8 Enzymology of milk and milk products

8.1 Introduction

Like all other foods of plant or animal origin, milk contains several indigenous enzymes which are constituents of the milk as secreted. The principal constituents of milk (lactose, lipids and proteins) can be modified by exogenous enzymes, added to induce specific changes. Exogenous enzymes may also be used to analyse for certain constituents in milk. In addition, milk and most dairy products contain viable micro-organisms which secrete extracellular enzymes or release intracellular enzymes after the cells have died and lysed. Some of these enzymes may cause undesirable changes, e.g. hydrolytic rancidity of milk and dairy products, bitterness and/or age gelation of UHT milks, bittiness in cream, malty flavours or bitterness in fluid milk, or they may cause desirable flavours, e.g. in ripened cheese.

This chapter is devoted mainly to the significance of indigenous enzymes in milk. The principal applications of exogenous enzymes have been dealt with in other chapters, e.g. rennets and lipases in cheese production (Chapter 10), β-galactosidase to modify lactose (Chapter 2). Some minor or potential applications of exogenous enzymes are presented here. Enzymes derived from contaminating bacteria, which may be significant in milk and some dairy products, will not be discussed. The interested reader is referred to McKellar (1989) for a comprehensive review of enzymes produced by psychrotrophs which are the principal spoilage microorganisms in refrigerated milk and milk products. The significance of enzymes from microbial cultures in cheese ripening is discussed in Chapter 10.

8.2 Indigenous enzymes of bovine milk

8.2.1 Introduction

As many as 60 indigenous enzymes have been reported in normal bovine milk. With the exception of α-lactalbumin, which is an enzyme modifier in lactose synthesis (Chapter 2) most, if not all, of the indigenous enzymes in milk have no obvious physiological role. They arise from three principal sources:

- the blood via defective mammary cell membranes;

- secretory cell cytoplasm, some of which is occasionally entrapped within fat globules by the encircling fat globule membrane (MFGM) (Chapter 3);
- the MFGM itself, the outer layers of which are derived from the apical membrane of the secretory cell, which, in turn, originates from the Golgi membranes (Chapter 3); this is probably the principal source of indigenous enzymes.

Thus, most enzymes enter milk due to peculiarities of the mechanism by which milk constituents, especially the fat globules, are excreted from the secretory cells. Milk does not contain substrates for many of the enzymes present, while others are inactive in milk owing to unsuitable environmental conditions, e.g. pH.

Many indigenous milk enzymes are technologically significant from five viewpoints:

1. Deterioration (lipase (commercially, probably the most significant enzyme in milk), proteinase, acid phosphatase and xanthine oxidase) or preservation (sulphydryl oxidase, superoxide dismutase) of milk quality.
2. As indices of the thermal history of milk: alkaline phosphatase, γ-glutamyl transpeptidase, lactoperoxidase.
3. As indices of mastitic infection: catalase, *N*-acetyl-β-D-glucosaminidase, acid phosphatase; the concentration of several other enzymes increases on mastitic infection.
4. Antimicrobial activity: lysozyme, lactoperoxidase (which is exploited as a component of the lactoperoxidase – H_2O_2 – thiocyanate system for the cold pasteurization of milk).
5. As commercial source of enzymes: ribonuclease, lactoperoxidase.

With a few exceptions (e.g. lysozyme and lactoperoxidase), the indigenous milk enzymes do not have a beneficial effect on the nutritional or organoleptic attributes of milk, and hence their destruction by heat is one of the objectives of many dairy processes.

The distribution of the principal indigenous enzymes in milk and their catalytic activity are listed in Table 8.1. In this chapter, the occurrence, distribution, isolation and characterization of the principal indigenous enzymes will be discussed, with an emphasis on their commercial significance in milk.

8.2.2 Proteinases (EC 3.4.–.–)

The presence of an indigenous proteinase in milk was suggested by Babcock and Russel in 1897 but because it occurs at a low concentration or has low activity in milk, it was felt until the 1960s that the proteinase in milk may be of microbial origin. Recent changes in the dairy industry, e.g. improved hygiene in milk production, extended storage of milk at a low temperature

Table 8.1 Indigenous enzymes of significance to milk

Enzyme	Reaction	Importance
Lipase	Triglycerides + H_2O → fatty acids + partial glycerides + glycerol	Off flavours in milk; flavour development in Blue cheese
Proteinase (plasmin)	Hydrolysis of peptide bonds, particularly in β-casein	Reduced storage stability of UHT products; cheese ripening
Catalase	$2H_2O_2 \rightarrow O_2 + 2H_2O$	Index of mastitis; pro-oxidant
Lysozyme	Hydrolysis of mucopolysaccharides	Bacteriocidal agent
Xanthine oxidase	Aldehyde + $H_2O + O_2 \rightarrow$ Acid + H_2O_2	Pro-oxidant; cheese ripening
Sulphydryl oxidase	$2RSH + O_2 \rightarrow RSSR + H_2O_2$	Amelioration of cooked flavour
Superoxide dismutase	$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	Antioxidant
Lactoperoxidase	$H_2O_2 + AH_2 \rightarrow 2H_2O + A$	Index of pasteurization; bacteriocidal agent; index of mastitis; pro-oxidant
Alkaline phosphomonoesterase	Hydrolysis of phosphoric acid esters	Index of pasteurization
Acid phosphomonoesterase	Hydrolysis of phosphoric acid esters	Reduce heat stability of milk; cheese ripening

at the farm and/or factory and altered product profile, e.g. UHT processing of milk, have increased the significance of indigenous milk proteinase which has, consequently, been the focus of considerable research.

Milk contains at least two proteinases, plasmin (alkaline milk proteinase) and cathepsin D (acid milk proteinase) and possibly several others, i.e. two thiol proteinases, thrombin and an aminopeptidase. In terms of activity and technological significance, plasmin is the most important of the indigenous proteinases and has been the subject of most attention. The relevant literature has been reviewed by Grufferty and Fox (1988) and Bastian and Brown (1996).

Plasmin (EC 3.4.21.7)

The physiological function of plasmin (fibrinolysin) is to dissolve blood clots. It is part of a complex system consisting of plasmin, its zymogen (plasminogen), plasminogen activators, plasmin inhibitors and inhibitors of plasminogen activators (Figure 8.1). In milk, there is about four times as much plasminogen as plasmin and both, as well as plasminogen activators, are associated with the casein micelles, from which they dissociate when the pH is reduced to 4.6. The inhibitors of plasmin and of plasminogen activators are in the milk serum. The concentration of plasmin and plasminogen in milk increase with advancing lactation, mastitic infection and number of lactations.

Plasmin is usually extracted from casein at pH 3.5 and purified by precipitation with $(NH_4)_2SO_4$ and various forms of chromatography, including affinity chromatography. Plasmin is optimally active at about pH 7.5 and 35°C; it exhibits *c.* 20% of maximum activity at 5°C and is stable over the pH range 4 to 9. Plasmin is quite heat stable: it is partially inactivated by heating at 72°C × 15 s but its activity in milk increases following HTST pasteurization, probably through inactivation of the indigenous inhibitors of plasmin or, more likely, inhibitors of plasminogen activators. It partly survives UHT sterilization and is inactivated by heating at 80°C × 10 min at pH 6.8; its stability decreases with increasing pH in the range 3.5–9.2.

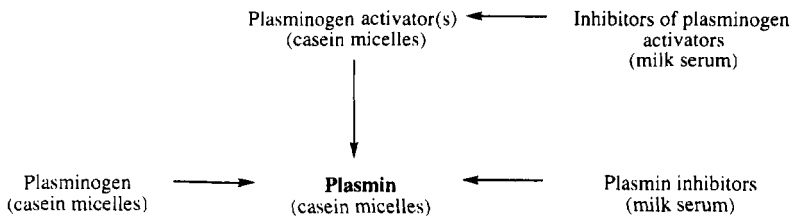


Figure 8.1 Schematic representation of the plasmin system in milk.

Plasmin is a serine proteinase (inhibited by diisopropylfluorophosphate, phenylmethyl sulphonyl fluoride and trypsin inhibitor) with a high specificity for peptide bonds to which lysine or arginine supplies the carboxyl group. Its molecular weight is about 81 Da and its structure contains five intramolecular disulphide-linked loops (kringles) which are essential for its activity.

Activity of plasmin on milk proteins. β-Casein is the most susceptible milk protein to plasmin action; it is hydrolysed rapidly at Lys_{28}–Lys_{29}, Lys_{105}–His_{106} and Lys_{107}–Glu_{108}, to yield 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 f1–105/7), PP8 slow (β-CN f29–105/7) and PP8 fast (β-CN f1–29) (Chapter 4). In solution, β-casein is also hydrolysed at Lys_{113}–Tyr_{114} and Lys_{183}–Asp_{184}, but it is not known if these bonds are hydrolysed in milk. γ-Caseins normally represent about 3% of total N in milk but can be as high as 10% in late lactation milk; the concentration of proteose peptones is about half that of the γ-caseins.

α_{s2}-Casein in solution is also hydrolysed very rapidly by plasmin at bonds Lys_{21}–Gln_{22}, Lys_{24}–Asn_{25}, Arg_{114}–Asn_{115}, Lys_{149}–Lys_{150}, Lys_{150}–Thr_{151}, Lys_{181}–Thr_{182}, Lys_{187}–Thr_{188} and Lys_{188}–Ala_{189} (see Bastian and Brown, 1996) but it is not known if it is hydrolysed in milk. Although less susceptible than α_{s2}- or β-caseins, α_{s1}-casein in solution is also readily hydrolysed by plasmin (see Bastian and Brown, 1996) but it does not appear to be hydrolysed to a significant extent in milk although it has been suggested that λ-casein is produced from α_{s1}-casein by plasmin. Although κ-casein contains several Lys and Arg residues, it appears to be quite resistant to plasmin, presumably due to a relatively high level of secondary and tertiary structure. β-Lactoglobulin, especially when denatured, inhibits plasmin, presumably via sulphydryl–disulphide interactions which rupture the structurally important kringles.

Significance of plasmin activity in milk. Plasmin and plasminogen accompany the casein micelles on the rennet coagulation of milk and are concentrated in cheese in which plasmin contributes to primary proteolysis of the caseins, especially in cheeses with a high-cook temperature, e.g. Swiss and some Italian varieties, in which the coagulant is totally or largely inactivated (Chapter 10). Plasmin activity may contribute to age gelation in UHT milk produced from high-quality raw milk (which contains a low level of *Pseudomonas* proteinase). It has been suggested that plasmin activity contributes to the poor cheesemaking properties of late-lactation milk but proof is lacking. The acid precipitability of casein from late lactation milk is also poor but evidence for the involvement of plasmin is lacking. Reduced yields of cheese and casein can be expected to result from plasmin action since the proteose peptones are, by definition, soluble at pH 4.6.

Cathepsin D (EC 3.4.23.5). It has been known for more than 20 years that milk also contains an acid proteinase, (optimum pH $\approx$ 4.0) which is now known to be cathepsin D, a lysozomal enzyme. It is relatively heat labile (inactivated by 70°C $\times$ 10 min). Its activity in milk has not been studied extensively and its significance is unknown. At least some of the indigenous acid proteinase is incorporated into cheese curd; its specificity on α_{s1}- and β-caseins is quite similar to that of chymosin but it has very poor milk-clotting activity (McSweeney, Fox and Olson, 1995). It may contribute to proteolysis in cheese but its activity is probably normally overshadowed by chymosin, which is present at a much higher level.

Other proteinases. The presence of low levels of other proteolytic enzymes in milk has been reported (see Fox and McSweeney, 1996). Most of these originate from somatic cells, and their level increases during mastitic infection. The presence of cathepsin D, a lysozomal enzyme, in milk suggests that all the lysozomal proteinases are present in milk although they may not be active. These minor proteinases are considered to be much less significant than plasmin, but more work on the subject is necessary.

8.2.3 Lipases and esterases (EC 3.1.1.–)

Lipases catalyse the development of hydrolytic rancidity in milk, and, consequently, lipases and lipolysis in milk have been studied extensively.

Milk contains three types of esterase:

1. A-type carboxylic ester hydrolases (arylesterases; EC 3.1.1.2), which hydrolyse aromatic esters, e.g. phenylacetate; they show little activity on tributyrin, and are not inhibited by organophosphates.
2. B-type esterases (glycerol tricarboxyl esterases, aliphatic esterases, lipases; EC 3.1.1.3): they are most active on aliphatic esters although they show some activity on aromatic esters; they are inhibited by organophosphates.
3. C-type esterases (cholinesterase; EC 3.1.1.7; EC 3.1.1.8): they are most active on choline esters but hydrolyse some aromatic and aliphatic esters slowly; they are inhibited by organophosphates.

In normal milk, the ratio of A : B : C esterase activity is about 3 : 10 : 1 but the level of A-esterase activity increases considerably on mastitic infection. A and C esterases are considered to be of little technological significance in milk.

Classically, lipases hydrolyse ester bonds in emulsified esters, i.e. at a water/oil interface, although some may have limited activity on soluble esters; they are usually activated by blood serum albumin and Ca^{2+} which bind free fatty acids, which are inhibitory. Little lipolysis normally occurs in

milk because more than 90% of the lipase is associated with the casein micelles while the triglyceride substrates are in fat globules surrounded, and protected, by the fat globule membrane (MFGM). When the MFGM is damaged, lipolysis occurs rapidly, giving rise to hydrolytic rancidity.

Lipase was first isolated from skim milk and characterized by Fox and Tarassuk in 1967. The enzyme was optimally active at pH 9.2 and 37°C and found to be a serine enzyme (inactivated by organophosphates). A lipoprotein lipase (LPL; activated by lipoprotein co-factors) was demonstrated in milk by Korn in 1962 and was isolated by Egelrud and Olivecrona in 1972. LPL is, in fact, the principal indigenous lipase in milk and most recent work has been focused accordingly. The molecule has been characterized at the molecular, genetic, enzymatic and physiological levels (see Olivecrona *et al.*, 1992).

In addition to LPL, human milk contains a bile salts-activated lipase, which probably contributes to the metabolism of lipids by breast-fed babies who have limited pancreatic lipase activity. Bovine milk and milks from other dairy animals do not contain this enzyme.

The lipolytic system in most milks becomes active only when the milk MFGM is damaged by agitation, homogenization or temperature fluctuations. However, some individual cows produce milk which becomes rancid spontaneously, i.e. without apparent activation. Spontaneous rancidity was considered to be due to a second lipase, termed **membrane lipase**, which was believed to be associated with the MFGM, but recent evidence suggests that LPL is responsible for spontaneous rancidity following activation by a lipoprotein (co-lipase) from blood serum; normal milk will become spontaneously rancid if blood serum is added, suggesting that 'spontaneous milks' contain a higher than normal level of blood serum. Dilution of 'spontaneous milk' with normal milk prevents spontaneous rancidity, which consequently is not normally a problem with bulk herd milks; presumably, dilution with normal milk reduces the lipoprotein content of the mixture to below the threshold necessary for lipase adsorption.

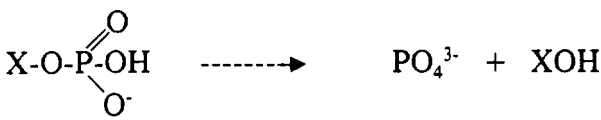
Natural variations in the levels of free fatty acids in normal milk and the susceptibility of normal milks to lipolysis may be due to variations in the level of blood serum in milk.

Significance of lipase. Technologically, lipase is arguably the most significant indigenous enzyme in milk. Although indigenous milk lipase may play a positive role in cheese ripening, undoubtedly the most industrially important aspect of milk lipase is its role in hydrolytic rancidity which renders liquid milk and dairy products unpalatable and eventually unsaleable. Lipolysis in milk has been reviewed extensively (Deeth and Fitz-Gerald, 1995). As discussed in Chapter 3, all milks contain an adequate level of lipase for rapid lipolysis, but become rancid only after the fat globule membrane has been damaged.

8.2.4 Phosphatases

Milk contains several phosphatases, the principal ones being alkaline and acid phosphomonoesterases, which are of technological significance, and ribonuclease, which has no known function or significance in milk. The alkaline and acid phosphomonoesterases have been studied extensively (see Andrews (1993) for references).

Alkaline phosphomonoesterase (EC 3.1.3.1). The existence of a phosphatase in milk was first recognized in 1925. Subsequently characterized as an alkaline phosphatase, 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 destroy *Mycobacterium tuberculosis*, then the target micro-organism for pasteurization. The enzyme is readily assayed, and a test procedure based on alkaline phosphatase inactivation was developed for routine quality control of milk pasteurization. Several major modifications of the test have been developed. The usual substrates are phenyl phosphate, *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 release of inorganic phosphate may be assayed but the other product is usually determined. Phenol is colourless but forms a coloured complex on reaction with one of several reagents, e.g. 2,6-dichloroquinonechloroimide, with which it forms a blue complex. *p*-Nitrophenol is yellow while phenolphthalein is red at the alkaline pH of the assay (10) and hence the concentration of either of these may be determined easily.

Isolation and characterization. Alkaline phosphatase is concentrated in the fat globule membrane and hence in cream. It is released into the buttermilk on phase inversion; consequently, buttermilk is the starting material for most published methods for the purification of alkaline phosphatase. Later methods have used chromatography on various media to give a homogeneous preparation with 7440-fold purification and 28% yield. The characteristics of milk alkaline phosphatase are summarized in Table 8.2. The enzyme appears to be similar to the alkaline phosphatase of mammary tissue.

Table 8.2 Characteristics of milk alkaline phosphatase

Characteristic	Conditions
pH optimum	Casein: 6.8 *p*-nitrophenylphosphate: 9.65 *p*-nitrophenylphosphate: 10.5
Temperature optimum	37°C
K_m	0.69 mM on *p*-nitrophenylphosphate
Activators	Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}
Molecular weight	170–190 kDa
Association/dissociation	2 subunits of molecular weight 85 kDa formed on heating (100°C for 2 min or acidification to pH 2.1)
Polymorphic forms	4

Reactivation of phosphatase. Much work has been focused on a phenomenon known as 'phosphatase reactivation', first recognized by Wright and Tramer in 1953, who observed that UHT-treated milk was phosphatase-negative immediately after processing but became positive on standing; microbial phosphatase was shown not to be responsible. Bulk HTST milk never showed reactivation, although occasional individual-cow samples did; HTST pasteurization after UHT treatment usually prevented reactivation and reactivation was never observed in very severely heated milk. Reactivation can occur following heating at temperatures as low as 84°C for milk and 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 by fat but this has not been substantiated. Mg^{2+} and Zn^{2+} strongly promote reactivation; 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. The role of Mg^{2+} or Zn^{2+} is seen as causing a conformational change in the denatured enzyme, necessary for renaturation.

Reactivation of alkaline phosphatase is of considerable practical significance since regulatory tests for pasteurization assume the absence of phosphatase activity. An official AOAC method used to distinguish between renatured and residual native alkaline phosphatase is based on the increase in phosphatase activity resulting from addition of Mg^{2+}: the activity of renatured alkaline phosphatase is increased about 14-fold but that of the native enzyme is increased only two-fold.

Although it can dephosphorylate casein under suitable conditions, as far as is known, alkaline phosphatase has no direct technological significance

in milk and milk products; perhaps its pH optimum is too far removed from that of milk; it is also inhibited by inorganic phosphate.

Acid phosphomonoesterase (EC 3.1.3.2). Milk contains an acid phosphatase which has a pH optimum at 4.0 and is very heat stable (LTLT pasteurization causes only 10–20% inactivation and 30 min at 88°C is required for full inactivation). Denaturation of acid phosphatase under UHT conditions follows first-order kinetics. When heated in milk at pH 6.7, the enzyme retains significant activity following HTST pasteurization but does not survive in-bottle sterilization or UHT treatment. The enzyme is not activated by Mg^{2+} (as is alkaline phosphatase), but it is slightly activated by Mn^{2+} and is very effectively inhibited by fluoride. The level of acid phosphatase activity in milk is only about 2% that of alkaline phosphatase; activity reaches a sharp maximum 5–6 days post-partum, then decreases and remains at a low level to the end of lactation.

Milk acid phosphatase has been purified to homogeneity by various forms of chromaotgraphy, including affinity chromatography; purification up to 40 000-fold has been claimed. The enzyme shows broad specificity on phosphate esters, including the phosphoseryl residues of casein. It has a molecular mass of about 42 kDa and an isoelectric point of 7.9. Many forms of inorganic phosphate are competitive inhibitors, while fluoride is a powerful non-competitive inhibitor. The enzyme is a glycoprotein and its amino acid composition is known. Milk acid phosphatase shows some similarity to the phosphoprotein phosphatase of spleen but differs from it in a number of characteristics.

Although casein is a substrate for milk acid phosphatase, the major caseins, in the order $\alpha_s(\alpha_{s1} + \alpha_{s2}) > \beta > \kappa$, also act as competitive inhibitors of the enzyme when assayed on *p*-nitrophenylphosphate, 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).

Significance. Although acid phosphatase is present in milk at a much lower level than alkaline phosphatase, its greater heat stability and lower pH optimum may make it technologically significant. Dephosporylation of casein reduces its ability to bind Ca^{2+}, to react with κ-casein, to form micelles and its heat stability. Several small partially dephosphorylated peptides have been isolated from Cheddar and Parmesan cheese. However, it is not known whether indigenous or bacterial acid phosphatases are mainly responsible for dephosphorylation in cheese. Dephosphorylation may be rate-limiting for proteolysis in cheese ripening since most proteinases and peptidases are inactive on phosphoproteins or peptides. It has been suggested that phosphatase activity should be included in the criteria for starter selection.

The acid phosphatase activity in milk increases by a factor of 4–10 during mastitic infection; three isoenzymes are then present, only one of which is indigenous milk acid phosphatase, the other two being of leucocyte origin; these latter isoenzymes are more thermolabile and are inactivated by HTST pasteurization.

8.2.5 Lysozyme (EC 3.2.1.17)

Lysozyme (muramidase, mucopeptide *N*-acetylmuramylhydrolase) is a widely distributed enzyme which lyses certain bacteria by hydrolysing the β(1-4)-linkage between muramic acid and *N*-acetylglucosamine of mucopolysaccharides of the bacterial cell wall.

Lysozyme was isolated from human milk in 1961 by Jolles and Jolles, who believed that bovine milk was devoid of lysozyme. Milks of many species have since been shown to contain lysozyme and several have been isolated and characterized. Human and equine milks are an exceptionally rich source, containing 130 mg l^{-1} (3000 times the level of bovine milk) and about 800 mg l^{-1}, respectively (see Farkye, 1992).

The pH optima of human milk lysozyme (HML), bovine milk lysozyme (BML) and egg-white lysozyme (EWL) are 7.9, 6.35 and 6.2, respectively. BML has a molecular weight of 18 kDa compared with 15 kDa for HML and EWL. The amino acid composition of BML is reported to be considerably different from that of HML or EWL. All lysozymes are relatively stable to heat at acid pH values (3–4) but are relatively labile at pH greater than 7. Low concentrations of reducing agents increase the activity of BML and HML by about 330%.

Significance. Presumably, the physiological role of lysozyme is to act as a bactericidal agent; in the case of milk it 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 nutritionally significant. Breast-fed babies generally suffer less enteric problems than bottle-fed babies. While there are many major compositional and physicochemical differences between bovine and human milks which may be responsible for the observed nutritional characteristics (Chapter 4), it has been suggested that the disparity in lysozyme content may be significant. A number of investigators have recommended fortification of bovine milk-based infant formulae with EWL, especially for premature babies. Feeding studies are equivocal on the benefits of this practice and recent trials failed to demonstrate any beneficial effect due to inactivation of EWL in the human stomach.

No beneficial effects from lysozyme on the shelf-life of milk have been reported. Addition of lysozyme to milk reduces its heat stability but the level

of indigenous lysozyme is probably too low to contribute to the natural variations in the heat stability of milk.

8.2.6 N-Acetyl-β-D-glucosaminidase (EC 3.2.1.30)

N-Acetyl-β-D-glucosaminidase (NAGase) hydrolyses terminal, non-reducing *N*-acetyl-β-D-glucosamine residues from glycoproteins. It is a lysosomal enzyme and originates mainly from somatic cells and mammary gland epithelial cells. Consequently, NAGase activity increases markedly and correlates highly with the intensity of mastitis. 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 yellow *p*-nitrophenol. NAGase is optimally active at 50°C and pH 4.2 and is inactivated by HTST pasteurization (70–71°C × 15–18 s) (see Farkye, 1992).

8.2.7 γ-Glutamyl transpeptidase (transferase) (EC 2.3.2.2)

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

$$\gamma\text{-glutamyl-peptide} + X \rightleftharpoons \text{peptide} + \gamma\text{-glutamyl-}X,$$

where X is an amino acid.

GGT, which has been isolated from the fat globule membrane, has a molecular mass of about 80 kDa and consists of two subunits of 57 and 26 kDa. It is optimally active at pH 8–9, has a pi of 3.85 and is inhibited by iodoacetate, diisopropylfluorophosphate and metal ions, e.g. Cu^{2+} and Fe^{3+}

It plays a role in amino acid transport in the mammary gland. γ-Glutamyl peptides have been isolated from cheese but since γ-glutamyl bonds do not occur in milk proteins, their synthesis may be catalysed by GGT. The enzyme is relatively heat stable and has been proposed as a marker enzyme for milks pasteurized in the range 72–80°C × 15 s. GGT is absorbed from the gastrointestinal tract, resulting in high levels of GGT activity in the blood serum of newborn animals fed colostrum or early breast milk. Since GGT is inactivated by the heat treatment to which infant formulae are subjected, the level of GGTase activity in infants can be used to distinguish breast-fed from formula-fed infants (see Farkye, 1992).

8.2.8 Xanthine oxidase (EC 1.2.3.2)

It has been recognized for about 80 years that milk contains an enzyme capable of oxidizing aldehydes and purines. The enzyme is now generally referred to as xanthine oxidase (XO); milk is a very good source of XO, at

least part of which is transported to the mammary gland via the bloodstream. A similar enzyme is found in various animal tissues and several bacterial species (Farkye, 1993).

Isolation. Numerous methods have been developed for the purification of XO from milk; since the enzyme is concentrated in the MFGM, in which it is one of the principal proteins, all methods employ cream as starting material, use a dissociating agent to liberate XO from membrane lipoproteins and some form of chromatography for further purification.

Milk XO has a molecular weight of *c.* 300 kDa and consists of two subunits. The pH optimum is about 8.5 and the enzyme requires flavin adenine dinucleotide (FAD), Fe, Mo and an acid-labile compound as co-factors; cows deficient in Mo have low XO activity. The amino acid composition of XO has been determined by a number of workers; at least five genetic polymorphic forms have been reported.

Activity in milk. Various processing treatments affect the XO activity of milk. Activity is increased by about 100% on storage at 4°C for 24 h, by 50–100% on heating at 70°C for 5 min and by 60–90% on homogenization. These treatments cause the transfer to XO from the fat phase to the aqueous phase, rendering the enzyme more active. The heat stability of XO is very dependent on whether it is a component of the fat globules or is dissolved in the aqueous phase; ageing and homogenization increase susceptibility and explain the inconsistency of early work in which the history of the sample was unknown or unrecorded. XO is most heat stable in cream and least in skim milk. Homogenization of concentrated milk prepared from heated milk (90.5°C for 15 s) partially reactivates XO, which persists on drying the concentrate, but no reactivation occurs following more severe heating (93°C for 15 s); apparently, homogenization releases potentially active, undenatured XO from the MFGM. All the major milk proteins can act as either activators or inhibitors of XO, depending on their concentration, and may have some significance in the activation, inactivation and reactivation of the enzyme.

Significance of xanthine oxidase

Lipid oxidation

XO, which can excite stable triplet oxygen ($^3O_2$), is a pro-oxidant. Milk which undergoes spontaneous rancidity contains about 10 times the normal level of XO, and spontaneous oxidation can be induced in normal milk by the addition of XO to about four times normal levels. Heat-denatured or flavin-free enzyme is ineffective and the susceptibility of unsaturated fatty acids to oxidation increases with the degree of unsaturation.

Atherosclerosis

It has been suggested that XO from homogenized milk enters the vascular system and may be involved in atherosclerosis via oxidation of plasmalogens (Appendix 3B) in cell membranes. However, the experimental evidence in support of this view is very weak and the hypothesis has been disclaimed (see Farkye, 1992).

8.2.9 Sulphydryl oxidase (EC 1.8.3.–)

Milk contains an enzyme, sulphydryl oxidase (SO), capable of oxidizing sulphydryl groups of cysteine, glutathione and proteins to the corresponding disulphide (reviewed by Farkye, 1992). The enzyme is an aerobic oxidase which catalyses the following reaction:

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

It undergoes marked self-association and can be purified readily by chromatography on porous glass. The enzyme has a molecular weight of about 89 kDa, a pH optimum of 6.8–7.0, and a temperature optimum of 35°C. Its amino acid composition, its requirement for iron but not for molybdenum and FAD, and the catalytic properties of the enzyme, indicate that sulphydryl oxidase is a distinct enzyme from xanthine oxidase and thiol oxidase (EC 1.8.3.2).

SO is capable of oxidizing reduced ribonuclease and restoring enzymic activity, suggesting that its physiological role may be the non-random formation of protein disulphide bonds, e.g. during protein biosynthesis.

SO immobilized on glass beads has the potential to ameliorate the cooked flavour arising from sulphydryl groups exposed upon protein denaturation, but the commercial viability of this system is not known.

The production of sulphur compounds is believed to be very important in the development of Cheddar cheese flavour. Residual sulphydryl oxidase activity may play a role in initially reoxidizing sulphydryl groups exposed upon heating cheesemilk; the sulphydryl groups thus protected may be reformed during the ripening process.

8.2.10 Superoxide dismutase (EC 1.15.1.1)

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

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

The H_2O_2 formed may be reduced by catalase, peroxidase or suitable reducing agents. SOD has been identified in many animal and bacterial cells; its biological function is to protect tissue against oxygen free radicals in anaerobic systems (reviewed by Farkye, 1992).

SOD, isolated from bovine erythrocytes, is a blue-green protein due to the presence of copper, removal of which by treatment with EDTA results in loss of activity, which is restored by adding Cu^{2+}; it also contains Zn^{2+}, which does not appear to be at the active site. The enzyme, which is very stable in 9 M urea at neutral pH, consists of two identical subunits of molecular weight 16 kDa held together by one or more disulphide bonds. The amino acid sequence has been established.

Milk contains trace amounts of SOD which has been isolated and characterized; it appears to be identical to the bovine erythrocyte enzyme. SOD inhibits lipid oxidation in model systems. The level of SOD in milk parallels that of XO (but at a lower level), suggesting that SOD may be excreted in milk in an attempt to offset the pro-oxidant effect of XO. However, the level of SOD in milk is probably insufficient to explain observed differences in the oxidative stability of milk. The possibility of using exogenous SOD to retard or inhibit lipid oxidation in dairy products has been considered.

SOD is more heat stable in milk than in purified preparations; in milk it is stable at 71°C for 30 min but loses activity rapidly at even slightly higher temperatures. Slight variations in pasteurization temperature are therefore critical to the survival of SOD in heated milk products and may contribute to variations in the stability of milk to oxidative rancidity.

8.2.11 Catalase (EC 1.11.1.6)

Indigenous milk catalase was first recognized in 1907. Although about 70% of the catalase activity of whole milk is in the skim-milk phase, cream has a higher specific activity; the pellet obtained from buttermilk on centrifugation at 10 000 g is a particularly rich source, from which catalase has been highly purified (reviewed by Farkye, 1992).

Milk catalase is a haem protein with a molecular weight of 200 kDa, and an isoelectric pH of 5.5; it is stable between pH 5 and 10 but rapidly loses activity outside this range. Heating at 70°C for 1 h causes complete inactivation. Like other catalases, it is strongly inhibited by Hg^{2+}, Fe^{2+}, Cu^{2+}, Sn^{2+}, CN^- and NO_3^-.

Catalase activity in milk varies with feed, stage of lactation and especially with mastitic infection, of which it may be used as an index. It may act as a lipid pro-oxidant via its haem iron.

8.2.12 Lactoperoxidase (EC 1.11.1.7)

The occurrence of a peroxidase, lactoperoxidase (LPO), in milk was recognized as early as 1881. It is one of the most heat-stable enzymes in milk; its destruction was used as an index of flash pasteurization (now very rarely used) and is now used as an index of super-HTST pasteurization.

LPO was first isolated in 1943; several isolation procedures have since been published (reviewed by Björck, 1993).

LPO is a haem protein containing about 0.07% Fe, with an absorbance peak (Soret band) at 412 nm ($A_{412}/A_{280} \sim 0.9$); the pH optimum is around 8.0; its molecular weight is 77.5 kDa and it consists of two identical subunits. Two principal forms (A and B) occur, each of which exhibits micro-heterogeneity with regard to amide groups (glutamine and/or asparagine) and carbohydrate content, giving a total of 10 variants.

Significance. Apart from its exploitation as an index of flash or super-HTST pasteurization, LPO is also technologically significant for a number of other reasons:

1. It is a possible index of mastitic infection; although the level of LPO in milk increases on mastitic infection, it is not well correlated with somatic cell count.
2. LPO causes non-enzymic oxidation of unsaturated lipids, probably acting through its haem group; the heat-denatured enzyme is more active than the native enzyme.
3. Milk contains bacteriostatic or bactericidal substances referred to as lactenins. One of these is LPO, which requires H_2O_2 and thiocyanate (SCN^-) to cause inhibition. The nature, mode of action and specificity of the LPO–H_2O_2–SCN^- system has been widely studied. LPO and thiocyanate, which is produced in the rumen by enzymic hydrolysis of thioglycosides from *Brassica* plants, occur naturally in milk, but H_2O_2 does not. However, H_2O_2 can be generated metabolically by catalase-negative bacteria, or produced *in situ* through the action of exogenous glucose oxidase on glucose, or it may be added directly.

   The peroxidase system has been found to have good bactericidal efficiency for the cold pasteurization of fluids or sanitization of immobilized enzyme columns. The generation of H_2O_2 *in situ* through the action of immobilized glucose oxidase on glucose is effective against Gram-negative bacteria in thiocyanate and glucose-enriched milk and whey. A self-contained LPO–H_2O_2–SCN^- system using coupled β-galactosidase and glucose oxidase, immobilized on porous glass beads, to generate H_2O_2 *in situ* from lactose in milk containing 0.25 mM thiocyanate has been developed. Indigenous xanthine oxidase, acting on added hypoxanthine, may also be exploited to produce H_2O_2 for the LPO–H_2O_2–SCN^- system. The bactericidal effects of the LPO–H_2O_2–SCN^- system may be used to cold pasteurize milk in situations where refrigeration and/or thermal pasteurization is lacking. LPO is cationic at the pH of milk and may be readily isolated on cation-exchange resins. Addition of isolated LPO to milk replacers for calves or piglets reduces the incidence of enteritis.

Table 8.3 Other enzymes that have been isolated from milk and partially characterized but which are of no known significance (Farkye, 1992)

Enzyme		Reaction catalysed	Comment
Glutathione peroxidase	EC 1.11.1.9 2	$GSH + H_2O \rightleftharpoons GSSH$	Contains Se
Ribonuclease	EC 3.1.27.5	Hydrolysis of RNA	Milk is a very rich source; similar to pancreatic RNase
α-Amylase	EC 3.2.1.1	Starch	
β-Amylase	EC 3.2.1.2	Starch	
α-Mannosidase	EC 3.2.1.24		Contains Zn^{2+}
β-Glucuronidase	EC 3.2.1.31		
5′-Nucleotidase	EC 3.1.3.5 5′	Nucleotides + $H_2O \rightleftharpoons$ ribonucleosides + P_i	Diagnostic test for mastitis
Adenosine triphosphatase	EC 3.6.1.3	$ATP + H_2O \rightleftharpoons ADP + P_i$	
Aldolase	EC 4.1.2.13	Fructose 1,6 diP $\rightleftharpoons$ glyceraldehyde-3-P + dihydroxyacetone-P	

4. Acid production in milk by some starters is reported to be retarded by severe heat treatment of milk (77–80°C for 10 min) but can be restored by addition of LPO; the mechanism involved is unknown.

8.2.13 Other enzymes

In addition to the enzymes described above, a number of other indigenous enzymes (Table 8.3) have been isolated and partially characterized (see Farkye, 1992). Although fairly high levels of some of these enzymes occur in milk, they have no apparent function in milk and will not be discussed further.

Nearly 40 other enzymic activities have been detected in milk but have not been isolated and limited information on their molecular and biochemical properties in milk are available; some of these are listed in Table 8.4.

8.3 Exogenous enzymes in dairy technology

8.3.1 Introduction

Crude enzyme preparations have been used in food processing since prehistoric times; classical examples are rennets in cheesemaking and papaya leaves to tenderize meat. Added (exogenous) enzymes are attractive in food processing because they can induce specific changes, in contrast to chemical or physical methods which may cause non-specific undesirable changes. For some applications, there is no viable alternative to enzymes, e.g. rennet-coagulated cheeses, whereas in some cases, enzymes are preferred

Table 8.4 Partial list of minor enzymes in milk (modified from Farkye, 1992)

Enzyme	Reaction catalysed	Source	Distribution in milk
EC 1.1.1.1 Alcohol dehydrogenase	Ethanol + NAD^+ ⇌ acetaldehyde + NADH + H^+		
EC 1.1.1.14 L-Iditol dehydrogenase	L-Iditol + NAD^+ ⇌ L-sorbose + NADH	–	SM
EC 1.1.1.27 Lactate dehydrogenase	Lactic acid + NAD^+ ⇌ pyruvic acid + NADH + H^+		
EC 1.1.1.37 Malate dehydrogenase	Malate + NAD^+ ⇌ oxaloacetate + NADH	Mammary gland	SM
EC 1.1.1.40 Malic enzyme	Malate + $NADP^+$ ⇌ pyruvate + CO_2 + NADH	Mammary gland	SM
EC 1.1.1.42 Isocitrate dehydrogenase	Isocitrate + $NADP^+$ ⇌ 2-oxoglutarate + CO_2 + NADH	Mammary gland	SM
EC 1.1.1.44 Phosphoglucuronate dehydrogenase (decarboxylating)	6-Phospho-D-gluconate + $NADP^+$ ⇌ D-ribose-5-phosphate + CO_2 + NADPH	Mammary gland	SM
EC 1.1.1.49 Glucose-6-phosphate dehydrogenase	D-Glucose-6-phosphate + $NADP^+$ ⇌ D-glucono-1,5-lactone-6-phosphate + NADPH	Mammary gland	SM
EC 1.4.3.6 Amine oxidase (Cu-containing)	$RCH_2NH_2 + H_2O + O_2 \rightleftharpoons RCHO + NH_3 + H_2O_2$	–	SM
– Polyamine oxidase	Spermine → spermidine → putrescine	–	SM
– Fucosyltransferase	Catalyses the transfer of fucose from GDP L-fucose to specific oligosaccharides and glycoproteins	–	SM
EC 1.6.99.3 NADH dehydrogenase	NADH + acceptor ⇌ NAD^+ + reduced acceptor	–	FGM
EC 1.8.1.4 Dihydrolipomide dehydrogenase (diaphorase)	Dihydrolipomide + NAD^+ ⇌ lipoamide + NADH	–	SM/FGM
EC 2.4.1.22 Lactose synthetase A protein: UDP-galactose: D-glucose, 1-galactosyltransferase; B protein: α-lactalbumin	UDP-galactose + D-glucose ⇌ UDP + lactose	Golgi apparatus	SM
EC 2.4.1.38 Glycoprotein 4-β-galactosyltransferase	UDP-galactose + *N*-acetyl-D-glucosaminyl-glycopeptide ⇌ UDP + 4,β-D-galactosyl-*N*-acetyl-D-glucosaminyl-glycopeptide	–	FGM
EC 2.4.1.90 *N*-Acetyllactosamine synthase	UDP-galactose + *N*-acetyl-D-glucosamine ⇄ UDP *N*-acetyllactosamine	Golgi apparatus	–
EC 2.4.99.6 CMP-*N*-acetyl-*N*-acetyl-lactosaminide α-2,3-sialyltransferase	CMP-*N*-acetylneuraminate + β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-glycoprotein ⇌ CMP + α-*N*-acetylneuraminyl-1,2,3-β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-glycoprotein	–	SM

EC 2.5.1.3 Thiamin-phosphate pyrophosphorylase	2-Methyl-4-amino-5-hydroxymethylpyrimide diphosphate + 4-methyl-5-(2-phosphono-oxyethyl)-thiazole $\rightleftharpoons$ pyrophosphate + thiamine monophosphate	–	FGM
EC 2.6.1.1 Aspartate aminotransferase	L-Aspartate + 2-oxoglutarate $\rightleftharpoons$ oxaloacetate + L-glutamate	Blood	SM
EC 2.6.1.2 Alanine aminotransferase	L-Alanine + 2-oxoglutarate $\rightleftharpoons$ pyruvate + L-glutamate	Blood	SM
EC 2.7.5.1 Phosphoglucomutase	–	–	–
EC 2.7.7.49 RNA-directed DNA polymerase	*n* Deoxynucleoside triphosphate $\rightleftharpoons$ *n* pyrophosphate + DNA_n	–	SM
EC 2.8.1.1 Thiosulphate sulphur transferase	Thiosulphate + cyanide $\rightleftharpoons$ sulphite + thiocyanate	–	SM
EC 3.1.1.8 Cholinesterase	An acylcholine + H_2O $\rightleftharpoons$ choline + a carboxylic acid anion	Blood	FGM
EC 3.1.3.9 Glucose-6-phosphatase	D-Glucose-6-phosphate + H_2O $\rightleftharpoons$ D-glucose + inorganic phosphate	–	FGM
EC 3.1.4.1 Phosphodiesterase	–	–	–
EC 3.1.6.1 Arylsulphatase	Phenol sulphate + H_2O $\rightleftharpoons$ phenol + sulphate	–	–
EC 3.2.1.21 β-Glucosidase	Hydrolysis of terminal non-reducing β-D-glucose residues	Lysosomes	FGM
EC 3.2.1.23 β-Galactosidase	Hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides	Lysosomes	FGM
EC 3.2.1.51 α-Fucosidase	An α-L-fucoside + H_2O $\rightleftharpoons$ an alcohol + L-fucose	Lysosomes	–
EC 3.4.11.1 Cytosol aminopeptidase (leucine aminopeptidase)	Aminoacyl-peptide + H_2O $\rightleftharpoons$ amino acid + peptide	–	SM
EC 3.4.11.3 Cystyl-aminopeptidase (oxytocinase)	Cystyl-peptides + H_2O $\rightleftharpoons$ amino acid + peptide	–	SM
EC 3.4.21.4 Trypsin	Hydrolyses peptide bonds, preferentially Lys-X, Arg-X	–	SM
EC 3.6.1.1 Inorganic pyrophosphatase	Pyrophosphate + H_2O $\rightleftharpoons$ 2-orthophosphate	–	SM/FGM
EC 3.6.1.1 Pyrophosphate phosphorylase	–	–	–
EC 3.6.1.9 Nucleotide pyrophosphorylase	A dinucleotide + H_2O $\rightleftharpoons$ 2 mononucleotides	–	SM/FGM
EC 4.2.1.1 Carbonate dehydratase	H_2CO_3 $\rightleftharpoons$ $CO_2 + H_2O$	–	SM
EC 5.3.1.9 Glucose-6-phosphate isomerase	D-Glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate	–	SM
EC 6.4.1.2 Acetyl-CoA carboxylase	ATP + acetyl CoA + HCO_3 $\rightleftharpoons$ ADP + orthophosphate + malonyl CoA	–	FGM

SM, Skim milk; FGM, fat globule membrane.

over chemical methods because they cause fewer side-reactions and consequently give superior products, e.g. hydrolysis of starch.

Although relatively few enzymes are used in the dairy industry on a significant scale, the use of rennets in cheesemaking is one of the principal of all industrial applications of enzymes.

The applications of exogenous enzymes in dairy technology can be divided into two groups:

1. Technological, in which an enzyme is used to modify a milk constituent or to improve its microbiological, chemical or physical stability.
2. Enzymes as analytical reagents. Although the technological applications are quantitatively the more important, many of the analytical applications of enzymes are unique and are becoming increasingly important.

Since the principal constituents of milk are proteins, lipids and lactose, proteinases, lipases and β-galactosidase (lactase) are the principal exogenous enzymes used in dairy technology. Apart from these, there are, at present, only minor applications for glucose oxidase, catalase, superoxide dismutase and lysozyme. Lactoperoxidase, xanthine oxidase and sulphydryl oxidase might also be included, although at present the indigenous form of these enzymes is exploited.

The application of enzymes in food technology has been widely reviewed (Fox, 1991; Nagodawithana and Reed, 1993). Reviews on applications of exogenous enzymes in dairy technology include Fox and Grufferty (1991), Fox (1993) and Brown (1993).

8.3.2 Proteinases

There is one major (rennet) and several minor applications of proteinases in dairy technology.

Rennets. The use of rennets in cheesemaking is the principal application of proteinases in food processing and is second only to amylases among industrial applications of enzymes. The sources of rennets and their role in milk coagulation and cheese ripening are discussed in Chapter 10 and will not be considered here.

Accelerated cheese ripening. Cheese ripening is a slow, expensive and partially uncontrolled process; consequently, there is increasing interest, at both the research and industrial levels, in accelerating ripening. Various approaches have been investigated to accelerate ripening, including a higher ripening temperature (especially for Cheddar-type cheese which is usually ripened at 6–8°C), exogenous proteinases and peptidases, modified starters (e.g. heat-shocked or lactose-negative) and genetically engineered starters or

starter adjuncts (Fox *et al.*, 1996). The possible use of exogenous proteinases and peptidases attracted considerable attention for a period but uniform distribution of the enzymes in the cheese curd is a problem. Microencapsulation of enzymes offers a possible solution but is not commercially viable at present. Exogenous proteinases/peptidases are not used commercially in natural cheeses but are being used to produce 'enzyme modified cheese' for use in processed cheese, cheese dips and sauces. Selected genetically modified and adjunct cultures appear to be more promising.

Protein hydrolysates. Protein hydrolysates are used as flavourings in soups and gravies and in dietetic foods. They are generally prepared from soy, gluten, milk, meat or fish proteins by acid hydrolysis. Neutralization results in a high salt content which is acceptable for certain applications but may be unsuitable for dietetic foods and food supplements. Furthermore, acid hydrolysis causes total or partial destruction of some amino acids. Partial enzymatic hydrolysis is a viable alternative for some applications but bitterness due to hydrophobic peptides is frequently encountered. Bitterness may be eliminated or at least reduced to an acceptable level by treatment with activated carbon, carboxypeptidase, aminopeptidase, ultrafiltration, hydrophobic chromatography or by the plastein reaction. Caseins yield very bitter hydrolysates but the problem may be minimized by the judicious selection of the proteinase(s) (so as to avoid the production of very bitter peptides) and by using exopeptidases (especially aminopeptidases) together with the proteinase.

A novel, potentially very significant, application of proteinases in milk protein technology is the production of biologically active peptides (Chapter 4). Carefully selected proteinases of known specificity are required for such applications, but the resulting products have high added value.

Modification of protein functionality. The functional properties of milk proteins may be improved by limited proteolysis. Acid-soluble casein, free of off-flavour and suitable for incorporation into beverages and other acid foods (in which casein is insoluble) has been produced by limited proteolysis. The antigenicity of casein is destroyed by proteolysis and the hydrolysate is suitable for use in milk protein-based foods for infants allergic to cows' milk formulations. Controlled proteolysis improves the meltability of directly acidified cheese but excessive proteolysis causes bitterness. Partial proteolysis of lactalbumin (heat-coagulated whey proteins), which is insoluble and has very poor functional properties, yields a product that is almost completely soluble above pH 6; although the product is slightly bitter, it appears promising as a food ingredient. Limited proteolysis of whey protein concentrate reduces its emulsifying capacity, increases its specific foam volume but reduces foam stability and increases heat stability.

8.3.3 β-Galactosidase

β-Galactosidases (commonly referred to as lactase), which hydrolyse lactose to glucose and galactose, are probably the second most significant enzyme in dairy technology. Twenty years ago, β-galactosidase was considered to have very considerable potential but this has not materialized although there are a number of significant technological or nutritional applications. The various aspects of lactose and applications of β-galactosidase are considered in Chapter 2.

8.3.4 Lipases

The principal application of lipases in dairy technology is in cheese manufacture, particularly hard Italian varieties. The characteristic 'piccante' flavour of these cheeses is due primarily to short-chain fatty acids resulting from the action of lipase(s) in the rennet paste traditionally used in their manufacture. Rennet paste is prepared from the stomachs of calves, kids or lambs slaughtered after suckling; the stomachs and contents are held for about 60 days and then macerated. The product, which has proteolytic (rennet) and lipolytic activities, is considered to be unhygienic and its use is not permitted in some countries. The lipase in rennet paste, generally referred to as pregastric esterase (PGE), is secreted by a gland at the base of the tongue, which is stimulated by suckling; the secreted lipase is washed into the stomach with the ingested milk. The physiological significance of PGE, which is secreted by several species, is to assist in lipid digestion in the neonate which has limited pancreatic function. The considerable literature has been reviewed by Nelson, Jensen and Pitas (1977) and Fox and Stepaniak (1993). PGE shows a high specificity for short-chain fatty acids, especially butanoic acid, esterified on the *sn*-3 position of glycerol, although some interspecies differences in specificity have been reported.

Semi-purified preparations of PGE from calf, kid and lamb are commercially available and give satisfactory results; slight differences in specificity renders one or other more suitable for particular applications. Connoisseurs of Italian cheese claim that rennet paste gives superior results to semipurified PGE, and it is cheaper.

Rhizomucor miehei secretes a lipase that is reported to give satisfactory results in Italian cheese manufacture. This enzyme has been characterized and is commercially available as 'Piccantase'. Lipases secreted by selected strains of *Penicillium roqueforti* and *P. candidum* are considered to be potentially useful for the manufacture of Italian and other cheese varieties.

Extensive lipolysis also occurs in Blue cheese varieties in which the principal lipase is secreted by *P. roqueforti* (Chapter 10). It is claimed that treatment of Blue cheese curd with PGE improves and intensifies its flavour but this practice is not widespread. Several techniques have been developed

for the production of fast-ripened Blue cheese-type products suitable for use in salad dressings, cheese dips, etc. Lipases, usually of fungal origin, are used in the manufacture of these products or to pre-hydrolyse fats/oils used as ingredients in their production.

Although Cheddar cheese undergoes relatively little lipolysis during ripening, it is claimed that addition of PGE, gastric lipase or selected microbial lipases improves the flavour of Cheddar, especially that made from pasteurized milk, and accelerates ripening. It is also claimed that the flavour and texture of Feta and Egyptian Ras cheese can be improved by adding kid or lamb PGE or low levels of selected microbial lipases to the cheese milk, especially if milk concentrated by ultrafiltration is used.

Lipases are used to hydrolyse milk fat for a variety of uses in the confectionary, sweet, chocolate, sauce and snack food industries and there is interest in using immobilized lipases to modify fat flavours for such applications (Kilara, 1985). Enzymatic interesterification of milk lipids to modify rheological properties is also feasible.

8.3.5 Lysozyme

As discussed in section 8.2.5, lysozyme has been isolated from the milk of a number of species; human and equine milks are especially rich sources. In view of its antibacterial activity, the large difference in the lysozyme content of human and bovine milks may have significance in infant nutrition. It is claimed that supplementation of baby food formulae based on cows' milk with egg-white lysozyme gives beneficial results, especially with premature babies, but views on this are not unanimous.

Nitrate is added to many cheese varieties to prevent the growth of *Clostridium tyrobutyricum* which causes off-flavours and late gas blowing. However, the use of nitrate in foods is considered to be undesirable because of its involvement in nitrosamine formation, and many countries have reduced permitted levels or prohibited its use. Lysozyme, which inhibits the growth of vegetative cells of *Cl. tyrobutyricum* and hinders the germination of its spores, is an alternative to nitrate for the control of late gas blowing in cheese but is not widely used at present. Lysozyme also kills *Listeria* spp. Lysozyme addition permits the use of lower temperatures in food sterilization. Co-immobilized lysozyme has been proposed for self-sanitizing immobilized enzyme columns; although the technique may be uneconomical for large-scale operations, it was considered feasible for pilot-scale studies, especially on expensive enzymes.

8.3.6 Catalase

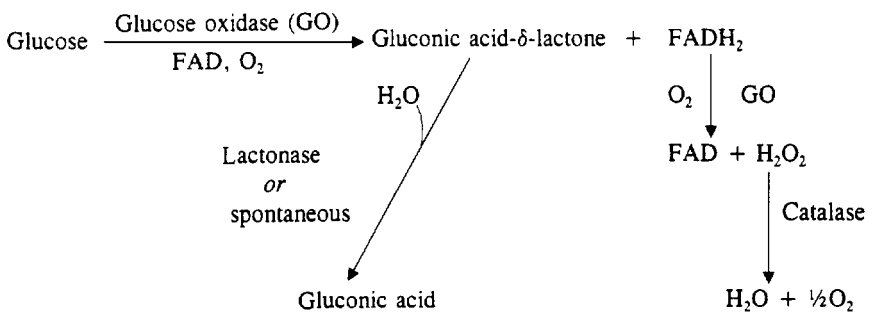
Hydrogen peroxide is a very effective chemical sterilant and although it causes some damage to the physicochemical properties and nutritional value

of milk protein, principally by oxidizing methionine, it is used as a milk preservative, especially in warm countries lacking refrigeration, and is permitted in the US for treatment of cheesemilk. Excess H_2O_2 may be reduced following treatment by soluble exogenous catalase (from beef liver, *Aspergillus niger* or *Micrococcus lysodeiktieus*). Immobilized catalase has been investigated for this purpose but the immobilized enzyme is rather unstable.

As discussed in section 8.3.7, catalase is frequently used together with glucose oxidase in many of the food applications of the latter; however, the principal potential application of glucose oxidase in dairy technology is for the *in situ* production of H_2O_2, for which the presence of catalase is obviously undesirable.

8.3.7 Glucose oxidase

Glucose oxidase (GO) catalyses the oxidation of glucose to gluconic acid (via gluconic acid-δ-lactone) according to the following reaction:



The H_2O_2 formed is normally reduced by catalase present as a contaminant in commercial GO preparations (from *P. notatum*, *P. glaucum* or *A. niger*) or added separately. Glucose oxidase, which has a pH optimum of about 5.5, is highly specific for D-glucose and may be used to assay specifically for D-glucose in the presence of other sugars.

In the food industry, glucose oxidase has four principal applications:

1. Removal of residual trace levels of glucose. This application, which is particularly useful for the treatment of egg white prior to dehydration (although alternative procedures using yeast fermentation are used more commonly), is of little, if any, significance in dairy technology.
2. Removal of trace levels of oxygen. Traces of oxygen in wines and fruit juices cause discolouration and/or oxidation of ascorbic acid. Chemical

reducing agents may be used to scavenge oxygen but enzymatic treatment with GO may be preferred. Glucose oxidase has been proposed as an antioxidant system for high-fat products such as mayonnaise, butter and whole-milk powder, but it does not appear to be widely used for this purpose, probably because of cost *vis-à-vis* chemical antioxidants (if permitted) and the relative effectiveness of inert gas flushing in preventing lipid oxidation in canned milk powder.

3. Generation of H_2O_2 *in situ*. The H_2O_2 generated by glucose oxidase has a direct bactericidal effect (which appears to be a useful side-effect of GO applied to egg products) but its bactericidal properties can be much more effectively exploited as a component of the lactoperoxidase – H_2O_2 – SCN^- system. Glucose required for GO activity may be added or produced by the action of β-galactosidase on lactose (both β-galactosidase and glucose oxidase have been immobilized on porous glass beads). H_2O_2 may also be generated *in situ* by the action of xanthine oxidase on added hypoxanthine. It is likely that exogenous H_2O_2 will be used in such applications rather than H_2O_2 generated by glucose oxidase or xanthine oxidase.
4. Production of acid *in situ*. Direct acidification of dairy products, particularly cottage and Mozzarella cheeses, is fairly common. Acidification is normally performed by addition of acid or acidogen (usually gluconic acid-δ-lactone) or by a combination of acid and acidogen. *In situ* production of gluconic acid from added glucose or from glucose produced *in situ* from lactose by β-galactosidase or from added sucrose by invertase has been proposed; immobilized glucose oxidase has been investigated. However, it is doubtful whether immobilized glucose oxidase could be applied to the acidification of milk because of the high probability of fouling by precipitated protein, even at low temperatures, which would lead to less casein precipitation. We are not aware if glucose oxidase in any form is used commercially for direct acidification of milk. Production of lactobionic acid from lactose by lactose dehydrogenase has also been proposed for the direct acidification of dairy and other foods.

8.3.8 Superoxide dismutase

Superoxide dismutase (SOD), an indigenous enzyme in milk, was discussed in section 8.2.10. A low level of exogenous SOD, coupled with catalase, was shown to be a very effective inhibitor of lipid oxidation in dairy products. It has been suggested that SOD may be particularly useful in preserving the flavour of long-life UHT milk which is prone to lipid oxidation. Obviously, the commercial feasibility of using SOD as an antioxidant depends on cost, particularly *vis-à-vis* chemical methods, if permitted.

8.3.9 Exogenous enzymes in food analysis

Exogenous enzymes have several applications in food analysis (Whitaker, 1991). One of the principal attractions of enzymes as analytical reagents is their specificity, which eliminates the need for extensive clean-up of the sample and makes it possible to quantify separately closely related molecules, e.g. D- and L-glucose, D-and L-lactic acid, which are difficult to quantify by chemical or physical methods. Enzymatic assays can be very sensitive; some can detect concentrations at the picomole level. Enzymes can be immobilized as enzyme electrodes and as such can be used continuously to monitor changes in the concentration of a substrate in a product stream. Disadvantages of enzymes as analytical reagents are their relatively high cost, especially when few samples are to be analysed, relatively poor stability (due to denaturation or inhibition) and the need to use highly purified enzymes.

Enzymes are rarely used by industrial food laboratories but find regular application in more specialized analytical or research laboratories. Important applications are summarized in Table 8.5 (see Boehringer Mannheim (1986) for methods). There are alternative chemical and/or physical methods, especially some form of chromatography, for all these applications, but extensive clean-up and perhaps concentration may be required.

The use of luciferase to quantify ATP (Blum and Coulet, 1994) in milk is the principle of modern rapid methods for assessing the bacteriological quality of milk based on the production of ATP by bacteria. Such methods have been automated and mechanized.

Table 8.5 Some examples of compounds in milk that can be analysed by enzymatic assays

Substrate	Enzyme
D-Glucose	Glucose oxidase; glucokinase; hexokinase
Galactose	Galactose dehydrogenase
Fructose	Fructose dehydrogenase
Lactose	β-Galactosidase, then analyse for glucose or galactose
Lactulose	β-Galactosidase, then analyse for fructose or galactose
D- and L-Lactic acid	D- and L-Lactate dehydrogenase
Citric acid	Citrate dehydrogenase
Acetic acid	Acetate kinase + pyruvate kinase + lactate dehydrogenase
Ethanol	Alcohol dehydrogenase
Glycerol	Glycerol kinase
Fatty acids	Acyl-CoA synthetase + Acyl-CoA oxidase
Amino acids	Decarboxylases; deaminases
Metal ions (inhibitors or activators)	Choline esterase; luciferase; invertase
ATP	Luciferase
Pesticides (inhibitors)	Hexokinase; choline esterase
Inorganic phosphate	Phosphorylase a
Nitrate	Nitrate reductase

Enzyme-linked immunosorbent assays. An indirect application of enzymes in analysis is as a marker or label in enzyme-linked immunosorbent assays (ELISA). In ELISA, the enzyme does not react with the analyte; instead, an antibody is raised against the analyte (antigen or hapten) and labelled with easily assayed enzyme, usually a phosphatase or a peroxidase. The enzyme activity is proportional to the amount of antibody in the system, which in turn is proportional, directly or indirectly depending on the arrangement used, to the amount of antigen present (Morris and Clifford, 1984).

Either of two approaches may be used: competitive and non-competitive, each of which may be used in either of two modes.

Competitive ELISA

On the basis of enzyme-labelled antigen

The antibody (Ab) is adsorbed to a fixed phase, e.g. the wells of a microtitre plate. An unknown amount of antigen (Ag, analyte) in the sample to be assayed, together with a constant amount of enzyme-labelled antigen (Ag–E), are then added to the well (Figure 8.2b). The Ag and Ag–E compete for the fixed amount of Ab and amount of Ag–E bound is inversely proportional to the amount of Ag present in sample. After washing away the excess of unbound antigen (and other materials), a chromogenic substrate is added and the intensity of the colour determined after incubation for a fixed period. The intensity of the colour is inversely proportional to the concentration of antigen in the sample (Figure 8.2b).

On the basis of enzyme-labelled antibody

In this mode, a fixed amount of unlabelled antigen (Ag) is bound to microtitre plates. A food sample containing antigen is added, followed by a fixed amount of enzyme-labelled antibody (Ab–E) (Figure 8.2a). There is competition between the fixed and free antigen for the limited amount of Ab–E. After an appropriate reaction time, unbound Ag (and other materials) are washed from the plate and the amount of bound enzyme activity assayed. As above, the amount of enzyme activity is inversely proportional to the concentration of antigen in the food sample.

Noncompetitive ELISA. The usual principle here is the sandwich technique, which requires the antigen to have at least two antibody binding sites (epitopes). Unlabelled antibody is first fixed to microtitre plates; a food sample containing antigen (analyte) is then added and allowed to react with the fixed unlabelled antibody (Figure 8.3). Unadsorbed material is washed out and enzyme-labelled antibody then added which reacts with a second site on the bound antigen. Unadsorbed Ab–E is washed off and enzyme activity assayed; activity is directly related to the concentration of antigen.

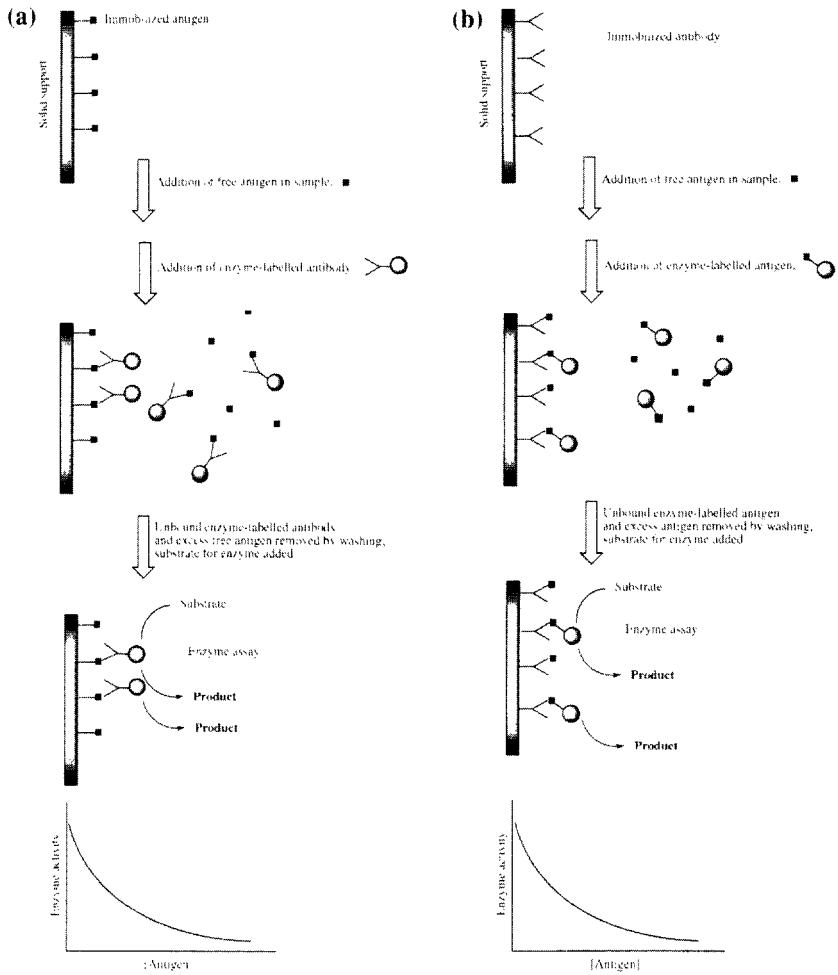


Figure 8.2. Schematic representation of a competitive enzyme-linked immunosorbent assay using (a) immobilized antigen or (b) immobilized antibody.

Examples of the use of ELISA in dairy analyses include:

- quantifying denaturation of β-lactoglobulin in milk products (native and denatured β-lg react differently with antibodies);
- detection and quantitation of adulteration of milk from one species with that from other species, e.g. sheep's milk by bovine milk;
- authentication of cheese, e.g. sheep's milk cheese;
- detection and quantitation of bacterial enzymes in milk, e.g. from psychrotrophs;

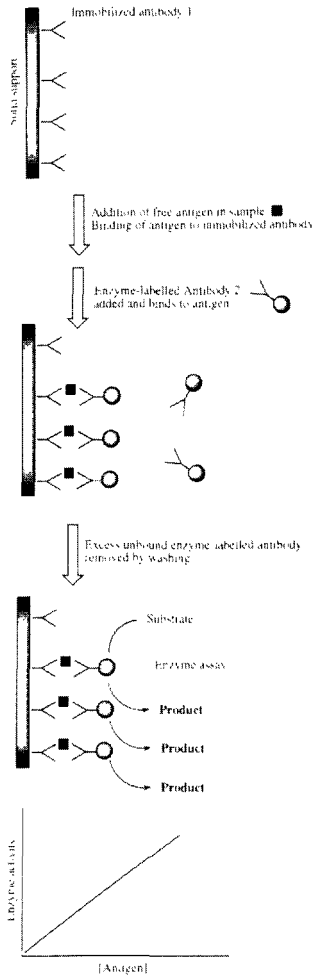


Figure 8.3. Schematic representation of a non-competitive enzyme-linked immunosorbent assay using the 'sandwich' technique.

- quantitation of antibiotics;
- potential application of ELISA includes monitoring proteolysis in the production of protein hydrolysates or in cheese.

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