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Enzymes in Milk and Cheese Industries

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2.1 INTRODUCTION

Milk and milk related products form an important part of human nutrition, and are essential both to improve health and support the growth process in children. Milk contains more than 60 different indigenous enzymes [1]. Current achievements in separation techniques in the dairy industry and enzyme technology offer opportunities to isolate, concentrate or modify these compounds, so that their application in functional foods, dietary supplements, nutraceuticals and medical foods has become possible [2]. Among the most significant features of indigenous milk enzymes are those used as indices of animal health, the thermal history of milk, deterioration of product quality, and the ability to create desirable changes in dairy products and protective effects [1]. The principal exogenous 'dairy' enzymes are proteinases and lipases of microbial and animal origin. The aim of this chapter is to emphasize current trends in the enzymology of the milk and cheese industries. The chapter is divided into two parts, which distinguish selected enzymes involved in milk processing and in cheese production. The main enzymes in milk and cheese industries as well as their applications are summarized in *Table 2.1*.

Table 2.1 Enzymes in milk and cheese industries

Enzymes	Applications
Lactoperoxidase	Milk preservation at ambient temperature (antimicrobial effect)
β-Galactosidase/Lactase	Hydrolysis of lactose to glucose and galactose in whey and whole milk

- *Membrane vesicles*: The secretion of various membrane vesicles into the extracellular space is a frequent phenomenon described in normal and tumour cells [4]. Two types of vesicles are secreted by cells. Exosomes (typically 40–100 nm in diameter) originate from endocytic multivesicular bodies, and are released in an exocytic manner. Although the functions of exosomes remain largely unresolved, they are thought to play immunoregulatory and antitumoral roles. Microvesicles (with a diameter in the range 100–1000 nm) originate from the cell surface membrane and are shed directly into the extracellular space, a process that seems to be important for membrane turnover, tumor ganglioside metabolism and vascular regulation.
- *Milk cells*: The milk of various mammals contains a heterogeneous population of cells, commonly referred to as somatic cells (SC). In most species, the predominant cells are leukocytes, composed of lymphocytes, polymorphonuclear neutrophils (PMNs) and macrophages, which serve as important components in the mammary defense against potential pathogens, mostly bacteria.

About 70 indigenous enzymes have been reported in normal bovine milk [5]. The indigenous enzymes are constituents of milk as secreted and arise from four principal sources: blood plasma, secretory cell cytoplasm, the milk fat globule membrane and somatic cells.

2.2 ENZYMES IN MILK PROCESSING

2.2.1 Milk Preservation

2.2.1.1 Endogenous milk enzymes

The preservation methods applied to milk depend on whether it is preserved in liquid form or as a processed dairy product, e.g. cheese or yogurt. Proliferation of contaminating bacteria may render milk unfit for consumption within a relatively short time. The bacterial growth can be retarded by refrigeration, but this may not always be readily available, particularly in developing countries. Milk contains an indigenous antimicrobial defence in the form of lactoperoxidase (LP), which is present in bovine milk at relatively high concentrations (30 mg ml^{-1}). This varies from species to species, being approx. 5% of this in human milk [6]. The concentration is highest in colostrum and decreases during the first few days of lactation. LP is a haem-containing protein, which binds calcium and is glycosylated. It is activated on addition of low concentrations of hydrogen peroxide and thiocyanate, which are present in milk at varying concentrations. The three together comprise the lactoperoxidase system. The antimicrobial activity in drawn milk is quite weak, but this can be re-activated by adding low concentrations of thiocyanate and hydrogen peroxide (15 mg kg^{-1} SCN^- and 8.5 mg kg^{-1} H_2O_2) [7]. This can extend the shelf life at 30°C to 7–8 hours, which allows milk producers in tropical regions to transport their product to a milk processing centre in the absence of refrigeration [8]. However, use of this method does not abrogate the requirement for good hygiene standards by milk producers. The active oxidation products are then removed by pasteurisation. Catalase can also be added to degrade any remaining H_2O_2.

The antimicrobial activities of the LP system against both Gram-positive and Gram-negative bacteria include growth inhibition, decreased oxygen uptake and lactic acid production, inhibition of microbial enzymes and structural damage to membranes [9]. The system extends the storage time for raw milk at 10°C by retarding the growth of psychrotrophic microorganisms [6]. LP is relatively thermostable, retains activity after pasteurisation at 72°C for 15 s, and is a contributory factor to the preservation of pasteurised milk [10]. One application for the lactoperoxidase system is addition to reconstituted infant formula, in which it can inhibit the enteropathogen *Enterobacter sakazakii, w*hich can cause neonatal bacteraemia and meningitis [11]. Xanthine oxidoreductase (XO) produces H_2O_2, a highly reactive antimicrobial agent, as a by-product of the metabolism of xanthine, thereby contributing to the bactericidal capability of milk. It also converts nitrate to nitrite, which can be metabolised to NO [3]. XO is the most abundant enzyme in milk, followed by LP [5]. These components may be regarded as part of the mammary innate immune system, which has bactericidal and bacteriostatic effects and protects the mammary gland from infection during milk stasis [3].

Lysozyme also comprises part of the indigenous antimicrobial defences of mammalian milk. Lysozyme hydrolyzes of the β1-4 linkages between *N*-acetyl muramic acid and *N*-acetyl glucosamine in the peptidoglycan layers of the cell wall of Gram-positive bacteria [12]. High hydrostatic pressure homogenization, coupled with addition of lactoperoxidase and lysozyme, led to enhanced antimicrobial efficacy against a selected group of Gram-positive and Gram-negative species inoculated in skim milk. It was suggested that the high pressure induced conformational changes in the enzymes, which enhanced their activity [13]. Lysozyme can preserve milk by reducing bacterial counts without reducing the *Lactobacillus bifidus* activity [14].

Preservation of milk products using exogenous enzymes: The preservation of milk is frequently carried out by converting it to a range of dairy products, including cheese and fermented foods e.g. yogurt and kefir. Bovine chymosin (rennet) is used in cheese manufacture to curdle milk and this function is now carried out by microbial rennet-like proteases for approximately one-third of cheese production worldwide. A range of microbial proteases, including those from *Rhizomucor pusillus*, *R. miehei*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lactis* are used in cheese manufacture [14]. Recombinant calf chymosin is also available, and a method for producing this has been patented by Sudershan Biotech Limited [8]. Studies have also been carried out to investigate the use of recombinant lamb chymosin, which has comparable properties to the recombinant calf enzyme [15].

2.2.1.2 Indigenous enzymes involved in antimicrobial and antiviral activity of milk

Lysozyme, xanthine oxidase, ribonucleases, abzymes (catalytic antibodies) with nuclease activity [16] and also some minor recently identified proteins, angiogenin, lactogenin and glycolactin, with RNAse activity [17] are directly antibacterial and/or antiviral. Lactoferrins from bovine, human and caprine milk are antibacterial and antiviral proteins, but pepsin and chymosin release cationic, broad-spectrum antimicrobial peptides from lactoferrins called lactoferricins, which may have 100 times higher antimicrobial potency than lactoferrins [18]. Pepsin also released two antimicrobial peptides from bovine α_{s2}-CN. Digestion of α-lactalbumin with

trypsin yielded a hydrolysate containing two antimicrobial peptides, and one antimicrobial peptide was identified in the chymotrypsin hydrolysate of β-lactoglobulin [19,20].

Xanthine oxidase (XO) is a complex metalloenzyme that acts on many substrates. In cheese, XO reduces nitrate to nitrite, which inhibits the germination of *Cl. tyrobutiricum* spores [21]. Human and bovine milk inhibit the metabolic activity of *E. coli*. Inhibition was dependent on both XO activity and on the presence of nitrite, implying that XO-generated nitric oxide functions as an antibacterial agent [22]. Nucleases are both antibacterial and antiviral. Catalytic antibodies (abzymes) from human milk and bovine colostrum hydrolyze both DNA and RNA. They also hydrolyzed polyadenylic acid, which is not hydrolyzed by RNAses [23].

2.2.2 Hydrolysis of Lactose in Milk and Whey

2.2.2.1 Lactose intolerance in humans

Lactose is the most abundant sugar in milk from most mammalian species, comprising approximately 7% (w/v) of human milk and 5% (w/v) of bovine milk. This sugar is degraded in the mammalian gut by lactase, alternatively known as lactase/phlorizin hydrolase, bound to microvilli on the apical surface of intestinal epithelial cells. The enzyme hydrolyzes dietary lactose to glucose and galactose, which are transported across the membrane into the cytosol of intestinal epithelial cells. The monosaccharides are then absorbed and distributed in the blood. Levels of this enzyme tend to be high in neonatal mammals, but decline as the animal's diet changes from being milk-based.

A relationship between cow's milk consumption and diarrhoea has been recognised for over a century. The prevalence of lactose intolerance depends on ethnic and racial origin, being relatively uncommon in people of northern European descent and more common in people of African and Asian descent [24]. The prevalence of lactose intolerance in different ethnic groups has been studied extensively [25,26] and is shown in *Table 2.2*. The undigested lactose in the large intestine acts as a fermentable substrate for the bacteria in the colon, as well as being osmotically active and drawing in water. The sugars are fermented by the colonic microflora, producing CO_2, hydrogen and methane, leading to the characteristic symptoms of diarrhoea, cramping, nausea, bloating, and intestinal gas [27]. However, the development of symptoms appears to depend on various factors, e.g. the quantity of lactose consumed, gastric emptying, intestinal transit time, hydrolysis by colonic microbial β-galactosidases, and the food in which lactose was consumed [28].

Table 2.2 Worldwide prevalence of lactose intolerance in adult subjects

Ethnic group	Lactase deficiency (%)
Northern European	1 – 5
Middle European (English, Russian)	10 – 20
Mediterranean (Greeks, Jews)	60 – 90
African and African-American	70 – 100
Native Americans	80 – 100
Asian	80 – 100
Mexican American	50 – 80
Bedouin (Saudi Arabia, Jordan)	< 25

Source: Ref. No. 25, 26.

permeate through the column of enzyme-derivatized beads at 30–50°C. This leads to removal of 80% of the lactose after 15–25 min. Valio Ltd. in Finland uses β-galactosidase immobilized on a phenol-formaldehyde resin in a fixed-bed column reactor to remove lactose [32]. An alternative approach to lactose removal is acid hydrolysis. This can be carried out either by adding acid or passing the whey over an acid cation-exchange resin. However, this cannot be applied to solutions containing protein, due to acid denaturation of protein, the whey must be demineralized to prevent acid inactivation, and undesirable by-products can be formed [37].

Bioremediation technologies for cheese whey, associated with reduction of chemical oxygen demand (COD) of the treated waste at elevated temperatures, were developed [38,39]. This novel approach is a potential application of the standards for food industry environmental management systems, notably ISO 14000. The bioremediation processes for the treatment of cheese whey involved use of a mixed population, including *Bacillus* sp. isolated from fruit-vegetable wastes, at elevated temperatures (55, 60 and 65°C) in an aerated system. High COD removal efficiencies (up to 93% at 55°C, and up to 70% at 65°C) were achieved.

Lactose removal is not only desirable for whey bioremediation but also low-lactose wheys have various applications in food products. For example, lactose in ice-cream manufacture can give rise to ice cream with a 'sandy' texture due to crystallization of lactose, and crystallized lactose can form in confectionery made with whey solids. Higher levels of whey solids can be used if these are low in lactose. Low-lactose whey can also be used to replace ordinary whey or milk powder in baked goods. This can improve dough development and crumb texture. The main application is in whey fermentation, but the yield of alcohol is limited by the slow metabolism of galactose [32].

2.2.2.3 Lactose hydrolysis in milk

Lactase action on milk products can arise by two main pathways: addition of exogenous enzyme in biotechnological processes, or by fermentation. The commonest fermented products derived from milk are yogurt and kefir. Yogurt is made by adjusting the composition of the solids, pasteurizing the mixture, and adding a fermentation culture. Commonly, two species of lactic acid bacteria are added: *Streptococcus salivarius* (ssp. *thermophilus*) and *Lactobacillus delbrueckii* (sp. *bulgaricus*). These microorganisms produce a wide range of enzymes, including proteases and various glycosidases, including β-galactosidases. *S. thermophilus* grows initially at pH 4.2-4.4, and this is followed by *Lactobacillus* growth at pH values as low as 3.5–3.8. This adds aroma and flavour to the mixture [40]. The conversion of lactose to lactic acid by these microorganisms gives these products their characteristic acidic taste. Kefir, a traditional fermented milk drink from the Caucasus made with kefir grains, in contrast contains not only fermentative bacteria but a yeast, *Torula,* which produces ethanol and CO_2. The ethanol content can be as high as 1%.

Hydrolysis of lactose in whole milk and milk products is accomplished by the action of lactase, which is a β-galactosidase (*Figure 2.2*). Beta-galactosidases are classified as EC 3.2.1.2 3/108 and are grouped in 4 separate glycosyl hydrolase families [41].

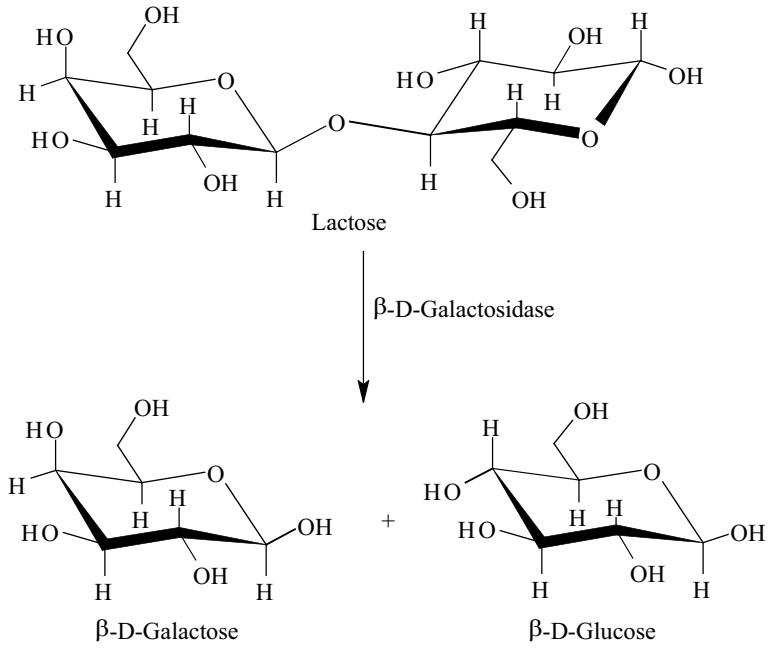

Figure 2.2 Hydrolysis of lactose by β-D-Galactosidase

Some of these enzymes use lactase as their natural substrate (GH 1 and 2), whereas β-galactosidases from other families hydrolyze a diversity of β-linked galactose residues (GH35 and GH42). All lactases can be classified as β-galactosidases, whereas not all β-galactosidases hydrolyze lactose. Many β-galactosidases produced by plant and mammalian cells hydrolyze cell wall polysaccharides, galactolipids or glycoproteins and do not hydrolyze lactose to any significant extent. Family GH1 includes the intestinal enzyme, which is a multidomain, membrane-bound glycosylated enzyme. One domain (domain III) is active towards glycosides, whereas domain IV specifically hydrolyzes lactose [42]. This family also includes the lactic acid β-glycosidases and bacterial cellobiases. Family GH2 includes various microbial lactases, including the *Escherichia coli* enzyme and fungal lactases. The *E. coli* enzyme, encoded by the *lac*Z gene, has been extensively studied, and has served as a valuable model for understanding the enzyme's mechanism of action, as well as immobilization studies. However, as *E. coli* is a coliform bacterium, the enzyme is not considered appropriate for use in food.

Commercially available lactases are derived from a variety of sources, including fungi, bacteria, plants and animals. However, safety considerations limit the microorganisms that can be used, and common commercial sources include *Aspergillus niger*, *A. oryzae*, and *K. lactis*, which are well-characterized. *Table 2.3* illustrates a range of microbial sources of β-galactosidases known to act on lactose [32]. The properties of these enzymes depend on the source. Fungal lactases generally have acidic pH optima (2.5-4.5), whereas the bacterial enzymes tend to have optima tending towards more neutral pH values (6.5-7.5) and those of yeasts tend to be closer to 6-7. These variations make lactases from different sources suitable for different applications. The acidic pH optimum of the fungal enzymes makes them suitable for use in acid whey processing, whereas the yeast and bacterial enzymes are more suited to processes taking place at higher pH values, e.g. milk and sweet whey processing [14]. The sensitivity to inhibition by galactose varies from enzyme to enzyme. For example, the *A. niger* enzyme is more strongly inhibited by galactose than the *A. oryzae* lactase [43].

Lactases also have varying temperature optima, depending on source. Thermostable enzymes have the advantage of activity at higher temperatures that are less liable to microbial contamination, as well as higher conversion rates. The enzyme from *K. lactis*, although widely used, is not thermostable, which is a drawback to its use. This enzyme is well suited to hydrolysis of lactose in milk, which contains K^+ and Mg^{2+} ions needed for its activity, but its requirement for moderate temperatures also means that microbes readily grow during the process. To minimize this, high concentrations of the enzyme are used for short periods (2-3 h) or the incubations are carried out at lower temperatures (4-6°C) overnight [32]. β-galactosidases from thermophiles have been explored as catalysts for lactose hydrolysis, e.g. that from *Thermotoga maritima*, which as a temperature optimum of 80°C for lactose hydrolysis [44].

For some purposes, cold-adapted β-galactosidases have an advantage, as these enzymes are active at lower temperatures that are suitable for shipping and storage of dairy products. Interest in such enzymes for milk processing has promoted research into β-galactosidases from microorganisms found in permanently cold environments, e.g. the Antarctic. Turkiewicz *et al.* [45] showed that β-galactosidase from the cold-adapted and halotolerant bacterium *Pseudoalteromonas* sp. 22b had 11-35% of its maximum activity at 0-20°C and was capable of degrading lactose in refrigerated milk and milk products. The enzyme was activated by Na^+ and was not inhibited by Ca^{2+} or galactose. This enzyme was immobilized on glutaraldehyde-treated chitosan beads and maintained activity for at least 40 days of continuous lactose hydrolysis at 15°C. It was stable for over 12 months at 4°C and active at 4-30°C [46]. Other *Pseudoalteromonas* isolates have been explored as sources of cold-active β-galactosidase [47] and an enzyme capable of lactose hydrolysis was isolated and immobilized.

Various processes have been developed to hydrolyze lactose in milk. The enzyme is commonly added to pasteurized milk in a tank at 5°C, left to hydrolyze the lactose until 70-100 % hydrolysis is obtained, and the milk is then re-heated to inactivate the enzyme and packed for sale. This also extends the milk's shelf life. Milk that is sterilized by UHT treatment is treated with *K. lactis* enzyme that has been sterilized by ultrafiltration just before aseptic packaging. This process requires very low concentrations of enzyme, and can produce complete hydrolysis over 7-10 days at room temperature. This process is relatively inexpensive and limits the enzyme and processing costs, but the β-galactosidase needs to be very pure and devoid of proteolytic activity, otherwise milk protein hydrolysis can occur during storage. Immobilized enzymes have also been used to deplete milk of lactose. For example, a batch process has been developed in the Centro Sperimentale del Latte in Milan, Italy using *K. lactis* β-galactosidase immobilized in porous cellulose acetate fibres that allow lactose diffusion but retain the enzyme in solution in the microcavities of the fibre. The process stabilizes the enzyme and retails other milk proteins, but is slow [32].

Lactose-hydrolyzed milk has found application in various food products, particularly those in which lactose crystallization affects texture or flavour. Its use can give rise to increased sweetness in flavoured milks, yoghurt and ice cream, allowing the concentration of added sucrose to be reduced. In yoghurt manufacture, use of low-lactose milk allows faster carbohydrate fermentation, accelerated acid development in starter cultures, and a faster set time. It also allows the more rapid growth of starter organisms in cheese production, thereby leading to a reduction in ripening time. This process also yields low-lactose whey as a by-product [32].

2.2.3 Bioactive Peptides

Milk proteins are the main source of various biologically active peptides. These are encrypted in the sequences of casein and whey proteins and can be released by proteolysis during gastrointestinal digestion, food processing or breakdown by microbial enzymes, e.g. those found in lactic acid bacteria. Their function is hormone-like, and they have potential nutraceutical and pharmaceutical applications [48]. Among the best-studied are inhibitors of angiotensin-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1), which raises blood pressure by converting angiotensin I released from angiotensinogen by renin into angiotensin II, a potent vasoconstrictor. ACE also degrades bradykinin, a vasodilator, and stimulates aldosterone release. As a result, ACE inhibitors are used clinically as antihypertensive agents. A summary of these peptides, derived largely from α-lactalbumin and β-lactoglobulin, and their properties, is given in [49,50]. Caseins are also a rich source of antithrombotic, antihypertensive, and opioid peptides. The major, and first, opioid peptides discovered were the so-called β-casomorphins, derived from β-casein, but a_{s1}, γ, and κ-caseins also give rise to various opioid-like peptide sequences [51]. One a_{s1} casein-derived peptide showed anxiolytic-like stress-relieving properties in an animal model and human studies [52]. Milk protein hydrolysates and milk-derived peptides can enhance immune functions, e.g. lymphocyte proliferation, antibody synthesis and lymphocyte proliferation [53] and in particular, peptides released by lactic acid bacteria can down-regulate production of some cytokines and stimulate phagocytic activity of macrophages [54].

Milk proteins are also a rich source of antimicrobial peptides, of which lactoferricin, a fragment of lactoferrin, is the best-known, possessing more potent antimicrobial properties than lactoferrin itself [55]. Upon oral administration, bioactive peptides, may affect the major body systems, namely, the cardiovascular, digestive, immune and nervous systems (*Figure 2.3*), depending on their amino acid sequence. In the future, milk-derived bioactive peptide may be important components in food sustaining health and in the prevention of diseases and conditions such as cardiovascular diseases, obesity, osteoporosis, and stress [56].

or all cheese varieties. The gene for calf chymosin (or prochymosin) has been cloned in selected prokaryotic and eukaryotic microorganisms; thus an unlimited supply of high-quality microbial rennet is now available [57, 58].

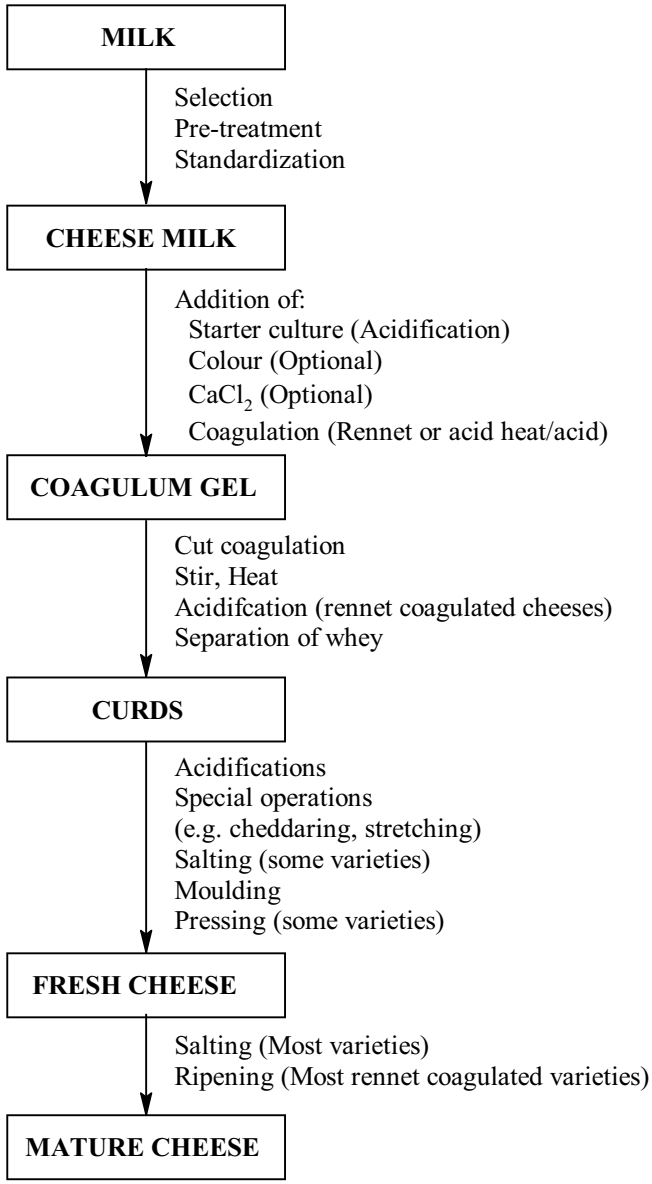

Figure 2.4 Cheese production and separation of whey. *Source*: Ref. No. 70.

During cheese ripening, microbiological and biochemical changes occur that result in the development of the flavour and texture characteristic of the particular variety [59,60]. The basic cheese-ripening biochemistry is shown in *Figure 2.5*. The biochemical changes in cheese during ripening may be grouped into primary (lipolysis, proteolysis and metabolism of residual

lactose and of lactate and citrate) or secondary (metabolism of fatty acids and of amino acids) events [60]. The biochemistry of cheese ripening is an active area of research and aspects of ripening have been reviewed extensively [61-67]. As an initial biochemical event of ripening, residual lactose is metabolized rapidly to lactate. Lactate is an important precursor for a series of reactions including racemization, oxidation or microbial metabolism. Citrate metabolism is of great importance in certain varieties. Lipolysis in cheese is catalyzed by lipases from various sources, particularly the milk and cheese microflora, and, in varieties where this coagulant is used, by enzymes from rennet paste.

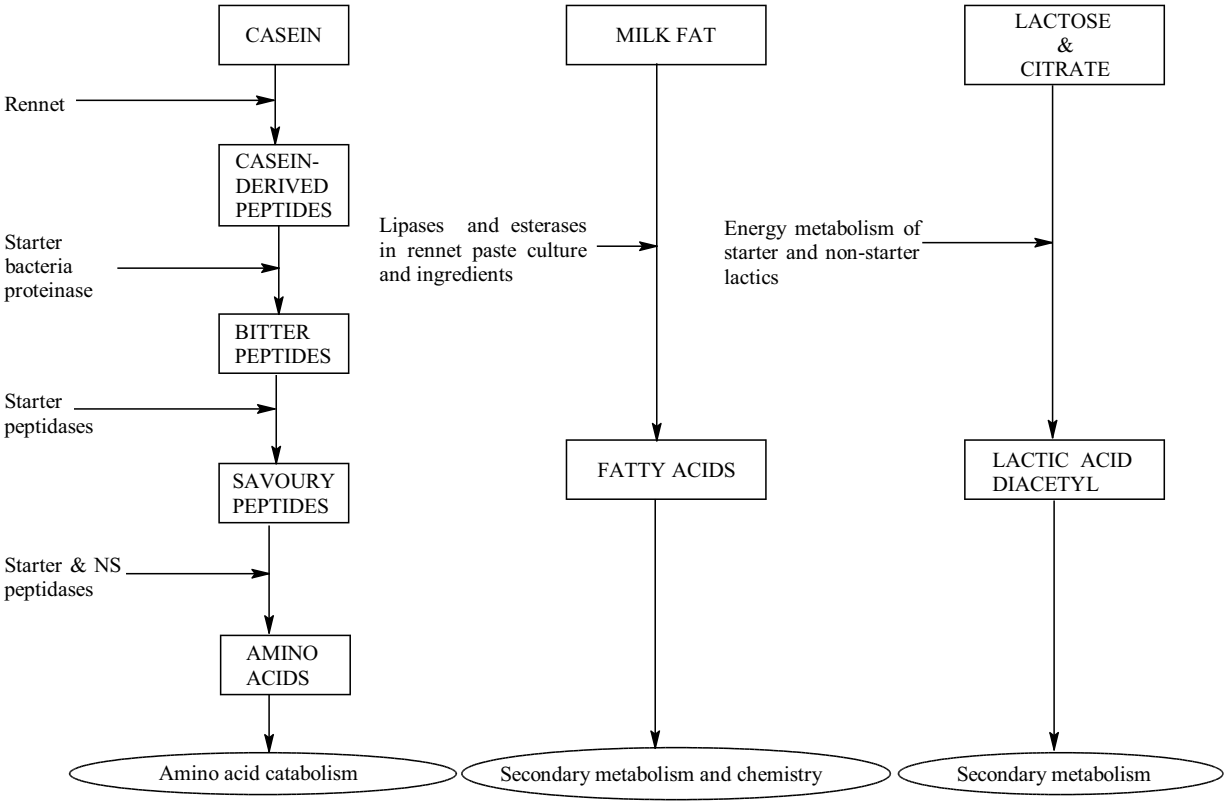

Figure 2.5 Basic cheese ripening biochemistry. *Source*: Ref. No. 59; reproduced with permission of Springer Sci. & Business Media.

2.3.2 Lipolysis

Milk contains a potent indigenous lipase, lipoprotein lipase (LPL), with a molecular mass of 55 kDa that exists in milk as a homodimer. The physiological role of this enzyme is in the metabolism of plasma triglycerides, and it enters milk from the blood [68]. Under optimum conditions, the levels of LPL in milk are enough to cause perceptible rancidity in milk within about 10 s [69]. This does not happen under normal circumstances as milk fat is protected from the action of LPL by the milk fat globule membrane (MFGM) and about 90% of LPL is

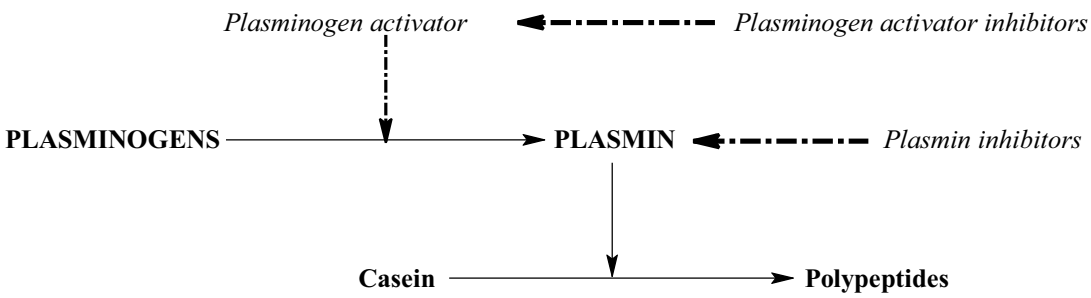

Figure 2.6 Plasmin/plasminogen system in milk. *Source*: Ref. No. 83, reproduced with permission.

2.3.5 Proteinases from Somatic Cells

Milk also contains other indigenous proteinases originating from the leucocytes of somatic cells. Polymorphonuclear leucocytes (PMNs) are the predominant somatic cells that enter milk during mastitis. Lysosomes of somatic cells contain aspartyl (acid) proteinases cathepsins D and E, cysteine (thiol) neutral proteinase cathepsin B, L and H and neutral serine-type proteinases, cathepsin G and elastase [83]. Cathepsin D, cathepsin G and elastase are major proteinases of PMNs. Five molecular forms of cathepsin D were isolated from acid whey, the major forms being 46- and 45-kDa procathepsin D. Cathepsin G, with a molecular mass of 24–26 kDa, can occur as three iso-forms; the enzyme is present in milk even at low somatic cell counts. PMN elastase has a molecular mass of 24–30 kDa [84].

The pattern of proteolysis in many varieties may be summarized as follows: the caseins are hydrolyzed initially by residual coagulant activity retained in the curd and by plasmin (and perhaps other indigenous proteolytic enzymes) to a range of large and intermediate-sized peptides that are hydrolyzed by proteinases and peptidases from the starter LAB, NSLAB and perhaps secondary microflora to shorter peptides and amino acids [59-61].

2.3.6 Indigenous Phosphatases

Phosphatases catalyze the hydrolysis of the C–O–P linkage of a wide variety of phosphate esters. Milk contains one major indigenous acid phosphatase, one major indigenous alkaline phosphatase and two minor acid phosphatases; the minor enzymes may originate from leucocytes. Indigenous milk alkaline phosphatase consists of two subunits of 85 kDa each. This enzyme is inhibited by metal chelators. Indigenous milk acid phosphatase (AP) is a glycoprotein of molecular mass ~ 42 kDa. NaF and NaH_2PO_4 are examples of many known inhibitors, while ascorbic acid and L-cysteine are examples of several stimulators of this enzyme [85]. The velocity of peptide bond cleavage of non-phosphorylated synthetic peptide by trypsin has been compared with cleavage velocity of the same bond of synthetic peptide phosphorylated on serine residue located in closed proximity to the specifically cleaved peptide bond. Enzymatic cleavage was shown to be inhibited by phosphorylation of the serine residue [86]. APs may indirectly accelerate cheese ripening and the dephosphorylation of

microorganism showed considerable intracellular and/or extracellular proteolytic and lipolytic activities. Enzyme preparations from *B. linens* may therefore be used for flavour intensification in hard cheeses, especially in low-fat cheeses and for production of EMCs. Methionine γ-lyase was isolated from *B. linens*, *Pseudomonas* and many other bacteria [63,94].

2.4 SUMMARY AND FUTURE PROSPECTS

More than a century of study of milk enzymes has revealed the presence of a wide range of indigenous activities. Many of these have been the subject of intensive investigation for decades and some have found biotechnological application, but nevertheless significant gaps in our knowledge in relation to their nature, biological function and significance remain. The best characterized of these are the proteinases and peptidases. In order to control cheese ripening, protein-degrading enzymes from LAB have been well characterized, both biochemically and genetically. These, and other proteinases, may find application in the preparation of functional and bioactive protein hydrolysates, with potential applications in value-added or functional dairy or non-dairy foods. Other enzymes may also have a range of applications: for example, there is current interest in controlling amino acid catabolism in cheese, and controlling phosphorylation and fatty acid catabolism may also be important in future. Proteinases and peptidases are the most extensively investigated endogenous milk enzymes, and plasmin in particular is well characterized in terms of its control, stability, variation and impact on dairy products. The other indigenous proteinases in milk are less well studied and are less well understood: even their number remains unclear [95]. Some of these enzymes may have biotechnological applications, and knowledge of their action may also lead to a better understanding of the innate immune competence of milk, as they may have anti-bacterial and anti-viral properties. It is for this reason that further study of these enzymes may yield significant and interesting results.

Progress in the general field of dairy enzymology has also been accelerated by recombinant DNA technology. Cloning and modification of enzymes are likely to be more widely applied in future to facilitate isolation and purification of enzymes and to improve their catalytic properties and stability in industrial processes. Synthetic enzymes (synzymes) and catalytic antibodies (abzymes), which may be tailored for any synthetic activity, may also find application in the dairy industry. The development of new, non-thermal techniques is potentially challenging to dairy microbiologists and enzymologists. Enzymes from cold-adapted microorganisms, e.g. β-galactosidases or proteases, may be used in dairy processing, and such enzymes are being explored for lactose hydrolysis.

In conclusion, further exploration of bovine milk should be encouraged. It may lead to a more profound knowledge of its wholesomeness, as a source of potential novel biocatalysts, and as a source of novel health-promoting food ingredients, nutraceuticals, and antimicrobials.

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