ASPECTS OF PROTEOLYSIS IN CHEESE

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ABSTRACT

The purpose of the present study was to elaborate methods for the detailed examination of proteolysis pathways in cheese (reviewed in Chapter 1) and to demonstrate their usefulness. Many techniques, including solvent fractionation, chromatographic separation and electrophoresis have been used previously and were revisited in this study.

Gel electrophoresis can be a powerful technique and was examined in detail. The methods investigated were: 1) a slab gel system using the apparatus of the E-C Apparatus Corporation and a polyacrylamide gel in a Tris-EDTA-borate buffer at alkaline pH and containing urea; 2) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide stacking and resolving gel with a discontinuous (Tris-chloride/Tris-EDTA-borate) buffer system that contained urea; 3) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide stacking and resolving gel and acetic acid-ammonium acetate buffers at acidic pH that contained urea; 4) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide gel with a stacking and resolving gel in Tris-HCl buffers containing sodium dodecyl sulphate (SDS) and a Tris-chloride-glycine electrode buffer.

The mini-slab alkaline urea polyacrylamide gel electrophoresis (PAGE) method was considered to be the most suitable for monitoring the loss of intact casein during cheese ripening. However, SDS-PAGE gave good resolution of para-κ-casein, β-lactoglobulin and α-lactalbumin and it could therefore be used for the analysis of cheese in which whey proteins have been incorporated or for monitoring the breakdown of para-κ-casein (Chapter 4) in cheese. Two-dimensional PAGE revealed the presence of more bands than were visible using any single method of electrophoresis. Traces of protein were found to lie beneath the α_{s1}-casein band and this explained why, even after considerable proteolysis, some α_{s1}-casein appeared to remain.

Storing cheese samples in such a way that there is a minimum of further change was examined using several different storage methods and temperatures, including storage as: freeze-dried powder at 4°C in the dark, frozen at -9, -16, -35, -75 and -100°C, and dissolved in 6 M urea solution and stored at 4 and -16°C. The trial ran for 6 months and involved the multiple sampling and detailed analysis of three Cheddar cheeses by reversed phase fast protein liquid chromatography (RP-FPLC) for the water-soluble fraction (WSF) and alkaline urea-PAGE for the protein fraction.

None of the methods used to store the cheese samples was completely satisfactory. Cheese stored at temperatures of -9 and -16°C was unstable, with proteolysis discernible after 66 days. Storage of cheese samples at these temperatures is, therefore, not recommended. Cheese stored at temperatures of -35, -75 and -100°C was unstable after 94 days, although the samples stored at -100°C were more stable. This lack of stability probably arose during thawing as well as during storage of the frozen cheese samples. Storage of freeze-dried samples at 4°C in the dark was equivalent to storing the frozen cheese at -100°C. Storage of samples in alkaline urea sample buffer was better at -16°C than at 4°C but should be for no longer than 1 month.

An indication of the differences to be expected within the normal range of Cheddar cheese was determined using three very similar Cheddar cheeses ripened at 5 and 13°C (Chapter 3, part II). Cheeses ripened at 5°C for 6 months were similar to those ripened at 13°C for 2 months and the proteolytic pathways appeared to the same at both temperatures.

The proteolytic pathways in Cheddar and Mozzarella cheeses, manufactured according to standard protocols, ripened at 13°C and sampled at regular intervals over a six month period were examined using a variety of techniques: total nitrogen (TN), non-protein nitrogen (NPN), water-soluble nitrogen (WSN), alkaline urea-PAGE, low molecular weight (LMW) SDS-PAGE, RP-FPLC and size exclusion high performance liquid chromatography (SE-HPLC). The TN and NPN analyses were done at the time of sampling whereas the other assays were done on samples that had been stored at $<$-75°C so that they could be analysed simultaneously.

The increase in WSN and NPN was greater in Cheddar cheese than in Mozzarella cheese and reflected the greater microbial enzyme activity in this cheese type.

Alkaline urea-PAGE revealed that there was more α_{s1}-casein hydrolysis (with the formation of α_{s1}-casein-I) in Cheddar cheese than in Mozzarella cheese, indicating that rennet activity was greater in Cheddar cheese. The presence of peptides believed to be β-I- (β-casein f1-189/192) and β-II-casein (β-casein f1-165) indicated that rennet may have hydrolysed β-casein. The amount of β-casein hydrolysis (and γ-casein formation) was greater in Mozzarella cheese, reflecting the greater plasmin activity in this cheese type. Both LMW SDS-PAGE and SE-HPLC of the whole cheese provided little additional information.

Examination of the WSF of each cheese by PAGE analysis showed that many of the

larger peptides may have been present in both cheese types. The different concentrations of these peptides in each cheese type were consistent with different rennet and plasmin activities and suggested that they may have been products of these enzymes. RP-FPLC and SE-HPLC analysis of the WSF of Cheddar cheese revealed that, although the larger peptides continued to accumulate during ripening, there was also a large increase in the amount of small peptides and amino acids in the cheese. In the Mozzarella cheese, the larger peptides accumulated and there was little evidence of their further hydrolysis to small peptides and amino acids.

The present studies indicate that SE-HPLC using a Toyo-Soda SW 2000 column and a 36% acetonitrile/0.1% trifluoroacetic acid solvent system is a promising new technique that may be useful in determining cheese type and maturity and in relating changes in the molecular weight distribution of the peptides to changes in the textural, functional and flavour characteristics of cheese.

It was concluded that the results are consistent with the concept that differences in the manufacture of Cheddar and Mozzarella cheeses result in the formation of two cheeses, each with different amounts of similar enzymes (rennet, plasmin, and the enzymes of the starter and non-starter lactic acid bacteria), and that these differences in enzyme concentration, combined with the modifying effect of pH, temperature, moisture content and S/M, result in different enzyme activities and patterns of proteolysis in the two types of cheese and these, in turn, result in cheeses with different functional properties.

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CHAPTER 1

PROTEOLYSIS IN CHEESE: A GENERAL REVIEW

SUMMARY

The following chapter reviews the progress made in understanding proteolysis and its effect on the texture, functional properties and flavour characteristics of cheese.

Differences in the manufacture of the various types of cheese result in the inclusion of different amounts of rennet, plasmin (and other indigenous proteases) as well as the enzymes of the starter microorganisms, non-starter lactobacilli and secondary microflora in the curd and create differences in the cheese environment (*e.g.*, pH, moisture, salt, salt-in-moisture, calcium) that result in differing enzyme activities in the ripening cheese.

The enzymes in the cheese initially breakdown the protein matrix to peptides and this contributes to changes in cheese texture and functional properties. The further breakdown of these peptides to smaller peptides, amino acids and the end-products of amino acid catabolism contributes to the development of cheese flavour. The cumulative effect of the cascading action of the contributing enzymes and the relevance of individual peptides to cheese properties has not yet been fully determined.

1 INTRODUCTION

There are between 500 and 1000 varieties of cheese (Burkhalter, 1981; Sandine and Elliker, 1981) produced throughout the world, created by differences in milk source, fermentation and ripening conditions as well as pressing, size and shape.

The basic principles for cheesemaking are the same for nearly all varieties of cheese. Their manufacture involves the removal of water from milk with a consequent six to ten-fold (Fox, 1989) concentration of the protein, fat, minerals and vitamins. Cheeses can be classified as either fresh or ripened. Fresh cheeses are made by the coagulation of milk, cream or whey with acid or a combination of acid and heat. They must be consumed soon after manufacture before any spoilage can occur. Ripened cheeses have undergone three major changes. Lactose has been metabolized to lactic acid (glycolysis) and also acetic and propionic acids, carbon dioxide, esters and alcohol by the starter microorganisms added to the milk. The lipids have been broken down (lipolysis) to form free fatty acids, ketones, lactones and esters. Proteins have been gradually broken down (proteolysis) to form peptides, amino acids, amines, aldehydes, alcohols and sulphur compounds.

1.1 CHEESE MANUFACTURE

The manufacture of most varieties of cheese involves:

(i) *Acidification* of the milk, usually by the addition of starter microorganisms that convert lactose to lactic acid. For some varieties of cheese, an acidifying agent such as citric acid, malic acid, acetic acid or gluconic acid-δ-lactone is added directly to the milk to achieve the desired pH. The amount of acid present has a marked effect on the level of proteolysis seen in the resultant cheese. The activity of the coagulant enzyme, the amount of enzyme remaining in the curd, and as a consequence, the amount of proteolysis, are dependent on the amount of acid produced in the initial stages of cheesemaking. The pH also controls the level of moisture which in turn affects proteolysis in the cheese. 'The rate of pH decline determines the extent of dissolution of colloidal calcium phosphate which modifies the susceptibility of the caseins to proteolysis during manufacture and influences the rheological properties' (such as texture) of the cheese (Fox, 1987a).

Acidification also controls the growth of many non-starter bacteria including pathogenic species and those that produce CO_2 and undesirable or excessive proteolysis (Fox, 1987a).

(ii) *Coagulation* of the casein fraction to form a gel. This can be achieved by lowering the pH to 4.6 which precipitates the casein, lowering the pH to ~5.2 and applying heat, or more commonly by the addition of an enzyme such as rennet (Fox, 1987a).

(iii) *Dehydration* or *syneresis* of the coagulum which is the loss of whey as the result of cutting, stirring, cooking, salting, or pressing the curd.

(iv) The formation of the final cheese shape by moulding and pressing.

(v) The addition of *salt* which acts as a preservative by controlling microbial growth and enzyme activity. The presence of salt affects the syneresis of the curd with a loss of moisture which in turn affects the rate of enzyme activity.

Salt is also involved in physical changes in cheese proteins which influence texture, protein solubility and conformation. Other important functions of salt in cheese are to act as both a flavour and a flavour enhancer and as a source of dietary sodium.

The manufacturing procedures which create distinct classes of cheeses are (i) the application of heat to the curd, which gives rise to the 'plastic curd' cheeses with a low level of proteolysis such as Mozzarella and Provolone and (ii) the addition of moulds such as *Penicillium camemberti* used in the manufacture of Camembert and *Penicillium roqueforti* in the manufacture of blue-veined cheeses such as Roquefort and Stilton. These cheeses typically have a high level of proteolysis.

2 THE PROTEOLYTIC ENZYMES IN CHEESE

The proteins in cheese undergo a series of complex, sequential changes during ripening that are caused by proteinases from milk, milk-clotting enzymes, the enzymes from lactic starter cultures and other microorganisms that are adventitious or added (Grappin *et al.*, 1985).

Proteolysis in cheese proceeds according to the following general pathway (Law, 1987).

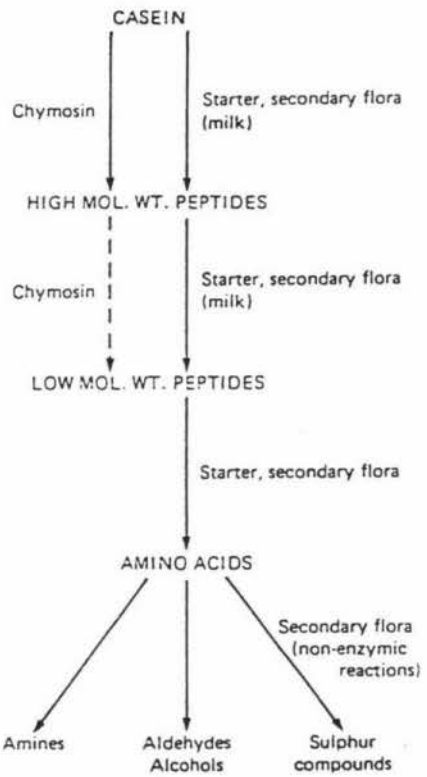


FIGURE 1.1 Breakdown of casein during cheese ripening: involvement of proteinases from various sources.

The casein fraction of cheese constitutes 99% of the protein and consists of the α_{s1}-group of caseins (199 amino acid residues) which is a mixture of α_{s1}-casein with 8 PO_4 groups and α_{s0}-casein with 9 PO_4 groups per molecule; the α_{s2}-group of caseins (207 amino acid residues) which is composed of α_{s2}-, α_{s3}-, α_{s4}-, α_{s6}-, and α_{s5}-caseins (α_{s5}-casein is a dimer of α_{s3}- and α_{s4}-casein) with 10-13 PO_4 groups per molecule; β-casein (209 amino acid residues) with 5 PO_4 residues; the κ-casein group (169 amino acid residues) with 1 PO_4 residue and seven different forms (K1 - K7) with different degrees of glycosylation and para-κ-casein (105 amino acid residues) with no PO_4 groups (Grappin *et al.*, 1985).

2.1 THE COAGULANT ENZYMES

Most varieties of cheese are enzymatically coagulated by rennet. The rennet can be of animal or microbial origin or a recombinant 'nature identical' rennet. The rennet incorporated in the cheese curd breaks down the proteins to peptides and amino acids during the maturation of the cheese.

2.1.1 Animal Rennet

Chymosin, an acid (aspartic) proteinase, is the traditional enzyme used in cheesemaking. It is obtained from the stomach of milk-fed calves. When substances other than milk enter the diet of the calf, chymosin is replaced by another acid proteinase known as pepsin which is also used in the manufacture of cheese. Rennet produced in New Zealand by The New Zealand Rennet Company Ltd (Eltham, New Zealand) has a minimum chymosin content of 92%. There are at least three genetically different variants of each of these enzymes.

Chymosin or pepsin from other species of animals is also used in the manufacture of cheese. Calf rennet has the highest milk clotting to proteolytic activity ratio of all the animal rennets. The proteolytic activities of the animal rennets decrease in the following order: porcine pepsin > ovine pepsin > bovine pepsin > calf rennet (Fox, 1969, 1987b; Green, 1972; O'Leary and Fox, 1972; Green *et al.*, 1984; Gouda *et al.*, 1988). A combination of chymosin and pig pepsin, usually in the ratio of 80/20, has commonly been used in the USA (L. Creamer, personal communication). An increased proportion of pepsin to chymosin results in a decrease in the rate of protein breakdown during maturation (Fox and Walley, 1971; Phelan, 1985). It is believed that this 'reflects the lower pH and heat stability of bovine pepsin during cheese manufacture and the consequent lower level of residual rennet activity in the curd' (Guinee and Wilkinson, 1992).

2.1.2 Recombinant Rennet

There are three genetically engineered or 'nature identical' rennets available commercially. Each of these is prepared from a different species of microorganism into which the appropriate gene sequence (obtained from either calf abomasum or synthesised) has been inserted and that was subsequently cloned. The first commercially available genetically engineered rennet was Chy-max (Pfizer and Co. Inc., USA). This enzyme is produced in an inactive form by *Escherichia coli* K12. The enzyme is contained in inclusion bodies within the organism and is chemically

extracted and activated to form chymosin (genetic variant A). A second genetically engineered rennet, known as Maxiren (Royal Gist Brocades NV, Delft, Netherlands), is secreted into the growth medium as prochymosin by the host organism *Kluyveromyces lactis* (a species of yeast). The prochymosin is activated to form a chymosin (genetic variant unknown) that contains a sugar molecule but is otherwise identical to natural chymosin. McSweeney *et al.* (1993a) and McSweeney (1993b) showed that the action of Maxiren and calf chymosin on α_{s1}- and α_{s2}-casein produced identical peptides. The third genetically engineered rennet is known as Chymogen (Genencor International, San Francisco, CA, USA and Chr. Hansen Ltd (Chr. Hansen's Laboratorium, Danmark A/S, Denmark)) and is produced by *Aspergillus niger* (a species of fungus). The organism secretes inactive prochymosin into the growth medium. The prochymosin is extracted and activated to form a chymosin (genetic variant B) that also contains a sugar molecule but is otherwise identical to native chymosin. Chymogen is also available in a form that contains added pepsin of animal origin and is known as Chymogen S (information supplied by Dr K. Turner, The New Zealand Rennet Company Ltd, Eltham, New Zealand). Cheese incorporating recombinant rennet has been shown to be indistinguishable from cheese made with calf rennet (Green *et al.*, 1985; Koch *et al.*, 1986; Hicks *et al.*, 1988; Prokopek *et al.*, 1988). These products have not gained acceptance in New Zealand although approximately 50% of cheese made in the U.S.A. is made from genetically engineered chymosin.

2.1.3 Microbial Rennet

Several different microbial enzymes are available commercially and can be used as rennet substitutes. To enable a rennet substitute to be used successfully in cheese it must possess the following characteristics: a high ratio of milk clotting-to-proteolytic activity; a milk clotting activity that is not very pH-dependent in the pH region of 6.5-7.0; a similar stability to calf rennet in the pH and temperature range used in the manufacture of cheese; low thermostability during whey processing; produce cheese that has the desired flavour, body and texture characteristics; produce cheese that has stability on storage and ease of handling (Scott, 1973; Phelan, 1977).

A frequently used enzyme known by tradenames such as Rennilase (Novo Industrie, Bagsvaerd, Denmark), Fromase (Royal Gist Brocades), Hanilase or Marzyme (Miles Laboratories, Madison, Wisconsin, USA) is obtained from *Mucor miehei*, a species of fungus (Foltmann, 1987). This enzyme has a ratio of clotting activity to proteolytic activity that is similar to chymosin and pepsin. However, the enzyme has a different

specificity to chymosin or pepsin and produces a different array of peptides. Rennilase is used commercially in the large scale manufacture of Feta cheese. An enzyme with similar activity and known as Emporase (Dairyland Food Laboratories, WI, USA), Meito (Meito Sangyo Co. Ltd, Japan) or Noury (Vitex, Paris, France) is produced by *M. pusillus* (Foltmann, 1987). In Cheddar cheese this enzyme has been shown to have a similar specificity to the *M. miehei* proteinase. The specificity is different to that of either calf rennet or a 50:50 bovine pepsin: porcine pepsin blend (Phelan, 1985 as reported by Guinee and Wilkinson, 1992). An examination of Cheddar cheese (at 26 weeks) incorporating either of these microbial enzymes revealed more extensive degradation of β-casein than in cheese made with calf rennet (Phelan, 1985 as reported by Guinee and Wilkinson, 1992). Similar findings were reported by Creamer *et al.* (1988b) who compared casein degradation in a pair of Cheddar cheeses made using either calf rennet or a microbial coagulant (Rennilase). They found that α_{s1}-casein degraded slightly faster in the calf rennet cheeses and β-casein degraded much more rapidly in the Rennilase cheeses.

Surecurd is a milk coagulant produced from *Endothia parasitica* (Pfizer) that contains lipase as well as protease activity and it is sometimes used in the manufacture of Cottage, Swiss and Mozzarella cheeses. It has been shown to be excessively proteolytic, hydrolysing α_{s1}-casein more rapidly than κ-casein (Vanderpoorten and Weckx, 1972; Tam and Whitaker, 1972; Phelan, 1977) and producing relatively high N losses in cheese wheys (Emmons, 1990).

2.1.4 Rennet as a Coagulant

The four main groups of proteins in milk are the α_{s1}-, α_{s2}-, β- and κ-caseins as previously discussed. These caseins exist in milk in the presence of calcium (35 mM) as micelles of ~ 100 nm in size and containing several thousand molecules of each type of casein.

The more hydrophobic regions of the phosphoproteins are believed to be located inside the micelle with the more hydrophilic κ-casein on the outside (McGann *et al.*, 1980; Donnelly *et al.*, 1984). The negatively charged carboxy-terminal of the κ-casein molecules is thought to protrude 'hair-like' from the micelle and repel other casein micelles (charge stabilization). In addition to this, the 'hair-like' macropeptide portions of κ-casein are unable to interpenetrate (steric stabilization). These two mechanisms are thought to enable the micelles to stay in solution as colloidal particles (Holt, 1975; Walstra *et al.*, 1981).

Rennet (includes all the acid proteinases) acts on κ-casein and cleaves the Phe_{105} - Met_{106} bond (Delfour *et al.*, 1965). The release of the hydrophilic carboxy-terminal peptide (glycomacropeptide) results in destabilization of the micelles which become less negatively charged and more hydrophobic. These micelles then aggregate (in the presence of calcium and at a temperature of >15°C (Berridge, 1942)) due in part to van der Waals attraction and hydrophobic interaction (Dalgleish, 1993).

The hydrolysis of κ-casein to form a coagulum is known as the primary stage of rennet action, and the aggregation of the micelles, as the secondary stage. After approximately 86-90% (Dalgleish, 1979) of the κ-casein has been hydrolysed the two events occur simultaneously and coagulation can be well advanced before all of the κ-casein is hydrolysed (Green and Marshall, 1977). The rate of enzyme action is dependent on temperature.

The presence of calcium ions is necessary for the coagulation of milk and the rate of both the enzymic reaction and aggregation is dependent on their concentration (Green and Marshall, 1977). It is thought that the negatively charged phosphoserine residues or carboxylic acid groups (Lucey, 1990), exposed by the loss of the micelle-stabilizing macropeptide, bind calcium ions. This results in an overall reduction of micelle charge and a decrease in repulsion resulting in aggregation.

A rennet coagulum consists of a continuous matrix of protein in which fat globules, water, minerals, lactose and micro-organisms are entrapped.

2.1.4.1 Factors that affect the coagulation properties of milk

The rennet coagulation time and the gel strength deteriorate in late lactation milk (White and Davies, 1958; O'Keeffe *et al.*, 1982; O'Keeffe, 1984) resulting in a cheese with a lower curd strength and syneresis rate and a higher moisture content (O'Keeffe *et al.*, 1982; O'Keeffe, 1984), poor body and texture, and poor melting and stretching properties (Fox and Stepaniak, 1993). This has been attributed to the activity of plasmin in the milk, however, extensive proteolysis must occur before any significant effect on the coagulation properties is seen (Grufferty and Fox, 1988b).

A study by Macheboeuf *et al.* (1993) confirmed the existence of considerable differences in milk coagulation properties according to breed (Storry *et al.*, 1983), the genetic variants of β-lactoglobulin and κ-casein as well as the nutritional status of the cow. Their study showed that inter-breed differences were almost totally explained by

the differences in casein contents associated with the distribution of κ-casein variants. They observed a beneficial effect of the κ-casein BB variant on the rheological properties of the coagulum (rennet clotting time, curd firmness, curd firming time) and found that even after the casein content of the two variants had been adjusted to the same level, marked differences existed between milk containing each of the two variants. It was thought that these differences may be linked to more evenly distributed micelles of smaller mean diameter which merge more tightly to produce a firmer coagulum. This improvement may also be due to the higher proportion of κ-casein in the total casein of the B-type milks (9.5% for the BB genotype, 6.7% for the AA genotype and 8.3 for the AB genotype, $P<0.01$). When the effect of the κ-casein variant was taken into account, the effect of the β-lactoglobulin variant was marked only on the soluble protein content (10% higher in the AA genotype, compared with the BB genotype, $P<0.05$) and the clotting time (25% longer at the natural milk pH and 15% longer at the standardised pH (pH 6.6) in the BB genotype compared with the AA genotype, $P<0.05$). In a similar study on the effect of κ-casein variants on the rennetability of milk carried out by Delacroix-Buchet *et al.* (1993) the same conclusions were reached.

The application of heat to milk (prior to the addition of rennet) at normal concentration, at ≤ pH 6.6 (Singh and Fox, 1985) and at temperatures that denature β-lactoglobulin results in an increase in clotting time (Morrissey, 1969). This is believed to be due to the formation of κ-casein-β-lactoglobulin (heat denatured) complexes on the outside of the casein micelle that make the Phe-Met bond inaccessible to rennet. It has also been proposed that rennet action may proceed, but because the heat denatured β-lactoglobulin binds to the para-κ-casein portion of κ-casein, coagulation is prevented (Dalgleish, 1993).

Alternatively, heating may result in changes to the micelle structure that prevent rennet action and/or coagulation. Heating is known to alter the distribution of calcium between the micelle and the serum phase of milk (Jenness and Patton, 1959). It is also known that the effect of this heating action can be almost totally reversed by lowering the pH of the milk to 5.8 (to partially dissociate the calcium phosphate) and then bringing it back up to pH 6.6 prior to the addition of rennet (Banks *et al.*, 1987; Singh *et al.*, 1988). The effectiveness of preacidification suggests that heat induced precipitation of calcium and phosphate (Schmidt and Both, 1987), may be one factor affecting gel strength, curd syneresis and cheese functional properties. The addition of Ca^{2+} can also be used to reverse, at least partially, the effect of this heat treatment

(Dalgleish, 1987).

Storage of milk at low temperatures prior to the addition of rennet results in an increase in coagulation time (Qvist, 1979; Ali *et al.*, 1980). This is believed to be due to the solubilisation of colloidal calcium phosphate (Jenness and Patton, 1959; Davies and White, 1960) and the diffusion of β-casein and κ-casein from the micelles. This is not completely reversed by holding at the renneting temperature ($\sim$32°C). A short heat treatment at 72°C/15 seconds (Van Hooydonk *et al.*, 1984) reverses this effect although recovery is not complete (Dalgleish, 1987).

The homogenisation of milk prior to the addition of rennet does not appear to adversely affect the rate at which the coagulum is formed. Homogenisation breaks up the milkfat globules and the newly formed milkfat globules, stabilised by casein adsorbed at the surface (Wiese and Palmer, 1932; Buchheim *et al.*, 1986), tend to behave as large casein micelles. Normally a coagulum consists of a casein matrix containing entrapped fat. These casein coated fat globules, acting as large casein micelles, form part of the casein matrix which results in changes in the properties of the coagulum (Shaker *et al.*, 1990).

2.1.5 Rennet and Proteolysis

The tertiary phase of rennet action involves proteolysis of the caseins in the cheese curd. The amount of rennet retained (usually about 6% of the initial amount added (Fox and Stepaniak, 1993)) and the rate and extent of rennet activity depends on the initial rennet level, the cook temperature, the curd pH at draining, the salt and moisture content of the curd and the storage temperature of the cheese.

All of the rennets are acid proteinases and show specificity for peptide bonds to which hydrophobic residues supply the carboxyl group.

2.1.5.1 Chymosin action on α_{s1}-casein

During the first two months of Cheddar cheese maturation, $\sim$80% of the α_{s1}-casein disappears (Johnston *et al.*, 1994). α_{s1}-Casein is cleaved at Phe_{23} - Phe_{24} and/or Phe_{24} - Val_{25} to produce α_{s1}-I-casein (Phe_{24}/Val_{25} - Trp_{199}) (Creamer and Richardson, 1974). This action is primarily due to chymosin but other enzymes may also be involved. It has been shown that plasmin cleaves α_{s1}-casein (in solution in 0.05 M NH_4HCO_3 buffer at pH 8.0, 37°C) at Arg_{22} - Phe_{23} (Le Bars and Gripon, 1993) and it is possible that the larger peptide formed has an electrophoretic mobility similar to that of α_{s1}-I-casein. An

acid proteinase from milk with a specificity similar to chymosin may also contribute to the formation of α_{s1}-I-casein (Kaminogawa and Yamauchi, 1972; Kaminogawa *et al.*, 1980; McSweeney, 1993b). The cleavage of α_{s1}-casein by chymosin is thought to be responsible for changes in cheese texture during the early stages of ripening (de Jong, 1976, 1977, 1978; Creamer and Olson, 1982; Johnston *et al.*, 1994).

The hydrolysis of α_{s1}-casein by rennet (with the consequent formation of α_{s1}-I-casein) has been shown to occur in solution under a wide range of pH (7.0 to 2.2 (at low pH, urea was used to solubilise the protein) (Fox and Walley, 1971; Mulvihill and Fox, 1977, 1979). In Na caseinate and in solution, the optimum pH for the hydrolysis of α_{s1}-casein is 5.8 (Fox (1969) and Mulvihill and Fox (1977), respectively). α_{s1}-I-Casein is further hydrolysed in solution to α_{s1}-II-casein (Phe_{24}/Val_{25} - Leu_{169}) and α_{s1}-III/IV (Phe_{24}/Val_{25} - Leu_{149}/Phe_{150}) at pH 5.8 to 7.0, α_{s1}-V-casein (Pro_{29}/Gly_{33} - Trp_{199}) at pH 4.0 to 5.2 in the absence of NaCl and α_{s1}-VII/VIII-casein at pH 5.2 in the presence of 5% NaCl (Mulvihill and Fox, 1977; Mulvihill and Fox, 1979). Mulvihill and Fox (1980), subsequently identified α_{s1}-V and α_{s1}-VII/VIII in Cheddar cheese.

In cheese, there is considerable variability in the rate of α_{s1}- and α_{s1}-I-casein hydrolysis and some of this variability has been attributed to the effect of salt concentration. Thomas and Pearce (1981) examined proteolysis in commercially produced Cheddar cheese, after one month at 10°C, and found a linear relationship between salt concentration and the extent of proteolysis. At 4% salt-in-moisture (S/M), approximately 95% of the α_{s1}-casein was hydrolysed, while at 6% and 8% S/M, the amount of α_{s1}-casein that was hydrolysed was 70% and 40%, respectively.

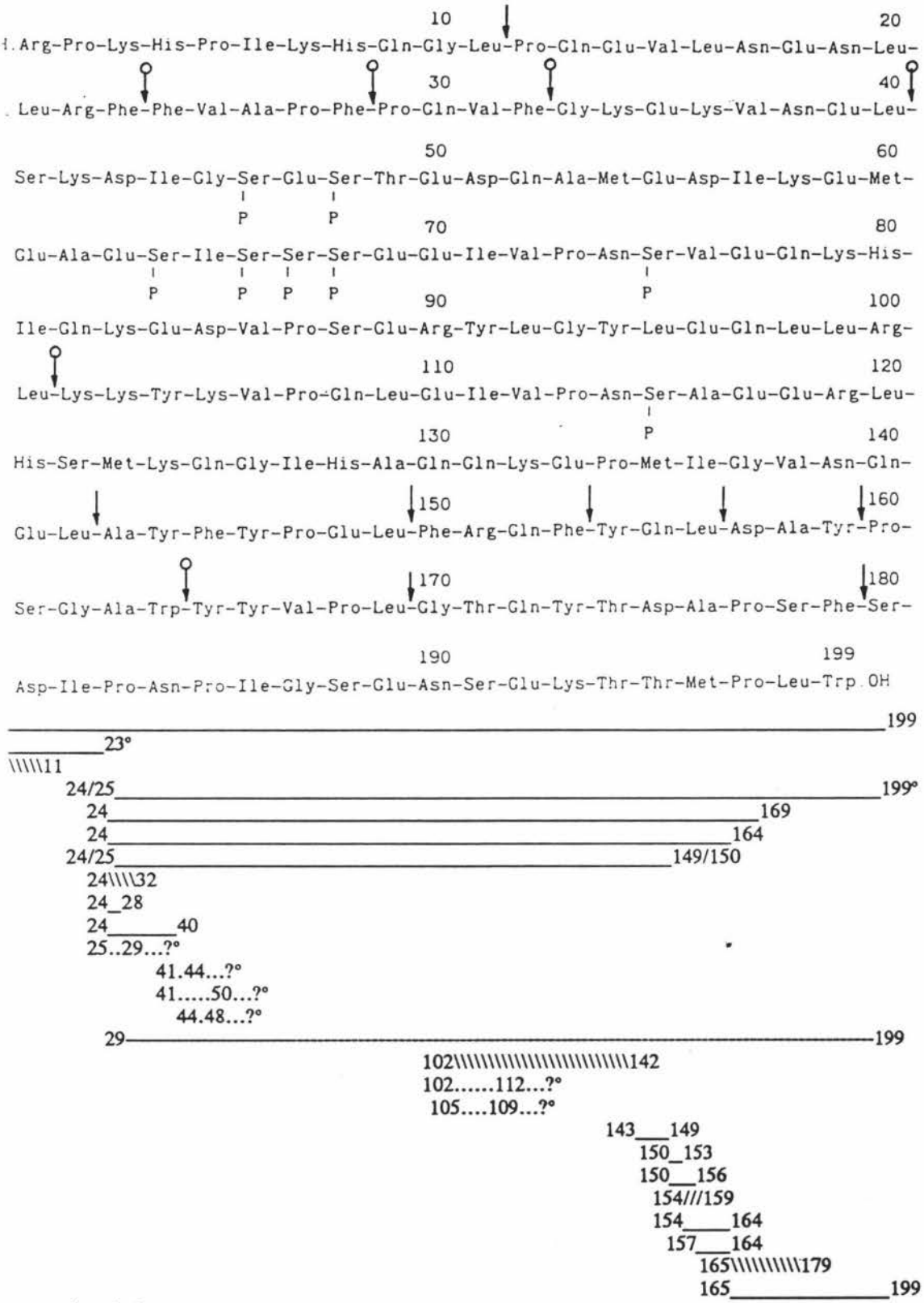
McSweeney *et al.* (1993a), investigated the proteolytic specificity of chymosin on bovine α_{s1}-casein in 100 mM potassium phosphate buffer at 30°C, pH 6.5 and at pH 5.2 in the presence of 5% (*w/v*) NaCl. The peptides were isolated by RP-HPLC and identified by amino acid sequence which was confirmed by mass spectrometry and/or amino acid composition. The following pH 4.6 soluble peptides were formed at pH 6.5; Arg_1 - Phe_{23}, Phe_{24} - Phe_{28}, Phe_{24} - Leu_{40} (?), Phe_{150} - Phe_{153}, Phe_{150} - Leu_{156}, Tyr_{154} - Tyr_{159}, Tyr_{154} - Trp_{164}, Asp_{157} - Trp_{164} and Tyr_{165} - Trp_{199}. The same peptides, except Tyr_{154} - Trp_{164}, were produced at pH 5.2 in the presence of 5% NaCl and, in addition, the peptides Arg_1 - Leu_{11}, Phe_{24} - Phe_{32}, Lys_{102} - Leu_{142}, Ala_{143} - Leu_{149}, and Tyr_{165} - Phe_{179}. Under each set of conditions the first peptides to be generated were Arg_1- Phe_{23} and Tyr_{165} - Trp_{199}. It is unclear whether it is the difference in pH or level of NaCl that is responsible for the differences observed. The identity of these peptides is in

agreement with the cleavage sites identified by Pelissier *et al.* (1974), with the exception of Arg_1 - Leu_{11}. Pelissier *et al.* identified several cleavage sites that were not identified in this study. A study by Pahkala *et al.* (1989a) investigated the action of chymosin on a mixture of α_{s1}- and α_{s2}-casein and tentatively identified, by amino acid analysis, several cleavage sites for α_{s1}-casein. Six of the sites were later confirmed by McSweeney *et al.* (1993a).

Singh *et al.* (personal communication, 1993) have isolated and identified water soluble peptides, produced by the action of chymosin on α_{s1}-casein, from a mature Cheddar cheese. The following cleavage sites were identified; Phe_{23} - Phe_{24}, Phe_{28} - Pro_{29}, Phe_{32} -Gly_{33}, Leu_{40} - Ser_{41}, Leu_{101} - Lys_{102} and Trp_{164} - Tyr_{165}. All of these sites had previously been identified *in vitro* (Pelissier *et al.*, 1974; Pahkala *et al.*, 1989a; McSweeney *et al.*, 1993a). Several cleavage sites identified by these authors were not identified in cheese. However, the results of Singh (1993) are preliminary and several peptides remain to be identified. The peptides that were tentatively identified were: Val_{25} - Pro_{29}..?, Ser_{41} - Ile_{44}..?, Ser_{41} - Ser_{48}..?, Ser_{41} - Glu_{50}..?, Lys_{102} - Val_{112}..? and Lys_{105} - $Leu_{109..?}$ Five peptides originating from the Arg_1 - Phe_{23} fragment of α_{s1}-casein were also isolated and identified. The cleavage sites were identified as consistent with the activity of a PI-cell wall-associated proteinase (Singh *et al.*, 1994) (Section 2.3).

Pannell (1992) examined the water soluble extracts of two commercial Cheddar cheeses and identified 13 of the 31 peptides isolated as originating from the Arg_1 - Phe_{23} or Gln_{78} - Leu_{92} fragments of α_{s1}-casein. (Hydrolysis of the Arg_1 - Phe_{23} fragment of α_{s1}-casein by cell free extracts of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* at pH 5.2 with 4.6% NaCl and at pH 6.5 indicated that some of the cleavage sites were consistent with the activity of X-prolyl dipeptidyl aminopeptidase (Section 2.3)).

The cleavage sites and peptides of α_{s1}-casein that have been identified are summarised in Figure 1.2.



in solution
in solution at pH 5.2 only in the presence of 5% NaCl
in solution at pH 4.0 to 5.2 only in the absence of salt
in solution at pH 6.5 only
cleavage sites and peptides identified from cheese
? denotes incomplete sequence

FIGURE 1.2 Chymosin action on α_{s1}-casein.

2.1.5.2 Chymosin action on β-casein

Chymosin activity on β-casein is closely related to both pH and NaCl concentration (Mulvihill and Fox, 1978; Lawrence *et al.*, 1987) and their effect on the conformation of the protein. The extent of salt induced aggregation of β-casein, and the consequent inaccessibility of the chymosin-sensitive bonds, increases as the salt in the moisture phase (S/M) increases and the water activity decreases (Creamer, 1976a).

In solution, β-casein (209 amino acid residues) has been shown to be hydrolysed by chymosin to β-I-casein (Arg_1 - Ala_{189}/Leu_{192}), β-II-casein (Arg_1 - Leu_{165}/Gln_{167}) and β-IIIa-casein (Arg_1 - Leu_{139}) (Pelissier *et al.*, 1974; Creamer, 1976a). Visser and Slangen (1977) identified the following cleavage sites of β-casein in 0.05 M sodium acetate buffer at pH 5.4; Leu_{127} - Thr_{128}, Leu_{139} - Leu_{140}, Leu_{163} - Ser_{164}, Leu_{165} - Ser_{166}, Gln_{167} - Ser_{168}, Ala_{189} - Phe_{190} and Leu_{192} - Tyr_{193}. Three of these cleavage sites were identified by Pelissier *et al.* (1974). Pahkala *et al.* (1989b) verified four cleavage sites and identified the following additional sites; Lys_{176} - Ala_{177}, Asp_{184} - Met_{185}, Tyr_{193} - Gln_{194}, Leu_{191} - Leu_{192} and Ile_{207} - Ile_{208}. Some of these sites indicate a specificity different from that previously demonstrated (S. Visser, personal communication). Guillou *et al.* (1991) compared the proteolytic action of chymosin and pepsin A in 100 mM phosphate-citrate buffer at pH 3.5 and 5.5 at 37°C, and isolated and identified the peptides produced by their action. They identified the following additional cleavage sites due to the action of chymosin; Ser_{57} - Leu_{58}, Leu_{58} - Val_{59}, Met_{93} - Gly_{94}, Glu_{121} - Ser_{122}, Leu_{140} - Gln_{141}, Ser_{142} - Trp_{143} and Trp_{143} - Met_{144}.

It is believed that while chymosin is able to hydrolyse β-casein in solution, it is not able to do so in cheese. Fox and Walley (1971) demonstrated that the action of chymosin on β-casein was inhibited by NaCl (while its action on α_{s1}-casein was shown to be optimal at 5% (*w/v*) NaCl). It is thought that the concentration of NaCl in most cheese varieties contributes to the inhibition of chymosin action on β-casein. However, even in the absence of NaCl the level of β-casein degradation is small (Phelan *et al.*, 1973). It is believed that only monomeric β-casein is susceptible to chymosin action (Creamer, 1976a) and that it is likely that temperature-dependent hydrophobic association of β-casein may make potential cleavage sites inaccessible to the active site of chymosin (Creamer, 1976a; Fox *et al.*, 1993). Microbial rennets degrade α_{s1}- and β-casein in cheese at approximately the same rate (Creamer *et al.*, 1988a,b; Phelan, 1985) and are less sensitive to NaCl (Phelan, 1985). An examination after 7 days, of Cheddar cheese made at the NZDRI (Coker, 1991a) according to a standard Cheddar cheese manufacture, revealed the presence of a peptide (at very low levels) with the same

electrophoretic mobility as β-I-casein. In the same cheese the presence of a peptide thought to be β-II-casein was noted at 3 months but no β-III-casein was observed. At 6 months approximately 40% of the β-casein had been hydrolysed by rennet and other enzymes present. Gripon *et al.* (1975) also reported the presence of β-I-casein after 20 days at 12°C in brined aseptic curd at pH 5.2.

The cleavage sites and peptides identified from the action of chymosin on β-casein are summarised in Figure 1.3.

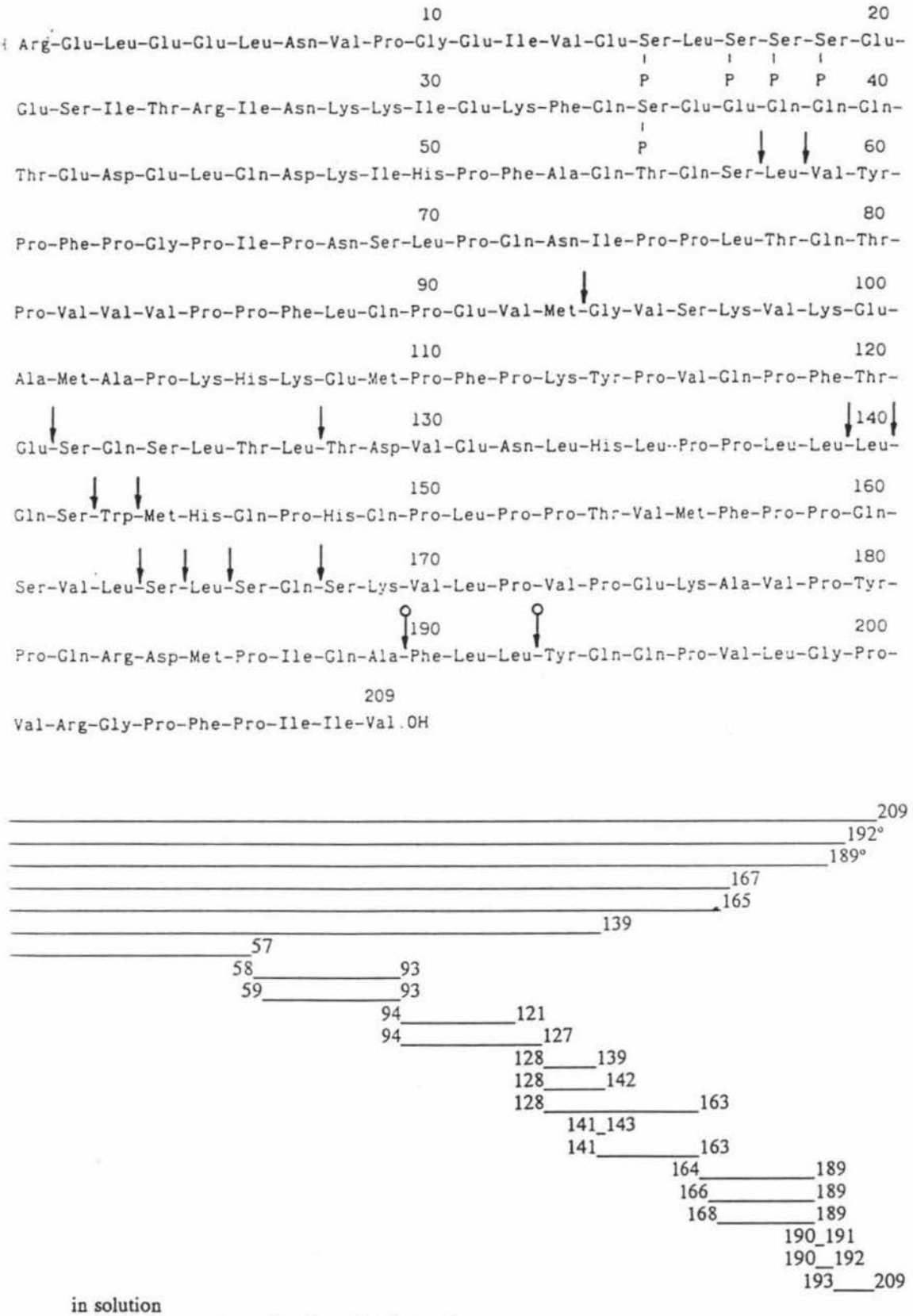


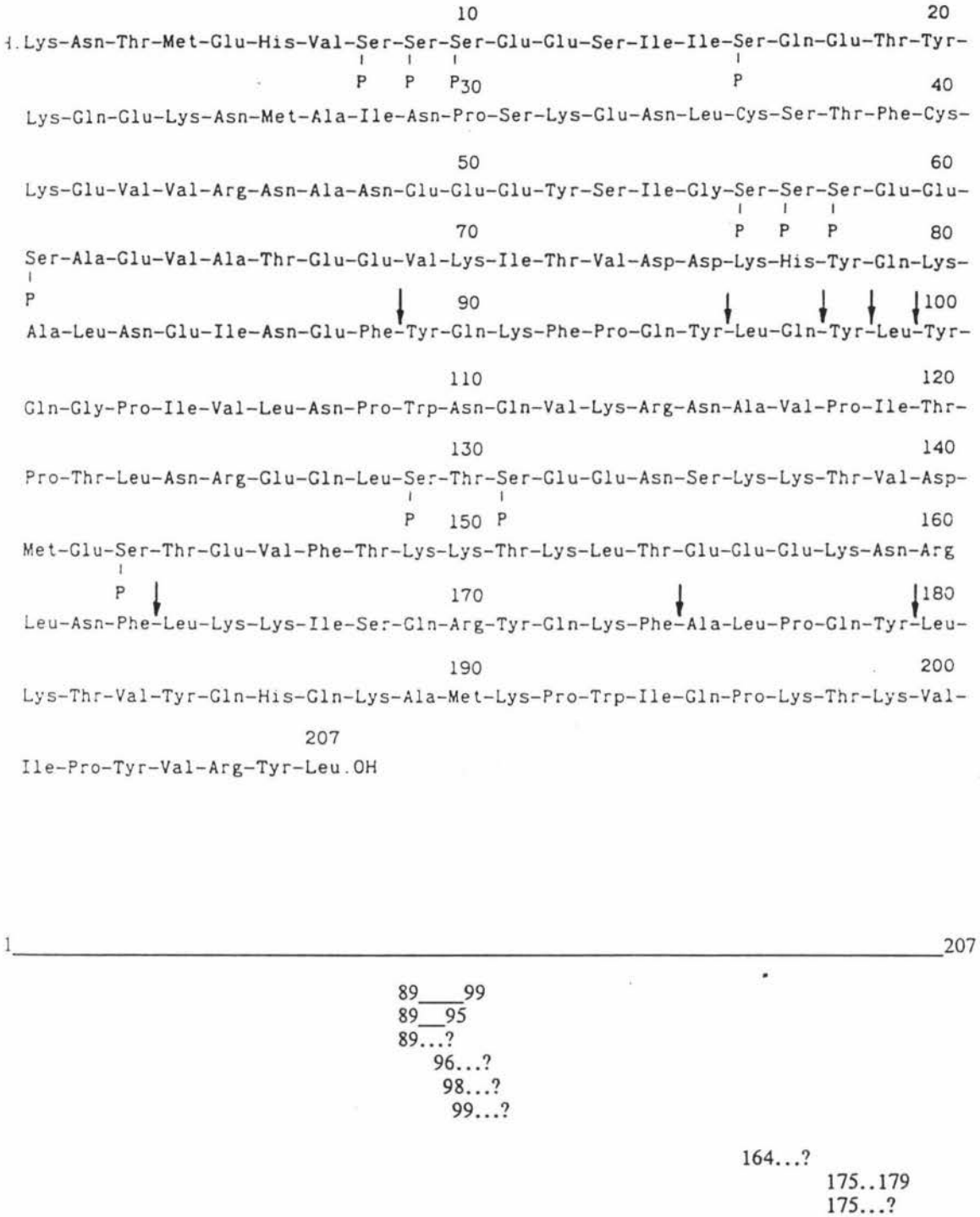
FIGURE 1.3 Chymosin action on β-casein.

2.1.5.3 Chymosin action on α_{s2}-casein

The action of chymosin on bovine α_{s2}-casein in 100 mM sodium phosphate buffer, pH 6.5, at 30°C was investigated by McSweeney (1993a). Peptides soluble in sodium acetate buffer at pH 4.6 were isolated by RP-HPLC and identified from their amino acid sequence. The peptides identified were Tyr_{89} - Tyr_{95}, Tyr_{89} - Leu_{99}, Tyr_{89} - ?, Leu_{96} - ?, Tyr_{98} - ?, Leu_{99} - ?, Leu_{164} - ?, Ala_{175} - Tyr_{179} and Ala_{175} - ?.

McSweeney (1993b) compared the specificity of chymosin and cathepsin D (the native acid milk proteinase) on α_{s1}-, α_{s2}-, β- and κ-casein in solution. They found that for α_{s1}- and κ-casein the peptide profiles and the order in which the peptides were produced was similar for both enzymes and that Cathepsin D may contribute to the formation of α_{s1}-I-casein (Noomen, 1978). The specificity of the enzymes on β-casein was similar with a number of peptides being produced by both enzymes. The specificity of the enzymes on α_{s2}-casein was substantially different. McSweeney (1993b) determined the activity of cathepsin D in the presence of various levels of salt and over a range of pH and predicted that the enzyme would be active in many young cheeses.

The cleavage sites and peptides identified from the action of chymosin on α_{s2}-casein are summarised in Figure 1.4.



_ in solution
...? denotes incomplete sequence

FIGURE 1.4 Chymosin action on α_{s2}-casein.

The remaining milk proteins, para-κ-casein, β-lactoglobulin and α-lactalbumin (present at low levels in most cheese) are thought to be more resistant to rennet action.

2.2 PLASMIN

Bovine milk contains ~60 (Fox and Stepaniak, 1993) indigenous milk proteinases most of which are present at very low levels and have little proteolytic activity (Fox and Morrissey, 1981).

The major indigenous proteolytic enzyme present in bovine milk, is a serine proteinase known as plasmin (milk alkaline proteinase). This enzyme is normally found in blood where it is responsible for solubilising fibrin clots. Plasminogen, the precursor of plasmin, is also present in bovine milk with a ratio of plasminogen to plasmin of ~8:1 (Korycka-Dahl *et al.*, 1983) and is associated with the casein fraction of milk (de Rham and Andrews, 1982; Korycka-Dahl *et al.*, 1983; Richardson, 1983a). According to Richardson (1983b) the concentrations of plasmin and plasminogen are greater in late lactation milk, presumably because of an increased permeability of the blood vessels in the mammary gland.

Freshly secreted milk contains both plasminogen activators (PAs) and plasmin inhibitors. The PAs are serine proteinases and are very specific in their action, catalysing the hydrolysis of the Arg_{557} - Ile_{558} bond in bovine plasminogen (Schaller *et al.*, 1985) to give plasmin (Lu and Nielsen, 1993). In mammals, there are two types of PAs, tissue-type (tPA) and urokinase-type (uPA) that both convert the pro-enzyme plasminogen to plasmin (Saksela, 1985). Deharveng and Nielsen (1990) detected at least four PAs in bovine milk. Two of these were isolated and purified and were believed to be of the uPA type. Indeed, most or all of the plasminogen activators in bovine milk are believed to be of the uPA-type (Lu and Nielsen, 1993). The main plasmin inhibitor is thought to be α_2-antiplasmin (α_2-AP) (Wiman and Collen, 1978). After pasteurization, plasmin activity in milk increases and one possible explanation is that this may be due to the destruction of the plasmin inhibitor (Noomen, 1975). This is thought to be unlikely as the heat stabilities of plasmin, plasminogen and the trypsin inhibitor are very similar (Richardson, 1983a). As there is also an increase in the activation of plasminogen after pasteurization it is possible that this could be due to the destruction of an inhibitor of the plasminogen activator (Richardson, 1983a).

2.2.1 Plasmin and Proteolysis of Milk Proteins

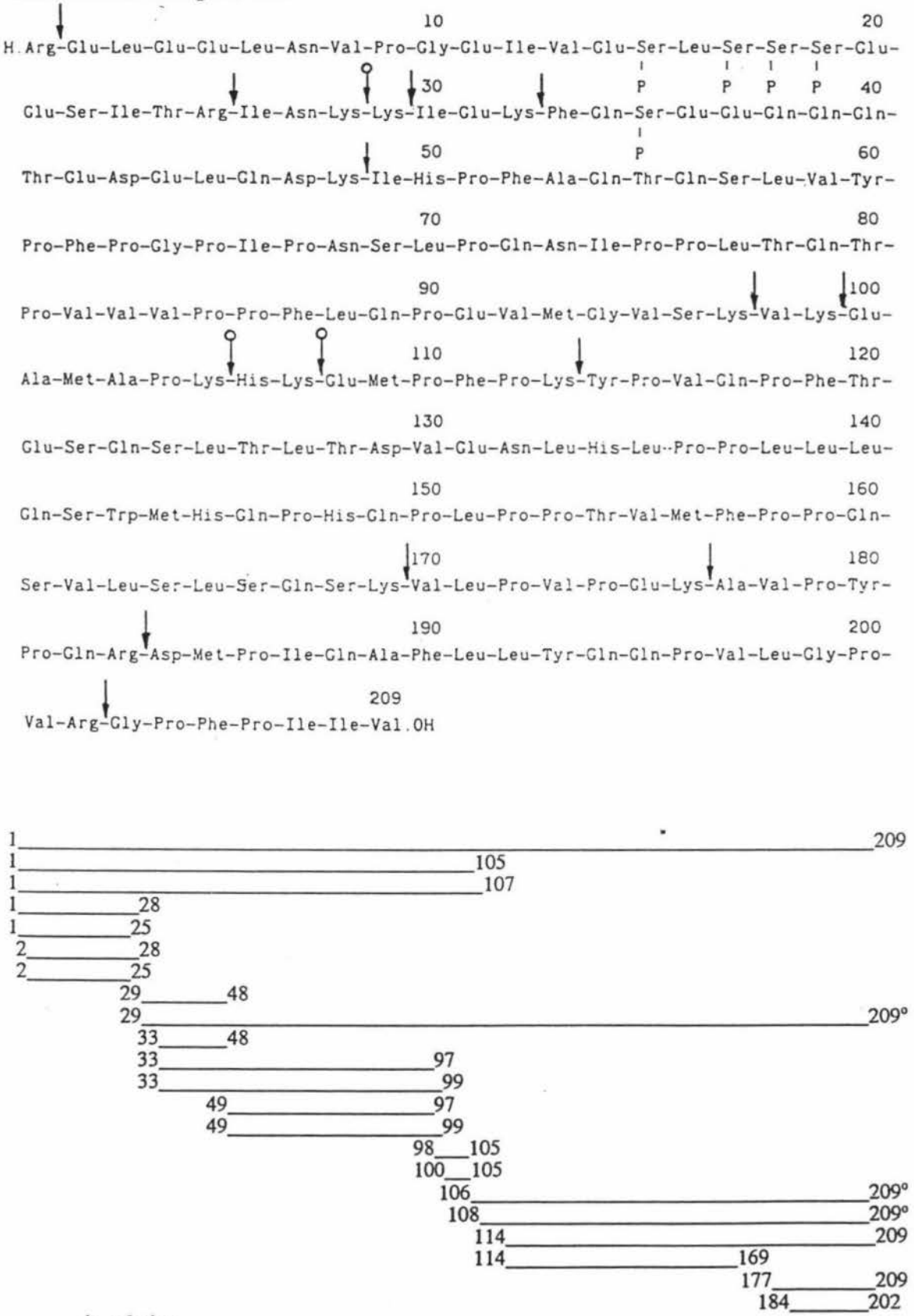
Plasmin has a specificity for Lys-X and Arg-X bonds and hydrolyses β-casein and α_{s2}-casein at approximately the same rate (Snoeren and van Riel, 1979; Richardson, 1983a), while α_{s1}-casein is hydrolysed more slowly (Chen and Ledford, 1971; Kaminogawa *et al.*, 1972; Noomen, 1975; de Rham and Andrews, 1982) and κ-casein is more resistant to its action (Chen and Ledford, 1971). It has an optimum activity at 37°C and a pH optimum of 7.5-8.0 (Chen and Ledford, 1971; Kaminogawa *et al.*, 1972). A linear relationship between β-casein hydrolysis and NaCl concentration (S/M) was demonstrated by Thomas and Pearce (1981). It is likely that this effect is due to the inhibition of plasmin (and starter enzyme) activity.

2.2.1.1 Plasmin action on β-casein in solution

Plasmin is responsible for the rapid hydrolysis of β-casein (in milk), with the production of the γ-caseins (γ_1, residues Lys_{29} -Val_{209}, γ_2, residues His_{106} - Val_{209}, γ_3, residues Glu_{108} - Val_{209} (Groves *et al.*, 1972, 1973) and other peptides. These peptides, often referred to as proteose peptones, are those peptides and proteins that are soluble at pH 4.6 and heat stable. The proteose peptones known as PP5 and PP8 have been identified as residues Arg_1 - $Lys_{105}(Lys_{107})$ and Arg_1 - Lys_{28}, respectively and are generated from the N-terminal of β-casein by the action of plasmin (Andrews, 1978a,b). Andrews and Alichanidis (1983) reported 38 proteose peptones in milk with 25 of them thought to arise from the action of plasmin on casein. They reported that of the total proteose peptone fraction, 52% arises from β-casein, 29% from α_{s1}-casein, 9% from α_{s2}-casein and 4% from κ-casein as a result of the action of plasmin. The remaining 6% could not be attributed unequivocally to any individual caseins.

Visser *et al.* (1989a) isolated 16 peptides produced by the action of plasmin on β-casein in 0.2 M ammonium acetate buffer, pH 7.4, at 37°C. The following peptides were identified by their N-terminal sequence and amino acid analysis and together account for virtually all the potential cleavage sites of β-casein; Arg_1 - Arg_{25}, Arg_1 - Lys_{28}, Glu_2 - Arg_{25}, Glu_2 - Lys_{28}, Lys_{29} - Lys_{48}, Lys_{33} - Lys_{48}, Lys_{33} - Lys_{97}/Lys_{99}, Ile_{49} - Lys_{97}, Ile_{49} - Lys_{99}, Val_{98} - Lys_{105}, Glu_{100} - Lys_{105}, His_{106} - Val_{209}, Tyr_{114} - Lys_{169}, Tyr_{114} - Val_{209}, Ala_{177} - Val_{209}, Asp_{184} - Arg_{202}. The N-terminal half of β-casein appeared to be more sensitive to hydrolysis by plasmin than the rest of the molecule. Pahkala *et al.* (1989b) identified three cleavage sites within the β-casein molecule that had not previously been demonstrated. They were Lys_{29} - Ile_{30}, Met_{93} - Gly_{94} and Val_{59} - Tyr_{60}. The last two cleavage sites are not compatible with the predicted specificity of plasmin.

The cleavage sites and peptides arising from the action of plasmin on β-casein are summarised in Figure 1.5.



— in solution

o cleavage sites and peptides identified from cheese

FIGURE 1.5 Plasmin action on β-casein.

2.2.1.2 Plasmin action on α_{s2}-casein in solution

The hydrolysis of α_{s2}-casein (in milk) (at the same rate as β-casein) can also be attributed to plasmin action.

Visser *et al.* (1989b) isolated 15 peptides produced by the action of plasmin on α_{s2}-casein in 0.2 M NH_4HCO_3 buffer at pH 8.0, 37°C. The peptides were identified by N-terminal sequence and amino acid analysis as Lys_1 - Lys_{21}, Lys_1 - Lys_{24}, Asn_{115} - Lys_{149}, Asn_{115} - Lys_{150}, Asn_{115} - Leu_{207}, Lys_{150} - Leu_{207}, Lys_{150} - Lys_{181}, Thr_{151} - Lys_{188}, Thr_{151} -Lys_{197}, Thr_{151} - Leu_{207}, Thr_{182} - Lys_{197}, Thr_{182} - Leu_{207}, Ala_{189} - Lys_{197}, Ala_{189} - Leu_{207} and Thr_{198} - Leu_{207}.

Le Bars and Gripon (1989a,b) identified 10 peptides produced by the action of plasmin on α_{s2}-casein (in solution in 0.05 M NH_4HCO_3 buffer at pH 8.4, 37°C) by N- and C-terminal sequence and amino acid analysis; Lys_1 - Lys_{21}, Lys_1 - Lys_{24}, Asn_{115} - Lys_{149}, Asn_{115} - Lys_{150}, Lys_{150} - Leu_{207}, Thr_{151} - Leu_{207}, Thr_{182} - Lys_{188}, Thr_{182} - Leu_{207}, Ala_{189} - Leu_{207}, Thr_{198} - Leu_{207}.

Pahkala *et al.* (1989a) investigated the action of plasmin on a mixture of α_{s1}- and α_{s2}-casein. They identified several of the peptides (Lys_1 - Lys_{21}, Lys_1 - Lys_{24}, Asn_{115} - Lys_{149}, Thr_{182} - Lys_{188}, Thr_{182} - Leu_{207}, Ala_{189} - Lys_{197}, Ala_{189} - Leu_{207}, Thr_{198} - Leu_{207}) identified by Visser *et al.* (1989b) and Le Bars and Gripon (1989a,b). They isolated and identified two additional cleavage sites, at Lys_{166} - Ile_{167} and Lys_{173} - Phe_{174}, when they isolated and identified the peptide, Ile_{167} - Lys_{173}. Two more peptides were isolated, but they did not conform with the predicted specificity of plasmin.

The cleavage sites and peptides arising from the action of plasmin on α_{s2}-casein are summarised in Figure 1.6.

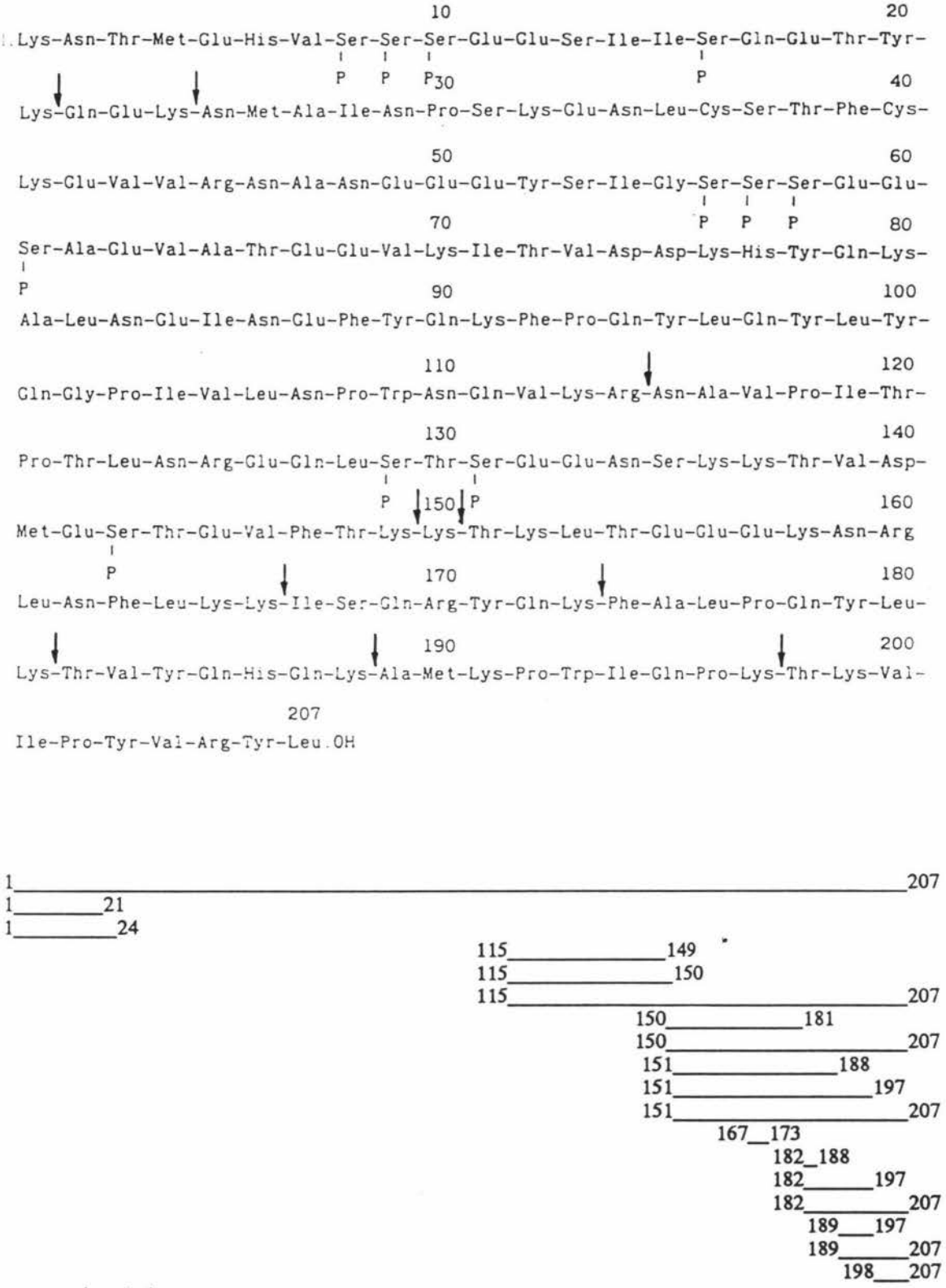


FIGURE 1.6 Plasmin action on α_{s2}-casein.

2.2.1.3 Plasmin action on α_{s1}-casein in solution

α_{s1}-Casein (in milk) is hydrolysed more slowly than β-casein by plasmin. It has been shown that plasmin cleaves α_{s1}-casein (in solution in 0.05 M NH_4HCO_3 buffer at pH 8.0, 37°C) and of the peptides generated, fifteen have been identified by their N-terminal sequence and amino acid composition as Arg_1 - Arg_{22}, Arg_1 - Lys_{34}, Glu_{35} - Arg_{90}, His_{80} - Arg_{90}, His_{80} - Lys_{103}, Tyr_{91} - Arg_{100}, Tyr_{91} - Lys_{103}, Lys_{103} - Lys_{124}, Tyr_{104} - Lys_{124}, Tyr_{104} - Trp_{199}, Val_{106} - Lys_{124}, Val_{106} - Trp_{199}, Gln_{125} - Trp_{199}, Gln_{152} - Trp_{199} and Thr_{194} - Trp_{199} (Le Bars and Gripon, 1993).

McSweeney *et al.* (1993c) studied the action of plasmin on bovine α_{s1}-casein in 50 mM ammonium bicarbonate buffer, pH 8.4, at 37°C. They identified seven of the nine cleavage sites found by Le Bars and Gripon (1993) as primary cleavage sites (Phe_{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}) and isolated seven of the fifteen peptides (Arg_1 - Arg_{22}, Glu_{35} - ?, His_{80} - Arg_{90}, Tyr_{91} - Arg_{100}, Lys_{103} - ?, Val_{106} - Lys_{124}, Thr_{194} - Trp_{199}), as well as the following additional peptides; Arg_1 - Lys_7, His_4 - Arg_{22}, Phe_{23} - Lys_{34}, Phe_{23} - Lys_{36}*, Phe_{23} - Lys_{42}?*, Phe_{23} - Ile_{44}*, Glu_{35} - Lys_{58}*, Val_{37} - Lys_{58}, His_{80} - Arg_{100}*, Leu_{101} - Lys_{105}, Lys_{103} - Lys_{105}, Tyr_{104} - Lys_{105}, Tyr_{104} - Arg_{119}*, Tyr_{104} - Lys_{124}*, Tyr_{104} - Lys_{132}?*, Val_{106} - Arg_{119}*, Gln_{125} - Lys_{132} and Gln_{125} - Arg_{151}* (where * denotes an incomplete sequence). With the exception of the cleavage site identified at Ile_{44} - Gly_{48}, the remainder of the sites are in agreement with the predicted specificity (Lys-X and Arg-X) for plasmin.

Pahkala *et al.* (1989a) investigated the action of plasmin on a mixture of α_{s1}- and α_{s2}-casein and while one of the peptides (His_{80} - Arg_{90}) was later identified by Le Bars and Gripon (1993), others (Arg_1 - Phe_{23}, Lys_{193} - Trp_{199}, Arg_{151} - Pro_{197}) did not fit the predicted cleavage sites for plasmin action on α_{s1}-casein.

The cleavage sites and peptides produced by the action of plasmin on α_{s1}-casein are summarised in Figure 1.7.

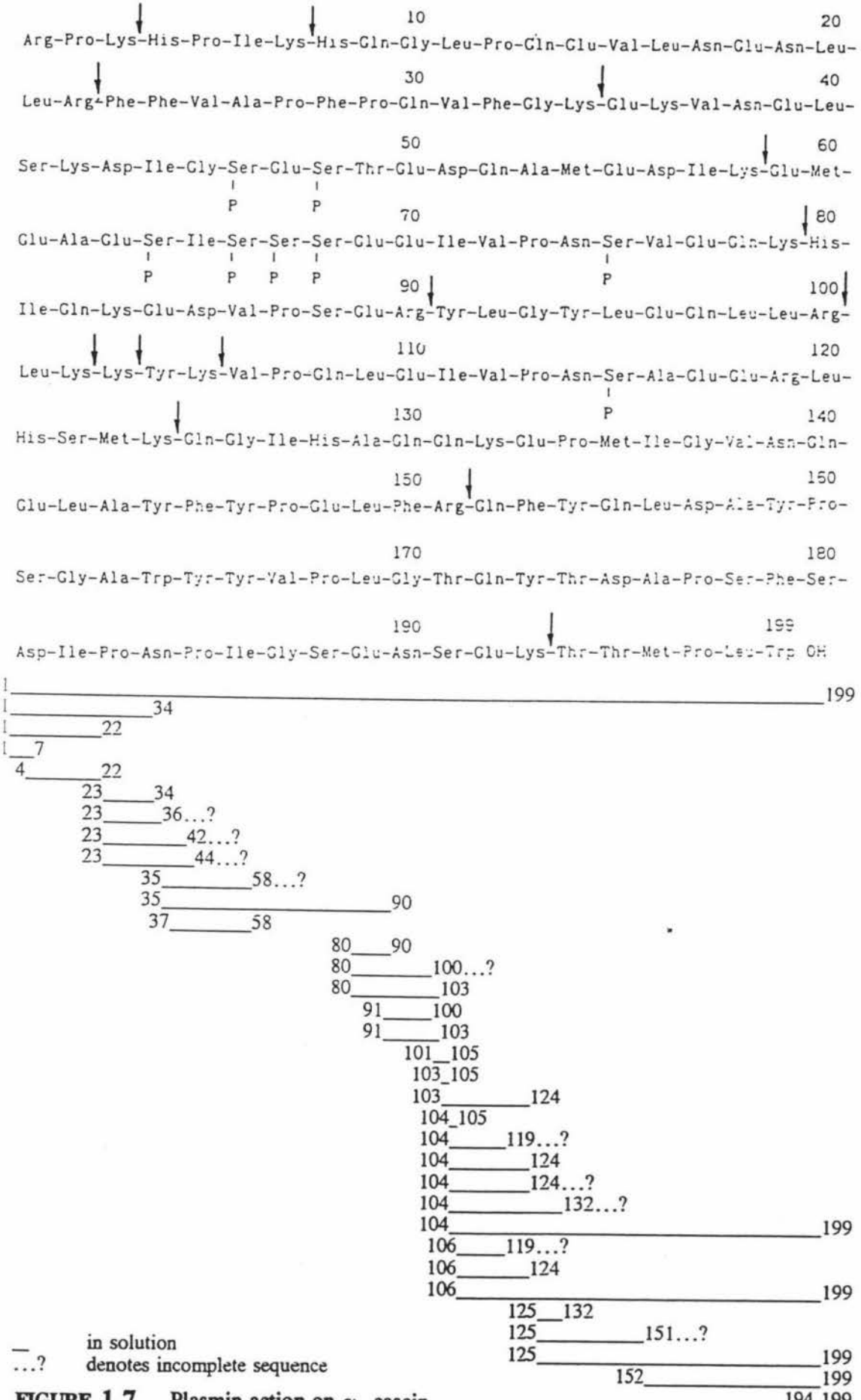


FIGURE 1.7 Plasmin action on α_{s1}-casein.

2.2.1.4 Plasmin action on γ-casein

The stability of the casein micelles in milk is related to the integrity of κ-casein. Eigel (1977) found that plasmin did not hydrolyse κ-casein in 0.05 M sodium tetraborate buffer at pH 8.4. Andrews and Alichanidis (1983) found that κ-casein was hydrolysed by plasmin in 0.05 M sodium phosphate buffer at pH 7.0 after 1 min at 37°C. Stepaniak *et al.* (1989) demonstrated that plasmin hydrolyses κ-casein in synthetic milk serum ultrafiltrate at pH 6.6, 37°C. It is believed that (in milk) the Phe_{105} - Met_{106} bond of κ-casein is resistant to plasmin and that plasmin is therefore not involved in the coagulation of milk during the manufacture of cheese.

2.2.1.5 Plasmin action and the whey proteins

Both β-lactoglobulin and α-lactalbumin are resistant to plasmin. Heat denatured β-lactoglobulin has been shown to have an inhibitory effect on plasmin (Grufferty and Fox, 1986; Alichanidis *et al.*, 1986). A possible mechanism for the inhibitory effect may be that the free sulphydryl group that becomes available when β–lactoglobulin is denatured may interact with plasmin, via thiol-disulphide interchange between the activated sulphydryl group of the denatured β-lactoglobulin and the disulphide bonds of plasmin, with a resultant loss of enzyme activity (Alichanidis *et al.*, 1986; Grufferty and Fox, 1988a). Bastian *et al.* (1993) showed that both native and denatured β-lactoglobulin inhibit the action of plasmin on casein especially when the reaction mixture was heated (60°C for 15 min). In a recent study Politis *et al.* (1993) demonstrated that β-lactoglobulin A, BSA and α-lactalbumin, at concentrations usually found in milk, had an inhibitory effect on plasmin activity while β-lactoglobulin B was a less potent inhibitor.

2.2.2 Plasmin and Proteolysis in Cheese

Plasmin plays an important role in cheese ripening, affecting the hydrolysis of β-casein, α_{s2}-casein and to a lesser extent, α_{s1}-casein. Plasmin is thought to contribute to both the formation (Farkye and Fox, 1991) and degradation of water soluble peptides in cheese (Farkye and Fox, 1992; Farkye and Landkammer, 1992). Farkye and Fox (1992) and Farkye and Landkammer (1992) showed that increasing the level of plasmin (approximately three-fold) in Cheddar cheese consistently resulted in a cheese of superior quality (to the control cheese) with no trace of the bitterness that is often attributed to plasmin activity (Le Bars and Gripon, 1989b). The cheese showed no significant differences in body but had superior flavour.

Plasmin, plasminogen and the plasminogen activator are associated with the casein

micelles (Reimerdes and Klostermeyer, 1974; de Rham and Andrews, 1982; Richardson, 1983a; Korycka-Dahl *et al.*, 1983) and are likely to be incorporated into the cheese (Grufferty and Fox, 1988c), whereas, the plasmin inhibitors and the plasmin activators are in the serum and are lost in the whey (Korycka-Dahl *et al.*, 1983). The addition of 1 M NaCl to milk results in the release of plasmin from the casein micelles and it has been suggested that the method used to salt the cheese may affect the level of plasmin incorporated in the cheese (Grufferty and Fox, 1988c). Farkye and Fox (1990) examined Cheddar and Emmental cheeses and found that the method of salting did not affect the retention and activity of plasmin. This conclusion is consistent with that (Coker, 1991b) from a similar study at the NZDRI in which the dry salting of Gouda was compared with brine salting. In the pH range 4.6-6.6, plasmin is associated with the casein micelles and it has been suggested that, within the normal pH range for draining the curd, plasmin retention in cheese is not affected by pH (Grufferty and Fox, 1988c).

The activity of plasmin in cheese is influenced by the heat treatment of either the cheese milk or the cheese curd. A range of cooking temperatures is used in the manufacture of the different varieties of cheese. It has been found that the activity of plasmin increases with increased cooking temperature within the range of 31-52°C in Cheddar cheese and 30-60°C in rennet curd (Farkye and Fox, 1990). In the same study it was shown that the level of plasmin activity (7-amido-4-methyl coumarin (AMC) units / g cheese) in Emmental cheese (~52°C) was ~2.5 times higher than that in Cheddar cheese (~38°C) (Richardson and Pearce, 1981; Farkye and Fox, 1990) and that plasmin activity (AMC units / g cheese) in Cheshire cheese (~33°C) was lower than in either Emmental or Cheddar cheese (Lawrence *et al.*, 1983; Farkye and Fox, 1990). The values obtained for Gouda cheese were intermediate between Emmental and Cheddar cheese.

The contribution made by plasmin in cheese is dependent on both the pH and NaCl concentration (Noomen, 1975) which affect both the level of plasmin (and moisture) incorporated in the cheese and the activity of the enzyme. The ripening temperatures also influence the activity of plasmin in cheese, with elevated temperatures resulting in an increased rate of proteolysis.

In Cheddar cheese, with a pH of 4.95, proteolysis attributable to plasmin is slow. Thomas and Pearce (1981) in their experiment with Cheddar cheese with a NaCl gradient, found that 50% of the β-casein was hydrolysed when the S/M was 4%, but

only 5% of the β-casein was hydrolysed when the S/M was 8%. The hydrolysis of β-casein was not attributed to either rennet or plasmin. Richardson and Pearce (1981) found a close relationship between the amount of β-casein hydrolysed and the amount of plasmin in Cheddar cheese. Noomen (1978) found that in simulated soft cheese the amount of plasmin activity was dependent on both pH and NaCl concentration. In cheese containing 4% S/M, the amount of β-casein degradation increased when the pH was raised from 4.9 to 6.2. The degradation of β-casein was five to six times greater at pH 6.3 than at pH 5.4 after 28 days storage at 13°C, with the maximum degradation (60% of the β-casein) occurring in 2% S/M. Although the addition of 2-4% S/M was found to increase plasmin activity, at higher concentrations of NaCl the activity of plasmin was reduced. At high salt concentration, providing the pH is sufficiently high (*e.g.* 6.3) plasmin is still active.

In Gouda cheese, with a higher pH (~5.38) and a higher moisture content than Cheddar cheese, hydrolysis of β-casein to form γ-caseins is more rapid (Creamer, 1975). Although the initial cleavage of α_{s1}-casein to α_{s1}-I-casein is primarily due to the action of rennet, Visser and de Groot-Mostert (1977) reported that analysis by PAGE of a rennet-free and starter-free Gouda at 6 months showed a peptide with similar electrophoretic mobility to α_{s1}-I-casein. This peptide may be produced by an indigenous acid proteinase that has chymosin-like specificity (Kaminogawa and Yamauchi, 1972; Kaminogawa *et al.*, 1980). It is also possible that the peptide is the same as that formed as a result of the cleavage of α_{s1}-casein by plasmin (in 0.05 M NH_4HCO_3 buffer at pH 8.0, 37°C) at Arg_{22} - Phe_{23}, one of the sites identified by Le Bars and Gripon (1993).

Plasmin plays an important role in the ripening of Camembert-type cheeses where there is a higher pH near the surface (Trieu-Cuot and Gripon, 1982) and a higher moisture content. This is discussed in greater detail in Section 4.5.

The level of plasmin activity in Swiss-type cheeses is two to three times greater than in Cheddar cheese (Richardson and Pearce, 1981). A high level of active plasmin is incorporated in the cheese (as previously discussed) and its activity is enhanced by the higher pH and lower salt and moisture contents of the cheese.

Mulvihill and McCarthy (1993) examined the role of plasmin in the ripening of cheese analogues made with rennet casein. They found that the β-casein and pH 4.6-soluble N contents of the cheese that had been ripened at 4°C for 6 months were significantly

correlated both with each other and with plasmin.

Recent reviews of this area have been written by Grappin *et al.* (1985), Grufferty and Fox (1988a), Fox (1989), Fox (1991), Fox *et al.* (1993), Fox and Stepaniak (1993) and Visser (1993).

2.3 ENZYMES OF STARTER AND NON-STARTER ORGANISMS

The enzymes of starter and non-starter organisms contribute to the formation of peptides in Cheddar cheese. They are also involved in the breakdown of peptides produced by the action of the coagulant enzymes, indigenous milk proteinases such as plasmin and starter and non-starter proteinases, to smaller peptides and amino acids. These small peptides and amino acids may contribute directly to the flavour of cheese or they may be catabolised to form flavour components in cheese.

Starter bacteria can be divided into two categories on the basis of their ability to coagulate milk at 22°C in 24 hours. Those strains of micro-organisms able to coagulate milk in 24 hours are designated Prt^+ (Pearce *et al.*, 1974). The remainder lack the plasmid that encodes the proteinase and are designated Prt^-. The lack of proteinase in the Prt- strains means that the growth rate is limited by the amount of small peptides and free amino acids present in the growth medium (Mills and Thomas, 1978; Thomas and Mills, 1981; Oberg *et al.*, 1990). Prt^- starters undergo autolysis and, when heat shocked, can be used in cheese as enzyme carriers (Exterkate and Stadhouders, 1971; Pettersson and Sjostrom, 1975; El Abboudi *et al.*, 1991). Heat shock and freeze shock treatments aim to destroy the ability of the cell to utilise lactose but leave the proteolytic enzyme system intact. This allows the incorporation of more enzyme into the cheese to enhance ripening without influencing the pH development during cheese manufacture.

.3.1 Enzymes of Starter Organisms

The principle role of the lactic acid bacteria used as starters in cheese is to convert lactose to lactic acid. Two types of starter are used. Mesophilic starters *e.g.* *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Leuconostoc* species, with an optimum temperature of ~30°C, are used in most common varieties of cheese such as Cheddar, Gouda, Edam, Blue vein and Camembert. Thermophilic starters *e.g.* *Lactobacillus delbruekii* subsp. *bulgaricus*, *Lactobacillus helveticus* and

Streptococcus salivarius subsp. *thermophilus*, with an optimum temperature of ~45°C, are used in the manufacture of Swiss and Italian cheeses which are cooked to much higher temperatures (50-55°C).

These organisms have an absolute requirement for certain essential amino acids in order to sustain growth. To provide these essential amino acids this group of organisms contains a range of proteinases and peptidases capable of hydrolysing caseins to peptides and amino acids. These enzymes are believed to contribute to cheese ripening and facilitate the conversion of the caseins to peptides and amino acids. The conversion of peptides to amino acids by peptidases is considered to be the rate-limiting step (Law *et al.*, 1993). There is considerable variation in the type and quantity of proteinase and peptidase possessed by different starter organisms. The enzymes can be extracellular and bound to either the cell wall or the cell membrane, or intracellular and found within the cell cytoplasm. Not all of the enzymes are possessed by each strain of lactic acid bacteria. Many of the proteolytic enzymes in the starter and non-starter bacteria are also present in mammalian systems where they have been widely studied and a classification scheme has been published (McDonald and Barrett, 1986). Figure 1.8 outlines the broad classes of proteinases and peptidases and divides them into two groups based on their mode of action; the exopeptidases which cleave at the N- or C-terminal of the peptide and the endopeptidases that cleave at other sites on the peptide chain depending on the particular specificity of the peptidase. Not all of these classes of enzymes will be present in starter and non-starter bacteria. Some of the enzymes possessed by these organisms and their specificities are shown in Figures 1.9 and 1.10.

The proteolytic enzyme system of the lactobacilli catalyses the hydrolysis α_{s1}-casein, β-casein, α_{s2}-casein and κ-casein, as well as the whey proteins, β-lactoglobulin and α-lactalbumin (Khalid and Marth, 1990).

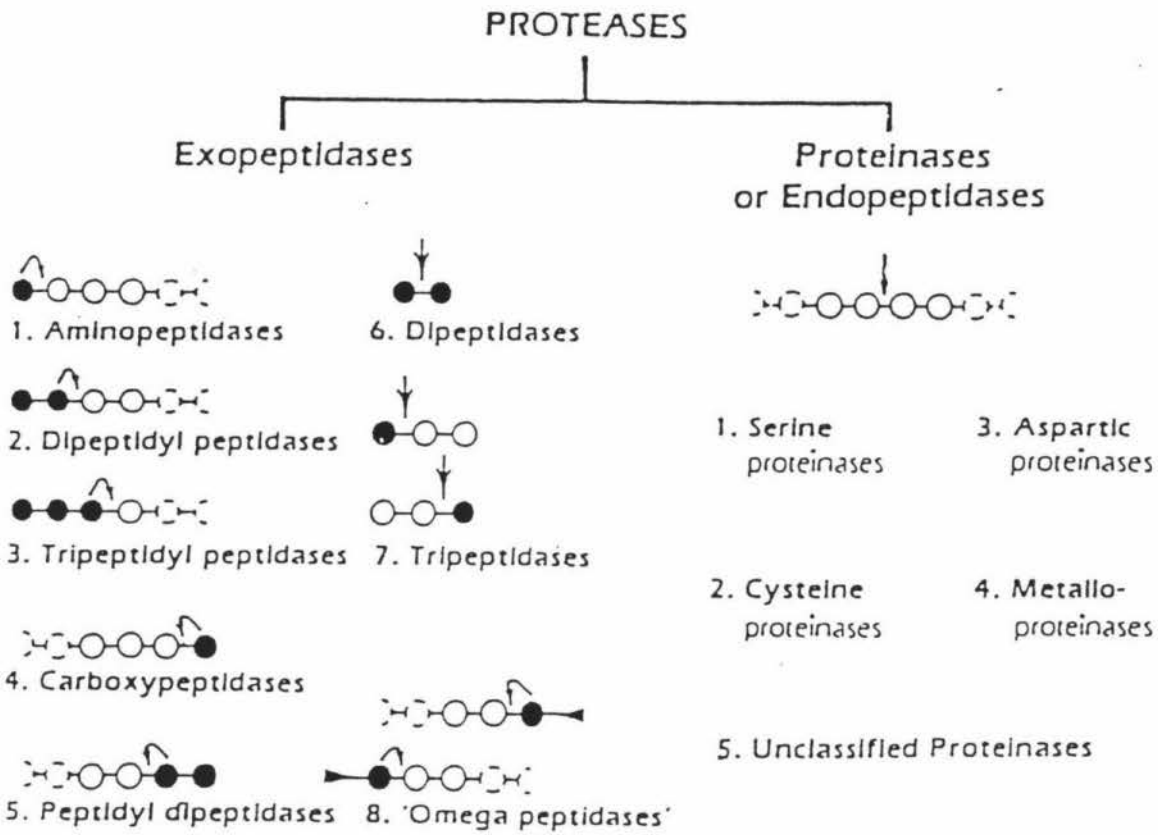


FIGURE 1.8 Classes of proteinases (from McDonald and Barrett, 1986).

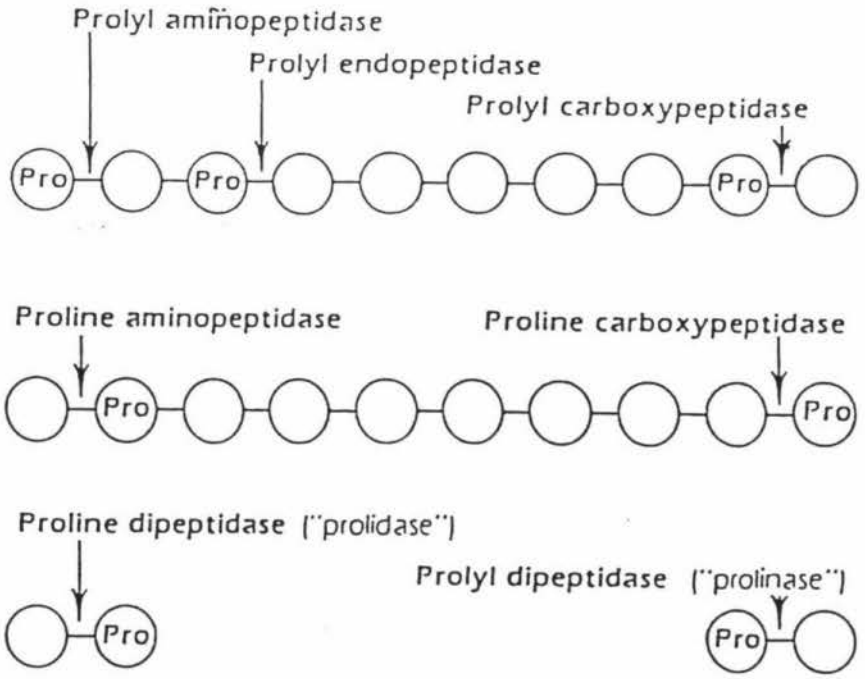


FIGURE 1.9 Designation of peptidases (from McDonald and Barrett, 1986).

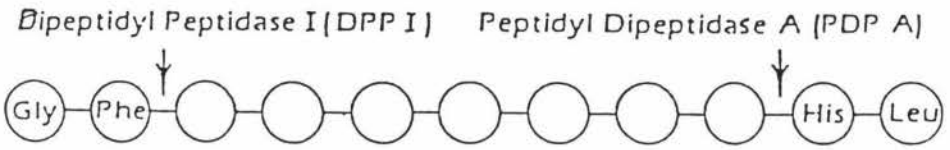


FIGURE 1.10 Action of dipeptidyl peptidase and peptidyl peptidase (from McDonald and Barrett, 1986).

The location of these enzymes within the bacterial cell is shown in Figure 1.11.

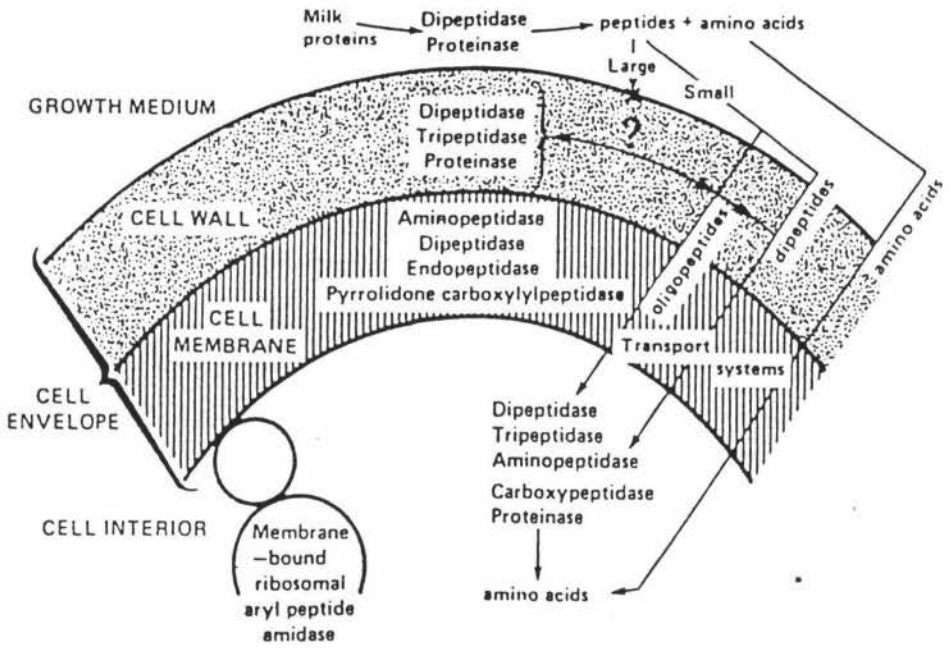


FIGURE 1.11 The interrelationship between proteolytic systems and amino acid transport systems in lactic acid bacteria (from Davies and Law, 1977).

It is a generally held view that the death of these cells is accompanied by lysis with the release of enzymes into the cheese matrix, although the nature of the cheese matrix means that physical constraints are imposed on the movement of the enzymes.

2.3.1.1 The Proteinases and Peptidases of *Lactococcus lactis* species

The proteinases and peptidases of this group of organisms are involved in the ripening of several common varieties of cheese (as previously discussed) and are therefore worthy of further consideration.

2.3.1.1.1 The Proteinases

The proteinases of these species are mainly associated with the cell wall and can be released by extraction in Ca^{2+}-free buffer or in the presence of metal-chelating agents such as EDTA. It has been proposed that the enzyme is anchored in the cell wall and that its conformation is stabilised by Ca^{2+}. The loss of Ca^{2+} is thought to alter the conformation of the molecule and expose a specific cleavage site which can then undergo autoproteolysis. This enables the release of the active proteinase into the medium (Laan and Konings, 1989). Alternatively, it has been proposed that Ca^{2+} mediated interactions link a heterodimeric proteinase complex to an anchor protein located within the cell wall and that the complex is able to dissociate from the anchor protein and cell wall when the Ca^{2+} is removed (Exterkate and de Veer, 1987, 1989).

Three types of proteinase activity have been found to exist and have been given the designations P_I-, P_{II}- and P_{III}-type proteinases (Visser *et al.*, 1986). The P_I-type (also called HP-type) proteinase acts preferentially on β-casein and produces peptides with similar electrophoretic mobilities to those produced by chymosin. The proteinase has also been shown to break down κ-casein and to a lesser extent α_{s1}-casein (Visser, 1993). The P_{II}-type proteinase is a heat labile form of the P_I-type proteinase. The P_{III}-type (or AM_1-type) proteinase acts preferentially on β-casein (but with different specificity to the P_I-type enzyme) and produces peptides with a similar electrophoretic mobility to the γ-caseins produced by the action of plasmin on β-casein (Visser *et al.*, 1986). The proteinase also breaks down κ-casein and α_{s1}-casein. Some strains of *L. lactis* contain either the P_I-type proteinase or the P_{III}-type proteinase, but others contain different levels of mixed P_I-type and P_{III}-type proteinase specificity. Neither of these proteinases produce α_{s1}-I-casein (Visser, 1993) but both are able to break down the Arg_1 - Phe_{23} peptide (Exterkate *et al.*, 1991) produced principally as a result of chymosin action on α_{s1}-casein.

A recent study by McSweeney *et al.* (1993d) investigated the activity of the cell wall-associated proteinases of *L. lactis* subsp. *cremoris* HP and *L. lactis* subsp. *lactis* JL 521 on β-casein *in vitro* and in Cheddar cheese. Peptides produced *in vitro* were not isolated from 6 month old cheese and it was suggested that the cell wall-associated

proteinases are not significant in the primary proteolysis of β-casein. This is thought to be due to the hydrophobic interaction of β-casein in cheese which makes the susceptible bonds inaccessible to the enzyme. The inability of rennet to hydrolyse β-casein in cheese was also attributed to the hydrophobic interaction of β-casein (Section 2.1.5.2).

2.1.1.1.2 The Peptidases

Most of the peptidases of *L. lactis* strains are metalloenzymes that can be inactivated by metal chelating agents such as EDTA. The peptidases can be either extracellular or intracellular and depending on their mode of action can be further divided into endopeptidases and exopeptidases. Several endopeptidases have been identified (Yan *et al.*, 1987a,b; Tan *et al.*, 1991; Baankreis, 1993). They have all been shown to exhibit size-limited substrate specificity with none being able to break down intact caseins. Of the exopeptidases, none has been shown to exhibit carboxypeptidase activity (Hickey *et al.*, 1983; Visser, 1993). The peptidases isolated include prolyl aminopeptidase (formerly known as proline iminopeptidase), prolyl dipeptidase (known as proline iminodipeptidase or prolinase), proline aminopeptidase (formerly known as aminopeptidase P), proline dipeptidase (also known as imidodipeptidase or prolidase) and X-prolyl dipeptidyl aminopeptidase (XPDAP (a dipeptidyl peptidase)) (Exterkate and Stadhouders, 1971; Mou *et al.*, 1975; Exterkate, 1977; Schmidt *et al.*, 1977; Law, 1979b; Kaminogawa *et al.*, 1984; Kolstad and Law, 1985; Thomas and Pritchard, 1987; Kok, 1990; Booth *et al.*, 1990). Caseins are rich in proline and bonds containing proline are resistant to most proteinases and peptidases. Those exopeptidases showing specificity for these bonds are therefore important for the degradation of peptides in cheese. Other exopeptidases isolated include aminopeptidase A and pyroglutamyl aminopeptidase which cleave the N-terminal glutamic acid or aspartic acid and the N-terminal pyroglutamic acid (formed after cyclization of the N-terminal glutamic acid) respectively (Baankreis, 1993), as well as alanyl, leucyl and lysyl aminopeptidases (Exterkate, 1984). Figures 1.9 and 1.10 demonstrate the cleavage sites of some of these peptidases.

2.3.2 Enzymes of Secondary Microflora

There are several varieties of cheese which owe their characteristic flavour and texture to the presence of secondary microflora.

Propionibacteria (*Propionibacterium shermanii*), added with the starter culture in Emmental and Gruyère cheese, grow only after the cheese has been manufactured.

They are thought to contribute to proteolysis by the release of peptidases which release proline in particular (Langsrud *et al.*, 1977, 1978).

Cheeses such as Danbo and Limburg are colonized at their surface by *Brevibacterium linens* and related coryneforms which contribute to their characteristic flavour. These organisms possess a range of proteinases (Friedman *et al.*, 1953) and peptidases (Sorhaug 1981). The major proteinase is a serine proteinase which is active at the surface of smear-ripened cheeses. *B. linens* possesses both intracellular and extracellular peptidases which hydrolyse a range of dipeptide substrates (Law, 1987). The catabolism of amino acids by *B. linens* also contributes to the flavour of these cheeses.

In Camembert and Brie cheeses the surface mould *Penicillium candidum* or *Pe. camemberti* is known to be highly proteolytic. The enzymes produced do not penetrate far into the cheese matrix and their action will be discussed further in Section 4.5.

Pe. roqueforti in blue cheeses produces a similar array of enzymes which dominate proteolysis in these cheeses. The action of these enzymes is discussed in Section 4.6.

2.3.3 Non-starter Lactic Acid Bacteria in Cheese

The majority of non-starter lactic acid bacteria (NSLAB) in cheese are lactobacilli although pediococci and micrococci are also present and can contribute, to a lesser extent, to cheese ripening (Fryer, 1969; Reiter and Sharpe, 1971; Chapman and Sharpe, 1981). The predominant species are *Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus brevis*. The numbers of these adventitious bacteria in cheese are affected by the numbers in raw milk (Peterson and Marshall, 1990) and the extent of post-pasteurization contamination (Thomas, 1986). Typically their numbers are in the range of 10-10^4/g in 10 day old Cheddar cheese, 10^6-10^7/g in 20 day old cheese and plateau at 10^6 to 10^8/g between two and nine months of age (Naylor and Sharpe, 1958a,b). Because of their ability to grow under the highly selective conditions of the ripening Cheddar cheese, lactobacilli are the dominant flora beyond three months of age. The precise role of lactobacilli in cheese has not yet been determined although they are thought to contribute to flavour development. These organisms have very specific amino acid requirements for their growth and possess a range of extracellular and intracellular proteinases and peptidases to provide them. Peptidases produced by *L. casei* possess dipeptidase, tripeptidase, carboxypeptidase, aminopeptidase (leucine, valine and cysteine aminopeptidases) (Requena *et al.*, 1991) and endopeptidase activities

(El Soda *et al.*, 1978). *L. plantarum* has been shown to possess intracellular aminopeptidase and dipeptidase activities and to lack endopeptidase and carboxypeptidase activities (El Soda *et al.*, 1983). Figure 1.11 shows the probable location of these enzymes. Figure 1.8 outlines the different classes and Figures 1.9 and 1.10 shows the cleavage modes of this group of enzymes.

Research into the role and possible exploitation of these organisms, either to improve flavour or decrease bitterness, in accelerated cheese ripening is ongoing. Several authors (*e.g.*, Tittsler *et al.*, 1948; Yates *et al.*, 1955; Bullock and Irvine, 1956) have described *L. casei* as a starter adjunct in Cheddar cheese. Most of the strains used have been reported to increase the rate and extent of proteolysis. Generally the cheeses have been shown to have better flavour. However some of them had a butterlike flavour and many had an acid flavour in the later stages of ripening. The butterlike flavour is due to the production of appreciable quantities of diacetyl from the fermentation of citrate by *L. casei* (Khalid and Marth, 1990). The use of *L. plantarum* in place of *L. casei* has been shown to improve cheese flavour without the acid flavour (Tittsler *et al.*, 1947, 1948). The use of *L. brevis* as a starter adjunct resulted in a cheese with objectionable off-flavours (Tittsler *et al.*, 1947). Peterson and Marshall (1990) found that two out of the four strains of *L.casei* they used as starter adjuncts with 'bitter' strains of streptococci in Cheddar cheese significantly reduced bitterness.

Microbially induced defects or spoilage, other than those previously discussed, can occur as a result of NSLAB activity in cheese. The formation of calcium lactate crystals resulting from lactate racemization is caused by both NSLAB and pediococci (Pearce *et al.*, 1973; Thomas and Crow, 1983).

The formation of toxic amines from amino acids by NSLAB has been implicated in outbreaks of food poisoning. Mature Swiss cheese may contain as much as 370 mg histidine/100 g as the result of proteolysis (Hintz *et al.*, 1956). *Lactobacillus buchneri* has the ability to generate histamine by decarboxylation of histidine and has been implicated in an outbreak of histamine poisoning following the consumption of Swiss cheese (Sumner *et al.*, 1985). Histamine production has also been reported for *L. brevis*, however, none of the *Lactobacillus* starter cultures have been found to produce histamine or histidine decarboxylase (Rice and Koehler, 1976; Voigt and Eitenmiller, 1977). *L. brevis* in Gouda has been shown to produce tyramine in cheese which has undergone extensive proteolysis (Joosten, 1987; Joosten and Northolt, 1987). Tyramine, formed by decarboxylation of tyrosine, may be present at high levels in very mature cheese and is thought to cause migraine.

2.3.4 Psychrotrophic Bacteria in Cheese

"The storage and handling of milk at refrigeration temperature is selective for psychrotrophic bacteria which have considerable spoilage potential" (Law, 1979a). Although most psychrotrophs are heat-sensitive Gram-negative rods, some species produce heat-resistant extracellular enzymes capable of surviving severe heat treatments and degrading important milk constituents. Heat resistant Gram-positive species have been isolated (Shehata and Collins, 1971; Bhadsavle *et al.*, 1972; Washam *et al.*, 1977). These organisms can cause spoilage in milk products. *Flavobacterium* and *Pseudomonas* species have been reported to degrade α_{s1}-caseins. *Ps. fluorescens* has been shown to produce an α_{s1}-I-casein-like material (Law *et al.*, 1977). Richardson and Te Whaiti (1978) examined the specificity of heat-stable extracellular proteases in refrigerated raw milk and found that they all hydrolysed casein by similar pathways, with the initial hydrolysis of κ-casein and β-casein followed by extensive casein degradation.

Psychrotrophic organisms, present in refrigerated raw milk in high numbers ($\sim 10^6$ cfu/ml), have been shown to cause a loss in soft cheese yield due to the action of their enzymes on casein (Feuillat *et al.*, 1976). Law *et al.* (1979) found that only two out of six strains of psychrotrophs growing in refrigerated raw milk to levels of 10^7 cfu/ml produced detectable proteolysis and that this was insufficient to influence Cheddar cheese yields. A similar lack of correlation between the number of psychrotrophs present in raw milk and cheese yield was reported by Nelson and Marshall (1977) and Cousin and Marth (1977a).

The proteolytic action of psychrotrophs in milk prior to processing results in the formation of small peptides and amino acids required by the starter organisms for growth. Therefore their growth in milk has a stimulatory effect on the rate of starter growth and lactic acid formation by the starter organisms (Koburger and Claydon, 1961; Cousin and Marth, 1977b). Cousin and Marth (1977a,c) found that the manufacturing times for Cheddar cheese were shorter when the level of psychrotrophs in the milk was 10^6 cfu/ml.

Lipases are also produced by psychrotrophic organisms and spoilage by rancidity is likely to be evident before proteinase activity becomes discernable. The enzymes of these bacteria are mostly metalloproteins requiring divalent cations (Zn^{2+}, Co^{2+}, Ca^{2+}) for activity and stability (Law, 1987).

3 TEXTURE AND FLAVOUR DEVELOPMENT IN CHEESE

Fresh cheese curd consists of a sponge-like protein matrix containing both fat globules and an aqueous phase in which various compounds (eg. salts) are dissolved. The initial properties of the protein matrix are strongly dependent on the pH at which the whey is drained which affects both the level of calcium and moisture within the matrix. As the pH falls, much of the calcium phosphate and some of the casein in the casein micelles becomes soluble (Roefs *et al.*, 1985) and calcium and phosphate are lost in the cheese whey. The resulting changes in the size and nature of the micelles allow them to absorb more water (Tarodo de la Fuente and Alais, 1975; Snoeren *et al.*, 1984; Creamer, 1985; Roefs *et al.*, 1985) and maximum hydration occurs at pH 5.35. Casein hydration in renneted milk also increases in the presence of NaCl between pH 5.0 and 5.4 (Creamer, 1985). Also, at any given pH, solubilisation of the micelles by NaCl decreases as the calcium concentration in the solution increases (Creamer, 1985). This explains the observation that a higher Ca^{2+}/Na^{+} results in a firmer cheese (Walstra and van Vliet, 1982). In most types of cheese, as the pH falls below 5.5 there is a progressive dissociation of the micelles into smaller casein aggregates (Hall and Creamer, 1972; de Jong, 1978; Roefs *et al.*, 1985). However in Gouda, which has a pH of 5.2 after brining, the structural units in the protein matrix appear to be in the same form as in the original casein micelles in the cheese milk (Hall and Creamer, 1972; de Jong, 1978). The caseins at low pH (approaching the isoionic point), stabilised by strong ionic and hydrophobic interactions, are more closely associated within the casein aggregates and therefore the moisture content of the casein matrix is low and the cheese becomes shorter in texture (Figure 1.13). At a higher pH there is less dissolution of the calcium phosphate in the casein micelles and the cheese curd has a higher calcium and phosphate content. The caseins, at a higher pH, have a net negative charge and absorb moisture resulting in a casein matrix with a high moisture content (Creamer and Olson, 1982; Lawrence *et al.*, 1987; Lucey, 1990). The following diagram shows the relationship between pH and calcium in traditionally made cheese (Figure 1.12).

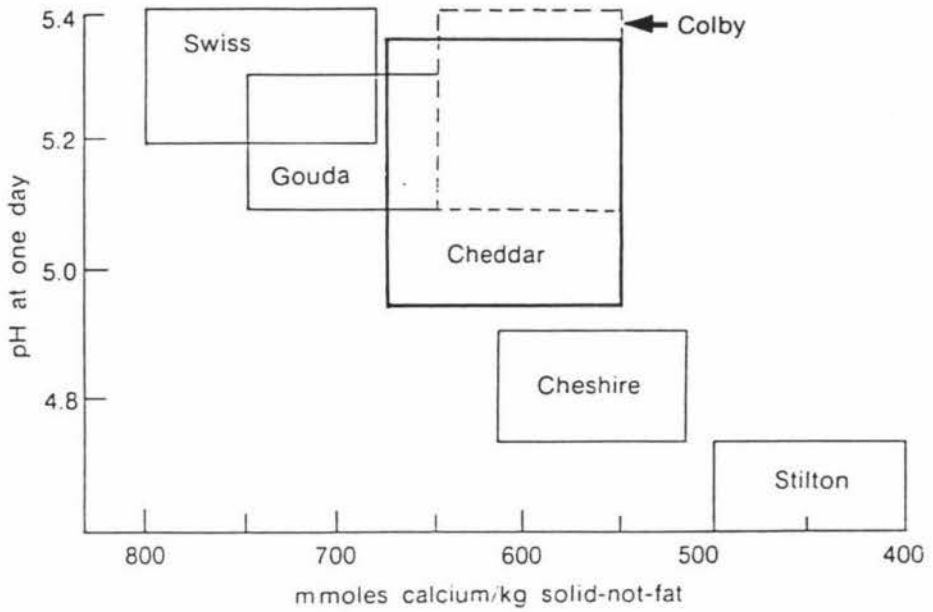


FIGURE 1.12 Classification of traditionally manufactured cheese varieties by their characteristic ranges of ratio of calcium/solid-non-fat and pH (Lawrence *et al.*, 1993).

The effect of pH and calcium content on the microstructure and texture of cheese is shown in Figure 1.13 (Lawrence *et al.*, 1987).

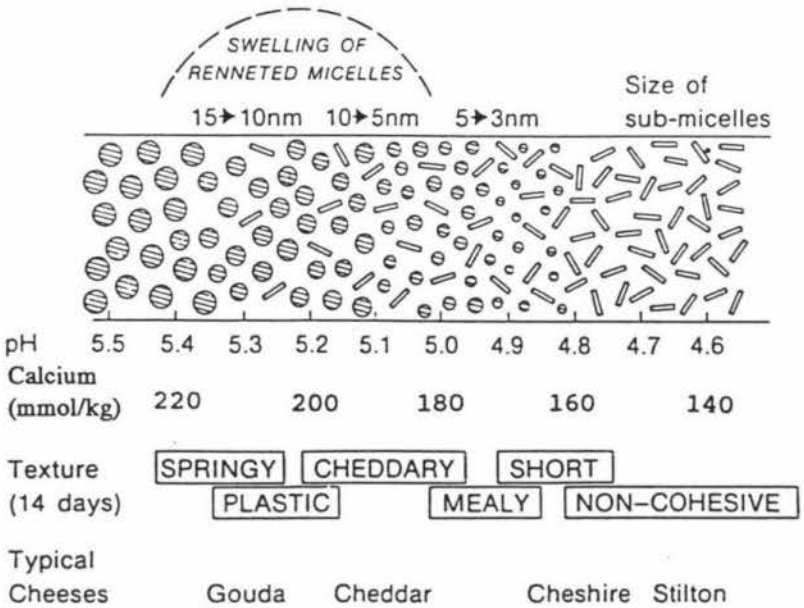


FIGURE 1.13 Diagrammatic representation of the effect of pH and calcium on cheese microstructure and texture (an adaptation of a diagram produced by Lawrence *et al.*, 1993).

The rate and extent of breakdown of the protein matrix is characteristic of the variety of cheese. The differing activities of a range of enzymes are dependent on factors such as the level of residual rennet and the quantity of plasmin, the type of microorganisms involved (including NSLAB and other adventitious species) and their enzyme complement, the pH, moisture content, calcium content, NaCl level and processing and ripening temperatures. Proteolysis has been identified as the key factor influencing the rate of texture and flavour development in most varieties of cheese.

3.1 PROTEOLYSIS AND TEXTURE

In the early stages of cheese ripening, for most varieties of cheese, the action of rennet on α_{s1}-casein is the major event. In cheeses that have been subjected to moderate temperatures ($<40°C$) during their manufacture most of the α_{s1}-casein is hydrolysed to yield α_{s1}-I-casein by two months. There is a close relationship between the amount of α_{s1}-casein hydrolysis and the consistency of the cheese (de Jong, 1976, 1977, 1978; Creamer and Olson, 1982; Johnston *et al.*, 1994). The effect of proteolysis on the consistency of the cheese is related closely to the pH at draining, which (as previously discussed) determines the mineral and moisture contents of the cheese. The higher pH of cheeses such as Swiss or Gouda (with their higher moisture and calcium contents) permits softening of the elastic curd as proteolysis progresses. The pH, calcium and moisture content of Cheddar cheese (intermediate between that of Gouda and Cheshire) allows the disintegration of the casein network during proteolysis and the cheese becomes smoother and less curdy in texture. Low pH cheese such as Cheshire (with its low moisture and calcium content) becomes more crumbly and brittle as proteolysis progresses and the protein matrix is broken down. The low pH, (which is close to the iso-electric point of the caseins) combined with the low moisture content prevents the caseins from becoming soluble (Creamer and Olson, 1982; Lawrence *et al.*, 1987; Creamer *et al.*, 1988a; Lucey, 1990; Lawrence *et al.*, 1993). The pH gradient established at the surface of cheeses (Section 4.5) such as Camembert is responsible for the diffusion of calcium phosphate to the surface (Le Graet *et al.*, 1983; Noomen, 1983). The loss of the protein stabilizing calcium and calcium phosphate allows the cheese to soften.

The contribution of plasmin and plasminogen to the texture or flavour of cheese is unknown. As previously discussed in Section 2.2, plasminogen is associated almost exclusively with the casein micelles and therefore it should all be retained in the cheese

curd. Cheeses in which there is a higher pH and moisture content, such as Gouda and Camembert (near the surface), show higher levels of plasmin activity. The highest levels of plasmin activity are seen in cheeses which have been subjected to higher manufacturing temperatures (eg. Swiss and Mozzarella). Much of the disappearance of β- and α_{s2}-casein in these cheeses is attributable to plasmin, and it is thought to play a major, but as yet undefined, role in their ripening.

3.2 PROTEOLYSIS AND FLAVOUR

The perception of flavour is related to both taste and smell. Thus, the flavour of cheese is dependent on the composition of both the non-volatile fraction (taste) and the odiferous volatile fraction. The presence of salt is believed to enhance flavour perception in cheese (Aston and Creamer, 1986).

The second stage of cheese ripening involves the breakdown of peptides produced mainly by the action of rennet and plasmin. The peptidases of the microorganisms present in cheese are thought to play the major role and were discussed in detail in Section 2.1. These enzymes are responsible for the conversion of larger peptides to small peptides and amino acids from which the flavour of cheese originates (Mulder, 1952; Marth, 1963). McGugan *et al.* (1979) demonstrated the importance of the water soluble fraction to the flavour of Cheddar cheese. Aston and Creamer (1986) fractionated the non-volatile water soluble fraction of Cheddar cheese using gel filtration chromatography in an endeavour to determine the contribution of its amino acid and peptide components to cheese flavour. They found that the fraction with the most flavour also contained most of the NaCl and some of the glutamic acid. Both of these substances are known to be flavour enhancers (Ikeda, 1909; Solms, 1969; Gillette, 1985). Cliffe *et al.* (1993) also investigated the water soluble fraction of Cheddar cheese with gel filtration chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC). The low molecular weight fractions with a savoury flavour eluted early from the RP-HPLC column and were believed to contain the more hydrophilic peptides and amino acids. The bitter, higher molecular weight fractions were found to elute later and were thought to contain the more hydrophobic peptides.

The release of proline, thought to be due to the action of the peptidases of propionibacterium (Langsrud *et al.*, 1977), in Swiss cheese is related to its sweet flavour (Langler *et al.*, 1967). Some peptides have been linked with 'brothy' and

'nutty' flavours in Swiss cheese (Biede and Hammond, 1979a,b).

The amino acids produced by these peptidases are also important flavour precursors and undergo decarboxylation, deamination, desulphurylation and demethiolation to produce compounds that contribute to cheese flavour. The catabolism of amino acids and their end products is outlined in Figure 1.14.

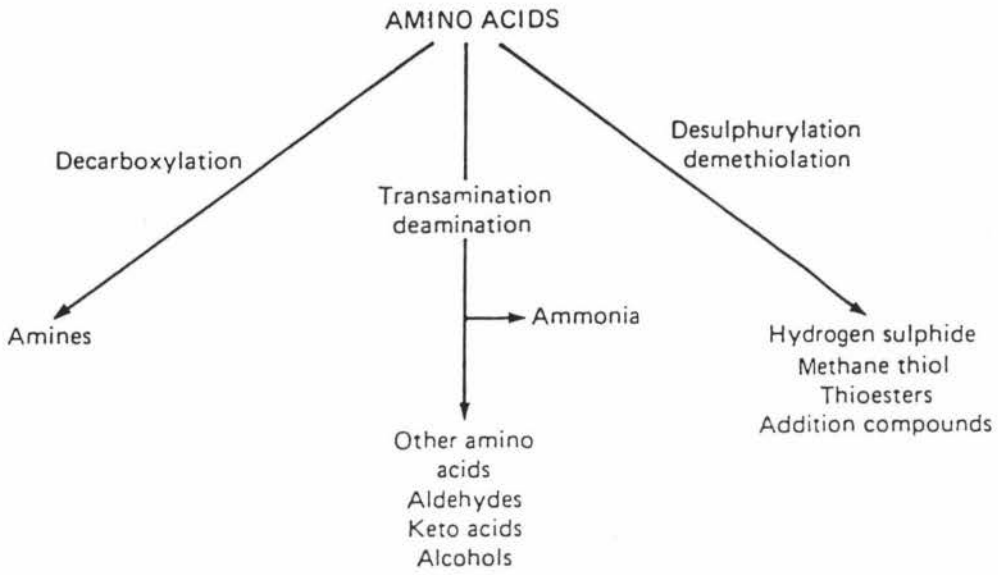


FIGURE 1.14 General pathways of amino acid catabolism in cheese (Law, 1987).

In Camembert and other mould-ripened cheeses methyl ketones and their alcohols are the most abundant neutral compounds in the volatile fraction and contribute to cheese flavour. The ability of *Pe. Camemberti* to produce methyl ketones varies greatly among strains. The mushroom note in Camembert is caused by oct-1-en-3-ol. However too much oct-1-en-3-ol causes flavour problems. Sulphur compounds are responsible for the garlic flavour in traditional (raw milk) Camembert. Compounds such as methylsulphide, methyl-disulphide and 3-methylthiopropanol, which are present in other cheeses also, give a basic cheese note, while 2,4-dithiapentane, 2,4,5-tri-thiahexane and 3-methylthio-2,4-dithiapentane are observed in typical Camembert. Coryneform bacteria are usually thought to be key contributors to the formation of sulphur compounds in surface-mould cheeses. *Pe. camemberti* possesses a demethiolase which converts methionine to methanethiol (Gripon, 1990). Several sulphur compounds that develop from methanethiol by non-enzymatic reactions are known to have cheese-like aromas (Bosch *et al.*, 1982).

The level of methanethiol in Cheddar cheese correlates with flavour intensity and its selective removal from the head space destroys the typical aroma of the cheese (Manning and Price, 1977). The organisms capable of producing methanethiol are not usually present in Cheddar cheese and it is believed that H_2S produced in the cheese can release methanethiol from undegraded casein or methionine by an addition or substitution (Manning, 1979).

Strains of *B. linens*, found at the surface of some varieties of cheese are also able to release methanethiol from methionine. These strains produce a wide variety of end-products of catabolic reactions involving amino acids such as leucine, phenylalanine and methionine. End-products include 3-methyl-1-butanol, phenylethanol and 3-methylthiopropanol (Hemme *et al.*, 1982). Many micro-organisms at the surface of cheese produce phenylethanol from phenylalanine (Lee and Richard, 1984).

Propionibacteria, by decarboxylation of amino acids, produce CO_2 for eye formation in Swiss type cheese. The decarboxylation of histidine and tyrosine to form toxic amines in Swiss and Gouda cheeses has been discussed in a previous section (Section 2.3.3). Reactions between amino acids and carbonyl compounds such as glyoxal, methyl glyoxal, diacetyl, dihydroxyacetone, acetoin and ethanol are believed to play a role in the formation of Swiss cheese flavour (Griffith and Hammond, 1989). These reactions may also occur in other varieties of cheese (Visser, 1993).

3.2.1 Bitterness in Cheese

Bitterness is a problem in many cheese varieties and is associated with the production of bitter peptides of 2-12 amino acids long and containing many hydrophobic residues (phenylalanine, isoleucine, leucine, valine and proline). Hydrophobic regions of both β-casein (Hamilton *et al.*, 1974; Visser *et al.*, 1983b) and α_{s1}-casein (Hodges *et al.*, 1972; Richardson and Creamer, 1973) have been implicated in bitterness. The principle sources of bitter peptides arising from β-casein are residues Val_{84} - Gln_{89} and Tyr_{193} - Val_{209} of the C-terminal end. The bitter peptides of α_{s1}-casein arise from the region Glu_{14} - Lys_{34} near the chymosin sensitive Phe_{23} - Phe_{24} bond. The production of bitter peptides has been attributed to both rennet action and/or the action of cell wall-bound microbial proteinases. P_{III}-type proteinases which initially cleave large fragments from the C-terminal of β-casein are believed to generate less bitterness than P_I-type proteinases which produce a relatively small and very bitter C-terminal fragment (Visser *et al.*, 1983b; Visser *et al.*, 1988; Visser *et al.*, 1991). All starter organisms are thought to contain membrane associated enzymes capable of hydrolysing bitter peptides

(Visser *et al.*, 1983a). Salt may contribute to bitterness by decreasing the permeability of the starter cells and increasing the hydrophobic association of bitter peptides. Both of these factors result in reduced availability of the substrate for the membrane-associated enzyme (Visser *et al.*, 1983a). An extensive review of this subject, containing tables of bitter peptides isolated and identified from the hydrolysis of α_{s1}-casein, α_{s2}-casein and β-casein, has been published by Lemieux and Simard (1992).

4 CHEESE VARIETIES

Each variety of cheese has a characteristic proteolytic profile. The extent and nature of proteolysis during the ripening of a particular cheese variety is dependent on its enzyme complement and other physical and chemical factors such as cook temperature, pH, calcium, salt, moisture, ripening temperature and age. An examination of proteolysis in some of the common varieties of cheese is presented in the following sections. Much of the information presented is a summary of information scattered throughout the preceding sections and some repetition is therefore unavoidable.

4.1 PROTEOLYSIS IN CHEDDAR CHEESE

Cheddar cheese is traditionally manufactured according to the following process: milk containing rennet and starter is coagulated; the coagulum is then cut; the cut curd is heated and stirred during the production of acid; whey is removed and the curd is then cheddared, milled, salted, pressed, packaged and ripened (Lawrence *et al.*, 1993).

The enzymes present in Cheddar cheese are rennet, plasmin (and to a lesser extent the other indigenous enzymes), those of the starter bacteria and the non-starter lactic acid bacteria.

The level of chymosin incorporated into the cheese curd is dependent on the initial level of chymosin and the pH at whey draining (with more rennet being incorporated into the cheese at lower pH) (Holmes *et al.*, 1977; Lawrence *et al.*, 1983; Creamer *et al.*, 1985). Cheddar cheese is subjected to moderate temperatures (<40°C) during its manufacture and the residual chymosin will therefore remain active. In the early stages of cheese ripening the action of chymosin on α_{s1}-casein with the production of α_{s1}-I-casein is the dominant event and is associated with a rapid change in texture as the α_{s1}-

casein network is weakened (Creamer and Olson, 1982; Johnston *et al.*, 1994). Chymosin action on β-casein is not believed to be significant. As previously discussed, the pH, calcium and moisture content of Cheddar cheese allows the disintegration of the casein network during proteolysis (Lawrence *et al.*, 1983) and the cheese becomes smoother and less curdy in texture (Johnston *et al.*, 1994).

Plasmin is active in Cheddar cheese where it hydrolyses β-casein, α_{s2}-casein and to a lesser extent, α_{s1}-casein. Plasmin is believed to contribute to both the formation (Farkye and Fox, 1991) and degradation of the peptides in cheese (Farkye and Fox, 1992; Farkye and Landkammer, 1992). Plasmin is associated with the casein micelles in the pH range 4.6-6.6 and is believed to be retained in the curd at whey draining (Grufferty and Fox, 1988c). The method of salting is not believed to affect plasmin retention and activity in Cheddar cheese (Farkye and Fox, 1990). However, as the S/M is increased above 4% the hydrolysis of β-casein decreases (Thomas and Pearce, 1981) and this is believed to be attributable (at least in part) to the action of plasmin. The pH of Cheddar cheese (5.0-5.3) is significantly lower than the optimum for plasmin (7.5-8.0) and its action is therefore limited. Any increase in the moisture content of the cheese (eg. in accelerated ripening) will lead to an increase in enzyme activity (Lawrence *et al.*, 1993). Similarly, the use of elevated ripening temperatures will significantly increase the level of enzyme activity in Cheddar cheese.

The inability of the proteinases of most starter organisms to hydrolyse α_{s1}-casein means that they are considered to be of secondary importance to rennet in the formation of cheese texture (Lawrence *et al.*, 1987). More importance is attached to their peptidases which produce small peptides and amino acids that either contribute directly to flavour or are flavour precursors (Marth, 1963; Mulder, 1952).

As previously discussed (see section 2.3.3) the numbers of non-starter lactic acid bacteria in Cheddar cheese increase dramatically to between 10^6 and 10^8/g of cheese between 3 and 4 months of age (Naylor and Sharpe, 1958a,b). The predominant organisms in ripening cheese at 3 to 4 months are lactobacilli (or pediococci) and these organisms possess a range of peptidases which are released from the cell as a result of lysis on cell death. These enzymes are able to hydrolyse α_{s1}-casein, β-casein, α_{s2}-casein, κ-casein, β-lactoglobulin and α-lactalbumin (Peterson and Marshall, 1990). Most of the strains examined have been shown to contribute to cheese ripening by increasing the rate and extent of proteolysis and improving the cheese flavour (Section 2.3.3).

Small numbers of Gram-negative rods, micrococci and Group D streptococci may also be present in the ripening cheese and contribute to proteolysis (Law, 1987).

The manufacture of Colby cheese is similar to Cheddar cheese until the whey is drained. At this point some of the whey is drained off and replaced with water before the curd is pressed. The temperature of the curd/whey/water mix is used to control the moisture content of the cheese. The wash stage results in a slightly lower calcium level and a small increase in pH of 0.1 - 0.2 pH units. Ripening is likely to be similar to that of the other washed curd type cheeses such as Gouda and Edam with proteolysis being enhanced by the higher pH and moisture content. Colby cheese has a more plastic texture and better melting properties than Cheddar cheese and these differences are due to the higher pH (>5.2) and moisture level (39-40%) and lower mineral content (Lawrence *et al.*, 1993).

A high level of acid is produced during the manufacture of Cheshire cheese prior to the addition of rennet. Low pH (<4.9 at 1 day) cheese such as Cheshire (with its low moisture, calcium and phosphate content) becomes more crumbly as proteolysis progresses and interstitial water is bound by newly generated ionic groups (Creamer and Olson, 1982). The low pH, (which is close to the iso-electric point of the caseins) combined with the low moisture content prevents the caseins from becoming soluble (Creamer and Olson, 1982; Lawrence *et al.*, 1987; Creamer *et al.*, 1988a; Lucey, 1990; Lawrence *et al.*, 1993).

4.2 PROTEOLYSIS IN DUTCH-TYPE CHEESE

Dutch-type cheeses are hard or semi-hard cheeses manufactured according to the following general process: Milk is standardised and pasteurised; starter (mesophilic), $CaCl_2$ and rennet are added and the milk is left to set at 31°C; the curd is cut and stirred; part of the whey is removed and stirring is continued; the temperature is increased to 35-38°C (scalding) by adding heated whey or water (washing); part of the salt may be added at this stage to inhibit the growth of undesirable microorganisms; when the pH and moisture content of the curd reach the desired level stirring is stopped and the curd is left to settle; a continuous mass of curd is formed and the whey is removed, the curd mass is placed into moulds and pressed; the cheese is brined (it takes several weeks for the salt to penetrate to the centre of the block, with the time being dependent on the block size), dried and may be coated with wax (or a synthetic film)

to prevent moisture loss prior to ripening (Scott, 1986; Walstra *et al.*, 1993).

Proteolysis in Dutch-type cheeses follows a similar pattern to Cheddar cheese, with rennet playing the major role in the early stages of ripening and the enzymes of the starter organisms being more important in the later stages. A low level of plasmin activity occurs throughout ripening. Gouda and Edam are washed curd cheeses and therefore have a lower calcium level, a lower lactose content and a higher pH and moisture content than Cheddar cheese. This, along with the gradation of salt concentration, influences the enzyme activity and texture formation in the cheese. The higher pH and moisture content and the moderate salt content (1-3% S/M) allows a higher level of rennet and plasmin activity than occurs in Cheddar cheese. α_{s1}-Casein is rapidly broken down by rennet (~80%) in one month and β-casein is degraded more slowly by plasmin (~50% in 6 months) (Visser and de Groot-Mostert, 1977). The peptidases of the microorganisms further hydrolyse the peptides produced by the action of rennet and plasmin. The small peptides and amino acids that are produced contribute either directly or indirectly to cheese flavour.

The effect of proteolysis on cheese texture is determined by the pH, calcium, phosphate and moisture contents. As maturation progresses the following textural changes occur: the apparent elastic modulus of the cheese increases, the deformation at which fracture occurs decreases and the fracture stress at first decreases and then increases again (Luyten, 1988, cited by Walstra *et al.*, 1993). Only the deformation at which fracture occurs has been shown to correlate well with maturation (Oortwijn, 1984 cited by Walstra *et al.*, 1993). In some cheese varieties, the changes in cheese texture that occur during maturation provide the consistency that is a prerequisite for the formation of holes.

Proteolysis is also responsible for changes in the flavour and aroma of Dutch-type cheeses. The array of peptides and amino acids formed may contribute directly to cheese flavour or be further broken down to form many different compounds that together (with the peptides and amino acids) provide the cheese with its characteristic flavour and aroma.

4.3 PROTEOLYSIS IN SWISS-TYPE CHEESES

Swiss-type cheeses such as Emmental or Gruyère (smaller with fewer and smaller holes) are manufactured according to the following general process (Scott, 1986): Cow's milk (3.2% fat) for Gruyère or standardised milk (3.0-3.15%) for Emmental cheese is pasteurised; $CaCl_2$, starter and rennet are added at 31 to 33°C; at 25-35 minutes the curd is cut (5-10 mm cubes for Gruyère and ~6 mm cubes for Emmental, the curd particle size affects the rate of loss of whey, the rate at which scalding can be done and, therefore, the rate of acid development) stirred gently as the temperature is slowly raised to 52-54°C for Gruyère and 50-53°C for Emmental cheese and stirring is continued for 40-50 min for Gruyère and 30-60 min for Emmental; the curds are then lifted from the whey, moulded, pressed, salted, dried off and ripened. The two varieties are subjected to different ripening temperatures. Gruyère cheese is ripened at 10°C for 3 weeks and then at 15-20°C for 2-3 months during which time the holes are formed. The cheese is then moved to 12-15°C for 8-12 months until it is ripe. Emmental cheese is ripened at 20-24°C for 3-6 weeks to allow the formation of holes. The cheese is then removed to a lower temperature until it is ripe (6-12 months depending on the temperature) (Scott, 1986). Emmental cheese has 37-42% moisture, 43-50% (minimum) FDM and 0.5-1.2% S/M, while Gruyère has 39-42% moisture, 40-45% (minimum) FDM and ~4.5% S/M (Scott, 1986; Steffen *et al.*, 1993).

The texture of these cheeses is close and the body is firm but elastic enough to allow the formation of the characteristic eyeholes without the formation of slits. Gruyère cheese is more firm and fatty (rather than moist), has smaller eyeholes, develops acid more quickly and consequently has a stronger flavour than Emmental (Scott, 1986). Initially the cheese has a very firm and long body with a tough and rubbery consistency but, after an initial equilibration period (35 days) during which time the body becomes longer as the curd particles fuse, proteolysis progresses and the protein network is weakened causing the cheese body to become shorter and less tough (Steffen *et al.*, 1993). Eberhard (1985) showed strong correlations between the temperature, time and amount of water soluble nitrogen formed during ripening and the compression at breaking point.

The high temperature that these cheeses are subjected to during their manufacture is believed to result in a reduction in rennet activity (Matheson, 1981) and the indigenous milk enzymes and those of the starter bacteria play a more important role in their ripening (Richardson and Pearce, 1981). As previously described (Section 2.2), these

temperatures promote the conversion of plasminogen to plasmin and result in the incorporation of ~2.5 times more plasmin in Emmental cheese than in Cheddar cheese (Richardson and Pearce, 1981; Farkye and Fox, 1990). The high rate of β- and α_{s2}-casein hydrolysis can therefore be attributed to the higher cook temperature as well as the higher pH and lower salt and moisture contents.

The starters used in Swiss-type cheeses contain both thermophilic lactococci and lactobacilli (*e.g. Lactobacillus delbruekii* subsp. *bulgaricus*, *Lactobacillus helveticus* and *Streptococcus salivarius* subsp. *helveticus*) with an optimum temperature for growth of 38-45°C and mesophilic lactococci (*Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, as well as *Propionibacterium shermanii*. These organisms, the most proteolytic of which are believed to be the thermophilic lactobacilli, produce proteinases and peptidases which contribute to the characteristic proteolytic profiles of this group of cheeses. The very small peptides and amino acids that they produce are responsible for the 'nutty' or 'brothy' flavour of these cheeses. The propionibacteria are added with the starter culture and grow only after the cheese has been made. They transform lactate to propionate, acetate and carbon dioxide and are thought to produce peptidases which release proline (Langsrud *et al.*, 1977) to give the cheese its characteristic sweet flavour (Langler *et al.*, 1967). The presence of Ca^{2+} and Mg^{2+} is essential for the production of sweetness (Biede and Hammond, 1979a,b). The amino acids are further catabolised to form compounds that also contribute to the flavour and aroma of the mature cheese.

4.4 PROTEOLYSIS IN MOZZARELLA CHEESE

Mozzarella cheese is a cohesive, elastic cheese with a low level of hardness, that is used mainly for melting on pizza. The properties of the cheese must be such that there is no clumping of the cheese after shredding and it has the desired melt characteristics on pizza.

Low moisture Mozzarella pizza cheese is manufactured according to the following general method: the milk is standardised and pasteurised; starter (usually thermophilic) is added and is mixed with the milk; rennet is added after ~30 min (pH 6.45) and the milk is left to set; the curd is cut at pH 6.40, scalded and drained at pH 6.10; at pH 5.90 the curd is cheddared (at 42°C) to pH 5.2 and milled; the milled curd is kneaded and plasticised (stretched) in hot water at ~70°C, moulded and brined at 4°C, and

stored at low temperature (Kindstedt, 1993). The cheddaring step may be eliminated and the granular curd soaked in warm water until the desired pH is reached (Kielsmeier and Leprino, 1970), or the partially acidified granular curd may be washed in cool water and transferred to cool storage overnight while the pH falls to the desired level (Kielsmeier, 1976), or the drained curd may be subjected to continuous stirring until the desired pH is reached (Nilson and LaClair, 1976). The curd is then stretched as previously described.

A high moisture Mozzarella cheese can be made using a smaller inoculum and lower vat temperature to give slower acid formation and higher moisture retention. After the curd is cut, the whey is drained and the curds are left to mat together. They are then washed in cold water, refrigerated overnight in cloth bags, warmed to room temperature, acidified to pH 5.2, stretched at 70°C, moulded, cooled in chilled water and brined (Kindstedt, 1993).

The stretch characteristics of melted Mozzarella cheese are related to the relatively high levels of intact casein and a critical concentration of calcium and phosphate (Lawrence *et al.*, 1987; Lucey, 1990). The amount of calcium incorporated in the curd is related to the pH at whey draining (Kiely *et al.*, 1992; Kindstedt *et al.*, 1993). Initially, Mozzarella melts to a tough elastic consistency that is not suitable for pizza. After ~one week at 4°C the cheese melts to the desired consistency. However, even at 4°C the cheese is only suitable for use on pizza for a relatively short period of time (3-4 weeks) and eventually becomes soft and does not melt to the desired consistency.

The high initial apparent viscosity of Mozzarella cheese, which appears to be inversely related to the moisture content of the non-fat substance (Kindstedt *et al.*, 1988), decreases rapidly during the first two weeks at 4°C and continues to decrease at a slow rate until the cheese is too soft for use on pizza (Kindstedt and Kiely, 1990a).

Guinee (unpublished results, reported by Guinee and Wilkinson, 1992) found that the melt and stretch characteristics of Mozzarella cheese improve as the casein is hydrolysed, up to ~11-12% water soluble nitrogen (WSN) (as a percentage of total nitrogen (TN)) and remain fairly constant until above 20% WSN/TN when, depending on the pH and calcium level, the cheese loses its stretch and melt characteristics and forms free oil.

The amount of free oil formed on heating, while being related to the amount of fat in

the cheese, also increases rapidly during the first one to two weeks at 4°C. Kindstedt and Kiely (1990b) found that the amount of free oil formed on melting a low moisture part-skim Mozzarella cheese stored at 4°C increased from 25% at one day to 50% at three weeks.

The easier melt and loss of stretch, the decrease in the apparent viscosity and the increase in free oil formation are believed to be caused by the action of enzymes on the protein matrix (Lawrence *et al.*, 1987). The enzymes active in the cheese are plasmin and a low level of rennet (Matheson, 1981) that is dependent on the temperature profile of the curd during stretching (Creamer, 1976b). The level of residual rennet activity, as evidenced by the hydrolysis of α_{s1}-casein, is intermediate between that seen in Cheddar and Gouda cheeses (Creamer, 1976b). The activity of plasmin is higher than in Cheddar cheese but not as high as in Gouda cheese. As mentioned previously, heat is believed to inactivate the inhibitor of the plasminogen activator resulting in a greater conversion of plasminogen to plasmin and hence in a higher level of plasmin activity and elevated rate of β- and α_{s2}-casein hydrolysis. The enzymes of the starter bacteria may also be active in Mozzarella cheese and are believed to affect the stretch and melting properties of the cheese (Oberg *et al.*, 1989).

An electrophoretic examination of proteolysis in four low moisture Mozzarella cheeses showed that the levels of intact α_{s1}- and β-casein decreased by 20-40% during storage at 4°C for four weeks (Kindstedt *et al.*, 1988). Farkye *et al.* (1991) used gel electrophoresis and WSN formation to measure differences in proteolysis within the salt gradient of high moisture brine salted Mozzarella cheese stored at 4°C for 14 days. Between 1 and 14 days post-manufacture, the level of WSN increased from 4.07% to 9.66% and at the same time the amount of intact α_{s1}- and β-casein decreased by 26.4 and 40.2%, respectively. They were unable to demonstrate significant differences in the rate of proteolysis within a S/M range of 0.59-1.59.

Kindstedt *et al.* (1991) investigated the effect of the stage of lactation on the functional properties (hardness, elasticity, free oil and melting properties) of Mozzarella cheese and concluded that changes in salt balance and proteolytic damage to casein (determined by the increase in WSN and band intensity on urea-PAGE), are factors related to the severe defects in functionality that occur in Mozzarella cheese made from late lactation milk.

4.5 PROTEOLYSIS IN CAMEMBERT AND BRIE CHEESES

Camembert and Brie are soft French cheeses that may be a creamy white to a yellow colour on the inside, have a close smooth body without gasholes, and a mild, mushroomy, slightly acid flavour that may be stronger and more piquant (ammoniacal) in the mature cheese (Scott, 1986).

They are manufactured according to the following general method: The milk may be standardised to 3.2-3.5% fat to give a firmer cheese and it is then pasteurised. Starter (and maybe a yellow dye (Brie)) is added. The mould spores may be added at this point or after salting if desired. Rennet is added and the curd is left to set at 30-32°C. The curd is cut and poured or ladled into hoops. It is then drained for 24-36 hours (depending on the type of cheese) to give a firm, demineralised curd with a pH of 4.5-4.6). The curd is then salted prior to ripening (Scott, 1986). The storage regime is dependent on the manufacturer, but generally the cheese is held in a warm room (80-85% RH) until the mould growth is established and then transferred to a lower temperature for ripening.

The cheese are ripened for 30 to 40 days (Camembert) during which time the protein is rapidly degraded. At the surface of the ripened raw-milk Camembert up to 35% of the total nitrogen (TN) is water soluble (Lenoir, 1962) but within the cheese only 25% of the nitrogen is water soluble. The water soluble fraction contains many small peptides, amino acids and ammonia (formed as a result of extensive deamination of amino acids) (Gripon, 1990). The enzymes responsible for the ripening of Camembert and Brie are rennet and plasmin and those of the lactic acid bacteria (usually *L. lactis* subspp. *lactis* and *cremoris*) and *Pe. camemberti* (*Pe. caseicolum* was until recently considered to be a distinct species of Penicillium but is now considered to be a white mutant form of *Pe. camemberti*).

Rennet, with the cleavage of α_{s1}-casein, is responsible for most of the early proteolysis (Trieu-Cuot & Gripon, 1982). The low initial pH prevents softening of the rennet degraded cheese due to the insolubility of caseins near their iso-electric points. The growth of the mould at the surface with its consumption of lactic acid (which diffuses from the centre) and production of ammonia causes the pH to rise at the surface (Noomen, 1983). As a consequence, calcium phosphate precipitates at the surface causing a concentration gradient which results in diffusion of calcium phosphate from the centre to the surface (Le Graet *et al.*, 1983). The combined effect, of chymosin

action and the pH gradient established by mould growth, is on the texture of the cheese. As the casein solubilizes there is a gradual softening of the cheese from the outside to the inside (Noomen, 1983).

Plasmin becomes active during the latter stages of ripening (21-35 days) as the pH rises to about 7.0 (near its optimum pH ~7.5-8.0) and is very active in the hydrolysis of β-casein (Trieu-Cuot and Gripon, 1982).

Enzymes produced by *Pe. camemberti* are active at the surface of the cheese and play a major in flavour formation. These enzymes include an extracellular acid (aspartate) proteinase with similar substrate specificity to that of chymosin and a neutral metalloproteinase. These proteinases are known to cleave α_{s1}-casein, β-casein and κ-casein (Trieu-Cuot *et al.*, 1982; Gripon, 1990). The relative activity ratio of the aspartate proteinase on α_{s1}-, β- and κ-casein is 1: 0.7: 0.6 (Gripon, 1990). Trieu-Cuot *et al.* (1982) identified the following cleavage sites in β-casein produced by the action of the aspartate proteinase: Lys_{29} - Ile_{30}, Lys_{97} - Val_{98} and Lys_{99} - Glu_{100}. They also identified the following cleavage sites in β-casein produced by the action of the metalloproteinase: Lys_{28} - Lys_{29}, Pro_{90} - Glu_{91} and Glu_{100} - Ala_{101}. In addition, *Pe. camemberti* produces both extracellular and intracellular carboxypeptidases and aminopeptidases. The acid carboxypeptidases contribute to the hydrolysis of bitter peptides by releasing hydrophobic amino acids. A proposed sequence of events for those enzymes whose function has been determined (Trieu-Cuot *et al.*, 1982) is presented in Figure 1.15 (Law, 1987).

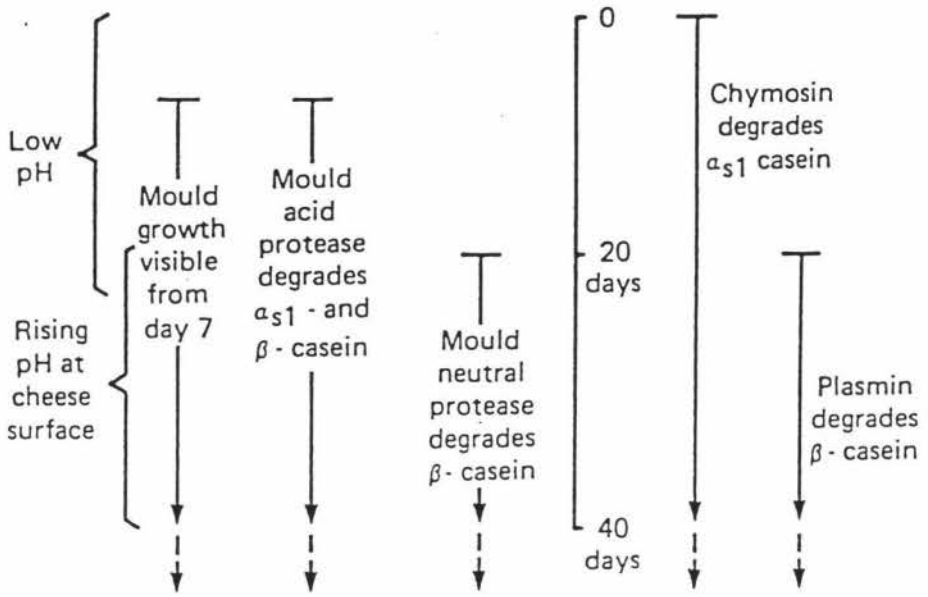


FIGURE 1.15 Sequence of proteolysis in Camembert cheese.

4.6 PROTEOLYSIS IN BLUE CHEESE

The manufacture of blue cheese is similar to Camembert and an acid curd, that has undergone no scalding is produced. The curd is cut and slowly drained with enough stirring to prevent pools of whey from forming. When the curd is sufficiently drained and the desired pH is reached it is put into moulds that are turned repeatedly to assist drainage. The curd is not pressed so that sufficient spaces remain between the curd particles for mould growth to occur. Blue cheese is inoculated with *Pe. roqueforti* which has a very potent proteolytic and peptidolytic enzyme system. The cheese has holes pierced in it to provide oxygen for the mould growth that occurs along the holes and in any gaps in the curd radiating from them. Brining of the cheese prevents mould growth on the outside of the cheese due to the high salt concentration.

Proteolysis is very intense in blue veined cheeses and in the mature Roquefort more than 50% of the TN is soluble at pH 4.6 (Devoyod *et al.*, 1968), while in Danish Blue ~65% is soluble (Hewedi and Fox, 1984). The soluble fraction contains a large number of small peptides (~30% of the TN is non-protein nitrogen (Godinho and Fox, 1982)) and amino acids (~10% of the TN (Ismail and Hanson, 1972)). Extensive

breakdown of both α_{s1}- and β-casein occurs and in the mature cheese very little of either of these caseins remains (Trieu-Cuot and Gripon, 1983; Hewedi and Fox, 1984; Marcos *et al.*, 1979).

Rennet is the major proteolytic enzyme active during the first 10-16 weeks of ripening (Hewedi and Fox, 1984). Also active, and of major significance during this period are extracellular (aspartate) acid and neutral proteinases, as well as several aminopeptidases and carboxypeptidases produced by *Pe. roqueforti*. These enzymes degrade both α_{s1}- and β-casein (Le Bars and Gripon, 1981; Trieu-Cuot *et al.*, 1982). The β-casein cleavage sites for these enzymes were identified as the same as those produced by the enzymes of *Pe. camemberti* (Trieu-Cuot *et al.*, 1982). In addition to these enzymes, those of the starter bacteria are also active. After 4-5 weeks of ripening the pH of the cheese reaches 5.5-6.0 in the inner regions (Godinho and Fox, 1982; Trieu-Cuot and Gripon, 1983) and this is likely to allow some plasmin activity to occur. In blue cheese the rate of proteolysis increases sharply between the 10th and 16th week. This corresponds to the maximum growth of the mould and it is believed that the sharp increase in proteolysis may be related to the release of intracellular enzymes (by lysis or leakage) from the mould (Hewedi and Fox, 1984).

The brine salting of blue cheese causes a salt concentration gradient to occur within the cheese. Mould growth is optimal between 1-3% salt (Godinho and Fox, 1981a,b) which corresponds to a region between the centre and the surface of the cheese. In this region no α_{s1}-casein remains after 16 weeks. The degradation of β-casein is less rapid. Proteins at the surface are the least degraded because of inhibition of chymosin action and mould growth by the higher salt concentration and the lower moisture content.

5 CONCLUSION

Proteolysis in cheese contributes to changes in the texture, functional properties and flavour characteristics of the ripening cheese.

The differences in manufacture of the various types of cheese result in the inclusion of different amounts of rennet, plasmin (and other indigenous proteases) as well as the enzymes of the starter microorganisms, non-starter lactobacilli and secondary microflora in the curd and create differences in the cheese environment (*e.g.*, pH, moisture, salt, S/M, calcium) that result in differing enzyme activities in the cheese.

The drain pH is particularly important, affecting the amount of calcium, the incorporation of rennet and the activity of the enzymes in the cheese and ultimately the texture and functional properties and probably the flavour of the cheese.

The use of high temperatures during the manufacture of the cheese (*e.g.*, 50-54°C for Swiss-type cheeses and ~70°C for Mozzarella-type cheeses) results in a reduction in rennet and probably microbial enzyme activity as well as in the activation of plasminogen to plasmin and influences the textural and functional properties of the cheese.

The storage temperature of the cheese is used to manipulate the rate of maturation as well as the growth of microorganisms in some cheese types (*e.g.*, Emmentaler).

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CHAPTER 2

GEL ELECTROPHORESIS: A COMPARISON OF METHODS SELECTED TO STUDY PROTEOLYSIS IN CHEESE

SUMMARY

Four different electrophoretic methods were selected to study proteolysis in different types of cheese and their relative usefulness was assessed. The methods investigated were: 1) a slab gel system using the apparatus of the E-C Apparatus Corporation and a polyacrylamide gel in a Tris-EDTA-borate buffer at alkaline pH and containing urea; 2) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide stacking and resolving gel with a discontinuous (Tris-chloride/Tris-EDTA-borate) buffer system that contained urea; 3) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide stacking and resolving gel and acetic acid-ammonium acetate buffers at acidic pH that contained urea; 4) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, a polyacrylamide gel with a stacking and resolving gel in Tris-HCl buffers containing sodium dodecyl sulphate (SDS) and a Tris-chloride-glycine electrode buffer. Two-dimensional polyacrylamide gel electrophoresis (PAGE) was used to assist in band identification in both the acid urea-PAGE and the SDS-PAGE systems and to determine whether the proteins and peptides on the alkaline urea mini-slab gel used in the first dimension were homogeneous.

The large-slab alkaline urea-PAGE method produced good resolution of the proteins and peptides in cheese but the method was considered to be too time consuming and unwieldy for the routine monitoring of proteolysis in cheese.

The mini-slab alkaline urea-PAGE method was more suitable for monitoring the loss of intact casein during cheese ripening. This method was relatively fast, gave good resolution of the caseins and produced bands with a uniform shape, thus allowing more accurate interpretation and densitometry to be carried out. Traces of protein were found to lie beneath the α_{s1}-casein band and this explained why, even after considerable proteolysis, some α_{s1}-casein appeared to remain.

The caseins were not well resolved using the SDS-PAGE method and it was therefore concluded that it was not a suitable method for monitoring their disappearance during

cheese ripening. However, good resolution of para-κ-casein, β-lactoglobulin and α-lactalbumin was obtained using this method and it could therefore be used for the analysis of cheese in which whey proteins have been incorporated or for monitoring the breakdown of para-κ-casein (Chapter 4) in cheese. Many well resolved peptides were retained using SDS-PAGE and it is likely to be a useful method for monitoring their presence in cheese (Chapter 4).

The acid urea-PAGE method gave very little information that could not be obtained by a combination of alkaline urea-PAGE and SDS-PAGE.

Two-dimensional PAGE revealed the presence of more bands than were visible using any single method of electrophoresis.

1 INTRODUCTION

The hydrolysis of the proteins and the consequent formation of peptides during the ripening of cheese can be monitored by various electrophoretic techniques. The proteins, separated on the basis of charge and/or size and conformation, are stained and can be quantitated using various types of densitometry (de Jong, 1975). It is important that the proteins and peptides are well resolved and that they form bands that are uniform in shape so that they may be interpreted and quantitated accurately.

In the following study three polyacrylamide gel electrophoresis (PAGE) methods currently in use at the NZDRI and a fourth method that required some adaption to enable it to be used in the mini-slab gel system were evaluated to determine their usefulness in the study of the ripening of cheese. A range of cheese varieties, with different amounts and types of enzyme activity and therefore with different band patterns, was analysed in order to determine the usefulness of each of the gel methods.

The following generalised methods were investigated:

1) a slab gel (11.5 cm x 16 cm) system using the apparatus of the E-C Apparatus Corporation (University City, Philadelphia, Pa. USA) and based on the methods and instructions that accompanied the apparatus. The gel was 7.5% (*w/v*) acrylamide (5% C, where % C (% cross-linking) is the amount of bisacrylamide expressed as a percentage of the total acrylamide plus bisacrylamide) (Sigma "gelling agent") in a Tris-EDTA-borate buffer (Peterson, 1963; Thompson *et al.*, 1964),at pH 8.4 (Peacock *et al.*, 1965) and containing urea. The chamber buffer was a Tris-EDTA-borate buffer;

2) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, California, USA) and a polyacrylamide gel system with a stacking and a resolving gel, that is a combination of the Davis (1964) and Ornstein (1964) system, in a discontinuous buffer system at alkaline pH that is similar to that of Peterson (1963), Thompson *et al.* (1964) and Peacock *et al.* (1965). The chamber buffer was a Tris-EDTA-borate buffer;

3) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus, and a polyacrylamide gel system with a stacking and a resolving gel (Davis, 1964 and Ornstein, 1964) in an acetic acid-ammonium acetate buffer system at acidic pH and containing urea. Acetic acid (6%) was used in the buffer chamber;

4) a mini-slab gel system using the Bio-Rad mini-Protean II apparatus and the SDS-PAGE system of Giulian and Graham (1988) that is designed to capture low molecular weight peptides. This method has a stacking gel and a resolving gel in Tris-HCl buffers containing SDS, and a Tris-chloride-glycine chamber buffer containing SDS.

Two dimensional gel electrophoresis, with the first dimension being alkaline urea-PAGE and the second either acidic urea-PAGE or low molecular weight SDS-PAGE, was used to assist with band identification and to determine whether the bands were homogeneous.

Some of the photographs produced for this chapter were used in a review of gel electrophoresis of cheese written by Creamer (1991).

2 LITERATURE REVIEW

2.1 GEL ELECTROPHORESIS

Polyacrylamide gels are widely used for the analysis of cheese. They are strong and transparent, with less endosmotic flow of buffers and have a more uniform pore size than was attainable with the earlier starch gel system (Peterson, 1971). Urea is often incorporated into the gel system to dissociate the casein aggregates and allow the separation of the casein monomers (Hipp *et al.*, 1952). Tris (tris[hydroxymethyl]-amino-methane), often used in conjunction with citrate, borate, glycinate or chloride, is the most commonly used buffer salt and has replaced the early buffer systems containing diethylbarbituric acid or phosphate. The disadvantage of using either of these substances is that diethylbarbituric acid is a drug and therefore not always available for use in the laboratory and phosphate binds with cations such as calcium. The use of dithiothreitol, dithioerythritol or mercaptoethanol allows proteins linked by disulphide bonds to be separated.

There are several different PAGE methods and each has many variations.

2.1.1 Polyacrylamide Gel Electrophoresis in Alkaline Conditions

Two polyacrylamide gel systems with many different minor modifications have been widely used. They are the discontinuous tube gel system of Davis (1964) and Ornstein (1964) and the continuous system of Raymond and Nakamichi (1962).

The continuous system of Raymond and Nakamichi (1962), as modified by Peacock *et al.* (1965) and containing 5% (*w/v*) acrylamide (5% C) in a Tris-EDTA-borate buffer at pH 8.3 to 8.4 (with no urea) that is also used for the chamber buffer is often referred to as the method of Peterson (1963) (7% (*w/v*) Cyanogum-41 (95% acrylamide and 5% bisacrylamide) (the minimum he found to be necessary for resolution of α_s-casein) in a Tris-EDTA-borate buffer (also used as chamber buffer) containing 4.5 M urea (the level of urea required to give good resolution of caseins with higher levels being found not to improve resolution)) or Thompson *et al.* (1964) (7% (*w/v*) Cyanogum (5% C) in a Tris-EDTA-borate buffer at pH 9.1-9.3 containing 4.5 M urea). All of these methods are modifications of the methods provided with the gel equipment of the E-C Apparatus Corporation (E-C Apparatus Corporation, 1966). The continuous system was also used by Davies and Law (1977, 1987) who used 4.5% (*w/v*) acrylamide gels containing 4.5 M urea in a Tris-EDTA-barbitone buffer at pH 7.9 $\pm$ 0.1 to obtain very good separation of the caseins.

The system of Davis (1964) and Ornstein (1964) (with a resolving gel containing 7% (*w/v*) acrylamide (2.6% C) dissolved in a Tris-HCl buffer at pH 8.9, a stacking gel containing 2.5% (*w/v*) acrylamide (20% C) in Tris-HCl buffer at pH 6.7 and a Tris-glycine chamber buffer at pH 8.3), has a discontinuity between the large pore size of the stacking and the small pore size of the resolving gel, the pH of the stacking and resolving gel and the buffers of the gels and the chamber buffer (the chloride used as the anion in the resolving gel was replaced by the glycinate anion from the chamber buffer) resulting in the protein constituents of the sample being sharpened into a series of very narrow bands a few microns thick before they enter the small pore gel (Andrews, 1988). In this method the samples were set into a tube gel with the same large pore size as the spacer gel. Samples are now applied to the gel in a buffer of higher density containing sucrose or glycerol. The method of Davis (1964) and Ornstein (1964) was used by several authors who added different levels of urea. Grindrod and Nickerson (1967) used 4.5 M urea, Lawrence and Creamer (1969) used 6.6 M urea, 4.0 M urea was used by El-Shibiny and Abd El-Salam (1976), Marcos *et al.* (1979), Furtado *et al.* (1984), Medrano and Sharrow (1989) and Fernandez-Salguero *et al.* (1989).

Melachouris and Tuckey (1966), using the method of Aschaffenburg (1964) (5% (*w/v*) acrylamide (2% C) in a veronal buffer at pH 8.6 in 5 M urea) but with higher concentrations of acrylamide (7% (*w/v*)), bisacrylamide (5% C) and urea (7 M), and Ledford *et al.* (1966, 1968) using the continuous system of Thompson *et al.* (1964) (Raymond and Nakamichi, 1962) were the first to use polyacrylamide gels to study cheese. The method of Thompson *et al.*, with minor modifications, was widely used to study cheese and other cheese related areas such as the effect of rennet on casein (Fox, 1968, 1969; Edwards and Kosikowski, 1969; Creamer, 1970, 1976a; Kiddy *et al.*, 1972; Phelan *et al.*, 1973; O'Keeffe *et al.*, 1975, 1976; Mulvihill and Fox, 1977; Anderson and Andrews, 1977; Sood and Kosikowski, 1979; Creamer *et al.*, 1982; Andrews, 1983; Andrews and Alichanidis, 1983; Shalabi and Fox, 1987; Wilkinson *et al.*, 1992).

De Jong (1975) used a discontinuous buffer system (to study proteolysis in cheese) that was a combination of the methods of Thompson *et al.* (1964) and Melachouris and Tuckey (1966), with a slab gel (with no spacer gel) composed of 7% (*w/v*) Cyanogum 41 (5% C) dissolved in a Tris-HCl buffer at pH 8.9 and containing 4.5 M urea. Tris-glycine at pH 8.5 was used as a chamber buffer and the samples were dissolved in a Tris-HCl buffer at pH 8.5 containing 8 M urea. This method was subsequently used

by Creamer (1976b), Noomen (1977), Visser (1977) and Visser and de Groot-Mostert (1977). A similar method was used by Andrews (1975, 1983) and Anderson and Andrews (1977). Their method was reported to be based on that of Kiddy *et al.* (1972) which was, in turn, based on that of the E-C Apparatus Corporation (1966). The method of Kiddy *et al.* comprised a resolving gel containing 7% (*w/v*) Cyanogum-41 in a Tris-HCl buffer at pH 8.9, a spacer gel containing 4% (*w/v*) Cyanogum-41 in a Tris-HCl buffer at pH 6.7 (Andrews (1975) using the same specifications found the pH to be 7.6), with a Tris-glycine chamber buffer at pH 8.3. This system contained no urea. Andrews (1975) using an adaptation of this method in which samples were dispersed in a 6.6 M urea Tris-chloride-EDTA buffer containing 2-mercaptoethanol and in which the vertical gel-slab electrophoresis cell was replaced with tube gels found that 4.5 M urea was the minimum needed to prevent the association of casein molecules. The polyacrylamide method of Andrews (1975) was used by Law and Wigmore (1982) to study accelerated cheese ripening and by Law *et al.* (1979) to study the effect of proteolytic raw milk psychrotrophs on Cheddar cheese-making with stored milk.

Andrews (1983) used a similar system in which the resolving gel comprised 12% (*w/v*) acrylamide (4% C) in a Tris-HCl buffer at pH 8.9 containing 4.5 M urea, the stacking gel comprised 4% (*w/v*) acrylamide (5% C) in a Tris-HCl buffer at pH 7.6 containing 4.5 M urea, and a Tris-glycine chamber buffer. This method was subsequently used by several researchers to study various aspects of cheese ripening (*e.g.*, Fernandez del Pozo *et al.*, 1988; Vafopoulou *et al.*, 1989). McSweeney (1993b) used this method to study the contribution of the indigenous microflora to the water soluble fraction (WSF) of the ripening Cheddar cheese and Law *et al.* (1993) used it to study the contribution of lactococcal starter proteinases to the WSF of Cheddar cheese during maturation.

In New Zealand two systems have been used routinely. A slab gel system using the apparatus of the E-C Corporation and a gel comprising 7.5% (*w/v*) acrylamide (5% C) in a Tris-EDTA-borate buffer (Peterson, 1963; Thompson *et al.*, 1964) at pH 8.4 (Peacock *et al.*, 1965) and containing 6 M urea. The same buffer system was used for the chamber buffer (Richardson and Pearce, 1981; Thomas and Pearce, 1981; Creamer *et al.*, 1985, 1987, 1988a,b). Since about 1990, electrophoresis has been carried out using the mini gel equipment supplied by Bio-Rad. The method used is a combination of the Davis (1964) and Ornstein (1964) system and the discontinuous system that is based on the methods of Peterson (1963), Thompson *et al.* (1964) and Peacock *et al.* (1965) and has a stacking and a resolving gel. The stacking gel comprised 3% (*w/v*) acrylamide (2.6% C) and 6.0 M urea in a Tris-EDTA-borate buffer at pH 8.4, with a

resolving gel that contains 12% (*w/v*) acrylamide (2.6% C) and 4.5 M urea (Peterson, 1963; Thompson *et al.*, 1964) in a Tris-chloride buffer at pH 8.8, and a Tris-EDTA-borate chamber buffer at pH 8.4. This method is currently used to study cheese ripening (*e.g.*, Johnston *et al.*, 1994).

2.1.2 Polyacrylamide Gel Electrophoresis in Acid Conditions

The use of an acidic (~pH 4.0) buffer system allows the caseins to be separated on the basis of the number of cationic charges.

Waugh *et al.* (1962) and Peterson *et al.* (1966) used gels set at low pH with acetic-formic acid buffer system to separate caseins. Medrano and Sharrow (1989) used a system in which the samples were dissolved in 6.6 M urea and analysed using 8% (*w/v*) acrylamide gels (2.55% C) in an acetic-formic acid buffer system at pH 3 containing 4 M urea to type the A variants of β-casein on mini-slab gel apparatus.

In our laboratories, a large slab gel system similar to the method of Peterson *et al.* (1966), has been used. The TEMED-ammonium persulphate catalyst system resulted in a gel that was slow to set and slow to run. This was replaced by the thiourea-hydrogen peroxide system of Basch and Farrell (1979). A more recent method was developed for use in the present study and will be discussed in this chapter.

2.1.3 SDS Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate (SDS) solubilises proteins and protein aggregates by adsorbing to hydrophobic or positively charged sites on the proteins thus rendering them more hydrophilic. The electrophoretic mobility of the protein:SDS complex is related to the size and possibly the configuration of the protein (Peterson, 1971). Electrophoresis has therefore often been used to determine the molecular weight of proteins and peptides (*e.g.*, Shapiro *et al.*, 1967; Green and Pastewka, 1976). The implication is that the size of the protein:SDS complex correlates with the molecular weight of the protein, however, some anomalies exist when electrophoretic mobility is used to determine the molecular weight of proteins. On SDS gels, the order of the position of α_{s1}-casein (M_r 23,600) and β-casein (M_r 24,000) is the reverse of that predicted by their molecular weights. Creamer and Richardson (1984), using the "ammediol"-chloride- glycinate system (Wyckoff *et al.*, 1977; Bury, 1981), showed that the maximum binding of SDS by both caseins was 1.3 g SDS/g protein. They found that the size of the α_{s1}-casein : SDS complex was larger than the β-casein:SDS complex at pH 6.8 or 7.0, but that they were similar in size at pH 2.9 or 3.0. The large hydrodynamic size of α_{s1}-casein is due

to electrostatic repulsion of adjacent areas of high negative charge within the molecule and results in an expanded structure. Caution must therefore be exercised when the SDS system is used to estimate the molecular weight of proteins and peptides.

Bury (1981), using 12.5% (*w/v*) acrylamide gels, compared the "ammediol"-chloride-glycinate buffer system (Wykcoff *et al.*, 1977) with the sulphate-borate-Tris buffer system (Neville, 1971) and the Tris-chloride-glycinate system (Laemmli, 1970). He concluded that the buffer system of Wykcoff *et al.* (1977) gave the best resolution of peptides (obtained from a tryptic digest of bovine serum albumin) over a wide range of molecular weights.

Thompson *et al.* (1987) modified (and simplified) the SDS-PAGE method of Laemmli (1970). Samples were solubilised in the SDS solvent system of Basch *et al.* (1985) (a Tris-glycine buffer at pH 8.3 containing 0.03% (*w/v*) SDS) that was also used as a chamber buffer. The gel method incorporated many of the essential features of other researchers, with the resolving gel containing 12% (*w/v*) acrylamide in a Tris-EDTA-chloride buffer system at pH 8.9 (with no stacking gel) that was used with a Tris-glycine chamber buffer at pH 8.3 containing 0.03% (*w/v*) SDS (to determine the presence of calmodulin in cows milk and found none).

Shalabi and Fox (1987) compared the method of Laemmli (1970) using 10% (*w/v*) and 12.5% acrylamide with the method of Tegtmeyer *et al.* (1975). They found the 20% (*w/v*) acrylamide system of Tegtmeyer *et al.* (1975) (Tris-sulphate-borate buffer system (Neville, 1971)) to be better than the system of Laemmli (1970) for investigating the WSF of cheese. However, they concluded that 'the resolution of the proteins in cheese was not as good in any of the three methods using SDS gels as in urea-containing gels' and did not recommend its use for cheese.

A modification of the method of Laemmli (1970), using a 16% (*w/v*) acrylamide gel and a 0.0625 M Tris-HCl sample buffer containing 2% (*w/v*) SDS and 5% mercapto-ethanol was used by Harper *et al.* (1989) in a study of the effect of whey proteins on proteolysis in Cheddar cheese slurries. The method of Laemmli has been used by various authors to study proteolysis (*e.g.*, Andrews and Alichanidis, 1983).

The "Phast-System" with SDS gels containing 20% acrylamide "in accordance with the manufacturer's instructions" was used by Calvo *et al.* (1992b) to monitor proteolysis during the ripening of Cheddar cheese made from overheated milk. Poor resolution of

the caseins was obtained, but resolution of the peptides and para-κ-casein was very good.

In New Zealand, two SDS-PAGE methods are currently in use. In the first method the resolving gel comprises 15.93% (*w/v*) acrylamide (2.6% C) and 0.1% (*w/v*) SDS in a Tris-HCl buffer at pH 8.8, the stacking gel comprises 3.88% (*w/v*) acrylamide (2.6% C) and 0.1% (*w/v*) SDS in a Tris-HCl buffer at pH 6.8, with a Tris-chloride-glycine chamber buffer (Laemmli, 1970) at pH 8.3 containing 0.1% (*w/v*) SDS. This method provides good separation of the caseins and whey proteins but does not capture many of the faster moving peptides. The second method is that of Giulian and Graham (1988) which has a resolving gel containing 20% (*w/v*) acrylamide (0.5% C) and 0.1% (*w/v*) SDS in a Tris-HCl buffer at pH 9.3, a stacking gel containing 10% (*w/v*) acrylamide (4.8% C) and 0.1% (*w/v*) SDS in a Tris-HCl buffer at pH 6.8, and a Tris-chloride-glycine chamber buffer at pH 8.5 containing 0.1% (*w/v*) SDS. This method captures and resolves many small peptides but does not separate the caseins well.

2.1.4 Two-Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis can be used to separate proteins and peptides in complex mixtures. Several gel combinations have been used. Starch gels with an acidic buffer for the first dimension and an alkaline buffer for the second dimension were used by Neelin *et al.* (1962) to identify and check the purity of casein fractions. Thin layer chromatography followed by alkaline urea gel electrophoresis was used to separate peptides produced by the action of plasmin on α_{s1}-casein (Aimutis and Eigel, 1982). An alkaline urea gel for the first dimension and SDS-PAGE for the second was used by Tutta *et al.* (1991) to resolve α_{s1}- and α_{s2}-casein in caprine milk. More commonly, a combination of isoelectric focusing and SDS-PAGE has been used. Trieu-Cuot and Gripon (1982) used two-dimensional gel electrophoresis (to study proteolysis in Camembert cheese) with isoelectric focusing in a 5% (*w/v*) acrylamide (0.5% bis) gel containing 2% ampholytes and 7 M urea used for the first dimension. The second dimension was a linear gradient gel of (0.4-28%) of a 16% (*w/v*) acrylamide (0.7% bis) gel solution containing 4.9 M urea, 0.1% (*w/v*) SDS in a Tris-HCl buffer at pH 8.8. Marshall and Williams (1988) investigated different varieties of cheese (that were solubilised by heating at 95°C in 2% (*w/v*) SDS and 5% 2-mercaptoethanol) using two-dimensional gel electrophoresis with SDS-PAGE and found the technique to give reproducible results.

2.2 SAMPLE PREPARATION

Most of the gel electrophoresis methods commonly used to analyse cheese incorporate urea and therefore the discussion on sample preparation is limited to methods containing urea.

It is necessary that samples analysed by gel electrophoresis should contain a minimal amount of fat so that streaking within the sample lanes of the gel does not occur. Several methods of cheese sample preparation have been used and they are all designed to remove the fat.

A Soxhlet apparatus was used by Melachouris and Tuckey (1966) to extract the fat from 5 g of cheese. The residue was then dialysed for 24 h at 5.5°C and dried with acetone and ether before being stored at 5.5°C. The most commonly used method for sample preparation is that of Ledford *et al.* (1966). In this method, the cheese is dispersed in a 7 M urea buffer and held at 37°C to facilitate the separation of the fat. The layer beneath the fat is then removed and treated with 2-mercaptoethanol 45 min prior to loading the sample on the gel. Several modifications of this method have been employed by different groups of workers. For example, the cheese may be dispersed in the urea buffer, warmed to 37°C and blended (in a stomacher or by an Ultra-Turrax) prior to the removal of the fat. Some workers have then centrifuged the samples at 4°C to obtain a more complete removal of the fat.

2.3 STAINING

There are several different dyes that can be used to stain the proteins and peptides in the gel. Amido black, in acetic acid with or without methanol/ethanol, has been used by many workers (*e.g.*, Wake and Baldwin, 1961; Aschaffenburg, 1964; Thompson *et al.*, 1964; Grindrod and Nickerson, 1967; Cheeseman, 1968; Kiddy *et al.*, 1972; Andrews, 1975; de Jong, 1975; El-Shibiny and Abd El-Salam, 1976; O'Keeffe *et al.*, 1976; Mulvihill and Fox, 1977; Thomas and Pearce, 1981; Creamer *et al.*, 1985; Wilkinson *et al.*, 1992). Most authors reported that the gels were destained in 5-7% acetic acid, with Wilkinson *et al.* (1992) using 7% acetic acid in 5% methanol.

Two very similar wool dyes that exhibit a greater intensity than Amido Black have been used more recently to stain proteins and peptides. They are Coomassie brilliant blue R-250 and Coomassie blue G-250. The use of Coomassie blue G-250 has been reported by many authors with several different methods (*e.g.*, Coomassie blue dissolved in

water (Furtado *et al.*, 1984), in 12.5% (*w/v*) TCA in 50% methanol (Anderson and Andrews, 1977; Andrews, 1983), in 9% acetic acid in 4.5% methanol (Thompson *et al.*, 1987)). A rapid staining method in which the background is not stained and the protein zones are visualised directly was described by Blakesley and Boezi (1977). The dye comprises a 0.2% (*w/v*) aqueous solution of PAGE blue G90 (Coomassie blue G250) mixed with an equal volume of 2 N H_2SO_4 that is allowed to stand for 3 h before filtering through Whatman No.1 filter paper. The filtrate is measured and a volume of 10 N KOH equal to 10% of the dye solution added. Trichloroacetic acid is added to a final concentration of 12% (*w/v*), the gels are stained for 3 h and transferred to water. This method has since been used by many workers (*e.g.*, Shalabi and Fox, 1987; McSweeney, 1993b; McSweeney *et al.*, 1993a,b,c,d). Trieu-Cuot and Gripon (1981, 1982) used Coomassie blue R-250 in 10% acetic acid in 50% methanol to study cheese ripening. Tieleman and Warthesen (1991) fixed the proteins in the gel with 50% methanol and 8% acetic acid overnight, stained with Coomassie blue R-250 and destained with a solution of 30% methanol and 8% acetic acid before storing the gels in 7% acetic acid. In New Zealand, isopropanol was used to replace methanol (due to its toxicity and volatility). The gels are stained in Coomassie blue R-250 in 10% acetic acid and 25% isopropanol (*e.g.*, Harper *et al.*, 1989; Johnston *et al.*, 1994) and destained in 10% acetic acid and 10% isopropanol.

Quantitation of the proteins can be achieved by measuring the amount of dye bound to the protein either by cutting out the dyed protein band and eluting the dye prior to measuring it spectrophotometrically (*e.g.*, El-Shibiny and Abd El-Salam, 1976; Marcos *et al.*, 1979) or by using a laser densitometer or a newer, and more accurate, image analysis densitometer. It has been found that within certain levels there is a linear relationship between the amount of dye bound and the amount of protein. McLean *et al.* (1984) determined the dye-binding of the casein components of milk. Creamer (1976a) estimated the dye-binding of the larger peptides released by the action of chymosin on β-casein. For most cheese studies the progress of proteolysis has been monitored by measuring the relative amounts of the different caseins as measured by the absorbance of the dye bound to the proteins (*e.g.*, Dulley, 1974; Thomas and Pearce, 1981; Creamer *et al.*, 1985, 1987, 1988a,b; Johnston *et al.*, 1994).

The reproducibility of gel electrophoresis at the NZDRI has been improved by employing a stringent staining (with Coomassie Brilliant Blue R-250) and destaining protocol over a fixed time period (Section 5.2). Hill (NZDRI, personal communication) found temperature dependent trends in the loss of dye from α-lactalbumin and β-

lactoglobulin bands on SDS-PAGE during destaining. These trends were believed to be related to fluctuations in room temperature and to temperature cycling in the cold room (6°C). We have found a large amount of variability in the purity of different brands of dye, as well as variability between different batches of dye from the same manufacturer and between different batches of stain made from the same batch of dye (M Tsao, NZDRI, personal communication).

3 OBJECTIVE

The objective of this work was to determine the usefulness of three existing gel electrophoretic methods and to adapt a fourth method to the mini-slab gel system to study proteolysis in cheese.

4 MATERIALS AND METHODS

4.1 CHEESE SAMPLES

Brie (both the outer "ripe" portion and the inner "unripe" portion) and Mozzarella cheeses were obtained from the Institute's pilot plant. "Mild" Cheddar cheese (Mainland brand), Mature Cheddar cheese (Mainland Vintage Tasty), Gruyère (Galaxy brand) and Feta made with cow's milk (Galaxy brand) were obtained from a local supermarket.

4.2 ALKALINE UREA-PAGE (SLAB GEL)

Proteins in the cheese were separated by alkaline urea-PAGE using a large slab gel (EC Corporation standard electrophoresis (EC 470) cell) and a Bio-Rad model 1000/500 power supply. This type of power supply allows either voltage, current or power to interchangeably become the limiting factor during electrophoresis.

Sample preparation: Cheese (0.500 g) was dispersed in 25 ml of sample buffer (0.0928 g EDTA, 1.08 g Tris, 0.55 g boric acid, 36.0 g urea dissolved in 50 ml water, adjusted to pH 8.4 with HCl and made up to 100 ml). The samples were heated to 40°C and held at that temperature for one hour, to get the fat into the liquid phase, prior to blending with an Ultra-Turrax T25 (Janke and Kunkel, IKA-Labortechnik, supplied by Labsupply Pierce, New Zealand) at approximately 24,000 rpm for 20 s. The warm samples were then centrifuged at 10,000 rpm at 4°C for 10 min to solidify and separate the fat. The aqueous subnatant (2 ml) was treated with 2-mercaptoethanol (10 μl/ml) and held for 18 h. Bromophenol blue (10 μl/ml of a 0.1% (*w/v*) solution) was added prior to loading 50 μl of the mixture into the gel slab.

Standard preparation: A casein standard was prepared by diluting 0.5 ml of a frozen solution of sodium caseinate (4.0% (*w/v*) made by dissolving freshly precipitated and washed casein at pH 7 with sodium hydroxide) with 4.0 ml of urea sample buffer. The standard was treated with 2-mercaptoethanol (10 μl/ml) and held for 18 h. Bromophenol blue (10 μl/ml of a 0.1% (*w/v*) solution) was added prior to loading 50 μl into the gel.

Gel preparation: 150 ml of a 7.5% (*w/v*) gel stock solution (15 g gelling agent (5% C) (Sigma) and 0.15 ml TEMED brought to 200 ml with urea sample buffer) was warmed to 20°C and degassed for 15 min by stirring on a magnetic stirrer while evacuating with a water pump. The temperature of the solution was readjusted to 17°C. Ammonium persulphate (0.15 g) was then added to the solution and was carefully but quickly mixed

in. The gel solution was poured into the apparatus immediately (at an angle of 40° to exclude air bubbles) and the comb inserted. The comb and excess gel were removed after the gel was set. Gels of 5% and 6% (*w/v*) acrylamide (5% C), but otherwise with the same composition, were also made.

Gel electrophoresis: The chamber buffer (21.58 g Tris, 10.96 g boric acid, 1.85 g EDTA in 1800 ml water, adjusted to pH 8.4 and brought to 2 litres with water) was added to the lower chamber (to a depth of 2-3 cm above the wire). The cheese samples (2% (*w/v*) cheese) (50 μl) and the casein standard (50 μl) were applied to the gel. Chamber buffer was carefully layered over the samples and the upper chamber was then filled (to 1 cm above the sample slots). The samples were electrophoresed with the power supply set at upper limits of 310 V, 120 mA and 20 W at a temperature of 17°C until the dye ran off the end of the gel (approximately 5 h).

Staining: The gel was stained with 500 ml Coomassie brilliant blue R solution (1.0 g Coomassie Blue R250 (Sigma), 500 ml isopropanol and 200 ml glacial acetic acid made up to 2 litres with water) for 15 hours, destained with four 500 ml changes of Coomassie destaining solution (200 ml isopropanol and 200 ml glacial acetic acid made up to 2 litres with water). The staining and destaining were done in sealed containers with continuous agitation. The gel was then photographed.

Photography: The gels were placed on a light box and were back-lit with fluorescent lighting. The emission spectra of the fluorescent light source has intense peaks at 406, 438 and 544 nm superimposed on broader emission bands. To achieve the greatest contrast between the background and the bands, the light must be restricted to a band of wavelengths that correspond to the area of maximum absorbance of the protein dye complexes, namely between 520 and 620 nm. This was achieved by using a combination of orange and green Hoya filters which restrict light at both high and low wavelengths (Creamer, 1992). The film (Kodak 100 ASA T-Max), which is sensitive to wavelengths from below 400 to about 620 nm, was exposed using a standard camera (Pentax K-1000 single lens reflex fitted with a 50 mm macro (close-focusing) lens) with the exposure controlled by varying the lens aperture from *f* 4 to 22 at a constant shutter speed of 1/15 s. For the colour photographs, Fuji Reala film was used. This film is preferable because it shows fluorescent light sources as white despite the spectral characteristics of the light (Creamer, 1992). The same camera set-up was used, but without the coloured filters.

4.3 ALKALINE UREA-PAGE (MINI GEL)

Proteins in the cheese were separated by alkaline urea-PAGE using the Bio-Rad Mini-Protean II system with 10 slot gels of 0.75 mm thickness and a Bio-Rad model 1000/500 power supply.

Sample preparation: Cheese samples were those prepared in Section 4.2. Aliquots (5 μl) of each sample were loaded into the gel slab.

Standard preparation: A casein standard was prepared by diluting 0.1 ml of a frozen solution of the sodium caseinate (described in Section 4.2) in 3.9 ml of urea sample buffer (described in Section 4.2). A skim milk standard was prepared by diluting 0.1 ml of skim milk with 3.9 ml of urea sample buffer. Each standard was treated with 2-mercaptoethanol (10 μl/ml) and held for 18 h. Bromophenol blue (10 μl/ml of a 0.1% (*w/v*) solution) was added to each standard prior to loading 10 μl of the mixture into the gel slab.

Gel preparation: The resolving gel was prepared by adding 8.0 ml of a 30% (*w/v*) stock solution of acrylamide/bis mixture (37.5:1, 2.6% C) to 11.9 ml of resolving gel buffer (9.2 g Tris, 0.8 ml HCl, 54.0 g urea in 100 ml water, adjusted to pH 8.8 with HCl and brought to 200 ml with water). The gel solution was warmed to 20°C and degassed for 15 min by stirring on a magnetic stirrer while evacuating with a water pump and 10 μl of TEMED was carefully mixed in. A 100 μl aliquot of ammonium persulphate solution (100 mg/1.0 ml water) was then added and the solution was carefully mixed. The gel solution (3.3 ml) was poured between two glass plates and overlaid with 400 μl of water. The water was removed after the gel was set and the stacking gel solution was applied. The stacking gel was prepared by adding 1.3 ml of the acrylamide/bis stock solution to 8.64 ml of urea sample buffer (Section 4.2). The gel solution was degassed (15 min/20°C) and 10 μl of TEMED was carefully mixed in. A 50 μl aliquot of ammonium persulphate solution (100 mg/ml of water) was then added and carefully mixed in prior to applying the solution to the top of the resolving gel and inserting a 10 slot comb. After the gel was set the comb was removed and the gel slots washed with water to remove unpolymerised gel solution and dried with filter paper.

Gel electrophoresis: A pair of gels was fitted into the electrode assembly and placed in the buffer chamber. A stock solution of chamber buffer (Section 4.2) was diluted 1:4 with water and 400 ml was used to completely fill the inner buffer chamber and partially fill the outer buffer chamber. The cheese samples (2% (*w/v*) cheese) and the

standards (5 μl and 10 μl respectively) were applied to the gel and electrophoresed with the upper limits of the power supply set at 210 V, 70 mA (an upper arbitrary value) and 6.5 W (setting for two gels electrophoresed simultaneously) for 1.7 h.

Staining: The gels were stained with 50 ml Coomassie brilliant blue R solution (Section 4.2) for 1 h and destained in 100 ml of Coomassie destain solution (Section 4.2) for 1 h. The destain was then replaced and destaining was continued for a further 19 h. The staining and destaining were done in sealed containers with continuous agitation.

Photography: The gels were photographed using the protocol described in Section 4.2.

4.4 ACID UREA-PAGE

Proteins in the cheese were separated by acid urea-PAGE using the Bio-Rad Mini-Protean II system with 10 slot gels of 0.75 mm thickness and a Bio-Rad model 1000/500 power supply.

An existing acid urea-PAGE method that was used in the large E-C Apparatus Corporation slab gel apparatus required considerable modification to make it suitable for use in the mini-slab gel system. An acidic sample buffer, a stacking gel and a resolving gel with a different pore size were required to achieve good resolution without streaking of the bands and the formation of 'horns' at the edges of each band.

Sample preparation: Cheese (0.500 g) was dispersed in 25 ml of sample buffer (2.82 g glacial acetic acid, 0.226 g ammonium acetate and 84 g urea made up to 200 ml (pH 4.16) and held at 40°C for one hour, to enable the fat to enter the aqueous phase, prior to blending with an Ultra-Turrax T25 at approximately 24,000 rpm for 20 s. The warm samples were then centrifuged at 10,000 rpm at 4°C for 10 min to separate and solidify the fat. The aqueous subnatant was diluted 1:1 with acid urea sample buffer and treated with 10 μl/ml of both 2-mercaptoethanol and basic fuchsin solution (3% w/v) prior to loading 5 μl of the mixture into the gel.

Standard preparation: A casein standard was prepared by diluting 0.1 ml of a frozen solution of sodium caseinate (Section 4.2) in 3.9 ml of acid urea sample buffer. A skim milk standard was prepared by diluting 0.1 ml of skim milk with 3.9 ml of acid urea sample buffer. Each standard was treated with 10 μl/ml of both 2-mercaptoethanol and basic fuchsin solution (3% w/v) prior to loading 5 μl of the mixture into the gel.

Gel preparation: The resolving gel was prepared by degassing 10 ml of a stock solution (9.75 g gelling agent (5% C) (Sigma), 40.36 g urea, 9.0 g glacial acetic acid, 0.72 g ammonium acetate and 0.26 g thiourea brought to 150 ml with water (pH 3.86)) for 15 min/20°C, cooling it to 17°C and carefully mixing in 30 μl of 30% hydrogen peroxide. The gel solution (3.3 ml) was poured between two glass plates and overlaid with 400 μl of water. The water was removed after the gel was set and the stacking gel solution was applied. The stacking gel was prepared by degassing 10 ml of a stock solution (5.00 g acrylamide (2.6% bis), 36.04 g urea, 0.123 g ammonium acetate and 1.41 g glacial acetic acid brought to 100 ml with water (pH 4.20)) for 15 min/20°C, cooling it to 17°C and carefully mixing in 30 μl of 30% hydrogen peroxide. The solution was applied to the top of the resolving gel and a 10 slot comb was inserted. After the gel was set the comb was removed and the gel slots washed with water to remove unpolymerised solution and dried with filter paper.

Gel electrophoresis: A pair of gels was fitted into the electrode assembly and placed in the buffer chamber. The chamber "buffer" (6% (*w/v*) glacial acetic acid) (500 ml) was added to completely fill the inner chamber and partially fill the outer chamber of the unit. The cheese samples (5 μl of the 1% (*w/v*) cheese solution) and the standards (5 μl) were applied to the gel. The samples were electrophoresed with the power supply set at 210 V, 70 mA (an upper arbitrary value) and 6.5 W (setting for two gels electrophoresed simultaneously) for 15 min after the dye front had left the gel. The normal direction of the current flow was reversed so that the positively charged proteins and peptides migrated towards the anode (rather than out the top of the gel).

Staining: The method of staining and destaining was the same as that described for alkaline urea gels (Section 4.3).

Photography: The gels were photographed using the protocol described in (Section 4.2).

4.5 LOW MOLECULAR WEIGHT SDS-PAGE

Proteins in the cheese were separated by "low molecular weight" (LMW) SDS-PAGE (a method that uses 20% (*w/v*) acrylamide (0.5% C) to enable the capture of small peptides) using the Bio-Rad Mini-Protean II system with 10 slot gels of 0.75 mm thickness and a Bio-Rad model 1000/500 power supply.

Sample preparation: Cheese (0.500 g) was dispersed in 25 ml of sample buffer (10 ml of 10% (*w/v*) SDS solution, 15 ml glycerol, 0.23 g dithiothreitol, 12.5 ml of 0.5 M Tris-HCl buffer (6.057 g Tris in 80 ml water, adjusted to pH 6.8 and brought to 100 ml with water) and 60.50 ml water) and held at 40°C for one hour, to enable the fat to enter the aqueous layer, prior to blending with an Ultra-Turrax T25 at approximately 24,000 rpm for 20 s. The warm samples were then centrifuged at 10,000 rpm at 4°C for 10 min to separate and solidify the fat. The aqueous subnatant was heated to 100°C using a heating block and held at that temperature for 90 s. Bromophenol blue (10 μl/ml of a 0.1% (*w/v*) solution) was added prior to loading 5 μl of the mixture onto the gel.

Standard preparation: A casein standard was prepared by diluting 0.1 ml of a frozen solution of sodium caseinate (Section 4.2) in 3.9 ml of LMW SDS sample buffer. A skim milk standard was prepared by diluting 0.1 ml of skim milk with 3.9 ml of LMW SDS sample buffer. The standards were heated to 100°C using a heating block and held at that temperature for 90 s. Bromophenol blue (10 μl/ml of a 0.1% (*w/v*) solution) was added to each standard prior to loading 10 μl of the skim milk standard and 5 μl of the casein standard onto the gel.

Gel preparation: The resolving gel solution was prepared with 9.07 ml of resolving gel stock acrylamide solution (37.5 g acrylamide and 0.1875 g bis brought to 100 ml with water), 1.70 ml glycerol, 4.24 ml of 3.0 M Tris-HCl buffer (36.342 g Tris in 80 ml water, adjusted to pH 9.3 with HCl and brought to 100 ml with water), 0.17 ml of a 10% (*w/v*) SDS solution and 1.75 ml of water. The gel solution was warmed to 20°C and degassed for 15 min by stirring on a magnetic stirrer while evacuating with a water pump. To prevent the loss of gel solution due to foaming of the SDS it was necessary to release the vacuum periodically during the first 2 min of evacuation. An aliquot (24 μl) of TEMED was carefully mixed in. An aliquot (48 μl) of ammonium persulphate solution (100 mg/1.0 ml water) was then added and the solution carefully mixed. The gel solution (3.3 ml) was poured between two glass plates and overlaid with 400 μl of water. The water was removed after the gel was set and the stacking gel solution was then applied. The stacking gel was prepared with 2.52 ml of a stacking gel stock acrylamide solution (37.5 g acrylamide and 1.875 g bis brought to 100 ml with water), 1.0 ml glycerol, 1.0 ml of 0.5 M Tris-HCl buffer, 0.1 ml of a 10% (*w/v*) SDS solution and 3.82 ml of water. The gel solution was degassed (15 min/20°C) and 24 μl of TEMED was carefully mixed in. An aliquot (30 μl) of ammonium persulphate solution (100 mg/ml of water) was then added and carefully

mixed in prior to applying the solution to the top of the resolving gel and inserting a 10 slot comb. After the gel was set the comb was removed, the gel slots were washed with water to remove unpolymerised solution and dried with filter paper.

Gel electrophoresis: A pair of gels was fitted into the electrode assembly and placed in the buffer chamber. A stock solution of chamber buffer (28.54 g glycine, 6.057 g Tris and 10.0 ml of a 10% (*w/v*) SDS solution in 800 ml water, adjusted to pH 8.4-8.6 and brought to 1 litre with water) was prepared and 300 ml was used to completely fill the inner buffer chamber and partially fill the outer buffer chamber. The cheese samples (2% (*w/v*) cheese) and the casein standard (5 μl and 10 μl respectively) were applied to the gel. The samples were electrophoresed with the power supply set at 210 V, 70 mA (an upper arbitrary value) and 6.5 W (setting for two gels electrophoresed simultaneously).

Staining: The method of staining and destaining was the same as that described for alkaline urea gels (Section 4.3).

Photography: The gels were photographed using the protocol described in (Section 4.2).

4.6 TWO-DIMENSIONAL PAGE

Samples of "Mature" Cheddar and Feta cheeses were selected to be examined using two dimensional PAGE. Alkaline urea-PAGE was selected for the first dimension and the second dimension was either LMW SDS-PAGE or acid urea-PAGE.

First dimension: A pair of alkaline urea gels were prepared according to the methods outlined in Section 4.3. The Feta cheese sample (Section 4.2) was loaded (a 10 μl aliquot was used as preliminary experiments showed that 5 μl was insufficient to allow much protein and peptide material to be visualised on the gel used for the second dimension) into the eight centre wells and 10 μl of the casein standard (Section 4.3) was loaded into the two outer wells of the first gel. The "Mature" Cheddar cheese sample (Section 4.2) and the casein standard were loaded into the second gel in the same manner. The gels were electrophoresed, removed from the glass plates and cut in half longitudinally. One half of each gel was stained, destained and photographed as previously described.

Second dimension for the "Mature" Cheddar cheese: Four single lanes from the portion of the gel containing the unstained "Mature" Cheddar cheese sample were excised. Two of the gel strips were pre-equilibrated in acid urea sample buffer and the other two in LMW SDS sample buffer for 5 min. (Preliminary experiments in which the gel strips were equilibrated for 20 min resulted in the loss of proteins and peptides from the gel with very little material entering the gel used for the second dimension. Equilibration for 5 min was found to be adequate.) Each gel strip was placed between a set of glass plates at the top of the smaller plate, to enable the resolving and stacking gels to be poured beneath it, and assembled in the gel apparatus.

An acid urea resolving gel solution was prepared (Section 4.4) and 3.3 ml was poured into each of the sets of plates containing the gel strips that had been equilibrated in acid urea sample buffer. The gel solution was overlaid with water (400 μl), taking care not to wet the gel strip.

A LMW SDS resolving gel solution was prepared (Section 4.5), poured and overlaid with water in the manner described in the preceding paragraph.

After the various resolving gels had set the water was removed and the appropriate stacking gel solution was poured (with the apparatus tilted at a 45° angle to prevent air bubbles from being trapped beneath the gel strips). A comb that had the central seven "teeth" removed was inserted into each of the stacking gels. After the gels were set the combs were removed, the gel slots were washed with water to remove unpolymerised gel solution and dried with filter paper. A casein standard (10 μl) was loaded into one of the outer wells of each gel and the corresponding cheese sample, in the appropriate buffer, was loaded into the other. The gels were electrophoresed, stained, destained and photographed using the methods previously described.

Second dimension for the Feta cheese: To improve resolution in the second dimension the following modifications were made to the above method. The gel strips were reduced in width, so that there was no gel (from between the lanes of the first gel) at the edges of the bands, to make room for a deeper stacking gel; the resolving gel volume was reduced to 3.0 ml to allow space for a deeper stacking gel; the "teeth" of comb were modified to make the wells shallower, in an endeavour to get the distance travelled by the caseins in the standards more similar to those travelled by the material from the gel strip. These improvements should be applied to further experiments involving other cheese types.

5 RESULTS

5.1 ALKALINE UREA-PAGE (SLAB GEL)

The band patterns of the different cheese types that were electrophoresed on the large slab alkaline urea gel made with 7.5% (*w/v*) acrylamide are presented in Figure 2.1. Three acrylamide concentrations (5, 6 and 7.5%) were compared, but only the gel containing 7.5% (*w/v*) acrylamide (*w/v*) (5% C) gave good resolution of the proteins and peptides with bands that were a uniform shape. This is consistent with the finding of Peterson (1963) that 7% (*w/v*) acrylamide was the minimum level required to give good separation of the α_s-caseins.

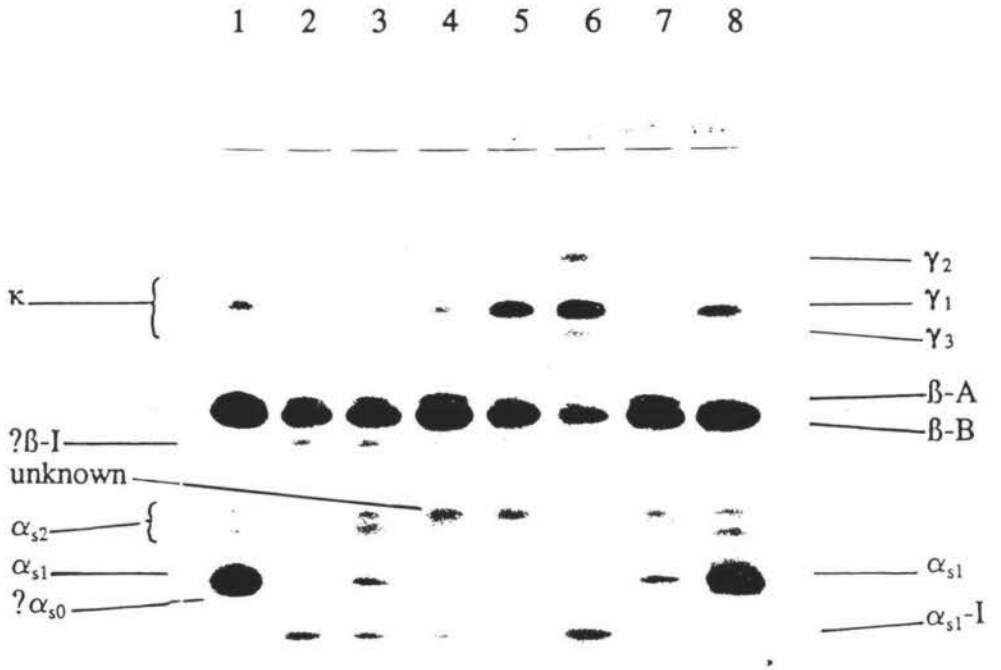

FIGURE 2.1 Polyacrylamide gel electrophoresis patterns of the different cheese varieties on the large alkaline urea (slab) gel. Lane 1 - Casein standard, lane 2 - Brie outer, lane 3 - Brie inner, lane 4 - Mild Cheddar, lane 5 - Mature Cheddar, lane 6 - Gruyère, lane 7 - Feta, lane 8 - Mozzarella.

5.2 ALKALINE UREA-PAGE (MINI GEL)

The band patterns of the different cheese varieties electrophoresed on the alkaline urea mini-slab gel are presented in Figure 2.2. The use of this method resulted in good resolution of the proteins and peptides in each of the cheese samples with the formation of bands of a uniform shape. (An unidentified band that migrates in the same position as α_{s2}-casein and has a bluer hue than the surrounding bands was observed). The position of the bands located between β- and α_{s1}-casein appears to differ in the large-slab (Figure 2.1) and the mini-slab (Figure 2.2) gel systems.

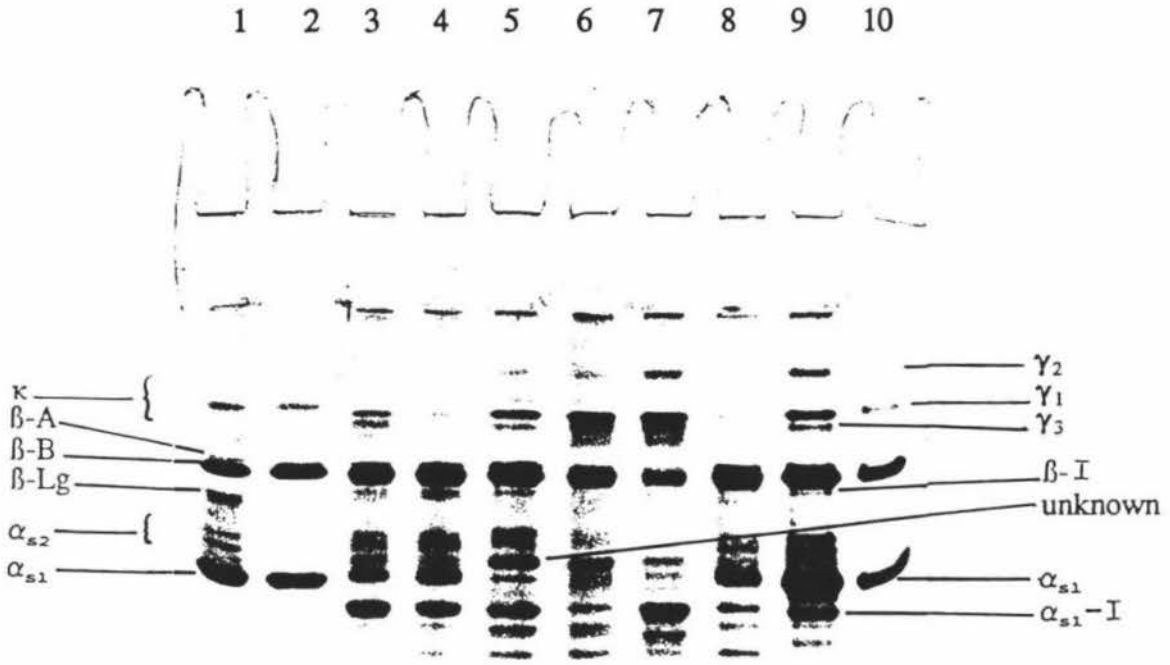

FIGURE 2.2 Polyacrylamide gel electrophoresis patterns of the different cheese varieties on the alkaline urea mini-slab gel. Lane 1 - Skim milk, lanes 2 and 10 - Casein standard, lane 3 - Brie outer, lane 4 - Brie inner, lane 5 - Mild Cheddar, lane 6 - Mature Cheddar, lane 7 - Gruyère, lane 8 - Feta, lane 9 - Mozzarella.

It was observed during the course of this study that the intensity of the stained bands continued to decrease if the gel was allowed to remain in the destain solution. Attempted restaining of the bands in the gel showed no improvement and hence this loss of colour intensity could be attributed to the proteins leaching from the gel. Based on these findings, a standard protocol was established where the gels were stained for 1 h with 50 ml of stain, destained for 1 h with 100 ml of destain and destained for 19 h with a further 100 ml of destain. The gels were then photographed. Strict adherence to these times was found to be necessary for reproducible results.

5.3 ACID UREA-PAGE

The results depicted in Figure 2.3A represent the culmination of the many developmental steps that led to this acid urea gel electrophoresis method. The method employs a continuous acidic buffer system with discontinuities between the buffer concentrations (0.25 M acetic acid sample and stacking gel buffers to 1.04 M acetic acid resolving gel buffer), the pore size of the gels (a large pore stacking gel (5% (*w/v*) acrylamide (2.6% C)) and a small pore resolving gel (6.5% (*w/v*) acrylamide (5% C)), and the urea concentrations of the sample buffer (7 M), the stacking gel (6 M) and the resolving gel (4.5 M).

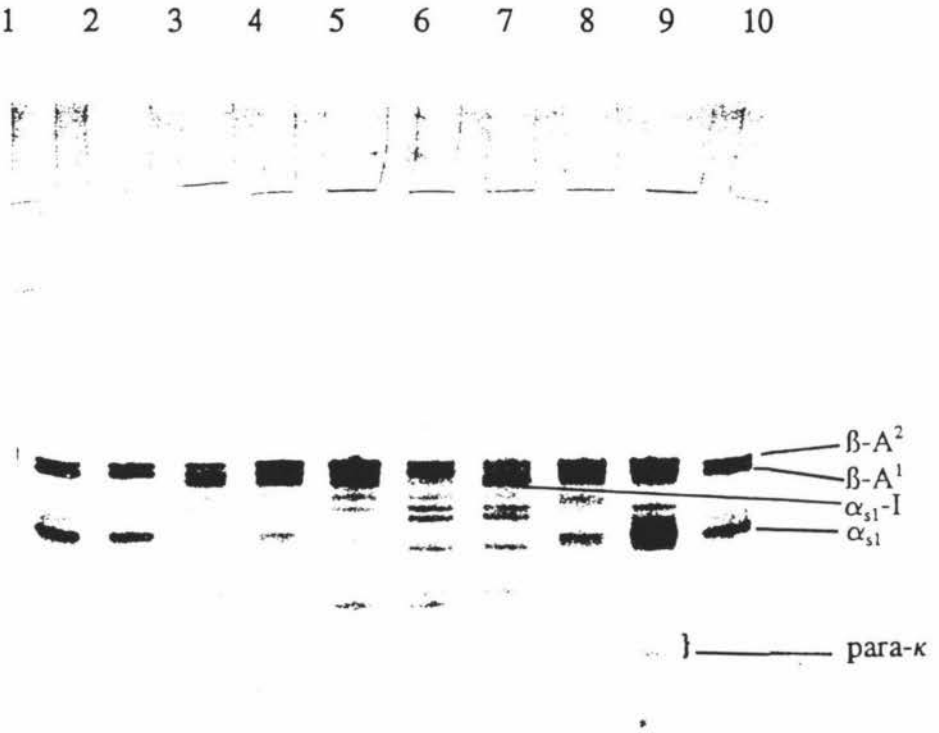

FIGURE 2.3A Polyacrylamide gel electrophoresis patterns of the different cheese varieties on the acid urea mini-slab gel. Lane 1 - Skim milk, lanes 2 and 10 - Casein standard, lane 3 - Brie outer, lane 4 - Brie inner, lane 5 - Mild Cheddar, lane 6 - Mature Cheddar, lane 7 - Gruyère, lane 8 -Feta, lane 9 - Mozzarella.

The proteins and peptides were well resolved and by referring to the alkaline urea gel (Figure 2.2) it was possible to tentatively determine the identity of the α_{s1}-casein, α_{s1}-casein-I and β-casein bands. Based on earlier unpublished work of K N Pearce (personal communication), a band was tentatively identified as para-κ-casein. Differences in the protein concentration of the samples, due to the different moisture

contents of the various cheese types, may account for some of the differences observed in the concentration of this and other bands on the gel. When more concentrated cheese samples were electrophoresed the gel system became overloaded and good separation of bands in the β-A^1A^2 region was not possible.

The original gel method was carried out in large, thick slab gels using the apparatus of the E-C Apparatus Corporation. This method consisted of an 8% (*w/v*) acrylamide gel (5% C) in an ammonium acetate buffer containing 4.5 M urea. Samples made up (according to the method) in the sample buffer used for alkaline gels were electrophoresed on the gel. Presumably the larger volume of acid in the thick slab gels allowed the alkaline samples to equilibrate during the initial stages of the long running time. When this method was transferred to the mini-slab gel system the gels were slow to set and were soft, the electrophoretic profiles were badly streaked and the proteins and peptides were poorly resolved.

To improve polymerisation, three different levels of 30% H_2O_2 (2, 3 and 4 μl of H_2O_2/ml of gel solution) were tested in the gel solution and it was found that 3 μl of H_2O_2/ml of gel solution resulted in a gel that set well within 30 min while lower levels of H_2O_2 resulted in gels that did not polymerise properly.

A large pore stacking gel was formulated to improve resolution. Selection of the level of acrylamide and bisacrylamide required to obtain an appropriate pore size was based on the work of Fawcett and Morris (1966) (cited by Gordon, 1969). Levels of acrylamide of 3, 4 and 5% (*w/v*) (2.6% C) were investigated and it was found that 5% (*w/v*) acrylamide was the minimum required for the gels to set. It was also noted that at lower pH (3.5) the stacking gel did not polymerise properly. The development of the stacking gel was done in conjunction with the development of an acidic sample buffer (following paragraph). Two levels of urea (6 M and 7 M) were evaluated in the stacking gel and it was found that the use of 6 M urea produced a discontinuity between the 7 M urea sample buffer and the stacking gel that improved the resolution of the bands. The final stacking gel comprised 6 M urea and 5% (*w/v*) acrylamide (2.6% C) in an acetic acid-ammonium acetate buffer at pH 4.2.

It was noted that streaking within lanes was less marked when smaller amounts of sample were loaded on the gel and also when the gels were run for a longer time by lowering the maximum voltage and power (105 V, 65 mA (upper arbitrary value) and 3.25 W), which suggested the possibility that the alkalinity of the sample buffer was

causing the problem. An acidic sample buffer (7 M urea (Peterson, 1963) in an acetic acid-ammonium acetate buffer at pH 4.16) was formulated and cheese samples were prepared in the same manner as those that had been prepared in the alkaline sample buffer. Both sets of samples were electrophoresed on the same gel, a 6.5% (*w/v*) acrylamide (5% C) resolving gel with a 5% (*w/v*) acrylamide (2.6% C) stacking gel, and it was found that dispersing the samples in the acidic sample buffer eliminated the streaking problem (Figure 2.3B). Lanes 2 and 6 demonstrate the deleterious effect of the alkaline sample buffer on the electrophoretic profiles of the samples, while lanes 3 - 5 and 7-9 clearly demonstrate the benefits of the acidic sample buffer.

Throughout these experiments it had been noted that there were often multiple bands migrating between the origin and β-casein. It was believed that these bands were dimers and trimers *etc.* of α_{s2}-casein that had arisen due to the incomplete reduction of this casein and that 2-mercaptoethanol may not be effective at reducing disulphide bonds at this acid pH (K N Pearce, personal communication). An experiment in which selected samples prepared without 2-mercaptoethanol, with the normal level (10 μl/ml) of 2-mercaptoethanol and with twice the normal level of 2-mercaptoethanol were electrophoresed on the same gel was performed (Figure 2.3B). Lanes 3 and 7 contained no 2-mercaptoethanol and the formation of multiple bands between the origin and β-casein was evident. The inclusion of 2-mercaptoethanol at both the normal level (lanes 4 and 8) and twice the normal level (lanes 5 and 9) prevented the formation of these bands. This work combined with the earlier observations suggests that to ensure that all disulphide bonds are effectively reduced, the samples should have 2-mercaptoethanol added to them approximately an hour before they are electrophoresed and that samples that had been prepared at an earlier time should have more 2-mercaptoethanol added to them. A further possibility would be to use dithiothreitol or dithioerythritol which are more effective.

An attempt to further improve the separation of the protein and peptide bands by decreasing the acrylamide concentration from 8% (*w/v*) (5% C) to 6.5% (5% C) was made. This resulted in a wider spread of the bands but made no difference to the band patterns and the band tentatively identified as α_{s1}-I-casein was not quite as well separated from the β-casein bands. The final resolving gel was composed of 4.5 M urea and 6.5% (*w/v*) acrylamide (5% C) in an acetic acid-ammonium acetate buffer at pH 3.86. (The gel method incorporating 6.5% acrylamide (*w/v*) (5% C) has become a standard method for the analysis of β-casein variants in our laboratories.)

FIGURE 2.3B Acid urea-PAGE. Lane 1 - casein standard in acidic buffer, lane 2 -"Mature" Cheddar in alkaline buffer, lane 3 - "Mature" Cheddar in acid buffer with no 2-mercaptoethanol, lane 4 - "Mature" Cheddar in acid buffer with the normal level (10 μl/ml) of 2-mercaptoethanol, lane 5 - "Mature" Cheddar in acid buffer with twice the normal level of 2-mercaptoethanol, lane 6 - Feta in alkaline buffer, lane 7 - Feta in acid buffer with no 2-mercaptoethanol, lane 8 - Feta in acid buffer with the normal level of 2-mercaptoethanol, lane 9 - Feta in acid buffer with twice the normal level of 2-mercaptoethanol, lane 10 - casein standard in alkaline buffer.

Multiple bands between the origin and β-casein were formed in the electrophoretic profiles of the samples with no 2-mercaptoethanol (lanes 3 and 7) but these bands, although initially sharp, faded rapidly and were difficult to capture on film.

5.3.1 Two-dimensional gel electrophoresis to identify bands separated by acid urea-PAGE

Two-dimensional gel electrophoresis, in which the first dimension was an alkaline urea gel (in which the identity of many of the bands was known) and the second dimension was an acid urea gel (8% acrylamide, to achieve better separation of the α_{s1}-casein-I from the β-casein), was used to assist in the identification of the proteins and peptides. Both the "Mature" Cheddar and the Feta cheese samples were selected to be electrophoresed in the two dimensions and Figures 2.4 and 2.5 show the gel electrophoresis profiles with the likely identity of the bands shown alongside the gel.

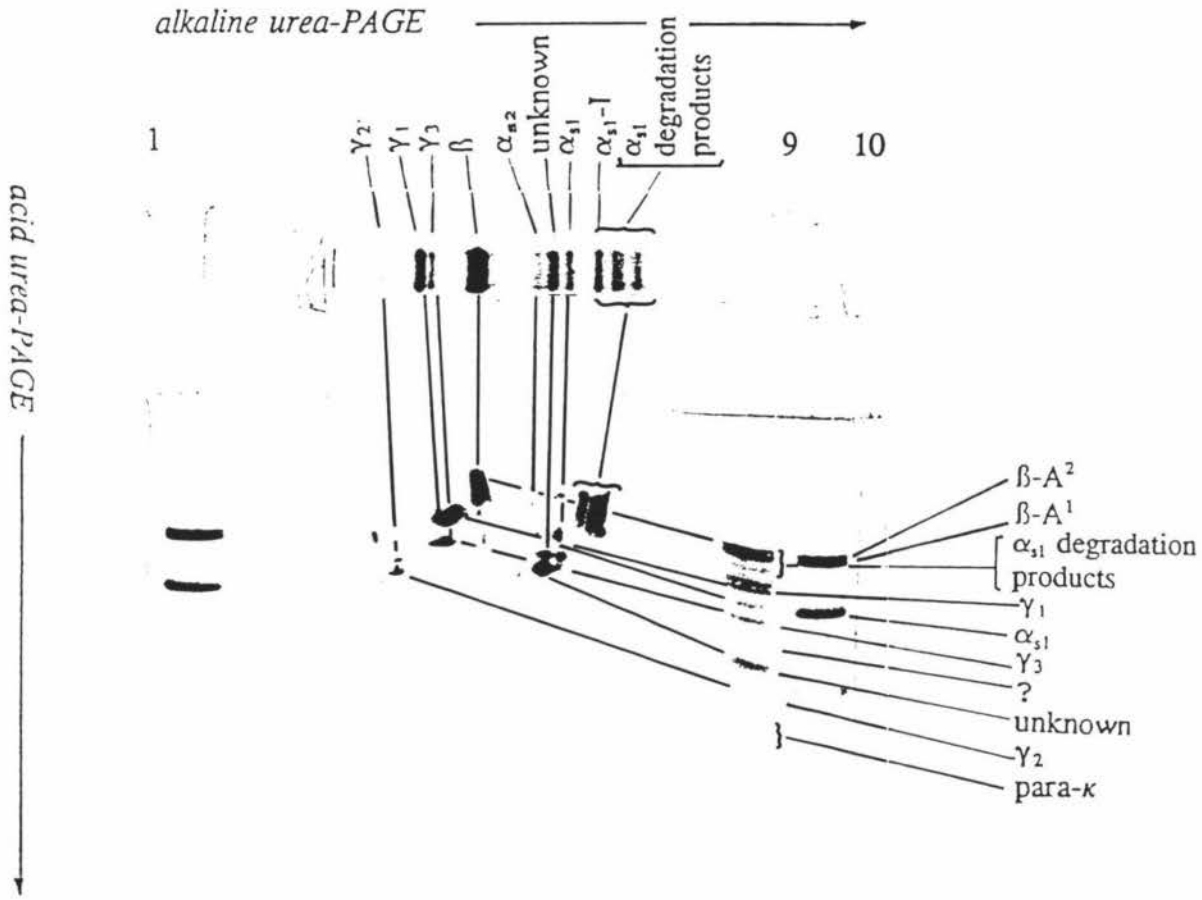

FIGURE 2.4 Two-dimensional polyacrylamide gel electrophoresis. "Mature" Cheddar cheese electrophoresed on an alkaline urea gel for the first dimension and an acid urea gel for the second dimension. Lanes 1 and 10 - Casein standard, lane 9 -"Mature" Cheddar cheese.

The use of two-dimensional PAGE to investigate the "Mature" Cheddar cheese enabled the detection of more bands on the acid urea gel than were visible on the alkaline urea gel. It was possible to tentatively identify the position of the $\gamma_{1\text{-}3}$-caseins and the "unknown" band based on the patterns obtained by two-dimensional gel patterns (Figure

2.4) which were interpreted in conjunction with the profiles obtained by alkaline urea and acid urea-PAGE (Figures 2.2 and 2.3A, respectively).

Modifications to the method, described in Section 4.6, enabled the sample (first dimension) to enter the stacking gel (second dimension) more rapidly and allowed the stacking gel size to be increased which resulted in a significant improvement in the resolution of the protein and peptide bands (Figure 2.5).

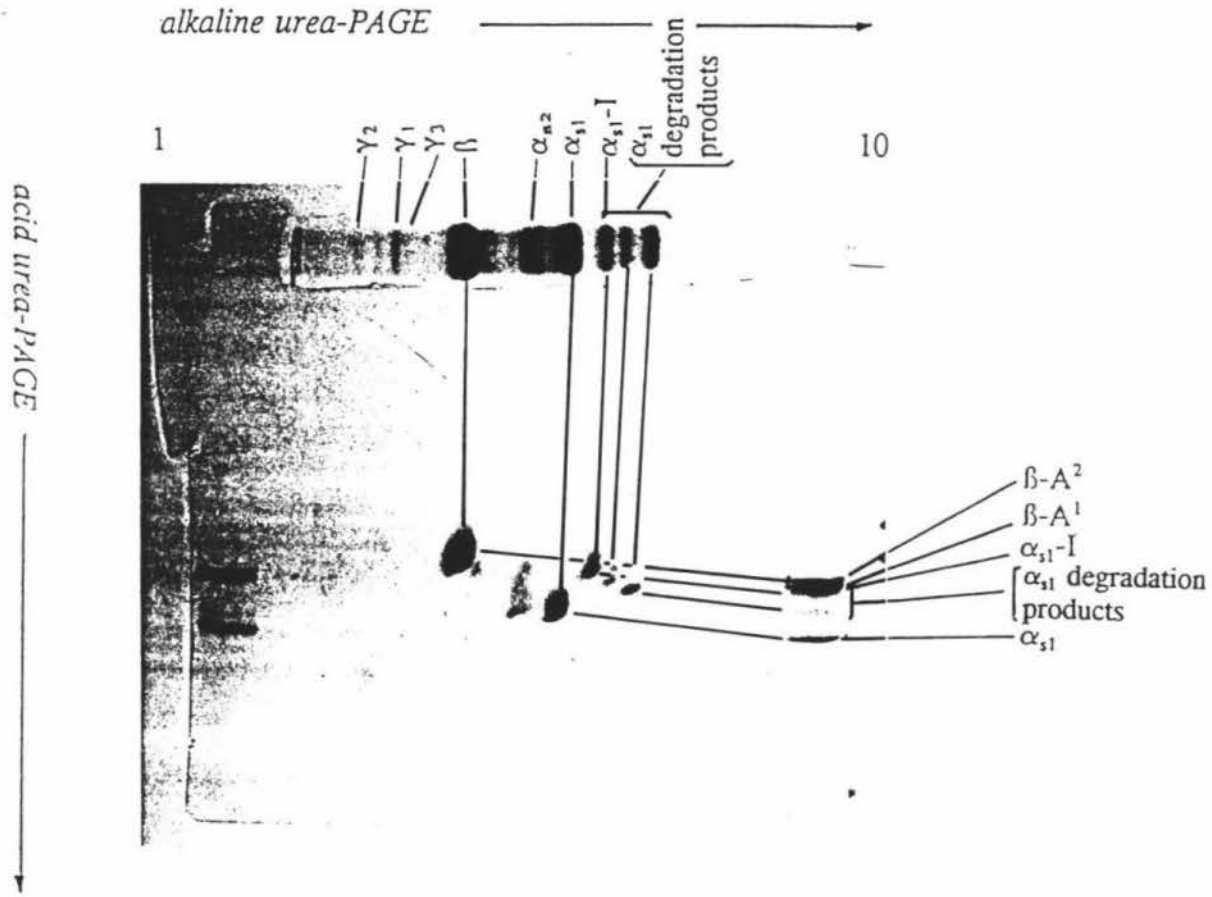

FIGURE 2.5 Two-dimensional polyacrylamide gel electrophoresis. Feta cheese sample electrophoresed on an alkaline urea gel for the first dimension and an acid urea gel for the second dimension. Lane 1 - Casein standard, lane 10 - Feta cheese.

The area in the acid urea gel that corresponds to the α_{s2}-casein region in the alkaline gel is diffuse and probably has several components.

5.4 LOW MOLECULAR WEIGHT SDS-PAGE

LMW SDS-PAGE was used to separate the proteins and peptides of the various cheese samples and the gel presented in Figure 2.6 shows the band patterns obtained.

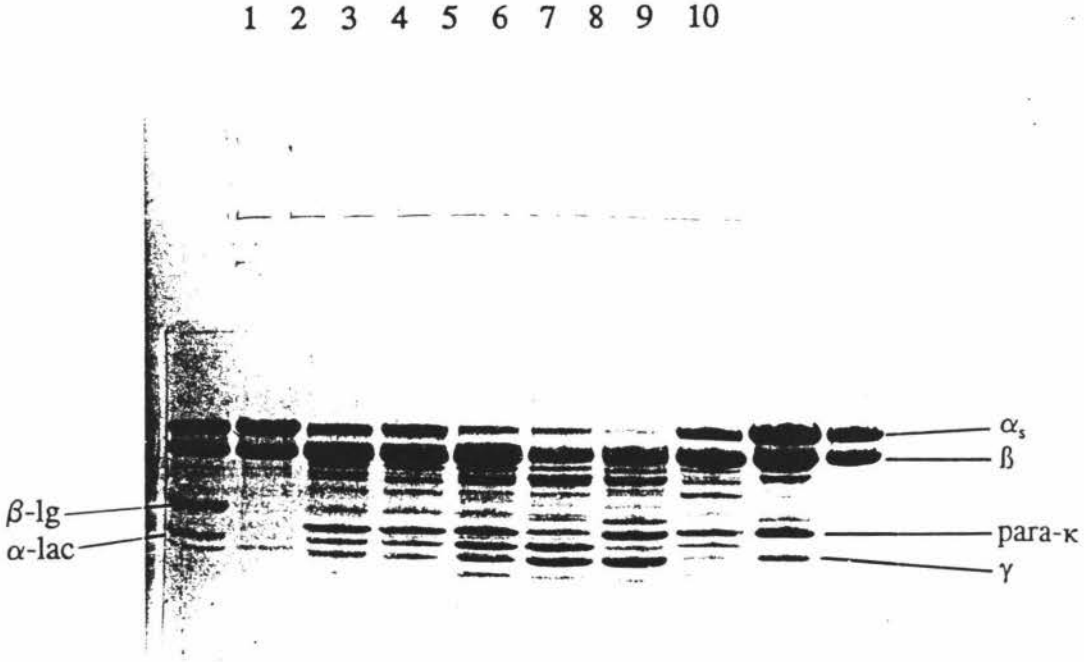

FIGURE 2.6 Polyacrylamide gel electrophoresis patterns of the different cheese varieties on the "low molecular weight" SDS mini-slab gel. Lane 1 -Skim milk, lanes 2 and 10 - Casein standard, lane 3 - Brie outer, lane 4 - Brie inner, lane 5 - Mild Cheddar, lane 6 - Mature Cheddar, lane 7 -Gruyère, lane 8 - Feta, lane 9 - Mozzarella.

Using this method, the proteins were poorly resolved and the protein bands were not a uniform shape. Good resolution of the peptides was achieved but the identity of most of the bands was unknown.

5.4.1 Two-dimensional electrophoresis to identify bands separated by LMW SDS-PAGE

To assist in establishing the identity of these bands the "Mature" Cheddar and Feta cheese samples were electrophoresed in two dimensions, with alkaline urea gels used for the first dimension and LMW SDS gels used for the second dimension. The resulting gels are presented in Figures 2.7 and 2.8 and the likely identity of the bands is recorded alongside the gel.

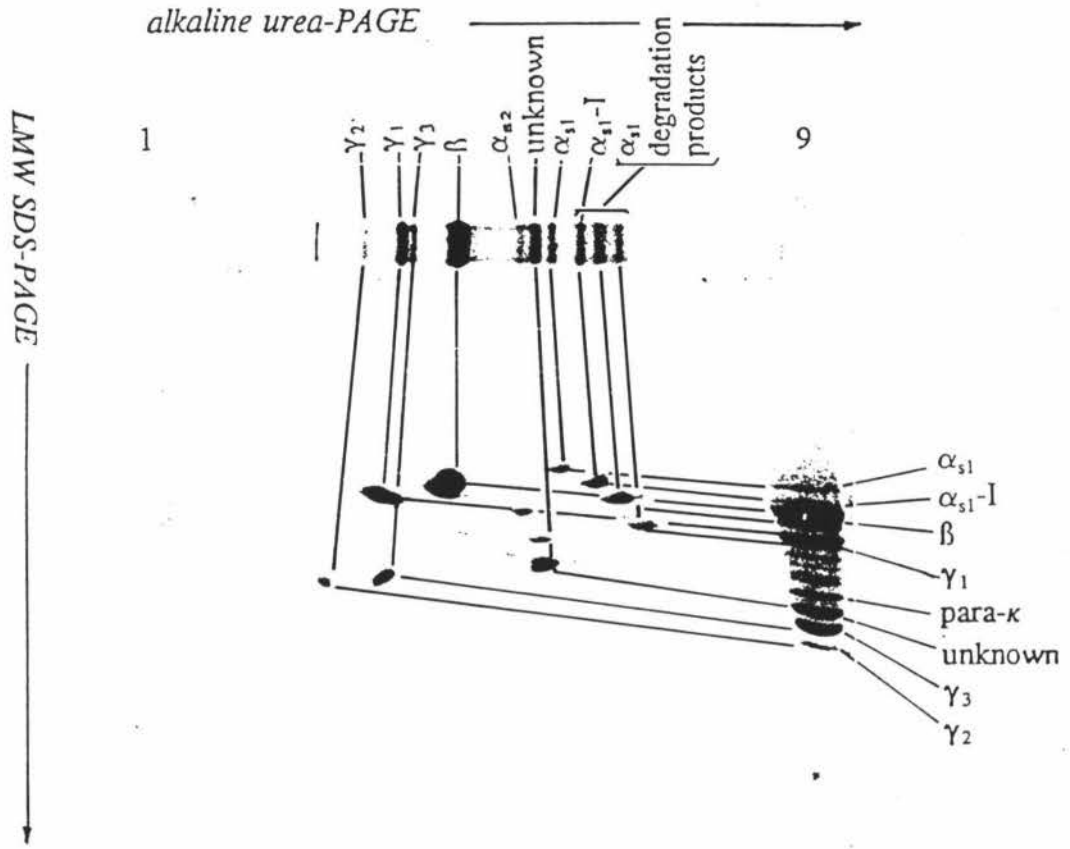

FIGURE 2.7 Two-dimensional polyacrylamide gel electrophoresis. "Mature" Cheddar cheese electrophoresed on an alkaline urea gel for the first dimension and a LMW SDS gel for the second dimension. Lane 1 - skim milk standard, lane 9 - "Mature" Cheddar cheese.

Very good separation of the γ_2- and γ_3-caseins was achieved using this method despite the very small difference between the two caseins. The γ_2-casein (His_{106} - Val_{209}) and γ_3-casein (Glu_{108} - Val_{209}) differ by only two amino acid residues, with γ_2-casein having an extra His and Lys residue. This confers a slightly greater positive charge on the γ_2-casein, enabling it to bind slightly more SDS, which was sufficient to give it a greater electrophoretic mobility and allow the two caseins (polypeptides) to be separated.

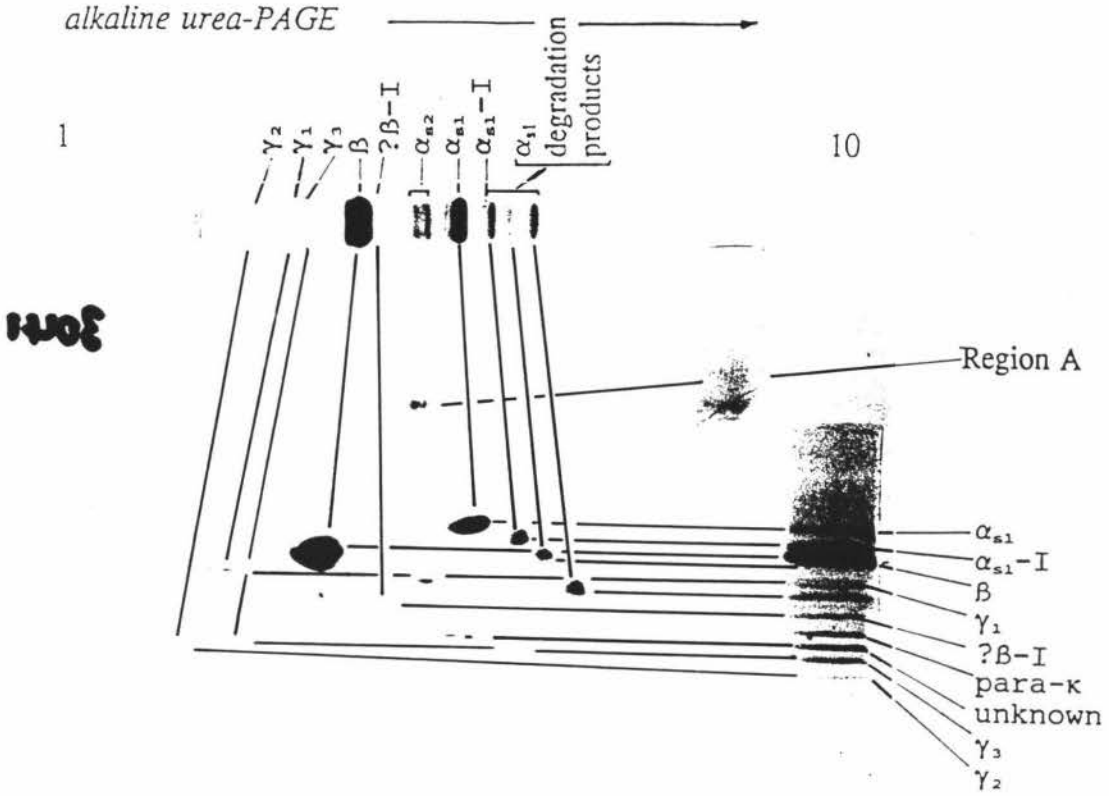

FIGURE 2.8 Two-dimensional polyacrylamide gel electrophoresis. Feta cheese electrophoresed on an alkaline urea gel for the first dimension and a LMW SDS gel for the second dimension. Lane 1 - Skim milk standard, lane 10 - Feta cheese.

The effects of the modifications outlined in section 4.6, that decreased the time taken for the sample (first dimension) to enter the stacking gel (second dimension) and increased the size of the stacking gel, are less dramatic on the longer running LMW SDS gels than on the faster running acid urea gels (Figures 2.4 and 2.5). However the bands are flatter, with less "tailing", and are better aligned with those of the standard.

The presence of a band in the same position as α_{s1}-casein on the alkaline urea gel was demonstrated by two-dimensional electrophoresis (Figure 2.7).

The region in which α_{s2}-casein was located in the alkaline urea gel was demonstrated, by two-dimensional PAGE, to have several components including one (Region A) that migrated very slowly and failed to enter the resolving gel.

6 DISCUSSION

The loss of intact casein and the formation of peptides can be monitored by various types of gel electrophoresis to obtain information on many aspects of cheese ripening. For quantitative purposes it is important that the protein and peptide bands are well resolved and a uniform shape.

The proteins and peptides in the cheese samples electrophoresed on the large-slab alkaline urea gel (Figure 2.1) were well resolved with bands that had a uniform width. The gel patterns were similar to those published by other authors (*e.g.*, de Jong, 1975; Creamer, 1985). However, the large-slab gel method was considered to be too time consuming and unwieldy to be useful for the routine analysis of cheese on the scale that was anticipated at the time the ripening study in Chapter 4 was planned.

The proteins and peptides in the cheese samples electrophoresed on the mini-slab alkaline urea gel (Figure 2.2) were apparently well resolved and were a uniform shape. In comparison with the large-slab gel method, the mini-slab gel method was fast, allowing a large number of samples to be analysed in a more reasonable time. A comparison of the profiles obtained using the large-slab (Figure 2.1) and the mini-slab (Figure 2.2) gels showed that the separation of the bands was very similar but revealed some differences in the location of the bands in the region between β- and α_{s1}-casein. An examination of the two-dimensional gel profiles (Figures 2.4, 2.7 and 2.8) revealed that approximately twice as many bands were present than were visible in the alkaline urea gels. However, none of these bands was sufficiently large to affect the quantitation of the proteins greatly. The presence of a band migrating in the same position as α_{s1}-casein was noted (Figure 2.7) and this explained why, despite extensive proteolysis in some of the cheese varieties, some α_{s1}-casein apparently remained. The limitations of this method are that the lower molecular weight peptides are not captured in the gel and that differences in dye-binding of the different proteins and peptides (McLean *et al.*, 1982) mean that, for the method to be truly quantitative, standard curves for each casein must be plotted. However, for studies of proteolysis in cheese it is usually sufficient to monitor the loss of intact casein (Chapter 4).

Acid urea-PAGE using the mini-slab gel system (Figure 2.3A) provided a rapid but not particularly helpful method for separating the proteins and peptides in cheese. The application of cheese samples of the concentration that was used for the alkaline urea-PAGE mini-slab method overloaded the system and separation of bands in the βA^1A^2

region was not possible. The small amount of sample that was found to be necessary to achieve good resolution of the proteins and peptides when using the acid urea-PAGE method meant that, from a quantitative aspect, the method was not considered to be particularly useful for studying proteolysis in cheese. Two-dimensional gel electrophoretic studies revealed that the α_{s1}-I-casein and some of the other α_{s1}-casein degradation products were located in the same region as β-casein on the acid urea mini-slab gel (Figures 2.4 and 2.5). Therefore, from both a qualitative and a quantitative aspect, acid urea-PAGE was not suitable for the study of proteolysis in cheese. The method is, however, useful for the identification of the different genetic variants of β-casein in milk. It was hoped that this method could be used to monitor para-κ-casein during cheese ripening, but the band tentatively identified as para-κ-casein is diffuse and may be composed of several bands (Figure 2.3A).

In agreement with Shalabi and Fox (1987) it was found that the 20% (*w/v*) polyacrylamide SDS gel did not produce good resolution of the caseins and was therefore not useful for monitoring the loss of casein during cheese ripening. The good resolution of the peptides that was obtained did indicate that this technique was likely to be useful in the study of the generation of peptides during ripening (Chapter 4). Good resolution of para-κ-casein, β-lactoglobulin and γ-caseins was also obtained. The SDS-PAGE method is therefore of use in the study of cheese in which whey proteins are included (Harper *et al.*, 1989; Calvo *et al.*, 1992a) and in the study of para-κ-casein in cheese (Calvo *et al.*, 1992b).

In summary, each of the gel electrophoresis methods showed that there are many bands (proteins and peptides) in cheese of a size that enables them to be seen by gel electrophoresis. The use of two-dimensional gel electrophoresis enabled many more bands to be differentiated and this technique could be used to study any particular cheese variety in greater depth. No single gel system allowed all of the bands that were present on the two-dimensional gels to be differentiated.

Of the four gel electrophoresis methods investigated in this study, only the alkaline urea mini-slab gel method was found to be useful for the routine monitoring of the loss of intact caseins during ripening. It was found, by two-dimensional PAGE, that there is a band lying beneath α_{s1}-casein and some caution must therefore be exercised in the interpretation of the results obtained. The alkaline urea mini-slab gel method was subsequently used to monitor the loss of intact casein in the ripening study in Chapter 4.

7 CONCLUSIONS

The large-slab alkaline urea method gives good resolution of the proteins and peptides but is a cumbersome and time consuming method not suited to the large scale monitoring of proteolysis in cheese.

The mini-slab gel system has the benefits of speed and resolution but this must be balanced against the loss of material from the bands that occurs during staining and destaining. Rigid control of both the staining and destaining processes must be exercised to achieve reproducible results. It may be possible to minimise the loss of material from the bands (as a percentage of the total band) by using a thicker mini-slab gel.

The mini-slab alkaline urea gel method gives good resolution of the proteins and peptides and for routine monitoring of the loss of intact casein during cheese ripening it was considered to be the best of the four methods evaluated. The lack of homogeneity of the bands was not considered likely to introduce any major source of error if the bands were to be quantitated.

The acid urea gel method gives very little information that cannot be obtained by a combination of alkaline urea-PAGE and SDS-PAGE.

The LMW SDS-PAGE method does not resolve the caseins well and is therefore not useful for monitoring the loss of intact casein during cheese ripening. The method enables the retention and separation of many small peptides and may be useful to study them. The method provides good resolution of the para-κ-casein, β-lactoglobulin and α-lactalbumin and may be used to monitor their presence in cheese.

Two-dimensional gel electrophoresis could be used to study any cheese variety in greater depth.

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CHAPTER 3

I. OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES DESTINED FOR QUANTITATIVE PEPTIDE AND PROTEIN ANALYSIS
and
II. THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

SUMMARY

PART I. OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES DESTINED FOR QUANTITATIVE PEPTIDE AND PROTEIN ANALYSIS

Longitudinal studies of cheese maturation involve determining changes in cheese component concentrations as a function of time. Usually, cheese samples are taken at pre-selected intervals, stored and then analysed by various techniques for the components of concern. In some earlier studies, gel electrophoresis patterns, for example, have not always been consistent for cheese stored as frozen grated samples. This may have been the result of continued protein hydrolysis. Such time-dependent sample alteration must be halted to obtain accurate data for quantitative interpretation of results.

The present trial examined several different storage methods and temperatures, including storage as: freeze-dried powder at 4°C in the dark, frozen at -9, -16, -35, -75 and -100°C, and dissolved in 6 M urea solution and stored at 4 and -16°C. The trial ran for 6 months and involved the multiple sampling and detailed analysis of three Cheddar cheeses by reversed phase fast protein liquid chromatography (RP-FPLC) for the water-soluble fraction (WSF) and alkaline urea-polyacrylamide gel electrophoresis (PAGE) for the protein fraction.

None of the methods used to store the cheese samples was completely satisfactory. Cheese stored at temperatures of -9 and -16°C was unstable, with proteolysis discernible after 66 days. Storage of cheese samples at these temperatures is, therefore, not recommended. Cheese stored at temperatures of -35, -75 and -100°C was unstable

after 94 days, although the samples stored at -100°C were more stable. This lack of stability probably arose during both freezing and thawing as well as during storage of the frozen cheese samples. Storage of freeze-dried samples at 4°C in the dark was equivalent to storing the frozen cheese at -100°C. Storage of samples in alkaline urea sample buffer was better at -16°C than at 4°C but should be for no longer than 1 month.

PART II. THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

The rate of maturation of cheese can be controlled by the storage temperature. The present trial investigated maturation in Cheddar cheese stored at 13, 5, -9 and -16°C.

The trial ran for 6 months and involved the multiple sampling and detailed analysis of three Cheddar cheeses by RP-FPLC for the WSF and alkaline urea-PAGE for the protein fraction, both before and after storage.

Proteolysis in cheese stored at 5°C was considerably slower than in cheese ripened at 13°C. After 6 months of ripening at 5°C, 76-79% of the α_{s1}-casein and 12-13% of the β-casein had been hydrolysed. Similar levels of hydrolysis were seen in the cheese ripened at 13°C after only 2 months (75-78% of the α_{s1}-casein and 13-16% of the β-casein had been hydrolysed). Storage of the cheese at -9 and -16°C resulted in a lower level of proteolysis.

1 INTRODUCTION

PART I

OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES DESTINED FOR QUANTITATIVE PEPTIDE AND PROTEIN ANALYSIS

The progress of proteolysis in the ripening cheese can be measured at various time intervals by a variety of techniques such as non-protein nitrogen, non-casein nitrogen, water-soluble nitrogen, ethanol soluble nitrogen, gel electrophoresis, high performance liquid chromatography, fast protein liquid chromatography (FPLC) and amino acid analysis.

To enable the comparison of results obtained by different techniques and to minimise experimental variation within a technique, which is particularly important when low levels of proteolysis are anticipated, it is desirable to carry out each type of analysis when all the cheese samples have been collected. This ensures that all of the samples are analysed under the same conditions within each technique. For this to occur, the samples must be stored under conditions that prevent further change. The primary focus of this study was the storage/preservation of samples for subsequent analysis by gel electrophoresis.

Previous researchers do not seem to have been concerned by or have not encountered problems with hydrolysis of protein in cheese samples stored at low temperature. However, several authors have reported that proteolysis occurs during the frozen storage of cheese. McDowall (1938) and Martin-Hernandez *et al.* (1990) reported an increase in soluble nitrogen during frozen storage of cheese. Kasprzak *et al.* (1994) reported an increase in trichloroacetic acid-soluble nitrogen during frozen storage of Cheddar cheese. Fuster (1970) used infrared analysis to examine milk stored at -20°C for 9-26 months and demonstrated a loss of double bonds and an increase in NH_2 and OH groups, indicating peptide bond breakage.

Fontecha *et al.* (1993) used Fourier transform infrared spectroscopy and Raman spectroscopy to study the effect of the rate of freezing and of frozen storage on the secondary structure of the caseins in cheese. They showed that the spectrum of caseins in cheese that was slowly frozen to
-20.5°C and then thawed was more similar to that of the control cheese held at 11°C

for 2 days than to that of the more rapidly frozen cheese. Further changes to the casein spectrum following storage of the cheese at -20.5°C for 4 months were only slight.

Several researchers have reported an increase in the rate of proteolysis following the thawing of frozen cheese (Alichanidis *et al.*, 1981; Ramos *et al.*, 1987; Martin-Hernandez *et al.*, 1990; Fontecha *et al.*, 1993; Kasprzak *et al.*, 1994).

The results of this study have been reported in part (Coker and Creamer, 1993).

PART II

THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

In New Zealand, there is a low volume of milk available for the manufacture of dairy products for approximately 3 months of each year, which creates a problem for the year-round supply of dairy products to the export market. As well as this, a large proportion of the cheese manufactured in New Zealand is used either as a food ingredient or in processed cheese. For many of these applications, it is necessary that the cheese be at a particular stage of maturity in order for it to possess the desired functional properties.

To satisfy these requirements, various compositional parameters (pH, salt, moisture, calcium, for example) can be altered, by changes in processing, to control the rate of ripening. In most instances, the effect of altering any of these parameters on the activity of the enzymes that contribute to cheese ripening differs for each enzyme and any alteration is likely to affect the delicate balance of reactions that occurs during cheese ripening. As the major enzymes involved in the early stages of cheese ripening are of animal origin and have similar temperature optima, one means of reducing the rate of ripening without upsetting the balance of the enzyme activities can be to lower the temperature at which the cheese is ripened and stored.

2 OBJECTIVES

PART I

OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES

The objective of the first part of this experiment was to select the most suitable conditions for the storage of cheese samples obtained during a ripening study so that they could be analysed after all the samples had been collected.

PART II

THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

The objective of the second part of this study was to gain information on the effect of the ripening temperature on proteolysis in Cheddar cheese.

3 EXPERIMENTAL PROCEDURE

Cheddar cheese was manufactured according to the same standard protocol in three vats, each containing different sets of starter strains (the effect of starter strain on cheese ripening was part of a project that was unrelated to this one).

The cheeses were ripened for 10 days at 10°C. They were then divided for (a) the low temperature storage trial, (b) the urea buffer storage trial and (c) further maturation at 5 and 13°C.

(a) A large representative sample of each cheese was grated, mixed thoroughly and divided into weighed subsamples (0.500 g) which were sealed in containers and stored at -9 (-9.5 to -11.5°C) or -16°C (-17 to -24°C) (Parts I and II), -35 (-24 to -35.5°C), -75 or -100°C (Part I) or freeze dried and stored at 4°C in the dark (Part I). These subsamples were removed at 38, 66, 94 and 180 days and analysed by alkaline urea polyacrylamide gel electrophoresis (PAGE). Each sample was electrophoresed in duplicate. The water-soluble fractions (WSFs) of samples stored for 38, 66 and 94 days were prepared and analysed by reversed phase FPLC (RP-FPLC).

(b) A cheese sample from each trial was dispersed in alkaline urea sample buffer (according to the protocol described in Section 4.2), divided into aliquots, stored at 4 and -16°C (Part I) and analysed in duplicate by alkaline urea-PAGE at 10 (immediately), 38, 66, 94 and 180 days of age.

(c) The remainder of each cheese block was then divided and ripening continued at 13 and 5°C (Part II). The cheeses were sampled at 10 (immediately), 38, 66, 94 and 180 days of age for analysis by alkaline urea-PAGE and at 38, 66 and 94 days of age for analysis of the WSF by RP-FPLC.

An investigation into the effect of storage temperature on the pH of the cheese milk, cheese exudate, alkaline urea sample buffer and the WSF of the cheese at 10 and 38 days was carried out after the above experiments had been completed in an endeavour to explain the unexpected results obtained for samples stored at very low temperatures.

4 MATERIALS AND METHODS

4.1 CHEDDAR CHEESE MANUFACTURE

Three vats of Cheddar cheese were manufactured, each with a different starter combination, according to a standard protocol (Johnston *et al.*, 1994).

4.2 ALKALINE UREA-PAGE (MINI-SLAB GEL)

Proteins in the cheese were separated by alkaline urea-PAGE using the Bio-Rad Mini-Protean II system and methods described in Chapter 2 (Section 4.3).

Gel analysis: The gels were scanned using a Personal Densitometer (Molecular Dynamics, Sunnyvale, California, USA) and photographed. The gel images were analysed using ImageQuant version 3.0 and Microsoft Excel 4.0 (Microsoft Corporation, Redmond, Washington, USA) software in conjunction with Microsoft Windows version 3.1.

4.3 RP-FPLC

The formation of water-soluble peptides during the storage of the cheese was investigated by RP-FPLC using a Pharmacia FPLC system (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected to a personal computer running FPLC Manager software. The effluent was monitored using a Pharmacia Model VWM 2141 variable wavelength monitor with VWM 2141 dual channel selection. The following settings were used.

Channel 1: 280 nm, Min -0.05 AU, Max 0.3 AU, Output 1000 mV/100%
Channel 2: 214 nm, Min -0.05 AU, Max 0.3 AU, Output 1000 mV/100%
Time constant: 1 s

The peptides were separated using two Pharmacia PepRPC HR 5/5 columns in series. A recirculating iced waterbath was used to maintain the sample temperatures at $<4°C$.

Preparation of the WSF of the cheese: Grated cheese (0.500 g) was dispersed in 5.0 ml of water and held at 40°C for 30 min prior to blending with an Ultra-Turrax T25 (Janke and Kunkel, IKA-Labortechnik, supplied by Labsupply Pierce) at approximately 24,000 rpm for 1 min. The warmed samples were then centrifuged at 10,000 rpm at

4°C for 10 min. The supernatant was filtered through a 0.80 μm/0.20 μm filter (purchased from Gelman Sciences, Ann Arbor, Michigan, USA) and stored at -100°C.

Preparation of amino acid and di- and tripeptide standards: Amino acid and di- and tri-peptide standards (1.0 mg/5 ml water) were filtered through a 0.80 μm/0.20 μm filter (Gelman Sciences) and 200 μl was loaded on to the column and eluted using the same method that was used for the WSF. The retention times were calculated for the individual standards as well as for a mixture of the amino acids (1 mg of each amino acid in 5 ml water, diluted 1:5 with water and filtered through a 0.80 μm/0.20 μm filter) and the results were used to identify the position of the tyrosine, phenylalanine and tryptophan peaks in the RP-FPLC profiles of the WSF of the cheese.

Preparation of casein and whey protein standards: α_{s1}-Casein and β-casein (supplied by R. Burr, NZDRI) were dissolved in water (1 mg/5 ml) at pH 10 and the pH was then reduced to 7. They were then treated in the same manner as the other standards. The β-lactoglobulin (supplied by R. Burr) and α-lactalbumin (Sigma) standards (0.5 mg/ml) were treated in the same manner as the amino acid standards.

Liquid chromatography gradient: The gradient consisted of 100% solvent A (5% acetonitrile (BDH, "Far UV" grade), 0.1% trifluoroacetic acid (TFA; "Far UV" grade) and 0.5% NaCl in distilled and deionised water) at a flow rate of 0.70 ml/min for 5 min, 0 to 90% solvent B (60% acetonitrile, 0.1% TFA and 0.5% NaCl in distilled and deionised water) over 75 min at 0.70 ml/min and 100% solvent A for 15 min at 0.7 ml/min.

A 1 ml aliquot of sample was removed from the freezer (-100°C), thawed and placed in the sample holder and a portion was drawn into a 200 μl sample loop. Fourteen samples were analysed as part of one automated sequence. A baseline was generated by running a gradient without a sample at the start and the finish of each automated sequence.

Each chromatogram was corrected by subtraction of the baseline chromatogram that was run under the same conditions. The area under each peak (above a threshold level of 2%) was integrated and the chromatogram plotted.

4.4 UNIVERSAL INDICATOR TEST FOR pH CHANGE

Universal Indicator (BDH Chemicals, Palmerston North, New Zealand) was added to samples of milk, cheese whey that was collected during the later stages of cheese pressing and alkaline urea sample buffer in 2 ml plastic vials. The samples were (a) frozen to -9, -16 and -35°C using an ethanol shell bath, (b) frozen to -100°C in the freezer used to store the cheese samples and (c) frozen to -40°C in the shell bath and then freeze-dried. Colour changes were compared with a standard chart supplied by the manufacturer and the pH was estimated.

5 RESULTS

PART I

OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES

5.1 THE STABILITY OF FROZEN CHEESE SAMPLES

Casein degradation and changes in the WSF of the cheese were monitored by alkaline urea-PAGE and RP-FPLC respectively.

5.1.1 The effect of frozen storage on casein degradation in cheese samples

The small changes in the amount of α_{s1}- and β-casein that were observed in the cheeses stored at low temperature were insufficient to show any unambiguous trends. The values for α_{s1}- and β-casein were adjusted using the values for the casein standards that were included on each gel but there were still no unambiguous trends. (It is possible that the casein standards, stored in urea sample buffer at -16°C, may have deteriorated). To overcome much of the experimental error that was likely to have occurred when samples (and casein standards) were run on different sets of gels, the ratio of the absorbances of the dyed bands of α_{s1}- to β-casein was calculated for each sample, and this allowed trends to be distinguished. This assumes that α_{s1}- and β-casein were equally affected by any differences between batches of gel and stain. The decision to use the ratio of α_{s1}- to β-casein was based on the very small amount of β-casein hydrolysis that occurred in the cheese during ripening at 5°C, which was in contrast to the large amount of α_{s1}-casein hydrolysis at 5°C (as can be expected in Cheddar cheese where the conditions are more favourable for rennet activity than for plasmin activity). If there was any hydrolysis of β-casein at lower temperatures, this would tend to minimise any trends observed.

The effect of sample storage temperature on the ratio of the dye bound to α_{s1}- and β-casein, in cheese from vats 1, 2 and 3, is illustrated in Figure 3.1. The samples were analysed in duplicate and there was very close agreement between the values that were obtained (mean error, 1.48%; standard deviation, 1.37%).

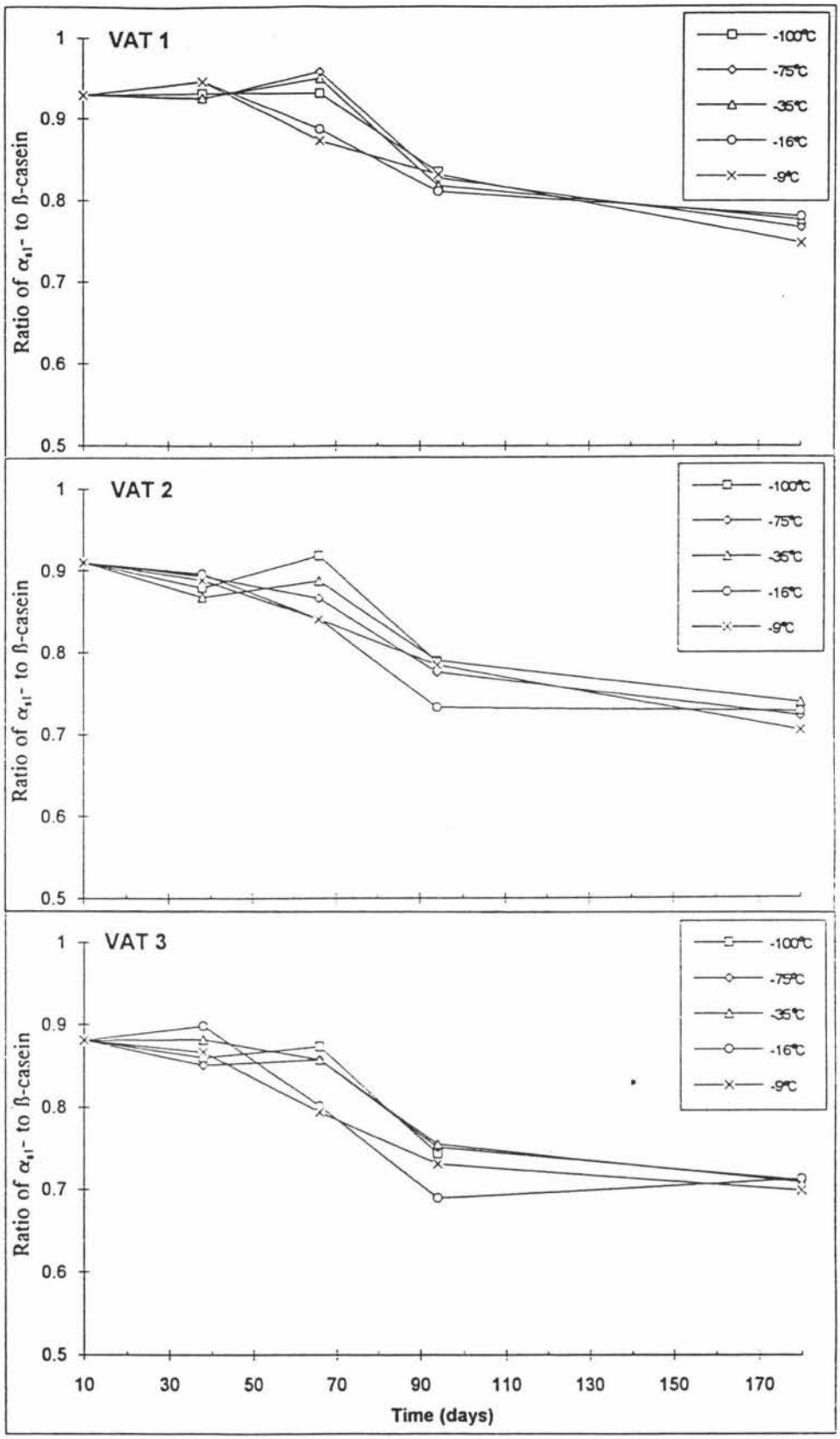
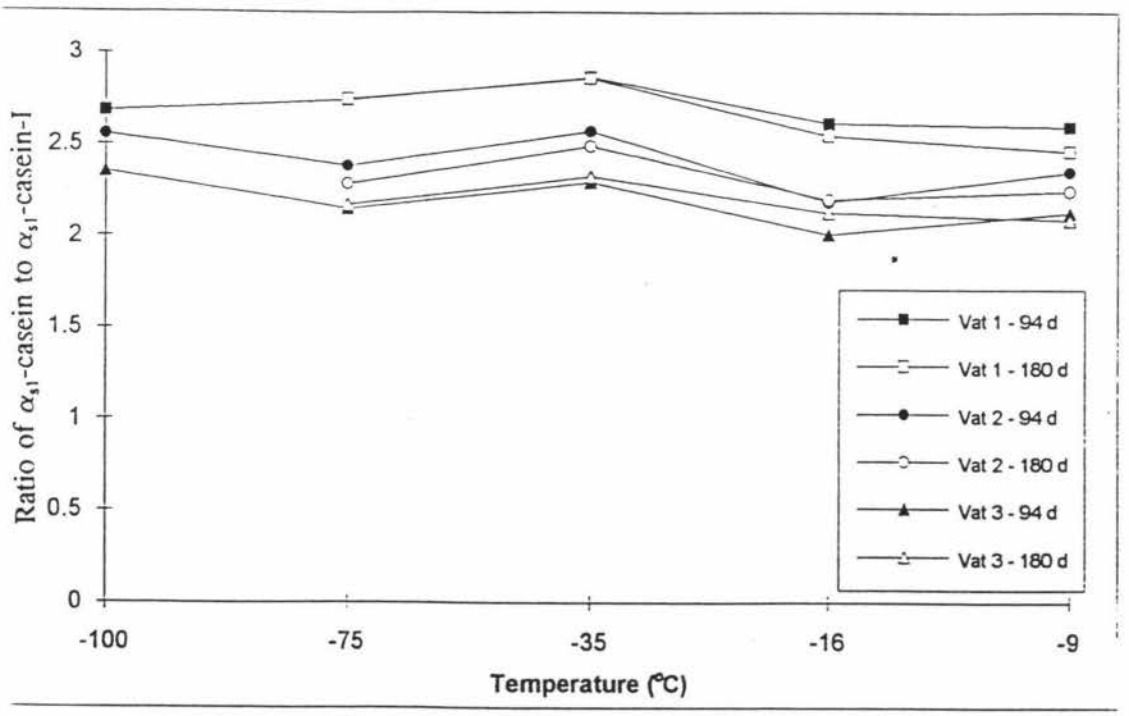


FIGURE 3.1 The effect of storage temperature on the ratio of α_{s1}- to β-casein in Cheddar cheese from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days. Aliquots (0.500 g) were stored at -100, -75, -35, -16 and -9°C and analysed by alkaline urea-PAGE at 10, 38, 66, 94 and 180 days.

The results indicate that at -100, -75 and -35°C the cheese was stable for 66 days, after which time a decrease in the ratio of α_{s1}-casein to β-casein (as determined by the amount of dye bound) was observed. At -9 and -16°C the cheese samples were stable for 38 days, after which time a decrease in the ratio of α_{s1}- to β-casein became evident. However, it was only at -9°C that the ratio of α_{s1}- to β-casein was consistently greater at 94 days than at 180 days, indicating that chymosin (or other aspartate proteinase) action was clear at -9°C, but not at other temperatures.

An examination of the ratio of α_{s1}-casein to α_{s1}-casein-I after 94 and 180 days of storage at -100, -75 and -35°C (Figure 3.2) indicates that the rates of loss of α_{s1}-casein and α_{s1}-casein-I were similar. A small decrease in the ratio of α_{s1}-casein to α_{s1}-casein-I at -9 and -16°C (Figure 3.2) (at 180 days the slope from -35°C to -9°C was -0.016 for vat 1, -0.011 for vat 2 and -0.009 for vat 3) indicates that the decrease in the ratio of α_{s1}- to β-casein was probably due to hydrolysis of the α_{s1}-casein by rennet and any other aspartate proteinases present. The rate of α_{s1}-casein hydrolysis was similar in each of the three cheeses despite each having been made with a different set of starters and it therefore appeared to be independent of starter type (in this group of cheeses).



GURE **3.2** The effect of storage temperature on the ratio of α_{s1}-casein to α_{s1}-casein-I in Cheddar cheese from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days. Aliquots (0.500 g) were stored at -100, -75, -35, -16 and -9°C and analysed by alkaline urea-PAGE after 94 and 180 days.

5.1.2 The effect of frozen storage on the WSF of Cheddar cheese samples

5.1.2.1 RP-FPLC of standards

Table 3.1 contains the RP-FPLC retention times of the amino acids, determined singly and as a mixture, the di- and tripeptides and the casein and whey protein standards. The RP-FPLC conditions were those used to analyse the WSF of the cheese.

TABLE 3.1 RP-FPLC retention times of amino acid (determined singly and as a mixture) (1 mg/5 ml), di- and tripeptide (1 mg/5 ml), casein (1 mg/5 ml) and whey protein (0.5 mg/ml) standards. The absorbance was monitored at 214 and 280 nm.

Standards	Retention time (min)
Isoleucine	2.98
Phosphoserine	3.02
Norleucine	3.02
Asparagine	3.03
Lysine	3.03
Alanine	3.08
Cysteine	3.10
Serine	3.11
Threonine	3.17
Glycine	3.22
Arginine	3.26
Proline	3.34
Histidine	4.37
Valine	4.56
Methionine	4.76
Tyrosine	6.97
Glutamine	9.79
Glutamic acid	9.79
Phenylalanine	11.22
Tryptophan	21.17
Leucine	n.d.
Amino acid mixture	
Peak 1 (mixture)	3.30
Peak 2 (mixture)	4.65
Peak 3 - Tyrosine	6.49
Peak 4 - Phenylalanine	10.54
Peak 5 - Tryptophan	20.22

Standards	Retention time (min)
Glycyl-L-asparagine	3.18
Glycyl-L-aspartic acid	3.50
Glycyl-glycine	3.49
Glycyl-DL-methionine	7.08
Glycyl-tyrosine	10.63
Glycyl-DL-phenylalanine	23.58
Glycyl-L-histidine	3.29
Glycyl-L-alanyl-L-alanine	4.63
Tri-L-serine	3.35
Tryptophyl-L-tyrosine	32.18
α_{s1}-casein	65.10 and 67.14
α_{s1}-casein-I	64.16 and 66.30
β-casein	70.13
β-lactoglobulin AB	70.45 and 71.50
α-lactalbumin	66.32

When all of the amino acids were combined and the mixture was analysed by RP-FPLC, only five peaks were visible. Most eluted in two peaks at 3.30 and 4.65 min. The three amino acids that eluted as single peaks were tyrosine, phenylalanine and tryptophan. Their retention times were used to locate these amino acids in the cheese traces. The profile obtained after the chromatogram of the solvent gradient was subtracted from the chromatogram of the amino acid standard mixture is shown in Figure 3.3.

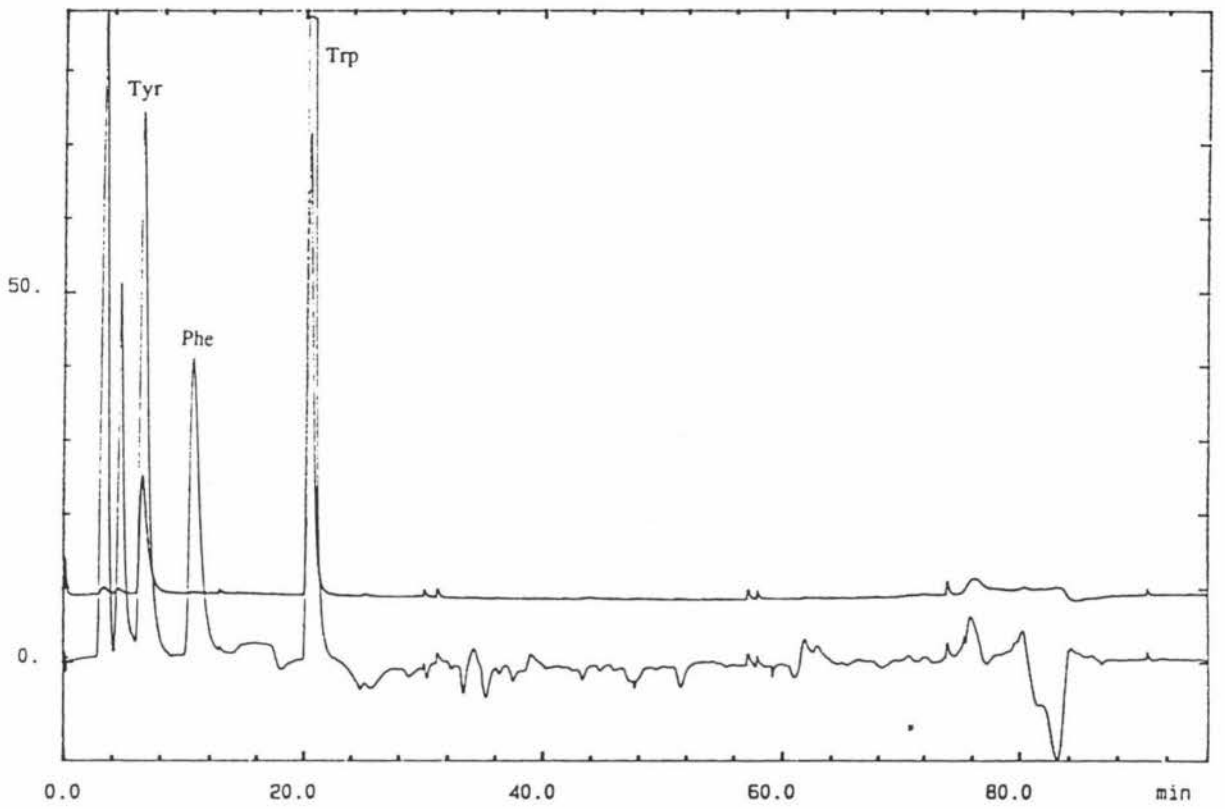


FIGURE 3.3 RP-FPLC profile of a mixture of amino acids. The absorbance was monitored at 214 and 280 nm.

5.1.2.2 RP-FPLC analysis of the WSF of the frozen cheese samples

The RP-FPLC traces obtained for the WSF of the frozen cheese samples are presented in Figure 3.4.

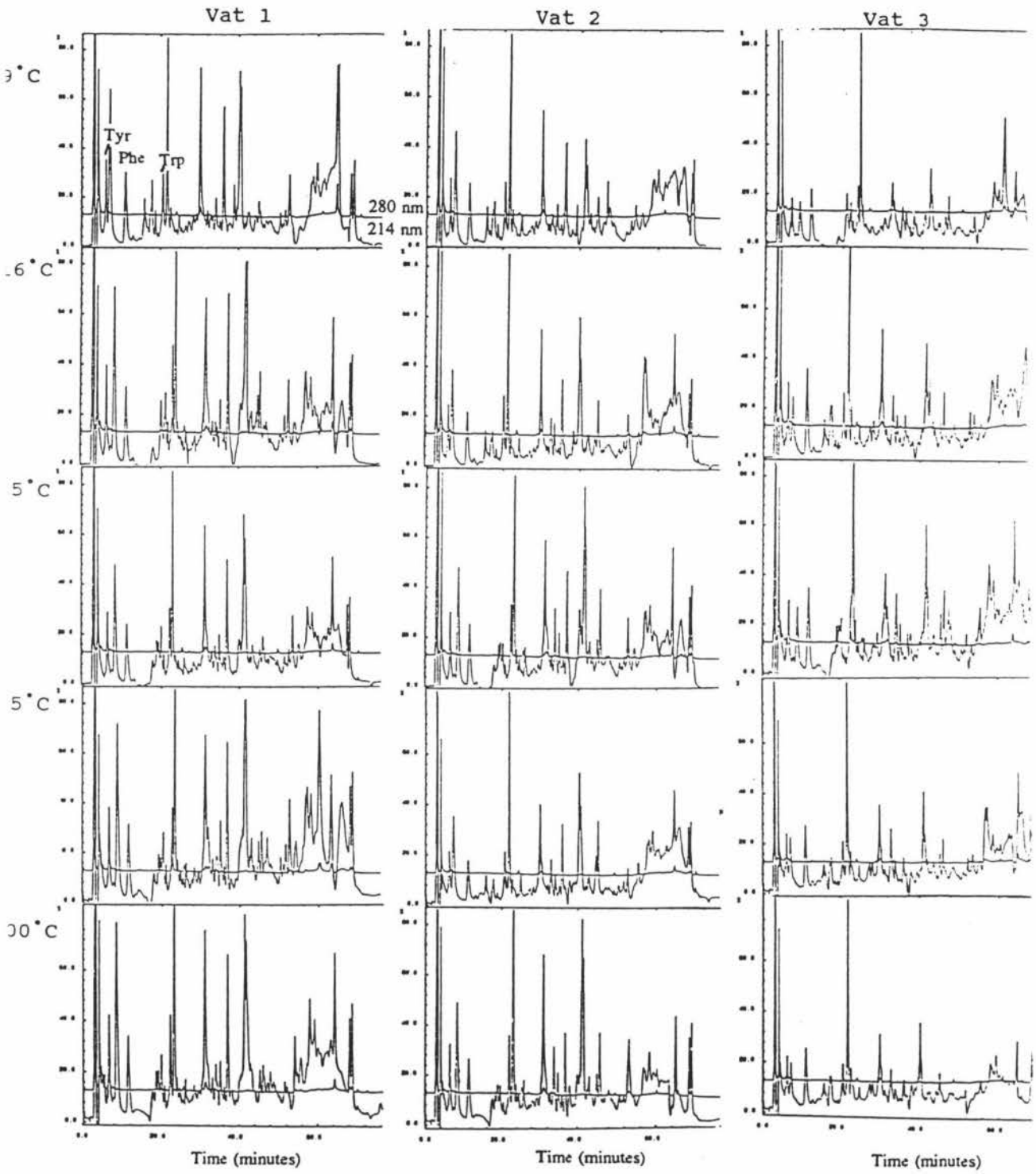


FIGURE 3.4 RP-FPLC. The effect of storage temperature on the peptide profiles of the WSF of Cheddar cheese from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days. Aliquots (0.500 g) were stored at -100, -75, -35, -16 and -9°C for 94 days and the WSF analysed. The absorbance was monitored at 214 and 280 nm.

Much of the difficulty experienced in the examination of the WSF of these cheeses was probably due to the very small amount of water-soluble material present in the cheese after 10 days of maturation.

A more detailed analysis of the RP-FPLC traces was necessary to determine whether there were any trends that could be related to the cheese storage temperature.

The amount of material that eluted between the tryptophan peak (exclusive) and 75 min was calculated as a percentage of the total amount of material and was plotted against the storage temperature, to determine whether or not proteolysis occurred in the cheese samples stored at freezing temperatures. The resultant graph is shown in Figure 3.6.

In the early stages of cheese ripening, when the proteolytic enzymes (mainly rennet, in Cheddar cheese) are more active than the peptidolytic enzymes, the amount of later eluting material, which is composed mainly of larger peptides, increases at a greater rate than the amount of early eluting material (Chapter 4).

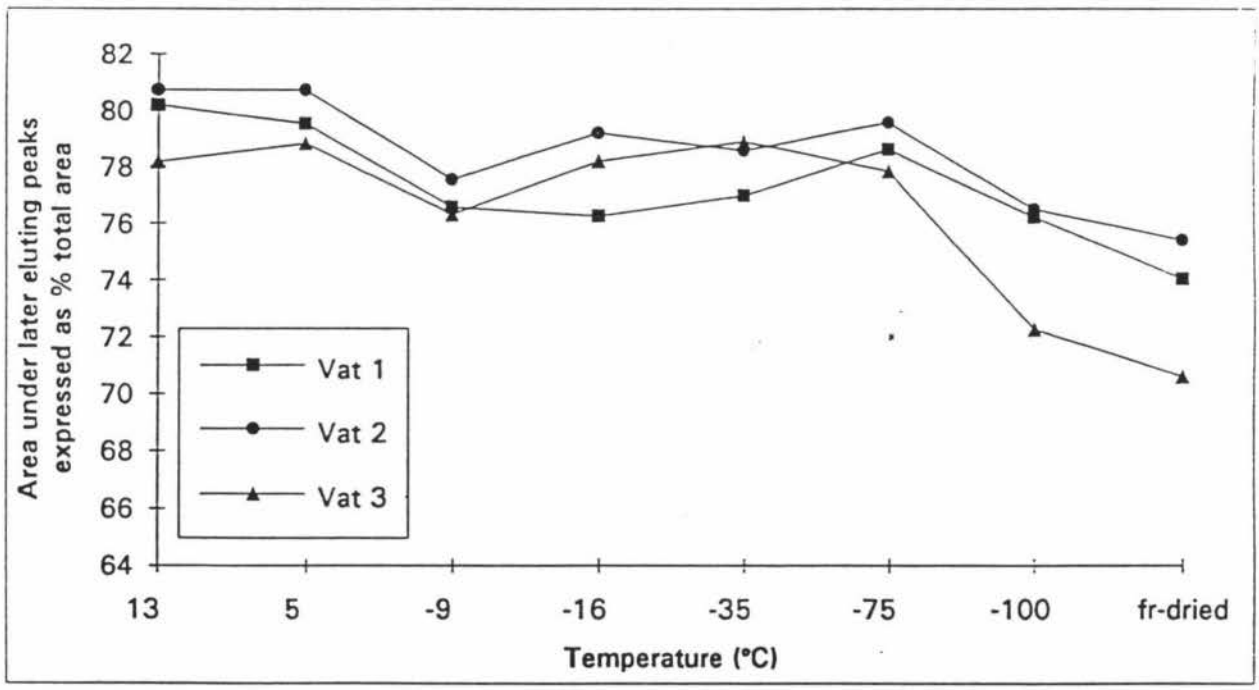


FIGURE 3.6 The effect of storage temperature (-100 to -9°C for 94 days) on the amount of material, with an absorbance at 214 nm, that eluted between the tryptophan peak and 75 min. The area under the profiles (Figure 3.4) from immediately after tryptophan (~22 min) to 75 min (expressed as a percentage of the total amount of material) is plotted against the storage temperature.

The amount of material in the later eluting (tryptophan to 75 min) peaks, when expressed as a percentage of the total amount of water-soluble material with an absorbance at 214 nm, was considerably less at -100°C than at temperatures between -9 and -75°C (Figure 3.6). This indicates that there was less proteolytic activity in the cheeses stored at -100°C than in the cheeses stored at higher temperatures, as could be expected. The lack of stability in the cheese samples stored at temperatures between -9 and -75°C, as indicated by the greater amount of later eluting material in the WSF of these cheeses, is likely to have been caused by the continued action of the proteolytic enzymes. The slight trend, in the cheese stored at temperatures between -75 and -9°C, towards less (apparent) material in the later eluting peaks of the cheese (when expressed as a percentage of the total amount of water-soluble material) was possibly due to an increase in the amount of material in the early eluting peaks as a consequence of greater enzyme activity at the warmer temperatures.

5.2 THE STABILITY OF FREEZE-DRIED CHEESE SAMPLES

5.2.1 The effect of storage time on casein degradation in freeze-dried Cheddar cheese samples

The ratio of dye bound to α_{s1}- and β-casein remained invariant for 66 days and then diminished between 66 and 94 days (Figure 3.7). This suggests that the protein may have been stable for the first 66 days.

The ratio of α_{s1}-casein to α_{s1}-casein-I at 94 days was 2.78, 2.47 and 2.26 and at 180 days was 2.83, 2.30 and 2.24 for vats 1, 2 and 3, respectively. These results are comparable with those obtained when the cheese was stored at temperatures of $\leq$-35°C (Figure 3.2) and indicate that rennet was probably inactive in the freeze-dried cheese.

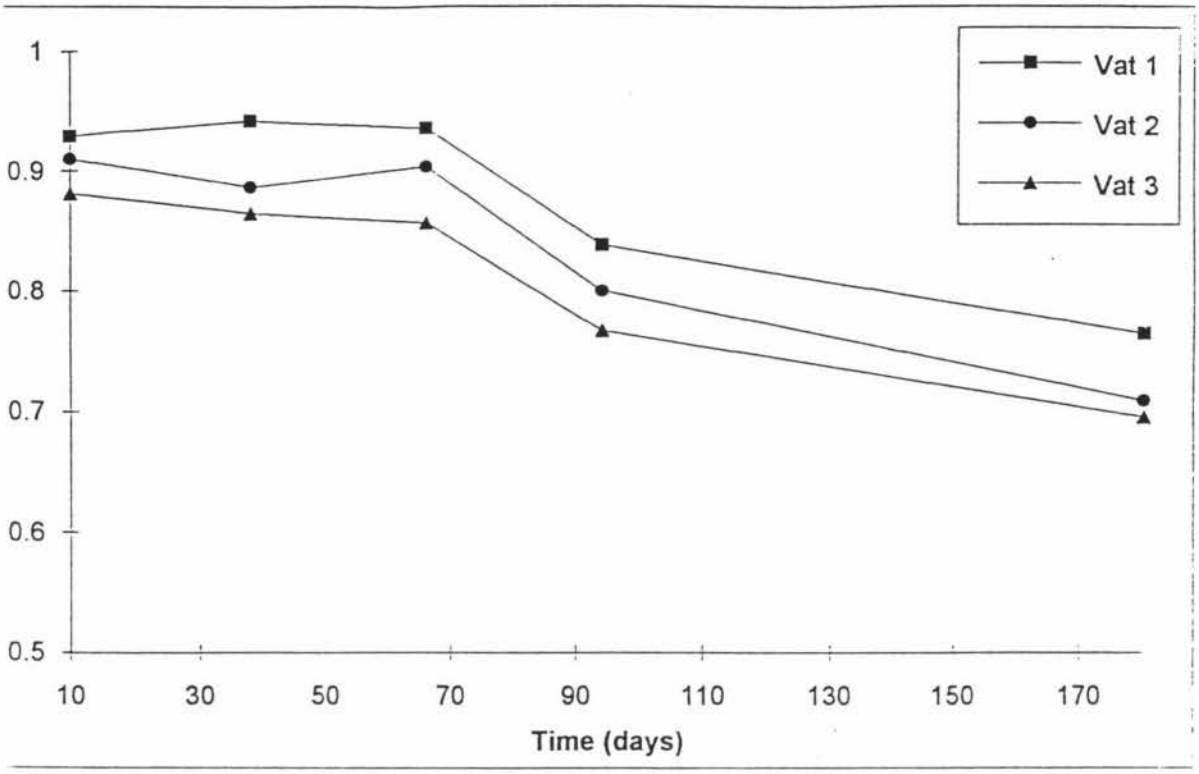


FIGURE 3.7 The effect of storage time on the ratio of α_{s1}- to β-casein in freeze-dried Cheddar cheese samples from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days. Aliquots (0.500 g) were freeze-dried, stored at 4°C and analysed by alkaline urea-PAGE after 10, 38, 66, 94 and 180 days.

5.2.2 The effect of storage time on the water-soluble fraction of freeze-dried Cheddar cheese

The effect of the storage of freeze-dried cheese samples on the formation of water-soluble peptides and amino acids was determined by comparing (a) the RP-FPLC profiles of the WSF (Figure 3.8) with those of the WSF of the frozen cheese samples (Figure 3.4), (b) the amount of material in the WSF with an absorbance at 214 nm with the amount in the WSF of the frozen cheese samples (Figure 3.5) and (c) the amount of later eluting material, expressed as a percentage of the total amount of water-soluble material, with the amount in the WSF of the frozen cheese (Figure 3.6).

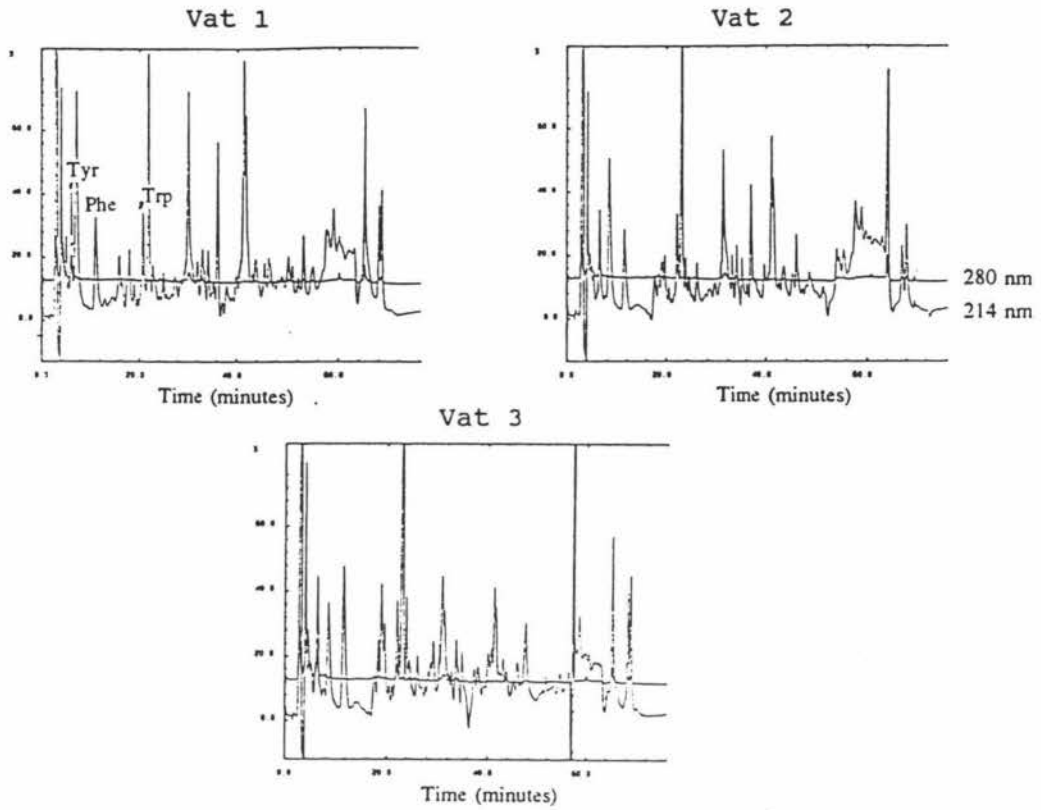


FIGURE 3.8 The effect of storage of freeze-dried cheese samples on the peptide profiles obtained by RP-FPLC analysis of the WSF of Cheddar cheese from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days. Aliquots (0.500 g) were freeze-dried and stored at 4°C for 94 days and the WSF analysed. The absorbance was monitored at 214 and 280 nm.

The RP-FPLC traces were similar to those of the frozen cheese (Figure 3.4). The total area of the peaks at 214 nm was 1057, 961 and 1010 / 200 mg of cheese for freeze-dried samples from vats 1, 2 and 3, respectively after 94 days. These were within, or very close to, the ranges (836-1199 for vat 1, 795-922 for vat 2 and 609-1123 for vat 3) observed in the frozen cheese samples (Figure 3.5).

The apparent amount of material in the later eluting peaks (tryptophan to 75 min), expressed as a percentage of the total amount of water-soluble material, was 74.1, 75.0 and 70.6% for the freeze-dried cheese samples from vats 1, 2 and 3, respectively. These values were slightly less than the values (74.5% for vat 1, 76.5% for vat 2 and 72.3% for vat 3) calculated for the cheese samples frozen at -100°C. This indicates that the freeze-dried cheese samples were more stable than any of the frozen cheese samples (Figure 3.6).

5.3 THE STORAGE OF CHEDDAR CHEESE SAMPLES IN UREA SAMPLE BUFFER

The cheese samples that were stored in alkaline urea sample buffer at both 4 and -16°C deteriorated during storage. As the samples aged, the casein bands separated by alkaline urea-PAGE became blurred and there was an apparent loss of α_{s2}-casein. The photograph in Figure 3.9 illustrates the effect of storage of the cheese samples in urea sample buffer on the casein bands. It can be seen that deterioration of the α_{s2}-casein band was particularly marked and was worse in the samples stored at 4°C than in those stored at -16°C. Both the α_{s1}- and β-casein bands were indistinct and this was more apparent at 4°C than -16°C. Although the samples depicted had been held for 180 days, deterioration was evident by 66 days. This photograph also shows the deterioration in the casein standards that were stored in alkaline urea sample buffer at -16°C.

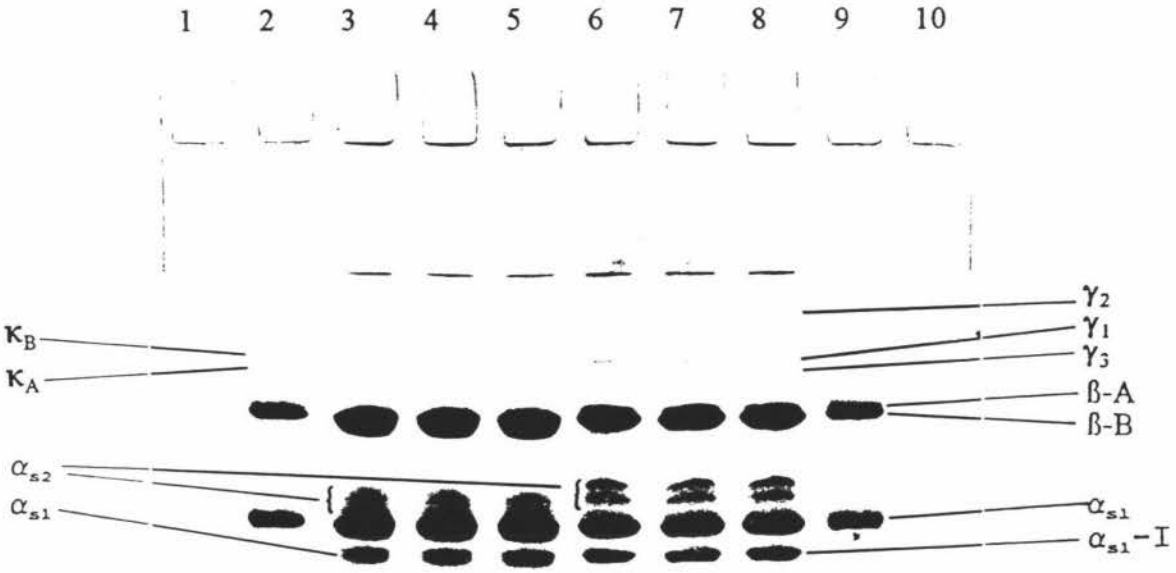


FIGURE 3.9 The effect of storage of Cheddar cheese samples from vats 1, 2 and 3 in alkaline urea sample buffer on the urea-PAGE patterns of the caseins. The cheeses were ripened at 10°C for 10 days. Cheese samples prepared in alkaline urea sample buffer and stored at 4 and -16°C were analysed after 180 days. Lanes 2 and 9 - casein standards; lanes 3, 4 and 5 - Cheddar cheese from vats 1, 2 and 3 in alkaline urea sample buffer at 4°C; lanes 6, 7 and 8 - Cheddar cheese from vats 1, 2 and 3 in alkaline urea sample buffer at -16°C.

Figure 3.10 demonstrates the effect of storing the samples in alkaline urea sample buffer at 4 and -16°C on the apparent ratio of α_{s1}- to β-casein. These results indicate that the samples were more stable when frozen and appeared to be stable for the first month, after which time deterioration (as determined by differences in the ratio of dye bound to α_{s1}- and β-casein) occurred.

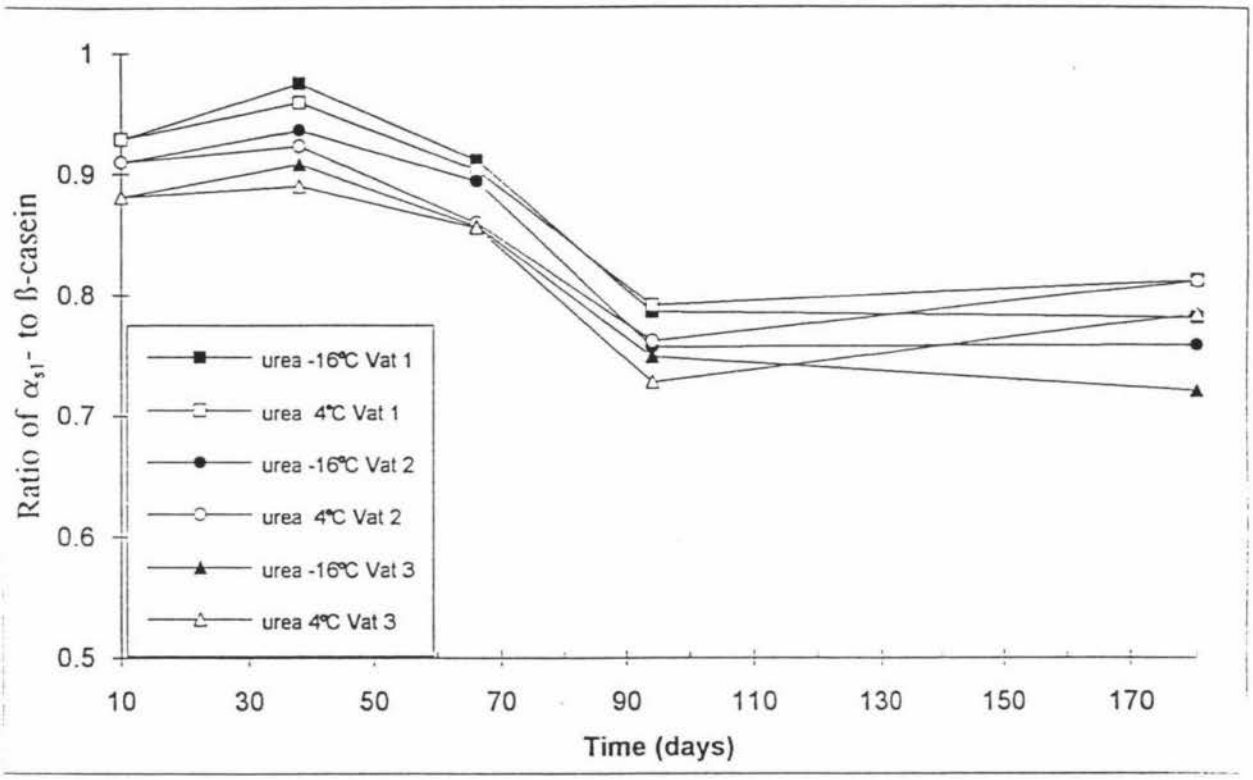


FIGURE 3.10 The effect of storage of Cheddar cheese samples from vats 1, 2 and 3 in alkaline urea sample buffer on the apparent ratio of α_{s1}- to β-casein. The cheeses were ripened at 10°C for 10 days. Cheese samples prepared in alkaline urea sample buffer and stored at 4 and -16°C were analysed after 10, 38, 66, 94 and 180 days.

5.4 THE EFFECT OF STORAGE CONDITIONS ON THE PH OF THE LIQUID PHASE OF CHEESE AND OTHER SAMPLES

During the freezing of buffered substances such as cheese, water is removed as ice and pockets of concentrated solutes form. Differential precipitation of the solutes in these pockets may occur and this can result in changes in the buffering capacity and in changes to the pH. It was suggested by J. Hill (NZDRI) that this could be one explanation for the apparent deterioration that occurred in the cheese samples stored at low temperatures.

To determine whether or not the pH changed during freezing, Universal Indicator solution was added to samples of milk, cheese, cheese whey (taken during the later stages of pressing), the WSF of cheese and to alkaline urea buffer, prior to freezing them in an ethanol shell bath at -9, -16 and -35°C, in a -100°C freezer and in liquid nitrogen. The colour of the frozen samples, which was unchanged for the first minutes of the thawing process, was compared with the colour chart (included with the indicator solution) and the pH estimated. The changes in the pH of the samples, that were caused by freezing at -9, -16, -35 and -100°C and by freezing to -40°C prior to freeze-drying, are recorded in Table 3.2. The minimum pH that could be measured was 4.0.

TABLE 3.2 The effect of freezing at different temperatures and freeze-drying on the pH of milk, cheese whey, alkaline urea sample buffer and the WSF of cheese.

	-9°C	-16°C	-35°C	-100°C	Liquid nitrogen	Freeze-dried
Milk	no change (pH ~6.5)	5 - 5.5	5 - 5.5	5.0+	≤ 4.0	5 - 5.5
Cheese whey	no change (pH ~6.0)	< 5.0	≤ 4.0	≤ 4.0	≤ 4.0	≤ 4.0
Urea buffer	no change (pH 8-8.5)	no change	7.5	-	regions at ≤ 4.0	-
WSF of cheese at 10 days	-	-	-	≤ 4.0	-	-

These pH changes were fully reversible; on thawing, the samples slowly went through a range of colour changes as the pH returned to the original pH.

PART II

THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

5.5 THE RIPENING TEMPERATURE OF CHEDDAR CHEESE

Casein hydrolysis and changes in the WSF of the ripening cheese were monitored by alkaline urea-PAGE and RP-FPLC, respectively.

5.5.1 The effect of ripening temperature on casein hydrolysis in Cheddar cheese

Figure 3.11 shows the effect of ripening temperature on proteolysis in cheese from vats 1, 2 and 3, respectively. The samples were analysed in duplicate and the values obtained were in close agreement (the mean error for α_{s1}-casein was 2.62% and the standard deviation was 2.18%; the mean error for β-casein was 1.60% and the standard deviation was 1.23%).

Casein hydrolysis in Cheddar cheese was slower at 5°C than at 13°C. It is probable that the band analysed as α_{s1}-casein at 180 days, which appeared to increase in concentration in vat 3, may in fact have been a degradation product with the same electrophoretic mobility as α_{s1}-casein. In mature Cheddar cheese, the presence of another band in this position was demonstrated by two-dimensional gel electrophoresis in Chapter 2 (Figure 2.7).

An examination of the decrease in α_{s1}-casein and β-casein in the cheese from vats 1, 2 and 3 after 66 days of maturation (Figure 3.11) showed that 48-56% of the α_{s1}-casein and 5-7% of the β-casein had been hydrolysed at 5°C, whereas at least 75-78% of the α_{s1}-casein and 13-16% of the β-casein had been hydrolysed at 13°C.

After 180 days, at least 76-79% of the α_{s1}-casein and 12-13% of the β-casein had been hydrolysed at 5°C, whereas at least 85-88% of the α_{s1}-casein and 27-33% of the β-casein had been hydrolysed at 13°C.

The amount of proteolysis observed in the cheese after 66 days at 13°C was not achieved until 180 days at 5°C.

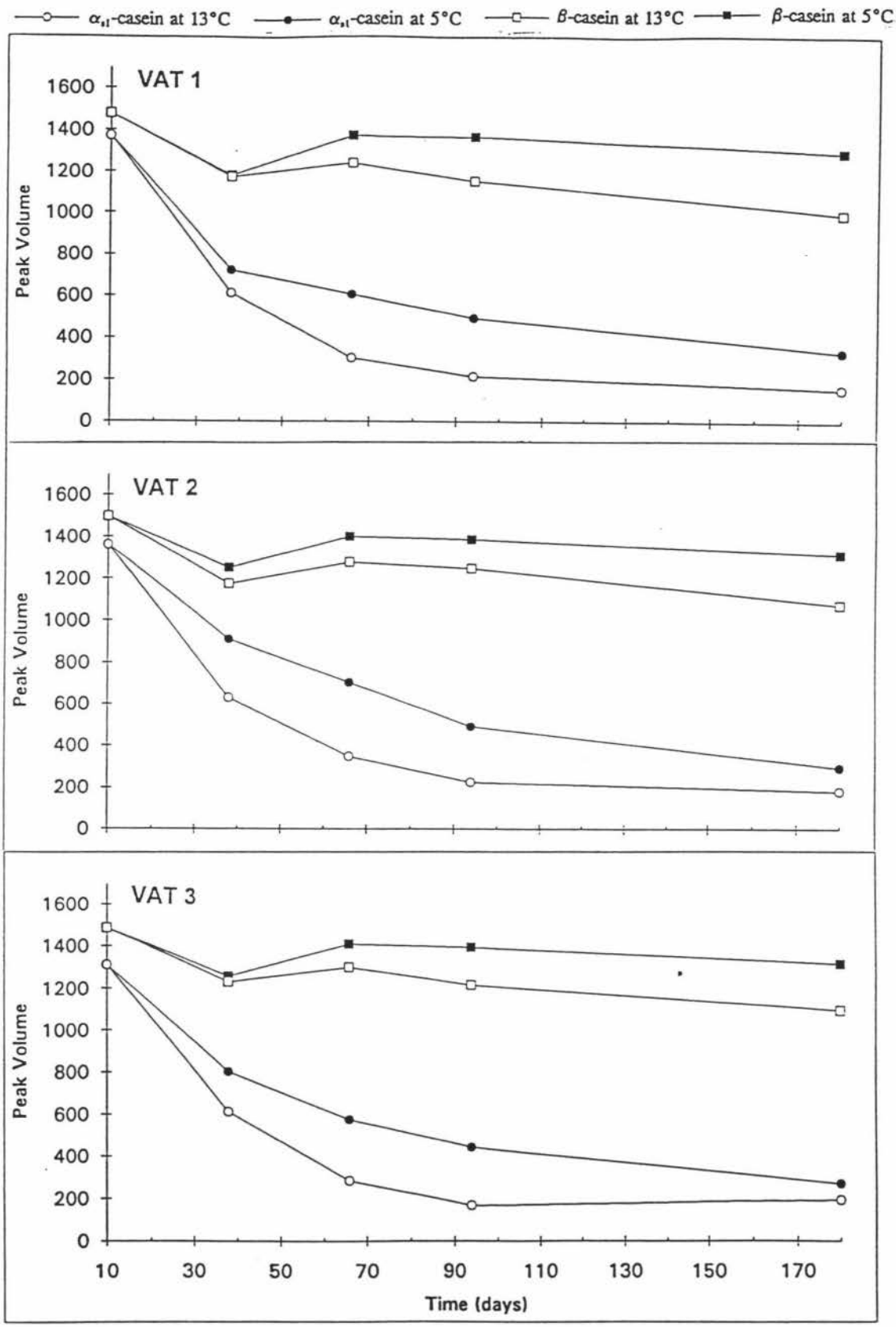


FIGURE 3.11 The effect of ripening temperature on proteolysis in Cheddar cheese from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days and then at 5 and 13°C. The peak volumes of α_{s1}- and β-casein were measured at 10, 38, 66, 94 and 180 days in duplicate and the average was plotted.

5.5.2 The effect of ripening temperature on the WSF of Cheddar cheese

The WSF of the ripening cheese from vats 1, 2 and 3 was prepared at the same concentration and analysed by RP-FPLC (Figure 3.12) under the conditions used for the frozen cheese, so that the two sets of profiles could be compared. This resulted in obvious overloading, particularly at 13°C.

The RP-FPLC profiles of the WSF of the cheese after 94 days of maturation (Figure 3.12) show the large increase in the amount of water-soluble peptide and amino acid material that occurred when the ripening temperature was increased from 5 to 13°C.

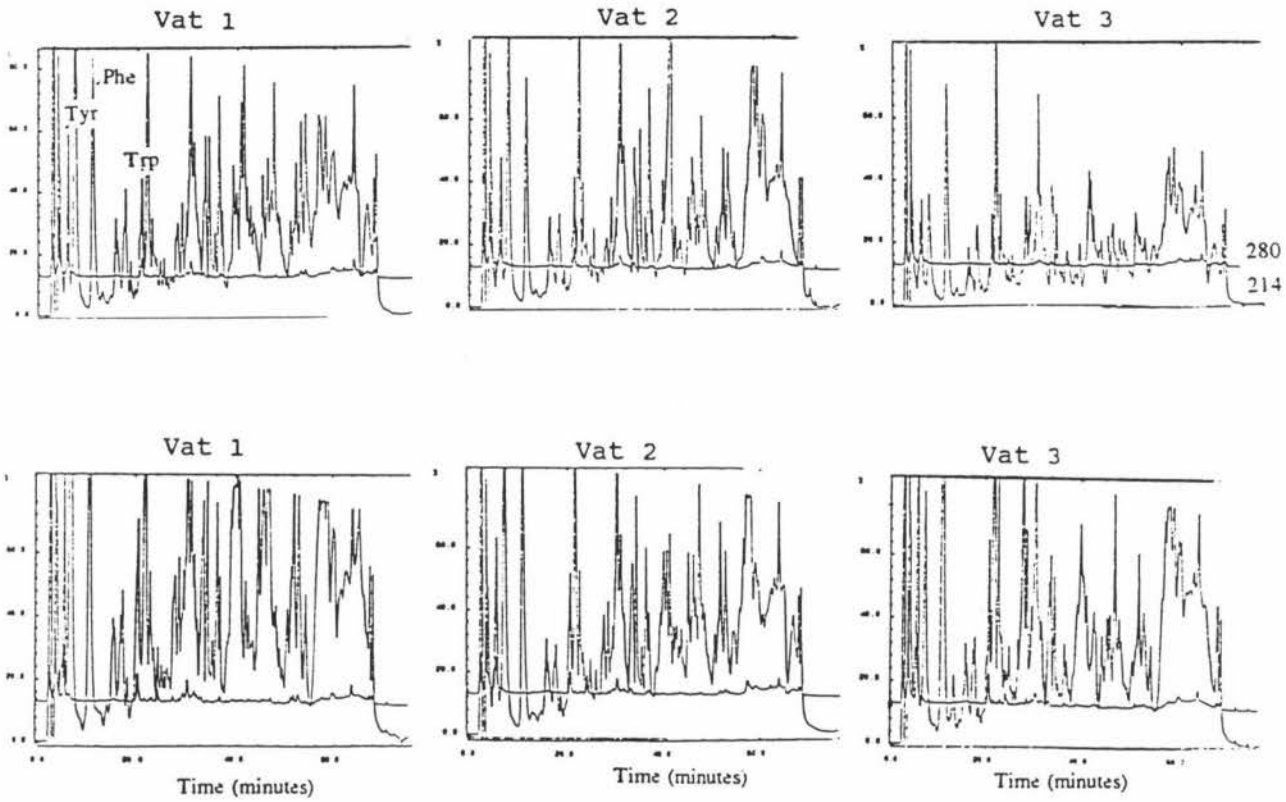


FIGURE 3.12 The effect of ripening temperature on the RP-FPLC profiles of the WSF of Cheddar cheese from vats 1, 2 and 3. The cheeses were ripened at 10°C for 10 days and then at 5 and 13°C for 94 days.

A large amount of water-soluble material formed in the cheese from vat 1 and the total peak area increased from 1957 units at 5°C to 2719 units at 13°C. Less water-soluble material was formed in the cheese from vat 2 and the total peak area increased from 1785 units at 5°C to 2119 units at 13°C. The amount of water-soluble material formed in the cheese from vat 3 at 5°C was less than in either of the other cheeses. The total peak area increased from 1212 units at 5°C to 2230 units at 13°C, which was similar to the amount formed in the cheese from vat 2.

The differences in the total amount of water-soluble material in each of the three cheeses were probably due to differences in the starter enzyme activities, as the only difference between each of the cheeses was in the starter combination used.

The number of peptides in the WSF of the cheese appears to have been the same regardless of the temperature at which the cheese was ripened. However, the amount of each peptide and the ratio of the peptides changed as more water-soluble peptides were formed due to the hydrolysis of the caseins and as the larger and more hydrophobic peptides were broken down to smaller peptides and amino acids.

The amount of material in the later eluting peaks (tryptophan to 75 min) was calculated as a percentage of the total amount of water-soluble material with an absorbance at 214 nm (Table 3.3).

TABLE 3.3 The effect of storage temperature (5 and 13°C) on the amount of material that eluted between tryptophan and 75 min, calculated as a percentage of the total amount of water-soluble material with an absorbance at 214 nm.

Vat	13°C	5°C
1	80.2%	79.5%
2	80.7%	80.7%
3	78.2%	78.8%
Average	79.7%	79.7%

The values obtained for the cheese ripened at 5 and 13°C were higher than for the frozen and freeze-dried cheese samples (Figure 3.6). The almost identical values obtained at both temperatures indicate that the ratio of proteolytic to peptidolytic activity was the same at both temperatures.

6 DISCUSSION

PART I

OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES

6.1 THE STORAGE OF CHEESE SAMPLES AT LOW TEMPERATURE

The cheese samples were not completely stable at any of the frozen storage temperatures.

The examination of the whole cheese by gel electrophoresis indicated that the samples stored at -9 and -16°C were similar and those stored at -35, -75 and -100°C constituted a second group.

In each of the cheese samples stored at -9 and -16°C, a decrease in the ratio of α_{s1}-casein to β-casein (Figure 3.1) with a corresponding small decrease in the ratio of α_{s1}-casein to α_{s1}-casein-I [found by Haasnoot *et al.* (1989) to be a suitable indicator of maturity in very young cheese ($\leq$ 4 weeks)] was measurable after 66 days (Figure 3.2), and this decrease continued during storage of the samples. This indicates that rennet (and possibly any other aspartate proteinases present) was active in the cheese at these temperatures.

According to Levine and Slade (1991), the stability of a substance stored at low temperature is related to the difference between T_f (temperature of the freezer) and T_g' (the subzero glass transition temperature of the amorphous solute/unfrozen water matrix surrounding the ice crystals in a maximally freeze-concentrated aqueous solution) and the storage life is limited when T_g' is lower than T_f. The T_g' for cheese is dependent on the stage of ripeness and is governed by the composite molecular weight of the water-soluble solids. The T_g' for Cheddar cheese was reported, by Levine and Slade (1989), to be -24.5°C (maturity not stated). At temperatures below T_g' any further rennet activity would occur at a rate that is too slow to be measurable.

A decrease in the ratio of α_{s1}- to β-casein was observed in the cheese samples that were held at -35, -75 and -100°C (Figure 3.1) for 94 and 180 days but no corresponding decrease in the ratio of α_{s1}-casein to α_{s1}-casein-I was observed (Figure 3.2). One interpretation of this result could be that it is unlikely that changes in the ratio of the caseins (based on the amount of dye bound by each casein) could be attributed to rennet activity and that these changes must have been due to some chemical modification of

the proteins that affected the amount of dye bound. As the error associated with using the ratio of α_{s1}- to β-casein was found to be small (mean error, 1.48%; standard deviation 1.37%), a second interpretation of these findings could be that any changes in the ratio of α_{s1}-casein to α_{s1}-casein-I were too small to be detected by gel electrophoresis and that the decrease in the ratio of α_{s1}- to β-casein could have been due to proteolytic activity.

The lack of stability observed in the frozen cheese samples that were analysed by gel electrophoresis is supported by the analysis of the RP-FPLC profiles of the WSF of the cheese samples (Figure 3.6), which indicates that, although the cheeses were more stable at -100°C than at any other temperature, they were not completely stable at any of the frozen storage temperatures. As the sample storage temperature increased from -100 to -9°C, there was a small overall increase in the amount of later eluting (tryptophan to 75 min) material, believed to be composed of the larger and more hydrophobic peptides (Cliffe *et al.*, 1993). This trend is consistent with the changes that occur, as the result of proteolysis, during the early stages of Cheddar cheese maturation (Chapter 4, Figure 4.10). Within this trend, a second trend was observed in the samples stored at temperatures of -75 to -9°C. As the sample storage temperature increased towards -16 and -9°C, there was a slight increase in the amount of material in the early eluting peaks and this was reflected in a slight decrease in the apparent amount of material in the later eluting peaks (when expressed as a percentage of the total amount of material with an absorbance at 214 nm). These results suggest that, although there were more large and hydrophobic peptides released by the proteolytic enzymes in the frozen cheese samples that were stored at temperatures between -9 and -75°C than in the cheese samples stored at -100°C, the amount of small peptides released by the proteolytic and peptidolytic enzymes increased as the sample storage temperature approached -16 and -9°C.

This apparent lack of stability of the proteins at low temperature is inconsistent with the glass transition theory if the changes occurred during frozen storage, rather than during freezing and thawing. These findings are consistent with the views of others within the food industry. Discussions with R. Winger (formerly of the Meat Industry Research Institute of New Zealand, currently Professor of Food Technology, Massey University, New Zealand) revealed that the activity of enzymes at very low temperatures affects the storage life of frozen meat and presents problems for the meat export industry. Deterioration in the texture of fish during storage at -40°C has been attributed to the action of enzymes (Jahncke *et al.*, 1992).

There are several factors, discussed in the following paragraphs, that may have resulted in continued proteolysis or hydrolysis, or in some modification of the proteins, during freezing and thawing or while the cheese was stored at low temperatures, that may have contributed to the apparent lack of stability in the frozen cheese samples.

The moisture phase of cheese contains various buffer salts including amino acids, phosphate, citrate, phosphate esters, carbonate, various carboxylic acids, various amines and lactic acid. The effect of freezing on the pH of buffers differs according to the type of buffer salts involved (Hill, 1988; Hill and Dickinson, 1989; Hill and Buckley, 1991). The pH of frozen Cheddar cheese whey, which is believed to be representative of the moisture phase of cheese, decreased to $\leq$4.0 (the lowest pH measurable by Universal Indicator) at temperatures of $\leq$-35°C. This decrease in pH is caused by the differential precipitation of the salts (van den Berg and Rose, 1959) as the water is removed as ice and pockets of concentrated solutes are formed. According to Hill and Buckley (1991), it is likely that chemical and enzymic reactions occur in these pockets of concentrated solutes during both freezing and thawing. The rate at which samples are frozen and the time spent in the frozen state are both important factors in determining the amount of protein degradation (Hill, 1988) (of aldehyde dehydrogenase). In the microenvironments within the cheese, the formation of pockets of concentrated solutes at low pH would have occurred during freezing, while the temperature was above T_g', and may have contributed to some chemical or enzymic modification of the proteins in the cheese samples that were stored above T_g' (-9 and -16°C, at least) and during freezing and following the onset of thawing in the samples stored at lower temperatures (-35, -75 and -100°C). It was observed that only the 94 and 180 day samples were affected and this could have been due to differences in the time taken for individual samples to freeze and thaw. As previously suggested, it seems more likely that the changes in the samples were progressive and were related both to the time spent in frozen storage and to the storage temperature. This is inconsistent with the glass transition theory, but perhaps this theory does not hold true in complex polymeric systems (O R Fennema, 1992, personal communication).

In summary, during the later stages of freezing, following the onset of thawing and perhaps during frozen storage, the proteins in cheese are in close proximity to one another in a low water environment and are surrounded by regions of concentrated NaCl, enzymes and buffer salts at low pH and this environment may have resulted in their modification or hydrolysis by the proteolytic enzymes that were present. The apparent lack of any new peptides (Figure 3.4) in the frozen cheese indicates that acid

hydrolysis of the caseins or peptides in cheese stored at low temperatures is unlikely to have occurred. The increase in the amount of later eluting material in the RP-FPLC profiles of the WSF of the frozen cheese (Figure 3.6) is consistent with further enzyme activity during storage and possibly during freezing and thawing of the cheese samples at -9 and -16°C and during frozen storage and/or during freezing and thawing of the samples stored at -35, -75 and -100°C.

6.2 THE STORAGE OF FREEZE-DRIED CHEESE SAMPLES

The temperature of the cheese samples that were freeze-dried was gradually reduced to -45°C during the early stages of freeze-drying. Therefore, the conditions to which these cheese samples were subject were similar to those experienced by the samples frozen at -35 to -100°C and they had similar stability. A decrease in the pH (to pH $\leq$4) of the cheese whey was demonstrated to occur during freeze-drying and it is believed that the freeze-concentration effect thought to occur in the frozen cheese samples would also have occurred within the cheese samples during freeze-drying.

An examination of the ratio of α_{s1}- to β-casein showed a decrease between 66 and 94 days at 4°C (Figure 3.7). Although the level of moisture in the freeze-dried samples may have been high enough to facilitate enzyme activity, there was no measurable increase in the amount of any of the casein degradation products (*e.g.* α_{s1}-casein-I). It is possible that the acid environment may have modified the caseins in such a way that the dye binding capacity was altered, giving rise to changes in the ratio of α_{s1}- to β-casein. Alternatively, gel electrophoresis may not be sensitive enough to detect small changes in the amount of α_{s1}-casein-I.

An examination of the WSF of the freeze-dried cheese samples (Figure 3.8) revealed no new peptides, and acid hydrolysis of the proteins or peptides is therefore thought to have been unlikely. The amount of material in the later eluting peaks, when expressed as a percentage of the total amount of material, was less than in any of the frozen cheese samples. This suggests that the freeze-dried cheese samples were more stable than many of the frozen cheese samples and were most similar to the cheese samples stored at -100°C. However, on the basis of the gel electrophoretic results, it is unlikely that the samples were completely stable.

6.3 THE STORAGE OF CHEESE SAMPLES IN UREA SAMPLE BUFFER

The samples stored in urea sample buffer at alkaline pH exhibited a time-dependent lack of stability (Figure 3.10). An examination of the pH of this buffer at -16°C (Table 3.2) showed that there was no change and therefore acid hydrolysis of the proteins during frozen storage could be discounted. Lysine and cysteine residues on the caseins react with cyanate formed from the urea at alkaline pH and the altered caseins have a slightly different electrophoretic mobility from the parent band (Cole and Mecham, 1966), thus giving rise to the loss of sharpness of α_{s2}-casein and the extra bands seen on the gel. This was particularly apparent in the samples that were stored at 4°C: there was at least one extra band in the region of the γ_1- and γ_2-caseins, each of the α_{s1}- and β-casein bands seemed to be composed of two equal sized bands and the α_{s2}-casein band was very indistinct. Similar but less obvious trends were apparent in the samples stored at -16°C (Figure 3.9).

Subsequent to the study reported above, alkaline urea-PAGE was used to determine whether proteolysis occurred during frozen storage (-18°C) of a Cheddar cheese designed to have reduced rennet activity (Coker, 1994). At regular time intervals, a large representative cheese sample was taken and stored at -86°C until all the samples had been collected. The samples were analysed on the same set of gels. A small amount of proteolysis was found to occur at -18°C, with approximately 4% of the α_{s1}-casein (as determined by the ratio of α_{s1}- to β-casein) having been hydrolysed over a 6 month period. This is above the 1.48% error (when samples are analysed on the same set of gels) associated with the method (Section 5.1.1). This result substantiates the finding that there is a small amount of proteolysis in cheese samples that are frozen at -9 and -16°C.

On the basis of these findings and the findings of the present study, which demonstrate that proteolysis occurs at low temperatures, it is recommended that all the samples for a particular experiment should be stored under conditions that prevent deterioration and should be analysed together on gels made at the same time, with the same batch of solutions and stained and destained with the same batch of stain and destain. As there was much less proteolytic activity in the cheese samples stored at -100°C than at any other frozen storage temperature (Figure 3.6), it is recommended that cheese samples should be stored at temperatures below -75°C and preferably closer to -100°C. A less practical alternative would be to freeze-dry the grated and weighed cheese samples and store them in the dark at low temperature.

PART II

THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

The reduction of the ripening/storage temperature from 13 to 5°C resulted in a substantial decrease in the rate of proteolysis, as measured by the decrease in peak volume of the α_{s1}- and β-casein bands on alkaline urea gels (Figure 3.11). A decrease from 13 to 5°C produced levels of proteolysis after 180 days (at least 76-79% of the α_{s1}-casein and 12-13% of the β-casein had been hydrolysed) that were comparable with those seen in the same cheese that had been ripened at 13°C for 66 days (75-78% of the α_{s1}-casein (a level that is consistent with the results of others (*e.g.* Calvo *et al.*, 1992)) and 13-16% of the β-casein had been hydrolysed). RP-FPLC analysis of the WSF of the cheeses stored at 5 and 13°C (Figure 3.12) indicated that the pattern of peaks produced from the cheeses ripened at both temperatures was the same, although there was more water-soluble material in the cheese ripened at the higher temperature. However, the patterns among the cheeses from the three different vats were quite different, indicating that a decrease in ripening temperature did not alter the ratio of activities of the various starter (different between vats) and other enzymes present. Further analysis of the RP-FPLC profiles revealed that the ratio of early eluting (zero-time to tryptophan, inclusive) to later eluting (from immediately after tryptophan to 75 min) material was the same in the cheese stored at both 5 and 13°C. Thus, for the purpose of controlling ripening so that Cheddar cheese would be available for export throughout the year, ripening at 5°C would seem to be an option that does not affect the balance of the peptides produced and is therefore unlikely to affect the characteristics of the cheese.

A product with predictable and relatively constant properties is required for some manufacturing purposes where cheese is used as an ingredient or where it is destined for the process kettle. Of the two freezing temperatures that were investigated, storage at -16°C resulted in a slightly less degraded product than storage at -9°C (Figures 3.1 and 3.6). According to O R Fennema (1992, personal communication), temperatures in the vicinity of -9 and -10°C are not recommended for the frozen storage of foodstuffs as there is a tendency for the formation of many large ice crystals, which would result in changes to the cheese structure. As New Zealand cheese factories have storage facilities at -18°C this would seem to be a practical cheese storage temperature to minimise protein degradation.

7 CONCLUSIONS

PART I

OPTIMUM STORAGE CONDITIONS FOR CHEDDAR CHEESE SAMPLES

None of the methods used to store the cheese samples was completely satisfactory.

Cheese stored at temperatures of -9 and -16°C was unstable, with proteolysis discernible after 66 days. Storage of cheese samples at these temperatures is therefore not recommended.

Cheese stored at temperatures of -35, -75 and -100°C was unstable after 94 days, although the samples stored at -100°C were more stable than the samples stored at higher temperatures. This lack of stability probably arises during both freezing and thawing as well as during frozen storage of the cheese samples.

Storage of freeze-dried samples at 4°C in the dark was, at least, equivalent to storing them at -100°C.

Storage of samples in alkaline urea sample buffer was better at -16°C than at 4°C but should be for no longer than 1 month. Storage of samples in alkaline urea sample buffer at 4°C cannot be recommended.

Any further experiments should investigate the effect of the rate of freezing and thawing on the proteins in cheese as this may be an important factor in determining the stability of the frozen cheese samples.

PART II

THE EFFECT OF STORAGE TEMPERATURE ON PROTEOLYSIS IN CHEDDAR CHEESE

The ripening of cheese can be achieved at temperatures that substantially slow the rate of proteolysis with little effect on the ratio of activities of the various enzymes present or on the balance of peptides produced. The use of lower temperatures to control the ripening of cheese would therefore seem to be a better solution than the alteration of variables such as salt-in-moisture or pH, which affect the enzyme activities in the cheese differently and are therefore likely to result in the generation of different peptide profiles and a cheese with different characteristics.

Proteolysis is markedly slowed by storage of the cheese at -9 and -16°C.

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CHAPTER 4

A COMPARISON OF PROTEOLYSIS IN CHEDDAR AND MOZZARELLA CHEESES USING DIFFERENT METHODS OF ANALYSIS

SUMMARY

The differences in the processing parameters for Cheddar and Mozzarella cheeses result in differences in the functional properties of each cheese type, which are probably a reflection of differences in the proteolytic pathways. Although the proteolytic pathways in Cheddar cheese have been studied in detail, comparatively little is known about those in Mozzarella cheese.

The purpose of this study was to examine and compare the action of the proteolytic enzymes in Cheddar and Mozzarella cheeses over a six month ripening period.

The proteolytic pathways in Cheddar and Mozzarella cheeses, manufactured according to standard protocols, ripened at 13°C and sampled at regular intervals over a six month period were examined using a variety of techniques: total nitrogen (TN), non-protein nitrogen (NPN), water-soluble nitrogen (WSN), alkaline urea polyacrylamide gel electrophoresis (alkaline urea-PAGE), low molecular weight sodium dodecyl sulphate-PAGE (LMW SDS-PAGE), reverse phase fast protein liquid chromatography (RP-FPLC) and size exclusion high performance liquid chromatography (SE-HPLC). The TN and NPN analyses were done at the time of sampling whereas the other assays were done on samples that had been stored at <-75°C so that they could be analysed simultaneously.

The WSN increased more rapidly in Cheddar cheese than in Mozzarella cheese, indicating that there was more proteolytic activity in Cheddar cheese.

The increase in NPN was greater in Cheddar cheese than in Mozzarella cheese and reflected the greater microbial enzyme activity in this cheese type. The small amount of NPN formed in the later stages of Mozzarella ripening was atypical and a consequence of the abnormally high (13°C) ripening temperature.

Alkaline urea-PAGE revealed that there was more α_{s1}-casein hydrolysis (with the

formation of α_{s1}-casein-I) in Cheddar cheese than in Mozzarella cheese, indicating that rennet activity was greater in Cheddar cheese. The presence of peptides believed to be β-I- (β-casein f1-189/192) and β-II-casein (β-casein f1-165) indicated that rennet may have hydrolysed β-casein. The amount of β-casein hydrolysis (and γ-casein formation) was greater in Mozzarella cheese, reflecting the greater plasmin activity in this cheese type. Both LMW SDS-PAGE and SE-HPLC of the whole cheese provided little additional information.

Examination of the water-soluble fraction (WSF) of each cheese by PAGE analysis showed that many of the larger peptides may have been present in both cheese types. The different concentrations of these peptides in each cheese type were consistent with different rennet and plasmin activities and suggested that they may have been products of these enzymes.

RP-FPLC and SE-HPLC analysis of the WSF of Cheddar cheese revealed that, although the larger peptides continued to accumulate during ripening, there was also a large increase in the amount of small peptides and amino acids in the cheese. In the Mozzarella cheese, the larger peptides accumulated and there was little evidence of their further hydrolysis to small peptides and amino acids.

The differences in the enzyme activities in the two cheese types were readily related to differences in the processing parameters. The greater rennet and microbial enzyme activities in the Cheddar cheese, despite the higher salt-in-moisture (S/M), were due to the greater initial rennet level, the lack of heat treatment and the favourable moisture content of the cheese. The low pH and high S/M contents of the Cheddar cheese were unfavourable for plasmin activity. The lower level of rennet activity in the Mozzarella cheese was mainly due to the lower initial level of rennet and to the effect of the high heat treatment on the survival of rennet, whereas the negligible amount of microbial enzyme activity reflected the effect of the heat treatment as well as the method of salting on the activity of the microbial enzymes. The greater plasmin activity in the Mozzarella cheese reflected the heat activation of plasminogen as well as the higher pH, the lower S/M and the higher moisture content of the cheese, conditions that favour plasmin activity.

It was concluded that rennet (and possibly milk acid protease) and plasmin contribute mainly to the formation of the larger and intermediate molecular weight peptides and contribute little to the formation of low molecular weight peptides and amino acids in cheese, and that it is the microbial enzymes (which are also damaged by the 68°C heat

treatment in Mozzarella cheese) that are largely responsible for the generation of low molecular weight peptides and amino acids in cheese.

The present studies indicate that SE-HPLC using a Toyo-Soda SW 2000 column and a 36% acetonitrile/0.1% trifluoroacetic acid solvent system is a promising new technique that may be useful in determining cheese type and maturity and in relating changes in the molecular weight distribution of the peptides to changes in the textural, functional and flavour characteristics of cheese.

It was concluded that the results are consistent with the concept that differences in the manufacture of Cheddar and Mozzarella cheeses result in the formation of two cheeses, each with different amounts of similar enzymes (rennet, plasmin, and the enzymes of the starter and non-starter lactic acid bacteria), and that these differences in enzyme concentration, combined with the modifying effect of pH, temperature, moisture content and S/M, result in different enzyme activities and patterns of proteolysis in the two types of cheese and these, in turn, result in cheeses with different functional properties.

1 INTRODUCTION

Proteolysis and its relation to the texture and flavour characteristics of Cheddar cheese has been widely studied by many researchers; however, comparatively little work has been carried out on proteolysis in Mozzarella cheese. Approximately 9000 tonnes of Mozzarella cheese is manufactured annually in New Zealand for use on pizza. The desirable functional properties of this cheese, and particularly its ability to form fibres or strings when hot (Kosikowski, 1966), are believed to be closely related to the level of intact casein (Creamer, 1976b; Guinee and Wilkinson, 1992) and to a critical level of calcium and phosphate (Lucey, 1990) which has been found to be influenced by the pH at whey draining (Kiely *et al.*, 1992; Kindstedt *et al.*, 1993).

The enzymes responsible for the loss of intact casein in cheese are rennet, plasmin (and other indigenous enzymes of milk) and, to a lesser extent, the enzymes of the starter and non-starter lactic acid bacteria (NSLAB). Rennet is responsible for the rapid hydrolysis of the Phe_{105} - Met_{106} bond of κ-casein (Delfour *et al.*, 1965) during cheese manufacture, resulting in destabilisation of the casein micelles and the formation of a coagulum. It is also responsible for the hydrolysis of α_{s1}-casein during the early stages of ripening (Creamer and Richardson, 1974) and its activity is believed to be related to changes in cheese texture (de Jong, 1976, 1977, 1978; Creamer and Olson, 1982; Creamer *et al.*, 1988; Johnston *et al.*, 1994). Plasmin hydrolyses β-casein (with the formation of γ-casein) and α_{s2}-casein (Snoeren and van Riel, 1979; Richardson, 1983), with α_{s1}-casein being hydrolysed more slowly (Chen and Ledford, 1971; Kaminogawa *et al.*, 1972; Noomen, 1975; de Rham and Andrews, 1982). The microbial enzymes, which hydrolyse β-casein and to a lesser extent κ- and α_{s1}-casein in solution, are not believed to hydrolyse β-casein in cheese (McSweeney *et al.*, 1993d) and contribute mainly to the breakdown of peptides.

Differences in the composition and manufacture (Creamer and Olson, 1982; Lawrence *et al.*, 1987; Lucey, 1990) of Cheddar and Mozzarella cheeses could be expected to result in differences in both the retention and the activity of these enzymes.

In Mozzarella cheese manufacture, lower initial rennet concentrations are used than in the manufacture of Cheddar cheese. This, combined with a higher pH at whey draining, ensures a lower level of rennet activity in Mozzarella cheese than in Cheddar cheese. There is also a high temperature heat treatment ($\sim 68°C$) during the stretching of Mozzarella which diminishes the rennet activity even further. The high temperature

heat treatment is also likely to result in a higher level of plasmin activity (Creamer, 1976b; Farkye and Fox, 1990), due to the inactivation of a heat sensitive inhibitor of the plasminogen activator (Richardson, 1983), and a lower level of microbial enzyme activity in Mozzarella than in Cheddar cheese. The high moisture content of Mozzarella cheese combined with a higher pH and lower salt-in-moisture (S/M), than in the Cheddar cheese, are likely to enhance the activity of the enzymes present. The temperature at which Mozzarella cheese is stored is usually much lower (4°C) than the temperature at which Cheddar cheese is ripened (10-13°C), and this reduces the rate of enzyme activity in Mozzarella cheese.

The only direct comparison of the ripening of Cheddar and Mozzarella cheeses was carried out by Creamer (1976b) who compared the total nitrogen (TN), non-casein nitrogen (NCN) and non-protein nitrogen (NPN) contents in Cheddar and Mozzarella cheeses at 12 weeks. He concluded that the action of the microbial enzymes, which are considered to be largely responsible for the production of NPN in cheese (O'Keeffe *et al.*, 1976), is considerably diminished in Mozzarella cheese. He also demonstrated the effect of pH on the activity of plasmin in Mozzarella cheese, dispersed in sodium citrate solution, and found a higher level of plasmin activity at the higher assay pH (8.5).

A review of the proteolytic enzymes in cheese can be found in Chapter 1, Section 2 and a review of proteolysis in Cheddar and Mozzarella cheeses can be found in Chapter 1, Sections 3.1 and 3.4, respectively.

2 OBJECTIVE

The purpose of this study was to examine and compare the patterns of proteolysis in Cheddar and Mozzarella cheeses over a six month ripening period.

3 MATERIALS AND METHODS

3.1 CHEESE MANUFACTURE

The Cheddar and Mozzarella cheeses used during this study were selected from a group of cheeses manufactured by members of the Cheese Technology Section (NZDRI). The selection was on the basis of normal manufacture and appropriate composition at day 1.

3.1.1 Cheddar cheese manufacture

Cheddar cheese was manufactured using mid-season milk (November) according to a standard protocol. Whole milk was standardised to a protein:fat ratio of 0.80, resulting in a final milk composition of 4.24% fat, 3.38% protein and 4.85% lactose. The standardised milk was thermised (65°C/15 s) and held overnight at 4.7°C prior to pasteurisation (72°C/15 s).

The vat was filled with milk and the temperature brought to 33°C prior to the addition of 2.5% starter (*Lactococcus lactis* subsp. *cremoris* strains). New Zealand standard strength (59 Rennin Units/ml, >95% pure chymosin) calf rennet (New Zealand Co-operative Rennet Company, Eltham, New Zealand) was added (16.0 ml/100 litres of milk) to the milk in the vat. The vat contents were mixed mechanically and the milk was left to set at 33°C. After 40 min, the coagulum was cut and stirring was commenced while the temperature was raised to 38°C and was continued during cooking. At pH 6.22, the curds and whey were pumped out on to the conveying system, where the whey was drained from the curd which was then cheddared, milled, salted, hooped and pressed. The next day, the cheese was vacuum sealed into 20 kg blocks. The cheese was ripened at 13°C.

3.1.2 Mozzarella cheese manufacture

Mozzarella cheese was manufactured using mid-season milk (November) according to a standard protocol (New Zealand). Whole milk was standardised to a protein:fat ratio of 1.46, resulting in a final milk composition of 2.37% fat, 3.47% protein and 5.01% lactose. The standardised milk was thermised (65°C/15 s) and held overnight at 3.9°C prior to pasteurisation (72°C/15 s).

The vat was filled with milk and the temperature brought to 33°C prior to the addition of 4.0% starter (*Lactococcus lactis* subsp. *cremoris* strains) and held at this temperature for 65 min (pH 6.3). New Zealand standard strength calf rennet was added (4.0 ml/100 litres of milk) to the milk in the vat. The vat contents were mixed mechanically and

the milk was left to set at 33°C. After 55 min, the coagulum was cut and stirring was commenced while the temperature was raised to 36°C and was continued during cooking. The whey was drained at pH 5.90, the curd was milled, dry salted (to gain early control of the growth of the microorganisms), stretched at 68°C, hooped and cooled. The next day, the cheese was vacuum sealed into 20 kg blocks. The cheese was ripened at 13°C, to enable comparisons of proteolysis to be made between the two varieties of cheese. (The normal ripening/storage temperature is 2-4°C.)

3.2 CHEESE SAMPLING

The cheeses were sampled after 1, 14, 28, 63, 91, 133 and 182 days and subjected to the following analyses.

-On day 1, each cheese was analysed for TN, NPN, fat, moisture, salt and calcium contents and pH. The cheese (three plugs) was stored at <-75°C and analysed 6 months after manufacture (it was believed that the large changes in the level of the caseins anticipated during ripening would outweigh any deleterious freezing effects) for water-soluble nitrogen (WSN) content and by alkaline urea polyacrylamide gel electrophoresis (alkaline urea-PAGE), low molecular weight sodium dodecyl sulphate-PAGE (LMW SDS-PAGE), size exclusion high performance liquid chromatography (SE-HPLC) and reverse phase fast protein liquid chromatography (RP-FPLC).

-After 14 days, each cheese was analysed for NPN. The cheese (three plugs) was stored at <-75°C and analysed 6 months after manufacture for WSN content and by alkaline urea-PAGE, LMW SDS-PAGE, SE-HPLC and RP-FPLC.

-After 28, 63, 91, 133 and 182 days, each cheese was analysed for NPN. The cheese (three plugs) was stored at <-75°C and analysed 6 months after manufacture for WSN content and by alkaline urea-PAGE, LMW SDS-PAGE, SE-HPLC and RP-FPLC.

3.3 ANALYSES

The cheese pH and cheese fat, moisture, salt and calcium contents were measured by the staff of the Analytical Chemistry Section (NZDRI).

3.3.1 Fat, moisture, salt and calcium contents and pH

Samples of cheese were analysed for TN, NPN, fat, moisture, salt and calcium contents and pH using standard methods (New Zealand Ministry of Agriculture and Fisheries, 1979). The S/M and moisture in the non-fat substance (MNFS) were derived from

these values.

3.3.2 TN, NPN and WSN contents

TN content: The grated and weighed cheese samples were analysed using a Kjelfoss (Foss Electric, Hillerod, Denmark) nitrogen analyser.

NPN content: The grated and weighed cheese samples were solubilised in 0.1 M NaOH. Trichloroacetic acid (TCA) (15%) was then added to give a final concentration of 12% TCA. The precipitated protein was removed using Whatman No. 42 filter paper and the filtrate analysed for nitrogen content using the Kjelfoss nitrogen analyser.

WSN content: The water-soluble fraction (WSF) (3.000 g of grated cheese dispersed in 30.0 ml of water) was prepared according to the method described in Chapter 3 (Section 4.3). An aliquot (approximately 20 ml) was weighed, freeze-dried, reweighed and sent to the Campbell Microanalytical Laboratory (Department of Chemistry, University of Otago) where it was analysed for TN using a Carlo Erba Elemental Analyser EA 1108 (Milan, Italy).

3.3.3 Alkaline urea-PAGE

Proteins and peptides in the cheese and peptides in the WSF of the cheese were separated by alkaline urea-PAGE using the equipment and methods described in Chapter 2 (Section 4.3). Each sample was analysed in duplicate on a separate gel. Following destaining, the gels were photographed and scanned (Chapter 3 (Section 4.2)). Modifications to these methods are described below.

Sample and standard preparation:

Whole cheese samples. The cheese samples were thawed and then prepared according to the methods described in Chapter 2 (Section 4.2) and 5 μl samples were loaded on to the gels.

WSF. The WSF of the cheese was prepared according to the methods in Chapter 3 (Section 4.3). The amount of cheese used was increased to 3.000 g per 30.0 ml of water to give a more representative sample. The WSF was divided into 1 ml aliquots that were stored at -100°C. After thawing, the WSF was diluted 1:1 with alkaline urea sample buffer, treated with 2-mercaptoethanol (10 μl/ml) and held for 18 h. Bromophenol blue (10 μl/ml of a 0.1% *(w/v)* solution) was added to the samples prior to loading 20 μl on the gel.

Water-insoluble fraction. The pellet obtained from the centrifugation of the WSF was resuspended in urea sample buffer and treated in the same manner as the whole cheese.

Rennet casein standard. A casein standard was prepared by dissolving 10 mg of a fresh high quality dried commercial rennet casein (selected on the basis of >85% protein) in 6 ml of alkaline urea sample buffer. The standard was diluted 1:1 with sample buffer and then treated in the same manner as the WSF prior to loading 10 μl on to the gel.

Gel electrophoresis: The samples were electrophoresed, in duplicate on separate gels, until the dye front began to elute from the bottom of the gels (~1.4 h).

Staining protocol for the WSF: To optimise the amount of material retained in the gel, it was necessary to reduce the total time that the gels spent in the stain and destain solutions. The gels were treated with 50 ml of a 5% TCA solution for 30 min. (The use of 5% TCA as a fixative resulted in sharper and more intense bands, whereas the use of 12% TCA caused the gels to soften and the bands were not as intensely stained.) The gels were then stained with 50 ml Coomassie brilliant blue R solution (Chapter 2, Section 4.2) for 30 min and destained in 100 ml of Coomassie destain solution (Chapter 2, Section 4.2) for 1 h. At this point, destaining of the WSF was complete, with continued destaining resulting in the progressive loss of material from the bands. The staining and destaining were done in sealed containers with continuous agitation.

Staining protocol for the whole cheese and the water-insoluble fraction: For the purposes of quantitation, it was necessary to use the staining protocol outlined in Chapter 2 (Section 4.3). The shorter staining times that were used for the peptides of the WSF were insufficient to completely stain the larger protein bands. The minor bands were less affected than the larger bands which had 20-30% less dye bound to the proteins than was bound to the corresponding proteins in the gels stained for 1 h.

Photography: The gels were photographed using the protocol described in Chapter 2, Section 4.2.

Gel analysis: The gels were scanned and the images were analysed using the equipment and computer programs described in Chapter 3, Section 4.2.

3.3.4 LMW SDS-PAGE

Proteins and peptides in the cheese and peptides in the WSF of the cheese were separated by LMW SDS-PAGE (a method that uses 20% (*w/v*) acrylamide (0.5% C, where % C (% cross-linking) is the amount of bisacrylamide expressed as a percentage of the total acrylamide plus bisacrylamide) to enable the retention of small peptides) using the methods and equipment described in Chapter 2 (Section 4.5). Each sample was analysed in duplicate on a separate gel.

Sample and standard preparation:

Whole cheese samples. The whole cheese samples (Section 3.3.3) were diluted 1:1 with LMW SDS sample buffer. The samples (2 ml) were then heated using a heating block and held at 100°C for 90 s. Bromophenol blue (10 μl/ml of a 0.1% (*w/v*) solution) was added to the samples prior to loading 10 μl on the gel.

WSF. The WSF (Section 3.3.3) was diluted 1:1 with LMW SDS sample buffer and treated in the same manner as the whole cheese samples prior to loading 20 μl on the gel.

Water-insoluble fraction. The pellet obtained from the centrifugation of the WSF was resuspended in urea sample buffer and treated in the same manner as the whole cheese.

Rennet casein standard. A casein standard was prepared by dissolving 10 mg of a fresh high quality dried commercial rennet casein (selected on the basis of >85% protein) in 6 ml of LMW SDS sample buffer and diluting it 1:1 with sample buffer. The standard was then treated in the same manner as the whole cheese samples prior to loading 10 μl on the gel.

Gel electrophoresis: The samples were electrophoresed, in duplicate on separate gels, until the dye front began to elute from the bottom of the gel.

Staining: The gels were stained using the protocol outlined in Chapter 2 (Section 4.5).

Photography: The LMW SDS resolving gel swelled during destaining and it was necessary to cut it away from the stacking gel, to allow the resolving gel to lie flat, in order to photograph and scan it. The gels were photographed as in Section 3.3.3.

Scanning: The gels were scanned and the images were analysed using the equipment and computer programs described in Chapter 3, Section 4.2.

3.3.5 RP-FPLC

The formation of water-soluble peptides during the ripening of both Cheddar and Mozzarella cheeses was investigated by RP-FPLC using the Pharmacia FPLC system and the columns and methods described in Chapter 3 (Section 4.3), with the following modifications.

Channel 1: 280 nm, Min -0.05 AU, Max 0.3 AU, Output 1000 mV/100%
Channel 2: 214 nm, Min -0.05 AU, Max 1.0 AU, Output 1000 mV/100%
Time constant: 1 s

A Pharmacia LKB 2219 MULTITEMP II thermostatic circulator was used to maintain the samples at a temperature of $<4°C$.

RP-FPLC of the WSF of the cheese: The WSF (Section 3.3.3) was prepared according to the method described in Chapter 3 (Section 4.3). A 1 ml aliquot was removed from the freezer (-100°C), rapidly thawed (at 45°C) and placed in the sample holder from which it was drawn through the 200 μl sample loop. All 14 samples were analysed as part of one automated sequence. A gradient was programmed to run at the start and finish of each automated sequence.

The area under each peak (above a threshold level of 2%) was integrated and the chromatogram plotted.

3.3.6 SE-HPLC

The breakdown of protein to peptides and amino acids and the formation of water-soluble peptides and amino acids during the ripening of both Cheddar and Mozzarella cheeses was investigated by SE-HPLC using the method of Motion (1992) which is based on the method of Swergold and Rubin (1983).

A Waters Associates Chromatograph (Millipore Corp., Waters Chromatography Division, Massachussetts, USA) with a Waters 700 Satellite Wisp autoinjector and Maxima 820 Gel Permeation Chromatography software was used. The detector was a Hewlett Packard 1040A multiwavelength detection system (Hewlett Packard Company, Camas, Washington, USA). The columns were a TSK-GEL G2000-SW column (7.5 mm I.D. x 60 cm) (Toyo Soda Manufacturing Co. Ltd, Tokyo, Japan) with a TSK-GEL

guard column SW (7.5 mm I.D. x 7.5 cm).

The whole cheese samples were prepared by diluting 200 μl of the urea-soluble fraction (Section 3.3.3) of the cheese with 10 ml of the mobile phase (36% "Far UV" grade acetonitrile, 0.1% trifluoroacetic acid (TFA; A.R. grade), in Milli-Q water) and filtering the sample through a 0.80 μm/0.20 μm filter (purchased from Gelman Sciences, Ann Arbor, Michigan, USA).

The WSF samples were prepared by diluting 200 μl of the WSF (Section 3.3.3) of the cheese with 10 ml of the mobile phase.

At this point, the samples were given to the operator of the HPLC dedicated to determining molecular weight distribution profiles (Ms J. Wilkinson, Analytical Services, Food Science Section, NZDRI).

Standards of known molecular weight were analysed and the elution time of each standard was plotted against the logarithm of molecular weight to form a calibration curve. The standards were:

1. Glyceraldehyde-3-phosphate dehydrogenase	MW	36,000
2. Carbonic anhydrase	MW	29,000
3. Soybean trypsin inhibitor	MW	20,100
4. Lysozyme	MW	14,300
5. Aprotinin	MW	6,500
6. Insulin	MW	5,730
7. Insulin B-chain	MW	3,400
8. Bacitracin	MW	1,420
9. Phenylalanine	MW	165

The column was equilibrated with the mobile phase at a flow rate of 0.5 ml/min. The standard or sample (50 μl) was injected on-to the column at a flow rate of 0.5 ml/min and elution was complete after 1 h. The absorbance was monitored at 205 nm.

The urea sample buffer was analysed to determine the effect of the buffer constituents on the chromatograms.

4 RESULTS

4.1 COMPOSITIONAL ANALYSIS

The pH and the fat, moisture, salt, calcium, S/M and MNFS contents of the cheeses are shown in Table 4.1. The Mozzarella cheese was lower in fat, higher in moisture, and had a higher pH and lower S/M and calcium contents than the Cheddar cheese.

TABLE 4.1 The compositional analyses of the Cheddar and Mozzarella cheeses

Analysis	Cheddar	Mozzarella
TN (% *w/w*)	3.83	4.27
Fat (% *w/w*)	35.0	20.0
Moisture (% *w/w*)	34.2	48.0
pH	5.26	5.48
Salt (% *w/w*)	1.73	1.43
Ca (mM/kg)	201	168
S/M (%)	5.06	2.98
MNFS (%)	52.6	60.0

These cheese compositions were typical of those normally manufactured in New Zealand, with the values for Cheddar cheese falling within the suggested range for first grade Cheddar cheese (Lawrence *et al.*, 1993).

The higher pH and moisture content and the lower S/M content of the Mozzarella cheese could be expected to favour the activity of the enzymes present in this cheese.

4.2 PROTEIN BREAKDOWN

The Cheddar and Mozzarella cheeses were both ripened at 13°C to enable the activity of the enzyme complement of each cheese type to be compared. The normal practice for Mozzarella cheese is to store it at 2-4°C.

4.2.1 TN, NPN and WSN contents of the cheese

Trends in the levels of NPN and WSN (expressed as a percentage of the TN) in the Cheddar and Mozzarella cheeses are shown in Figure 4.1.

The amounts of WSN and NPN increased steadily, and at a similar rate, during the ripening of the Cheddar cheese. After 182 days of ripening at 13°C, the amounts of WSN and NPN were 23.1% and 20.1% of the TN, respectively.

In the Mozzarella cheese, the amounts of WSN and NPN produced during ripening were less than in the Cheddar cheese. The amount of WSN in the Mozzarella cheese increased more rapidly than the amount of NPN. After 182 days of ripening at 13°C, the amounts of WSN and NPN were 14.8% and 5.2% of the TN, respectively.

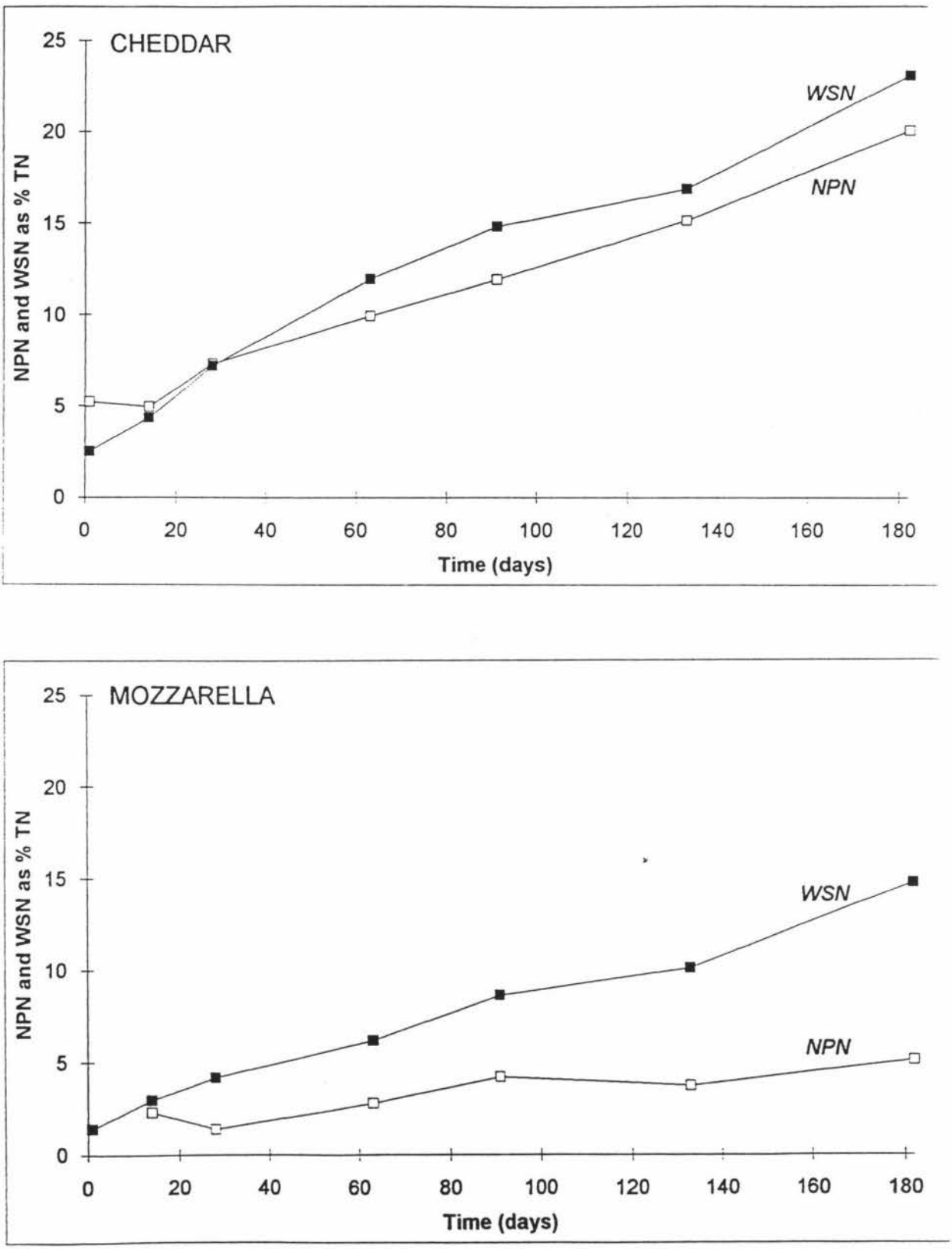


FIGURE 4.1 Changes in WSN and NPN (expressed as a percentage of the TN) in Cheddar and Mozzarella cheeses during maturation at 13°C.

4.2.2 Alkaline urea-PAGE of the whole cheese

The urea-soluble fraction (the whole cheese) of each cheese type was investigated by alkaline urea-PAGE. The breakdown of α_{s1}- and β-casein during the ripening of Cheddar and Mozzarella cheeses is depicted in the photographs shown in Figure 4.2.

After scanning the gels (Figure 4.2), analysing the scans and quantitating the results, the trends in the breakdown of α_{s1}- and β-casein in the ripening Cheddar and Mozzarella cheeses were plotted and are shown in Figure 4.3.

The extent of casein hydrolysis in the Cheddar cheese is typical of the variety. The hydrolysis of α_{s1}-casein by rennet, with the concomitant formation of α_{s1}-casein-I, was rapid, with 80% of the α_{s1}-casein hydrolysed by 63 days (Figure 4.3). The amount of α_{s1}-casein remained constant between 91 and 182 days and this may have been due to the presence of a peptide(s) migrating in the same position as α_{s1}-casein, as demonstrated by two-dimensional gel electrophoresis in Chapter 2 (Figures 2.4 and 2.7). The amount of α_{s1}-casein-I initially increased and then decreased progressively as the cheese matured (Figure 4.2). In the Cheddar cheese, β-casein was hydrolysed much more slowly than α_{s1}-casein, with only 12% hydrolysed by 63 days and 34% by 182 days (Figure 4.3). The products of β-casein hydrolysis (Figure 4.2) were the γ-caseins formed by plasmin action and the band tentatively identified as β-I-casein (β-casein f1-189/192) formed by rennet action. The band labelled "unknown" increased in intensity (Figure 4.2) during maturation of the Cheddar cheese and was more intense than in the Mozzarella cheese which suggests that it was a product of rennet (or microbial enzyme) action. The amount of α_{s2}-casein decreased during Cheddar cheese maturation (Figure 4.2) and this was probably due to plasmin action.

The extent of casein hydrolysis in the Mozzarella cheese was greater than would normally occur in this type of cheese because of the artificially high ripening temperature (13°C). The breakdown of α_{s1}-casein by rennet, in the Mozzarella cheese, was much slower than that in the Cheddar cheese, despite its lower S/M and higher moisture content (Table 4.1), with only 24% hydrolysed by 63 days (Figure 4.3). After 182 days, 70% of the α_{s1}-casein in the Mozzarella cheese had been hydrolysed. The lower rennet activity was due to the lower initial amount of rennet (see Section 3.1) as well as to the effect of the stretch temperature on rennet activity. During the early stages of maturation, β-casein was hydrolysed at the same rate as α_{s1}-casein with 23% hydrolysed after 63 days. After 182 days of maturation, 54.5% of the β-casein had been hydrolysed. The amount of α_{s2}-casein also decreased during maturation of the

Mozzarella cheese (Figure 4.2). The progressive hydrolysis of β-casein (with the concomitant formation of γ-caseins) and α_{s2}-casein was due to the action of plasmin.

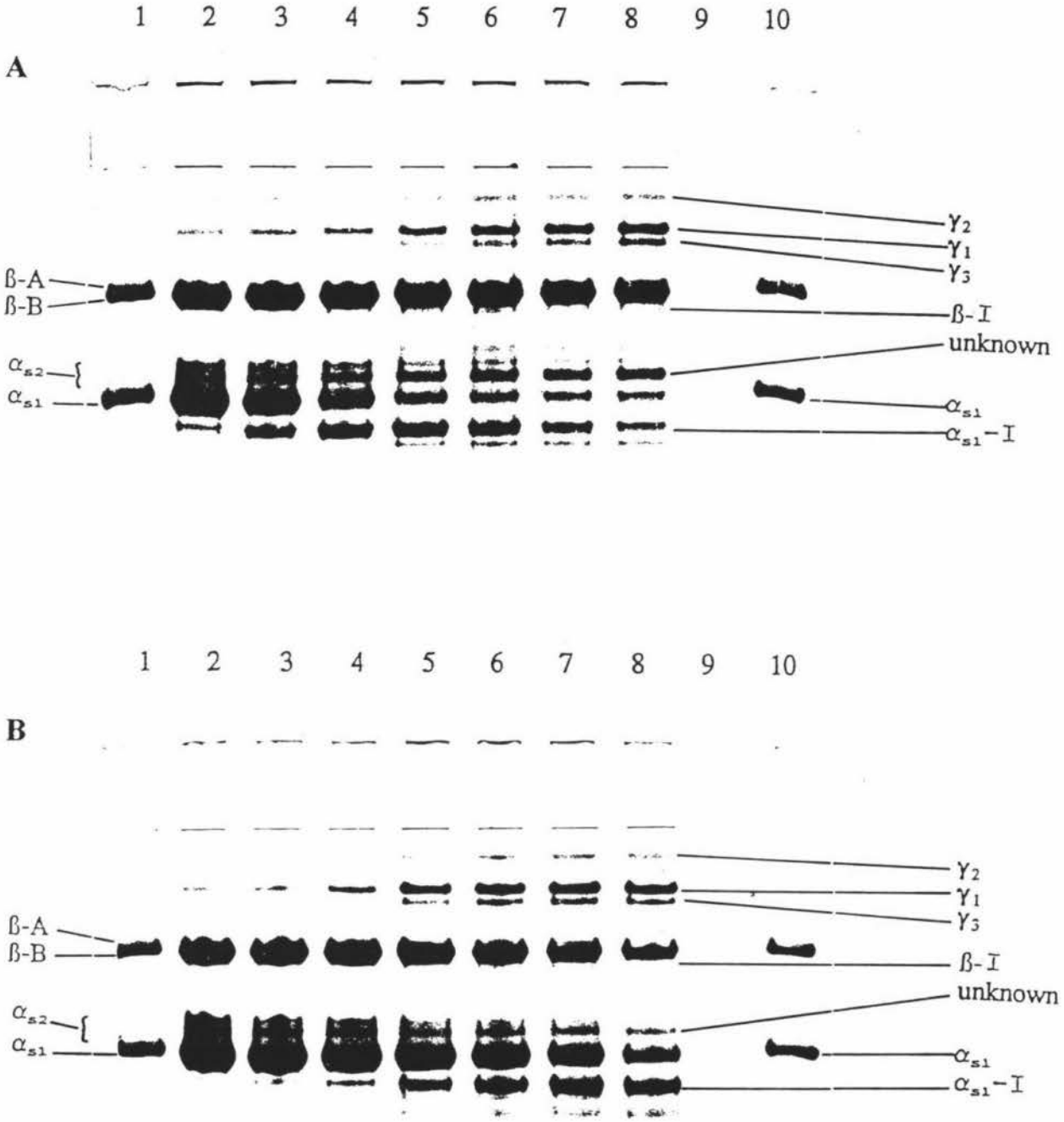


FIGURE 4.2 Alkaline urea-PAGE. Casein degradation in (A) Cheddar cheese and (B) Mozzarella cheese ripened at 13°C. Lanes 1 and 10, rennet casein standard; lanes 2 to 8, cheese (2% cheese, 5 μl sample volume) sampled after 1, 14, 28, 63, 91, 133 and 182 days of maturation, respectively.

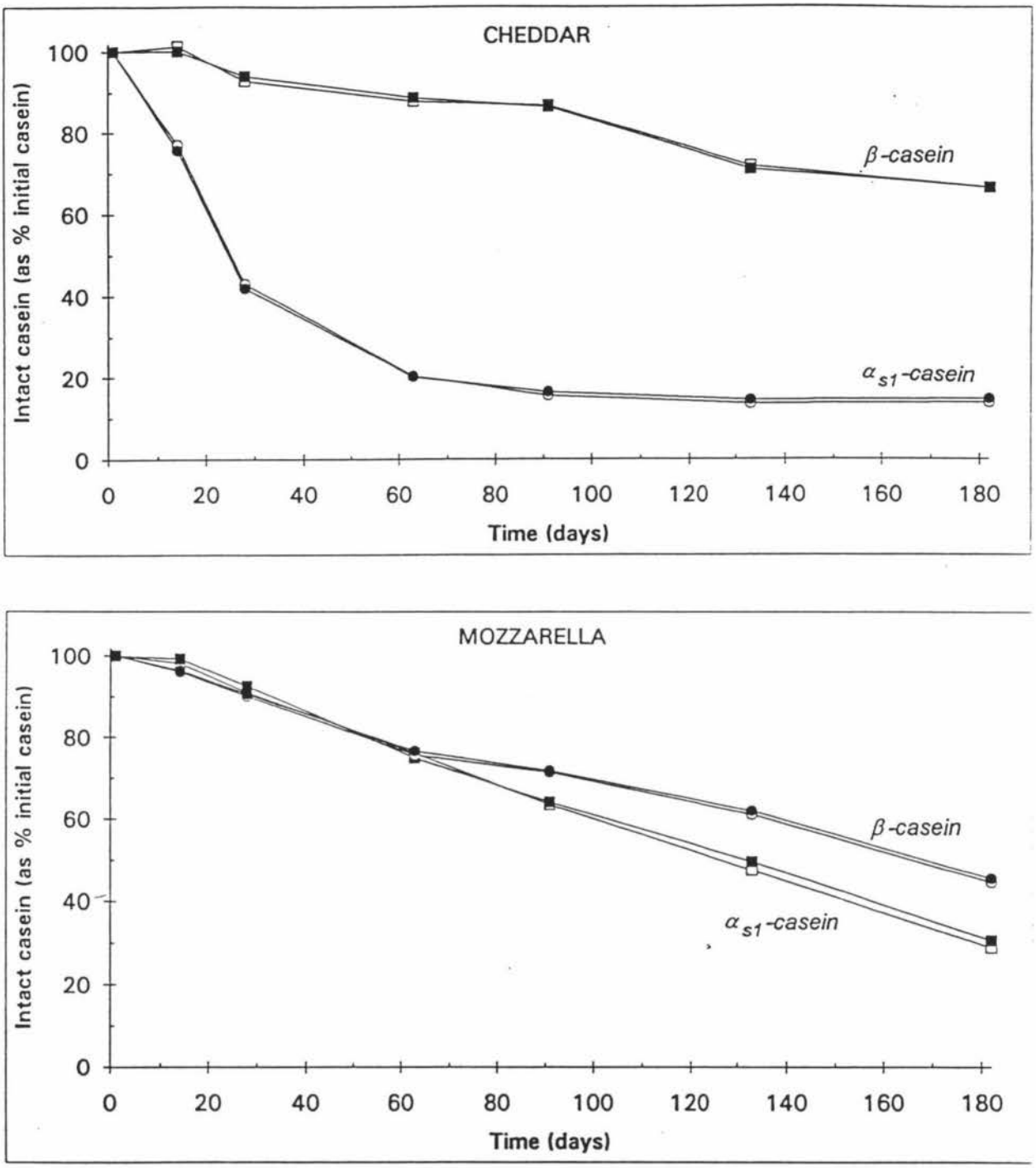


FIGURE 4.3 Trends in α_{s1}- and β-casein breakdown in Cheddar and Mozzarella cheeses ripened at 13°C, and sampled after 1, 14, 28, 63, 91, 133 and 182 days.

4.2.3 LMW SDS-PAGE of the whole cheese

Trends in proteolysis in Cheddar and Mozzarella cheeses can also be demonstrated by LMW SDS-PAGE (Figure 4.4). The identity of the bands was determined by two-dimensional gel electrophoresis in Chapter 2 (Figure 2.7).

This technique does not lend itself to quantitation of the caseins as they run in only two bands, the first of which contains the α_s-caseins and the second of which contains the κ- and β-caseins as well as α_{s1}-casein-I.

The intensity of the α_s (α_{s1} + α_{s2})-casein band decreased at a greater rate in the Cheddar cheese than in the Mozzarella cheese, which was consistent with the rate of loss of α_{s1}- and α_{s2}-casein identified by alkaline urea-PAGE (Figures 4.2 and 4.3).

The band migrating immediately ahead of the α_s-casein band comprised both α_{s1}-casein-I and β-casein. Although the intensity of this band decreased during the maturation of both cheese types, interpretation of this result is difficult because there were three events, identified in Section 4.2.2, that would each have affected the quantity of protein in this band. The three events were: the formation of α_{s1}-casein-I (as the α_{s1}-casein was hydrolysed), which would have resulted in an increase in the intensity of this band; the subsequent hydrolysis of α_{s1}-casein-I, which would have resulted in a loss of intensity of this band; and the hydrolysis of β-casein, which would also have resulted in a loss of intensity of this band.

The intensity of the γ_1- and γ_3-casein bands increased progressively during maturation of both the Cheddar cheese and the Mozzarella cheese. The band identified as γ_2-casein was barely visible in either cheese type.

The intensity of the "unknown" band increased at a greater rate in the Cheddar cheese than in the Mozzarella cheese, which is consistent with the findings in Section 4.2.2 (Figure 4.2).

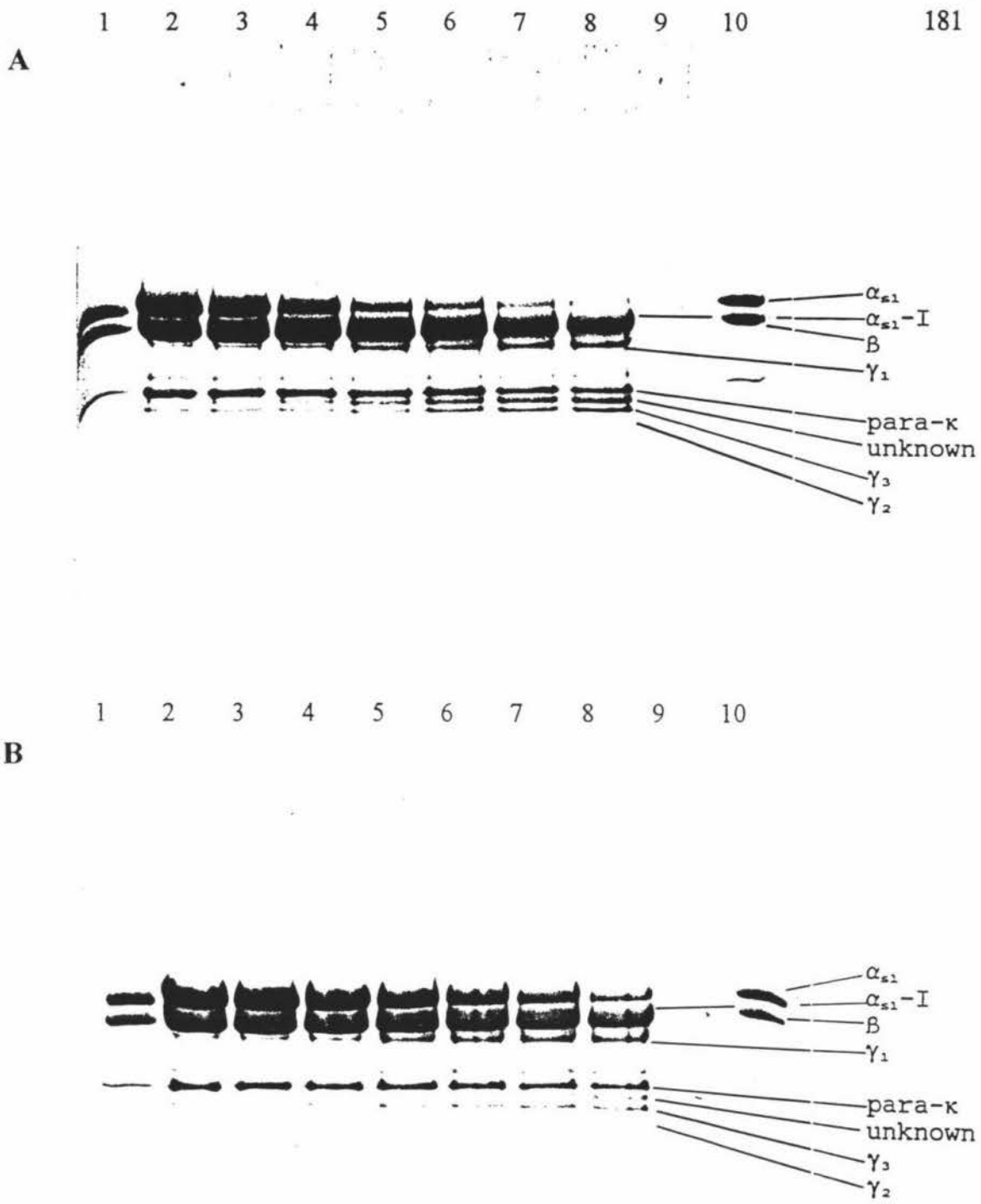


FIGURE 4.4 Proteolysis monitored by LMW SDS-PAGE in (A) Cheddar cheese and (B) Mozzarella cheese ripened at 13°C. Lanes 1 and 10, rennet casein standard; lanes 2 to 8, cheese (2% cheese in urea sample buffer, diluted 1:1 with SDS sample buffer, 10 μl loaded) sampled after 1, 14, 28, 63, 91, 133 and 182 days of maturation, respectively.

Although this technique was not suitable for monitoring the loss of intact caseins in the cheese, it was possible to monitor the formation of a band in the region of para-κ-casein. The band was quantitated using ImageQuant densitometry and the results are shown in Figure 4.5. The band was found to increase in intensity in each cheese type until 91 days. After 91 days, the intensity of the band decreased slightly, probably as a result of proteolysis. The band increased in intensity at the same rate in the Cheddar and Mozzarella cheeses despite the Mozzarella cheese having a lower level of rennet activity. It is therefore likely that this band, initially believed to be para-κ-casein, contained additional peptides. An examination of the two-dimensional gels (alkaline urea-PAGE and LMW SDS-PAGE) (Chapter 2, Figure 2.8) showed that there were several peptides located in the region in which para-κ-casein is normally found. Although para-κ-casein was not identifiable by two-dimensional electrophoresis, because it was not present on the alkaline urea gels used for the first dimension, the position it occupied was identified from the standards electrophoresed on the LMW SDS-PAGE gel used for the second dimension.

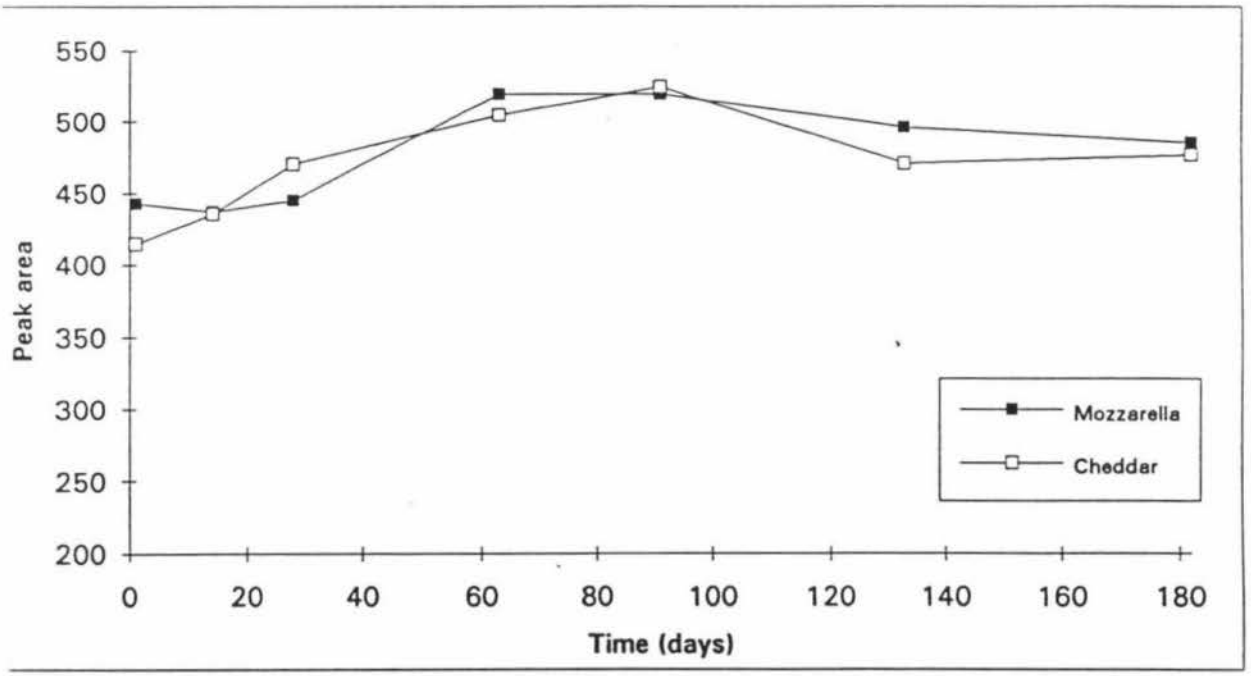


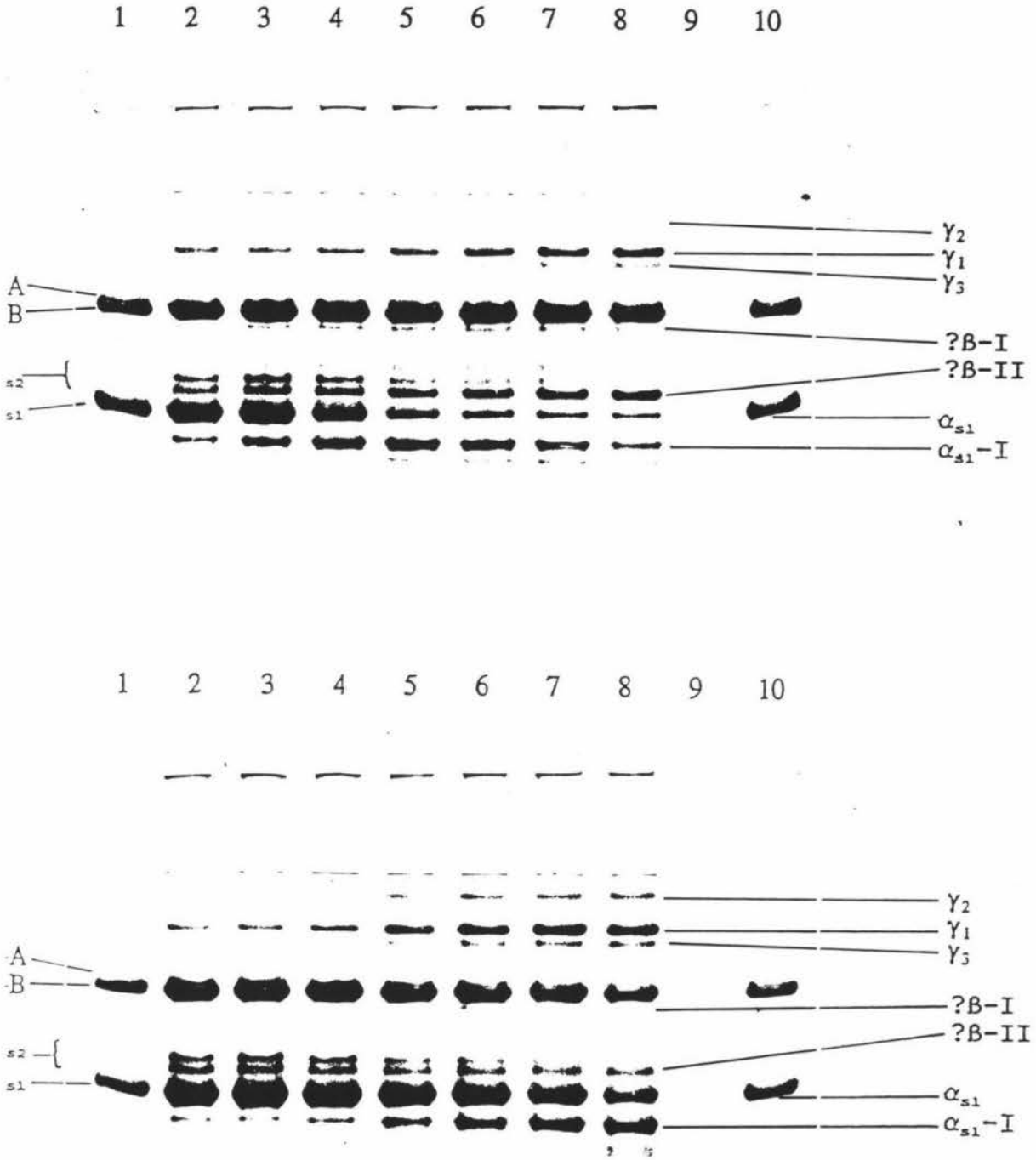
FIGURE 4.5 The increase in dye intensity in the region of para-κ-casein in Cheddar and Mozzarella cheeses ripened at 13°C, and sampled after 1, 14, 28, 63, 91, 133 and 182 days.

4.2.4 Alkaline urea-PAGE of the water-insoluble fraction of the cheese

The water-insoluble fraction was examined by alkaline urea-PAGE and the results are presented in Figure 4.6. This fraction was found to contain the same caseins and peptides as the urea-soluble fraction.

The band in the Cheddar and Mozzarella cheeses (Figure 4.6) that migrated immediately ahead of β-casein may have been β-I-casein (β-casein f1-189/192), formed by the action of rennet on β-casein. This band could be distinguished more clearly in the water-insoluble fraction of the cheese, in which the β-casein band was less intense, than in the whole cheese (Figure 4.2) and it appeared to increase in intensity during ripening. The band was more prominent in the Cheddar cheese than in the Mozzarella cheese which suggests that it may have arisen from the action of rennet.

The intensity of the "unknown" band (Figure 4.6) increased during ripening and was greatest in the Cheddar cheese. As rennet activity was greatest in the Cheddar cheese, it is possible that this band may have been β-II-casein (β-casein f1-165), formed by the action of rennet on β-casein.



IGURE **4.6** Alkaline urea-PAGE. The water-insoluble fraction of (A) Cheddar cheese and (B) Mozzarella cheese stored at 13°C. Lanes 1 and 10, rennet casein standard; lanes 2 to 8, cheese (~2% cheese, 5 μl sample volume) sampled after 1, 14, 28, 63, 91, 133 and 182 days of maturation, respectively.

4.2.5 LMW SDS-PAGE of the water-insoluble fraction of the cheese

The water-insoluble fraction was examined by LMW SDS-PAGE and the results are presented in Figure 4.7. This fraction was found to contain the same caseins and peptides as the urea-soluble fraction (examined by LMW SDS-PAGE) (Figure 4.4).

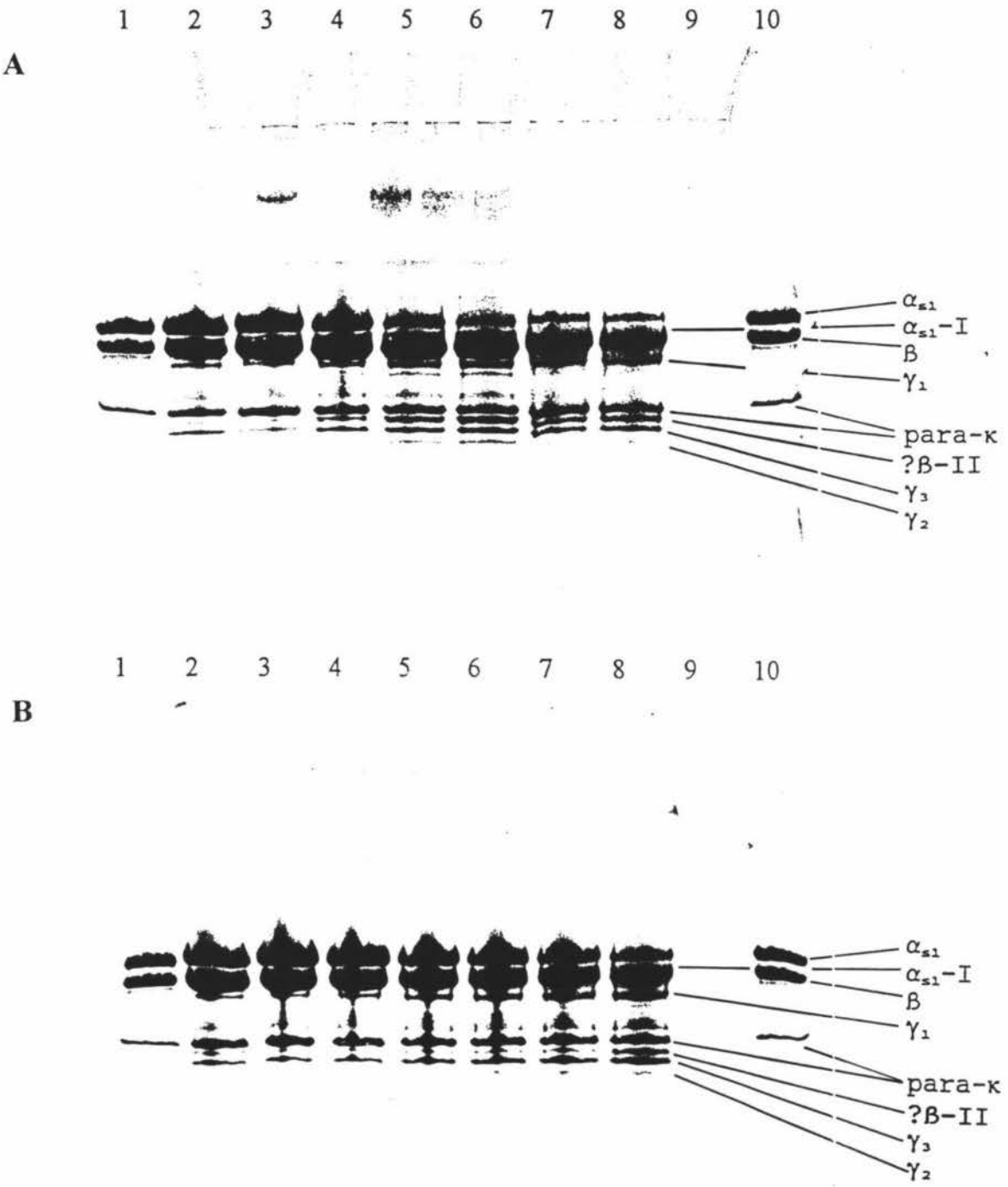


FIGURE 4.7 LMW SDS-PAGE. The water-insoluble fraction of (A) Cheddar cheese and (B) Mozzarella cheese stored at 13°C. Lanes 1 and 10, rennet casein standard; lanes 2 to 8, cheese (~2% cheese in urea sample buffer, diluted 1:1 with SDS sample buffer, 10 μl sample volume) sampled after 1, 14, 28, 63, 91, 133 and 182 days of maturation, respectively.

4.2.6 Alkaline urea-PAGE of the WSF of the cheese

Alkaline urea-PAGE of the WSF of the cheeses, at the concentration used for the urea-soluble fraction, revealed very little water-soluble material. Most of the larger peptides, observed in the urea-soluble fraction of the cheeses, were insoluble in water. Because of the low stain absorption and the small amount of material present, it was necessary to increase the concentration of the WSF by a factor of 10 (*i.e.* 20 μl of 5% cheese (1 mg) as opposed to 5 μl of 2% cheese (0.1 mg) for the urea-soluble (Figure 4.2) and water-insoluble (Figure 4.6) fractions of the cheeses) to make the peptides visible.

The WSF of the ripening cheese was analysed by alkaline urea-PAGE (Figure 4.8). The gels were examined and photographed after 1 h of destain as further destaining resulted in a decrease in the intensity of the bands. The relative mobility (R_f) of each band on the gels was calculated (Table 4.2).

In the PAGE patterns for Cheddar cheese (Figure 4.8A), the intensity of bands A (R_f 0.29) and B (R_f 0.33) appeared to vary a little during maturation of the cheese. Band A was located in the same region as the band tentatively identified as β-I-casein (β-casein f1-189/192) in the whole cheese (Figure 4.2) and the water-insoluble fraction (Figure 4.6) of the cheese. The intensity of band C (R_f 0.35) appeared to increase during the first 28 days of maturation and then rapidly decrease. Band D (R_f 0.44), which was located in the same position as the "unknown" band in the urea-soluble fraction of Cheddar cheese (Figure 4.2), increased in intensity during maturation. Band E (R_f 0.58) increased in intensity until 63-91 days but the intensity appeared to have decreased at 182 days. The remaining bands (bands F (R_f 0.63), G (R_f 0.66), H (R_f 0.72), J (R_f 0.86) and K (R_f 0.94)) gradually increased in intensity during maturation of the Cheddar cheese.

In the PAGE patterns for Mozzarella cheese (Figure 4.8B), the intensity of bands A (R_f 0.29) and B (R_f 0.33) varied considerably during maturation of the cheese. Band C (R_f 0.35) was very indistinct. Band D (R_f 0.44) increased in intensity during maturation of the Mozzarella cheese. Band E (R_f 0.58) initially increased in intensity but the intensity of this band had diminished at 182 days. The remaining bands (bands F (R_f 0.63), G (R_f 0.67), H (R_f 0.71), I (R_f 0.77), J (R_f 0.86) and K (R_f 0.94)) gradually increased in intensity during the 182 days that the cheese was held at 13°C. As the intensity of band J increased, it became apparent that there were three bands in this region.

The gel patterns of the WSF of the Cheddar and Mozzarella cheeses (Figure 4.8) and the relative mobilities of the bands on the gels (Table 4.2) were compared. With the exception of band I, which was present only in the Mozzarella cheese, each band had a very similar relative mobility on both gels. It is, therefore, possible that the bands on each of the gels may have corresponded to the same peptides in both cheese types, although the intensity of each of the bands was quite different in each type of cheese.

The bands in region A (between the origin and β-casein) increased in intensity at a greater rate in the Cheddar cheese than in the Mozzarella cheese, although the intensity of the bands was not great in either cheese type. The position of these bands corresponded to the position of the γ-caseins in the rennet casein standard that was included on the gel. It is probable that a small amount of the γ-caseins may have been incorporated in the WSF and that these bands may have been γ-casein. Alternatively, the bands may have been formed as a result of microbial enzyme action on β-casein. According to Visser *et al.* (1986), the action of the microbial enzymes on β-casein generates peptides that have similar electrophoretic mobilities to the γ-caseins.

Band A had the same relative mobility (Table 4.2) in the gels of both cheese types. This band was located in the same position as the peptide tentatively identified as β-I-casein (β-casein f1-189/192) and was present at a greater concentration in the Cheddar cheese than in the Mozzarella cheese. The variable quantities of this peptide, particularly obvious in the Mozzarella cheese (Figure 4.8), may indicate that the peptide had poor water solubility.

Band C, which was clearly apparent only in the Cheddar cheese pattern, was transient and appeared to be progressively broken down after 28 days.

Band D, present at a greater concentration in the Cheddar cheese than in the Mozzarella cheese, was located in the same position as the "unknown" band that was tentatively identified as β-II-casein (β-casein f1-165) in the gels of the cheese (Figures 4.2 and 4.6).

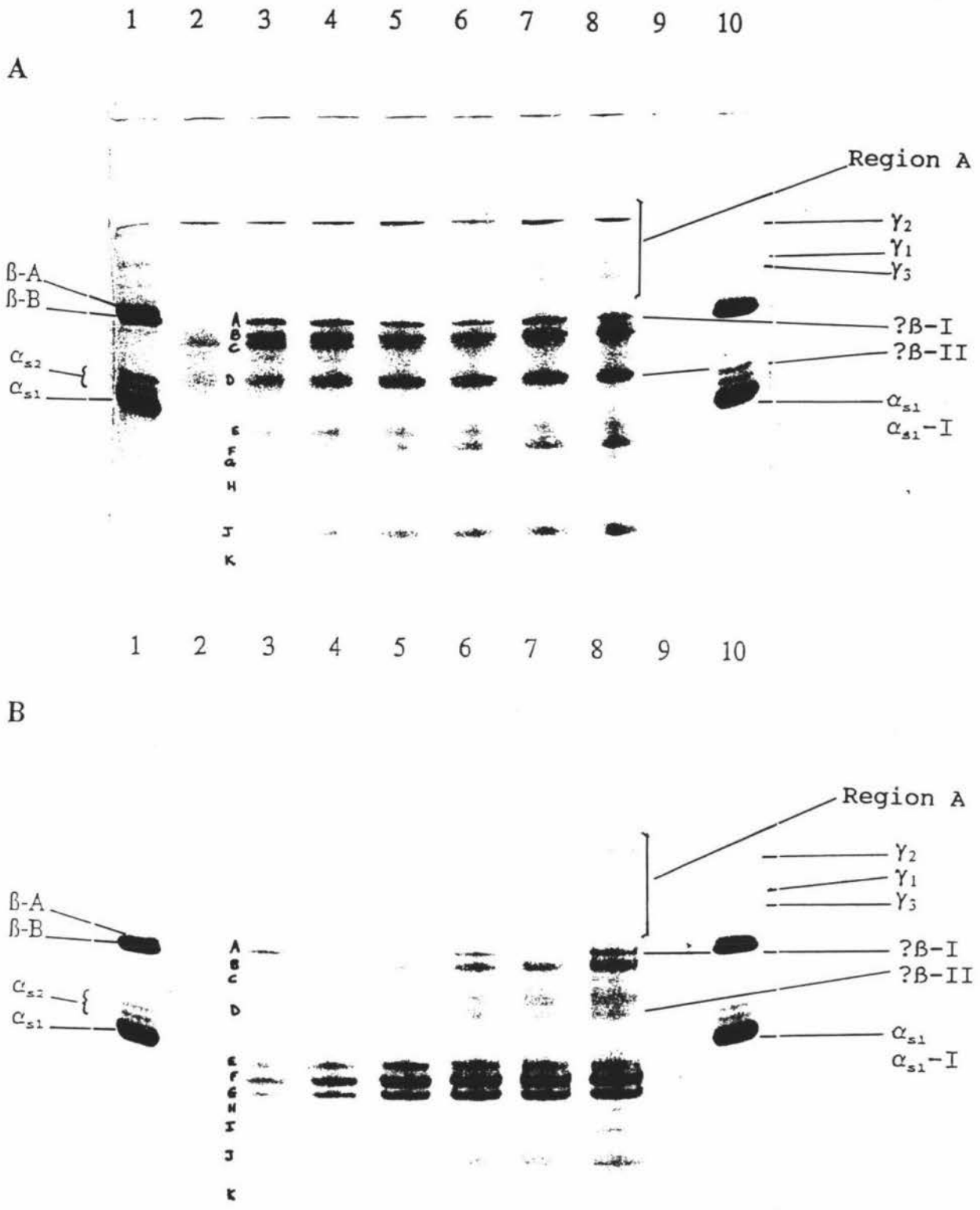


FIGURE 4.8 Alkaline urea-PAGE. The WSF of (A) Cheddar cheese and (B) Mozzarella cheese stored at 13°C. Lanes 1 and 10, rennet casein standard; lanes 2 to 8, cheese (10% cheese in water, diluted 1:1 with urea sample buffer, 20 μl sample volume) sampled after 1, 14, 28, 63, 91, 133 and 182 days of maturation, respectively.

TABLE 4.2 Alkaline urea-PAGE. The relative mobilities of the bands in the WSF of Cheddar and Mozzarella cheeses (Figure 4.8)

Relative mobility	Cheddar cheese	Mozzarella cheese
Band A	0.29	0.29
Band B	0.33	0.33
Band C	0.35	0.35
Band D	0.44	0.44
Band E	0.58	0.58
Band F	0.63	0.63
Band G	0.66	0.67
Band H	0.72	0.71
Band I	-	0.77
Band J (three bands?)	0.86	0.86
Band K	0.94	0.94

4.2.7 LMW SDS-PAGE of the WSF of the cheese

The WSF of the ripening Cheddar and Mozzarella cheeses was examined by LMW SDS-PAGE (Figure 4.9) and the relative mobility of each band on the gels was calculated (Table 4.3). These gels were stained and destained for longer than the alkaline urea gels of the WSF. There was no apparent loss of material from the bands in the LMW SDS gels during destaining, unlike the alkaline urea gels of the WSF (Section 4.2.6). After 1 h of destain, the intensity of the background of the LMW SDS gels remained high, resulting in poor differentiation of the bands from the background. Continued destaining for a further 19 h resulted in good differentiation of the bands from the background, with no apparent loss of material from the bands. Differences in the pore size of the two gels were likely to be responsible for the differences in both the retention of the peptides in the gel during destaining and the time taken to destain the gels. It was believed that the general blurriness of the bands may have been due to the presence of salts in the samples. However, this would seem unlikely as there was no improvement when the samples were dialysed against SDS sample buffer.

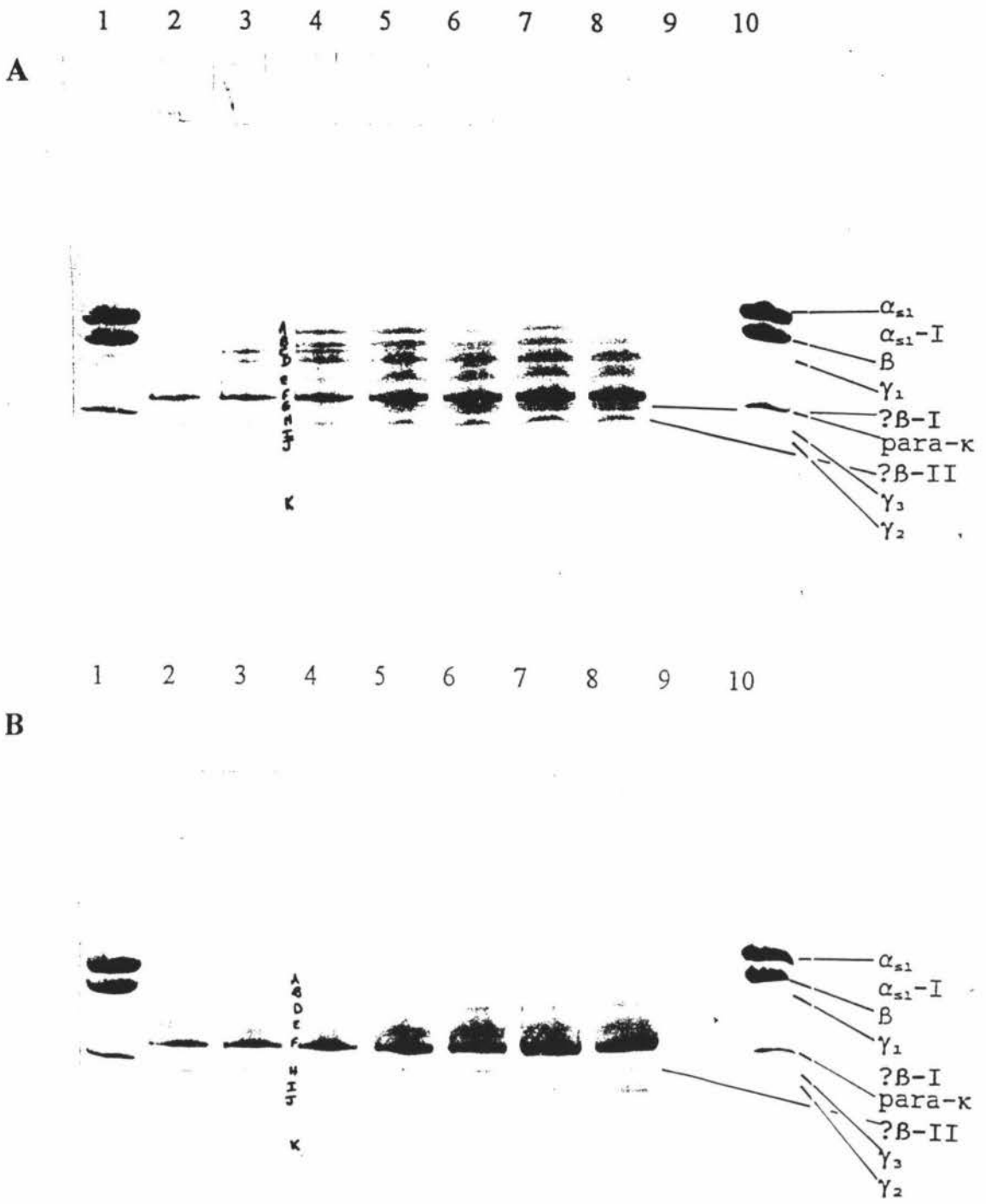


FIGURE 4.9 LMW SDS-PAGE. The WSF of (A) Cheddar cheese and (B) Mozzarella cheese stored at 13°C. Lanes 1 and 10, rennet casein standard; lanes 2 to 8, cheese (10% cheese (in water), diluted 1:1 with SDS sample buffer, 20 μl sample volume) sampled after 1, 14, 28, 63, 91, 133 and 182 days of maturation, respectively.

TABLE 4.3 LMW SDS-PAGE. The relative mobilities of the bands in the WSF of Cheddar and Mozzarella cheeses (Figure 4.9).

Relative mobility	Cheddar cheese	Mozzarella cheese
Band A	0.23	0.24
Band B	0.26	0.26
Band C	0.27	-
Band D	0.31	0.31
Band E	0.36	0.36
Band F	0.40	0.40
Band G	0.43	-
Band H	0.46	0.47
Band I	0.48	0.49
Band J	0.54	0.53
Band K	0.65	0.66

In the gel patterns for Cheddar cheese (Figure 4.9A), bands A (R_f 0.23) and B (R_f 0.26) appeared to vary in intensity. Band C (R_f 0.27) was transient and was present for only 28 days. Bands D (R_f 0.31), E (R_f 0.36), F (R_f 0.40), G (R_f 0.43), H (R_f 0.46), I (R_f 0.48), J (R_f 0.54) and K (R_f 0.65) appeared to increase in intensity during maturation.

In the gel patterns for Mozzarella cheese (Figure 4.9B), there was a general increase in the intensity of each of the bands. The bands present on the gel were bands A (R_f 0.24), B (R_f 0.26), D (R_f 0.31), E (R_f 0.36), F (R_f 0.40), H (R_f 0.47), I (R_f 0.49), J (R_f 0.53) and K (R_f 0.66).

The gel patterns of the WSF of the Cheddar and Mozzarella cheeses (Figure 4.9) and the relative mobilities of the bands on the gels (Table 4.3) were compared. Bands A, B, D, E, F, H, I, J and K had a similar relative mobility on both gels and it is possible that these bands may have corresponded to the same peptides in each cheese type. Bands C and G in the Cheddar cheese were absent in the Mozzarella cheese.

The intensity of most of the bands in the gels of the WSF of the Cheddar and Mozzarella cheeses gradually increased during ripening. In the Mozzarella cheese, there appeared to be no loss of any of the peptides as ripening progressed. This may be indicative of a lack of microbial activity in the cheese, but may also have been due to the lower rennet activity (indicated by the slower α_{s1}-casein degradation (Figure 4.2)).

Band A (Figure 4.9) was present in both cheese types at variable concentrations that appear to be unrelated to ripening. It is likely that this peptide had poor water solubility.

Band C (Figure 4.9), present only in the Cheddar cheese, appeared to be transient and had disappeared by 63 days. It is probable that this band corresponded to peptide C on the alkaline urea gels of the WSF of the Cheddar cheese (Figure 4.8), as each band was transient and present for approximately 28 days.

Band G (Figure 4.9), present in the Cheddar cheese, was absent in the Mozzarella cheese and therefore may have arisen as a result of the greater rennet or microbial enzyme activity that occurred in this cheese. An examination of the two-dimensional gel electrophoretogram (alkaline urea-PAGE and LMW SDS-PAGE (Chapter 2, Figure 2.8)) indicated that this band may have been β-I-casein (β-casein f1-189/192).

Band H (Figure 4.9) corresponded to the "unknown" peptide in the whole cheese (Figure 4.2). It was present at a greater concentration in the Cheddar cheese than in the Mozzarella cheese and may have been β-II-casein (β-casein f1-165) (band D in the alkaline urea gels (Figure 4.8).

Dialysis of the WSF diluted 1:1 with sample buffer (using dialysis tubing with a molecular weight cutoff of 3,500 Da (Spectra/Por)), against sample buffer, revealed little difference between the undialysed and dialysed peptide profiles, indicating that the molecular weight of the peptides observed by gel electrophoresis was probably above 3,500 Da.

4.2.8 RP-FPLC of the WSF of the cheese

The WSF of the Cheddar and Mozzarella cheeses, ripened for 1, 14, 28, 63, 91, 133 and 182 days, was examined by RP-FPLC. The peak areas (above a threshold level of 2%) were integrated and the profiles are shown in Figure 4.10.

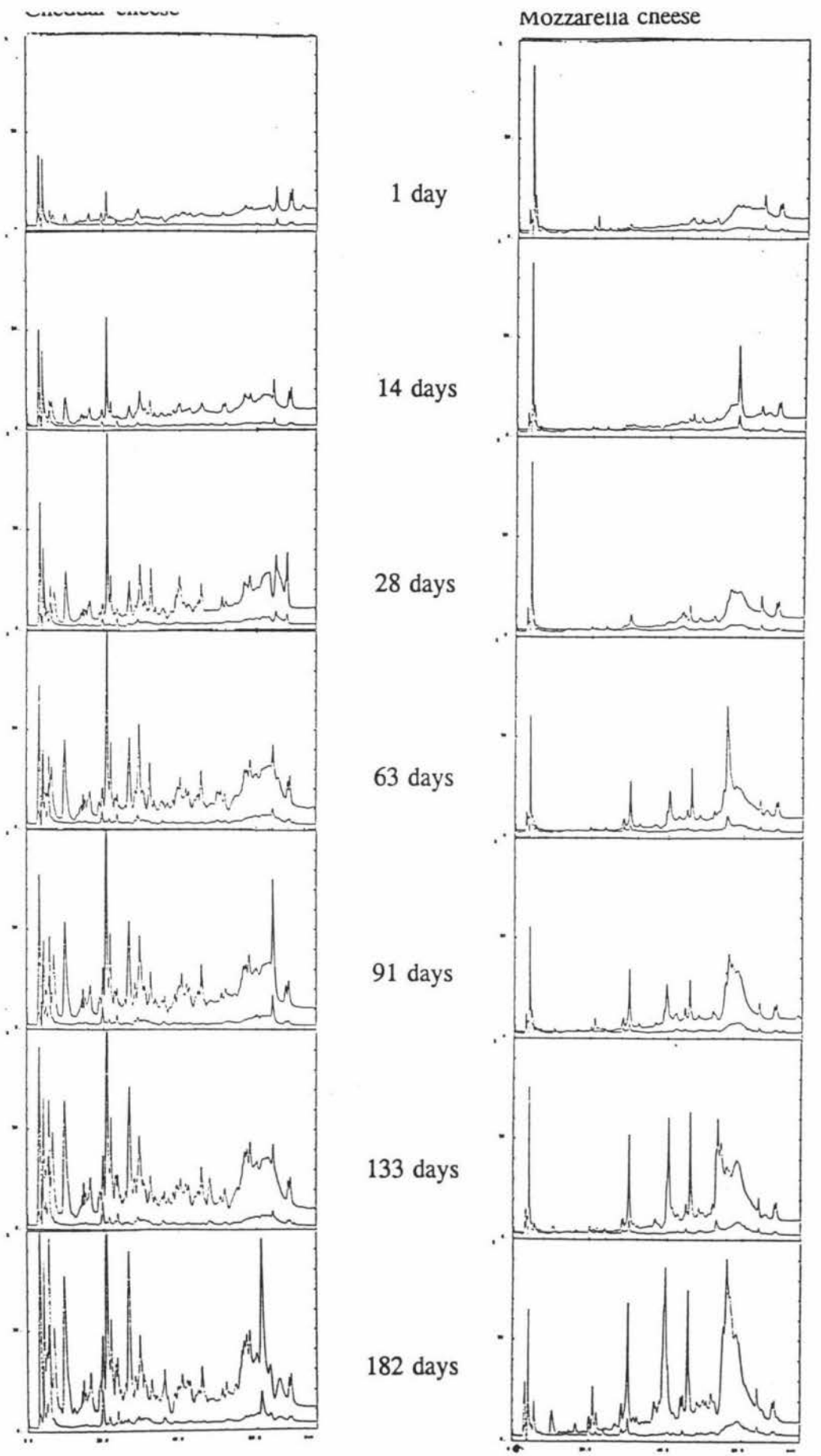
The retention times of the amino acids, using the RP-FPLC conditions that were used to analyse the WSF of the cheese, were determined in Chapter 3 (Section 5.1.2.1).

In both of the cheese varieties, there was a gradual increase in the later eluting (50-70 min) material reported to be composed of the more hydrophobic and higher molecular weight peptides (Cliffe *et al.*, 1993). The shift in position of some of the later eluting peaks was believed to be related to the lower solubility of the more hydrophobic peptides.

During the ripening of the Cheddar cheese, there was an increase in both the number and the size of the peaks in both the early eluting region (0-22 min) and the intermediate region (22-50 min) (Figure 4.10), indicating progressive hydrolysis of the larger peptides to smaller peptides and amino acids, due to the combined action of the enzymes present.

In the Mozzarella cheese profiles, there were only a few peptide peaks in the intermediate region of the profiles but they became progressively larger. This indicates the extent of proteolysis due to the combined action of rennet and plasmin as well as the lack of microbial enzyme activity that in the Cheddar cheese seemed to be responsible for the further hydrolysis of these peptides. There was no increase in material in the early eluting region in which the amino acids were shown to elute (Chapter 3, Section 5.1.2.1). In the Mozzarella cheese at 1 day, there seemed to be more amino acids present than in any of the samples taken at later times. These peaks gradually disappeared during ripening, perhaps in some catabolic reaction involving the surviving microorganisms.

The RP-FPLC profiles for cheese older than 91 days were atypical of normal Mozzarella cheese which has a shorter ripening time at lower temperatures (2-6 weeks at 4°C).



GURE **4.10** RP-FPLC of the WSF of Cheddar and Mozzarella cheeses after 1, 14, 28, 63, 91, 133 and 2 days maturation. Gradient: 100% solvent A for 5 min; 0-90% solvent B over 75 min; 100% solvent B r 15 min. Flow rate, 0.7 ml/min. The absorbance was measured at 214 and 280 nm (A_{max} 1.0).

The increase in the amount of material, in the WSF of each variety of cheese, that absorbs UV at 214 and 280 nm was monitored during ripening and is shown in Figure 4.11. In the WSF of the Cheddar cheese, it can be seen that, after an initial lag phase, there was an almost linear increase in the amount of material with an absorbance at 214 nm. During the lag phase, α_{s1}-casein was hydrolysed (Figure 4.2) and the products became available as substrates for further enzyme activity.

The amount of material in the WSF of the Mozzarella cheese, with an absorbance at 214 and 280 nm, increased slowly between 14 and 91 days and then more rapidly in the last 91 days. This rapid increase during the last 91 days, combined with the observation that there was positive pressure within the vacuum-sealed cheese bag at 182 days, suggests atypical ripening with the possibility of the growth of non-starter bacteria. Mozzarella cheese would not normally be kept beyond 91 days, nor would it be ripened at such a high temperature (13°C).

The aromatic amino acids, which were present in the Cheddar cheese, absorb strongly at 214 nm and would have contributed a disproportionate amount to the area under the peaks. This would have slightly amplified differences in the amount of water-soluble material in the two cheese types.

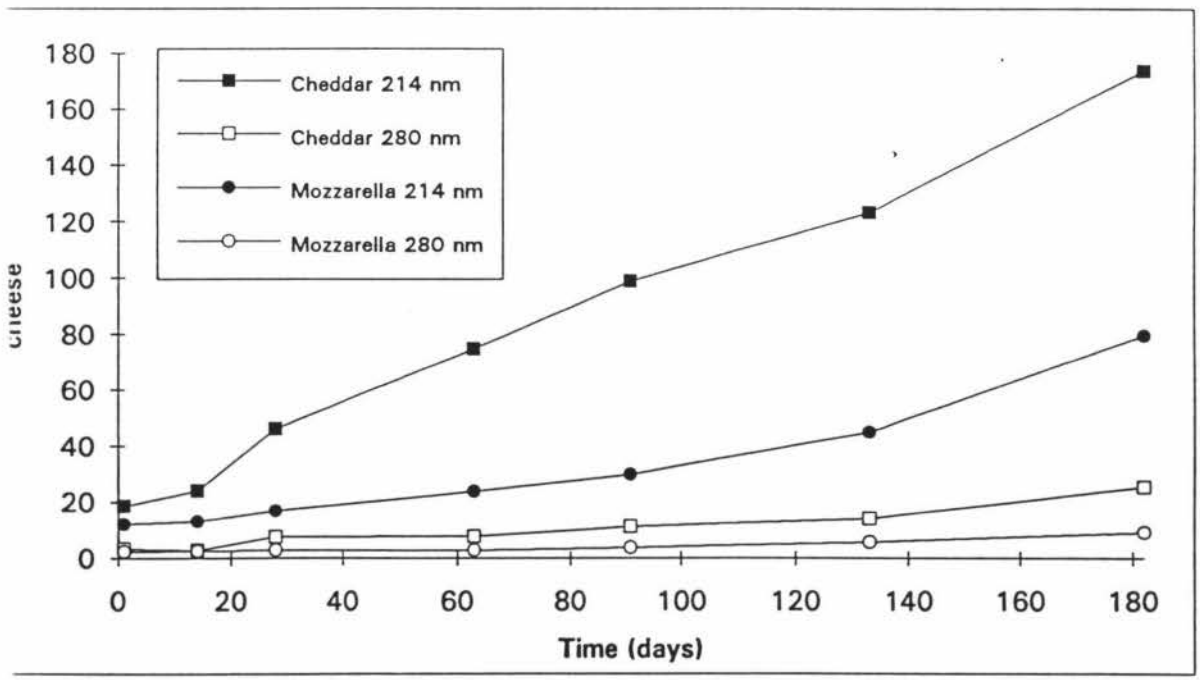


FIGURE 4.11 RP-FPLC. The total accumulated peak area, measured at 214 and 280 nm, for Cheddar and Mozzarella cheeses after 1, 14, 28, 63, 91, 133 and 182 days.

The steady increase in absorbance of the peaks, corresponding to tyrosine, phenylalanine and tryptophan in Cheddar cheese, was monitored during ripening and is shown in Figure 4.12. During the 182 day ripening period, the increase in phenylalanine, tryptophan and tyrosine concentrations appeared to be linear in the Cheddar cheese. The Phe-X bonds in α_{s1}-casein are rapidly hydrolysed by chymosin, leaving phenylalanine at the N- or C-terminal of the resultant peptides. The phenylalanine is then liberated by subsequent peptidase activity. Tyr-X and Trp-X bonds are not at the N- and C-terminals of early liberated peptides and were therefore liberated more slowly.

Mozzarella cheese, due to its lack of microbial enzyme activity and lower chymosin activity, showed little evidence of any of these peaks (tyrosine, tryptophan and phenylalanine). The microorganisms responsible for the production of the gas within the cheese bag were probably also responsible for the generation of the small amounts of tyrosine, tryptophan and phenylalanine that were formed between 91 and 182 days.

The 280 nm absorbances were used to locate the tyrosine and tryptophan peaks.

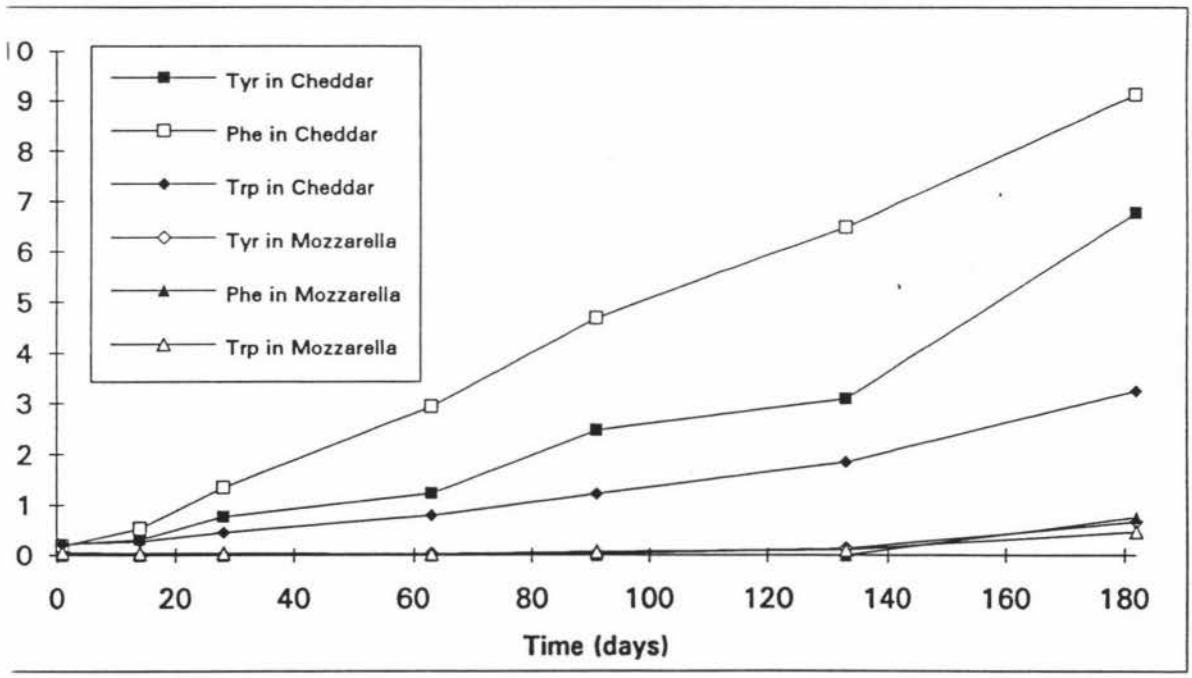


FIGURE 4.12 RP-FPLC. The absorbance at 214 nm of three peaks corresponding to tyrosine, phenylalanine and tryptophan in Cheddar and Mozzarella cheeses ripened at 13°C and sampled after 1, 14, 28, 63, 91, 133 and 182 days.

4.2.9 SE-HPLC of the whole cheese and the WSF of the cheese

Size exclusion or gel permeation chromatography enabled cheese ripening to be characterised according to the molecular weight distribution of the caseins, peptides and amino acids.

In order to assess the results, it is first necessary to consider the method employed to separate the proteins, peptides and amino acids. Using the low ionic strength and highly acidic solvent system described (Section 3.3.6), the molecular weight pattern is spread over a wide elution time. With few exceptions, proteins have been found to elute according to their molecular weight, with the larger species eluting first. Separation of the very small peptides (dipeptides, tripeptides and tetrapeptides) and amino acids is affected by both size and charge. In this acidic solvent system, most of the amino acids are positively charged and the basic amino acids carry a double positive charge. The number of positive charges affects the order of elution of the very small peptides and amino acids but has little effect on the larger peptides and proteins. It is possible that ion-pairing between the TFA anions (113 Da) and the positive charges on the amino acids may increase the apparent molecular weight of the small peptides and amino acids and be responsible for their order of elution (Motion, 1992). Although the silica matrix of the column is blocked to prevent interactions with the solute molecules, blocking is incomplete and at low pH the silanol groups on the column surface may take up a positive charge and repel the positively charged amino acids. This will cause the basic amino acids to elute first. It is probable that some mixture of these two mechanisms is responsible for the order of elution of the peptides.

In this experiment, both the urea-soluble fraction (representing the whole cheese) and the WSF were analysed by SE-HPLC and the results are shown in Figures 4.14 and 4.15.

The values on the *y*-axis are expressed in volts and it is the difference between the minimum and maximum voltage that is important rather than the absolute values. The 0-2 volt full scale signal corresponds to an absorbance range of 2 units.

The relationship between retention time and molecular weight was determined as part of the routine evaluation of column performance. A series of standards of known molecular weight was run and the retention time was plotted against the logarithm of the molecular weight of the standard, to form the calibration curve demonstrated in Figure 4.13. The values for molecular weight quoted in the text were read from this

calibration curve.

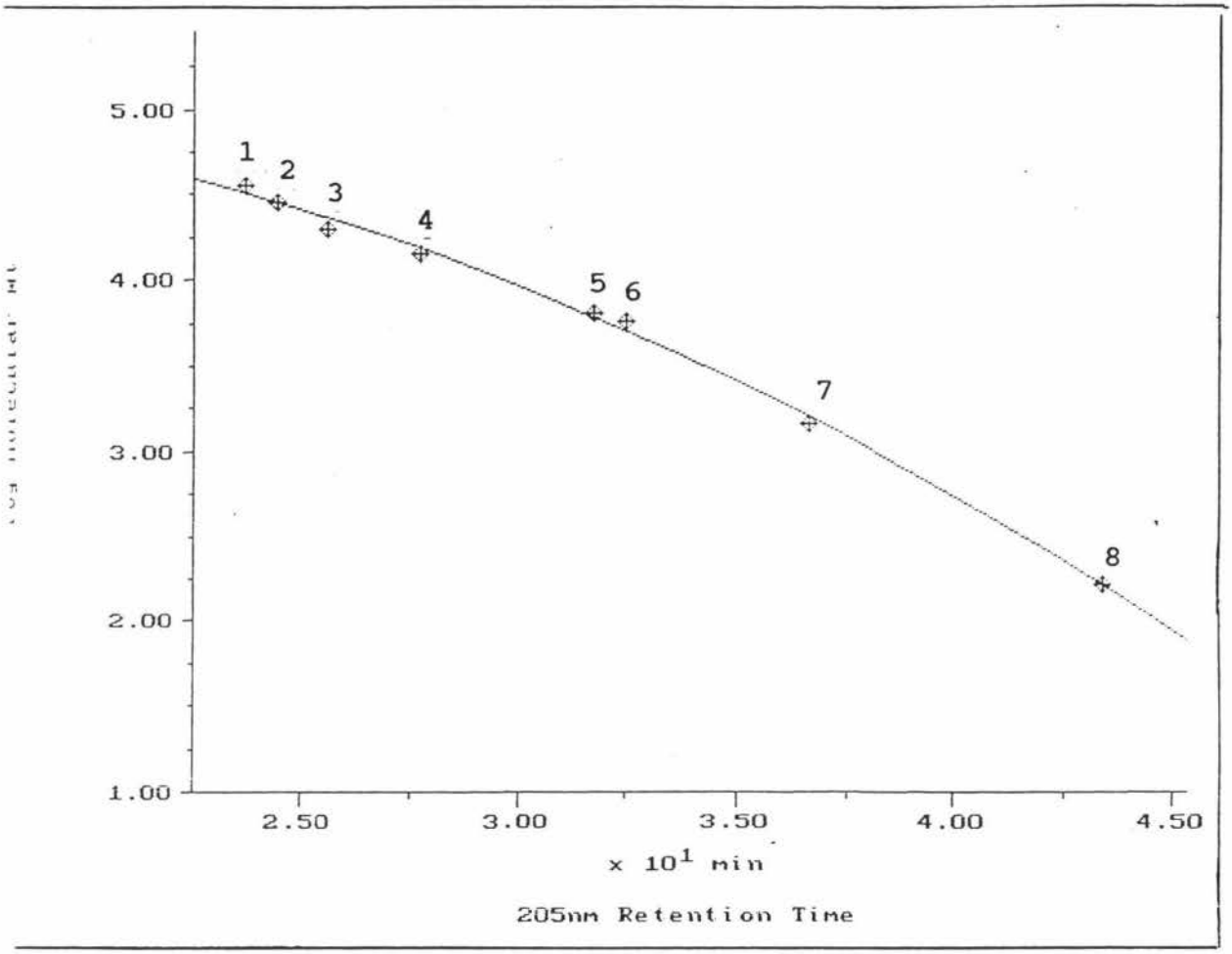


FIGURE 4.13 SE-HPLC calibration curve of standards. Logarithm of molecular weight versus retention time. (1) Glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), (2) Carbonic anhydrase (29,000 Da), (3) Soybean trypsin inhibitor (20,100 Da), (4) Lysozyme (14,300 Da), (5) Aprotinin (6,500 Da), (6) Insulin (5,730 Da), (7) Bacitracin (1,420 Da), (8) Phenylalanine (165 Da).

The whole cheese (urea-soluble fraction)

The urea-soluble fraction showed the change in molecular weight distribution as the high molecular weight caseins were hydrolysed to peptides and amino acids (Figure 4.14).

Urea sample buffer was analysed to determine the effect of the buffer constituents on the SE-HPLC profiles. Constituents of the sample buffer were found to contribute a very small amount to the peak identified as containing the amino acids that eluted at ~44 min. The main contribution of the sample buffer was to the absorbance of the solvent peak that eluted at 47 min.

In the SE-HPLC traces of the Cheddar cheese (Figure 4.14), there was a gradual decrease with time of the size of the major casein peaks eluting at 23.5 and 25.5 min. This was accompanied by increases in the intensity of the peaks eluting between 27 and 40 min. As ripening progressed, and the peptide bonds responsible for much of the absorbance (at 205 nm) were hydrolysed, there was an apparent decrease in the total amount of material.

The comparable traces of the SE-HPLC of ripening Mozzarella cheese are also shown in Figure 4.14. The chromatograms were very similar to those for Cheddar cheese, with a decrease in the early eluting peaks, an increase in the 27-40 min region but an overall loss of intensity for the whole chromatogram.

An attempt to scale up the region of the lower molecular weight material failed to produce any more peaks due to the low concentrations of these peptides in the cheese.

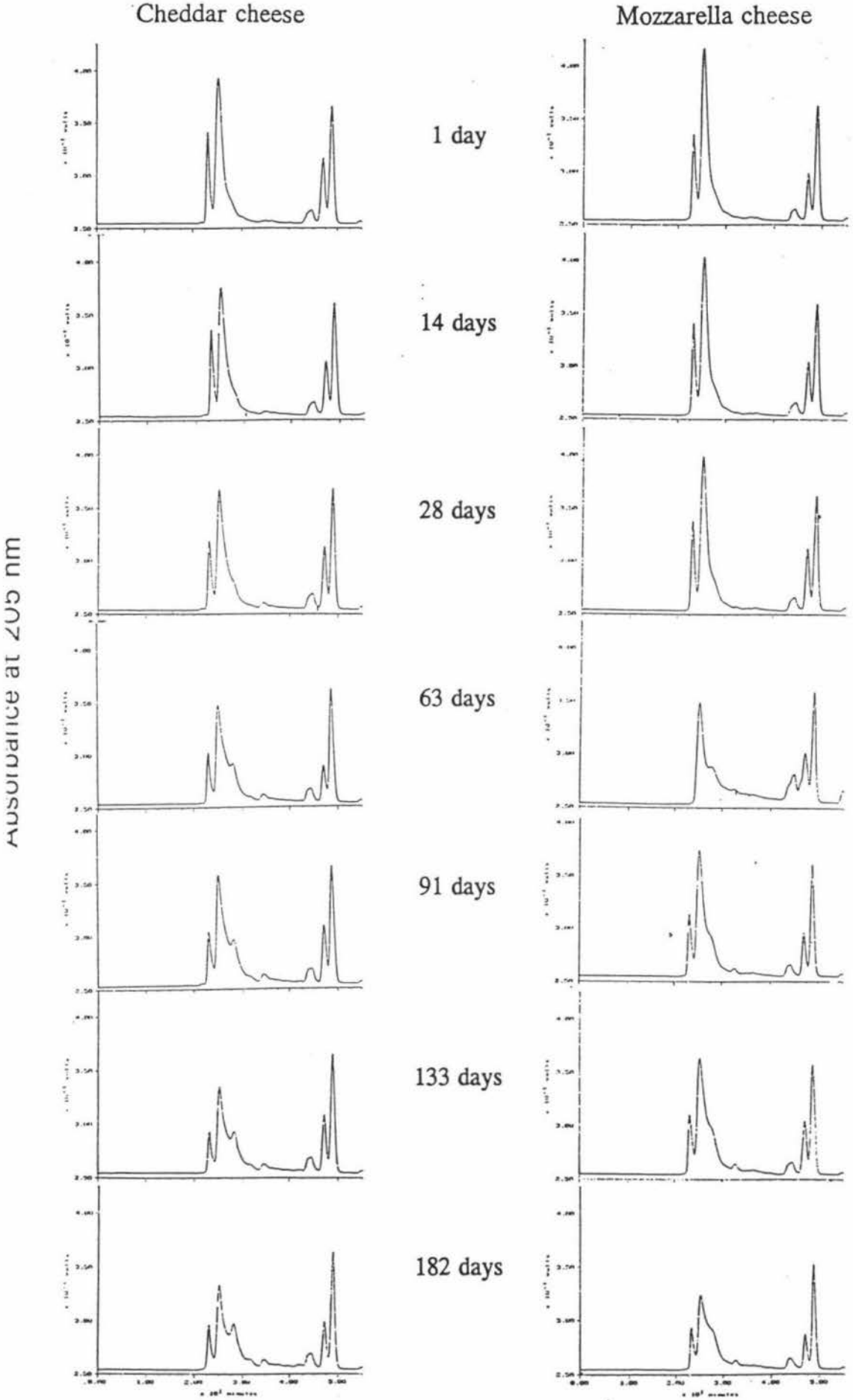


FIGURE 4.14 SE-HPLC of the urea-soluble fraction of Cheddar and Mozzarella cheeses.

The WSF

The WSF was taken from five times the amount of cheese used for the urea-soluble fraction (Figure 4.14).

For the Cheddar cheese samples (Figure 4.15), there was a steady increase in the amount of material with an absorbance at 205 nm during the 182 days of ripening. The increase in material occurred across the full range of molecular weights (165-21,000 Da) indicating a large amount of protease and peptidase activity. Peak A, which eluted at approximately 26.2 min, contained the higher molecular weight material of ~21,000 Da. Although this peak increased in size during maturation, it was small in relation to peaks C-L. The peptides in the WSF that were tentatively identified by gel electrophoresis as β-I-, β-II- and γ_1-casein have molecular weights in this region. Peak B, which eluted at approximately 28.5 min, was a broad peak that contained peptides with molecular weights of ~13,200 Da. This peak gradually increased in size during maturation but, like peak A, was small in relation to the remaining peaks. Many of the β-casein breakdown products, including γ_2- and γ_3-casein and the proteose peptones, have molecular weights in this (broad) region but most are quite hydrophobic and are unlikely to be found in the WSF in any quantity. Peak C, which eluted at 31 min, was a broad peak that increased in size more rapidly than the preceding peaks and comprised material with an approximate molecular weight of 7,400 Da. Peak D, which eluted at approximately 34.5 min, comprised material with an approximate molecular weight of 2,900 Da. α_{s1}-Casein f1-23, which was formed (along with α_{s1}-casein-I (f24-199)) by the action of rennet on α_{s1}-casein (Figure 4.3), has a molecular weight in this region, but it apparently does not accumulate in Cheddar cheese (Kaminogawa *et al.*, 1986; Exterkate *et al.*, 1991). Peaks E (37 min) and F (~38 min) increased in size during ripening and contained peptides with molecular weights of ~1,400 Da and ~1,000 Da, respectively. Peak G (~39 min), containing material with a molecular weight of ~700 Da, increased in size during ripening but was not discernible after 182 days because the neighbouring peaks had broadened and concealed it. Peak H (~40 min), containing material with an approximate molecular weight of 500 Da, increased in size steadily as the cheese matured. The remaining peaks, peak I (~41 min), peak J (~42 min), peak K (42.5 min) and peak L (~44 min), were thought to contain the tetra-, tri- and dipeptides and free amino acids. These peaks gradually increased in size during ripening. Peak L was thought to contain many of the free amino acids, including tyrosine, phenylalanine and tryptophan, which absorb strongly at this wavelength. The size of this peak was therefore believed to over-represent the actual amount of material present. Peak M (47 min) was identified as a solvent peak and was

thought to contain uncharged solutes, including some amino acids.

During the first 28 days of ripening of the Mozzarella cheese (Figure 4.15), there was comparatively little material, in relation to that in the Cheddar cheese, that was discernible by SE-HPLC. During the latter stages of ripening (28-182 days), a large amount of high molecular weight material (5000-20,000 Da) was generated. There was comparatively little material in the later eluting peaks believed to contain the lower molecular weight peptides and amino acids. This was quite different from the Cheddar cheese and was indicative of a narrow range of proteolytic specificity in the Mozzarella cheese.

Peak A (26.5 min), containing peptides with an approximate molecular weight of 20,000 Da, was a small peak on the shoulder of peak B that became evident after 91 days. Peak B (29 min), containing material with a molecular weight of ~12,000 Da, was the largest of the peptide peaks and increased in size steadily during maturation. Peak C (31 min), containing material with an approximate molecular weight of 7,400 Da, formed a shoulder on peak B that was discernible as a separate peak after 182 days. β-Casein f29-105, formed by the action of plasmin on β-casein, has a molecular weight of 8706 Da and may have been contained within this peak. Peak D (33 min), was a large peak that increased steadily in size during maturation and contained material with an approximate molecular weight of ~4,200 Da. Peak E (34.5 min) increased in size during ripening and contained material with an approximate molecular weight of 2,900 Da. As there was little microbial enzyme activity in this cheese, it is probable that α_{s1}-casein f1-23 accumulated during maturation and was contained within this peak. β-Casein f1-28, one of the N-terminal fragments released by the action of plasmin on β-casein, has a molecular weight of 3444 Da and was also probably contained within this peak. Peak F (36.5 min), although the same height as the preceding peak, was broader and contained material of ~1,600 Da. Peak G (38 min) increased in size during ripening and contained material of ~1,400 Da. Peak H (39.5 min) was smaller than the previous peaks and contained material with an approximate molecular weight of 630 Da. Peak I (42 min) was a very small peak that increased in size during the latter stages of maturation. It probably contained di- and tripeptides and possibly some amino acids. Peak J (44 min) increased in size a little between 133 and 182 days and probably contained amino acids. Also between 133 and 182 days, peak K (47 min) decreased in size considerably and it is likely that the material contained in this peak was catabolised by the non-starter bacteria believed to be present.

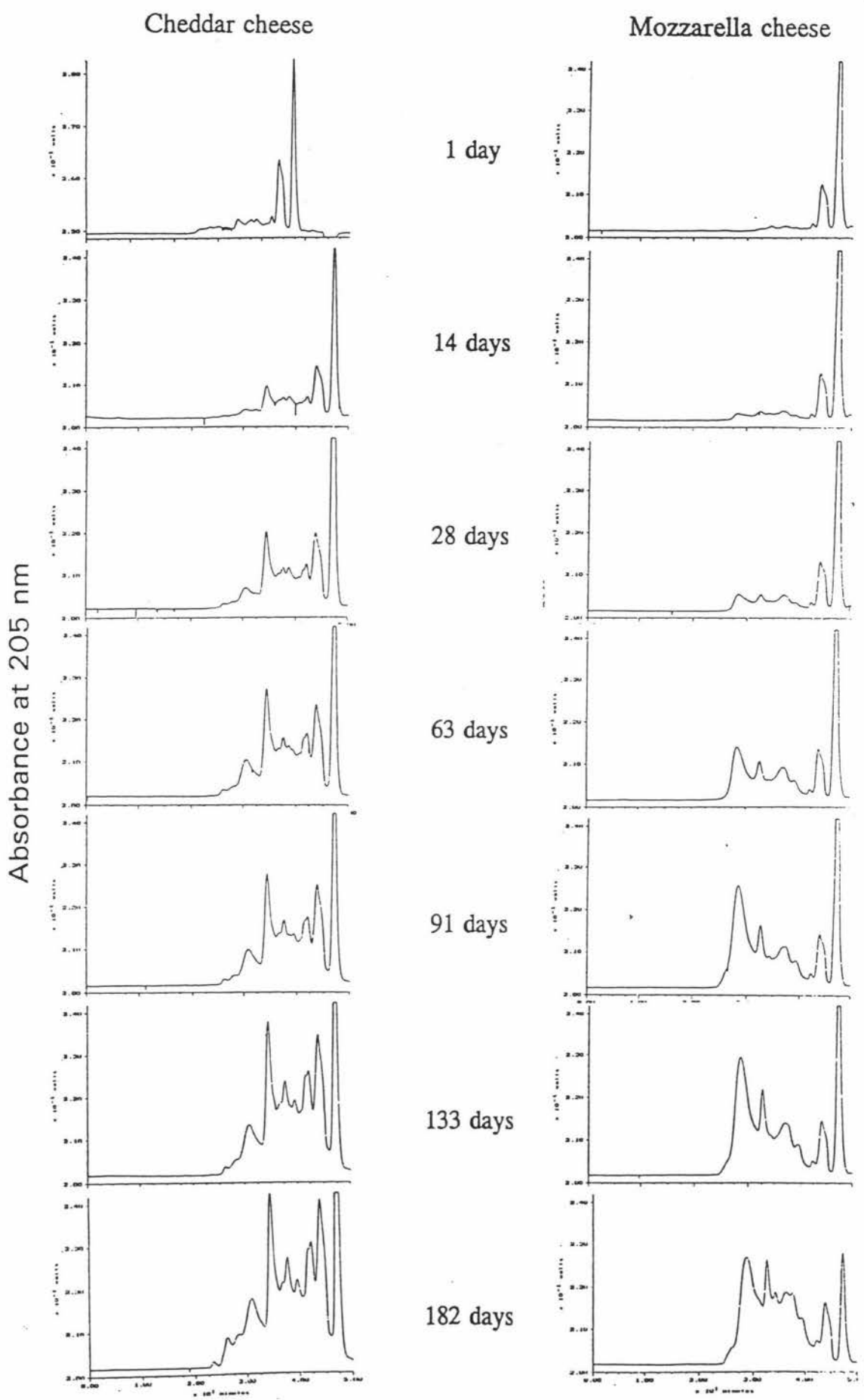


FIGURE 4.15 SE-HPLC of the WSF of Cheddar and Mozzarella cheeses.

5 DISCUSSION

The present results are consistent with the concept that differences in the manufacture of Cheddar and Mozzarella cheeses result in the formation of two cheeses, each with different amounts of similar enzymes (rennet, plasmin, and the enzymes of the starter and NSLAB), and that these differences in enzyme concentration, combined with the modifying effect of pH, temperature, moisture content and S/M (Lawrence *et al.*, 1983, Creamer *et al.*, 1988), result in different enzyme activities and patterns of proteolysis in the two types of cheese and these, in turn, result in cheeses with different functional properties.

The generally accepted notion is that, during proteolysis in cheese, the caseins are degraded to large peptides, then to small peptides, then to amino acids and finally to even smaller organic molecules and ammonia salts.

Each of the different analytical techniques used in this study provided insights into the differences between Cheddar and Mozzarella cheeses that added to the overall picture and enabled conclusions to be drawn about the type and extent of enzyme activities in the cheese.

5.1 NITROGEN ANALYSIS

Gross proteolysis in cheese can be detected adequately by a fractionation procedure followed by some general protein assay, *e.g.* Kjeldahl nitrogen. In the present study both the WSN and NPN contents were monitored during cheese ripening.

According to Rank *et al.* (1985), the fraction of cheese that is soluble in water "is a complex mixture of large, medium, and small peptides and amino acids" that results from the action of rennet, plasmin (and other indigenous enzymes) and the enzymes of the starter and NSLAB.

An examination of the level of nitrogen in the WSN and NPN fractions of each cheese type (Figure 4.1) revealed a steady increase during ripening, which is in agreement with the findings of other researchers for Cheddar cheese (*e.g.* Vakaleris *et al.*, 1960; Reville and Fox, 1978; Farkye and Landkammer, 1992; Wilkinson *et al.*, 1992; Law *et al.*, 1993) and for Mozzarella cheese (Farkye *et al.*, 1991; Kindstedt *et al.*, 1991).

The amount of WSN in the Cheddar cheese after 182 days of maturation was 23.1%

of the TN (Figure 4.1) which is in agreement with the findings of others (*e.g.* Vakaleris *et al.*, 1960; Wilkinson *et al.*, 1992). The amount of WSN formed in the Mozzarella cheese after 182 days was 14.8% of the TN (Figure 4.1) and was less than in the Cheddar cheese. These results indicate that there was a lower level of proteolytic activity in the Mozzarella cheese than in the Cheddar cheese. It is probable that the differences in the amount of WSN in the two cheese types were due mainly to the lower initial level of rennet used in the manufacture of the Mozzarella cheese and to the effect of the high stretch temperature (68°C) on the concentrations of active enzymes in the Mozzarella cheese.

At the same time of maturation (182 days), the amount of NPN (*ie.* soluble in 12% TCA) was 20.1% of the TN in the Cheddar cheese but only 5.2% of the TN in the Mozzarella cheese (Figure 4.1). The NPN comprises small peptides of 2 to 20 residues (Yvon *et al.*, 1989), amino acids and amino acid degradation products. The lower level of NPN in the Mozzarella cheese is probably a reflection of the low level of peptidase activity that remains in this cheese variety (Creamer, 1976b), with few of the microorganisms surviving the pasteurising heat treatment (68°C) to which the cheese is subject during stretching.

Although the measurement of nitrogen in the different fractions provided valuable information on the overall trends in proteolysis, it provided no information as to which caseins were hydrolysed or the nature of the peptides produced.

5.2 ALKALINE UREA-PAGE

The whole cheese

Alkaline urea-PAGE of the whole cheese was a more discerning technique than WSN and NPN measurement. When used to analyse the whole cheese (Figure 4.2), it provided information on the type and amount of casein hydrolysis (Figure 4.3).

Gel electrophoresis (Figure 4.2) revealed that 80% of the α_{s1}-casein in the Cheddar cheese was hydrolysed by 63 days, which is in agreement with the findings of Johnston *et al.* (1994). This was attributed mainly to the action of rennet which was shown by Creamer and Richardson (1974) to hydrolyse α_{s1}-casein in cheese with the concomitant formation of α_{s1}-casein-I. Conditions (high S/M (5.06), lower pH (5.26) (Table 4.1)) in the Cheddar cheese were less favourable for the activity of plasmin. However, 12%

of the β-casein was hydrolysed, largely by plasmin (with the concomitant formation of γ-caseins), after 63 days and 34% was hydrolysed after 182 days of maturation (Figure 4.3). It is possible that rennet also hydrolysed β-casein. The band that migrated immediately ahead of β-casein (Figures 4.2 and 4.6) is believed to be β-I-casein (β-casein f1-189/192), formed by the action of rennet (Creamer, 1976a). This band was more prominent in the Cheddar cheese than in the Mozzarella cheese which suggests that it may have arisen from the action of chymosin. McSweeney (1993b) identified a band that migrated immediately ahead of β-casein as β-I-casein, formed in solution by the action of chymosin on β-casein. A band in the same position in the water-insoluble fraction of 3 month old Cheddar cheese was tentatively identified as β-I-casein by McSweeney (1993c). The intensity of the "unknown" band (Figures 4.2 and 4.6) increased during ripening and was greatest in the Cheddar cheese. As rennet activity was also greatest in the Cheddar cheese, it is possible that this band was formed by the action of rennet and may have been β-II-casein (β-casein f1-165). McSweeney (1993b), in a study of the action of chymosin on β-casein in solution, identified a band in the same position as the "unknown" band as β-II-casein. A band in a similar position in the water-insoluble fraction of 3 month old Cheddar cheese was identified as β-casein f1-* by McSweeney (1993c), who believed it to be a proteose peptone. It is unlikely that the "unknown" band in the Cheddar and Mozzarella cheeses was a proteose peptone, formed by the action of plasmin on β-casein, as its intensity was greatest in the Cheddar cheese in which the γ-casein bands were less intense (than in the Mozzarella cheese).

In solution, the primary product of chymosin action on β-casein is β-I-casein (Creamer, 1976a, McSweeney, 1993b). In cheese, the intensity of the band tentatively identified as β-I-casein was low (Figures 4.2 and 4.6) and it is possible that it was rapidly hydrolysed to form the more intense band that was tentatively identified as β-II-casein. It seems likely that both β-I- and β-II-casein were formed and that β-I-casein was hydrolysed to form β-II-casein. It is also possible that the conformation of β-casein was different in the environment of the cheese and that the major product of chymosin action on β-casein was β-II-casein. Creamer (1976a) estimated apparent rate constants in solution and Phelan *et al.* (1973) and also Creamer (1976a) showed that polymeric β-casein, as it is in cheese, does not hydrolyse to β-I-casein as readily.

In the Mozzarella cheese, with its high moisture content (48%), high pH (5.48) and low S/M (2.98) (Table 4.1), both rennet (and/or milk acid protease) and plasmin were active and hydrolysed α_{s1}- and β-casein at approximately the same rate, as indicated by gel

electrophoresis (Figure 4.2). After 63 days, 24% of the α_{s1}-casein and 23% of the β-casein had been hydrolysed. The rate of α_{s1}-casein hydrolysis was slower and the amount of α_{s1}-casein hydrolysis was less than in the Cheddar cheese. This was due to the high final pH (5.48), the lower initial level of rennet (4 ml/100 litres of milk) and the denaturing effect of the high stretch temperature (68°C) on chymosin. These findings are in agreement with the earlier findings of low residual rennet activity in Mozzarella cheese by Creamer (1976b), Matheson (1981) and Farkye *et al.* (1991). The amount of β-casein hydrolysis was greater than in Cheddar cheese and this is also believed to be due to both the high final pH (5.48) (demonstrated in Meshanger-type cheese to affect plasmin activity (Noomen, 1978)) and to the effect of the high stretch temperature (68°C) on plasmin activity (Farkye and Fox, 1990) which is believed to result in the heat inactivation of an inhibitor of the plasminogen activator (Richardson, 1983). Farkye *et al.* (1991) reported a much higher level of β-casein hydrolysis and WSN formation and a similar level of α_{s1}-casein hydrolysis in high moisture Mozzarella cheese stored at 4°C for only 14 days. Although proteolysis was found by Farkye *et al.* (1991) to be unrelated to S/M within the range 0.59-1.59, these low levels are likely to permit higher levels of plasmin activity than was seen in our Mozzarella cheese which had a S/M of 2.98. Unfortunately, details of the initial level of rennet addition, stretch temperature and cheese pH were not reported and it is differences in these variables that were likely to be responsible for the much greater amount of proteolysis found in their cheese.

Alkaline urea-PAGE of the whole cheese was found to be an adequate method for quantitative analysis of the loss of intact casein during cheese ripening, provided all the samples were analysed within the same set of gels and were stained and destained with the same batches of stain and destain (as recommended in Chapter 3). The limitations of the method were that it showed only the larger and most abundant of the degradation products and provided limited information as to the extent of proteolysis.

The water-insoluble fraction of the cheese

There was no apparent benefit in examining the water-insoluble fraction of the cheese by alkaline urea-PAGE (Figure 4.6) as the gel profiles were essentially the same as those of the whole cheese (Figure 4.2).

The WSF of the cheese

Alkaline urea-PAGE of the WSF of the cheese (Figures 4.8) indicated definite trends in the generation and hydrolysis of peptides in the Cheddar and Mozzarella cheeses.

The electrophoretic mobility of most of the bands (A-H and J-K) was the same in both cheese types (Table 4.2), although this does not mean that the peptides were necessarily the same. The intensity of each of the bands was different in each cheese type and, once the identity of the peptides is known, these differences should provide information on the type and amount of enzyme activity in the cheese.

Although the concentration of the WSF was increased by a factor of 10 (*i.e.* 20 μl of a 5% cheese solution (1 mg) was loaded on the gel, as opposed to 5 μl of a 2% cheese solution (0.1 mg) for the urea-soluble fraction (Figure 4.2)), the larger peptides observed in the urea-soluble fraction of the cheese (Figure 4.2) were not present at the same (or greater) intensity in the WSF of the cheese (Figure 4.8) and it is therefore probable that they were quite insoluble in water.

In the WSF (Figure 4.8), the intensity of the bands in similar positions to the bands tentatively identified as β-I- and β-II-casein in the whole cheese and the water-insoluble fraction of the cheese (Figures 4.2 and 4.6) was greatest in the Cheddar cheese and increased during ripening. If the bands in the WSF and the water-insoluble fraction were formed by the same peptide, it must have only limited water solubility. Creamer and Berry (1975) showed that β-I- and β-II-casein did not self associate as strongly as β-casein and hence they could well be more easily dissociated from the cheese matrix.

The intensities of some of the bands were observed to diminish during destaining, whereas other bands disappeared altogether. It is likely that this was caused by lower molecular weight peptides diffusing out of the gel. It is probable that the smaller peptides were not retained in the gels in sufficient quantities to be seen.

The limitation of this method was that only the more abundant hydrophilic peptides of intermediate molecular weight were likely to be present in the WSF in sufficient quantities to be seen.

The method may, in time, provide more information than it does currently, but it would first be necessary to identify the peptides on the gel (either by using RP-FPLC to isolate the peptides from the cheese (or hydrolysates of pure caseins), sequencing the peptides and running them on the gel, or by running the cheese samples (or hydrolysates of pure caseins) on the gel and then extracting and sequencing the peptides).

5.3 LMW SDS-PAGE

The whole cheese

LMW SDS-PAGE of the whole cheese was not an adequate method for separating and quantitating the caseins because the four major caseins were contained within two bands (Figure 4.4).

The method was used to monitor para-κ-casein during cheese ripening. In both cheese types, there was an apparent increase in the amount of para-κ-casein during the first 91 days of ripening. This result must be treated with caution as there may be several peptides that migrate concurrently with para-κ-casein (Chapter 2, Figures 2.7 and 2.8) and an increase in the concentration of any of these peptides would cause the intensity of the para-κ-casein band to increase. After 91 days, the intensity of the para-κ-casein band decreased in both varieties in agreement with the findings of Calvo *et al.* (1992b).

In the future, it may be beneficial to use SDS gels with a larger pore size to monitor para-κ-casein and in this way it may be possible to selectively retain only the larger peptides.

SDS-PAGE can be used for detecting and monitoring β-lactoglobulin and α-lactalbumin in cheese made with ultrafiltered or heat treated milk (Harper *et al.*, 1989; Calvo *et al.*, 1992a). There was no evidence of β-lactoglobulin or α-lactalbumin in either the Cheddar cheese or the Mozzarella cheese.

The water-insoluble fraction of the cheese

There was no apparent benefit to be gained from analysing the water-insoluble fraction of the cheese by LMW SDS-PAGE (Figure 4.7) as the gel patterns were essentially the same as those of the whole cheese (Figure 4.4).

The WSF of the cheese

LMW SDS-PAGE of the WSF of the cheese (Figure 4.9) separated the peptides mainly on the basis of molecular weight. It is likely that each of the peptides on the gel had a molecular weight of ≥3,500 Da as dialysis of the WSF (dispersed in sample buffer) against sample buffer, using tubing with a 3,500 Da molecular weight cutoff, did not result in the loss of bands from the gel, although it is possible that peptides of lower molecular weight associated with each other and did not dialyse out.

None of the larger peptides that were present in the whole cheese (Figure 4.4) was present at the same, or greater, intensity in the WSF of the cheese (Figure 4.9) despite the concentration of the WSF having been increased by a factor of 10 (*i.e.* 20 μl of a 5% cheese solution (1 mg) was loaded on the gel, as opposed to 10 μl of a 1% cheese solution (0.1 mg) for the urea-soluble fraction in SDS buffer (Figure 4.4)). This indicates that these peptides were fairly insoluble in water.

The electrophoretic mobility of most of the bands (A, B, D-F and H-K) was the same in both cheese types although the concentration of each of these bands was different in each type of cheese.

Once the identity of the peptides in the cheese is established, this method should be useful for separating and quantitating their appearance and disappearance during ripening.

The limitation of this method was that only the more abundant hydrophilic peptides with a molecular weight that was likely to be $\geq$3,500 Da were retained in the gel.

5.4 RP-FPLC OF THE WSF

RP-FPLC of the WSF (Figure 4.10) separates the many peptides in the cheese and allows trends in their generation and hydrolysis to be seen. The mechanism for the separation is complex, but generally the smaller and more hydrophilic peptides elute first and the larger and more hydrophobic peptides elute later (Cliffe *et al.*, 1993).

In both the Cheddar cheese and the Mozzarella cheese, there was a gradual increase in the total amount of water-soluble material (Figure 4.11). In agreement with the findings for WSN (Figure 4.1), this increase was much greater in the Cheddar cheese than in the Mozzarella cheese and indicates that the total amount of enzyme activity was greater in the Cheddar cheese than in the Mozzarella cheese.

During the ripening of the Cheddar cheese, there was a gradual increase in the amount of material in the later eluting regions (after 50 min) of the RP-FPLC traces (Figure 4.10). This material is believed to comprise the more hydrophobic and larger peptides produced mainly by the action of rennet and plasmin. The greater amount of later eluting material in the Cheddar cheese than in the Mozzarella cheese probably reflects the greater rennet activity (as evidenced by the loss of intact α_{s1}-casein and the

concomitant formation of α_{s1}-casein-I (Figure 4.2)) and possibly microbial enzyme activity in the Cheddar cheese. Many of the larger peptides in Cheddar cheese appeared to be progressively hydrolysed during ripening and there was a gradual increase in the amount of material in the intermediate (22-50 min) and earlier (before 22 min) eluting peaks which are believed to comprise the more hydrophilic and smaller peptides as well as the amino acids. It is probable that the microbial enzymes were largely responsible for the formation of these smaller peptides and amino acids.

During the maturation of the Mozzarella cheese, there was also a gradual increase in the amount of material in the later eluting regions of the RP-FPLC traces (Figure 4.10). However, there were very few peptide peaks in the intermediate region of the RP-FPLC traces and those that were present gradually increased in size during maturation. The further breakdown of these peptides, believed to have been produced by rennet and plasmin, to the early eluting lower molecular weight peptides and amino acids did not occur to the same extent as in the Cheddar cheese. It is believed that this demonstrates the extent of rennet and plasmin activity in the Mozzarella cheese and reflects the effect of the pasteurising stretch temperature (68°C) on the starter and NSLAB in the Mozzarella cheese.

The large differences in the quantity of small peptides and amino acids produced in the Cheddar and Mozzarella cheeses are believed to account for the large differences in NPN (as % TN) (Figure 4.1) that were observed between the two cheese types.

These findings support the belief that the generation of low molecular weight peptides and amino acids is attributable mainly to the activity of the microbial enzymes (O'Keeffe *et al.*, 1976; Law *et al.*, 1993).

Once each of the peptides has been identified, this method is likely to provide valuable information on the flavour (taste) components of cheese, which have been demonstrated to reside in the WSF of Cheddar cheese (McGugan *et al.*, 1979; Aston and Creamer, 1986).

5.5 SE-HPLC

SE-HPLC was used to separate the proteins, peptides and amino acids in the Cheddar and Mozzarella cheeses on the basis of their apparent conformational size in the low ionic strength and highly acidic 36% acetonitrile system.

SE-HPLC of the urea-soluble fraction of the Cheddar and Mozzarella cheeses was not particularly useful (Figure 4.14). However, analysis of the WSF (Figure 4.15) provided a valuable source of information on the trends in the generation and breakdown of the larger peptides ($\leq$20,900 Da) to smaller peptides and amino acids. The increase in the amount of higher molecular weight material that was observed by SE-HPLC (Figure 4.15), during the ripening of both the Cheddar and Mozzarella cheeses, appears to correspond to the increase in size of the later eluting peaks (after 50 min) observed by RP-FPLC (Figure 4.10) and is believed to be related to the combined activities of rennet and plasmin in the cheese. The increase in the amount of these higher molecular weight peptides was greater in the Cheddar cheese than in the Mozzarella cheese and reflects the greater rennet activity in the Cheddar cheese, as evidenced by the greater rate of loss of α_{s1}-casein (with the formation of α_{s1}-casein-I) (Figure 4.2). Between 91 and 182 days there was a rapid increase in the amount of intermediate molecular weight material (650-5,000 Da) in the Mozzarella cheese (Figure 4.15). This appears to correspond to the rapid increase, observed by RP-FPLC (Figure 4.10), in the amount of the few peptides that eluted between 28 and 50 min. A rapid increase in the amount of intermediate molecular weight (as well as low molecular weight) material was also observed in the Cheddar cheese.

SE-HPLC of the Mozzarella cheese revealed that very few small peptides and amino acids were generated before 133 days of maturation (Figure 4.15). This appears to correspond to the very small amount of material that eluted in the first 20 minutes when the Mozzarella cheese was examined by RP-FPLC (Figure 4.10) and reflects the lack of microbial enzyme activity in the cheese. According to Oberg *et al.* (1989), the microbial enzyme action in Mozzarella cheese affects the stretch and melt properties of the cheese. However, there was little evidence of microbial enzyme activity in the Mozzarella cheese examined in this study, even after 91 days at 13°C, a temperature that is well above the normal storage temperature of 2-4°C. It is therefore unlikely, in this particular cheese, that the microbial enzymes would be responsible for any changes in the functional properties that may have occurred. In contrast with the Mozzarella cheese, a large amount of low molecular weight material was observed in the Cheddar cheese by SE-HPLC (Figure 4.15) which seems to correspond to the large amount of early eluting material observed by RP-FPLC (Figure 4.10) and reflects the amount of microbial enzyme activity in the cheese.

The trends in the molecular weight distribution observed during ripening indicate that this method may be useful for classifying different cheese varieties. Molecular weight

distribution profiles of the WSF of Cheddar cheese were recently used by Wilkinson *et al.* (1992) to study the effects of commercial enzymes on proteolysis and ripening in Cheddar cheese. Cliffe *et al.* (1993), using a combination of SE-HPLC and RP-HPLC, demonstrated that the higher molecular weight fractions obtained by SE-HPLC contained bitter peptides and corresponded to the later eluting peaks on the RP-FPLC traces, whereas the lower molecular weight fractions corresponded to the early eluting peaks. They used water to disperse the dried protein residue (after extraction with ethanol) and to elute the peptides and amino acids from the SE-HPLC column. It is therefore possible that the higher molecular weight material contained aggregates of smaller hydrophobic peptides.

The 36% acetonitrile/0.1% TFA solvent system appears to be unusual in its ability to separate the components of the WSF. Lemieux and Amiot (1990) and Lemieux *et al.* (1991) reported that SE-HPLC using a TSK G-2000 SW column was not a good method to characterise peptides. They used a different solvent system (0.1% TFA, 0.05 or 0.12 M phosphate buffer at pH 5.0 and 10% methanol) and reported a wide range of molecular weights in each of the fractions that were collected and examined.

Considerable importance has been attached to finding a reliable indicator of cheese type and maturity. The ratio of NPN (12% TCA-soluble nitrogen) to TN was found by Venema *et al.* (1987) to correlate better with cheese ripening than does the ratio of WSN to TN and has been widely used as an indicator of maturation. Haasnoot *et al.* (1989) found that the ratio of β-:γ-casein was a good indicator of age in mature Gouda and the ratio of α_{s1}-casein:α_{s1}-casein-I was the best indicator of maturity in young cheese. However, the ratio of β-:γ-casein takes into account only the amount of plasmin activity (and perhaps the microbial proteinase activity) in the cheese and disregards the effect of the other enzymes present. Investigation of the WSF of cheese by PAGE or RP-FPLC was considered by Fox (1993) to be more discriminating but many changes occur in the profiles as the cheese matures and the results are complex and difficult to interpret. It may be possible to use the molecular weight distribution profiles of the WSF (and perhaps an organic-solvent-soluble fraction to give an indication of the amount of more hydrophobic material) to determine the cheese type and maturity. These profiles were clearly different in the two types of cheese examined in this study and the molecular weight distribution changed progressively during the maturation of both cheese types (Figure 4.15). SE-HPLC profiles offer a much simpler method of classification than either PAGE or RP-FPLC as the peptides do not require identification.

6 CONCLUSIONS

The differences between Cheddar and Mozzarella functionality were likely to be a reflection of differences in the proteolytic pathways in the maturing cheeses which, in turn, were a reflection of differences in the processing parameters of the two cheese types.

It was concluded that:
(1) the rapid breakdown of α_{s1}-casein, despite the higher S/M,
(2) the slow breakdown of β-casein,
(3) the relatively rapid increase in WSN and NPN, despite the higher S/M,
(4) the large amount of small peptides and amino acids (on the RP-FPLC traces), and
(5) the large amount of low molecular weight material (on the SE-HPLC traces), in the Cheddar cheese were consequences of greater rennet activity, less plasmin activity and greater microbial enzyme activity in this cheese type than in Mozzarella cheese. The greater enzyme activities despite the higher S/M, in turn, reflect the higher initial level of rennet, the high moisture content and the lack of heat treatment.

It was concluded that:
(1) the slow breakdown of α_{s1}-casein, despite the low level of rennet and the high heat treatment,
(2) the comparable breakdown of α_{s1}- and β-casein,
(3) the slow increase in WSN and NPN, despite the high heat treatment,
(4) the accumulation of large and intermediate molecular weight peptides and the lack of very small peptides and amino acids (on the RP-FPLC traces), and
(5) the small amount of low molecular weight material (on the SE-HPLC traces), in the Mozzarella cheese were consequences of lower residual rennet activity, plasminogen activation, a lack of starter enzyme activity, as well as a small amount of atypical microbial enzyme activity in the later stages of maturation at the artificially high ripening temperature of 13°C. These enzyme activities, in turn, reflect the low initial level of rennet, the survival of rennet despite the heat treatment, the effect of the heat treatment on the activation of plasminogen and on the (lack of) survival of the starter enzymes, the high pH, the low S/M and the high moisture content of this cheese.

Rennet (and possibly milk acid protease) and plasmin contributed mainly to the formation of the larger and intermediate molecular weight peptides and contributed little to the formation of low molecular weight peptides and amino acids whereas the

microbial enzymes were largely responsible for the generation of low molecular weight peptides and amino acids in the cheeses.

The previously unreported technique of SE-HPLC using a Toyo-Soda SW 2000 column and a 36% acetonitrile/0.1% TFA solvent system is a promising new technique that has considerable potential for examining protein breakdown in cheese and could possibly be used in conjunction with PAGE to determine cheese type and extent of maturation.

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