Biochemistry of Milk Products

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Preface

The milk and dairy products sector forms a very major part of the whole food and agriculture industries, which worldwide take up the greatest share of all human activity. A large proportion of food and drink is consumed directly with little or no pretreatment and a great deal more following only minimal pretreatment, such as peeling, slicing, chopping, heating, etc. These are usually regarded as simple preparation steps and not thought of as manufacturing processes, so only quite a small percentage would generally be thought of as "processed". In this the dairying area is no exception and most milk is still consumed as such rather than being made into products. Nevertheless processing even only a part of the total milk production still represents an extremely important activity. It is worth remembering that historically the oldest manufacturing industries of all were geared to food and drink products, namely fermentation to give alcoholic beverages and the production of cheese from milk. Both of these industries function world wide and are performed on scales varying from amateur operations in the home, through small often specialist cottage industries, to major multinational companies with turnovers of hundreds of millions of dollars.

Cheesemaking began 8-9000 years ago, probably in the Middle East where the stomachs of animals were kept after slaughter and used as leather-type bottles for storing and transporting liquids. It is thought that the storage of milk in imperfectly cleaned stomachs which still contained traces of the digestive enzyme pepsin, aided perhaps by contaminating lactic bacteria, led to clotting of the milk and to the realisation that the resulting curds represented a convenient and concentrated form of most of the protein. Also because of the preservative action of the low pH generated by the fermentation of lactose to lactic acid the curds could be stored for considerable periods of time. In spite of this long history it is still cheesemaking that is the most active and fruitful area in dairy research today. This is very apparent from the contents of this book which represents the proceedings of a recent symposium on advances in dairy biochemistry. There are two principal topics in cheese research at present. Firstly, improvements in starter microorganisms to give better quality cheeses with superior flavour and texture, preferably developed in a shorter time to minimise expensive storage, and to facilitate the tailoring of cheese flavour to particular products and processes. Secondly, the production of new milk coagulants as alternatives to traditional rennet. Both of these topics nowadays lean heavily on molecular biological techniques, the former to develop new micoorganisms with enzyme profiles (peptidases, proteinases and to a lesser degree lipases) better suited to their tasks than current natural microorganisms, and the latter to produce from microorganisms, following genetic manipulation and expression, a purified proteinase of high specificity capable of coagulating milk without the formation of undesirable by-products such as bitter peptides.

In order to be successful in these objectives it is necessary to understand in fine detail at the molecular level all aspects of the cheesemaking process and of what takes place during a typical maturation, including especially the role of starter enzymes, residual coagulant enzymes and indigenous milk enzymes. The initial papers in this symposium were devoted to reviewing and extending knowledge in this area.

The second biggest field in current dairy research covers the functional behaviour of milk proteins. These have been used for many years as ingredients in a wide variety food products and in dietetic applications because of their desirable physical attributes and high nutritional quality. Only recently however have separation methods improved to the extent that the large-scale production of individual protein components with specific functional properties for particular applications has become a commercially viable route to new food ingredients. Again, such advances depend upon a thorough understanding at the molecular level of the processes involved, in this case of what molecular features make a protein a good emulsifying agent, enable it to form gels, or to stabilise foams, etc. Once these features can be identified it then becomes a practical proposition to alter proteins via the genetic engineering route to enhance or modify functional behaviour and so ultimately to produce tailor-made proteins designed to fulfil a particular task. For these reasons other invited papers in this symposium and a number of the supporting poster presentations cover not only the production and functional evaluation of natural proteins but also the effect that genetic substitution of particular amino acid residues has on functional behaviour. This should give us a greater understanding of the interactions involved, which should in turn lead later to proteins with improved performance.

Inevitably, as the proceedings of what was only a one-day symposium the selection of topics covered may appear to be somewhat limited, but we believe that the most active areas of dairy research are well represented and that the review-like nature of most of the invited papers means that the coverage is much less limited than would be expected. Many of the findings described, and certainly the techniques used, will undoubtedly be applicable not only elsewhere in the milk and dairy chemistry area but also widely outside it, especially in the microbiological and protein chemistry/protein engineering fields. These proceedings give a good state-of-the-art picture of current research which should be very valuable to research workers, lecturers, graduate students and final year undergraduates with interests in the practical applications of molecular biology, enzymology and protein chemistry, not just in improving the quality and performance of dairy foods and ingredients but also in a much wider context.

We should like to thank all those who made this symposium possible by both their physical and unflagging moral support, and especially all the contributors of papers and posters whose universally excellent quality manuscripts made our task as editors so straightforward and enjoyable.

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Proteolysis in Cheese during Ripening

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1. INTRODUCTION

The conversion of milk to cheese curd is only the first stage in the production of most cheese varieties. Essentially all hard, and many soft, cheeses are ripened for periods ranging from a few weeks to two years or longer. During this period, cheeses undergo numerous biochemical changes which lead to the development of the appropriate texture, flavour and aroma.

The biochemistry of cheese ripening is very complex, among the most complex of any food. It involves 3 primary processes: glycolysis, lipolysis and proteolysis, the relative importance of which depends on the variety. Proteolysis is the most complex of these phenomena and has been the subject of much research in recent years. Methods for assessing proteolysis have been reviewed extensively[1-5] and will not be discussed further here.

2. CHEESE RIPENING AGENTS AND THEIR CONTRIBUTION TO PROTEOLYSIS

The extent of proteolysis in cheese varies from very limited (e.g. Mozzarella) to very extensive (e.g. blue mould varieties). The products of proteolysis range in size from large polypeptides, comparable in size to intact caseins, through a range of medium and small peptides to free amino acids.

Proteolytic agents in cheese generally originate from 5 sources: the coagulant, the milk, starter bacteria, non-starter bacteria and adjunct starter. Enzymes from the first four sources are active in nearly all ripened cheeses. The adjunct starter (i.e. microorganisms added to cheesemilk for purposes other than acidification) can exert considerable influence on maturation in cheese varieties in which they are used (e.g. *Penicillium roqueforti*, *P. camemberti* in mould-ripened varieties or *Brevibacterium linens* in smear-ripened cheeses). Exogenous enzymes used to accelerate ripening could be added to the above list, and when present can be very influential.

Quantitation of the contribution of these agents individually or in various combinations has been attempted using three complimentary approaches: (1) model cheese systems from which the non-starter microflora have been eliminated by aseptic techniques, in which acidification may be accomplished using an acidogen (usually gluconic acid-δ-lactone) rather than starter, and in which coagulant and indigenous milk enzymes may be inactivated or inhibited; these techniques have been reviewed,[6]

(2) activity and specificity of the principal proteinases and peptidases on caseins or casein-derived peptides in solution, and (3) isolation of peptides from cheese and, based on the known specificity of the proteinases/peptidases on the caseins in solution, identificiation of their formative agent(s) in cheese.

The use of model systems has enabled the contribution of the principal ripening agents to proteolysis in cheese to be estimated fairly precisely. The subject has been reviewed[6] and may be summarized as follows. Using aseptic control cheeses, aseptic rennet-free cheeses, aseptic starter-free cheeses and aseptic, rennet-free, starter-free cheeses, Visser[7-10] and Visser and de Groot-Mostert[11] concluded that the coagulant is responsible for the initial hydrolysis of caseins, e.g. as shown by PAGE and the production of most of the water- or pH 4.6-soluble N in Gouda-type cheese, and that the actions of indigenous milk and starter proteinases are less important at this level of proteolysis. However, the production of small peptides and amino acids is due primarily to the action of starter bacteria or their enzymes. The results of other studies[12-17] on controlled-microflora cheese yielded generally similar results.

Direct evidence for the rôle of the principal indigenous milk proteinase, plasmin, in cheese is limited. Visser[10] found that approximately 5% of the total N in a 6 month-old aseptic starter-free, rennet-free cheese was soluble at pH 4.6, but very low levels of free amino acids were found. Farkye and Fox,[18] who eliminated the influence of plasmin in Cheddar cheese by an inhibitor, 6-aminohexanoic acid (AHA), found differences between electrophoretograms of these cheeses and those of controls; bands corresponding to the γ-caseins (produced from β-casein by plasmin) were less intense in cheese containing AHA. These authors also found that AHA reduced the level of water-soluble N, again suggesting a rôle for plasmin in the initial hydrolysis of caseins.

Milk also contains an indigenous acid proteinase (pH optimum, 4.0) which is probably cathepsin D (E.C. 3.4.23.5).[19-22] Cathepsin D is relatively heat-labile and is probably largely inactivated by pasteurization. The action of cathepsin D on the caseins is very similar to that of chymosin,[21, 23] and it has been suggested that a band with an electrophoretic mobility corresponding to α_{s1}-CN f24-199, i.e. the primary product of chymosin action on α_{s1}-casein, in aseptic Meshanger-type cheese is due to its action.[24] The contribution of cathepsin D to proteolysis in cheese has not been quantified.

Although non-starter lactic acid bacteria (NSLAB) usually dominate the microflora of Cheddar-type cheese during much of its ripening (see ref. 25), their influence on proteolysis in cheese has been neglected by most authors. Visser[8-10] used an aseptic control cheese to eliminate the influence of the NSLAB, as did Desmazeaud *et al.*[14] and O'Keeffe *et al.*[15-17] A wide range of proteolytic enzymes has been identified in NSLAB (see ref. 25), and thus it is likely that they play a rôle in proteolysis in cheese. In a comparative study on the ripening characteristics of Cheddar cheese made from raw, pasteurized or microfiltered milks, McSweeney *et al.*[26] concluded that non-starter lactobacilli were responsible for differences in proteolysis in the cheese made from the raw milk, particularly with regard to the formation of short peptides and free amino acids.

The progress of proteolysis in most ripened cheeses can be summarized as follows:- initial hydrolysis of caseins is caused primarily by residual coagulant, and to a lesser extent by plasmin and perhaps cathepsin D, resulting in the formation of large and intermediate-sized peptides which are subsequently degraded by the coagulant and enzymes from the starter and non-starter flora. The production of small peptides and free amino acids results from the action of bacterial proteinases and peptidases. This general outline of proteolysis can vary substantially between varieties where differences in manufacturing practices can have a profound influence on proteolysis. In Mozzarella, Swiss and other high-cook varieties, coagulant is extensively or completely denatured by the high cooking temperature. In these varieties, the

contribution of plasmin to the initial hydrolysis of caseins is more pronounced than in Cheddar and Dutch varieties. Likewise, in mould or bacterial surface-ripened varieties, proteinases and peptidases from the adjunct starter influence proteolysis strongly.

This presentation will focus on the specificity of the principal proteinases and peptidases in cheese on the individual caseins and casein-derived peptides and on the isolation and identification of peptides from Cheddar cheese.

3. SPECIFICITY OF THE PRINCIPAL PROTEINASES AND PEPTIDASES IN CHEESE

3.1. Proteinases from the Coagulant

Chymosin (E.C. 3.4.23.4) is the principal proteinase in traditional rennets used for cheesemaking. It is an aspartyl proteinase of gastric origin, secreted by the young of a number of mammalian species. The principal rôle of chymosin in cheesemaking is to coagulate the milk. However, about 6% of the chymosin added to cheese milk is retained in the curd for Cheddar and plays a major rôle in the initial proteolysis of caseins in many cheese varieties (see Section 2).

The action of chymosin on the B-chain of insulin indicates that it is specific for hydrophobic and aromatic amino acid residues.[27] Relative to many other proteinases, chymosin is weakly proteolytic; indeed, limited proteolysis is one of the characteristics to be considered in the selection of proteinases for use as rennet substitutes.[2]

The primary chymosin cleavage site in the milk protein system is the Phe_{105}-Met_{106} bond in κ-casein which is many times more susceptible to chymosin than any other bond in milk proteins; its hydrolysis leads to coagulation of the milk (see ref. 28). Cleavage of κ-casein Phe_{105}-Met_{106} yields para-κ-casein (κ-CN f1-105) and glycomacropeptides (GMPs; κ-CN f106-169). Most of the GMPs are lost in the whey but para-κ-casein remains attached to the casein micelles and is incorporated into the cheese.

Although considerably less susceptible than the Phe_{105}-Met_{106} bond of κ-casein, α_{s1}-, α_{s2}- and β-caseins are readily hydrolysed by chymosin under appropriate conditions. A number of authors have investigated the action of chymosin on β-casein.[29-32] In solution in 0.05 M Na acetate buffer, pH 5.4, chymosin cleaves β-casein at 7 sites: Leu_{192}-Tyr_{193} > Ala_{189}-Phe_{190} > Leu_{165}-Ser_{166} ≥ Gln_{167}-Ser_{168} ≥ Leu_{163}-Ser_{164} > Leu_{139}-Leu_{140} ≥ Leu_{127}-Thr_{128}.[31] The Michaelis parameters, K_m and k_{cat}, for the action of chymosin on the bond Leu_{192}-Tyr_{193} are 0.075 mM and 1.54 s^{-1}, respectively, for micellar β-casein and 0.007 mM and 0.56 s^{-1} for the monomeric protein.[32] NaCl inhibits the hydrolysis of β-casein by chymosin to an extent dependent on concentration and pH; the relative rates of hydrolysis of certain peptide bonds by chymosin are influenced by the ionic conditions of the solution.[33]

The primary site of chymosin action on α_{s1}-casein is Phe_{23}-Phe_{24}.[34, 35] Cleavage at this site has significance in producing a small peptide (α_{s1}-CN f1-23) which is further hydrolyzed by starter proteinases, and in the softening of cheese texture.[36] The specificity of chymosin on α_{s1}-casein in solution has been studied.[29,37-39] In 0.1 M phosphate buffer, pH 6.5, chymosin cleaves α_{s1}-casein at Phe_{23}-Phe_{24}, Phe_{28}-Pro_{29}, Leu_{40}-Ser_{41}(?), Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160} and Trp_{164}-Tyr_{165}.[39] These sites are also cleaved at pH 5.2 in the presence of 5% NaCl and, in addition, Leu_{11}-Pro_{12}, Phe_{32}-Gly_{33}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143} and Phe_{179}-Ser_{180}. The rates at which specific peptide bonds appear to be cleaved are dependent on the ionic conditions and differ between pH 6.5 and pH 5.2 in the presence of 5% NaCl, particularly Leu_{101}-Lys_{102}, which is cleaved far faster at the lower pH.[39]

Carles and Ribadeau-Dumas[35] found that the k_{cat} and K_m for chymosin at pH 6.2 and 30°C on the Phe_{23}-Phe_{24} bond of α_{s1}-casein were 0.66 s^{-1} and 0.37 mM, respectively. The influence of pH and urea on the hydrolysis of α_{s1}-casein by chymosin was studied by Mulvihill and Fox[40] who found that pH affected the pattern of proteolysis. Ionic conditions also affected proteolysis.[37] Dunn *et al.* [41] reported that the hydrolysis of synthetic octapeptides of the type Lys-Pro-Xxx-Yyy-Phe-(NO_2)Phe-Arg-Leu by chymosin is pH-dependent.

α_{s2}-Casein appears to be relatively resistant to proteolysis by chymosin but the specificity of chymosin, and indeed of other proteinases, on this protein has received little attention. Chymosin cleavage sites in α_{s2}-casein (pH 6.5) are generally restricted to the hydrophobic regions of the molecule, i.e. residues 90-120 and 160-207: Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Tyr_{98}, Tyr_{98}-Leu_{99}, Leu_{99}-Tyr_{100}, Phe_{163}-Leu_{164}, Phe_{174}-Ala_{175} and Tyr_{179}-Leu_{180}; the primary site appears to be Phe_{88}-Tyr_{89}.[42]

The extent of chymosin action on para-κ-casein (κ-CN f1-105) is unclear. Para-κ-casein migrates in the opposite direction to the other caseins in the alkaline urea PAGE gels widely used to study the initial proteolysis of caseins during ripening and thus is often ignored in such studies. Although it contains a number of potential chymosin cleavage sites, Green and Foster[43] found that para-κ-casein is resistant to chymosin action.

The parameter, k_{cat}/K_m, a measure of the affinity of an enzyme for a substrate, for the action of chymosin on the most susceptible bonds in κ-, β- and α_{s1}-caseins (Phe_{105}-Met_{106}, Leu_{192}-Tyr_{193} and Phe_{23}-Phe_{24}, respectively) has been estimated as 1405, 20.6 and 1.8 s^{-1} mM^{-1}, respectively,[32, 35] which suggests that the second most susceptible bond in the caseins to chymosin action is Leu_{192}-Tyr_{193} of β-casein.

Calf rennet contains about 10% bovine pepsin (E.C. 3.4.23.1) which contributes to proteolysis in Cheddar cheese;[44, 45] cleavage of the bond Leu_{109}-Glu_{110} in α_{s1}-casein appears to be due to its action.[45] Peptides produced from Na caseinate[46] or β-casein[47] by bovine pepsin appear to be generally similar to those produced by chymosin, although, as far as we are aware, the specificity of bovine or porcine pepsins on bovine caseins has not been determined rigorously.

For several years, the supply of calf rennet has been insufficient to meet demand and much effort has been expended on searching for suitable rennet substitutes (see refs. 48 and 49). Several enzymes have been studied, including bovine,[50] porcine,[46] ovine,[51] and chicken[52] pepsins. Microbial proteinases studied include those from *Cryphonectria parasitica*, *Rhizomucor pusillus*, *R. miehei*, *Penicillium janthinellum*, *Rhizopus chinensis* and *Aspergillus usameii*; the first 3 are used commercially, especially in the USA. The specificity of many of these enzymes on the oxidized B-chain of insulin was summarized by Green.[48] Their specificities on α_{s1}- and β-caseins differ from that of chymosin[49] but have not been established. The introduction of recombinant chymosins has limited the use of these enzymes.

Recombinant calf chymosins, expressed by *Aspergillus niger* var. *awamori*, *Kluveromyces marxianus* var. *lactis* and *Escherischia coli*, were introduced recently and, since their acceptance by regulatory authorities for use in cheese, they have been used widely for cheesemaking in many, but not all, countries. Cheesemaking trials, involving a number of varieties, have shown only small differences between cheese made with calf rennet or recombinant chymosins.[44, 53-57] Recombinant chymosins may contain only one genetic variant,[58] while calf rennet can contain three chymosin variants, A, B and C,[59] as well as bovine pepsin. Possible differences in specificity between chymosin variants have not been reported.

Residual coagulant activity in cheese is a function of a number of technological parameters, including the pH of the curd at whey drainage (which influences the amount of coagulant retained in the curd), final pH of the cheese and, especially, the

cook temperature. Cheeses which are cooked at a high temperature (e.g. Mozzarella and Swiss varieties) have relatively little coagulant activity. Chymosin and other enzymes from the coagulant act primarily on α_{s1}-casein in cheese. β-Casein is a good substrate for chymosin in solution but not in cheese, perhaps because the hydrophobic C-terminal region of the protein, which contains chymosin-susceptible sites, may be involved in hydrophobic interactions. Chymosin is probably inactive, or very weakly active, on α_{s2}-casein in cheese as this protein is not a good substrate for chymosin in solution.

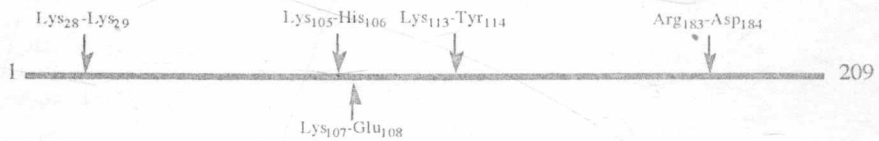
3.2. Indigenous Milk Proteinases

The presence of indigenous proteinases in milk has been recognized for nearly a century. The principal indigenous proteinase is plasmin, which is optimally active at pH 7.5, while the lesser, cathepsin D, has a pH optimum at ~4.0.

3.2.1. Plasmin. Plasmin (fibrinolysin, E.C. 3.4.21.7) has been the subject of much study (for review see ref. 60). The physiological rôle of plasmin is solubilization of fibrin clots, to achieve which there exists a complex fibrinolytic system consisting of the active enzyme, its zymogen, activators and inhibitors of the enzyme and zymogen activators, all of which are present in milk. Plasmin, plasminogen and plasminogen activators are associated with the casein micelles in milk, while the inhibitors are in the serum phase.[60, 61]

Plasmin, a trypsin-like proteinase with a high specificity for peptide bonds containing lysyl residues, is active on all caseins, but especially α_{s2}- and β-caseins.[60]

Plasmin cleaves β-casein in solution at 5 principal sites (Figure 1): Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, Lys_{107}-Glu_{108}, Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184} with the formation of the polypeptides, γ_1-CN (β-CN f29-209), γ_2-CN (f106-209), γ_3-CN (f108-209), γ_4-CN (f114-209) and γ_5-CN (β-CN f184-209) and proteose peptone 5 (β-CN f1-105 and f1-107), proteose peptone 8-slow (β-CN f29-105 and f29-107), proteose peptone 8-fast



PRODUCTS

Proteose Peptones		**γ-Caseins**
Known	***Probable***	
PP8f β-CN f1-28	β-CN f106-113	γ^1-CN (β-CN f29-209)
PP8s β-CN f29-105	β-CN f108-113	γ^2-CN (β-CN f106-209)
PP8s β-CN f29-107	β-CN f114-183	γ^3-CN (β-CN f108-209)
PP-T β-CN f29-113	β-CN f106-183	γ^4-CN (β-CN f114-209)
PP5 β-CN f1-105	β-CN f108-183	γ^5-CN (β-CN f184-209)
PP5 β-CN f1-107	β-CN f1-113 (unlikely)	

Figure 1 Specificity of plasmin on β-casein[62, 66, 67] (PP8f, proteose peptone 8 fast; PP8s, proteose peptone 8 slow; PP-T, proteose peptone T; PP5, proteose peptone 5).

(β-CN f1-28) and proteose peptone T (β-CN f29-113?) and probably the fragments β-CN f113-183, f106-113 and f108-113.[62-67]

Plasmin hydrolyses α_{s2}-casein in solution at 8 sites: Lys_{21}-Gln_{22}, Lys_{24}-Asn_{25}, Arg_{114}-Asn_{115}, Lys_{149}-Lys_{150}, Lys_{150}-Thr_{151}, Lys_{181}-Thr_{182}, Lys_{188}-Ala_{189} and Lys_{197}-Thr_{198},[68, 69] producing about 14 peptides, three of which are potentially bitter.[69]

Although plasmin is less active on α_{s1}- than on α_{s2}- or β-casein, the formation of λ-casein, a minor casein component, has been attributed to its action on α_{s1}-casein.[70] The principal plasmin cleavage sites in α_{s1}-casein are: Arg_{22}-Phe_{23}, Arg_{90}-Tyr_{91}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Lys_{124}-Glu_{125} and Arg_{151}-Gln_{152}.[71]

Although there are a number of potential plasmin cleavage sites in κ-casein, plasmin has low activity on this protein and its specificity does not appear to have been determined. Eigel[72] found no hydrolysis of κ-casein under conditions adequate for the complete hydrolysis of α_{s1}-casein, but Andrews and Alichanidis[65] reported that hydrolysis of κ-casein by plasmin accounted for 4% of the proteose peptone fraction produced by indigenous plasmin in pasteurized milk stored at 37°C for 7 days and detectable by PAGE.

3.2.2. Other Indigenous Milk Proteinses. The specificity of cathepsin D on the caseins has not been determined but electrophoretograms of caseins incubated with milk acid proteinase or exogenous cathepsin D indicate a specificity very similar to that of chymosin[19, 23] although the enzymes differed with respect to rates of cleavage of certain peptide bonds. Interestingly, κ-casein appears to be a poor substrate for cathepsin D which has very poor milk clotting properties.

The presence of other minor proteolytic enzymes in milk has been reported, including thrombin,[73] a lysine aminopeptidase[73] and proteinases from leucocytes,[74, 75] but they are considered not to be very significant.[74, 60] The occurrence of cathepsin D in milk suggests that other lysosomal proteinases are also present, although, as far as we are aware, none of these has yet been detected in milk.

3.3. Combined Action of Chymosin and Plasmin

The theoretical combined action of chymosin and plasmin on the principal cleavage sites of α_{s1}- and β-caseins is shown schematically in Figure 2. Theoretically,

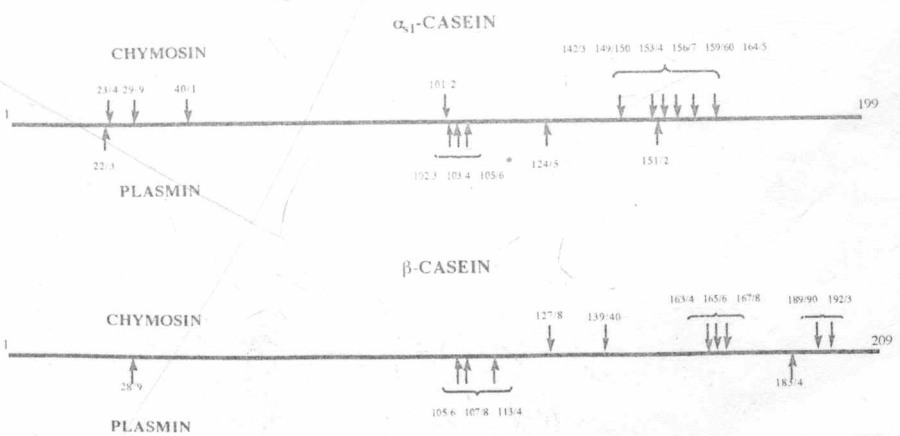


Figure 2 Potential combined action of chymosin and plasmin in cheese

their combined action could produce quite small peptides and their specificities are in fact complementary, especially on β-casein which chymosin cleaves primarily toward the C-terminal while plasmin cleaves mainly in the N-terminal region. As far as we know, the combined action of these enzymes on the isolated caseins has not been studied and it is not known whether they act in a concerted manner in cheese.

3.4. Proteolytic Enzymes from Starter

3.4.1. Proteinases of *Lactococcus* and *Lactobacillus* The starter cells are a major source of proteinases and peptidases in cheese. The proteolytic system of *Lactococcus*, the most widely used cheese starter, has been studied thoroughly, while those of thermophilic *Streptococcus* and *Lactobacillus* starters have recently attracted considerable attention.

The principal proteinase of the lactic acid bacteria is associated with the cell wall, where it has ready access to extracellular proteins. Cell wall-associated proteinases of *Lactococcus* can be classified into 3 groups, P_I-, P_{III}- and P_I/P_{III}-types. P_I-type proteinases degrade β- but not α_{s1}-casein at a significant rate, P_{III}-type proteinases readily hydrolyse both α_{s1}- and β-caseins,[76] while P_I/P_{III}-types have intermediate specificity. The nucleotide sequences of the genes for both P_I- and P_{III}-type proteinases have been established;[77-79] few differences are apparent and alteration of a few residues by site-directed mutagenesis can alter the specificity of the proteinase.[80] Exterkate[81] reported that a cation-binding site in P_I proteinases, but absent in P_{III}, was mainly responsible for the difference in specificity between the enzymes on positively-charged chromophoric peptides. The specificities of cell wall-associated proteinases from a number of strains of *Lactococcus* on α_{s1}-, α_{s2}-, β- and κ-caseins have been determined (Figures 3 to 5; refs. 82-88). Tan *et al.*[89] commented that P_I-type proteinases have a rather broad specificity on β-casein and that its cleavage

Figure 3 Amino acid sequence of *Bos* α_{s1}-casein showing the position of the cleavage sites of cell wall-associated proteinases of [1] *Lactococcus lactis* ssp. *cremoris* SK112 (ref. 87) and [2] *L. lactis* ssp. *lactis* NCDO 763 (ref. 84).

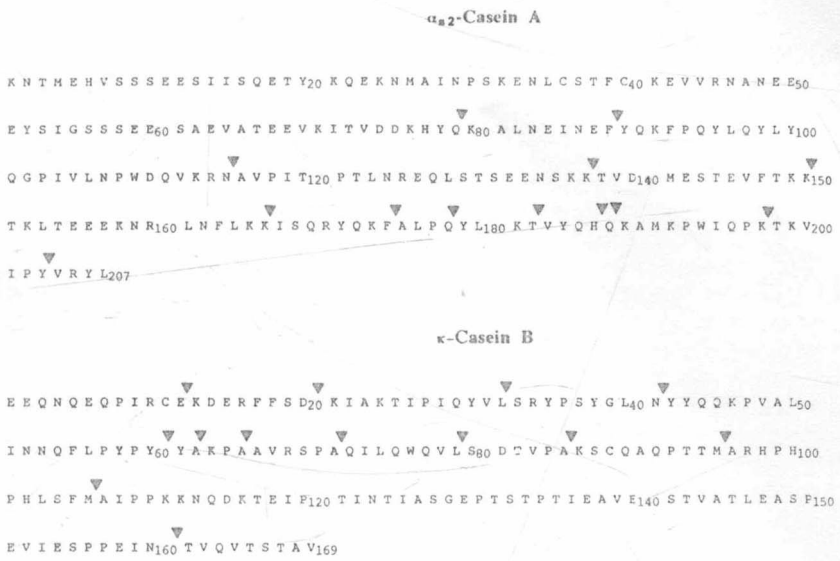


Figure 4 Amino acid sequences of *Bos* α_{s2}-casein A and κ-casein B showing the positions of the cleavage sites of cell wall-associated proteinase of *Lactococcus lactis* ssp. *lactis* NCDO 763 (ref. 84).

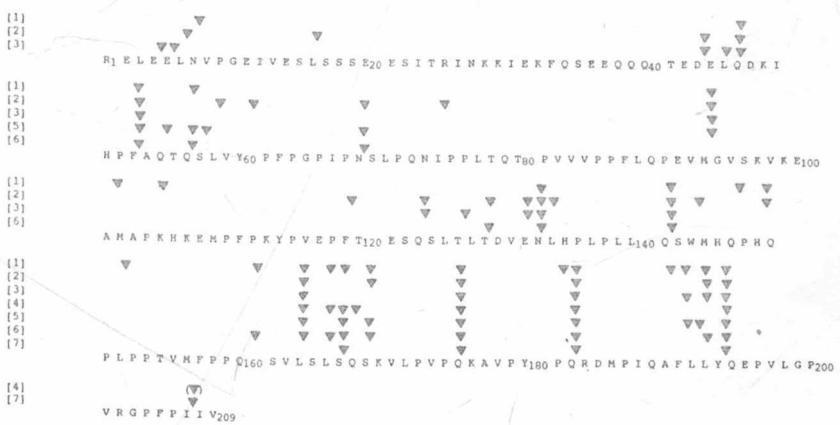


Figure 5 Amino acid sequence of *Bos* β-casein showing the cleavage sites of cell wall-associated proteinases of *Lactococcus*. [1] P_I-type, *L. lactis* ssp. *cremoris* H2 (ref. 88); [2] P_{III}-type, *L. lactis* spp. *cremoris* SK112 (ref. 88); [3] P_{III}-type, *L. lactis* ssp. *cremoris* AM_1 (ref. 86); [4] *L. lactis* ssp. *cremoris* HP (ref. 85); [5] *L. lactis* ssp. *cremoris* AC1 (ref. 83); [6] *L. lactis* ssp. *lactis* NCDO 763 (ref. 83); [7] *L. lactis* ssp. *lactis* NCDO 763 (ref. 82).

sites typically contained a Gln or Ser residue and are likely to lie in regions of the molecule which have high hydrophobicity, a high proline content and a low charge. On the other hand, P_{III}-type proteinases tend to cleave bonds of the type Glx-X or X-Glx, where X is generally a large, hydrophobic residue (Met, Phe, Leu or Tyr), while a hydrophobic residue is usually found at the P_2 or P'_2 position with a negatively-charged residue in the P_2-P_3 or P'_2-P'_3 regions. In general, P_{III}-type proteinases have broader specificities on β-casein than P_I-type enzymes,[89] which Visser *et al.*[86] suggested might explain why such strains produce less bitter peptides from casein than P_I-type strains.[90] The literature on lactococcal cell wall-associated proteinases has been reviewed extensively.[6, 89, 91] Thermophilic *Lactobacillus* spp. used as starters also possess a cell wall-associated proteinase (see ref. 91)

The primary rôle of lactococcal proteinases in cheese appears to be the hydrolysis of large and intermediate-sized peptides produced from caseins by chymosin or plasmin. A number of authors have investigated the action of lactococcal cell wall proteinases on such peptides (Figure 6). The cell wall-associated proteinase of *Lactococcus* does not appear to be important in the initial hydrolysis of β-casein in Cheddar as detected by urea-PAGE, perhaps because the primary cleavage sites on this

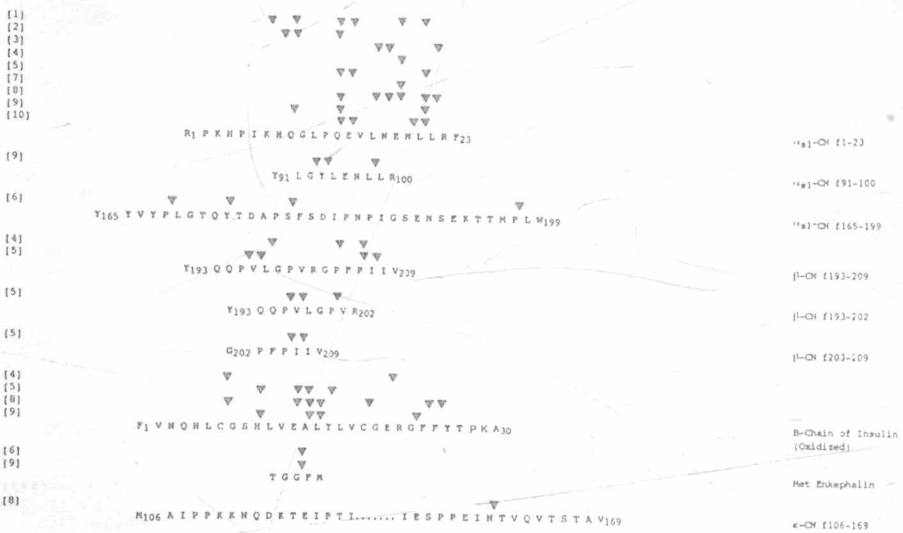


Figure 6 Amino acid sequence of a number of peptides from casein hydrolysates and other sources showing the specificity of lactococcal proteinases and peptidases. [1] Cell wall associated proteinase of *L. lactis* ssp. *cremoris* H61 (ref. 96); [2] cell wall-associated proteinase (P_I-type) of *L. lactis* ssp. *cremoris* HP (ref. 97); [3] cell wall-associated proteinase (P_{III}-type) of *L. lactis* ssp. *cremoris* AM_1 (ref. 97); [4] metalloendopeptidase from *L. lactis* ssp. *cremoris* HP (ref. 98); [5] neutral oligo-endopeptidase from *L. lactis* ssp. *cremoris* C13 (ref. 98); [6] LEP III-I from *L. lactis* ssp. *lactis* MG 1363 (ref. 99); [7] LEP I from *L. lactis* ssp. *cremoris* (ref. 100); [8] cell wall-associated proteinase of *L. lactis* ssp. *lactis* NCDO 763 (ref. 84); [9] LEP II from *L. lactis* ssp. *cremoris* H61 (ref. 101); [10] LEP III-I from *L. lactis* ssp. *lactis* MG 1363 (ref. 99)