Indigenous Enzymes of Milk

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12.1 Introduction

The indigenous enzymes in milk have been the subject of considerable research for 130 years. To date, about 70 indigenous enzymes have been reported in normal bovine milk (see Fox et al., 2003). With the exception of lipoprotein lipase (LPL) and xanthine oxidoreductase (XOR), most of the indigenous enzymes in milk have no obvious physiological role in the biosynthesis and secretion of milk, and only a few have an obvious function in milk post-secretion. LPL hydrolyses triglycerides in the chylomicrons in blood and supplies about 60% of the fatty acids and monoglycerides for the synthesis of TGs in the mammary gland; XOR plays a major role in the expression of lipid globules through the apical membrane of the mammocytes and is the second most abundant protein in the milk fat globule membrane (MFGM). As α-lactalbumin (α-La) modifies the specificity of UDP-galactosyltransferase (EC 2.4.1.22) in the synthesis of lactose and represents ~4% and ~40% of the protein in bovine and human milk, respectively, it is an enzyme modifier; it will not be discussed here, and the reader is referred to Chap. 8 for a comprehensive review.

The indigenous enzymes in milk arise from four principal sources:

- Blood plasma, through 'leaky junctions' between mammary cells.
- Secretory cell cytoplasm, some of which may be entrapped within some fat globules by the encircling MFGM during excretion from the cell.
- The MFGM itself, the outer layer of which is derived from the apical membrane of the mammary cell, and which, in turn, originates from the Golgi membranes; this is probably the source of most of the enzymes in milk.
- Somatic cells (leucocytes), which enter the mammary gland from the blood to fight bacterial infection (mastitis), and thereby enter milk.

Thus, most enzymes enter milk due to peculiarities of the mechanism by which milk constituents are excreted from the secretory cells. Milk does not contain substrates for many of the enzymes present, while others are inactive in milk due to unsuitable environmental conditions, e.g., pH or redox potential. However, many indigenous milk enzymes are significant from at least the following viewpoints:

- Deterioration, e.g., LPL (potentially, the most technologically significant enzyme in milk), proteinases, acid phosphatase and XOR.
- Indices of the thermal history of milk, e.g., alkaline phosphatase, lactoperoxidase (LPO), catalase, γ-glutamyl transferase, amylase and perhaps others.

This chapter is a modified and updated version of the reviews by Fox and Kelly (2006a, b).

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- Antimicrobial activity, e.g., lysozyme, XOR and LPO (which is exploited as a component of the LPO-H_2O_2-thiocyanate system for the cold pasteurisation of milk).
- Indices of mastitic infection, e.g., *N*-acetyl-β-D-glucosaminidase, catalase and acid phosphatase.
- Preservation of milk quality, e.g., sulphydryl oxidase (SHOx), superoxide dismutase (SOD).
- As a commercial source of enzymes, e.g., ribonuclease and LPO.
- Physiological functions in the neonate or in the mammary gland, e.g., bile salts-stimulated lipase and amylase, which are important in digestion by the human baby, and lysozyme, XOR and LPO, which have antimicrobial activity. However, these functions are not essential since the neonate can survive and thrive on heated milk in which these enzymes have been inactivated or on artificial formulae from which they are lacking.

Since the indigenous milk enzymes have no essential beneficial effect on the nutritional or organoleptic attributes of milk, their destruction by heat is one of the objectives of many dairy processes.

The first report of an enzyme, (lacto)peroxidase, in milk was by Arnold (1881), followed by reports of the presence of diastase (amylase) in the same decade. By 1902, the following enzymes had been reported in milk: peroxidase (oxidase), diastase (amylase), proteinase (galactase), 'fibrin ferment', lipase and 'salolase' (arylesterase) (Moro 1902). In 1902, Schardinger reported an enzyme in milk (now known as XOR) capable of oxidising aldehydes to acids but which also functions as a dehydrogenase. By the mid-1930s, the list of enzymes in milk was recognised as including proteinase, carbohydrase (amylase), esterases/lipases, peroxidase, xanthine oxidase (*aldehyd-katalase*) and catalase, with some reports of lactase (β-galactosidase) and salolase (arylesterase). By the 1950s, the list had been extended by the addition of alkaline phosphatase, lactase and coagulase (possibly thrombin). Probably reflecting the development of more sensitive assays, many new enzymes were detected in milk during the 1960s. For more detailed historical aspects of the discovery of these enzymes, the reader is referred to Fox and Kelly (2006a, b).

Andrews et al. (1992) reviewed the literature on 25 indigenous enzymes, and listed 34 other activities. Since 1992, the number of enzymes identified in milk has increased even further; Fox et al. (2003) described 32 enzymes and listed 37 other activities. It is likely that other enzymes, especially lysosomal enzymes, are present but have not been detected yet, perhaps due to the redox potential of milk, which is unsuitable for the action of some enzymes, or perhaps they have not even been assayed for. Barrett (1972) listed 53 enzymes that had been identified in lysosomes, only some of which have been reported in milk; it seems reasonable to assume that all lysosomal enzymes are present in milk. Multiple forms (isoenzymes) of many of the indigenous enzymes have also been reported.

Thus, the indigenous enzymes in bovine milk have attracted the attention of researchers for 130 years, and a very extensive literature has accumulated. In addition, the literature on the principal technologically significant enzymes has been reviewed individually (see the appropriate sections below).

There have been only occasional studies on the indigenous enzymes in the milk of other species, but some of the enzymes that have been studied show very marked differences from bovine milk. The indigenous enzymes in human milk also have been studied fairly extensively; human milk shows marked differences from bovine milk in the levels of several enzymes, e.g., a very high level of lysozyme, a bile salts-activated lipase (BSSL) in addition to the ubiquitous LPL, a high level of amylase but a low level of XOR and lacks LPO. Reviews include Hamosh (1988) and Hernell and Lonnerdal (1989). The indigenous enzymes in caprine and ovine milk have been reviewed by Moatsou (2010).

This article will review the literature on the principal indigenous enzymes in bovine milk and where possible in the milk of other species. These enzymes have been isolated and well characterised; they include the enzymes that were investigated during the early days of enzymology either because they were easily assayed or were technologically important. The minor enzymes are listed in Table 12.1; most of these have been identified

Table 12.1 Partial list of minor enzymes in milk, with associated EC numbers (modified from Farkye 2003)

Enzyme	EC Number
L-Iditol dehydrogenase	1.1.1.14
L-Lactate dehydrogenase	1.1.1.27
Malate dehydrogenase	1.1.1.37
Malic enzyme (oxaloacetate-decarboxylating) ($NADP^+$)	1.1.1.40
Isocitrate dehydrogenase ($NADP^+$)	1.1.1.42
Phosphogluconate dehydrogenase (decarboxylating)	1.1.1.44
Glucose-6-phosphate dehydrogenase	1.1.1.49
Amine oxidase	1.4.3.6
Polyamine oxidase	–
Fucosyltransferase	–
NADH dehydrogenase	1.6.99.3
Dihydrolipoamide dehydrogenase (diaphorase)	1.8.1.4
Lactose synthetase	2.4.1.22
Glycoprotein 4-β-galactosyltransferase	2.4.1.38
N-Acetyllactosamine synthase	2.4.1.90
CMP-*N*-acetylneuraminate-galactosyldiacylglycerol α-2,3-sialyltransferase	2.4.99.6
Thiamine-phosphate pyrophosphorylase	2.5.1.3
Aspartate aminotransferase	2.6.1.1
Alanine aminotransferase	2.6.1.2
RNA-directed DNA polymerase	2.7.7.49
Thiosulphate sulphurtransferase	2.8.1.1
Cholinesterase	3.1.1.8
Glucose-6-phosphatase	3.1.3.9
Phosphatidate phosphatase	3.1.3.4
Phosphodiesterase I	3.1.4.1
Arylsulphatase	3.1.6.1
β-Glucosidase	3.2.1.21
β-Galactosidase	3.1.1.23
α-Mannosidase	3.2.1.24
α-L-Fucosidase	3.2.1.51
Cytosol aminopeptidase (leucine aminopeptidase)	3.4.11.1
Cystyl-aminopeptidase (oxytocinase)	3.4.11.13
Trypsin	3.4.21.4
Inorganic pyrophosphatase	3.6.1.1
Adenosine triphosphatase	3.6.1.3
Thiamine pyrophosphatase (nucleoside diphosphatase)	3.6.1.6
Nucleotide pyrophosphatase	3.6.1.9
Fructose-bisphosphate aldolase	4.1.2.13
Carbonate dehydratase	4.2.1.1
Glucose-6-phosphate isomerase	5.3.1.9
Acetyl-CoA carboxylase	6.4.1.2

in milk only by their activity and have not been isolated from milk although similar enzymes have been isolated from other sources and, presumably, the enzymes in milk are generally similar. Some of these minor enzymes have been considered as indices of the heat treatment of milk.

12.2 Lactoperoxidase (EC 1.11.1.7)

Peroxidases, which are widely distributed in plant, animal and microbial tissues and secretions, catalyse the following reaction:

$$2HA + H_2O_2 \rightarrow A + 2H_2O$$

where HA is an oxidisable substrate or a hydrogen donor, which may be an aromatic amine, a (poly)phenol, an aromatic acid or a leuco dye. Many of these reducing agents are chromogenic, thus offering a method for detecting and quantifying peroxidase activity. The reducing substrates used most widely initially for the assay of LPO were guaiacol, pyrogallol and *p*-phenylenediamine, but 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] is now generally used, with measurement of A_{412}.

LPO was first demonstrated in milk by Arnold (1881), using *guajaktinctur* as reducing agent; he reported that the activity of LPO is lost on heating milk at 80°C. Louis Pasteur showed (1860–1864) that the spoilage of wine and beer could be prevented by heating at ~65°C for 30 min, and this process was first applied in 1891 to improve the quality of cream for buttermaking. As a means of controlling the spread of tuberculosis in cattle, legislation was introduced in Denmark in 1898 requiring that all skim milk returned by creameries to farmers should be flash (i.e. no holding period) pasteurised at 85°C (later changed to 80°C). Various tests were proposed to ensure that such milk was adequately pasteurised, but the most widely adopted was that developed by Storch (1898), who assayed LPO activity using *p*-phenylenediamine as reducing agent; the principle of the Storch test is still used to identify super-pasteurised milk, i.e. milk heated ≥76°C for 15 s. The original Storch test was purely qualitative, but

quantitative assays for LPO activity in milk were developed later. Because of the suitability of LPO as an indicator for super-pasteurised milk, its thermal denaturation has been intensively studied (e.g., Martin et al., 1990; Trujillo et al., 2007; Lorenzen et al., 2010).

The first study on the isolation of LPO was by Thurlow (1925), who obtained enriched preparations of LPO by fractional precipitation with $(NH_4)_2SO_4$. An improved method for the isolation of LPO from rennet whey by salting out, displacement chromatography and crystallisation was published by Polis and Shmukler (1953), who also characterised the enzyme. They reported that the enzyme is green in colour and that it was contaminated during the early stages of purification with a red protein, now called lactoferrin. LPO is a haem protein containing protoporphyrin IX with 0.069% Fe, a Soret band at 412 nm, an A_{412}:A_{280} ratio of 0.9, has a mass of 82 kDa and occurs as two isozymes, A and B.

During the following years, several methods for the isolation of LPO were published and knowledge on the characteristics of the enzyme was refined (e.g., Carlstrom 1969). Since LPO is cationic at the pH of milk, as are lactoferrin and some minor proteins, it can be easily isolated from milk or concentrated sweet (rennet) whey using a cationic exchange resin (e.g., Amberlite CG-50-NH_4) (Mitchell et al., 1994; Fweja et al., 2010) and further purified by a suitable technique, e.g., RP-HPLC (Carmen et al., 1990). The use of ultrasound-assisted ultrafiltration and aqueous two-phase extraction for recovery of LPO from whey was reported by Nandini and Rastogi (2011).

There are ten isozymes of LPO, arising from differences in the level of glycosylation and deamination of Gln or Asn. The mass of the enzyme is 78,030 Da, including sugars (8–10% of the mass of the enzyme) and the haem group. Carlstrom (1969) reported that LPO occurs as a homodimer but Sievers (1981), using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), found that the enzyme is a monomer. The primary structure of LPO was reported by Cals et al. (1991); it contains 612 amino acids and shows 55%, 54% and 45% identity with human myeloperoxidase, eosinophil peroxidase and thyroperoxidase, respectively. The LPO molecule is highly structured, with 65% β-structure, 23% α-helix and 12% unordered structure (Sievers 1980). A model of the tertiary structure of LPO, based on that of myeloperoxidase, was reported by de Wit and van Hooydonk (1996). LPO binds a Ca^{2+}, which has a major effect on its stability, including its heat stability. At pH below ~5.0, the Ca^{2+} is lost, with a consequent loss of stability.

LPO, which is synthesised in the mammary gland (Cals et al., 1991), was reported by Sievers (1980) and de Wit and van Hooydonk (1996) to be present also in human tears and saliva. Next to xanthine oxidase, LPO is the most abundant enzyme in milk, constituting ~0.5% of the total whey proteins (~0.1% of total protein; 30 mg/L). Hamosh (1988) reported that human milk is devoid of LPO but contains myeloperoxidase, which is generally similar to LPO, but Watanabe et al. (2000) reported a low level of LPO in human milk, about 5% of that in bovine milk. Apparently, human colostrum contains a high level of myeloperoxidase, derived from leucocytes, and a lower level of LPO. The level of myeloperoxidase decreases rapidly *post partum* and LPO is the principal peroxidase in mature human milk; it has been isolated and quantified by Shin et al. (2001). LPO has been suggested as a useful indicator of subclinical mastitis in goats (Seifu et al., 2007) but has been shown to be poorly suited to this purpose in the case of cows (Asadpour et al., 2008).

In the presence of low levels of H_2O_2 and SCN^-, LPO exhibits very potent bactericidal activity; this system is 50–100 times more effective than H_2O_2 alone. Most of the very extensive recent interest in LPO has focused on this aspect (see Björck 1992; Kussendrager and van Hooijdonk, 2000; Fox 2003; Pruitt 2003; Cankaya et al., 2010). Boulares et al. (2011a) showed significant increases in the refrigerated shelf life of raw ovine, bovine and caprine milk by activation of the LPO system through addition of sodium thiocyanate and sodium percarbonate. The impact of such treatments on the manufacture of cheese (Amornkul and Henning 2007; Boulares et al., 2011b) and yoghurt (Masud et al., 2010) has been studied. Boulares et al. (2011a, b) and Amornkul and Henning (2007) reported

alterations to manufacture and ripening of Saint-Paulin and Cheddar cheese, respectively, probably due to reduced growth of psychrotrophic bacteria in milk, following LPO treatment.

12.3 Catalase (EC 1.11.1.6)

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) catalyses the decomposition of H_2O_2, as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase also oxidises reducing agents, i.e. it has peroxidase activity. For a general review of catalases, see Wong and Whitaker (2003).

Catalase activity may be determined by quantifying the evolution of O_2 manometrically or by titrimetrically measuring the reduction of H_2O_2. Catalases are haem-containing enzymes that are distributed widely in plant, microbial and animal tissues and secretions; liver, erythrocytes and kidney are particularly rich sources. A catalase was among the first enzymes demonstrated in milk. Babcock and Russell (1897) reported that an extract of separator slime (somatic cells and other debris) could decompose H_2O_2, presumably indicating the presence of catalase.

Catalase activity in milk varies with feed, stage of lactation, and the level of activity increases markedly during mastitis (Johnson 1974). Catalase has been proposed as a useful indicator of mastitis (Kitchen 1981); however, it is now rarely used for this purpose; determination of somatic cell count (SCC), *N*-acetylglucosaminidase activity or electrical conductivity is used more frequently.

McMeekin and Polis (1949) reported that catalase is associated with casein but recent work indicates that it is concentrated in the cream and separator slime. According to Kitchen et al. (1970), 73% of the catalase in milk is in the skimmed milk, but the specific activity in the cream is 12-fold higher than that in skimmed milk; only about 8% of total catalase activity is in the ultracentrifugal casein pellet. Hence, the MFGM fraction is usually used as the starting material for the isolation of catalase from milk.

Considering that the level of catalase in milk is relatively high and that the enzyme is easily assayed, catalase was not isolated from milk until relatively recently. Various aspects of catalase in milk were reported by Prof. O. Ito in a series of papers during the period 1969–1983 (see Ito and Akuzawa 1983a), who isolated catalase from milk. The enzyme was purified 23,000-fold and crystallised and shown by gel permeation to have a molecular mass of 225 kDa. Ito and Akuzawa (1983b) reported that there were three isozymes in the catalase preparation isolated from cream. Ito and Akauzawa (1983c) reported that milk catalase was dissociated by SDS into five subunits ranging in molecular mass from 11 to 55 kDa. Bovine liver catalase is a homotetramer of 60–65 kDa subunits (total MW ~250 kDa). It seems likely that the structure of catalase in milk is similar to that enzyme and that the heterogeneity reported by Ito and Akuzawa (1983b) is due to proteolysis during isolation.

Catalase is relatively heat-labile (Farkye and Imafidon 1995; Hirvi et al., 1996) and was among the first indicators of pasteurisation investigated. More recently, the presence of active catalase has been considered as an indicator of cheese made from sub-pasteurised milk. There is general agreement that cheese made from raw milk ripens more quickly and develops a more intense (although not always a more desirable) flavour than cheese made from pasteurised milk (Fox et al., 2000). However, for public health reasons and in the interest of producing a consistent product, pasteurised milk is now generally used for cheesemaking. However, many varieties of cheeses are still made from raw milk, especially in southern Europe. Sub-pasteurised or thermised milk (e.g., that heated at 63–65°C for 16 s) has, in some cases, been considered as a compromise between raw and pasteurised milk for cheesemaking. The thermal inactivation of catalase was studied by Hirvi et al. (1996), and the possibility of using its inactivation as an index of thermised milk was investigated by Hirvi and Griffiths (1998). Although the inactivation of catalase was found to be a useful index of thermisation of milk (it being almost completely inactivated by heating at 65°C for 16 s), it was not suitable as an

index of cheese made from thermised milk owing to the production of catalase in the cheese during ripening, especially by coryneform bacteria and yeasts, if present

12.4 Xanthine Oxidoreductase [EC 1.1.3.22, 1.1.1.204]

In 1902, F. Schardinger showed that milk contains an enzyme capable of oxidising aldehydes to acids, accompanied by the reduction of methylene blue; this enzyme was then commonly called the 'Schardinger enzyme'. Morgan et al. (1922) showed that milk contains an enzyme capable of oxidising xanthine and hypoxanthine to uric acid, with the concomitant reduction of O_2 to H_2O_2; this enzyme was called xanthine oxidase (XO). Booth (1938) presented strong evidence that the Schardinger enzyme was, in fact, XO and partially purified it. Corran et al. (1939) isolated XO from whole milk and characterised its molecular properties and enzymatic activity. Ball (1939) showed that XO is concentrated in the cream phase of milk, from which they obtained highly purified preparations and showed that it requires FAD^+ for catalytic activity. XO can dehydrogenate xanthine under certain circumstances and is now usually called XOR.

An improved method for the isolation of XOR was published by Avis et al. (1955a) and the enzyme was crystallised and characterised by Avis et al. (1955a, b, c). XOR is concentrated in the MFGM, in which it is the second most abundant protein, after butyrophilin; it represents ~20% of the protein of the MFGM (~0.2% of total milk protein; ~120 mg/L). Isolation methods generally use washed cream as the starting material which is churned to yield a crude MFGM preparation. The early isolation methods used a proteinase (pancreatin) to solubilise XOR, but this causes limited proteolysis and changes the enzyme from a xanthine dehydrogenase (XDH) to an oxidase. Waud et al. (1975) used butanol to solubilise XO in milk followed by precipitation with $(NH_4)_2SO_4$ and chromatography on DEAE cellulose to isolate the enzyme; the effects of pancreatin on the properties of the enzyme were reported. Waud and Rajagopalan (1975, 1976) studied the interconversion of XO and XDH. Mangino and Brunner (1977) used deoxycholate to dissociate XOR from membrane lipoproteins and chromatography on hydroxylapatite to purify the enzyme. Plasmin, the principal indigenous proteinase in milk, was shown to hydrolyse XOR during isolation and explained the different values for the molecular mass of XOR reported by various authors. However, plasmin caused little hydrolysis of XOR in comparison with trypsin, chymotrypsin or papain (Cheng et al., 1988) and was considered to have little effect on XOR during isolation.

Silanikove and Shapiro (2007) reported that 33% of XOR activity in bovine milk is on the inner face of the MFGM, 20% on the inner face of skim milk membranes (which originate from the MFGM) and 47% is 'effectively soluble'. It was suggested by Silanikove et al. (2007) that the XOR on the inner membrane plays a non-enzymatic role in the expression of fat globules from the mammocytes while the extra-membranous XOR plays a role in the immune system of the mammary gland. However, Sharma et al. (2009) reported that only 5–10% of XO activity in bovine, buffalo and caprine milk is in the skim milk phase.

Because bovine milk is a very rich source of XOR, from which it is isolated relatively easily and because of its important and varied functions, XOR is a very well-characterised enzyme; currently it is probably the most studied of the indigenous milk enzymes. The extensive literature on XOR has been reviewed by Booth (1938), Whitney (1958), Fox and Morrissey (1981), Kitchen (1985), Massey and Harris (1997), Farkye (1992, 2003), Harrison (2004, 2006) and Fox and Kelly (2006a). XOR is a dimer of identical 146 kDa subunits, each containing ~1,330 amino acid residues. The gene for the enzyme from several sources has been cloned and shows a high degree of sequence conservation. Each XOR monomer contains 1 atom of Mo, 1 molecule of FAD^+ and 2 Fe_2S_2 redox centres. NADH acts as a reducing agent and the oxidation products are H_2O_2 and O_2. The milk of cows deficient in Mo has low XOR activity. Xanthine oxidase (XO; EC 1.1.3.22) and XDH (1.1.1.204) can be

interconverted by sulphydryl reagents, and XDH can be converted irreversibly to XO by specific proteolysis, e.g., by plasmin. The quaternary structure of XDH and XO was described by Enroth et al. (2000). Milk is a very good source of XOR, at least part of which is transported to the mammary gland *via* the blood stream. A similar enzyme is found in various animal tissues and in several bacterial species.

Early investigators reported that human milk lacks XOR, but Bradley and Gunther (1960), using a more sensitive assay, showed that human milk does contain XOR and that its level varies markedly during lactation. The XOR activity in human milk is low because 95–98% of the enzyme molecules lack Mo (Godber et al., 1997, 2005). Although XOR is a major protein in caprine MFGM (Cebo et al., 2010), the level of XOR activity in caprine and ovine milk is low (Atmani et al., 2004; Benboubetra et al., 2004; Gonzalez-Ronquillo et al., 2010). The level of XOR activity in human, ovine and caprine milk can be increased by supplementing the diet with Mo. A low level of XOR activity has been reported in camel milk (Al-Seeni 2009). XOR was identified in equine MFGM by Barello et al. (2008) but the level of activity was not reported.

12.4.1 Assay Methods

Xanthine oxidase activity can be assayed manometrically (uptake of O_2), potentiometrically, using a platinum electrode, polarographically or spectrophotometrically; the latter may involve the reduction of colourless triphenyltetrazolium chloride to a red product or the conversion of xanthine to uric acid which is quantified by measuring absorbance at 290 nm (see Fox and Kelly 2006a). XDH activity can be assayed by changes in NADPH concentration by absorbance at 290 nm.

12.4.2 Effect of Processing on XOR Activity in Milk

XOR activity in bovine milk varies substantially (Griffiths 1986). Processing treatments that damage or alter the MFGM affect the XOR activity in milk (Fox and Kelly 2006a). Measured activity is increased by storage at 4°C, heating at 70°C or by homogenisation. These treatments cause the release of XOR from the MFGM into the skim milk phase, rendering the enzyme more active. The heat stability and catalytic activity (Briley and Eisenthal 1974) of XOR are very dependent on whether it is a component of the MFGM or is dispersed in the aqueous phase of milk. Cold storage and homogenisation reduce the heat stability of XOR and explain the inconsistency of early work in which the history of the sample was unknown or unrecorded. XOR is most heat-stable in cream and least stable in skim milk. Homogenisation of concentrated milk prepared from heated (e.g., 90.5°C for 15 s) milk partially reactivates XOR, which persists on drying the concentrate; no reactivation occurs following more severe heating (e.g., 93°C for 15 s). Apparently, homogenisation releases potentially active, undenatured XOR from the MFGM. All the major milk proteins can act as either activators or inhibitors of XOR, depending on their concentration, and may have some significance in the activation, inactivation and reactivation of the enzyme (Hwang et al., 1967). Studies on the heat stability of XOR have been reviewed by Griffiths (1986), who investigated its stability in a pilot-scale high-temperature-short-time (HTST) pasteuriser; the enzyme was not completely inactivated by heating at 80°C for 120 s and a *Z*-value of 6.8°C was calculated.

12.4.3 Significance of Xanthine Oxidoreductase in Milk and Dairy Products

XOR has many functions in milk and dairy products:

- *As an index of heat treatment*: Andrews et al. (1987) suggested that XOR is a suitable indicator of milk heated in the temperature range 80–90°C, but Griffiths (1986) considered the natural variability in the level of XOR activity in milk to be too high for its use as a reliable index of heat treatment. Sharma et al. (2009)

suggested that XOR may be a suitable marker of heat treatment sufficient to kill *M. avium* ssp. *paratuberculosis*.

- *Lipid oxidation*: XOR can excite stable triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$), a potent pro-oxidant. Some milk samples from individual cows, which undergo oxidative rancidity spontaneously (i.e. without contamination with metals or exposure to light), contain about 10× the normal level of XOR, and oxidation can be induced in normal milk by the addition of XOR to ~4× the normal level (Aurand et al., 1967, 1977). Heat-denatured or FAD-free enzyme is not a pro-oxidant.
- *Atherosclerosis*: It has been suggested that XOR enters the vascular system from homogenised milk and may be involved in atherosclerosis *via* oxidation of plasmalogens in cell membranes; this aspect of XOR attracted considerable attention in the early 1970s but the hypothesis has been discounted (see Clifford et al., 1983; Deeth 1983; Harrison 2002).
- *Reduction of nitrate in cheese*: Sodium nitrate is added to milk for many cheese varieties to prevent the growth of *Clostridium tyrobutyricum*, which causes flavour defects and late gas blowing in these cheeses; XOR reduces nitrate to nitrite, which is bactericidal, and then to NO.
- *Bactericidal activity*: XOR has strong antibacterial activity in the human intestine, and probably in the mammary gland *via* the production of peroxynitrite ($ONOO^-$) (Stevens et al., 2000; Godber et al., 2000; Atmani et al., 2005). XOR activity may contribute to the lower level of gastrointestinal infection in breast-fed babies compared to those bottle-fed. Indigenous XOR is inactivated in the production of infant formulae and the supplementation of such formulae with exogenous XOR (e.g., MFGM) has been proposed (see Harrison 2006). XOR, along with lysozyme and lactoferrin, is part of the innate immune system which evolved prior to the evolution of antibodies and is frequently referred as a housekeeping molecule (Vorbach et al., 2003).
- *Secretion of milk fat globules*: Probably the most important role of XOR in milk is now considered to be in the secretion of milk fat globules from the mammary secretory cells. The triglycerides in milk are synthesised in the endoplasmic reticulum (ER), where the TGs are formed into micro-lipid droplets and released through the involvement of a protein, acidophilin, which surrounds the globules. The ADPH-covered globules move towards the apical membrane of the cell, probably through a microtubular/microfilament system, and acquire additional coat material, cytoplasmic proteins and phospholipids. At the apical membrane, ADPH forms a disulphide-linked complex with two other proteins, butyrophilin, a transmembrane protein in the apical membrane of the cell, and dimeric XOR. Somehow, XOR causes blebbing of the fat globule through the membrane, and it is eventually pinched off and released into the alveolar lumen (McManaman et al., 2002, 2007; Vorbach et al., 2002). In the secretion of milk fat globules, XOR does not function as an enzyme. It is proposed that the secretion of milk fat globules is controlled by butyrophilin (Robenek et al., 2006).
- *Evolution of mammals*: Since XOR is a component of the innate immune system, it must have existed from a very early stage in evolution. It has been suggested (Vorbach et al., 2002, 2003, 2006) that the evolution of mammary glands (and hence mammals) was made possible through the function of XOR in the excretion of fat globules from the mammocytes. A second component of the innate immune system, lysozyme, evolved to become α-lactalbumin, the regulator of lactose synthesis. Thus, the production of the two principal sources of energy in milk, lipids and lactose, is possible through the involvement of two major components of the innate immune system. It is argued that the nutritional value of milk evolved subsequently to its immunological function and that the mammary gland evolved as a mucus skin gland, potentially with the objective of protecting the newly evolving mammalian skin from infectious diseases or to protect the surface of soft-shelled eggs or the newborn against dehydration and infection.

It is assumed that the newborn licked some secretion from the sebaceous glands and thus inadvertently obtained nutritional benefit. Various aspects of the origin and structure of the MFGM have been described by Aoki (2006) and Keenan and Mather (2006).

12.5 Superoxide Dismutase (EC 1.15.1.1)

SOD scavenges superoxide radicals, $O_2^{-\cdot}$ according to the reaction:

$$2\ O_2^{-\cdot} + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

The H_2O_2 formed may be reduced to $H_2O + O_2$ by catalase, peroxidase or a suitable reducing agent. The biological function of SOD is to protect tissue against free radicals of oxygen in anaerobic systems. Although oxygen radical-scavenging proteins had been isolated from cells previously, the significance of these proteins was not recognised until the work of J.M. McCord and I. Fridovich, in 1968–1969, which showed that the scavenging protein was an enzyme, which they called superoxidase dismutase. Since then, SOD has been identified in many animal and bacterial cells; the work has been reviewed by Fridovich (1975), McCord and Fridovich (1977) and Hara et al. (2003).

There are four isoforms of SOD, Cu/Zn-SOD, extracellular (EC) SOD, Mn-SOD and Fe-SOD. Cu/Zn-SOD is the most common form in mammals and has been isolated from a number of tissues, including bovine erythrocytes. It is a blue-green protein due to the presence of Cu (1 atom per monomer), removal of which by EDTA results in the loss of activity, which is restored by adding Cu^{2+}; it also contains 1 atom of Zn per monomer, which appears not to be involved in catalysis. The enzyme, which is very stable in 9 M urea at neutral pH, consists of two identical subunits of molecular weight 16 kDa (153 amino acid residues), linked by one or more disulphide bonds. The SOD in bovine milk is a Cu-Zn enzyme but human colostrum and mature milk contain both Cu-Zn and Mn types, the latter being eight and two times higher in human colostrum and milk, respectively (Kiyosawa et al., 1993). The amino acid sequence of Cu/Zn-SOD from several species has been reported (see Hara et al., 2003). The tertiary structure of Cu-Zn SOD from bovine erythrocytes was reported by Tainer et al. (1982). Mn-SOD and EC-SOD are tetrameric enzymes of 20 and 35 kDa subunits, respectively.

Bovine milk contains a low level of SOD (150 times less than in blood), which is present exclusively in the skim milk fraction; the SOD activity in bovine milk varies between animals and breeds (Holbrook and Hicks 1978; Granelli et al., 1995). The SOD in milk appears to be identical to the bovine erythrocyte enzyme (Hill 1975; Hicks et al., 1975; Keen et al., 1980). Assay methods for SOD are described by Stauffer (1989), Granelli et al. (1995) and Hara et al. (2003).

12.5.1 Significance

SOD inhibits lipid oxidation in model systems. The level of SOD in milk parallels that of XOR (but at a lower level), suggesting that SOD may offset the effect of the pro-oxidant XOR. Attempts to correlate the stability of milk to oxidative rancidity with indigenous SOD have been equivocal (Holbrook and Hicks 1978). Milk contains several pro- and antioxidants, the precise balance of which, rather than any single factor, determines oxidative stability (see Hicks 1980; Lindmark-Mansson and Akesson 2000).

SOD is more heat-stable in milk than in purified preparations. In milk it is stable to heating at 71°C for 30 min but it loses activity rapidly at slightly higher temperatures (Hicks 1980). Therefore, slight variations in pasteurisation temperature are critical to the survival of SOD in heated milk products and may contribute to variations in the stability of milk to oxidative rancidity. Homogenisation has little effect on the distribution of SOD in milk.

The possibility of using exogenous SOD to retard or inhibit lipid oxidation in dairy products has been considered. A marked improvement in the oxidative stability of milk was achieved by adding a low level of SOD (Aurand et al., 1977).

However, SOD is too expensive in comparison with chemical antioxidants for commercial use.

12.6 Sulphydryl Oxidase (EC 1.8.3-)

Gould (1940) reported that glutathione (GSH) added to raw or low-temperature heated milk disappeared quickly but was stable in milk that had been heated ≥80°C, suggesting that an enzyme was responsible for the destruction of GSH. An enzyme capable of oxidising the sulphydryl group of cysteine, GSH and proteins to disulphide bonds according to the following reaction:

$$2RSH + O_2 \rightarrow RSSR + H_2O_2$$

was first detected in milk by Kiermeier and Petz (1967) and purified by Janolino and Swaisgood (1975, 1978). The above reaction can also be catalysed by glutathione oxidase (GSHOx, EC 1.8.3.3) and γ-glutamyltransferase (GGT). Schmelzer et al. (1984) reported that antibodies raised against milk SHOx can immunoprecipitate GSHOx but not GGT. SHOx differs from thiol oxidase (EC 1.8.3.2) which requires FAD, and microbial SHOx. SHOx is widely distributed in cell membranes, including those of the mammary gland, kidney and pancreas but was not found in intestine, brain, heart, liver, lung, spleen or thymus (Clare et al., 1984). SHOx has been found in bovine, caprine, porcine, human and rat milk; Ouchterlony immunodiffusion showed that the enzyme from bovine and caprine milk and bovine kidney cross-reacted but the enzyme from human milk did not (Clare et al., 1984). There are several widely distributed enzymes called SHOx that oxidise sulphydryl groups in small molecules and/or proteins. Many of these enzymes found in mammalian tissues require FAD as a cofactor (see Tury et al., 2006), as does the SHOx of egg white (Hoober et al., 1996). An SHOx from *Aspergillus niger* does not require FAD (Vignaud et al., 2002); the properties of this enzyme are quite different from those of SHOx isolated from milk in terms of substrate specificity, molecular mass and isoelectric point (Janalino and Swaisgood 1992).

Milk SHOx is a glycoprotein (~10% carbohydrate) containing ~0.5 atoms of Fe per monomer (89 kDa) and does not require FAD. It has a strong tendency to associate, which makes it easy to isolate from whey by permeation chromatography on agarose or porous glass. A relatively simple, reproducible method for quantification of SHOx from milk involving covalent chromatography on cysteinylsuccinamidopropyl glass was published by Sliwkowski et al. (1983). The enzyme is optimally active at ~pH 7 and 35°C and is inhibited by metal chelators and sulphydryl-blocking reagents (Swaisgood and Janolino 2003). The kinetics of the enzyme were described by Sliwkowski et al. (1984).

SHOx oxidises reduced RNase and restores enzymatic activity, suggesting that its physiological function is the formation of specific disulphide bonds during the post-synthesis processing of proteins. It can convert XDH to xanthine oxidase (XO) by oxidising a sulphydryl group in the former (Clare et al., 1981; Blakistone et al., 1986). As discussed above, xanthine oxidase is involved in the expression of fat globules from the mammocytes and is a major protein in the MFGM; thus, it is possible that SHOx is involved in the expression of fat in the mammary gland.

The principal technological significance of SHOx in the dairy industry is in its ability to oxidise sulphydryl groups exposed and activated during high-temperature processing and which are responsible for the cooked flavour of such products. SHOx immobilised on glass beads reduces the cooked flavour of UHT-treated milk and remains active over a long period; this process has been patented (see Swaisgood and Janolino 2003) but has not been used commercially. Apparently, oxidation of the sulphydryl groups renders the product more stable to lipid oxidation, although sulphydryl groups *per se* are antioxidants.

SHOx activity is usually assayed on GSH at pH 7, by reacting with dithiodinitrobenzene, with which GSH forms a yellow product which is quantified by measuring absorbance at 412 nm (Janolino and Swaisgood 1975).

12.7 Glutathione Peroxidase (EC 1.11.1.9)

Glutathione peroxidase (GSHPOx) catalyses the reaction:

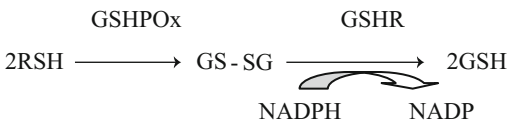
$$2GSH + ROOH \rightarrow GS\text{-}SG + ROH + H_2O$$

where GSH is glutathione (γ-Glu-Cys-Gly) and ROOH is a peroxide, including H_2O_2.

GSHPOx is widespread in the cytoplasm of animal tissues, especially erythrocytes from which it has been isolated. Its function is to protect the cell against the damaging effects of peroxides, as part of an antioxidative system which includes SOD. There are at least two forms of GSHPOx, cellular and extracellular (plasma) GSHPOx in mammals which are kinetically, structurally and antigenically distinct (see Douglas 1987; Avissar et al., 1991).

GSHPOx is a tetrameric protein of four identical subunits (21 kDa), each of which contains one atom of Se bound to a cysteine residue. The molecule has been well characterised, including elucidation of its primary, secondary and tertiary structures (see Epp et al., 1983; Liu and Luo 2003).

GSHPOx is assayed by a coupled reaction with glutathione reductase (GSHR; EC 1.6.4.2):



The loss of NADPH is quantified by measuring A_{340}. The nonenzymatic oxidation of NADPH is a problem (Chen et al., 2000). Alternatively, the decrease in the concentration of GSH can be quantified by reaction with dithiodinitrobenzoic acid or polarographically.

Milk contains a low level (27 ng/mL) of GSHPOx, more than 90% of which is the extracellular type. The level of GSHPOx in milk varies with the species (human> caprine> bovine) and diet (Debski et al., 1987). GSHPOx has no known enzymatic function in milk, in which it binds 30% of the total Se, an important trace element in the diet. Lindmark-Masson and Akesson (2001) reported the isolation of GSHPOx from bovine milk and developed an ELISA assay for the enzyme protein. Lindmark-Masson et al. (2001) reported that GSHPOx was stable to heating at 72°C for 2 min at the pH of milk and was stable for several days at 8°C. However, Stagsted (2006) claimed that the assay method used by the above and other authors measured superoxidase dismutase rather than GSHPOx and concluded that bovine milk does not contain GSHPOx activity.

A related enzyme GSHOx (EC 1.8.3.3), which catalyses the reaction:

$$2GSH + O_2 \rightarrow GSSG + H_2O_2$$

has not been reported in milk although antibodies raised against milk SHOx can immunoprecipitate GSHOx (Schmelzer et al., 1984).

12.8 γ-Glutamyl Transferase (EC 2.3.2.2)

γ-Glutamyl transferase (GGT) catalyses the transfer of γ-glutamyl residues from γ-glutamyl-containing peptides:

$$\gamma\text{-glutamyl-peptide} + X \rightarrow \text{peptide} + \gamma\text{-glutamyl-}X$$

where X is an amino acid.

GGT is associated with the membranes of a number of epithelial cells. Tate and Meister (1976) isolated GGT from rat kidney by affinity chromatography of a detergent extract of the tissue on concanavalin A. The enzyme is a glycoprotein and isoelectric focusing showed 12 isozymes, which differed in sialic acid content. SDS-PAGE showed that the enzyme is a dimer of subunits of molecular weight 46 and 22 kDa.

In milk, GGT is found in the membrane material in skim milk (~70%) or in the MFGM, from which it can be dissociated by detergents or organic solvents. The enzyme, which has been purified from the MFGM, has a molecular weight

of ~80 kDa and consists of two subunits of 57 and 25 kDa (determined by SDS-PAGE), both of which are glycoproteins (Baumrucker 1979, 1980). The enzyme, which associates strongly (Tate and Meister 1976; Kenny 1977), is optimally active at pH 8.5–9 and ~45°C and has an isoelectric point of 3.85. It is strongly inhibited by diisopropylfluorophosphate, iodoacetamide and metals, e.g., Cu^{2+} and Fe^{3+} (see Farkye 2003). GGT activity in human and bovine milk varies during lactation, being highest in colostrum; variation in its activity in buffalo milk over lactation was reported by Pero et al. (2006).

GGT functions in the regulation of cellular GSH and may be involved in the transport of amino acids from blood into the mammary gland *via* the so-called γ-glutamyl cycle (Meister 1973; Kenny 1977) and thus may be involved in the biosynthesis of milk proteins (Baumrucker and Pocius 1978).

GGT is usually assayed using γ-glutamyl-*p*-nitroanilide as substrate; the liberated *p*-NA can be determined by measuring the absorbance at 410 nm or by reaction with naphthylethylenediamine and measuring the absorbance at 540 nm (McKellar et al., 1991).

From a dairy technologist's viewpoint, GGT is of interest mainly because of its heat stability characteristics, as for many other enzymes. As discussed earlier, alkaline phosphatase is the test enzyme usually used to evaluate the effectiveness of HTST pasteurisation; however, as discussed, reactivation of AlP in UHT-treated products poses problems in the interpretation of the test. Based on a comparative study on the heat-stability characteristics of a number of indigenous enzymes in milk, Andrews et al. (1987) concluded that GGT is appropriate for monitoring heat treatments in the range of 70–80°C for 16 s. This conclusion has been confirmed in pilot-scale studies (Patel and Wilbey 1989; Carter et al., 1990). In whole or skim milk, GGT is completely inactivated by heating at 78°C for 15 s (Patel and Wilbey 1989) or 77°C for 16 s (McKellar et al., 1991). No reactivation was found under various conditions and little seasonal variation occurs. As little as 0.1% or 0.25% raw milk could be detected in pasteurised skim or whole milk, respectively (McKellar et al., 1991).

Linear models for the thermal inactivation of GGT and LPO in a HTST pasteuriser were developed by McKellar et al. (1996). The relationship between % inactivation and pasteurisation equivalent was more linear than the relationship for AlP, possibly due to the presence of more than one isozyme of AlP (McKellar et al., 1996). GGT was ~9 times more stable in ice cream mix than in whole milk (McKellar 1996). Thus, it appears that GGT is a suitable enzyme for estimating the intensity of heat treatment of milk in the range 72–77°C for 15 s; this was recently proposed for camel milk (Wernery et al., 2008). The pressure-resistance of GGT was reported by Pandey and Ramaswamy (2004).

GGT is absorbed from the gastrointestinal tract, resulting in high GGT activity in the blood serum of newborn animals fed colostrum or early-lactation breast milk. Since GGT is inactivated by the heat treatment to which infant formulae are subjected, the level of serum GGT activity in infants can be used to distinguish breast-fed from formula-fed infants (see Farkye 2003).

γ-Glutamyl peptides have been isolated from Comté (Roudot-Algaron et al., 1994) and Gouda and Blue (Toelstede and Hofmann 2009) cheese; since casein contains no γ-glutamyl bonds, the presence of these peptides in cheese may suggest GGT activity in cheese but there appear to be no data to support this hypothesis.

12.9 Lipases and Esterases

Lipase is, potentially, the most important indigenous enzyme technologically. Its activity causes hydrolytic rancidity, an off-flavour defect that has been recognised since the pre-industrialisation of dairying: Dunkley (1946) cited the description by Lawrence in 1726 of a bitter flavour in milk and cream, which Dunkley considered to be hydrolytic rancidity, caused by a lipase. Hydrolytic rancidity has been a major problem for many years, especially after the introduction of pipeline milking machines in the 1950s (see Herrington 1954). The influence of various factors on the development of rancidity was described by Dunkley (1946), Herrington (1954), Jensen

(1964), Downey (1975), Deeth and Fitz-Gerald (1976, 1995, 2006) and Deeth (2006).

In addition to causing off-flavours in milk and dairy products, lipolysis, by reducing the surface activity of milk, reduces its foaming capacity, e.g., in cappuccino coffee, and its whipping and churning time (Deeth and Fitz-Gerald 2006). However, milk lipase contributes positively to the flavour of raw-milk cheese (Collins et al., 2003; Deeth and Fitz-Gerald 2006).

The presence of lipolytic activity in milk was reported by Moro (1902) and this view was confirmed by several workers (see Palmer 1922; Corbin and Whittier 1965; Fox and Kelly 2006a). Palmer (1922), who critically reviewed the earlier studies on milk lipase and lipolysis in milk, concluded that most of the earlier studies were defective because of failure to include proper controls and/or because soluble esters were used as substrate; he found no evidence of lipolytic activity in normal bovine milk but Rice and Markley (1922) presented strong evidence indicating the presence of lipase in milk. Herrington (1954) compiled an extensive review on milk lipase but concentrated on various aspects of lipolysis, rather than on the enzyme, which at that stage had not been isolated and had been characterised only in general terms.

Tarassuk and Frankel (1957) claimed that there were at least two lipases in milk, 'plasma lipase' and 'membrane lipase'; the latter was considered to be responsible for spontaneous lipolysis (see Corbin and Whittier 1965). Gel permeation and ion-exchange chromatography indicated that there are several lipases in milk, but it seems likely that these multiple forms of lipase were due the self-association of lipase or to association with other milk proteins (Fox and Kelly 2006a). At least 90% of the lipase in milk is associated with the casein micelles (Gaffney et al., 1966), but it can be dissociated from the micelles by treatment with 1 M NaCl (Downey and Andrews 1966), dimethylformamide (Fox et al., 1967) or heparin (Hoynes and Downey 1973).

A very low molecular weight (~8 kDa) lipase was purified from separator slime by Chandan and Shahani (1963a, b). This lipase probably originated from somatic cells and it was considered to be only a minor lipase in milk (Castberg et al., 1975).

Quigley et al. (1958) and Korn (1962) reported that milk contains a lipoprotein lipase (LPL). A lipase was isolated from skimmed milk by Fox and Tarassuk (1968) and characterised by Patel et al. (1968). This enzyme was inhibited by organophosphates and had a molecular mass of 210 kDa (by gel permeation chromatography). Fox and Flynn (1980) showed that the lipase isolated from milk by Fox and Tarassuk (1968) is an LPL.

Brockerhoff and Jensen (1974) and Jensen and Pitas (1976) proposed that milk contains both lipase and LPL. LPL was isolated from milk by Egelrud and Olivecrona (1972); it was found to be a homodimer (molecular mass ~90 kDa) with each monomer containing 450 amino acid residues. It originates from the vascular endothelial surfaces, where it is bound by heparin sulphate chains and plays a very important role in the synthesis of lipids in the mammary gland (see McBride and Korn 1963; Liesman et al., 1988; Olivecrona et al., 2003; Palmquist 2006). Askew et al. (1970) and Castberg et al. (1975) showed that the LPL isolated from mammary tissue was the same as that isolated from milk.

LPL is strongly involved in the biosynthesis of milk lipids: all of the C18 acids and ~50% of the C16 acids in ruminant milk lipids are derived from dietary lipids which are transported to the mammary gland in chylomicrons, from which FAs are released by mammary LPL and incorporated into TGs in the mammocytes (see Barber et al., 1997 and Palmquist 2006).

Owing to its importance for lipid metabolism in animal tissue, including the absorption of dietary lipids, the biosynthesis of lipids, including milk lipids, and cardiovascular diseases (Goldberg 1996; Mead et al., 2002; Glisic et al., 2008), LPL is very well characterised (see Olivecrona and Bengtsson 1984; Senda et al., 1987; Olivecrona and Bengtsson-Olivecrona 1991; Auwerx et al., 1992; Wong and Schotz 2002; Olivecrona et al., 2003). LPL has been isolated from several tissues of several species (Cryer 1987). Milk is a rich source of LPL (~1 mg/L) from which it can be isolated relatively easily by affinity chromatography on heparin agarose.

The enzyme is a homodimer; each monomer of human LPL consists of 448 amino acid residues, with a molecular mass of 50,394 Da and containing 8% carbohydrate. The isoelectric point of the protein is 8.91; it contains 10 cysteine residues, all of which are in disulphide linkages and two *N*-glycosylation sites, Asn_{44} and Asn_{361} (Yang et al., 1989).

The secondary and tertiary structures of LPL have not been determined, but its 3D structure has been deduced by analogy with the structure of pancreatic lipase with which LPL has a high level of homology. LPL is a member of a family of lipases, which evolved from a common ancestral lipase; the principal members are pancreatic lipase (PL) which hydrolyses emulsified triglycerides, and two lipoprotein-metabolising lipases, LPL and hepatic lipase (HL) (see Borgstrom and Brockman 1984). The amino acid sequences of human LPL and human PL show 30% homology (van Tilbeurgh et al., 1994). PL requires colipase, a protein which contains 96 amino acid residues, which anchors the enzyme at the lipid/water interface (see Wong 2003), while LPL is activated by apolipoprotein CII, a small protein containing 79 amino acid residues. Hepatic lipase, which is produced in the liver, plays a major role in lipid metabolism; it is a glycoprotein of mass ~62 kDa and is activated by apolipoprotein AII but inhibited by apolipoproteins AI, CI, CII and CIII (Jahn et al., 1983). The literature on HL has been reviewed by Perret et al. (2002) and Jansen et al. (2002). The three lipases have Ser, Asp and His at the active site.

Pancreatic lipase, which is regarded as the typical lipase, is stimulated by bile salts, which serve as emulsifiers or to complex with, and remove, the liberated fatty acids, which inhibit the enzyme. Other molecules that bind or react with fatty acids, e.g., calcium salts, blood serum albumin or β-lactoglobulin, also stimulate PL. Pancreatic lipase is a well-characterised monomeric glycoprotein with a molecular mass of ~50 kDa (450 amino acid residues) and is optimally active at ~pH 9 (Van Tilbeurgh et al., 1992, 1994).

Reflecting its importance in the biosynthesis of milk fat and its role in hydrolytic rancidity, mammary/milk lipase/LPL has been the subject of several reviews, including those by Herrington (1954), Chandan and Shahani (1964), Shahani et al. (1973), Brockerhoff and Jensen (1974), Jensen and Pitas (1976), Olivecrona and Bengtsson (1984), Olivecrona and Bengtsson-Olivecrona (1991), Olivecrona et al. (1992, 2003) and Deeth and Fitz-Gerald (1995, 2006).

The pH and temperature optima of LPL are ~9°C and 37°C, respectively. Under optimum conditions, the k_{cat} of LPL is ~3,000 s^{-1} and milk contains sufficient lipase (1–2 mg/L; 10–20 nM) to cause hydrolytic rancidity in 10 s. However, in most milk samples, LPL causes hydrolytic rancidity only if the MFGM is damaged, e.g., by agitation, foaming, cooling/warming, freezing or homogenisation. The various factors that activate lipolysis cause a shift of LPL from the casein micelles to the cream phase (Cartier and Chilliard 1989; Cartier et al., 1989). The milk of some cows undergoes spontaneous lipolysis, i.e. without the need for an activation step. Initially, it was proposed that such milks contained a second (membrane) lipase (Tarassuk and Frankel 1957). However, it now appears that they contain either a high level of apolipoprotein CII, which activates LPL, or that normal milk has a higher level of proteose peptone eight, which inhibits LPL (see Deeth 2006).

According to de Foe et al. (1982), caprine milk contains only ~4% as much lipolytic activity as bovine milk and contains two LPL isozymes, both with a higher molecular mass than bovine LPL. The yield of LPL is increased markedly by chromatography on hydroxyapatite and heparin-Sepharose 4B, probably due to the removal of an inhibitor(s) (a much smaller increase in yield was obtained for ovine LPL). Badaoui et al. (2007) identified two polymorphisms in caprine LPL, one of which occurred in the signal peptide and which may affect the expression of the enzyme and which is breed-dependent.

Most (~80%) of the LPL in bovine milk is associated with the casein micelles, with <10% in the cream phase, but in caprine milk <10% of the LPL is associated with the micelles, with 45% each in the cream and serum phases. The differences in the distribution pattern of LPL may explain the greater susceptibility of caprine milk

to spontaneous lipolysis and the characteristic flavour of goat milk, which is due to minor branched-chain fatty acids, 4-methyl- and 4-ethyl-octanoic acids. The lipolytic system in caprine milk and its significance for various aspects of caprine milk were reviewed by Chilliard et al. (1984, 2003). Ovine milk contains only ~10% of the lipolytic activity of bovine milk (Chandan et al., 1968). Ovine LPL has been described by Edwards et al. (1993) and Bonnet et al. (2000).

Equine milk contains about the same level of lipolytic activity as bovine milk and is due to an LPL (Chillard and Doreau 1985) which has not been isolated or characterised. Guinea pig milk contains high LPL activity but rat milk has low activity (Hamosh and Scow 1971). Guinea pig LPL was purified by Wallinder et al. (1982) and found to be similar to the LPL of bovine milk.

12.9.1 Bile Salts-Stimulated Lipase (EC 3.1.1.3)

It has been known since the early years of the twentieth century that human milk has considerably higher lipolytic activity than bovine milk (see Palmer 1922). In fact, human milk, and that of some other species, contains a second lipase in addition to LPL, i.e. bile salts-stimulated lipase (BSSL) which is similar to the broad-specificity pancreatic carboxylic ester hydrolase (CEH; also called cholesterol ester hydrolase; see Chen et al., 1998). This enzyme was studied by E. Freudenberg during the period 1927–1953 (see Freudenberg 1953), but the significance of these studies was not generally appreciated at that time. BSSL is considered to be very important for the digestion of lipids by human babies who secrete low levels of both pancreatic lipase and bile salts (see Shahani et al., 1980; Hernell and Bläcksberg 1991). The significance of pre-duodenal lipases (lingual lipase, pre-gastric esterase and gastric lipase) in fat digestion by human infants was described by Hamosh (1990). Fatty acids released by either LPL or BSSL may have an antibacterial effect (see Hamosh 1988).

BSSL was isolated from human milk by Blackberg and Hernell (1981); it is synthesised in the mammary gland and represents ~1% of the total protein in human milk. The enzyme is inactivated by pasteurisation, as a result of which the absorption of lipids by preterm infants is reduced by ~30%. The gene for human BSSL was cloned by Nilsson et al. (1990), who compared its derived amino acid sequence with that of CEH. The sequence consists of 722 amino acid residues; it has a total molecular mass of ~105 kDa, including 15–20% carbohydrate (molecular mass of the polypeptide, 76,271 Da; Nilsson et al., 1990). BSSL shows high homology with lysophospholipase from rat pancreas and acetylcholine esterase, as well as to CEH. BSSL has been found only in the milk of higher primates. The structure of pancreatic CEH, a monomeric protein of ~65.5 kDa, has been described by Rudd and Brockman (1984) and Chen et al. (1998). The structure of the human CEH gene was reported by Kumar et al. (1992). There is quite an extensive literature on BSSL, which was reviewed by Olivecrona and Bengtsson (1984), Corry (2004) and Deeth and Fitz-Gerald (2006).

12.9.2 Phospholipase

Bulk herd milk was reported by Shukla and Tobias (1970) to possess significant phospholipase-D activity, which, it was suggested, might increase the resistance of milk to oxidative rancidity; however, Chen et al. (1978) failed to identify phospholipase-D in milk. O'Mahony and Shipe (1972) reported that phospholipase-C stabilised milk to oxidation.

12.9.3 Esterases

Esterases are distinguished from lipases by their preference for soluble rather than emulsified ester substrates. As discussed by Palmer (1922), early studies on milk lipase did not distinguish between lipases and esterases. Milk contains several esterases (Kitchen 1985), the most significant of which are arylesterases (EC 3.1.1.7), cholinesterase (EC 3.1.1.8) and carboxylesterase (3.1.1.1). Arylesterase (also called

solalase) was among the first enzymes reported in milk (see Moro 1902). It has been isolated from milk and characterised (Kitchen 1971). Arylesterase activity is high in colostrum and during mastitis but it probably has no technological significance (see Kitchen 1985).

12.10 Proteinases

Babcock and Russell (1897) extracted from separator slime a trypsin-like proteinase, which they called 'galactase' (derived from *gala*, Greek for milk; genative, *galaktos*). They proposed that this enzyme is involved in cheese ripening, and it seems likely that it originated from leucocytes (somatic cells), which are rich in cathepsins, and was not the principal indigenous milk proteinase, plasmin, which is associated with the casein micelles, rather than somatic cells. The milk of several species (cow, goat, sheep, horse, donkey, bison, pig and human) was subsequently shown to contain proteolytic activity, porcine milk being a particularly rich source (Babcock et al., 1898).

The presence of an indigenous proteinase in milk, mainly in separator slime, was confirmed by Tatcher and Dahlberg (1917). However, the presence of an indigenous proteinase in milk was doubted for many years, contaminating bacteria being considered as a possible source of the activity detected. Warner and Polis (1945) reported a low level of proteolytic activity in acid casein which caused a decrease in the viscosity of sodium caseinate during storage, with a concomitant increase in pH 4.6-soluble N. Using aseptically drawn, low-bacterial-count milk with added antibiotics, Harper et al. (1960) finally showed that milk does indeed contain an indigenous proteinase(s), although the authors considered the level to be so low as to be insignificant.

12.10.1 Plasmin (EC 3.4.21.7)

Milk is now known to contain several indigenous proteinases, the principal of which is plasmin (fibrinolysin). In fact, milk contains the complete plasmin system found in blood: plasmin, plasminogen, plasminogen activators (PAs) and inhibitors of both PAs and plasmin. This system enters milk from blood, and plasmin activity increases in situations where there is an increased influx of blood constituents into milk, i.e. during mastitic infection and in late lactation. Plasmin activity has been linked to the physiology of milk secretion in the udder (Silanikove et al., 2006); in particular, products of the hydrolysis of β-casein by plasmin (proteose peptone 8f, fragment β-casein f1-28) have been shown to be able to downregulate milk secretion in the udder.

In milk, plasminogen, plasmin and PAs are associated with the casein micelles and are concentrated in rennet-coagulated cheese curds and casein, while the inhibitors of PAs and plasmin are soluble in the milk serum (Ismail et al., 2006). During storage of milk, activation of plasminogen to plasmin can occur, even at refrigeration temperatures (Schroeder et al., 2008; Lu et al., 2009). Owing to changes in practices in the dairy industry, e.g., improved bacterial quality, extended storage on farms and factories and the introduction of high-temperature processed milk (plasmin is very heat-stable), plasmin has become a very significant enzyme in milk and, consequently, the subject of considerable research. The literature has been reviewed regularly, e.g., Humbert and Alais (1979), Grufferty and Fox (1988), Bastian and Brown (1996), Kelly and McSweeney (2003) and Ismail and Nielsen (2010).

Plasmin is a very well-characterised proteinase, as are the various components of the plasmin system (see Kelly and McSweeney 2003). Bovine plasminogen is a single-chain glycoprotein containing 786 amino acid residues, with a calculated molecular mass of 88,092 Da; the polypeptide exists as five disulphide-linked loops ('kringles'). Plasminogen is converted to plasmin by cleavage of the Arg_{557}-Ile_{558} bond by specific proteinases, of which there are two types, urokinase-type and tissue-type PA. The impact of heating milk on the distribution of PA and activation of plasminogen was studied by Burbrink and Hayes (2006), Prado et al. (2006) and Wang et al. (2007). Plasmin is optimally active at pH 7.5 and 37°C; it is quite heat-stable

and partially survives UHT processing and other high-temperature processes (Newstead et al., 2006; van Asselt et al., 2008). Recent studies have examined the effect of new processing technologies on plasmin, including ultra-high-pressure homogenisation (Iucci et al., 2008), high-pressure treatment (Hurpertz et al., 2004; Moatsou et al., 2008a) and microfiltration (Aaltonen and Ollikainen 2011).

Plasmin is highly specific for peptide bonds containing Lys or Arg at the N-terminal side. The specificity of plasmin on α_{s1}-, α_{s2}- and β-caseins in solution has been determined (see Kelly and McSweeney 2003); it has little or no activity on κ-casein (CN), β-lg or α-la (in fact, denatured β-lg is an inhibitor; Grufferty and Fox 1986). In milk, the principal substrate for plasmin is β-CN, from which it produces γ^1- (β-CN f29-209), γ^2- (β-CN f106-209) and γ^3- (β-CN f108-209) caseins and proteose peptone (PP)5 (β-CN f1-105/107), $PP8_{slow}$ (β-CN f29-105/107) and $PP8_{fast}$ (β-CN f1-29).

Long et al. (1958) isolated a proteinaceous fraction, which they called λ-caseins, by ultracentrifugation of a crude κ-casein preparation. El-Negoumy (1973) prepared this fraction from milk by ammonium sulphate precipitation in the presence of *N*,*N*-dimethyl formamide and, by electrophoretic and chromatographic techniques, identified at least nine components with a higher electrophoretic mobility than α_{S1}-casein. Aimutis and Eigel (1982) concluded that many of the peptides in this fraction are produced from α_{S1}-casein by plasmin. O'Flaherty (1997) studied the λ-casein fraction of milk and identified several peptides that could have been produced from α_{s1}-casein by either plasmin or cathepsin D.

Plasmin contributes to primary proteolysis in cheese, most significantly in high-cooked varieties in which the coagulant is extensively denatured (Sheehan et al., 2007); it may cause age gelation of UHT-sterilised milk (although this has not been proven unequivocally; Newstead et al., 2006; Gaucher et al., 2009), can affect the coagulation properties of milk (Srinivasan and Lucey 2002) and may reduce the yield of cheese and casein owing to the loss of proteose peptones in whey (Mara et al., 1998). Cheesemaking parameters such as salting and cooking temperature can influence plasmin activity in cheese, and hence the contribution of the enzyme to ripening (Choi et al., 2006); increasing the temperature at which curds are cooked increases plasmin activity in the cheese, and the concomitant increased inactivation of chymosin further increases the relative contribution of plasmin to primary proteolysis of the caseins.

12.10.2 Cathepsin D (EC 3.4.23.5)

The second proteinase identified in milk was cathepsin D (Kaminogawa and Yamauchi 1972), which originates from lysosomes but is present in acid whey (Larsen et al., 1996). As with plasmin, cathepsin D is part of a complex system, including inactive precursors (for review, see Hurley et al., 2000). The major form of cathepsin D in milk is the inactive zymogen, procathepsin D, although milk also contains low levels of the mature form of the enzyme. The level of cathepsin D in milk is correlated significantly with SCC (O'Driscoll et al., 1999), although it is not clear whether this reflects increased production of cathepsin D and/or increased activation of precursors (see Hurley et al., 2000).

Kaminogawa et al. (1980) and McSweeney et al. (1995) showed that partially purified cathepsin D from milk hydrolysed α_{s1}-casein to a peptide with the same molecular mass or electrophoretic mobility as α_{s1}-CN (f24-199), which is one of the primary peptides produced from α_{s1}-casein by chymosin. The proteolytic specificity of cathepsin D on β-casein is also similar to that of chymosin. Cathepsin D can cleave κ-casein but has very poor milk clotting properties (McSweeney et al., 1995; Larsen et al., 1996). Two cleavage sites of cathepsin D on α-lactalbumin have been identified, but native β-lactoglobulin is resistant to cleavage by this enzyme (Larsen et al., 1996). Hayes et al. (2001) and Moatsou et al. (2008a,b) reported on the heat and pressure resistance of cathepsin D in milk, and the former concluded that some cathepsin D activity could survive heat treatments such as HTST pasteurisation and the cooking applied during the manufacture of Swiss-type cheese.

12.10.3 Other Proteinases

Somatic cells contain several other proteinases, including cathepsins B (EC 3.4.22.1), L (EC 3.4.22.15) and G (EC 3.4.21.20), and elastase (EC 3.4.21.36), which have received limited attention to date (see Kelly and McSweeney 2003). The lysosomal cysteine proteinases were reviewed by Kirschke et al.(1998); in addition to cathepsins B, L and G, these include cathepsins S (EC 3.4.22.27), K (EC 3.4.22.38), T (EC 3.4.22.24), N and O, dipeptidyl peptidase I (EC 3.4.14.1) and legumain (EC 3.4.22.34) (in legumes). Magboul et al. (2001) presented evidence for the presence of cysteine protease activity, most likely that of cathepsin B (based on immunological analysis), in milk; the specificity of this enzyme on the caseins, which is very broad and shared some bond preferences with both plasmin and chymosin, was determined by Considine et al. (2004).

Presumably, most of these enzymes are present in milk but are inactive owing to the high redox potential of milk, under which conditions the active-site sulphydryl group would be oxidised; the authors do not know if attempts have been made to assay these enzymes under reducing conditions.

12.10.4 Relative Significance of Proteinases in Milk

The relative significance of the different proteinases in milk to hydrolysis of proteins clearly depends on a number of factors, in particular the health status of the cow. In milk from a healthy cow, the predominant activity is that of plasmin, but as the SCC increases, the relative importance of lysosomal proteinases increases. A number of other recent studies have teased out the complex system of proteolytic enzymes in high SCC milk (Le Roux et al., 2003; Somers et al., 2003; Larsen et al., 2004, 2006, 2010a; Haddadi et al., 2006; Wedholm et al., 2008; Albenzio et al., 2009; Santillo et al., 2009). These studies have collectively suggested strong evidence of the activity of elastase, cathepsin B and several other lysosomal enzymes in milk with either naturally or artificially high SCC; a number of recent studies have used bacteria-free systems where mastitis is induced by infusion of a bacterial antigen such as lipopolysaccharide or lipoteichoic acid, which allows the enzymology of the resulting milk to be studied in the absence of confounding bacterial activities. Using such an approach, Larsen et al. (2010b) found evidence of amino- and carboxypeptidase activity in high SCC milk, perhaps originating from cathepsin H. The specific contribution of lysosomal proteases from polymorphonuclear leucocytes (PMN), the main type of somatic cell recruited during mastitic infection, has been elucidated by Le Roux et al. (2003) and Haddadi et al. (2006).

12.10.5 Proteinases in Human Milk

Greater proteolytic activity in human than in bovine milk was reported by Storrs and Hull (1956), Hernell and Lonnerdal (1989) and Heegaard et al. (1997). However, Korycka-Dahl et al. (1983) reported that the level of plasmin is about the same in human and bovine milk but that the former contains about four times more plasminogen. Ferranti et al. (2004) identified several casein-derived peptides, some of which may be biologically active, in human milk. Their results suggest that human milk contains several proteinases and peptidases in addition to plasmin, including amino- and carboxypeptidases. The mechanisms of proteolysis in human milk were further studied in detail, in particular in the context of milk from mothers giving birth prematurely, by Armaforte et al. (2010); the milk from mothers of premature infants was found to have higher plasmin activity and consequently greater proteolysis of casein than that of term mothers, which may be to provide more vulnerable newborn infants with a higher level of easily digested proteins and peptides, or result from the mammary gland producing milk under stressed conditions.

12.11 Alkaline Phosphatase (EC 3.1.3.1)

12.11.1 Introduction

Milk contains several phosphatases, the principal ones being alkaline and acid phosphomonoesterases, which are of technological significance. Milk

also contains ribonuclease, which has no known function or significance in milk, although it may be significant in the mammary gland. The alkaline and acid phosphomonoesterases in milk have been studied extensively; the literature has been reviewed by Fox and Morrissey (1981), Kitchen (1985), Andrews et al. (1992), Shakeel-Ur-Rehman et al. (2003), Fox (2003) and Fox and Kelly (2006b).

The occurrence of a phosphatase in milk was first recognised in 1925 by F. Demuth (see Whitney 1958). Subsequently characterised as an alkaline phosphatase indigenous to milk (Graham and Kay 1933), it became significant when it was shown that the time-temperature combinations required for the thermal inactivation of alkaline phosphatase were slightly more severe than those required to kill *Mycobacterium tuberculosis*, then the target microorganism for pasteurisation (Kay and Graham 1933). The enzyme is readily assayed, and a test procedure based on the inactivation of AlP was developed as a routine quality control test for HTST pasteurisation of milk (Kay and Graham 1935).

The AlP activity of bovine milk varies considerably between individuals and herds, and throughout lactation (minimum at ~1 week and maximum at ~28 weeks); activity varies inversely with milk yield but is independent of fat content, breed and feed (Haab and Smith 1956). The variability in AlP activity in human milk was described by Stewart et al. (1958).

12.11.2 Isolation and Characterisation

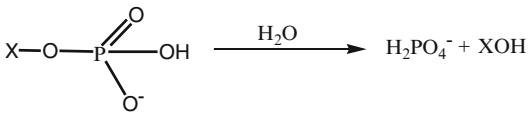
Kay and Graham (1933) observed that AlP is concentrated in cream and released into buttermilk on churning (in fact about 50% of AlP is in the skimmed milk but the specific activity is higher in cream). Zittle and DellaMonica (1952) partially purified AlP from whey, and Morton (1950) showed that lipoprotein particles, which he called 'microsomes' (Morton 1953), are a rich source of AlP and many other indigenous enzymes (Morton 1953; Zittle et al., 1956). AlP can be released from the microsomes by treatment with *n*-butanol (Zittle and DellaMonica 1952; Morton 1953) which, combined with salting-out and ion-exchange or gel permeation chromatography, formed the basis of all early methods for the isolation of AlP from milk (Zittle and DellaMonica 1952; Morton 1953, 1954; Gammack and Gupta 1967; Le Franc and Han 1967; Buruiana and Marin 1969; Linden et al., 1974). Chromatography of *n*-butanol extracts of MFGM on Concanavalin A Agarose/Sepharose 4B/Sephacryl S-200 has been used in a number of methods developed recently for the isolation of AlP from milk (Vega-Warner et al., 1999; see Shakeel-ur-Rehman et al., 2003). Bingham and Malin (1992) reported that AlP is released from the phospholipids of the MFGM by treatment of milk with phosphatidylinositol-specific phospholipase C, indicating that AlP is bound to the mammary cell membranes and the MFGM *via* phosphatidylinositol. This is the common form of linkage of AlP to membranes (see Moss 1992).

AlP is well characterised; it is optimally active at pH 10.5 when assayed on *p*-nitrophenylphosphate but at ~6.8 on caseinate, its optimum temperature is ~37°C. The enzyme is a homodimer of two identical subunits, each of molecular weight ~85 kDa; it contains four atoms of Zn which are essential for activity and is also activated by Mg^{2+} (Linden et al., 1974; Linden and Alais 1976, 1978). AlP is inhibited by metal chelators; the apoenzyme may be reactivated by the addition of one of a number of metals, which is used as the principle of methods to determine very low concentrations of zinc in biological systems. It is also inhibited by inorganic phosphate. The amino acid composition of milk AlP was reported by Linden et al. (1974). It appears that the amino acid sequence of milk AlP has not been reported, but the sequences of human placental and germ cell AlPs show 98% homology (see Hoylaerts and Millan 1991). The sequence of *E. coli* AlP has also been determined and shows 35% homology with human placental AlP and the sequence around the active site is fully conserved. Although milk AlP does not belong to either the placental or intestinal groups of AlP, it is likely that its sequence is generally similar. Models of the tertiary and quaternary structures of *E. coli* AlP were developed by Kim and Wyckoff (1990) and Hoylaerts and Millan (1991). It is likely that the structure of milk AlP is generally similar to that of *E. coli* AlP.

The indigenous AlP in milk is similar to the enzyme in mammary tissue (O'Keefe and Kinsella 1979). The AlP in human milk is similar, but not identical, to human liver AlP (i.e. tissue non-specific type); the difference between the two AlPs is due to variations in the sialic acid content (Hamilton et al., 1979). Unfortunately, a similar comparative study between mammary and liver AlPs has not been reported. Most of the AlP in the mammary gland is in the myoepithelial cells, which may suggest a role in milk secretion; there is much lower AlP activity in the epithelial secretory cells and in milk (Leung et al., 1989; Bingham et al., 1992). The results of the work by Bingham et al. (1992) suggest that there are two AlPs in milk, one of which is from sloughed-off myoepithelial cells, the other originating from lipid microdroplets and acquired intracellularly. The latter is probably the AlP found in the MFGM but unlike XOR it is not a structural component of the MFGM (Leung et al., 1989). Most or all studies on milk AlP have been on AlP isolated from cream/MFGM, i.e. the minor form of AlP in milk; a comparative study of AlP isolated from skimmed milk with that isolated from the MFGM is warranted.

12.11.3 Assay Methods

Kay and Graham (1933, 1935) developed a method based on the inactivation of AlP as an indicator for the adequate pasteurisation of milk. The principle of this method is still used throughout the world and several modifications have been published. The usual substrates are phenylphosphate, *p*-nitrophenyl phosphate or phenolphthalein phosphate, which are hydrolysed to inorganic phosphate and phenol, *p*-nitrophenol or phenolphthalein, respectively:



where XOH = phenol, *p*-nitrophenol or phenolphthalein. The liberated phosphate could be measured but the increase is small against a high background of phosphate in milk. Therefore, in all practical methods, the liberated alcohol is quantified. Reflecting the widespread assay of AlP in routine dairy laboratories, coupled with the need for speed and accuracy, there are more analytical methods for AlP than for any other indigenous milk enzyme. The principal methods are:

- Scharer (1938) used phenyl phosphate as substrate and quantified the liberated (colourless) phenol after reaction with 2,6-dibromoquinonechloroimide, with which it forms a blue complex. The method of Scharer, modified by Sanders and Sager (1946) for application to cheese as well as to milk, uses 2,6-dichloroquinonechloroimide for colour development; this is still the reference method in the USA.
- Kosikowski (1964) modified the method of Sharer by using dialysis rather than a protein precipitant to clarify the phenol-containing solution.
- Aschaffenburg and Mullen (1949) used *p*-nitrophenylphosphate as substrate; the liberated *p*-nitrophenol is yellow at the pH of assay (~10.0). This method, which was modified by Tramer and Wight (1950) by the incorporation of reference coloured standards, is used throughout Europe and in many other countries.
- Huggins and Talalay (1948) and Kleyn (1978) used phenolphthalein phosphate as substrate; the liberated phenolphthalein is red at the alkaline pH of the assay (~10) and hence is easily quantified.
- O'Brien (1966) reacted the phenol liberated from phenylphosphate with 4-aminoantipyrine to form a colourless product which forms a red complex on reaction with potassium ferricyanide; the absorbance of the solution at 505 nm may be determined in an autoanalyser.
- Reynolds and Telford (1967) also developed an automated method based on the dialysis principle of Kosikowski (1964) but using *p*-nitrophenylphosphate as the substrate.
- A fluorogenic aromatic orthophosphoric monoester, Fluorophos (Advanced Instruments, Inc., Needham Heights, MA, USA), has been developed for the determination of AlP in milk and milk products. Hydrolysis of this ester yields a fluorescent compound, 'Fluoroyellow',

the concentration of which is determined fluorometrically (excitation, 439 nm; emission, 560 nm). Fluorometric methods are 100–1,000 times more sensitive than colorimetric assays. A dedicated fluorometer has been developed for the analysis (Advanced Instruments, Inc.). Studies on the fluorometric assay for AlP include Rocco (1990), Eckner (1992), Yoshitomi (2004) and Rampling et al. (2004).

- A chemiluminescent assay for ALP, using adamantyl-1,2-dioxetane phenylphosphate as substrate, was developed for measuring AlP activity in milk by Girotti et al. (1994). A chemiluminescent assay (Paslite) from Charm Sciences Inc., Lawrence, MA, USA, was recently approved by the International Association for Standardisation/International Dairy Federation (ISO 22160/IDF 209) and is (along with the fluorometric test method) documented in the 2009 Pasteurised Milk Ordinance (DHHS-FDA 2007) as acceptable for AlP testing of grade A milk products.
- A rapid, highly sensitive electrochemical method for the determination of AlP using a coupled tyrosinase biosensor was published by Serra et al. (2005). The phenol liberated by AlP is oxidised to quinone by tyrosinase immobilised in a graphite-Teflon-composite electrode containing a Ag/AgCl/KCl reference electrode. The quinone is reduced to catechol at the electrode surface, giving rise to a current that is measured amperometrically. The catechol is reoxidised by tyrosinase to quinone, setting up a redox cycle and giving sensitive detection of AlP. Total analysis time is 5 min, without the need for pre-incubation; the detection limit is 6.7×10^{-14} M AlP.
- The standard colorimetric, fluorometric and chemiluminescent methods (outlined above) for measurement of AlP activity in milk are non-specific, i.e. they are unable to differentiate between bovine AlP and microbial AlP (Painter and Bradley 1997). Enzyme-linked immunosorbent assays (ELISA) have been developed with the objective of differentiating between microbial and milk AlP (Vega-Warner et al., 1999; Chen et al., 2006; Geneix et al., 2007). This type of analytical approach, if used in conjunction with other established approaches (e.g., Fluorophos method), may be useful in determining the level of AlP from microbial sources in milk and dairy products. To the authors' knowledge, there are no approved immunochemical assays available for routine assessment of AlP activity in milk or dairy products; however, such assays would be expected to offer significant potential in the routine, rapid and accurate measurement of AlP activity and in the differentiation of residual native, thermally denatured and reactivated AlP.

A comprehensive review of the various analytical approaches available for measurement of AlP in milk and dairy products, with a focus on validation of milk product pasteurisation was compiled by Rankin et al. (2010).

Most of the studies conducted to date on the quantification of AlP in milk have focused on bovine milk—presumably mainly for commercial reasons, However, the limited research conducted on interspecies comparisons has shown considerable variations in AlP content and activity between species, breeds and individual animals (Raynal-Ljutovaca et al., 2007). Caprine milk has lower AlP activity than bovine milk (Mathur 1974; Williams 1986), while ovine milk is reported to have AlP activity two to three times higher than that of bovine milk, with levels increasing throughout lactation (Scintu et al., 2000). Studies have also shown that AlP in caprine and ovine milks is more susceptible to denaturation on thermal processing than that in bovine milk (Anifantakis and Rosakis 1983; Vamvakaki et al., 2006).

12.11.4 Reactivation of Alkaline Phosphatase

Much work has been focussed on a phenomenon known as 'phosphatase reactivation', first recognised by Wright and Tramer (1953a, b, 1954, 1956), who observed that UHT-treated milk was phosphatase-negative immediately after processing but became positive on storage; microbial phosphatase was shown not to be responsible.

HTST-pasteurised bulk milk does not show reactivation, although some samples from individual cows may. HTST pasteurisation after UHT treatment usually prevents reactivation, which is never observed in in-container sterilised milk. Reactivation can occur following heating at a temperature as low as 84°C for milk or 74°C for cream. The optimum storage temperature for reactivation is 30°C, at which reactivation is detectable after 6 h and may continue for up to 7 days. The greater reactivation in cream than in milk may be due to protection of the enzyme by fat but this has not been substantiated.

A number of attempts have been made to explain the mechanism of reactivation of AlP (see Lyster and Aschaffenburg 1962; Kresheck and Harper 1967; Copius Peereboom 1970; Murthy et al., 1976; Linden 1979; Fox and Morrissey 1981; Andrews et al., 1992; Fox 2003; Fox et al., 2003; Shakeel-Ur-Rehman et al., 2003). There is evidence that the form of the enzyme which becomes reactivated is membrane-bound and several factors which influence reactivation have been established. Mg^{2+} and Zn^{2+} strongly promote reactivation but Sn^{2+}, Cu^{2+}, Co^{2+} and EDTA are inhibitory, while Fe^{2+} has no effect. Sulphydryl (SH) groups appear to be essential for reactivation; perhaps this is why phosphatase becomes reactivated in UHT milk but not in HTST milk. The role of SH groups, supplied by denatured whey proteins, is considered to be chelation of heavy metals, which would otherwise bind to SH groups of the enzyme (also activated on denaturation), thus preventing renaturation. It has been shown that in UHT milk the reactivation rate of AlP is inversely related to oxygen content (Gallusser and Bergner 1981); it was proposed that at high oxygen concentrations, the free SH groups created during the heat treatment are oxidised during storage, preventing SH cross-linking reactions which are critical for the activity of AlP. It has also been proposed that Mg^{2+} or Zn^{2+} cause a conformational change in the denatured enzyme, which is necessary for renaturation. Maximum reactivation occurs in products heated at ~104°C, adjusted to pH 6.5, containing 64 mM Mg^{2+} and incubated at 30°C; homogenisation of products before heat treatment reduces the extent of reactivation (Murthy et al., 1976).

Reactivation of alkaline phosphatase is of considerable practical significance since regulations for HTST pasteurisation specify the absence of phosphatase activity. Methods for distinguishing between renatured and residual native alkaline phosphatase are based on the increase in phosphatase activity resulting from addition of Mg^{2+} to the reaction mixture; various versions of the test have been proposed (see Fox 2003). The official AOAC method is based on that of Murthy and Peeler (1979); however, difficulties are experienced in the interpretation of this test when applied to cream or butter (Kwee 1983; Karmas and Kleyn 1990). Reactivation of AlP is also of significance in the manufacture and analysis of nutritional beverages (e.g., infant formula) fortified with 5′-mononucleotides. In such nutritional beverages, particularly those processed by UHT, reactivated AlP has the potential to convert added nucleotides to nucleosides by dephosphorylation (Gill and Indyk 2007). For this reason, there has been a move in recent years towards development and implementation of HPLC techniques for simultaneous quantification of nucleotides and nucleosides in nucleotide-fortified infant nutritional products.

12.11.5 Significance

Alkaline phosphatase in milk is significant mainly because it is used universally as an index of HTST pasteurisation. However, the enzyme may not be the most appropriate for this purpose (McKellar et al., 1994) because:

- Reactivation of alkaline phosphatase under certain conditions complicates interpretation of the test.
- The enzyme appears to be fully inactivated by temperature *x* time combinations (e.g., 70°C × 16 s), less severe than full HTST conditions (72°C × 15 s).
- The relationship between $\log_{10}$ % initial activity and pasteurisation equivalent deviates slightly from linearity in contrast to the relationship for LPO or GGT (McKellar et al., 1996).

Study of the inactivation kinetics of AlP in milk, as part of the development, evaluation and validation of novel (some being nonthermal) processing technologies, has received attention in recent years, due to its potential use as a marker of the severity of the treatment. These processing technologies have/are being evaluated for the production of extended shelf-life milk or as alternatives to traditional pasteurisation in the production of premium drinking milk products with superior taste and flavour characteristics. Examples of such novel/nonthermal processing technologies include, but are not restricted to, high hydrostatic pressure (HHP), high-pressure homogenisation, pulsed electric fields (PEF), sonication and high-intensity light pulses. High-pressure homogenisation of raw whole bovine milk at 150, 200 or 250 MPa results in 71%, 98% and 100%, respectively, inactivation of AlP of raw milk (Hayes et al., 2005). AlP is quite resistant to HHP, with no inactivation in raw milk after treatment at 400 MPa for 60 min at 20°C (Lopez-Fandino et al., 1996; Huppertz et al., 2005; Rademacher and Hinrichs 2006); HHP at higher temperatures generally increases inactivation of AlP (Seyderhelm et al., 1996; Ludikhuyze et al., 2000). Shamsi et al. (2008) showed that PEF treatment (28–37 kV cm^{-1}) of raw, skimmed milk at 15°C resulted in 24–42% inactivation of AlP. Given the resistance of AlP to inactivation by many of these novel processing technologies, it is likely that new indices of the severity of the treatments will need to be developed for the rapid validation of their effectiveness in the inactivation of microorganisms and/or enzymes.

AlP has the ability to dephosphorylate casein under suitable conditions (Lorient and Linden 1976), but as far as is known, it has no direct technological significance in milk. Perhaps its pH optimum is too far removed from that of milk, especially in acid milk products, although the pH optimum on casein is reported to be ~7 (Lorient and Linden 1976). Moreover, the activity of AlP on casein is inhibited by inorganic phosphate (Lorient and Linden 1976) and whey proteins, particularly β-lactoglobulin (Jasinska et al., 1985). Research conducted over the past 30 years or so focusing on the role of dephosphorylation on the technological properties of casein and casein-based ingredients (e.g., emulsifying and foaming properties) has been done mainly with potato acid phosphatase or calf intestinal alkaline phosphatase (Bingham et al., 1976; Li-Chan and Nakai 1989; Darewicz et al., 2000; Tezcucano Molina et al., 2007; Hiller and Lorenzen 2009).

Proteolysis is a major contributor to the development of the flavour and texture of cheese during ripening. Most of the small water-soluble peptides in cheese are derived from the N-terminal region of α_{s1}- or β-casein; many are phosphorylated and show evidence of phosphatase activity (i.e. they are partially dephosphorylated; see Fox 2003). In cheese made from pasteurised milk, both indigenous acid phosphatase and bacterial phosphatase are probably responsible for dephosphorylation (which is the more important is not clear), but in cheese made from raw milk, e.g., Parmigiano Reggiano or Grana Padano, milk alkaline phosphatase appears to be particularly important (Pellegrino et al., 1997). Further work on the significance of indigenous alkaline and acid phosphatases in the dephosphorylation of phosphopeptides in cheese is warranted.

A recent study by Shakeel-Ur-Rehman et al. (2006) showed that the addition of alkaline phosphatase (of bovine intestinal origin) to pasteurised cheese milk had no quantitative effect on the levels of primary or secondary proteolysis (as measured by water-soluble nitrogen or total free amino acids) in Cheddar-type cheese made therefrom. However, there were qualitative differences in the RP-HPLC peptide profiles of the water-soluble fractions, indicating that AlP activity caused the release of different peptides in the cheese during ripening. This is significant as dephosphorylation of caseins and phosphopeptides can lead to increased peptidase activity during ripening (Ferranti et al., 1997).

12.12 Acid Phosphatase (EC 3.1.3.2)

The occurrence of an acid phosphomonoesterase (AcP) in milk was first reported by Huggins and Talalay (1948) and confirmed by Mullen (1950), who reported that AcP was optimally active at pH

4.0 and was very heat-stable (heating at 88°C for 10 min is required for complete inactivation). The enzyme is not activated by Mg^{2+} (as is AlP), but it is activated slightly by Mn^{2+} and is very strongly inhibited by fluoride. The level of AcP activity in milk is only ~2% that of AlP; activity reaches a maximum 5–6 days *post partum*, then decreases and remains at a low level to the end of lactation (see Andrews et al., 1992).

12.12.1 Isolation and Characterisation

About 80% of the AcP in milk is found in the skimmed milk but the specific activity is higher in cream. Acid phosphatase in milk has been purified to homogeneity by various forms of chromatography, including affinity chromatography (Bingham et al., 1961; Bingham and Zittle 1963; Andews and Pallavicini 1973; Andrews 1976); purification factors of 10,000–1,000,000 have been reported. Adsorption onto Amberlite IRC50 resin is a very effective first step in purification. According to Andrews (1976), all the acid phosphatase activity in skim milk is adsorbed by Amberlite IRC50. However, Flynn (1999) found that only ~50% of the total acid phosphatase in skim milk was adsorbed by Amberlite IRC50, even after re-extracting the skim milk with fresh batches of Amberlite, suggesting that skim milk may contain at least two AcP isozymes. About 40% of the AcP in skim milk partitioned into the whey on rennet coagulation and this enzyme did not adsorb on Amberlite IRC50. The enzyme was partly purified from whey by Flynn (1999).

Flynn (1999) attempted to purify AcP from the MFGM by gel permeation chromatography; sonication and nonionic detergents failed to dissociate the enzyme from the membrane (in agreement with Kitchen 1985). The MFGM enzyme, which does not adsorb on Amberlite IRC50, was much less heat-stable than the acid phosphatase isolated from whey or from skim milk by adsorption on Amberlite IRC50. Overall, it appears that milk contains more than one acid phosphatase.

The AcP activity in milk increases four to tenfold during mastitis. Three isoenzymes are then present, two of which are of leucocyte origin (Andrews and Alichanidis 1975). Using a zymogram technique, Andrews and Alichanidis (1975) reported that milk from healthy cows contains one AcP isozyme while that from mastitic cows contains two additional isozymes which are of leucocyte origin. This may explain the heterogeneity observed by Flynn (1999). The leucocyte isozymes are more thermolabile than the MFGM enzyme and are inactivated by HTST pasteurisation.

Fleming (2000) resolved, by ion-exchange chromatography on DEAE cellulose, the AcP in skimmed milk that adsorbed on Amberlite IRC50 into two fractions, I and II in the proportions of 95:5. These isozymes were generally similar and distinctly different from that isolated from the MFGM by Flynn (1999).

The AcP isolated from skim milk by adsorption on Amberlite IRC50 has been well characterised. It is a glycoprotein with a molecular weight of ~42 kDa and a pI of 7.9. It is inhibited by many heavy metals, F^-, oxidising agents, orthophosphates and polyphosphates and activated by thiol-reducing agents and ascorbic acid; it is not affected by metal chelators (Andrews 1976). It contains a high level of basic amino acids and no methionine.

Since milk AcP is quite active on phosphoproteins, including caseins, it has been suggested that it is a phosphoprotein phosphatase. Although casein is a substrate for milk AcP, the major caseins, in the order α_s $(\alpha_{s1}+\alpha_{s2})>\beta>\kappa$, also act as competitive inhibitors of the enzyme when assayed on *p*-nitrophenylphosphate (Andrews 1974), probably due to binding of the enzyme to the casein phosphate groups (the effectiveness of the caseins as inhibitors is related to their phosphate content).

12.12.2 Assay Methods

Acid phosphatase may be assayed at pH *ca*. 5, on the same substrates as used for AlP. If *p*-nitrophenol phosphate or phenolphthalein phosphate is used, the pH must be adjusted to >8 after incubation to induce the colour of the product, i.e. *p*-nitrophenol or phenolphthalein.

12.12.3 Significance

Although AcP is present in milk at a much lower level than AlP, its greater heat stability and lower pH optimum may make it technologically significant. Andrews (1974) showed that while AcP retains significant activity after HTST pasteurisation at pH 6.7, it does not withstand in-container or UHT sterilisation; however, thermal stability was shown to increase with decreasing pH. Dephosphorylation of casein reduces its heat stability and its ability to bind Ca^{2+}, to react with κ-casein and to form micelles (Bingham et al., 1976; Tezcucano Molina et al., 2007).

As discussed under AlP, several small partially dephosphorylated peptides have been isolated from Cheddar, Parmigiano Reggiano and Grana Padano cheese (de Noni et al., 1997; Ferranti et al., 1997; Pellegrino et al., 1997; Singh et al., 1997). However, it is not known whether indigenous or bacterial acid phosphatase is mainly responsible for dephosphorylation in cheese made from pasteurised milk. It is claimed (see Fox 2003; Shakeel-Ur-Rehman et al., 2003; Akuzawa and Fox 2004) that alkaline phosphatase is mainly responsible for dephosphorylation of peptides in raw milk cheese. Dephosphorylation may be rate-limiting for proteolysis in ripening cheese since most proteinases and peptidases are inactive on phosphoproteins or phosphopeptides (Schormuller et al., 1960).

Given that AcP activity in milk increases four to tenfold during mastitic infection, the enzyme could be used as an index of mastitis; however, other enzymes (e.g., *N*-acetylglucosaminidase) are more effective markers.

The suitability of AcP as an indicator enzyme for super-pasteurisation of milk has been assessed (Griffiths 1986; Andrews et al., 1987); it is not as useful for this purpose as some alternatives, e.g., γ-glutamyl transpeptidase or LPO.

AcP is much less resistant to HHP than AlP, with the majority of AcP activity being lost within 10 min during treatment at 500 MPa, whereas AlP requires pressures in excess of 800 MPa to be inactivated at room temperature. This difference has been suggested by Balci et al. (2002) as useful in discriminating between heat- and pressure-treated milks.

12.13 Nucleases

12.13.1 Ribonuclease (EC 3.1.4.22)

Ribonucleases (RNase) catalyse cleavage of the phosphodiester bond between the 5′-ribose of a nucleotide and the phosphate group attached to the 3′ position of ribose of an adjacent pyrimidine nucleotide, forming a 2′, 3′ cyclic phosphate, which is then hydrolysed to the corresponding 3′-nucleotide phosphate. RNases of various origin and with different biological functions have been purified and characterised. They form a superfamily, which has been the subject of several reviews, including those by Barnard (1969), Adams et al. (1986), D'Alessio and Riordan (1997) and Bientema and Zhao (2003) and in a series of articles in the journal, *Cellular and Molecular Life Sciences* (Anon 1998). RNase occurs in various tissues and secretions, including milk (see Barnard 1969). Bovine pancreatic RNase A has been studied in great detail; it was the first enzyme to have its complete amino acid sequence determined (Smyth et al., 1963), and early studies on its tertiary structure were reported by Kartha et al. (1967). It contains 124 amino acid residues, with a calculated molecular weight of 13,683 Da, and has a pH optimum of 7.0–7.5.

Although Zittle and DellaMonica (1952) reported that fractions of bovine milk showed phosphodiesterase activity when RNA was used as substrate, the first study on the indigenous RNase in milk appears to be that of Bingham and Zittle (1962). These authors reported that bovine milk contains a much higher level of RNase than the blood serum or urine of human, rat or guinea pig and that most or all of the activity is in the serum phase; bovine milk could potentially serve as a commercial source of RNase. Like pancreatic RNase, the RNase in milk is optimally active at pH 7.5 and is more heat-stable at acid pH values than at pH 7; in acid whey, adjusted to pH 7, 50% of RNase activity was lost on heating at 90°C for 5 min and 100% after 20 min, but it was completely stable in whey at pH 3.5 when heated at 90°C for 20 min (Bingham and Zittle 1962). The enzyme was purified 300-fold by adsorption

on Amberlite IRC-50 resin with desorption by 1 M NaCl, followed by precipitation with cold (4°C) acetone (46–66% fraction). The partially purified enzyme showed no phosphodiesterase activity on Ca [*bis* (*p*-nitrophenyl phosphate)]$_2$ as substrate.

The RNase in bovine milk was further purified from acid whey by Bingham and Zittle (1964), using the same general procedure but with elution from Amberlite IRC-50 using a NaCl gradient, which resolved two isoenzymes, A and B, at a ratio of about 4:1, as for pancreatic RNase. Amino acid analysis, electrophoresis and immunological studies showed that milk RNase is identical to pancreatic RNase (Bingham and Zittle 1964). It is presumed that the RNase in milk originates in the pancreas and is absorbed through the intestinal wall into the blood, from which it enters milk. Intestinal absorption of pancreatic RNase (13,683 Da) was demonstrated in rats by Alpers and Isselbacher (1967), showing that it is possible for proteins of this size to be absorbed into the blood stream, although the level of RNase activity in milk is considerably higher than in blood serum, which suggests active transport (Bingham and Zittle 1962).

Ribonucleases A and B were isolated from bovine milk by Bingham and Kalan (1967) essentially by a scaled-up version of the procedure of Bingham and Zittle (1964) and including a gel permeation step. Two other isoenzymes, C and D, were demonstrated but not purified. Milk RNase A was shown by various criteria to be identical to pancreatic RNase A, but milk RNase B was shown to differ from both milk and pancreatic RNase A and pancreatic RNase B. All four isozymes had the same amino acid composition, but the two RNase B isozymes are glycoproteins, which differed in sugar content and chromatographic behaviour; both RNase A isozymes were free from carbohydrate.

Chandan et al. (1968) reported that bovine milk contains about three times as much RNase as human, ovine or caprine milk and that porcine milk contains a very low level of RNase. The same group (Dalaly et al., 1970) purified RNase from human milk; the principal isozyme contained no carbohydrate but the minor one was a glycoprotein. The enzyme hydrolysed RNA, polycytidylic and polyuridylic acids, but not polyadenylic or polyguanylic acids or DNA. Dalaly et al. (1970) considered milk RNase to be generally similar to bovine pancreatic RNase. Further characterisation of the two human milk isozymes was reported by Dalaly et al. (1980).

Gupta and Mathur (1989a) reported a single peak of RNase activity for goats' milk following chromatography on Amberlite IRC-50 resin; both bovine and buffalo milks showed two peaks of activity after the same procedure. The molecular weight of goat milk RNase was reported to be 29,500 Da and the enzyme showed maximum activity at 50°C and pH 9; the large differences between these values and the corresponding characteristics of bovine RNase have not been explained. According to Gupta and Mathur (1989b), goats' milk contains about one third as much RNase as bovine or buffalo milk. The literature on nucleases, including RNase, in milk has been reviewed by Stepaniak et al. (2003).

The possible immunological and nutritional effects of RNase in milk were investigated by Meyer et al. (1987a). Three isoenzymes were isolated from bovine milk by cation exchange chromatography on phosphocellulose: RNase A and B, previously reported by Bingham and Zittle (1964), and an isoenzyme termed RNase II-1, in the ratio 70:30:1. RNase II-1 differed from A and B in being more heat-stable and also in its inability to hydrolyse polycytidylate. [The classification nomenclature used by Meyer et al. (1987a) for RNases was based on immunological reaction and conflicts with that of the International Union of Biochemistry, which designates pancreatic ribonuclease (and milk RNase) as Ribonuclease I (EC 3.1.27.5)]. Meyer et al. (1987a) reported that bovine colostrum has three times as much total RNase activity as mature milk and 10–15 times more RNase II-1. RNase activity is also elevated in mastitic milk, to more than twice the normal level. Considering that tissue RNases also increase during infection, Meyer et al. (1987b) suggested that the RNase in milk may play a role in protecting the neonate against microbial infection.

Little or no RNase activity survives UHT heat treatment (121°C for 10 s) but about 60% survives

heating at 72°C for 2 min (Meyer et al., 1987a) or at 80°C for 15 s (Griffiths 1986). RNase activity in raw or heat-treated milk is stable to repeated freezing and thawing and to frozen storage for at least a year (Meyer et al., 1987a).

A high molecular weight (80 kDa) RNase (hmRNase) was purified from human milk by Ramaswamy et al. (1993) and characterised as a single-chain glycoprotein, with a pH optimum in the range 7.5–8.0. It was more heat-labile than bovine RNase A and was considered to be an isoform of lactoferrin, due to similarities in physical, chemical and antigenic properties; however, RNase has no iron-binding capacity and lactoferrin has no RNase activity. It was speculated that hmRNase is synthesised in the mammary gland and passes into milk, rather than being transferred from blood, as are RNase A and B (Bingham and Zittle 1964).

Ramaswamy et al. (1993) reported that the incidence of breast cancer is about three times higher in Parsi women in Western India than in other Indian communities and that the level of RNase in their milk is lower than normal. It was suggested that RNase may serve as a marker for the risk of breast cancer.

Research has intensified in recent years on the antiviral and antitumour activities of RNases. With the knowledge that the antitumour activity of bull semen RNase depends on its dimeric structure, Piccoli et al. (1999) engineered human pancreatic RNase from a monomeric to a dimeric form. The engineered protein was enzymatically active and selectively cytotoxic for several malignant mouse and human cell lines. This could offer a less toxic alternative to chemotherapeutic agents in the treatment of cancer patients. An amphibian RNase, called onconase, has shown success in clinical trials on cancer patients (Mikulski et al., 1993; Saxena et al., 2002). Recent research using recombinant DNA technology has shown that mouse and human RNase have bactericidal activity, suggesting a role for RNase activity in host defense in the intestinal epithelium (Hooper et al., 2003).

Lee-Huang et al. (1999) identified RNase in the urine of pregnant women as a factor responsible for activity against type 1 HIV virus. Pancreatic RNase was also effective in blocking HIV replication, creating an exciting new avenue for research on the treatment of AIDS. McCormick et al. (1974) found that RNase protects milk from viruses by inhibiting the action of RNA-dependent DNA polymerase and thus preventing viral replication. Perhaps RNase can inhibit bacteriophage, which inhibits the growth of starter cultures in cheesemaking; such a study seems warranted. Based on the similarity of its structure to angiogenin, a protein which induces blood vessel formation in tumours, Roman et al. (1990) suggested that growth promotion may be a biological function of RNase in milk and colostrum. Although RNase has no technological significance in milk, which contains very little RNA, it may have significant biological functions.

12.13.2 Catalytic Antibodies (Abzymes) with Oligonuclease Activity

The term abzyme is used to describe antibodies with enzymatic activity, including nuclease activity. The first catalytic antibodies were produced in 1986 (Lerner et al., 1991), with the term abzyme, derived from 'antibody enzyme' being used routinely to describe such antibodies. Catalytic antibodies are relatively slow catalysts, with turnover numbers 10^3–10^6 times lower than is common for enzymes (Kirby 1996). Catalytic antibodies capable of catalysing a broad spectrum of chemical reactions have been produced (Lerner et al., 1991; Janda 1994; Shchurov 1997). The selectivity of antibodies is usually higher than that of enzymes, which has important implications for the biological function of abzymes. The abzymes produced to date are generally produced by immunisation with a transition state analogue coupled as a hapten to a carrier protein (Kirby 1996; Fletcher et al., 1998). The presence of DNA- and RNA-hydrolysing antibodies has been demonstrated in the milk of healthy women (Kanyshkova et al., 1997; Buneva et al., 1998). Human milk also contains secretory immunoglobulin A (sIgA) which can catalyse the hydrolysis of RNA and DNA (Kit et al., 1995; Nevinsky et al., 2000). It is likely that such abzymes are present in human milk to confer a protective role

(e.g., antibacterial or antiviral activity) for infants, given that the immune system of infants is not fully developed in the early stages of life. The literature on catalytic antibodies has been reviewed by Lerner et al. (1991), Benkovic (1992), Suzuki (1994), Kirby (1996), Shchurov (1997) and Stepaniak et al. (2003).

12.13.3 5′-Nucleotidase (EC 3.1.3.5)

5′-Nucleotidase catalyses the hydrolysis of 5′-nucleotides; the enzyme is a component of the MFGM (Patton and Trams 1971) and has been purified from acid whey (Caulini et al., 1972) and from the MFGM (Huang and Keenan 1972). The enzyme may be purified from the MFGM using detergent treatment, $(NH_4)_2SO_4$ fractionation, heat treatment, sonication and chromatography on Sepharose-4B. Such an approach yields two fractions (designated V and VI) with 5′-nucleotidase activity (Huang and Keenan 1972). The two isoenzymes differ in phospholipid content, substrate specificity and kinetic properties. 5′-Nucleotidease has optimum activity at pH 7.0–7.5 (Caulini et al., 1972; Huang and Keenan 1972) and 69°C (Huang and Keenan 1972) and it does not require a metal cofactor. 5′-Nucleotidase activity is suitable for use as a marker in studying secretory mechanisms for the milk fat globules and biogenesis of MFGM material. Heating at 60°C for 30 min or 75°C or 80°C for 15 s reduces the activity of 5′-nucleotidase by 20%, 40% and 97%, respectively (Huang and Keenan 1972; Andrews et al., 1987). While milk contains 5′-mononucleotides (i.e. substrates for 5′-nucleotidase activity), such indigenous nucleotides appear to be resistant to dephosphorylation (Gill and Indyk 2007), albeit by mechanisms which are still poorly understood.

12.14 Lysozyme (EC 3.1.2.17)

According to Whitney (1958), Shahani et al. (1962) and Chandan et al. (1965), the presence of natural antibacterial factor(s) in fresh raw bovine milk was reported by Kitasoto in 1889 and by Fokker in 1890. These inhibitors are now called lactenins, one of which is LPO.

Fleming (1922, 1929) identified an antibacterial agent in nasal mucus, tears, sputum, saliva and other body fluids which caused lysis of many types of bacteria (*Micrococcus lysodeikticus* was used for assays). He showed that it was an enzyme, which he called lysozyme. [According to Jolles and Jolles (1967), it had been known since 1893 that tears possessed bactericidal activity.] Fleming (1922, 1929) found that chicken egg white is a particularly rich source of lysozyme; it constitutes ~3.5% of egg-white protein and is the principal commercial source of lysozyme. Chicken egg-white lysozyme (EWL) is referred to as lysozyme 'c'; a second type of lysozyme, 'g', is present in the egg white of the domestic goose; the two lysozymes differ in molecular weight and amino acid composition. EWL is easily purified and has been studied extensively as a model protein for structure, dynamics and folding; the literature has been reviewed by Kato (2003).

Fleming (1922, 1929) did not include milk among the several fluids in which he found lysozyme but Bordet and Bordet (1924) reported that the milk of several species contains lysozyme and that human milk is a comparatively rich source. The situation regarding bovine milk was less clear; some workers, including Fleming (1932), reported that bovine milk contains lysozyme but others did not find it (see Shahani et al., 1962). Lysozyme has been isolated from the milk of a wider range of species than any other milk enzyme. This may reflect the perceived importance of lysozyme as a protective agent in milk or it may be because it can be isolated from milk relatively easily. While the milk lysozymes are generally similar, there are substantial differences, even between closely related species, e.g., cow and buffalo.

Lysozyme (also called muramidase, mucopeptide *N*-acetyl-muramyl hydrolase) is a widely distributed enzyme which lyses certain bacteria by hydrolysing the β $(1 \rightarrow 4)$-linkage between muramic acid and *N*-acetylglucosamine of mucopolysaccharides in the bacterial cell wall. The presence and activity of lysozyme is normally assayed for by the lysis of a culture of *M. lysodeikticus*, measured by a decrease in

turbidity (e.g., Manas et al., 2006), but it can also be assayed by enzyme-linked immunosorbent assay techniques using monoclonal or polyclonal antibodies (Rauch et al., 1990; Yoshida et al., 1991; Besler 2001; Schneider et al., 2010b), reversed-phase high-performance liquid chromatography with fluorescence detection (Pellegrino and Tirelli 2000), liquid chromatography-mass spectrometry (LC-MS), immunocapture mass spectrometry or surface-enhanced mass spectrometry (Schneider et al., 2010a). In recent years, the increasing incidence of cases describing allergic reactions to lysozyme present in food products has refocused attention on comparison and development of rapid, specific, sensitive and reliable methods for the detection and quantification of lysozyme in food matrices (Kerkaert et al., 2010; Jimenez-Saiz et al., 2011; Schneider et al., 2011).

Lysozyme was isolated from human milk by Jolles and Jolles (1961), who believed that bovine milk was devoid of lysozyme; human milk lysozyme (HML) was found to be generally similar to EWL. Variability in the level of lysozyme in human milk and its heat stability were studied by Chandan et al. (1964) and the isolation procedure was improved by Jolles and Jolles (1967) and Parry et al. (1969); a method for the simultaneous isolation of RNase and lysozyme from human milk was reported by Dalaly et al. (1970).

According to Chandan et al. (1965), lysozyme had by then been found in the milk of many other species, e.g., donkey, horse, dog, sow, cat, rat, rabbit, llama and rhesus monkey, but no lysozyme or only traces were found in the milk of goat, sheep and guinea pig; they did not mention bovine milk although a low and variable level of lysozyme had been found in bovine milk by Shahani et al. (1962). According to Chandan et al. (1968), porcine milk is devoid of lysozyme but this has not been confirmed (see Wagstrom et al., 2000). Equine milk has a very high ability to inhibit bacterial growth, which is probably due to its high level of lysozyme activity.

Bovine milk lysozyme (BML) was isolated and characterised by Chandan et al. (1965), Dalaly et al. (1970) and Eitenmiller et al. (1971, 1974, 1976). Equine milk lysozyme was isolated and characterised by Jauregui-Adell (1971, 1975) and Jauregui-Adell et al. (1972). Human and equine milks are exceptionally rich sources of lysozyme, containing 400 and ~800 mg/L, respectively (3,000 and 6,000 times the level in bovine milk); these levels represent ~4% and ~3% of the total protein in human and equine milk, respectively (Chandan et al., 1968; Jauregui-Adell 1975). Asinine milk contains about the same level of lysozyme as equine milk (Civardi et al. 2002). Although lysozyme is a lysosomal enzyme, it is found in soluble form in many body fluids (tears, mucus, egg white) and the lysozyme in milk is usually isolated from whey, indicating that it is in solution.

In addition to the lysozyme in human, equine and bovine milk, lysozyme has been isolated and partially characterised from the milk of several other species: baboon (Buss 1971), camel (see Benkerroum et al., 2004), buffalo (Priyadarshini and Kansal 2002, 2003) and dog (Watanabe et al., 2004). The reported properties of these lysozymes are generally similar to those of HML, but there are substantial differences, even between the lysozymes of closely related species, e.g., cow and buffalo.

The pH optimum of HML, BML and EWL is 7.9, 6.35 and 6.2, respectively (Chandan et al., 1965; Parry et al., 1969). According to Eitenmiller et al. (1971, 1976) and Friend et al. (1972), BML has a molecular weight of 18 kDa compared with 15 kDa for HML and EWL, and its amino acid composition and immunological properties are considerably different from those of the latter two lysozymes. White et al. (1988) isolated BML and found that it resembled closely the BML studied by Chandan et al. (1965), including a mass of ~18 kDa. However, when they analysed their preparation by RP-HPLC it resolved into two peaks, only the smaller of which had lysozyme activity; the larger peak was inactive and had a high molecular weight. White et al. (1988) suggested that the apparent relatively high molecular weight of BML reported by Eitenmiller et al. (1971, 1974, 1976) was due to a high molecular weight impurity. A more thorough study of a homogeneous preparation of BML appears warranted.

The complete amino acid sequence of HML and EWL were reported by Jolles and Jolles (1972). Although highly homologous, the sequences showed several differences; HML consists of 130 amino acid residues, compared with 129 in EWL, the extra residue in the former being Val_{100}. The amino acid sequence of equine milk lysozyme was reported by McKenzie and Shaw (1985); the molecule consists of 129 amino acid residues, like EWL, with a mass of 14,647 Da. It showed only 51% homology with HML and 50% homology with EWL. The partial sequence of BML reported by White et al. (1988) showed differences between EWL, HML and BML and from lysozymes of other animal tissues (Ito et al., 1993). The three-dimensional structure of EWL was reported by Blake et al. (1965); Johnson (1998) reviewed further studies on the structure of lysozyme.

The amino acid sequence of lysozyme is highly homologous with that of α-lactalbumin (α-la), a whey protein which is an enzyme modifier in the biosynthesis of lactose. The similarities in primary structure, gene sequence and three-dimensional structure of α-la and c-type lysozymes are described by McKenzie and White (1991). α-La binds a Ca^{2+} in an Asp-rich loop but most c-type lysozymes do not bind a Ca^{2+}, equine and canine milk lysozymes being exceptions (Tada et al., 2002; Watanabe et al., 2004).

All lysozymes are relatively stable to heat at acid pH values (3–4) but are relatively labile at pH >7. More than 75% of the lysozyme activity in bovine milk survives heating at 75°C × 15 min or 80°C × 15 s, and therefore it is affected little by HTST pasteurisation. HML and BML are inactivated by mercaptoethanol; the reduced enzyme can be reactivated by diluting the desalted reduced protein in 0.1 M Tris–HCl buffer (pH 8.5). The activity of reoxidised BML and HML were ~330% and 84%, respectively, of the native enzyme (Friend et al., 1972).

The effects of specifically modifying residues in EWL, HML and BML showed that the first two behaved generally similarly but BML appeared to be quite different; e.g., modifying Trp strongly inhibited EWL and HML but BML was inhibited only slightly (Friend et al., 1975). These authors concluded that BML differs from most lysozymes of animal origin but resembled plant lysozymes, especially those from fig or papaya. These differences do not seem to have been investigated further.

The most significant physiological role of lysozyme is to act as a bactericidal agent, evidence of which has been available for decades (e.g., Fleming 1922). The bactericidal effect of lysozyme against Gram-positive microorganisms is partially dependent on its lytic activity on the cell wall (Jimenez-Saiz et al., 2011), while research conducted over the last 10 years or so also implicates a nonenzymatic mechanism of action (Ibrahim et al., 2002; Masschalck and Michiels 2003). Much research has also been conducted on various means of enhancing the antibacterial activity of lysozyme against Gram-negative bacteria; examples of such approaches include HHP (Masschalck et al., 2002), ultrafiltration (Cegielska-Radziejewska et al., 2003), disulphide bond reduction and covalent attachment of various components such as polysaccharides, fatty acids and peptides (Masschalck and Michiels 2003). Research on this topic has suggested that the nonenzymatic mechanism of action of lysozyme may involve disruption of normal electrostatic interactions between divalent cations and components of the outer cell membrane of Gram-negative bacteria (Ibrahim 1998; Ibrahim et al., 1997). In addition to bactericidal activity, biological functions of lysozyme, such as immunomodulatory, antiviral and anti-inflammatory activity, have also been reported (Lesnierowski and Kijowski 2007).

In the case of milk, lysozyme may simply be a 'spill-over' enzyme or it may have a definite protective role. If the latter is true, then the exceptionally high level of lysozyme in human and equine milk may be significant. The specific activity of human lysozyme is approximately ten times greater than that of bovine lysozyme. HML also has approximately three times more lytic activity than that of EWL due to the fact that it possesses a greater positive charge than the latter (Parry et al., 1969). Breast-fed infants generally

experience a lower incidence and severity of infections and gastrointestinal difficulties than formula-fed infants. The role of HML in reducing microbial infections in the gastrointestinal tract of breast-fed infants has been studied extensively (Lonnerdal 1985). Addition of lysozyme to infant formula has been shown to reduce the incidence of gastroenteritis and allergies and to increase the beneficial gastrointestinal microflora (Birch and Parker 1980). Given these significant biofunctionalities, but due to the lack of availability, some recent research has focused on transgenic production of lysozyme (Maga et al., 1994; Yu et al., 2006; Scharfen et al., 2007; Yang et al., 2011).

One might expect that, owing to its bactericidal effect, indigenous milk lysozyme would have a beneficial effect on the shelf-life of milk; there are limited reports to support this expectation, e.g., a study conducted on the shelf-life of goat milk containing recombinant human lysozyme (Maga et al., 2006). Research is also underway on the effects of lysozyme addition as part of an overall nonthermal hurdle approach to microbiological control of milk and dairy products. Early research has demonstrated synergistic effects of combining lysozyme with high-intensity PEF technology for inactivation of *Staphylococcus aureus* in skim milk (Sobrino-Lopez and Martin-Belloso 2008). Other preliminary research has shown that the bactericidal activity of chicken EWL against *Listeria monocytogenes*, in media, may be enhanced by high-pressure homogenisation at 100 MPa (Iucci et al., 2007).

Exogenous lysozyme may be added to milk in the manufacture of several hard and semihard cheese varieties, e.g., Gouda, Edam, Emmental and Parmigiano Reggiano, as an alternative to nitrate to prevent the growth of *Clostridium tyrobutyricum* which can cause late gas blowing and off-flavour defects during ripening. This is probably the most widespread commercial application for chicken EWL, although it is also used to control malolactic fermentation of wine (Proctor and Cunningham 1988; Tirelli and De Noni 2007). As eggs and egg products are major food allergens, EC legislation (2003/89/EC, Annex IIIa amending Directive 2000/13/EC) mandates that the use of lysozyme as an additive needs to be declared on the ingredient/product label.

12.15 Amylase (α-Amylase EC 3.2.1.1, β-Amylase EC 3.2.1.2)

Amylase (diastase) was one of be first indigenous enzymes identified in milk; according to Sato (1920), Bechamp 'isolated' an amylase from human milk in 1883. During the next 40 years, several workers reported that the milk of several species contains an amylase but several other authors reported that they do not. Sato (1920) reported that all samples of raw milk and cheese assayed by him contained amylase and he concluded that the enzyme is produced in the mammary gland. Richardson and Hankinson (1936) also concluded that the amylase in milk is indigenous and that α-amylase is the principal enzyme, with a lesser amount of β-amylase; the enzymes partition mainly into skimmed milk and whey. A highly concentrated preparation of α-amylase was obtained from whey by Guy and Jenness (1958). There appears to have been no further work on the isolation of amylase from bovine milk. Milk amylase is quite labile to heat and inactivation was proposed as a reliable index of the intensity of heat treatment applied to milk (Orla-Jensen 1929; Gould 1932).

Human milk and colostrum contain 25–40 times more α-amylase than bovine milk; however, there is at least a tenfold variation in the amylase activity in individual cow milk samples (Stejskal et al., 1981; deWit et al., 1993). The α-amylase in milk is similar to salivary amylase but different from pancreatic amylase; it appears that during pregnancy, the production of α-amylase is switched from saliva to the mammary gland (Stejskal et al., 1981). The amylase in human milk is a major contributor to the ability of human breast-fed infants to digest starch.

α-Amylase was purified from human milk by gel permeation chromatography and its stability to pH and pepsin determined (Lindberg and Skude 1982). These investigators reported that

the level of α-amylase in human milk is 15–140 times higher in human milk than in blood plasma, suggesting that it is not transferred from the blood but is synthesised in the mammary gland. Since bovine milk contains no starch and only low levels of oligosaccharides, the function of amylase in milk is unclear. Human milk, also, does not contain starch but it contains up to 130 oligosaccharides, at a total concentration up to 150 mg/L (e.g., Newburg and Neubaurer 1995; Miller and McVeigh 1999; Urashima et al., 2001). These oligosaccharides are built up from lactose and contain unusual monosaccharides (e.g., fucose and *N*-acetylneuraminic acid) linked by unusual glycosidic bonds; therefore, it is unlikely that α-amylase, which is highly specific for α (1→4) glycosidic bonds linking glucose molecules, will hydrolyse the oligosaccharides in milk (Gnoth et al., 2002). Since human babies secrete low levels of salivary and pancreatic amylases (0.2–0.5% of the adult level; Hamosh 1988), the high level of amylase activity in human milk may enable them to digest starch in infant formulae (Lindberg and Skude 1982; Heitlinger et al., 1983; Hamosh 1988; deWit et al., 1993). By hydrolysing the polysaccharides in the cell wall of bacteria, it has been suggested that milk amylase may have antibacterial activity (see Lindberg and Skude 1982). The amylase activity of human milk is an active area of research at present but there appears to be little or no recent research on the amylase in bovine milk or that of other species. It seems reasonable to suggest that the isolation, characterisation and significance of amylase from the milk of other species warrant investigation. El-Fakharany et al. (2009) isolated and characterised a β-amylase from camel milk [they appear not to have assayed for α-amylase activity]; it had a molecular mass of 61 kDa, which differed from that of other animal amylases, including camel pancreatic amylase. Moatsou (2010) reported that there are no publications on amylase in ovine or caprine milk and the current authors have found no reports on amylase in the milk of other species.

Human milk also contains abzymes [antibodies with enzymatic activity] with amylotic activity, especially in the IgG and IgA fractions (Kulminskaya et al., 2004).

12.16 β-*N*-Acetylglucosaminidase (E.C. 3.2.1.30)

β-*N*-Acetylglucosaminidase (NAGase) hydrolyses terminal, nonreducing *N*-acetyl-β-D-glucosamine residues from *N*-acetyl-β-D-glucosaminides, including glycoproteins and fragments of chitin. However, NAGase is not specific for *N*-acetyl-β-D-glucosaminides; since it can also hydrolyse *N*-acetyl-β-D-galactosaminides, it has been recommended (Cabezas 1989) that the enzyme should be called *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52).

NAGase is thought to be a lysosomal enzyme (Sellinger et al., 1960) which originates principally from mammary gland epithelial cells and, to a lesser extent, from somatic cells. The first report on NAGase in milk appears to be that of Mellors (1968), who purified (~10-fold increase in specific activity) the enzyme from separator slime. More than 95% of NAGase in milk is in the skimmed milk. The enzyme is optimally active at 50°C and pH 4.2. Mellors (1968) suggested that NAGase should be a convenient index of mammary gland infection. The effectiveness of NAGase as an indicator of mastitis was demonstrated by Kitchen (1976), Kitchen and Midleton (1976) and Kitchen et al. (1978). Since then, there have been numerous studies on the reliability of NAGase as a marker of mastitis (Mattila 1985; Pyörälä and Pyörälä 1997; Bansal et al., 2005; Larsen et al., 2010a, b; Barth et al., 2010). A field test for mastitis based on NAGase activity has been developed using chromogenic *N*-acetyl-β-D-glucosamine-*p*-nitrophenol as substrate; hydrolysis yields *p*-nitrophenol, which is yellow at alkaline pH (Kitchen and Midleton 1976). NAGase activity is also high in colostrum.

NAGase is inactivated by HTST pasteurisation (70–71°C × 15–18 s) and Andrews et al. (1987) proposed that NAGase would be a suitable indicator enzyme for assessing heat treatment in the range 65–75°C × 15 s. With the objective of developing a test to determine the heat load to which cheese milk had been subjected, Ardo et al. (1999) compared the thermal inactivation of alkaline phosphatase (AlP), NAGase and GGT. As AlP was considered to be

too heat-sensitive and GGT too heat-stable to meet the objective, NAGase was considered to be the most suitable.

Although NAGase is a lysosomal enzyme, it occurs mainly in the whey fraction (82% of total activity; Kitchen et al., 1978), from which it has been isolated by various forms of chromatography. Two isozymes of NAGase, A and B, differing in molecular weight, i.e. 118 and 234 kDa, respectively, and charge were isolated from bovine mammary tissue by Kitchen and Masters (1985). Each isoenzyme dissociates into two dissimilar subunits of mass 55 and 25 kDa, on treatment with 2-mercaptoethanol and sodium dodecyl sulphate.

12.17 Aldolase (EC 4.1.3.13)

Aldolase reversibly hydrolyses fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate; it is a key enzyme in the glycolytic pathway. The presence of aldolase in milk was first reported by Polis and Shmukler (1950), who partially purified it. Although most (66%) of the aldolase in milk is in the skimmed milk (Kitchen et al., 1970), it is also found in the cream/MFGM (Polis and Shmukler 1950; Erwin and Randolph 1975; Keenan et al., 1988; Keenan and Dylewski 1995; Keenan and Mather 2006). According to Blanc (1982), the aldolase is located in the cytoplasm of the mammary cells, from which the enzyme in milk presumably originates, although some may be from blood.

It has been suggested (Dwivedi 1973) that aldolase plays a role in flavour development in dairy products. There appear to have been no recent publications on milk aldolase. The aldolase from rabbit muscle is a homotetramer of 161 kDa (4×40 kDa) with a pH optimum of ~7.0. The literature on aldolase was reviewed by Horecker et al. (1972).

12.18 Conclusions

As a biological fluid, it is not surprising that milk contains enzymes—approximately 70 have been identified to date. Many of these enzymes are present at quite high concentrations, and those that are easily assayed were studied long before the proteinaceous nature of enzymes was recognised, even before the term 'enzyme' was coined. Being a fluid, it was relatively easy to purify and study the indigenous enzymes of milk. Some of the indigenous enzymes in milk are significant for the protection and/or nutrition of the neonate but most are not important and none is essential. However, many are very significant in dairy technology as a cause of spoilage or as indicators of quality or history. The lipids and proteins of milk are susceptible to the action of milk enzymes, generally with negative effects; however, lactose is not a substrate for any of these enzymes.

Although the indigenous enzymes in milk have been studied since 1881, they are still very active research subjects. The focus of attention has changed many times during the past 130 years, mainly as dairy processing technologies changed and new dairy-based products were developed. Not surprisingly, most research has been on bovine milk, with human milk also receiving considerable attention. Some enzymes in the milk of other commercially important domesticated species have been studied but little or no research has been done on the enzymes of most species. Available data indicate that there are some very large interspecies differences in the levels of many enzymes, some of which are physiologically and/or technologically important. The reasons for these interspecies differences are unknown and may reflect differences in the mechanism of synthesis or secretion of milk constituents.

Considering the great diversity of mammals, it is not an exaggeration to suggest that the study of milk enzymology offers an almost inexhaustible number of research projects.

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