

Biochemistry of cheese ripening

PAUL L H McSWEENEY

Department of Food and Nutritional Sciences, University College, Cork, Ireland

Rennet-coagulated cheeses are ripened for periods ranging from about two weeks to two or more years depending on variety. During ripening, microbiological and biochemical changes occur that result in the development of the flavour and texture characteristic of the variety. Biochemical changes in cheese during ripening may be grouped into primary (lipolysis, proteolysis and metabolism of residual lactose and of lactate and citrate) or secondary (metabolism of fatty acids and of amino acids) events. Residual lactose is metabolized rapidly to lactate during the early stages of ripening. Lactate is an important precursor for a series of reactions including racemization, oxidation or microbial metabolism. Citrate metabolism is of great importance in certain varieties. Lipolysis in cheese is catalysed by lipases from various source, particularly the milk and cheese microflora, and, in varieties where this coagulant is used, by enzymes from rennet paste. Proteolysis is the most complex biochemical event that occurs during ripening and is catalysed by enzymes from residual coagulant, the milk (particularly plasmin) and proteinases and peptidases from lactic acid bacteria and, in certain varieties, other microorganisms that are encouraged to grow in or on the cheese. Secondary reactions lead to the production of volatile flavour compounds and pathways for the production of flavour compounds from fatty acids and amino acids are also reviewed.

Keywords Amino acid catabolism, Cheese flavour, Cheese ripening, Citrate metabolism, Lipolysis, Metabolism of fatty acids, Metabolism of lactate, Proteolysis.

INTRODUCTION

Rennet-coagulated cheeses are ripened for periods ranging from about two weeks (e.g. mozzarella) to two or more years (e.g. Parmigiano-Reggiano or extra-mature Cheddar). The ripening process of cheese is very complex and involves microbiological and biochemical changes to the curd resulting in the flavour and texture characteristic of the particular variety. Microbiological changes to cheese during ripening include the death and lysis of starter cells, the growth of an adventitious flora ('nonstarter lactic acid bacteria', principally facultatively heterofermentative lactobacilli) and, in many varieties, the development of a secondary microflora, e.g. *Propionibacterium freudenreichii* in Swiss cheese, moulds in mould-ripened varieties and a complex Gram-positive bacterial flora in smear cheeses, which is often of great importance to the flavour and, in some instances, the texture of these varieties. Microbial changes during ripening have been discussed by Beresford and Williams (2004) and are not discussed further here. Cheese texture softens during ripening as a consequence of hydrolysis of the casein micelle by proteolysis and changes to the water-binding ability of the curd and changes in pH (which in turn may cause other changes such as the migration and precipitation of calcium phosphate). Cheese texture and rheology have been discussed by O'Callaghan and Guinee (2004).

The biochemical changes occurring during ripening may be grouped into primary events that include the metabolism of residual lactose and of lactate and citrate (often, though erroneously, referred to collectively as 'glycolysis'), lipolysis and proteolysis. Following these primary events, secondary biochemical events are very important for the development of many volatile flavour compounds and include the metabolism of fatty acids and of amino acids. The biochemistry of cheese ripening is an active area of research and aspects of ripening have been reviewed extensively (e.g. Grappin *et al.* 1985; Rank *et al.* 1985; Fox 1989; Fox *et al.* 1990, 1993, 1994, 1995, 1996; Fox and Law 1991; Fox and McSweeney 1996a,b, 1997; Fox and Wallace 1997; McSweeney and Sousa 2000; Sousa *et al.* 2001; Yvon and Rijnen 2001; Smit *et al.* 2002; Collins *et al.* 2003b, 2004; Curtin and McSweeney 2004; McSweeney 2004; McSweeney and Fox 2004; Upadhyay *et al.* 2004).

INITIAL BIOCHEMICAL EVENTS

Metabolism of residual lactose

As cheese is a fermented dairy product, a key feature of its manufacture is the metabolism of lactose to lactate by selected cultures of lactic acid bacteria (LAB) known as starters. The rate and extent of acidification influence the initial texture of the curd by controlling the rate of demineralization

E-mail:
p.mcsweeney@ucc.ie

(see McSweeney and Fox 2004). Demineralization has been reported to increase the susceptibility of casein micelles to proteolysis (O'Keeffe *et al.* 1975) but the effect of demineralization on proteolysis during ripening requires further study. The pH of cheese curd is determined by the extent of acidification during manufacture, the buffering capacity of cheese curd and, in some cases, deacidification during ripening. The pH of cheese affects the texture of curd directly by influencing the solubility of the caseins; all else being equal, high pH cheeses are softer than more acid cheeses. pH also affects texture and flavour indirectly by affecting the activity of enzymes important to ripening and, in the case of the coagulant, the retention of enzyme in the curd during manufacture (Holmes *et al.* 1977; Stadhouders *et al.* 1977; Visser 1977; Creamer *et al.* 1985; Garnot *et al.* 1987).

Most of the lactose in milk is lost in the whey as lactose or lactate during cheese manufacture. However, low levels of lactose remain in the curd at the end of manufacture (e.g. 0.8–1.0% for Cheddar at milling; Huffman and Kristoffersen 1984). The complete fermentation of lactose is important in cheese to avoid the development of an undesirable secondary microflora. Residual lactose is metabolized quickly to L-lactate during the early stages of ripening at a rate largely determined by temperature and the salt-in-moisture (S/M) levels of the curd by the action of starter bacteria (Turner and Thomas 1980; Parente and Cogan 2004). As S/M levels in Cheddar and other dry-salted varieties increase rapidly on salting, starter activity is stopped

very quickly at the end of manufacture. Lactose that remains unfermented by the starter is probably metabolized by nonstarter lactic acid bacteria (NSLAB) flora (McSweeney and Fox 2004). At high NSLAB populations, considerable amounts of D-lactate are formed by NSLAB by fermentation of residual lactose or by racemization of L-lactate to DL-lactate (Turner and Thomas 1980).

Lactose metabolism in Swiss cheese is quite complex (Turner *et al.* 1983; Fox *et al.* 1990; McSweeney and Fox 2004). Residual lactose trapped in the curd after moulding is rapidly metabolized as the curd cools by *Streptococcus thermophilus*, which metabolizes glucose with the production of L-lactate. Galactose accumulates initially, but this sugar and any remaining lactose are metabolized by the lactobacilli present in the starter giving a mixture of D- and L-lactate (Turner *et al.* 1983; Fox *et al.* 1990; McSweeney and Fox 2004). As discussed later, lactate is metabolized by propionic acid bacteria during the ripening of Swiss cheese to propionate, acetate, H₂O and CO₂.

Metabolism of lactate

Lactate produced from lactose by the growth of the starter is an important substrate for a range of reactions that occur in cheese during ripening (Figure 1). D-Lactate may be formed directly from lactose by starter lactobacilli or NSLAB (Fox *et al.* 2000) or by racemization of L-lactate. The rate at which L-lactate is racemized depends on the composition of the NSLAB flora, for example pediococci racemize lactate faster than lactobacilli (Thomas and Crow 1983) and racemization is probably faster in raw milk cheese than in cheese made from pasteurized milk (McSweeney and Fox 2004). The pathway for lactate racemization probably involves oxidation of L-lactate by L-lactate dehydrogenase to form pyruvate, which is then reduced to D-lactate by the action of D-lactate dehydrogenase. Racemization of lactate is significant because the solubility of Ca-DL-lactate is lower than that of Ca-L-lactate (Thomas and Crow 1983; Dybing *et al.* 1988) and thus racemization favours the formation of Ca-DL-lactate crystals, which are manifested in cheese as white specks, particularly on cut surfaces. These crystals are harmless but they may cause consumers to reject cheese as being mouldy or containing foreign particles (Dybing *et al.* 1988). Increased levels of lactose favour the growth of NSLAB and thus crystal formation (Pearce *et al.* 1973; Sutherland and Jameson 1981) as do factors increasing the release of casein-bound Ca (e.g. low pH or high NaCl) or reducing the solubility of Ca-lactate (e.g. low ripening temperatures; McSweeney and Fox 2004).

Lactate can be oxidized by LAB in cheese to products including acetate, ethanol, formate and CO₂ (see Fox *et al.* 2000). However, the extent to

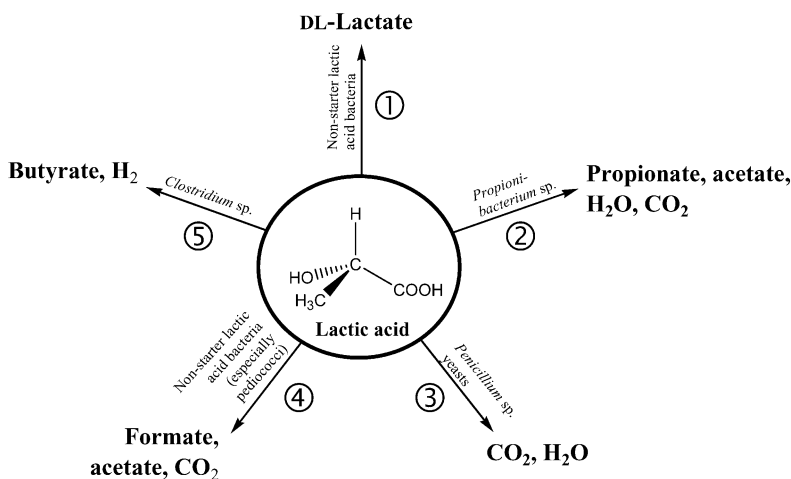


Figure 1 Pathways by which lactate is metabolized in cheese during ripening. 1) racemization, 2) metabolism by *Propionibacterium freudenreichii* in Swiss cheese, 3) oxidative metabolism of lactate, 4) conversion to formate, ethanol and acetate and 5) anaerobic metabolism of lactate to butyrate and H₂, which leads to late gas blowing (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). McSweeney P L H & Fox P F. Metabolism of residual lactose and of lactate and citrate, pp 361–371, Copyright 2004, with permission from Elsevier).

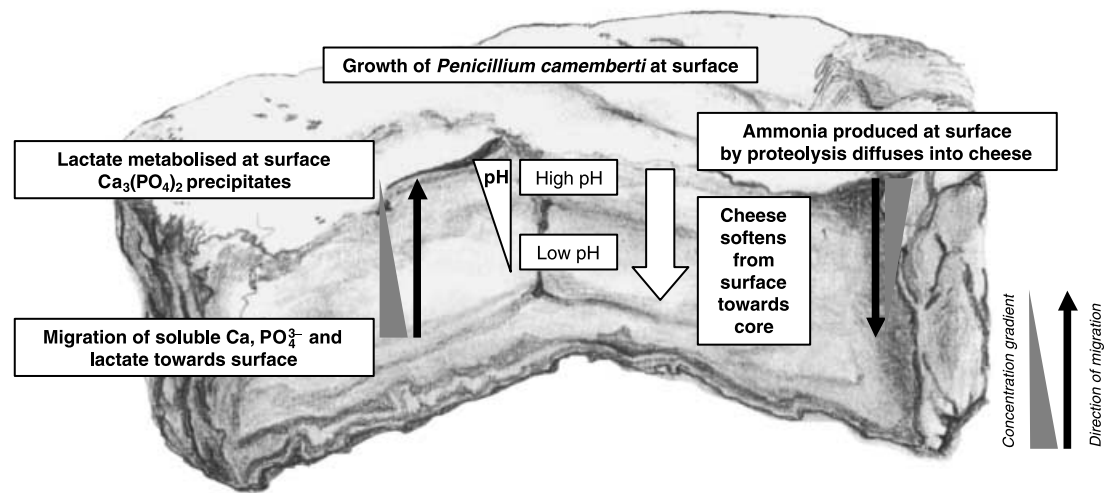


Figure 2 Schematic representation of the changes that occur in Camembert-type cheese during ripening as a consequence of the growth of *Penicillium camemberti* at the surface (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). McSweeney P L H & Fox P F. Metabolism of residual lactose and of lactate and citrate, pp 361–371, Copyright 2004, with permission from Elsevier).

which this pathway occurs in cheese depends on the NSLAB population and the availability of O_2 , which in turn is determined by the size of the block and the oxygen permeability of the packaging material (Thomas 1987), and hence oxidation of lactate by LAB occurs to a very limited extent in cheese wrapped in film due to the low level of O_2 available (McSweeney and Fox 2004).

Anaerobic metabolism of lactate by *Clostridium tyrobutyricum* to butyrate and H_2 results in a defect known as late gas blowing, which causes cracks to appear in the cheese during ripening and the development of off-flavours (Fox *et al.* 1990; McSweeney and Fox 2004). Late gas blowing is a problem principally in brine-salted cheeses because of the time required for NaCl to diffuse into the cheese and to reach inhibitory concentrations (Kleter *et al.* 1984); NaCl levels increase rapidly in Cheddar and other dry-salted varieties and thus these varieties are not susceptible to late gas blowing. Strategies to avoid late gas blowing usually involve minimizing spore numbers in milk (e.g. good hygiene, avoidance of silage, inhibition of spore germination and the growth of vegetative cells, for example by the use of lysozyme or nitrate) or the physical removal of spores by bactofugation or microfiltration (see McSweeney and Fox 2004).

Metabolism of lactate is essential to the development of Emmental and related varieties characterized by the development of large eyes during ripening. Low numbers of *Pr. freudenreichii* are added to the milk or gain access to the vat from the environment and grow during the hot-room step of ripening where they metabolize lactate to propionate, acetate, CO_2 and H_2O (Piveteau 1999). The first two compounds contribute to the flavour of the cheese while CO_2 migrates through the cheese curd until it reaches a point of weakness where

it accumulates. If the partial pressure of CO_2 is sufficient, an eye will form. Eye formation in Swiss-type cheese depends on the rate and quantity of CO_2 production, the number and size of loci suitable for eye development, CO_2 pressure and diffusion rate and cheese texture and temperature (Steffen *et al.* 1993). Relatively little CO_2 produced during the propionic acid fermentation remains trapped in the eyes; most remains dissolved in the cheese mass or is lost to the atmosphere (Steffen *et al.* 1993). As *Pr. freudenreichii* preferentially metabolizes L-lactate (Crow 1986), D-lactate accumulates initially before being metabolized later during ripening.

Lactate metabolism is perhaps of most importance in the ripening of surface mould-ripened cheeses (e.g. Camembert and Brie). Soon after manufacture the surface of these cheeses becomes colonized by secondary microorganisms. Initially, *Geotrichum candidum* and yeasts grow, but they are soon followed by a dense growth of *Penicillium camemberti* and, particularly in artisanal cheese, low numbers of Gram-positive organisms similar to those of the surface flora of smear cheeses (McSweeney and Fox 2004). *G. candidum* and *P. camemberti* rapidly metabolize lactate oxidatively to CO_2 and O_2 , thus deacidifying the cheese surface. As is summarized in Figure 2, deacidification causes a pH gradient to develop from the centre of the cheese to its surface and lactate diffuses from the centre towards the surface of the cheese. In very mature cheese, NH_3 is produced at the surface from proteins and diffuses into the curd. As the pH of the surface of the cheese increases, calcium phosphate precipitates as a layer of $Ca_3(PO_4)_2$, which results in a calcium phosphate gradient from centre to surface and the migration of calcium phosphate towards the surface. Reduction in the concentration of calcium phosphate, together

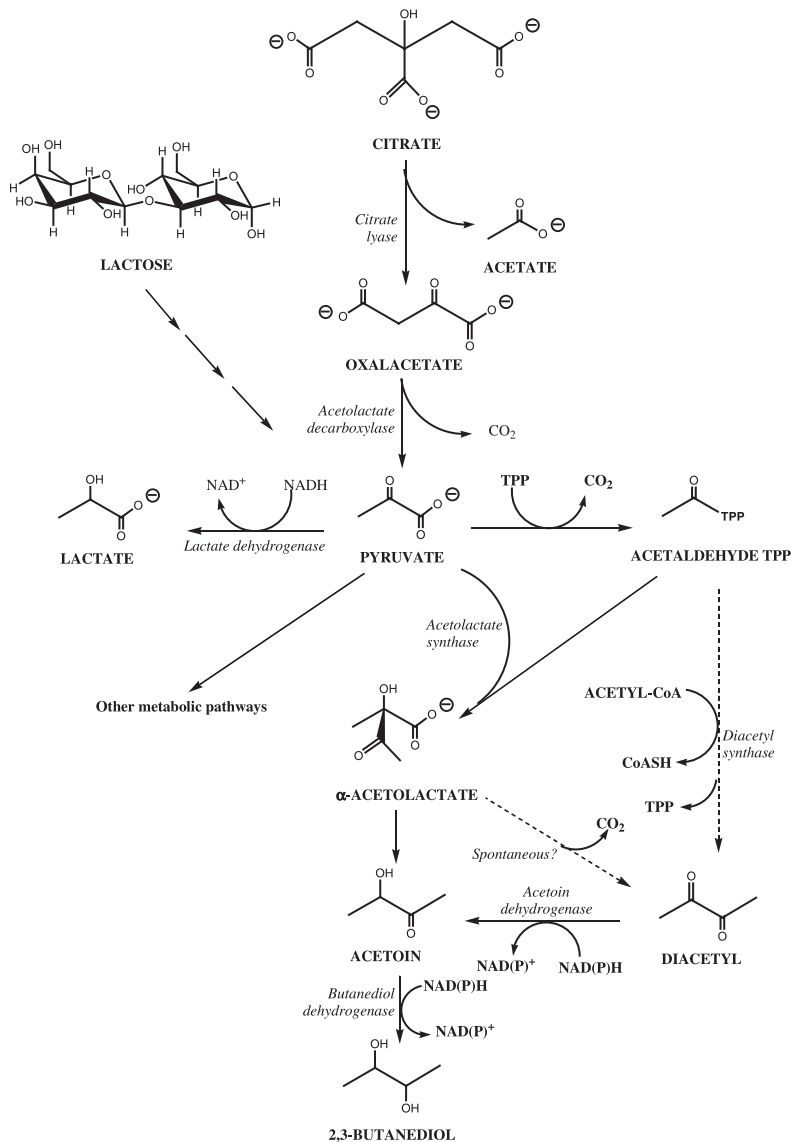


Figure 3 Pathways for citrate-positive strains of *Lactococcus* and *Leuconostoc* sp. (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). McSweeney P L H & Fox P F. Metabolism of residual lactose and of lactate and citrate, pp 361–371, Copyright 2004, with permission from Elsevier).

with increased pH and proteolysis, results in softening of the interior, which is characteristic of mature Camembert-type cheese (McSweeney and Fox 2004). Changes to the cheese matrix may also influence the rates of migration of flavour compounds through the curd or the release of volatile flavour compounds (Engel *et al.* 2001). Metabolism of lactate and the increase in pH at the surface of these cheeses has a major indirect effect on proteolysis by increasing plasmin action. Plasmin and residual coagulant are mainly responsible for proteolysis during the ripening of these cheeses as enzymes produced by *P. camemberti* remain at the surface and do not diffuse into the cheese to any appreciable extent (McSweeney and Fox 2004).

However, small molecules, including flavour compounds, can diffuse through the cheese curd.

Metabolism of citrate

Milk contains approximately 1750 mg citrate per litre, most of which is in the soluble phase and thus is lost on whey drainage (Fox *et al.* 1993). Levels of citrate in cheese curd are approximately three times higher than in the whey (Fryer *et al.* 1970), presumably due to the concentration of colloidal citrate. Cheddar cheese curd typically contains 0.2–0.5% citrate (McSweeney and Fox 2004). Citrate is an important precursor for flavour compounds in certain varieties made using mesophilic starter cultures (Fox *et al.* 1990; Cogan and Hill 1993; Parente and Cogan 2004; McSweeney and Fox 2004).

Citrate is metabolized (Figure 3) by citrate-positive (Cit^+) strains of lactococci, previously referred to as '*Streptococcus diacetylactis*' or '*Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*', which contain a plasmid for citrate transport. *Leuconostoc mesenteroides* ssp. *cremoris* and *Ln. lactis* also metabolize citrate. Citrate is not metabolized by other LAB used as starters (i.e. thermophilic lactobacilli, *Sc. thermophilus* and most strains of lactococci). Citrate is co-metabolized with a fermentable carbohydrate by Cit^+ lactococci and *Leuconostoc* spp. by the pathway shown in Figure 3. The products of citrate metabolism include CO_2 , which is responsible for the small eyes often found in Dutch-type cheeses, and important flavour compounds, particularly diacetyl, which contribute to the flavour of these cheeses.

Citrate may also be metabolized by the pathway shown in Figure 3 by some strains of NSLAB to acetoin, acetate and probably diacetyl (Palles *et al.* 1998). Levels of citrate in Cheddar cheese curd decline to trace levels during the first 6 months of ripening, presumably due to NSLAB action (Thomas 1987).

Lipolysis

Lipids in foods may undergo hydrolytic or oxidative degradation. However, in cheese, oxidative changes are very limited due to the low oxidation/reduction potential (about -250 mV) (Fox and Wallace 1997; McSweeney and Sousa 2000; Collins *et al.* 2003b). However, triglycerides in all cheese varieties undergo hydrolysis by the action of indigenous, endogenous and/or exogenous lipases, which result in the liberation of fatty acids in cheese during ripening. The triglycerides of ruminant milk fat are rich in short-chain fatty acids that, when liberated, have low flavour thresholds that contribute significantly to the flavour of many cheese varieties. Although some lipolysis occurs in most or all cheeses, it is most extensive in some hard Italian varieties and in blue cheese. Low levels

Table 1 Concentration (mg/kg cheese) of free fatty acids in some selected cheese varieties (modified from Collins *et al.* 2003b)

Cheese type	C _{2:0}	C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Total	Reference
Parmesan		1055	451	243	440	439	1540	3896	1171	3471	123		13697	de la Feunte <i>et al.</i> (1993)
Cheddar	476	952	143	175	159	571	952	1556	794	2841	635	238	9492	Kilcawley <i>et al.</i> (2001)
	1587	952	191	159	175	619	746	1253	508	1476	413	175	8254	
	1270	794	111	111	48	238	397	619	270	667	206	111	4842	
Swiss		170	90	45	122	208	311	1904	1427 ^a				4277	Woo <i>et al.</i> (1984)
		345	21	25	53	88	267	930	1197 ^a				2926	
Edam		60	8	9	14	47	39	122	57 ^a				356	Woo <i>et al.</i> (1984)
Mozzarella		54	7	1	120	12	27	76	66 ^a					Woo and Lindsay (1984)
Provolone		782	308	81	172	122	120	199	334 ^a					
Camembert		35	5	14	35	43	69	270	210 ^a				681	Woo <i>et al.</i> (1984)
Camembert	208	101	58				448	1028		1421				Lesage <i>et al.</i> (1993)
Camembert		361	287	160	225	298	622	1442	303	1043			5066	de la Feunte <i>et al.</i> (1993)
Roquefort		961	626	707	2280	1295	3185	6230	2241	6282	896		25969	de la Feunte <i>et al.</i> (1993)
Port salut		41	4	8	54	33	86	275	199 ^a				700	Woo <i>et al.</i> (1984)
Limburger		1475	688	24	50	92	602	565	709 ^a					
Münster		163	102	66	154	206	704	2057	833	1412	59	504		de Leon-Gonzalez <i>et al.</i> (2000)

^aC_{18:0} congeners

of lipolysis contribute to the ripening of Cheddar, Gouda and Swiss cheese, but excessive levels of lipolysis are undesirable and result in rancidity (McSweeney and Sousa 2000; Collins *et al.* 2003b). Typical levels of free fatty acids in some cheese varieties are given in Table 1. In addition to their direct contribution to cheese flavour, as discussed later, fatty acids are also important precursors for the production of volatile flavour compounds.

Lipolytic agents in cheese generally originate from the milk, the coagulant (in the case of rennet paste) and the cheese microflora (starter, nonstarter and adjunct microorganisms). Milk contains a potent indigenous lipase, lipoprotein lipase (LPL), with a molecular mass of 55 kDa, that exists in milk as a homodimer. The physiological role of this enzyme is in the metabolism of plasma triglycerides and it enters milk from the blood (Olivecrona *et al.* 2003). Under optimum conditions, the levels of LPL in milk are enough to cause perceptible rancidity in milk within about 10 s (Walstra and Jenness 1984). This does not happen under normal circumstances as milkfat is protected from the action of LPL by the milk fat globule membrane (MFGM) and about 90% of LPL is associated with the casein micelles. However, if the MFGM suffers mechanical damage, for example by homogenization, agitation or foaming, significant lipolysis may occur quickly, resulting in the development of off-flavours (Fox *et al.* 2000). LPL exhibits a preference for hydrolysis of triglycerides containing medium-chain fatty acids (C₆–C₁₂) and acts preferentially at the *sn*-1 and *sn*-3 positions of triglycerides (Olivecrona *et al.* 2003; Collins *et al.* 2003b). LPL activity is of most significance in raw milk cheeses as the enzyme is largely inactivated

by pasteurization, although 78°C × 10 s is required to inactivate this enzyme completely (Driessen 1989).

Commercial rennet extracts used for the manufacture of most cheese varieties are free from lipase activity. However, rennet pastes used in the manufacture of certain hard Italian cheese varieties, such as Provolone and the various Pecorino cheeses, and often in traditional Greek feta, contain much lipase activity. Rennet pastes are manufactured, often on the farm, by macerating the stomach and its contents of the young dairy animal (calf, lamb or kid) into a paste that contains, in addition to chymosin, high levels of pregastric esterase (PGE), an enzyme that is produced by glands at the base of the tongue and is washed into the stomach with milk as the animal suckles. Although the term 'esterase' suggests that PGE is active only on soluble substrates, it is in fact a lipase that, depending on species of origin, is active at 32–42°C, pH 4.8–5.5 in the presence of 0.5 M NaCl (Collins *et al.* 2003b). PGE is specific for short-chain fatty acids esterified at the *sn*-3 position of triglycerides and is the agent responsible for the high levels of lipolysis in some hard Italian cheeses.

Many internal bacterially ripened varieties (e.g. Cheddar and Gouda) made from pasteurized milk have no strongly lipolytic agent although lipolysis progresses during ripening as a result of the action of enzymes from the starter and nonstarter microflora. Cheeses chemically acidified using gluconic acid- δ -lactone develop very low levels of free fatty acids during ripening (Reiter *et al.* 1967). Evidence for a link between lipolysis in Cheddar cheese and its starter microflora was given by Collins *et al.* (2003a), who showed that lipolysis was

higher in cheese made using a fast-lysing starter culture. Although weakly lipolytic in comparison with some other cheese-related microorganisms (e.g. *Penicillium*), LAB possess intracellular esterolytic/lipolytic enzymes. As starter and nonstarter LAB are present in cheese in high numbers, enzymes from these organisms are responsible for the liberation of significant levels of fatty acids during the long ripening period of many internal bacterially ripened cheeses (Collins *et al.* 2003b 2004). Lipolytic enzymes from LAB are intracellular (Fernandez *et al.* 2000), and hence are released into the cheese matrix on lysis. The enzymes are optimally active at pH 7–8.5 (Kamaly *et al.* 1990; Gobetti *et al.* 1996; Chich *et al.* 1997). With few exceptions (e.g. Chich *et al.* 1997), the lipolytic enzymes of LAB have temperature optima at around 35°C and are most active on substrates containing short-chain fatty acids (see Collins *et al.* 2003b, 2004).

Levels of lipolysis in cheeses in which a secondary microflora develop are often related to the lipolytic ability of the adjunct starter. Bacterial surface (or smear) ripened varieties are often characterized by high levels of lipolysis. Although not studied in detail, it appears that coryneform bacteria (e.g. *Brevibacterium linens*) associated with the surface smear are quite lipolytic (Sørhaug and Ordal 1974). Lipases from the yeast *G. candidum*, which develops on the surface of these cheeses and is responsible for much deacidification during the early stages of ripening, have also been studied (Baillargeon *et al.* 1989; Jacobsen and Poulsen 1995) and found to be active at pH 7 and 37°C (Baillargeon *et al.* 1989). *Propionibacterium freudenreichii* is more lipolytic than LAB (Dupuis 1994) and possesses an intracellular lipase with pH and temperature optima of 7.2 and 47°C, respectively, and is most active on triglycerides with short-chain fatty acids (Oterholm *et al.* 1970). However, the most lipolytic organisms associated with cheese are *Penicillium* spp., which grow on or in mould-ripened varieties. Despite their importance, these enzymes have not been studied extensively in recent years. *Penicillium roqueforti*, which causes the extensive lipolysis in blue cheese, possesses two lipases with pH optima of 7.5–8 and 9–9.5, respectively (Morris and Jezeski 1953; Kman *et al.* 1966; Niki *et al.* 1966). *Penicillium camemberti* produces an extracellular lipase that is optimally active on tributyrin at pH 9 and 35°C (Lamberet and Lenoir 1976).

Proteolysis

Proteolysis is the most complex and, in most varieties, the most important of the primary biochemical events that occur in most cheeses during ripening. Because of its importance, proteolysis and the enzymes responsible for this process have

been reviewed extensively over the past two decades (Grappin *et al.* 1985; Rank *et al.* 1985; Fox 1989; Fox and Law 1991; Fox *et al.* 1993, 1994, 1995a, 1996; Fox and McSweeney 1996a,b, 1997; Sousa *et al.* 2001; Upadhyay *et al.* 2004). Proteolysis contributes to the softening of cheese texture during ripening due to hydrolysis of the casein matrix of the curd and through a decrease in the water activity (a_w) of the curd due to changes in water binding by the new carboxylic acid and amino groups formed on hydrolysis. Proteolysis has a direct influence on flavour through the production of short peptides and amino acids, some of which are flavoured (often bitter), by facilitating the release of sapid compounds from the cheese matrix and, probably most importantly, by providing free amino acids that are substrates for a series of catabolic reactions that generate many important flavour compounds (discussed later). The proteinases and peptidases that catalyse proteolysis in cheese during ripening originate from six primary sources, namely, the coagulant, the milk, starter LAB, NSLAB, secondary starters (e.g. *Pr. freudenreichii* in Swiss-type cheese, *P. roqueforti* in blue cheese, *P. camemberti* in Camembert and Brie-type cheeses and a complex Gram-positive bacterial microflora on the surface of smear cheeses, plus, in certain cases, exogenous proteinases or peptidases added to the milk or curd to accelerate ripening.

A major source of proteolytic enzymes in many cheese varieties is the residual coagulant, often chymosin, that remains trapped in the curd on whey drainage. Up to 30% of the coagulant activity added to the milk remains active in the curd depending on factors such as enzyme type, cooking temperature and pH at whey drainage (Upadhyay *et al.* 2004). The specificity of chymosin on all of the caseins is now known. In solution, chymosin cleaves β -casein at seven sites (Visser and Slangen 1977), many of which are located near the hydrophobic C-terminal β -casein, and cleavage of these sites (e.g. Leu₁₉₂-Tyr₁₉₃) can result in the production of short hydrophobic peptides, which are bitter. The primary site of chymosin action on α_{s1} -casein (α_{s1} -CN) is Phe₂₃-Phe₂₄ (McSweeney *et al.* 1993), which results in the production of a small peptide (α_{s1} -CN f1–23) that is hydrolysed rapidly by starter proteinases. Chymosin cleaves α_{s1} -casein at a number of other sites, particularly Leu₁₀₁-Lys₁₀₂, which is hydrolysed in many cheeses during ripening. α_{s2} -Casein is more resistant to hydrolysis by chymosin than is α_{s1} -casein; cleavage sites of chymosin on α_{s2} -casein are restricted to the hydrophobic regions of the molecule (sequences 90–120 and 160–207) (McSweeney *et al.* 1994). Although *para*- κ -casein has several potential chymosin cleavage sites, it does not appear to be hydrolysed either in solution or in cheese (Green and Foster 1974). Presumably, this

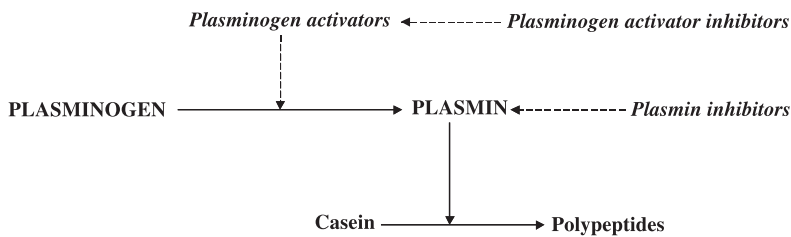


Figure 4 Plasmin/plasminogen system in milk (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). Upadhyay *et al.* Proteolysis in cheese during ripening, pp 391–433, Copyright 2004, with permission from Elsevier).

reflects the relatively high level of secondary structure in κ -casein compared to the other caseins (see Swaisgood 1992). The action of other enzymes that may be found in coagulants (e.g. pepsin, *Rhizomucor* proteinases, *Cryphonectria parasitica* proteinases or proteinases from *Cynara cardunculus*) on the caseins have also been studied (see Fox and McSweeney 1996b; Sousa *et al.* 2001; Upadhyay *et al.* 2004).

Milk itself is an important source of proteolytic enzymes. The principal indigenous proteinase in milk is plasmin, which is a trypsin-like serine proteinase originating in the blood and optimally active at approximately pH 7.5 and 37°C. The physiological role of plasmin in the blood is in the degradation of fibrin clots during the blood-clotting process. Hence, the activity of plasmin in blood must be under tight control and it is thus produced from an inactive precursor, plasminogen, through the action of plasminogen activators (PAs). Inhibitors of plasmin and PAs also form part of this system, all of which is found in milk (Figure 4). In milk, plasmin, plasminogen and PAs are mainly associated with the casein micelle while plasmin inhibitors and inhibitors of PA are found in the serum and thus are lost on whey drainage. The specificity of plasmin is restricted to peptide bonds of the type Lys–X, to a lesser extent Arg–X, and it degrades the caseins in the order β -casein \approx α_{s2} -casein $>$ α_{s1} -casein; κ -casein seems to be resistant to the action of this proteinase (Bastian and Brown 1996). The specificity of plasmin on β -, α_{s2} - and α_{s1} -caseins is known (see Fox and McSweeney 1996b; Upadhyay *et al.* 2004) but its most important substrate in cheese is β -casein, which plasmin hydrolyses at three sites, Lys₂₈–Lys₂₉, Lys₁₀₅–His₁₀₆ and Lys₁₀₇–Glu₁₀₈, to produce γ_1 -CN (β -CN f29–209), γ_2 -CN (β -CN f106–209), γ_3 -CN (β -CN f108–209), proteose peptone PP8 fast (β -CN f1–28), PP8 slow (β -CN f29–105 and f29–107) and PP5 (β -CN f1–105 and 1–107). α_{s2} -Casein is also very susceptible to plasmin action and it is likely that the disappearance of this protein, which is often observed in cheese during ripening, is due to plasmin action (Fox

and McSweeney 1996b), although this hypothesis remains to be proved. Plasmin activity is of most significance in cheese varieties that are cooked to high temperatures, e.g. Swiss-type cheeses. In these varieties, plasmin, which is a relatively heat-stable enzyme, survives the cooking temperature ($\sim 55^\circ\text{C}$) while much chymosin activity is lost. There is also some activation of plasminogen to plasmin at high cooking temperatures, probably due to the thermal inactivation of inhibitors of PAs and of plasmin (Farkye and Fox 1990). Plasmin is also very important in mould-ripened and smear cheeses in which the pH increases during ripening, moving away from the pH optimum of chymosin and towards that of plasmin (Upadhyay *et al.* 2004).

Milk also contains other indigenous proteinases originating from the leucocytes of somatic cells. Somatic cells contain many proteinases including cathepsins B, D, G, H, L and elastase (Kelly and McSweeney 2003). However, only the presence of cathepsins B and D has been confirmed in milk although it is highly likely that other indigenous somatic cell enzymes remain to be discovered. It has been shown that milk contains indigenous thiol proteinase activity (O'Driscoll *et al.* 1999) and the presence in milk of cathepsin B has been confirmed recently (Magboul *et al.* 2001). However, the significance of indigenous cathepsin B in milk to proteolysis in cheese during ripening is unknown, although this enzyme has a wide specificity on the caseins (Considine *et al.* 2004). The indigenous aspartyl proteinase, cathepsin D, has received considerable attention in recent years (see reviews by Hurley *et al.* 2000a; Kelly and McSweeney 2003). This enzyme is an aspartyl proteinase with temperature and pH optima of 37°C and 4.0, respectively. Cathepsin D is a typical mammalian proteinase and is produced autocatalytically from a precursor, procathepsin D, to pseudocathepsin D and thence by thiol proteinases to a number of mature forms (Kelly and McSweeney 2003). The specificity of cathepsin D on the caseins, particularly α_{s1} -casein, is very similar to that of chymosin, although cathepsin D is poor at coagulating milk (McSweeney *et al.* 1995). Because of the similarity of its specificity to that of chymosin, it is difficult to assess the role of cathepsin D in the ripening of rennet-coagulated cheeses. The action of cathepsin D in acid-curd cheeses made without the addition of rennet has been demonstrated (Wium *et al.* 1998; Hurley *et al.* 2000b). However, the contribution of this enzyme to the ripening of most cheese varieties, particularly those made from pasteurized milk, is likely to be limited. Cathepsin D is found in the serum phase in milk, and thus most is lost on whey drainage and most ($\sim 92\%$) cathepsin D activity is lost on pasteurization (Hayes *et al.* 2001).

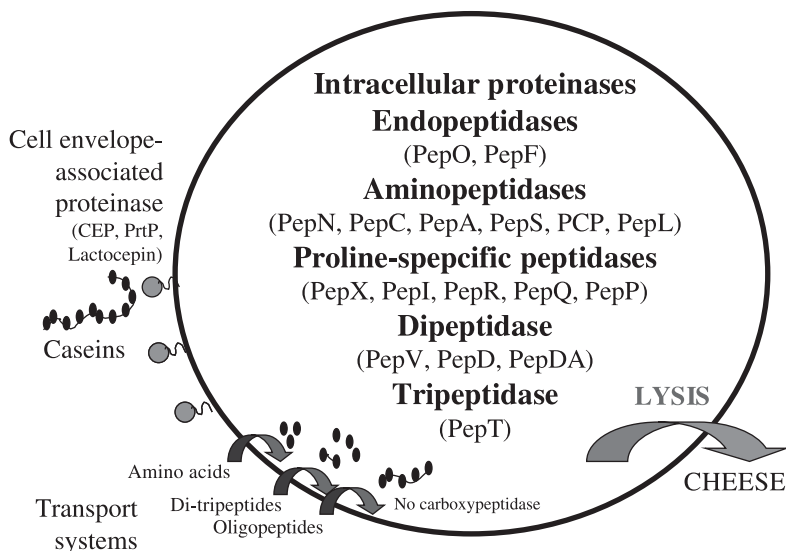


Figure 5 Summary of the proteolytic system of *Lactococcus*. The proteolytic systems of other lactic acid bacteria are generally similar (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). McSweeney P L H. *Biochemistry of cheese ripening: introduction and overview*, pp 347–360, Copyright 2004, with permission from Elsevier).

LAB require many amino acids and thus have complex proteolytic systems to liberate the amino acids necessary for growth from the proteins in their environment. The proteinases and peptidases of LAB have been the subject of active study over the past two decades and the extensive literature on this topic has been reviewed frequently (e.g. Pritchard and Coolbear 1993; Kunji *et al.* 1996; Law and Haandrikman 1997; Christensen *et al.* 1999; Upadhyay *et al.* 2004). The proteolytic system of *Lactococcus* has been studied most thoroughly, followed by those of the thermophilic lactobacilli, and the systems of the facultatively heterofermentative lactobacilli that dominate the NSLAB flora have received less attention. The proteolytic system of *Lactococcus* (Figure 5) is composed of a cell envelope-associated proteinase [variously called lactocepin, cell envelope-associated proteinase (CEP) or PrtP] and a range of intracellular proteinases and, more importantly, peptidases. The proteinases and peptidases of LAB are essential for the ripening of cheese.

The principal proteolytic enzyme of *Lactococcus* is lactocepin, which is loosely attached by Ca^{2+} to the cell surface. Lactocepin is a serine proteinase (~140 kDa, pH optimum 5.5–6.5), the gene for which is plasmid-encoded. The lactocepins from a number of strains of *Lactococcus* have been studied biochemically and genetically. Lactocepins were first classified into two broad groups: P_I- and P_{III}-type proteinases (Fox and McSweeney 1996b). The former act rapidly on β -casein but only slowly on α_{s1} - and κ -caseins whereas P_{III}-type enzymes

hydrolyse β -casein differently to P_I-type proteinases and act rapidly on α_{s1} - and κ -caseins. However, enzymes from all lactococcal strains are closely related genetically and subsequently it became apparent that classification into two groups was insufficient and other schemes have been proposed (e.g. Exterkate *et al.* 1993). The primary role of lactocepin is to degrade the caseins to provide short peptides to permit the lactococcal cell to grow in milk. However, in cheese, it acts primarily to degrade intermediate-sized peptides produced from the casein by the action of chymosin (e.g. α_{s1} -CN fl–23) or plasmin. The specificities of the lactocepins from a range of LAB on the caseins are known (see Fox and McSweeney 1996b; Upadhyay *et al.* 2004). Lactococci also possess a number of intracellular proteinases (see Upadhyay *et al.* 2004), whose role in cheese ripening is unclear although they are probably not as significant as lactocepin.

LAB also contain intracellular peptidases that are very important for the final stages of proteolysis in cheese during ripening and the ultimate liberation of free amino acids as substrates for catabolic reactions. As the LAB are auxotrophic for a range of amino acids, they must get these compounds preformed from their environment and hence contain a wide range of peptidases (Figure 6). LAB produce three types of oligoendopeptidases (Kunji *et al.* 1996; Upadhyay *et al.* 2004): PepO and PepF, monomeric metalloendopeptidases with molecular masses of about 70 kDa, differ in their action on various peptide substrates; PepE is a thiol-dependent endopeptidase (Fenster *et al.* 1997) that has not been studied extensively. Tripeptidases of LAB are generally di- or trimeric metalloenzymes with broad substrate specificity. LAB produce a range of aminopeptidases (see Kunji *et al.* 1996; Law and Haandrikman 1997; Christensen *et al.* 1999; Upadhyay *et al.* 2004). PepN is a broad specificity monomeric metalloaminopeptidase of 85–98 kDa while PepC is a multimeric thiol aminopeptidase with broad specificity and a subunit molecular mass of about 40–50 kDa. PepG is a cysteine aminopeptidase related structurally to PepC but with different substrate specificity (Klein *et al.* 1997). Other aminopeptidases produced by LAB include PepA, a multimeric metalloaminopeptidase specific for Glu/Asp residues, and PepL, a leucyl aminopeptidase and pyrrolidone carboxyl peptidease that removes pyroglutamic residues from the N-terminus of peptides (see Upadhyay *et al.* 2004). LAB do not appear to produce carboxypeptidases.

Caseins are rich in the imino acid proline. Because of its unique cyclical structure, specialized peptidases are needed to hydrolyse proline-containing peptides and the LAB possess such enzymes in abundance to enable them to use the caseins fully as growth substrates. PepX releases

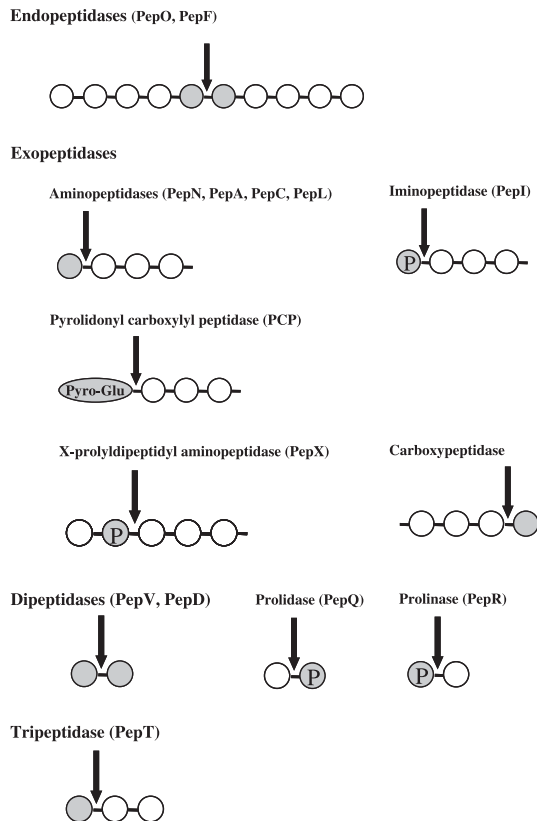


Figure 6 Schematic representation of the action of peptidases found in lactic acid bacteria (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). Upadhyay *et al.* Proteolysis in cheese ripening, pp 391–433, Copyright 2004, with permission from Elsevier).

X-Pro dipeptides from the N-terminus of peptides and is the best characterized proline-specific peptidase of the LAB (see Kunji *et al.* 1996; Upadhyay *et al.* 2004). PepXs from most strains are dimeric serine dipeptidylaminopeptidases with molecular masses of 117–200 kDa. PepI catalyses the release of an N-terminal proline residue from a peptide. The PepI from *Lc. lactis* ssp. *lactis* HP is a dimeric metalloiminopeptidase with a molecular mass of 110 kDa (Baankreis and Exterkate 1991) although PepIs from *Lactobacillus* spp. have different properties (see Upadhyay *et al.* 2004). PepP is a monomeric metalloaminopeptidase that catalyses the removal of the N-terminal amino acid from peptides with the sequence X-Pro-Pro-(X)_n or X-Pro-(X)_n. LAB also possess two specialized dipeptidases that cleave proline-containing dipeptides. Prolinase (PepR) cleaves Pro-X dipeptides while prolidase (PepQ) hydrolyses X-Pro dipeptides.

Although enzymes of starter and nonstarter LAB contribute to the ripening of nearly all cheeses, proteolysis in varieties in which a secondary flora is encouraged to grow is often affected greatly by enzymes from these secondary organisms. *Propionibacterium* sp. used as secondary

starters in Swiss-type cheeses are weakly proteolytic but are highly peptidolytic (see Gagnaire *et al.* 1999). The proteolytic systems of *P. camemberti* and *P. roqueforti* are generally similar; both organisms synthesize aspartyl and metalloproteinases. *G. candidum* is often found associated with mould- and smear-ripened cheeses although its enzymes have not been studied in great detail. A very complex bacterial flora develops at the surface of smear-ripened cheeses, which at the end of ripening consists of a range of Gram-positive organisms from genera including *Brevibacterium*, *Arthrobacter*, *Micrococcus*, *Staphylococcus* and *Corynebacterium*. Enzymes from *Br. linens* have been characterized most thoroughly (Rattray and Fox 1999). This organism produces extracellular proteinases and aminopeptidases in addition to a range of intracellular enzymes. Enzymes from other microorganisms associated with the smear microflora have received much less attention (see Upadhyay *et al.* 2004).

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

However, the pattern and extent of proteolysis varies considerably between varieties because of differences in manufacturing practices (particularly cooking temperature) and ripening protocols that cause differences in ripening time, moisture content, residual coagulant activity, activation of plasminogen to plasmin, and possibly the development of a highly proteolytic secondary microflora. The pattern of proteolysis (i.e. the relative concentrations of different peptides and amino acids) is very variable and is essentially unique to a particular variety. The differences in pH 4.6-soluble N content (a widely used index of proteolysis) are due to differences in moisture content, temperature and pH, length of ripening, cooking temperature and pH at draining; peptides in this fraction are produced mainly by the action of chymosin and to a lesser extent of plasmin (Fox and McSweeney 1996b). A short ripening period (~3 weeks) and extensive denaturation of chymosin during the high-temperature stretching step during the manufacture of mozzarella cheese explain the low level of soluble N, whereas extensive proteolysis is characteristic of blue cheese and some smear-ripened varieties, caused by the action chymosin, plasmin and proteinases from their characteristic secondary microflora. In addition, differences in the action of these proteolytic agents causes in differences in peptide profiles.

As discussed by Upadhyay *et al.* (2004), primary proteolysis is similar during the ripening of most cheeses; chymosin readily hydrolyses the Phe₂₃-Phe₂₄ bond of α_{s1} -casein except in cheeses that are cooked at a high temperature ($\sim 55^\circ\text{C}$, e.g. Swiss cheese), where the hydrolysis of this protein is slow and in which plasmin is the principal proteolytic agent. Enzymes from *P. roqueforti*, after its sporulation in blue-veined cheeses, hydrolyse α_{s1} -CN (f24-199) and other peptides, changing the peptide profile (Gripon 1993). In many cheeses, α_{s1} -casein is hydrolysed faster than β -casein (Sousa *et al.* 2001). In blue-veined cheeses, both α_{s1} - and β -caseins are completely hydrolysed at the end of ripening. In Swiss-type cheeses, β -casein is hydrolysed faster than α_{s1} -casein, with concomitant increases in γ -caseins, indicating a role for plasmin and denaturation of chymosin during cooking. However, α_{s1} -CN (f24-199) is produced slowly in Swiss cheese, suggesting the survival of some chymosin during cooking or perhaps the activity of cathepsin D. During the ripening of mozzarella cheese, α_{s1} -CN (f24-199) is produced slowly and γ -caseins more rapidly, indicating weak chymosin activity and fairly high plasmin activity (Kindstedt 1993). Plasmin and

Lactobacillus proteinases are mainly responsible for extensive proteolysis in Parmigiano-Reggiano cheese, which is ripened for a long period (~ 24 months) at elevated temperatures (~ 18 - 20°C) (Battistotti and Corradini 1993). The high cooking temperature used during the manufacture of Parmigiano-Reggiano cheese denatures most of the chymosin. Several peptides from Cheddar, Parmigiano-Reggiano, blue, Swiss and feta cheeses have been isolated and characterized. Of these varieties, the peptide profile of Cheddar cheese is the best characterized (see Upadhyay *et al.* 2004).

SECONDARY EVENTS LEADING TO THE PRODUCTION OF VOLATILE FLAVOUR COMPOUNDS

Metabolism of free fatty acids

While short-chain fatty acids contribute directly to cheese flavour, free fatty acids (FFAs) also contribute indirectly to cheese flavour by acting as precursors for the production of volatile flavour compounds through a series of reactions known collectively as metabolism of fatty acids. Pathways for the metabolism of FFAs in cheese during ripening are summarized in Figure 7.

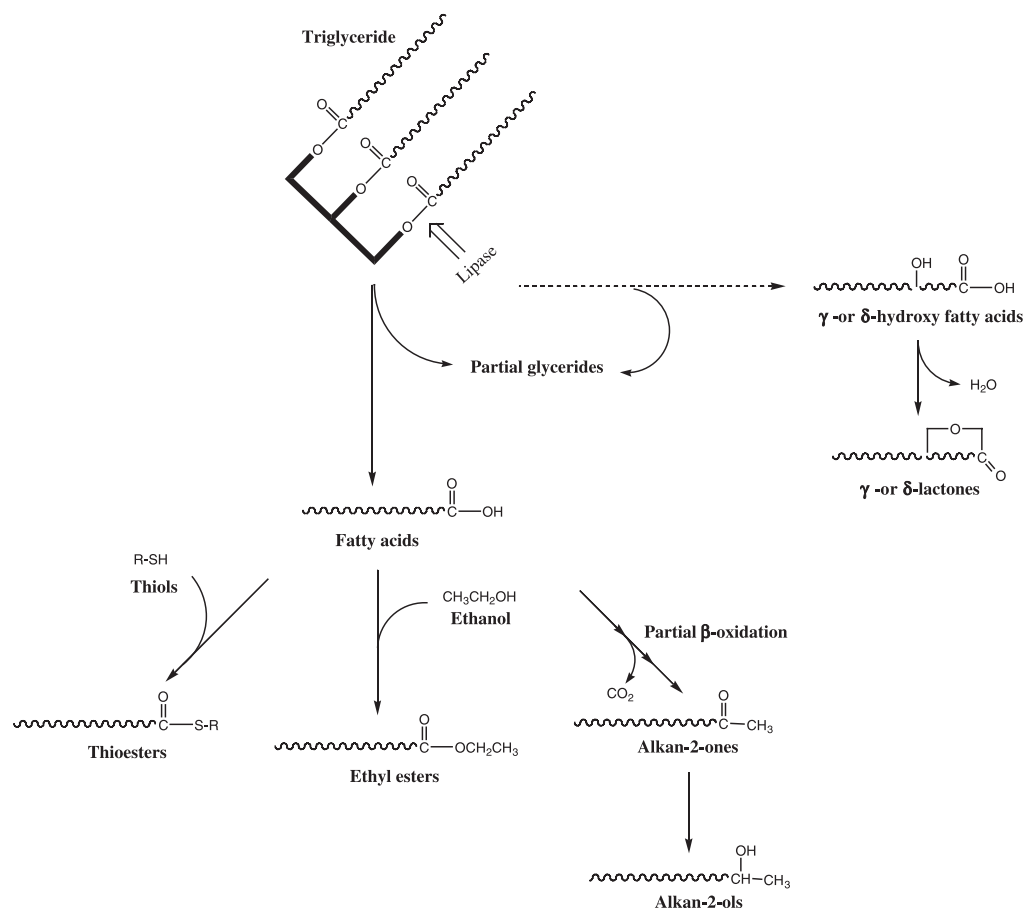


Figure 7 Pathways for the production of flavour compounds from fatty acids during cheese ripening (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). McSweeney P L H. Biochemistry of cheese ripening: introduction and overview, pp 347-360, Copyright 2004, with permission from Elsevier).

Esters are found commonly in many cheese varieties and are produced by the reaction of an FFA with an alcohol. While methyl, propyl and butyl esters have been found in cheese (Meinhart and Schreier 1986), the most common alcohol available for this reaction is ethanol and hence ethyl esters are the dominant esters in cheese. Ethanol is the limiting reactant in the production of esters; this alcohol is derived from the fermentation of lactose or from amino acid catabolism. Holland *et al.* (2002) suggested that esters are formed during cheese ripening by transesterification of an FFA from partial glycerides to ethanol. Thioesters are compounds formed by the reaction of FFAs with sulphhydryl compounds, usually methanethiol (CH_3SH ; thus forming methylthioesters) (McSweeney and Sousa 2000; Collins *et al.* 2003b, 2004).

Lactones are cyclic compounds formed from hydroxyacids following intramolecular esterification. Both γ - and δ -lactones (with five- and six-sided rings, respectively) have been found in cheese. The production of lactones during ripening is limited by the levels of their precursor compounds, hydroxyacids. The mammary gland is reported to possess a δ -oxidation system for fatty acids, or hydroxyacids may be produced by reduction of

ketones (Collins *et al.* 2003b, 2004). The presence of large amounts of high molecular weight lactones in rancid Cheddar cheese (Wong *et al.* 1975) has led to suggestions that lactones may also originate from pathways other than the release of hydroxyacids from triglycerides. Dodecalactone may be produced from long-chain unsaturated fatty acids by *P. roqueforti* while hydroxyacids may be produced by the action of lipoxygenases and other enzymes present in members of the rumen microflora (Collins *et al.* 2003b, 2004).

FFA metabolism is of most significance in blue-mould cheese in which FFAs are converted to 2-methyl ketones (alkan-2-ones) via a pathway corresponding to the early stages of β -oxidation caused by the action of spores and vegetative mycelia of *P. roqueforti* (Urbach 1997; Chalier and Crouzet 1998; Collins *et al.* 2003b, 2004) and perhaps other fungi (e.g. *P. camemberti* or *G. candidum*) (Lamberet *et al.* 1982; Cerning *et al.* 1987; Molimard and Spinnler 1996). The rate of production of methyl ketones is affected by a number of factors, including temperature, physiological state of the mould and concentration of precursor FFA. The rate of production is maximal between pH 5 and 7; this range of pH values encompasses most blue cheeses (Gripion 1993). Although up to 11 methyl ketones have been identified in cheese (Collins *et al.* 2004), the most common are pentan-2-one, heptan-2-one and nonan-2-one (Collins *et al.* 2004). Methyl ketones may be reduced to the corresponding secondary alcohol (e.g. pentan-2-ol, heptan-2-ol and nonan-2-ol) by the action of enzymes of *P. roqueforti* (Martelli 1989; Engels *et al.* 1997).

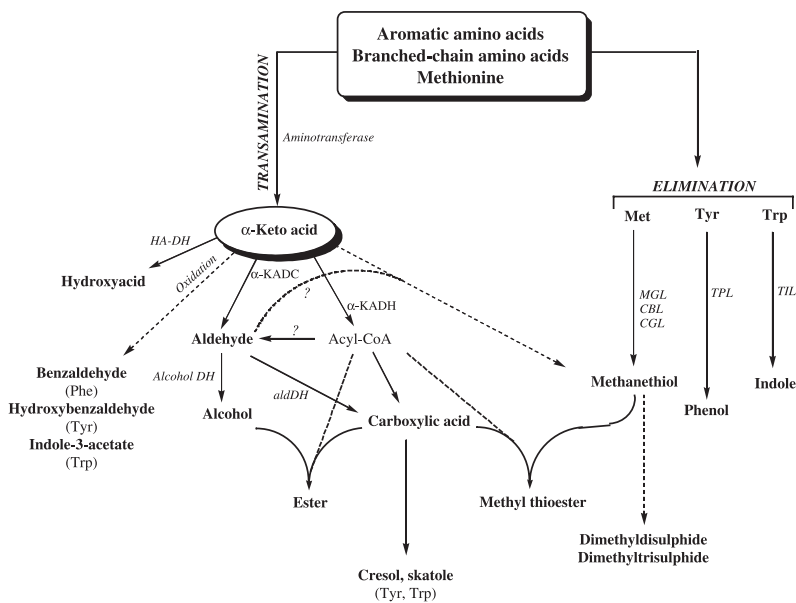


Figure 8 Schematic diagram of pathways for amino acid catabolism found in different microorganisms and some chemical reactions (dotted lines) occurring in cheese during ripening (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). Curtin A C and McSweeney P L H. Catabolism of amino acids in cheese during ripening, pp 435–454. Copyright 2004, with permission from Elsevier). HA-DH, hydroxyacid dehydrogenase; α -KADH, α -keto acid dehydrogenase; α -KADC, α -keto acid decarboxylase; aldDH, aldehyde dehydrogenase; alcohol DH, alcohol dehydrogenase; MGL, methionine- γ -lyase, CGL, cystathionine- γ -lyase; CBL, cystathionine- β -lyase; TPL, tyrosine-phenol lyase; TIL, tryptophan-indole lyase.

Metabolism of free amino acids

Pathways for the catabolism of free amino acids during ripening produce many flavour compounds and this has been an active area of research in recent years (see reviews by McSweeney and Sousa 2000; Yvon and Rijnen 2001; Smit *et al.* 2002; Curtin and McSweeney 2004). Indeed, it is now thought that the principal contribution of proteolysis to the development of cheese flavour is through the liberation of amino acids, which act as precursors for catabolic reactions. Pathways for amino acid catabolism in cheese remain to be elucidated fully, although recent work has provided much clarification in this area. Amino acids in cheese appear to be catabolized by one of two major pathways (Yvon and Rijnen 2001) initiated by the action of an aminotransferase or a lyase, although other catabolic pathways (e.g. deamination or decarboxylation) also occur (Figure 8).

The first pathway is initiated by the action of aminotransferases (EC 2.6.1.x), pyridoxal-5'-phosphate (PLP)-dependent enzymes, which convert an amino acid to the corresponding α -ketoacid (and in turn transfer the amino group of the amino

flavour compounds in cheese during ripening (Curtin and McSweeney 2004), and hence a number of attempts have been made to improve cheese flavour by the addition of α -ketoglutarate to cheese to enhance aminotransferase activity. Yvon *et al.* (1998) added α -ketoglutarate to St Paulin-type cheese and observed faster rates of amino acid catabolism than in the control but found that α -ketoacids produced by aminotransferase action accumulated in the cheese, suggesting that their degradation to aroma compounds may be more rate-limiting than their production from amino acids. Banks *et al.* (2001) and Shakeel-Ur-Rehman and Fox (2002) supplemented Cheddar cheese curd with α -ketoglutarate and found better flavour development than in untreated control cheeses. As an alternative to exogenous addition, α -ketoglutarate may be produced from Glu by the action of glutamate dehydrogenase, and Rijnen *et al.* (2000) cloned the gene for this enzyme from *Peptostreptococcus asaccharolyticus* into *Lactococcus* and

found that α -ketoglutarate was produced by the modified *Lactococcus* strain in a model system under cheese ripening conditions.

Volatile sulphur-containing compounds are known to be important for the flavour of a number of cheese varieties (Fox and McSweeney 1996a,b; McSweeney and Sousa 2000; Curtin and McSweeney 2004). As levels of Cys in the caseins are low, sulphur compounds in cheese originate principally from the catabolism of Met. Pathways for the catabolism of Met in cheese are shown in Figure 10 and important sulphur-containing flavour compounds include methanethiol, dimethyldisulphide, dimethyltrisulphide, methional (β -methyl mercaptopropionaldehyde) and thioesters (produced by the reaction of a thiol, often methanethiol, and a carboxylic acid) and the enzymes involved in the production of these and other sulphur-containing compounds have been studied actively (see Yvon and Rijnen 2001; Curtin and McSweeney 2004, for references). In particular, methionine- γ -lyase (which converts Met to α -ketobutyrate, methanethiol and ammonia) cystathionine- β -lyase (which converts cystathionine to homocysteine, pyruvate and ammonia) and cystathionine- γ -lyase (which converts cystathionine to cysteine, α -ketobutyrate and ammonia) have received much attention (see Curtin and McSweeney 2004).

Pathways for the catabolism of the aromatic amino acids Trp, Tyr and Phe have also been studied in detail (see Curtin and McSweeney 2004). The pathway for the catabolism of Phe is shown in Figure 11; corresponding pathways are known for the other aromatic amino acids. The first step in the catabolism of these amino acids is catalysed by aminotransferase activity producing the α -ketoacids indole-3-pyruvate, *p*-hydroxyphenyl pyruvate and phenyl pyruvate from Trp, Tyr and Phe, respectively. Aromatic amino acid aminotransferases have been studied in a number of cheese-related microorganisms including lactococci (Gao *et al.* 1997; Yvon *et al.* 1997; Gao and Steele 1998), lactobacilli (Nierop Groot and de Bont 1998; Gummalla and Broadbent 2001) and *Br. linens* (Lee and Desmazeaud 1985; Hayashi *et al.* 1993). The α -ketoacids produced from aromatic amino acids degrade by a number of pathways to produce volatile compounds important to cheese flavour. The branched-chain amino acids Leu, Ile and Val are degraded by aminotransferases, producing a corresponding α -ketoacid that can then be degraded to other compounds (see Figure 12 for Leu). Aminotransferases capable of transaminating branched-chain amino acids have been studied in lactococci (Engels 1997; Yvon *et al.* 1997, 2000; Atiles *et al.* 2000), *Lb. paracasei* ssp. *paracasei* (Hansen *et al.* 2001) and *Pr. freudenreichii* (Thierry *et al.* 2002).

Amino acids may also be degraded by deamination reactions involving the action of

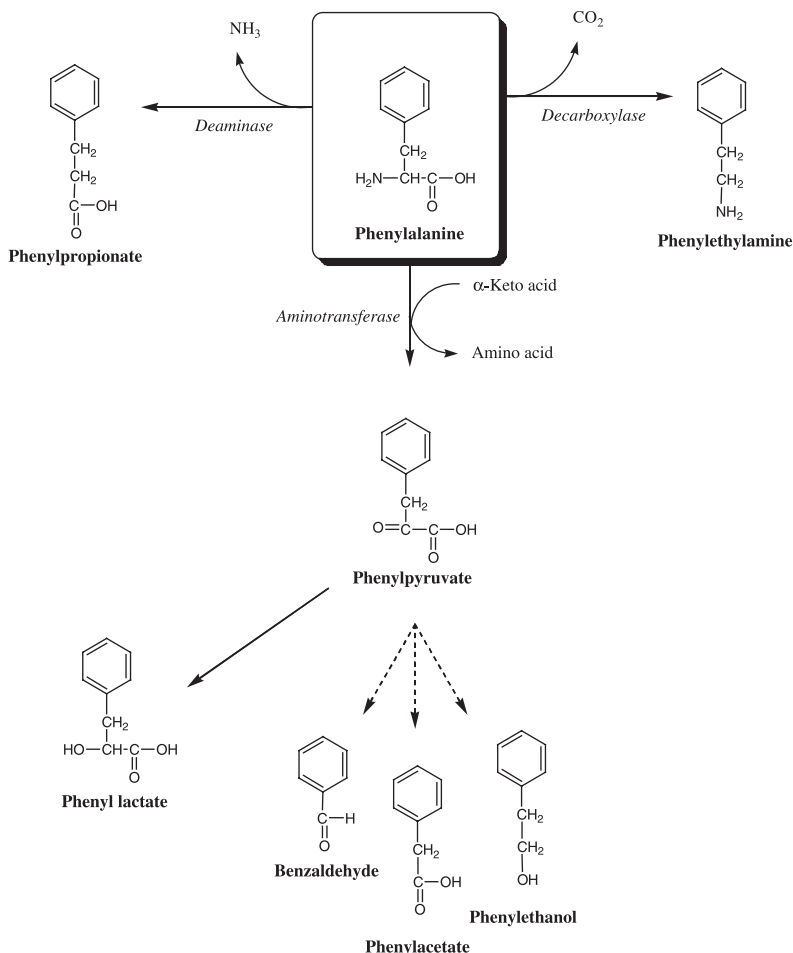


Figure 11 Pathways for the catabolism of phenylalanine (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). Curtin A C and McSweeney P L H. Catabolism of amino acids in cheese during ripening, pp 435–454, Copyright 2004, with permission from Elsevier).

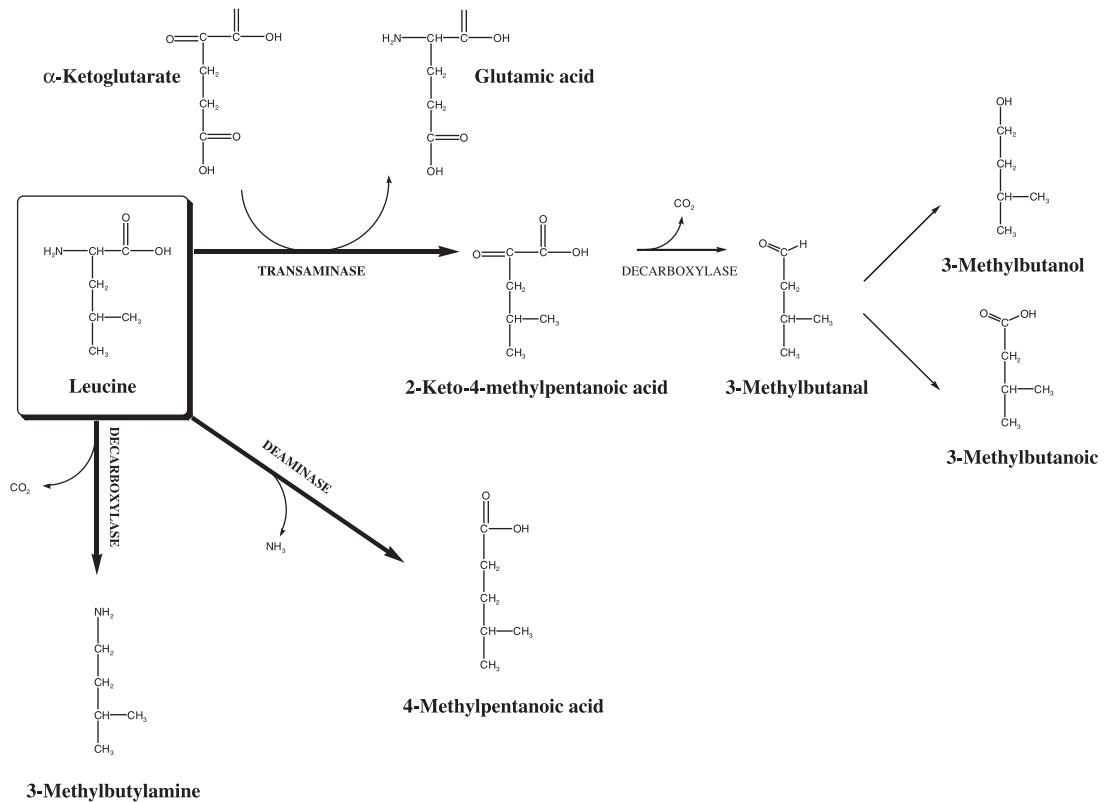


Figure 12 Catabolism of leucine initiated by transaminase, deaminase or decarboxylase action and volatile flavour compounds that may be formed from this amino acid. Similar catabolic pathways operate for the other branched-chain aliphatic amino acids (isoleucine and valine) (Reprinted from *Cheese: Chemistry, Physics and Microbiology*, Vol 1 (3rd edition) Fox P F, McSweeney P L H, Cogan T M & Guinee T P (eds). McSweeney P L H. Biochemistry of cheese ripening: introduction and overview, pp 347–360, Copyright 2004, with permission from Elsevier).

dehydrogenases (which use NAD^+ as the electron acceptor and produce an α -ketoacid and ammonia) or oxidases (which use oxygen as the electron acceptor and form aldehydes and ammonia) (Curtin and McSweeney 2004). Ammonia produced by deamination contributes to the flavour of certain varieties including smear cheeses, certain Swiss-type cheeses that develop a surface microflora (e.g. Gruyère and Comté) and perhaps mature Camembert-type cheeses (McSweeney and Sousa 2000), and that, if produced in sufficient quantities, may contribute to an increase in pH during ripening.

Decarboxylation of amino acids is of particular significance as the amines produced often have strong and unpleasant aromas and, more importantly, some (the 'biogenic amines') cause adverse physiological effects in susceptible consumers. Decarboxylases generally have an acid pH optimum (about pH 5.5) and usually require PLP as cofactor (Hemme *et al.* 1982). The rate of production of amines in cheese depends on the concentration of precursor amino acids and, more importantly, the cheese microflora, which in turn may be affected by factors such as ripening temperature, pH and salt concentration (Joosten 1988; Curtin and McSweeney 2004). As starter strains with high decarboxylase activities are not used,

nonstarter lactobacilli and enterococci have been implicated in the production of high levels of biogenic amines in most cheese varieties (Joosten and Northolt 1987; Broome *et al.* 1990; Gardini *et al.* 2001; Roig-Sagues *et al.* 2002) and certain strains of *Br. linens* may be able to reduce the levels of biogenic amines in smear cheese (Leuschner and Hammes 1998).

CONCLUSION

Cheese ripening clearly involves a very complex series of interrelated events, resulting in the development of the flavour and texture characteristic of the cheese variety. The biochemical pathways through which lactose, lactate, milkfat and caseins are converted to flavour compounds are now known in general terms, although much remains to be discovered, particularly with respect to secondary reactions, amino acid catabolism, and in the area of the interactions of products of various secondary reactions. Despite the wealth of knowledge available on the ripening of certain cheese varieties, it remains impossible to guarantee premium quality cheese from each day's production. This, together with the characterization of minor cheese varieties, remains a challenge for future research in this area.

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