SYMPOSIUM
CONTRIBUTION

Dairy enzymology

LESZEK STEPANIAK
Department of Chemistry, Biotechnology and Food Science, Agricultural University of Norway, Ås, Norway

The properties and the significance of the principal indigenous enzymes in milk, milk coagulants, enzymes from dairy microorganisms participating in cheese ripening, and spoilage enzymes are discussed. In particular, the properties of plasmin, lipases, phosphatases, enzymes from somatic cells, enzymes involved in antimicrobial and antiviral systems in milk, enzymes from lactic acid bacteria, propionibacteria, and microorganisms involved in smear- and mould-ripened cheese are reviewed. Some assay methods and the impact of some processing factors on selected enzymes are also discussed.

Keywords Dairy microorganisms, Enzymes, Milk.

INTRODUCTION

The aim of this review is to highlight current trends in dairy enzymology and only selected enzymes are discussed. The use of recent reviews and papers does not give sufficient recognition to the earlier works of Professor Fox, in whose honour the Symposium was organized, and the works of many other researchers.

Basic knowledge of enzymes, their structure and functions in relation to food enzymology, is presented by Whitaker *et al*. (2003). Milk contains more than 60 different indigenous enzymes (Fox 2003). The principal exogenous 'dairy' enzymes are proteinases and lipases of microbial and animal origin. 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 (Fox 2003). A comprehensive review by Fox and Wallace (1997) gives an insight into the complexity and significance of enzymatic reactions involved in cheese ripening by indigenous and exogenous enzymes.

PLASMIN SYSTEM IN MILK

Plasminogen is the zymogen of the serine-type proteinase, plasmin. Plasminogen and plasmin are glycoproteins. Plasminogen consists of an N-terminal preactivation peptide (PAP) followed by five 'kringle' structures and the serine proteinase domain. It also contains the only phosphorylated site. Plasmin activation is a key event in the fibrinolytic system, which results in the dissolution of blood clots. Plasmin also has a number of other physiologically important functions such as activation of some metalloproteinases and participation in wound healing (Parry *et al*. 2000). Milk contains the full plasmin system: plasmin, plasminogen, plasmin inhibitors, plasminogen activators of urokinase (uPA) and tissue (tPA) types, as well as inhibitors of plasminogen activators. The concentration of plasmin in fresh bovine milk is in the range 0.1–0.7 mg/L. The ratio of plasminogen to plasmin in milk has been reported to range from 50 : 1 to 2 : 1. The concentration of plasmin in blood is *c.* 200 mg/L. As revealed by comparison of the amino acid sequence, immunological and biochemical properties, the enzyme long known as alkaline milk proteinase is identical to plasmin from bovine blood plasma. Bovine milk apparently contains both 80- and 85-kDa plasminogens. Activation of bovine plasminogen by uPA or tPA involves cleavage of the Arg557–Ile558 bond. Autolytic cleavage at the Lys77–Arg78 bond releases the PAP fragment. Human plasmin is activated by cleavage of the Arg561–Val562 bond (Fox 1989; Benfeldt *et al*. 1995; Grappin and Beuvier 1997; Parry *et al*. 2000; Nielsen 2002; Kelly and McSweeney 2003). In the bovine system, autolytic cleavage of Arg342–Met343 also occurs, resulting in formation of a truncated form of plasmin, called midi-plasmin (see Benfeldt *et al*. 1995; Kelly and McSweeney 2003 for figures illustrating plasminogen structures and its activation).

Most bacterial species do not possess plasmin activators. However, some invasive human pathogens have evolved nonenzymatic protein activators (e.g. staphylokinase and streptokinase) of human blood plasminogen. Streptokinase activates plasminogen by binding to it, forming a 1 : 1 complexes. After binding to plasmin, staphylokinase changes its specificity (Parry *et al*. 2000).

Native plasminogen and plasmin possess a specific affinity for lysine residues. This affinity has been ascribed to 'kringle' structures. Chromatography

*Author for correspondence: E-mail: leszek.stepaniak@ikbm.nlh.no

on lysine–Sepharose was used to isolate plasminogen from milk (Benfeldt *et al*. 1995).

In milk, plasmin, plasminogen and plasminogen activators are associated with the casein (CN) micelles and are incorporated into rennet-coagulated cheese. Plasmin inhibitors and inhibitors of plasminogen activators are in the serum and are lost in whey (Fox and Stepaniak 1993; Grappin and Beuvier 1997). Proteinases from pseudomonads may disrupt casein micelles and release the plasmin system components into whey (Nielsen 2002).

When assayed on *N*-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin, inhibition of plasmin activity by whey proteins was observed at pH 6.5, while at pH 5.2 in the presence of 5% NaCl whey proteins apparently increased plasmin activity (Hayes *et al*. 2002).

Specificity and significance in dairy products

Plasmin is most active on β- and α_{s2}-CNs. It positively influences cheese ripening (Farkye and Fox 1992). One of most important effects of plasmin is degradation of β-CN to γ-caseins and proteose peptones. The occurrence of β-CN f29–209 (γ_1-CN), γ-CN f106–209 (γ_2-CN) and γ-CN f106–209 (γ_3-CN) in urea–polyacrylamide gel electrophoresis (PAGE) patterns is indicative of plasmin activity in cheese during ripening (Farkye and Fox 1992; Fox and McSweeney 1996; Grappin and Beuvier 1997). Plasmin can also degrade some peptides released by chymosin (Lane and Fox 1999).

Activation of plasminogen to plasmin in high temperature short time (HTST)-pasteurized milk is greater than in control milk. Swiss and Cheddar cheeses contain 6–13 and 3–4.6 μg plasmin/g, respectively. Hydrolysis of β-casein to γ-caseins is more rapid in Gouda and Swiss cheeses than in Cheddar. Replacement of some whey by water would be expected to remove more plasmin inhibitors from Gouda cheese. Higher pH and higher moisture content favours plasmin activity in Gouda cheese; the higher cooking temperature used for Swiss cheese appears to explain the high level of plasmin activity in these cheeses (Farkye and Fox 1992). Changes in viscosity observed in some non-dairy products supplemented with caseinate were attributed to active plasmin. Residual plasmin may cause defects of ultrahigh temperature (UHT)-treated milk (Grappin and Beuvier 1997; Nielsen 2002). Limited hydrolysis of casein in milk by plasmin had no clear effect on the rheology of rennet-coagulated gels (Considine *et al*. 2002b).

Measurement of plasmin and plasminogen activity in milk and cheese by enzymatic methods and infrared spectroscopy

In classic assay methods, a chromogenic substrate, such as D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide (Rollema *et al*. 1983), or a fluorogenic substrate, such as *N*-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (Saint-Denis *et al*. 2001), is used to assay plasmin activity. Plasminogen content is measured after addition of urokinase. The sensitivity and accuracy of assay methods was improved by addition of milk clarifying agents (Saint-Denis *et al*. 2001). Fourier-transform infrared spectroscopy in combination with multivariate statistical analysis was developed to determine concentrations of plasminogen and plasmin. Fourier self-deconvolution of subtracted water allowed individual quantification of plasmin and plasminogen added to a protein solution in the concentration range 0.38–1.8 mg/mL (Ozen *et al*. 2003).

PROTEINASES FROM SOMATIC CELLS

Bovine blood contains several different types of white blood cells, commonly called 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. 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 isoforms; the enzyme is present in milk even at low somatic cell counts. PMN elastase has a molecular mass of 24–30 kDa (McSweeney *et al*. 1995; Chitpinityol and Crabbe 1998; Considine *et al*. 1999, 2000, 2002a,b; Kelly and McSweeney 2003). The specificity of cathepsin D on α_{s1}- and β-CN was similar to that of chymosin. The enzyme hydrolysed all casein fractions and was more active on α_{s1}-CN than on β-CN and showed poor milk-clotting activity (McSweeney *et al*. 1995). Cathepsin G cleaved α_{s1}-casein at a minimum of 16 sites and β-CN at a minimum of 21 sites, some of which were also cleavage sites for chymosin, plasmin, elastase, cathepsin B or the cell envelope-associated proteinase (CEP) of *Lactococcus* (Considine *et al*. 2002a). Elastase cleaved β-casein at several sites. Some of the sites were identical with those cleaved by chymosin, plasmin or the CEP of *Lactococcus* (Considine *et al*. 1999). Elastase also showed broad specificity on α_{s1}-CN. The protein was cleaved at 25 identified sites (Considine *et al*. 2000). Cathepsin D was active in quarg (Hurley *et al*. 2000). Both elastase (which coagulated milk with concomitant proteolysis of casein) and cathepsin B influenced the rheology of chymosin-induced gels (Considine *et al*. 2002b).

MILK LIPOPROTEIN LIPASE

Milk contains a lipoprotein lipase (LPL) and bile salt-stimulated lipase (BSSL); the later enzyme is also present in pancreatic juice. LPL is well characterized; bovine LPL is a noncovalent homodimer of glycosylated subunits with 450 amino acid residues, five disulphide bridges and two oligosaccharide chains (Olivecrona *et al*. 2003).

The main substrates for LPL in blood are triglycerides associated with very low density lipoproteins and chylomicrons. LPL is relatively nonspecific and liberates fatty acids from *sn*-1 and *sn*-3 positions of mono-, di- or triglycerides and from the *sn*-1 position of phospholipids. LPL is activated by apolipoprotein C II both *in vitro* and *in vivo*. Hydrolysis of phospholipids is less dependent on the activator than triglyceride hydrolysis (Lambert *et al*. 2000). The majority (> 80%) of LPL in bovine milk is associated with the casein micelles. LPL is subject to strong product inhibition. Milk probably also contains LPL inhibitors. Thus, potential LPL activity in milk is more than 500 times higher than the actual activity.

'Spontaneous lipolysis' in cold-stored milk is due to the action of LPL, which can be stimulated by agitation, foaming and especially by rapid temperature changes. The LPL activity is lower in caprine than in bovine milk, although more caprine LPL is bound to the fat globules and better correlated to spontaneous lipolysis (Chilliard *et al*. 2003).

Bile salt-stimulated lipase in human milk

Human milk provides neonates with several physiologically important proteins not found in cows' milk, including BSSL, which compensates for the reduced lipolytic capability of the newborn intestine. BSSL enzyme is also present in gorillas', cats' and dogs' milks. The gene coding for human BSSL was successfully cloned and expressed in *Pichia pastoris* (Murasugi *et al*. 2001).

PHOSPHATASES: INDIGENOUS AND MICROBIAL

Phosphatases catalyse 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 (see Shakeel-ur-Rehman *et al*. 2003). The velocity of peptide bond cleavage of nonphosphorylated 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 (Hynek *et al*. 2002). APs may indirectly accelerate cheese ripening and the dephosphorylation of casein or phosphopeptides, thus making them susceptible to proteolysis. Milk AP is capable of dephosphorylating caseins but the role and the origin of indigenous and microbial APs in cheese are uncertain (Akuzawa and Fox 1998; Deutsch *et al*. 2000; Shakeel-ur-Rehman *et al*. 2003).

AP from the cell membrane of *Lactococcus* is probably a large spheroidal molecule of molecular mass 430 kDa, stable in 6 M urea. It released inorganic phosphate from phosphocasein peptides but it did not dephosphorylate sodium caseinate (Akuzawa and Fox 1998).

A tetrameric, ~26 kDa subunit, intracellular AP was isolated from *Lactobacillus curvatus*. The N-terminal sequence of the enzyme was found to be highly homologous with AP from *Lactobacillus plantarum* and less homologous with phosphatases from mammals, yeasts and *Escherichia coli*. The enzyme showed little dephosphorylating activity on casein (Magboul and McSweeney 1999b). The tetrameric, ~27 kDa subunit, AP from *Lb. plantarum* had very similar properties to AP from *Lb. curvatus* (Magboul and McSweeney 1999a). Bovine κ-casein was totally dephosphorylated at pH 7.5 by the catalytic subunit of a type 2A protein phosphatase from the yeast *Yarrowia lipolytica*; the dephosphorylation increased plasminolysis (Jolivet *et al*. 2000).

INDIGENOUS ENZYMES INVOLVED IN ANTIMICROBIAL AND ANTIVIRAL ACTIVITY OF MILK

Colostrum, milk and cheese whey are rich sources of immunoglobulins, and other biologically active proteins that may be used by the pharmaceutical industry as novel antimicrobial and antiviral therapeutic agents and by the food industry for preservation of foods other than milk (Walzem *et al*. 2002; Floris *et al*. 2003). Indigenous lactoperoxidase is a component of the well-known lactoperoxidase system, which, after activation by addition to milk of less than 10 mg/L of thiocyanate and hydrogen peroxide, inhibits both Gram-positive and Gram-negative bacteria (Pruitt 2003). Lysosyme, xanthine oxidase, ribonucleases, abzymes (catalytic antibodies) with nuclease activity (Wilkins and Board 1989; Fox and Stepaniak 1993; Stepaniak *et al*. 2003) and also some minor recently identified proteins, angiogenin, lactogenin and glycolactin,

with RNAse activity (Wang *et al.* 2002) are directly antibacterial and/or antiviral. Lactoferrins from bovine, human and caprine milk are antibacterial and antiviral proteins, but pepsin and chymosin release from lactoferrins, cationic, broad-spectrum antimicrobial peptides called lactoferricins, which may have 100 times higher antimicrobial potency than lactoferrins. Bactericidal fragments of bovine lactoferricins are resistant to digestion by gastric enzymes and are immunostimulative (Walzem *et al.* 2002). The most potent bovine and human lactoferricins are fragments 17–41(42) and 1–47 of bovine and human lactoferrin, respectively. Pepsin also released two antimicrobial peptides from bovine α_{s2}-CN. Digestion of α-lactalbumin with trypsin yielded hydrolysate containing two antimicrobial peptides, and one antimicrobial peptide was identified in the chymotrypsin hydrolysate of β-lactoglobulin (Floris *et al.* 2003; Schanbacher *et al.* 2003). Proteinases from lactic acid bacteria (LAB) released a number of bioactive peptides; proteinase from *Lactobacillus helveticus* released an antimicrobial peptide from human β-CN (Minervini *et al.* 2003).

Bovine milk is rich in lactoperoxidase, nucleases and xanthine oxidase but, compared to human milk, it contains very little lysozyme. Acting synergistically, lysozyme increases the antibacterial activity of lactoferrin. Commercial preparations of lysozyme are an interesting alternative to nitrate as an antisporulating agent, preventing the growth of *Clostridium tyrobutiricum* in cheese.

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 (Wilkins and Board 1989; Fox and Stepaniak 1993; Pruitt 2003). 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 (Hancock *et al.* 2002).

Nucleases are both antibacterial and antiviral. Catalytic antibodies (abzymes) from human milk and bovine colostrum hydrolyse both DNA and RNA. They also hydrolysed polyadenylic acid, which is not hydrolysed by RNAses (Stepaniak 2002; Stepaniak *et al.* 2003).

Table 1 Activity of some aspartyl proteinases on haemoglobin, synthetic substrate and inhibition by pepstatin (adapted from Kageyama 2002)

	Relative hydrolytic activity (%)		*Inhibition by pepstatin*[a]
Enzyme	*Haemoglobin*[b]	*APDT*[b]	K_i *(nM)*
Bovine chymosin	7–14[c]	6	0.05–1[c]
Porcine pepsin A	55–59[c]	70	70
Human gastricin	100	0	100

[a]Determined at pH 3–4
[b]Determined at pH 2
[c]Different values reported, see References in Kageyama (2002)
APDT, *N*-acetyl-L-phenylalanyl-L-diiodotyrosine

COAGULANTS

The manufacture of rennet-coagulated cheese is essentially an enzymatic process. With a few exceptions, the traditional rennets used in the principal dairying countries were prepared from the stomach of young calves, kids or lambs, the major enzyme being chymosin. Many proteinases can induce the coagulation of milk but most are too proteolytic or have an incorrect specificity and hence cause a reduced cheese yield or defective cheese.

Beside chymosin, bovine, porcine and chicken pepsins and aspartyl proteinases from *Rhizomucor pusillus*, *R. miehei* and *Cryphonectria parasitica* have been found to be more or less satisfactory for some 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 (Fox and Stepaniak 1993; Chitpinityol and Crabbe 1998; Aikawa *et al.* 2001).

Prochymosin is the zymogen for chymosin. Together with pepsinogens A, B and F and progastricins, which are precursors of pepsins A, B, F and gastricins, respectively, prochymosin belongs to a group of pepsinogens. Prochymosin and pepsinogen F are the main forms in the infant, while the three other pepsinogens predominate in adult animals (Kageyama 2002). Two aspartates in the centre of the active cleavage site, Asp32 and Asp215, function as catalytic residues; therefore, pepsins and chymosins are classified as aspartyl proteinases. Studies on the kinetics of pepsin action on long-chain synthetic peptides suggest that the catalytic site is an extended structure. Similar properties were found for chymosin. The secondary structure of chymosin consists mainly of β-sheets with a few α-helical segments. Since 1975, the three-dimensional structures of pepsins and chymosin were determined by X-ray diffraction techniques, greatly extending our insight into the mechanism of the catalytic action of these enzymes. That knowledge has led to the design of new inhibitors of aspartyl proteinases. The crystal structure of recombinant bovine chymosin has been determined using X-ray data, extending to 2.3 Å resolution. The resulting model includes all 323 amino acid residues, as well as 297 water molecules. The enzyme has an irregular shape with approximate maximum dimensions of $40 \times 50 \times 65$ Å (Chitpinityol and Crabbe 1998; Fruton 2002; Kageyama 2002). An indication of the specificity of some aspartyl proteinases is given in Table 1.

The major cleavage site in the oxidized insulin B chain by chymosin at pH 2 is Leu15–Tyr16. Contrary to pepsin A and gastricin, the cleavage of other bonds in this peptide by chymosin is very slow (Kageyama 2002). Activity on haemoglobin, synthetic substrate and sensitivity to an inhibitor of aspartyl proteinases (Table 1) reveals further differences between the three enzymes.

The specificity of chymosin on caseins and the coagulation mechanism have been studied extensively (McSweeney *et al*. 1993; Chitpinityol and Crabbe 1998; Mooney *et al*. 1998; Soeryapranata *et al*. 2002; Hyslop 2003). Milk coagulation is initiated by cleavage of the Phe105–Met106 bond of κ-casein (κ-CN) by chymosin and many other coagulants. This divides κ-CN into *para*-κ-casein, which remains with the casein micelles, and hydrophilic glycopeptides (caseino-macropeptide), which pass into the whey. In cheese, the Phe23–Phe24 bond of α_{s1}-CN is hydrolysed rapidly. Apart from α_{s1}-CN f1–23, chymosin releases several other peptides from α_{s1}-CN; α_{s1}-CN f101–199 is released fairly rapidly from α_{s1}-CN in cheese, a large amount of α_{s1}-CN f165–199 was found in solutions of α_{s1}-CN treated with chymosin. Chymosin is also active on β-CN in solution, releasing several peptides including bitter β-CN f193–209 (McSweeney *et al*. 1993; Mooney *et al*. 1998; Minkiewicz *et al*. 2000).

Modern mass spectrophotometry techniques allow rapid identification of peptides released by chymosin and other proteinases (Minkiewicz *et al*. 2000; Soeryapranata *et al*. 2002).

Coagulants of plant origin

Crude extracts from the flowers of *Cynara cardunculus* L. have been used since ancient times in the farm-level manufacture of traditional cheeses from raw ovine or caprine milk in Portugal and Spain. The extracts contain two aspartyl proteinases named cardosin A and B. Cardosin A, like chymosin, cleaves the Phe105–Met106 bond of κ-CN, while the specificity of cardosin B is similar to that of pepsin (Veríssimo *et al*. 1995). Cheeses manufactured with coagulant blends containing cardosins and chymosin had higher levels of pH 4.6-soluble nitrogen than control cheeses made using chymosin. The extent of breakdown of α_{s1}-CN was also greater in cheeses made using blends of cardosins and chymosin (O'Mahony *et al*. 2003).

A milk-coagulating proteinase, 'lettucine', was extracted from lettuce leaves (*Lactuca sativa* L. cv. Romana). The serine-type proteinase probably hydrolysed other bonds of κ-CN than the Phe105–Met106 bond and was active on α- and β-CNs; its milk clotting activity was not stimulated by addition of calcium (Lo Piero *et al*. 2002).

Modification of milk coagulants

The following selected examples show that important cheesemaking properties of chymosin and other coagulants can be altered markedly using site-directed mutagenesis. Elimination of Cys45–Cys50 in bovine chymosin caused a large reduction in thermostability and alteration of substrate specificity (Zhang *et al*. 1997). Deletion of Cys206–Cys210 in recombinant chymosin induced conformational change, resulting in a perturbation of the local conformation around the active site cleft, and in turn altered its specificity (Chen *et al*. 2000). When the amino acid residues Gly-Thr-Prp-Pro at positions 21–24 of chymosin were replaced by Gly-Gly, the inactivation temperature of the resulting pseudochymosin was reduced by 15–20°C (Li *et al*. 1998). The modified *R. pusillus* proteinase showed a fivefold increase in the ratio of clotting to proteolytic activity without significant loss of clotting activity (Aikawa *et al*. 2001).

APPLICATIONS OF COMMERCIAL PREPARATIONS OF PROTEINASES, PEPTIDASES LIPASES/ESTERASES AND TRANSGLUTAMINASE

Proteinases, peptidases (including general and specific aminopeptidases, carboxypeptidases, oligopeptidases), transglutaminases and lipases have been used in the dairy industry for different applications. A number of proteinase and aminopeptidase preparations from microbial and animal sources are available commercially. Proteinases are used for the production of casein and whey protein hydrolysates with improved functional properties and reduced allergenicity, for the production of hydrolysates rich in bioactive peptides and the production of enzyme-modified cheeses (EMCs). They also have potential use for accelerated cheese ripening. Commercial microbial proteinases are derived from *Bacillus*, *Aspergillu*s spp. or from *Rhizomucor niveus*. Both the extent of proteolysis and the specificity of a proteinase are important for reducing the allergenicity of milk proteins (Fox and Grufferty 1991; Stepaniak *et al*. 1998; Darewicz *et al*. 2000; Kilcawley *et al*. 2002a,b).

The main application of aminopeptidases and carboxypeptidase is debittering and flavour generation in protein hydrolysates as well as the production of EMCs. A number of commercial preparations of general (nonspecific) aminopeptidases from LAB and *A. oryzae* are available. There is limited availability of peptidases that can hydrolyse proline-containing bonds. X-proline dipeptidyl proteinase preparations from *Lc. lactis* and proline endopeptidase from *Flavobacterium* spp. are available commercially (Kilcawley *et al*. 1998, 2002a,b; Raksakulthai and Haard 2003).

Lipolytic preparations used for the production of Italian-type cheeses and EMCs contain mainly lamb calf and kid pregastric esterases and/or

fungal lipases from several mould species (Fox and Gufferty 1991; Fox and Stepaniak 1993). The definition of lipases and esterases remains unclear. Kilcawley *et al*. (2002a) proposed that lipase activity preferentially releases medium- and long-chain fatty acids, while esterase activity refers to the preferential release of short-chain fatty acids.

Commercial enzyme preparations are not usually pure because of the high costs involved in purification. All 23 commercial preparations of proteinases, seven preparations of peptidases and 17 preparations of lipases and esterases were found to contain enzyme activities in addition to their stated main activity (Kilcawley *et al*. 2002a,b). Esterolytic activity was found in all preparations of proteinases.

Transglutaminases (TGases) introduce covalent cross-links between the γ-carboxyamide group of peptide-bound glutamine residues and the ε-amino group of lysine, resulting in the formation of an ε-(γ-glutamyl)lysine isopeptide bond. Water molecules may also act as acyl acceptors, resulting in the deamidation of glutamine to glutamic acid. Incorporation of amines, cross-linking or deamidation reactions can modify the solubility, hydration, gelation, rheological, emulsifying, rennetability and heat stability properties of a variety of food proteins. Microbial TGase from *Streptoverticillium* spp. is commercially available (O'Sullivan *et al*. 2002; Flanagan and FitzGerald 2003). Casein hydrolysate obtained using proteinase from *Bacillus* was incubated with TGase. The treatment improved emulsifying properties and increased foamability (Flanagan and FitzGerald 2003). An inhibitor of streptoverticillium TGase was found in bovine milk (De Jong *et al*. 2003).

Enzymatic debittering

Enzymatic hydrolysis of proteins frequently results in a bitter taste, due to the formation of low molecular mass peptides, composed mainly of hydrophobic amino acids. Bitterness can be reduced by adding 'masking' proteins or fat, removing bitter peptides by extraction with alcohol, treatment with activated carbon, or by using chromatographic techniques. Biobased methods include the hydrolysis of bitter peptides with proteolytic enzymes, especially with aminopeptidases and carboxypeptidases, or condensation of bitter peptides using proteinases. Bitterness is common in low-fat cheeses. Bitterness in cheese may be controlled by selecting starter or adjunct microflora (Saha and Hayashi 2001; Raksakulthai and Haard 2003). The degradation rate of β-CN f193–209, as measured by mass spectrometry, was proposed as an index for screening cheese microflora for debittering activity (Soeryapranata *et al*. 2002).

Proline-specific endo- and exopeptidases may play an important role in cheese ripening and debittering, either by degrading proline-containing peptides, which are often bitter, or by making peptides accessible to the action of other peptidases by removing proline. Dipeptides containing proline have been reported to be bitter (Baankreis and Exterkate 1991; Martínez-Cuesta *et al*. 2001). Aminopeptidases, types PepN, PepP and PepX, and carboxypeptidases are considered to be efficient debittering enzymes for milk protein hydrolysates, cheeses and EMCs. Some strains of *Lactobacillus* spp., especially *Lb. casei*, are nonstarter microorganisms having an enzyme system of potent debittering activity (Martínez-Cuesta *et al*. 2001; Saha and Hayashi 2001). Interestingly, proteinase and aminopeptidase from the spoilage psychrotroph *Pseudomonas fluorescens* showed debittering activity (Gobbetti *et al*. 1995; Koka and Wimer 2000). The extracellular proteinase degraded bitter α_{s1}-CN f1–9 and β-CN fl193–209 (Koka and Wimer 2000), while the intracellular aminopeptidase degraded synthetic bitter peptides (Gobbetti *et al*. 1995). The enzyme specifically hydrolysed peptidic bonds involving Leu, Trp and Val, usually identified as major components of bitter peptides.

ENZYMES FROM MICROORGANISMS THAT ARE IMPORTANT FOR CHEESE RIPENING

Over 300 different volatile and nonvolatile compounds have been implicated in cheese flavours. Enzymes of the exogenous microflora contribute essentially to the formation of sapid and aromatic compounds typical for cheese flavours. The biochemical pathways for the formation of key cheese flavour compounds originating from proteolysis, lipolysis, glycolysis, citrate and lactate metabolism have been reviewed (Fox *et al*. 1995; Fox and Wallace 1997; Christensen *et al*. 1999; McSweeney and Sousa 2000; Yvon and Rijnen 2001). In this section only proteolytic, lipolytic and selected amino acid catabolizing enzymes are discussed briefly.

Proteolytic system of LAB and propionibacteria

Caseolytic CEPs from starter lactococci have been studied extensively. Originally, three types of CEP were differentiated by their relative specificity on CNs. They were CEP type PI (or lactocepin I), PIII and intermediate type PI/III. More recently, CEPs have been classified on the basis of enzymatic specificity on α_{s1}-CN f1–23. Lactocepin groups a–g have been recognized and nine key amino acids in the lactocepin molecule thought to be responsible for controlling this specificity have been identified (Kunji *et al*. 1996; Pillidge *et al*. 2003).

Experiments with strains of lactococci resistant to autolysis were performed to limit the participation of intracellular enzymes in protein breakdown and emphasize the role of lactocepins in cheese maturation. Isogens of *Lc. lactis* lacking the gene coding for the major autolysin and producing three different types of lactocepins were genetically constructed and used to produce low-fat Cheddar cheese. Cheeses made using isogenic starters that produced group a, e, or h lactocepins were significantly more bitter than cheese made using the lactocepin-negative strain. The propensity for bitterness was highest in cells that produced type h lactocepins (Broadbent *et al*. 2002).

Lactobacilli also produce diverse CEPs with different specificity on caseins and α_{s1}-CN f1–23 (Kunji *et al*. 1996; Oberg *et al*. 2002).

Intracellular turnover of proteins and peptides is an essential function in all living cells. Complete hydrolysis of proteins or oligopeptides to free amino acids requires many specific and nonspecific proteolytic enzymes. An evolutionary relationship was found between some LAB and mammalian endopeptidases (Orlowski 1990; Rawling and Barret 1995). LAB possess a relatively weak but complex intracellular proteolytic system. As in other bacteria, intracellular proteolytic enzymes of LAB include:

- aminopeptidases, which release free amino acids or dipeptides at a high rate from the N-terminal of peptides containing usually no more than 5–7 amino acid residues (longer peptides are hydrolysed slowly and proteins are essentially not hydrolysed by peptidases);
- carboxidases, which release amino acids from the C-terminal of peptides;
- dipeptidases and tripeptidases, which hydrolyse di- and tripeptides, respectively;
- noncaseinolytic oligopeptidases, which hydrolyse peptides containing between five and 30 amino acids, and caseinolytic proteinases are also intracellular enzymes found in LAB.

LAB and propionibacteria contain peptidases with broad or narrow specificity. Specificity is determined by high specificity for a particular amino acid and/or its position in the peptide; however, peptidases claimed as specific usually slowly degrade some 'nonspecific' substrates, and many aminopeptidases show dipeptidase activity. Hydrolysis of proline-containing peptide bonds requires strictly specific proline peptidases. Oligopeptidases show substrate size-recognizing specificity. During cheese ripening, peptidases are responsible for secondary proteolysis, i.e. degradation of peptides released by plasmin or chymosin, removing bitterness and producing free amino acids.

The same types of aminopeptidases and oligopeptidases were found in lactobacilli, lactococci and propionibacteria, although it is believed that the total proteolytic system, caseinolytic activity and intracellular proteolytic systems of lactobacilli are more active than those of lactococci and that the caseinolytic activity of propionibacteria is weak. Multicatalytic proteinases (proteosomes) important for intracellular protein turnover were found in the cytoplasm of many mammalian, yeasts and bacterial cells (Orlowski 1990) but they have not been reported in LAB or propionibacteria.

Some properties of the major peptidases from lactococci and lactobacilli are summarized in Table 2. Oligopeptidases from lactococci and lactobacilli were most active on methionine enkephalin and more slowly on α_{s1}-CN f1–23, hydrolysed β-CN f193–209 and 194–209 very slowly, but did not hydrolyse β-CN f58–72, a peptide which inhibited both lactococcal PepO and PepN. A number of differences were also observed in the specificity of peptidases from these two groups of LAB. For example, metallooligopeptidases, PepO, from *Lactococcus* and *Lactobacillus* had identical molecular masses (70 kDa) but the enzymes were immunologically unrelated and had different specificity both on bitter β-CN f193–209 and on bitter β-CN f194–209, which is produced by lactocepins (Stepaniak and Fox 1995; Tobiassen 1996; Christensen *et al*. 1999; Stepaniak and Sørhaug 2001; Stepaniak *et al*. 2001; Gobbetti *et al*. 2002). The specificities of lactococcal lactocepins and PepOs on α_{s1}-CN f1–23 were different (Stepaniak and Fox 1995).

Several LAB peptidases have been cloned within and between species. Genetically modified LAB, which overproduced or lacked the gene for some peptidases, were engineered to study their effect on cheese ripening (Christensen *et al*. 1999). Characteristic differences between the intracellular system of propionibacteria and LAB are that the relative activity of PepI is low in LAB and high in propionibacteria, while the opposite is usually true for PepX (Tobiassen 1996; Stepaniak *et al*. 2001). Free proline generated in high amounts, mainly by PepI from propionibacteria, is responsible for the typical sweet and nutty flavour of Swiss cheese. The gene coding PepI in propionibacteria was cloned and multiplied in the chromosome of *Lc. lactis* (Leenhouts *et al*. 1998). No prolyl endopeptidases were reported in LAB or propionibacteria.

The information on carboxypeptidases from LAB is scarce. The evidence for carboxypeptidase activity in *Lb. helveticus* and *Streptococcus thermophilus* was supported by the presence in β-CN hydrolysates, treated with enzymes from these organisms, of two sets of peptides differing by a single amino acid at the C-terminal position (Deutsch *et al*. 2000).

Genetic modification of LAB for the release of intracellular enzymes without affecting their growth

All known peptidases in LAB are intracellular. Different approaches have been proposed to accelerate

Table 2 Selected peptidases in lactic acid bacteria (adapted from Christensen *et al*. 1999)

Peptidase	*Molecular mass*[a] *(kDa)*	*Structure*[b]	*Class*[c]	*Specificity, good substrates*[d]
Aminopeptidase PepC	50–54	Hexamer, tetramer	C	B, different AA-*p*NA, chromogenic substrates and peptides except Pro-Xaa
Aminopeptidase PepN	89–95	Monomer	M	B, very high on Lys-*p*NA, also high on different peptides except Pro-Xaa
Aminopeptidase PepA	40–45	Octamer, hexamer,		N, Glu-*p*NA
X-prolyl dipeptidyl aminopeptidase PepX	82–95	Monomer, trimer	S	N, Xaa-Pro-*p*NA, bradykinin
Dipeptidase PepD	53	Octamer	C	B, various dipeptides
Dipeptidase V	46–52	Monomer	M	B, various dipeptides, does not hydrolyse AA-*p*NA substrates
Prolyl iminopeptidase PepI	33–50	Dimer, trimer	S	S, Pro-*p*NA, highly specific
Prolidase PepQ	41	Monomer	M	N, dipeptides Xaa-Pro Some Xaa-Pro dipeptides are not hydrolysed
Prolinase PepR	32–35	Dimer, tetramer	S	N, dipeptides Pro-Xaa
Tripeptidase PepT	23–55	Monomer, dimer, trimer	M	B, tripeptides including Pro-Gly-Gly
Oligopeptidases PepO	70–71	Monomer	M	Peptides containing 5–30 AA residues, some casein-derived peptides are not hydrolysed
Oligopeptidase PepF	70	Monomer	M	Peptides with not less than seven and not more than 17 AA residues

[a]Molecular mass calculated from known amino acid sequence or by SDS–PAGE
[b]Predicted from comparison between molecular mass obtained by gel filtration and SDS–PAGE or from X-ray crystal analysis
[c]Catalytic class: C, cysteine; M, metallo; S, serine peptidases
[d]For details see References in Christensen *et al*. (1999); Kilcawley *et al*. (2002a,b). B, broad specificity; N, narrow specificity; AA, amino acid; Xaa, different, unspecified amino acids; *p*N, *para*-nitroanilide

cheese ripening by stimulating the lysis of LAB cells using various phage- and autolysin-based mechanisms.

A novel, 'nondestructive' intracellular enzyme delivery system has been created to excrete β-galactosidase from *Lc. lactis* cells without the need for export signals and without affecting the cell growth. Approximately 85% of β-galactosidase activity was detected in the supernatant of 'leaky' lactococci without evidence of hindered growth, cell lysis or membrane damage. The system is based on the controlled expression of integrated pro-phage gene coding holin and lysin cassettes via a lactococcal bacteriophage transcriptional activator (Tac31A) that resides on a high-copy plasmid (Walker and Klaenhammer 2001). The lactococcal phage-based protein release mechanism had, however, no effect or facilitated very little the secretion of the lactococcal or lactobacillus peptidases PepA, PepC, PepN, PepO and PepX (Tuler *et al*. 2002).

Catabolism of amino acids by LAB

The current research on amino acid catabolism in cheese is focused mainly on elucidating the role of aminotransferases and cystathionine lyases isolated from lactococci and lactobacilli. Some of these enzymes have been characterized at both the molecular and the genetic level (Dobric *et al*. 2000; Yvon and Rijnen 2001; Rijnen *et al*. 2003). Aminotransferases initiate the first step in amino acid catabolism by converting amino acids into the corresponding α-keto acid. Aminotranferases from LAB have been found to transaminate Asp, branched amino acids (isoleucine, leucine and valine), aromatic amino acids (phenylalanine, tyrosine and tryptophan) and methionine. The resulting α-keto acids are transformed via different enzymatic and chemical reactions to a range of aroma compounds. Phenylacetic acid, phenylacetaldehyde, benzaldehyde, cresol, phenol and indole produced from the aromatic amino acids

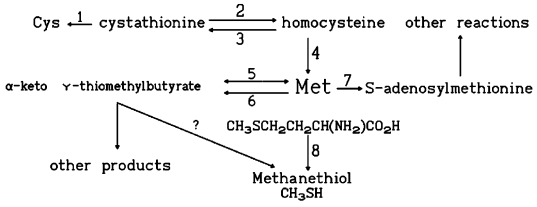

Figure 1 Metabolic pathways for methionine conversion showing the primary intermediates and enzymes. Enzymes (1–8) involve: 1, cystathionine γ-lyase; 2, cystathionine β-lyase; 3, cystathionine β-synthase; 4, homocysteine methyltransferase; 5, aromatic aminotransferase (AraT) or branched chain aminotransferase (BcaT); 6, amino acid oxidase; 7, Met adenosyltransferase; 8, Met γ-lyase (adapted from Dias and Weimer 1998a).

(phenylalanine, tyrosine and tryptophan) have a floral or phenolic aroma, whereas isovaleric acid, isobutyric acid, 2- and 3-methylbutan(al/ol) and 2-ethylpropan(al/ol), produced from branched-chain amino acids (isoleucine, leucine and valine), have a ripened cheese, fruity or malty aroma. Transamination requires the presence of an α-ketoacid acceptor for the amino group. Aminotransferases of LAB use as acceptor mainly α-ketoglutarate (α-KG), which is transformed into glutamic acid.

Cystathionine lyases from various bacteria show broad substrate specificity; their major activity is the conversion of cystathionine but they can also produce methanethiol from methionine (Fig. 1).

The γ-lyase converts cystathionine to cysteine, α-oxobutyrate and ammonia via an α,γ-elimination reaction. The β-lyase converts cystathionine to homocysteine, pyruvate and ammonia via an α,β-elimination reaction (Dobric *et al*. 2000).

The volatile compounds produced from methionine such as methanethiol and dimethyldisulphide have an aroma of garlic, cheese and sulphur compounds. Pyridoxal-5′-phosphate (PLP)-dependent cystathionine lyase from LAB is thus regarded as the enzyme that generates methanethiol in cheese, a compound believed to be important for typical cheese flavour. Methionine γ-lyase (Fig. 1) has not been reported in LAB (Yvon and Rijnen 2001).

Rijnen *et al*. (2003) suggested that control of amino acid catabolism by LAB could allow the production of cheeses with a diverse range of aromas. Different strategies have been examined. The amount of α-ketoacid in cheese is not sufficient for efficient transamination (Tanous *et al*. 2002). Pyruvate is used for the synthesis of some amino acids. Cheddar cheese curds were supplied with α-KG, PLP and pyruvic acids to increase the activity of aminotransferases, lyases and production of amino acids, respectively. The results from analysis of mature cheeses suggested that α-KG and PLP enhanced the degradation of most amino acids, while pyruvic acid promoted the formation of amino acids (Ur-Rehman and Fox 2002).

Some strains of lactobacilli show glutamate dehydrogenase (GDH) activity required for conversion of glutamate, which is abundant in cheese, to α-KG. These strains were capable of degrading leucine or phenylalanine to aroma compounds in a culture medium at pH 5.5 (Tanous *et al*. 2002). Aromatic amino acid aminotransferase (AraT) and branched-chain aminotransferase (BcaT) were isolated from lactococci. The AraT was a PLP-dependent enzyme composed of two identical subunits of 43.5 kDa (Yvon *et al*. 1997). BcaT isolated from the same strain of *Lactococcus* catalysed the transamination of the three branched-chain amino acids. The enzyme was identified as a class IV PLP-dependent aminotransferase. In contrast to most of the bacterial BcaTs, which are hexameric, the enzyme was homodimeric, comprising two identical 38 kDa subunits (Yvon *et al*. 2000). Two active methionine BcaT-type aminotransferases were separated on MonoQ from the cytoplasm of another strain of *Lactococcus*. Both enzymes were homodimers, each unit having molecular mass of *c.* 40 kDa (Engels *et al*. 2000).

Cystathionine lyases were isolated from three different strains of lactococci and two species of lactobacilli. The β-lyase from *Lactococcus* consisted of four identical subunits of 35–40 kDa (Alting *et al*. 1995). A lactococcal γ-lyase apparently consisted of six identical subunits of 20 kDa, a structure atypical for γ-lyases from other species of bacteria (Bruinenberg *et al*. 1997). The enzyme produced methanethiol. A unique β/γ-lyase was isolated from *Lactococcus* and overexpressed in *E. coli*. The enzyme carried out both α,β-elimination and α,γ-elimination reactions. Its molecular mass calculated from the gene sequence was 40.937 kDa (Dobric *et al*. 2000). Apparently homotetrameric, ~35–40 kDa subunit, γ-lyases were isolated from *Lactobacillus fermenti* and *Lactobacillus reuteri* (Smacchi and Gobbetti 1998; De Angelis *et al*. 2002). Typically for lactococcal lyases, the two enzymes were inhibited by carbonyl reagents such as semicarbazide or phenylhydrazine. The transaminases and lyases from LAB discussed above were most active at pH > 6.5 and a temperature of 35–45°C but all showed noticeable activity under cheese-ripening conditions.

Reactions of free amino acids with other compounds in cheese have been reviewed (Fox and Wallace 1997).

Esterases from LAB

Weak intracellular lipolytic activity in LAB, in comparison to typical lipolytic bacteria, was detected about three decades ago. As in the case of proteolytic enzymes, the primary role of lipolysis is likely to be in meeting the physiological and metabolic functions of the organisms, as lactococcal lipase is most active on the lactococcal neutral

lipids (cf. Holland and Coolbear 1996). The intracellular lactococcal esterase could be involved in (phospho)lipid metabolism or cellular detoxification or both (Fernández *et al*. 2000). Cell-bound esterase from *Lactobacillus* has also been characterized (Gobbetti *et al*. 1997b). Although several esterases from lactococci and lactobacilli were purified, characterized biochemically and genetically, evidence that these enzymes contribute to the accumulation of free fatty acids (FFAs) and formation of esters in cheeses has only recently been obtained. Cheddar cheeses were produced using strains of *Lactococcus* with similar level of lipolytic and esterolytic activity but with different propensity to autolysis. Levels of caprylic, myristic, palmitic and stearic acids were significantly higher in cheeses produced with the strain, which lysed more extensively in cheese (Collins *et al*. 2003). In cheese made from prelipolysed milk and therefore enriched with mono- and diglycerides, which are good substrates for LAB esterases, the rate of FFA accumulation during cheese ripening increased dramatically (Holland *et al*. 2002). In a model system simulating Parmesan cheese-ripening conditions and containing FFAs and alcohols, purified esterases (EstA, EstB and EstC) from *Lactococcus* and *Lactobacillus* spp. accumulated favourable ethyl esters (Fenster *et al*. 2003c). The production of esters was also demonstrated using wild, EstA-positive, *Lactococcus* and its esterase-negative genetically constructed mutant (Nardi *et al*. 2002). Esterases from LAB may have multiple activities, depending on substrate available. Their activities are routinely measured on synthetic chromogenic ester substrates, which may have little relevance to the impact of the enzyme on milk fat substrates. The enzymes generally show preference for glyceryl esters of short-chain fatty acids, are most active on monoglycerides but show some activity on diglycerides, tributyrin and milk fat, which complicates their classification (Holland and Coolbear 1996; Gobbetti *et al*. 1997a; Holland *et al*. 2002). The major esterases identified in *Lactococcus* and *Lactobacillus* were termed tributyrin esterases. The single intracellular esterase from *Lactococcus* was a homotetramer of 29 kDa subunits (Holland and Coolbear 1996).

One major and one minor esterase were identified in the cytoplasmic fraction from *Lb. plantarum.* The major, monomeric 85-kDa, serine-class esterase hydrolysed β-naphthyl esters of fatty acids from C2 to C10 in preference to β-naphthyl butyrate and was less active on tributyrin than the minor enzyme (Gobbetti *et al*. 1997a). The cell-bound, monomeric, 67-kDa esterase from *Lb. fermenti* (Gobbetti *et al*. 1997b) had biochemical properties similar to the major esterase from *Lb. plantarum.* Two of three intracellular esterases from *Str. thermophilus* were purified to homogeneity. One of the esterases was probably monomeric, of molecular mass ~34 kDa, while the other was dimeric of subunit molecular mass ~60 kDa (Liu *et al*. 2001).

More recently, four recombinant esterases were obtained from LAB. Genomic libraries of *Lb. helveticus* (Fenster *et al*. 2000), *Lb. casei* (Fenster *et al*. 2003a) or *Lc. lactis* (Fernández *et al*. 2000) were constructed using an *E. coli* expression vector, and esterases expressed in *E. coli* were purified and characterized. Named after the corresponding gene, EstA was found in *Lb. helveticus* and *Lc. lactis*, while two recombinant esterases (EstB and EstC) were produced by *Lb. casei* (Fenster *et al*. 2003a,b,c). The serine-dependent EstA from *Lb. helveticus* had a monomeric molecular mass of 22.6–25.1 kDa, while the serine-dependent EstC from *Lb. casei* was a dimeric, 28.9-kDa subunit enzyme. Some differences in specificity for the hydrolysis of synthetic substrates, pH, temperature and NaCl concentration required for maximal activity and ability for the synthesis of esters were found between the four enzymes. Lactococcal EstA was overproduced in *Lc. lactis*. Its unusual property was the ability to hydrolyse short- and long-chain synthetic phospholipids (Fernández *et al*. 2000).

Proteolytic, lipolytic and amino acid converting enzymes from smear bacteria on surface-ripened cheeses

The surface microflora of various smear surface-ripened cheeses, such as Limburger, Trappist, Münster, Brick, Appenzeller and Tilsiter, includes yeasts, moulds and bacteria. At a later stage of ripening, aerobic, halotolerant *Brevibacterium linens* become a dominating bacterium. Other coryno-bacteria species such as *Arthrobacter nicotianae* and micrococci also comprise a large part of the smear microflora (Rattray and Fox 1997, 1999; Gobbetti *et al*. 2001).

The capacity of *B. linens* to hydrolyse casein and milk fat and to produce aromatic compounds from amino acids and FFAs is higher than that of LAB. Moreover, some strains of the microorganism showed considerable intracellular and/or extracellular proteolytic and lipolytic activities. Overall, the distribution of activities was strain dependent. Production of more than one extracellular proteinase and aminopeptidase was reported. Cell-associated peptidases and lipases were also reported. 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 (Hayashi 1996; Dias and Weimer 1998a,b; Rattray and Fox 1997, 1998, 1999; McSweeney and Sousa 2000; Yvon and Rijnen 2001).

Methionine γ-lyase from *B. linens* was LPL-dependent, consisting of four identical 43 kDa subunits. Its activity was inhibited by carbonyl reagents. The enzyme was active under the salt and pH conditions found in ripening Cheddar cheese but was susceptible to degradation by intracellular proteinases (Dias and Weimer 1998b).

B. linens possesses two intracellular esterases, one of which was purified to homogeneity. The enzyme is a tetramer of total molecular mass 200 kDa, it is strongly inhibited by thiol blocking agents and shows a high specificity for short-chain fatty acids with no activity on tributyrin or milk fat (Rattray and Fox 1998). A carboxylesterase that was converting 1,4-butanediol diacrylate to 4-hydroxybutylacrylate was found in another strain of *B. linens* (Sakai *et al*. 1999). The cell-associated lipase of *B. linens* is a serine-class enzyme. Cell-associated activity was released from the cells by treatment with lysozyme (Adamitsch and Hampel 2000).

B. linens may secrete as many as five different extracellular proteinases (Hayashi 1996). The extracellular serine proteinase from *B. linens* characterized by Rattray *et al*. (1995) is a dimer consisting of 56-kDa subunits. The enzyme had broad specificity on the amino acid residues at the P_1-P_1' position of α_{s1}- and β-CN (Rattray *et al*. 1996, 1997). The proteinase did not produce the typical bitter peptides from β-CN but produced β-CN (f191–209), which could be further degraded to bitter peptides by the extracellular aminopeptidases of this microorganism (Rattray *et al*. 1997). Extracellular proline iminopeptidase (PepI) from *Arthrobacter nicotianae* was active at low temperature and at NaCl concentrations up to 7.5%. The enzyme is a monomer of 53 kDa and is sensitive to serine proteinase inhibitors (Smacchi *et al*. 1999). Purified serine extracellular PepI from *Corynobacterium variabilis* was also active at the temperature of cheese ripening and an NaCl concentration of 7.5%. The monomeric enzyme had a molecular mass of 45 kDa (Gobbetti *et al*. 2001).

Proteolytic and lipolytic enzymes from moulds used for the production of roquefort- and camembert-type cheeses

Advanced proteolysis and lipolysis characterize mould-ripened cheese. High concentrations of methyl ketones are associated with catabolism of FFAs by *Penicillium roqueforti* (Fox and McSweeney 1996; Fox and Wallace 1997; McSweeney and Sousa 2000). Production and general properties of both extra- and intracellular proteinases and lipases from *P. camemberti* and *P. roqueforti* were studied extensively by French workers in the 1970s (for references see Chrzanowska *et al*. 1995; Fox and McSweeney 1996).

The proteolytic system of *P. roqueforti* comprises extracellular aspartyl proteinase, a metalloproteinase, aminopeptidases and carboxypeptidases. High carboxypeptidase activity seems to be characteristic of *P. roqueforti* and *P. camemberti* (Gente *et al*. 1997; Mechakra *et al*. 1999; Geurts *et al*. 2003). *Penicillium camemberti* produces an aspartyl proteinase and an acid carboxypeptidase in a whey medium (Mechakra *et al*. 1999).

The extracellular aspartyl proteinase from *P. camemberti* was a 33.5-kDa monomer. The enzyme was inhibited by pepstatin. Its specificity at low pH was similar to that of pepsin. The enzyme hydrolysed the peptide bond Leu7–Met8 of squash trypsin inhibitor at low pH and resynthesized it at neutral pH (Chrzanowska *et al*. 1995).

The extracellular aspartyl proteinase produced by *P. roqueforti* was a 36–37-kDa monomer (Bracq *et al*. 1997). Production of the enzyme was induced by casein added to the medium and the gene encoding the enzyme was cloned and characterized (Gente *et al*. 1997). Extracellular lipase from *P. roqueforti* had a molecular mass of 25 kDa by sodium dodecyl sulphate (SDS)–PAGE, and showed high specificities towards short-chain fatty acid esters (Mase *et al*. 1995).

HYDROLASES FROM SPOILAGE MICROORGANISMS

Cold storage of raw milk and perishable dairy products is a prerequisite of the modern dairy industry. The shelf life of dairy products during cold storage is limited by the growth of psychrotrophic microorganisms, mainly pseudomonads. Enzymatic spoilage without concomitant bacterial growth concerns mainly UHT-treated milk. Pseudomonads are highly evolved microorganisms, capable of degrading more than 100 different organic compounds, and in the spoilage of milk and dairy products the production and properties of extracellular proteinases and lipases by *Pseudomonas* spp., especially *Ps. fluorescens*, have been studied extensively. Phospholipases from pseudomonads and hydrolases from psychrotrophic aerobic *Bacillus* spp. have been characterized to a lesser extent (see Sørhaug and Stepaniak 1991, 1997).

Most pseudomonad hydrolases retain marked activity at 4–7°C and at pH 5.0. Simultaneous action of pseudomonas proteinases, phospholipases and glycosidases may enhance lipolysis by damaging the fat globule membrane. Phospholipase C from *Bacillus cereus* was associated with formation of fat clumps (bittiness) in cream. Proteinases from pseudomonads degrade all major casein fractions and cause gelation of UHT milk. Gelation is preceded by development of a stale, unclean and bitter flavour. Bitterness occurred in quarg produced from milk containing 5 ng/mL of

metalloproteinase from *Ps. fluorescens* after 3 weeks of storage at 7°C. Lipoprotein-like activity and lack of positional specificity on triglycerides was found for some pseudomonas lipases.

The secretion of one extracellular proteinase was reported most frequently but some *Pseudomonas* strains secrete two or three immunologically unrelated proteinases. Most proteinases from *Pseudomonas* spp. are monomeric 40–55-kDa metalloenzymes containing one zinc and four to eight calcium atoms. However, some of them were not inhibited by phosphoramidon, a typical inhibitor of metalloproteinases, and showed activity on synthetic trypsin substrates. Early reports showed that lipases from pseudomonads were serine catalytic class enzymes, with a wide range of molecular masses, many forming large self-aggregates (Sørhaug and Stepaniak 1991, 1997).

Recent research on hydrolases from pseudomonads summarized below has been stimulated by the potential use of these enzymes in biotechnology. An extracellular proteinase from *Pseudomonas* spp., unstable above 60°C, showed both lytic activity against cells of *Xanthomonas campestris* and proteolytic activity on casein. The enzyme was a 35-kDa serine proteinase associated with a 15-kDa nonenzymatic protein (Shastry and Prasad 2002). An extracellular, heat-stable, 59-kDa lipase from *Ps. fluorescens* did not hydrolyse di- and triglycerides (Sakiyama *et al*. 2001). Extracellular lipase from another strain of *Ps. fluorescens* was cloned and characterized at the molecular level. Nucleotide sequence analysis revealed an open reading frame of 1854 bp encoding the enzyme. The lipase showed significant sequence similarity to lipase from *Serratia marcescens* (Kojima *et al*. 2003). The extracellular phosphatidylcholine-hydrolysing phospholipase C from *Ps. fluorescens* is a 40-kDa monomer; as for other types of bacterial phospholipase C, the enzyme required Ca^{2+} and Zn^{2+} for activity (Preuss *et al*. 2001).

THERMAL DENATURATION OF ENZYMES IN MILK

Indigenous enzymes

There are large differences in the heat stability of indigenous enzymes. Differences occur within enzymes originating from blood plasma, somatic cells and associated with milk fat globule membrane. Although values reported in literature vary considerably, it can be concluded generally that HTST pasteurization inactivates very little of plasmin and plasminogen, ribonucleases, β-amylase, aspartate aminotransferase, acid phosphatase and DNA and RNA hydrolysing abzymes. Pasteurization causes moderate inactivation of lactoperoxidase, xanthine oxidase, cathepsin D and lysosyme. Residual activity of plasmin and nucleases can be detected in UHT treated milk. No or very little activity of LPL, alkaline phosphatase (ALP), γ-glutamyl transferase or α-L-fucosidase remains in milk after HTST pasteurization (Farkye and Imafidon 1995; Grappin and Beuvier 1997; McKellar and Piyasena 2000; Stepaniak 2002; Stepaniak *et al*. 2003).

Measurement of indigenous ALP is the principle of the official test to determine whether milk has been properly pasteurized or cheeses and other dairy products were made from properly pasteurized milk. Some other enzymes that occur in milk at sufficient concentration and whose activity can be measured by a simple method have been assessed as indices of the thermal history of milk. Because of renaturation, postpasteurization activity of ALP and some other enzymes may be partially restored during storage. Therefore, measurement of the activity of γ-glutamyl transferase, which is only slightly more stable than ALP, was proposed as an alternative to the ALP index of pasteurization. Measurement of residual lactoperoxidase activity was proposed to indicate if heat treatment of milk exceeded HTST conditions (Farkye and Imafidon 1995). Heat treatment at the upper range of thermization can be detected by measuring the activity of β-L-fucosidase, which is almost completely destroyed at 62°C after 16 s (McKellar and Piyasena 2000).

Bacterial enzymes

The preparation of attenuated cells from LAB is usually accomplished by adjustment of cultured milk to pH 7.0 and heating at 60–68°C for several seconds. This treatment inactivates cell growth but retains the activity of enzymes important for cheese ripening. Lactococcal intracellular oligopeptidases and leucine aminopeptidase will be unlikely to survive HTST pasteurization but this heat treatment may only partially inactivate proteinase from *Lb. murinus*, ALP from *S. liquefaciens* and penicillinase from *B. cereus* (Fox and Stepaniak 1993; Stepaniak and Sørhaug 1995). Lactocepins from lactococci are generally less thermostable than intracellular proteolytic enzymes. Lysozyme released two proteinases from cells of *Lb. delbrueckii* ssp. *bulgaricus*. The serine-type proteinase could survive thermization, but the metalloproteinase was rapidly inactivated at 55°C (Stefanitsi *et al.* 1995)

Acid phosphatase from *Lactococcus* spp. retained 23% of its activity after heating for 30 min at 70°C (Akuzawa and Fox 1998). An esterase from *Lactobacillus* had a decimal reduction value (*D* value) at 70°C of 2.5 min (Gobbetti *et al*. 1997a), but an esterase from *Lactococcus* was inactivated within 15 min at 65°C (Holland and Coolbear 1996). The β-lyase from *Lactococcus* was stable at 60°C up to 10 min (Alting *et al*. 1995). Extracellular

proteinases, lipases and phospholipase C from psychrotrophic pseudomonads are scarcely inactivated by UHT treatment. At 150°C, the time needed to reduce 90% activity (*D*-value) for these enzymes is usually above 20 s. Proteinases from some aerobic, spore-forming *Bacillus* spp. are also very heat stable and survive UHT treatment. Proteinases from *Ps. fluorescens* are not inactivated by HTST pasteurization but are rapidly inactivated at 50–60°C in milk and buffers due to autodigestion and probably interaction with casein micelles. Lipases from *Ps. fluorescens* are also sensitive to low-temperature inactivation (LTI) at subpasteurization temperatures but phospholipases are not. The LTI mechanism of inactivation of pseudomonads lipases is not well understood. Extracellular glycosidases and intracellular aminopeptidases from pseudomonads are markedly less stable than extracellular proteinases, lipases and phospholipase C (Stepaniak and Sørhaug 1995; Sørhaug and Stepaniak 1997).

EFFECT OF NONTHERMAL TECHNIQUES ON ENZYMES. HIGH HYDROSTATIC PRESSURE AND PULSED ELECTRIC FIELD

While heat remains the most extensively used method for killing microorganisms, there has been increasing interest recently in the development of alternative approaches in response to the desire of consumers for products that are less organoleptically and nutritionally damaged during processing and less reliant on additives than previously. The new approaches mostly involve technologies that offer full or partial alternatives to heat for the killing of microorganisms. High hydrostatic pressure (HHP) is being used commercially to nonthermally pasteurize a number of foods. Other physical procedures such as high-voltage pulsed electric field (PEF), high-intensity pulsed light, high-intensity magnetic-field pulses, treatment with accelerated electrons or X-rays and 'manothermosonication' (the combination of mild heating with ultrasonication and a moderately raised pressure) are in various stages of development and commercial evaluation (Gould 2001). HHP has significant and, in many cases, unique effects on many constituents of milk (Datta and Deeth 1999; Huppertz *et al.* 2002). Under conditions having a biocidal effect comparable to that of HTST pasteurization, HHP and PEF treatments at temperatures not exceeding 30°C have variable effects on a number of indigenous milk enzymes and bacterial enzymes. HHP up to 100 MPa stimulates the activity of most enzymes (Hendrickx *et al*. 1998; Bendicho *et al.* 2002). HHP treatment of milk at 400–600 MPa and 20–30°C destroys most vegetative microorganisms (Datta and Deeth 1999). The application of 'hurdle technology' achieved by combined use of HHP with lysozyme, with the activated lactoperoxidase system or with peptides derived from lysozyme or lactoferrin has been shown to increase the microbiocidal effect of HHP or to achieve the required effect under reduced pressure (García-Graells *et al*. 2003).

HHP at 500–600 MPa for 5 min had little effect on the activity of plasmin or on LPL, which is inactivated by pasteurization. Goat cheese made from HHP-treated milk underwent significantly greater lipolysis than cheese made from pasteurized milk (Buffa *et al*. 2001). More than 50% of the activity of chymosin survived treatment at 600 MPa for 5 min (Malone *et al*. 2003). Lactoperoxidase seems to be markedly more sensitive to treatment at 600 MPa for 2 min than to HTST pasteurization (Hendrickx *et al*. 1998). The effect of HHP applied for 5 min was assessed on a number of proteolytic and glycolytic enzymes from *Lactococcus* (Malone *et al*. 2003). Activity of proteinases was stimulated by pressure up to 100 MPa but inactivated at 400 MPa. A pressure of 300 MPa significantly inactivated aminopeptidase PepN but had no effect on the proline-specific aminopeptidase PepX. The activity of PepC was not affected, even by a pressure of 800 MPa. Treatment of lactococcal cells by 200 MPa slightly increased the production of lactic acid, but cells treated at 400 MPa produced very little lactic acid. These data indicate that HHP may be used to prepare attenuated starter cells or lactococcal enzyme solutions having different profiles of proteolytic and glycolytic activities.

PEF in the range 15–45 kV inactivated up to 90% of the plasmin in milk after application of 20 pulses. Depending on the conditions used, 30–65% of ALP, LPL, lactoperoxidase, lipases and proteinases from *Ps. fluorescens* could be inactivated by PEF. Plasmin seems to be more sensitive to inactivation by PEF than LPL and microbial lipases (Bendicho *et al*. 2002).

IMMUNOLOGICAL METHODS FOR IDENTIFYING AND QUANTIFYING ENZYMES AND THEIR INHIBITORS AND MEASUREMENT OF PROTEOLYSIS

The immunological methods for the detection of enzymes and their inhibitors in milk and cheeses have been reviewed by Stepaniak *et al*. (1998). Simple agar immunodiffusion methods using specific polyclonal antibodies can be used to detect and identify chymosin, pepsin and rennet substitutes in commercial preparations and for identification of intracellular peptidases from LAB in cytoplasmic extracts. Immunochemical assays, especially the enzyme-linked immunosorbent assay (ELISA), could detect nanogram per gram quantities of

chymosin and plasmin + plasminogen, heat-stable proteinases, lipases and phospholipase from *Pseudomonas* spp., aspartyl proteinase from *P. roqueforti*, and some peptidases from LAB in milk or cheeses. The methods were used to study the distribution of some of these enzymes between curd and whey and their denaturation, or association with casein micelles. Natural and recombinant chymosins are immunologically indiscernible. Therefore, antibodies were raised to proteinous components of the medium or cells present in commercial preparations of recombinant rennets and used to identify recombinant rennets produced by four different microorganisms. ELISA was used to measure concentrations of seven enzyme inhibitors in milk and colostrum. The concentration of α_{s2}-plasmin inhibitor could be measured individually. Antibodies raised to elastase and trypsin/plasmin inhibitors cross-reacted and therefore only total concentrations of the two inhibitors were measured (Bracq *et al*. 1997; Stepaniak *et al*. 1998, 2001).

Antibodies to specific intracellular enzymes were used as markers to detect the lysis of *Lactobacillus* and *Propionibacterium* spp. directly in cheese (Valence *et al*. 1998). Anti-peptide antibodies that recognize the sequence around the cleavage site for a particular proteinase may be used to monitor, individually, proteolysis by different proteolytic enzymes. As long as the cleavage site is intact, the antibody will bind to the protein containing the peptide sequence. However, after cleavage of the peptide bond by the enzyme, the antibody will no longer recognize the substrate. The antipeptide antibodies were tested for monitoring of proteolysis in cheese by plasmin (Dupont *et al*. 2003). Polyclonal antibodies specific for peptides representing fragments of β-CN and containing plasmin-sensitive bonds were not fully satisfactory for monitoring degradation of β-CN by plasmin. The authors assume that the use of monoclonal antibodies may allow specific measurement of β-CN hydrolysis by this enzyme.

CONCLUSIONS

Many indigenous milk enzymes categorized as 'minor', which have been reviewed by Farkye (2003), may in future be recognized as deserving more attention. New important functions have already been attributed to recently characterized proteinases from somatic cells and to 'old enzymes' such as xanthine oxidase and nucleases characterized 30–40 years ago. Tranglutaminase is a 'newcomer' to dairy technology. Whey is an interesting source of lactoperoxidase and RNAse that may find application in other foods.

In order to control cheese ripening, proteinases and peptidases from LAB have been very well characterized, both biochemically and genetically. These and other enzymes may find application in the preparation of functional and bioactive protein hydrolysates with potential use in value-added or functional dairy and nondairy foods. There is current interest in controlling amino acid catabolism in cheese. Controlling dephosphorylation and fatty acid catabolism may become important tasks in the future. The progress in genetics is accelerating the progress in dairy enzymology. Genetic techniques are likely be widely used in the future to facilitate isolation and characterization of enzymes and to improve their properties. General progress in enzymology should be expected to influence the development of dairy enzymology. Synthetic enzymes (synzymes) and catalytic antibodies (abzymes), which can be tailored for any enzymatic activity, may find application in the dairy industry.

The development of new nonthermal techniques is challenging to both dairy microbiologists and enzymologists.

Finally, methods based on immunochemistry, biosensors or spectrometry may find new applications in analytical dairy enzymology.

ACKNOWLEGEMENTS

I am grateful for 3 years' supervision provided by Professor Patrick Fox in UCC, Cork, Ireland, and for the subsequent collaboration that began in 1982, after my affiliation with the Agricultural University of Norway. Over the period of 22 years I have received much good advice and hundreds of suggestions.

REFERENCES

Adamitsch B F and Hampel W A (2000) Formation of lipolytic enzymes by *Brevibacterium linens*. *Biotechnology Letters* **22** 1643–1646.

Aikawa J, Park Y N, Sugiyama M, Nishiyama M, Horinouchi S and Beppu T (2001) Replacements of amino acid residues at subsites and their effects on the catalytic properties of *Rhizomucor pusillus* pepsin, an aspartic proteinase from *Rhizomucor pusillus*. *Journal of Biochemistry* **129** 791–794.

Akuzawa R and Fox P F (1998) Purification and characterization of an acid phosphatase from cell membrane fraction of *Lactococcus lactis* ssp. *lactis* 303. *Food Science International* **31** 157–165.

Alting A C, Engels W J M P, van Schalkwijk S and Exterkate F A (1995) Purification and characterization of cystathionine β-lyase from *Lactococcus lactis* subsp. *cremoris* B78 and its possible role in flavor development in cheese. *Applied and Environmental Microbiology* **61** 4037–4042.

Baankreis R and Exterkate F A (1991) Characterization of a peptidase from *Lactococcus lactis* ssp. *cremoris* HP that hydrolyses di- and tripeptides containing proline or hydrophobic residues as the aminoterminal amino acid. *Systematic Applied Microbiology* **14** 317–323.

Bendicho S, Barbarosa-Cánovas G V and Martín O (2002) Milk processing by high intensity pulsed electric fields. *Trends in Food Science and Technology* **13** 195–204.

Benfeldt C, Larsen L B, Rasmussen J T, Andreasen P A and Petersen T E (1995) Isolation and characterization of plasminogen and plasmin from bovine milk. *International Dairy Journal* **5** 517–592.

Bracq E, Levieux A and Levieux D (1997) Purification and immunochemical quantitation of *Penicillium roqueforti* acid aspartyl proteinase. *Journal of Dairy Research* **64** 105–113.

Broadbent J R, Barnes M, Brennand C, Strickland M, Houck K, Johnson M E and Steele J (2002) Contribution of *Lactococcus lactis* cell envelope proteinase specificity to peptide accumulation and bitterness in reduced-fat Cheddar cheese. *Applied and Environmental Microbiology* **68** 1778–1785.

Bruinenberg P G, de Roo G and Limsowtin G K Y (1997) Purification and characterisation of cystathionine γ-lyase from *Lactococcus lactis* subsp. *cremoris* SK11: possible role in flavour compound formation during cheese maturation. *Applied and Environmental Microbiology* **63** 561–566.

Buffa M, Guamis B, Pavia M and Trujillo A J (2001) Lipolysis in cheese made from raw, pasteurized or high-pressure-treated goats' milk. *International Dairy Journal* **11** 175–179.

Chen H J, Zhang G B, Zhang Y Y, Dong Y C and Yang K Y (2000) Functional implications of disulfide bond, Cys206–Cys210, in recombinant prochymosin (chymosin). *Biochemistry* **39** 12140–12148.

Chilliard Y, Ferlay A, Rouel J and Lamberett G (2003) A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. *Journal of Dairy Science* **86** 175–1770.

Chitpinityol S and Crabbe M J C (1998) Chymosin and aspartic proteinases. *Food Chemistry* **61** 395–418.

Christensen J E, Dudley E G, Pederson J A and Steele J L (1999) Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **76** 217–246.

Chrzanowska J, Koaczkowska M, Dryjański M, Stachowiak D and Polanowski A (1995) Aspartic proteinase from *Penicillium camemberti*. Purification, properties, and substrate specificity. *Enzyme and Microbial Technology* **17** 719–724.

Collins Y F, McSweeney P L H and Wilkinson M G (2003) Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening. *Journal of Dairy Research* **70** 105–113.

Considine T, Healy A, Kelly A L and McSweeney P L H (1999) Proteolytic specificity of elastase on bovine β-casein. *Food Chemistry* **66** 463–470.

Considine T, Healy A, Kelly A L and McSweeney P L H (2000) Proteolytic specificity of elastase on bovine α_{s1}-casein. *Food Chemistry* **69** 19–26.

Considine T, Geary S, Kelly A L and McSweeney P L H (2002a) Proteolytic specificity of cathepsin G on bovine α_{s1}- and beta-caseins. *Food Chemistry* **76** 59–67.

Considine T, McSweeney P L H and Kelly A L (2002b) The effect of lysosomal proteinases and plasmin on the rennet coagulation properties of skim milk. *Milchwissenschaft* **57** 425–428.

Darewicz M, Dziuba J and Caessens P W J R (2000) Effect of enzymatic hydrolysis on emulsifying and foaming properties of milk proteins — a review. *Polish Journal of Food and Nutrition Sciences* **9** 3–8.

Datta N and Deeth H C (1999) High pressure processing of milk and dairy products. *Australian Journal of Dairy Technology* **54** 41–48.

De Angelis M, Curtin A C, McSweeney P L H, Faccia M and Gobbetti M (2002) *Lactobacillus reuteri* DMS 20016. purification and characterization of a cystathionine γ-lyase and use as adjunct starter in cheesemaking. *Journal of Dairy Research* **69** 255–267.

De Jong G A H, Wijngaards G and Koppelman S J (2003) Transglutaminase inhibitor from milk. *Journal of Food Science* **68** 820–825.

Deutsch S M, Molle D, Gagnaire V, Piot M, Atlan D and Lortal S (2000) Hydrolysis of sequenced β-casein peptides provides new insight into peptidase activity from thermophilic lactic acid bacteria and highlights intrinsic resistance of phosphapeptides. *Applied and Environmental Microbiology* **66** 5360–5367.

Dias B and Weimer B (1998a) Conversion of methionine to thiols by lactococci, lactobacilli and brevibacteria. *Applied and Environmental Microbiology* **64** 3320–3326.

Dias B and Weimer B (1998b) Purification and characterization of L-methionine γ-lyase from *Brevibacterium linens*. *Applied and Environmental Microbiology* **64** 3327–3331.

Dobric N, Limsowtin G K Y, Hillier A J, Dudman N P B and Davidson B E (2000) Identification and characterisation of a cystathionine β/γ-lyase from *Lactococcus lactis* susp. *cremoris* MG1363. *FEMS Microbiology Letters* **182** 249–254.

Dupont D, Rolet-Repecaud O and Senocq D (2003) A new approach to monitoring proteolysis phenomena using antibodies specifically directed against the enzyme cleavage site on its substrate. *Analytical Biochemistry* **317** 240–246.

Engels W J M, Alting A C, Arntz M M T G, Gruppen H, Voragen A G J, Smit G and Visser S (2000) Partial purification and characterization of two aminotransferases from *Lactococcus lactis* subsp. *cremoris* B78 involved in the catabolism of methionine and branched-chain amino acids. *International Dairy Journal* **10** 443–452.

Farkye N Y (2003) Other enzymes. In *Advanced Dairy Chemistry*, Vol. 1, Part A, pp 581–603. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Farkye N Y and Fox P F (1992) Contribution of plasmin to Cheddar cheese ripening: effect of added plasmin. *Journal of Dairy Research* **59** 209–216.

Farkye N Y and Imafidon G I (1995) Thermal denaturation of indigenous milk enzymes. In *Heat-Induced Changes in Milk*, pp 331–348. Fox P F, ed. Brussels: International Dairy Federation.

Fenster K M, Parkin K L and Steele J L (2000) Characterization of an arylesterase from *Lactobacillus helveticus* CNRZ32. *Journal of Applied Microbiology* **88** 572–583.

Fenster K M, Parkin K L and Steele J L (2003a) Intracellular esterase from *Lactobacillus casei* LILA. Nucleotide sequencing, purification and characterization. *Journal of Dairy Science* **86** 1118–1129.

Fenster K M, Parkin K L and Steele J L (2003b) Nucleotide sequencing, purification and biochemical properties of

an arylesterase from *Lactobacillus casei* LILA. *Journal of Dairy Science* **86** 2547–2557.

Fenster K M, Rankin S A and Steele J L (2003c) Accumulation of short n-chain ethyl esters by esterases of lactic acid bacteria under conditions simulating ripening Parmesan cheese. *Journal of Dairy Science* **86** 2818–2825.

Fernández L, Beerthuyzen M M, Brown J, Siezen R J, Coolbear T, Holland R and Kuipers O P (2000) Cloning, characterization, controlled overexpression, and inactivation of the major tributyrin esterase gene of *Lactococcus lactis*. *Applied and Environmental Microbiology* **66** 1360–1368.

Flanagan J and FitzGerald R J (2003) Functional properties of *Bacillus* proteinase hydrolysates of sodium caseinate incubated with transglutaminase pre- and post-hydrolysis of sodium caseinate incubated with transglutaminase pre- and post-hydrolysis. *International Dairy Journal* **13** 135–143.

Floris R, Recio I, Berkhout B and Visser S (2003) Antibacterial and antiviral effects of milk proteins and derivatives thereof. *Current Pharmaceutical Design* **9** 1257–1275.

Fox P F (1989) Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science* **72** 1379–1400.

Fox P F (2003) Indigenous enzymes in milk. In *Advanced Dairy Chemistry*, Vol. 1, Part A, pp 467–471. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Fox P F and Grufferty M D (1991) Exogenous enzymes in dairy technology. In *Food Enzymology*, Vol. 1, pp 219–270. Fox P F, ed. London: Elsevier.

Fox P F and McSweeney P L H (1996) Proteolysis in cheese during ripening. *Food Reviews International* **12** 457–509.

Fox P F and Stepaniak L (1993) Enzymes in cheese technology. *International Dairy Journal* **3** 509–530.

Fox P F and Wallace J M (1997) Formation of flavour compounds in cheese. *Advances in Applied Microbiology* **45** 1785.

Fox P F, Singh T K and McSweeney P L H (1995) Biogenesis of flavour compounds in cheese. In *Chemistry of Structure–Function Relationships in Cheese*, pp 59–98. Malin E L and Tunick M H, eds. London: Plenum Press.

Fruton J S (2002) A history of pepsin and related enzymes. *Quarterly Review of Biology* **77** 127–147.

García-Graells C, Van Opstal I, Vanmuysen S C M and Michiels C W (2003) The lactoperoxidase system increases efficacy of high-pressure. *International Journal of Food Microbiology* **81** 211–221.

Gente S, Durand N, Poussereau N and Fevre M (1997) Controls of the expression of aspA, the aspartyl protease gene from *Penicillium roqueforti*. *Molecular and General Genetics* **256** 557–565.

Geurts T J, Lettink F J and Wouters J T M (2003) Lipase in milk, curd and cheese. *Milchwissenschaft* **58** 61–65.

Gobbetti M, Cossignani L, Simonetti M S and Diamani P (1995) Effect of aminopeptidase from *Pseudomonas fluorescens* ATTC-948 on synthetic bitter peptides, bitter hydrolysates of UHT milk proteins and on the ripening of Italian Caciotta-type cheese. *Lait* **75** 169–179.

Gobbetti M, Fox P F and Stepaniak L (1997a) Isolation and characterization of a tributyrin esterase from *Lactobacillus plantarum* 2739. *Journal of Dairy Science* **80** 3099–3106.

Gobbetti M, Smacchi E and Corsetti A (1997b) Purification and characterization of a cell surface-associated esterase from *Lactobacillus fermentum* DT41. *International Dairy Journal* **7** 13–21.

Gobbetti M, Smacchi E, Semeraro M, Fox P F, Lanciotti R and Cogan T (2001) Purification and characterization of an extracellular proline iminopeptidase from *Corynebacterium variabilis* NCDO 2101. *Journal of Applied Microbiology* **90** 449–456.

Gobbetti M, Stepaniak L, De Angelis M, Corsetti A and Di Cagno R (2002) Latent bioactive peptides in milk proteins. Proteolytic activation and significance in dairy processing. *Critical Reviews in Food Science and Nutrition* **42** 223–239.

Gould G W (2001) New processing technologies: an overview. *Proceedings of the Nutrition Society* **60** 463–474.

Grappin R and Beuvier E (1997) Possible implications of milk pasteurization on the manufacture and sensory quality of ripened cheese. *International Dairy Journal* **7** 751–761.

Hancock J T, Salisbury V, Ovejero-Boglione MC, Cherry R, Hoare C, Eisenthal R and Harrison R (2002) Antimicrobial properties of milk. Dependence on presence of xanthine oxidase and nitrite. *Antimicrobial Agents and Chemotherapy* **46** 3308–3310.

Hayashi K (1996) Accelerated maturation of cheese by proteolytic enzymes produced by *Brevibacterium linens*. *Japan Agricultural Research Quarterly* **30** 129–137.

Hayes M G, McSweeney P L H and Kelly A L (2002) The influence of native and heat-denatured whey proteins on enzyme activity. 1. Plasmin. *Milchwissenschaft* **57** 208–211.

Hendrickx M, Ludikhuyze L, Van der Broeck I and Weemaes C (1998) Effect of high pressure on enzymes related to food quality. *Trends in Food Science and Technology* **9** 197–203.

Holland R and Coolbear T (1996) Purification of tributyrin esterase from *Lactococcus lactis* subsp. *cremoris* E8. *Journal of Dairy Research* **63** 131–140.

Holland R, Liu S Q, Wang T, Bennett M, Norris G, Delabre M L, Lubbers M W, Dekker J W and Crow V L (2002) Esterases of lactic acid bacteria. *Australian Journal of Dairy Technology* **57** 116–116.

Huppertz T, Kelly A L and Fox P F (2002) Effects of high pressure on constituents and properties of milk. *International Dairy Journal* **12** 561–572.

Hurley M J, Larsen L B, Kelly A L and McSweeney P L H (2000) Cathepsin D activity in quarg. *International Dairy Journal* **10** 453–458.

Hynek R, Zuzalkova L, Sikac Z and Kas J (2002) Influence of phosphorylation on proteolytic cleavage. *Journal of Liquid Chromatography and Related Technologies* **25** 2307–2314.

Hyslop D B (2003) Enzymatic coagulation of milk. In *Advanced Dairy Chemistry*, Vol. 1 Part B, pp 839–878. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Jolivet P, Macedo I Q, Wu M and Meunier J C (2000) Plasmin digestion of bovine beta-casein dephosphorylated with one protein phosphatase type 2A purified from *Yarrowia lipolytica*. *Lait* **80** 517–526.

Kageyama T (2002) Pepsinogens, progastricins and prochymosins: structure, function, evolution and development. *Cellular and Molecular Life Sciences* **59** 288–306.

Kelly A L and McSweeney P L H (2003) Indigenous proteinases in milk. In *Advanced Dairy Chemistry*, Vol. 1, Part B, pp 495–521. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Kilcawley K N, Wilkinson M G and Fox P F (1998) Enzyme-modified cheese. *International Dairy Journal* **8** 1–10.

Kilcawley K N, Wilkinson M G and Fox P F (2002a) Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. *Enzyme Microbiology and Technology* **31** 310–320.

Kilcawley K N, Wilkinson M G and Fox P F (2002b) Properties of commercial microbial proteinase preparations. *Food Biotechnology* **16** 29–55.

Kojima Y, Kobayashi M and Shimizu S (2003) A novel lipase from *Pseudomonas fluorescens* HU380. Gene cloning, overproduction, renaturation–activation, two-step purification, and characterization. *Journal of Bioscience and Bioengineering* **96** 242–249.

Koka R and Wimer B C (2000) Investigation of the ability of a purified protease from *Pseudomonas fluorescens* RO98 to hydrolyze bitter peptides from cheese. *International Dairy Journal* **10** 75–79.

Kunji E R S, Mierau I, Hagting A, Poolman B and Konings W N (1996) The proteolytic system of lactic acid bacteria. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **70** 187–221.

Lambert D A, Smith L C, Pownall H, Sparrow J T, Nicolas J P and Gotto A M (2000) Hydrolysis of phospholipids by purified milk lipoprotein lipase — effect of apoprotein CII, CIII, A and E, and synthetic fragments. *Clinica Chimica Acta* **291** 19–33.

Lane C N and Fox P F (1999) The individual or combined action of chymosin and plasmin on sodium caseinate or β-casein in solution: effect of NaCl and pH. *Lait* **79** 423–434.

Leenhouts K, Bolhuis A, Boot J, Deutz I, Toonen M, Venema G, Kok J and Ledeboer A (1998) Cloning, expression, and chromosomal stabilization of the *Propionibacterium shermanii* proline iminopeptidase gene (pip) for food-grade application in *Lactococcus lactis*. *Applied and Environmental Microbiology* **64** 4736–4742.

Li H Y, Zhang Y Y, Dong Y C and Yang K Y (1998) Functional implications of the 21–24 loop in recombinant prochymosin. *Biochimica et Biophysica Acta* **1384** 121–129.

Liu S Q, Holland R and Crow V L (2001) Purification and properties of intracellular esterases from *Streptococcus thermophilus*. *International Dairy Journal* **11** 27–35.

Lo Piero A R, Puglisi I and Petrone G (2002) Characterization of 'lettucine', a serine-like protease from *Lactuca sativa* leaves, as a novel enzyme for milk clotting. *Journal of Agricultural and Food Chemistry* **50** 2439–2443.

Magboul A A A and McSweeney P L H (1999a) Purification and characterization of an acid phosphatase from *Lactobacillus plantarum* DPC2739. *Food Chemistry* **65** 15–22.

Magboul A A A and McSweeney P L H (1999b) Purification and characterization of an acid phosphatase from *Lactobacillus curvatus* DPC2024. *International Dairy Journal* **9** 849–855.

Malone A S, Wick C, Shellhammer T H and Courtney P D (2003) High pressure effects on proteolytic and glycolytic enzymes involved in cheese manufacturing. *Journal of Dairy Science* **86** 1139–1146.

Martínez-Cuesta MC, de Palencia P F, Requena T and Peláez C (2001) Enzymatic ability of *Lactobacillus casei* subsp. *casei* IFPL731 for flavour development in cheese. *International Dairy Journal* **11** 577–588.

Mase T, Matsumiya Y and Matsuura A (1995) Purification and characterization of *Penicillum roqueforti* IAM-7268 lipase. *Bioscience, Biotechnology and Biochemistry* **59** 329–330.

McKellar R C and Piyasena P (2000) Predictive modelling of inactivation of bovine milk α-L-fucosidase in a high-temperature short-time pasteurizer. *International Dairy Journal* **10** 1–6.

McSweeney P L H and Sousa M J (2000) Biochemical pathways for the production of flavor compounds in cheeses during ripening: a review. *Lait* **80** 293–324.

McSweeney P L H, Olson N F, Fox P F, Healy A and Højrup O (1993) Proteolytic specificity of chymosin on bovine α_{s1}-casein. *Journal of Dairy Research* **60** 401–412.

McSweeney P L H, Fox P F and Olson N F (1995) Proteolysis of bovine caseins by cathepsin D: preliminary observations and comparison with chymosin. *International Dairy Journal* **5** 321–336.

Mechakra A, Auberger B, Remeuf F and Lenoir J (1999) Optimization of a culture medium for acid proteolytic enzyme production by *Penicillium camemberti*. *Sciences des Aliments* **19** 663–675.

Minervini F F, Algaron F, Rizzello G G, Fox P F, Monnet V and Gobbetti M (2003) Angiotensin I-converting-enzyme-inhibitory and antimicrobial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species. *Applied and Environmental Microbiology* **69** 5297–5305.

Minkiewicz P, Slangen C J, Dziuba J, Visser S and Mioduszewska H (2000) Identification of peptides obtained via hydrolysis of bovine casein by chymosin using HPLC and mass spectrometry. *Milchwissenschaft* **55** 14–17.

Mooney J S, Fox P F, Healy A and Leaver J (1998) Identification of the principal water-insoluble peptides in Cheddar cheese. *International Dairy Journal* **9** 813–818.

Murasugi A, Asami Y and Mera-Kikuchi Y (2001) Production of recombinant human bile salt-stimulated lipase in *Pichia pastoris*. *Protein Expression and Purification* **23** 282–288.

Nardi M, Fiez-Vandal C, Tailliez P and Monnet V (2002) The EstA esterase is responsible for the main capacity of *Lactococcus lactis* to synthesize short chain fatty acid esters *in vitro*. *Journal of Applied Microbiology* **93** 994–1002.

Nielsen S S (2002) Plasmin system and microbial proteases in milk. Characteristics, roles and relationship. *Journal of Agricultural and Food Chemistry* **50** 6628–6634.

Oberg C J, Broadbent J R, Strickland M and McMahon D J (2002) Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Letters in Applied Microbiology* **34** 455–460.

Olivecrona T, Vilaró S and Olivecrona G (2003) Lipases in milk. In *Advanced Dairy Chemistry*, Vol. 1, Part A, pp 473–494. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

O'Mahony J A, Sousa M J and McSweeney P L H (2003) Proteolysis in miniature Cheddar-type cheeses made using blends of chymosin and *Cynara cardunculus* proteinases as coagulant. *International Journal of Dairy Technology* **53** 52–58.

Orlowski M (1990) The multicatalytic proteinase complex, a major extra-lysosomal proteolytic system. *Biochemistry* **29** 10289–10297.

O'Sullivan M M, Kelly A L and Fox P F (2002) Influence of transglutaminase treatment on some physico-chemical

properties of milk. *Journal of Dairy Research* **69** 433–442.

Ozen B F, Hayes K D and Mauer L J (2003) Measurement of plasminogen concentration and differentiation of plasmin and plasminogen using Fourier-transform infrared spectroscopy. *International Dairy Journal* **13** 441–446.

Parry M A A, Zhang X C and Bode W (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends in Biochemical Sciences* **25** 53–59.

Pillidge C J, Crow V L, Coolbear T and Reid J R (2003) Exchanging lactocepin plasmids in lactococcal starters to study bitterness development in Gouda cheese: a preliminary investigation. *International Dairy Journal* **13** 345–354.

Preuss I, Kaiser I and Gehring U (2001) Molecular characterization of a phosphatidylcholine-hydrolyzing phospholipase C. *European Journal of Biochemistry* **268** 5081–5091.

Pruitt K (2003) Lactoperoxidase. In *Advanced Dairy Chemistry*, Vol. 1, Part A, pp 545–561. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Raksakulthai R and Haard N F (2003) Exopeptidases and their application to reduce bitterness in food: a review. *Critical Reviews in Food Science and Nutrition* **43** 401–445.

Rattray F P and Fox P F (1997) Purification and characterisation of an intracellular aminopeptidase from *Brevibacterium linens* ATCC 9174. *Lait* **77** 169–180.

Rattray F P and Fox P F (1998) Recently identified enzymes of *Brevibacterium linens* ATCC 9174 — a review. *Journal of Food Biochemistry* **22** 353–373.

Rattray F P and Fox P F (1999) Aspects of enzymology and biochemical properties of *Brevibacterium linens* relevant to cheese ripening: a review. *Journal of Dairy Science* **82** 91–908.

Rattray F P, Bockelmann W and Fox P F (1995) Purification and characterization of an extracellular proteinase from *Brevibacterium linens* ATCC 9174. *Applied and Environmental Microbiology* **61** 3454–3456.

Rattray F P, Fox P F and Healy A (1996) Specificity of an extracellular proteinase from *Brevibacterium linens* ATCC 9174 on bovine α_{s1}-casein. *Applied and Environmental Microbiology* **62** 501–506.

Rattray F P, Fox P F and Healy A (1997) Specificity of an extracellular proteinase from *Brevibacterium linens* ATCC 9174 on bovine β-casein. *Applied and Environmental Microbiology* **63** 2468–2471.

Rawling N D and Barret A J (1995) Evolutionary families of metallopeptidases. *Methods in Enzymology* **248** 183–228.

Rijnen L, Yvon M, van Kranenburg R, Courtin P, Verheul A, Chambellon E and Smit G (2003) Lactococcal aminotransferases AraT and BcaT are key enzymes for the formation of aroma compounds from amino acids in cheese. *International Dairy Journal* **13** 805–812.

Rollema H S, Visser S and Poll J K (1983) Spectrophotometric assay of plasmin and plasminogen in bovine milk. *Milchwissenschaft* **38** 214–217.

Saha B and Hayashi K (2001) Debittering of protein hydrolyzates. *Biotechnology Advances* **19** 355–370.

Saint-Denis T, Humbert G and Gaillard J L (2001) Enzymatic assays for native plasmin, plasminogen and plasminogen activators in bovine milk. *Journal of Dairy Research* **68** 437–449.

Sakai Y, Ishikawa J, Fukasaka S, Yurimoto H, Mitsui R, Yanase H and Kato N (1999) A new carboxylesterase from *Brevibacterium linens* IFO 12171 responsible for the conversion of 1,4-butanediol diacrylate to 4-hydroxybutyl acrylate: purification, characterization, gene cloning, and gene expression in *Escherichia coli*. *Bioscience, Biotechnology and Biochemistry* **63** 688–697.

Sakiyama T, Yoshimi T, Miyake A, Umeoka M, Tanaka A, Ozaki S and Nakanishi K (2001) Purification and characterization of a monoacylglycerol lipase from *Pseudomonas* sp. LP7315. *Journal of Bioscience and Bioengineering* **91** 27–32.

Schanbacher F L, Talhouk R S, Murray F A, Gherman L I and Willet L B (2003) Milk-borne bioactive peptides. *International Dairy Journal* **8** 393–404.

Shakeel-ur-Rehman, Fleming C M, Farkye N Y and Fox P F (2003) Indigenous phosphatases in milk. In *Advanced Dairy Chemistry*, Vol. 1, Part A, pp 523–543. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Shastry S and Prasad M S (2002) Extracellular protease from *Pseudomonas* sp (CL 1457) active against *Xanthomonas campestris*. *Process Biochemistry* **37** 611–621.

Smacchi E and Gobbetti M (1998) Purification and characterization of cystathionine γ-lyase from *Lactobacillus fermentum* DT41. *FEMS Microbiology Letters* **166** 197–202.

Smacchi E, Gobbetti M, Lanciotti R and Fox P F (1999) Purification and characterization of an extracellular proline iminopeptidase from *Arthrobacter nicotianae* 9458. *FEMS Microbiology Letters* **178** 191–197.

Soeryapranata E, Powers J R, Hill H H, Siems W F, Al-Saad K and Weller K M (2002) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method for the quantification of β-casein fragment (f193–209). *Journal of Food Science* **67** 534–538.

Sørhaug T and Stepaniak L (1991) Microbial enzymes in spoilage of milk and dairy products. In *Food Enzymology*, Vol. 1, pp 169–218. Fox P F, ed. London: Elsevier.

Sørhaug T and Stepaniak L (1997) Psychrotrophs and their enzymes in milk and dairy products: quality aspects. *Trends in Food Science and Technology* **8** 35–41.

Stefanitsi D, Sakellaris G and Garel J R (1995) The presence of two proteinases associated with the cell wall of *Lactobacillus bulgaricus*. *FEMS Microbiology Letters* **128** 53–58.

Stepaniak L (2002) Isolation and partial characterization of catalytic antibodies with oligonuclease activity from bovine colostrum. *Preparative Biochemistry and Biotechnology* **32** 17–28.

Stepaniak L, Fleming C M, Gobbetti M, Corsetti A and Fox P F (2003) Indigenous nucleases in milk. In *Advanced Dairy Chemistry*, Vol. 1, Part A, pp 545–561. Fox P F and McSweeney P L H, eds. New York: Kluwer Academic.

Stepaniak L and Fox P F (1995) Characterization of the principal intracellular endopeptidase from *Lactococcus lactis* sbsp. *lactis* MG1363. *International Dairy Journal* **5** 699–713.

Stepaniak L and Sørhaug T (1995) Thermal inactivation of bacterial enzymes in milk. In *Heat-Induced Changes in Milk*, pp 349–363. Fox P F, ed. Brussels: International Dairy Federation.

Stepaniak L and Sørhaug T (2001) Degradation of β-casein fragments 193–209 and 194–209 by oligopeptidases and cytoplasms from *Lactococcus* and *Lactobacillus*. *Natural Sciences (Poland)* **8** 145–152.

Stepaniak L, Jedrychowski L, Grabska J, Wróblewska B and Sørhaug T (1998) Application of immunoassays for protein and peptides in milk and dairy products. *Recent Research Developments in Agricultural and Food Chemistry* **2** 673–687.

Stepaniak L, Wróblewska B, Jedrychowski L and Sørhaug T (2001) Immunorelatedness of oligopeptidases from *Lactobacillus* and *Lactococcus* and proline iminopeptidase from *Propionibacterium* using double immunodiffusion assay. *Food and Agricultural Immunology* **13** 183–187.

Tanous C, Kieronczyk A, Helinck S, Chambellon E and Yvon M (2002) Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **82** 271–278.

Tobiassen R O (1996) *Endopeptidases of Dairy Microorganisms*. PhD Thesis, Agricultural University of Norway, Ås.

Tuler T R, Callanan M J and Klaenhammer T R (2002) Overexpression of peptidases in *Lactococcus* and evaluation of their release from leaky cells. *Journal of Dairy Science* **85** 2438–2450.

Ur-Rehman S and Fox P F (2002) Effect of added α-ketoglutaric acid, pyruvic acid or pyridoxal phosphate on proteolyis and quality of Cheddar cheese. *Food Chemistry* **76** 21–26.

Valence F, Richoux R, Thierry A and Lortal S (1998) Autolysis of *Lactobacillus helveticus* and *Propionibacterium freudenreichii* in Swiss cheese: first evidence using species-specific lysis markers. *Journal of Dairy Research* **65** 609–620.

Veríssimo P, Esteves C, Faro C and Pires E (1995) The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnology Letters* **17** 621–626.

Walker S A and Klaenhammer T R (2001) Leaky *Lactococcus* cultures that externalize enzymes and antigens independently of culture lysis and secretion and export pathways. *Applied and Environmental Microbiology* **67** 251–259.

Walzem R L, Dillard C J and German J B (2002) Whey components: millennia of evolution create functionalities for mammalian nutrition. What we know and what we may be overlooking. *Critical Reviews in Food Science and Nutrition* **42** 353–375.

Wang X, Ye X and Ng T B (2002) First demonstration of an inhibitory activity of milk proteins against human immunodeficiency virus-1 reverse transcriptase and the effect of succinylation. *Life Sciences* **67** 2745–2752.

Whitaker J R, Voragen A G J and Wong D W S (2003) *Handbook of Food Enzymology*. New York: Marcel Dekker.

Wilkins K M and Board R G (1989) Natural antimicrobial systems. In *Mechanisms of Action of Food Preservation Procedures*, pp 285–362. Gould G W, ed. London: Elsevier.

Yvon M, Chambellon E, Bolotin A and Roudot-Algaron F (2000) Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. *Applied and Environmental Microbiology* **66** 571–577.

Yvon M and Rijnen L (2001) Cheese flavour formation by amino acid catabolism. *International Dairy Journal* **11** 185–201.

Yvon M, Thirouin L, Rijnen D, Fromentier D and Gripon J C (1997) An aminotransferase from *Lactococcus lactis* initiates conversion of aromatic amino acids to flavor compounds. *Applied and Environmental Microbiology* **63** 414–419.

Zhang Y Y, Li H S, Wu H, Don Y C, Liu N J and Yang K Y (1997) Functional implications of disulfide bond, Cys45–Cys50, in recombinant prochymosin. *Biochimica et Biophysica Acta* **1343** 278–286.