripening characteristics of lactic acid starter

bacteria: The levels of subcellular distributions of peptidase and esterase activities. Int. Dairy J. 4: 723–742

Crow VL, Martley FG, Coolbear T & Roundhill SJ (1996) The influence of phage-assisted lysis of *Lactococcus lactis* subsp *lactis* ML8 on Cheddar cheese ripening. Int. Dairy J. 6: in press

Crow VL, Coolbear T, Holland R, Pritchard GG & Martley FG (1993) Starters as finishers: Starter properties relevant to cheese ripening. Int. Dairy J. 3: 423–460

Daeschel MA (1993) Applications and interactions of bacteriocins from lactic acid bacteria in foods and beverages. In: Hoover DG & Steenson LR (Eds) Bacteriocins of Lactic Acid Bacteria (pp 63–91). Academic Press, New York

Dulley JR (1976) The utilization of cheese slurries to accelerate the ripening of Cheddar cheese. Aust. J. Dairy Technol. 31: 143–148

Efthymiou CC & Mattick JF (1964) Development of a domestic Feta cheese. J. Dairy Sci. 47: 593–598

El Neshawy AA, Abdel Baky AA & Farahat SM (1982) Enhancement of Domiati cheese flavour with animal lipase preparations. Dairy Ind. Intern. 47(2): 29, 31

El Shibiny S, Soliman MA, El-Bagoury E, Gad A & Abd El-Salam MH (1978) Development of volatile fatty acids in Ras cheese. J. Dairy Res. 45: 497–500

El-Soda M (1993) Accelerated maturation of cheese. Int. Dairy J. 3: 531–544

El-Soda M & Pandian S (1991) Recent developments in accelerated cheese ripening. J. Dairy Sci. 74: 2317–2335

El Soda M, Desmazeaud MJ, Abou Donia S & Badran A (1982) Acceleration of cheese ripening by the addition of extracts from *Lactobacillus helveticus, Lactobacillus bulgaricus* and *Lactobacillus lactis* to the cheese curd. Milchwissenschaft 37: 325–327

El Soda M, Desmazeaud MJ, Abou Donia S & Kamal N (1981) Acceleration of cheese ripening by the addition of whole cells or cell free extracts from *Lactobacillus casei* to the cheese curd. Milchwissenschaft 36: 140–142

Exterkate FA (1979) Effect of membrane perturbing treatments on the membrane-bound peptidases of *Streptococcus cremoris* HP. J. Dairy Res. 46: 473–484

Exterkate FA (1984) Location of peptidases outside and inside the membrane of *Streptococcus cremoris*. Appl. Environ. Microbiol. 47: 177–185

Exterkate FA & Alting AC (1995) The role of starter peptidases in the initial proteolytic events leading to amino acids in Gouda cheese. Int. Dairy J. 5: 15–28

Exterkate FA & de Veer GJCM (1987) Efficient implementation of consecutive reactions by peptidases at the periphery of the *Streptococcus cremoris* membrane. Appl. Environ. Microbiol. 53: 1482–1486

Exterkate FA, Alting AC & Slangen CJ (1995) Conversion of α_{s1}-casein-(24–199)-fragment and β-casein under cheese conditions by chymosin and starter peptidases. System. Appl. Microbiol. 18: 7–12

Farkye NY & Fox PF (1991) A preliminary study of the contribution of plasmin to proteolysis in Cheddar cheese: cheese containing plasmin inhibitor, 6aminohexanoic acid. J. Agric. Food Chem. 39: 786–788

Farkye NY & Fox PF (1992) Contribution of plasmin to Cheddar cheese ripening: effect of added plasmin. J. Dairy Res. 59: 209–216

Farkye NY & Landkammer CF (1992) Milk plasmin activity influence on Cheddar cheese quality during ripening. J. Food Sci. 57: 622–624, 639

Fedrick IA (1987) Technology and economics of the accelerated ripening of Cheddar cheese. Proc. Ann. Conf. Victorian Div. Dairy Ind. Assoc. Aust., Melbourne

Fedrick IA & Dulley JR (1984) The effect of elevated storage temperatures on the rheology of Cheddar cheese. N.Z. J. Dairy Sci. Technol. 19: 141–150

Fedrick IA, Aston JW, Durward IF & Dulley JR (1983) The effect of elevated ripening temperatures on proteolysis and flavour development in Cheddar cheese. I. High temperature storage midway during ripening. N.Z. J. Dairy Sci. Technol. 18: 253–260

Fedrick IA, Aston JW, Nottingham SM & Dulley JR (1986a) The effect of neutral fungal protease on Cheddar cheese ripening. N.Z. J. Dairy Sci. Technol. 21: 9–19

Fedrick IA, Cromie SJ, Dulley JR & Giles JE (1986b) The effects of increased starter populations, added neutral proteinase and elevated temperature storage on Cheddar cheese manufacture and maturation. N.Z. J. Dairy Sci. Technol. 21: 191–203

Feirtag JM & McKay LL (1987a) Isolation of *Streptococcus lactis* C2 mutants selected for temperature sensitivity and potential use in cheese manufacture. J. Dairy Sci. 70: 1773–1778

Feirtag JM & McKay LL (1987b) Thermoinducible lysis of temperature sensitive *Streptococcus cremoris* strains. J. Dairy Sci. 70: 1779–1784

Ferchichi M, Hemme D, Nardi M & Pamboukjian N (1985) Production of methanethiol from methionine by *Brevibacterium linens* CNRZ918. J. Gen. Microbiol. 131: 715–723

Folkertsma B, Fox PF & McSweeney PLH (1996) Acceleration of Cheddar cheese ripening at elevated temperatures. Int. Dairy J. 6: in press

Fox PF (1988/89) Acceleration of cheese ripening. Food Biotechnol. 2: 133–185

— (1989) Proteolysis during cheese manufacture and ripening. J. Dairy Sci. 72: 1379–1400

Fox PF & McSweeney PLH (1996a) Proteolysis in cheese during ripening. Food Rev. Int., in press

Fox PF & McSweeney PLH (1996b) Rennets: their role in milk coagulation and cheese ripening. In: Law BA (Ed) The Microbiology and Biochemistry of Cheese and Fermented Milk, 2nd edn., Chapman & Hall, London, in press

Fox PF & Stepaniak L (1993) Enzymes in cheese technology. Int. Dairy J. 3: 509–530

Fox PF, McSweeney PLH & Singh TK (1995) Methods for assessing proteolysis in cheese. In: Malin EL & Tunick MH (Eds) Chemistry of Structure Function Relationships in Cheese (pp 161–194). Plenum Press, New York

Fox PF, Singh TK & McSweeney PLH (1994) Proteolysis in cheese during ripening. In: Andrews AT & Varley J (Eds) Biochemistry of Milk Products (pp 1–31). Royal Society of Chemistry, Cambridge

Fox PF, Singh TK & McSweeney PLH (1995) Biogenesis of flavour compounds in cheese. In: Malin EL & Tunick MH (Eds) Chemistry of Structure/Function Relationships in Cheese (pp 59–98). Plenum Press, New York

Fox PF, Law J, McSweeney PLH & Wallace J (1993) Biochemistry of cheese ripening. In: Fox PF (Ed) Cheese: Chemistry, Physics and Microbiology, Vol 1 (pp 389–438). Chapman & Hall, London

Fox PF, O'Connor TP, McSweeney PLH, Guinee TP & O'Brien NM (1996) Cheese: Physical, chemical, biochemical and nutritional aspects. Adv. Food Nutr. Res. 39: 163–328

Frick CM, Hicks CL & O'Leary J (1984) Use of fungal enzymes to accelerate cheese ripening. J. Dairy Sci. 67: suppl. 1, 89

Fryer TF (1982) The controlled ripening of Cheddar cheese. Proc. XXI Intern. Dairy Congr. (Moscow) 1(1): 485

Fullbrook P (1987) Biotechnology and related developments as they apply to cheese and other dairy products. Soc. Dairy Technol. Spring Conf., Apr. 26–29

Gaya P, Medina M, Rodriguez-Marin MA & Nunez M (1990) Accelerated ripening of ewes' milk Manchego cheese: The effect of elevated ripening temperatures. J. Dairy Sci. 73: 26–32

Gobbetti M & Fox PF (1996) Isolation and characterization of a dipeptidase from *Pseudomonas fluorescens* ATCC 948. J. Dairy Sci., in press

Gobbetti M, Corsetti A & Fox PF (1995) Purification and characterization of an intracellular aminopeptidase from *Pseudomonas fluorescens* ATCC 948. J. Dairy Sci. 78: 44–54

Grappin R, Rank TC & Olson NF (1985) Primary proteolysis of cheese proteins during ripening. J. Dairy Sci. 68: 531–540

Grieve PA & Dulley JR (1983) Use of *Streptococcus lactis* lac$^-$ mutants for accelerating Cheddar cheese ripening. 2. Their effect on the rate of proteolysis and flavour development. Aust. J. Dairy Technol. 38: 49–54

Gripon J-C, Monnet V, Lamberet G & Desmazeaud MJ (1991) Microbial enzymes in cheese ripening. In: Fox PF (Ed) Food Enzymology, Vol 1 (pp 131–169). Elsevier Applied Science, London

Guinee T, Wilkinson M, Mulholland E & Fox PF (1991) Influence of ripening temperature, added commercial enzyme preparations and attenuated, mutant (lac$^-$) *Lactococcus lactis* starter on the proteolysis and maturation of Cheddar cheese. Ir. J. Food Sci. Technol. 15: 27–51

Harte BR & Stine CM (1977) Effects of process parameters on formation of volatile acids and free fatty acids in quick-ripened Blue cheese. J. Dairy Sci. 60: 1266–1272

Heap HA & Lawrence RC (1976) The selection of starter strains for cheesemaking. N.Z. J. Dairy Sci. Technol. 11: 16–20

Hemme D, Bouillanne C, Metro F & Desmazeaud M-J (1982) Microbial catabolism of amino acids during cheese ripening. Sci. des Aliments 2: 113–123

Hickey MW, van Leeuwen H, Hillier AJ & Jago GR (1983) Amino acid accumulation in Cheddar cheese manufactured from normal and ultrafiltered milk. Aust. J. Dairy Technol. 38: 110–113

IDF (1991) Chemical Methods for Evaluation of Proteolysis in Cheese Maturation. Bulletin 261. International Dairy Federation, Brussels

IDF (1992) Fermentation-produced Enzymes and Accelerated Ripening in Cheesemaking. Bulletin 269. International Dairy Federation, Brussels

Ismail AA & Hansen K (1972) Accumulation of free amino acids during cheese ripening of some types of Danish cheese. Milchwissenschaft 27: 556–559

Jensen F (1970) Free fatty acids in lipase activated cheese. Proc. XVIII Intern. Dairy Congr. (Sydney) IE: 365 (abstr.)

Johnston KA, Dunlop FP, Coker CJ & Wards SM (1994) Comparisons between the electrophoretic pattern and textural assessment of aged Cheddar cheese made using various levels of calf rennet or microbial coagulant (Rennilase 46L). Int. Dairy J. 4: 303–327

Jordan KN & Cogan TM (1993) Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. Ir. J. Agric. Food Res. 32: 47–55

Kilara A (1985) Enzyme-modified lipid food ingredients. Process. Biochem. 20(2): 35–45

Kalinowski L, Frackiewicz E & Janiszewska L (1982) Acceleration of cheese ripening by the use of complex enzyme preparation. Proc. XXI Intern. Dairy Congr (Moscow). I (Book 2): 500

Kalinowski L, Frackiewicz E, Janiszewska L, Pawlik A & Kikolska D (1979) Enzymic preparation for ripening milk protein products. US Patent 4, 158, 607

Kelly AL (1995) Variations in Total and Differential Milk Somatic Cell Counts and Plasmin Levels and their Role in Proteolysis and Quality of Milk and Cheese. PhD Thesis, National University of Ireland, Cork

Kowalewska J, Zelazowska H, Babuchowski A, Hammond EG, Glatz BA & Ross F (1985) Isolation of aroma-bearing material from *Lactobacillus helveticus* culture and cheese. J. Dairy Sci. 68: 2165–2171

Kristoffersen T, Mikolajcik EM & Gould IA (1967) Cheddar cheese flavor. IV. Directed and accelerated ripening process. J. Dairy Sci. 50: 292–297

Kuchroo CN & Fox PF (1983) A fractionation scheme for the water-soluble nitrogen in Cheddar cheese. Milchwissenschaft 38: 389–391

Lane CN & Fox PF (1996) Contribution of starter and added lactobacilli to proteolysis in Cheddar cheese during ripening. Int. Dairy J. (in press)

Law BA (1978) The accelerated ripening of cheese by the use of non-conventional starters and enzymes - a preliminary assessment. Doc. 108 (pp 40–48). International Dairy Federation, Brussels

— (1980) Accelerated ripening of cheese. Dairy Ind. Int. 45(5): 15, 17, 19, 20, 22, 48

— (1981) The formation of aroma and flavour compounds in fermented dairy products. Dairy Sci. Abstr. 43: 143–154

— (1982) Cheeses. In: Rose AH (Ed) Economic Microbiology, Vol 7, Fermented Foods (pp 147–198). Academic Press, London

— (1984) The accelerated ripening of cheese. In: Davies FL & Law BA (Eds) Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk (pp 209–228). Elsevier Applied Science Publishers, London

— (1987) Proteolysis in relation to normal and accelerated cheese ripening. In: Fox PF (Ed) Cheese: Chemistry, Physics and Microbiology, Vol 1 (pp 365–392). Elsevier Applied Science, London

Law BA & King JS (1985) Use of liposomes for proteinase addition to Cheddar cheese. J. Dairy Res. 52: 183–188

Law BA & Sharpe ES (1978) Formation of methanethiol by bacteria isolated from raw milk and Cheddar cheese. J. Dairy Res. 45: 267–275

Law BA & Wigmore A (1982a) Accelerated cheese ripening with food grade proteinases. J. Dairy Res. 49: 137–146

— (1982b) Microbial proteinases as agents for accelerated cheese ripening. J. Soc. Dairy Technol. 35: 75–76

— (1983) Accelerated ripening of Cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. J. Dairy Res. 50: 519–525

— (1985) Effect of commercial lipolytic enzymes on flavour development in Cheddar cheese. J. Soc. Dairy Technol. 38: 86–88

Law BA, Castanon MJ & Sharpe ME (1976) The contribution of starter streptococci to flavour development in Cheddar cheese. J. Dairy Res. 43: 301–311

Law BA, Hosking ZD & Chapman HR (1979) The effect of some manufacturing conditions on the development of flavour in Cheddar cheese. J. Soc. Dairy Technol. 32: 87–90

Law J & Haandrikman A (1996) Proteolytic enzymes of lactic acid bacteria. Int. Dairy J. 6 (in press)

Law J, Fitzgerald GF, Daly C, Fox PF & Farkye NY (1992) Proteolysis and flavor development in Cheddar cheese made with the single starter strains *Lactococcus lactis* ssp *lactis* UC317 or *Lactococcus lactis* ssp *cremoris* HP. J. Dairy Sci. 75: 1173–1185

Le Bars D & Gripon J-C (1993) Hydrolysis of α_{s1}-casein by bovine plasmin. Le Lait 73: 337–344

Le Bars D, Desmazeaud MJ, Gripon J-C & Bergere JL (1975) Etude du role des micro-organismes et de leurs enzymes dans la maturation des fromages. I. -Fabrication aseptique d'un caille modelle. Le Lait 55: 377–389

Lee BH, Laleye LC, Simard RE, Munsch MH & Holley RA (1990) Influence of homofermentative lactobacilli on the microflora and soluble nitrogen components in Cheddar cheese. J. Food Sci. 55: 391–397

Lee CR, Lin CR & Melachouris N (1986) Process for preparing intensified cheese flavor product. US Patent 4, 594, 595

Lemieux L & Simard RE (1991) Bitter flavour in dairy products. I. A review of the factors likely to influence its development, mainly in cheese manufacture. Le Lait 71: 599–636

— (1992) Bitter flavour in dairy products. II. A review of bitter peptides from the caseins: their formation, isolation and identification, structure masking and inhibition. Le Lait 72: 335–382

Lin JCC, Jeon IJ, Roberts HA & Milliken GA (1987) Effects of commercial food grade enzymes on proteolysis and textural changes in granular Cheddar cheese. J. Food Sci. 52: 620–625

Lõpez-Fandiño R & Ardö Y (1991) Effect of heat treatment on the proteolytic/ peptidolytic enzyme system of a *Lactobacillus delbrueckii* subsp *bulgaricus* strain. J. Dairy Res. 58: 469–475

Lowrie RJ & Lawrence RC (1972) Cheddar cheese flavour. IV. A new hypothesis to account for the development of bitterness. N.Z. J. Dairy Sci. Technol. 7: 51–53

Lynch CM, McSweeney PLH, Fox PF, Cogan TM & Drinan FD (1996a) Contribution of starter and non-starter lactobacilli to proteolysis in Cheddar cheese with a controlled microflora. J. Dairy Res. (submitted)

Lynch CM, McSweeney PLH, Fox PF, Cogan TM & Drinan FD (1996b) Manufacture of Cheddar cheese under controlled microbiological conditions, with or without adjunct lactobacilli. Int. Dairy J. (in press)

Mabbitt LA, Chapman HR & Berridge NJ (1955) Experiments in cheesemaking without starter. J. Dairy Res. 22: 365–373

Mabbitt LA, Chapman HR & Sharpe ME (1959) Making Cheddar cheese on a small scale under controlled bacteriological conditions. J. Dairy Res. 26: 105–112

Madkor SA & Fox PF (1994) Ripening of Cheddar cheese containing added trypsin. Egypt. J. Dairy Sci. 22: 93–106

Manning DJ (1979) Cheddar cheese flavour studies: II. Relative flavour contributions of individual volatile components. J. Dairy Res. 46: 523–529

Manning DJ & Price JC (1977) Cheddar cheese aroma - the effect of selectively removing specific compounds from cheese headspace. J. Dairy Res. 44: 357–361

Marsili R (1985) Monitoring chemical changes in Cheddar cheese during aging by high performance liquid chromatography and gas chromatography techniques. J. Dairy Sci. 68: 3155–3161

Martley FG & Lawrence RC (1972) Cheddar cheese flavour. II. Characteristics of single strain starters associated with good or poor flavour development. N.Z. J. Dairy Sci. Technol. 7: 38–44

McGarry A, El-Kholi A, Law J, Coffey A, Daly C, Fox PF & Fitzgerald GF (1994) Impact of manipulating the lactococcal proteolytic system on ripening and flavour development in Cheddar cheese. Proc. 4^{th} Meeting BRIDGE T-Project, Oviedo, Spain, p 32 (abstr.)

McGarry A, Law J, Coffey A, Daly C, Fox PF & Fitzgerald GF (1995) Effect of genetically modifying the lactococcal proteolytic system on ripening and flavor development in Cheddar cheese. Appl. Environ. Microbiol. 60: 4226–4233

McKellar RC (Ed) (1989) Enzymes of Psychrotrophs in Raw Food. CRC Press Inc., Boca Raton, FL

McSweeney PLH & Fox PF (1993) Cheese: Methods of chemical analysis. In: Fox PF (Ed) Cheese: Chemistry, Physics and Microbiology, Vol 1, 2nd edn. (pp 341–388). Chapman and Hall, London

McSweeney PLH, Fox PF & Olson NF (1995) Proteolysis of bovine caseins by cathepsin D: preliminary observations and comparison with chymosin. Int. Dairy J. 5: 321–336

McSweeney PLH, Fox PF, Lucey JA, Jordan KN & Cogan TM (1993a) Contribution of the indigenous microflora to the maturation of Cheddar cheese. Int. Dairy J. 3: 613–634

McSweeney PLH, Olson NF, Fox PF, Healy A & Hϕjrup P (1993b) Proteolytic specificity of chymosin on bovine α_{s1}casein. J. Dairy Res. 60: 401–412

— (1993c) Proteolytic specificity of plasmin on bovine α_{s1}casein. Food Biotechnol. 7: 143–158

McSweeney PLH, Pochet S, Fox PF & Healy A (1994a) Partial identification of peptides from the water-insoluble fraction of Cheddar cheese. J. Dairy Res. 61: 587–590

McSweeney PLH, Walsh EM, Fox PF, Cogan TM, Drinan FD & Castelo-Gonzalo M (1994b) A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. Ir. J. Agric. Food Res. 33: 183–192

Mierau I, Junji ERS, Venema G, Poolman B & Kok J. (1996). Peptidases and growth of *Lactococcus lactis* in milk. Le Lait 76: 25–32

Monnet V, Chapot-Chartier MP & Gripon J-C (1993) Les peptidases des lactocoques. Le Lait 73: 97–108

Morgan SM, Ross RP & Hill C (1995) Bacteriolytic activity due to the presence of novel lactococcal plasmid encoding lactococcins A, B and M. Appl. Environ. Microbiol. 61: 2995–3001

Moskowitz GJ & Noelck SS (1987) Enzyme-modified cheese technology. J. Dairy Sci. 70: 1761–1769

Muir DD, Banks JM & Hunter EA (1996) Sensory properties of Cheddar cheese: Effect of starter type and adjunct. Int. Dairy J. 6: 407–432

Nelson JH, Jensen RG & Pitas RE (1977) Pregastric esterase and other oral lipases - A review. J. Dairy Sci. 60: 327–362

Niland EJ & Fox PF (1996) Use of *Pseudomonas tolaasii* as an adjunct for Cheddar cheese. Int. Dairy J. (in press)

Oberg CJ, Davis LH, Richardson GH & Ernstrom CA (1986) Manufacture of Cheddar cheese using proteinase-negative mutants of *Streptococcus cremoris*. J. Dairy Sci. 69: 2875–2981

O'Donovan C (1994) An Investigation of the Autolytic Properties of Different Strains of Lactococci during Cheddar Cheese Ripening. MSc Thesis, National University of Ireland, Cork

O'Keeffe AM, Fox PF & Daly C (1978) Proteolysis in Cheddar cheese: role of coagulant and starter bacteria. J. Dairy Res. 45: 465–477

O'Keeffe RB, Fox PF & Daly C (1976a) Manufacture of Cheddar cheese under controlled bacteriological conditions. Ir. J. Agric. Res. 15: 151–155

— (1976b) Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. J. Dairy Res. 43: 97–107

O'Sullivan M & Fox PF (1990) A scheme for the partial fractionation of cheese peptides. J. Dairy Res. 57: 135–139

Pettersson HE & Sjostrom G (1975) Accelerated cheese ripening: a method for increasing the number of lactic starter bacteria in cheese without detrimental effect on the cheesemaking process and its effect on the cheese ripening. J. Dairy Res. 42: 313–326

Pritchard G & Coolbear T (1993) The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. 12: 179–206

Puchades R, Lemieux L & Simard RD (1989) Evolution of free amino acids during the ripening of Cheddar cheese containing added lactobacilli strains. J. Food Sci. 54: 885–888

Rank TC, Grappin R & Olson NF (1985) Secondary proteolysis of cheese during ripening: A review. J. Dairy Sci. 68: 801–805

Richardson GH, Emstrom CA, Kim JM & Daly C (1983) Proteinase negative variants of *Streptococcus cremoris* for cheese starters. J. Dairy Sci. 66: 2278–2286

Shamsuzzaman K & McKellar RC (1987) Peptidases of 2 strains of *Pseudomonas fluorescens*: partial purification, properties and action in milk. J. Dairy Res. 54: 283–293

Shchedushnov EV & D'Yachenko PF (1974) Activation of the enzymatic processes in the manufacture of cheese. Proc. XIX Intern. Dairy Congr. (New Delhi), IE: 696–697

Singh S & Kristoffersen T (1970) Factors affecting flavor development in Cheddar cheese slurries. J. Dairy Sci. 53: 533–536

— (1971a) Accelerated ripening of Swiss cheese curd. J. Dairy Sci. 54: 349–354

— (1971b) Influence of lactic culture and curd milling acidity on flavor of Cheddar curd slurries. J. Dairy Sci. 54: 1589–1594

— (1972) Cheese flavor development using direct acidified curd. J. Dairy Sci. 55: 744–749.

Singh TK, Fox PF & Healy A (1995) Water-soluble peptides in Cheddar cheese: Isolation and identification of peptides in the UF retentate of water-soluble fractions. J. Dairy Res. 62: 629–640

— (1996) Water-soluble peptides in Cheddar cheese: Isolation and identification of peptides in the UF retentate of water-soluble fractions. 2. J. Dairy Res. 63 (in press)

Singh TK, Fox PF, Hϕjrup P & Healy A (1994) A scheme for the fractionation of cheese nitrogen and identification of principal peptides. Int. Dairy J. 4: 111–122

Skeie S (1994) Developments in microencapsulation science applicable to cheese research and development. A review. Int. Dairy J. 4: 573–595

Stadhouders J (1960) De eiwithydrolyse tijdens de kaasrijping de enzymes die het eiwit in kaas hydrolyseren. Neth. Milk Dairy J. 14: 83–110

Stadhouders J, Hup G, Exterkate FA & Visser S (1983) Bitter flavor in cheese. I. Mechanism of the formation of the bitter flavour defect in cheese. Neth. Milk Dairy J. 37: 157–167

Stepaniak L, Gobbetti M & Fox PF (1995) Isolation and characterization of intracellular proteinases from *Lactococcus lactis* subsp *lactis* MG1363. Proc. Conf. on Lactic Acid Bacteria, 22–26 October 1995, p 111, Cork, Ireland

Sutherland BJ (1975) Rapidly ripened cheese curd slurries in processed cheese manufacture. Aust. J. Dairy Technol. 30: 138–142

Talbott LL & McCord C (1981) The use of enzyme modified cheeses for flavoring processed cheese products. Paper No. 1981–14, Proc. 2^{nd} Biennial Marshall International Cheese Conference. Madison, WI, September 15–18

Tan PST, Poolman B & Konings WN (1993) The proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. 60: 269–286

Tan PST, Chapot-Chartier MP, Pos KM, Rosseau M, Boquien CY, Gripon J-C & Konings WN (1992) Localization of peptidases in lactococci. Appl. Environ. Microbiol. 58: 285–290

Teuber M (1990) Production of chymosin (EC 3.4.23.4) by microorganisms and its use in cheesemaking. Bulletin 251. International Dairy Federation, Brussels, pp 3–15

Thomas TD & Pearce K (1981) Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. N.Z. J. Dairy Sci. Technol. 16: 253–259

Thomas TD & Pritchard G (1987) Proteolytic enzymes and dairy starter cultures. FEMS Microbiol. Rev. 46: 245–268

Torres N & Chandan RC (1981) Flavor and texture development in Latin American White cheese. J. Dairy Sci. 64: 2161–2169

van Belkum MJ, Hayema BJ, Geis A, Kok J & Venema G (1989) Cloning of two bacteriocin genes from a lactococcal bacteriocin plasmid. Appl. Environ. Microbiol. 55: 1187–1191

van Belkum MJ, Hayema BJ, Jeeninga RE, Kok J & Venema G (1991a) Organisation and nucleotide sequence of two lactococcal bacteriocin plasmid. Appl. Environ. Microbiol. 57: 492–498

van Belkum MJ, Kok J, Venema G, Holo H, Nes IF, Konings WN & Abee T (1991b) The bacteriocin lactococcin A specifically increases the permeability of lactococcal cytoplasmic membranes in a voltage- independent, protein mediated manner. J. Bacteriol. 173: 7934–7941

Venema K, Abee T, Haandrikman AJ, Leenhouts KJ, Kok J, Konings WN & Venema G (1993) Mode of action of lactococcin B, a thiol- activated bacteriocin from *Lactococcus lactis*. Appl. Environ. Microbiol. 59: 1041–1048

Venema K, Haverkart RE, Abee T, Haandrikman AJ, Leenhouts KJ, de Leij L, Venema G & Kok J (1994) Mode of action of LciA,yhe lactococcin A immunity protein. Mol. Microbiol. 14: 521–532

Visser FMW (1977a) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 1. Description of cheese and aseptic cheesemaking techniques. Neth. Milk Dairy J. 31: 120–133

— (1977b) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 2. Development of bitterness and cheese flavour. Neth. Milk Dairy J. 31: 188–209

— (1977c) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino acid fractions. Neth. Milk Dairy J. 31: 210–239

Visser FMW & de GrootMostert AEA (1977) Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 4. Protein breakdown: a gel electrophoretical study. Neth. Milk Dairy J. 31: 247–264

Visser S (1993) Proteolytic enzymes and their relation to cheese ripening and flavor: an overview. J. Dairy Sci. 76: 329–350

von Bockelmann I & Lodin LO (1974) Use of a mixed microflora of ripened cheese as an additive to starter cultures for hard cheese. Proc. XIX Intern. Dairy Congr. (New Delhi). IE: 441 (abstr.)

Wainwright T, McMahon JF & McDowell J (1972) Formation of methional and methanethiol from methionine. J. Sci. Food Agric. 23: 911–914

Wallace JM & Fox PF (1994) Comparison of different methods for quantitation of peptides/amino acids in the water-soluble fraction of Cheddar cheese. Proc. FLAIR-SENS 3: 41–58

— (1996) Effect of adding free amino acids to Cheddar cheese curd on proteolysis, flavour and texture development. Int. Dairy J., submitted

Wilkinson MG (1993) Acceleration of cheese ripening. In: Fox PF (Ed) Cheese: Chemistry, Physics and Microbiology, Vol 1, 2^{nd} edn., (pp 523–555). Chapman & Hall, London

Wilkinson MG, Guinee TP & Fox PF (1994a) Factors which may influence the determination of autolysis of starter bacteria during Cheddar cheese ripening. Int. Dairy J. 4: 141–160

Wilkinson MG, Guinee TP, O'Callaghan DM & Fox PF (1994b) Autolysis and proteolysis in different strains of starter bacteria. J. Dairy Res. 61: 249–262

— (1992) Effect of commercial enzymes on proteolysis and ripening in Cheddar cheese. Le Lait 72: 449–459

Wood AF, Aston JW & Douglas GK (1985) The determination of free amino acids in cheese by capillary column gas-liquid chromatography. Aust. J. Dairy Technol. 40: 166–169

Zerfiridis G & Kristoffersen T (1970) Accelerated ripening of Feta curd. Proc. 18^{th} Intern. Dairy Congr. (Sydney). IE: 351 (abstr.)