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Primary Proteolysis of Caseins in Cheddar Cheese

Thesis Presented by

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for the degree of

Doctor of Philosophy

in

Food Chemistry

April, 1993

For My Parents

SUMMARY

McSweeney, P. L. H., (1993), *Primary Proteolysis of Caseins in Cheddar Cheese*, Ph.D. Thesis, The National University of Ireland, Cork.

Studies were undertaken to investigate proteolysis of the caseins during the initial stages of maturation of Cheddar cheese. Isolated caseins were hydrolyzed by enzymes thought to be of importance during cheese ripening and the resulting peptides isolated and identified. Large peptides were also isolated from Cheddar cheese and identified, thus enabling the extent to which casein degradation studies could be extrapolated to cheese to be established.

The proteolytic specificity of chymosin on bovine α_{s1}- and α_{s2}-caseins and of plasmin on bovine α_{s1}-casein were determined. The action of cathepsin D, the principal indigenous acid milk proteinase, on caseins was studied and its pH optimum and sensitivity to NaCl determined. The action of cathepsin D on α_{s1}-, α_{s2}-, β- and κ-caseins was compared with that of chymosin and was found to be generally similar for the hydrolysis of α_{s1}- and κ-caseins but to differ for α_{s2}- and β- caseins.

β-Casein in solution was hydrolyzed by cell wall-associated proteinases from three strains of *Lactococcus lactis*; comparison of electrophoretograms of the hydrolyzates with those of Cheddar cheese indicated that no peptides produced by cell wall-associated proteinases were detectable in the cheeses.

All the major peptides in the water-insoluble fraction of Cheddar cheese were isolated and identified. It was found that β-casein was degraded primarily by plasmin and α_{s1}-casein by chymosin. Initial chymosin and plasmin cleavage sites in α_{s1}- and β-casein, respectively, identified in these and other studies corresponded to the peptides isolated from cheese.

The importance of non-starter lactic acid bacteria (NSLAB) to the maturation of Cheddar was studied in cheeses manufactured from raw, pasteurized or microfiltered milks. NSLAB were found to strongly influence the quality and patterns of proteolysis.

Results presented in this thesis are consistent with the hypothesis that primary proteolysis in Cheddar is catalysed primarily by the action of chymosin and plasmin on intact α_{s1}- and β-caseins, respectively. The resulting large peptides so produced are subsequently degraded by these enzymes and by proteinases and peptidases from the starter and NSLAB.

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1

CHEESE: AN OVERVIEW

HISTORICAL ASPECTS

Cheesemaking in Antiquity: Cheese has been defined as the curd of milk separated from its whey and variously prepared as a food; the word originates from the Latin *caseus* through the Old English *cese.*[1] Cheesemaking appears to have originated about 6,000 to 7, 000 BC, a period when a number of other important agricultural developments occurred in the region of present-day Iraq, between the rivers Tigris and Euphrates[2]. Early Sumerian friezes have been found which depict the milking process and the coagulation of milk[2]. Cheesemaking probably originated by accident; bags made from animal skins would have been a convenient method of storing liquids by nomadic tribes. The manufacture of foods from an acid coagulum of milk probably predated those made from an enzymatic (rennet) coagulum. In warm climates, milk sours quickly due to the action of its indigenous lactic flora; this fermentation process is used, under controlled conditions, in the manufacture of all cheese varieties and fermented milks. The use of proteolytic enzymes to produce a coagulum probably originated through the use of animal stomachs to store milk: during storage, the gastric proteinases (chymosin and pepsin) would be extracted into the milk. Another possible source of proteolytic enzymes used in cheese manufacture may have been plant leaves or extracts added to milk as a flavouring or preservative. The coagulum may have been broken by agitation of an animal skin bag and the whey separated from the curd; the whey may have been drunk as a refreshing drink and the curds salted (and perhaps moulded and dried) to provide a relatively non-perishable source of protein for the community. The practice of using goat or lamb skins as containers for milk or cheese is still practiced by nomadic peoples in Iran[3] and is mentioned in the Bible[4].

Scott[2] considers it likely that the development of cheese and fermented milks occurred in two directions. The first led to products such as yoghurt, laban, koumis and kefir, while the other led to the production of cheese by the separation of whey through cloths or perforated bowls or baskets, leaving curds which were then salted, moulded, and perhaps dried, to produce cheese. Cheesemaking methods spread from the Sumerian plains like the spokes of a wheel northwards to the steppes of Russia, westwards to Europe and eastwards to India and Tibet[2].

Biblical References: There are numerous references to cheese and other foods in the Bible[5]. Milk and dairy products formed an important part of the diet of peoples of the Near-East during Biblical times, and indeed Palestine was described as "a land flowing with milk and honey[6]". Animals kept during Biblical times for milk included goats[7], sheep[8] and possibly camels[9]. Goats milk was considered to be the best[7] ; cows' milk is rarely specified in the Bible, perhaps because of the unsuitability for cows of pastures in Biblical lands[5]. In addition to milk, other foods of dairy origin mentioned in the Bible include cream[5, 10], fermented milks[5, 11] and butter[12]. Cheese is mentioned in the Books of Samuel[13, 14] (*ca.* 1170 to 1017 BC) as a delicacy sent by Jesse to his sons and as gifts presented to David. Biblical references to the cheesemaking process are, necessarily, lacking in detail, although in the Book of Job (*ca.* 1520 BC), Job remarks to God "did Thou not pour me out like milk and curdle me like cheese?"[15]

Classical References: The most complete descriptions of cheesemaking in ancient times are those of Classical Greek and Roman authors. Homer mentions cheesemaking in the Odyssey[16] (*ca.* 1184 BC). The "Cyclops", Polyphenus, was described as making cheese in a cave from goats' and ewes' milks. The milk was placed in "well-made dairy vessels" and curdled in "pails swimming with whey" before the whey was drained by means of plaited baskets. This cheese may have been the ancestor of Feta[2]. Later, the Greek historian Herodotus (484 to 408 BC) referred to "Scythian" cheese made from mares' milk[2] and Aristotle (384 to 322 BC) noted that "Phrygian" cheese was made from mares' and asses' milks[2].

Roman treatises on agriculture and related practices give detailed accounts of cheesemaking. Marcus Terentius Varro (116 to 27 BC), in a treatise on agriculture,[17] considered that cows' milk cheeses were the most nutritious, followed by those from ewes' and goats' milk. Varro also distinguished between soft and ripened cheese varieties and considered the Spring and Summer to be the best seasons for cheesemaking. According to Varro, milking should be done early in the morning and coagulation achieved by addition of a piece of rennet about "the size of an olive" to two *congii* (about 9 l) of milk. Rennet from the hare or kid was preferred to that from lambs, although coagulant from the fig tree or acid coagulation using vinegar were mentioned as alternatives and rock-salt was preferred to sea-salt for sprinkling on the cheese. Pliny[18] (23 to 79 AD) discussed the nutritional aspects of cows', mares' and goats' milk cheese and also described the manufacture of lactic butter.

The most complete classical description of cheesemaking is that of Lucius Junius Moderatus Columella (*ca.* 50 AD) in the treatise *De Re Rustica*[19]. Despite its age, it reads as an accurate account of artisanal cheesemaking and it would be possible to manufacture a cheese sucessfully with no other directions than those provided by this author. Columella considerd that cheese manufacture was important as a method of preserving milk in rural areas and distinguished between soft cheeses (which must be consumed shortly after preparation) and hard cheeses (which may be stored). Milk should be as fresh as possible and lamb or kid rennets should be used to coagulate the milk, although other rennet preparations, including seeds of the safflower, exudate from the fig tree or flowers of the wild thistle (the latter coagulant is still used in the manufacture of a number of Spanish and Portuguese varieties) could be used. However, Columella reported that the best cheese was that produced with the least amount of coagulant, which should be limited to the weight of one silver *denarius* per pail of milk. The milk should be heated slightly, but not too much, during coagulation. On coagulation, the whey should be drained quickly by means of wicker baskets and whey drainage could be accelerated by pressing the curd. The curd was then placed in a cool place and its surface sprinkled with salt so that it might exude more whey. When the curd had hardened, it was pressed strongly, sprinkled with salt and pressed for nine days before being washed. Cheeses should be ripened on wicker shelves. This type of ripened cheese had a long shelf life and could be exported. Columella described textural defects and their remedies, the manufacture of a soft-cheese variety and techniques such as brine-salting, smoking and washing the curds with hot water.

The emperor Diocletian (284 to 305 AD) fixed a maximum price for cheese, including a variety, *Lunar*, which is thought to be the predessor of Parmesan[2].

Cheesemaking in the Middle Ages and into Modern Times: After the decline of the Roman Empire, cheesemaking techniques spread further by the migration of peoples, including the Crusades and contact between monasteries.

Monasteries played an important rôle in the development of cheese varieties and in safeguarding knowledge on cheesemaking. Wenslydale was developed in Rievaulx Abbey (England), Saint Paulin in the Monastery of Notre Dame du Port du Salut (France) and Trappist in the Maria Stern Monastery (Bosnia). Muenster cheese is said to have been brought by monks from Ireland to the Netherlands where it became an established variety[2]. The names of many common cheese varieties date

Table 1.1: First recorded usage of names of some cheese varieties (adapted from Scott[2].)

Variety	*Year, AD*	*Variety*	*Year, AD*
Gorgonzola	879	Gouda	1679
Roquefort	1070	Gloucester	1783
Grana	1200	Stilton	1785
Cheddar	1500	Camembert	1791
Parmesan	1579	Saint Paulin	1816

from the distant past (Table 1.1), although these may have changed in character throughout the years.

During the 17th and 18th centuries, cheese manufacture spread with European emigrants and colonists to the Americas, Australia and New Zealand. Cheesemaking remained largely a farmhouse operation until the middle of the 19th century when it was industrialized following the development of the co-operative movement.

There are some 800 "official" cheese varieties with many local variations and there are about 2,000 named cheeses, which can be grouped into about 20 families and 5 major classes (Table 1.2). Most varieties were not standardized until the mid-19th century; Cheddar and Cheshire were standardized by Joseph Harding (1805 to 1876). Cheesemaking was not studied scientifically until the latter part of the 19th century.

Cheesemaking in Ireland: Cheese and other dairy products were consumed in ancient Ireland and constituted a substantial portion of the diet[20]. References to cheeses are relatively common in Gaelic literature, but only rarely is information other than the name of the cheese available and thus it is not possible to give a complete description of native Irish cheese varieties. Cheese varieties mentioned in Gaelic literature were described by Ó Sé[20]:-

Pressed Cheeses

Cáis : clearly originating from the Latin *caseus* , is also the modern Irish word for cheese and when used without qualification means a pressed cheese.

Tanag : thought to be a hard cheese, perhaps made from skim milk. There is an account of how Maeve, Queen of Connaught, was killed by her nephew, who fired a piece of *tanag* from a sling.

Grus : appears to be similar to *tanag*.

Faisce Grotha : literally meaning "a compression of curds", appears to refer to a relatively small curd-cheese which was moulded and probably consumed when fresh. It is said that an attempt was made to kill St. Patrick by giving him a piece of this cheese which had been poisoned.

Unpressed Cheeses

Gruth : this is the modern Irish word for "curd", and appears to refer to an unpressed curd cheese made from skim milk or buttermilk, similar to Cottage cheese.

Tath : made from sour milk curds, probably heated and stirred during manufacture.

Millsen : a sweet cheese prepared from a rennet curd.

Maothal : apparently a soft cheese-like product prepared by heating colostrum until coagulation.

Mulchan : a cheese made from buttermilk until relatively recent times (*ca.* 1500 AD). The curd was probably heated and "beaten" into a smooth cheese before being moulded.

Gruthnuis : curd or curd-cheese made from colostrum.

None of the ancient Irish cheese varieties has survived into modern times. Reasons for the disappearence of cheesemaking in Ireland are related to the political history of the country. The Feudal system, with its associated self-contained communities with designated duties on manorial farms, did not develop to any great extent in Ireland. Unlike their counterparts in continental Europe, Irish monasteries developed and declined earlier and were more schloastic and missionary in outlook and thus contributed little to agricultural development. Cheesemaking was one of the many elements of Celtic civilization lost during the economic decline of the native population subsequent to the English conquest of this country in the 16th and 17th centuries.

Cheesemaking in Ireland declined throughout the 17th and 18th centuries as a result of political instability and the imposition of the Cattle Acts of 1663 to 1669, which imposed tariffs on the export of cattle from Ireland to Britain, and had the effect of encouraging the production of butter and pigs[21]. Abigail Greenfield of Cork called on the members of the Royal Dublin Society in 1732 to encourage the practice of cheesemaking[2]. Arthur Young, an English agriculturalist found that, between 1776 and 1779, cheese was produced in parts of Counties Offaly, Wexford and Cork. However, cheese manufacture in Ireland remained negligible until the early part of the 20th century. A Department of Agriculture and Technical Instruction for Ireland was established in 1900 and there then followed a period of experimentation and industrialization of cheese production[21]. Cheese production was substantial for a brief period during World War I but declined thereafter until about 1950 when production began to increase reaching 70,800 tonnes in 1990. The development of the Irish cheese industry was reviewed by McCarthy[21].

OVERVIEW OF CHEESE MANUFACTURE

As discussed above, cheesemaking originated as a form of food preservation, allowing peoples who kept dairy animals to store food for use during periods of shortage, particularly during Winter when the seasonality of lactation would cause a considerable decrease in milk production. The preservation of cheese is as a result of the combined action of dehydration, addition of NaCl and the production of acid, anaerobic conditions and antibiotics by the starter.

Cheesemaking is esentially a dehydration process in which the fat and casein in milk are concentrated 6 to 12 fold, depending on the variety. Cheese is a medium-moisture food, containing from 30 to 50%, w/w, water: the stability of cheese is inversely related to its moisture content. In nearly all cheese varieties, dehydration occurs by coagulating the casein enzymatically, isoelectrically or by a combination of heat and acid. When the coagulum is cut or broken, it contracts under the influence of acid and heat, expressing whey; the curd is further processed into cheese. Lactose, whey proteins, approximately 50% of milk salts and 90% of water are removed in the whey. The a_w of cheese is usually >0.95 and is highly correlated with NaCl concentration, especially in young cheeses.

The basic steps in the manufacture of rennet-coagulated cheeses are summarized in Fig. 1.1.

Table 1.2: Major classes of cheese

Class	*Varieties*
Rennet coagulated	Most varieties
Acid coagulated	Cottage, Quarg, Queso Blanco
Heat, heat/acid	Ricotta
Concentration/crystallization (whey "cheeses")	Mysost, Gjetost
Ultrafiltered	Various

Milk for cheesemaking is now normally pasteurized and should be of good chemical and microbiological quality. Proteolysis and lipolysis can occur in cheese milk pre-manufacture, due to the action of indigenous milk enzymes and bacterial proteinases and lipases (particularly from psychrotrophs)[22].

Milk is acidified by the fermentation of lactose to lactic acid by the starter. Prior to about 1880, fermentation was as a result of the indigenous milk microflora, leading to variable and often poor quality cheese. The primary starters now used are *Lactococcus lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus* spp. The species of bacterium used depends on variety and the primary function of the starter is to produce acid at an appropriate rate during manufacture. Adjunct starters are used in some cheese varieties for specific functions, e.g. citrate fermentation to produce eyes and flavour compounds in Dutch varieties (*Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*), propionic acid fermentation to produce eyes and flavour compounds in Swiss varieties (*Propionibacterium shermanii*), surface growth in Limburger, Tilsit and Muenster (*Brevibacterium linens*), mould growth in Blue cheeses (*Penicillium roqueforti*) and Camembert and Brie (*Penicillium camemberti*).

Within 6 to 24 h of manufacture, the pH of curd decreases to ~5 for most varieties. Acidification influences the activity of the coagulant, its retention in the curd, gel strength, control the non-starter microflora and demineralization of the curd by solubilization of colloidal calcium phosphate and thus affects cheese texture. Acidification also influences syneresis of the rennet coagulum and thus moisture levels, as well as influencing proteolysis and other biochemical changes during ripening.

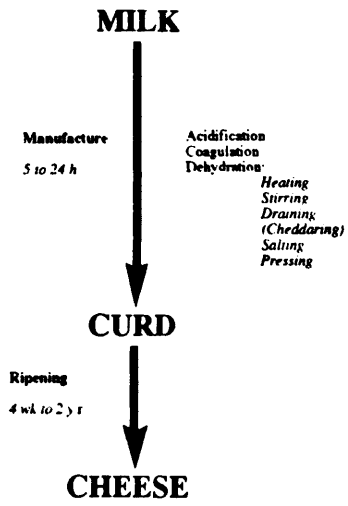

Fig. 1.1: Basic stages in the manufacture of a rennet-coagulated cheese

Pigments (usually annato) are added to milk for certain cheese varieties to give the impression of "richness" (high fat).

Casein micelles in milk are stabilized by κ-casein. In rennet-coagulated cheeses, coagulation is induced by the hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein. Hydrolysis of this bond releases a strongly hydrophilic peptide (glycomacropeptide, κ-CN f106-169) and destroys the colloidal stability of the micelles. When ~85% of the total κ-casein has been hydrolyzed, micelles begin to aggregate due to reduction in ζ-potential, destruction of steric stabilization, and reduction in solvation.

The rennet coagulation of milk is a two stage process. The first involves the enzymatic hydrolysis of κ-casein to *para*-κ-casein and glycomacropeptides, while the second involves the aggregation of the rennet-altered micelles in the presence of Ca^{2+} and at >20°C (in practice ~30°C) to form a gel (coagulum).

When it has reached the desired firmness (strength), the coagulum is cut and the temperature of the curds-whey mixture increased ("cooking") to promote syneresis. The size of curd pieces affects syneresis; fine cutting (e.g. Swiss varieties) promotes curd syneresis and thus reduces the moisture content of cheese. High cooking temperatures (e.g. ~52°C for Swiss varieties) also promote curd

syneresis. In addtion to these factors, syneresis is influenced by the rate of rennet action, type of rennet, pH, concentration of Ca^{2+}, colloidal calcium phosphate, protein and fat, and manufacturing processes including stirring during cooking, dry stirring, cheddaring, salting, and pressing.

After cooking and stirring, the curds are separated from the whey and, for Cheddar and related varieties, the curd is "cheddared". In most other varieties, the curds are placed in moulds where acidification and drainage of whey continues. In certain varieties, pressure may by applied to aid whey drainage.

In Pasta-filata varieties (e.g. Mozzarella), the acidified curd is stretched in hot water to develop the correct texture.

All cheeses are salted, at a level characteristic of the variety, from 0.7 to 11%, w/w. Cheddar and related varieties are salted before moulding by the addition of dry salt to curd pieces. Other varieties are salted by immersion in brine or application of dry salt to the surface of the cheese. Salt affects cheese flavour and curd syneresis and controls the growth of undesirable bacteria and enzyme activity.

Rennet-coagulated cheeses are ripened under controlled temperature conditions (from 3 to 25°C, depending on variety) and possibly under controlled humidity (particularly for bacterial surface-ripened and mould-ripened varieties). The duration of ripening varies from 3 weeks (e.g. Mozzarella) to 2 years or longer (e.g. Parmesan).

Immediately after manufacture, the curds for most varieties are very similar and generally lack flavour. The characteristics of the different cheese varieties develop during ripening as a result of the extensive biochemical changes (see Chapter 2).

CHEDDAR CHEESE

Cheddar is a semi-hard, directly-salted, internal bacterially ripened cheese variety, named after a village of the same name in the Mendip hills in Somerset in the South-West of England. Originally a British variety, Cheddar is now amongst the most important cheese varieties in terms of worldwide production.

A flow diagram for the production of Cheddar cheese is given in Fig. 1.2 and its typical composition in Table 1.3. The starters used are mesophilic lactococci; strains which can ferment citrate are not used as the CO_2 produced on the fermentation of citrate would produce undesirable gas holes in the cheese. Milk is renneted and the coagulum cut, the curds/whey mixture cooked to 37-39°C and the whey drained. The next manufacturing step, cheddaring, is unique to Cheddar and related varieties. The process of cheddaring traditionally refers to the process of piling and repiling warm curd blocks in the bottom of the cheese vat for about 2 h. Today, in large cheese factories, the curd is not piled in the traditional way but is

Table 1.3: Typical composition (%) of milk and Cheddar cheese.

Constituent	*Milk*	*Cheddar*
Fat	3.5	34
Protein	3.2	-
Casein	2.5	25
Whey Proteins	0.7	0
Lactose	4.6	0
Ash	0.7	2
Water	88	37
NaCl	-	2

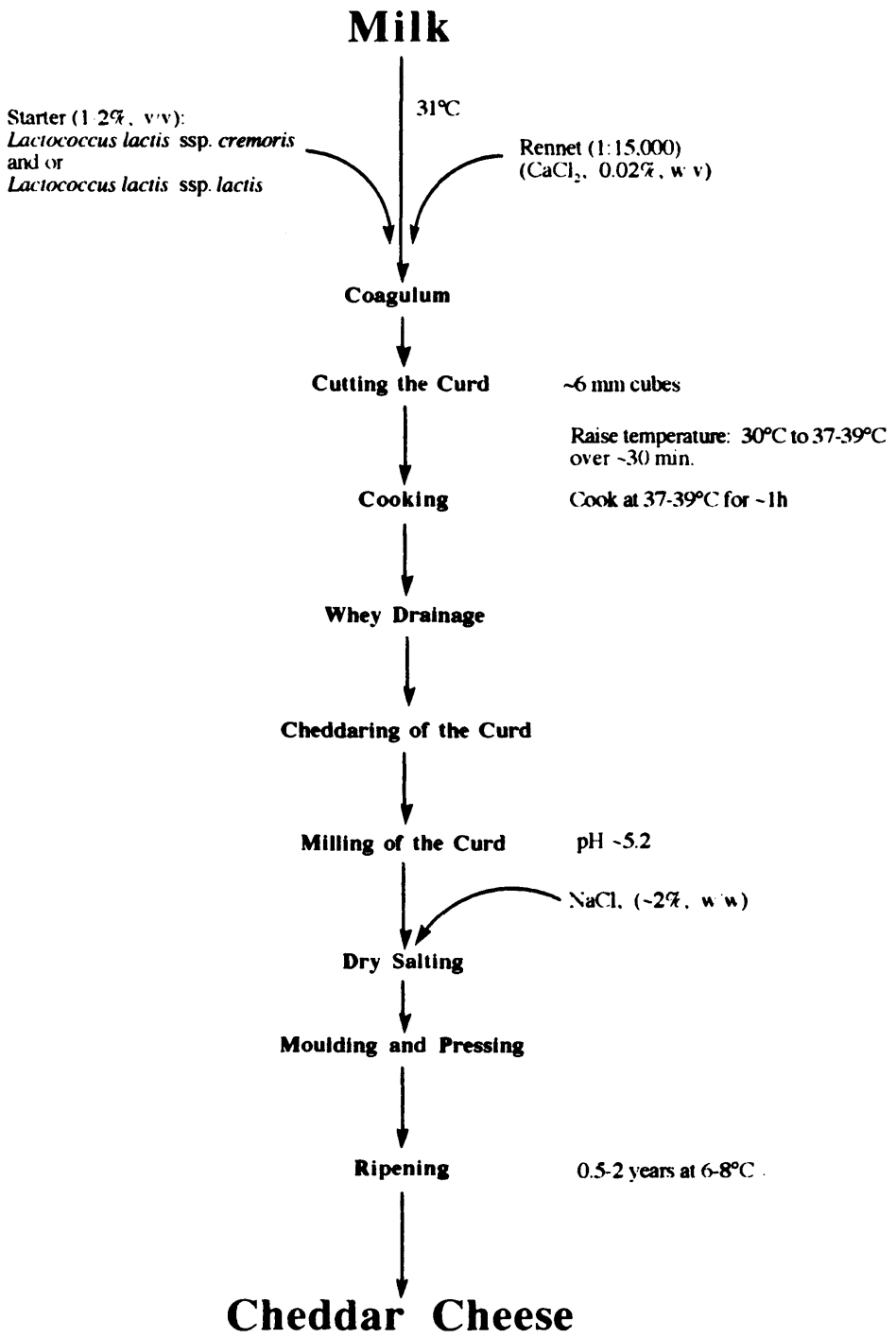

Fig. 1.2: Protocol for the manufacture of Cheddar cheese.

compressed in towers, mainly to facilitate acid development and solubilization of colloidal calcium phosphate which are necessary for the development of the correct texture. Curd blocks are then milled into pieces (3-5 cm long, 1-2 cm cross section); dry salt is added and well mixed with the curd. The salted curds are placed in moulds (usually 20 kg) and pressed before packaging and ripening, usually at 6-8°C.

The characteristic flavour of Cheddar develops during ripening (6 months to 2 years) as a result of considerable biochemical changes (see Chapter 2).

THE CHEMISTRY OF MILK

Lactose: After water, lactose (4-O-β-D-glactopyranosyl-D-glucopyranose) is the major constituent of bovine milk (~48 g l^{-1}). Lactose has been found in milks of nearly all species studied to date and essentially no where else in nature[22a]. Lactose plays an important rôle during the maufacture of cheese as the primary substrate for the growth of the starter which ferments it to lactic acid. The majority of lactose is lost in the whey; residual lactose is quickly fermented to lactate by the starter and non-starter lactic acid bacteria. The rate at which this process occurs is influenced by the NaCl concentration, until after ~1 month of ripening, the lactose concentration in the cheese is reduced to ~0%.

Milk Lipids: Bovine milk contains ~35 g l^{-1} fat, consisting of ~98% triglycerides with smaller amounts of free fatty acids, mono- and diglycerides, phospholipids, sterols and hydrocarbons. Milk fat exists almost entirely in the form of globules (0.1 to 15 μm in diameter) stabilized by phospholipids.[22a] During cheese manufacture, the majority (~90%) of milk fat globules are incorpoated into the curd.

Milk Salts: The ash content of bovine milk is ~7 g l^{-1}. Milk salts include both organic and inorganic ions and is not equilavent to either minerals or ash. The principal cations are Na^+, K^+, Ca^{2+} and Mg^{2+} while PO_4^{3-}, citrate, Cl^-, CO_3^{2-} and SO_4^{2-} are the principal anions. Milk salts play an important rôle in casein micelle stability and in the development of cheese texture. The degree of demineralization during cheese manufacture is characteristic of variety

The Milk Protein System: Normal mid-lactation bovine milk contains approximately 30 to 35 g l^{-1} protein. The milk protein system (Fig. 1.3) consists of two major families; the caseins and whey proteins. Whey proteins (5 to 7 g l^{-1}) are defined as the proteins that remain in the whey (serum) after precipitation of the caseins at pH 4.6 and 20°C. The principal whey proteins are β-lactoglobulin, α-lactalbumin, blood serum albumin, immunoglobulins and proteose peptones, although the latter group consists primarily of peptides produced from β-casein by plasmin and therefore are grouped in the β-casein family[23]. During cheese manufacture, whey proteins are lost in the whey, although small amounts may be occuluded in the curd.

Originally, the caseins (24 to 28 g l^{-1}) were defined as phosphoproteins which precipitate when milk is acidified to pH 4.6 at 20°C but they are now classified according to their primary structure. The family consists of 4 gene products, designated α_{s1}-, α_{s2}-, β- and κ-caseins. Caseins undergo post-translational phosphorylation, and in the case of κ-casein, glycosylation. Genetic variants of each casein have been identified (Fig. 1.3). The nomenclature of the caseins was revised by Eigel *et al.*[24], who recommended that they be identified by a lower-case Greek character, with or without subscript, preceeding the class name ("casein" or "CN"). The genetic variant is indicated by an upper-case Latin letter, with subscript if necessary. Post-translational modifications then follow. For example, β-CN B-5P (f1-105) indicates that this protein belongs to the β-casein family, the B genetic variant with 5 post-translational phosphorylations and is the fragment

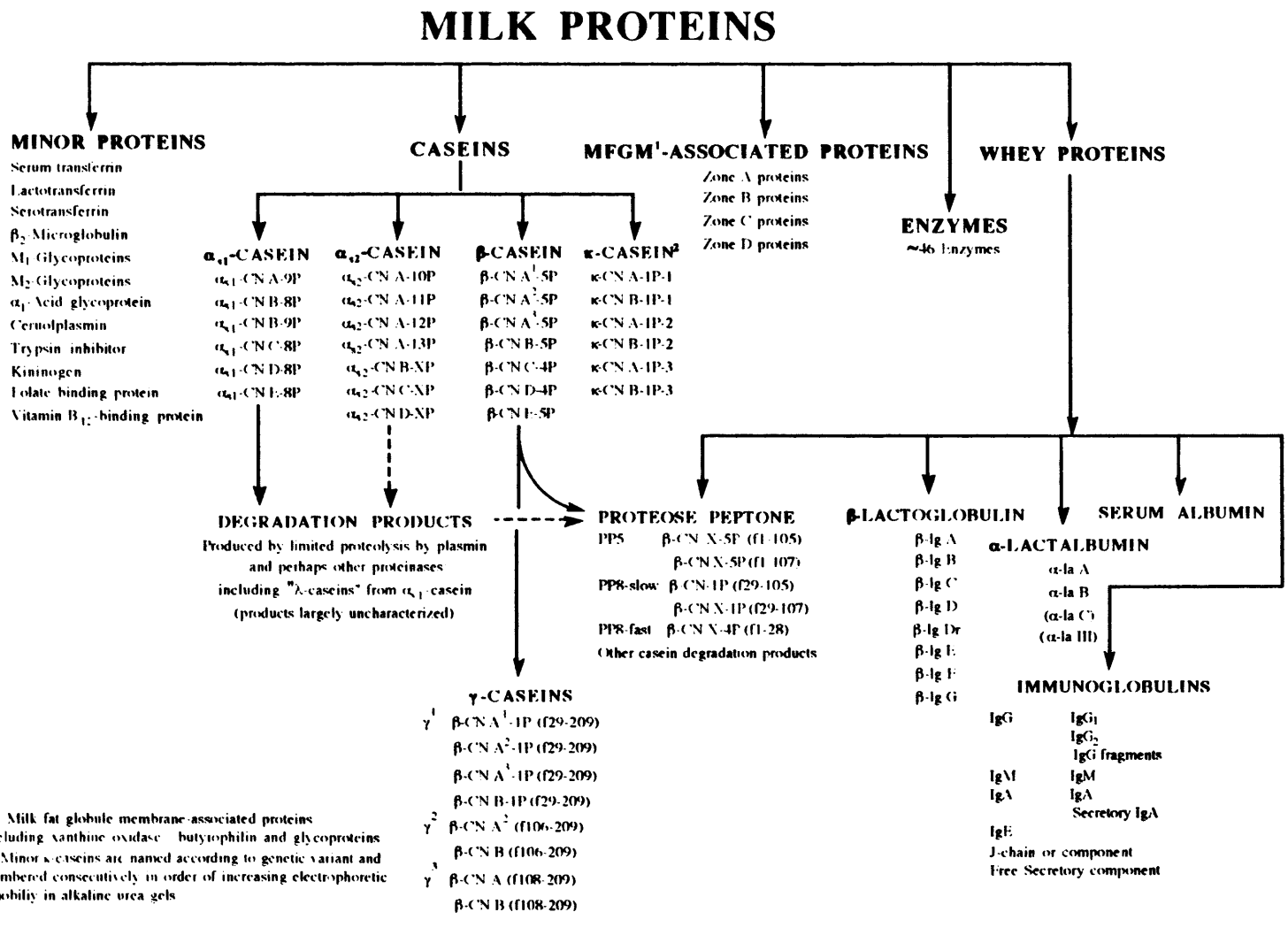

Fig. 1.3: Distribution of proteins and fragments thereof in milk of the genus Bos

of the entire β-casein B sequence from residue 1 to 105. The chemistry of the caseins has been reviewed extensively (e.g. refs 23 to 25).

Unlike the whey proteins, which exist as individual molecules or as small oligomers, the caseins possess considerable quaternary structure. Caseins exist as "micelles", spherically-shaped aggregates with an average dimeter of 140 nm. The micelles are, in turn, built up from sub-micelles (10-15 nm) held together by colloidal calcium phosphate. Sub-micelles at or near the surface of the micelles are relatively rich in κ-casein which stabalizes the structure especially against precipitation by Ca^{2+}, to which the α_{s1}-, α_{s2}- and β-caseins are very sensitive. Sub-micelles at the core of the micelle are richer in hydrophobic caseins (reviews on casein micelle structure include refs. 26 and 27).

REFERENCES

1. Collins English Dictionary, 1991, 3rd. Edn., Harper Collins Publishers, Glasgow.

2. Scott, R., 1981, *Cheesemaking Practice*, Applied Science Publishers Ltd., Barking, Essex.

3. Kosikowski, F. V., 1982, *Cheese and Fermented Milk Foods*, 2nd Edn., F. V. Kosikowski and Associates, Brookdondale, New York.

4. Judges IV xiii

5. MacAlister, A., 1904, Food, In: *A Dictionary of the Bible Dealing with its Language, Literature and Contents, Including Biblical Theology*, Vol. 2, p. 36, J. Hastings (ed.), T. & T. Clark, Edinburgh.

6. Exodus III viii

7. Proverbs XXVII xxvii

8. Deuteronomy XXII xiv

9. Genesis XXXII xv

10. Isaiah VII xxii

11. Genesis XVIII viii

12. Psalms LV xxi

13. I Samuel XVII xviii

14. II Samuel XVII xxix

15. Job X x

16. Homer, 1980, *The Odessey* , IX 215-282, (p. 104), W. Shewring (translator), Oxford University Press.

17. Varro, 1912, *Libri Rerum Rustiarum*, L. Storr-Best (translator), Bell and Sons, London.

18. Pliny, 1975, *Natural History*, XXVIII, 34-35, W. H. S. Jones (translator), The Loeb Classical Library, Harvard University Press and Heinemann, London.

19. Columella, 1944, *De Re Rustica*, E. S. Forster and E. H. Heffner (translator), The Loeb Classical Library, Harvard University Press and Heinemann, London.

20. Ó Sé, M., 1948, Old Irish cheeses and other milk products, *J. Cork His. Arch. Soc.*, **53**, 82.

21. McCarthy, J., 1992, History of the development of the Irish cheese industry, Proc. 3rd Cheese Symp., Moorepark, Fermoy, Co. Cork, Ireland, p. 1.

22. Fox, P. F., 1989, Proteolysis during cheese manufacture and ripening, *J. Dairy Sci.*, **72**, 1379.

22a. Wong, N. P. (ed.), *Fundamentals of Dairy Chemistry*, 3rd edn., Van Nostrand Reinhold (Publ.), New York.

23. Whitney, R. McL., 1988, Proteins of Milk, In: *Fundamentals of Dairy Chemistry*, 3rd edn., p. 81, N. P. Wong (ed.), Van Nostrand Reinhold (Publ.), New York.

24. Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M., Jr., Harwalkar, V. A., Jenness, R., Whitney, R. McL., 1984, Nomenclature of the proteins of cows' milk: Fifth revision, *J. Dairy Sci.* **67**, 1599.

25. Swaisgood, H. E., 1992, Chemistry of the caseins, In: *Advanced Dairy Chemistry*, Vol. 1 Proteins, p. 63, P. F. Fox (ed.), Elsevier Applied Science Publishers, London.

26. Farrell, H. M., Jr., 1988, Physical equlibria: Proteins, In: *Fundamentals of Dairy Chemistry*, 3rd edn., p. 461, N. P. Wong (ed.), Van Nostrand Reinhold, New York.

27. Rollema, H. S., 1992, Casein association and micelle formation, In: *Advanced Dairy Chemistry*, Vol. 1 Proteins, p. 111, P. F. Fox (ed.), Elsevier Applied Science Publishers, London.

2

BIOCHEMISTRY OF CHEESE RIPENING†

1. INTRODUCTION

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

The procedure for the production of cheese curd is generally similar for many varieties of cheese and the curds for Cheddar, Gouda, Swiss and many other varieties are quite similar on the day of manufacture. It is during ripening that the individual characteristics of cheese varieties develop.

The biochemistry of cheese ripening is very complex, amongst the most complex of any food. It involves 3 primary processes: glycolysis, lipolysis and proteolysis; the relative importance of each depends on the variety. Changes in cheese during ripening have been the subject of extensive study and will be reviewed in this chapter.

2. CHEESE RIPENING AGENTS: ASSESSMENT OF CONTRIBUTION TO RIPENING

Ripening agents in cheese generally originate from 5 sources: the coagulant, the milk, starter bacteria, adjunct starter, non-starter bacteria.

The residual coagulant and enzymes from the starter, and probably the non-starter microorganisms, are common to nearly all ripened cheeses. The adjunct starter (i.e. microorganisms added to cheesemilk for purposes other than acidification) can exert considerable influence on maturation in cheese varieties in which they are used (e.g. *Penicillium roqueforti*, *P. camemberti* in mould-ripened varieties or *Brevibacterium linens* in smear-ripened cheeses). Exogenous enzymes used to accelerate ripening could be added to the above list, and when present can exert great influence.

2.1. Model Cheese Systems

The rôle of the individual ripening agents during cheese maturation has been studied by the development of a number of model cheese systems in which the action of one or more of these agents is eliminated and the resulting cheese studied. The most comprehensive studies of this nature involved the manufacture of cheese under controlled microbiological conditions, with the inactivation of the rennet and/or chemical acidification, allowing the effect of individual ripening agents to be studied.

† *Parts published in Fox, P. F., Law, J., McSweeney, P. L. H. and Wallace, J, 1993, Biochemistry of Cheese Ripening, In:* Cheese: Chemistry, Physics and Microbiology, *2nd ed., vol. 1, P. F. Fox (ed.), Elsevier Applied Science Publishers, London and Fox, P. F., O'Connor, T. P., McSweeney, P. L. H., Guinee, T. P. and O'Brien, N. M., 1993, Cheese: Physical, Chemical, Biochemical and Nutritional Aspects,* Adv. Food Nutr. Res. *(in press).*

A milk supply of exceptionally high bacteriological quality is essential to eliminate non-starter bacteria from aseptic cheese. Sterile teat cups, clusters and bucket milking plant have been used by a number of authors (Perry and McGillivray, 1964; O'Keeffe *et al.*, 1976a) to obtain milk with an initial bacterial count of *ca.* 10^2 cfu ml^{-1}. Kleter and de Vries (1974) included a cooling coil between the cluster and bucket and obtained counts averaging 46 cfu ml^{-1}. Reiter *et al.* (1969) withdrew milk aseptically by means of a teat cannula but the quantities obtained were sufficient only to produce cheeses of about 100 g.

Heat-treatment of aseptically-drawn milk is necessary to further reduce bacterial counts. Perry and McGillivray (1964) used batch pasteurization (68°C x 5 min) in a steam-jacketed cheese vat. LTLT pasteurization (63°C x 30 min) was also used by Reiter *et al.* (1969) and O'Keeffe *et al.* (1976a). Chapman *et al.* (1966), who did not use an aseptic milking technique, used HTST pasteurization (71.6°C x 17 s) to produce low count milk. Other authors who used HTST pasteurization include Visser (1976, 1977a), Kleter (1976) and Paulsen *et al.* (1980). UHT-treated milk was used by Le Bars *et al.* (1975) who employed $CaCl_2$, a higher rennet concentration and higher setting temperature to offset the ill-effects of the high heat treatment on the rennet coagulability of milk. Recently, McSweeney *et al.* (unpublished results), used a pasteurization regime of 78°C x 15 s with the addition of $CaCl_2$ to produce milk of high bacteriological quality for a study involving the manufacture of cheese under aseptic conditions to study the influence of non-starter lactobacilli on cheese ripening.

The thermal destruction of a number of strains of bacteria with reference to the production of milk for aseptic cheesemaking was investigated by Turner *et al.* (1986) who concluded that a heat treatment of 83°C x 15 s or 72°C x 58 s would be necessary to ensure a reduction of 10^8, which was deemed necessary to produce cheese with non-starter counts of < 10 cfu kg^{-1} cheese from milk with an initial count of 10^3 cfu ml^{-1}. However, these authors concluded that HTST pasteurization (72°C x 15 s) is sufficient for milk with an initial count of 10 cfu ml^{-1}.

The manufacture of cheese under aseptic conditions has generally been performed in enclosed cheese vats (Mabbitt *et al.*, 1959; Kleter, 1976; Visser, 1977a; Paulsen *et al.*, 1980). Le Bars *et al.* (1975) made cheese in a sterile room while O'Keeffe *et al.* (1976a, b) used a laminar air-flow unit. Enclosed vats with rubber gauntlets were used by Mabbitt *et al.* (1959) and modified by Perry and McGillivray (1964) to include an overpressure of sterile air. Chapman *et al.* (1966) and Reiter *et al.* (1969) used similar techniques. Le Bars *et al.* (1975) made cheese in an aseptic room (5 x 3 m) with a filtered air supply and the cheeemakers were clothed in sterile garments. The technique used by O'Keeffe *et al.* (1976a, b) is probably the most simple, involving thermostatically controlled waterbaths placed in laminar air-flow units.

In the absence of starter, acidification is generally achieved using an acidogen, usually gluconic acid-δ-lactone (GDL), which is hydrolyzed in aqueous solution to gluconic acid at a predictable rate (O'Keeffe *et al.*, 1975), although this rate is faster than occurs in biologically acidified cheese. Although direct acidification of cheesemilk is used in the manufacture of certain cheese varieties, it is not suitable for the production of hard ripened cheese and early workers had difficulty in controlling pH when dilute acid was used for acidification. The rate of acid production influences proteolysis in the curd (O'Keeffe *et al.*, 1975) and these authors suggested that cheesemilk be partially acidified with lactic acid prior to renneting and during cutting and cooking. Solid GDL was added when the curd was drained and further GDL was added with the salt. The protocol was deemed to be closer to biological acidification than previous methods.

To determine the rôle of the coagulant in cheese ripening, it is necessary to produce cheese in which coagulant is inactivated. Three techniques have been developed to achieve this. The first approach, developed by Visser (1976), involves separation of the first and second stages of rennet action: milk was depleted of Ca^{2+} and Mg^{2+} by treatment with an ion-exchange resin, followed by

renneting of the milk, which did not coagulate in the absence of Ca^{2+}. The renneted milk was then heated (72°C x 20 s) to inactivate the rennet, cooled to < 5°C and $CaCl_2$ added. The milk was then heated dielectrically to avoid agitation and allowed to coagulate. In an alternative approach, O'Keeffe *et al.* (1977) used procine pepsin as coagulant. After coagulation, the curds-whey mixture were titrated to pH 7.0 to inactivate the pepsin, which is very sensitive to pH denaturation. Mulvihill *et al.* (1979) demonstrated the potential of piglet gastric proteinase for use in aseptic cheesemaking. This enzyme hydrolyzes bovine κ-casein, but has little activity on α_{s1}- or β-casein. However, its suitability was demonstrated only in small-scale cheesemaking trials. The use of immobilized rennets could provide a fourth method for producing rennet-free curd, but leaching of enzyme from the support causes difficulties with this approach.

Most early attempts to assess the relative importance of the individual cheese ripening agents made no attempt to eliminate the action of the indigenous alkaline milk proteinase, plasmin (fibrinolysin, E.C. 3.4.21.7), and thus plasmin action in the control cheeses represented a "background" level of proteolysis against which the action of other agents was assessed. Eliminating plasmin action in cheese presents considerable problems and thus its action has been assessed indirectly in most cases. However, one of a number of possible approaches should permit direct assessment of the contribution of plasmin in isolation.

The non-competitive plasmin inhibitor, 6-aminohexanoic acid (AHA), was used by Farkye and Fox (1991) to study the significance of plasmin in cheese ripening. This compound inhibits plasmin but not chymosin or bacterial proteinases and peptidases and thus allowed the assessment of plasmin action in normal cheese made with lactic starter and without aseptic precautions. However, it was necessary to use a relatively high concentration of AHA which influenced curd syneresis and thus affected the moisture content of the final cheese. Also, since AHA contains N, the background level of soluble N was increased greatly.

The high heat stability of plasmin and the finding that its activity is increased by high cooking temperatures (Farkye and Fox, 1990), suggest that it may be possible to develop a model system in which an aseptic curd is made, the rennet denatured by a suitable cooking temperature and the curd acidified by GDL. Such a system would allow plasmin to act in isolation.

Plasmin is inhibited by soy-bean trypsin inhibitor, which may be suitable for use as a plasmin inhibitor in cheese. Other specific plasmin inhibitors include dichloroisocoumarin (Harper *et al.*, 1985). However, neither of these approaches has been invesitgated.

2.2. Contribution of Individual Agents to Ripening

2.2.1. Proteinases

Proteolysis during cheese maturation is an important biochemical event in most cheese varieties. The extent of proteolysis varies from very limited (eg. Mozzarella) to very extensive (eg. blue mould varieties). The products of proteolysis range in size from large polypeptides, comparable in size to intact caseins, through a range of medium and small peptides to free amino acids. Clearly, no one proteolytic agent can be responsible for such a wide range of products.

Much of the information on the relative importance of individual proteolytic agents has been gleaned from studies involving aseptic model cheese systems as described above. The most comprehensive of these studies to date is that of Visser (1976, 1977 a, b, c) and Visser and de Groot-Mostert (1977) in which the relative importance of enzymes from rennet, starter bacteria and milk to proteolysis in Gouda cheese was assessed. It is generally accepted that the results for Gouda can be extrapolated to other internal bacterially-ripened varieties, such as Cheddar. These authors made aseptic control cheeses, aseptic rennet-free cheeses, aseptic

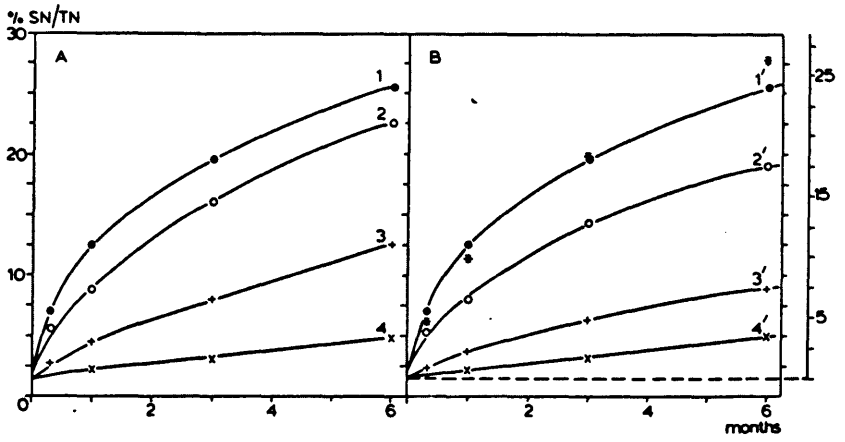

Fig. 2.1 *(a) Average soluble N values developed during the ripening of normal aseptic cheeses (1), aseptic starter-free cheeses (2), aseptic rennet-free cheeses (3) and aseptic rennet and starter-free cheeses (4) and (b) net contribution of rennet (2'), starter bacteria (3') and plasmin (4') to soluble N production as compared with the net production of soluble N in normal aseptic cheese (from Visser, 1977c).*

starter-free cheeses and aseptic, rennet-free, starter-free cheese. The results of these studies indicated that rennet is responsible for the initial hydrolysis of caseins and the production of most of the water- or pH 4.6-solubleN in these cheeses (Fig. 2.1a, b), and that the action of starter bacteria is of lesser importance at this level of proteolysis. However, the production of amino acid-N was found to be due primarily to the action of starter bacteria or their enzymes (Fig. 2.2). The results of other studies on controlled-microflora cheese (Reiter *et al.*, 1969; Gripon *et al.*, 1975; Desmazeaud *et al.*, 1976; O'Keeffe *et al.*, 1976a, b, 1978) yielded generally similar results. Direct evidence for the rôle of plasmin in cheese is less plentiful. Visser (1977c) found that approximately 5% of the total N of a 6 month-old aseptic starter-free, rennet-free cheese to be soluble at pH 4.6, but very low levels of free amino acids were found. These results suggest that chymosin, and to a lesser extent plasmin, is primarily responsible for the hydrolysis of caseins to large and intermediate-sized peptides, although Reiter *et al.* (1969) suggested that plasmin contributed to the formation of amino acid N in their aseptic cheeses.

Farkye and Fox (1991), who eliminated the influence of plasmin in Cheddar cheese by use of the inhibitor, 6-aminohexanoic acid (AHA), found differences in electrophoretograms of the cheeses and water-soluble extracts therefrom. It was found that bands corresponding to the γ-caseins (produced from β-casein by plasmin) were less intense in cheese containing AHA. These authors also found that AHA decreased the production of water-soluble N, again suggesting a rôle for plasmin in the initial hydrolysis of caseins.

The presence of a second indigenous proteinase in bovine milk, with an acid pH optimum, has been recognized (Kaminogawa and Yamauchi, 1972, 1978; Kaminogawa *et al.*, 1980). These authors suggested that this acid proteinase might be the lysosomal acid proteinase, cathepsin D, and recently its zymogen, procathepsin D, has been identified in bovine milk (Petersen and Nielsen, personal

communication). This enzyme does not survive pasteurization (Kaminogawa and Yamauchi, 1972), although it may play a rôle in proteolysis in raw milk cheeses. The action of cathepsin D on the caseins is very similar to that of chymosin (Kaminogawa *et al.*, 1980) and it has been suggested that the presence of a band with an electrophoretic mobility corresponding to the peptide α_{s1}-CN f24-199, i.e. the primary product of chymosin action on α_{s1}-casein, in aseptic Meshanger-type cheese is due to the action of cathepsin D (Noomen, 1978).

The influence of adjunct starters, e.g *Penicillium roqueforti*, on proteolysis can be great in varieties in which they are used. Desmazeaud *et al.* (1976) found that *P. roqueforti* degraded α_{s1}- and β-caseins simultaneously and released large amounts of small peptides and free amino acids.

Despite the finding that non-starter lactic acid bacteria (NSLAB) can dominate the microflora of Cheddar-type cheeses during much of its ripening period (see Peterson and Marshall, 1990), their influence on proteolysis in cheese has been neglected by most authors. Visser (1977 a, b, c) used an aseptic control cheese to eliminate the influence of the NSLAB, as did Desmazeaud *et al.* (1976) and O'Keeffe *et al.* (1976, 1978). A wide range of proteolytic enzymes have been identified in NSLAB (see review by Peterson and Marshall, 1990), and thus it is likely that they play a rôle in proteolysis in cheese.

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

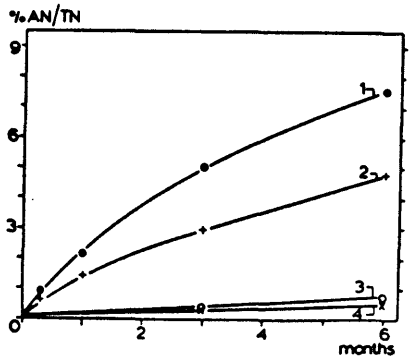

Fig. 2.2 . Average values of amino acid N developed during the ripening of normal aseptic cheeses (1), aseptic rennet-free cheeses (2), aseptic starter-free cheeses (3) and aseptic rennet and starter-free cheeses (4) (from Visser, 1977c).

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

Direct evidence for the above model of proteolysis in cheese is now becoming available. Kaminogawa *et al.* (1986) identified a number of low molecular weight peptides in Gouda cheese as products of the action of cell wall-associated proteinases of *Lactococcus* on the peptide α_{s1}-CN f 1-23, produced by the action of chymosin. Singh (personal communication) found peptides α_{s1}-CN f1-9, f1-13 and f11-? originating from the chymosin-produced α_{s1}-CN f1-23 in Cheddar cheese and also found peptides originating at the chymosin cleavage site Leu_{101}-Lys_{102} in α_{s1}-casein.

2.2.2. Lipases

Lipases in cheese originate from the same sources as proteinases, *viz.*, milk, rennet preparation (paste), starter, adjunct starter or non-starter bacteria. The degree of lipoysis in cheese varies widely between varieties, from 6.14 meq free fatty acid / 100 g fat in Gouda to 45.34 meq / 100 g fat in Danish Blue (Gripon, 1987). Lipolysis in internal bacterially-ripened varieties, such as Gouda, Cheddar and Swiss, is limited, but in mould-ripened and certain Italian varieties lipolysis is extensive. In general, in varieties in which lipolysis is extensive, lipases originate from the coagulant (Italian varieties, rennet paste), or from the adjunct starter (mould-ripened varieties).

Milk contains an indigenous lipoprotein lipase (Olivecrona and Bengtsson-Olivecrona, 1991; Olivecrona *et al.*, 1992) as well as a number of esterases (Deeth and Fitz-Gerald, 1983). The indigenous lipolytic activity of milk is inactivated by heating $\geq$ 78°C x 10s (Driessen, 1989) and is therefore of less consequence in cheeses made from pasteruized milk but may play a significant rôle in cheeses made from raw milk.

Lipases originating from the coagulant are limited to certain Italian varieties (e.g. Romano and Provalone) for which the rennet preparation used as coagulant contains a lipase, pregastric esterase, which dominates lipolysis in these cheeses (Section 2.5.1.2).

The essential lipolytic agents in mould-ripened cheeses originates *Penicillium camemberti* or *P. roquforti* and *Geotricium candidum*, which produce a range of lipases (Gripon, 1987).

The presence of weak lipolytic activity in *Lactococcus* has been recognized for many years. In the absense of a strongly lipolytic surface flora, e.g. Cheddar or Dutch varieties, the low level of lipolysis can be attributed to this source. *Propionibacterium shermanii* also contains lipase(s) (Paulsen *et al.*, 1980) and contributes to lipolysis in Swiss varieties.

Thus, the degree of lipolysis in cheese is highly dependent on individual factors unique to each variety, but there is a low background of lipolysis in all cheeses due to the starter. In the varieties in which extensive lipolysis is desired, the lipolytic agents of significance originate in either the coagulant or the adjunct starter.

2.2.3. Glycolytic Enzymes

Glycolysis is the third primary biochemical event in cheese during ripening. The low level of indigenous glycolytic activity in the milk or coagulant and the fact that glycolysis is an intracellular event in living cells mean that the glycolytic agents in cheese are of microbial origin. The primary glycolytic event, the conversion of lactose to lactate, is normally mediated by the starter during cheese manufacture. In cases where the glycolysis has not been completed by the starter, non-starter

bacteria may play an important rôle in such changes in cheese, in particular racemization of lactate (Fox *et al.*, 1990).

3. RIPENING AGENTS

3.1. Glycolytic Agents

The metabolism of lactose, lactate and citrate during cheese ripening is significant to the flavour and texture of cheese; the subject has been discussed by Fox *et al.* (1990, 1993).

During the manufacture of cheese, lactose is converted to L-lactate by mesophilic starters. The rate and extent of acidification is of significance due to differences in the extent of casein demineralization and to the retention of coagulant activity. In Cheddar-type cheeses, most of the lactate is produced in the cheese vats prior to salting, whereas in most other varieties acidification occurs primarily after the curds have been moulded. Cheese pH decreases to *ca.* pH 5 within 12 h of the start of cheesemaking (Fig. 2.3).

Cheddar cheese curd contains *ca.* 1% residual lactose which is normally metabolized quickly at a rate determined by the NaCl in moisture content. Lactose metabolism in Swiss-type varieties is complex. *Streptococcus salivarius* ssp. *thermophilus* utilizes only the glucose moiety of lactose; the galactose is excreted and metabolized by Gal$^+$ *Lactobacillus helveticus* to a mixture of D and L-lactate.

Although, lactococci produce only L-lactate, Cheddar cheese contains considerable amounts of the D-isomer. The production of D-lactate could be as a result of the metabolism of residual lactose by lactobacilli or the racemization of L-lactate, *via* pyruvate. Lactate racemization may have adverse nutritional consequences and impact on cheese quality due to the lower solubility of Ca-D-

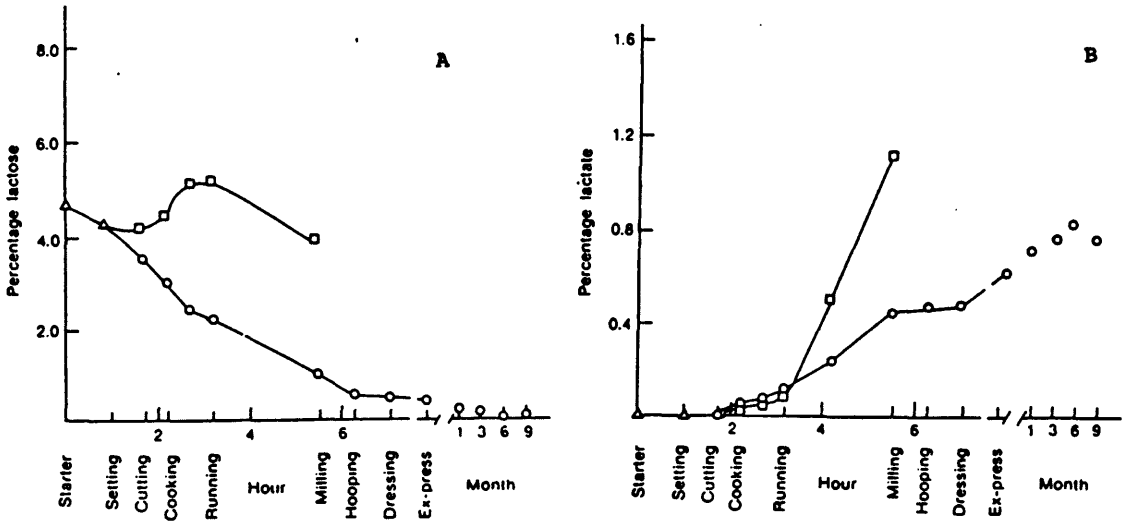

Fig. 2.3. Changes in the concentration of lactose (A) and lactate (B) in milk (Δ) and curd (O) during the manufature and ripening of Cheddar cheese (from Huffman and Kristoffersen, 1984)

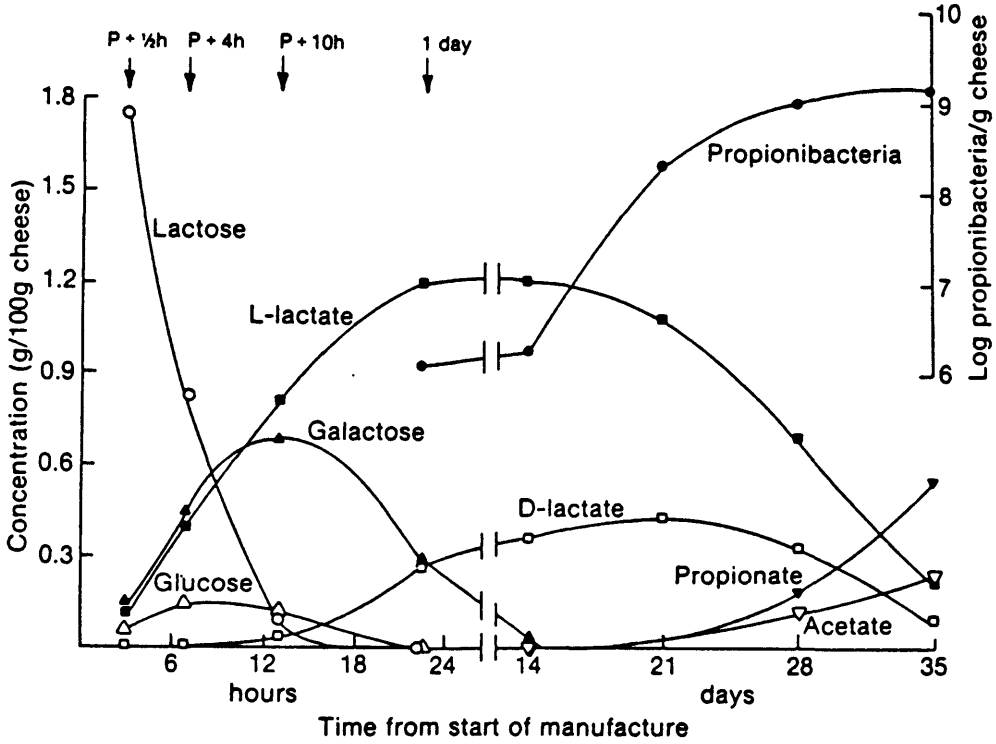

Fig. 2.4 *Lactose and lactate metabolism in Swiss-type cheese manufactured with an inoculum of 0.005%* Lactobacillus helveticus *strain 5001. Lactose (○), glucose (△), galactose (▲), D-lactate (□), L-lactate (■), acetate (▽), propionate (▼) and propionibacterium count (●). Arrowed positions P+½h, P+ 4h and P+10h indicate sampling times 0.5, 4 and 10h after the commencement of pumping (from Turner et al., 1983).*

lacate which may crystallize if its solubility is exceeded. Lactate can also be metabolized to acetate and CO_2 by *Pediococcus*.

Oxidation of lactate to acetate by non-starter lactic acid bacteria (NSLAB) depends on cell numbers and the availability of O_2, which in turn is influenced by the size of the block and the O_2 permeability of the wrapping material.

Metabolism of lactate is very extensive in surface mould-ripened varieties (e.g. Camembert, Brie) and lactate is rapidly metabolized to CO_2 and H_2O by the surface flora, causing an increase in pH.

In Swiss-type cheeses lactate is metabolized to propionate, acetate and CO_2, the latter being responsible for eye formation; carbohydrate metabolism in Swiss-type cheese is summarized in Fig. 2.4.

Milk contains low concentrations of citrate (*ca.* 8mM), which is, nevertheless, of considerable importance in many cheeses. Citrate is not metabolized by *Lactococcus lactis* spp. *lactis* or *L. lactis* ssp. *cremoris* but is metabolized by *L. lactis* ssp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. and by a number of *Lactobacillus* spp. by a pathway outlined in Fig. 2.5. Citrate metabolism is responsible for eye formation in Dutch-type cheeses and for undesirable defects in Cheddar and Cottage cheeses. Citrate concentrations in Cheddar decrease to almost zero during ripening, presumably due to metabolism by Cit^+ NSLAB, which can dominate the microflora of such cheeses. Although the pathway shown in Fig. 2.5 is used by the majority of *Leuconostoc* spp., some strains appear to be able to reduce pyruvate produced from citrate to lactate and do not produce diacetyl or acetoin.

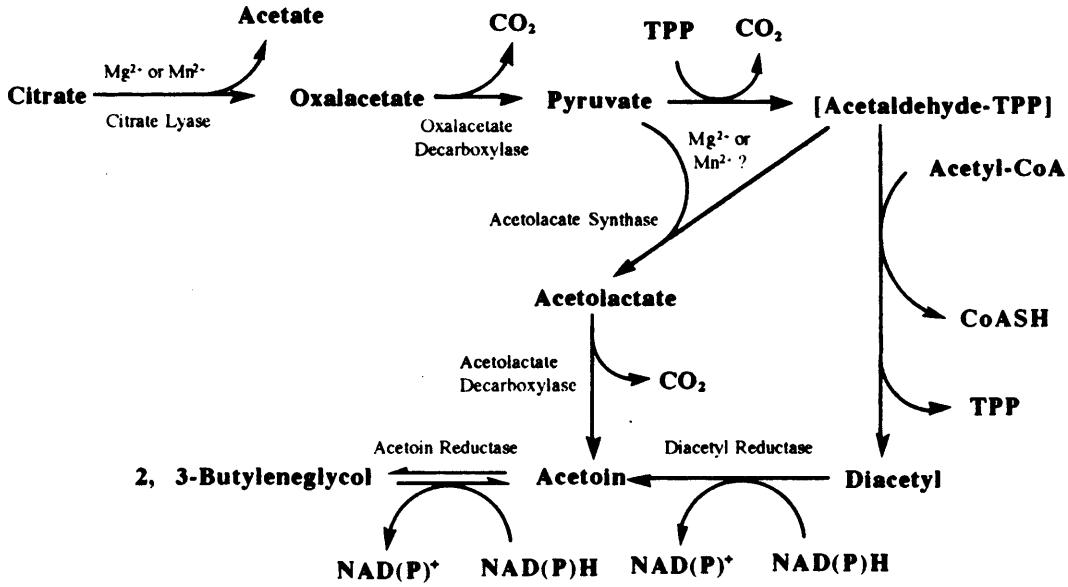

Fig. 2.5 *Pathway for the metabolism of citrate by* Lactococcus lactis *ssp.* lactis *biovar* diacetylactis *and* Leuconostoc *spp.*

3.2. Proteolytic Agents

The relative importance of the various proteolyic agents was discussed in Section 2.2.1. Proteolysis is the primary biochemical event during the ripening of most cheese varieties. The identification of individual peptides, without an understanding of the action of the various proteolytic agents, will not permit identification of the agent(s) responsible for the production these peptides. Thus, considerable effort has been expended on the study of the action of proteinases and peptidases of significance in cheese ripening.

3.2.1. Proteinases from the Coagulant

Chymosin (E.C. 3.4.23.4) is the principal proteinase in traditional rennets used for cheesemaking (Rothe *et al.*, 1977). Chymosin is an aspartyl proteinase of gastric origin, secreted by the young of a number of species of mammals. The principal rôle of chymosin in cheesemaking is to coagulate the milk. However, about 6% of the chymosin added to cheese milk is retained in the curd for Cheddar and plays a major rôle in the initial proteolysis of caseins in many cheese varieties (Fox, 1989).

The action of chymosin on the B-chain of insulin indicates that the enzyme is specific for hydrophobic and aromatic amino acid residues (Fish, 1957). Relative to many other proteinases, chymosin is weakly proteolytic; indeed, limited proteolysis is one of the characteristics to be considered in the selection of proteinases for used as rennet subtitutes (Fox, 1989).

The primary chymosin cleavage site in the milk protein system is the Phe_{105}-Met_{106} bond in κ-casein. This bond is many times more susceptible to chymosin action than any other bond in milk proteins (Vreeman *et al.*, 1986) and its hydrolysis leads to coagulation of the milk. Cleavage of κ-casein Phe_{105}-Met_{106} yields para-κ-casein (κ-CN f1-105) and glycomacropeptides (GMP; κ-CN f106-169). Most of the GMP is lost in the whey but the para-κ-casein remains attached to the casein micelles and is incorporated into the cheese. It migrates in the opposite direction to the other caseins in the alkaline urea gels widely used to study the initial proteolysis of caseins during ripening and thus is often ignored in studies on cheese ripening. Despite the presence of a number of potential chymosin cleavage sites in

its sequence, Green and Foster (1974) found that para-κ-casein is resistant to chymosin action.

Chymosin is also active on α_{s1}-, α_{s2}- and β-caseins. The parameter, k_{cat}/K_m, a measure of the specificity of an enzyme for a substrate, has been determined for the action of chymosin on the most susceptible bonds in κ-, β- and α_{s1}-caseins (Phe_{105}-Met_{106}, Leu_{192}-Tyr_{193} and Phe_{23}-Phe_{24}, respectively). The parameter k_{cat}/K_m of chymosin on the primary cleavage sites of κ-, β- and α_{s1}-caseins have been estimated as 1405, 20.6 and 1.8 s^{-1} mM^{-1}, respectively (Carles and Ribadeau-Dumas, 1984, 1985), which suggest that the next most susceptible bond to chymosin action in the caseins is Leu_{192}-Tyr_{193} of β-casein.

A number of authors have investigated the action of chymosin on β-casein (Pelissier *et al.*, 1974; Creamer, 1976a; Visser and Slangen, 1977; Carles and Ribadeau-Dumas, 1984). In solution in 0.05 M Na acetate buffer, pH 5.4, chymosin cleaves β-casein at 7 sites (Fig. 2.6): Leu_{192}-Tyr_{193} > Ala_{189}-Phe_{190} > Leu_{165}-Ser_{166} ≥ Gln_{167}-Ser_{168} ≥ Leu_{163}-Ser_{164} > Leu_{139}-Leu_{140} ≥ Leu_{127}-Thr_{128} (Visser and Slangen, 1977). Creamer (1976a) established the order of production of large polyeptides from β-casein by chymosin, as did Carles and Ribadeau-Dumas (1984) who determined the Michaelis parameters, K_m and k_{cat}, for the action of chymosin on the bond Leu_{192}-Tyr_{193} to be 0.075 mM and 1.54 s^{-1}, respectively, for micellar β-casein and 0.007 mM and 0.56 s^{-1} for the monomeric protein.

NaCl inhibits the hydrolysis of β-casein by chymosin to an extent dependent on pH (Mulvihill and Fox, 1978).

The primary site of chymosin action on α_{s1}-casein, ie. Phe_{23}-Phe_{24}, is well established (Hill *et al.*, 1974; Carles and Ribadeau-Dumas, 1985). Cleavage at this site has significance in producing a small peptide (α_{s1}-CN f1-23) which is further hydrolyzed by starter proteinases and in softening the cheese. The specificity of chymosin on α_{s1}-casein in solution (Fig. 2.7) was studied by Pellissier *et al.* (1974), Mulvihill and Fox (1979), Pahkala *et al.* (1989) and McSweeney *et al.* (1992).

In 0.1 M phosphate buffer, pH 6.5, chymosin cleaves α_{s1}-casein at the sites Phe_{23}-Phe_{24}, Phe_{28}-Pro_{29}, Leu_{40}-Ser_{41}(?), Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160} and Trp_{164}-Tyr_{165} (McSweeney *et al.*, 1992).

Carles and Ribadeau-Dumas (1985) found the k_{cat} and K_m for chymosin action on the Phe_{23}-Phe_{24} bond of α_{s1}-casein to be 0.7 s^{-1} and 0.37 mM, respectively. The influence of pH and urea on the hydrolysis of α_{s1}-casein by chymosin were studied by Mulvihill and Fox (1977) who found that pH affected the pattern of proteolysis. Ionic conditions also affected proteolysis (Mulvihill and Fox, 1979). Dunn *et al.* (1987) reported that the hydrolysis of synthetic octapeptide substrates of the type Lys-Pro-Xxx-Yyy-Phe-(NO_2)Phe-Arg-Leu by chymosin is pH-dependent.

α_{s2}-Casein appears to be relatively resistant to proteolysis by chymosin but the specificities of chymosin, and indeed of other proteinases, on this protein has received little attention.

Calf rennet contains about 10% bovine pepsin (E.C. 3.4.23.1, Rothe *et al.*, 1977). The proteolytic products produced from Na caseinate by bovine pepsin are similar to those produced by chymosin (Fox, 1969), although as far as we are aware, the specificity of bovine or porcine pepsins on bovine caseins has not been rigorously determined.

For several years, the supply of calf rennet has been insufficient to meet demand and much effort has been expended on searching for suitable rennet substitutes for cheesemaking (see Green, 1977; Phelan, 1986). A number of enzymes have been studied, including bovine (Fox and Walley, 1971), porcine (Fox, 1969) and ovine (O'Leary and Fox, 1973) pepsins. Microbial enzymes studied include proteinases from *Cryphonectria parasitica*, *Rhizomucor pusillus*, *R. miehei*, *Penicillium janthinellum*, *Rhizopus chinensis* and *Aspergillus usameii*. The specificities of many of these enzymes on the oxidized B-chain of insulin were

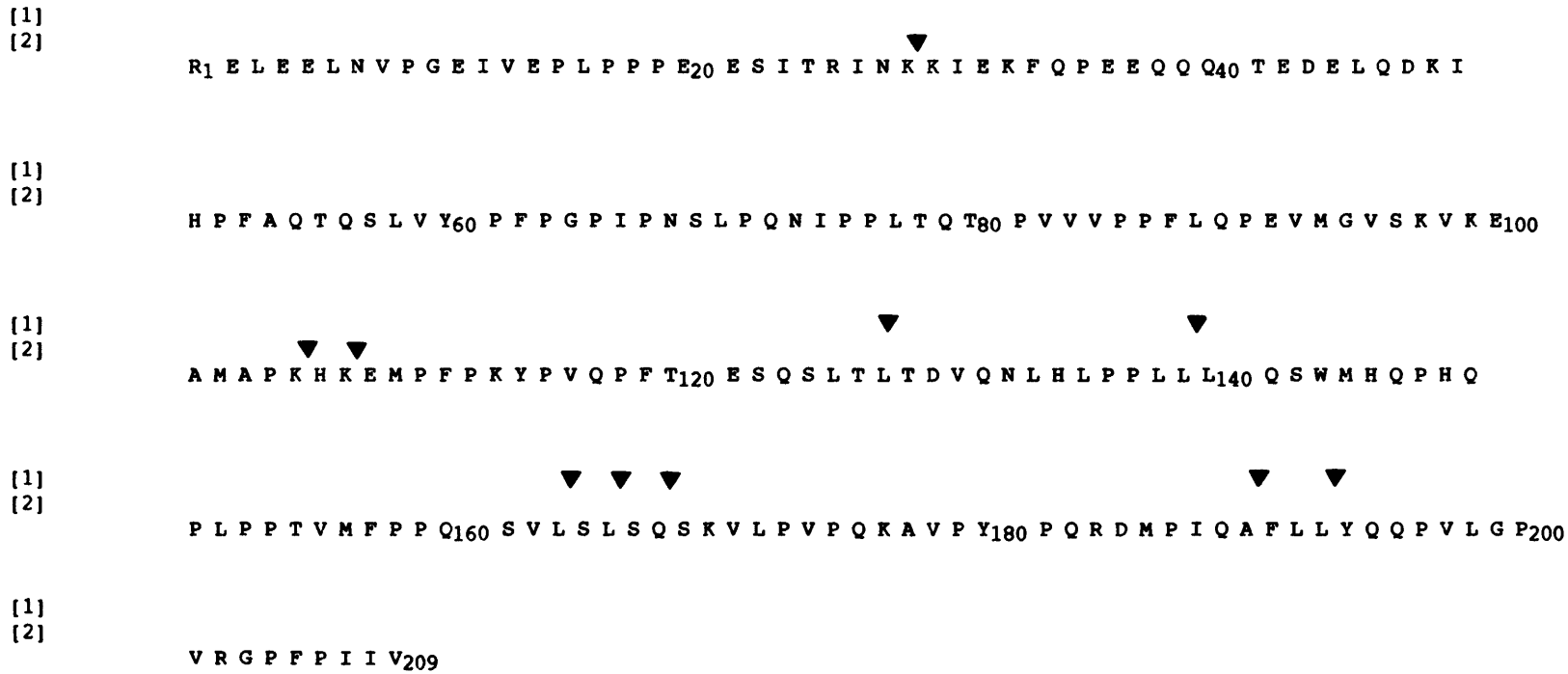

Fig. 2.6: *Amino acid sequence of* Bos *β-casein showing the position of the cleavage sites of chymosin [1] Visser and Slangen (1977) and plasmin [2] Eigel (1981, 1984).*

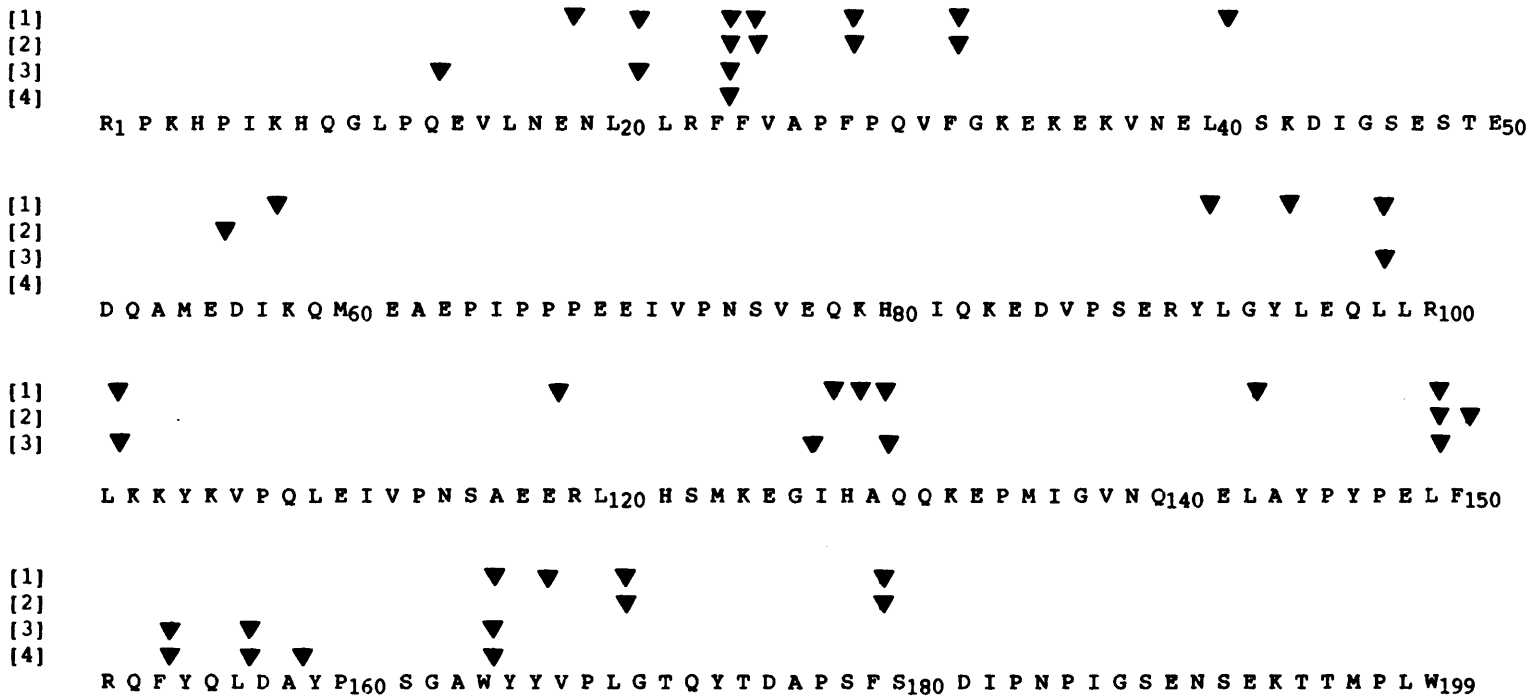

Fig. 2.7: *Amino acid sequence of* Bos α_{s1}*-casein showing the position of the cleavage sites of chymosin identified by [1]Pelissier* et al. *(1974); [2] Mulvihill and Fox (1979); [3] Pahkala* et al. *(1989); [4] McSweeney* et al. *(1992).*

summarized by Green (1977). However, the introduction of recombinant calf chymosins has limited the use of these enzymes.

Recombinant calf chymosins, expressed in *Aspergillus niger* var. *awamori*, *Kluveromyces marxianus* var. *lactis* and *Escherischia coli*, were introduced recently and since their acceptance by regulatory authorities for use in foods, they have been used widely for cheesemaking. Recombinant chymosin expressed in *K. marxianus* var. *lactis* is immunologically indistinguishable from purified calf chymosin (van den Berg and de Koning, 1990). Cheesemaking trials, involving a number of cheese varieties, have shown only small differences between cheese made with calf rennet or recombinant chymosins (Green *et al.*, 1985; Hicks *et al.*, 1988; Bines *et al.*, 1989; van den Berg and de Koning, 1990; O'Sullivan and Fox, 1991; Nuñez *et al.*, 1992). Recombinant chymosins contain only one genetic variant of this enzyme (Harboe, 1992), while calf rennet can contain three chymosin variants (A, B and C; Teuber, 1990) as well as bovine pepsin.

3.2.2. Indigenous Milk Proteinases

The presence of indigenous proteinases in milk and their possible rôle in cheese ripening has been recognized for nearly a century (Babcock and Russell, 1897). Two main indigenous proteinases are now recognized, the principal of which, plasmin, has an alkaline pH optimum while the less active, cathepsin D, has an acid pH optimum.

3.2.2.1. Plasmin: Plasmin (fibrinolysin, E.C. 3.4.21.7) has been the subject of much study (for review see Grufferty and Fox, 1988). The physiological rôle of plasmin is solubilization of fibrin clots, and to achieve this there exists a complex fibrinolytic system consisting of the active enzyme, its zymogen, activators and inhibitors of the enzyme and its activators, all of which are present in milk. Plasmin, plasminogen and plasminogen activators are associated with the casein micelles in milk, while the inhibitors are in the serum phase. (see Grufferty and Fox, 1988; Fox and Law, 1991).

Plasmin is a trypsin-like proteinase with a pH optimum at about 7.5 and a high specificity for peptide bonds involving lysyl residues (Weinstein and Doolittle, 1972). Plasmin is active on all caseins, but its preferred substrates are α_{s2}- and β-caseins (Grufferty and Fox, 1988).

The action of plasmin on β-casein is of particular interest as it results in the formation of the γ-caseins (Gordon *et al.*, 1972; Eigel, 1977a); complementary peptides constitute part of the proteose peptone fraction of milk (Eigel, 1981; Andrews and Alichanidis, 1983). Plasmin cleaves β-casein in solution at 3 primary sites (Fig. 2.6): Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}, with the formation of the polypeptides, β-CN f29-209 (γ_1-CN), f106-209 (γ_2-CN) and f108-209 (γ_3-CN) Gordon *et al.*, 1972; Eigel *et al.*, 1984); proteose peptone 5 (β-CN f1-105 and f1-107), proteose peptone 8-slow (β-CN f29-105 and f29-107) and proteose peptone 8-fast (β-CN f1-28). (Eigel *et al.*, 1984).

Plasmin cleaves α_{s2}-casein in solution at 8 sites: Lys_{21}-Gln_{22}, Lys_{24}-Asn_{25}, Arg_{114}-Asn_{115}, Lys_{149}-Lys_{150}, Lys_{150}-Thr_{151}, Lys_{181}-Thr_{182}, Lys_{188}-Ala_{189} and Lys_{197}-Thr_{198} (Visser *et al.*, 1989; Le Bars and Gripon, 1989), producing about 14 peptides, three of which are potentially bitter (Le Bars and Gripon, 1989).

Although plasmin is less active on α_{s1}-casein than on α_{s2}- and β-caseins (see Grufferty and Fox, 1988), the formation of λ-casein, a minor casein component, has been attributed to its action on α_{s1}-casein (Aimutis and Eigel, 1982). The action of plasmin on α_{s1}-casein was also studied by Eigel (1976).

Plasmin has very low activity on κ-casein; Eigel (1977b) found no hydrolysis of κ-casein A under conditions adequate for the hydrolysis of α_{s1}-casein. However, Andrews and Alichanidis (1983) reported hydrolysis of κ-casein by plasmin and found that 4% of the peptides produced by indigenous plasmin in pasteurized milk stored at 37°C for 7 days and detecable by PAGE originated from κ-casein. There are a number of potential plasmin cleavage sites in κ-casein, but the specificity of plasmin on this protein does not appear to have been determined.

3.2.2.2. Other Indigenous Milk Proteinses: The second indigenous proteinase in milk has received little attention. The presence of an indigenous milk proteinase with an acid pH optimum was recognized by Kaminogawa and Yamauchi (1972), who isolated and characterized the enzyme and considered it to be similar to the lysosomal acid proteinase, cathepsin D (E.C. 3.4.23.5). The presence of its zymogen, procathepsin D, in milk has been confirmed recently (Petersen and Nielsen, personal communication). Cathepsin D is relatively heat-labile (completely inactivated by 70°C x 10 min) and has a pH optimum of 4.0 (Kaminogawa and Yamauchi, 1972).

The specificity of cathepsin D on the caseins has not been determined, although electrophoretograms of caseins incubated with milk acid proteinase indicate a specificity very similar to that of chymosin (Kaminogawa and Yamauchi, 1972).

The presence of other minor proteolytic enzymes in milk has been reported, including thrombin and a lysine aminopeptidase (Reimerdes, 1983) and proteinases from leucocytes (Grieve and Kitchen, 1985; Verdi and Barbano, 1991), but they are considered not to be very significant (Grieve and Kitchen, 1985; Grufferty and Fox, 1988).

3.2.3. Proteolytic Enzymes from Starter

3.2.3.1. Proteinases from Lactococcus: The starter is major source of proteinases and peptidases in cheese. The genus most widely used as a cheese starter is *Lactococcus* and its proteolytic system has been studied most thoroughly.

Cell wall-associated proteinases of *Lactococcus* can be classified into 3 groups, P_I- and P_{III}-types and mixed-type strains. P_I-type proteinases degrade β- but not α-casein at a significant rate, while P_{III}-type proteinases rapidly degrade both α- and β-caseins (Visser *et al.*, 1986). The P_{II}- designation is not used; proteinases which had previously been classified as P_{II}- are now designated as P_I-type. The nucleotide sequences of the genes for both P_I- and P_{III}-type proteinases are now known (Kok *et al.*, 1988; Vos *et al.*, 1989a, b); few differences are apparent and alteration of a few residues by site-directed mutagenesis can alter the specificity of the proteinase (Kok, 1990).

Monnet *et al.* (1986) studied the specificity of a cell wall-associated proteinase from *L. lactis* subsp. *lactis* NCDO 763 on β-casein. Cleavage sites identified were in the C-terminal region of the molecule (Fig. 2.8). Of the 5 peptides identified (β-CN f176-182, 183-193, 194-207, 194-209 and 167-175), 4 are considered to be potentially bitter. The majority of cleavage sites identified by Monnet *et al.* (1986) had a hydrophobic residue at position P_3 to the labile bond, although the authors commented that it was difficult to assess the specificity of the proteinase due to limited data.

Visser *et al.* (1988) reported that the specificity of the cell wall proteinase of *L. lactis* ssp. *cremoris* HP (P_I-type) on β-casein was the same as those found for *L. lactis* ssp. *lactis* NCDO 763 by Monnet *et al.* (1986) but they also identified sites at Leu_{163}-Ser_{164}, Leu_{165}-Ser_{166} and Gln_{167}-Ser_{168} (Fig. 2.8) Visser *et al.* (1988) commented that the majority of scissile bonds contained Gln and noted that 3 of the bonds were also susceptible to chymosin action.

The specificities of cell wall-associated proteinases of *L. lactis* ssp. *cremoris* AC1 and L. lactis ssp. *lactis* NCDO 763 on β-casein were determined by Monnet *et al.* (1989). Both enzymes had 9 cleavage sites in common (Fig. 2.8). In addition, the proteinase of strain NCDO 763 cleaved at 6 additional sites, while *L. lactis* ssp. *cremoris* AC1 cleaved at 5 other sites (Fig. 2.8). The authors commented the the proteinases appeared to have no clear specificity although *ca.* 25% of the cleavage sites were of the type Gln-X, where X was a basic residue or serine. Pro was often observed in the P_2, P_4 or P'_4 positions. The authors also suggested that substrate binding may involve hydrophobic interactions at positions P_2 to P_4 and P'_2 to P'_4.

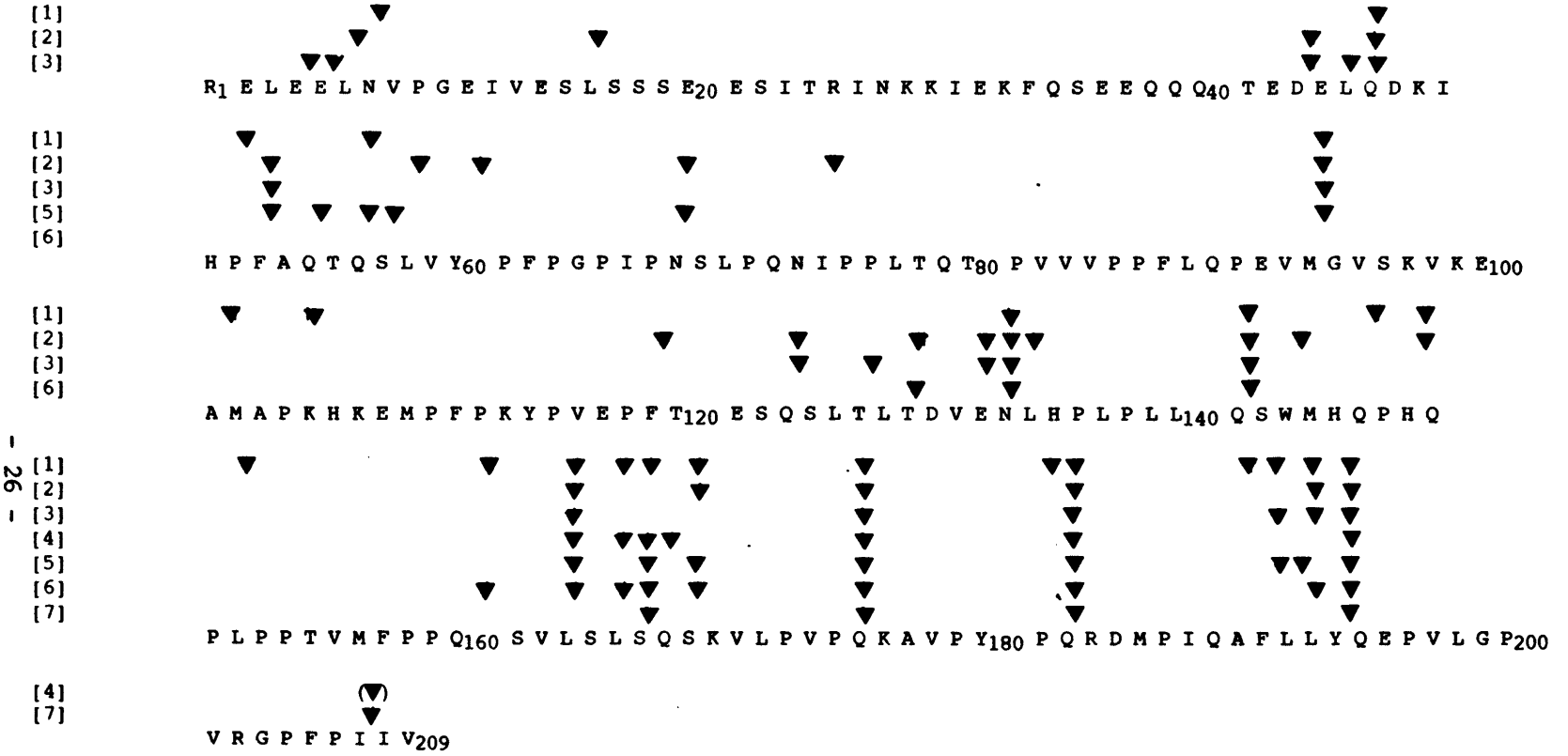

Fig. 2.8: *Amino acid sequence of* Bos *β-casein showing the position of the cleavage sites of cell wall-associated proteinases of* Lactococcus. *[1] P_I-type proteinase from* L. lactis *ssp.* cremoris *H2(Reid* et al., *1991b); [2] P_{III} type proteinase from* L. lactis *spp.* cremoris *SK112 (Reid* et al., *1991b); [3] P_{III}-type proteinase from* L. lactis *ssp.* cremoris *AM1 (Visser* et al., *1991); [4] proteinase from* L. lactis *ssp.* cremoris *HP (Visser* et al., *1988); [5] proteinase from* L. lactis *ssp.* cremoris *AC1 (Monnet* et al., *1989); [6] proteinase from* L. lactis *ssp.* lactis *NCDO 763 (Monnet* et al., *1989); [7] proteinase from* L. lactis *ssp.* lactis *NCDO 763 (Monnet* et al., *1986).*

The specificity of the cell wall-associated proteinase from *L. lactis* subsp. *cremoris* AM1 (P_{III}-type) on β-casein was investigated by Visser *et al.* (1991). In a majority of the cleavage sites (Fig. 2.8), a Glx residue was present (9 out of 16 sites) or the bond contained at least one large hydrophobic residue (10 cases). In 13 out of the 16 sites there was a hydrophobic residue at position P_2 or P'_2 and in all cases there was at least one side chain potentially involved in hydrogen bonding at the P_2-P_3 and/or P'_2-P'_3 positons. Thus, these authors suggest that hydrophobic, electrostatic or hydrogen bonding played a rôle in enzyme-substrate interaction.

The specificities of P_I and P_{III}-type proteinases on β-casein from *L. lactis* subsp. *cremoris* H2 (P_I) and SK112 (P_{III}) were studied by Reid *et al.* (1991b). About half the cleavage sites identified were hydrolyzed by both types of proteinase (Fig. 2.8). In addition, the P_I-type strain (H2), cleaved at an additional 12 sites, while the P_{III}-type strain (SK112) cleaved at 13 sites. These authors speculate that the differences observed between P_I and P_{III} proteinases are more quantitative than qualitative. The authors also concluded that despite there being no particular residue or sequence which clearly defined specificity, a Gln residue was more likely to be at the P_1 position for the P_I-type than for the other and that cleavage sites for this type of proteinase lie generally in the region of β-casein with high hydrophobicity and proline content. In the case of P_{III}-type enzymes, Leu is more likely to occur at the P_1 position and Pro residues are common at the P_2 position, but are absent from the P_1 and P_3 positions. It was also observed that Glu residues were common at the P_1 position .

The specificity of a cell wall-associated proteinase from *L. lactis* subsp. *lactis* SK11 (P_{III}-type) was determined on α_{s1}-casein by Reid *et al.* (1991a). The proteinase cleaved α_{s1}-casein in solution at 19 sites (Fig. 2.9), producing 16 trifluoroacetic-acid soluble peptides (α_{s1}-CN f1-17, 17-23, 34-37, 75-84, 89-98, 122-130, 122-142, 131-142, 140-148, 143-148, 149-146, 149-157, 151-156, 157-161, 162-169 and 170-199). Despite the large number of cleavage sites identified, these authors came to no conclusions concerning the type of bond most susceptible to hydrolysis.

Exterkate (1990) reported that a cation-binding site in P_I proteinases but absent in P_{III} was mainly responsible for the difference between the specificity of the enzymes on chromophoric peptides with positive charge.

The specificity of the cell-wall associated proteinase of *L. lactis* ssp. *lactis* NCDO 763 towards caseins was studied by Monnet *et al.* (1992). The specificity of the cell wall-associated proteinase on α_{s1}-, α_{s2}- and κ-casein, the peptide α_{s1}-CN f1-23, the oxidized B-chain of insulin and on the glycomacropeptide (κ-CN f106-169) was determined (Figs. 2.10, 2.12). These authors statistically studied the susceptibility of bonds in these substrates to cleavage. The authors noted that the apparent preference of cell wall-associated proteinases for the hydrophobic region of β-casein was not confirmed for other caseins less hydrophobic than this protein. Residues at positions P_6 to P'_2 were important in proteinase-substrate binding. The most stastically important residues were Phe, Tyr and Pro at the P_4, P_3 and P_2 positions, respectively, and was negatively correlated with Pro at positions P_1 and P'_1 (Fig. 2.11). Hydrophobic residues were generally found at position P_4 and P'_2, while neutral amino acids were favoured at the P_1 position to the scissile bond. Monnet *et al.* (1992) also found that the action of the proteinase on short peptide substrates suggested that the two Cys residues in the B-chain of insulin were more susceptible to cleavage than they were by other subtilisin-like proteinases. The correlations between scissile bond and amino acid residues in its vicinity which were determined for the caseins were not observed for short peptide substrates, perhaps as such peptides offer only a poor choice of amino acid sequences. Monnet *et al.* (1992) also comment that the apparent specificity of the cell wall-associated proteinase from NCDO 763 depended om the substrate used; its action on α_{s1}-CN f1-23 appeared to be P_{III}-like, on β-casein to be P_I-like and apparently P_{III}-like on α_{s1}-casein. The authors speculated that the binding area of the enzyme was more complex than that of subtilisin.

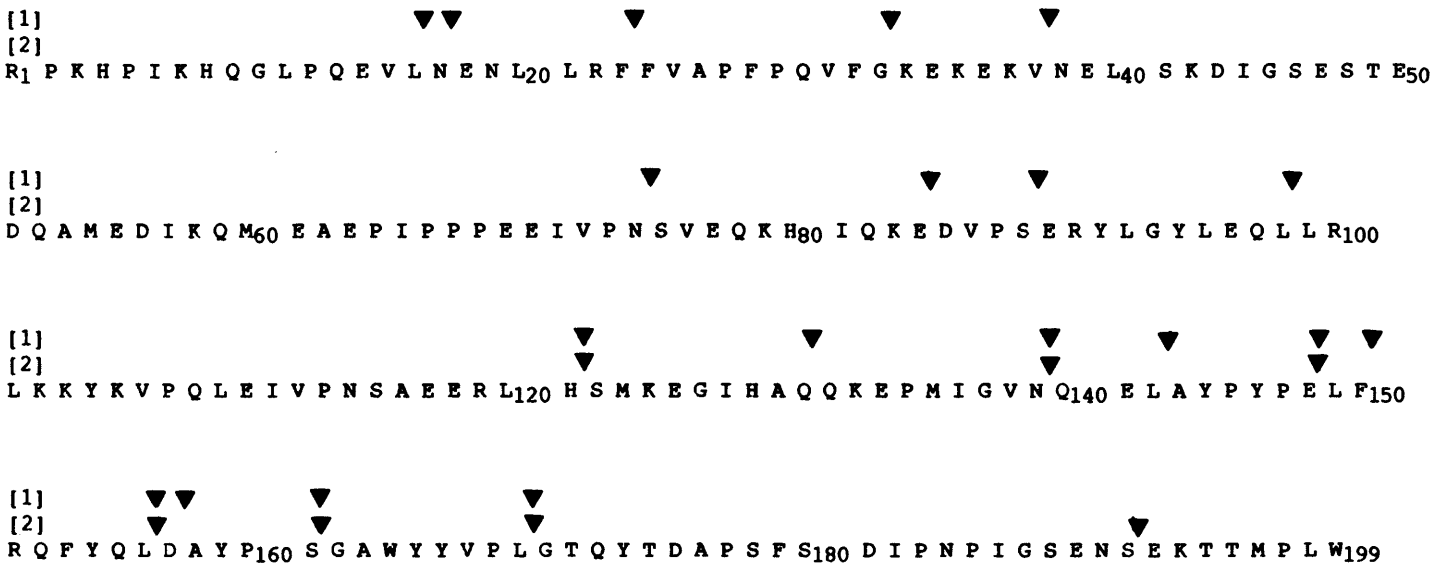

Fig. 2.9: *Amino acid sequence of* Bos α_{s1}*-casein showing the position of the cleavage sites of cell wall-associated proteinases of [1]* Lactococcus lactis *ssp.* cremoris *SK112 identified by Reid* et al. *(1991a) and [2]* L. lactis *ssp.* lactis *NCDO 763 identifed by Monnet* et al. *(1992).*

α_{s2}-Casein A

K N T M E H V S S S E E S I I S Q E T Y_{20} K Q E K N M A I N P S K E N L C S T F C_{40} K E V V R N A N E E_{50}

E Y S I G S S S E E_{60} S A E V A T E E V K I T V D D K H Y Q K_{80} A L N E I N E F Y Q K F P Q Y L Q Y L Y_{100}

Q G P I V L N P W D Q V K R N A V P I T_{120} P T L N R E Q L S T S E E N S K K T V D_{140} M E S T E V F T K K_{150}

T K L T E E E K N R_{160} L N F L K K I S Q R Y Q K F A L P Q Y L_{180} K T V Y Q H Q K A M K P W I Q P K T K V_{200}

I P Y V R Y L_{207}

κ-Casein B

E E Q N Q E Q P I R C E K D E R F F S D_{20} K I A K T I P I Q Y V L S R Y P S Y G L_{40} N Y Y Q Q K P V A L_{50}

I N N Q F L P Y P Y_{60} Y A K P A A V R S P A Q I L Q W Q V L S_{80} D T V P A K S C Q A Q P T T M A R H P H_{100}

P H L S F M A I P P K K N Q D K T E I P_{120} T I N T I A S G E P T S T P T I E A V E_{140} S T V A T L E A S P_{150}

E V I E S P P E I N_{160} T V Q V T S T A V_{169}

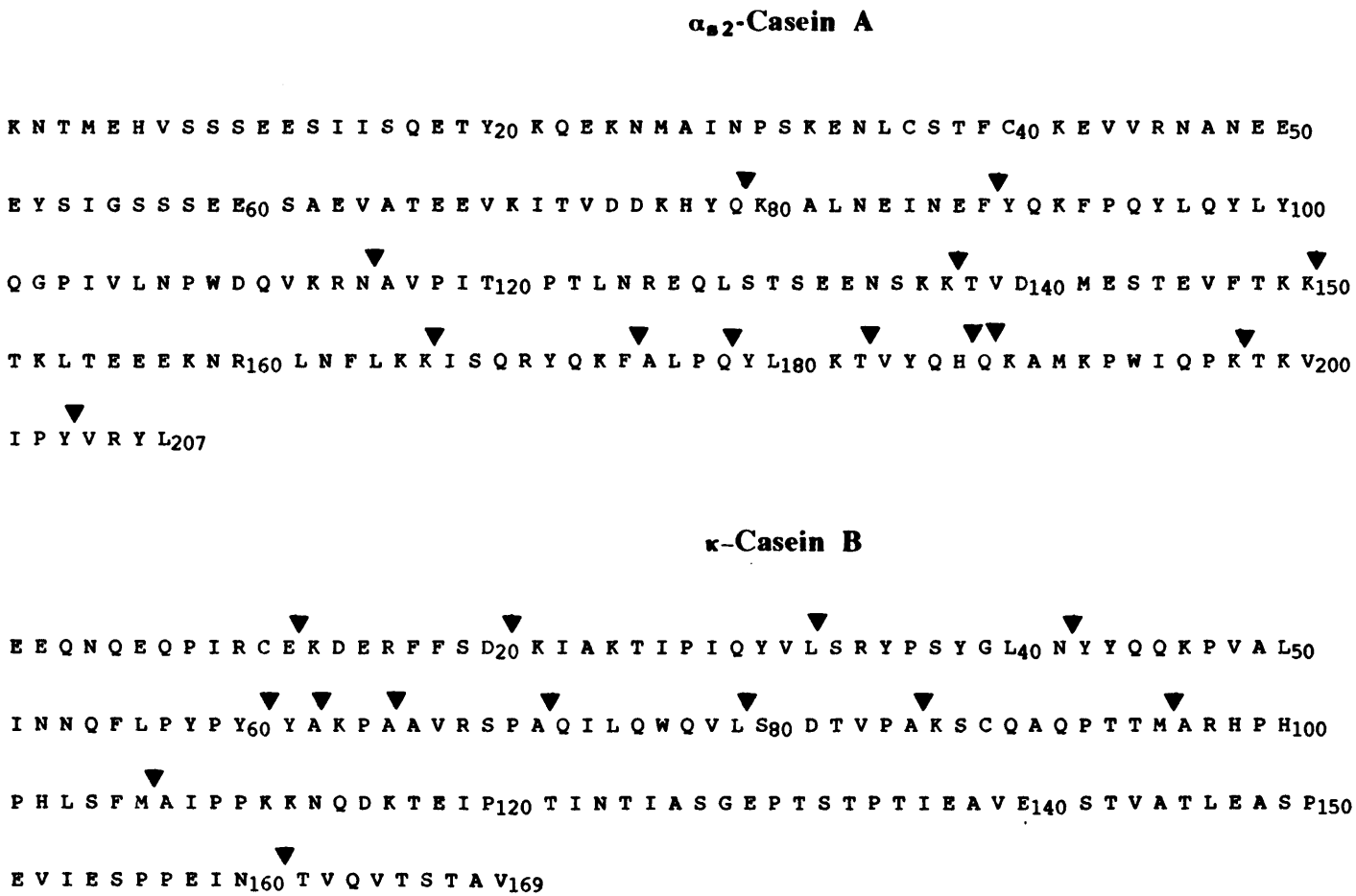

Fig. 2.10: *Amino acid sequences of* Bos *α_{s2}-casein A and κ-casein B showing the positions of the cleavage sites of cell wall-associated proteinase of* Lactococcus lactis *ssp.* lactis *NCDO 763 identified by Monnet* et al. *(1992).*

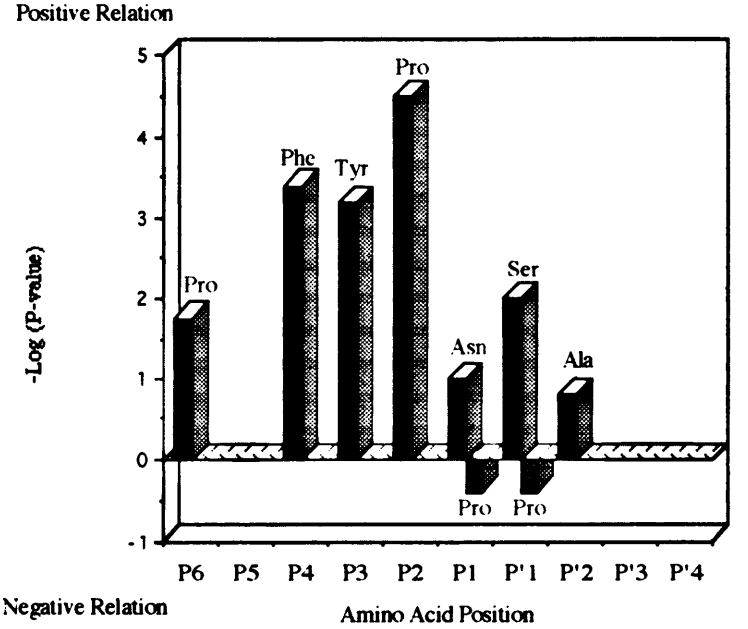

Fig. 2.11. Statistically significant relationships between amino acids and positions in the regions of bonds in caseins cleaved by the cell wall-associated proteinase of Lactococcus lactis *ssp.* lactis *NCDO 763 (Monnet* et al., *1992).*

The primary rôle of proteinases and peptidases from the starter in cheese appears to be in the degradation of large and intermediate-sized peptides produced from caseins by chymosin and plasmin. A number of authors have investigated the action of bacterial cell wall proteinases on the peptide α_{s1}-CN f1-23 (produced by chymosin). Kaminogawa *et al.* (1986) found that the cell wall- associated proteinase of *L. lactis* subsp. *cremoris* H61 produced the peptides f1-7, 1-18, 1-20 and 14-23 from the f1-23 fragment (Fig. 2.12) and identified a number of low molecular weight peptides in Gouda-type that cheese originated from this peptide through the action of starter proteinases (α_{s1}-CN f1-9, 1-13 and 1-14. Exterkate *et al.* (1991) found that a P_I proteinase from *L. lactis* subsp. *cremoris* HP cleaved f1-23 primarliy at positions His_8-Gln_9, Gln_9-Gly_{10} and Gln_{13}-Glu_{14} while a P_{III} proteinase from *L. lactis* subsp. *cremoris* AM_1 cleaved primarliy at Leu_{16}-Asn_{17}, Asn_{17}-Glu_{18} and Leu_{21}-Arg_{22}. Exterkate *et al.* (1992) reported that pH and NaCl alter the specificity of cell wall-associated proteinases on f1-23.

Zevaco and Desmazeaud (1980) described an intracellular neutral proteinase from *L. lactis* ssp. *lactis* biovar *diacetylactis*. The enzyme cleaved β-casein slowly at Pro_{186}-Ile_{187} and Ala_{189}-Phe_{190}. These authors also investigated the cleavage of a number of chymosin-derived peptides by this intracellular proteinase. The peptide β-CN f165-189 was rapidly cleaved at Lys_{169}-Val_{170} and Lys_{176}-Ala_{177} and the peptide β-CN f193-209 was also cleaved quickly, at Pro_{206}-Ile_{207}. This intracellular proteinase also cleaved bradykinin. Muset *et al.* (1989) isolated an intracellular proteinase from a cell wall proteinase-deficient variant of *L. lactis* ssp. *lactis* NCDO 763. This metalloenzyme (MW 93 kDa) was optimally active at pH 7.5 and 45°C, showed thermolysin-like specificity on the B-chain of insulin and hydrolyzed β-casein slowly.

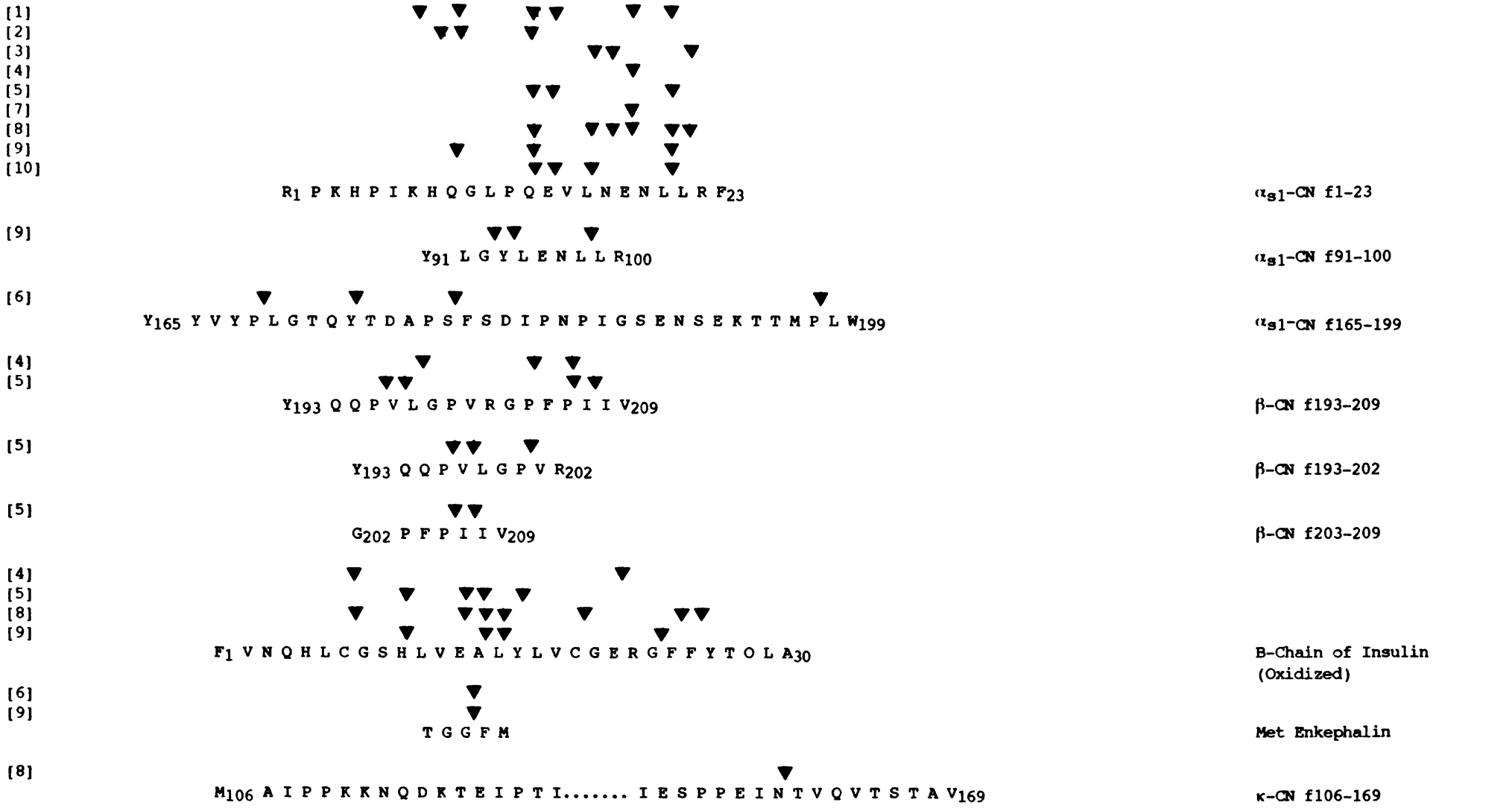

Fig. 2.12: Amino acid sequence of a number of peptides from casein hydrolysates and other sources showing the specificity of Lactococcus *proteinases and peptidases. [1] Cell wall associated proteinase of* L. lactis *ssp.* cremoris *H61 (Kunugawa* et al., *1986); [2] cell wall-associated proteinase (PI-type) of* L. lactis *ssp.* cremoris *HP (Exterkate* et al., *1991); [3] cell wall-associated proteinase (PIII-type) of* L. lactis *ssp.* cremoris *AM1 (Exterkate* et al., *1991); [4] metalloendopeptidase from* L. lactis *ssp.* cremoris *HP (Baankreis, 1992); [5] neutral oligo-endopeptidase from* L. lactis *ssp.* cremoris *C13 (Baankreis, 1992); [6] LEP III I from* L. lactis *ssp.* lactis *MG 1363 (Stepanak and Fox, 1992); [7] LEP I from* L. lactis *ssp.* cremoris *(Yan* et al., *1987b); [8] cell wall-associated proteinase of* L. lactis *ssp.* lactis *NCDO 763 (Monnet* et al., *1992); [9] LEP II from* L. lactis *ssp.* cremoris *H61 (Yan* et al., *1987a); [10] LEP III I from* L. lactis *ssp.* lactis *MG 1363 (Stepanak and Fox, unpublished)*

3.2.3.2. Peptidases from Lactococcus

Many of the peptides discussed above are too large to permit their transport into the bacterial cell (Law, 1978) and further proteolysis is necessary before the cell can utilize the amino acids. *Lactococcus* spp. possess a very wide range of peptidases, *viz.* amino-, di- and tri-peptidases and proline-specific peptidases. However, *Lactococcus* spp. do not appear to possess a carboxypeptidase. The range of peptides which might be produced from β-casein in solution by the action of the proteolytic and peptidolytic system of Lactococcus was described by Smid *et al.* (1991).

The nomenclature of lactococcal peptidases is somewhat confused and a systematic scheme has been proposed (Tan 1992). Peptidases which have not been characterized at the genetic level are indicated by their biochemical name (or abbreviation) followed by a numeral indicating the chronological order of their description (eg. LEP I, II and III). Peptidases which have been characterized at the genetic level are given the abbreviation "Pep" followed by a capital letter denoting the specific enzyme (e.g. PepN).

Lactococcal peptidases which have been isolated are summarized in Table 2.1.

Aminopeptidases: Mou *et al.* (1975) found a general aminopeptidase in *Lactococcus* while Law (1979) isolated an aminopeptidase of MW *ca.* 49 kDa with a neutral pH optimum and a temperature optimum of 37°C which was inhibited by pCMB and slightly by EDTA. Desmazeaud and Zevaco (1979) isolated 2 metalloaminopeptidases from *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* CNRZ 267. One of the peptidases (AMP I) had a wide substrate specificity and hydrolyzed tripeptides and β-naphtyl amino acids and was optimally active at pH 6.5 and 35°C. The second enzyme (TRP I) hydrolyzed only certain tripeptides and was optimally active at pH 7.0 and 35°C. Based on the aminopeptidase activity of cell-free extracts of *Lactococcus*, Kaminogawa *et al.* (1984a) classified the 11 strains studied into 3 groups. Aminopeptidases from a representative of each group was partially purified and the influence of pH, metal ions, N-ethylmalemide and 2-mercaptoethanol on activity was studied. These authors identified a range of peptidase activities, including a dipeptidase specific for hydrophobic amino acid-X, dipeptidase specific for basic amino acid residue-X, proline diaminopeptidase, prolidase (PRD), a general aminopeptidase and a tripeptidase.

Exterkate and de Veer (1987) isolated a membrane-bound aminopepetidase A (L-α-glutamyl [aspartyl] peptide hydrolase, GAP I) from *Lactococcus lactis* ssp. *cremoris* HP; it had a MW of *ca.* 130 kDa and consisted of catalytically inactive subunits (MW *ca.* 43 kDa). The enzyme was inactivated by dithiothreitol, metal chelating agents, Cu^{2+}, Hg^{2+} and pCMB.

An intracellular aminopeptidase (PepC) from *Lactococcus lactis* ssp. *cremoris* AM_2 was isolated by Neviani *et al.* (1989) who found it to be a hexamer (MW ~300 kDa) and to be optimally active at 40°C and pH 7. The enzyme, which had a broad specificity, was a not a metalloenzyme, but was inhibited by sulphydryl blocking agents.

An aminopeptidase (PepN) isolated from *Lactococcus lactis* ssp. *cremoris* Wg2 had a MW of ~95 kDa and was optimally active at 40°C and pH 7 (Tan and Konings, 1990). It was inactivated by pCMB, chelating agents, Cu^{2+} and Cd^{2+}. Niven (1991) isolated an aminopeptidase (GAP II) from *L. lactis* spp. *lactis* NCDO 712. The enzyme was hexameric (MW: 245 kDa, subunits: 41 kDa) and was a metalloenzyme with optimal activity on Glu-*p*-NA at 65°C and pH 8. Baankreis (1992) isolated an aminopeptidase (GAP III, MW ~520 kDa) from *L. lactis* ssp. *cremoris* HP.

Chapot-Chartier *et al.* (1993) cloned and sequenced the gene for the cysteine aminopeptidase, pepC, from *L. lactis* ssp. *cremoris* AM2. These authors found no singnal sequence for the gene, indicating an intracellular location of this enzyme.

Table 2.1 Peptidases of *Lactococcus lactis* (adapted from Baankreis, 1992, with the provisional nomenclature for lactococcal peptidases proposed by Tan, 1992)

Peptidase	Substrate	MW/kDa	Optimum Activity	Class	Reference
Endopeptidases					
LEP I	peptides	98	pH 7-7.5, 40°C	metalloenzyme	Yan *et al.* (1987b)
LEP II	peptides	80	pH 6, 37°C	metalloenzyme	Yan *et al.* (1987a)
LEP III	peptides	70	pH 6-6.5, 30-38°C	neutral peptidase	Tan *et al.* (1991)
MEP (LEP)	peptides	180	pH 8-9, 42°C	metalloenzyme	Baankreis (1992)
NOP (LEP)	peptides	70	-	neutral peptidase	Baankreis (1992)
LEP III-1	peptides	-	-		Stepaniak and Fox (1993)
Aminopeptidases					
AMP I	Lys-*p*-NA	85	-	metalloenzyme	Desmazeaud and Zevaco (1979)
AMP III	Lys-p-NA	35	-	metalloenzyme	Geiss *et al.* (1985)
PepN	Leu-Lys-*p*-NA	95	pH 7, 40°C	metalloenzyme	Tan and Koning (1990)
PepN	Leu-Lys-*p*-NA	-	-	metalloenzyme	Baankreis (1992)
GAP I	Glu-Asp-*p*-NA	130	pH 7, 40°C	metalloenzyme	Exterkate and de Veer (1987)
GAP II	Glu-*p*-NA	300	pH 7, 40°C	metalloenzyme	Niven (1991)
PepN (GAP III)	Glu-Asp-*p*-NA	520	pH 8, 50°C	metalloenzyme	Baankreis (1992)
PepX	X-Pro-*p*-NA	90	-	serine peptidase	Kiefer-Partsch *et al.* (1989)
PepX	X-Pro-*p*-NA	190	pH 8.5, 40-45°C	serine peptidase	Zevaco *et al.* (1990)
XAP I	X-Pro-*p*-NA	117	pH 6-9	serine peptidase	Booth *et al.* (1990b)
XAP II	X-Pro-*p*-NA	150	pH 6-9	serine peptidase	Lloyd and Pritchard (1990)
PCP	Pyr-*p*-NA	-	-	serine peptidase	Exterkate (1977)
PCP	pyr-*p*-NA	25 and 80	pH 8 8.5, 37°C	serine peptidase	Baankreis (1992)
PepC	Leu-Lys-*p*-NA	300	pH 7, 40°C	thiol enzyme	Neviani *et al.* (1989)
Di-/Tripeptidases					
DIP	dipeptides	25 and 34	pH 7, 30°C	metalloenzyme	Law (1979)
DIP I	dipeptides	50	-	metalloenzyme	Desmazeaud and Zevaco (1977)
DIP II	dipeptides	100	pH 8	metalloenzyme	Hwang *et al.* (1981)
DIP III	dipeptides	49	pH 8, 50°C	metalloenzyme	van Boven *et al.* (1988)
TRP I	tripeptides	75	-	metalloenzyme	Desmazeaud and Zevaco (1979)
TRP II	tripeptides	103-105	pH 7.5, 55°C	metalloenzyme	Bosman *et al.* (1990)
PRD	X-Pro dipeptides	43	-	metalloenzyme	Kaminogawa *et al.* (1984b)
PRD	X-Pro dipeptides	42	pH 7.35-9.0	metalloenzyme	Booth *et al.* (1990c)
PIP	Pro-X-(Y) peptides	50	pH 8.5, 37°C	metalloenzyme	Baankreis (1992)

Dipeptidases: Law (1979) found 2 dipeptidases (MW: 25 and 34 kDa) with optimum activity at pH 7 and 30°C which were inhibited by EDTA. A dipeptidase, DIP II, isolated by Hwang *et al.* (1981) had an MW of 100 kDa and a pH optimum at pH 8;. the enzyme was inhibited by EDTA and was active on a wide range of substrates.

Van Boven *et al.* (1988) isolated a dipeptidase (DIP III) with a MW of 49 kDa from *L. lactis* ssp. *cremoris* Wg2 which was active on a wide range of substrates. The enzyme was considered to be a Mn-metalloenzyme and was optimally active at pH 8 and at 50°C and was also inhibited by thiol reducing agents. The enzyme was inactive on tripeptides, tetrapeptides or on dipeptides containing Pro, His, Gly or Glu as the N-terminal residue. These authors also found a tripeptidase but did not study it.

Tripeptidases: Mou *et al.* (1975) partially purified a tripeptidase active on Leu.Gly.Gly, while Law (1979) found cell-bound di- and tripeptidases in *L. lactis* ssp. *cremoris* and ssp. *lactis*. The tripeptidase had a MW of ~38 kDa, optimum activity at 30°C and neutral pH and was inhibited by EDTA. Kolstad and Law (1985) found a tripeptidase in zymograms of cell wall fraction.

Bosman *et al.* (1990) purified a tripeptidase (TRP II) from a cell-free extract of *L. lactis* ssp. *cremoris* Wg2. The enzyme (MW 103-105 kDa) was a dimer consisting of two identical subunits (52 kDa) and was only capable of hydolyzing the N-terminal residue from tripeptides but not dipeptides or oligopeptides. The tripeptidase was optimally active at pH 7.5 and 55°C and was inactivated by EDTA and reactivated after removal of EDTA by Zn^{2+}, Mn^{2+} and Co^{2+} (partially). The enzyme was also inhibited by dithiothreitol, 2-mercaptoethanol and Cu^{2+}.

Endopeptidases: Exterkate (1975) identified 2 endopeptidases in *L. lactis* ssp. *cremoris* with temperature optima at 37°C and 50°C and which showed different activities on N-glutaryl-L-phenylalanine-4-nitroanalide. Two intracellular endoepetidases (LEP I and LEP II) were demonstrated by Yan *et al.* (1987a) in *L. lactis* ssp. *cremoris* H61. LEP II was studied in detail and found to be a dimer (MW 80kDa) of identical subunits and to be maximally active at pH 6.0 and 37°C. LEP II was unstable at acid pH and was a metalloenzyme inhibited by EDTA and phenthroline but not by inhibitors specific for acid proteinases. The enzyme could be reactivated by Zn^{2+} when the chelating agent was removed. LEP II did not degrade α_{s1}-, β- or κ-casein, α-lactalbumin, β-lactoglobulin or the polypeptides α_{s1}-CN f1-54, 61-123 or 136-196 but could hydrolyze the peptides α_{s1}-CN f1-23 and f91-100. As the hydrolysis of α_{s1}-CN f1-23 was not competitively inhibited by α_{s1}-casein, the authors considered that larger polypeptides and proteins did not associate with the active site of the enzyme.

LEP I was purified and studied by Yan *et al.* (1987b). This enzyme was found to be a monomer (98 kDa) and to be optimally active at pH 7 to 7.5 and 40°C. LEP I was unstable at acid pH and inhibited by EDTA and phenthroline and reactivated by Mn^{2+} after removal of the chelating agent. LEP I hydrolyzed α_{s1}-CN f1-23 (to f1-18 and f19-23) and f91-100 but not the polypeptides f1-54, f61-123 or α_{s1}-casein. LEP I was more specific for short peptides than was LEP II, e.g. LEP II could hydrolyse the oxidized B-chain of insulin but LEP I could not.

An endopeptidase from *Lactococcus lactis* ssp. *cremoris* Wg2 was isolated by Tan *et al.* (1991). The enzyme had a molecular mass of ~70 kDa and had no amino-, carboxy-, di-, tri- or tetrapeptidase activity and did not hydrolyse α-, β- or κ-caseins or the peptides β-CN f33-48, β-caseinomorphin, penta- or hexaalanine. Peptides with less than 5 amino acid residues were not hyrolyzed and the largest peptide hydrolyzed was the oxidized B-chain of insulin. The enzyme was maximally active at pH 6.0 to 6.5 and between 30 to 38°C and was inactivated by 1,10-phenanthroline, EDTA, 2-mercaptoethanol and phenylmethylsulphonyl fluoride (PMSF). Inactivation by metal chelating agents could be reversed Co^{2+}.

TABLE 2.2 Proline-specific exopeptidases

Enzyme	*Bond-Type Cleaved*
Aminopeptidase P	X-Pro-Y-Z...
Proline iminopeptidase	Pro-X-Y-Z...
Proline iminodipeptidase (prolinase)	Pro-X
Imidodipeptidase (prolidase)	X-Pro
X-Prolyl dipeptidylaminopeptidase (XPDA)	X-Pro-Y-Z...

Immunological techniques showed that the enzyme was present in other *Lactococcus* spp., *Lactobacillus* spp. and *Streptococcus salivarius* ssp. *thermophilus*.

Baankreis (1992) isolated a thermolysin-like neutral oligo-endopeptidase from *L. lactis* ssp. *cremoris* C13. The enzyme was monomeric (70 kDa) and could hydrolyse peptides up to 30 residues in length, including the B-chain of insulin and α_{s1}-CN f1-23 on which it exhibited thermolysin-like specificity, different from those of LEP I or II. It could not efficiently hydrolyse α_{s1}-, β- or κ-casein. The enzyme was inhibited by EDTA and reactivated after removal of EDTA by Zn^{2+}, Co^{2+} and less efficiently by Mn^{2+}.

Proline Specific Peptidases: As caseins have a relatively high Pro content and as many peptidases cannot hydrolyse at or near Pro-containing bonds, the hydrolysis of caseins during cheese ripening leads to the formation of proline-containing peptides which, unless they are to accumulate, must be metabolized further. The accumulation of free Pro in cheese points to the rôle played by proline-specific peptidases.

Five types of proline exopeptidases have been identified in the lactococci (Table 2.2). Possible mechanisms by which free Pro can be released from oligopeptides containing a single Pro residue or an internal Pro-Pro sequence are outlined in Figs. 2.13 and 2.14.

Prolyl iminopeptidase, prolyl iminodipeptidase and aminopeptidase P activities were found by Mou *et al.* (1975) in a number of strains of lactococci (*L. lactis* ssp. *cremoris* SK 11 and C2 and *L. lactis* ssp. *lactis* biovar *diacetylactis* DRC 1). Zevaco *et al.* (1990) isolated an intracellular XPDA (PepX) from *L. lactis* ssp. *lactis* NCDO 763. The enzyme was a dimer (MW: 190 kDa), consisting of identical subunits and was optimally active at pH 8.5 and 40 to 45°C. It was a serine-type enzyme, inhibited by organophosphates, and it hydrolysed β-caseinomorphin-7 (Y.P.F.P.G.P.I) to G.P, Y.P, G.P.I, F.P and F.P.G.P.I.

Booth *et al.* (1990a) studied the proline-specific enzymes in *L. lactis* ssp. *cremoris* AM2. XPDA and prolidase were found to be predominantly cytoplasmic. The prolidase, which was isolated by Booth *et al.* (1990c), had a MW 42 kDa, a pH optimum of pH 7.35 to 8.25 in citrate, phosphate or borate buffers, or between pH 8.3 to 9.0 in universal buffer. The enzyme was strongly inhibited by EDTA, 1, 10-phenanthroline and 8-hydroxyquinoline. It was also inhibited by dithiothreitol and was active on all amino acyl-proline substrates (including Pro-Pro) except Gly-Pro and Glp-Pro.

The XPDA (XAP I) from *L. lactis* ssp. *lactis* AM2 was isolated by Booth *et al.* (1990b). The enzyme had a broad pH optimum (6 to 9) and a MW of 117 kDa; it was not inhibited by EDTA but was inhibited by PMSF and could release Pro-Pro from P.P.G.F.S.P.

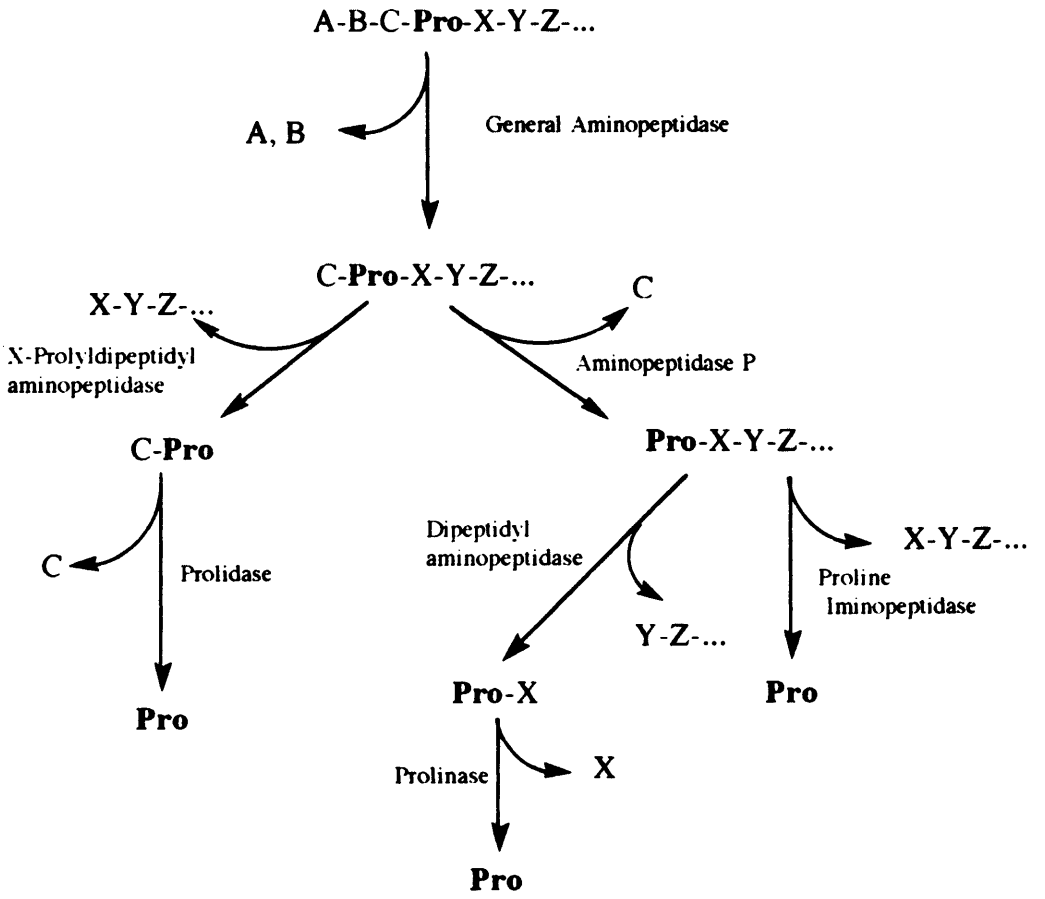

Fig. 2.13. Possible sequences of enzyme activities leading to the release of free proline from oligopeptides containing a single proline residue (from Booth et al., *1990a)*

An XPDA (XAP II) was isolated from *L. lactis* ssp. *lactis* H1 by Lloyd and Pritchard (1991). The enzyme was inhibited by PMSF but not by metal chelating agents and a molecular mass of 150 kDa and had a broad pH optimum between 6 to 9.

Baankreis (1992) isolated a peptidase from *L. lactis* ssp. *cremoris* HP which was active against di- and tripeptides containing an amino terminal Pro. The peptidase was a dimeric (50 kDa subunits) metalloenzyme depending on Mn^{2+}, Co^{2+} or Zn^{2+} for activity and was optimally active at pH 8.5 and 37°C.

Pyrrolidone Carboxylyl Peptidases:
Pyrrolidone carboxylyl peptidases (PCP, 5-oxoprolyl peptide hydrolase, E.C. 3.4.19.3) are enzymes which hydrolyze peptide bonds following an N-terminal pyroglutamic acid residue. Pyroglutamic acid is formed by the spontaneous cyclicization of an N-terminal glutamyl residue. Aminopeptidases of general specificity cannot hydrolyze adjacent to pyro-Glu residues which could be formed in cheese peptides, due to the relatively high concentrations of Glu in caseins. κ-Casein has a pyro-Glu residue as its N-terminus. Baankreis (1992) found PCP activity in all but 2 of the 25 strains of *Lactococcus lactis* spp. *cremoris*, *L. lactis* ssp. *lactis*, *L. lactis* ssp. *lactis* biovar *diacetylactis* and *Lactobacillus helveticus* studied.

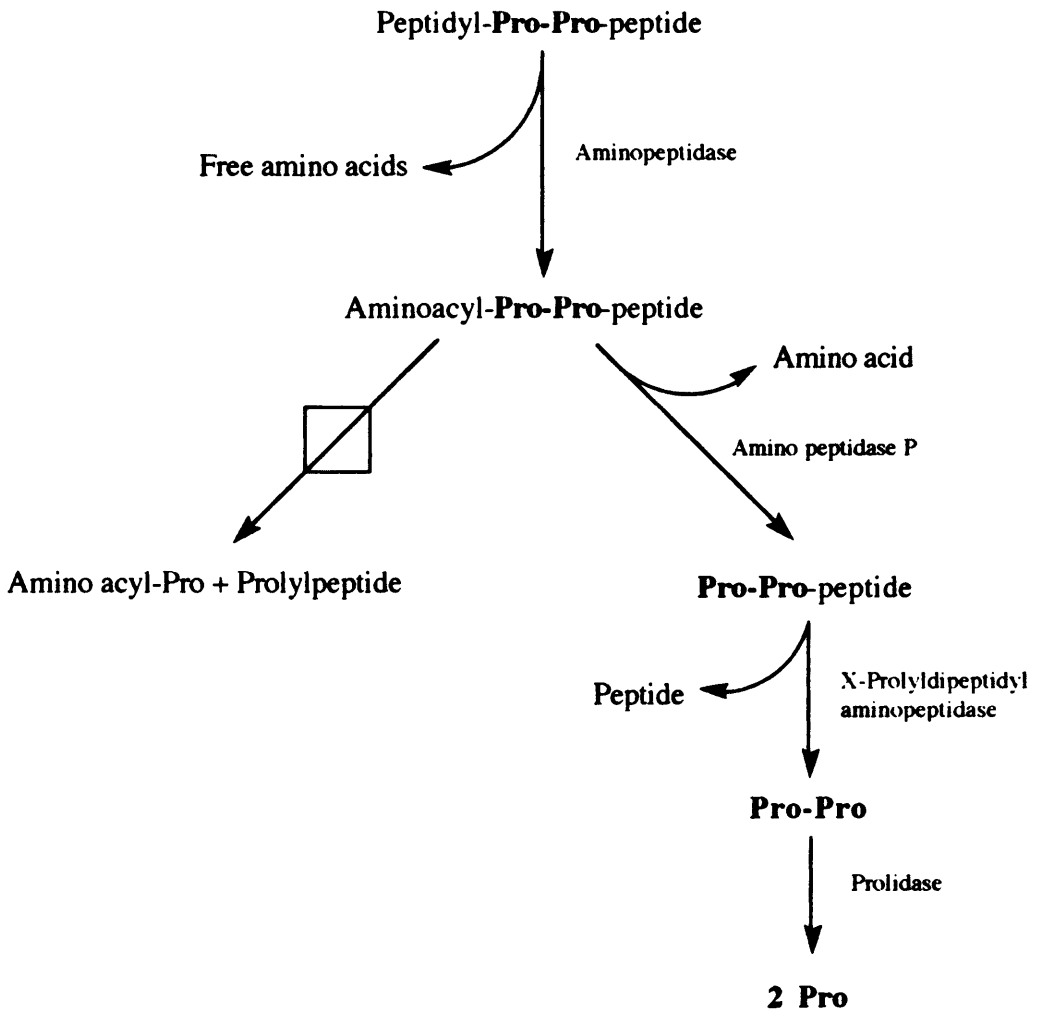

Fig. 2.14. The rôle of post-proline dipetidylaminopeptidase in releasing free proline from oligopeptides containing an internal Pro-Pro sequence (from Booth et al., *1990b)*

Exterkate (1977) studied PCP activity from *Lactococcus lactis* spp. *cremoris* HP and found that the enzyme was associated with the cell membrane. This enzyme (from the same strain) was partially purified by Baankreis (1992) who found 2 serine PCPs (a monomeric enzyme ~25 kDa and an enzyme of molecular mass 80 kDa which was dimeric). The 80 kDa enzyme had a pI of 4.5 and was optimally active on Pyr-*p*-NA at pH 8 to 8.5 and 37°C.

3.2.4 Proteinases from Adjunct Starter

Proteinases and peptidases from the adjunct starter can play an important rôle in proteolysis in cheese varieties where such adjuncts are used. A number of authors (eg. Broome *et al.*, 1990, 1991) have used lactobacilli as adjuncts in the manufacture of Cheddar; their effect on proteolysis is presumably the same as non-starter lactobacilli, as was discussed above. In this section, enzymes from traditional adjuncts, ie. *Penicillium* spp. (mould-ripened varieties), *Brevibacterium linens* (smear-ripened varieties) and *Propionibacterium* spp. (Swiss varieties) will be discussed.

Blue-veined cheese are characterized by the growth of *Penicillium roqueforti* throughout the cheese and Camembert and Brie by the growth of *P. camemberti* on the surface. These moulds produce aspartyl and metalloproteinases which have generally similar specificities on α_{s1}- and β-caseins (Trieu-Cuot and Gripon, 1981). The aspartyl proteinases from both species cleave β-casein at positions Lys_{29}-Ile_{30}, Lys_{97}-Val_{98} and Lys_{99}-Glu_{100}, releasing 5 peptides: β-CN f98-209, f100-209, f1-97/99, f30-209 and f1-29 (Trieu-Cuot *et al.*, 1982). The aspartyl proteinase of *Penicillium* spp. also cleaves α_{s1}-casein at a number of sites; according to Trieu-Cuot and Gripon (1982), the primary site is at or near the primary site of chymosin action, i.e. Phe_{23}-Phe_{24}.

The metalloproteinase of *Penicillium roqueforti* and *P. camemberti* are generally similar; the primary cleavage sites for the enzyme from *P. camemberti* on β-casein are Lys_{28}-Lys_{29}, Pro_{90}-Glu_{91} and Glu_{100}-Ala_{101} (Trieu-Cuot *et al.*, 1982).

Penicillium spp. also produce intracellular proteinases, carboxypeptidases and extracellular aminopeptidases (see Cerning, 1987; Gripon *et al.*, 1991).

Brevibacterium linens, which is used as an adjunct culture in the production of smear-ripened varieties, has a strong proteolytic activity (Gripon *et al.*, 1991). Foissy (1974) found both intra- and extracellular proteinases; an extracellular aminopeptidase was purified by Foissy (1978). Sørhaug (1981) described a number of peptidase activities in cell-free extracts of *B. linens*.

Propionibacterium shermanii is used as an adjunct starter in the production of Swiss cheese varieties. There are few studies on the proteinase and peptidase activities of this bacterium but Langsrud *et al.* (1977, 1978) studied the release of Pro in growth media caused by *Propionibacterium*.

3.2.5 Proteolytic Enzymes of the Non-Starter Microflora

Despite the findings by a number of authors that the NSLAB can dominate the microflora of Cheddar-type cheese during much of its ripening, the proteolytic system of NSLAB has received little attention compared with that of *Lactococcus*. The proteolytic specificity of proteinases from NSLAB on the caseins has not been determined.

Lactobacillus (the genus to which the majority of NSLAB appear to belong), possess a cell wall-associated and intracellular proteinases. A range of peptidases, including intracellular peptidases, dipeptidases, aminopeptidases and endopeptidases, have been identified in *Lactobacillus* (see reviews by Khalid and Marth, 1990a and Peterson and Marshall, 1990). Interestingly, carboxypeptidase activity, which has not been found in lactococci, has been identified in *Lactobacillus casei* (El Soda *et al.*, 1978).

β-Casein is preferentially degraded by a number of strains of *Lactobacillus plantarum* and *L. casei*, but some strains degraded α_{s1}-casein also (Khalid and Marth, 1990b).

NSLAB also include *Micrococcus* and *Pediococcus*. *Micrococcus* spp. produce extracellular proteinases with alkaline pH optima. Membrane-associated and intracellular proteinases have also been found (see review by Bhowmik and Marth, 1990a). There are few reports on the proteolytic activity of *Pediococcus*: Tzanetakis and Litopoulou-Tzanetaki (1989) found Leu and Val aminopeptidase activities in *P. pentosaceus* and El-Soda *et al.* (1991) reported aminopeptidase and dipeptidase activity in *Pediococcus* sp. LR.

Yeasts and moulds can grow on the surface of many soft cheeses (principally of the genera *Kluveromyces*, *Debaryomyces* and *Saccharomyces*) and these microorganisms have primarily intracellular proteolytic activity (see Gripon *et al.*, 1991). *Geotrichum candidum* can grow on the surface of Camembert made from raw milk and produces extra- and intracellular proteinases (Guéguen and Lenoir, 1976).

3.2.6. Catabolism of Amino Acids and Related Events

Free amino acids in cheese result from proteolysis of the caseins by the action of various proteinases and peptidases, as discussed in Sections 3.2.1 to 3.2.5. Catabolism of free amino acids probably plays some rôle in all cheese varieties but is of particularly significant in mould- and smear-ripened varieties.

The first stage in amino acid catabolism involves decarboxylation, deamination, transamination, desulphuration, and perhaps hydrolysis of the amino acid side chains. The second stage involves the resulting compounds (amines and α-ketoacids), as well as amino acids themselves being converted to aldehydes, primarily by the action of deaminases on amines. The final level of amino acid catabolism is the reduction of the aldehydes to alcohols, or their oxidation to acids (Hemme *et al.*, 1982). Sulphur-containing amino acids can undergo extensive conversion, leading to the formation of a number of compounds, including methane thiol and other sulphur derivatives. General pathways of amino acid catabolism are summarized in Figs. 2.15 and 2.16. The catabolism of amino acids has been reviewed by Hemme *et al.* (1982) and Law (1984, 1987).

Decarboxylation involves the conversion of an amino acid to the corresponding amine with the loss of CO_2. The presence of primary amines (e.g. tyramine) in cheese can be explained in terms of simple decarboxylation, although the presence of secondary and tertiary amines is more difficult to explain. The principal primary amine in cheese is tyramine (Law, 1987). The decarboxylase activities of microorganisms of significance in cheese ripening are discussed by Hemme *et al.* (1982). Deamination results in the formation of NH_3 and α-ketoacids. Ammonia is an important constituent of a number of cheeses such as Camembert, Gruyère and Comté (Hemme *et al.*, 1982). Ammonia can also be formed by the

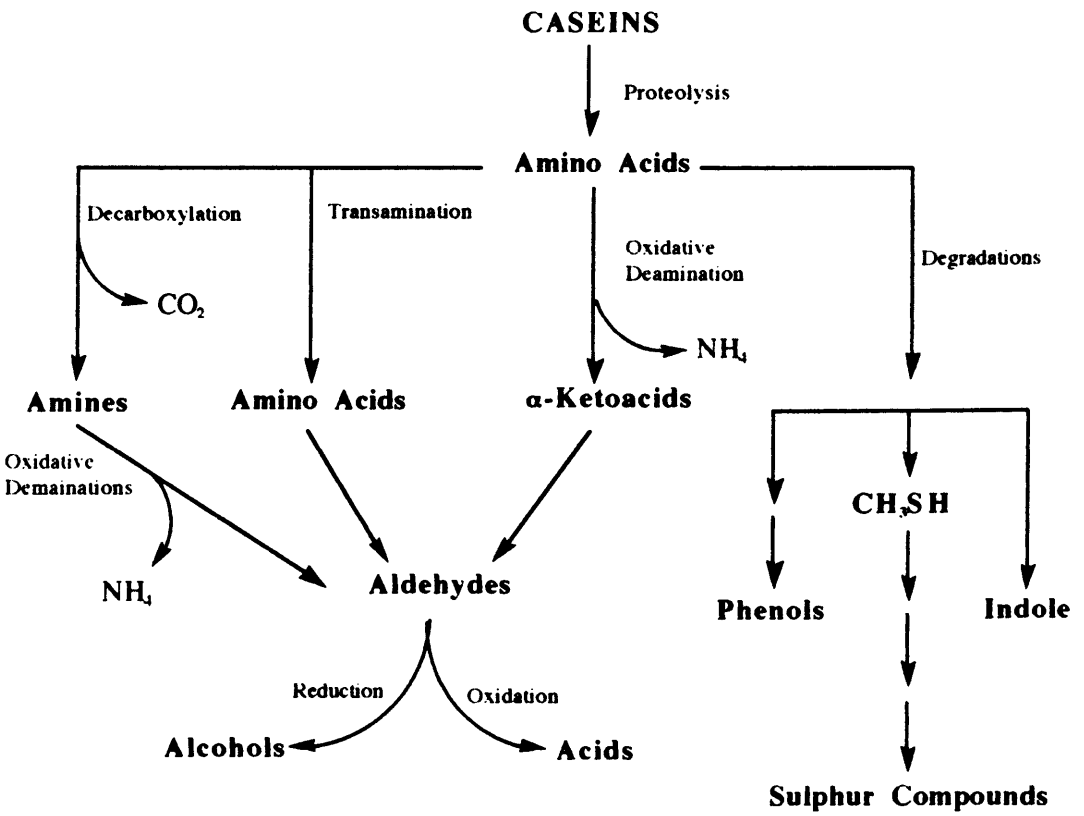

Fig. 2.15. General pathways of microbial catabolism of amino acids during cheese ripening (from Hemme et al., *1982).*

oxidative deamination of amines (yielding aldehydes), and products of complex reactions involving amino acid side chains can also be deaminated. Transamination results in the formation of other amino acids by the action of transaminases. Aldehydes formed by the above processes can then be oxidized to acids or reduced to the corresponding alcohols.

Amino acid side chains can also be modified in cheese. Hydrolases can release ammonia from Asn or Gln or by the partial hydrolysis of the guanidino group of Arg, forming citrulline or its degradation to ornithine (Hemme *et al.*, 1982). Phenol and indole, in addition to pyruvate and ammonia, can be produced by the action of C-C lyases on Tyr and Trp.

Volatile sulphur compounds are found in most cheeses and can be important flavour constituents. The origin of sulphur-containing compounds is generally thought to be the sulphur-containing amino acids, methionine and cysteine (Law, 1987). As Cys is rare in the caseins (occurring at low levels only in α_{s2}- and κ-caseins), the origin of sulphur compounds must be primarily Met. Reactions involving Met are summarized in Fig. 2.16. Sulphur compounds formed from Met include H_2S, dimethylsulphide and methanethiol. The importance of methanethiol and related compounds in cheese aroma is discussed by Law (1987).

A number of amines produced in cheese are biologically active, including tyramine, histamine, tryptamine, putrescine, cadaverine and phenylethylamine. These biogenic amines can have important physiological effects for susceptible individuals, including migrane headaches and hypertension. Concentration of biogenic amines shows great variation in cheese and depends on such factors as variety , ripening period and microbial flora (Flynn, 1992; Joosten and van Boekel, 1988; T. P. O'Connor and N. M. O'Brien, pers. comm.).

3.3. Lipolysis

3.3.1. Indigenous Lipases

It has been recognized for many years that milk contains substantial amounts of an indigenous lipoprotein lipase (LPL, Olivecrona and Bengtsson-Olivecrona, 1991). The physiological rôle of LPL is in the metabolism of plasma triglycerides and, although it has been generally believed that LPL occurs in milk as a result of leakage, it may have a function in milk (see Olivecrona and Bengtsson-Olivecrona, 1991). Bovine LPL is well characterized (see reviews by Olivecrona and Bengtsson-Olivecrona, 1991; Olivecrona *et al.*, 1992). It is rather non-specific and readily hydrolyzes the 1 and 3 positions of mono-, di- and triglycerides and the 1 position of glycerophospholipids. However, lipolysis in milk leads to preferential release short and medium chain acids, because in milk triglycerides, short chain acids are generally esterified at the *sn*-3 position, and may orient themselves so that they are more readily accessible to hydrolysis, or because longer chain acids may be more susceptible to re-incorporation into triglycerides by LPL-catalyzed synthesis. The apparent specificity may also be related to the greater solubility and mobility of triglycerides containing such acids rather than any factor relating to the active site.

In bovine milk, more than 80% of the LPL is associated with the casein micelles (Olivecrona *et al.*, 1992) and so presumably is incorporated into cheese. LPL probably causes significant lipolysis in raw milk cheese but it may also contribute to lipolysis in pasteurized milk cheese as heating ≥ 78°C x 10 s is required for complete inactivation of LPL (Driessen, 1989).

3.3.2. Lipases from Rennet

Rennet extract should contain no lipolytic activity. Rennet pastes used in the manufacture of hard Italian varieties (e.g. Romano, Provolone) contain a potent lipase, pregastric esterase (PGE), which is responsible for the extensive lipolysis

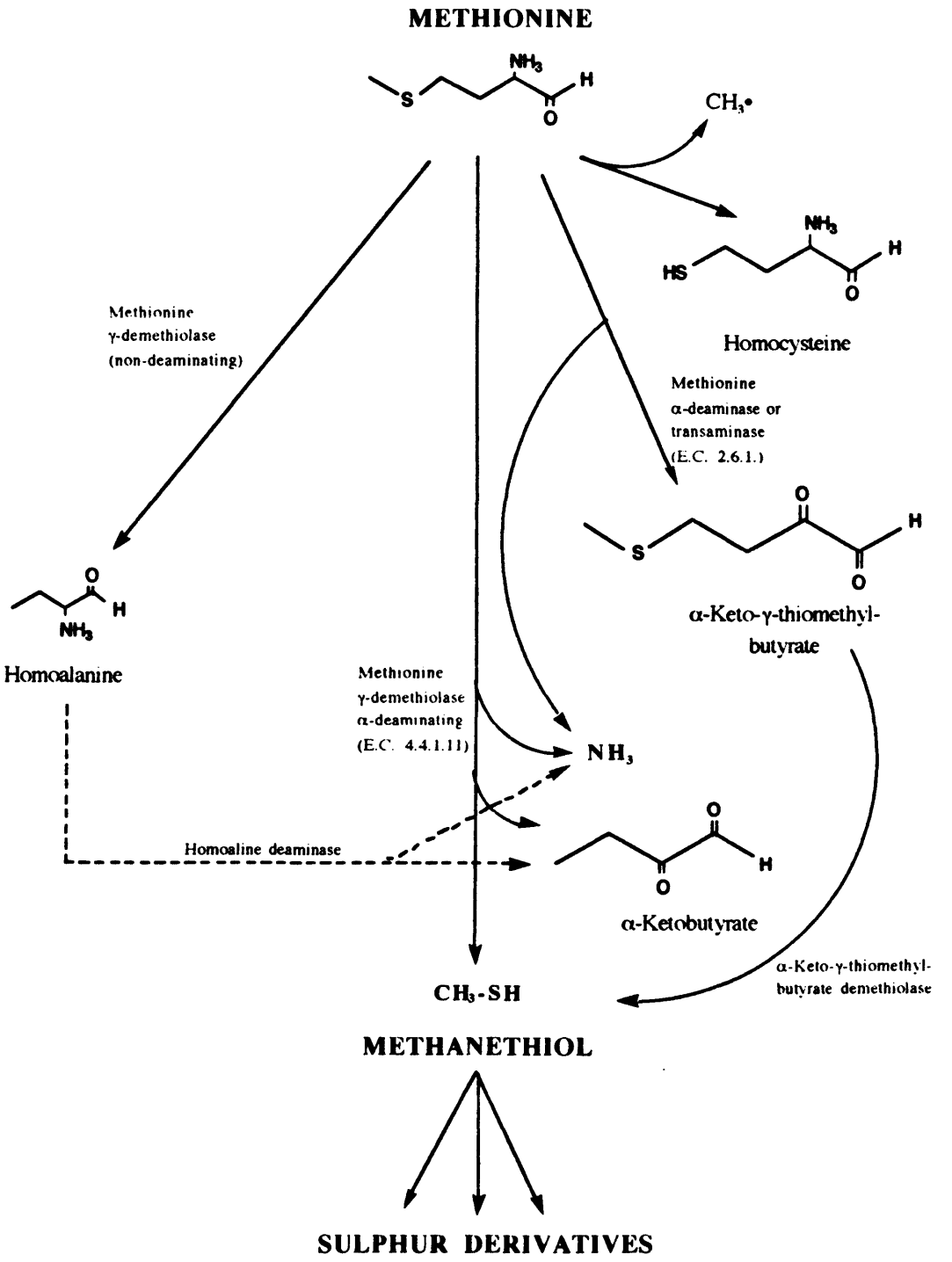

Fig. 2.16. Pathways of methanethiol fromation from methionine (from Hemme et al., *1982).*

which causes the "piccante" flavour characteristic of such varieties. The literature on PGE was comprehensively reviewed by Nelson *et al.* (1977).

PGE, also called lingual or oral lipase, is secreted by glands at the base of the tongue. Its secretion is stimulated by suckling and it is subsequently washed into the abomasum with milk and siliva. Rennet paste is prepared from the abomasa of

calves, kids or lambs slaughered immediately after suckling. The abomasa are partially dried and ground into a paste which is slurried with milk or water before being added to cheesemilk. PGE can also be added to cheesemilk in a partially purified form.

Calf, kid and lamb PGEs were partially purified from commercial preparations (Lee *et al.*, 1980) and calf PGE from oral tissue (Sweet *et al.*, 1984; Bernback *et al.*, 1985). The enzyme appears to be a glycoprotein with a pI of 7.0 and a MW of about 49 kDa. The gene for rat lingual lipase has been cloned and sequenced and the primary structure of the enzyme deduced (Docherty *et al.*, 1985). PGE is highly specific for short chain acids esterfied at the *sn*-3 position (Nelson *et al.*, 1977). Since short chain acids in milk triglycerides are predominately at the *sn*-3 position, the action of PGE leads to the release of high concentrations of short and medium chain acids. The slight specificity differences between calf, lamb and kid PGEs allow the manufacture of Italian cheeses with slightly different flavour characteristics. Most other lipases are unsuitable for the manufacture of Italian cheeses because of incorrect specificity but it has been claimed that certain fungal lipases may be acceptable alternatives (see Fox, 1988/9). The use of PGE to accelerate of the ripening of other cheese varieties was discussed by Fox (1988/9).

3.3.3. Microbial Lipases

Lactococcus spp. and *Lactobacillus* spp. have low lipolytic activities when compared with other genera of bacteria (e.g *Pseudomonas*). Fryer *et al.* (1967) considered that, although weakly lipolytic, lactococci will hydrolyze milk fat to a significant extent if present at high numbers for long periods of time (e.g. during cheese ripening). Small aseptic cheeses acidified with GDL instead of starter had lower FFA concentrations which did not increase during ripening (Reiter *et al.*, 1967). Umemoto *et al.* (1968) found that the cell-free extracts of various dairy lactic acid bacteia were most active on tributyrin at pH between 6 to 8 and at 37°C. Singh *et al.* (1973) found intracellular, but no extracellular, lipases in *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* ; the lipolytic activity of lactococci was considerably less than that of other bacteria (e.g. *Pseudomonas* and *Micrococcus*) and was more active on tributyrin than tripalmitate or triolein. Harper *et al.* (1980) reported that *Lactococcus lactis* ssp. *lactis* had a more complex esterase system than *L. lactis* ssp. *cremoris*; the former showed 2 bands on a zymogram stained with α-naphtyl acetate, while the latter showed only one. In a comparative study of the lipase activities of a number of strains of lactic acid bacteria, including *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*, Singh *et al.* (1981) found that mutants prepared by UV treatments produced more long chain acids than the parent strains. Piatkiewicz (1987) found inter-strain differences between lipases and esterases which are influenced by the composition of the growth medium and the physiological age of the culture. This author also reported that lactococci were more lipolytic than lactobacilli. Kamaly *et al.* (1990) studied the lipolytic activities of a number of strains of *Lactococcus*. Lipases, in cell-free extracts, were most active at 37°C and at pH 7 to 8.5 (tributyrin substrate) or 7.0 (milk fat emulsion). Lipases were more active on triglycerides containing short chain acids ($C_{4:0}$ to $C_{10:0}$) than long chain acids ($C_{12:0}$ to $C_{18:1}$). Starter bacteria can to liberate FFA from mono- and diglycerides in milk produced by other lipases, e.g. LPL or lipases from Gram-negative bacteria (Stadhouders and Veringa, 1973).

Kamaly *et al.* (1988) studied the esterolytic activities of lactococci on *p*- and *o*-nitrophenyl esters of fatty acids and found some quantative differences between strains.

The esterase / lipase system of *Lactococcus* has recieved relatively little attention in comparison with its proteolytic system. Unlike the situation with lactococcal proteinases and peptidases, little is known about the genetics of the lactococci. Isolation of lipase/esterase negative variants of *Lactococcus* would permit the significance of these enzymes in cheese ripening to be assessed.

There has been considerable recent interest in the lipolytic / esterolytic activities of *Lactobacillus*. An esterase of *Lactobacillus plantarum* was purified by Oterholm *et al.* (1972) who found that the enzyme was maximally active at pH 6.7 and 40°C (triacetin substrate). The enzyme was not affected by heavy metals or dilute solutions of cyanide or azide but was inhibited by concentrated solutions of the latter compounds. The enzyme preferentially hydrolyzed acetylesters and exhibited a strong preference of substrates in solution rather than emulsion.

The intracellular esterases of *L. helveticus*, *L. delbreuckii* ssp. *bulgaricus* and *L. lactis* were studied on *o*- and *p*-nitrophenyl derivatives of fatty acids by El Soda *et al.* (1986). The optimum temperature for lipase production was 40 to 45°C and the cells had little esterolytic activity if grown at 35 or 55°C. The esterase(s) of these strains was complex and generally specific for short chain acids.

Piatkiewicz (1987), who investigated the lipase and esterase activities of *L. casei*, found that cells had higher activities in the logarithmic than in the stationary phase of cell growth. Khalid *et al.* (1990) studied the esterases of *L. helveticus* CNRZ 32 (a strain thought to have potential in accelerated cheese ripening), *L. helveticus* ATCC10797 and *L. delbreuckii* ssp. *bulgaricus*; all strains had active esterases, especially *L. delbreuckii* ssp. *bulgaricus*.

Other non-starter genera (e.g. *Micrococcus* and *Pediococcus*) also produce lipases. It is generally believed that lipases from *Micrococcus* spp., when present in cheese, can contribute to lipolysis during ripening (Bhowmik and Marth, 1990a). The lipolytic activities of a number of strains of micrococci were studied by Peterson and Johnson (1949) who observed intracellular lipolytic activity which was active on butterfat between pH 5 and 6. The lipase of *M. freudenreichii* was strongly inhibited by organophosphates and divalent metal ions, but less so by EDTA or pCMB (Lawrence *et al.*, 1967).

Bhowmik and Marth (1990b) studied the esterases of 5 strains of *Micrococcus*, all of which showed esterolytic activity; most strains hydrolyzed *p*-nitrophenyl derivatives of fatty acids faster than *o*-nitrophenyl derivatives. Esterases were found in cell-fre extracts of the strains studied. The esterase of *Micrococcus* spp. ATCC 8459 was studied in detail; it was optimally active at pH 8.0 and 40°C and was inhibited by organphosphates, divalent metal ions, NaCl and redox reagents.

The lipase / esterase of *Pediococcus* spp. has received little attention. Tzanetakis and Litopoulou-Tzanetaki (1989) found only weak esterase and lipase activities in a number of strains of *P. pentosaceus* of dairy origin by means of the API-ZYM™ system. Bhowmik and Marth (1989) found esterase activity in 6 strains of *P. pentosaceus* but none in 2 strains of *P. acidilactici*. The lipases of *Propionibacterium shermanii* were studied by Oterholm *et al.* (1970) who found maximum activity at pH 7.2 and 47°C on tributyrin; the enzymes showed a high preference for tripropionate and tributyrin. The lipases were inhibited by Hg^{2+} and Na_2HAsO_4 but not by pCMB or EDTA. Some esterase activity was observed but the enzyme was more active on emulsified than on soluble substrates.

Lipases and esterases of *Brevibacterium linens* were described by Sørhaug and Ordal (1974) and Foissy (1974). Sørhaug and Ordal (1974) found only intracellular lipase and esterase activities in *B. linens*. Most of the strains studied were more active on tributyrin than on triacetin or methyl butyrate. Foissy (1974) found intracellular esterase activity in 15 *B. linens* isolates but also found weak extracellular activity, which, based on zymogram patterns, may have been of intracellular origin.

Extensive lipolysis occurs in mould-ripened cheese, particularly blue varieties. In some cases, up to 25% of the total FFA may be liberated (see Gripon, 1987, 1993). However, the impact of FFA on the flavour of blue mould-ripened cheeses is less than that for hard Italian varieties, possibly due to neutralization as the pH increases during the ripening and to the dominant influence of methyl ketones on the flavour of blue cheese.

Lipolysis in mould-ripened varieties is due primarily to the lipases of *Penicillium roqueforti* or *P. camemberti* which secrete potent extracellular lipases. *Penicillium* lipases are well characterized (see Kinsella and Hwang, 1976; Gripon, 1987, 1993). *P. camemberti* appears to excrete only one lipase with an optimum pH at *ca.* 9.0 and a temperature optimum at *ca.* 35°C. *P. roqueforti* excretes two lipases, one with a pH optimum at 7.5 to 8.0 (or perhaps 9.0 to 9.5), the other at pH 6.0 to 6.5. The acid and alakaline lipases exhibit different specificities.

Psychrotrophs, which can dominate the microflora of refrigerated milk, are a potentially important source of potent lipases in cheese. Cousins *et al.* (1977) considered that active lipase would be present in cheese if psychrotroph numbers exceed 10^7 ml^{-1}. Many psychrotroph lipases are heat stable and thus may cause rancidity in cheese over a long ripening time. The subject of psychrotroph enzymes in cheese was discussed by Mottar (1989). Unlike psychrotroph proteinases, which are largely lost in the whey, psychotroph lipases adsorb onto the fat globules and are thus concentrated in the cheese.

3.3.4. Patterns and Levels of Lipolysis in Selected Cheeses

Lipolysis is considered to be undesirable in most cheese varieties; Cheddar, Gouda and Swiss-type cheeses containing even a moderate level of free fatty acids would be considered rancid; however, certain cheese varieties are characterized by extensive lipolysis (e.g. Romano, Parmesan and Blue cheeses). Bills and Day (1964) quantified free fatty acids (FFA) ($C_{2:0}$ to $C_{18:3}$) in 14 Cheddar cheeses with wide variations in flavour but found only small differences, qualitatively or quantitatively, between cheeses of different flavour. The proportions of FFAs ($C_{6:0}$ to $C_{18:3}$) in cheese was found to be similar to that in milk fat, indicating that these FFA were released non-specifically manner. However, free butyric acid was found at a higher concentration than could be explained by its proportion in milk fat, suggesting that it was selectively liberated or synthesized by the cheese microflora. Lipolysis in hard Italian varietes is extensive and due primarily to the action of PGE in the rennet paste used in the manufacture of these cheeses. Lipolysis in blue cheese varieties is extensive due to the action of lipases from *Penicillium* spp. and is characterized by the production of methyl ketones. Free fatty acid levels in a number of cheese varieties are listed in Table 2.3.

3.3.5. Catabolism of Fatty Acids

The flavour and aroma of blue cheese is dominated by saturated *n*-methyl ketones. A homologous series of methyl ketones with odd-numbered carbon chains from C_3 to C_{15}, as well as a number of even-numbered carbon chains (including C_4 to C_{10}), are found in blue cheese (Patton, 1950). Concentrations of methyl ketones in blue cheese fluctuate, presumable due to reduction to secondary alcohols (Dartley and Kinsella, 1971). Heptan-2-one, nonan-2-one and undecan-2-one are the dominant methyl ketones in blue cheese during most of its ripening (Dartley and Kinsella, 1971). Jolly and Kosikowski (1975) that C_5, C_7, C_9 and C_{11} were the dominant methyl ketones in a blue cheese flavour concentrate.

The metabolism of fatty acids in cheese by *Penicillium* spp. involves four main steps: (1) release of fatty acids by the lipolytic systems discussed above (Sections 3.3.1-3.3.4), (2) oxidiation to β-ketoacids, (3) decarboxylation to form a methyl ketone with one less carbon atom, and (4) reduction of methyl ketones to the corresponding secondary alcohol (Hawke, 1966); step 4 is reversable under aerobic conditions (Adda *et al.*, 1982). The concentration of methyl ketones is related to lipolysis. Methyl ketones can also be formed by the action of the mould on the ketoacids naturally present at low concentrations in milk fat (*ca.* 1% of total fatty acids). Methyl ketones could also be formed by the oxidation of monounsaturated

Table 2.3: Typical concentrations of free fatty acids (FFA) in some cheese varieties (Woo *et al.*, 1984; Woo and Lindsay, 1984))

Variety	FFA (mg kg^{-1})	Variety	FFA (mg kg^{-1})
Sapsago	211	Gjetost	1658
Edam	356	Provolone	2118
Mozzerella	363	Brick	2150
Colby	550	Limburger	4187
Camembert	681	Goat's Milk	4558
Port Salut	700	Parmesan	4993
Monterey Jack	736	Romano	6754
Cheddar	1028	Blue (US)	32230
Gruyere	1481	Roquefort	32453

acids; Adda *et al.* (1982) discussed the implications of such a pathway for methyl ketone formation in cheese and suggested that the evidence for such a pathway is equivocal.

A number of factors affect the rate of methyl ketone production in cheese, including temperature, pH, the physiological state of the mould and the ratio of concentration of fatty acid to the dry weight of spores (Adda *et al.*, 1982). Fan *et al.* (1976) found that both resting spores and fungal mycelium were capable of producing methyl ketones. The rate of production of methyl ketones does not depend directly on the concentrations of FFA precursors; indeed high concentrations of FFA are toxic to *P. roqueforti*.

Lactones are cyclic esters resulting from the intramolecular esterification of a hydroxyacid through the loss of water to form a ring structure. α- and β-lactones are highly rective and are used or occur as intermediates in organic synthesis; γ- and δ-lactones are stable and have been found in cheese. Lactones possess a strong aroma, which although not specifically cheese-like, may be important in the overall cheese flavour impact.

Eriksen (1975) concluded that γ- and δ-lactones in freshly secreted milk originated from the corresponding γ- and δ-hydroxyacids esterified in triglycerides. Dimick *et al.* (1969) reported a δ-oxidation system for fatty acid catabolism in the mammary gland of ruminants and thus oxidation within the mammary gland is the primary source of lactone precursors. The potential for lactone production depends on such factors as feed, season, stage of lacation and breed (Dimick *et al.*, 1969). The formation of γ- or δ-lactones is spontaneous following release of the corresponding hydroxyacid from triglycerides (Christie, 1983) and it would therefore appear that lactone production should correlate with the hydrolysis of triglycerides.

Wong *et al.* (1975) found disproportionate concentrations of high molecular weight lactones in Cheddar and suggested two further pathways for their formation; i.e. reduction of the corresponding keto acid or the microbial metabolism of homoricinolec acid to shorter chain hydroxyacids and lactones.

4. SIGNIFICANCE OF GLYCOLYSIS, PROTEOLYSIS AND LIPOLYSIS TO CHEESE TEXTURE

The development of cheese texture during maturation is an important aspect of cheese ripening. Cheese consists of a casein matrix with a continuous aqueous phase, interspersed with fat globules. The structural unit in the protein matrix in cheese varies between varieties, as does the state of the fat globules. In Gouda, the protein matrix consists of small globular units (10 to 15 nm in diameter) while in

Cheshire, protein consists of strands and aggregates, 3 to 4 nm in diameter (Hall and Creamer, 1972). The structure of the cheese is modified by pH and the protein aggregates present in high pH cheeses are probably derived from casein sub-micelles (Hall and Creamer, 1972). The basic structure of cheese varies considerably according to the degree of demineralization of the sub-micelles, from Swiss, with essentially the same globular form as sub-micelles to acid varieties, such as Feta and Cheshire and mould-ripened cheeses. The basic structure of cheese consists of a micellar framework of α_{s1}-casein and colloidal phosphate, β-casein in the sub-micelles having largely dissociated on the reduction of $[Ca^{2+}]$ (Creamer and Olson, 1982; Lawrence *et al.*, 1983). The rheological properties of cheese vary with age and a number of factors including pH, retention of coagulant, activity of coagulant, water activity and water sorption which are influenced by the manufacturing protocol of the cheese variety (for reviews on cheese rheology see Prentice, 1987; IDF, 1991; Prentice and Marshall, 1993). The relationship between rheological properties and proteolysis is unclear. Since the basic structure of cheese is determined largely by its protein fraction, breakdown of the matrix by proteolysis is likely to affect the texture of cheese. De Jong (1976) found that the decrease in the consistency of a Meshanger-type cheese was due to the degradation of α_{s1}-casein and this was confirmed by de Jong (1977) who found no softening without hydrolysis of α_{s1}-casein. Meshanger-type cheese softens from the inside-out and for a given composition, proteolysis (due to rennet action) correlates with consistency; plasmin appears to play a minor rôle in the development of cheese texture (Noomen, 1977). The softening of Meshanger-type cheese from the inside out can be explained in terms of the initially lower NaCl concentration at the centre of the cheese which permits starter growth with a decrease in pH; a low pH and low salt concentration favours chymosin action on α_{s1}-casein.

Softening of Cheddar cheese early in ripening is reported by Creamer and Olson (1982) to be due to cleavage of α_{s1}-casein at Phe_{23}-Phe_{24} by chymosin; it is likely that further proteolysis during ripening further modifies cheese texture. Although Mozzarella has a pH and calcium concentration similar to Cheddar and Gouda, it retains its stretching properties longer than the other cheeses (Lawrence *et al.*, 1983). The high heat treatment used in the production of Mozzarella denatures residual rennet and therefore this cheese has relatively high concentrations of intact caseins and large peptides which are believed to be responsible for its stretchability (Creamer, 1976b).

Textural changes in surface-ripened cheese (e.g. Camembert) were investigated by Noomen (1983) who found that very little migration of enzymes from the suface flora occurred. It was also found that little softening occurred in rennet-free cheese or renneted cheese without a surface flora despite the extensive degradation of α_{s1}-casein in the latter cheese and only a thin soft layer developed in rennet-free cheese (with a sufrace flora). When renneted cheese was placed in an NH_3 atmosphere, it softened quickly. These results underline the relationship between proteolysis and other factors (e.g., pH) in the development of cheese texture. Proteolysis or an increase in pH alone does not cause softening in surface-ripened cheeses, which soften only when both events occur (Noel and Lefier, 1991).

Fat plays an important rôle in the development of texture, fat percentage, fat globule size and the fatty acid profile being important parameters. Although the influence of lipolysis on cheese texture has been the subject of little research, it is considered not to play an important rôle (Adda *et al.*, 1982).

5. BIOCHEMISTRY OF CHEESE FLAVOUR AND AROMA

"There is a cheese for every taste preference and a taste preference for every cheese" (Olson, 1990). Starting from milk, a substrate which although not entirely flavourless, is rather bland, a cheesemaker can make any of the 800 varieties of

cheese listed by the IDF, each variety having a unique flavour and aroma. Cheese flavour and aroma have attracted much attention for many years and have been the subject of hundreds of investigations and reviews. However, despite this attention, only limited information is available on the chemistry of the flavour of most varieties and the flavour of none is characterized sufficiently to permit its reproduction by mixtures of pure compounds. Cheese curd is relatively flavourless and in the case of most varieties flavour and aroma develop during ripening. The biogenesis of cheese flavour is as a direct or indirect consequence of the action of the ripening agents discussed in Section 3.

The study of cheese flavour is clearly important, both from the aspect of mimicing cheese flavour in products such as cheese analogues, acceleration and control of ripening, but more importantly, from the point of view of promoting good flavour development and avoiding off-flavours. The extensive literature on the subject of cheese flavour has been review by Reiter *et al.* (1966), Kristoffersen (1973, 1985), Horwood (1974), McGugan (1975), Aston and Dulley (1982), Adda *et al.* (1982), Lawrence *et al.* (1983), Olson (1990), Steele and Ünlü (1992) and Urbach (1993).

The biochemistry of cheese flavour and aroma is inseparable from the biochemistry of the cheese. Over 300 different volatile and non-volatile chemical compounds have been implicated in cheese flavour and aroma (Kristoffersen, 1973). These compounds originate from the action of the cheese microflora and its complement of enzymes on the lactose, lipids and proteins of cheese curd. Further flavour compounds can be formed through the chemical reaction of precursor compounds formed by microorganisms or enzymes. A number of compounds which have been identified in Cheddar are listed in Table 2.4

As is apparent from Table 2.4, many of the potential flavour compunds in cheese result from the processes of gylcolysis, lipolysis and proteolysis. The biochemistry of these processes and the generation of the above compounds has been discussed above and will not be discussed here further.

5.2. Physico-Chemical Aspects of the Development of Cheese Flavour

NaCl has been added as a preservative to cheese since ancient times. This constituent is very important both in its direct influence on cheese taste, but also due to its effect on a_w, and thus on factors such as bacterial growth and proteolysis. Salt distribution in directly-salted varieties (e.g. Cheddar and related cheeses) is relatively uniform, but in brine-salted varieties (e.g. Gouda, Mozzarella, Swiss-type cheeses, Camembert), an NaCl gradient develops from the outside towards the centre, thus influencing the rate of ripening and hence flavour development.

The fermentation of lactose to lactate by the starter, and the consequent decrease in pH, is of fundamental importance in the manufacture of all cheese varieties. The decrease in pH influences the physical structure of the cheese by solubilizing colloidal calcium phosphate and by promoting curd syneresis (Fox *et al.*, 1990). The pH of a cheese affects enzyme activity and thereby has a direct influence on cheese flavour, directly by contributing to the acid taste of many cheeses and indirectly by influencing bacterial growth and enzyme activity as well as the degree of dissociation of free fatty acids (see above).

Growth of the stater changes the redox potential (E_h) of the cheese curd from *ca.* +300 mV (milk) to *ca.* -200 mV in cheese. Indeed, the reducing conditions in cheese appear to be essential for the production of a number of flavour compounds from sulphur amino acids (Manning, 1979a) and probably also inhibits lipid oxidation to which milk fat is normally very susceptible.

Downey (1969) is one of the few authors who investigated lipid oxidation in cheese. This author found that Cheddar wrapped in transparent cellophane and exposed to light in an illuminated display cabinet developed detectable oxidation on the surface of the cheese facing the light after 48 h and flavour was deemed to be objectionable after 72 h illumination. Little oxidation was observed inside the

Table 2.4: Compounds which have been identified in Cheddar cheese (from Urbach, 1993)

acealdehyde	dimethyl disulphide	3-methylbutanol
acetoin	dimethyl trisulphide	3-methyl-2-butanone
acetone	δ-dodecalactone	3-methylbutyric acid
acetophenone	ethanol	2-nonanone
β-angelicalatone	ethyl acetate	δ-octalactone
1, 2-butanediol	2-ethyl butanol	n-octanoic acid
n-butanol	ethyl butyrate	2-octanol
2-butanol	ethyl hexanoate	2, 4-pentanediol
butanone	2-heptanone	n-pentanoic acid
n-butyl acetate	n-hexanal	2-pentanol
2-butyl acetate	n-hexanoic acid	pentan-2-one
n-butyl butyrate	n-hexanol	n-propanol
n-butyric acid	2-hexanone	propanal
Carbon dioxide	hexanethiol	propenal
p-cresol	2-hexenal	n-propyl butyrate
γ-decalactone	isobutanol	tetrahydrofuran
δ-decalactone	isohexanal	thiophen-2-aldehyde
n-decanoic acid	methane thiol	2-tridecanone
diacetyl	methional	2-undecanone
diethyl ether	methyl acetate	
dimethyl sulphide	2-methylbutanol	

cheese. This author suggested that, like butter, consumer portions of cheese should be wrapped in a light-impervious material.

5.3. Contribution of Glycolysis and Related Events to Flavour

The metabolism of lactose and citrate in cheese leads to the formation of a number of compounds of potential significance to cheese flavour, including lactate, acetate, propionate and butyrate.

The metabolism of lactate to propionate, acetate and CO_2 by *Propionibacterium shermanii* is significant to the flavour of Swiss cheese, while metabolism of citrate to diacetyl, acetoin and 2,3-butylene glycol is important to the flavour of Dutch cheese varieties and may also be of significant in Cheddar. Manning and Robinson (1973) identified diacetyl in a distillate of Cheddar cheese and considered that it contributed to cheese aroma. Barlow *et al.* (1989) found that lactate and acetate were amongst the chemical constituents (which also included volatile sulphur compounds, butanone and water soluble N) of Cheddar which correlated most strongly with flavour scores. Biede and Hammond (1979a) correlated the "burned" flavour of Swiss cheese with lactic acid concentrations, although they considered it more likely that this flavour note was due to medium-sized peptides.

5.4. Contribution of Proteolysis and Related Events to Flavour Development

Peptides produced by plasmin and chymosin are probably not important in the development of typical cheese flavour, although these enzymes can produce very hydrophobic, and hence bitter, peptides (see Section 5.6). The breakdown in cheese structure *per se* may also influence the perception of flavour by the release of flavour compounds which were previously bound to the protein (McGugan *et al.*,

1979). However, compounds can be produced in the later stages of proteolysis which are themselves important to cheese flavour or act as substrates for the production of further compounds.

McGugan *et al.* (1979) found that non-volatile water extractable material, and hence products of proteolysis was important for the flavour of Cheddar cheese. Likewise, Aston and Creamer (1986) found that the water-soluble fraction of Cheddar contributed most to the flavour of the cheese. Intermediate-sized peptides are probably not major contributors to cheese flavour, although they may contribute to a brothy flavour, e.g. in Swiss cheese (Biede, 1977). Short peptides can have important flavour characteristics (e.g. aspartame or bitter peptides), although the very heterogeneous nature of cheese peptides makes the correlation between flavour and specific peptides dificult. The contribution of small peptides to cheese flavour is probably similar to that of free amino acids (Aston and Dulley, 1982). It has been suggested that the typical sweet flavour of Swiss-type cheeses may result from the interaction of Ca and Mg with small peptides and that small peptides and amino acids may contribute to the brothy-nutty flavour note in this cheese (Biede and Hammond, 1979a). Biede and Hammond (1979a) failed to correlate the acid taste of a fraction of Swiss cheese with lactic acid or pH, but did correlate the perception of this taste with concentrations of small peptides and free amino acids

Proteolysis ultimately results in the formation of free amino acids. A number of early workers did not consider free amino acids to be important for cheese flavour (see Aston and Dulley, 1982, for references), although correlations between amino acid N and cheese flavour were found by Aston *et al.* (1983).

Although the acceleration of proteolysis has been shown to accelerate ripening (see Fox, 1989), it is unlikely that the flavour of Cheddar or Gouda results from amino acids and peptides only (Olson, 1990). However, the further catabolism of amino acids can result in the formation of flavour compounds. Amino acids can react with carbonyls to form a number of flavour compounds. Kowalewska *et al.* (1985) identified a number of flavourful compounds from *Lactobacillus helveticus* cultures and from cheese and attributed a "burned" flavour to carbonyls released on the degradation of 2,5-dimethyl-4-hydroxy-3[2H] furfanone. Griffith and Hammond (1989) studied the generation of flavour compounds arising from the reaction of amino acids with carbonyls. Glyoxal, methylglyoxal, dihydroxyacetone and ethanal were deemed to be prominant carbonyls and flavour-generating reactions were observed with amino acids including Val, Leu, Ile, Met, Cys, Phe, Pro and Lys. These authors suggest that the final stages in flavour development in Swiss cheese were chemical rather than enzymatic.

As discussed previously, catabolism of amino acids can result in a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols, all of which may contribute to cheese flavour.

Development of flavour compounds in Cheddar resulting from the degradation of sulphur amino acids has been the subject of much attention. Manning and Robinson (1973) found H_2S, methanethiol and dimethyl suphide in distillates of Cheddar cheese. Manning (1974) compared the sulphur-containing volatiles from a number of cheeses and found that methanethiol was found only in distillates from cheeses having Cheddar flavour. The importance of methanethiol to Cheddar flavour was also found by Manning *et al.* (1976) who correlated its concentration with Cheddar flavour intensity. Manning and Price (1977) considered the presence of H_2S in Cheddar to have a beneficial effect on flavour, although its concentration may not be critical. The origin of methanethiol in Cheddar was studied by Law and Sharpe (1978) who concluded that it was unlikely that this compound was formed by the direct action of the cheese microflora and possible routes for the chemical formation of methanethiol were discussed by Manning (1979a). Manning (1979b) and Manning and Moore (1979) correlated Cheddar flavour with 2-pentanone, methanethiol and methanol. The latter authors studied a variety of hard cheeses and found that varieties containing low concentrations of sulphur compounds had little aroma and derived most of their

flavour from taste. The importance of methanethiol in a number of cheese varieties was discussed by Hemme *et al.* (1982). Methanethiol is produced from methioine and in cheese can be produced via two pathways. The enzyme, demethiolase, produced by coryneform bacteria and some Gram-negative rods, can produce methanethiol directly. However, cornyforms are normally absent from Cheddar cheese and a second, chemical, pathway is thought to operate. In this pathway, H_2S, released from cysteine/cystine by a reducing agent, reacts with methionine to form methanethiol (Manning, 1979a). Although Barlow *et al.* (1989) found that H_2S correlated well with Cheddar flavour, the relationship between methanethiol and cheese flavour has been questioned by Olson (1990).

Aston and Creamer (1986) found that the subfraction of a water-soluble extract of Cheddar contining NaCl, free Met and free Glu contributed most to the flavour of the water-soluble extract.

5.5. Contribution of Lipolysis to Flavour

The fat fraction of cheese is important for the perception and development of flavour. Cheddar cheese made from skim milk does not develop a typical flavour even after 12 months of ripening (Ohren and Tuckey, 1967). The poor development of flavour and texture in low-fat cheeses is a considerable technological problem which limits the market for such products (Banks *et al.*, 1992). Replacement of milk fat by vegetable or mineral oils improves the flavour perception of Cheddar over cheese made from skim milk (Foda *et al.*, 1975) which suggests that one important action of fat is as a solvent for flavour compounds. Foda *et al.* (1975) also found that natural milk fat emulsion gave higher flavour scores than milk fat emulsified into skim milk, suggesting that the fat-water interface plays an important rôle in the development of cheese flavour and that milk fat gave higher flavour scores than mineral or vegetable oils, suggesting that the composition of milk fat is also important for the development of flavour.

As in all high-fat foods, the fat in cheese can undergo degradation *via.* lipolysis (enzymatic) or oxidation (chemical). The degree of lipid oxidation in cheese is probably limited, due to the low redox potential present in cheese and the presence of natural antioxidants. However, there is some evidence that limited lipid oxidation does occur in cheese (Downey, 1969).

Lipolysis can contribute directly to the delelopment of flavour by the formation of fatty acids. Fatty acids are very important flavour compounds in hard Italian and mould-ripened varieties but probably not in varieties such as Cheddar and Gouda. Bills and Day (1964) point out that the pH of cheese can exert a considerable influence on the flavour impact of FFA. At the pH of Cheddar (*ca.* pH 5.2), a considerable portion of FFAs are likely to be present as their salts, thus reducing their flavour impact. Biede and Hammond (1979a) found that the acid flavour of Swiss cheese correlated well with the concentrations of total water-insoluble fatty acids in the aqueous phase of the cheese. In extra-mature cheese, FFAs probably make a positive contribution to flavour, provided that they are properly balanced by products of proteolysis and other reactions.

FFAs also contribute to cheese flavour by providing substrates for the formation of other compounds. The catabolism of FFA to methyl ketones in mould-ripened varieties is of great importance. Methyl ketones of intermediate chain length are the amongst the principal flavour
compounds in blue cheese (Dartley and Kinsella, 1971). Wong *et al.* (1973, 1975) considered that lactones of the fatty acids may be significant for the flavour Cheddar cheese as they were present in concentrations in excess of their flavour threshold. Other products of lipolysis which may have a significant effect on flavour include esters and amides (Adda *et al.*, 1982).

Dimos (1992) found a significant linear relationship between Cheddar flavour and a the concentration of number of chemical constituents (Equation 2.1).

Flavour = $b_0 + b_1 \log [H_2S] + b_2 \log [\text{heptan-2-one}] + b_3 \log [\text{butanone}] + b_4 \log [\delta\text{-decalactone}] + b_5 \log [\text{propan-2-ol}]$

R^2=0.8435 (Adjusted flavour)
R^2=0.8604

b_0 to b_5, constants.

Equation 2.1. Relationship between Cheddar cheese flavour and concentrations of various chemical constituents. (Dimos, 1992).

5.6. Chemistry of Cheese Off-Flavours

Bitterness in cheese results from the accumulation of hydrophobic, short chain peptides which can originate from both α_{s1}- and β-caseins. Certain sequences in the caseins are particularly hydrophobic and when excised by the action of proteinases can lead to bitterness.

The action of chymosin has been suggested in the formation of bitter peptides in cheese (Czulak, 1959; Stadhouders, 1962) and thus factors affecting the retention of rennet in the curd may influence the development of bitterness. Lawrence *et al.* (1972) pointed out the importance of both starter strain and rennet type and suggested that the major rôle of rennet in the production of bitter peptides may be in the production of long-chain peptides which are subsequenly degraded to small bitter peptides by starter. These authors also found a higher concentration of free amino acids in cheeses made with non-bitter starters, suggesting greater peptidase activity in these strains. It has been recognized that certain strains of starter and *Penicillium* spp. are associated with the development of bitterness (see Adda *et al.*, 1982) and it would appear that the development of bitterness in cheese is as a result of the action of chymosin on casein with the release of bitter peptides. These peptides accumulate in bitter cheese due to the inability of "bitter" starters to hydrolyze them to non-bitter compounds due to a deficiency in peptidase activity (Stadhouders and Hup, 1975).

The majority of studies in which bitter peptides were identified were conducted in model systems consisting of isolated casein incubated with various enzymes. Bitter peptides have been identified in hydrolyzates of α_{s1}-, α_{s2}- and β-caseins. Para-κ-casein is a potential source of bitter peptides (Visser, 1981), although the few studies which investigated the hydrolysis of para-κ-casein found it to be resistant to proteolysis (Section 3.2). Bitter peptides identified in casein hydrolysates and originating from α_{s1}-casein include f22-43 and f45-50 (see Sullivan and Jago, 1972). Hill and van Leeuwen (1974) found the peptides α_{s1}-casein f23-34, f91-100 and f145-151 in a tryptic digest of casein to be bitter. LeBars and Gripon (1989) considered that 3 short peptides produced from the C-terminal region of α_{s2}-casein by plasmin (f198-207, 182-207 and f189-207) may be bitter. Bitter peptides found in hydrolysates and originating from β-casein include f203-208, f195-209, f201-209 (see Sullivan and Jago, 1972) and f53-79 (Clegg *et al.*, 1974).

A number authors have described the isolation of bitter peptides from cheese (e.g. Harwalkar and Elliot, 1971; Visser, 1977c), but there is some disagreement as to the source of these peptides (Visser, 1981). However, unlike α_{s1}-, the initial products of the hydrolysis of β-casein by chymosin are probably bitter and thus the limited hydrolysis of this protein may contribute to bitterness. NaCl concentration has a major effect on the hydrolysis of β-casein by chymosin in solution (Fox and Walley, 1971) and in cheese (Kelly, 1993), and thus may also be a factor in the development of bitterness. Kelly (1993) found the formation of the peptide, β-casein f193-209 (the primary product of chymosin action on β-casein and potentially bitter), was inhibited by increasing NaCl concentrations. The primary action of plasmin on β-casein probably does not result in the formation of bitter peptides. The fate of α_{s2}-casein in cheese is unclear, but plasmin can release

potentially bitter peptides from this protein in solution (Le Bars and Gripon, 1989). Guigoz and Solms (1974) found the bitter dipeptide α_{s1}-CN f198-199 in Alpkäse cheese. Stepaniak and Fox (1993) demonstrated the production of this peptide from α_{s1}-CN f165-199 by the action of a lactococcal endopeptidase (LEP III-I).

In addition to peptides, a number of other subsances can contribute to bitterness in cheese, including amino acids, amines, amides, substituted amides, long-chain ketones, some monoglycerides and probably other compound (Adda *et al.*, 1982).

The origin of "unclean" and related flavours in Cheddar was investigated by Dunn and Lindsay (1985). These authors quantified a number of Strecker-type compounds, including phenylacetaldehyde, phenethanol, 3-methyl butanol, 2-methyl propanol, phenol and *p*-cresol. Phenylacetaldehyde concentrations were elevated in cheeses with an "unclean-rosy" off-flavour and the addition of this compound to clean-flavoured mild Cheddar reproduced this defect. At higher concentrations (> 500 ppb), phenylacetaldehyde contributed astringent, bitter and stinging flavours to cheese. Concentrations of phenethanol were similar in most of the cheeses studied (*ca.* 100 ppb), which was considered to be low. *p*-Cresol was found to impart an "utensil"-type flavour when present at high concentrations. Dunn and Lindsay (1985) also discussed the potential of short chain fatty acids to potentiate the flavour impact of *p*-cresol. Branched chain Strecker-type aldehydes (3-methyl butanal, 2-methyl butanal and 2-methyl propanal) were found not to cause flavour defects when added to clean-flavoured cheese at concentrations below 200 ppb. Phenol was found not to contribute to the unclean flavour of cheese and indeed phenol enhanced the sharpness of Cheddar flavour. Dunn and Lindsay (1985) included quinine in cheese to simulate the bitterness often associated with the Strecker-type compounds and this addition enhanced the perception of these compounds.

6. REFERENCES

Adda, J., Gripon, J.-C. and Vassal, L., 1982, *Food Chem.* **9**, 115.

Aimutis, W. R. and Eigel., W. N., 1982, *J. Dairy Sci.* **65**, 175.

Andrews, A.T. and Alichanidis, E., 1983, *J. Dairy Res.* **50**, 275.

Aston, J.W. and Dulley, J.R., 1982, *Aust. J. Dairy Technol.* **37**, 59.

Aston, J. W, Durward, I. G. and Dulley, J. R, 1983, *Aust. J. Dairy Technol.* **38**, 55.

Aston, J. W. and Creamer, L. K., 1986, *N. Z. J. Dairy Sci. Technol.* **21**, 229.

Baankreis, R., 1992, *The Role of Lactococcal Peptidases in Cheese Ripening*, Ph.D. thesis, University of Amsterdam.

Babcock, S. M. and Russell, H. L., 1897, 14th Ann. Report. Wisc. Agr. Exp. Stat., p. 161.

Banks, J., Muir, D. D., Brechany, E. Y. and Law, A. J. R., 1992, In Proc. 3rd Cheese Symposium, Moorepark, Fermoy, Co. Cork, Ireland.

Barlow, I., Lloyd, G. T., Ramshaw, E. H., Miller, A. J. and McCabe, G. P., 1989, *Aust. J. Dairy Technol.* **44**, 7.

Bernback, S., Hernell, O., Blackberg, L., 1985, *Eur. J. Biochem.* **148**, 233.

Bhowmik, T. and Marth, E.H., 1989, *J. Dairy Sci.* **72**, 2869.

Bhowmik, T. and Marth, E.H., 1990a, *J. Dairy Sci.* **73**, 859.

Bhowmik, T. and Marth, E.H., 1990b, *J. Dairy Sci.* **73**, 33.

Biede, S. L., 1977, *A Study of the Chemical and Flavour Profiles of Swiss Cheese*, Ph.D. thesis, Iowa State Univ., cited from Adda, J., Gripon, J.-C. and Vassal, L., 1982, *Food Chem.* **9**, 115.

Biede, S.L. and Hammond, E.G., 1979a, *J. Dairy Sci.* **62**, 238.

Biede, S.L. and Hammond, E.G., 1979b, *J. Dairy Sci.* **62**, 227.

Bills, D.D. and Day, E.A., 1964, *J. Dairy Sci.* **47**, 733.

Bines, V. E., Young, P. and Law, B. A., 1989, *J. Dairy Res.* **56**, 657.

Booth, M., Donnelly, W. J., Ní Fhaoláin, I., Jennings, P. V. and O'Cuinn, G., 1990a, *J. Dairy Res.* **57**, 79.

Booth, M., Ní Fhaoláin, I., Jennings, P.V. and O'Cuinn, G., 1990b, *J. Dairy Res.* **57**, 89.

Booth, M., Jennings, P.V., Ní Fhaoláin, I. and O'Cuinn, G., 1990c, *J. Dairy Res.* **57**, 245.

Bosman, B.W., Tan, P.S.T. and Konings, W.N., 1990, Appl. Environ. Microbiol. **56**, 1839.

Broome, M. C., Krause, D. A. and Hickey, M. W., 1990, *Aust. J. Dairy Technol.* **45**, 67.

Broome, M. C., Krause, D. A. and Hickey, M. W., 1991, *Aust. J. Dairy Technol.* **46**, 6.

Carles, C. and Ribadeau-Dumas, B., 1984, *Biochemistry* **23**, 6839.

Carles, C. and Ribadeau-Dumas, B., 1985, *FEBS Lett.* **185**, 282.

Cerning, J., Gripon, J. C., Lambert, G. and Lenoir, J., 1987, *Lait* **67**, 3.

Chapman, H.R., Mabbitt, L.A. and Sharpe, M.E., 1966, Proc. XVII Intern. Dairy Congr. (Munich) **D**, 55.

Chapot-Chartier, M.-P., Nardi, M., Chopin, M.-C., Chopin, A. and Gripon, J.-C., 1993, *Appl. Environ. Microbiol.* **59**, 330.

Christie, W.W., 1983, In: *Developments in Dairy Chemistry - 2 - Lipids*, P.F. Fox, ed., p 1. Applied Science Publishers, London.

Clegg, K. M., Lim, C. L and Manson, W., 1974, *J. Dairy Res.* **41**, 283.

Cousins, C. M., Sharpe, M. E and Law, B. A., 1977, *Dairy Ind. Int.* **42**, 12.

Creamer, L. K., 1976a, *N. Z. J. Dairy Sci. Technol.* **11**, 30.

Creamer, L. K., 1976b, *N. Z. J. Dairy Sci. Technol.* **11**, 130.

Creamer, L.K. and Olson, N.F., 1982, *J. Food Sci.* **47**, 631.

Czulak, J., 1959, *Aust. J. Dairy Technol.* **14**, 177.

Dartley, C. K. and Kinsella, J. E., 1971, *J. Agr. Food Chem.* **19**, 771.

Deeth, H. C. and Fitz-Gerald, C. H., 1983, *Aust. J. Dairy Technol.* **31**, 53.

de Jong, L., 1976, *Neth. Milk Dairy* J. **30**, 242.

de Jong, L., 1977, *Neth. Milk Dairy J.* **31**, 314.

Desmazeaud, M.J., Gripon, J.-C., Le Bars, D. and Bergere, J.L., 1976, *Lait* **56**, 379.

Desmazeaud, M. J. and Zevaco, C., 1977, *Ann. Biol. Anim. Bioch. Biophys.* **17**, 723.

Desmazeaud, M. J. and Zevaco, C., 1979, *Milchwissenschaft* **34**, 606.

Dimick, P.S., Walker, N.J. and Patton, S., 1969, *Biochem. J.* **111**, 395.

Dimos, A., 1992, *A Comparative Study by GC/MS of the Flavour Volatiles Produced During the Maturation of Full-Fat and Low-Fat Cheddar Cheese.* M.Sc. Thesis, Latrobe Univ., Bundoora, Victoria, Australia, cited from Urbach, G., 1993, *Intern. Dairy J.* (in press).

Docherty, A. J. P., Bodiner, M. W., Angal, S., Verger, R., Riviere, C., Lowe, P. A., Lyons, A., Emtage, J. S. and Harris, T. J. R., 1985, *Nucleic Acid Res.* **13**, 1891.

Downey, W. K., 1969, *J. Soc. Dairy Technol.* **22**, 154.

Driessen, F.M., 1989, In: *Heat-induced Changes in Milk*, Bull. 238. International Dairy Federation, Brussels.

Dunn, H. C. and Lindsay, R. C., 1985, *J. Dairy Sci.* **68**, 2859.

Dunn, B. M., Valler, M. J., Rolph, C. E., Foundling, S. I., Jimenez, M. and Kay, J., 1987, *Biochim. Biophys. Acta* **913**, 122.

Eigel, W. N., 1977a, *Int. J. Biochem.*, **8**, 187.

Eigel, W. N., 1977b, *J. Dairy Sci.* **60**, 1399.

Eigel, W. N., 1981, *Int. J. Biochem.*, **13**, 1081.

Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M., Jr., Harwalkar, V. R., Jenness, R. and Whitney, R. McL., 1984, *J. Dairy Sci.* **67**, 1599.

El-Soda, M., Bergere, J.L. and Desmazeaud, M.J., 1978, *J. Dairy Res.* **45**, 519.

El-Soda, M., El-Wahab, H.A., Ezzat, N., Desmazeaud, M.J. and Ismail, A., 1986, *Lait* **66**, 431.

El-Soda, M., Macedo, A. and Olson, N.F., 1991, *Milchwissenschaft* **46**, 223.

Eriksen, S., 1975, *Milchwissenschaft* **31**, 549.

Exterkate, F.A., 1975, *Neth. Milk Dairy J.* **29**, 303.

Exterkate, F. A., 1977, *J. Bacteriol*, **129**, 1281.

Exterkate, F.A. and de Veer, G.J.C.M., 1987, *Appl. Environ. Microbiol.* **53**, 577.

Exterkate, F. A., 1990, *Appl. Microbiol. Biotechnol.* **33**, 401.

Exterkate, F. A., Alting, A. C. and Slangen, C. J., 1991, *Biochem. J.* **273**, 135.

Exterkate, F. A., Alting, A. C and Engels, W. J., 1992, *Conversion of the (1-23)-fragment of α_{s1}-casein in cheese*, report, NIZO, Ede, the Netherlands.

Fan, T. Y., Hwang, D. H. and Kinsella, J. E., 1976, *J. Agr. Food Chem.* **24**, 443.

Farkye, N.Y. and Fox, P.F., 1990, *J. Dairy Res.* **57**, 413.

Farkye, N.Y. and Fox, P.F., 1991, *J. Agric. Food Chem.* **39**, 786.

Fish, J. S., 1957, *Nature* **180**, 345.

Flynn, A., 1992, In Proc. 3rd. Cheese Symp., Moorepark, Fermoy, Co. Cork., Ireland., 28-29 Oct. 1992.

Foda, E. A., Hammond, E. G., Reinhold, G. W. and Hotchkiss, D. K., 1974, *J. Dairy Sci.* **57**, 1137.

Foissy, H., 1974, *J. Gen. Microbiol.* **80**, 197.

Foissy, H., 1978, *Milchwissenschaft* **33**, 221. 223.

Fox, P. F., 1969, *J. Dairy Res.* **36**, 427.

Fox, P.F. and Walley, B.F., 1971, *J. Dairy Res.* **38**, 165.

Fox, P.F., 1988/89, *Food Biotechnol.* **2**, 133.

Fox, P.F., 1989, *J. Dairy Sci.* **72**, 1379.

Fox, P.F., Cogan, T.M. and Lucey, J.A., 1990, *CRC Crit. Rev. Food Sci. Technol.* **29**, 237.

Fox, P. F. and Law, J., 1991, *Food Biotechnol.* **5**, 239.

Fox, P. F., Law, J., McSweeney, P. L. H. and Wallace, J., 1993, In: *Cheese: Chemistry, Physics and Microbiology*, 2nd. edn., vol. 1, P. F. Fox (ed.), Elsvier Applied Publishers Ltd., London.

Fryer, T., Reiter, B. and Lawrence, R.C.,1967, *J. Dairy Sci.* **50**, 388.

Geis, A., Bockelmann, W. and Teuber, M., 1985, *Appl. Microbiol. Biotechnol.* **23**, 79.

Gordon, W. G., Groves, M. L., Greenberg, R., Jones, S. B., Kalan, E. B., Peterson, R. F. and Townend, R. E., 1972, *J. Dairy Sci.* **55**, 261.

Green, M.L., 1977, *J. Dairy Res.* **44**, 159.

Green, M. L. and Foster, P. D. M., 1974, *J. Dairy Res.* **41**, 269.

Green, M. L., Angal, S., Lowe, P. A. and Marston, F. A. O., 1985, *J. Dairy Res.* **52**, 281.

Grieve, P. A. and Kitchen, B. J., 1985, *J. Dairy Res.* **52**, 101.

Griffith, R., Hammond, E. G., 1989, *J. Dairy Sci.* **72**, 604.

Gripon, J.-C., 1987, In: *Cheese: Chemistry, Physics and Microbiology*, 1st edn., vol. 2, p. 121, P. F. Fox (ed.), Elsiver Applied Science Publishers Ltd., London.

Gripon, J.-C., Monnet, V., Lambert, G and Desmazeaud, M. J., 1991, In Food Enzymology, vol. 1, p. 131, P. F. Fox (ed.), Elsevier Applied Science Publishers, London.

Gripon, J.-C., 1993, In *Cheese: Chemistry, Physics and Microbiology*, 2nd edn., P. F. Fox (ed.), Elsevier Appleid Science Publishers, London (in press).

Grufferty, M.B. and Fox, P.F., 1988, *J. Dairy Res.* **55**, 609.

Guéguen, M. and Lenoir, J., 1975, *Lait* **55**, 145.

Guigoz, Y. and Solms, J., 1974, *Lebensmittelwiss. Technol.* **7**, 356.

Hall, D. M. and Creamer, L. K., 1972, *N. Z. J. Dairy Sci. Technol.* **7**, 95.

Harboe, M. K., 1992, In: Bulletin 269, p.3, Intern. Dairy Fed., Brussels.

Harper, W. J., Carmona de Catril, A. and Chen, J. L., 1980, *Milchwissenschaft*, **35**, 129.

Harper, J.W., Hemmi, K. and Powers, J.C., 1985, *Biochemistry* **24**, 1831.

Harwalker, V. R. and Elliott, J. A., 1971, *J. Dairy Sci.* **54**, 8.

Hawke, J. C., 1966, *J. Dairy Res.* **33**, 225.

Hemme, D., Bouillanne, C., Metro, F. and Desmazeaud, M.J., 1982, *Sci. Aliments* **2**, 113.

Hicks, C. L., O'Leary, J. and Bucy, J., 1988, *J. Dairy Sci.* **71**, 1127.

Hill, R. D. and van Leeuwen, H., 1974, *Aust. J. Dairy Technol.*, **29**, 33.

Hill, R. D., Lahav, E. and Givol, D., 1974, *J. Dairy Res.* **41**, 147.

Horwood, J. F., 1974, *Aust. J. Dairy Technol.* **35**, 13.

Huffman, L.M. and Kristoffersen, T., 1984, *N.Z. J. Dairy Sci. Technol.* **19**, 151.

Hwang, I.K., Kaminogawa, S. and Yamauchi, K., 1981, *Agric. Biol. Chem.* **45**, 159.

IDF 1991, *Rheological and Fracture Properties of Cheese*, Bulletin 268, Intern. Dairy Fed., Brussels.

Jolly, R.C. and Kosikowski, F.V., 1975, *J. Agric. Food Chem.* **23**, 1175.

Joosten, H. M. L. J. and van Boekel, M. A. J. S., 1988, *Neth. Milk Dairy J.* **42**, 3

Kamaly, K. M., El Soda, M. and Marth, E. H., 1988, *Milchwissenschaft*, **43**, 346.

Kamaly, K.M., Takayama, K. and Marth, E.M., 1990, *J. Dairy Sci.* **73**, 280.

Kaminogawa, S. and Yamauchi, K., 1972, *Agr. Biol. Chem.* **36**, 2351.

Kaminogawa, S. and Yamauchi, K., 1978 Proc. 20th Intern. Dairy Congr., Paris, p. 313.

Kaminogawa, S., Yamauchi, K., Miyazawa, S. and Koga, Y., 1980, *J. Dairy Sci.* **63**, 710.

Kaminogawa, S., Ninomiya, T. and Yamauchi, K., 1984a, *J. Dairy Sci.* **67**, 2483.

Kaminogawa, S., Ninomya, T and Yamauchi, K., 1984b, *Agric. Biol. Chem.* **48**, 3035.

Kaminogawa, S., Yan, T.R., Azuma, N. and Yamauchi, K., 1986, *J. Food Sci.* **51**, 1253.

Kelly, M. 1993, *The Effect of Salt and Moisture Contents on Proteolysis in Cheddar Cheese During Ripening*, M.Sc. thesis, National University of Ireland, Cork.

Khalid, N.M. and Marth, E.H., 1990a, *J. Dairy Sci.* **73**, 2669.

Khalid, N. M. and Marth, E. H., 1990 b, *J. Dairy Sci.* **73**, 3068.

Khalid, N.M., El-Soda, M. and Marth, E.H., 1990, *J. Dairy Sci.* **73**, 2711.

Kiefer-Partch, B., Bockelman, W., Geis, A. and Teuber, M., 1989, *Appl. Microbiol. Biotechnol.* **31**, 75.

Kinsella, J.E. and Hwang, D.M., 1976, *CRC Crit. Rev. Food Sci. Nutr.* **8**, 191.

Kleter, G., 1976, *Neth. Milk Dairy J.* **30**, 254.

Kleter, G. and de Vries, Tj., 1974, *Neth. Milk Dairy J.* **28**, 212.

Kok, J., Leenhouts, K.J., Haandrikman, A.J. and Ledeboer, A.M. and Venema, G., 1988, *Appl. Environ. Microbiol.* **54**, 231.

Kok, J., 1990, *FEMS Microbiol. Rev.* **87**, 15.

Kolstad, J. and Law, B. A., 1985, *J. Appl. Bacteriol.* **58**, 449.

Kowalewska, J., Zelazowska, H., Babuchowski, A., Hammond, E.G., Glatz, B.A. and Ross, F., 1985, *J. Dairy Sci.* **68**, 2165.

Kristoffersen, T., 1973, *J. Agric. Food Chem.* **21**, 573.

Kristoffersen, T., 1985, *Milchwissenschaft* **40**, 197.

Langsrud, T., Reinbold, G.W. and Hammond, E.G., 1977, *J. Dairy Sci.* **60**, 1.

Langsrud, T., Reinbold, G.W. and Hammond, E.G., 1978, *J. Dairy Sci.* **61**, 303.

Law, B. A., 1978, *J. Gen. Microbiol.* **105**, 113.

Law, B. A., 1979, *J. Appl. Bacteriol.* **46**, 455.

Law, B.A., 1984, In: *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*, F.L. Davies and B.A. Law, eds, Elsevier Science Publishers, London, p 187.

Law, B.A., 1987, In: *Cheese: Chemistry, Physics and Microbiology*, vol. 1, P.F. Fox (ed.), Elsevier Applied Science, London, p 365.

Law, B.A. and Sharpe, M.E., 1978, *J. Dairy Res.* **45**, 267.

Lawrence, R.C., Fryer, T.F. and Reiter, B., 1967, *J. Gen. Microbiol.* **48**, 401.

Lawrence, R. C., Creamer, L. K., Gilles, J. and Martley, F. G., 1972, *N. Z. J. Dairy Sci. Technol.* **7**, 32.

Lawrence, R.C. Gilles, J. and Creamer, L.K., 1983, *N.Z. J. Dairy Sci. Technol.* **18**, 175.

LeBars, D., Desmazeaud, M.J., Gripon, J.-C. and Bergere, J.L., 1975, *Lait* **55**, 377.

LeBars, D. and Gripon, J.-C., 1989, *J. Dairy Res.* **56**, 817.

Lee, H. J., Olson, N. F. and Ryan, D. S., 1980, *J. Dairy Sci.* **63**, 1834.

Lloyd, R. J. and Pritchard, G. G., 1991, *J. Gen. Microbiol.* **137**, 49.

Mabbitt, L.A., Chapman, H.R. and Sharpe, M.E., 1959, *J. Dairy Res.* **26**, 105.

Manning, D.J., 1974, *J. Dairy Res.* **41**, 81.

Manning, D.J., 1979a, *J. Dairy Res.* **46**, 531.

Manning, D.J., 1979b, *J. Dairy Res.* **46**, 523.

Manning, D. J. and Robinson, H. M., 1973, *J. Dairy Res.* **40**, 63.

Manning, D. J., Chapman, H. R. and Hosking, Z. D., 1976, *J. Dairy Res.* **43**, 313.

Manning, D. J. and Price, J. C., 1977, *J. Dairy Res.* **44**, 357.

Manning, D. J. and Moore, C., 1979, *J. Dairy Res.* **46**, 539.

McGugan, W.A., 1975, *J. Agric. Food Chem.* **23**, 1047.

McGugan, W.A., Emmons, D.B. and Larmond, E., 1979, *J. Dairy Sci.* **62**, 398.

McSweeney, P. L. H., Olson, N. F and Fox, P. F., 1992, *J. Dairy Sci.* **75** (Suppl. 1), 121 (Abstr.).

Monnet, V., Le Bars, D. and Gripon, J.-C., 1986, *FEMS Microbiol. Lett.* **36**, 127.

Monnet, V., Bockelmann, W., Gripon, J. C. and Teuber, M., 1989, *Appl. Microbiol. Biotechnol.* **31**, 112.

Monnet, V., Ley, J. P. and Gonzàlez, S., 1992, *Int. J. Biochem.* **24**, 707.

Mottar, J. F., 1989, In: *Enzymes of Psychrotrophs of Raw Foods*, p. 227, R. C. McKellar (ed.), CRC Press, Boca Raton, FL, USA.

Mou, L., Sullivan, J.J. and Jago, G.R., 1975, *J. Dairy Res.* **42**, 147.

Mulvihill, D.M. and Fox, P.F., 1977, *J. Dairy Res.* **44**, 533.

Mulivhill, D.M. and Fox, P.F., 1978, *Ir. J. Food Sci. Technol.* **2**, 135.

Mulvihill, D.M. and Fox, P.F., 1979, *J. Dairy Res.* **46**, 641.

Mulvihill, D.M., Collier, T.M. and Fox, P.F., 1979, *J. Dairy Sci.* **62**, 1567.

Muset, G., Monnet, V. and Gripon, J.-C., 1989, *J. Dairy Res.* **56**, 765.

Nelson, J.H., Jensen, R.G. and Pitas, R.E., 1977, *J. Dairy Sci.* **60**, 327.

Neviani, E., Boquien, C.Y., Monnet, V., Phan Thanh, L. and Gripon, J.-C., 1989, *Appl. Environ. Microbiol.* **55**, 2308.

Niven, G. W., 1991, *J. Gen. Microbiol.* **137**, 1207.

Noel, Y., Lefier, D., 1991, Bulletin 268, p. 44, Intern. Dairy Fed., Brussels.

Noomen, A., 1977, *Neth. Milk Dairy J.* **31**, 75.

Noomen, A., 1978, *Neth. Milk Dairy J.* **32**, 26.

Noomen, A., 1983, *Neth. Milk Dairy J.* **37**, 229.

Nuñez, M., Medina, M., Gaya, P., Guillen, A. M. and Rodríguez-Marín, M. A., 1992, *J. Dairy Res.* **59**, 81.

Ohren, J. A. and Tuckey, S. L., 1969, *J. Dairy Sci.* **52**, 598.

O'Keeffe, A.M., Fox, P.F. and Daly, C., 1977, *J. Dairy Res.* **44**, 335.

O'Keeffe, A.M., Fox, P.F. and Daly, C.,1978, *J. Dairy Res.* **45**, 465.

O'Keeffe, R.B., Fox, P.F. and Daly, C., 1975, *J. Dairy Res.* **42**, 111.

O'Keeffe, R.B., Fox, P.F. and Daly, C., 1976a, *Ir. J. Agric. Res.* **15**, 151.

O'Keeffe, R.B., Fox, P.F. and Daly, C., 1976b, *J. Dairy Res.* **43**, 97.

O'Leary and Fox, 1973, *Ir. J. Agric. Res.* **12**, 267.

Olivecrona, T. and Bengtsson-Olivecrona, G., 1991, In: *Food Enzymology*, vol. 1, P.F. Fox (ed.), p 63, Elsevier Applied Science, London.

Olivecrona, T., Vilaró, S. and Bengtsson-Olivecrona, G., 1992, In: *Advanced Dairy Chemistry - 1 - Proteins*, P.F. Fox, ed., Elsevier Applied Science, London, p. 292.

Olson, N. F., 1990, *FEMS Microbiol. Rev.* **87**, 131.

O'Sullivan, M. and Fox, P. F., 1991, *Food Biotechnol.*, **5**, 19.

Oterholm, A., Ordal, Z.J. and Witter, L., 1970, *Appl. Microbiol.* **20**, 16.

Oterholm, A., Witter, L.D. and Ordal, Z.J.,1972, *J. Dairy Sci.* **55**, 8.

Pahakala, E., Pihlanto-Leppälä, A., Laukkanen, M. and Antila, V., 1989, *Meijeritietleenllinen Aikakauskirja* **47**, 39.

Patton, S., 1950, *J. Dairy Sci.* **33**, 680.

Paulsen, P. V., Kowalewska, J., Hammond, E. G. and Glatz, B. A.,, 1980, *J. Dairy Sci.* **63**, 912.

Pelissier, J. P., Mercier, J. C., Ribadeau-Dumas, B., 1974, *Ann. Biol. Anim. Biochem. Biophys.* **14**, 343.

Perry, K.D. and McGillivray, W.A., 1964, *J. Dairy Res.* **31**,155.

Peterson, M. H. and Johnson, M. J., 1949, *J. Bacteriol.* **58**, 701.

Peterson, S.D. and Marshall, R.T., 1990, *J. Dairy Sci.* **73**, 1395.

Phelan, J. A., 1986, Milk Coagulants: *An Evaluation of Alternative to Standard Calf Rennet*, Ph.D. thesis, National University of Ireland, Cork.

Piatkiewicz, A., 1987, *Milchwissenschaft* **42**, 561.

Prentice, J. H., 1987, In: *Cheese, Chemistry Physics and Microbiology*, 1st edn., vol. 1, p. 299, P. F. Fox (ed.), Elsevier Applied Science Publishers, London.

Prentice, J. H. and Marshall, R. J., 1993, In: *Cheese, Chemistry Physics and Microbiology*, 2nd Ed., Vol. 1, P. F. Fox (ed.), Elsevier Applied Science Publishers Ltd., London, (in press).

Reid, J. R., Moore, C. H., Midwinter, G. G. and Pritchard, G. G., 1991a, *Appl. Microbiol. Biotechnol.* **35**, 222.

Reid, J. R., Ng, K. H., Moore, C. H., Coolbear, T. and Pritchard, G. G., 1991b, *Appl. Microbiol. Biotechnol.* **36**, 344.

Reimerdes, E. H., 1983, *J. Dairy Sci.* **66**, 1591.

Reiter, B., Fryer, T.F. and Sharpe, M.E., 1966, *J. Appl. Bacteriol.* **29**, 231.

Reiter, B., Fryer, T. F., Pickering, A., Chapman, H. R., Lawrence, R. C. and Sharpe, M. E., 1967, *J. Dairy Res.* **34**, 257.

Reiter, B., Sorokin, Y., Pickering, A. and Hall, A.J., 1969, *J. Dairy Res.* **36**, 65.

Rothe, G. A. L., Harboe, M. K. and Martiny, S. C., 1977, *J. Dairy Res.* **44**, 73.

Singh, A., Srinivasan, R. A. and Dudani, A. T., 1973, *Milchwissenschaft* **28**, 164.

Singh, J. B., Ranaganathan, B. and Chandler, H., 1981, *Milchwissenschaft* **36**, 742.

Smid, E.J., Poolman, B. and Konings, W.N., 1991, *Appl. Environ. Microbiol.* **57**, 2447.

Sørhaug, T., 1981, *Milchwissenschaft* **36**, 137.

Sørhaug, T. and Ordal, Z.J., 1974, *Appl. Microbiol.* **25**, 607.

Stadhouders, J., 1962, Proc. XVI Intern. Dairy Congr., Copenhagen, **B**, 353.

Stadhouders, J. and Veringa, H. A., 1973, *Neth. Milk Dairy J.* **27**, 77.

Stadhouders, J. and Hup, G., 1975, *Neth. Milk Dairy J.* **29**, 335.

Steele, J. L. and Ünlü, G., 1992, *Food Technol.* **46** (11), 128.

Stepaniak, L. and Fox, P. F., 1993, Proc. 29th Norwegian Biochemical Soc. Meeting, Beito, Jan. 21-24, 1993, p. 97.

Sullivan, J. J., Jago, G. R., 1972, *Aust. J. Dairy Technol.* **27**, 98.

Sweet, B. J., Mathews, L. C. and Richardson, T., 1984, *Arch. Biochem. Biophys.* **234**, 144.

Tan, P. S. T, 1992, *The Biochemical, Genetic and Physiological Properties of Aminopeptidase N from* Lactococcus lactis, Ph.D. thesis, University of Groningen.

Tan, P.S.T. and Konings, W.N., 1990, *Appl. Environ. Microbiol.* **56**, 526.

Tan, P. S. T., Kos, K. M. and Konings, W. N., 1991, *Appl. Environ. Microbiol.* **57**, 3539.

Teuber, M., 1990, In IDF Bull. 251, p. 3, Inetrn. Dairy Fed., Brussels.

Trieu-Cout, P. and Gripon, J. C., 1981, *Neth. Milk Dairy J.* **35**, 353.

Trieu-Cuot, P. and Gripon, J.-C., 1982, *J. Dairy Res.* **49**, 501.

Trieu-Cuot, P., Archiere-Haze, M. J. and Gripon, J. C., 1982, *J. Dairy Res.* **49**, 487.

Turner, K.W., Morris, H.A. and Martley, F.G., 1983, *N.Z. J. Dairy Sci. Technol.* **18**, 117.

Turner, K.W., Lawrence, R.C. and Lelievre, J., 1986, *N.Z. J. Dairy Sci. Technol.* **21**, 249.

Tzanetakis, N. and Lipopoulou-Tzanetaki, E., 1989, *J. Dairy Sci.* **72**, 859.

Umemoto, Y., Sato, Y. and Kito, J., 1968, *Agric. Biol. Chem.* **42**, 227.

Urbach, G., 1993, *Intern. Dairy J.* (in press).

van Boven, A., Tan, P.S.T. and Konings, W.N., 1988, *Appl. Environ. Microbiol.* **54**, 43.

van den Berg, G. and de Koning, P. J., 1990, *Neth. Milk Dairy J.* **44**, 189.

Verdi, R. J. and Barbano, D. M., 1991, *J. Dairy Sci.* **74**, 2077.

Visser, F.M.W., 1976, *Neth. Milk Dairy J.* **30**, 41.

Visser, F.M.V., 1977a, *Neth. Milk Dairy J.* **31**, 120.

Visser, F.M.W., 1977b, *Neth. Milk Dairy J.* **31**, 188.

Visser, F.M.W., 1977c, *Neth. Milk Dairy J.* **31**, 210.

Visser, F.M.W. and de Groot-Mostert, A.E.A., 1977, *Neth. Milk Dairy J.* **31**, 247.

Visser, S., 1981, *Neth. Milk Dairy J.* **35**, 65.

Visser, S. and Slangen, K.J., 1977, *Neth. Milk Dairy J.* **31**, 16.

Visser, S., Exterkate, F. A., Slangen, C. J. and de Veer,G. J. C. M., 1986, *Appl. Environ. Microbiol.* **52**, 1162.

Visser, S., Slangen, C. J., Exterkate, F. A. and de Veer, G. J. C. M., 1988, *Appl. Microbiol. Biotechnol.* **29**, 61.

Visser, S., Slangen, K.J., Alting, A.C. and Vreeman, H.J., 1989, *Milchwissenschaft* **44**, 335.

Visser, S., Robben, A. J. P. M. and Slangen, C. J., 1991, *Appl. Microbiol. Biotechnol.* **35**, 477.

Vos, P., Simons, G., Siezen, R.J. and de Vos, W.M., 1989a, *J. Biol. Chem.* **264**, 13579.

Vos, P., van Asseldonk, M., van Jeveren, F., Siezen, R., Simons, G. and de Vos, W.M., 1989b, *J. Bacteriol.* **171**, 2795.

Vreeman, H. J., Visser, S., Slangen, C. J. and van Reel, J. A. M., 1986, *Biochem. J.* **240**, 87.

Weinstein, M. J. and Doolittle, R. F., 1972, *Biochim. Biophys. Acta* **258**, 577.

Wong, N. P., Ellis, R., La Croix, D. E. and Alford, J. A., 1973, *J. Dairy Sci.* **56**, 636.

Wong, N.P., Ellis, R. and La Croix, D.E., 1975, *J. Dairy Sci.* **58**, 1437.

Woo, A. H. and Lindsay, R. C., 1984, *J. Dairy Sci.* **67**, 960.

Woo, A. H., Kollodge, S. and Lindsay, R. C., 1984, *J. Dairy Sci.* **67**, 874.

Yan, T.-R., Azuma, N., Kaminogawa, S. and Yamauchi, K., 1987a, *Eur. J. Biochem.* **163**, 259.

Yan, T.-R., Azuma, N., Kaminogawa, S. and Yamauchi, K., 1987b, *Appl. Environ. Microbiol.* **53**, 2296.

Zevaco, C. and Desmazeaud, M. J., 1980, *J. Dairy Sci.*, **63**, 15.

Zevaco, C., Monnet, V. and Gripon, J.-C., 1990, J. Appl. Bacteriol. **68**, 357.

3

CHEESE: METHODS OF CHEMICAL ANALYSIS†

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1. INTRODUCTION

Cheese is subjected to chemical analysis for a variety of reasons, eg., to ascertain its composition for nutritional purposes, to ensure its compliance with standards of identity, to assess the efficiency of production or as an index of quality (see ref.1). Chemical analyses are of critical importance to the dairy scientist involved in cheese research, to analysts working on quality assurance and for regulation of the production process. This chapter will review the principal methods available for the chemical analysis of cheese, with particular reference to the ripening process and to techniques used in research. As far as we are aware, the methodology used to monitor the biochemistry of cheese ripening has not been comprehensively reviewed, although some aspects have been, eg. proteolysis (2-5).

2. COMPOSITIONAL ANALYSIS

Gross compositional analysis of cheese is conducted in accordance with standard methods published by the International Dairy Federation and the Association of Official Analytical Chemists. Standard methods for moisture, ash, protein, fat, acidity and anion analysis are listed in Table 3.1 and will not be discussed further. Enzyme assays for lactose, lactic acid and citrate have been introduced recently (6-9). The enzymatic assay for lactate is particularly useful since it permits determination of the D- and L-isomers, the proportions of which provide useful information on the activity of starter and non-starter lactic acid bacteria in cheese during ripening. There does not appear to be a standard method for determining the pH of cheese. The method used in our laboratory is as follows; grated cheese (10 g) is thoroughly blended with 10 ml H_2O using a mortar and pestle and the pH of the resulting slurry measured potentiometrically. However, it may be preferable to measure the pH of the cheese directly (eg. ref 10) to minimise changes caused by alteration of the salt balance in the cheese.

Calcium can be quantified by titration with ethylenediamine tetraacetic acid (EDTA), using ammonium purpurate (murexide) as indicator or by means of oxalate precipitation, atomic absorption spectrophotometry or ion-specific electrodes (it should be noted that the later method measures calcium ion activity and not total concentration).

The concentration of Na^+ can be quantified specifically by ion-selective electrodes, atomic absorption or flame spectrophotometry.

The water activity (A_w) of cheese can be determined by a variety of methods, including psychrometry, cryoscopy, dew-point hygrometry and isopiestic equilibration. A number of regression equations have been developed to predict A_w from chemical composition for various cheeses (see refs 11 and 12). Other

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Table 3.1. Standard Methods for Compositional Analysis of Cheese.

Constituent	*Method*
Total Solids (Moisture)	IDF 4A:1982 (15) AOAC 926.08, 948.12, 969.19, 977.11 (1990) (16)
Ash	AOAC 935.42 (1990) (16)
Fat	IDF 5B:1986 (15) AOAC 933.05 (1990) (16)
Protein (Total Nitrogen)	IDF 25:1964 (15) AOAC 920.123 (1990) (16)
Chloride	IDF 88:1979 (15) AOAC 935.43, 983.14 (1990) (16)
Salt (NaCl)	AOAC 975.20 (1990) (16)
Citrate	IDF 34B:1971 (15) AOAC 976.15 (1990) (16)
Phosphorus	IDF 33C:1987 (15)
Nitrate/Nitrite	IDF 84A:1984 (15) AOAC 976.14 (1990) (16)
Acidity	AOAC 920.124 (1990) (16)

methods of gross compositional analysis have been proposed, eg. Frank and Birth (13) applied near infra-red reflectance spectroscopy to measure fat, protein, moisture and moisture in non-fatty substances in a range of cheese varieties. Cronin and McKenzie (14) quantified fat in a variety of foodstuffs, including Cottage and Cheddar cheeses, by infrared transmittance spectrophotometry of a solvent extract of cheese, measuring the carbonyl ester stretch of the triglyceride at 5.72 μm relative to a methyl silicone internal standard.

3. METHODS USED TO MONITOR CHEESE RIPENING

3.1. Assessment of Proteolysis

3.1.1. Extraction and Fractionation of Cheese Nitrogen

Introduction: Proteolysis in cheese involves a complex and dynamic series of events. It is desirable and sometimes necessary to separate the various proteins, peptides and amino acids which result from the action of the proteolytic enzymes from the milk, rennet, starter and non-starter bacteria. Various approaches have been used to extract and fractionate cheese nitrogen, as an aspect of the study of proteolysis in general and to prepare extracts for the identification and quantitation of peptides and free amino acids.

Water Soluble Extracts: Quantitation and characterization of nitrogen in a water extract of cheese is a commonly used index of cheese ripening (see ref 3) and several procedures have been developed (see ref 17). Water-soluble extracts are also frequently used for the isolation of peptides and amino acids (3).

Mabbitt (18) quantified the free amino acids in the aqueous phase of Cheddar cheese, prepared from cheese press juice by the removal of fat and proteinaceous material, by ion-exchange chromatography. In preparation for another chromatographic study of free amino acids in Cheddar, Bullock and Irvine (19) homogenized cheese samples in a Waring blendor, followed by adjustment to pH 6.2

and filtration; the filtrate was further fractionated using ethanol. Stadhouders (20) macerated Edam cheese with water in a mortar at 50°C and diluted the paste to volume, taking onto consideration the moisture content of the cheese. The homogenates were held at 25°C for 16 h and centrifuged; the supernatants were filtered and analyzed for nitrogen by the Kjeldahl method. Water soluble nitrogen was used as an index of proteolysis in aseptic cheese by Reiter *et al.* (21).

McGugan *et al.* (22) prepared a water-soluble extract of cheese by centrifugally defatting the cheese, extraction of the defatted residue with methanol/water/methylene chloride, followed by repeated water extractions of the residue. A water-soluble extract prepared according to this method was analyzed by HPLC by Pham and Nakai (23) and the procedure was also adopted by Aston and Creamer (24) who omitted extraction with methanol/water/methylene chloride.

Kuchroo and Fox (25) compared various extraction procedures for Cheddar cheese. All but one of several homogenization techniques yielded essentially similar results; a stomacher was used for routine work. Homogenization temperature had little effect on the level of extractable N, which increased with the ratio of water to cheese; a ratio of 2:1 was recommended and 90% of the potentially water-extractable N was obtained in two extractions. The final procedure recommended is: grated cheese is homogenized in a stomacher at 20°C for 10 min with twice its weight of water; the slurry is held at 40°C for 1 h, centrifuged and the supernatant filtered through glass wool and Whatman No. 113 filter paper. The residue can be re-extracted to increase the yield of extract. This procedure has been used by a number of workers (26-31). The water-soluble fraction of Cheddar cheese is very heterogeneous (30)

The preparation of a water-soluble extract is an efficient procedure for separating the small peptides from proteins and larger peptides in cheese (17).

Extraction at pH 4.6: pH 4.6-soluble N is also widely used as an index of cheese ripening. The pH of a water extract of internal bacterially ripened cheese (eg. Cheddar, Swiss, Dutch) is approximately 5.2 (4). Thus, for these cheeses there is little difference between levels of N soluble in water or in pH 4.6 buffers (25). However, for cheeses which are characterized by a significant rise in pH during ripening, water soluble N may be far higher than the levels of N soluble at pH 4.6 (4).

Dahlberg and Kosikowsky (32) extracted Cheddar with a buffer intended to maintain the pH of the cheese and to mimic the ionic composition of the aqueous phase. Kuchroo and Fox (25) reported that the level of N extracted by this method was only 30% of that in a water-soluble extract. It was suggested that this low extraction rate was due to the presence of $CaCl_2$ in the buffer, which might precipitate otherwise soluble casein-derived peptides. An extraction method using sodium citrate, in which the extract had a pH of 4.40±0.05, was proposed by Mogensen (33). This procedure was adopted by Vakaleris and Price (34) and Vakaleris *et al.* (35): cheese was dispersed in 0.5 M sodium citrate, and the pH of the dispersion adjusted to 4.4 with HCl. This approach has also been adopted by a number of other workers (36-38). Kuchroo and Fox (25) found that this method gave slightly lower values for soluble N than extraction with water. The extraction is more difficult to perform but may be easier to standardize (4). Christensen *et al.* (17) favoured this approach over that of Dahlberg and Kosikowsky (32) because of the better dispersion of cheese at pH 7.

O'Keeffe *et al.* (39) adjusted the pH of a water extract of cheese to 9.0 to inactivate rennet prior to adjustment of the pH to 4.6; some dissociation of the peptides was also achieved. This approach was also used by O'Keeffe *et al.* (40).

Extraction at pH values about the isoelectric point of casein is used to isolate small and medium sized peptides (3). O'Keeffe *et al.* (39) found that pH 4.6-soluble N was produced mainly by the activity of rennet. Proteose peptones and whey proteins are also soluble at pH 4.6, but their contribution to pH 4.6-soluble nitrogen is small. γ-Caseins are precipitated at pH 4.6 (see ref 3).

The pH 4.6-soluble fraction of cheese is very heterogeneous (25). O'Keeffe *et al.* (40) found whey proteins, proteose peptones and a variety of peptides in a pH 4.6-soluble extract. Reville and Fox (41) reported that about 20% of the total N in a 6 month old Cheddar was soluble at pH 4.6 and they concluded that fractionation at pH 4.6 was the most suitable extraction method for young cheeses.

Fractionation with $CaCl_2$: $CaCl_2$ has been used to fractionate cheese nitrogen by a number of workers. Dahlberg and Kosikowsky (32) included $CaCl_2$ at 0.02 M in their extraction buffer. This results in the recovery of only 30% of water-soluble N, presumably due to precipitation of casein-derived peptides by $CaCl_2$ (25).

Noomen (42) and Venema *et al.* (43) homogenized cheese in a $CaCl_2$ (0.137 M)/NaCl(0.684 M) solution; the homogenate was adjusted to pH 5.1, acidified with 0.25 M HCl (final pH *ca.* 1.6), held at 55°C for 30 min, then overnight at room temperature and filtered. Visser (44) homogenized cheese in a 0.55% $CaCl_2$-4% NaCl solution; the homogenate was defatted and adjusted to pH 7.5, centrifuged and the supernatant clarified by filtration.

Kuchroo and Fox (25) found that only 40% of the water soluble nitrogen was soluble in 0.1 M $CaCl_2$. Increasing the concentration of $CaCl_2$ above 0.05% at or above pH 7.0 had little influence on extraction (42). The increase in $CaCl_2$-soluble N correlates with the age of the cheese and the extracts contain whey proteins, peptides and amino acids, while the $CaCl_2$- insoluble fraction contains caseins and high molecular weight peptides, similar to those in the water-insoluble fraction (17).

Fractionation with NaCl: Fractionation of cheese N with a 5% NaCl brine was employed by Chakravorty *et al.* (45) and Gupta *et al.* (46). Reville and Fox (41) found that 93% of the total N of a 12 month old Cheddar cheese was soluble in 5% NaCl and concluded that fractionation with NaCl is suitable only for very young cheeses. NaCl (5%)-soluble N and unfractionated cheese are indistinguishable electrophoretically (41); thus α-, β- and γ-caseins are extracted as well as peptides (3).

Inclusion of $CaCl_2$ in the NaCl extraction solution reduces the percentage N extracted (see ref 4). Addition of $CaCl_2$ to 0.1 M to a water extract of cheese and adjusting the pH to 4.6 precipitates 60% of the water soluble N (25).

Christensen *et al.* (17) concluded that fractionation with 5% NaCl is not as descriminating for the fractionation of cheese N as water.

Fractionation with Chloroform/Methanol: Harwalkar and Elliott (47) extracted the bitter and astringent peptides in Cheddar with chloroform/methanol. Freeze dried cheese was blended with chloroform/methanol (2:1, v/v) and filtered. Water was added to the filtrate and the mixture allowed to separate. The chloroform layer, containing lipids, was discarded. The methanol was evaporated from the aqueous methanolic layer, resulting in the formation of a precipitate; the supernatant was very bitter and astringent. This approach was also used by Visser (48) and Visser *et al.* (49). The latter used a freeze dried water- or dilute NaCl-soluble extract as starting material. The chloroform phase was extracted with methanol/water (50:30, v/v); the extract was freed of methanol and the aqueous residue diafiltered using 500 Da membranes; the retentate contained the bitter peptides.

The chloroform/methanol procedure consistantly extracts more N than water which is understandable considering the relatively hydrophobic nature of many casein-derived peptides but both extracts yield identical chromatograms on Sephadex G-25 (3). Extraction with chloroform/methanol is useful for isolating the more hydrophobic peptides in cheese.

Butan-1-ol: Butanol has been used to extract bitter peptides from casein hydrolysates but does not appear to have been applied to the fractionation of cheese (4).

Fractionation with Trichloroacetic Acid (TCA): The protein precipitant, TCA, has been applied by a number of workers to fractionate cheese nitrogen. The concentrations used have varied from 2% or 2.5% (25, 26, 30, 41) to 12% (25, 26, 40 ,41, 43). Most authors have used TCA to fractionate water-soluble N, but O'Sullivan and Fox (30) used 2% TCA to fractionate the UF retentate of a water-soluble fraction.

Reville and Fox (41) found that about 14% of the total N of a 6 month old Cheddar cheese was soluble in 2.5% TCA. It was noted that 2.5% TCA was the best extractant for mature cheese, but other methods are recommended if further characterization of peptides is to be performed. Kuchroo and Fox (26) found that 90% of the water soluble N in Cheddar was soluble in 2% TCA while O'Sullivan and Fox (30) reported that 50-60% of a UF retentate (10,000 Da membranes) was soluble in this solvent. Ion-exchange chromatography confirmed the heterogeneity of the TCA soluble and insoluble fractions (30).

Rennet is responsible for the production of some of the12% TCA-soluble N but the N levels in this fraction are higher in cheeses acidified by starter than in chemically acidified cheeses (21, 39), indicating that starter peptidases are responsible for the formation of some of the 12% TCA soluble N. Reville and Fox (41) found that about 13% of total N in mature Cheddar was soluble in 12% TCA; this fraction, which is very heterogeneous (26), increases with age and Venema *et al.* (43) reported the ratio 12% TCA soluble N : total N is a better index of maturity than water soluble N : total N.

There is no "precipitation threshold" relating peptide size to solubility in TCA but all peptides studied by Yvon *et al.* (50) containing less than 7 amino acid residues were soluble in 12% TCA; peptide solubility is related to hydrophobicity.

A disadvantage of TCA for peptide fractionation is the necessity to remove it prior to further analysis of the fractions, eg. by chromatography or electrophoresis. Small peptides and free amino acids will be lost on dialysis. Other methods for removal of TCA include repeated ether extraction, gel permeation and ion-exchange chromatography (4). The use of 70% ethanol, which gives similar precipitation levels, is preferrable because ethanol can be readily removed by evaporation (4).

Ethanol: Precipitation of proteins and peptides by ethanol is a classical protein fractionation method and has been used extensively to fractionate cheese (see ref 17). However, various ethanol concentrations have been used, presumably with different results. Edwards and Kosikowski (51) used about 50% while Ismail and Hansen (36) extracted amino acids from a sodium citrate/HCl-soluble fraction using ethanol at 80%. Peptide fractions soluble in 2% or12% TCA were precipitated by 80% ethanol or 75% acetone acidified to pH 4.6 with HCl (52). Gonashvily (53) used 65% ethanol while Aston and Creamer (24) precipitated peptides from the water-soluble fraction of Cheddar with methanol in the ratio of 2:1, extract : methanol.

However, 70% ethanol has been used most widely (25-27, 41). Reville and Fox (41) found that 12% TCA and 70% ethanol had approximately similar extraction levels. The ethanol-soluble extracts contained a considerable amount of nitrogenous material in addition to free amino acids, but no protein bands could be detected in 70% ethanol- or 12% TCA-soluble fractions by gel electrophoresis. Material soluble in 12% TCA or 70% ethanol gave similar patterns on high voltage electrophoresis, but differences were detected in the insoluble fractions (26). The 70% ethanol-insoluble fraction contains proteins, large and medium peptides (27).

Fractionation of a UF retentate (10,000 Da) of a water-soluble extract of Cheddar using increasing concentrations of ethanol (30-80%) showed that most of the precipitable peptides were precipitated by 30% ethanol at pH 5.2; fractionation of UF retentate with 30 % ethanol at pH 6.5, followed by adjustment of the filtrate to pH 5.5 is quite effective (53a).

Ethanol precipitation may be used to fractionate cheese or to sub-fractionate water-soluble extracts and should be preferred to the largely equivalent 12% TCA owing to the ease of removal of ethanol by evaporation (17).

Phosphotungstic Acid (PTA): PTA-H_2SO_4 is a very descriminating protein precipitant. Free amino acids, except dibasic amino acids, are soluble in 5% PTA but peptides greater than about 600 Da are precipitated (54). PTA (2.5% or 5%)- soluble nitrogen has been widely used as an index of free amino acids in cheese (20, 37, 54-60). PTA-soluble nitrogen increases with age (56) and is produced primarily by the action of microbial peptidases. The free amino acid profile of the soluble fraction has been determined (54). Reiter *et al.* (21) reported that 5% PTA soluble-N constituted 3.0% of total N of Edam at 3 mth.

Sulphosalicilic Acid (SSA): SSA is a descriminating protein precipitant (4). Water-soluble extracts of cheese have been prepared for amino acid analysis by treatment with 3% SSA (21). SSA has also been used to prepare samples for amino acid analysis (61). However, Kuchroo and Fox (25) found that 2.5% SSA precipitated only 10% of the water-soluble N. The water-soluble proteins and peptides in the fractions obtained with SSA have not been characterized (see ref 17).

Picric Acid: Picric acid is supposed to be the most descriminating protein precipitant (4) and Reville and Fox (41) found that 0.85% picric acid-soluble extracts of cheese contained the lowest levels of N of the methods examined. It was considered to be the most suitable extractant for amino acids but small peptides are also soluble in picric acid (62).

However, picric acid interferes with the determination of N by Kjeldahl or spectrophotometric methods (4, 41).

$Ba(OH)_2/ZnSO_4$: Free amino acids were extracted from Cheddar by Hickey *et al.* (63) using $Ba(OH)_2/ZnSO_4$. Cheese was macerated in a 0.15 M $Ba(OH)_2$ - 0.14 M $ZnSO_4$ solution, filtered and residual fat removed by extraction with cholorform/ethanol (1:1). The aqueous phase was filtered and the filtrate freeze dried and suspended in a sodium citrate buffer prior to amino acid analysis. No data were given on extraction levels or whether peptides were soluble. This reagent does not appear to be widely used as an extractant for N from cheese.

Ethylenediaminetetraacetic Acid (EDTA): Kuchroo and Fox (26) fractionated a water-soluble extract of Cheddar with 0.1 M EDTA; approximately 30% of the water-soluble N was precipitated.

Dialysis and Ultrafiltration: While many authors have attempted to fractionate water soluble extracts of cheese by exploiting differences in the relative solubility of its components in various reagents, others have used fractionation methods based on molecular size, using dialysis or ultrafiltration.

Kuchroo and Fox (26) found that exhaustive dialysis (96 h, 4 changes) was a simple method for partitioning water soluble peptides and that it was suitable for relatively large samples. It gave clean-cut fractionation and appeared more effective than many other fractionation techniques. About 50% of the water-soluble N in a mature Cheddar was dialyzable; the diffusate was completely soluble and the retentate 50% soluble in 70% ethanol. This technique was applied by Kuchroo and Fox (28) as a step in their fractionation scheme.

Diafiltration through membranes with a nominal molecular weight cut-off of 500 Da was used by Visser *et al.* (49, 64) to remove very low molecular weight components from a bitter extract of Gouda. Aston and Creamer (24) used ultrafiltration with 1000 Da cut-off membranes to fractionate water-soluble N, while O'Sullivan and Fox (30) used 10,000 Da membranes. The 10,000 Da permeate contained no peptides detectable by PAGE and 40-50% of water-soluble N was permeable. All peptides in the permeate are soluble in 2% TCA but some peptides in the UF retentate are insoluble.

Rejection of hydrophobic peptides by UF membranes and aggregation of small peptides are potential limitations in this approach. However, UF allows fractionation

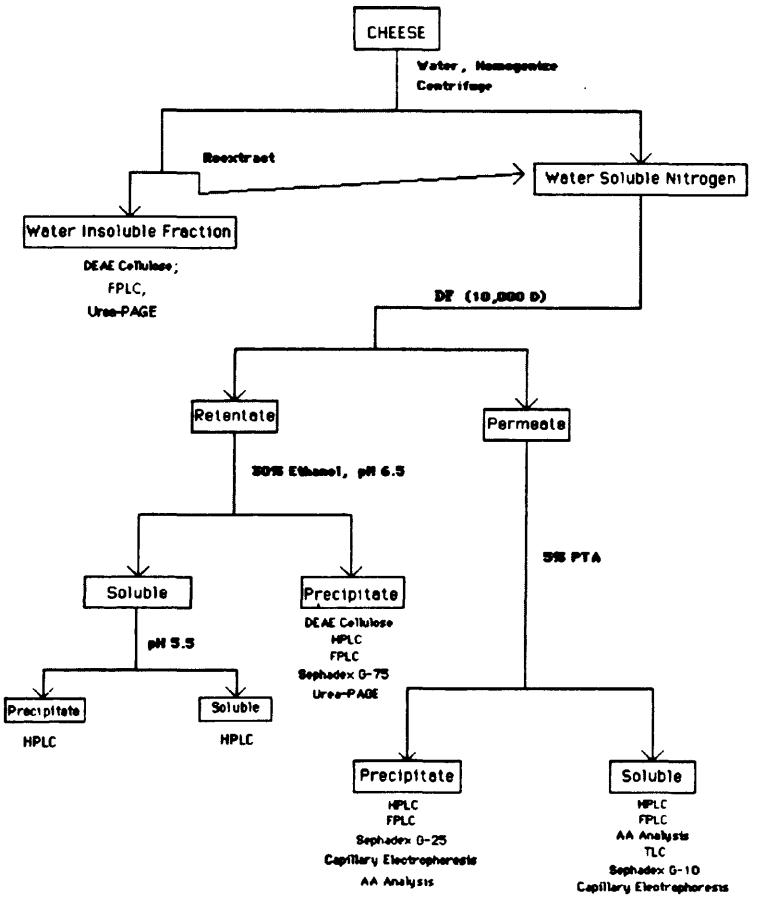

Fig. 3.1 Fractionation scheme for cheese nitrogen (adapted from ref. 53a).

of large samples and does not require solvents, both of which facilitate taste panel work (see ref 4).

Trifluoroacetic Acid/Formic Acid: Following a comparison of various extractants/precipitants for the fractionation of cheese nitrogen, Bican and Spahni (283) recommended the following procedure: homogenize cheese (2g) dispersed in 25 ml of an extractant consisting of 1% (v/v) NaCl and 1 M HCl in a Polytron-Kinematica homogenizer at 4°C. Centrifuge the homogenate at 9750 g for 30 min at 4°C. Filter the supernatant and load onto a Sep-Pak C_{18} cartridge, wash with 0.1% TFA and elute the peptides with 80% aqueous acetonitrile containing 0.1% TFA. The peptide fraction was analysed by HPLC and fingerprinting by thin-layer chromatography and high volatge electrophoresis on silica gels.

Fractionation Schemes: Since proteolysis in cheese involves a complex and dynamic series of events, it is desirable to fractionate the heterogeneous mixture of hydrolysis products to facilitate analysis. Various fractionation schemes have been proposed (4, 24, 28, 30, 53a, 288).

A modification of the fractionation scheme proposed by Fox (4) is summarized in Fig. 3.1. A water soluble extract is prepared according to the method of Kuchroo and Fox (25). The extract is diafiltered through 10,000 Da membranes, the retentate sub-fractionated by ethanol (30%) at pH 6.5 and 5.5 and the permeate by 5% PTA. All the fractions prepared by this scheme are heterogeneous and other reagents/techniques, eg. fractionation with ethanol, acetone, chloroform, PTA, SSA, gel filtration, HPLC, FPLC, preparative gel electrophoresis or TLC are required to isolate homogeneous peptides (4).

3.1.2. Methods for the Direct Measurement of Proteolysis in Cheese

Introduction: Many methods used to assess proteolysis in cheese (eg. Kjeldahl determination of the N content of various fractions) are time-consuming and therefore it is desirable to develop direct methods to estimate proteolysis. Direct approaches adopted include estimation of the tyrosine and tryptophan content of cheese fractions by absorbance at 280 nm, protein dye-binding, liberation of ammoniacal nitrogen and, most importantly, determination of free amino groups by methods such as the formol titration, reaction with TNBS, ninhydrin, OPA or fluorescamine. There is a growing demand for such rapid methods for assessing the degree of cheese maturation (65).

Formation of Ammoniacal Nitrogen: This was used as an index of proteolysis in Ulloa cheese by Ordonez and Burgos (66). Ammoniacal nitrogen levels, measured by direct Nesslerization of a non-protein N preparation, constituted about 4 mg g^{-1} dry matter and increased slightly during ripening.

Measurement of Tryptophan and Tyrosine: Measurement of the "soluble" tyrosine and tryptophan content of cheese is a well-established method for assessing proteolysis. Hull (67) used the Folin-phenol reagent to assess proteolysis in milk. When compared on 12 % TCA filtrates, the Folin reagent was less sensitive than TNBS for the assessment of proteolysis in cheese (68). Singh and Ganguli (69) also applied the Folin reagent to TCA-soluble fractions of cheese.

Vakaleris and Price (34) measured the A_{280} of a sodium citrate-HCl extract (pH 4.6) of cheese as an index of proteolysis.

Dye-Binding Methods: At about pH 2, anionic dyes, eg. amido-black, acid orange 12 and orange G, react with the positively charged lysyl and arginyl residues of proteins, leading to precipitation. The precipitate is removed by centrifugation or filtration and the excess dye measured spectrophotometrically. The amount of dye bound is proportional to the concentration of protein. However, low molecular weight proteins and peptides react only slowly, leading to poor separation and turbid filtrates or centrifugal supernatants (70).

A decrease in the dye-binding capacity of a dispersion of Cheddar cheese with age was demonstrated by Ashworth (70). Kroger and Weaver (71) reported that a dye binding method could be used as an index of proteolysis in cheese but Kuchroo *et al.* (29) concluded that the dye-binding method of McGann *et al.* (72), using amido black, is not a suitable method for assessing proteolysis in cheese.

Formol Titration: The formol titration is a simple method for estimating amino groups in milk and may be used to measure the extent and rate of proteolysis. The method involves adding a formaldehyde solution, neutralized with NaOH, to a sample of milk and titrating to the phenolphthalein end-point (73). This procedure has been used to estimate proteolysis in cheese (eg. ref 35) but it is now considered obsolete owing to difficulties caused by variations in the buffering capacity of cheese (65).

Buffering Capicity: The buffering capacity of cheese increases during ripening owing to the formation of ammonia, amino and imino groups. This principle was used by Ollikainen (74) to assess proteolysis in cheese; it was claimed that the method is rapid and convenient and as accurate as colorimetric methods.

p-*Benzoquinone:* Reaction with *p*-benzoquinone provides the basis of a colorimetric procedure for assaying peptide bond cleavage. The method of Lorenz (75) was compared by Ollikainen (74) with the above titrimetric procedure.

Trinitrobenzenesulphonic Acid: 2,4,6-Trinitrobenzenesulphonic acid (TNBS) reacts quantatively with primary amines, producing a chromophore which absorbs

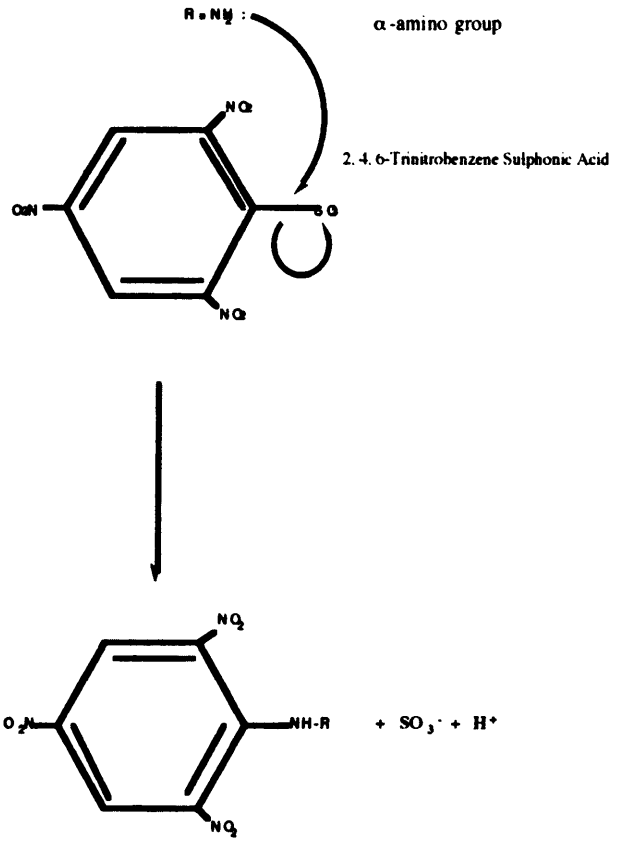

Fig. 3.2 *Reaction of 2,4,6-trinitrobenzenesulphonic acid with α-amino groups (81).*

maximally at 420 nm. The chromophore remains attached to the amino acid, peptide or protein (Fig. 3.2).

The reagent, which was introduced by Satake *et al.* (76), was used by Fields (77) to quantify amino groups in proteins and peptides. The reaction is performed at an alkaline pH and stopped by lowering the pH. TNBS also reacts slowly with the hydroxyl ion, a reaction which is catalyzed by light. Since ammoniacal nitrogen produces only 20% of the absorbance of amino groups when reacted in equimolar concentrations with TNBS (78), this reagent might underestimate proteolysis in cheeses which have undergone significant deamination (79). Clegg *et al.* (78) concluded that TNBS is not as sensitive as ninhydrin for assessing proteolysis in cheese but is preferable owing to the simple analytical procedure: they proposed a correction factor for ammoniacal nitrogen. A disadvantage of the TNBS method is that the dry powder is explosive and prolonged storage leads to high blank values (65).

Habeeb (80) and Adler-Nissen (81) measured the absorbance of the TNBS-amino group complex at 340 nm whereas in the procedure of Fields (77), the absorbance of a sulphite-TNBS-amino group complex is measured at 420 nm. Barlow *et al.* (82) reported that the latter is preferable since the TNBS-amino group complex itself gave an unstable colour complex and the procedure is liable to operator error. Alder-Nissen (81) developed a procedure to assess the degree of hydrolysis of food proteins using TNBS. A linear relationship was found between the concentration of α-amino groups and A_{420} but the relationship varied between proteins, owing to variations in the concentration of ε-amino groups.

The spectroscopic method of Hull (67) was compared with the TNBS method by Samples *et al.* (68) who found that the latter was a better index of proteolysis in cheese. Jarrett *et al.* (54) used the TNBS assay to quantify free amino acids in 5% PTA-soluble fractions of cheese. Kuchroo *et al.* (29) found that the TNBS method was reproduceable for monitoring proteolysis in cheese and concluded that while the method could be applied to unfractionated cheese, it is more sensitive if applied to pH 4.6- or 12% TCA-soluble extracts owing to a lower background colour, caused by reaction of TNBS with ε-amino groups. A strong correlation was found by Madkor *et al.* (83) between water soluble N and the A_{420} of the TNBS complex for a water-soluble extract of Stilton cheese.

Barlow *et al.* (82) considered that application of the TNBS method of Fields (77) to a water soluble extract, prepared according to Kuchroo and Fox (25), would provide a simple routine method for the determination of soluble N in cheese. Whole cheese was analyzed by Polychroniadou (84) who found a good correlation between the TNBS method and the ratio of water-soluble N to total N, as determined by the Kjeldahl procedure. A method was described for the assessment of proteolysis in whole cheese and fractionation of the cheese prior to analysis was considered to be unnecessary.

Humbert *et al.* (85) developed a new solvent (a patented mixture of organic acids and detergents) to clarify the sample after the TNBS reaction.

Ninhydrin: The use of ninhydrin to assay for proteolysis in cheese is based on the spectrophotometric estimation of the chromophore formed when ninhydrin reacts with free amino groups (Fig. 3.3.) The purple chromophore, named Ruhemann's purple after its discoverer (86), does not remain attached to the protein or peptide and thus is not precipitated during procedures necessary to clarify the sample prior to spectrophotometric analysis at 570 nm (65). This is considered to be a major advantage of the technique (79). Ninhydrin reacts with ammonia almost as readily as with amino groups and thus ninhydrin values are consistantly higher than those found by the TNBS procedure, the discrepancy being due to ammonia (78). Ninhydrin is more sensitive than TNBS, but the latter was preferred by Clegg *et al.* (78) because the analytical procedure is simpler.

Moore and Stein (87) applied the ninhydrin reaction to quantify amino acids in chromatographic eluates. The procedure was modified by Moore and Stein (88) and further modified by Moore (89) who used aqueous dimethyl sulphoxide as solvent.

Doi *et al.* (90) described a number of ninhydrin-based assays for peptidase activity. Two were modifications of the methods of Moore and Stein (87, 88) and two were based on the Cd-ninhydrin assay of Tsarichenko (91). It was found that the Cd-ninhydrin reagent was more selective for the amino group of free amino acids than the amino groups of peptides or proteins and was the most sensitive of several ninhydrin reagents, including Sn-ninhydrin.

Ninhydrin-reactive lysine in food proteins was measured by Friedman *et al.* (92) who optimized the production of chromophore. Pearce *et al.* (79) developed a Li-ninhydrin assay for proteolysis in ripening cheese based on that of Freidman *et al.* (92). Cheese was dispersed in a citrate buffer and an aliquot of this solution heated with aqueous dimethyl sulphoxide, ninhydrin and lithium acetate solutions at pH 5.2. The mixture was diluted and A_{570} determined. The method correlated well with conventional amino acid analyses.

Folkertsma and Fox (93) applied the Cd-ninhydrin assay of Doi *et al.* (90) to the assay of proteolysis in cheese; the reagent was found to be about 5 times as sensitive as TNBS for the measurement of amino acid nitrogen and could be performed on citrate-, water- or PTA-soluble fractions but not on TCA-soluble fractions as the latter appeared to interfere with colour development.

Fluorescamine: Fluorescamine (4-phenylspiro [furan-2 (3H), 1'-phthalan]-3,3'-dione), is used to quantify amino acids and peptides in the picomole range. Fluorecamine, which was introduced by Weigele *et al.* (94), reacts with primary amino groups to produce a fluorophor which is assayed at 390 nm excitation and 475

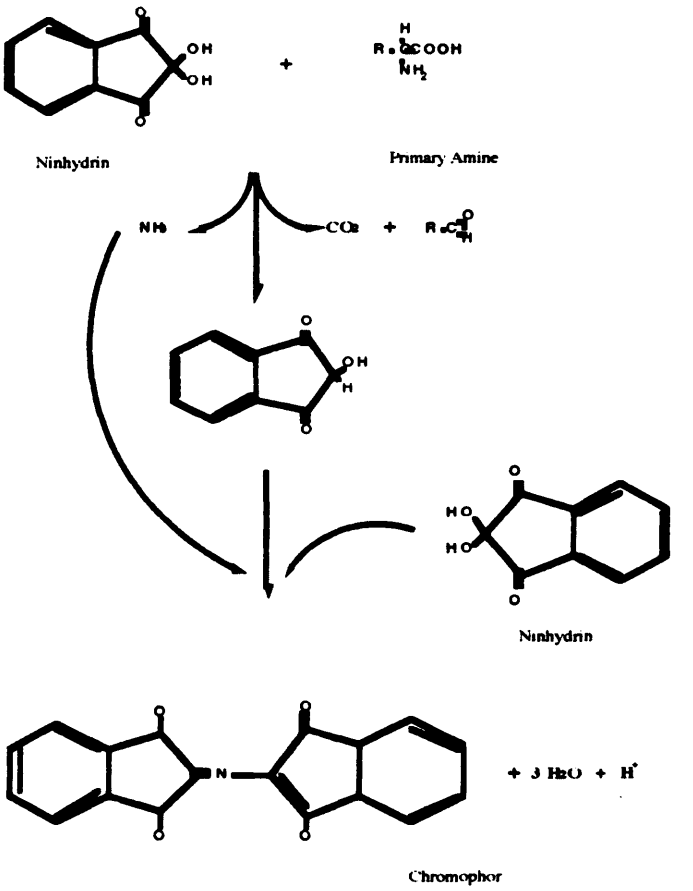

Fig. 3.3 The ninhydrin reaction

nm emission (Fig. 3.4.). It reacts at room temperature with water or a primary amine, the latter reaction being far faster (95). Fluorometric methods of analysis are desirable because of their high sensitivity.

Pearce (96) used this reagent to measure the release of glycomacropeptide from κ-casein by rennet action at an excitation wavelength of 395 nm and emission wavelength of 480 nm. Aliquots of TCA filtrate were mixed with 4% borax solution and an aliquot of fluorescamine solution in acetone added rapidly. The reaction product was protected from light to prevent decomposition prior to measurement of the fluorescence intensity. The hydrolysis of κ-casein was also studied using fluorescamine by Beeby (97), who found that the procedure could be used to estimate the concentration of this protein in milk.

This reagent was used by Creamer *et al.* (98) to quantify acid-soluble proteins, peptides and amino acids from cheese; they noted that the fluorescamine procedure appeared to give more consistant results than did the TNBS method.

o-*Phthaldialdehyde:* *o*-Phthaldialdehyde (OPA) reacts with β-mercaptoethanol and primary amines to form a fluorescent complex (1-alkylthio-2-alkylisoindole), which also absorbs strongly at 340 nm (Fig. 3.5) (99). Church *et al.* (99), who used OPA to quantify proteolysis in milk protein systems, noted that the method is more accurate than the measurement of A_{280} and is more convenient than the ninhydrin, TNBS or fluorescamine procedures. OPA has been applied by Frister *et al.* (100) to monitor proteolysis in cheese.

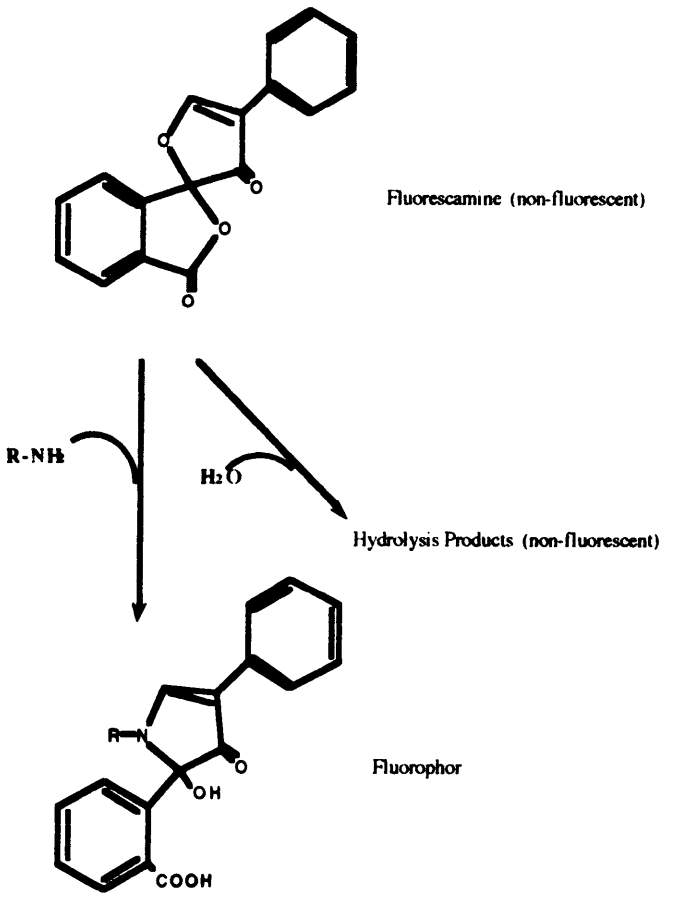

Fig. 3.4. Reaction of fluroescamine with water and a primary amine (95)

3.1.3. Electrophoretic Analysis of Cheese

Electrophoresis has been applied widely to study primary proteolysis in cheese (4). Since only proteins and large peptides can be visualized by staining, the technique is limited to the assessment of casein loss and the formation and subsequent hydrolysis of the primary products of casein proteolysis. However, it is a powerful technique for studying proteolysis during the early stages of cheese ripening (101). The peptides in a 10,000 Da UF permeate do not stain on urea-PAGE, but the retentate of the WSN contains several detectable peptides (30).

The first application of electrophoresis to the study of cheese ripening appears to be that of Lindqvist *et al.* (102) who used paper electrophoresis to study the ripening process in a number of cheese varieties. Lindqvist and Storgards (103) used free boundrary electrophoresis for this purpose. Isoelectric focusing was used by Trieu-Cuot and Gripon (104), as was two-dimensional electrophoresis (isoelectric focusing in one dimension, SDS-PAGE in the other). High-voltage paper electrophoresis (eg. ref 26) is considered to be of limited value (see ref 4).

Although starch gel electrophoresis has been used by some workers, electrophoresis in polyacrylamide gels (PAGE) has found the most widespread application (see ref 4). The literature was reviewed by Creamer (101) and Shalabi and Fox (105). The latter compared several electrophoretic procedures for the analysis of cheese and strongly recommended the stacking gel system of Andrews (106) in gels containing 6 M urea. The direct staining procedure of Blakesley and Boezi (107) with Page Blue G90 gave very satisfactory results. It was concluded that

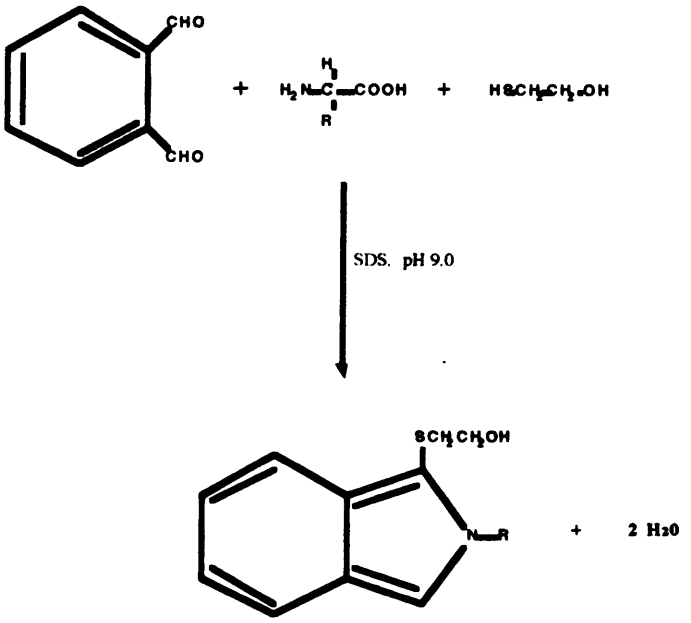

Fig. 3.5. Reaction of o-phthaldialdehyde with a primary amine (99)

SDS-PAGE was inferior to urea-PAGE for cheese analysis and its use was not recommended.

After staining, the electrophoretograms are usually recorded by photography, although densitrometry or excision and elution of the stained bands followed by spectrophotometric quantitation have also been used (see ref 101).

The difficulty in obtaining quantitative data is a serious limitation of electrophoresis. Creamer (101) recommends that several control samples be included in each gel and that comparisons should only be made between samples on the same gel. He emphasised that band dimensions are critical for densitometry and that dye uptake is a function of the protein as well as staining and destaining protocol.

3.1.4. Chromatographic Methods to Assess Proteolysis in Cheese

As discussed in Section 3.1.5. paper chromatography has been used by many workers to quantify free amino acids in cheese (45, 108-111).

Various forms of chromatography have been used to characterize peptides in cheese. O'Keeffe *et al.* (40) used single dimension paper chromatography (butanol/acetic acid/water/pyridine, 30:6:24:20) to separate peptides from fractions obtained by gel filtration. Kuchroo and Fox (26) characterized the peptides in a water-soluble extract of Cheddar cheese by paper chromatography with butanol/pyridine/acetic acid/water (35:25:10:30, v/v) as solvent. Kuchroo and Fox (27) also used these chromatographic conditions to characterize peptides in fractions from gel permeation and hydrophobic chromatography. Paper chromatography is cheap and straightforward but suitable only for assessing the complexity of systems containing only small peptides (111).

Thin layer chromatography on silica gel has been used to characterize peptides in cheese fractions (26-28, 49, 51). Different solvent systems have been used, eg. n-propanol/water (70/30, v/v) and n-propanol/acetic acid/water (5:1:3). Ninhydrin is normally used to develop the plates, although UV fluorescence of the spots was used by Edwards and Kosikowski (51). Preparative TLC has been used (51) to isolate

bitter peptides from Cheddar. TLC is a simple, straightforward method for separating peptides and is normally superior to paper chromatography, although Kuchroo and Fox (27) obtained better resolution of peptides by PC in contrast to TLC.

Column chromatography on silica gel was used by Visser *et al.* (113) to study the bitter peptides from rennet-treated casein. Peptide fractions were eluted with n-propanol/water (7:3, v/v) and monitored at 206 and 280 nm. The authors point to the potential of the technique but it does not appear to have been widely used.

Metal chelate chromatography on Cu-Sephadex was used by Ney (114) to isolate peptides from Cheddar. This technique was developed further by Mojarro-Guerra *et al.* (284) for the separation peptides from amino acids; the peptides were further fractionated on Aminex A6 or Durrum D6 resins. Kuchroo and Fox (27) separated the peptides in a water-soluble extract of Cheddar by hydrophobic chromatography on phenyl- and octyl- Sepharose CL-4B. Chromatography on Duolite S-571 was unsuccessful since the peptides in the water-soluble fraction of the cheese did not adsorb onto this medium. Visser *et al.* (49) isolated bitter peptides by hydrophobic chromatography on Sephadex LH 20.

Gel permeation chromatography has been used widely to study cheese peptides. When a column has been calibrated, the technique allows the estimation of molecular weights. The majority of workers have used Sephadex gels of varous pore sizes (Sephadex G 10 will resolve molecules with molecular weights of 700 to 1500 Da; G25, 1000-5000 Da; G 50, 1500-30,000 Da and G 75, 3000-80000 Da; ref 3). Cheese preparations chromatographed have varied from defatted cheese (115) to fractions of the dialyzate of a water-soluble extract (28). A wide variety of eluents have been used. Chromatograms are generally quantified by spectrophotometry at UV wavelengths; 280 nm is generally used and is suitable for fractions containing large peptides but for fractions containing smaller peptides, absorbance of the carbonyl group in the peptide bond at a lower wavelength, eg. 220 nm (116, 117), is preferable, since smaller peptides may not contain aromatic residues, which are relatively scarce in caseins. Measurement of the amino functional group by reaction with ninhydrin (eg. ref 118) or TNBS are alternatives. Studies in which classical gel permeation chromatography was used are summarized in Table 3.2.

A number of early workers quantified free amino acids by classical ion-exchange chromatography and auto analysers based this technique are widely used for this purpose (see below). Although ion-exchange chromatography on DEAE-cellulose is widely used to fractionate milk proteins, this medium has had limited use for the fractionation of cheese peptides (4). Creamer and Richardson (125) isolated α_{s1}-I casein from Cheddar by chromatography on DEAE-cellulose. Chromatography on DEAE-cellulose is suitable for fractionating 2% TCA soluble and insoluble fractions of a 10,000 Da UF retentate of a water-soluble extract of Cheddar cheese (30). Ion-exchange chromatography on DEAE-cellulose has considerable potential for fractionating water-insoluble peptides in cheese (53a). Mulvihill and Fox (129) used phosphocellulose to fractionate peptides produced from α_{s1}-casein by chymosin, but this medium does not appeared to have been used for cheese analysis. A few authors have used ion-exchange chromatography on Dowex 50 resins to fractionate cheese peptides (eg. ref 123). Studies on cheese in which classical ion-exchange chromatography has been used are summarized in Table 3.3.

The introduction of gel-permeation and ion-exchange HPLC and FPLC in recent years will reduce considerably the work-load involved in these forms of chromtography, although these methods are not truly preparative.

Reversed-phase high performance liquid chromatography (RP-HPLC) is being used increasingly to characterize peptides in casein hydrolyzates (eg. ref 131) and gel-permeation HPLC has been used to characterize caseins, whey proteins and peptides (132-134). HPLC analysis of cheese peptides is becoming increasingly popular, especially on reversed-phase columns. Water extracts have generally been studied but other preparative techniques have included pH 4.6 -soluble and -insoluble fractions and classical gel-permeation chromatography (28). Gradient elution with water/acetonitrile and trifluoroacetic acid as an ion-pair reagent is most commonly

used but isocratic conditions have been used by some workers. Detection is generally by UV spectrophotometry, usually at a wavelength in the region of 200-230 nm (which measures the carbonyl in the peptide bond), although 280 nm has been used in cases where larger peptides, which are more likely to contain aromatic residues, are expected. Fluorescence detection has also found limited use. Studies involving reversed-phase HPLC are summarized in Table 3.4.

To date, HPLC has been confined to the research laboratory; difficulties remain with data interpretation and the development of a solvent system which will permit tasting of the peptide fractions (112). However, Smith and Nakai (135) discuss the potential of HPLC for the routine assessment of cheese quality.

Fast protein liquid chromatography (FPLC) on Mono-S or Mono-Q columns gives better resolution of bovine milk proteins than classical chromatography on DEAE-cellulose (136, 137). Wilkinson *et al.* (138) fractionated press juice from Cheddar by gel permeation FPLC on a Superose-12 column; changes in the peptide profile were evident during ripeneing. Preliminary results indicate that gel permeation FPLC with a Superose-12 column is valuable for the characterization of peptides in fractions obtained from Cheddar on application of the fractionation scheme shown in Fig. 3.1 (53a). FPLC is suitable for small-scale preparative work; fractions can be collected, freeze-dried and analyzed by PAGE. Preliminary results indicate that preparative ion exchange FPLC is valuable in studying larger peptides from Cheddar (53a). Ion exchange and gel permeation FPLC greatly reduce the work-load involved in these forms of column chromatography, while increasing speed and reproducability. Thus, it seems likely the FPLC will achieve more widespread application for monitoring proteolysis in cheese.

3.1.5. Free Amino Acids in Cheese

Analysis of Free Amino Acids: Paper chromatography was used by many early workers to semi-quantify amino acids in cheese (19, 108-110, 147, 148). Solvent systems used have included phenol/water-collidine-lutidine (108, 147) or a combination of various buffered solvents. Chromatograms were generally developed with ninhydrin and amino acids quantified by reference to spot area and colour intensity or by a photoelectric scanner. However, at best, paper chromatography is semi-quantitative and identification of all the protein amino acids is difficult. It has thus been rendered obsolete for quantitative amino acid analysis although it may still be useful for qualitative work.

Mabbitt (18) used ion-exchange chromatography on Dowex-50 columns to quantify a number of amino acids in press juice from Cheddar cheese. Short (15 cm) columns of Dowex-50 were employed by Hintz *et al.* (149) to study free amino acids in a water soluble extract of Swiss cheese. Ali and Mulder (150) also used a method based on that of Moore and Stein (151) to separate amino acids using Dowex-50. They found that the free amino acid pattern of mature Edam did not vary significantly between samples, that amino acids contribute only to the basic taste and that the flavour of Edam connot be attributed solely to free amino acids.

Classical ion-exchange chromatography is laborious and has been superseded by automated amino acid analysers based on ion-exchange chromatography which has greatly facilitated the analysis of free amino acids in cheese. Amino acid analysis is relatively simple, accurate and quantitative and requires little sample preparation. However, the capital cost of the instrument is higher than those for other methods for amino acid analysis.

Free amino acids were one of the indices of proteolysis considered by Reiter *et al.* (21) who used automated by ion-exchange chromatography of a water-soluble extract of cheese, deproteinized by sulphosalicilic acid. Visser (44) used a technique similar to that of Kosikowsky (147), i.e. the ethanol soluble fraction of a water-soluble extract of cheese. A similar preparation method was used by Polychroniadou and Vlachos (152). Ion-exchange chromatography was used by Weaver *et al.* (153) to prepare samples for amino acid analysis; cheese samples were homogenized in a

Table 3.2: Studies Involving Gel Permeation Chromatography of Cheese Peptides

Cheese	Medium[1]	Fraction Studied	Eluent	Detection	Reference
Cheddar	G 10 G 25 G 50 G 150	Freeze-dried water-soluble N	water	UV, 280 nm	27
Cheddar	G 10 G 25	Fractions of diffusate of water-soluble N	water	UV, 280 nm	28
Cheddar	G 50	Water-soluble N	pyridine-acetic acid	UV, 280 mn	39, 122
Cheddar	G 25 G 50	Bitter fraction	water	UV, 280 nm	47
Cheddar	G 25	Bitter extracts	0.1 N NH_4OH	UV, 254 nm	51
Limburger	G 100	Defatted cheese dissolved in 0.033 M acetate buffer, pH 7	0.033M acetate buffer	UV, 280 nm	115
Cheddar	G 100	Cheese dissolved in slovent: 0.1M tris-citrate, pH 8.6, 1 mM Na_2-EDTA, 1 mM NaN_3, 6 M urea, 1 mM dithiothreitol aqueous fraction prepared centrifugally	solvent	UV, 280 nm, UV, 220 nm	116, 117, 126
Aseptic cheese	G 25	pH 4.6 soluble	1 M acetic acid	UV, 280 nm; Ninhydrin	118

[1] Sephadex gels unless otherwise specificied

Table 3.2: Cont'd

Cheese	Medium[1]	Fraction Studied	Eluent	Detection	Reference
Svecia	G 25	Cheese homogenized in water, EDTA, 1N NaOH; defatted centrifugally	1. NH_4OH 2. 0.1 N Sigma "7-9" 3. Glycine buffer, pH 10.5 4. 0.1 N NaCl	UV, 253.7 nm Conductivity	119
Svecia Adelost Hard cheese (37 yr old)	G 25 G 200	Na-citrate extract, dialysis	-	UV; Conductivity	120
Domiati	G 25	Cheese homogenized in 1 M acetic acid, defatted centrifugally	1 M acetic acid	UV, 280 nm; Ninhydrin	121
Butterkase	G 25 G 50	Water-soluble N	water	UV 280 nm	123
Unspecified	G 100	Cheese homogenized in water, EDTA, 1N NaOH, defatted centrifugally	phosphate buffer G/2=.06, pH 7.1	UV	124
Cheddar	Sepharose 6B	High molecular weight peptides	6 M guanidine-HCl	UV, 280 nm	125
Aseptic cheese	G 25 Biogel A5m	pH 4.6 soluble and insoluble N	1 M acetic acid	UV, 280 nm, UV, 254.5 nm	127

[1] Sephadex gels unless otherwise specificied

Table 3.2: Cont'd

Cheese	Medium[1]	Fraction Studied	Eluent	Detection	Reference
Provolone	G 10 G 25 G 50 G 150 Sepharose 6B	Freeze-dried water-soluble N	water	UV, 280 nm	128
Gomonedo	G10	PTA soluble N	water	UV, 280 nm	146
Cabrales	G10	PTA soluble N	water	UV, 280nm	285

[1] Sephadex gels unless otherwise specified

Table 3.3: Studies involving Ion-Exchange Chromatography of Cheese Peptides

Cheese	Medium	Fraction Studied	Elution Conditions	Detection	Reference
Cheddar	DEAE-cellulose	70% ethanol-soluble and insoluble fractions of water-soluble N retentate	0.02 N Na phosphate buffer, pH 6.5, NaCl gradient: 0 to 0.5 M	UV ,280 nm	28
Cheddar	DEAE-cellulose	2% TCA-soluble fractions of 10,000 Da UF retentate of water soluble extract	0.02 M Na phosphate buffer, pH 6.4, gradient: 0 to 0.5 M NaCl	UV, 280 nm	30
Butterkase	Dowex 50Wx2	Fraction from gel permeation chromatography	linear gradient: 2.5 l 0.2 M pyridine acetate, pH 3.1 2.5 l 2 M pyridine acetate, pH 5	ninhydrin	123
Cheddar	DEAE-cellulose	Cheese dispersed in Na-citrate solution, defatted, urea, mercaptoethanol added	4.5 M urea, pH 5.5, 0 to 0.5 M NaCl gradient	UV, 280 nm	125
Provolone	cationic resin on auto-analyser	Fractions from gel-permeation chromatography	linear pyridine gradient, 0.2 to 2 M, pH 2.8 to 6.5	ninhydrin	128
Cheddar Gouda	DE 52	Cheese dispersed in 6 M urea	6 M urea, pH 7.0 linear gradient: 0-.05 M NaCl	UV, 280 nm	130
		Fractions from ion-exchange chromatogram	6 M urea, pH 8.5 linear gradient: 0-0.3 M NaCl	UV, 280 nm	

Table 3.4: Studies Involving Reversed-Phase HPLC of Cheese Peptides

Cheese	Extract	Column	Elution Contitions	Detection	Reference
Cheddar	Water extract	Adsorbo-sphere C_8; Spherisorb S_5 C_8; Vydac C_{18}	Isocratic: 0.1 M phosphate buffer, pH 6.0	UV, 220 nm	23
Cheddar Cheshire Gouda	Water extract	Spheri-5 RP-18	Gradient: TFA: H_2O: CH_3CN	UV, 214 nm, UV, 280 nm	24, 141
Cheddar	Fractions from gel-permeation chromatography	Waters RP-18	Isocratic: 0.01 M KH_2PO_4 pH 4.3	UV, 200 nm	28
Edam Gouda Swiss Cheddar Parmesan	Extract according to ref 22	Adsorbosphere C_8	Ternary gradient 0.1% TFA: CH_3CN: CH_3OH	UV, 220	135
Cheddar	Water extract	Adsorbo-sphere C8	Gradient: TFA, H_2O, CH_3CN	UV, 220 nm	139
Gouda	pH 4.6 soluble extract	Zorbax ODS	Gradient: TFA, H_2O, CH_3CN	UV, 230 nm	140

RP-HPLC Reversed-Phase High Performance Liquid Chromatography
TFA Trifluoroacetic Acid

Table 3.4. Cont'd.

Cheese	Extract	Column	Elution Conditions	Detection	Reference
Danbo Havarti	pH 4.6 insoluble extract	Nucleosil $10C_{18}$	Gradient H_2O, TFA: H_20, CH_3CN	UV, 280 nm	142
Cheddar-type	Water extract treated with methanol, hexane and permeation chromatography on Sephadex G25	Pharmacia Pep HR 5/5	Gradient, H_2O, TFA: CH_3CN, TFA	UV, 214 nm	143, 144
Cheddar	Water extract	Pharmacia Pep HR 5/5	Gradient, H_2O, TFA: CH_3CN, TFA	UV, 214 nm	145
Blue	Water, 5% PTA soluble extracts	Ultrasphere ODS	Gradient, H_2O, TFA: CH_3CN, TFA	UV, 230 nm	146
Appenzel	1% TFA- 5% formic acid 1% NaCl 1% HCl	Vydec 218 TP54	TFA, CH_3CN	UV, 215 nm	283

RP-HPLC Reversed-Phase High Performance Liquid Chromatography
TFA Trifluoroacetic Acid

picric acid solution and an internal standard added. The samples were then centrifuged and the supernatant passed through a column of Dowex 2X8-200.HCl; the eluate was used for amino acid analysis. An essentially similar approach was adopted by Shindo *et al.* (154). Omar (155) used sulphosalicilic acid to deproteinize a Na-citrate dispersion of Ras cheese for amino acid analysis, while Hickey *et al.* (63) used $Ba(OH)_2/ZnSO_4$ to deproteinize cheese.

Polo *et al.* (156) and Ramos *et al.* (61) used reverse-phase HPLC to measure free amino acids in cheese as *o*-phthaldialdehyde derivatives.

Amino acids can be quantified by gas chromatography after derivatization with heptafluorobutyric anhydride (HFBA) to yield n-heptafluorobutyryl isobutyl derivatives. However, packed GC columns do not give adequate resolution (eg. ref 157) and apparently has not been used for cheese analysis. However, Wood *et al.* (158) and Laleye *et al.* (159) applied capilliary GC to quanitify free amino acids (as HFBA derivatives) in cheese. This technique gave good recovery of added amino acids; all protein amino acids could be resolved in a single chromatogram and speed and accuracy were comparable to that of automated amino acid analysers but with far reduced equipment costs. However, this technique has not been used as widely as automated amino acid analysers based on ion-exchange chromatography.

Amino Acid Analyses of Various Cheese Varieties: Studies in which the free amino acids in cheese have been quantified are summarized in Table 3.5. Amino acid concentrations found in selected varieties are given in Table 3.6.

Weaver *et al.* (153) found that the total concentration of free amino acids in an 8 mth old Cheddar was 9967 $\mu g\ g^{-1}$. Val, Tyr, Phe, Glu and Leu accounted for about 80% of the free amino acids at all stages of ripening; Leu showed the largest increase during ripening. The findings of Wood *et al.* (158) were in general agreement with these results but Hickey *et al.* (63) found Glu and Asp to be relatively low.

Wood *et al.* (158) noted that the relative proportions of free amino acids in a Swiss-type cheese were similar to those in Cheddar with the exception of Pro, which is high in Swiss cheese in which it is an important flavour compound.

Kosikowsky and Dahlberg (148) reported that Roquefort-type cheeses have higher concentrations of free amino acids than Camembert and this was confirmed by Ismail and Hansen (36).

Most of the studies on free amino acid in bacterial surface-ripened cheeses have been performed using paper chromatography. Kosikowsky and Dahlberg (148) found lower levels of free amino acids in Limburger-type cheese than in Roquefort or Emmental this result was unexpected.

Polychroniadou and Vlachos (152) found that Leu (18% of total free amino acids), Phe, Lys and Val were the principal free amino acids in Teleme cheese, together making up 50% of the total in a 4 mth-old cheese; the development of flavour was correlated with amino acid levels. Mahon cheese was studied by Polo *et al.* (156) who found that Phe, Val, Pro, Glu and Ile were the principal free amino acids, contributing 67 to 80% of the total. The free amino acid profile of Ras cheese was similar to that of some other hard cheese varieties (eg. Emmental): the young cheese contained mainly Phe, Val, His, Ala, Pro and Lys while a 4 mth-old cheese contained more Glu, Leu, Phe, Val and less Asp, Tyr, Gly, His and Ser (155).

3.2. Assessment of Lipolysis

3.2.1. Introduction

The degree of lipolysis in cheese depends on the variety and varies from slight to extensive. Extensive lipolysis in internal bacterially-ripened cheeses (eg. Cheddar, Gouda, Swiss) is undesirable, while in mould-ripened cheeses, lipolysis is

Table 3.5: Studies on Free Amino Acids in Cheese.

Variety	*Analytical Method*	*Reference*
Cheddar	Ion-X	18
Cheddar, Edam	AAA	21
Samsoe, Maribo, Danbo, Havarti, Danish Camembert, Danablue	AAA	36
Cheddar	AAA	39
Gouda	AAA	44
Cheddar	PC	45
Cabrales	HPLC	61
Cheddar	AAA	62
Cheddar	AAA	63
Cheddar	PC	108, 147
Cheddar, Emmental, Edam, Gouda, Svecia, Herrgard, Roquefort, Domestic Blue, Gorgonzola, Limburger, Brick, Liederkranz, Asiago, Provolone, Primost	PC	109
Tilsit	PC	110
Mansoura	PC	111
Emmental, Domestic Swiss, Roquefort, Gorgonzola, Blue, Liederkranz, Limburger, Brick, Camembert, Asiago, Provolone, Gouda, Gruyere-type, Primost-type	PC	148
Domestic Swiss	Ion-X	149
Edam	Ion-X	150
Teleme	AAA	152
Cheddar	AAA	153
Gouda	AAA	154
Ras	AAA	155
Mahon	AAA?	156
Cheddar, Swiss-Type, Edam, Gouda, Jarlsberg	Cap-GC	158
Cheddar	Cap-GC	159
Rokpol	AAA	160
Bryndza	-	161
Fior de Latte	-	162
Cheddar	-	163
Feta	AAA	164
Kashkawal	-	165
Emmental, Gruyere, Appenzel, Camembert, Brie	-	166
Teleme	-	167
Parmesan	-	168
Cheddar	HPLC	169
Cheddar	-	170
Ras	-	171

AAA Automated Amino Acid Analyzer
Cap-GC Capilliary Gas Chromatography
HPLC High Performance Liquid Chromatography
Ion-X Ion Exchange ChromatographyPC Paper Chromatography

Table 3.6: Free Amino Acid Composition of Selected Cheeses

Amino Acid	*Variety*				
	Cheddar		Swiss	Gouda	Blue[e]
	mol%[a]	mmol/g[b]	mmol/g[c]	mg/g[d]	
Ala	4.0	4.04	5.66	0.50	1729
Arg	1.5	6.37	nd	1.18	445
Asn	-	-	-	-	-
Asp	2.3	11.65	5.40	0.45	860
Cys	-	.37	nd	-	-
Gln	-	-	-	-	-
Glu	19.7	21.62	9.79	3.38	4775
Gly	3.2	4.14	1.74	0.37	466
His	-	-	-	0.22	449
Ile	2.2	3.58	-	0.65	1929
Leu	18.7	21.19	15.72	3.34	3015
Lys	11.8	7.8	1.12	2.02	2231
Met	1.9	2.95	.42	0.66	1111
Phe	7.7	9.02	6.40	1.67	1785
Pro	3.4	2.95	2.41	-	3076
Ser	13.2	4.00	2.65	0.54	1045
Thr	-	3.78	2.80	1.55	1123
Trp	-	-	-	-	552
Tyr	2.6	3.75	1.03	1.00	1154
Val	7.6	9.47	8.36	1.13	2408
Orn	-	nd	nd	-	77
Asn, Gln					876
Citrulline					423
γ-ABA					221

nd= not detected
- = not analysed
γ-ABA= g-amino butyric acid
[a] Salji and Kroger (62); 3 wk old cheese analyzed by amino acid analyzer, individual amino acids expressed as % of total.
[b] Wood *et al.* (158) 8 mth old cheese analyzed by capilliary GC.
[c] Wood *et al.* (158) 2 mth old Swiss-type cheese analyzed by capilliary GC.
[d] Visser (44) 6 mth old cheese made with starter *Lactococcus lactis* spp. *cremoris* E8 under aseptic conditions and analyzed by amino acid analyzer.
[e] Ismail & Hansen (36) 248 d old Danablue cheese analyzed by amino acid analyzer, results expressed as mg amino acid residue/15.7 g total N

essential for flavour development. A number of procedures have been developed to quantify lipolysis.

3.2.2. Copper Soaps Methods

Spectrophotometric methods based on the formation of copper soaps for the estimation of free fatty acids in milk were described by Koops and Klomp (172) and Shipe *et al.* (173). However, Bynum *et al.* (174) obtained low recovery of free fatty acids from Cheddar cheese by the above versions of the Cu-soaps method which they modified (below) for application to cheese.

Cheese samples (200 mg) are mixed with 0.4 ml 0.7 M HCl and 7.5 ml copper soaps reagent [100 ml 1.0 M $Cu(NO_3)_2.2.5H_2O$ plus 50 ml triethanolamine diluted to 1 litre with saturated NaCl and adjusted to pH 8.3 with 1.0 M NaOH], incubated at 60°C for 10 min, cooled and mixed vigorously. Copper soaps of free fatty acids are extracted with 20 ml chloroform/heptane/methanol (49:49:2, v/v/v) by shaking gently for 30 min, followed by centrifugation. An aliquot (5 ml) of the solvent layer is added to 0.2 ml 0.5% (w/v) sodium diethyldithiocarbamate in butan-1-ol. The colour of the resulting complex is measured spectrophotometrically at 440 nm. A standard curve is prepared using palmitic acid, which is one of the principal free fatty acids in cheese (174).

The copper soaps of free fatty acids partition between the aqueous and apolar solvent phases, and therefore short chain fatty acids (< C_{10}) may not be extracted fully. According to Bynum *et al.* (174), the recovery of octanoic acid was 60% of the predicted level and the authors suggest that the recovery of shorter chain free fatty acids would be lower. However, Woo and Lindsay (175) showed that short chain fatty acids as a percentage of total free fatty acids were similar for rancid and non-rancid Cheddar cheese and that long chain fatty acids were dominant, although they may not have as significant an impact on flavour as short chain acids. Therefore, Bynum *et al.* (174) considered that the poor recovery of short chain fatty acids was not a serious limitation of the copper soaps procedure. Inclusion of methanol (2%) in the extraction solvent minimizes interference from copper complexes of phospholipids (172).

3.2.3. Acid Degree Value

The acid degree value (ADV) has been used for many years as an index of lipolysis in dairy products. Free fat is obtained by the combined action of detergent, ion-exchange and heat and is separated from the aqueous phase by centrifugation. Aliquots of the free fat are weighed, dissolved in solvent and the free fatty acids titrated with alcoholic KOH (*ca.* 0.02 M) using methanolic phenolphthalein as indicator (176).

The Bureau of Dairy Industry (BDI) method, developed by Thomas *et al.* (177), was applied to Cheddar cheese by Dulley and Grieve (178). Cheese samples were dispersed in 2% sodium citrate prior to deemulsification and titration. Salji and Kroger (62) used a similar method to measure the fat acidity of Cheddar cheese. A finely ground sample of cheese (10 g) was placed in a Babcock cream butyrometer and 20 ml BDI reagent (30 g Triton X-100, 70 g Na tetraphosphate, H_2O to 1 litre) added. The mixture was then heated at 100°C for 20 min and centrifuged to liberate the fat. Aqueous methanol was added to bring the fat into the neck of the butyrometer. An aliquot (1 ml) of free fat was titrated with alcoholic KOH.

Deeth and Fitz-Gerald (176) consider the ADV method to be tedious, but to be reliable in the hands of a competent operator for foods containing >2% fat; they report that Cheddar cheese with an ADV >3.0 will have a rancid off-flavour.

3.2.4. Gas Chromatography

Analysis of free fatty acids by gas chromatography has been applied widely to cheese. A preparative procedure is generally necessary and in many cases the esters of free fatty acids are chromatographed.

Aqueous and solvent extractions have been used (eg. ref 179, 180) but this approach has been critised owing to partitioning effects (181) and the extraction of compounds which interfere with analysis (179, 180). Martin-Hernandez *et al.* (182) described a simple extraction-derivitization procedure for free fatty acids in cheese: cheese is acidified and homogenized with diethyl ether; the organic phase is dried

with anhydrous Na_2SO_4 and the fatty acids methylated by treatment with 20% tetramethylammonium hydroxide in methanol prior to GC analysis (182).

Anion-exchange resins have been used to prepare samples for free fatty acid analysis in cheese (eg. ref 183) but these methods have been criticised because of incomplete recovery, glyceride hydrolysis and they are time consuming (175).

In the method of McNeill and Connolly (184), free fatty acids were extracted from Cheddar and Emmental with diethyl ether/ HCl and the extract passed through a column of silicic acid to remove phosoholipids. Free fatty acids were adsorbed on Amberlyst A-26 ion-exchange resin and methylated with a 5% solution of HCl in methanol. The methyl esters were recovered by salting out from a mixture of the ion-exchange resin and diethyl ether with saturated aqueous NaCl. The organic layer was dried with anhydrous Na_2SO_4 prior to GC analysis.

Alkaline arrestant columns have also been used to prepare samples of GC analysis (175). McCarthy and Duthie (185) introduced such a column which has been applied to cheese by a number of workers, eg. Thakur *et al.* (186), who chromatographed fatty acids as butyl esters, and by Harte and Stine (187), but the method causes glyceride hydrolysis (175). An improved silicic acid-KOH column was developed and applied to butter by Woo and Lindsay (188) but the lactic acid in cheese interferes with the analysis and the relatively large amounts of water induced glyceride hydrolysis (175). However, Woo and Lindsay (175) developed a technique for Cheddar cheese involving an ethylene glycol pre-column (to remove lactic acid) and an arrestant column modified to remove water. Ha and Lindsay (189) chromatographed volatile ($< C_{12}$) free and total branched-chain fatty acids from Parmesan cheese as butyl esters and identifed them by GC-MS.

Triglycerides and partial glycerides in cheese have been studied by Contarini *et al.* (190) who extracted fat from Gorgonzola by hexane and diethyl ether; aliquots were subjected to TLC and the chromatograms developed with 2, 7-dichlorofluorescin in ethanol. The bands corresponding to mono-, di- and triglycerides were recovered and extracted with chloroform and diethyl ether. Mono- and diglycerides were chromatographed by capilliary GC as trimethylsilane derivatives and triglycerides were chromatographed directly. Triglyceride analysis gave no useful information on cheese ripening but comparisons between partial glycerides were valuable. Studies involving the analysis of shorter chain acids include refs 169, 178-180.

3.3. Assessment of Glycolysis

The metabolism of lactose to lactic acid by starter is fundamental to most, if not all, varieties of cheese. L-Lactate is produced by mesophilic and some thermophilic starters while a mixture of D- and L-isomers is produced by other theromphiles. In certain varieties (eg. Swiss), the lactate produced serves as a substrate for further microbial metabolism and in many varieties the L-isomer produced by the starter is converted to a racemic mixture by the non-starter flora of the cheese (see ref 191). Citrate is an important substrate and its metabolism leads to flavour compounds (diacetyl, acetoin and 2,3-butylene glycol) in some cheeses. Many of the products of glycolysis are quantified by enzyme assay and in these cases the enzymatic procedure has superseded earlier methods because of increased sensitivity and specificity. Only enzymatic methods can distinguish between D- and L-lactate.

Lactose can be measured by an enzyme assay, as can D-galactose and D-glucose. Suppliers of test kits include Boehringer Mannheim GmbH (Mannheim, Germany). Lactose is hydrolyzed by β-galactosidase to D-glucose and D-galactose. The galactose produced is then oxidized by treatment with galactose dehydrogenase with the concomitant conversion of NAD^+ to NADH, which is monitored spectrophotometrically at 334, 340 or 365 nm and is stoichiometrically related to the concentration of lactose. Glucose is phosphorylated by ATP and hexokinase to glucose-6-phosphate (G6P) which is then oxidized by $NADP^+$ in the presence of G6P dehydrogenase; the NADPH formed is quantified spectrophotometrically (6, 7).

Enzyme assay test kits for D- and L-lactate are available (eg. Boehringer Mannheim) which use L- and/or D-lactate dehydrogenase (LDH) and glutamate-pyruvate transaminase (GPT). Lactate is oxidized by NAD^+ in the presence of LDH to pyruvate and NADH. The equilibrium of this reaction normally lies in favour of lactate but the pyruvate formed reacts with L-glutamate in the presence of GPT, shifting the equilibrium in favour of NADH. The concentration of NADH is measured spectrophotometrically and is stoichiometrically related to the concentration of D- or L- lactate (8).

Acetic acid in cheese can also be measured by an enzyme assay (eg. from Boehringer Mannheim). Acetyl-coenzyme A is formed from acetate and ATP in the presence of acetyl-CoA synthetase. Acetyl-CoA reacts with oxaloacetate and water in the presence of citrate synthetase to form citrate and free CoA. The oxaloacetate necessary for this reaction is formed *via* the action of malate dehydrogenase from malate and NAD^+ with the concomitant formation of NADH, the concentration of which is related, non-linearly, to the acetate concentration (192).

Citrate in cheese can be quantified by chemical methods (IDF 34B:1971, ref 15; AOAC 920.126, 976.15, 1990, ref 16) or by an enzyme assay (eg. from Boehringer Mannheim). In the latter, citrate is converted to oxaloacetate and acetate by citrate lyase. In the presence of malate dehydrogenase and L-LDH, oxaloacetate and its decarboxylation product, pyruvate, are reduced to L-malate and L-lactate, respectively, with the concomitant oxidation of NADH to NAD^+, which is measured spectrophotometrically (9).

Diacetyl can be quantified in milk by chemical methods. Walsh and Cogan (193, 194) described a quantitative colorimetric method for diacetyl and acetoin in milk. Diacetyl is extracted by steam distillation and treated with hydroxylamine to form dimethylglyoxime which is converted to a pink ammono-ferrous dimethylglyoximate complex by reaction with $FeSO_4$ in alkaline solution. Essentially all the diacetyl is recovered in the first 10 ml of distillate, thus effectively separating it from acetoin and enabling their seperate estimation (193). Keen and Walker (195) measured diacetyl in a steam distillate of Cheddar colorimetrically by reaction with 3, 3'-diaminobenzidine tetrahydrochloride and HCl. Acetoin and 2, 3-butylene glycol were quantified by extraction with methylene chloride; the extract was separated from the residue, dried with anhydrous Na_2SO_4 and reduced in volume by rotary evaporation. The extract was then equilibrated with water when acetoin and 2, 3-butylene glycol passed into the aqueous phase which was clarified by treatment with $BaCl_2/NaOH/ZnSO_4$. Acetoin was quantified directly in the aqueous phase by treatment with α-naphtol. 2, 3-Butylene glycol was quantified in an aliquot of clarified aqueous phase: traces of acetoin and diacetyl were removed by ion-exchange chromatography and the 2, 3-butylene glycol oxidized to acetoin with bromine water and quantified as acetoin by reaction with α-naphtol. 2-Butanol in a cheese homogenate was quantified by gas chromatography (195). Thornhill and Cogan (196) described a gas chromatographic method for quantifying acetic acid, acetoin, racemic and *meso*-2,3-butylene glycol in broth media.

Keen and Walker (180) described a gas chromatographic method for the determination of propionate in cheese. This was applied to Swiss cheese by Turner *et al.* (197). Marsili (169) measured pyruvic, lactic, acetic and propionic acids by ion-exchange HPLC in aqueous extracts prepared by $ZnSO_4$ and $Ba(OH)_2$ of Cheddar and monitored acetone, 2-butanone, ethanol, 2-pentanone, 2-butanol and n-propanol by GC of headspace gasses.

4.1. Hydroxy Acids and Fatty Acid Lactones in Cheese

Although it is generally agreeed that lactones may play a role in the overall flavour of cheese, they have received little attention. They may be quantified by gas chromatography and various extraction and preparation procedures have been developed. O'Keefe *et al.* (198) separated cheese lipids by centrifugation at 40°C and

subjected the lipid fraction to molecular distillation at 70°C and 10^{-5} mm Hg. Free fatty acids in the distillate were then removed by saponification and the lactones quantified by GC-MS. Wong *et al.* (199) developed an adsorption chromatographic method using a column of aluminium oxide, sodium sulphate and Celite 545 with which a cheese sample was mixed. The column was eluted with acetonitrile and the eluate concentrated prior to GC. This procedure was modified by Wong *et al.* (200): the acetonitrile extract was evaporated under nitrogen, sodium sulphate was added and the residue re-extracted with acetonitrile and passed through a small column of alumina before final concentration and analysis by GC.

4.2. Methyl Ketones

Patton (201) prepared a fraction rich in methyl ketones by steam distillation and ether extraction; the methyl ketones in the extract were separated by fractional distillation. Morgan and Anderson (202) reacted a distillate from a cheese/water suspension with 2,4-dinitrophenylhydrazine (DNPH) and identified the 2,4-dinitrophenylhydrazones by paper chromatography. Sato *et al.* (203) also employed a steam distillation step. Kanisawa and Itoh (204) measured the methyl ketones produced by fungi in lipolysed milk-fat by steam distillation and gas chromatography.

Schwartz *et al.* (205) described a method for the quantitative isolation of monocarbonyls from fats and oils by reaction with DNPH and adsorption of the derivatives onto activated magnesia; most of the lipid material was removed by elution with hexane and the DNPH derivatives then eluted with nitromethane/cholorform and fractionated on activated alumina into 4 classes (methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals) which were identified by chromatographic procedures. This method has been applied to Blue, Camembert and Roquefort (206-209). Godinho and Fox (210) also used this method but measured the DNPHs of total carbonyls spectrophotometrically.

Dartley and Kinsella (211) prepared DNPH derivatives from a hexane extract of Blue cheese and fractionated them into classes on activated Celite-Sea Sorb 43. Methyl ketones were then regenerated from the dinitrophenylhydrazones by treatment with H_2SO_4 and analyzed by gas chromatography. Keen and Walker (195) quantified 2-butnone in a cheese/water homogenate by refluxing, followed by GC. Manning (212) measured 2-pentanone in Cheddar headspace gas directly by GC.

4.3. Biogenic Amines

Decarboxylation of free amino acids leads to the formation of amines, many of which are biologically active and have physiological importance; the principal amines in cheese are histamine, tyramine, tryptamine, putrescine, cadaverine and phenylethylamine (213).

Spector *et al.* (214) developed a fluorometric assay for tyramine in tissue, involving homogenization and extraction followed by the formation of a fluorophor by reaction with 1-nitroso-2-naphtol. This assay was applied to Gruyere by Price and Smith (215). Voigt *et al.* (216) measured tyramine, tryptamine and histamine in a variety of cheeses by TLC; the plates were visualized using 0.2% 7-chloro-4-nitribenzofuran with which the amines formed fluorescent spots which were scraped off the plate and quantified spectofluorometrically. Colonna and Adda (217), using a different extraction procedure, quantified tyramine, histamine and tryptamine in a number of French cheeses by TLC, as described by Voigt *et al.* (216). Taylor *et al.* (218) considered the chromatographic separation step to be cumbersome and described a simplified method for the determination of histamine in foods, involving selective extraction, reaction with *o*-phthalaldehyde and spectrofluorometric assay. They determined the histamine content of Cheddar cheese by this method, which was also used by Antila *et al.* (219) to determine the histamine content of 10 varieties of cheese consumed in Finland. Kaplan *et al.* (220) considered such fluorometric assays

for amines to be laborious and prone to error owing to long extraction procedures; GC was perferred.

Evans *et al.* (221) quantified tyramine in a number of cheese varieties by TLC; the chromatograms were visualized by a sulphanilic acid-containing reagent and the spots corresponding to tyramine eluted and quantified spectrophotometrically.

Gas chromatographic methods have been used by a number of workers (159, 214, 220, 222). Spector *et al.* (214) measured tyramine in tissue extracts directly by GC. Kaplan *et al.* (220), who considered direct GC methods to be unsuitable for the determination of amines, recommended derivitization with trifluoroacetic acid prior to GC analysis. Laleye *et al.* (159) extracted a number of biogenic amines from Cheddar and formed N-heptafluorobutyryl-isopropyl derivatives which were quantified by capilliary GC. Staruszkiewicz and Bond (222) analyzed cadaverine, putrescine and histamine in a number of foods, including several cheese varieties, by GC of perfluoropropionyl derivatives.

Staruszkiewicz (223) quantified the *o*-phthalicdicarboxaldehyde derivative of histamine by HPLC with fluorescent detection at 350 nm excition and 444 nm emission. Chambers and Staruszkiewicz (224) used this method to quantify amines in a number of cheeses. Tyramine, phenylethylamine, histamine and tryptamine were analysed in extracts of a number of cheese varieties by Koehler and Eitenmiller (225) and Chang *et al.* (226) using HPLC without derivitization and with UV detection. Suhren *et al.* (227) adapted an autoanalyzer to measure histamine and tyramine in dairy products. Antila *et al.* (228) reacted the amines in cheese extracts with dansyl chloride and quantified the derivatives by HPLC with UV detection. A HPLC procedure with amperometric detection was used by Takeba *et al.* (229) to determine tyramine in cheese; it was claimed that this detection procedure was 25 times more sensitive than fluorometric detection. Sumner *et al.* (230) used a variation of the AOAC standard procedure for measuring histamine in seafoods (AOAC 18.067-18.071, 1984, ref 231) to study the production of histamine in Swiss cheese; cheese was homogenized with methanol, heated to 60°C x 10 min and cooled to room temperature; aliquots were chromatographed on Dowex I-X8 and the histamine quantified fluorimetrically by reaction with *o*-phthalicdicarboxaldehyde. An other AOAC standard method for histamine determination in seafoods involves a bioassay using guinea pig intestine (AOAC 18.060-18.063, 1984, ref 231) but this does not appear to have been applied to cheese.

4.4 Sulphur Compounds

Volatile sulphur compounds, eg. H_2S, methanethiol, methional, dimethylsulphide, occur in cheese at levels characteristic of the variety and ripening conditions and have been implicated in cheese flavour (232, 233).

Walker (234) heated an aqueous dipersion of shredded Cheddar cheese under reflux conditions and flushed the headspace with a nitrogen stream. The gas from the reflux was passed through a series of traps to remove specific classes of compounds (crystalline lead acetate to remove H_2S; $HgCl_2$ solution [3%] for sulphides and disulphides and $Hg(CN)_2$ solution [3%] for mercaptans). The odour of the gas was noted after each trap to establish the effect of removing each class of compound from the gas stream. Tsugo and Matsuoka (235) used a similar series of traps, but with 4% $Hg(CN)_2$ solution, to separate and identified various sulphur compounds from a semi-soft white mould-type cheese by chemical means. Lawrence (236) flushed an aqueous slurry of cheese with nitrogen, trapped the H_2S in a 1% Zn-acetate/NaOH solution and quantified it by the development of methylene blue from *p*-diamino-NN-dimethylaniline hydrochloride and $FeCl_3$ which was measured spectrophotometrically at 678 nm. McGugan *et al.* (237) stripped volatiles from centrifugally defatted Cheddar, made with or without starter, by vacuum distillation and trapping in liquid nitrogen. The distillates were then analyzed by GC for a number of volatiles, including dimethylsulphide. Manning and Robinson (238) used headspace trapping, methanol extraction and reduced pressure distillation to prepare samples for GC.

Headspace gases were considered to contain the essential components of cheese aroma in the correct proportions and the sampling conditions were such that labile components were not lost or damaged. Methanol extraction was not considered suitable and distillation under low pressure was the preferred method of sample preparation because of drawbacks in the headspace analysis techniques used. Dimthylsulphide and H_2S were analyzed by this method. Manning (239) modified the distillation apparatus of Manning and Robinson (238) and quantified H_2S, methanethiol and dimethylsulphide by GC. Manning *et al.* (240) developed an improved technique for headspace analysis: a plug of cheese was removed and the entrance to the hole immediately sealed with a metal cap fitted with a septum; the atmosphere was allowed to equilibrate for 1 h and 5 ml aliquots of the headspace gas then removed by inserting a syringe needle through the septum and used for GC analysis. This method was used by a number of workers (212, 240-244) to analyze cheese volatiles.

4.5. Detection of Adulteration of Ovine and Caprine Milks and Cheeses

Adulteration of ewes' or goats' milk for cheesemaking with less expensive bovine milk has lead to the development of techniques which permit the detection of mixed-species milks. This topic has been reviewed by Ramos and Juarez (245, 246).

Various authors have used differences in fatty acid profiles to detect adulteration (eg. refs 247, 248). Such chromatographic methods have limited sensitivity and will not detect adulteration with skim milk, but they can be applied to mature cheese since relatively small changes occur in fat composition during maturation (245). Ramos and Juarez (246) suggest that differences in triglycerides might be used to identify milks from different species. The absence of β-carotene from sheeps' and goats' milks may permit the detection of bovine milk in caprine or ovine milks (246).

Differences between HPLC profiles of tryptic hydrolyzates of caseins from different species have been demonstrated (249); however, the effect of proteolysis during cheese ripening was not studied. The amino acid compositions of caprine (250) and ovine (251) caseins differ from bovine casein.

Electrophoretic techniques have been developed to detect mixtures of milks from different species. Aschaffenburg and Dance (252) showed that bovine α_{s1}-casein had a faster electrophoretic mobility than caprine α-casein while Ramos *et al.* (248) demonstrated differences between ovine and bovine α_{s1}-caseins which have been exploited by a number of workers to detect mixed-species milks (see ref 246). Proteolysis during cheese ripening interferes with this procedure although Pierre and Portmann (253) achieved satisfactory results up to 15 days of ripening for Saint-Maure and Chabichou cheeses. However, Ramos and Juarez (246) considered that the detection of bovine milk in industrially-produced sheeps' cheeses by electrophoresis is difficult. Differences between ovine and bovine para-κ-caseins permitted the detection by isoelectric focusing of bovine milk in ewes' milk Pecorino cheese after 5 months of ripening (254). Addeo *et al.* (255) also used isoelectric focusing and densitometry to study the composition of cheeses made with caprine and ovine milks. Krause *et al.* (256) applied isoelectric focusing to determine interspecies differences in the γ-caseins.

Interspecies differences in the electrophoretic mobility of whey proteins may be used to identify mixed-species milks but not cheeses.

The higher xanthine oxidase activity of bovine milk was used by Monget *et al.* (257) to detect adulteration of goats' milk. The test was reported to be simple, rapid and could be applied to raw or pasteurized (< 75°C x 20 s) milk or fresh cheese and allowed detection of 2% added bovine milk.

Pollman (258) found differences in the Ca/Mg ratio in cheeses made from cows' or sheeps' milk.

Immunological methods are well suited for analysis of mixed-species milks because of their sensitivity, specificity and their availability in kit form. However,

when preparing antisera to proteins from a particular milk, the antisera will react not only with that milk but with the milks of many other species' also; however, techniques have been developed to overcome this (see ref 246). Ramos and Juarez (246) report that Durand *et al.* (259) detected bovine milk in ewes' milk by immunodiffusion in agar with antisera to cow blood serum and Levieux (260) choose immunoglobulin G_1 as the antigen. Caseins have relatively low antigenicity but to overcome this difficulty, Aranda *et al.* (261) used an immunodotting technique to detect bovine caseins in ewes' milk and cheese. Rodriguez *et al.* (262) developed an indirect enzyme-linked immunosorbent assay (ELISA) for bovine caseins and used it to assay for bovine milk in sheeps' milk and cheese.

5. OBJECTIVE INDICES OF CHEESE RIPENING AND QUALITY

5.1. Introduction

As discussed in above, chemical analyses of cheese are performed for one of several reasons. Possibly the most ambitious is the development of objective methods for assessing cheese quality. The organoleptic qualities of cheese are determined by the physical and chemical changes which occur during ripening and are traditionally assessed by sensory evaluation of flavour, body and texture, finish and overall quality by experienced judges or trained or untrained consumer panelists. However, sensory analyses are subjective, and although at present the best index of consumer acceptability, they provide data that are difficult to evaluate scientifically and to compare between laboratories or studies. Therefore, chemical and physical analyses of cheese are used to objectively monitor ripening and to assess quality, usually to complement sensory evaluation. While it may never be possible to assess cheese quality by chemical criteria alone, they can provide very valuable indices of quality. The following is an attempt to describe how some of the analytical methods described in the preceeding sections may be valuable for assessment of quality. The subject was reviewed by Farkye & Fox (263).

5.2. Chemical Indices

5.2.1. Proteolysis

For most hard and semi-hard cheeses, proteolysis is the most commonly used index of maturity. Most, if not all, nitrogenous compounds that contribute to cheese flavour are soluble in aqueous solvents. Solubility in water or at pH 4.6 is probably most widely used for initial fractionation of cheese N or as a crude index of proteolysis; although relatively simple to perform, it may not be sufficiently discriminating as an index of quality. Both PTA and 12% TCA-soluble N correlate significantly ($p < .001$) with the age and flavour intensity of Cheddar cheese (56). Kroger and Weaver (71) reported that the dye binding capacity of cheese correlated strongly ($r = 0.85$) with the concentration of free amino acids and suggested its use in determining the chemical age of cheese; however, Kuchroo *et al.* (29) found that a dye-binding technique using amido black was not very useful for this purpose. Since the concentration of free amino acids in cheese is reported (56) to correlate strongly with the age and maturity of cheese, chemical indicators of free amino acid concentration, e.g. TNBS (29, 54, 68, 78, 82) or ninhydrin-reactive groups (79, 93) should be useful indicators of cheese age and quality; while there is a substantial amount of information relating these indices to age, there is much less relating them to quality. Marsili (169) reported that the best predictors of the proteolytic age of Cheddar were leucine ($r^2 = 0.532$), methionine ($r^2 = 0.525$) and glutamic acid ($r^2 = 0.477$).

Pham and Nakai (23) analyzed water soluble N (WSN) from 41 commercial Cheddar cheeses of different ages by reverse phase-HPLC. They observed 13 chromatographic peaks, the relative areas of which varied with the age of the cheese, suggesting that discriminate HPLC analysis of WSN should be a good method for objective evaluation of cheese maturity. Santa-Maria *et al.* (264) successfully applied linear discriminant analysis of 18 parameters of proteolysis for the age classification of Manchego cheese. Polyacrylamide gel electrophoresis (PAGE) is widely used to monitor primary proteolysis in cheese and is mostly related to the activities of residual coagulant and plasmin. α_{s1}-Casein is hydrolyzed faster than β-casein by all commercial coagulants but the plant rennets produce very different products (265, 266). Thus, PAGE can provide information on the type of coagulant used, which may be indicative of quality. The level of residual α_{s1}-casein is a good index of the level of general proteolysis in relatively young cheeses (e.g. < 3 months for Cheddar or Gouda) but is probably not sufficiently discriminating to be a useful index of quality. Chymosin, bovine and porcine pepsins hydrolyze only α_{s1}-casein in cheese while proteolysis of β-casein in bacterially-ripened cheeses is due mainly to plasmin activity, which appears to be directly related to cooking temperature (263). Plasmin is active in most cheeses and is considered to make a significant contribution to proteolysis in Romano-type (267), Swiss and Dutch-type cheese (268); its activity is clearly indicated by the level of γ-casein but the relevance of this for assessment of cheese quality is not apparent although it is clearly indicative of age.

5.2.2. Lipolysis

The extent of lipolysis during cheese ripening varies among varieties (269, 270). In Cheddar, Emmental, Gruyere and Gouda-type cheeses, the level of lipolysis is low, while in Blue mould-ripened and hard Italian varieties, extensive lipolysis occurs. Obviously, the concentration of free fatty acids increases during ripening and has a major impact on cheese flavour (175, 268, 271-273). A certain concentration of free fatty acids in the correct balance appears to be necessary for optimum flavour, but an excess of fatty acids or the incorrect balance leads to offflavours, especially in mild cheese varieties. As far as we are aware, the only attempt to correlate the "lipolytic age" of Cheddar cheese with the concentrations of individual free fatty acids is that of Marsili (169) who found that C_{10} (r^2= 0.375), C_{14} (0.227), C_{12} (r^2 = 0.198) and C_{16} (r^2 = 0.129) were the best indicators although considerably less useful as indicators of age than certain products of glycolysis and proteolysis. Such correlations for the more highly lipolyzed cheeses, i.e. Blue and Italian, appear to be lacking although the flavour intensity and quality of Italian cheese is strongly influenced by the concentration and balance of free fatty acids (270).

Liberated fatty acids may be modified to products which may influence cheese quality. This is particularly true for methyl ketones which are responsible for the characteristic flavour of blue mould cheeses; their formation has been extensively studied (see ref. 274). The concentration and ratios of methyl ketones are useful indicators of the maturity and quality of blue-cheeses. A homologous series of δ-lactones (C_{10}-C_{18}) occurs in Cheddar (and probably other) cheese and their concentrations increase during ripening up to about 2 months after which they remain constant or decline slightly (200). Although the concentration of δ-lactones did not correlate with cheese flavour, they may be a useful index of age up to 2 months. The concentration of γ-dodecalactone was considerably lower than those of the δ-lactones but increased in parallel with the intensity of cheese flavour and hence may be a useful index of maturity. Further work in this area appears warranted.

5.2.3. Lactose Fermentation and Changes in Lactate

Fermentation of lactose to lactic acid during cheesemaking reduces the pH of cheese curd to values which prevent the growth of pathogenic bacteria. The rate and extent of acidification of cheese strongly influence cheese quality (see refs 191, 275) and hence the pH of cheese is a useful indirect index of quality. However, the pH of cheese increases during ripening (see below) and hence the pH of mature cheese may not reflect that of fresh curd. Although approx. 98% of the lactose of milk is removed in the whey as lactose or lactic acid, cheese curd contains 0.7-1.5% lactose (see ref. 191). The concentration of residual lactose in cheese curd depends on the method of manufacture, the type and activity of the starter and especially on the concentration of salt in the moisture phase. Although considerable changes occur in the concentrations of lactose and lactic acid during ripening (see ref. 191), these are rarely, or not at all, used as indices of ripening. Except in cases where the concentration of salt is so high (> approx. 6% salt-in-moisture) as to prevent bacterial growth, the lactose concentration in Cheddar decreases to essentially zero within about 2 weeks. In Dutch-type cheeses, lactose is completely fermented to lactic acid within 24 h. In high-cooked varieties in which a thermophilic starter (*S. thermophilus* and a *Lactobacillus*) is used, the concentration of lactose also decreases to essentially zero within 1 week. In these cheeses, galactose accumulates initially (due to the inability of *S. thermophilus* to metabolize galactose) but it, also, rapidly reaches zero (see ref. 191). In mould ripened cheeses, lactose is completely metabolized within a short period. Therefore, lactose and its constituent monosaccharides are of little value as indices of cheese maturity.

In most cheese varieties, lactose is metabolized to L-lactic acid. In Cheddar, Dutch and many other varieties, L-lactate is isomerized to D-lactate by non-starter lactic acid bacteria (NSLAB) so that a racemic mixture of lactic acid exists after 3-4 months (see ref. 191). The rate of racemization of lactate depends on the NSLAB population and hence is an index of their numbers and activity rather than of cheese maturity. Although insufficient studies have been undertaken on the rate of racemization of L-lactate in cheese, it is unlikely to be a reliable index of cheese maturity due to variations in NSLAB populations.

In Swiss-type cheese, lactate is converted to propionate, acetate and CO_2 (3 lactate = 2 propionate + 1 acetate + 1 CO_2) when the growth of propionic acid bacteria is initiated on transfer to the hot room (see ref. 191). During certain periods, the concentrations of lactate, propionate and acetate are good indices of the activity of propionic acid bacteria and hence of correct maturation; the molar ratio of propionate and acetate should be 2:1 and is a good index of the quality of Swiss-type cheese but is less useful as an index of its age.

In surface mould-ripened cheeses, lactate is metabolized to CO_2 and H_2O by the metabolic activity of *P. camemberti*. Thus, the decrease in lactate concentration may be a useful index of the maturity of these cheeses during a certain period.

5.2.4. Changes in pH

The formation of lactic acid during manufacture and the metabolism of residual lactose during the initial stages of ripening reduces the pH of cheese to approx. 5 ± 0.3, depending on variety, after all the lactose has been metabolized. During ripening, the pH of cheese rises due to the formation of alkaline nitrogenous compounds and/or catabolism of lactic acid. The pH of Cheddar increases by only approx. 0.1 unit after 6 months of ripening (268); the pH of Gruyere increases from pH 5.35 to 5.95 (272) and that of Gouda from approx. 5.1 to 5.3-5.9 (the extent of the increase appears to depend on the season of manufacture for unidentified reasons; 268). The pH of Camembert increases from approx. 4.8 to as high as 7.5 (104, 276) while the pH of Blue cheese increases from approx. 4.8 to approx. 7 (210). Obviously, changes in pH of this magnitude during ripening are useful indicators of the age of cheeses,

especially mould ripened varieties, but pH is of little use as an index of the maturity of Cheddar. The increase in pH is essential for the desirable textural changes in surface-ripened cheeses but we are not aware of studies on the correlation between the quality of other varieties and their final pH.

5.2.5. Volatile Organic Compounds

Volatile organic compounds, e.g. acids, carbonyls, lactones, H_2S, methanethiol, dimethylsulphide, etc, occur in cheese at concentrations depending on the variety and ripening conditions. The contribution of volatile compounds to cheese flavour was reviewed by Aston and Dulley (232). Volatile compounds are responsible for cheese aroma and contribute to both background and characteristic flavour, but their concentrations in cheese are variable (243). McGugan *et al.* (22) noted that volatiles from un-deodorized cheese fat contributes to flavour but when the volatiles were reconstituted in fat from mild or aged cheeses, the intensity of cheese flavour did not increase significantly, suggesting that quantitation of volatile compounds as an indicator of ripening may underestimate the chemical age of cheese. Marsili (169) reported that the concentrations of alcohols, aldehydes and ketones were poor indicators of the age of Cheddar. However, he found that acetate ($r^2 = 0.395$) and especially propionate ($r^2 = 0.705$) were good indices of the glycolytic age of Cheddar cheese: the concentration of propionate was the best single index of cheese maturity. The mechanism and pathway for the formation of propionate in Cheddar are not obvious; Keen & Walker (180) found no propionate in Cheddar and Dutch-type cheeses.

5.3. Physical Indices

5.3.1. Cheese Rheology

In many respects, the rheological properties (body and texture) of cheese are as important as its flavour and are routinely assessed (organoleptically) in cheese grading. Cheese rheology has been reviewed by Prentice (286) and Prentice and Marshall (287) and textural changes in cheese during ripening has been discussed by Lawrence *et al.* (268). There are relatively few data on the rheological changes in cheese during ripening. Creamer and Olson (277) showed that for Cheddar, yield strain decreased linearly with logarithm of age (days). It is thought thatthe breakdown of α_{s1}-casein to α_{s1}-I is the most significant reaction responsible for the initial softening of cheese body and texture (277, 278), indicating that textural changes during ripening are related to proteolysis, particularly by residual rennet. Indeed the hardness and springiness of Cheddar are correlated with proteolysis (279). The large and obvious changes in the texture of Brie and Camembert are due in part to the increase in pH but an appropriate level of proteolysis is also required for proper texture development (280, 281).

Obviously, physical methods for assessing the body and texture of cheese, to replace subjective, sensoric methods, are desirable. However, Green *et al.* (282) cautioned against instrumental measurements of cheese texture unless these involve fracture and mechanisms similar to those that occur during normal grading and consumption. One of the principal difficulties in physically measuring the rheological properties of cheese is obtaining representative samples: many varieties, e.g. Cheddar, Cheshire, some Blue cheeses, are heterogeneous and fractured, while the eyes in Swiss-type cheese create obvious problems.

5.4. Conclusions

Cheese ripening is a complex phenomenon. Attempts to understand this phenomenon have led to the development of objective measurements which, hopefully, will be able to predict the final quality of cheese. Sensory evaluation of cheese relies on the ability of a grader to examine a small random sample from a batch of cheese and use his skills to predict its quality at a later date. The results of sensory evaluation are subjective. Various objective chemical and rheological approaches to evaluating cheese ripening have been developed and assessed. However, in order to predict accurately the ripening process by chemical and rheological methods, the manufacturing history of the cheese, including the types and activities of starter and rennet used and ripening conditions, need to be taken into consideration. While chemical and physical parameters may be good indicators of maturity, their ability to assess cheese quality is less certain, at least until much more precise knowledge of the biochemistry of cheese ripening has been accumulated.

REFERENCES

1. Lawrence, R.C. and Gilles, J., 1987, In *Cheese: Chemistry, Physics and Microbiology*, 1st edn., Vol. 2, P.F. Fox ed., Elsevier Applied Science Publishers, London, p 1.

2. Grappin, R., Rank, T.C. and Olson, N.F., 1985, *J. Dairy Sci.*, **68**, 531.

3. Rank, T.C., Grappin, R. and Olson, N.F., 1985, *J. Dairy Sci.*, **68**, 801.

4. Fox, P.F., 1989, *J. Dairy Sci.*, **72**, 1379.

5. International Dairy Federation, 1990, F-Doc 173, Brussels.

6. Boehringer Mannheim, 1986, *Methods of Biochemical Analysis and Food Analysis*, Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany, p 66.

7. Boehringer Mannheim, 1986, *Methods of Biochemical Analysis and Food Analysis*, Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany, p 70.

8. Boehringer Mannheim, 1986, *Methods of Biochemical Analysis and Food Analysis*, Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany, p 62.

9. Boehringer Mannheim, 1986, *Methods of Biochemical Analysis and Food Analysis*, Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany, p 20.

10. Marcos, A., Esteban, M.A. and Alcala, M., 1990, *Food Chem.*, **38**, 189.

11. Esteban, M.A. and Marcos, A., 1990, *Food Chem.*, **35**, 179.

12. Esteban, M.A., Marcos, A., Alcala, M. and Gomez, R., 1991, *Food Chem.*, 1991, **40**, 147.

13. Frank, J.F. and Birth, G.S., 1982, *J. Dairy Sci.*, **65**, 1110.

14. Cronin, D.A. and McKenzie, K., 1990, *Food Chem.*, **35**, 39.

15. International Dairy Federation, Standard Methods, Brussels.

16. Official Methods of Analysis, 1990, 15th edn., Vol. 2, Association of Official Analytical Chemists, Washington, DC.

17. Christensen, T.M.I.E., Bech, A.-M and Werner, H., 1990, In F-Doc, 173, International Dairy Federation, pp 720, Brussels.

18. Mabbitt, L.A., 1955, *J. Dairy Res.*, **22**, 224.

19. Bullock, D.H and Irvine, O.R.,1956, *J. Dairy Sci.*, **39**, 1229.

20. Stadhouders, J., 1960, *Neth. Milk Dairy J.*, **14**, 83.

21. Reiter, B., Sorokin, Y., Pickering, A. and Hall, A.J., 1969, *J. Dairy Res.*, **36**, 65.

22. McGugan, W.A., Emmons, D.B. and Larmond, E., 1979, *J. Dairy Sci.*, **62**, 398.

23. Pham, A.M. and Nakai, S., 1984, *J. Dairy Sci.*, **67**, 1390.

24. Aston, J.W. and Creamer, L.K., 1986, *N.Z. J. Dairy Sci. Technol.*, **21**, 229.

25. Kuchroo, C.N. and Fox, P.F., 1982, *Milchwissenschaft*, **37**, 331.

26. Kuchroo, C.N. and Fox, P.F., 1982, *Milchwissenschaft*, **37**, 651.

27. Kuchroo, C.N. and Fox, P.F., 1983, *Milchwissenschaft*, **38**, 76.

28. Kuchroo, C.N. and Fox, P.F., 1983, *Milchwissenschaft*, **38**, 389.

29. Kuchroo, C.N., Rahilly, J. and Fox, P.F., 1983, *Ir. J. Food Sci.Technol.*, **7**, 129.

30. O'Sullivan, M. and Fox, P.F., 1990, *J. Dairy Res.*, **57**, 135.

31. Farkye, N.Y. and Fox, P.F., 1991, *J. Agric. Food Chem.*, **39**, 786.

32. Dahlberg, A.C. and Kosikowsky, F.V., 1947, *J. Dairy Sci.*, **30**, 165.

33. Mogensen, M.T.S., 1947, Assk. Alnarps. Lantsbruk., Mejeri-Tradgardsinst, 279, cited from *Chem. Abstr.* 1948, **42**, 8987.

34. Vakaleris, D.G. and Price, W.V., 1959, *J. Dairy Sci.*, **42**, 264.

35. Vakaleris, D.G., Olson, N.F., Price, W.V. and Knight, S.G., 1960, *J. Dairy Sci.*, **43**, 1058.

36. Ismail, A.A. and Hansen, K., 1972, *Milchwissenschaft*, **27**, 556.

37. Gripon, J.C., Desmazeaud, M.J., Le Bars, D. and Bergere, J.C.,1975, *Lait* **55**, 502.

38. Furtado, M.M. and Partridge, J.A., 1988, *J. Dairy Sci.*, **71**, 2877.

39. O'Keeffe, R.B., Fox, P.F. and Daly, C., 1976, *J. Dairy Res.*, **43**, 97.

40. O'Keeffe, A.M., Fox, P.F. and Daly, C., 1978, *J. Dairy Res.*,**45** , 465.

41. Reville, W.J. and Fox, P.F., 1978, *Ir. J. Food Sci. Technol.*, **2**, 67.

42. Noomen, A., 1977, *Neth. Milk Dairy J.*, **31**, 163.

43. Venema, D.P., Herstel, H. and Elenbaas, H.L., 1987, *Neth. Milk Dairy J.*, **41**, 215.

44. Visser, F.M.W., 1977, *Neth. Milk Dairy J.*, **31**, 210.

45. Chakravorty, S.C., Spinivasan, R.A., Babbar, I.J., Dudani, A.T., Burde, S.D. and Iya, K.K., 1966, Proc. XVII Intern. Dairy Congr. (Munich), **D**, 187.

46. Gupta, S.K., Whitney, R.M. and Tuckey, S.L., 1974, *J. Dairy Sci.*, **57**, 540.

47. Harwalkar, V.R. and Elliott, J.A., 1971, *J. Dairy Sci.*, **54**, 8.

48. Visser, F.M.W., 1977, *Neth. Milk Dairy J.*, **31**, 265.

49. Visser, S., Slangen, K.J., Hup, G. and Stadhouders, J., 1983, *Neth. Milk Dairy J.*, 37, 181.

50. Yvon, M., Chabanet, C. and Pelisser, J.-P., 1989, *Intern. J. Peptide Protein Res.*, **34**, 166.

51. Edwards, J. and Kosikowski, F.V., 1983, *J. Dairy Sci.*, **66**, 727.

52. Poznanski, S., Habaj, B., Rymaszewski, J. and Rapczynski, T., 1966, Proc. XVII Intern. Dairy Congr. (Munich), **D**, 555.

53. Gonashvily, Sh., 1966, Proc. XVII Intern. Dairy Congr. (Munich), **D**, 289.

53a. Breen, E. D., 1992, *Fractionation and Identification of Water-Soluble and Water-Insoluble Cheese Nitrogen*, M.Sc. Thesis, National University of Ireland, Cork.

54. Jarrett, W.D., Aston, J.W. and Dulley, J.R., 1982, *Aust. J. Dairy Technol.*, **37**, 55.

55. Kleter, G., 1976, *Neth. Milk Dairy J.*, **30**, 254.

56. Aston, J.W., Durward, I.G. and Dulley, J.R., 1983, *Aust. J. Dairy Technol.*, **38**, 55.

57. Aston, J.W., Grieve, P.A., Durward, I.G. and Dulley, J.R., 1983, *Aust. J. Dairy Technol.*, **38**, 59.

58. Nunez, M., Garcia-Aser, C., Rodriguez-Martin, M.A., Medina,M.and Gaya, P., 1986, *Food Chem.*, **21**, 115.

59. Fernandez del Pozo, B., Gaya, P., Medina, M., Rodriguez-Martin, M.A. and Nunez, M., 1988, *J. Dairy Res.*, **55**, 457.

60. Ardo, Y. and Pettersson, H.-E., 1988, *J. Dairy Res.*, **55**, 239.

61. Ramos, M., Caceres, I., Polo, C., Alonso, L. and Juarez, M., 1987, *Food Chem.*, **24**, 271.

62. Salji, J.P. and Kroger, M., 1981, *J. Food Sci.*, **46**, 1345.

63. Hickey, M.W., van Leeuwen, H., Hiller, A.J. and Jago, G.R., 1983, *Aust. J. Dairy Technol.*, **38**, 110.

64. Visser, S., Hup, G., Exterkate, F.A. and Stadhouders, J., 1983,*Neth. Milk Dairy J.*, **37**, 169.

65. Ardo, Y. and Meisel, H., 1990, In: Doc 173, International Dairy Federation, pp 21, Brussels.

66. Ordonez, J.A. and Burgos, J., 1977, *Lait*, **57**, 150.

67. Hull, M.E., 1947, *J. Dairy Sci.*, **30**, 881.

68. Samples, D.R., Richter, R.L. and Dill, C.W., 1984 *J. Dairy Sci.*, **67**, 60.

69. Singh, A. and Ganguli, N.C., 1972,*Milchwissenschaft*, **27**, 412.

70. Ashworth, U.S., 1966, *J. Dairy Sci.*, **49**, 133.

71. Kroger, M. and Weaver, J.C., 1979, J. Food Sci.,**44**, 304.

72. McGann, T.C.A., Mathiassen, A. and O'Connell, J.A., 1972, *Lab. Pract.*, **21**, 628.

73. Davis, J.G., 1965, Cheese, Vol. 1., Basic Technology, J. & A. Churchill Ltd., London, pp 308.

74. Ollikainen, P., 1990, *J. Dairy Res.*, **57**, 149.

75. Lorenz, K., 1974, *Z. Analytische Chemie*, **269**, 182.

76. Satake, K., Okuyama, T., Ohashi, M. and Shinoda, T., 1960, *J. Biochem.*, **47**, 654.

77. Fields, R.,1971, *Biochem. J.*, **124**, 581.

78. Clegg, K.M., Lee, Y.K. and McGilligan, J.F., 1982, *J. Food Technol.*, **17**, 517.

79. Pearce, K.N., Karahalios, D. and Friedman, M., 1988, *J. Food Sci.*, **53**, 432.

80. Habeeb, A.F.S.A., 1966, *Anal. Biochem.*, **14**, 328.

81. Adler-Nissen, J., 1979, *J. Agric. Food Chem.*, **27**, 1256.

82. Barlow, I.E., Lloyd, G.T. and Ramshaw, E.H., 1986, *Aust. J. Dairy Technol.*, **41**, 79.

83. Madkor, S.A., Fox, P.F. and Metwally, N.H., 1984, *Ir. J. Food Sci. Technol.*, **8**, 84.

84. Polychroniadou, A., 1988, *J. Dairy Res.*, **55**, 585.

85. Humbert, G., Guingamp., M.-F., Kouomegne, R. and Linden, G., 1990, *J. Dairy Res.*, **57**, 143.

86. Ruheman, S., 1910, *J. Chem. Soc.*, **98**, 2025.

87. Moore, S. and Stein, W.H., 1948, *J. Biol. Chem.*, **176**, 367.

88. Moore, S. and Stein, W.H., 1954, *J. Biol. Chem.*, **211**, 907.

89. Moore, S., 1968, *J. Biol. Chem.*, **243**, 6281.

90. Doi, E., Shibata, D. and Matoba, T., 1981, *Anal. Biochem.*, **118**, 173.

91. Tsarichenko, A.P., 1966, Nauch. Tr. Krasnodar. Gos. Pedagog. Inst. No. **70**, 86, cited from Chem. Abstr. **67**, No. 79479c.

92. Friedman, M., Pang, J. and Smith, G.A., 1984, *J. Food Sci.*, **49**, 10.

93. Folkertsma, B. and Fox, P.F., 1992, *J. Dairy Res.*, **59**, 217

94. Weigele, M., De Bernardo, S.L., Tengi, J.P. and Leimgruber, W., 1972, *J. Am. Chem. Soc.*, **94**, 5927.

95. Udenfried, S., Stein, S., Bohlen, P., Darman, W., Leimgruber, W. and Weigele, M., 1972, *Science*, **178**, 871.

96. Pearce, K.N., 1979, *N.Z. J. Dairy Sci. Technol.*, **14**, 233.

97. Beeby, R., 1980, *N.Z. J. Dairy Sci. Technol.*, **15**, 99.

98. Creamer, L.K., Lawrence, R.C. and Gilles, J.,1985, *N.Z. J. Dairy Sci. Technol.*, **20**, 185.

99. Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L., 1983, *J.Dairy Sci.*, **66**, 1219.

100. Frister, H., Meisel, H. and Schlimme., E., 1989, *Kierler Milchwirtschaftliche Forschungsberichte*, **41**, 237.

101. Creamer, L.K., 1990, In: F-Doc 173, International Dairy Federation, p 27, Brussels.

102. Lindqvist, B., Storgards, T., Goransson, M.-B., 1953, Proc. XIII Intern. Dairy Congr. (the Hague), **3**, 1261.

103. Lindqvist, B. and Storgards, T., 1959, Proc. XV Intern. Dairy Congr. (London), **2**, 679.

104. Trieu-Cuot, P. and Gripon, J.-C., 1982, *J. Dairy Res.*, **49**, 501.

105. Shalabi, S.I. and Fox, P.F., 1987, *Ir. J. Food Sci. Technol.*, **11**, 135.

106. Andrews, A.T., 1983, *J. Dairy Res.*, **50**, 45.

107. Blakesley, R.W. and Boezi, J.A., 1977, *Anal. Biochem.*, **82**, 580.

108. Kosikowsky, F.V., 1951, *J. Dairy Sci.*, **34**, 235.

109. Storgards, T. and Lindqvist, B., 1953, *Milchwissenschaft*, **8**, 5.

110. Clemens, W., 1954, *Milchwissenschaft*, **9**, 195.

111. Mahmoud, S.A.Z., Khader, A.E., Salem, O.M., Moussa, A.M. and Kebary, K.M., 1983, *Minufiya J. Agric. Res.*, **6**, 207, cited from *Food Sci. Technol. Abstr.*, 1984, **16**, 6 P1446.

112. Ardo, Y. and Gripon, J.C., 1990, In: F-Doc 173, International Dairy Federation, pp 59-68, Brussels.

113. Visser, S., Slangen, K.J. and Hup, G., 1975, *Neth. Milk Dairy J.*, **29**, 319.

114. Ney, K.H., 1985, *Milchwissenschaft*, **40**, 207.

115. Tokita, F. and Hosono, A., 1968, *Milchwissenschaft*, **23**, 758.

116. Foster, P.M.D. and Green, M.L., 1974, *J. Dairy Res.*, **41**, 259.

117. Green, M.L. and Foster, P.M.D., 1974, *J. Dairy Res.*, **41**, 269.

118. Gripon, J.C., Desmazeaud, M.J., Le Bars, D. and Bergere, J.L., 1977, *J. Dairy Sci.*, **60**, 1532.

119. Lindqvist, B., 1962, Proc. XVI Intern. Dairy Congr. (Copenhagen), **B**, 673.

120. Lindqvist, B., 1963, Lindberg, I. and Molin, H., *Milchwissenschaft*, **18**, 12.

121. Abd-El-Salam, M.H. and El-Shibiny, S., 1972, *J. Dairy Res.*, **39**, 219.

122. Nath, K.R. and Ledford, R.A., 1973, *J. Dairy Sci.*, **56**, 710.

123. Huber, L. and Klostermeyer, H., 1974, *Milchwissenschaft*, **29**, 449.

124. Bachmann, M. and Schaub, W., 1974, Proc. XIX Intern. Dairy Congr. (New Delhi), **1E**, 271.

125. Creamer, L.K. and Richardson, B.C., 1974, *N.Z. J. Dairy Sci.Technol.*, **9**, 9.

126. Green, M.H. and Stackpoole, A., 1975, *J. Dairy Res.*, **42**, 297.

127. Gripon, J.C., Desmazeaud, M.J., Le Bars, D. and Bergere, J.L., 1975, *Lait*, **55**, 502.

128. Santoro, M., Leone, A.M., La Notte, E. and Liuzzi, V.A., 1987, *Milchwissenschaft*, **42**, 709.

129. Mulvihill, D.M. and Fox, P.F., 1979, *J. Dairy Res.*, **46**, 641.

130. Creamer, L.K., 1975, *J. Dairy Sci.*, **58**, 287.

131. Bican, P., 1983, *J. Dairy Sci.*, **66**, 2195.

132. Van Hooydonk, A.C.M. and Olieman, C., 1982, *Neth. Milk Dairy J.*, **36**, 153.

133. Bican, P. and Blanc, B., 1982, *Milchwissenschaft*, **37**, 592.

134. Vreeman, H.J., Visser, S., Slangen, C.J. and van Riel, J.A.M., 1986, *Biochem. J.*, **240**, 87.

135. Smith, A.M. and Nakai, S., 1990, *Can. Inst. Food Sci. Technol. J.*, **23**, 53.

136. Barrefors, P., Ekstrand, B., Fagerstam, L., LarsonRaznikiewicz, M., Scharr, J. and Steffner, P., 1985, *Milchwissenschaft*, **40**, 257.

137. St. Martin, M. and Paquin, P., 1990, *J. Dairy Res.*, **57**, 63.

138. Wilkinson, M.G., Guinee, T.P., O'Callaghan, D. M. and Fox, P.F., 1992, Lait, **72**, 449.

139. Amantea, G.F., Skura, B.J. and Nakai, S., 1986, *J. Food Sci.*, **51**, 912.

140. Kaminogawa, S., Yan, T.R., Azuma, N. and Yamauchi, K., 1986, *J. Food Sci.*, **51**, 1253.

141. Creamer, L.K., Aston, A. and Knighton, D., 1988, *N.Z. J. Dairy Sci. Technol.*, **23**, 185.

142. Christensen, T.M.I.E., Kristiansen, K.R. and Madsen, J.S., 1989, *J. Dairy Res.*, **56**, 823.

143. Cliffe, A.J., Revell, D. and Law, B.A., 1989, *Food Chem.*, **34**, 147.

144. Cliffe, A.J. and Law, B.A., 1990, *Food Chem.*, **36**, 73.

145. Cliffe, A.J. and Law, B.A., 1991, *Food Biotechnol.*, **5**, 1.

146. Gonzalez de Llano, D., Polo, C. and Ramos, M., 1991, *J. Dairy Res.*,**58**, 363.

147. Kosikowsky, F.V., 1951, *J. Dairy Sci.*, **34**, 228.

148. Kosikowsky, F.V. and Dahlberg, A.C., 1954, *J. Dairy Sci.*, **37**, 167.

149. Hintz, P.C., Slatter, W.L. and Harper, W.J., 1956, *J. Dairy Sci.*, **39**, 235.

150. Ali, L.A.M. and Mulder, H., 1961, *Neth. Milk Dairy J.*, **15**, 377.

151. Moore, S. and Stein, W.H., 1954, *J. Biol. Chem.*, **211**, 831.

152. Polychroniadou, A. and Vlachos, J.,1979, *Lait*, **59**, 234.

153. Weaver, J.C., Kroger, M. and Thompson, M.P., 1978, *J. Food Sci.*, **43**, 579.

154. Shindo, K., Sakurada, K., Niki, R. and Arima, S., 1980, *Milchwissenschaft*, **35**, 527.

155. Omar, M.M., 1984, *Food Chem.*, **15**, 19.

156. Polo, C., Ramos, M. and Sanches, R., 1985, *Food Chem.*, **16**, 85.

157. March, J.F., 1975, *Anal. Biochem.*, **69**, 420.

158. Wood, A.F., Aston, J.W. and Douglas, G.K., 1985, *Aust. J. Dairy Technol.*, **40**, 166.

159. Laleye, L.C., Simard, R.E., Grosselin, C., Lee, B.H. and Giroux, R.N., 1987, *J. Food Sci.*, **52**, 303.

160. Kostyra, H. and Damicz, W., 1979, *Zes. Naukowe. Akadem. Rolniczo Tehnic. Olsztynie Technol. Zywnosci.*, **15**, 91; cited from *Food Sci. Technol. Abstr.*, 1981, **13**, 6P1024.

161. Desyatnikova, O.I. and Alexaev, N.S., 1979, *Izvestiya Vysshikh Uchebnykh Zavedeii Pishchevaya Tekhnologiya*, **5**, 29; cited from *Food Sci. Technol. Abstr.*, 1981, **13**, 2P309.

162. Notte, E.La, Santoro, M., Leone, A.M. and Vitagliano, M., 1980, *Sci. Tec. Lattiero-Casearia*, **31**, 19; cited from *Food Sci. Technol. Abstr.*, 1980, **12**, 21P2047.

163. Ali, M.M., Al-Dahhan, D.H. and Abo-Elnaga, I.G., 1980, Zanco, A.*Pure Appl. Sciences*, **6**, 97, cited from *Food Sci.Technol. Abstr.*, 1984, **16**, 9P2064.

164. Ucuncu, M., 1981, *Molkerei-Zeitung wett der Milch*, **35**, 634, cited from *Food Sci. Technol. Abstr.*, 1982, **14**, 2P298.

165 Buruiana, L.M. and Zeidan, A.N.,1982, *Egypt. J. Dairy Sci.*, **10**, 209; cited from *Food Sci. Technol. Abstr.*, 1984, 16, 2P334.

166. Lavanchy, P. and Buhlmann, C., 1983, *Schweiz. Milchwirtschaft. Forsch.*, **12**, 3; cited from *Food Sci. Technol. Abstr.*, 1984, **16**, 3P623.

167. Buruiana, L.M. and Farag, S.I., 1983, *Egypt. J. Dairy Sci.*, **11**, 53; cited from *Food Sci. Technol. Abstr.*, 1984, **16**, 8P1828.

168. Resmini, P., Pellegrino, L., Pazzaglia, C. and Hogenboom, J.A., 1985, *Scienza Tec. Lattiero-Casearia*, **36**, 557; cited from *Food Sci. Technol. Abstr.*, 1988, **20**, 12P126.

169. Marsili, R., 1985, *J. Dairy Sci.*, **68**, 3155.

170. Hwang, J.H., Huh, J.W. and Yu, J.H., 1987, *Korean J. Dairy Sci.*, **9**, 101; cited from *Food Sci. Technol. Abstr.*, 1988, **20**, 4P98.

171. Attia, I.A. and Gooda, E., 1987, *Egypt. J. Dairy Sci.*, **15**, 135; cited from *Food Sci. Technol. Abstr.*, 1987, **19**, 11P82.

172. Koops, J. and Klomp, H., 1977, *Neth. Milk Dairy J.*, **31**, 56.

173. Shipe, W.F., Senyk, G.F. and Fountain, K.B., 1980, *J. Dairy Sci.*, **63**, 193.

174. Bynum, D.G., Senyk, G.F. and Barbano, D.M., 1984, *J. Dairy Sci.*, **67**, 1521.

175. Woo, A.H. and Lindsay, R.C., 1982, *J. Dairy Sci.*, **65**, 1102.

176. Deeth, H.C. and Fitz-Gerald, C.H., 1976, *Aust. J. Dairy Technol.*, **31**, 53.

177. Thomas, E.L., Nielson, A.J. and Olson, J.C. Jr., 1955, *Am. Milk Rev.*, **77**, 50.

178. Dulley, J.R. and Grieve, P.A., 1974, *Aust. J. Dairy Technol.*, **29**, 120.

179. Ledford, R.A., 1969, *J. Dairy Sci.*, **52**, 949.

180. Keen, A.R. and Walker, N.J., 1974, *J. Dairy Res.*, **41**, 397.

181. Humbert, E.S. and Lindsay, R.C., 1969, *J. Dairy Sci.*, **52**, 1862.

182. Martin-Hernandez, M.C., Alonso, L., Juarez, M. and Fontecha, J., 1988, *Chromatographia*, **25**, 87.

183. Bills, D.D., Khatri, L.L. and Day, E.A., 1963, *J. Dairy Sci.*, **46**, 1342.

184. McNeill, G.P. and Connolly, J.F., 1989, *Ir. J. Food Sci. Technol.*, **13**, 119.

185. McCarthy, R.D. and Duthie, A.H., 1962, *J. Lipid Res.*, **3**, 117.

186. Thakur, M.K., Kirk, J.R. and Hedrick, T.I., 1975, *J. Dairy Sci.*, **58**, 175.

187. Harte, B.R. and Stine, C.M., 1977, *J. Dairy Sci.*, **60**, 1266.

188. Woo, A.H. and Lindsay, R.C., 1980, *J. Dairy Sci.*, **63**, 1058.

189. Ha, J.K. and Lindsay, R.C., 1990, *J. Dairy Sci.*, **73**, 1988.

190. Contarini, G., Galliena, C., Toppino, P.M. and Amelotti, G., 1990, Proc. 11th Intl. Symp. Capilliary GC. Monterey, CA, USA, pp 407-420.

191. Fox, P.F., Lucey, J.A. and Cogan, T.M., 1990, *CRC Crit. Rev. Food Sci. Technol.*, **29**, 237.

192. Boehringer, Mannheim,. 1986 Methods of Biochemical Analysis and Food Analysis, p 8, Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany.

193. Walsh, B. and Cogan, T.M., 1974, *J. Dairy Res.*, **41**, 25.

194. Walsh, B. and Cogan, T.M.,1974, *J. Dairy Res.*, **41**, 31.

195. Keen, A.R. and Walker, N.J., 1974, *J. Dairy Res.*, **41**, 65.

196. Thornhill, P.J. and Cogan, T.M., 1984, *Appl. Environ. Microbiol.*, **47**, 1250.

197. Turner, K.W., Morris, H.A. and Martley, F.G., 1983, *N.Z. J. Dairy Technol.*, **18**, 117.

198. O'Keefe, P.W., Libbey, L.M. and Lindsay, R.C., 1969, *J. Dairy Sci.*, **52**, 888.

199. Wong, N.P., Ellis, R., La Croix, D.E. and Alford, J.A., 1973, *J. Dairy Sci.*, **56**, 636.

200. Wong, N.P., Ellis, R. and La Croix, D.E., 1975, *J. Dairy Sci.*, **58**, 1437.

201. Patton, S., 1950, *J. Dairy Sci.*, **33**, 680.

202. Morgan, M.E. and Anderson, E.O., 1956, *J. Dairy Sci.*, **39**, 253.

203. Sato, M., Honda, T., Yamada, Y., Takada, A. and Kawanami, T., 1966, Proc. XVII Intern. Dairy Congr, (Munich), **D**, 539.

204. Kanisawa, T. and Itoh, H., 1984, *J. Jap. Soc. Food Sci. Technol.*, **31**, 477; cited from *Food Sci. Technol. Abstr.*, 1986, **18**, 2P137.

205. Schwartz, D.P., Haller, H.S. and Keeney, M., 1963, *Anal. Chem.*, **35**, 2191.

206. Schwartz, D.P. and Parks, O.W., 1963, *J. Dairy Sci.*, **46**, 989.

207. Schwartz, D.P. and Parks, O.W., 1963, *J. Dairy Sci.*, **46**, 1136.

208. Schwartz, D.P., Parks, O.W. and Boyd, E.N., 1963, *J. Dairy Sci.*, **46**, 1422.

209. Jolly, R.C. and Kosikowski, F.V., 1975, *J. Dairy Sci.*, **58**, 846.

210. Godinho, M. and Fox, P.F.,1981, *Milchwissenschaft*, **36**, 476.

211. Dartley, C.K. and Kinsella, J.E., 1971, *J. Agr. Food Chem.*, **19**, 771.

212. Manning, D.J., 1978, *J. Dairy Res.*, **45**, 479.

213. Renner, E., 1987, In: Cheese: Chemistry, Physics and Microbiology, 1st edn., (P.F. Fox, ed.), Elsevier Applied Science Publishers, London, p 345.

214. Spector, S., Melmon, K., Lovenberg, W. and Sjoerdsma, A., 1963, *J. Pharmacol. Exper. Ther.*, **140**, 229.

215. Price, K. and Smith, S.E., 1971, *Lancet*, **1**, part 1, 130.

216. Voigt, M.N., Eitenmiller, R.R., Koehler, P.E. Hamdy, H.K., 1974, *J. Milk Food Technol.*, **37**, 377.

217. Colonna, P. and Adda, J., 1976, *Lait*, **56**, 143.

218. Taylor, S.L., Lieber, E.R. and Leatherwood, M., 1978, *J. Food Sci.*, **43**, 247.

219. Antila, P., Luomanpera, E. and Antila, V., 1982, Proc. XXI Intern. Dairy Congr. (Moscow), **1**, book 1, 465.

220. Kaplan, E.R., Sapeika, N. and Moodie, I.M., 1974, *Analyst*, **99**, 565.

221. Evans, C.S., Gray, S. and Kazim, N.O., 1988, *Analyst*, *113*, 1605.

222. Staruszkiewicz, W.F. and Bond, J.F., 1981, *J. Assoc. Off. Anal. Chem.*,**64**, 584.

223. Staruszkiewicz, W.F., 1977, *J. Assoc. Off. Anal. Chem.*, **60**, 1131.

224. Chambers, T.L. and Staruszkiewicz, W.F., 1978, *J. Assoc. Off. Anal. Chem.*, **61**, 1092.

225. Koehler, P.E. and Eitenmiller, R.R., 1978, *J. Food Sci.*, **43**, 1245.

226. Chang, S.F., Ayres, J.W. and Sandine, W.E., 1985, *J. Dairy Sci.*, **68**, 2840.

227. Suhren, G., Heeschen, W. and Tolle, A., 1982, *Milchwissenschaft*, **37**, 143.

228. Antila, P., Antila, V., Mattila, J. and Hakkarainen, H., 1984, *Milchwissenschaft*, 39, 81.

229. Takeba, K., Maruyama, T., Matsumoto, M. and Nakazawa, H., 1990, *J. Chromatog.*, **504**, 441.

230. Sumner, S.S., Roche, F. and Taylor, S.L., 1990, *J. Dairy Sci.*, **73**, 3050.

231. Official Methods of Analysis, 1984, 14th edn., p 339, Association of Official Analytical Chemists, Washington, DC.

232. Aston, J.W. and Dulley, J.R., 1982, *Aust. J. Dairy Technol.*, **37**, 59.

233. Adda, J., Gripon, J.C. and Vassal, L., 1982, *Food Chem.*, **9**, 115.

234. Walker, J.R.L., 1959, *J. Dairy Res.*, **26**, 273.

235. Tsugo, T. and Matsuoka, H., 1962, Proc. XVI Intern. Dairy Congr (Copenhagen), **B**, 385.

236. Lawrence, R.C., 1963, *J. Dairy Res.*, **30**, 235.

237. McGugan, W.A., Howsam, S.G., Elliott, J.A., Emmons, D.B., Reiter, B. and Sharpe, M.E., 1968, *J. Dairy Res.*, **35**, 237.

238. Manning, D.J. and Robinson, H.M., 1973, *J. Dairy Res.*, **40**, 63.

239. Manning, D.J., 1974, *J. Dairy Res.*, **41**, 81.

240. Manning, D.J., Chapman, H.R. and Hosking, Z.D., 1976, *J. Dairy Res.*, **43**, 313.

241. Manning, D.J. and Price, J.C., 1977, *J. Dairy Res.*, **44**, 357.

242. Manning, D.J., 1979, *J. Dairy Res.*, **46**, 531.

243. Manning, D.J. and Moore, C., 1979, *J. Dairy Res.*, **46**, 539.

244. Aston, J.W. and Douglas, K., 1983, *Aust. J. Dairy Technol.*, **38**, 66.

245. Ramos, M. and Juarez, M., 1984, Bull. 181, International Dairy Federation, p 3, Brussels.

246. Ramos, M. and Juarez, M., 1986, Bull. 202, International Dairy Federation, p 175, Brussels.

247. Palo, V., 1975, A 7-Doc 3, International Dairy Federation, Brussels.

248. Ramos, M., Martinez-Castro, I. and Juarez, M., 1977, *J. Dairy Sci.*, **60**, 870.

249. Kaiser, K.P. and Krause, I., 1985, *Z. Lebensm. Unters. Forsch.*, **180**, 181.

250. Lavoille, B., 1976, *Ann. Fals. Exp. Chim.*, **69**, 535.

251. Sawaya, W.N., Safi, W.J., Al-Shalhat, A.F. and AlMohammad, H.M., 1984, *Milchwissenschaft*, **39**, 90.

252. Aschaffenburg, R. and Dance, J.E., 1968, *J. Dairy Res.*, **35**, 383.

253. Pierre, A. and Portmann, A., 1970, *Ann. Technol. Agric. Technol. Agric.*, **19**, 107.

254. Addeo, F., Anelli, G., Stingo, C., Chianese, L., Petrilli, P. and Scudiero, A., 1984, *Latte*, **9**, 37.

255. Addeo, F., Anelli, G. and Chianese, L., 1986, Bull. 202, International Dairy Federation, p 191, Brussels.

256. Krause, I., Belitz, H.D. and Kaiser, K.P., 1982, *Z. Lebensm. Unters. Forsch.*, **174**, 195.

257. Monget, D., Gelin, M. and Laviolette, P., 1979, *Lait*, **59**, 117.

258. Pollman, R.M., 1984, *J. Assoc. Off. Anal. Chem.*, **67**, 1062.

259. Durand, M., Meusnier, M., Delahaye, J. and Prunet, P., 1974, *Boll. Ac. Vet.*, **47**, 247.

260. Levieux, D., Proc. XX Intern. Dairy Congr. (Paris), Paper 15 ST, 1978.

261. Aranda, P., Oria, R. and Calvo, M., 1988, *J. Dairy Res.*, **55**, 121.

262. Rodriguez, E., Martin, R., Garcia, T., Hernandez, P.E. and Sanz, B., 1990, *J. Dairy Res.*, **57**, 197.

263. Farkye, N.Y. and Fox, P.F., 1990, *Trends Food Sci. Technol.*, **1**, 37.

264. Santa-Maria, G., Ramos, M. and Ordonez, J.A., 1986, *Food Chem.*, **19**, 225.

265. Phelan, J.A., 1985, *Milk Coagulants - an evaluation of alternatives to standard calf rennet*. Ph.D. Thesis, National University of Ireland, Cork.

266. Yiadom-Farkye, N.A., 1986, *Role of Chymosin and Porcine Pepsin in Cheddar Cheese Ripening*. Ph.D. Thesis, Utah State University, Logan, UT, USA.

267. Guinee, T.P. and Fox, P.F., 1984, *Ir. J. Food Sci. Technol.*, **8**, 105.

268. Lawrence, R.C., Creamer, L.K. and Gilles, J., 1987, *J. Dairy Sci.*, **70**, 1748.

269. Woo, A.H., Kollodge, S. and Lindsay, R.C., 1984, *J. Dairy Sci.*, **67**, 874.

270. Woo, A.H. and Lindsay, R.C., 1984, *J. Dairy Sci.*, **67**, 960.

271. Arnold, A.G., Shahani, K.M. and Dwivedi, B.K., 1975, *J. Dairy Sci.*, **58**, 1127.

272. Zerfiridis, G., Zafopoulou-Mastrogiannaki, A. and Litopoulou-Tzanekati, E., 1984, *J. Dairy Sci.*, **67**, 1397.

273. Barbano, D.M., 1985, In: *New Dairy Products via New Technology*, Proc. IDF Seminar, Atlanta, GA, USA, p 31.

274. Kinsella, J.E. and Hwang, D.H., 1976, *CRC Crit. Rev. Food Sci. Nutr.*, **8**, 191.

275. Lawrence, R.C., Heap, H.A. and Gilles, J., 1984, *J. Dairy Sci.*, **67**, 1632.

276. Karahadian, G. and Lindsay, R.C., 1987, *J. Dairy Sci.*, **70**, 909.

277. Creamer, L.K. and Olson, N.F., 1982, *J. Food Sci.*, **47**, 631.

278. de Jong, L., 1976, *Neth. Milk Dairy J.*, **30**, 242.

279. Fedrick, I.A. and Dulley, J.R., 1984, *N.Z. J. Dairy Sci. Technol.*, **19**, 141.

280. Noomen, A., 1983, *Neth. Milk Dairy J.*, **37**, 229.

281. Lenoir, J., 1984, Bull. 171, International Dairy Federation, pp 3-20, Brussels.

282. Green, M.L., Marshall, R.J. and Brooker, B.E., 1985, *J. Texture Studies*, **16**, 351.

283. Bican, P. and Spahni, A., 1991, *Lebensm. Wiss. u. Technol.*, **24**, 315.

284. Mojarro-Guerre, S. H., Amado, R., Arrigoni, G., and Solms, J., 1991, *J. Food Sci.*, **56**, 943.

285. Gonzalez de Llano, D., Ramos, M. and Polo, C., 1987, *Chromatographia*, **23**, 764.

286. Prentice, J. H., 1987, In: Cheese, Chemistry, Physics and Microbiology, 1st edn., Vol. 1, p. 299, P. F. Fox (ed)., Elsevier Applied Science Publishers, London.

287. Prentice, J. H. and Marshall, , 1987, In: Cheese, Chemistry, Physics and Microbiology, 2nd edn., Vol. 1, P. F. Fox (ed)., Elsevier Applied Science Publishers, London.

288. Singh, T. K., Fox, P. F., Højrup, P. and Healy, A., 1993, *Intern. Dairy J.* (in press)

4

PROTEOLYTIC SPECIFICITY OF CHYMOSIN ON BOVINE α_{s1}-CASEIN†

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The proteolytic specificity of chymosin (E.C. 3.4.23.4) on bovine α_{s1}-casein at 30°C in phosphate buffer, pH 6.5, and at pH 5.2 in the presence of 5% (w/v) NaCl was investigated. Peptides (pH 4.6-soluble) were isolated by reversed phase-HPLC and identified from their amino acid sequence; the identity of some peptides was confirmed by mass spectrometry and/or amino acid composition. The small peptides produced at pH 6.5 were: 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 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}. The rate of production of individual peptides differed under the two conditions studied but Arg_1-Phe_{23} and Tyr_{165}-Trp_{199} were the first and second peptides produced under both conditions. Pathways are proposed to interpret the proteolysis of α_{s1}-casein in solution under the conditions of this study.

INTRODUCTION

The aspartyl proteinase, chymosin (E. C. 3.4.23.4), is secreted in the stomach of young mammals. Its natural function is to coagulate milk in the stomach of the neonate, thereby increasing digestive efficiency. However, chymosin is the principal enzyme in calf, kid and lamb rennets used to coagulate milk for cheesemaking. The primary action of chymosin in the coagulation of milk is the specific hydrolysis of κ-casein (CN) at the Phe_{105}-Met_{106} bond (Delfour *et al.*, 1965), which destabalizes the casein micelles which coagulate in the presence of Ca^{2+}. Most of the coagulant added to cheese milk is lost in the whey but about 6% of the added enzyme is retained in the curd; the amount of chymosin activity retained in the curd is dependent on a number of factors (see Fox, 1988). The residual chymosin activity in the curd has a considerable impact on proteolysis in the resulting cheese and consequently on its quality (Fox, 1989). It has been demonstrated that chymosin is the main agent responsible for the initial proteolysis in most cheese varieties and that the formation of water-soluble N in such cheeses is largely a consequence of chymosin action (O'Keeffe *et al.*, 1976, 1978).

Chymosin is active on both α_{s1}-CN and β-CN in solution but in cheese it appears to hydrolyse primarily α_{s1}-CN which is extensively degraded in Cheddar and Dutch-type cheeses, primarily due to the action of chymosin (Fox, 1989).

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Proteolysis by the coagulant is believed to be responsible for softening of the cheese early in ripening via the hydrolysis of α_{s1}-CN at the bond Phe_{23}-Phe_{24} (Creamer & Olson, 1982) and it is likely that proteolysis of α_{s1}-CN f24-199 by the coagulant and other proteinases further modifies the texture of the cheese. It appears that the proteinases and peptidases of the starter bacteria act primarily on large primary and intermediate sized peptides produced from casein by the coagulant and perhaps plasmin, to yield a range of peptides, amino acids and other compounds which are essential to the development of cheese flavour (Olson, 1990). Therefore, the action of chymosin on α_{s1}-CN is fundamentally important in many aspects of cheese ripening.

A number of authors have investigated the proteolysis of α_{s1}-CN in solution by chymosin. Pelissier *et al.* (1974) found that more than 20 peptides were produced, some of which were found to be bitter. The authors proposed a number of cleavage sites based on the amino acid composition of peptides isolated. Mulvihill & Fox (1979) studied the proteolysis of α_{s1}-CN by chymosin in aqueous solutions at pH 6.5 and at pH 5.2 in the presence of 5% NaCl. The latter conditions were chosen to mimic the ionic conditions present in many young cheeses, i.e. pH of *ca.* 5.2 and a salt-in-moisture concentration of about 5%. These authors investigated the production of various polypeptides which they detected by electrophoresis and proposed a number of potential cleavage sites based primarily on the amino acid composition of the polypeptides isolated by preparative polyacrylamide gel electrophoresis. Mulvihill & Fox (1977) studied the influence of NaCl and urea on proteolysis of α_{s1}-CN by chymosin. Pahkala *et al.* (1989) incubated a crude α_s-CN preparation, containing α_{s1}- and α_{s2}-CN, with chymosin and identified a number of the peptides produced based on their amino acid composition.

However, the results of these studies are conflicting and the sites at which chymosin cleaves α_{s1}-CN in solution remain to be determined definitively. This study was undertaken in order to clarify the identity of peptides produced from α_{s1}-CN by chymosin under different ionic conditions.

MATERIALS AND METHODS

Enzyme: Recombinant chymosin B (E. C. 3.4.23.4, expressed in *Kluveromyces marxianus* var. *lactis*) was obtained from Gist Brocades Ltd., Delft, the Netherlands. Prior to use, the enzyme preparation was dialyzed against two 20-volume changes of distilled H_2O at 4°C overnight and freeze-dried. The freeze-dried powder was resuspended in 100 mM K-phosphate buffer, pH 6.5. This solution had an activity of approximately 70 chymosin units (CU) ml^{-1} (1 CU is the activity necessary to coagulate 10 ml bovine milk, pH 6.5, in 100 s at 30°C). Crystalline calf chymosin was obtained from Sigma Chemical Co., St. Louis, MO, USA.

Substrate: Whole casein was prepared by isoelectric precipitation from a sample of bulk bovine skim milk obtained from the Dairy Plant, University of Wisconsin-Madison, according to the method of Hill (1963). α_{s1}-CN [approximately 97% B allele, calculated from the composition of breeds in the University herd according to the data of Ng-Kwai-Hang *et al.* (1990) and Van Eenennaam & Medrano (1991)] was prepared by ion-exchange chromatography (Creamer, 1974) on DEAE-cellulose (Whatman DE-52) using 10 mM imidazole buffer, pH 7.0, containing 4.5 M urea and 0.1% (v/v) 2-mercaptoethanol. Proteins were eluted from the ion-exchanger by a linear NaCl gradient (0 to 0.5 M). Fractions corresponding to α_{s1}-CN were combined, dialyzed against 5 changes of distilled H_2O and freeze-dried.

Hydrolysis Conditions: α_{s1}-Casein was dissolved (10 mg ml^{-1}) in 100 mM K-phosphate buffer at pH 6.5 or at pH 5.2 in the presence of 5% (w/v) NaCl. Sodium azide (0.05%, w/v) was added to inhibit bacterial growth. Chymosin was added (approximately 0.5 units ml^{-1} at pH 6.5 and 0.7 units ml^{-1} at pH 5.2) and the solution

was incubated at 30°C. Aliquots were taken periodically for analysis by reversed-phase (RP-) HPLC or urea-PAGE. Peptides were isolated from 12 and 24 h hydrolysates.

Reversed-Phase HPLC: Samples for RP-HPLC were heated at 64°C for 30 min to inactivate the chymosin. Samples (1 ml) were adjusted to approximately pH 4.6 by the addition of 30 ml acetic acid (33.3%, w/v), held at room temperature for 10 min and 30 ml Na acetate (3.33 M) added (McGann *et al.*, 1972). The samples were centrifuged at 16,000 **g** for 10 min and the supernatant used for analysis. RP-HPLC was performed on a Beckman HPLC system (Beckman, San Ramon, CA, USA, consisting of autosampler, model 507, programmable solvent module, model 126AA, and programmable detector, model 166, interfaced with a personal computer using Beckman System Gold software). A LiChrosorb RP-18 (5 μm) column, 250 x 4 mm (E. Merck, Darmstadt, Germany), was used with an Adsorbosphere C_{18} guard column (Alltech Associates, Deerfield, IL, USA). Elution was by means of a gradient prepared using solvent A [0.1 % trifluoroacetic acid (TFA) in H_2O] and solvent B [0.1% TFA in acetonitrile (Aldrich Chemical Co., Milwaukee, WI, USA)] as follows: 100% A for 5 min, 0 to 50% B at a rate of 1.65 % B min^{-1} followed by 50 % B for 5 min. Flow rate was 1 ml min^{-1} and detection was at 215 nm.

Urea-PAGE: Samples of hydrolysates were prepared for urea-PAGE by the addition of an equal volume of buffer of the following composition : 1.5g tris (hydroxymethyl) methylamine, 48g urea, 0.8 ml 9.86 M HCl, 1.4 ml 2-mercaptoethanol and 50 mg bromophenol blue, made up to 100 ml (modified from Andrews, 1983). Discontinuous alkaline urea-PAGE was performed according to the method of Andrews (1983), 12% T (acrylamide plus bisacrylamide), 4% C (bisacrylamide as a percentage of T), pH 8.9 with direct staining using Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977).

Isolation of Peptides: Peptides were isolated by RP-HPLC as described above. In one case, further separation was performed using the same apparatus at neutral pH (Le Bars & Gripon, 1989). Solvent A was 50 mM K-phosphate, pH 7.0, while solvent B was composed of buffer A (40%, v/v) and acetonitrile (60%, v/v). Gradient elution was 0 to 70 % B at a rate of 2.33% B min^{-1} followed by 70 to 100% B at 6 % B min^{-1}; flow rate 1 ml min^{-1}. The two peptides separated under these neutral conditions were rechromatographed using an acetonitrile/H_2O gradient to desalt the peptides prior to sequence analysis.

Before sequence analysis all peptides were rechromatographed using the acetonitrile/water gradient (as for isolation) to assess their purity. The acetonitrile was removed by evaporation under nitrogen and the peptides freeze-dried.

Amino Acid Composition: The amino acid composition of one peptide (no. 6, Fig. 4.1) was determined after hydrolysis with 6 M HCl (Sequanal grade, Pierce, Rockford, IL, USA). Amino acids were quantified by cation-exchange HPLC on the above system with a Spherogel AA-NA+ column (Beckman, San Ramon, CA, USA, 250 x 3 mm; column temperature, 54°C) with post-column ninhydrin derivitization.

Sequence Analysis: Peptides were sequenced at the National Food Biotechnology Centre, University College, Cork, Ireland, by Edman degradation on an automated pulsed liquid-phase protein/peptide sequencer (Applied Biosystems Inc., Foster City, CA, USA, model 477A.). Liberated amino acids were detected as their phenylthiohydantoin derivatives by means of a model 120A analyser (Applied Biosystems Inc.).

The carboxy-terminal sequence of one peptide (no. 9, Fig.4.1) was determined by a time-course hydrolysis with carboxypeptidase Y (E. C. 3.4.16.1,

protein sequencing grade, Sigma Chemical Co., St. Louis, MO, USA). Hydrolysis conditions were as described by the supplier; aliquots (5µl) were taken and the reaction stopped by addition to 95 µl amino acid analysis sample diluent (pH 2.20, $[Na^+]$= 0.27 M, Beckman, San Ramon, CA, USA). Amino acids were quantified as described above.

Mass Spectrometry: Mass spectrometry was performed using a BioIon 20K plasma desorption time-of-flight instrument (Applied Biosystems AB, Uppsala, Sweden). The peptide samples were dissolved in 0.1% TFA and applied to a nitrocellulose-covered target, spin-dried and micro-rinsed as described by Nielsen *et al.* (1988). Spectra were recorded at 14 kV for 10^6 primary fission events. Identification of peptides by by mass was based on partial sequence data combined with mass searches using the GPMAW program (Lighthouse Data, DK-5250 Odense SV, Denmark).

RESULTS

The peptides produced from α_{s1}-CN by recombinant chymosin were compared to those produced by crystalline calf chymosin. Identical peptide profiles by RP-HPLC were obtained for recombinant and calf chymosin under the conditions of this study (results not shown).

The pH 4.6-soluble peptide profiles of the hydrolysates at pH 6.5 and at pH 5.2 in the presence of 5%, w/v, NaCl had a number of peptides in common (Fig. 4.1a, b), although one peptide (peak no. 4, f154-164) was detected only in the pH 6.5 hydrolyzate while 5 peptides (peak nos. 10-14) were detected only in the pH 5.2 hydrolyzate. The activity of chymosin added at each pH was chosen so as to give approximately equal rates of proteolysis of α_{s1}-CN and α_{s1}-CN (f24-199) at pH 5.2 and 6.5. All the major pH 4.6-soluble peptides in the pH 6.5 hydrolysate were identified. In the case of the hydrolysate at pH 5.2 in the presence of 5%, w/v, NaCl, only those peaks which were not present in the pH 6.5 hydrolysate were analyzed and identified. The reversed-phase gradient used was adequate to separate most of the peptides but it was necessary to further separate two peptides (peaks 7 and 8), which were poorly resolved by this gradient, by reversed-phase chromatography at a neutral pH.

The isolated peptides were sequenced by an automated Edman procedure to an apparent C-terminus in most cases. Peptide no. 6 (f1-23) was identified from its 9 N-terminal residues and from its amino acid composition. As the primary chymosin cleavage site in α_{s1}-CN, i.e. Phe_{23}-Phe_{24}, is well established (Hill *et al.*, 1974; Carles & Ribadeau-Dumas, 1985), it was decided that identification by N-terminal sequence and amino acid composition was sufficient in this case. Peptide no. 9 was identified as α_{s1}-CN f165-199, i.e. the C-terminus of α_{s1}-CN, based on the sequence of the 10 N-terminal residues and the time-course of the release of the 4 C-terminal residues by carboxypeptidase Y. Due to limited amounts of samples or washout from the sequencer, it was impossible to determine the C-terminus of 2 peptides (no. 11 and 12, Fig.1). In these cases, the size of the peptide was determined by mass spectrometry, thus establishing the C-terminal cleavage site (Table 4.1). It was not possible to determine the extent of peptide 7 due to limited sample size; this peptide was equenced to Gly_{33} and probably extends beyond this residue, perhaps to Leu_{40} since the next likely chymosin cleavage site is Leu_{40}-Ser_{41}. Therefore, peptide 7 is tentatively identified as Phe_{24}-Leu_{40}.

The sequences determined for the peptides are summarized in Table 4.1 and their position in the α_{s1}-CN molecule indicated in Fig. 4.2. The bonds identified as being susceptible to hydrolysis by chymosin under the conditions of this study were:- Leu_{11}-Pro_{12}, Phe_{23}-Phe_{24}, Phe_{28}-Pro_{29}, Phe_{32}-Gly_{33}, Leu_{40}-Ser_{41}(?), Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160}, Trp_{164}-Tyr_{165} and Phe_{179}-Ser_{180}.

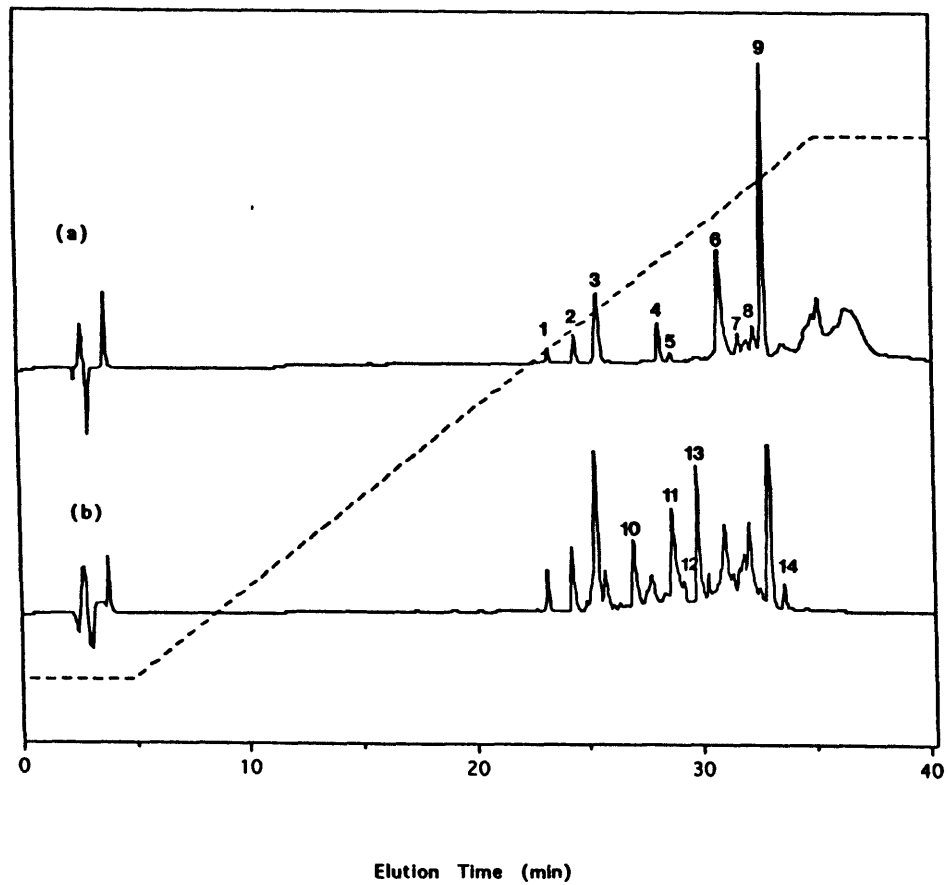

Fig. 4.1. Reversed-phase HPLC profiles of pH 4.6-soluble peptides from bovine α_{s1}-casein (10 mg ml^{-1}) hydrolyzed by chymosin at 30°C for 24 h in 100 mM K-phosphate buffer, as described in the text, (a) at pH 6.5 and (b) at pH 5.2 in the presence of 5% NaCl. Solvent A was 0.1% trifluoroacetic acid (TFA) in H_2O, solvent B 0.1% TFA in acetonitrile, gradient (0-50% CH_3CN) as indicated (--------). Absorbance detected at 215 nm.

Table 4.1: *Identity of the pH 4.6-soluble peptides produced from bovine α_{s1}-casein (CN) by the action of chymosin at pH 6.5 or at pH 5.2 in the presence of 5% NaCl.*

HPLC Peak No.[†]	*Position in α_{s1}-CN sequence*	*Sequence determined*
1	154 - 159	H_2N-Tyr-Gln-Leu-Asp-Ala-Tyr-COOH
2	150 - 153	H_2N-Phe-Arg-Gln-Phe-COOH
3	157 - 164	H_2N-Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp-COOH
4	154 - 164	H_2N-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp-COOH
5	150 - 156	H_2N-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-COOH
6	1 - 23	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-.....[§]
7	24 - ?	H_2N-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-.....
8	24 - 28	H_2N-Phe-Val-Ala-Pro-Phe-COOH
9	165 - 199	H_2N-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr..... .....Met-Pro-Leu-Trp-COOH[¶]
10	1-11	H_2N-Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-COOH
11	102-142	H_2N-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val...[††]
12	165-179	H_2N-Tyr-Tyr-Val-Pro-Leu-Gly...[#]
13	143-149	H_2N-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-COOH
14	24-32	H_2N-Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-COOH[§§]

[†] See Fig. 4.1.

[§] Amino acid composition (mol residue / mol peptide): Asp=2.17 (2); Thr=0.16 (0); Ser=0.28 (0); Glu=4.21 (4); Pro=3.01 (3); Gly=1.10 (1); Ala=0.13 (0); Cys=0.00 (0); Val=1.07 (1); Met=0.00 (0); Ile=0.80 (1); Leu=3.66 (4); Tyr=0.06 (0); Phe=1.03 (1); Lys=1.96 (2); His=1.79 (2); Arg=1.75 (2); Trp=not determined (0)
Values in parentheses indicate molar ratio of amino acids in peptide α_{s1}-CN (f1-23).

[¶] C-terminal sequence determined by enzymic hydrolysis with carboxypeptidase Y, deduced sequence ...Met-Pro-Leu-Trp-COOH

[††] Identity confirmed by mass spectrometry. Mass of peptide f102-142= 4809.1 Da (experimental), 4808.5 Da (theoretical).

[#] Identity confirmed by mass spectrometry. Mass of peptide f165-199= 1720.3 Da (experimental), 1721.9 Da (theoretical).

[§§] Identity confirmed by mass spectrometry. Mass of peptide f24-32=1051.8 Da (experimental), 1052.2 Da (theoretical).

The pH 4.6-soluble peptides identifed as being produced by chymosin from α_{s1}-CN at pH 6.5 are listed in Table 4.1. All of these, except f154-164, were also produced at pH 5.2 in the presence of NaCl, and in addition f1-11, f24-32, f102-142, f143-149 and f165-179. Although peptides with the same elution time as those found at pH 6.5 were not isolated and identified from the hydrolysate at pH 5.2, it is likely that peptides with the same elution times were the same. The production of the peptide f160-164 would be expected from the above cleavage sites, but it was not dentified in the RP-HPLC elution profile. Likewise, the peptides f12-23 and f180-199 were not identified in the hydrolysate at pH 5.2 in the presence of 5% (w/v) NaCl. Five of the peptides identified in this study agree with those reported by Pahkala *et al.* (1989), but most of the peptides found by these authors were not identified.

Changes in the relative peak areas of the major peptide peaks with time are shown in Fig. 4.3 (pH 6.5) and Fig. 4.4 (pH 5.2 in the presence of 5%, w/v, NaCl). The progress of proteolysis differed under the two conditions studied. At pH 6.5, f1-23 was produced first and reached a plateau after *ca.* 3 h. Peptide f165-199 was formed next but did not reach a plateau during the period of the study (24 h); its relative peak area did increase until at 12 h it was the major peak on the RP-HPLC profile of the hydrolysate. The other peptides appeared at slower rates in the approximate order: f157-154, f154-164, f154-159, f24-28 and f150-153. Peptide f150-156 remained a minor component in the RP-HPLC elution profile throughout. Peptide f24-28 appears to increase at a more rapid rate than the peptides from the region 150-164 and eventually became a relatively major component in the RP-HPLC profile.

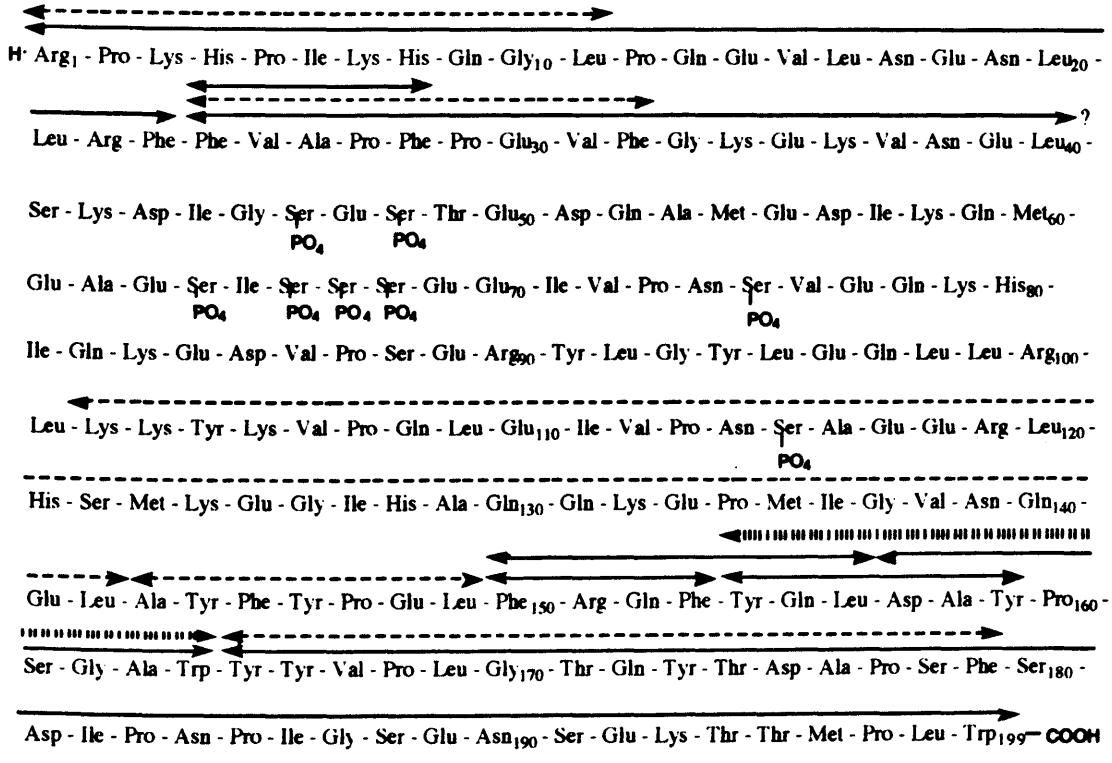

Fig. 4.2. Primary structure of Bos *α_{s1}-casein B 8-P (Mercier* et al., *1971; Grosclaude* et al., *1973) showing the position of the pH 4.6 soluble peptides produced by hydrolysis with chymosin at pH 6.5 or at pH 5.2 in the presence of 5% (w/v) NaCl. Peptides identified in hydrolyzates at both pH values are indicated as ——; those found only in hydrolyses at pH 5.2 in the presence of 5% (w/v) NaCl are indicated as -------. The peptide f157-164 was identified only in the hydrolysate at pH 6.5 ( ).*

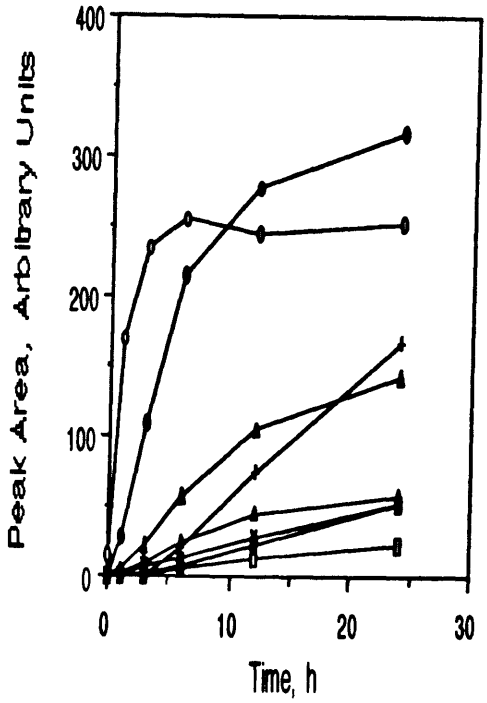

Fig. 4.3. Time course of changes in the area of the HPLC peaks representing the major peptides produced on hydrolysis of bovine α_{s1}-casein by chymosin at 30°C in 100 mM K-phosphate buffer, pH 6.5. f 1-23, (○); f 165-199, (●); f 157-164, (△); f154-164, (▲); f 154-159, (□); f 150-153, (■); f 24-40(?), (X); f 24-28 (+). Experimental details are given in the text.

At pH 5.2, peptides f1-23 and f165-199 appeared to be produced at similar rates, although f1-23 was hydrolyzed subsequently. Peptide f157-164 was also produced quickly, as were peptides f24-28 and f24-40(?). The remaining peptides were produced in the approximate order: f102-142, f143-149, f1-11, f150-153 and f154-159.

Electrophoretograms of hydrolyzates of α_{s1}-CN over time at pH 6.5 and at pH 5.2 in the presence of NaCl are shown in Fig. 4.5. Under both conditions, α_{s1}-CN was hydrolysed completely within 3 h. The primary hydrolysis product detected by PAGE in the pH 5.2 and 6.5 hydrolyzates, "α_{s1}-I", i.e. f24-199 (Creamer & Richardson, 1974; Mulvihill & Fox, 1979), was completely hydrolysed subsequently under both conditions but the hydrolysis pattern differed with the ionic conditions.

DISCUSSION

A number of authors (Barbano & Rasmussen, 1992; O'Sullivan & Fox, 1991; van den Berg & de Koning, 1990; Bines *et al.*, 1989; Hicks *et al.*, 1988; Green *et al.*, 1985) have found that the characteristics of cheeses made with recombinant chymosin, expressed in *Kluveromyces marxianus* var. *lactis* or *Escherichia coli*, were generally similar to those made with calf rennet; any differences observed could be as a result of the presence of *ca.* 10% bovine pepsin (E. C. 3.4.23.1) in commercial rennets (Rothe *et al.*, 1977) or the presence of some factor in calf rennet preparations which stimulates starter bacteria (Berridge, 1956). O'Sullivan & Fox (1991) showed that the enzyme used in this study (Maxiren™) behaved in a similar manner to calf rennet with respect to temperature, pH, $CaCl_2$ and enzyme concentration. Furthermore,van den Berg and de Koning (1990) showed that Maxiren™ was immunologically identical to highly purified calf chymosin. Some characteristics of recombinant chymosin from *K. marxianus* var. *lactis* have been reported (Meisel, 1987, 1988). In this study it was demonstrated that the recombinant chymosin used and calf chymosin had similar action on α_{s1}-CN under the conditions studied.

Peptide bonds in α_{s1}-CN found to be susceptible to hydrolysis by chymosin generally had an aromatic or hydrophobic residue at the N-terminal side of the scissile bond. The bonds most susceptible to cleavage by chymosin (Phe_{23}-Phe_{24} and Trp_{164}-Tyr_{165}) had aromatic residues at both sides of the bond. These findings are in general agreement with the specificity of chymosin on the B-chain of insulin (Fish, 1957) and β-CN (Visser & Slangen, 1977).

The peptide sequences found, together with the order in which they were produced, suggest the following pathway of proteolysis of α_{s1}-CN at pH 6.5 (Fig. 4.6). Chymosin first cleaves at the Phe_{23}-Phe_{24} bond, producing the peptide f1-23 and the complementary polypeptide f24-199. The next bond cleaved is Trp_{164}-Tyr_{165}, which produces the peptide f165-199. As the peptide f1-23 is produced quickly, it seems likely that chymosin acts primarily on the polypeptide f24-199, rather than on intact α_{s1}-CN, at this stage. The removal of the peptide f165-199 exposes a region of the α_{s1}-CN molecule (residues 149-164) which has 4 cleavage sites, and 5 peptides (f157-164, 154-164, 154-159, 150-153 and 150-156) are produced, approximately in that order, although the peptide f150-156 did not accumulate to any great extent. Since the concentrations of the peptides f154-159 and f157-164 continued to increase during the course of the study, it is likely that chymosin did not act on these peptides, although they each include a chymosin-susceptible bond (Leu_{156}-Asp_{157} and Tyr_{159}-Pro_{160}, respectively). The presence of a chymosin-susceptible bond in f150-156 (i.e. Phe_{153}-Tyr_{154}) may explain why this peptide did not accumulate to any great extent although the peptide f154-156 was not detected. The increase in the concentrations of the peptides f24-28 and f24-40(?) with time suggests that chymosin acted at the N-terminal end of the polypeptide f24-164 (or smaller polypeptides derived from it by hydrolysis in the C-terminal region) simultaneously with hydrolysis in the region 149-164. The bond Phe_{28}-Pro_{29} was more susceptible to hydrolysis than Leu_{40}-Ser_{41}. As the peptide f29-40 was not identified, and since the concentrations of the peptides f24-28 and f24-40(?) did not decrease, it is likely that the bond Phe_{28}-Pro_{29} in the peptide f24-40(?) was not susceptible to hydrolysis by chymosin.

The primary cleavage site of chymosin on α_{s1}-CN, ie. Phe_{23}-Phe_{24}, has been identified previously (Hill *et al.*, 1974; Carles & Ribadeau-Dumas, 1985). Four other cleavage sites (Phe_{28}-Pro_{29}, Phe_{32}-Gly_{33}, Leu_{149}-Phe_{150} and Phe_{179}-

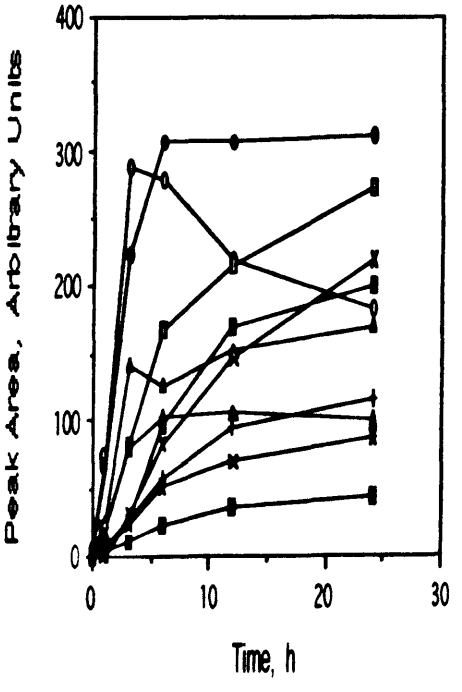

Fig. 4.4. Time course of changes in the area of the HPLC peaks representing the major peptides produced on hydrolysis of bovine α_{s1}-casein by chymosin at 30°C in 100 mM K-phosphate buffer, pH 5.2 in the presence of 5% w/v NaCl. f 1-23, (○); f 165-199, (●); f 24-28, (△); f 24-40(?), (▲); f 157-164, (□); f 102-142, (■); f 143-149, (✕); f 1-11, (+); f 150-153, (✱); f 154-159, (⊞). Experimental details are given in the text.

Fig. 4.5. *Urea-polyacrylamide gel electrophoretograms of whole casein (C) and bovine α_{s1}-casein (10 mg ml^{-1} in 100 mM K-phosphate buffer) hydrolyzed at 30°C by chymosin, pH 6.5 (0.49 chymosin units ml^{-1}) for 0, 1, 3, 6, 12 and 24 h (1, 2, 3, 4, 5 and 6) or at pH 5.2 in the presence of 5% w/v NaCl (0.7 chymosin units ml^{-1}) for 1, 3, 6, 12 and 24 h (7, 8, 9, 10 and 11).*

Ser_{180}) agree with the findings of Mulvihill & Fox (1979) who identified potential cleavage sites based on the amino acid composition of the large polypeptides isolated by preparative gel electrophoresis However, nearly half of the cleavage sites identified by those authors were found not to be susceptible to cleavage by chymosin under the conditions of this study. Pelissier *et al.* (1974) also found that a number cleavage sites identified in this study were susceptible to chymosin but the majority of the cleavage sites reported by those authors were found not to be susceptible to chymosin action under the conditions of this study. Differences between the results of previous authors and those of this study may be due to differences in reaction conditions (particularly with regard to Pelissier *et al.*, 1974, who appeared to use a more extensive hydrolysate than in this study) or due to superior techniques used to identify peptides in this study.

Many of the peptides produced at pH 5.2 in the presence of 5% (w/v) NaCl, i.e. the conditions in many young cheeses, were also produced at the higher pH. However, the relative rates of formation were markedly different and several additional peptides were also produced at pH 5.2. The initial bonds in α_{s1}-CN cleaved at pH 5.2 were similar to those at pH 6.5, although Trp_{164}-Tyr_{165} was hydrolyzed more rapidly and the peptides f24-28 and f24-40(?) were produced somewhat earlier. However, proteolysis towards the C-terminal of the polypeptides f24/29/41(?)-164 was quite different. The peptide f157-164 was produced first from this region, as in the case of pH 6.5, but subsequent proteolysis did not proceed towards the N-terminal, as at pH 6.5. The peptide f102-142 appeared produced next, necessitating initial cleavage towards the centre of the

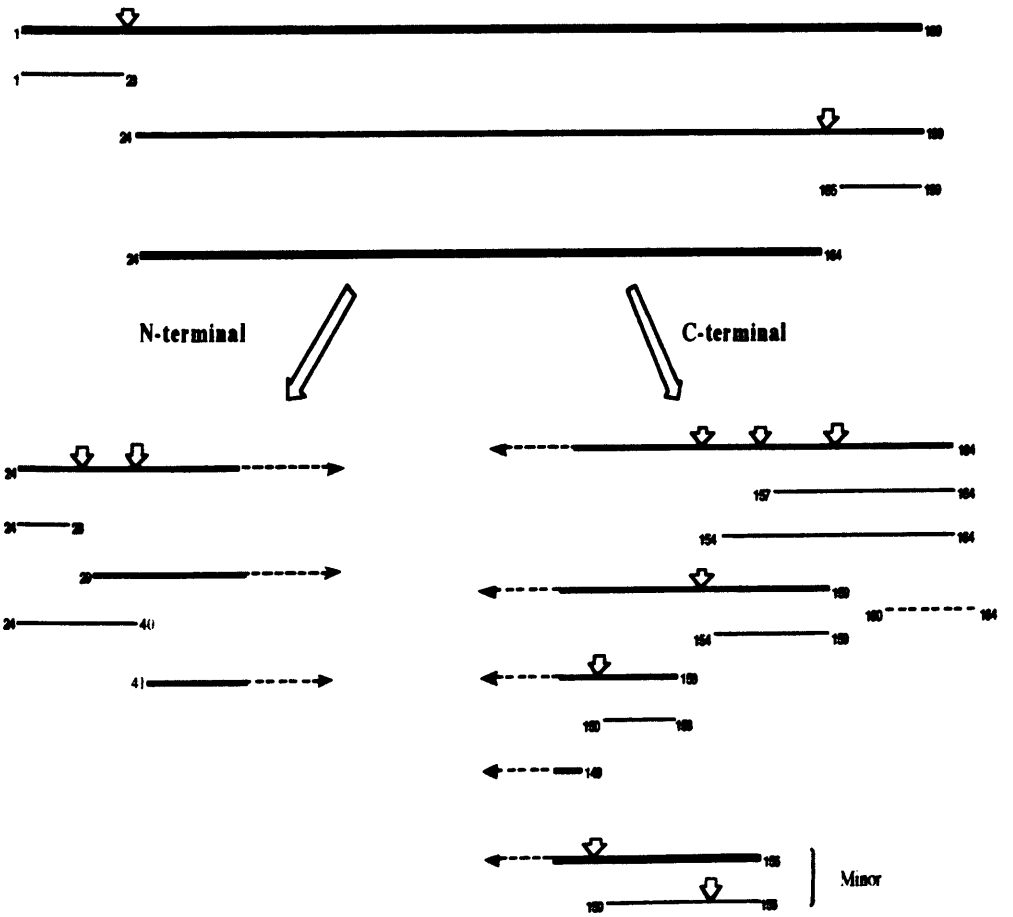

Fig. 4.6. *Pathway proposed for the proteolysis of* Bos α_{s1}*-casein with chymosin in solution in 100 mM K-phosphate buffer, pH 6.5.*

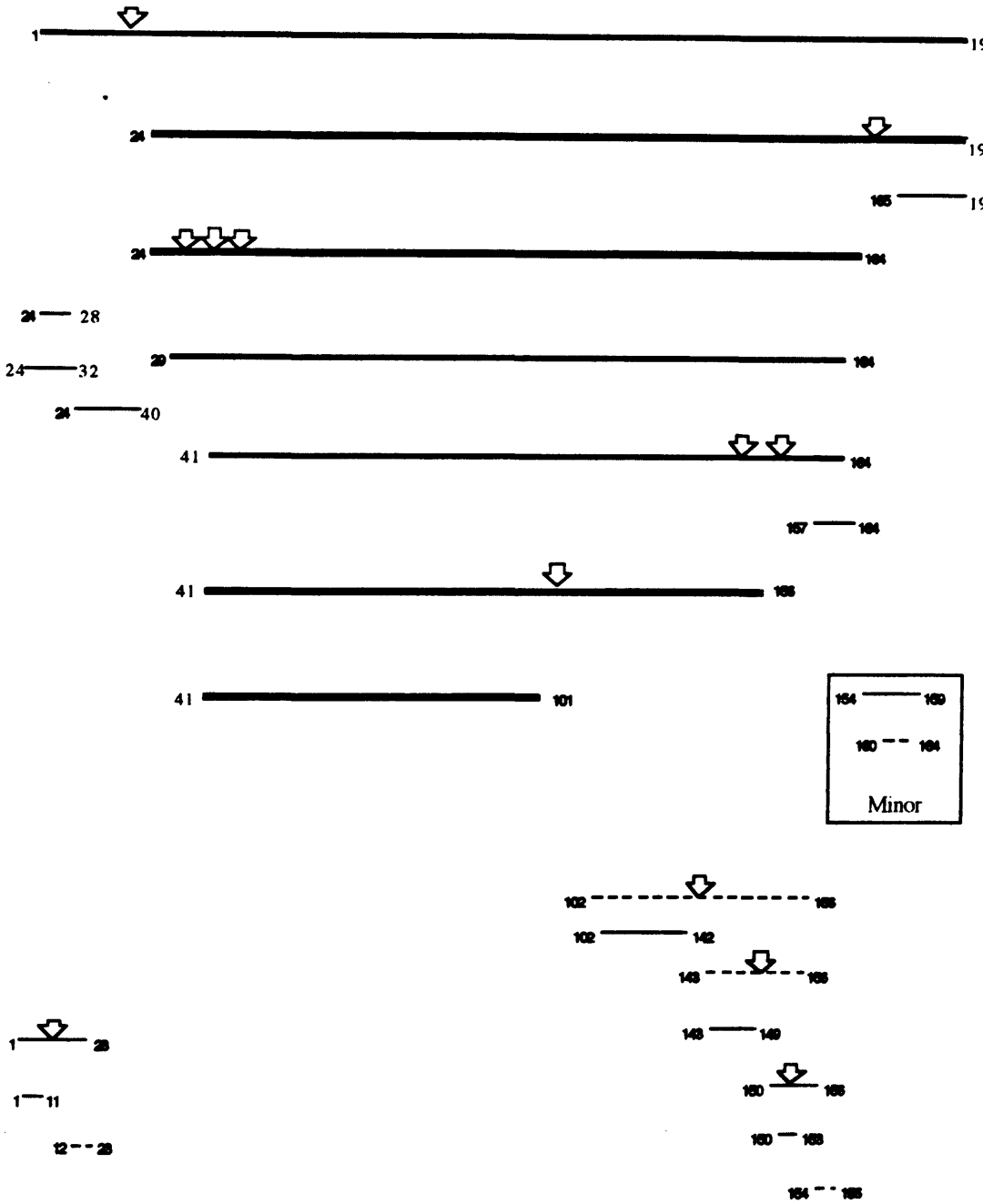

Fig. 4.7. Pathway proposed for the proteolysis of Bos α_{s1}*-casein with chymosin in solution in 100 mM K-phosphate buffer, pH 5.2 in the presence of 5%, w/v, NaCl.*

polypeptides f29/41(?)-153/156/159, i.e. at Leu_{101}-Lys_{102}, followed by further cleavage of one of the peptides produced, e.g. f102-156. The order in which the peptides f143-149, f150-153 and f154-159 were produced suggested that the proteolysis of the peptide f102-156 proceeded generally towards the C-terminal (Fig. 4.7).

Variations in the kinetic parameters, k_{cat} and K_M were observed with pH (Dunn *et al.*, 1987) for the action of chymosin on single peptide bonds in synthetic octapeptide substrates. Likewise, Mulvihill & Fox (1977; 1979) demonstrated that

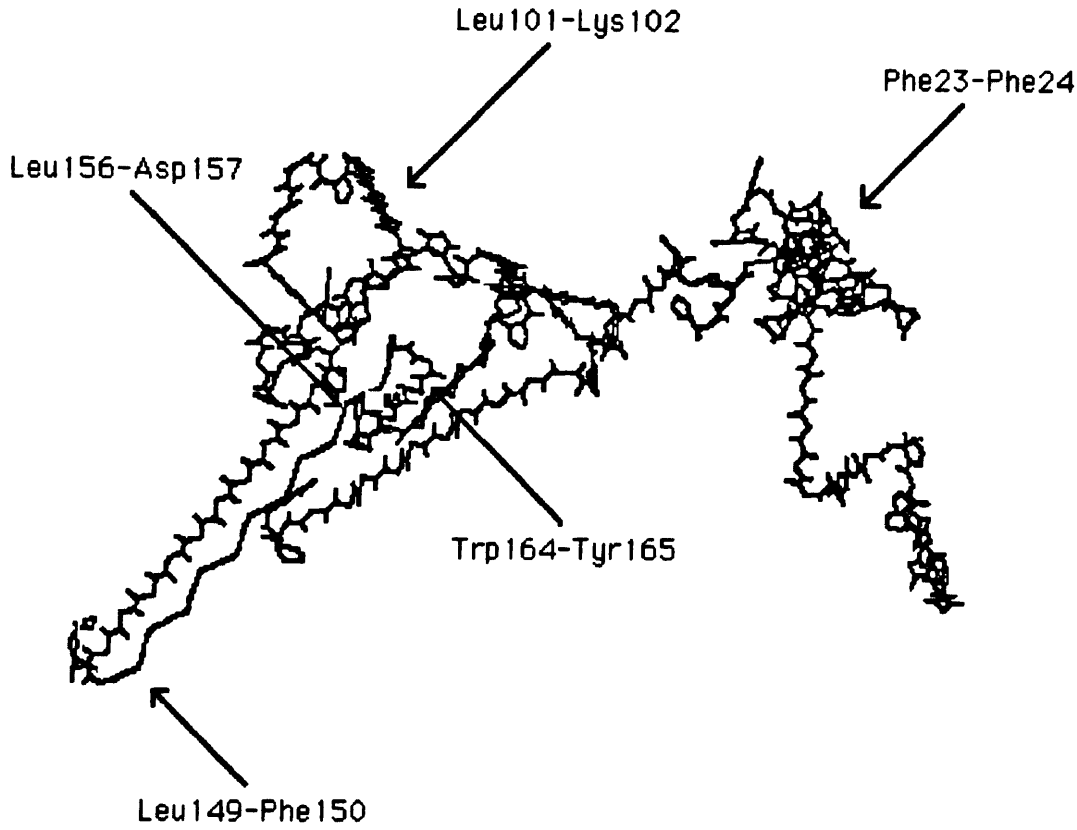

Fig. 4.8. *Model of the 3-dimensional structure of* Bos α_{s1}*-casein (Kumosinski* et al., *1991) showing the position of the primary sites of chymosin action identified in this study.*

the specificity of chymosin on α_{s1}-CN was dependent on the reaction pH and the state of aggregation of the substrate (Mulvihill & Fox, 1977). Likewise, in this study the action of chymosin on bovine α_{s1}-CN was found to depend on the ionic conditions used. However, further work remains to be done to establish whether variation in pH or NaCl concentration caused the differences observed in this study.

Electrophoretograms of α_{s1}-CN hydrolyzed by chymosin at pH 6.5 and at pH 5.2 in the presence of NaCl are shown in Fig. 4.5. In electrophoretograms of hydrolysates at pH 6.5, the 4 bands with mobilities immediately grater than f24-199 (ca. 5.5 to 6 cm) may correspond to the polypeptides f24-149, f24-153, f24-156 and f24-159 produced by hydrolysis at the C-terminus of f24-149. The remaining bands appeared in pairs, with the band having a slower mobility being more intense in each pair. These may be as a result of hydrolysis of the peptides f24-149, f24-153, f24-156 and f24-159 at N-terminal sites (Phe_{28}-Pro_{29} and Leu_{40}-Ser_{41}). At pH 5.2 in the presence of NaCl, few bands with intermediate electrophoretic mobilities were apparent in the electrophoretograms of the hydrolysates (Fig. 4.5) which may be explained by cleavage of peptides at Leu_{101}-Lys_{102} to yield species with high relative mobility (*ca.* 10 cm).

It has not been possible to crystallize caseins and thus their tertiary structures have not been determined experimentally by X-ray diffraction. However, a possible 3-dimensional structure for α_{s1}-CN, predicted from its amino acid sequence, has been proposed recently (Kumosinski *et al.*, 1991). The position of the primary chymosin cleavage sites identified in this study are shown on this model in Fig. 4.8. The primary sites of chymosin action appear to be near the surface of the molecule. A number of sites in the region 149 to 164 appeared not to be cleaved before the

production of the peptide f165-199 (Fig. 4.3). The hydrolysis of the bond Tyr_{164}-Trp_{165} removes a large peptide (f165-199) which covers a region of β-sheet secondary structure (residues 147-160) and may render potential chymosin cleavage sites in this region inaccessible to hydrolysis due to steric inhibition.

ACKNOWELDGEMENTS

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REFERENCES

ANDREWS, A. T. 1983 Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research* **50** 45-55

BARBANO, D. M. & RASMUSSEN, R. R. 1992 Cheese yield performance of fermentation-produced chymosin and other milk coagulants. *Journal of Dairy Science* **75** 1-12

BERRIDGE, N. J. 1956 Crystalline versus crude rennet in cheese-making *Proceedings of the XIV International Dairy Congress*, Rome, **2** 59-66

BINES, V. E., YOUNG, P. & LAW, B. A. 1989 Comparison of Cheddar cheese made with a recombinant calf chymosin and with standard calf rennet. *Journal of Dairy Research* **56** 657-664

BLAKESLEY, R. W. & BOEZI, J. A. 1977 A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G250. *Analytical Biochemistry* **82** 580-581

CARLES, C. & RIBADEAU DUMAS, B. 1985 Kinetics of the action of chymosin (rennin) on a peptide bond of bovine α_{s1}-casein. *Federation of European Biochemical Societies Letters* **185** 282-286

CREAMER, L. K. 1974 Preparation of α_{s1}-casein A. *Journal of Dairy Science* **57** 341-344

CREAMER, L. K. & RICHARDSON, B. C. 1974 Identification of the primary degradation product of α_{s1}-casein in Cheddar cheese. *New Zealand Journal of Dairy Science and Technology* **9** 9-13

CREAMER, L. K. & OLSON, N. F. 1982 Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science* **47** 631-636, 646

DELFOUR, A., JOLLES, J., ALAIS, C. & JOLLES, P. 1965 Caseino-glycopeptides: Characterization of a methionine residue and of the N-terminal sequence. *Biochemical and Biophysical Research Communications* **19** 452-455

DUNN, B. M., VALLER, M. J., ROLPH, C. E. , FOUNDLING, S. I. , JIMENEZ, M. & KAY, J. 1987 The pH dependence of the hydrolysis of chromogenic substrates of the type, Lys-Pro-Xaa-Yaa-Phe-(NO_2)-Arg-Leu, by selected aspartic proteinases: Evidence for specific interactions in subsites S_3 and S_2. *Biochimica et Biophysica Acta* **913** 122-130

FISH, J. S. 1957 Activity and specificity of rennin. *Nature* **180** 345

FOX, P. F. 1988 Rennets and their action in cheese manufacture and ripening. *Biotechnology and Applied Biochemistry* **10** 522-535

FOX, P. F. 1989 Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science* **72** 1379-1400

GREEN, M. L., ANGAL, S., LOWE, P. A. & MARSTON, F. A. O. 1985 Cheddar cheesemaking with recombinant calf chymosin synthesized in *Escherichia coli*. *Journal of Dairy Research* **52** 281-286

GROSCLAUDE, F., MAHÉ, M.-F. & RIBADEAU-DUMAS, B. 1973 [Primary structure of bovine α_{s1}- and β-casein. Corrective]. *European Journal of Biochemistry* **40** 323- 324

HICKS, C. L., O'LEARY, J. & BUCY, J. 1988 Use of recombinant chymosin in the manufacture of Cheddar and Colby cheese. *Journal of Dairy Science* **71** 1127-1131

HILL, R. D. 1963 The preparation of κ-casein. *Journal of Dairy Research* **30** 101-107

HILL, R. D., LAHAV, E. & GIVOL, D. 1974 A rennin-sensitive bond in α_{s1} B-casein. *Journal of Dairy Research* **41** 147-153

KUMOSINSKI, T. F., BROWN, E. M. & FARRELL, H. M. 1991 Three-dimensional molecular modeling of bovine caseins: α_{s1}-casein. *Journal of Dairy Science* **74** 2889-2895

Le BARS, D. & GRIPON, J.-C. 1989 Specificity of plasmin towards bovine α_{s2}-casein. *Journal of Dairy Research* **56** 817-821

McGANN, T. C. A., MATHIASSEN, A. & O'CONNELL, J. A. 1972 Applications of the Pro-Milk Mk. II. Part III. Rapid estimation of casein in milk and protein in whey. *Laboratory Practice* **21** 628-631, 650

MEISEL, H. 1987 [Characterization of rennet preparations produced by genetic engineering in comparison to calf rennet. 1. Materials and methods] *Milchwissenschaft* **42** 787-789

MEISEL, H. 1988 [Characterization of rennet preparations produced by genetic engineering in comparison to calf rennet. 2. Results] *Milchwissenschaft* **43** 71-75

MERCIER, J.-C., GROSCLAUDE, F. & RIBADEAU-DUMAS, B. 1971 [Primary structure of bovine α_{s1}-casein]. *European Journal of Biochemistry* **23** 41-51

MULVIHILL, D. M. & FOX, P. F. 1977 Proteolysis of α_{s1}-casein by chymosin: Influence of pH and urea. *Journal of Dairy Research* **44** 533-540

MULVIHILL, D. M. & FOX, P. F. 1979 Proteolytic specificity of chymosin on bovine α_{s1}-casein. *Journal of Dairy Research* **46** 641-651

NG-KWAI-HANG, K. F., MONARDES, H. G. & HAYES, J. F. 1990 Association between genetic polymorphism of milk proteins and production traits during three lactations. *Journal of Dairy Science* **73** 3414-3420

NIELSEN, P.E., KLARSKOV, K., HØJRUP, P. & ROEPSTORFF, P. 1988 Optimization of sample preparation for plasma desorption mass spectrometry of proteins and peptides using nitrocellulose matrix. *Biomedical and Environmental Mass Spectrometry* **17** 355-362

O'KEEFFE, A. M., FOX, P. F. & DALY, C. 1978 Proteolysis in Cheddar cheese: Role of coagulant and starter Bacteria. *Journal of Dairy Research* **45** 465-477

O'KEEFFE, R. B., FOX, P. F. & DALY, C. 1976 Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. *Journal of Dairy Research* **43** 97-107

O'SULLIVAN, M. & FOX, P. F. 1991 Evaluation of microbial chymosin from genetically engineered *Kluveromyces lactis*. *Food Biotechnology* **5** 19-32

OLSON, N. F. 1990 The impact of lactic acid bacteria on cheese flavor. *Federation of European Microbiology Societies Microbiology Reviews* **87** 131-148

PAHKALA, E., PIHLANTO-LEPPÄLÄ, A., LAUKKANEN, M. & ANTILA, V. 1989 Decomposition of milk proteins during the ripening of cheese. 1. Enzymatic hydrolysis of α_s-casein. *Meijeritieteellinen Aikakauskirja* **47** 39-47

PÉLISSIER, J.-P., MERCIER, J.-C. & RIBADEAU-DUMAS, B. 1974 [Study of the proteolysis of bovine α_{s1}- and β-caseins by rennet.] *Annales de Biologie Animale Biochimie Biophysique* **14** 343-362

ROTHE, G. A. L., HARBOE, M. K. & MARTINY, S. C. 1977 Quantification of milk-clotting enzymes in 40 commercial bovine rennets, comparing rocket immunoelectrophoresis with an activity ratio assay. *Journal of Dairy Research* **44** 73-77

van den BERG, G. & De KONING, P. J. 1990 Gouda cheesemaking with purified calf chymosin and microbially produced chymosin. *Netherlands Milk and Dairy Journal* **44** 189-205

van EENENNAAM, A. & MEDRANO, J. F. 1991 Milk protein polymorphisms in California dairy cattle. *Journal of Dairy Science* **74** 1730-1742

VISSER, S. & SLANGEN, K. J. 1977 On the specificity of chymosin (rennin) in its action on bovine β-casein. *Netherlands Milk and Dairy Journal* **31** 16-30

5

PROTEOLYTIC SPECIFICITY OF CHYMOSIN ON BOVINE α_{S2}-CASEIN

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Proteolysis of bovine α_{S2}-casein by chymosin (E.C. 3.4.23.4) in solution in 100 mM Na phosphate buffer, pH 6.5, at 30°C was studied by reversed-phase (RP)-HPLC and urea-PAGE. Chymosin hydrolyzed α_{S2}-casein in solution to approximately 11 peptides detectable by urea-PAGE.

Peptides soluble in an acetate buffer, pH 4.6, were isolated by RP-HPLC on a C_{18} column using an acetonitrile/water gradient and identified from their amino acid sequence.

Peptides α_{S2}-casein f89-95, f89-99, f89-?, ff96-?, 98-?, f99-?, f164-?, f175-179, f175-?, corresponding to chymosin cleavage sites in positions Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Leu_{98}, Tyr_{98}-Leu_{99}, Leu_{99}-Tyr_{100}, Phe_{163}-Leu_{164}, Phe_{174}-Ala_{175} and Tyr_{179}-Leu_{180}. The bond-type in α_{S2}-casein cleaved by chymosin was in agreement with that found to be susceptible to chymosin in other casein substrates. The order of production of the peptides was consistent with early hydrolysis of Phe_{88}-Tyr_{89}.

INTRODUCTION

Chymosin (E.C. 3.4.23.4) is an aspartyl proteinase secreted into the stomachs of a number of mammalian species. Its natural substrates is κ-casein and chymosin is the principal active constituent in traditional rennet preparations used to coagulate milk during cheesemaking.

The primary function of chymosin in cheese manufacture is hydrolysis of the micelle-stabilizing component, κ-casein, as a result of which the micelles coagulate in the presence of Ca^{2+} at temperatures >20°C. However, chymosin also plays an important rôle in the primary proteolysis of caseins during cheese ripening. It is responsible for much of the initial hydrolysis of caseins in internal bacterially-ripened cheese varieties such as Cheddar (O'Keeffe *et al.*, 1976, 1978). The resulting large peptides are hydrolyzed by starter proteinases and peptidases to a range of small peptides and free amino acids, which probably contribute to cheese flavour. Chymosin action in cheese is responsible for much of the softening of cheese early in ripening (Creamer and Olson, 1982).

The specificity of chymosin has been determined on α_{S1}-, β- and κ-caseins (Delfour *et al.*, 1965; Visser and Slangen, 1977; Mulvihill and Fox, 1979; McSweeney *et al.*, 1993), but as far as we are aware, the specificity of chymosin on α_{S2}-casein has not been determined.

α_{S2}-Casein constitutes approximately 10% of the total casein in bovine milk and is the most hydrophilic of the caseins. α_{S2}-Casein consists of a single polypeptide containing 207 amino acid residues. It undergoes extensive post-translational phosphorylation and exists in 4 forms containing 10 to 13 phosphate

groups and molecular weights of 25, 157 to 25, 400 Da. The primary structure of α_{s2}-casein has a number of similar amino acid sequences and thus appears to have resulted from gene duplication. Two areas of the molecule are relatively hydrophobic, i.e. 90-120 and residues 160-207 (Swaisgood, 1992).

The fate of α_{s2}-casein during cheese ripening is unclear. On the urea-PAGE gels widely used to characterize the initial stages of proteolysis in cheese, α_{s2}-casein probably co-migrates with one of the proteose peptones produced from β-casein by plasmin (Chapter 9) making it difficult to quantify α_{s2}-casein. α_{s2}-Casein is readily hydrolysed by plasmin and its specificity is known (Le Bars and Gripon, 1989; Visser *et al.*, 1989). None of the major bands on electrophoretograms of a 3-month old Cheddar originated from α_{s2}-casein (Chapter 9) and, as far as we are aware, no peptides in cheese originating from α_{s2}-casein have been identified. Although data suggests that bands in the α_{s2}-casein region of electrophoretograms of Cheddar do decrease during ripening (van den Berg and de Koning, 1990).

The objectives of this study were to investigate the proteolysis of α_{s2}-casein by chymosin in solution and to determine its specificity.

MATERIALS AND METHODS

Recombinant chymosin (E. C. 3.4.23.4, expressed in *Kluveromyces marxianus* var. *lactis*) was obtained from Gist Brocades Ltd., Delft, the Netherlands. Prior to use, the enzyme preparation was dialyzed against two 20-volume changes of distilled H_2O at 4°C overnight and freeze-dried. The freeze-dried powder was resuspended in 100 mM K-phosphate buffer, pH 6.5. This solution had an activity of approximately 70 chymosin units (CU) ml^{-1} (1 CU is the activity necessary to coagulate 10 ml bovine milk, pH 6.5, in 100 s at 30°C). Crystalline calf chymosin was obtained from Sigma Chemical Co., St. Louis, MO, USA.

Whole casein was prepared by isoelectric precipitation from a sample of bulk bovine skim milk obtained from the Dairy Plant, University of Wisconsin-Madison, according to the method of Hill (1963). α_{s2}-Casein was prepared by ion-exchange chromatography (Creamer, 1974) on DEAE-cellulose (Whatman DE-52) using 10 mM imidazole buffer, pH 7.0, containing 4.5 M urea and 0.1% (v/v) 2-mercaptoethanol. Proteins were eluted from the ion-exchanger by a linear NaCl gradient (0 to 0.5 M). Fractions corresponding to α_{s2}-casein were combined, dialyzed against 5 changes of distilled H_2O and freeze-dried.

α_{s2}-Casein was dissolved (10 mg ml^{-1}) in 100 mM Na-phosphate buffer, pH 6.5. Sodium azide (0.05%, w/v) was added to inhibit bacterial growth. Chymosin was added (approximately 1.0 units ml^{-1}) and the solution incubated at 30°C. Aliquots were taken periodically for analysis by reversed-phase (RP)-HPLC or urea-PAGE. Peptides were isolated from 24 h hydrolysates.

Samples for RP-HPLC were heated at 64°C for 30 min to inactivate chymosin. Samples (1 ml) were adjusted to approximately pH 4.6 by the addition of 30 ml acetic acid (33.3%, w/v), held at room temperature for 10 min and 30 ml Na acetate (3.33 M) added (McGann *et al.*, 1972). The samples were centrifuged at 16,000 **g** for 10 min and the supernatant used for analysis. RP-HPLC was performed on a Beckman HPLC system (Beckman, San Ramon, CA, USA, consisting of autosampler, model 507, programmable solvent module, model 126AA, and programmable detector, model 166, interfaced with a personal computer using Beckman System Gold software). A LiChrosorb RP-18 (5 μm) column, 250 x 4 mm (E. Merck, Darmstadt, Germany), was used with an Adsorbosphere C_{18} guard column (Alltech Associates, Deerfield, IL, USA). Elution was by means of a gradient prepared using solvent A [0.1 % trifluoroacetic acid (TFA) in H_2O] and solvent B [0.1% TFA in acetonitrile (Aldrich Chemical Co., Milwaukee, WI, USA)] as follows: 100% A for 5 min, 0 to 50% B at a rate of 0.5 % B min^{-1}, followed by 50 % B for 5 min. The flow rate was 1 ml min^{-1} and detection was at 215 nm.

Samples of hydrolysates were prepared for urea-PAGE by the addition of an equal volume of double-concentrated urea-PAGE sample buffer (McSweeney *et al.*, 1993). Discontinuous alkaline urea-PAGE was performed according to the method of Andrews (1983) [12% T (acrylamide plus bisacrylamide), 4% C (bisacrylamide as a percentage of T), pH 8.9]. Gels were directly stained using Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977).

Peptides were isolated by RP-HPLC as described above. Before sequence analysis, all peptides were rechromatographed using the above gradient to assess their homogeneity. The acetonitrile was removed by evaporation under nitrogen and the peptides freeze-dried.

Peptides were sequenced at the National Food Biotechnology Centre, University College, Cork, Ireland, by Edman degradation on an automated pulsed liquid-phase protein/peptide sequencer (Applied Biosystems Inc., Foster City, CA, USA, model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives by means of a model 120A analyser (Applied Biosystems Inc.).

RESULTS AND DISCUSSION

RP-HPLC profiles of the pH 4.6-soluble peptides produced from α_{s2}-casein by the recombinant chymosin (Maxiren™) used in this study were identical to those produced by crystalline calf chymosin under the conditions of this study (results not shown). Van den Berg and de Koning (1990) found that Maxiren™ was immuniologically indistinguishible from purified calf chymosin and McSweeney *et al.* (1993) found that Maxiren™ and calf chymosin produced identical peptides from α_{s1}-casein.

Electrophoretograms of α_{s2}-casein hydrolyzed by chymosin over time are shown in Fig. 5.1; under the conditions of this study, about 11 peptides detectable by urea-PAGE were produced. α_{s2}-Casein was less susceptible to hydrolysis by chymosin than α_{s1}-casein, requiring about twice the proteolytic activity to achieve an approximately equal extent of hydrolysis (McSweeney *et al.*, 1993). The electrophoretogram of a control, incubated for 24 h (Fig. 5.1, lane 7), was similar to that of the protein at the begining to hydrolysis (lane 1), indicating that the α_{s2}-casein preparation used appeared to be free of indigenous proteolytic activity.

The HPLC profile of pH 4.6-soluble peptides of α_{s2}-casein hydrolyzed by chymosin for 24 h is shown in Fig. 5.2. Approximately 25 peaks were resolved, nearly all of which were isolated and sequenced. Although, the α_{s2}-casein preparation used in this study appeared to be free of α_{s1}-casein and containing only a small amount of β-casein (Fig. 5.1, lane 1), a number of peptides were found to have originated from α_{s1}-casein and one from β-casein, formed by the preferential hydrolysis of these proteins by chymosin. Only the peptides which originated in α_{s2}-casein are indicated on Fig. 5.2 and their sequences summarized in Table 5.1. The position of the pH 4.6-soluble peptides produced by chymosin from α_{s2}-casein are indicated on Fig. 5.3.

Chymosin cleavage sites in α_{s2}-casein were identified at Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Leu_{98}, Tyr_{98}-Leu_{99}, Leu_{99}-Tyr_{100}, Phe_{163}-Leu_{164}, Phe_{174}-Ala_{175} and Tyr_{179}-Leu_{180}. All cleavage site were in the two hydrolphobic regions (residues 90-120 and 160-207) of the protein identified by Swaisgood (1992).

The majority of cleavage sites identified were in the central hydrophobic region of the molecule (residues 90-120). Three peptides (α_{s2}-CN f164-?, f175-199 and f175-?) which originated from C-terminal hydrophobic region (residues 160-207) were identified. The bond type in α_{s2}-casein found to be susceptible to chymosin action usually had an aromatic amino acid residue at its N-terminal side and an aromatic or hydrophobic residue at its C-terminal side and thus was similar to those cleaved by chymosin in α_{s1}-, β- and κ-caseins (Delfour *et al.*, 1965; Visser and Slangen, 1977; Mulvihill and Fox, 1979; McSweeney *et al.*, 1993) and the B-chain of insulin (Fish, 1957).

Fig. 5.1. *Urea-polyacrylamide gel electrophoretograms of whole casein (C) and bovine α_{s2}-casein (10 mg ml^{-1} in 100 mM Na-phosphate buffer, pH 6.5) hydrolyzed at 30°C by chymosin for 0, 1, 3, 6, 12 or 24 h (lanes 1 to 6) and control (lane 7) incubated for 24h without chymosin.*

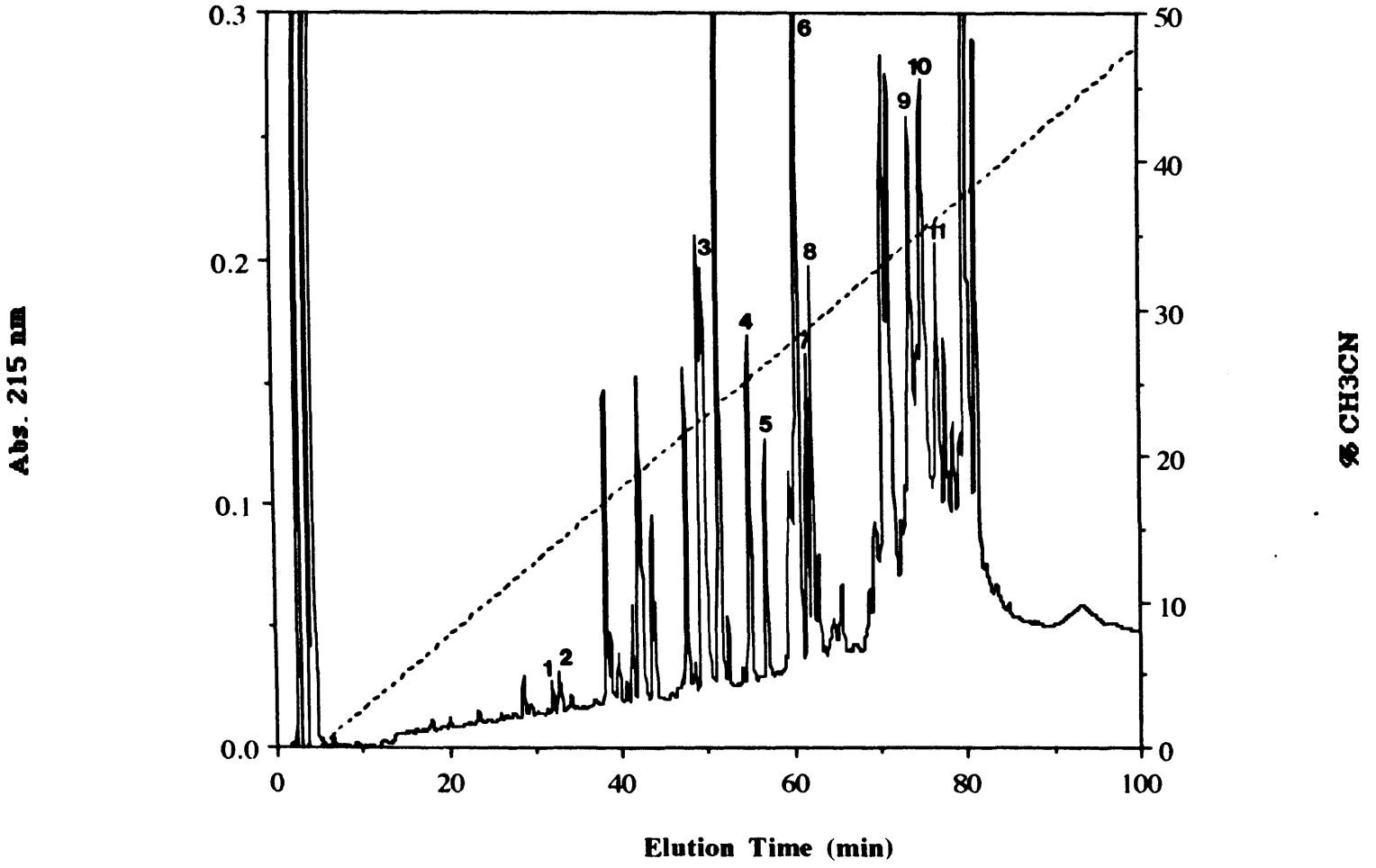

Fig. 5.2. *Reversed-phase HPLC elution profile of the pH 4.6-soluble peptides produced by chymosin from bovine α_{s2}-casein (10 mg ml^{-1}) at 30°C for 24 h in 100 mM Na-phosphate buffer, pH 6.5. Solvent A was 0.1% trifluoroacetic acid (TFA) in H_2O, solvent B 0.1% TFA in acetonitrile, gradient 0 to 50% CH_3CN (----------). Absorbance detected at 215 nm.*

Changes in relative peak area of the major peptide peaks with time are shown in Fig. 5.4. Since peptides f89-? and f89-95 were produced early, it is likely that the primary site of chymosin action on α_{s2}-casein is in the central hydrophobic region of the molecule, probably at Phe_{88}-Tyr_{89}.

The results of this study indicate that α_{s2}-casein is susceptible to proteolysis by chymosin and its specificity on this protein has been determined.

Table 5.1: Identity of the pH 4.6-soluble peptides produced from α_{s2}-casein by chymosin.

HPLC Peak No.	*Sequence*	*Identity*
1	Y.Q.K.F.P.Q.Y.L.Q.Y.L	α_{s2}-CN f89-98
2	L.K.K.I.S.Q*	α_{s2}-CN f164-*
3	Y.Q.K.F.P*	α_{s2}-CN f99-*
4	Y.Q.K.F.P.Q.Y	α_{s2}-CN f89-98
5	A.L.P.Q.Y	α_{s2}-CN f175-179
6	Y.Q.K.F.P.Q.Y.L.Q.Y*	α_{s2}-CN f89-*
7	Y.Q.K.F.P.Q.Y.L.Q.Y*	α_{s2}-CN f89-*
	A.L.P.Q.Y.L.K.T*	α_{s2}-CN f175-*
8	A.L.P.Q.Y.L.K*	α_{s2}-CN f175-*
9	Y.L.Y.Q.G.P*	α_{s2}-CN f98-*
10	L.Q.Y.L.Y.Q*	α_{s2}-CN f96-*
11	L.Y.Q.G.P*	α_{s2}-CN f99-*

* C-terminus not determined

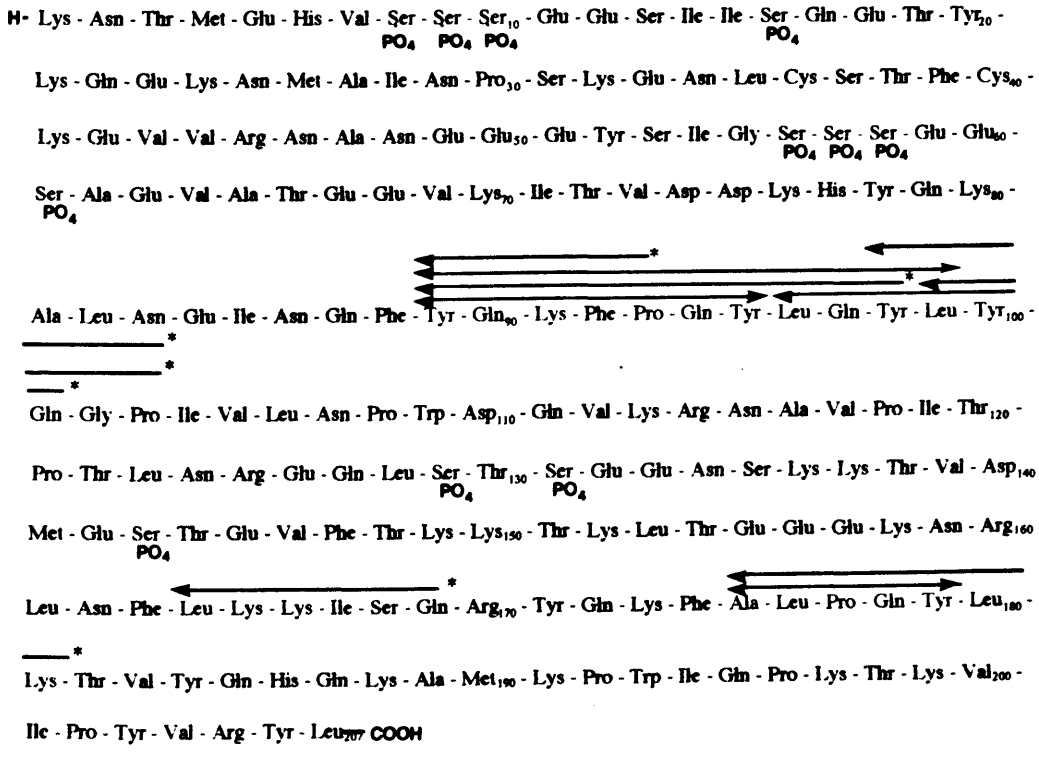

Fig. 5.3. Primary structure of Bos α_{s2}*-casein A-11P (from Swaisgood, 1993) showing the position of the pH 4.6-soluble peptides produced by hydrolysis with chymosin at pH 6.5. Peptides are indicated as* ———— . *(* Incomplete sequence)*

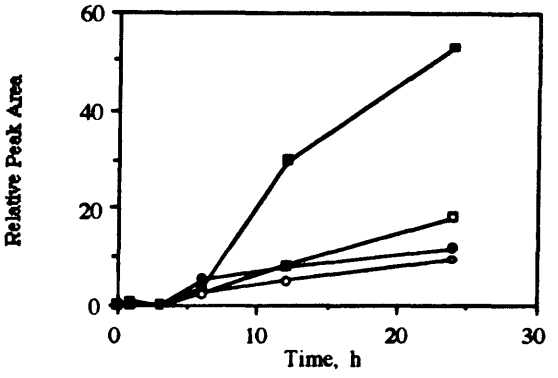

Fig. 5.4. Time course changes in the area of the HPLC peaks respersenting the major peaks produced on hydrolysis of bovine α_{s2}-casein by chymosin at 30°C in 100 mM Na-phosphate buffer, pH 6.5. (■), f89-?; (□), f89-95; (●); f89-? and f175-?; (○), f175-179.

ACKNOWLEDGEMENTS

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REFERENCES

ANDREWS, A. T. 1983 Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research* **50** 45-55

BLAKESLEY, R. W. & BOEZI, J. A. 1977 A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G250. *Analytical Biochemistry* **82** 580-581

CREAMER, L. K. 1974 Preparation of α_{s1}-casein A. *Journal of Dairy Science* **57** 341-344

CREAMER, L. K. & OLSON, N. F. 1982 Rheological evaluation of maturing Cheddar cheese. *Journal of Food Science* **47** 631-636, 646

DELFOUR, A., JOLLES, J., ALAIS, C. & JOLLES, P. 1965 Caseino-glycopeptides: Characterization of a methionine residue and of the N-terminal sequence. *Biochemical and Biophysical Research Communications* **19** 452-455

FISH, J. S. 1957 Activity and specificity of rennin. *Nature* **180** 345

HILL, R. D. 1963 The preparation of κ-casein. *Journal of Dairy Research* **30** 101-107

Le BARS, D. & GRIPON, J.-C. 1989 Specificity of plasmin towards bovine α_{s2}-casein. *Journal of Dairy Research* **56** 817-821

McGANN, T. C. A., MATHIASSEN, A. & O'CONNELL, J. A. 1972 Applications of the Pro-Milk Mk. II. Part III. Rapid estimation of casein in milk and protein in whey. *Laboratory Practice* **21** 628-631, 650

McSWEENEY, P. L. H., OLSON, N. F., FOX, P. F., HEALY, A. & HØJRUP, P. 1993. Proteolytic specificity of chymosin on bovine α_{s1}-casein. *Journal of Dairy Research* (in press).

MULVIHILL, D. M. & FOX, P. F. 1979 Proteolytic specificity of chymosin on bovine α_{s1}casein. *Journal of Dairy Research* **46** 641-651

O'KEEFFE, A. M., FOX, P. F. & DALY, C. 1978 Proteolysis in Cheddar cheese: Role of coagulant and starter bacteria. *Journal of Dairy Research* **45** 465-477

O'KEEFFE, R. B., FOX, P. F. & DALY, C. 1976 Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. *Journal of Dairy Research* **43** 97-107

SWAISGOOD, H. E. 1992. Chemistry of the caseins. In *Advanced Dairy Chemistry*, Vol. 1, pp. 63-110, P. F. Fox (Ed.), Elsevier Applied Science Publishers, London.

van den BERG, G. & De KONING, P. J. 1990 Gouda cheesemaking with purified calf chymosin and microbially produced chymosin. *Netherlands Milk and Dairy Journal* **44** 189-205

VISSER, S. & SLANGEN, K. J. 1977 On the specificity of chymosin (rennin) in its action on bovine β-casein. *Netherlands Milk and Dairy Journal* **31** 16-30

VISSER, S., SLANGEN, K. J., ALTING, A. C. & VREEMAN, H. J. 1989. Specificity of plasmin in its action on bovine α_{s2}-casein. *Milchwissenschaft* **44** 335-339

6

PROTEOLYTIC SPECIFICITY OF PLASMIN ON BOVINE α_{s1}-CASEIN†

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The proteolytic specificity of plasmin (fibrinolysin, E. C. 3.4.21.7, from bovine plasma) on bovine α_{s1}-casein was determined in solution in 50 mM ammonium bicarbonate buffer, pH 8.4, at 37°C.

Peptides, isolated by RP-HPLC on a C_{18} column or by electroblotting from urea-PAGE gels, were identified from their amino acid sequence and mass.

The principal plasmin cleavage sites were found at Arg_{22}-Phe_{23}, Arg_{90}-Tyr_{91}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Lys_{124}-Glu_{125} and Arg_{151}-Gln_{152}. The initial cleavage sites and the order of production of small (pH 4.6-soluble) peptides suggest that α_{s1}-casein was cleaved initially towards the centre of the molecule.

INTRODUCTION

Bovine plasmin (fibrinolysin, E. C. 3.4.21.7), the principal indigenous proteinase in milk, is a trypsin-like enzyme with a pH optimum at about 7.5 and a high specificity for peptide bonds involving lysyl residues. α_{s1}-Casein is susceptible to proteolysis by plasmin in solution (Eigel, 1977; Andrews and Alichanidis, 1983) but α_{s2}- and β-caseins are the preferred substrates (Grufferty and Fox, 1988). Plasmin is closely associated with the casein micelles (Grufferty and Fox, 1988; Fox and Law, 1991) and its activity in milk results in the formation of proteose peptones and γ- and λ-caseins. γ-Caseins result from the proteolysis of β-casein by plasmin, the complementary peptides forming part of the proteose peptone fraction of milk (Andrews and Alichanidis, 1983). λ-Casein was described by Long *et al.* (1958), who prepared it from a κ-casein preparation by high speed centrifugation. Swaisgood and Brunner (1961) prepared λ-casein by fractionating "α-casein" with concentrated trichloroacetic acid-urea. The dissociating agent, N,N-dimethylformamide, was used by El-Negoumy (1973), followed by chromatography on DEAE-cellulose and preparative electrophoresis, to prepare λ-casein from whole casein. This author described some of the properties of λ-casein and concluded that it was a smaller protein than α_{s1}-casein, had a higher phosphorus content and was glycosylated. However, the λ-casein fraction was subsequently shown to be an artifact consisting of peptides produced from α_{s1}-casein by plasmin (Aimutis and Eigel, 1982). Andrews and Alichanidis (1983)

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found that peptides produced from α_{s1}-casein by plasmin were present in the total proteose peptone fraction of milk.

Plasmin is specific for peptide bonds of the type Lys-X and to a lesser degree, Arg-X (Weinstein and Doolittle, 1972). The specificity of plasmin on β-casein is well documented (Gordon *et al.*, 1972; Eigel, 1977, 1981) and its specificity on α_{s2}-casein has been determined recently (Le Bars and Gripon, 1989; Visser *et al.*, 1989). However, it appears that the specificity of plasmin on bovine α_{s1}-casein has not been reported and was the subject of this study.

MATERIALS AND METHODS

Whole casein was prepared by isoelectric precipitation according to the method of Hill (1963) from a sample of bulk bovine milk obtained from the Dairy Plant, University of Wisconsin-Madison. α_{s1}-Casein [approximately 97% B allele, calculated from the composition of breeds in the University herd according to the data of Ng-Kwai-Hang *et al.* (1990) and Van Eenennaam and Medrano (1991)] was prepared by ion-exchange chromatography (Creamer, 1974) on DEAE-cellulose (Whatman DE-52) using10 mM imidazole buffer, pH 7.0, containing 4.5 M urea, and 0.1%, v/v, 2-mercaptoethanol. Proteins were eluted from the ion-exchanger by a linear NaCl gradient (0 to 0.5 M). Fractions corresponding to α_{s1}-casein were combined, dialyzed against 5 changes of distilled H_2O and freeze-dried. Plasmin (fibrinolysin, E.C. 3.4.21.7, from bovine plasma) was obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

α_{s1}-Casein (10 mg ml^{-1}) was dissolved in 50 mM ammonium bicarbonate buffer, pH 8.4, containing 0.05%, w/v, sodium azide. Plasmin (0.125 U ml^{-1}) was added and the solution incubated at 37°C. Samples were taken periodically for analysis by reversed-phase high performance liquid chromatography (RP-HPLC) or urea-PAGE. Peptides were isolated from a 6 h hydrolysate.

Samples for RP-HPLC were heated at 100°C x 10 min to inactivate plasmin, cooled to 20°C and adjusted to pH 4.6 using an acetate buffer (McSweeney *et al.*, 1993); the pH 4.6-soluble fraction was used for analysis. RP-HPLC was performed on a Beckman HPLC system (Beckman, San Ramon, CA, USA) consisting of autosampler, model 507, programmable solvent module, model 126AA, and programmable detector, model 166, interfaced with a personal computer using Beckman System Gold software. A LiChrosorb RP-18 (5 μm) column, 250 x 4 mm (E. Merck, Darmstadt, Germany), was used with an Adsorbosphere C_{18} guard column (Alltech Associates, Deerfield, IL, USA). Elution was by means of a gradient using:- Solvent A, 0.1 % trifluoroacetic acid (TFA) in H_2O, Solvent B, 0.1% TFA in acetonitrile (Aldrich Chemical Co., Milwaukee, WI, USA). The gradient consisted of 100% A for 5 min, 0 to 25% B at a rate of 1.39 % B min^{-1}, followed by 25 % B for 20 min; acetonitrile concentration was then increased to 33.5% B at a rate of 1.39% B min^{-1} and held at this concentration for 20 min before being increased to 50% B at a rate of 1.65% B min^{-1}. Acetonitrile concentration was held at 50% B for 5 min before being increased to 95% B at a rate of 9% B min^{-1} and was held at 95% B for 5 min before returning to the starting conditions. The eluate was monitored at 215 nm.

Samples of hydrolysates were prepared for urea-PAGE by addition of an equal volume of double-concentrated sample buffer (McSweeney *et al.*, 1993). Discontinuous alkaline urea-PAGE was performed according to the method of Andrews (1983) (12% T, 4% C, pH 8.9), with direct staining using Coomassie Brilliant Blue G250 (Blakesley and Boezi, 1977).

Peptides (pH 4.6-soluble) were isolated by RP-HPLC, as described above. Before sequence analysis, peptides were rechromatographed to assess purity. The acetonitrile was removed by evaporation under nitrogen and the peptides freeze-dried.

pH 4.6-Insoluble peptides were isolated by electroblotting from urea-PAGE gels using a mini Trans-Blot™ electrophoretic transfer cell (BioRad, San Ramon, CA, USA). Transfer of peptides was at 100 V for 1 h in transfer buffer (25 mM tris, 192 mM glycine in 20 %, v/v, methanol) onto polyvinylidene difluoride membranes, pore size 0.22 μm (ProBlot™, Applied Biosystems, Inc., Foster City, CA, USA). Membranes were stained using 0.2% (w/v) Ponceau S in 1% (v/v) acetic acid for 10 min followed by destaining in water. Bands corresponding to peptides were excised from membranes and sequenced.

Peptides were sequenced at the National Food Biotechnology Centre, University College, Cork, Ireland, by an Edman degradation automated by an Applied Biosystems model 477A protein/peptide sequencer (Applied Biosystems Inc., Foster City, CA, USA). Liberated amino acids were detected as their phenylthiohydantoin derivatives by a model 120A analyser.

Mass spectrometry was performed at the Department of Molecular Biology, University of Odense, Denmark, using a Biolon 20K plasma desorption time-of-flight instrument (Applied Biosystems AB, Uppsala, Sweden). The peptide samples were dissolved in 0.1% trifluoroacetic acid and applied to a nitrocellulose-covered target, spin-dried and micro-rinsed as described by Nielsen *et al.* (1988). Spectra were recorded at 14 kV for 10^6 primary fission events. Identification of peptides by mass was based on partial sequence data combined with mass searches using the GPMAW program (Lighthouse Data, DK-5250 Odense SV).

RESULTS AND DISCUSSION

Electrophoretograms of α_{s1}-casein hydrolysed by plasmin are shown in Fig. 6.1. α_{s1}-Casein was degraded by plasmin to 12 or 13 peptides detectable by PAGE, 3 or 4 of which accumulated. A number of bands were produced at approximately equal rates, although 4 of the polypeptides with lower relative electrophoretic mobilities were subsequently degraded. The general pattern of proteolysis detectable by PAGE was similar to that found by Andrews and Alichanidis (1983). The α_{s1}-casein preparation used appeared to be pure by electrophoresis (lane 1), but the sequences of 6 peptides identified in the pH 4.6-soluble fraction of its hydrolysate indicated that they originated from α_{s2}-casein.

Six peptides (A to F, Fig. 6.1) were isolated by electroblotting from electrophoretograms of plasmin hydrolyzates of α_{s1}-casein and their N-terminal sequence determined, thus allowing identification of the primary sites of plasmin action on the protein (Table 6.1).

Primary sites of plasmin action were found at Arg_{22}-Phe_{23}, Arg_{90}-Tyr_{91}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Lys_{124}-Glu_{125} and Arg_{151}-Gln_{152}. These sites are generally towards the centre of the molecule and the resulting polypeptides would thus be insoluble at pH 4.6 (with the probable exception of α_{s1}-CN f1-22) and would not be present in the RP-HPLC profile (Fig. 6.2).

The reversed-phase HPLC elution profile of the pH 4.6-soluble fraction of a 6h plasmin hydrolyzate of α_{s1}-casein is shown in Fig. 6.2. The profile was quite complex and preliminary experiments were necessary to select a gradient to achieve adequate separation of the peptides. The gradient finally chosen was such that the majority of peptides eluted during two isocratic periods (at 25 and 33.5% CH_3CN). However, in a number of cases single peaks were found to contain more than one peptide on sequence analysis. The results of sequence and mass analysis (Table 6.2) showed considerable overlap between peptide sequences which may account for difficulties encountered in isolating individual peptides in some cases.

Changes in RP-HPLC elution profiles of α_{s1}-casein hydrolyzed by plasmin over time are shown in Fig. 6.3a-e. The RP-HPLC elution conditions chosen separated the peptides into 2 distinct groups. The first group, with elution times of 15 to 40 min, were produced more quickly than the second group (elution times from 55 to 70 min). The peptides eluting in the hydrophobic region (after 80 min)

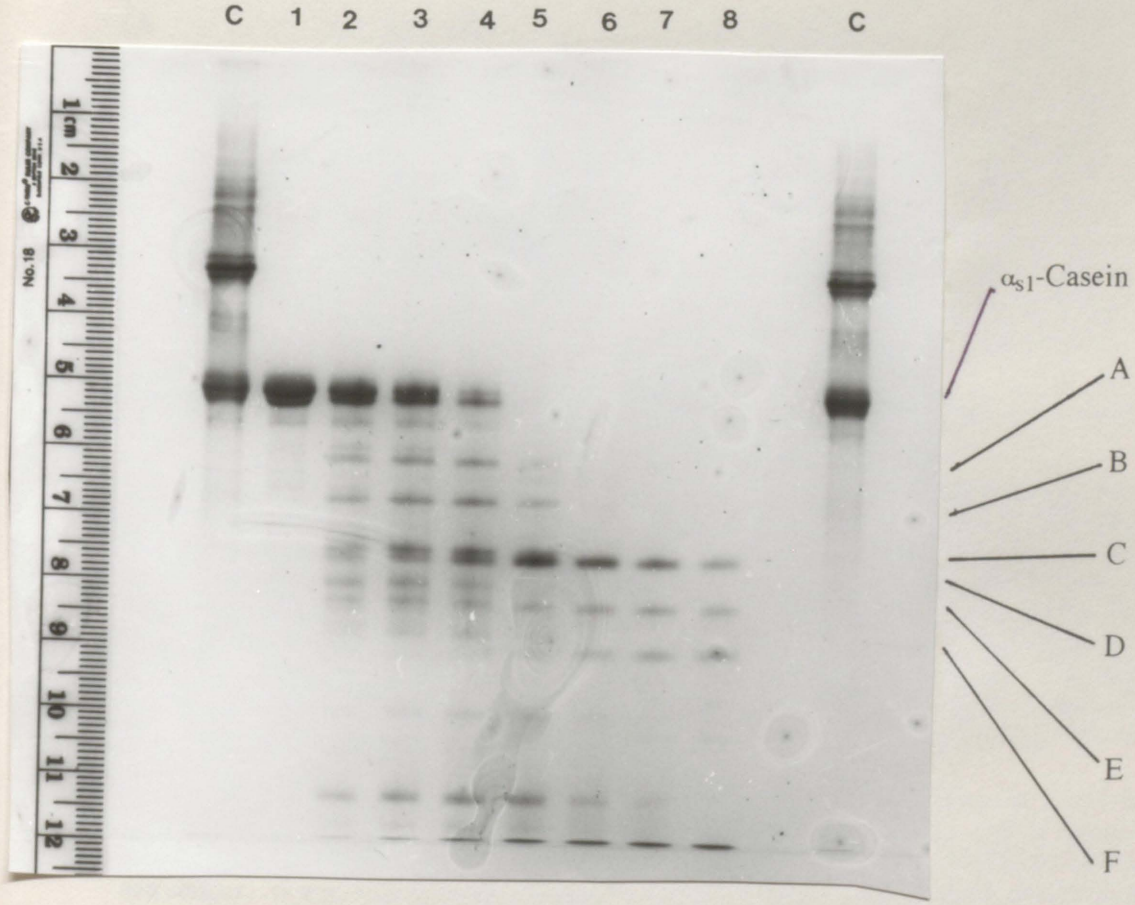

Fig. 6.1. *Urea-polyacrylamide gel electrophoretograms (12%T, 4%C, pH 8.9) of whole casein (C) and bovine α_{s1}-casein (10 mg ml^{-1}) hydrolysed by plasmin (0.125 U ml^{-1}) in solution in 50 mM ammonium bicarbonate buffer, pH 8.4, at 37°C for 0, 0.5, 1, 2, 4, 6, 8 or 12 h (lanes 1-8).*

TABLE 6.1: Identity of peptides detectable by urea-polyacrylamide gel electrophoresis produced from α_{s1}-casein (CN) by plasmin (see Figure 6.1 for location of peptides).

Peptide	N-Terminal Sequence		Identity	Cleavage Site
A	K.Y.K.V.P.Q		α_{s1}-CN f103-*	Lys_{102}-Lys_{103}
B	Y.K.V.P.Q	(major)	α_{s1}-CN f 104-*	Lys_{103}-Tyr_{104}
	F.F.V.A.P	(minor)	α_{s1}-CN f 23-*	Arg_{22}-Phe_{23}
C	E.G.I.H.A	(major)	α_{s1}-CN f 125-*	Lys_{124}-Glu_{125}
	V.P.Q.L.E	(major)	α_{s1}-CN f 106-*	Lys_{105}-Val_{106}
	Y.L.G.Y.L	(minor)	α_{s1}-CN f 91-*	Arg_{90}-Tyr_{91}
D	E.G.I.H.A	(major)	α_{s1}-CN f125-*	Lys_{124}-Glu_{125}
	Q.F.Y.Q.L	(minor)	a_{s1}-CN f152-*	Arg_{151}-Gln_{152}
E	V.P.Q.L		α_{s1}-CN f106-*	Lys_{105}-Val_{106}
F	F.F.V.A.P.F		α_{s1}-CN f23-*	Arg_{22}-Phe_{23}

*Incomplete Sequence

were not resolved by the HPLC conditions used and peptides from this region were not identified. The peptides produced very quickly (visible in the 0 h hydrolysate in Fig. 6.3a) were subsequently shown to be produced from α_{s2}-casein, present in the α_{s1}-casein preparation, which was preferentially degraded. During the course of hydrolysis of α_{s1}-casein by plasmin, a relatively large number of peptides were produced at an early stage. In the 2 h hydrolyzate (Fig. 6.3b), approximately 13 peptides are apparent and this number increased in the 4 h hydrolyzate. This rapid appearance of a relatively large number of peptides may be as a result of the initial cleavage of α_{s1}-casein towards its centre, producing a number of large pH 4.6-insoluble polypeptides and thus facilitating further proteolysis.

The sequences and masses of the isolated peptides are shown in Table 6.2. The locations of the peptides and plasmin cleavage sites on the α_{s1}-casein sequence are summarized in Fig. 6.4. Due to limited amounts of sample or washout from the sequencer, it was not possible to determine the sequence of several peptides to the C-terminus. In most of these cases, the mass of the peptide was determined by mass spectrometry (see Table 6.2). α_{s1}-Casein was extensively degraded and peptides were identified from all regions of the molecule except from Gln_{59} to Lys_{79} and Gln_{152} to Lys_{193}. The former sequence contains 7 of the 8 phosphoserine residues in α_{s1}-casein B 8-P. Relatively few pH 4.6-soluble peptides from the C-terminal region of the molecule were identified, which reflects the relative paucity of potential plasmin cleavage sites in this region. A major cleavage site appears to be at Arg_{22}-Phe_{23}; two peptides terminated and 6 originated at this site. The presence of this site adjacent to the primary chymosin cleavage site on α_{s1}-casein, Phe_{23}-Phe_{24} (Hill et al., 1974; Carles and Ribadeau-Dumas, 1985; McSweeney et al., 1993), has implications for the possible rôle of plasmin in the degradation of α_{s1}-casein during cheese ripening. Plasmin is active in a number of cheeses (Farkye and Fox, 1990) where it appears to hydrolyse primarily β-casein (Grufferty and Fox, 1988; Fox, 1989). The hydrolysis of Phe_{23}-Phe_{24} by chymosin is rapid (McSweeney *et al.*, 1993) and thus would deny plasmin one of its primary cleavage sites.

The order of production of the pH 4.6-soluble peptides confirmed the primary cleavage sites identified above (Fig. 6.5a, b). Peaks containing peptides α_{s1}-CN f 106-124, f104-124, f1-22, f23-44 (and other peptides originating from cleavage at Phe_{22}-Phe_{23}) and f80-90, corresponding to cleavage sites Lys_{105}-Val_{106}, Lys_{124}-Glu_{125}, Arg_{22}-Phe_{23}, Lys_{103}-Tyr_{104} and Arg_{90}-Tyr_{91}, accumulated early

TABLE 6.2: Identity of pH 4.6-soluble peptides produced from bovine α_{s1}-casein by plasmin.

HPLC Peak No.	Sequence Determined	Mass (kDa)	Probable Identity
1	Y.K		α_{s1}-CN(f104-105)
2	K.Y.K		α_{s1}-CN (f103-105)
3	L.N.E.I.N.E*		α_{s2}-CN (f182-?)
	Q.G.I.H.A.Q.Q.K		α_{s1}-CN(f125-132)
4	L.K.K.Y.K		α_{s1}-CN (f101-105)
	I.S.Q.R.Y.Q.K		α_{s2}-CN (f167-173)
5	H.I.Q.K.E.D.V.P.S.E.R	1337.6	α_{s1}-CN(f80-90)
6	K.N.T.M.E.H.V*		α_{s2}-CN(f1-?)
7	V.P.E.L.E.I*	1661.8	α_{s1}-CN (f106-119)
8	A.M.K.P.W.I.Q.P.K		α_{s2}-CN(f189-197)
9	K.Y.KV.P.Q.L*		α_{s1}-CN (f103-?)
10	Y.K.V*	1952.2	α_{s1}-CN (f104-119)
11	E.K.V.N*		α_{s1}-CN (f35-?)
12	V.P.Q.L.E.I.V.P.N.S.A.E.E.R.L.H.S.M.K*	2257.3	α_{s1}-CN (f106-124)
	V.N.E.L.S.K.D.I.G.S.E.S.T.E.D.I.K		α_{s1}-CN (f37-58)
	E.K.V.N.E.L.S.K.D.I.G*		α_{s1}-CN (f35-58?)
13	Y.K.V.P.Q.L.E.I.V.P.N.S.A.E.E.R.L.H*	2548.7	α_{s1}-CN (f104-124)
14	R.P.K.H.P.I.K.H.Q.G.L.P.Q.E.V.L.N.E.N*	2617.5	α_{s1}-CN (f1-22)
	Y.K.V.P.Q.L.E.I.V.P.N.S.A.E.E.R.L.H.S*		α_{s1}-CN(f104-132?)
	F.A.L.P.Q.Y.L.K		α_{s2}-CN (f174-181)
15	T.T.M.P.L.W		α_{s1}-CN(f194-199)
	R.P.K.H.P.I.K		α_{s1}-CN(f1-7)
	N.M.A.I.N.P.S.K		α_{s2}-CN (f25-32)
	Not Determined	2235.6	α_{s1}-CN (f4-22)
16	F.F.V.A.P.F.P.Q.V.P.G.K.E.K.V*	2616.8	α_{s1}-CN(f23-44)
17	F.F.V.A.P.F.P.Q*		α_{s1}-CN(f23-?)
18	F.F.V.A.P.F.P.Q.V.F.G.K.E.K.V*		α_{s1}-CN(f23-42?)
19	F.F.V.A.P.F.P.Q.V.F.G.K.E.K.V*	1641.9	α_{s1}-CN (f23-36)
20	F.F.V.A.P.F.P.Q.V.F.G.K.E.K.V.N*		α_{s1}-CN(f23-?)
21	Y.L.G.Y.L.E.Q.L.L.R*	1267.6	α_{s1}-CN(f91-100)
22	F.F.V.A.P.F.P.Q.V.P.G.K	1384.6	α_{s1}-CN (f23-34)
23	H.I.Q.K.E.D.V.P.S.E.R.Y.L*	2587.1	α_{s1}-CN(f80-100)
	E.G.I.H.A.Q.K.E.P.M.I.G*	3207.7	α_{s1}-CN (f125-151)

* Indicates incomplete sequence

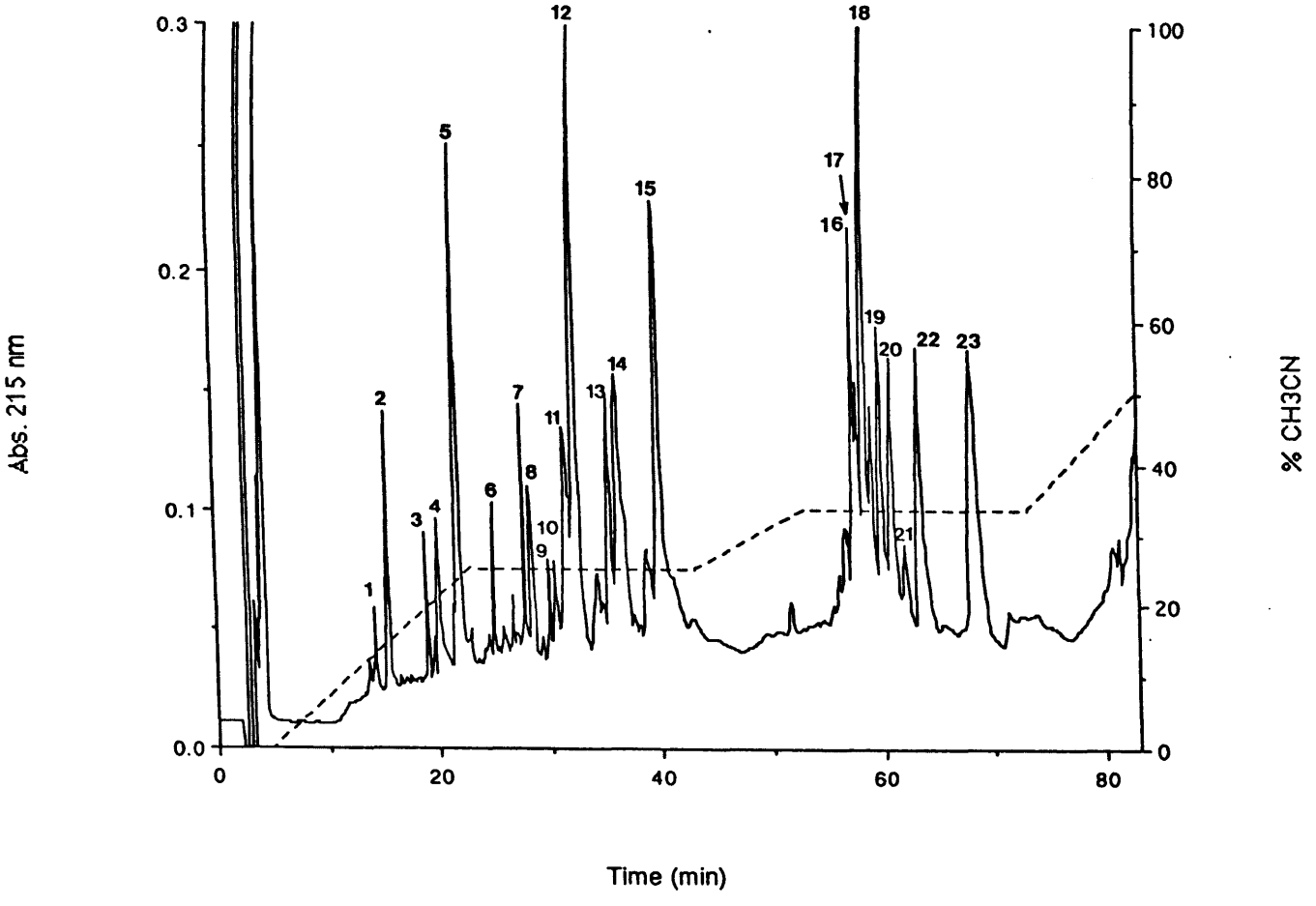

Fig. 6.2. Reversed-phase HPLC (C_{18}) elution profile of the pH 4.6-soluble peptides produced from bovine α_{s1}-casein (10 mg ml^{-1}) by plasmin (0.125 U ml^{-1}) in solution in 50 mM ammonium bicarbonate buffer, pH 8.4, at 37°C for 6h. Acetonitrile/H_2O gradient indicated by dotted line. Detection at 215 nm. Numbers indicate peaks which were isolated and identified.

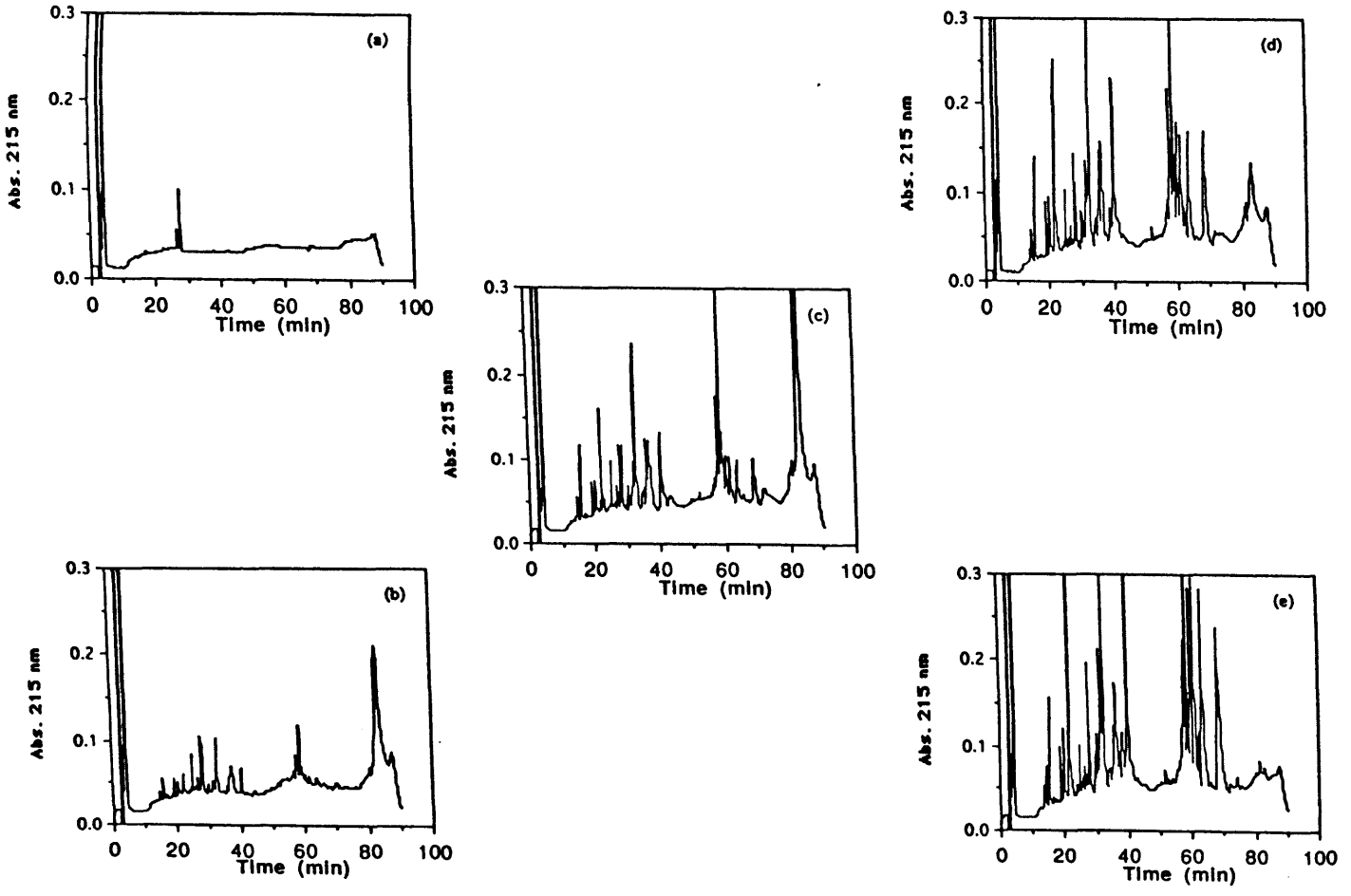

Fig. 6.3. Reversed-phase HPLC (C_{18}) elution profiles of pH 4.6-soluble peptides produced from α_{s1}-casein (10 mg ml^{-1}) by plasmin (0.125 U ml^{-1}) in solution in 50 mM ammonium bicarbonate buffer, pH 8.4, at 37°C for 0, 2, 4, 6 and 12 h (a, b, c, d and e).

during hydrolysis. The identity of the primary cleavage sites and the order of production of the small (pH 4.6-soluble) peptides suggest the following pathway of proteolysis of α_{s1}-casein by plasmin: Plasmin cleaves initially close to the centre of the molecule at positions 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 towards its N-terminal at Arg_{22}-Phe_{23}. Proteolysis of the resulting polypeptides proceeds at both their N- and C-terminii to produce a number of pH 4.6-soluble peptides.

Six peptides were identified as being produced from α_{s2}-casein present as a contaminant in the α_{s1}-casein preparation used in this study. The peptide, α_{s2}-casein f1-? corresponds to either α_{s2}-casein f1-24 or α_{s2}-casein f1-21 found by Le Bars and Gripon (1989) and Visser *et al.* (1989). The peptide α_{s2}-casein f189-197 was identified by Visser *et al.* (1989) and corresponds to cleavage sites (Lys_{188}-Ala_{189} and Lys_{197}-Thr_{198}) found by Le Bars and Gripon (1989). The peptide f174-181 was not identified by either of these groups but its C-terminal corresponds to a cleavage site, Lys_{181}-Thr_{182}, found by both groups. However, formation of this peptide would necessitate a further cleavage at Lys_{173}-Phe_{174}. Likewise, the peptide α_{s2}-casein f25-32 was not identified previously, although its N-terminus corresponds to the cleavage site at Lys_{24}-Asn_{25} but its formation necessitates a further cleavage at Lys_{32}-Glu_{33} which has not been reported.

The bond type found in this study to be cleaved by plasmin is in good agreement with the bond-type hydrolysed in fibrin (Weinstein and Doolittle, 1972), α_{s2}-casein (Le Bars and Gripon, 1989; Visser *et al.*, 1989) and β-casein (Gordon *et al.*, 1972; Eigel, 1977, 1981). At least 18 bonds are cleaved in α_{s1}-casein, 13 of the type Lys-X and 5 of the type Arg-X.

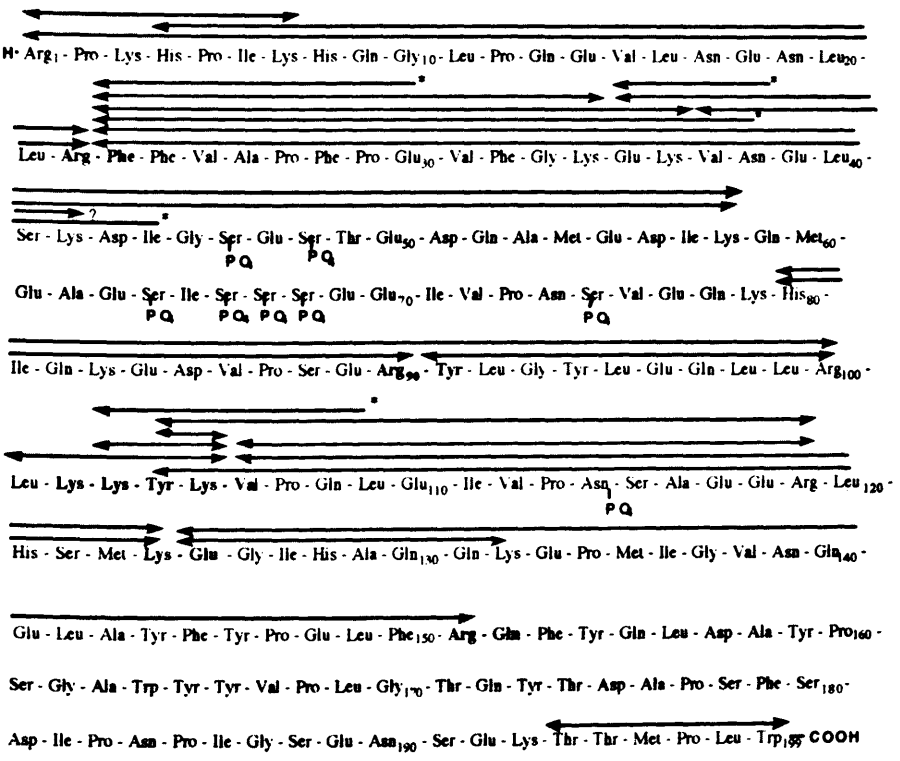

Fig. 6.4. *Primary structure of* Bos α_{s1}*-casein B 8-P (Mercier* et al., *1971; Grosclaude* et al., *1973), showing the positions of the pH 4.6-soluble peptides produced by hydrolysis by plasmin at pH 8.4. Primary sites of plasmin action are indicated in bold type. (* Incomplete Sequence)*

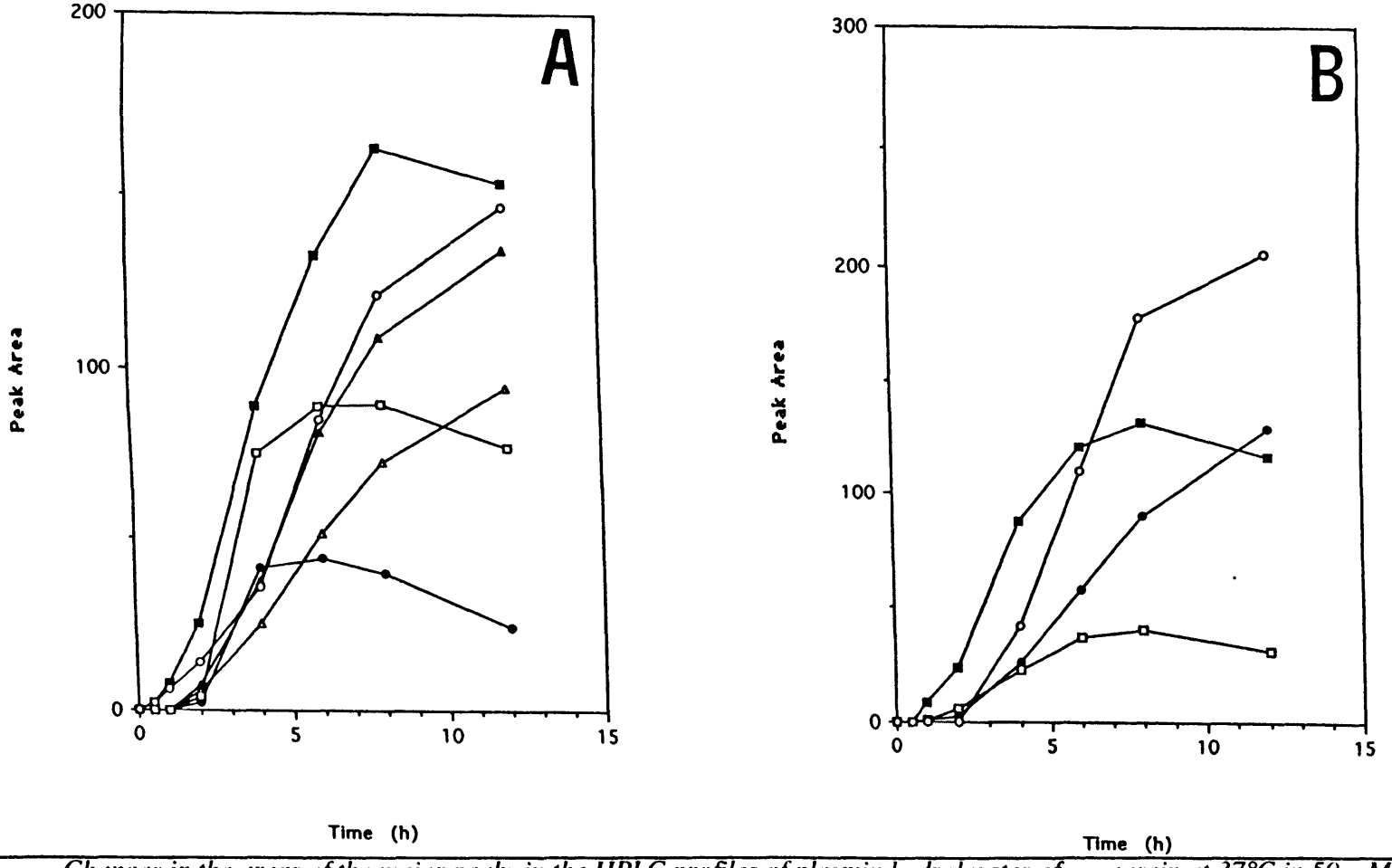

Fig. 6.5. *Changes in the areas of the major peaks in the HPLC profiles of plasmin hydrolyzates of α_{s1}-casein at 37°C in 50 mM ammonium acetate buffer, pH 8.4 (a) (■) peak 12, (□) peak 14, (●) peak 13, (○) peak 16, (▲) peak 5, (△) peak 11 and (b) (■) peak 18, (□) peak 17, (●) peak 20, (○) peak 23. For identification of peaks see Table 6.2.*

CONCLUSIONS

The proteolytic specificity of plasmin on bovine α_{s1}-casein in solution has been determined. Primary plasmin cleavage sites were found at 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}, but the molecule was also cleaved at 11 other sites. The position of the primary plasmin cleavage sites and the order of production of the small (pH 4.6-soluble) peptides suggested the the molecule is cleaved initially towards its centre and at Arg_{22}-Phe_{23}, with the subsequent hydrolysis of the polypeptides produced.

ACKNOWLEDGEMENTS

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REFERENCES

Aimutis, W. R. & Eigel, W. N., 1982, Identification of λ-casein as plasmin-derived fragments of bovine α_{s1}-casein, J. Dairy Sci. **65**, 175-181.

Andrews, A. T. & Alichanidis, E., 1983, Proteolysis of caseins and the proteose-peptone fraction of bovine milk, J. Dairy Res. **50**, 275-290.

Andrews, A. T., 1983, Proteinases in normal bovine milk and their actions on caseins, J. Dairy Res. **50**, 45-55.

Blakesley, R. W. & Boezi, J. A., 1977, A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250, Anal. Biochem. **82**, 580-581.

Carles, C. & Ribadeau-Dumas, B., 1985, Kinetics of the action of chymosin (rennin) on a peptide bond of bovine α_{s1}-casein, FEBS. Letts. **185**, 282-286.

Creamer, L. K., 1974, Preparation of α_{s1}-casein A, J. Dairy Sci. **57**, 341-344.

Eigel, W. N., 1977, Formation of γ_1-A^2, γ_2-A^2 and γ_3-A caseins by *in vitro* proteolyis of β-casein A^2 with bovine plasmin, Int. J. Biochem. **8**, 187-192.

Eigel, W. N., 1981, Identification of proteose-peptone component 5 as a plasmin-derived fragment of bovine β-casein, Int J. Biochem. **13**, 1081-1086.

El-Negoumy, A. M., 1973, Separation of lambda casein and some of its properties, J. Dairy Sci. **56**, 1486-1491.

Farkye, N. Y. & Fox, P. F., 1990, Observations on plasmin activity in cheese, J. Dairy Res. **57**, 413-418.

Fox, P. F., 1989, Proteolysis during cheese manufacture and ripening, J. Dairy Sci. **72**, 1379-1400.

Fox, P. F. & Law, J., 1991, Enzymology of cheese ripening, Food Biotechnol. **5**, 239-262.

Gordon, W. G., Groves, M. L., Greenberg, R., Jones, S. B., Kalan, E. B., Peterson, R. F. & Townend, R. E., 1972, Probable identification of γ-, TS-, R-, and S-caseins as fragments of β-casein, J. Dairy Sci. **55**, 261-263.

Grosclaude, F., Mahé, M.-F. & Ribeadeau-Dumas, B., 1973, Structure primaire de la caséine α_{s1} et de la caséine β bovines. Correctif, Eur. J. Biochem. **40**, 323-324.

Grufferty, M. B. & Fox, P. F., 1988, Reviews of the progress of Dairy Science. Milk alkaline proteinase, J. Dairy Res. **55**, 609-630.

Hill, R. D., 1963, The preparation of κ-casein, J. Dairy Res. **30**, 101-107.

Hill, R. D., Lahav, E. & Givol, D., 1974, A rennin-sensitive bond in α_{s1} B-casein, J. Dairy Res. **41**, 147-153.

Le Bars, D. & Gripon, J. C., 1989, Specificity of plasmin towards bovine α_{s2}-casein, J. Dairy Res. **56**, 817-821.

Long, J., Van Winkle, Q. & Gould, I. A., 1958, Isolation and identification of λ-casein, J. Dairy Sci. **41**, 317-318.

McSweeney, P. L. H., Olson, N. F., Fox, P. F. Healy, A. & Højrup, P., 1993, Proteolytic specificity of chymosin on bovine α_{s1}-casein, J. Dairy Res. (in press).

Mercier, J.-C., Grosclaude, F. & Ribadeau-Dumas, B., 1971, Structure primaire de la caséine α_{s1}-bovine. Séquence complète, Eur. J. Biochem. **23**, 41-51.

Ng-Kwai-Hang, K. F., Monardes, H. G. & Hayes, J. F., 1990, Association between genetic polymorphism of milk proteins and production traits during three lactations, J. Dairy Sci. **73**, 3414-3420.

Nielsen, P. E., Klarskov, K., Højrup, P. & Roepstorff, P., 1988, Optimization of sample preparation for plasma desorption mass spectrometry of proteins and peptides using nitrocellulose matrix, Biomed. Environ. Mass Spectr. **17**, 355-362.

Swaisgood, H. E. & Brunner, J. R., 1961, The isolation of λ-casein from concentrated TCA-urea solutions, J. Dairy Sci. **44**, 1163 (Abstr.).

Van Eenennaam, A. & Medrano, J. F., 1991, Milk protein polymorphisms in California dairy cattle, J. Dairy Sci. **74**, 1730-1742.

Visser, S., Slangen, K. J., Alting, A. C. & Vreeman, H. J., 1989, Specificity of plasmin in its action on bovine α_{s2}-casein, Milchwissenschaft **44**, 335-339.

Weinstein, M. J. & Doolittle, R. F., 1972, Differential specificities of thrombin, plasmin and trypsin with regard to synthetic and natural substrates and inhibitors, Biochim. Biophys. Acta **258**, 577-590.

7

PROTEOLYSIS OF BOVINE CASEINS BY CATHEPSIN D: PRELIMINARY OBSERVATIONS AND COMPARISON WITH CHYMOSIN

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Proteolysis of α_{s1}-, α_{s2}-, β- and κ-caseins by bovine cathepsin D (E.C. 3.4.23.5), the indigenous acid proteinase in milk, was studied by reversed-phase HPLC and urea-PAGE. The results indicate that cathepsin D hydrolyzed all casein fractions and was more active on α_{s1}- than on β-casein. α_{s1}-Casein was optimally hydrolysed at pH 4.0. Proteolysis of β-casein was more sensitive to inhibition by NaCl than was α_{s1}-casein. Comparison of the proteolysis of individual caseins with that by chymosin (E.C. 3.4.23.4) showed that the hydrolysis of α_{s1}-casein by the two enzymes was very similar, but the specificities on α_{s2}-casein differed somewhat. The initial stages of β-casein hydrolysis by cathepsin D were similar to that by chymosin and HPLC profiles showed a number of peptides in common. Although cathepsin D hydrolyzed κ-casein in solution and the HPLC profiles of hydrolysates were similar to those produced by chymosin although cathepsin D appeared to be unable to coagulate milk.

INTRODUCTION

The presence of indigenous proteolytic activity in milk has been recognized for many years. The principal indigenous proteinase in milk is plasmin (fibrinolysin, E.C. 3.4.21.7), is potimally active at ~pH 7.5, and has been studied intensively (see Visser, 1981; Grappin *et al.*, 1985; Grufferty & Fox, 1988; Fox, 1989; Fox & Law, 1991). The presence of a second indigenous proteinase in milk, with an acid pH optimum (~pH 4.0), is also recognized (Kaminogawa & Yamauchi, 1972, Kaminogawa *et al.*, 1978, 1980). Kaminogawa & Yamauchi (1972) described some properties of this "milk acid protease" and suggested that it was similar to the lysosomal proteinase, cathepsin D (E.C. 3.4.23.5). Recently, the zymogen, procathepsin D, has been demonstrated in milk and the identity of cathepsin D as an indigenous acid proteinase in milk has been confirmed (T. E. Petersen & L. B. Nielsen, pers. comm.).

The action of plasmin on bovine caseins has been the subject of many investigations (see above reviews), but the action of the indigenous acid proteinase on caseins has received little attention. Kaminogawa & Yamauchi (1972) found that the acid proteinase preferentially hydrolyzed α_{s1}-casein and that its specificity on α_{s1}-, β- and κ-caseins was similar to that of chymosin (Kaminogawa *et al.*, 1980).

The importance of plasmin in cheese ripening has been demonstrated (Farkye & Fox, 1990, 1991, 1992) and the possible significance of milk acid proteinase in cheese ripening was discussed by Kaminogawa & Yamauchi (1972). Kaminogawa *et al.* (1978, 1980). Noomen (1978) suggested that the indigenous acid proteinase may play a minor rôle in cheese ripening and that the presence of

α_{s1}-casein f24-199 in aseptic, chemically acidified rennet-free cheese may be due to its action.

Cathepsin D is a lysosomal enzyme (Goll *et al.*, 1983). Its physiological rôle involves intracellular digestion and turnover of proteins. While the enzyme has received little attention in the context of dairy foods, it has been investigated extensively in relation to meat and muscle biology where it may be involved in *post mortem* changes (Dutson, 1983). The proteolysis of collagen (Scott & Pearson, 1978) and myofibrillar proteins (Matsumoto *et al.*, 1983; Zeece *et al.*, 1986; Schwartz and Bird, 1977) has been investigated. Cathepsin D is susceptible to autolysis (Lah & Turk, 1982) and rabbit cathepsin D exists as multiple isomeric forms.

The presence of many enzymes in milk is accidental (Walstra & Jenness, 1984). Presumably, this is also the case with cathepsin D which plays no known physiological rôle in milk. Lysosomes in leucocytes may be a source of cathepsin D in milk. Lysosomal proteinases play a rôle in involution of the uterus *post partum* and of the mammary gland at the end of lactation, when large turnover of tissue occurs (Pitt, 1975). Lysosomes are present in the mammary gland and the concentration of lysosomal enzymes increases at the onset of pregnancy (Pitt, 1975); however, variations in the concentration of cathepsin D in milk with the stage of lactation are unknown.

The objectives of the present study were to characterize the action of cathepsin D on bovine caseins and to compare its action with that of chymosin.

MATERIALS AND METHODS

Whole casein was prepared from a sample of bulk bovine milk obtained from the University of Wisconsin-Madison Dairy Plant by isoelectric precipitation. It was fractionated by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) using 10 mM imidazole buffer, pH 7.0, containing 4.5 M urea and 0.1% (v/v) 2-mercaptoethanol (Creamer, 1974).

Bovine cathepsin D (E.C. 3.4.23.5) was obtained from Sigma Chemical Co. (St. Louis, MO.). Recombinant chymosin (E.C. 3.4.23.4, produced by *Kluveromyces marxianus* var. *lactis*), was obtained from Gist Brocades Ltd., Delft, the Netherlands, and was dialyzed against H_2O, freeze dried and resuspended in 100 mM K-phosphate buffer, pH 6.5, prior to use. The specificity of recombinant chymosin on α_{s1}-casein is the same as that of calf chymosin (McSweeney *et al.*, 1993) and the enzymes are immuniolgically indistinguishible (van den Berg & de Koning, 1990). Also, the peptides produced from α_{s2}-casein by the recombinant enzyme were the same as those produced by calf chymosin obtained from Sigma Chemical Co., St. Louis, MO, (Chapter 5).

The action of cathepsin D on micellar casein was studied in diluted skim milk (1:6 with water), adjusted to pH 5.5 with 1 M HCl and containing 0.05% (w/v) NaN_3. Cathepsin D was added (2×10^{-1} U ml^{-1}) and the samples incubated at 37°C [1 unit of cathepsin D activity will produce an increase in A_{280} of 1.0 min^{-1} ml^{-1} at pH 3.0 at 37°C measured as trichloroacetic acid-soluble products using haemoglobin as substrate (Sigma Chemical Co., St. Louis, MO)]. Samples for urea-PAGE were taken periodically.

α_{s1}-Casein, β-casein or κ-casein (5 mg ml^{-1}) was dissolved in 100 mM Na acetate buffer, pH 5.5, containing 0.05% NaN_3. Cathepsin D was added at the following concentrations: α_{s1}-casein, 1×10^{-2} U ml^{-1}; β-casein, 2×10^{-1} U ml^{-1}; κ-casein, 2.5×10^{-1} U ml^{-1} the samples incubated at 37°C. α_{s2}-Casein was dissolved in 100 mM Na phosphate buffer, pH 6.5, containing 0.05% NaN_3, 4×10^{-1} U ml^{-1} cathepsin D added. The corresponding concentrations (U ml^{-1}) of chymosin used were: α_{s1}-casein, 4×10^{-2}; α_{s2}-casein, 1.0; β-casein, 3×10^{-2}; κ-casein, 1×10^{-2}. Aliquots were taken periodically for analysis by urea-PAGE or

reversed-phase HPLC. Samples for urea-PAGE were prepared by the addition of an equal volume of double-strength sample buffer (McSweeney *et al.*, 1993).

The optimum pH for hydrolysis of α_{s1}-casein by cathepsin D was determined by dissolving α_{s1}-casein (1 mg ml^{-1}) in H_2O containing 0.05%, w/v, NaN_3 and adjusting the pH to values in the range 2 to 7 with 1 M HCl. Cathepsin D was added (1 x 10^{-3} U ml^{-1}) and incubated at 37°C for 12 h. Because protein precipitation occurred in the pH range 4-5, enzyme was added to these samples prior to pH adjustment. The influence of NaCl on the hydrolysis of α_{s1}- and β-caseins was studied by dissolving whole Na caseinate (5 mg ml^{-1}) in 100 mM Na acetate buffer, pH 5.5, containing 0.05% NaN_3 and NaCl concentrations from 0 to 20% (w/v). Cathepsin D was added (2 x 10^{-1} U ml^{-1}) and the mixture incubated at 37°C for 1 h; sub-samples were taken for urea-PAGE.

Discontinuous alkaline urea-PAGE was performed according to the method of Andrews (1983) (12% T, 4% C, pH 8.9) with direct staining using Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977). Samples for reversed-phase HPLC were heated at 100°C for 10 min to inactivate the proteinases and pH 4.6-insoluble peptides precipitated by the addition of a Na acetate buffer as described by McSweeney *et al.* (1993). For κ-casein, the reaction was stopped by the addition of an equal volume of 16% TCA; the 8% TCA soluble material used for analysis.

Reversed-phase HPLC was performed using a Beckman HPLC system (Beckman, San Ramon, CA, consisting of autosampler, model 507, programmable solvent module, model 126AA, and programmable detector, model 166, interfaced with a personal computer using Beckman System Gold software). A LiChrosorb RP-18 (5 μm) column (Metachem Technologies Inc., Redondo Beach, CA or E. Merck, Darmstadt, Germany) was used with an Adsorbosphere C_{18} guard column (Alltech Associates, Deerfield, IL). Elution was by means of a gradient using 0.1 % trifluoroacetic acid (TFA) in H_2O (Solvent A) and 0.1% TFA in acetonitrile (Aldrich Chemical Co., Milwaukee, WI) (Solvent B). The eluent consisted of 100% A for 5 min followed by a gradient from 0 to 50% B at a rate of 1.65 % B min^{-1}.

The ability of cathepsin D to coagulate milk was determined on a sample of raw milk obtained from the University of Wisconsin-Madison Dairy Plant. The milk was adjusted to pH 6.5, cathepsin D added (1 U ml^{-1}), incubated at 37°C and inspected periodically for clot formation.

RESULTS AND DISCUSSION

Urea polyacrylamide gel electrophoretograms of micellar casein (in diluted milk) hydrolysed by cathepsin D are shown in Fig. 7.1; α_{s1}-casein was completely degraded within 3 h, while there was still some residual β-casein after 24 h. Thus, α_{s1}-casein was preferentially degraded by cathepsin D, in agreement with the specificity of the acid proteinase isolated from bovine milk (Kaminogawa & Yamauchi, 1972; Kaminogawa *et al.*, 1978).

Electrophoretograms of α_{s1}-casein hydrolyzed by cathepsin D at pH values in the range 2 to 7 indicated that α_{s1}-casein was optimally hydrolyzed at about pH 4 (results not shown). Kaminogawa and Yamauchi (1972) found that the acid milk proteinase isolated by them had maximum activity at this pH and Schwartz and Bird (1977) reported that cathepsin D optimally degraded native myosin and actin at pH 4.0.

The influence of NaCl concentration on the hydrolysis of α_{s1}- and β-caseins is shown in Fig. 7.2. The hydrolysis of β-casein by cathepsin D was more susceptible to inhibition by NaCl than that of α_{s1}-casein (under the conditions of this study, hydrolysis of β-casein was strongly inhibited by 10%, w/v, NaCl; after 3 h α_{s1}-casein was completely hydrolyzed in the presence of 10%, w/v, NaCl and slight hydrolysis occurred in the presence of 20%, w/v). Fox and Walley (1971) reported that the hydrolysis of β-casein by chymosin was more strongly inhibited by NaCl

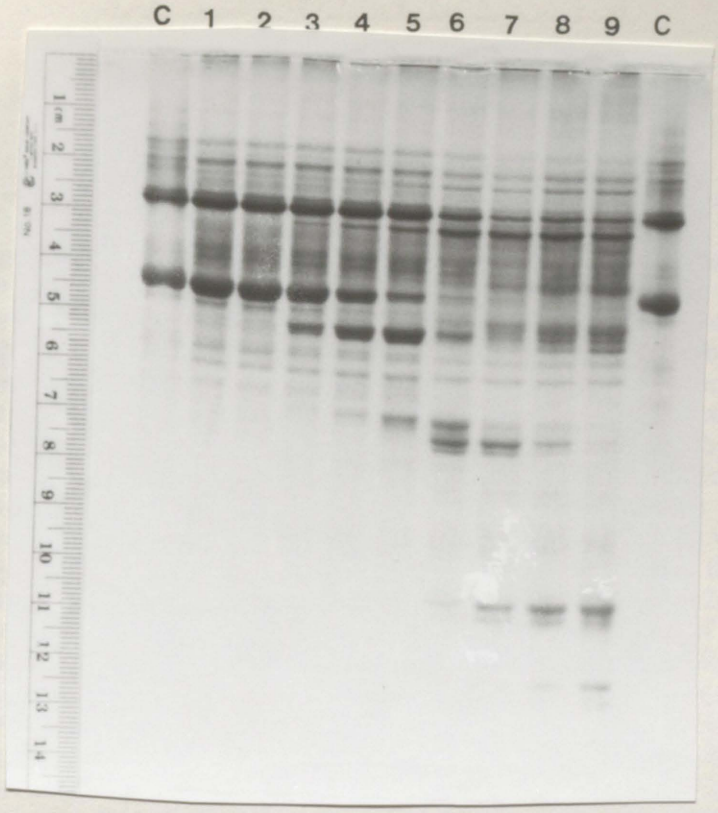


Fig. 7.1. *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of Na caseinate (C), micellar casein (1) and micellar casein hydrolysed by bovine cathepsin D (0.2 U ml^{-1}) at 37°C in 100 mM Na acetate buffer, pH 5.5, for 0, 0.25, 0.5, 1, 3, 6, 12 or 24 h (lanes 2-9).*

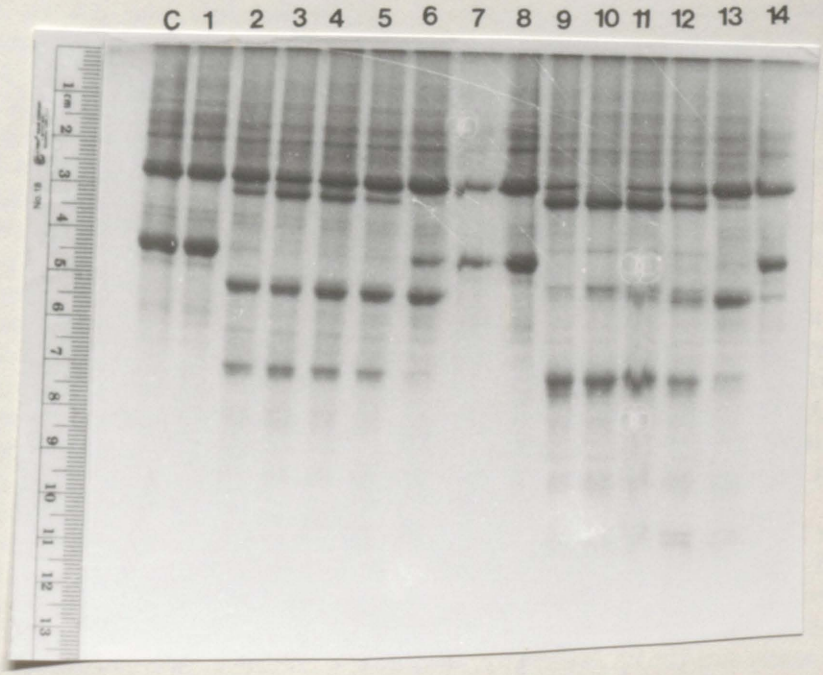


Fig. 7.2. *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of Na caseinate casein (C), Na caseinate control (1) and Na caseinate (5 mg ml^{-1}) hydrolysed by bovine cathepsin D (0.2 U ml^{-1}) at 37°C in 100 mM Na acetate buffer, pH 5.5, in the presence of 0, 1, 2.5, 5, 10 or 20% (w/v) NaCl for 1 h (lanes 2-7) and for 3 h (lanes 8-14).*

than that of α_{s1}-casein. Low concentrations of NaCl (1%) appeared to increase the activity of cathepsin D on both α_{s1}- and β-caseins (Fig. 7.2); Fox & Walley (1971) found that α_{s1}-casein was optimally degraded by chymosin in the presence of 5% (w/v) NaCl.

Kaminogawa *et al.*, (1978, 1980) found that there were similarities between the action of acid milk proteinase and chymosin on α_{s1}-, β- and κ-caseins. A peptide with an electrophoretic mobility identical to the primary product produced from α_{s1}-casein by chymosin (i.e. α_{s1}-CN f24-199) was produced by the action of acid milk proteinase (Kaminogawa *et al.*, 1980). These authors also demonstrated the production of a peptide with an electrophoretic mobility similar to β-I (β-CN f1-189/192), the primary large peptide produced from β-casein by chymosin, when β-casein was incubated with acid milk proteinase. It was therefore decided to compare proteolysis of bovine caseins by cathepsin D with that by chymosin. Electrophoretograms of α_{s1}-casein hydrolyzed by cathepsin D were very similar to those of chymosin-hydrolyzed α_{s1}-casein (Fig. 7.3), confirming the findings of Kaminogawa *et al.* (1980) with respect to the formation of α_{s1}-CN f24-199 and also demonstrate that the peptides produced on further hydrolysis of α_{s1}-casein by chymosin or cathepsin D had identical electrophoretic mobility. Reversed-phase HPLC of the pH 4.6-soluble peptides produced from α_{s1}-casein by cathepsin D and chymosin were also very similar (Fig. 7.4 a, b); the pH 4.6-soluble peptides produced from α_{s1}-casein by chymosin have been identified (McSweeney *et al.*, 1993). The order in which the pH 4.6-soluble peptides were produced from α_{s1}-casein by the two enzymes was also similar (Fig. 7.5 a, b); cathepsin D produced the peptide α_{s1}-casein f165-199 more quickly than did chymosin and the concentration of the peptide α_{s1}-CN f1-23 decreased on extended incubation.

Electrophoretograms of cathepsin D hydrolysates of α_{s2}-casein differed markedly from those of chymosin hydrolysates (Fig. 7.6). The major urea-PAGE-detectable peptide which accumulated in the chymosin hydrolysate (electrophoretic mobility of 4.8 cm) did not appear in electrophoretograms of cathepsin D hydrolysates. A group of peptides (mobilities 6.2 to 6.7 cm) were detectable in the cathepsin D hydrolysates but not in those produced chymosin. However, HPLC profiles of the pH 4.6-soluble peptides produced from α_{s2}-casein by cathepsin D or chymosin had a number of peptides with similar elution times (Fig. 7.7 a, b), although some of these peaks may have originated from α_{s1}-casein, present as a contaminant (Chapter 5).

Proteolysis of β-casein by cathepsin D was similar in some respects to that by chymosin (Fig. 7.8). Like chymosin, β-I (β-CN f1-189/192) was the primary product produced from β-casein by cathepsin D; β-II (β-CN f1-165) was also formed but did not accumulate in cathepsin D hydrolysates, in contrast to those produced by chymosin. Bands with electrophoretic mobilities in the region of β-III were present in both hydrolyzates, but differences between the specificities of the two enzymes were apparent in this region of the electrophoretogram. HPLC profiles of the pH 4.6-soluble peptides in hydrolysates of β-casein by cathesin D and chymosin had a number of peaks in common (Fig. 7.7 c, d), but differed in respect to peptides eluting in the region from 30 to 35 min.

Production of a peptide with an electrophoretic mobility identical to that of para-κ-casein by acid milk proteinase was shown by Kaminogawa *et al.* (1980). In this study, the HPLC profiles of the 8% TCA-soluble fraction of the hydrolysate of κ-casein by chymosin and cathepsin D were similar, indicating the production of glycomacropeptide (Fig. 7.9 a, b). However, cathepsin D did not coagulate milk within 2.5 h, even at a high enzyme levels (1 U ml^{-1}). The rapid hydrolysis of α_{s1}- and β- caseins by cathepsin D may cause changes in the micelles which render them unable to aggregate upon hydrolysis of κ-casein.

Fig. 7.3. *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of Na caseinate (C) and α_{s1}-casein (5 mg ml^{-1}) in 100 mM Na acetate buffer, pH 5.5, hydrolysed at 37°C by chymosin (4 x 10^{-2} U ml^{-1}) for 0, 1, 3, 6, 12 or 24 h (lanes 1-6) or bovine cathepsin D (1 x 10^{-2} U ml^{-1}) for 0, 1, 3, 6, 12 or 24 h (lanes 7-12).*

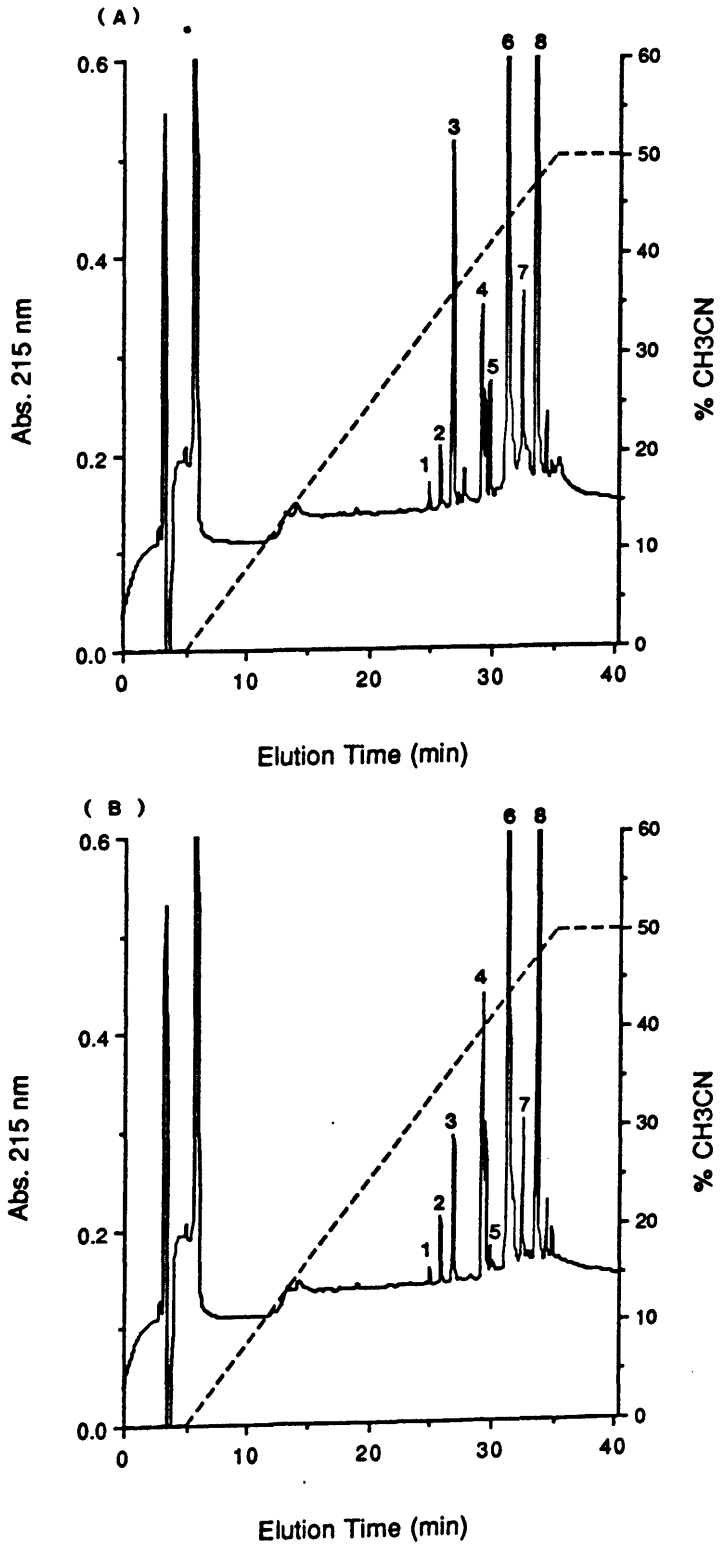


Fig. 7.4. *Reversed-phase HPLC chromatograms of pH 4.6-soluble peptides produced from bovine α_{s1}-casein (5 mg ml^{-1}) by (A) chymosin or (B) bovine cathepsin D in 100 mM Na acetate buffer, pH 5.5, for 6 h at 37°C. The identity of peptides was as follows:- (1) α_{s1}-casein f 154-158, (2) f 150-154, (3) f 157-164, (4) f 154-164, (5) f150-156, (6) f 1-23, (7) f 24-32, (8) f 165-199 (McSweeney et al., 1993). Acetonitrile gradient -------.*

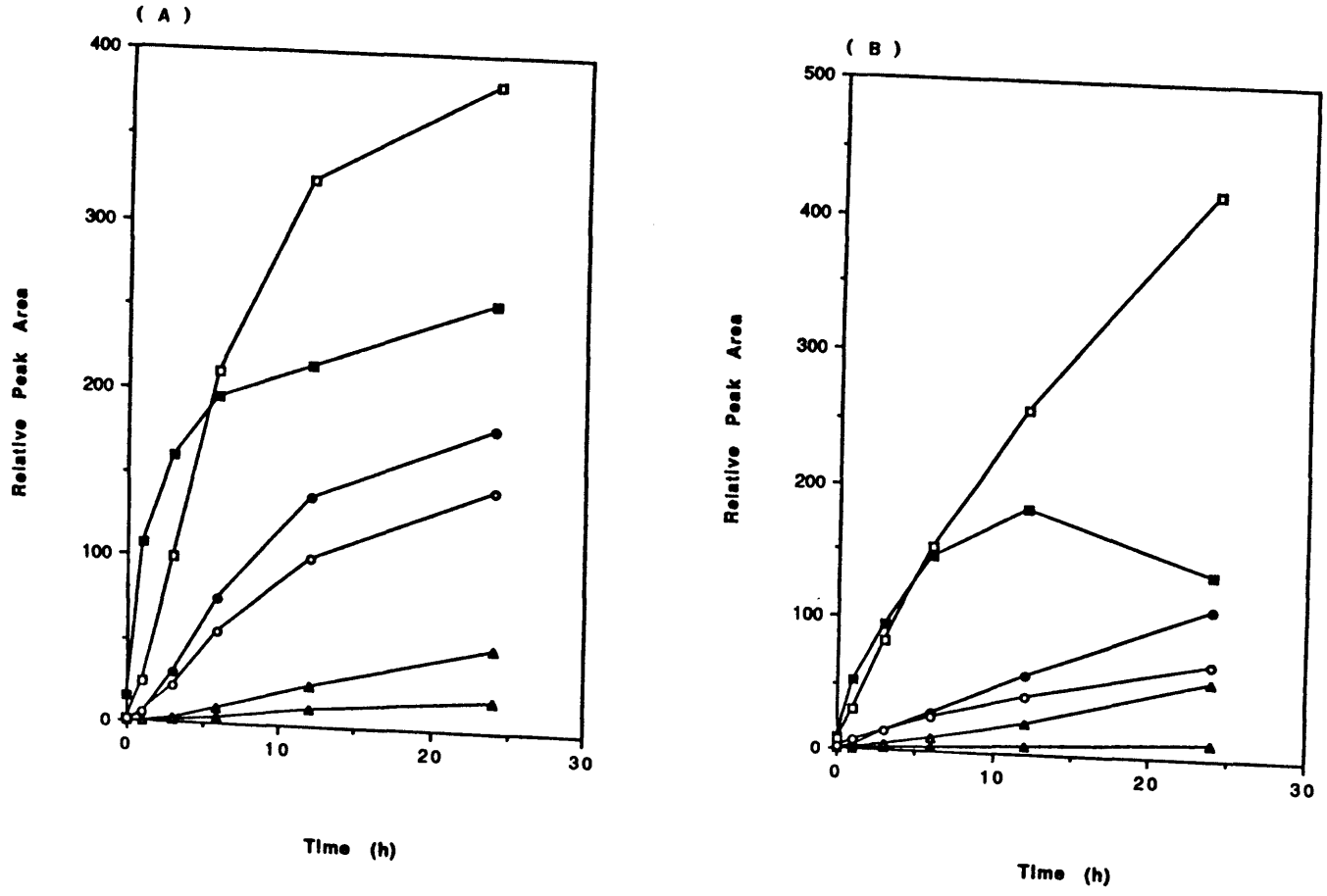


Fig. 7.5. *Changes in the areas of the HPLC peaks corresponding to the major pH 4.6-soluble peptides produced from α_{s1}-casein in solution in 100 mM Na acetate buffer, pH 5.5, by (A) chymosin or (B) bovine cathepsin D. (■) α_{s1}-casein f 1-23, (□) f 165-199, (●) f 157-164, (○) f 24-32, (▲) f 154-158 and (△) f 150-154.*

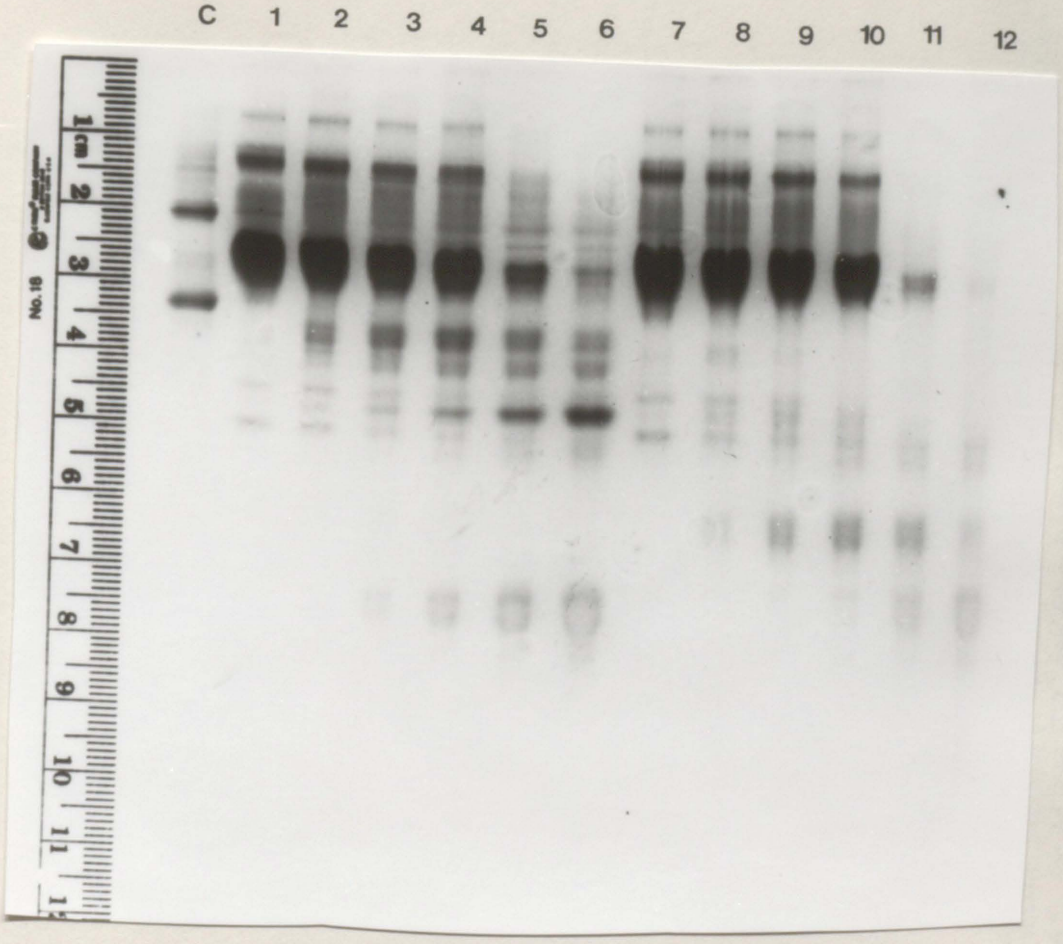


Fig. 7.6. *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of Na caseinate (C) and α_{s2}-casein (5 mg ml^{-1}) in 100 mM Na phosphate buffer, pH 6.5, hydrolysed by chymosin (1 U ml^{-1}) for 0, 1, 3, 6, 12 or 24 h (lanes 1-6) and bovine cathepsin D (4 x 10^{-1} U ml^{-1}) for 0, 1, 3, 6, 12 or 24 h (lanes 7-12) at 37°C.*

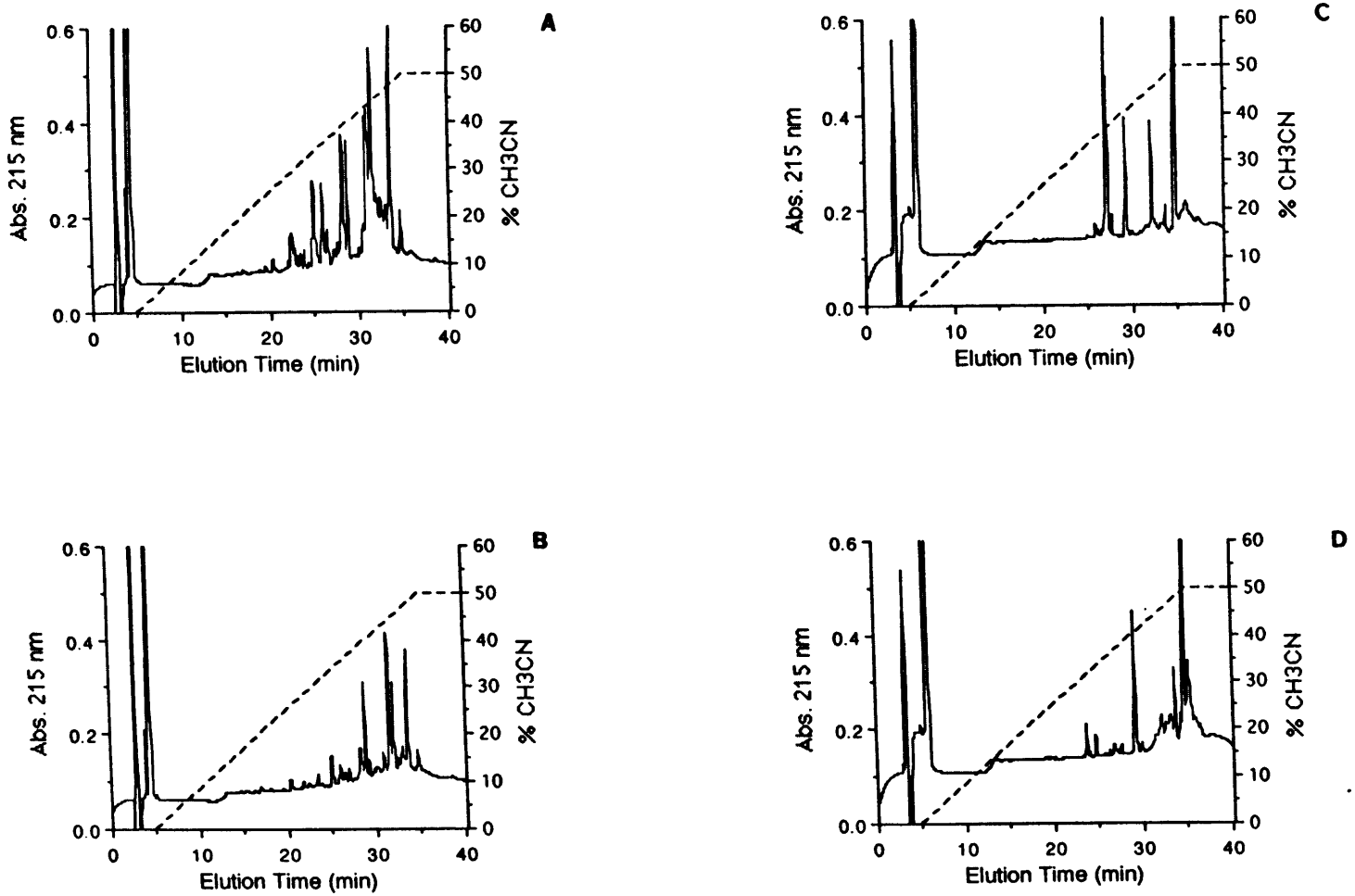


Fig. 7.7. Reversed-phase HPLC chromatograms of pH 4.6-soluble peptides produced from bovine α_{s2}-casein (5 mg ml^{-1}) by chymosin (A) or bovine cathepsin D (B) in 100 mM Na phosphate buffer, pH 6.5, for 24 h at 37°C and from bovine β-casein (5 mg ml^{-1}) by chymosin (C) or bovine cathepsin D (D) in 100 mM Na acetate buffer, pH 5.5 for 6 h.

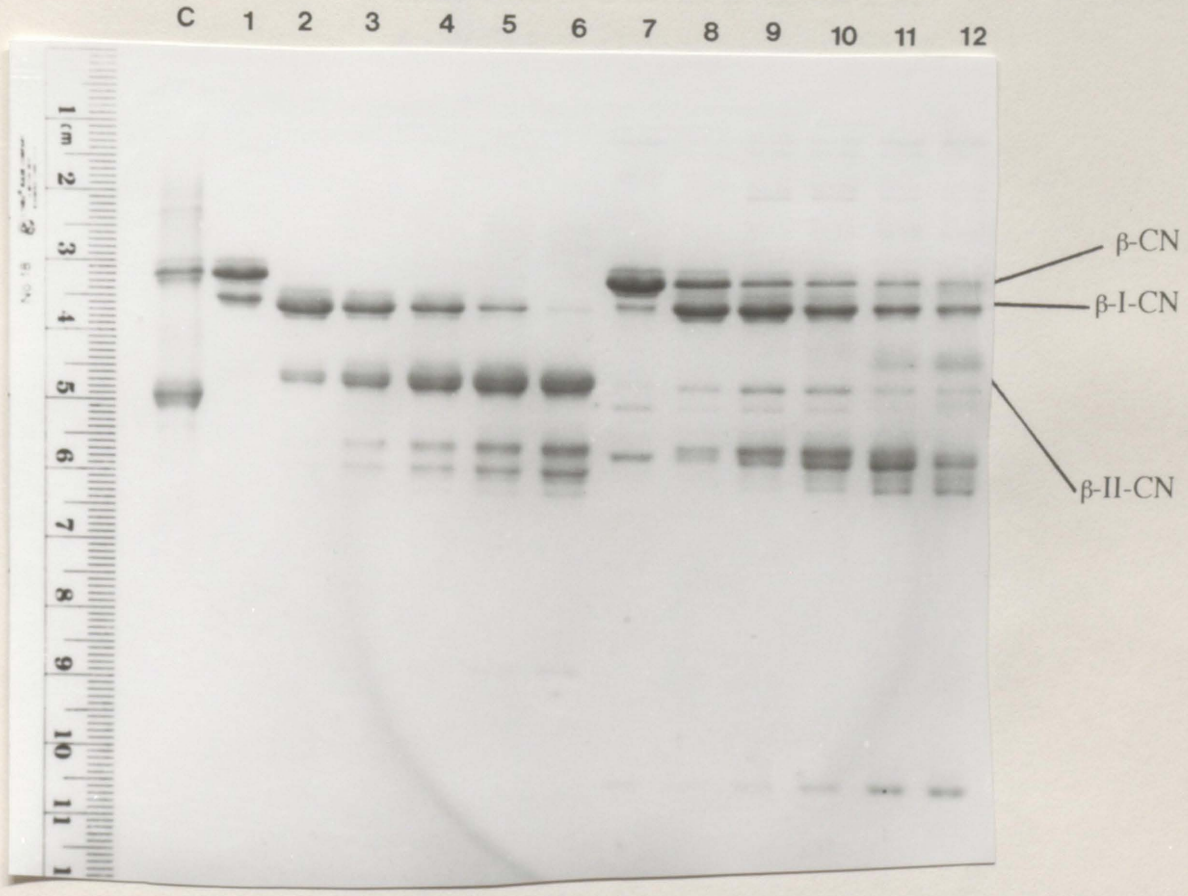
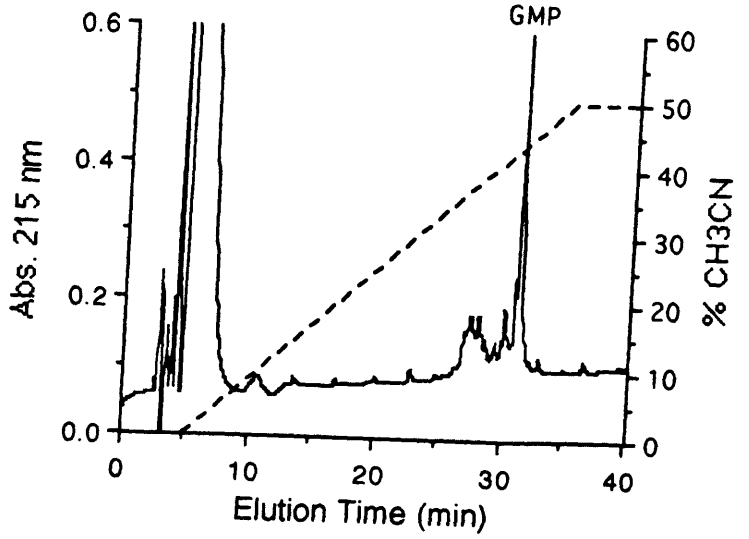


Fig. 7.8. *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of whole casein (C) and β-casein (5 mg ml^{-1}) in 100 mM Na acetate buffer, pH 5.5, hydrolysed at 37°C by chymosin (3 x 10^{-2} U ml^{-1}) for 0, 1, 3, 6, 12 or 24 h (lanes 1-6) and bovine cathepsin D (2 x 10^{-1} U ml^{-1}) for 0, 1, 3, 6, 12 and 24 h (lanes 7-12).*



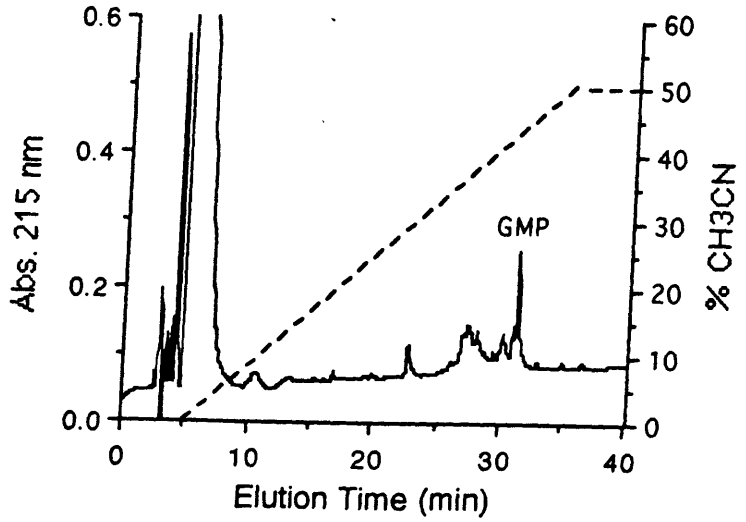


Fig. 7.9. Reversed-phase HPLC elution profiles of the 8% trichloroacetic acid-soluble peptides produced from κ-casein (5 mg ml^{-1}) by chymosin (A) or bovine cathepsin D (F) in 100 mM Na acetate buffer, pH 5.5, at 37°C for 20 min (GMP= κ-casein f 106-169). Acetonitrile gradient -------.

CONCLUSIONS

The results of this investigation indicate that all bovine caseins are susceptible to hydrolysis by cathepsin D *in vitro*, that α_{s1}-casein is more susceptible to proteolysis than β-casein and is maximally hydrolysed at pH 4.0 and that the proteolysis of β-casein is more susceptible to inhibition by NaCl than is α_{s1}-casein. The enzyme should be active under the ionic conditions present in many young cheeses (eg. Cheddar, pH 5.2 and *ca.* 5% NaCl-in-moisture) but its activity is probably masked by that of chymosin. The specificity of cathepsin D on α_{s1}- and κ-caseins is similar to that of chymosin, but there were substantial differences between the specificities of the enzymes on β- or α_{s2}-caseins.

ACKNOWLEDGEMENTS

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REFERENCES

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* **50**, 45-55.

Blakesley, R. W. & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Anal. Biochem.* **82**, 580-581.

Creamer, L. K. (1974). Preparation of α_{s1}-casein A. *J. Dairy Sci.* **57**, 341-344.

Dutson, T. R. (1983). Relationship of pH and temperature to disruption of specific muscle proteins and activity of lysosomal proteases. *J. Food Biochem.* **7**, 223-245.

Farkye, N. Y. & Fox, P. F. (1990). Observations on plasmin activity in cheese. *J. Dairy Res.* **57**, 413-418.

Farkye, N. Y. & Fox, P. F. (1991). Preliminary study on the contribution of plasmin to proteolysis in Cheddar cheese: cheese containing plasmin inhibitor, 6-aminohexanoic acid. *J. Agr. Food Chem.* **39**, 786-788.

Farkye, N. & Fox, P. F. (1992). Contribution of plasmin to Cheddar cheese ripening: effect of added plasmin. *J. Dairy Res.* **59**, 209-216.

Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *J. Dairy Sci.* **72**, 1379-1400.

Fox, P. F. & Law, J. (1991). Enzymology of cheese ripening. *Food Biotechnol.* **5**, 239-262.

Fox, P. F. & Walley, B. F. (1971). Influence of sodium chloride on the proteolysis of casein by rennet. *J. Dairy Res.* **38**, 165-170.

Goll, D. E., Otsuka, Y., Nagainis, P. A., Shannon, J. D., Sathe, S. K. & Muguruma, M. (1983). Role of muscle proteinases in maintenance of muscle integrity and mass. *J. Food Biochem.* **7**, 137-177.

Grappin, R., Rank, T. C. & Olson, N. F. (1985). Primary proteolysis of cheese proteins during ripening. A review. *J. Dairy Sci.* **68**, 531.

Grufferty, M. B. & Fox, P. F. (1988). Milk alkaline proteinase. *J. Dairy Res.* **55**, 609-630.

Kaminogawa, S. & Yamauchi, K. (1972). Acid protease of bovine milk. *Agr. Biol. Chem.* **36**, 2351-2356.

Kaminogawa, S. & Yamauchi, K. (1978). Degradation of casein components by milk acid protease. *Proc. 20th Intern. Dairy Congr.*, Paris, p. 313.

Kaminogawa, S., Yamauchi, K., Miyazawa, S. & Koga, Y. (1980). Degradation of casein components by acid protease of bovine milk. *J. Dairy Sci.* **63**, 701-704.

Lah, T. & Turk, V. (1982). Autolysis studies of cathepsin D. *Hoppe-Seyler's Zeit. Physiol. Chemie* **363**, 247-254.

Matsumoto, T., Okitani, A., Kitamura, Y. & Kato, H. (1983). Mode of degradation of myofibrillar proteins by rabbit muscle cathepsin D. *Biochim. Biophys. Acta* **755**, 76-80.

McSweeney, P. L. H., Olson, N. F., Fox, P. F., Healy, A. & Højrup, P. (1993). Proteolytic specificity of chymosin on bovine α_{s1}-casein. *J. Dairy Res.* (in press).

Pitt, D. (1975). *Lysosomes and Cell Function*. Longman, London and New York.

Schwartz, W. N. & Bird, J. W. C. (1977). Degradation of myofibrillar proteins by cathepsins B and D. *Biochem. J.* **167**, 811-820.

Scott, P. G. & Pearson, C. H. (1978). Cathepsin D: cleavage of soluble collagen and crosslinked peptides. *FEBS. Lett.* **88**, 41-45.

van den Berg, G. & de Koning, P. J. (1990). Gouda cheesemaking with purified calf chymosin and microbially produced chymosin. *Neth. Milk Dairy J.* **44**, 189-205.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. *Neth. Milk Dairy J.* **35**, 65-88.

Walstra, P. & Jenness, R. (1984). *Dairy Chemistry and Physics*. New York, Wiley.

Zeece, M. G., Katoh, K., Robson, R. M. & Parrish, F. C., Jr. (1986). Effect of cathepsin D on bovine myofibrils under different conditions of pH and temperature. *J. Food Sci.* **51**, 769-773,780.

8

CONTRIBUTION OF CELL WALL-ASSOCIATED PROTEINASES OF *LACTOCOCCUS* TO THE PRIMARY PROTEOLYSIS OF β-CASEIN IN CHEDDAR CHEESE†

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β-Casein was hydrolyzed in vitro *by cell wall-associated proteinases from* Lactococcus lactis *ssp.* cremoris *HP and* L. lactis *ssp.* lactis *JL 521 and JL 3601. Electrophoretograms of the hydrolyzates were compared with those of whole and water soluble extracts of Cheddar cheese made using these strains as starters. The peptides produced by hydrolysis of β-casein* in vitro *were not detected in the 6 month-old cheese or water extracts therefrom. Inter-strain differences were observed in the electrophoretograms of the cheeses, water extracts and β-casein hydrolyzates. The results of this study suggest that the cell wall-associated proteinases of the* Lactococcus *starters studied had little, if any, activity on intact β-casein in Cheddar cheese; it is postulated that this is due to hydrophobic interactions in cheese of the susceptible regions of β-casein.*

INTRODUCTION

Electrophoretic studies on Cheddar cheese have shown that β-casein is more resistant to protelysis than α_{s1}-casein throughout ripening (see ref. 7). The limited hydrolysis of β-casein that does occur in cheese has been attributed primarily to the action of plasmin (4). β-Casein is preferentially hydrolyzed by cell wall-associated proteinases of *Lactococcus* (12) and its specificity has been determined for both P_I- and P_{III}-type strains (11, 13). The primary cleavage sites of cell wall-associated proteinases of *Lactococcus* spp. on bovine β-casein are in its C-terminal region (11, 13). Although chymosin hydrolyses β-casein in solution, it does not do so in cheese, presumably because the susceptible peptide bonds of β-casein are inaccessible in cheese due to intermolecular hydrophobic interactions of segments of the polypeptide containing the cleavage sites (see ref. 7). The bonds in β-casein cleaved by chymosin, i.e. 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} (15) are in the same region as the primary sites hydrolyzed by cell wall-associated lactococcal proteinases, as identified by MONNET *et al.* (11) and REID *et al.* (13). Consequently, starter proteinases may not be active on β-casein in cheese. The present study was undertaken to determine whether peptides produced from β-casein *in vitro* by starter cell wall-associated proteinases could be detected in urea-polyacrylamide gel electrophoretograms of cheeses made using these starters and thus to determine the importance of the action of cell wall-associated proteinases of *Lactococcus* in the initial hydrolysis of β-casein in Cheddar cheese.

† *Milchwissenschaft* (in press)

MATERIALS AND METHODS

Lactococcus lactis ssp. *cremoris* HP and *L. lactis* ssp. *lactis* JL 521 and JL 3601 were obtained from the culture collection of the Department of Food Microbiology, University College, Cork. *L. lactis* ssp. *lactis* JL 521 and JL 3601 are proteinase-negative derivatives of *L. lactis* ssp. *lactis* UC 317 containing the cloned proteinase genes from *L. lactis* ssp. *cremoris* SK 11 (P_{III}-type) or *L. lactis* ssp. *lactis* UC 317 on a high copy number vector, respectively (8). The cleavage specificity of the cell wall-associated proteinase of *L. lactis* ssp. *lactis* UC 317 and *L. lactis* ssp. *lactis* NCDO 763 (P_I-type) on α- and β-caseins appear to be similar, although the former produces a proteinase which is a natural hybrid of P_I- and P_{III}-types (9). More proteinase was produced by the recombinant strains (JL 521 and JL 3601) than the wild-type strains (SK 11 and UC 317, respectively, ref. 8).

The strains were propagated overnight at 30 °C in a whey-based medium containing 1.9 % (wt/wt) β-glycerophosphate, 0.5 % (wt/vol) glucose and 0.1 % (wt/vol) casitone (16). Cells were harvested centrifugally (5,000 **g** x 10 min) and the cell wall-associated proteinases liberated by incubation in 50 m*M* Na acetate-phosphate buffer, pH 6.5, at 30°C essentially as described by EXTERKATE and de VEER (5).

β-Casein (minimum 90% β-casein by electrophoresis) was obtained from Sigma Corp. (St. Louis, MO, USA); solutions (2 mg ml^{-1}) were prepared in 50 m*M* Na acetate-phosphate buffer, pH 6.5. To 50 ml aliquots of β-casein solution were added 10, 20 or 50 ml of enzyme solution and the volume made up to 100 ml with the 50 m*M* Na acetate-phosphate buffer. The solutions were incubated at 30 °C for 1 h and the reaction stopped by the addition of 100 ml of electrophoresis sample buffer (1.5 g tris, 96 g urea, 0.8 ml conc. HCl, 1.4 ml β-mercaptoethanol, 0.1 g bromophenol blue made up to 200 ml in H_2O).

Urea-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of ANDREWS (2); staining and destaining were according to the procedure of BLAKESLEY and BOEZI (3)

Cheddar cheeses were made in 100 L vats as described by VAN SLYKE and PRICE (14). The individual single strain starters (see above) were propagated in sterilized (110°C for 10 min) reconstituted (10 % wt/wt) skim milk powder (Irish Dairy Bord, Dublin, Ireland). The cheeses were analyzed in duplicate for moisture, protein, NaCl and pH (1) and bacterial counts on M17 agar (Oxoid, Basingstoke, England). The cheeses were ripened at 8°C for 6 months.

Water-soluble extracts were prepared according to the method of KUCHROO and FOX (10).

RESULTS AND DISCUSSION

The compositions of the cheeses are given in Table 8.1. The composition of the HP cheese was within the range expected for Cheddar cheese but the moisture content of the cheeses made with starter strains *L. lactis* spp. *lactis* JL 521 or JL 3601, which were made from late lactation milk, were slightly high.

Comparison of the electrophoretograms of the cheese, water-soluble extracts therefrom and hydrolyzates of β-casein (Figure 8.1) indicate that the peptides produced from β-casein by the cell wall-associated proteinases from *L. lactis* ssp. *cremoris* HP and *L. lactis* ssp. *lactis* JL 3601 and JL 521 were not among the principal peptides in 6 months old Cheddar cheese made using these starters.

Comparison of the electrophoretograms of β-casein hydrolyzates prepared with varying amounts of the cell wall-associated proteinases (Figure 8.2) indicated that the peptides produced initially from β-casein in solution were quite resistant to subsequent proteolysis by cell wall-associated proteinases and were not broken down to peptides undetectable by PAGE. Inter-strain differences were apparent in the electrophoretograms of the cheeses, water soluble extracts therefrom and hydrolyzates of β-casein produced by cell wall-associated proteinases from these

Table 8.1. Composition[1] of Cheddar Cheeses made with *Lactococcus. lactis* ssp. *cremoris* HP, *L. lactis* ssp. *lactis* JL 3601 or JL 521.

Starter	Moisture	Protein	NaCl	pH	cfu g^{-1}
	----------%----------		----------Day 1----------		
HP	36.8	24.7	1.65	5.27	$2x\ 10^9$
JL 3601	39.8	23.2	1.65	5.20	$4x\ 10^9$
JL 521	39.2	22.8	1.65	5.10	$3x\ 10^9$

[1]Means of duplicates.

strains.

The results presented suggest that starter proteinases which readily hydrolyse β-casein in solution are unable to do so in cheese; at least large peptides produced from β-casein by starter cell wall-associated proteinases do not accumulate in cheese, as do those produced by chymosin, eg. α_{s1}-I-casein or plasmin, eg. γ-caseins. This is analogous to the situation with chymosin which readily hydrolzes β-casein in solution but not in cheese. Since the primary cleavage sites for chymosin and lactococcal proteinase cleavage sites in β-casein are in the hydrophobic C-terminal region, it appears reasonable to suggest that the failure of these enzymes to hydrolyze β-casein in cheese is due to the hydrophobic interaction of the β-casein in cheese as a result of which susceptible bonds are rendered inaccessible.

CONCLUSIONS

The findings of this study suggest that cell wall-associated starter proteinases are not significant in the primary proteolysis of β-casein in Cheddar cheese; at least none of the large peptides detected by PAGE in solutions of β-casein incubated with isolated cell wall-associated proteinase from *Lactococcus* spp. were amongst the principal peptides detectable in gel electrophoretograms of the 6 month old Cheddar cheese or in the water soluble fraction therefrom.

These preliminary results suggest that cell wall-associated proteinases of *Lactococcus* are not important in the primary hydrolysis of β-casein in Cheddar cheese and suggest that the limited proteolysis of this protein is due to some other proteinase, probably plasmin. These results do not preclude an important rôle for starter proteinases in the proteolysis of intermediate-sized peptides produced by chymosin or plasmin. Definative conclusions as to the importance of starter proteinases in the primary proteolysis of caseins in Cheddar will require identification of the primary hydrolysis products of caseins and comparison with the specificity of these proteinases. Studies with these objectives are currently underway in this laboratory (see Chapter 9).

REFERENCES

(1) AOAC (1984). Official Methods of Analysis of the Association of Official Analytical Chemists. S. Williams (Ed.), Association of Oficial Analytical Chemists Inc., Arlington, VA, USA.

(2) Andrews, A. T. *J. Dairy Res.* **50** 45-55 (1983)

(3) Blakesley, R. W., Boezi, J. A. *Anal. Biochem.* **82** 580-581 (1977)

(4) Creamer, L. K. *J. Dairy Sci.* **58** 287-292 (1975)

Fig. 8.1. *Urea-polyacrylamide gel electrophoretograms of Na-caseinate (C), β-casein (B), cheese made with* Lactococcus lactis *ssp.* cremoris *HP (1), hydrolysate of β-casein by HP proteinase (2), water-soluble extract of HP cheese (3), cheese made with* L. lactis *ssp.* lactis *JL 521 (4), hydrolysate of β-casein by JL 521 proteinase (5), water-soluble extract of JL 521 cheese (6), cheese made with* L. lactis *ssp.* lactis *JL 3601 starter (7), hydrolysate of β-casein by JL 3601 proteinase (8), water-soluble extract of JL 3601 cheese (9).*

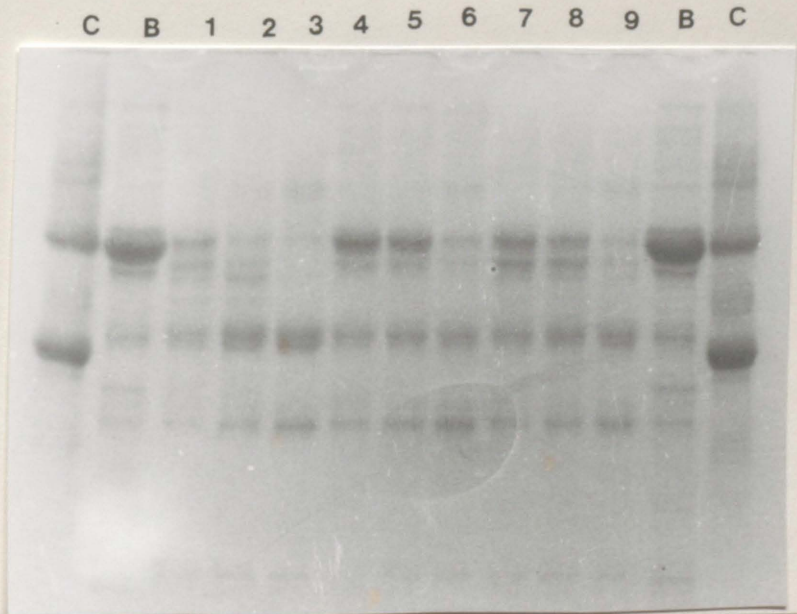

Fig. 8.2. *Urea-polyacrylamide gel electrophoretograms of Na-caseinate (C), β-casein (B), β-casein after incubation with 10, 20 or 50 µl* Lactococcus lactis *ssp.* cremoris *HP (1, 2, 3),* L. lactis *ssp.* lactis *JL 521 proteinase (4, 5, 6),* L. lactis *ssp.* lactis *JL 3601 proteinase (7, 8, 9).*

(5) Exterkate, F. A., de Veer, G. J. C. M. *Syst. Appl. Microbiol.* **9** 183-191 (1987)

(6) Farkye, N. Y., Fox, P. F., Fitzgerald, G. F., Daly, C. *J. Dairy Sci.* **73** 874-880 (1990)

(7) Fox, P. F. *J. Dairy Sci.* **72** 1379-1400 (1989)

(8) Law, J. M. Ph. D. Thesis, National University of Ireland, Cork, (1991).

(9) Law, J., Vos, P., Hayes, F., Daly, C., de Vos, W. M., Fitzgerald, G. *J. Gen. Microbiol.* **138** 709-718 (1992)

(10) Kuchroo, C. N., Fox, P. F. *Milchwissenschaft* **37** 331-335 (1982)

(11) Monnet, V., LeBars, D., Gripon, J. C. *FEMS Microbiol. Letts.* **36** 127-131 (1986)

(12) Monnet, V., LeBars, D., Neviani, E., Gripon, J. C. *Lait* **67** 51-61 (1987)

(13) Reid, J. R., Ng, K. H., Moore, C. H., Coolbear, T., Pritchard, G. G. *Appl. Microbiol. Biotechnol.* **36** 344-351 (1991)

(14) Van Slyke, L. L., Price, W. V. In *Cheese*, Orange Judd Publ. Co., New York, NY (1952)

(15) Visser, S., Slangen, K. J. *Neth. Milk Dairy J.* **31** 16-30 (1977)

(16) Vos, P., van Asseldonk, M., van Jeveren, F., Siezen, R., Simons, G., de Vos, W. M. J. Bacteriol. **171** 2795-2802 (1989)

9

FRACTIONATION AND IDENTIFICATION OF PEPTIDES FROM THE WATER-INSOLUBLE FRACTION OF CHEDDAR CHEESE

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Peptides from the water-insoluble fraction (WISF) of a 3 month-old Cheddar cheese made using Lactococcus lactis *subsp.* cremoris *SK 11 were fractionated by ion-exchange chromatography on DEAE-cellulose. Peptides were isolated by electroblotting from urea- and SDS-polyacrylamide electrophoretograms (PAGE) gels and identified from their N-terminal amino acid sequence and mass. Eleven peptides, 5 from β-casein and 6 from α_{s1}-casein, were identified. In most cases, the origin of the peptides could be explained based on the specificities of chymosin and plasmin. The results permit identification of the majority of bands on urea-PAGE gels of the WISF of Cheddar cheese.*

INTRODUCTION

Proteolysis is regarded as the most important biochemical event during the ripening of Cheddar cheese (Rank *et al.*, 1985; Fox, 1989). Although schemes have been developed for the fractionation of peptides in cheese (Kuchroo and Fox, 1983; O'Sullivan and Fox, 1989; Breen, 1992; Singh *et al.*, 1993), few peptides have been isolated from cheese and identified.

The primary proteolysis of caseins in Cheddar results from the action of chymosin and plasmin (Fox, 1989). The specificity of chymosin (Visser and Slangen, 1977; McSweeney *et al.*, 1993a), plasmin (Eigel, 1977, 1981; Visser *et al.*, 1989; Visser *et al.*, 1989; Le Bars and Gripon, 1989; McSweeney *et al.*, 1993b) and cell wall-associated proteinases of *Lactococcus* spp. (Monnet *et al.*, 1986; Visser *et al.*, 1991, Reid *et al.*, 1991a,b; Monnet *et al.*, 1992) on the caseins in solution have been established, but the extent to which these results can be extrapolated to cheese is not clear.

Electrophoresis in alkaline urea polyacrylamide gels is widely used to study proteolysis in cheese during ripening (Creamer, 1991). However, identification of bands on the gels is not possible, with the exception of the intact caseins and the primary product of chymosin on α_{s1}-casein (α_{s1}-CN f24-199, α_{s1}-I-CN) and of plasmin on β-casein (β-CN f29-209, f106-209 and f108-209; γ^1, γ^2 and γ^3). Most workers have concentrated on fractionation of the water-soluble fraction of cheese (see review by Fox *et al.*, 1993), and little attention has been focussed on the water-insoluble peptides.

This study was undertaken with the objectives of fractionating peptides in the water-insoluble fraction of Cheddar cheese, identifying these peptides by partial sequence analysis and mass spectrometry and thus establish the extent to which the results of casein hydrolysis studies in solution can be extrapolated to Cheddar cheese.

MATERIALS AND METHODS

Cheddar cheese was manufactured from whole milk according to a standard protocol (Kosikowski, 1982), using 10 strains of *Lactococcus lactis* subsp. *cremoris* or *L. lactis* spp. *lactis* as starter (317, HP, AM1, ML1, SK11, AM2, 147, C13, 952 and UC063) individually as starter. Cheese was ripened at 8°C for 3 months. Water-insoluble peptides were isolated from cheese made with *L. lactis* ssp. *cremoris* SK11.

The water-soluble (WSF) and insoluble (WISF) fractions of the cheese were prepared according to the method of Kuchroo and Fox (1982); the WISF was re-extracted, grated and freeze dried.

Peptides in the WISF of the cheese were fractioned by chromatography on a column of DEAE-cellulose (68.5 x 2 cm, Whatman International Ltd., Maidstone, England) using 0.02 M tris (hydroxymethyl) methylamine buffer, pH 8.6, containing 4.5 M urea (Breen, 1992). Prior to appliction, samples (50 mg ml^{-1}) were dissolved in 10 ml of chromatography buffer and dialyzed against 1 L of the same buffer for 24 h at 4°C. Elution was by a linear 0 to 0.3 M NaCl gradient. Eluate was monitored at 280 nm and fractions containing peptides were pooled, dialyzed overnight against H_2O at 4°C and freeze dried.

Urea-polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Andrews (1983) with direct staining using Coomassie brilliant blue G250 (Blaksley and Boezi, 1977). Sodium dodecylsulphate (SDS)-PAGE was performed in 13.6% acrylamide separating gels, pH 8.9 contining 0.1% (w/v) SDS with 5% acrylamide stacking gels, pH 6.5. Electrophoresis was performed at a constant voltage (200 V). Gels were fixed overnight in 25% (v/v) propan-2-ol and 10% (v/v) acetic acid in water. Gels were stained for 3 to 4 h in 0.1% (w/v) Coomassie blue R250 in methanol: water: acetic acid (5: 4: 1) and destained in 7.5% (v/v) methanol, 5% (v/v) acetic acid in water.

Peptides were isolated by electroblotting from urea- or SDS-PAGE gels using a mini Trans-Blot™ electrophoretic transfer cell (BioRad, San Ramon, CA, USA). Transfer of peptides was at 100 V for 1 h in 25 mM tris-192 mM glycine buffer in 20% (v/v) methanol onto polyvinlidene difluoride membranes, pore size 0.22 μm (ProBlot™, Applied Biosystems Inc., San Jose, CA, USA). Membranes were stained for 15 min using 0.2% Coomassie blue R250 (w/v) in 45% (v/v) methanol, 10% (v/v) acetic acid and destained for 30 min in 7% (v/v) acetic acid in 45% (v/v) methanol and 2 min in 7% (v/v) acetic acid in 90% (v/v) methanol. Alternatively, membranes were rinsed with water and stained in 0.2% (w/v) Ponceau S in 1% (v/v) acetic acid for 10 min followed by destaining in water. Bands corresponding to peptides were excised from membranes and sequenced.

The N-terminal sequence of peptides was determined by Edman degradation, automated by an Applied Biosystems model 477A protein sequencer (Applied Biosystems Inc., San Jose, CA, USA). Liberated amino acids were detected as their phenylthiohydantoin derivatives. Usually, the sequence of 6 amino acid residues from the N-terminus was determined.

Mass spectrometry of fractions obtained from chromatography on DEAE-cellulose was performed using a matrix-assisted laser desorption ionisation mass spectrometer (MALDI-MS). Identification of peptides by mass was based on partial sequence data combined with mass searches using the GPMAW program (Lighthouse Data, DK-5250 Odense SV, Denmark).

β-Casein was isolated by fractionation of Na caseinate by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) using 10 mM imidazole buffer, pH 7.0, containing 4.5 M urea and 0.1% (v/v) 2-mercaptoethanol (Creamer, 1974). β-Casein (2 mg ml^{-1}) was dissolved in 0.05 M NH_4HCO_3 buffer, pH 8.4, containing 0.05% NaN_3, and incubated with porcine plasmin (Sigma Chemical Co., St. Louis, MO, USA, 0.02 U ml^{-1}) at 30°C. Samples were taken periodically for analysis by urea-PAGE and the reaction stopped by the addition of an equal volume of double-concentrated sample buffer (McSweeney *et al.*, 1993a).

RESULTS AND DISCUSSION

Urea-polyacrylamide gel elelctrophoretograms of 3 month old cheeses made with single strains of *L. lactis* ssp. *lactis* or *L. lactis* ssp. *cremoris* are shown in Figure 9.1. All major peptide bands were present in all cheeses, indicating that if the starter contributed to the level of proteolysis detectable by PAGE, the effect was independent of strain, including differences in cell wall-associated proteinase type (e.g. P_I-type, HP and P_{III}-type, AM_1).

The ion-exchange chromatogram of the WISF of a 3 month-old Cheddar cheese made with *L. lactis* ssp. *cremoris* SK11 is shown in Figure 9.2. Nineteen fractions were collected which were subsequently analyzed by urea-PAGE (Figure 9.3a, b). Most of the chromatographic fractions were heterogeneous. The chromatogram consisted of 2 major peaks, corresponding primarily to unhydrolyzed β-casein (fraction 11) and α_{s1}-casein and its primary chymosin degradation product, α_{s1}-CN f24-199 (fractions 14 to 18). With the exception of fraction 5, the chromatographic fractions were too heterogeneous to warrant sequence analysis directly and therefore peptides were isolated from the electrophoretograms (urea- or SDS-PAGE) of the fractions by electroblotting.

N-Terminal sequences and masses of some of the peptides isolated from the WISF are summarized in Table 9.1 and their loctation on urea-PAGE gels indicated in Figure 9.4.

All peptides with electrophoretic mobilities slower than that of α_{s1}-casein (4.15 cm, Figure 9.4) originated from β-casein and all appeared to have been produced through the action of plasmin. The group of 3 peptides with mobilities of 1.35 to 2.00 cm (Figure 9.4) corresponded to the γ-caseins (β-CN f 29-209, f106-209 and f108-209, Eigel, 1977, 1981); the mass of putitative f108-209 remains to be confirmed. The identity of these bands as γ-caseins is generally recognized (Creamer, 1991), although the order in which they migrate (γ^2, γ^1 and γ^3) has been misinterpreted (e.g Farkye and Fox, 1991, 1992). The identity of the middle of these bands as γ^1-casein is consistant with its early production from β-casein by plasmin in solution (Fig. 9.5), and this band does not accumulate presumably because it is further hydrolyzed to γ^2 and γ^3.

Three bands were identified as having the N-terminal sequence of β-casein (electrophoretic mobilities of 2.60, 2.90 and 3.75 cm, Fig. 9.4). The peptide in fraction 1 (Fig. 9.3) with electrophoretic mobility of 2.60 cm had the N-terminal sequence of β-casein. This peptide may represent β-casein which did not adsorb onto the DEAE-cellulose, although this peptide eluted from the column at a much lower NaCl concentration (fractions 1 and 2, Fig. 9.3) than did β-casein (fraction 11). Alternatively, it may be an N-terminal fragment of β-casein, e.g. a proteose peptone, complimentary to one of the γ-caseins or some another fragment of β-casein produced by proteolysis. The peptide with an electophoretic mobility of 2.90 cm (Fig. 9.4) migrated in the same position as β-CN f1-189/192, (β-I-CN), the primary product of chymosin action on β-casein, and this peptide is thus tentatively identified as β-I. The peptide with an electrophoretic mobility of 3.75 and the N-terminal sequence of β-casein may be a proteose peptone.

The N-terminii of the majority of bands with electrophoretic mobilities faster than α_{s1}-casein corresponded to chymosin cleavage sites (McSweeney *et al.*, 1993a). Peptides with electrphoretic mobilities of 4.90 and 5.25 cm had N-terminal sequences of F.V.A.P.F and G.K.E.K (Table 9.1) originated at positions Phe_{23}-Phe_{24}, Phe_{32}-Gly_{33} in α_{s1}-casein both of which are cleaved rapidly by chymosin under the ionic conditions in Cheddar cheese (McSweeney *et al.*, 1993a). The peptide with electrophoretic mobility of 4.60 cm was identifed by mass spectrometry and from its N-terminal sequence and was identifed as α_{s1}-CN f102-149. Both the N- and C-terminii of this peptide correspond to chymosin cleavage sites (McSweeney *et al.*, 1993a); the cleavage site at its N-terminus (Leu_{101}-Lys_{102}) is amongst the primary sites of chymosin action on cheese. The peptide with a mobility of 4.90 cm was identified as α_{s1}-CN f24-199 (α_{s1}-I-CN) by mass

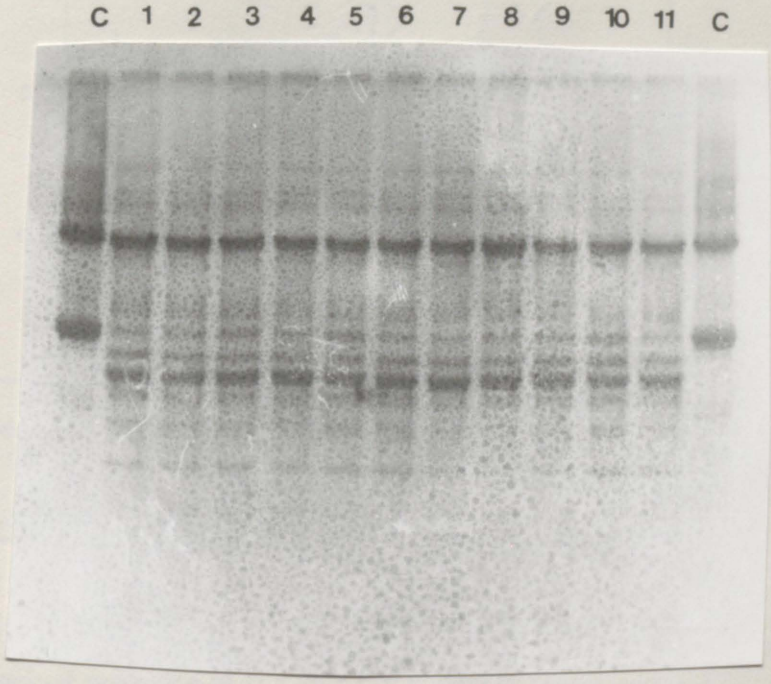


Fig. 9.1: *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of Na-caseinate (C) and Cheddar cheeses made with single strains of* Lactococcus lactis *spp.* lactis *or* L. lactis *spp.* cremoris *strains 317, HP, AM1, ML1, SK11, 317, AM2, 147, C13, 952 and UC063 (1 to 11).*

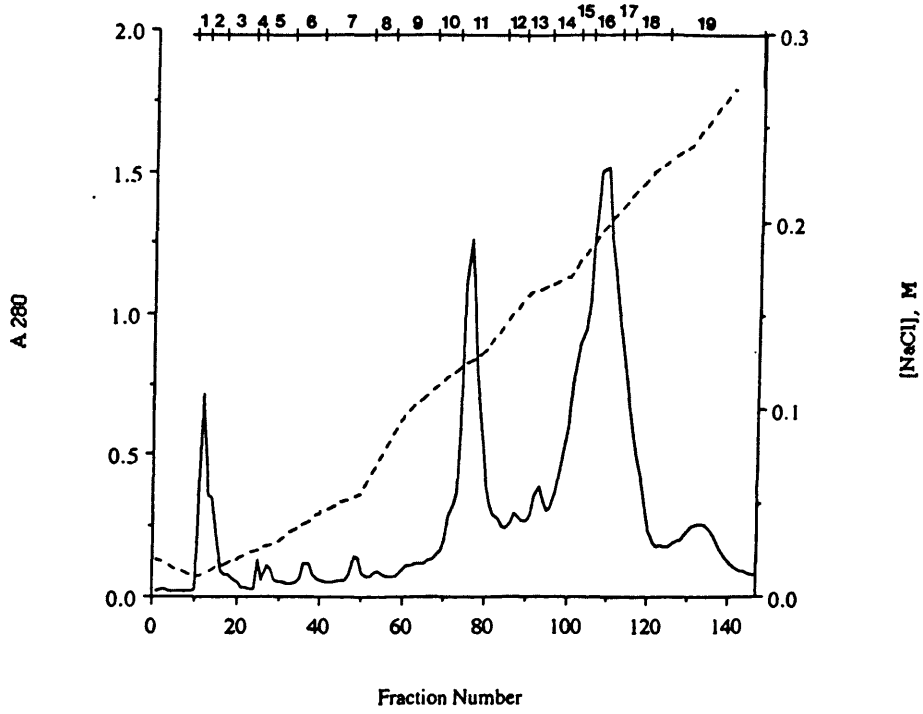


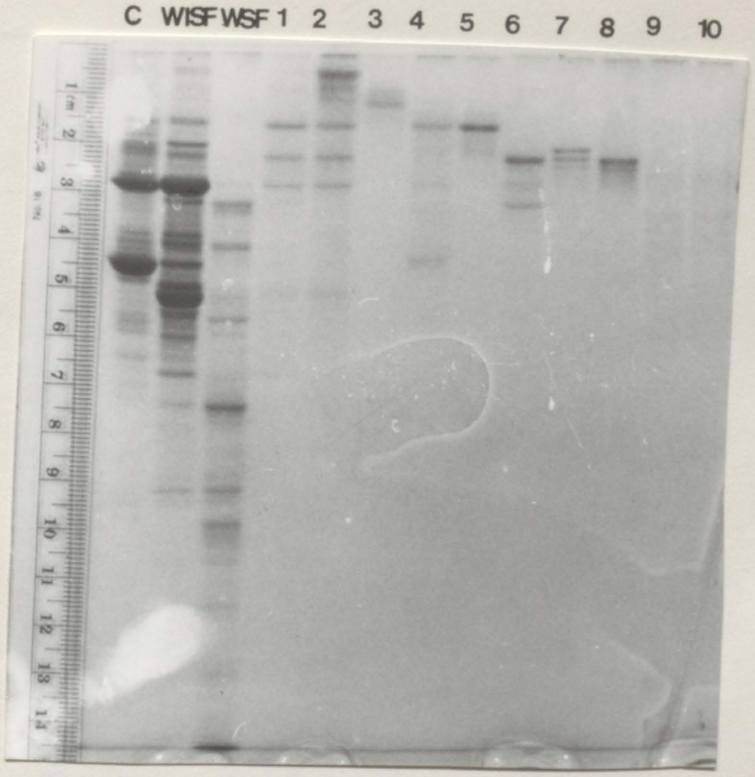
Fig. 9.2: Chromatogram of the water-insoluble fraction of 3 month-old Cheddar cheese made with Lactococcus lactis *subsp.* cremoris *SK 11 on DEAE-cellulose, 0.02 M tris (hydroxymethyl) methylamine buffer, pH 8.6, containing 4.5 M urea. Elution was by a 0 to 0.3 M NaCl gradient (----------). Fractions were pooled as indicated.*

spectrometry and from its N-terminal sequence and corresponds to the primary product of chymosin action on α_{s1}-casein. The peptide with a mobility of 8.75 cm had an N-terminal sequence of F.V.A.P.F.P (Table 9.1) and presumably originated from α_{s1}-CN f 24-199 *via* hydrolysis towards its C-terminal.

The origins of 2 peptides with N-terminal sequences of M.E.A.E.X.I and E.I.V.P.N.S.A (corresponding to cleavage of the bonds Gln_{59}-Met_{60} and Leu_{109}-Glu_{110} in α_{s1}-casein) do not correspond with the specificity of chymosin (McSweeney *et al.*, 1993a), plasmin (McSweeney *et al.*, 1993b) or the cell wall-associated proteinase of *L. lactis* subsp. *lactis* SK11 (Reid *et al.*, 1991a). Presumably, these 2 peptides are formed by the action of starter peptidases or non-starter proteinases and/or peptidases acting on peptides produced from α_{s1}-casein by chymosin (e.g produced by cleavage at Leu_{40}-Ser_{41} or Leu_{101}-Lys_{102}, or plasmin (e.g. following cleavage at Lys_{58}-Gln_{59} or Lys_{105}-Val_{106}). The bond Lys_{58}-Gln_{59} is not amongt the principal sites cleaved by plasmin in α_{s1}-casein (McSweeney *et al.*, 1993b), although it is possible that this bond may be rendered more accessible to plasmin action in cheese as much of α_{s1}-casein has been partially hydrolysed by chymosin.

None of the peptides identified in this study originated from α_{s2}-casein, the fate of which in cheese during ripening is ambiguous. Fox (1989) stated that α_{s2}-casein is very resistant to proteolysis during cheese ripening, although bands in the α_{s2}-casein region do decrease in concentration during ripening (van den Berg and de Koning, 1991; McSweeney *et al.*, 1993d). α_{s2}-Casein is susceptible to plasmin (Le Bars and Gripon, 1989; Visser *et al.*, 1989) and chymosin (Chapter 5) action and bands in the α_{s2} region of urea-PAGE electrophoretograms were more intense in cheeses containing plasmin inhibitor than in the control (Farkye and Fox, 1992), suggesting that α_{s2}-casein is degraded by plasmin during ripening. The detection of α_{s2}-casein in urea-PAGE gels is made difficult by the migration of a product of β-casein in the region normally occupied by α_{s2}-casein (*ca.* 3.50 to 4.00 cm, Figure 9.4).

(a)



(b)

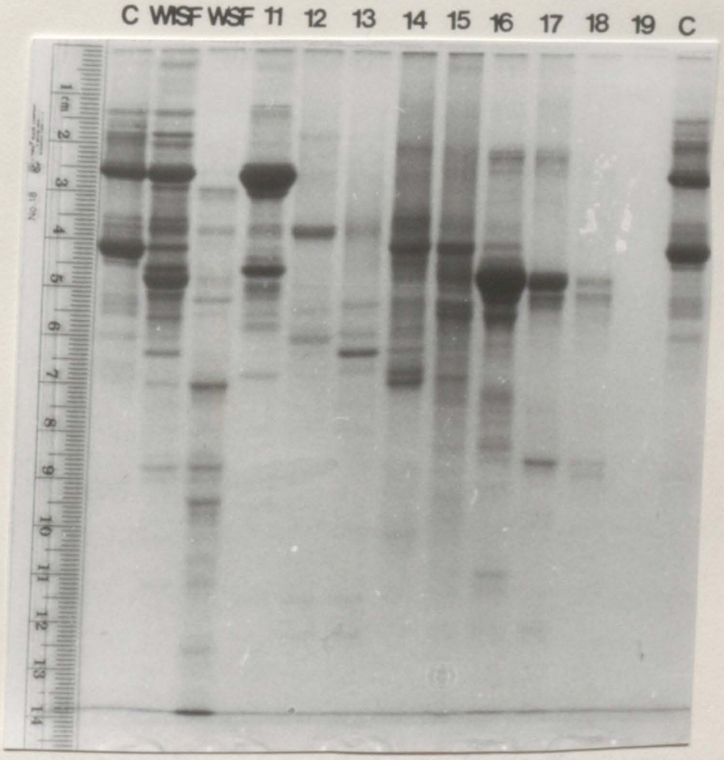


Fig. 9.3: *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of casein (C) and water-insoluble (WISF) and water-soluble (WSF) fractions of 3 month-old Cheddar cheese made with* Lactococcus lactis *subsp.* cremoris *SK 11 and fractions from chromatography of the WISF on DEAE-cellulose (a) 1 to 10, and (b) 11 to 19 (see Fig. 9.2).*

TABLE 9.1: Identity of peptides isolated from the water-insoluble fraction of Cheddar cheese.

Fraction No.	Electrophoretic. Mobility (cm)	N-Terminal Sequence	Mass (Da)		Position in Casein Sequence
			Experimental	Theoretical	
1	2.00	E.M.P.F.P.K			β-CN f 108
	2.60	R.E.L.E.E			β-CN f 1
	-		11,974	11,823	β-CN f 106-209
5	1.35	H.E.P.M.P.F			β-CN f 106
7	1.80	K.I.E.K.F	20,544	20,447	β-CN f 29-209
11	2.90	R.E.L.E.E			β-CN f 1
	4.60	K.K.Y.K.V.P	5707	5706	α_{s1}-CN f 102-149
12	3.75	R.E.L.E.E.L			β-CN f 1
13	6.35	E.I.V.P.N.S.A			α_{s1}-CN f 110
16	5.25	G.K.E.K			α_{s1}-CN f 33
	5.60	M.E.A.E.X.I			α_{s1}-CN f 60
17	4.90	F.V.A.P.F			α_{s1}-CN f 24
	8.75	F.V.A.P.F.P	20,956	20,929	α_{s1}-CN f 24-199

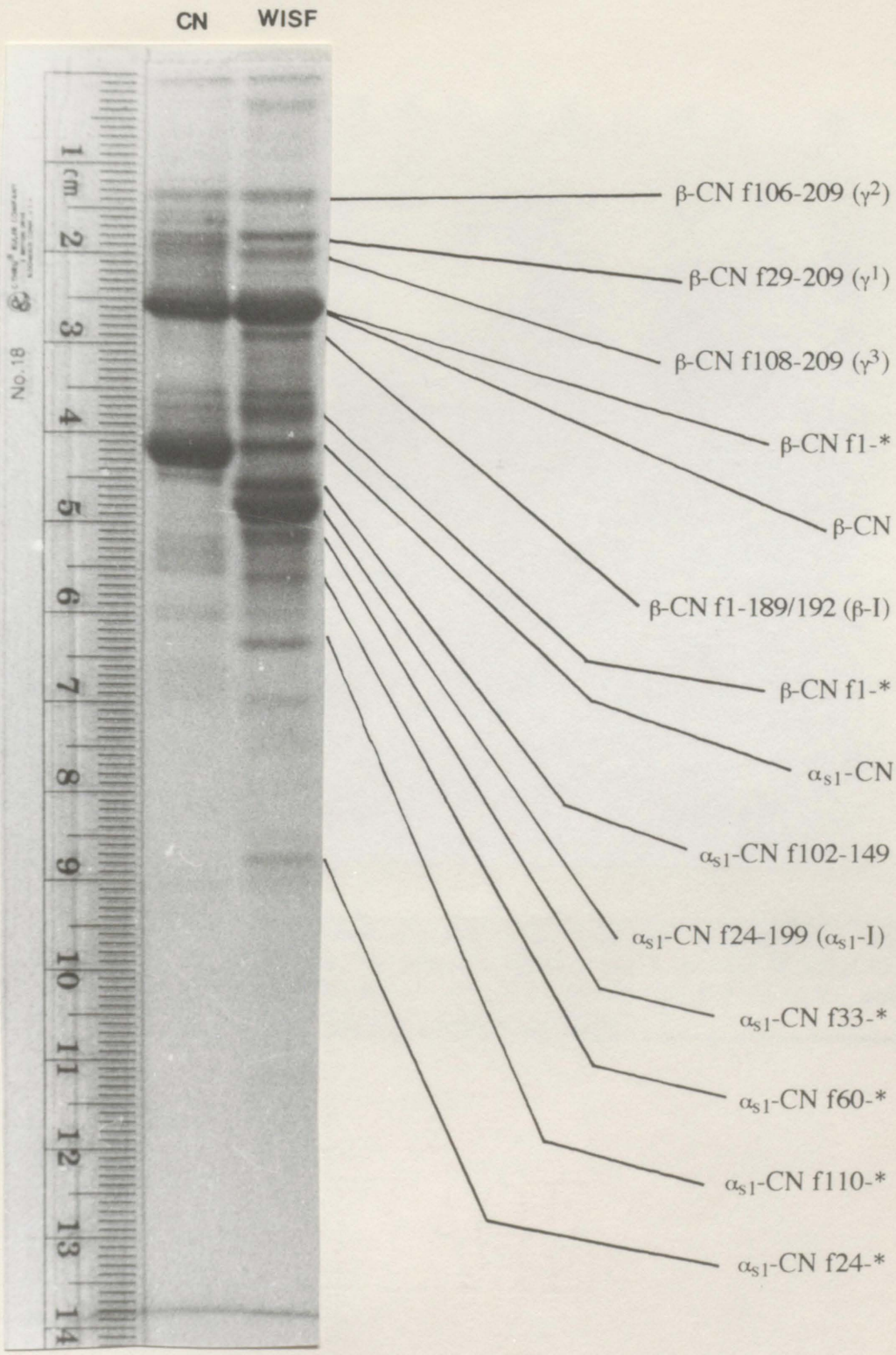


Fig. 9.4: *Urea-polyacrylamide gel electrophoretograms (12% T, 4% C, pH 8.9) of casein (CN) and water-insoluble (WISF) fraction of 3 month-old Cheddar cheese made with single strain of* Lactococcus lactis *subsp.* cremoris *SK 11 indicating the positions of the caseins and the N-terminal of the principal peptides identified in this study. (* Undetermined C-terminus)*

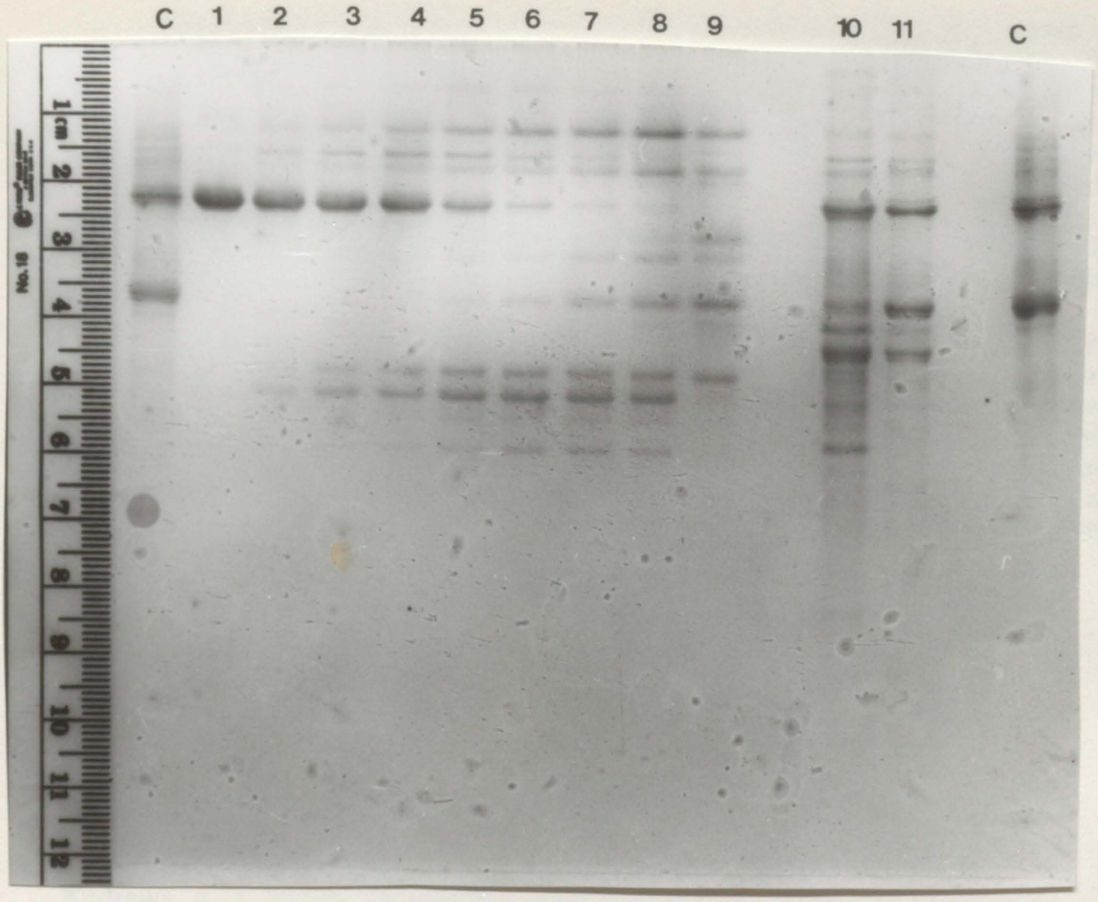


Fig. 9.5: Urea-polyacrylamide gel electrophoretograms of casein (C) and β-casein (2 mg ml^{-1}) hydrolyzed by plasmin (0.02 U ml^{-1}) in 0.05 M NH_4CO_3 buffer, pH 8.4 for 0, 5, 10, 15, 30, 60, 90 and 120 min and 6 h (1 to 9) and Cheddar cheese at 6 months and 45 days of ripening (10 and 11).

Three minor bands in the urea-PAGE electrophoretogram of 3 month-old Cheddar (mobilities 0.35, 3.55 and 7.00 cm, Figure 9.4) remain to be identified and further work is required to establish the C-terminus of the remainder of the peptides isolated in this study. The peptide α_{s1}-CN f24-164, which is produced during the hydrolysis of α_{s1}-casein by chymosin (McSweeney *et al.*, 1993a), was not identified. This peptide may have been produced and subsequently degraded rapidly (e.g. to peptides including α_{s1}-CN f102-149) or it may not be produced at all. The complementrary short peptide (α_{s1}-CN f165-199) has not been identified in cheese although α_{s1}-CN f198-199 was isolated from Alpkäse by Guigoz and Solms (1974).

The results of this study indicate that, in general, the level of proteolysis detectable by urea-PAGE can be explained by the action of chymosin and plasmin. The results are consistant with preliminary findings that indicated that starter cell wall-associated proteinases are not significant in the primary proteolysis of β-casein in Cheddar (McSweeney *et al.*, 1993c). The results also indiciate that the major cleavage sites of chymosin on α_{s1}-casein and of plasmin on β-casein are hydrolyzed in cheese. The results presented permit the identification of the majority of bands detectable in urea-PAGE gels of 3- month-old Cheddar cheese.

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REFERENCES

Andrews, A. T. and E. Alichanidis (1983). Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *Journal of Dairy Research* **50**: 275-290.

Blakesley, R. W. and J. A. Boezi (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry* **82**: 580-581.

Breen, E. D. (1992) *Fractionation of Water-Soluble and Water-Insoluble Cheese Nitrogen.* M.Sc. Thesis, National University of Ireland.

Creamer, L. K. (1974). Preparation of α_{s1}-casein A. *Journal of Dairy Science* **57**: 341-344.

Creamer, L. K. (1991). Electrophoresis of cheese. *IDF Bulletin 261* pp. 14-28.

Eigel, W. N. (1977). Formation of γ_1-A^2, γ_2-A^2 and γ_3-A caseins by in vitro proteolysis of β-casein A^2 with bovine plasmin. *International Journal of Biochemistry* **8**: 187-192.

Eigel, W. N. (1981). Identification of proteose-pepton component 5 as a plasmin-derived fragment of bovine β-casein. *International Journal of Biochemistry* **13**: 1081-1086.

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

Farkye, N. Y. and P. F. Fox (1991). Preliminary study on the contribution of plasmin to proteolysis in Cheddar cheese: cheese containing plasmin inhibitor, 6-aminohexanoic acid. *Journal of Agricultural and Food Chemistry* **39**: 786-788.

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

Fox, P. F., J. Law, P. L. H. McSweeney and J. Wallace (1993). Biochemistry of cheese ripening. In *Cheese: Chemistry, Physics and Microbiology*, 2nd Ed., Vol. 1, P. F. Fox (ed.), Elsevier Applied Science Publishers Ltd., London.

Guigoz, Y. and J. Solms (1974). Isolation of a bitter tasting peptide from Alpkäse, a Swiss mountain-cheese. *Lebensmittelwissenschaft und Technologie* **7**: 356-357.

Grufferty, M. B. and P. F. Fox (1988). Milk alkaline proteinase. *Journal of Dairy Research* **55**: 609-630.

Kosikowski, F. V. (1982). *Cheese and Fermented Milk Foods.*, F. V. Kosikowski and Associates, Brooktondale, NY.

Kuchroo, C. N. and P. F. Fox (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft* **37**: 331-335.

Kuchroo, C. N. and P. F. Fox (1983). A fractionation scheme for the water-soluble nitrogen in Cheddar cheese. *Milchwissenschaft* **38**: 389-391.

Le Bars, D. and J.-C. Gripon (1989). Specificity of plasmin towards bovine α_{s2}-casein. *Journal of Dairy Research* **56**: 817-821.

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

McSweeney, P. L. H., N. F. Olson, P. F. Fox, A. Healy and P. Højrup (1993b). Proteolytic specificity of plasmin on bovine α_{s1}-casein. *Food Biotechnology* (submitted).

McSweeney, P. L. H., Fox, P. F. and Law, J. (1993c). Contribution of cell wall-associated proteinases of *Lactococcus* to the primary proteolysis of β-casein in Cheddar cheese. *Milchwissenschaft* (in press).

McSweeney, P. L. H., Fox, P. F., Lucey, J. A., Jordan, K. N. and Cogan, T. M. (1993d). Contribution of the indigenous microflora to the maturation of Cheddar cheese. *International Dairy Journal* (in press).

Monnet, V., D. Le Bars and J.-C. Gripon (1986). Specificity of a cell-wall proteinase from *Streptococcus lactis* NCDO763 towards bovine β-casein. *Federation of European Microbiology Societies Microbiology Letters* **36**: 127-131.

Monnet, V., J. P. Ley and S. Gonzàlez (1992). Substrate specificity of the cell envelope-located proteinase of *Lactococcus lactis* subsp. *lactis* NCDO 763. *International Journal of Biochemistry* **24**: 707-718.

O'Sullivan, M. and P. F. Fox (1990). A Scheme for the partial fractionation of cheese peptides. *Journal of Dairy Research* **57**: 135-139.

Rank, T. C., R. Grappin and N. F. Olson (1985). Secondary proteolysis of cheese during ripening: a review. *Journal of Dairy Science* **68**:810-805.

Reid, J. R., C. H. Moore, G. G. Midwinter and G. G. Prichard (1991a). Action of a cell wall proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 on bovine α_{s1}-casein. *Applied Microbiology and Biotechnology* **35**: 222-227.

Reid, J. R., K. H. Ng, C. H. Moore, T. Coolbear and G. G. Prichard (1991b). Comparison of bovine β-casein hydrolysis by P_I and P_{III} -type proteinases from *Lactobacillus* [sic] *lactis* subsp. *cremoris*. *Applied Microbiology and Biotechnology* **36**: 344-351.

Singh, T. K., P. F. Fox, P. Højrup and A. Healy (1993). A scheme for the fractionation and identification of small peptides from Cheddar cheese. *International Dairy Journal* (in press).

van den Berg, G. and P. J. de Koning (1990). Gouda cheesemaking with purified calf chymosin and microbially produced chymosin. *Netherlands Milk and Dairy Journal* **44**: 189-205.

Visser, S. and K. J. Slangen (1977). On the specificity of chymosin (rennin) in its action on bovine β-casein. *Netherlands Milk and Dairy Journal* **31**: 16-30.

Visser, S., K. J. Slangen, A. C. Alting and H. J. Vreeman (1989). Specificity of bovine plasmin in its action on bovine α_{s2}-casein. *Milchwissenschaft* **44**: 335-339.

Visser, S., A. J. P. M. Robben and C. Slangen (1991). Specificity of a cell-envelope-located proteinase (P_{III}-Type) from *Lactococcus lactis* subsp. *cremoris* AM_1 in its action on bovine β-casein. *Applied Microbiology and Biotechnology* **35**: 477-483.

10

CONTRIBUTION OF THE INDIGENOUS MICROFLORA TO THE MATURATION OF CHEDDAR CHEESE†

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Cheddar cheeses were made from raw, pasteurized (72°C x 15 s) or microfiltered milk produced from skim milk which had been microfiltered using an Alfa-Laval MFS-1 MF unit and mixed with pasteurized cream (72°C x 30 s). Microfiltration (MF) reduced the total bacterial count (TBC) by >99% and MF cheesemilk had a lower TBC than pasteurized milk; counts of non-starter lactic acid bacteria (NSLAB) were < 1 ml^{-1}. Cheeses were ripened at 10°C.

Commercial graders and a trained taste panel found the pasteurized and MF cheeses to be of high and equivalent sensory quality but the raw milk cheese rapidly developed a strong and atypical flavour and was downgraded. NSLAB counts for the MF, pasteurized and raw milk cheeses were 43, 3.9 x 10^2 and 1.47 x 10^5 cfu g^{-1} after pressing and 9.3 x 10^6, 1.12 x 10^7 and 1.19 x 10^8 cfu g^{-1} after 11 weeks. The cheeses and their water-soluble extracts were indistinguishable by urea-polyacrylamide gel electrophoresis for up to 3 months, but slight differences were apparent at 6 months. Peptide profiles were similar for pasteurized and MF cheeses but different for the raw milk cheese. Water-soluble nitrogen levels in the cheeses were similar although amino acid nitrogen and free glutamic acid were consistently highest in the raw milk cheese. The raw milk cheese exhibited the highest concentration of free fatty acids throughout ripening. Although the non-starter microflora of the raw and pasteurized cheeses consisted predominantly of Lactobacillus *spp., they differed with respect to the species present.*

The results show that MF efficiently reduced the numbers of indigenous microorganisms in milk and cheese. The results also suggest that the indigenous microflora of milk markedly affects the quality of Cheddar cheese made from raw milk.

INTRODUCTION

Cheddar cheese made from raw milk tends to develop a stronger flavour and generally ripens more quickly than cheese made from pasteurized milk (Price and Call, 1969). Changes in cheesemilk that may be caused by pasteurization include denaturation of indigenous enzymes, slight denaturation of whey proteins and their interaction with caseins and destruction of the thermolabile members of the indigenous microflora, including non-starter lactic acid bacteria (NSLAB). It is possible that any of these, and perhaps other, changes influence the ripening process but alteration of the indigenous NSLAB microflora is probably most significant. To date, the exact rôle of NSLAB in cheese maturation and flavour development remains unclear.

As reviewed by Peterson and Marshall (1990), the starter bacteria in Cheddar cheese rapidly exhaust the growth substrate (lactose) and the starter cell numbers decline due to a combination of the absence of lactose, the low pH and the high NaCl

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concentration in the curd. Typically, the starter counts in cheese fall to *ca.* 1% of the maximum levels within 1 month. In contrast, NSLAB can increase from quite low numbers to become the dominant microorganisms in the cheese (e.g. 10^8 cfu g^{-1}). The non-starter flora of cheese is variable but consists mainly of members of the genera *Lactobacillus*, *Pediococcus* and *Micrococcus* (Peterson and Marshall, 1990). The non-starter flora of Irish Cheddar appears to consist almost entirely of *Lactobacillus* species (K. N. Jordan, unpublished results). Since lactobacilli dominate the flora of cheese throughout much of the ripening period, it seems likely that at least some of the many enzymes identified in the NSLAB (Khalid and Marth, 1990a) play a rôle in the biochemistry of cheese ripening.

A number of researchers have found differences between cheeses made from raw and pasteurized milk (Sherwood, 1936; Bullock and Irvine, 1956; Scarpellino and Kosikowski, 1962; Lau *et al.*, 1990; 1991); however the reason(s) for the observed differences are not clear (Lau *et al.*, 1991). Sherwood (1936) treated young cheeses with chloroform in an attempt to inhibit the growth of NSLAB and concluded that the differences observed between the raw and pasteurized milk cheeses were due largely to heat-induced chemical changes in milk constituents. In a more recent study, Lau *et al.* (1991) compared cheeses made from raw and pasteurized milk using modern analytical techniques. Both the extent and characteristics of proteolysis were altered by pasteurization and the authors suggest that this could be a result of heat-induced whey protein-casein interactions which reduced the accessibility of the caseins to enzyme action since pasteurization may cause a significant increase in the amount of whey protein retained in the cheese (Lau *et al.*, 1990). However, Lau *et al.* (1991), who did not study the microbiological conditions of milk or cheeses, did not preclude the possibility that the observed differences were a consequence of some inactivation of milk proteinases or NSLAB. They commented that their experimental procedure was not designed to establish the rôle of such proteinases or NSLAB in proteolysis.

Microfiltration (MF) is a membrane separation technique developed for use in the food and biotechnology industries. The membranes retain bacteria but allow casein micelles to pass through. Therefore, microfiltration offers a very efficient technique for removing the microorganisms from milk (Maubois, 1989). It can be used on a large scale to produce essentially sterile milk and provides a simpler approach than aseptic milking and pasteurization (Fox, 1989) to prepare cheese milk free from NSLAB without concomitant heat-induced changes, in order to study the contribution of NSLAB to cheese ripening.

The objectives of the present study were to compare the ripening characteristics of Cheddar cheeses made from raw, pasteurized or microfiltrated milks in order to assess the significance of the NSLAB in cheese ripening without concomitant heat-induced changes.

MATERIALS AND METHODS

Cheese Manufacture: Cheddar cheeses were manufactured in 450 L vats according to a standard protocol from raw, pasteurized (72°C x 15 s) or microfiltered milk at the National Dairy Products Research Centre, Moorepark, Co. Cork, Ireland. The microfiltered cheese milk was produced by recombining raw skim milk which had been microfiltered using an Alfa-Laval MFS-1 cross-flow microfiltration unit (Alfa-Laval Filtration Systems, Lund, Sweden, with ceramic membranes, pore size: 1.4 μm; flow rate 100 l h^{-1} at 0.3 bar giving a flux rate of 500 l m^{-2} h^{-1}) and pasteurized cream (72°C x 30 s). Cream and microfiltered skim were recombined to give the same fat percentage as the raw milk. Cheeses were ripened at 10°C. Composition of the cheeses was determined in accordance with standard methods.

Cheesemaking was performed in triplicate. The cheeses from only one experiment were analysed in detail and the results presented here.

Microbiological Analyses: The efficiency of the MF process for removal of bacteria from the milk was assessed by enumerating coliform (Violet Red Bile Agar, Oxoid, Basingstoke, UK), total bacteria (Tryptone-Glucose-Yeast Extract Agar, Oxoid, Basingstoke, UK) and NSLAB (*Lactobacillus* Selection Agar, LBS, Baltimore Biological Laboratories, Rockville, MD, USA) in the raw, pasteurized and MF milks.

During ripening, counts of coliform, starter (LM 17 agar, Terzaghi and Sandine, 1975) and NSLAB were determined in duplicate after the cheeses were removed from the press and after 6, 11,16 and 27 weeks of aging.

Fifty colonies each were selected at random from the LBS agar plates prepared from the 16 week old raw and pasteurized cheeses, incubated aerobically and cultured in de Man-Rogosa-Sharpe (MRS) broth (deMan *et al.*, 1960). Pure isolates were obtained by streaking twice on MRS agar and the pure strains then transferred into MRS broth. The broth cultures were observed microscopically and the cells separated centrifugally from 1 ml aliquots and frozen in MRS broth, containing 25% glycerol.

Stock cultures were prepared by culturing the frozen strains overnight in MRS broth and classified by the following characteristics:- colony morphology, thermophilic growth, utilization of mannitol, raffinose, lactose and citrate, growth in litmus milk, absence of catalase, end products of metabolism and the isomer of lactate produced. The strains were provisionally identified according to the schemes of Sharpe (1981) and Kandler and Weiss (1984).

Assessment of Proteolysis: Water-soluble extracts of the cheeses were prepared according to the method of Kuchroo and Fox (1982). Water-soluble nitrogen (WSN) was determined by the macro-Kjeldahl method. The liberation of free amino acids was determined in duplicate on the water extract by the Cd-ninhydrin method of Folkertsma and Fox (1992) and free glutamic acid was assayed using an enzyme assay kit with glutamate dehydrogenase and pig-heart diaphorase (Boehringer Mannheim, Mannheim, Germany). Aliquots of the water extracts were freeze dried for analysis by Reverse Phase HPLC (RP-HPLC) and electrophoresis.

Urea-polyacrylamide gel electrophoresis (PAGE) was performed on the cheeses and water-soluble extracts using the stacking gel system of Andrews (1983); the gels were stained using a modification of the method of Blakesley and Boezi (1977) with PAGE Blue G-90.

RP-HPLC was performed using an LC-9A solvent delivery system with FCV-9AL flow control valve and DGU-2A degassing unit (Shimadzu Corp., Kyoto, Japan) and a Rheodyne model 7125 syringe-loading sample injector (Rheodyne Inc., Cotati, CA, USA) fitted with a 20 µl sample loop. Ultrasphere-ODS guard (4.6 mm x 4.5 cm) and analytical (4.6 mm x 25 cm) columns (Beckman Instruments Inc., San Ramon, CA., USA) were used and detection was by means of an LC-75 spectrophotometric detector (Perkin Elmer, Norwalk, CT., USA) at 230 nm, interfaced with a C-R6A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan).

Chromatographic conditions were as follows :-

Solvent A: 0.1% trifluoroacetic acid (v/v, TFA, sequential grade, Sigma, St. Louis, MO, USA) in H_2O.

Solvent B: 0.1 % TFA (v/v) in CH_3CN/H_2O (50:50, v/v, HPLC grade acetonitrile: Rathburn Chemicals Ltd., Walkerburn, Scotland).

Samples (4 mg ml^{-1}) were dissolved in solvent A and filtered through a 0.45 µm cellulose acetate filter (Sartorius GmbH, Gottingen, Germany). Filtrate (20 µl) was applied to the column and eluted at a flow rate of 1 ml min^{-1} at 100% A for 5 min. A gradient of 0-100% B was then commenced (3.3% B min^{-1}) and the column was finally eluted with 100% solvent B for 5 min.

Table 10.1:	Bacterial Populations (cfu ml^{-1}) in Microfiltered (MF), Raw and Pasteurized Cheese Milks.[a]		
Milk	TBC[b]	Coliform	NSLAB[c]
MF skim and cream[d]	630	1	<1
Raw milk	63,000	320	191
Pasteurized milk	840	<1	<1

a : mean of duplicate analyses
b : Total bacterial counts
c: Non-starter lactic acid bacteria count
d: Total bacterial counts for skim milk exiting the MF unit were 16 cfu ml^{-1} and had risen to 380 cfu ml^{-1} before cream was added.

Assessment of Lipolysis: Total free fatty acids were determined by the Cu-soaps method of Bynum *et al.* (1984). Free fatty acid profiles of the cheese were determined by methylating diethyl ether extracts with tetra methyl ammonium hydroxide followed by GC analysis, as described by Martinez-Castro *et al.* (1986). The fatty acid methyl esters were resolved by a Pye Unicam PU 4500 gas chromatograph (Pye Unicam Ltd., Cambridge, England), containing a 10% Silar 10-C column (210 cm), interfaced with a Shimadzu CR3A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan).

Sensory Assessment: During ripening, cheeses were graded by a trained taste panel consisting of 9 members at the National Dairy Products Research Centre on a 0-8 (0=poorest: 8=best) scale considering 4 attributes (appearance; flavour and aroma; body; and overall quality). Cheeses were also assessed by two graders from the Irish Department of Agriculture and Food, using the same scale.

RESULTS

Microbiological Analyses

Milk: Total bacterial, coliform and NSLAB counts for the milks are summarized in Table 10.1. MF reduced the indigenous microflora with a very high efficiency (> 99.9%), producing an almost sterile milk. Although less so than MF, the efficiency of pasteurization was also quite high. Both MF and pasteurized milk showed no lactobacilli in 1 ml test samples.

Cheese: The composition of the cheeses (Table 10.2) was within the range typical for Cheddar.

The microbiological quality of the cheese during ripening is shown in Fig. 10.1. In agreement with earlier studies (Martley and Lawrence, 1972; Visser, 1977), starter counts decreased during ripening, by 1-2 log cycles in 27 weeks (Fig 10.1a) . Coliforms were virtually absent from the MF and pasteurized cheesemilk (Table 10.1); however, the cheeses made from these milks contained significant numbers of coliform bacteria, which decreased by 2 log cycles in the first 10 weeks of ripening (Fig 10.1c). The raw milk cheese contained substantial numbers of coliforms at day 1, but these also declined during ripening. The numbers of NSLAB were low in the MF and pasteurized milk cheeses ex-press but their

Table 10.2: Composition[a] of Cheddar Cheeses made from Microfiltered, Raw and Pasteurized Milk.

Milk	pH	NaCl	Moisture	Fat	Protein	Ash
		%				
MF	5.12	1.78	35.97	33.0	23.80	3.73
Raw	5.14	1.74	37.79	30.5	24.69	3.74
Pasteurized	5.10	1.68	37.63	31.0	24.25	3.69

[a] Mean of Duplicates

Table 10.3: Classification of Lactobacilli Isolated from Raw and Pasteurized Milk Cheeses[a].

Strain	% Isolates	
	Raw	Pasteurized
L. plantarum	8	0
L. casei ssp. *pseudoplantarum*	30	8
L. casei spp. *casei*	38	88
L. curvatus	16	0
others (incl. no identification)	8	4

[a] 50 isolates each were selected at random from LBS agar plates of 16 week-old raw or pasteurized milk cheeses.

number increased markedly during ripening (Fig 10.1b), with higher counts being consistantly obtained in the raw milk cheese. Presumably, this is a reflection of higher numbers of lactobacilli in the raw milk. All of the NSLAB were identified as lactobacilli (Table 10.3). More species were found in the raw milk cheese than in the pasteurized milk cheese. Care must be taken in interpreting the data on starter counts (Fig. 10.1a), since NSLAB will also grow on the M17 medium used to count them and, if present in sufficiently large numbers, result in overestimations. In the case of the MF and pasteurized milk cheese, this was not a problem since the counts of starter bacteria were $>10^8$ g^{-1} in most cases while the NSLAB were $<10^7$ g^{-1}. However, in the case of the raw milk cheese, counts of NSLAB and starter bacteria reached 10^8 g^{-1} within 11 weeks. This suggests that the counts of starter bacteria in this cheese may be overestimated.

One hundred isolates (50 from the raw milk cheese and 50 from the pasteurized cheese) were selected from the LBS agar plates used to count the NSLAB at 16 weeks. All the isolates were *Lactobacillus* but the species found in the raw milk cheese differed considerably from those in the pasteurized milk cheese (Table 10.3). *L. casei* ssp. *casei* was the dominant species in the pasteurized cheese but the microflora of the raw milk cheese was more heterogeneous. Presumably, the lactobacilli in the pasteurized cheese originated from the cheesemaking equipment, environment, personnel, etc., whereas the lactobacilli in the raw milk cheese originated from these sources as well as from the milk.

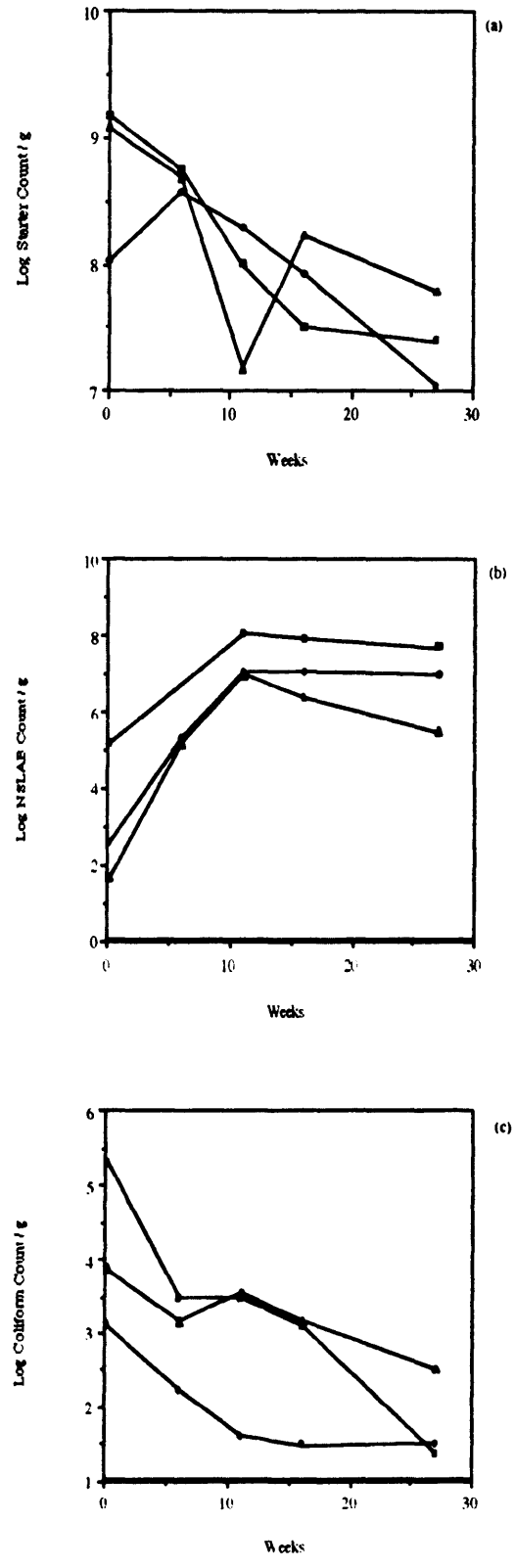

Fig. 10.1: *Counts of (a) starter, (b) NSLAB and (c) coliform bacteria in cheese made from raw (■), pasteurized (●) or microfiltered (▲) milk during ripening. Note different scales on each ordinate.*

Sensory Assessment: The scores for appearance, aroma and flavour, body and overall acceptability are summarized in Table 10.4 for each of the 3 cheese which were assessed by the two groups of judges; a 9-point scale (0=poorest: 8=best) was used for each attribute. The sensory quality of the cheeses was not consistent between experiments but the ranking was similar, i.e. the cheeses made from pasteurized and microfiltered milk were generally similar and different from that of the raw milk cheese, which was always the most strongly flavoured and was always regarded as atypical. The MF and pasteurized milk cheeses were considered to be of high quality and received generally equal grades from both sets of graders. Department of Agriculture and Food graders awarded somewhat higher grades to both cheeses than did the taste panel. The raw milk cheese was downgraded by both groups of graders, mainly on the basis of flavour and aroma which were considered atypical of Cheddar cheese currently available on the Irish market. The authors, who were not members of the taste panel, agreed that the raw milk Cheddar was atypical; however, they considered this intensly flavoured cheese may be attractive to connoisseurs of traditional Cheddar cheese.

Assessment of Proteolysis: Urea-PAGE electrophoretograms of the three cheeses were similar

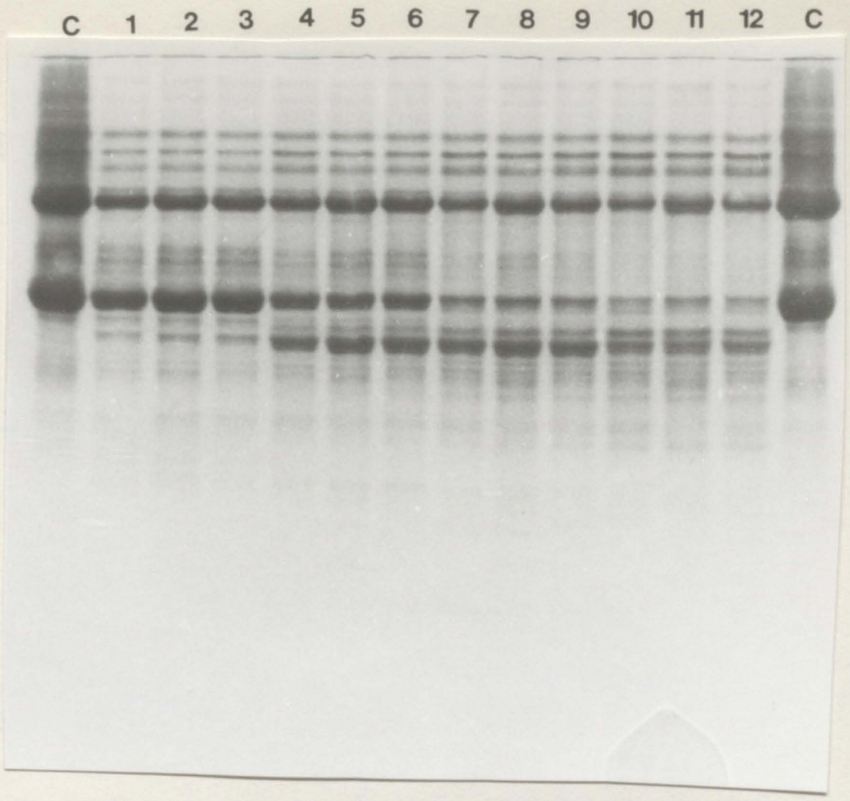

Fig. 10.2: *Urea-polyacrylamide gel electrophoretograms of Na-caseinate (C) and Cheddar cheeses made from MF, raw or pasteurized milks at 2 days (1, 2, 3), 2 weeks (4, 5, 6), 3 months (7, 8, 9) and 6 months (10, 11, 12).*

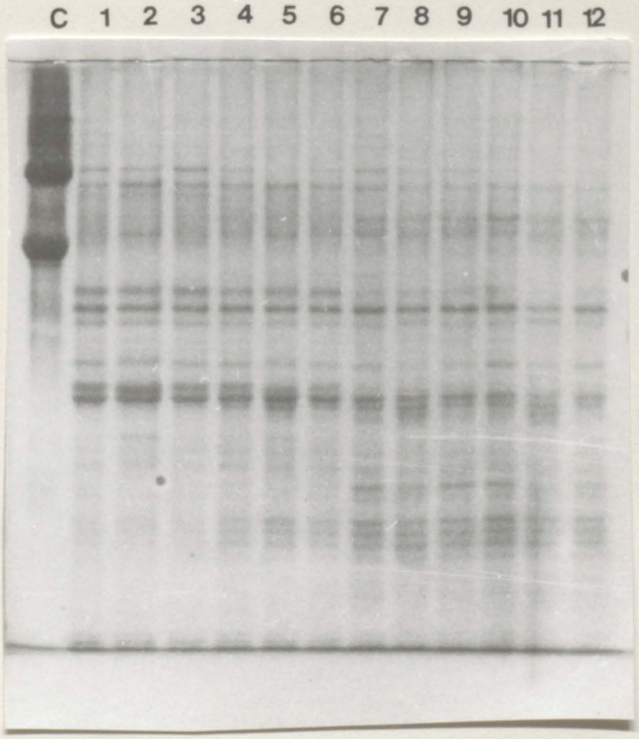

Fig. 10.3: *Urea-polyacrylamide gel electrophoretograms of Na-caseinate (C) and water-soluble extracts of Cheddar cheeses made from MF, raw and pasteurized milks at 2 days (1, 2, 3), 2 weeks (4, 5, 6), 3 months (7, 8, 9) and 6 months (10, 11, 12).*

Table 10.4: Grades for Quality of Cheddar Cheeses made from Microfiltered, Raw or Pasteurized Milks.

Attribute	Cheese: MF 1	MF 2	MF 3	Raw 1	Raw 2	Raw 3	Pasteurized 1	Pasteurized 2	Pasteurized 3
Appearance	5.0	1.27	**6.0**	5.1	1.32	**5.5**	5.9	1.16	**6.0**
Flavour & Aroma	4.8	1.12	**7.0**	3.7	1.82	**4.0**	4.9	1.50	**7.0**
Body	4.6	1.05	**4.0**	4.6	1.51	**4.0**	5.0	1.32	**4.0**
Overall Quality	4.7	1.0	**6.0**	4.1	1.54	**4.25**	4.0	1.05	**6.0**

1.=Mean and 2.= ± standard deviation of scores by a 9 member taste panel at 3 months (0=poorest; 8=best).
3.=Average of two Irish Department of Agriculture and Food graders at 4 months.

at each sampling time (Fig. 10.2). However, differences were apparent in urea-PAGE of WSN of the raw milk cheese at 6 months (Fig. 10.3), although the levels of WSN were essentially the same in all cheeses (Fig. 10.4).

Analysis of the water soluble extracts of the cheeses by the Cd-ninhydrin method (which is highly sensitive for free amino acids), showed that the raw milk cheese contained significantly higher concentrations of free amino acids than the MF and pasteurized milk cheeses throughout ripening (Fig. 10.5). The concentration of free Glu in the raw milk cheese was also higher than that in the other cheeses (Fig. 10.6). These results suggest a greater depth of proteolysis in the raw milk cheese, resulting from greater peptidase activity, presumably originating from the NSLAB.

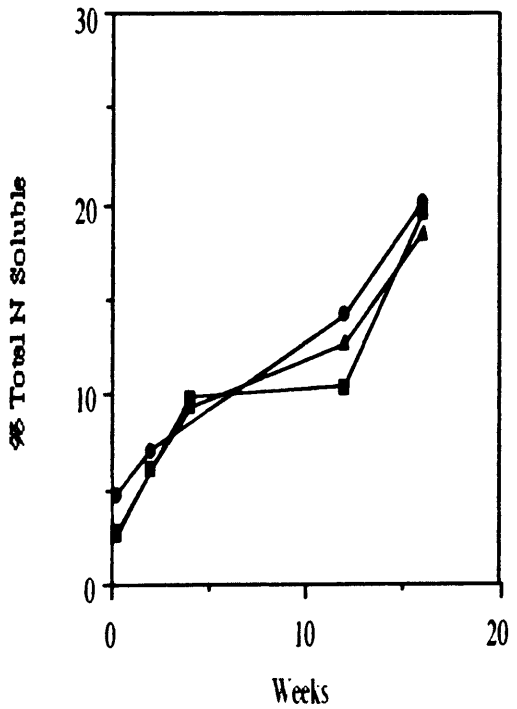

Fig. 10.4: Formation of water-soluble N in Cheddar cheeses made from raw (■), pasteurized (●) or microfiltered (▲) milk analysed in duplicate by the macro-Kjeldahl method.

R P - H P L C elution profiles of water-soluble extracts of the cheeses revealed considerable differences. Profiles of the cheeses at 2 days were similar with only

minor differences visible (Fig. 10.7 a, b, c). In each case, three major peaks (with elution times of *ca.* 3, 4.5 and 15 min) and minor peaks at *ca.* 7 min and in the region 18-28 min were present. Much of the peptide material was eluted in the hydrophobic region (30-40 min).

At 3 months, the RP-HPLC profiles of WSN from the MF and pasteurized cheeses were essentially similar but that from the raw milk cheese was quite different, both qualitatively and quantitatively (Fig. 10.7 d, e, f). Qualitatively, the principal differences were in the peptides that eluted between 24 and 28 min and to a lesser extent between 32 and 36 min (i.e. hydrophobic peptides). The MF and pasteurized milk cheeses contained considerably higher concentrations of peptides eluting in both of these regions than the raw milk cheese and different peptides were also present- at least the relative proportions of the peptides present were markedly different. The raw milk cheese contained higher concentrations of the peptides with elution times of 16, 22 (2 peaks) and 24 min.

The elution profiles at 6 months (Fig. 10.7 h, i, j) confirmed that the MF and pasteurized cheeses were similar (although quantitative differences are apparent in the peak eluting at 14 min) and that the profile of the raw milk cheese was considerably different from the others. Differences were apparent throughout the region 16-32 min and there were quantitative differences in the relative proportions of the peptide eluting at 14 min.

Assessment of Lipolysis: Total lipolysis (assessed by the Cu-soaps procedure) was greatest in the raw milk cheese at all stages throughout ripening (Fig. 10.8); at 6 months, the concentration of the free fatty acids in the raw milk cheese was nearly twice that in the other cheeses. The MF cheese showed greater lipolysis than the pasteurized milk cheese, but a greater rate of increase in free fatty acid concentrations was apparent in the pasteurized milk cheese and both cheeses were equivalent at 6 months.

The profiles of individual saturated free fatty acids in the 6 month old cheeses are shown in Fig. 10.9. The raw milk cheese contained the highest concentration of all free fatty acids. Caprylic ($C_{8:0}$) and caproic ($C_{6:0}$) were the dominant free fatty acids found.

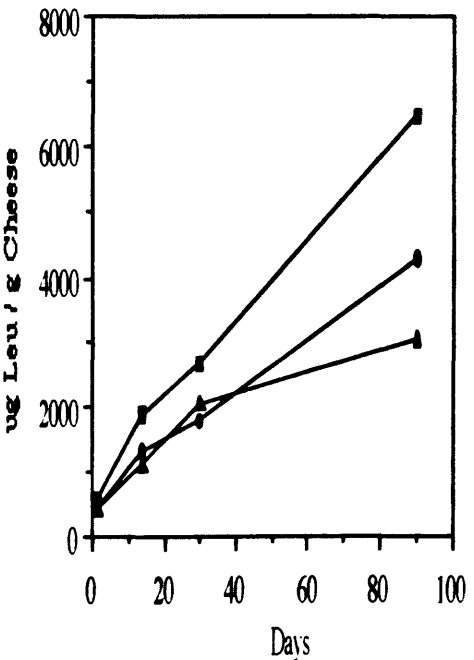

Fig. 10.5: Formation of total free amino acids in Cheddar cheeses made from raw (■), pasteurized (●) or microfiltered (▲) milk (as determined by the Cd-ninhydrin assay). Values are means of duplicate analysis

DISCUSSION

The results of this study demonstrate considerable differences between cheese made from raw milk and those made from pasteurized or microfiltered milk. The

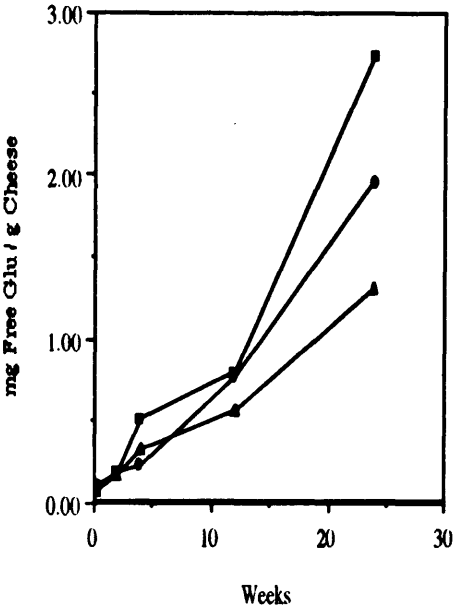

Fig. 10.6: Formation of free glutamic acid in Cheddar cheese made from raw (■), pasteurized (●) or microfiltered(▲) milk. Values are means of duplicate analyses.

pasteurized and microfiltered cheeses were found to be very similar in terms of sensory quality, peptide profiles by RP-HPLC and urea-PAGE, amino acid N, free Glu, water soluble N and free fatty acids.

Microfiltration reduced the indigenous microflora of the cheesemilk to very low levels without heat treatment. The MF cheese was similar in all respects to the cheese made from pasteurized milk; the differences observed between these cheeses and the cheese made from raw milk are presumably due to the NSLAB, which were consistently higher in the raw milk cheese. This suggests that the non-starter microflora has a significant effect on proteolysis, lipolysis and overall quality of Cheddar cheese made from raw milk. This conclusion is at variance with that of Sherwood (1936). A number of authors have used lactobacilli as adjunct cultures to the starter *Lactococcus* ; generally, a positive effect of lactobacilli on flavour was obtained although some authors found very little improvement (see Peterson and Marshall, 1990).

The raw milk cheese matured more quickly (as determined by the formation of total free amino acids, Glu and fatty acids) and rapidly developed a strong flavour. The finding that the raw milk cheese ripened more quickly agrees with those of Price and Call (1969) and Lau *et al.* (1991) and the accelerated formation of free amino acids agrees with the results of Bullock and Irvine (1956). Slight differences in the peptide profiles of the cheeses were indicated by urea-PAGE while RP-HPLC showed considerable differences between the peptide profiles of the pasteurized and MF cheeses, which were almost identical, and those of the raw milk cheese. Only the larger peptides in the water extract of Cheddar stain under the electrophoretic conditions used (O'Sullivan and Fox, 1990). Hence, the principal difference between the cheeses was in respect to small peptides (detectable by RP-HPLC but not by PAGE) and free amino acids, indicating a higher extent of proteolysis in the raw milk cheese, presumably due to proteinases and peptidases from NSLAB.

Lactobacillus spp. dominated the non-starter flora of the cheeses and the strains found in the raw milk cheese were much more heterogeneous than those present in the pasteurized milk cheese. The flora of the raw milk cheese contained *L. casei* ssp. *casei*, *L. casei* ssp. *pseudoplantarum* and *L. curvatus* while the pasteurized milk cheese contained mainly *L. casei* ssp. *casei*. A variety of proteolytic and peptidolytic enzymes have been identified in the NSLAB isolated from cheese (Khalid and Marth, 1990a, 1990b; Peterson *et al.*, 1990) and the differences in proteolysis observed in this study presumably reflect differences in the microflora, both quantitative and qualitative. It is likely that the lactobacilli present in the pasteurized milk cheese were the result of post-pasteurization contamination (see Peterson and Marshall, 1990). The coliform levels in the MF and pasteurized cheeses were rather high initially but all counts, including the raw cheese, decreased

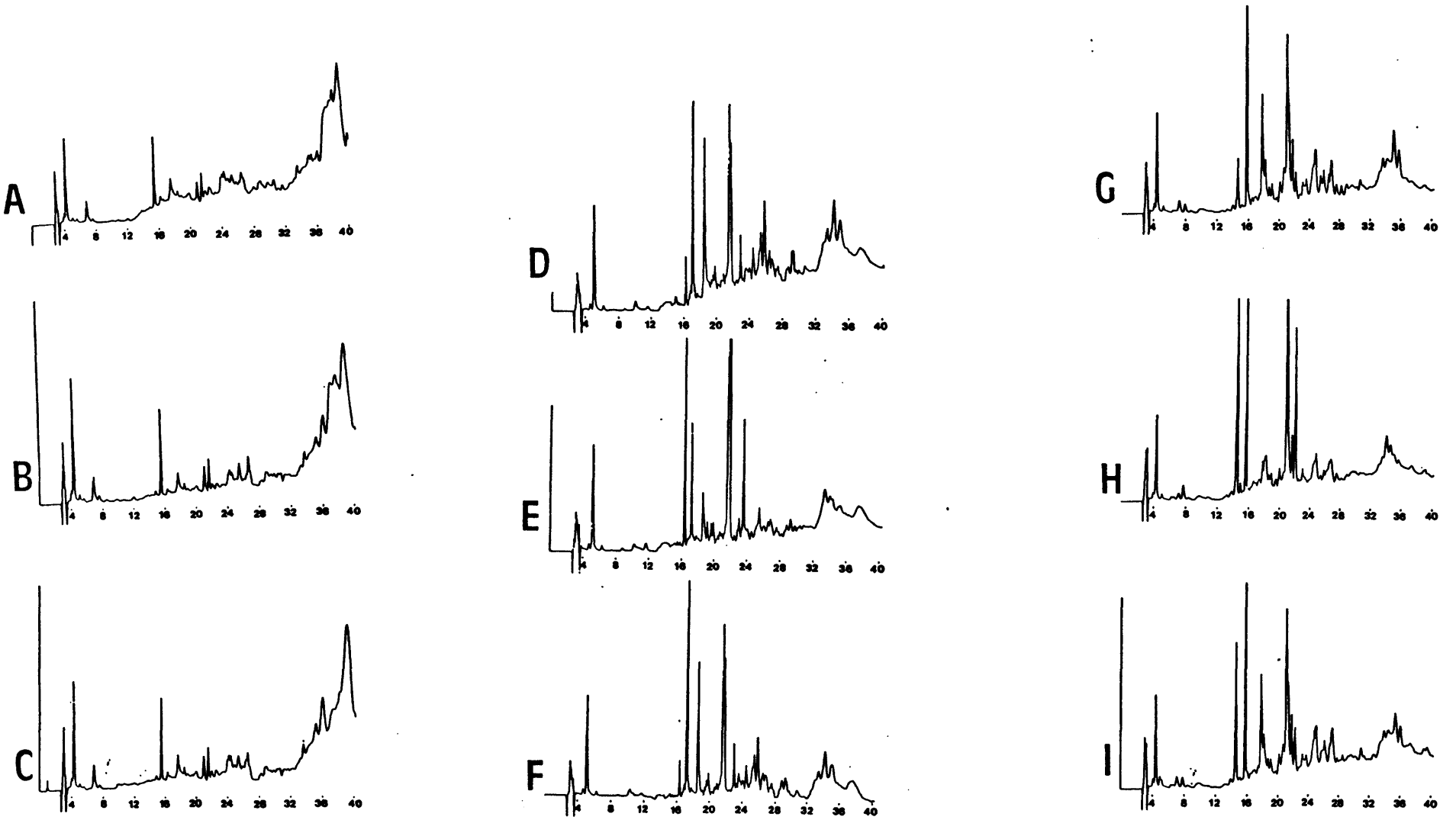

Fig. 10.7: Reversed-phase HPLC peptide profiles of water-soluble extracts from Cheddar cheeses made from microfiltered, raw or pasteurized milks at 2 days (A, B, C), 3 months (D, E, F) and 6 months (G, H, I).

during ripening. It is unlikely that levels of coliform of 10^4/g had any effect on proteolysis

The MF cheese was manufactured from milk prepared by recombining raw skim and pasteurized cream to give a fat percentage similar to that of the raw milk.

Many of the indigenous enzymes in milk are concentrated on the fat globule membrane (Kitchen, 1985) and some of these would have been inactivated or attenuated by pasteurization. It is thus possible that indigenous enzymes in the cream phase could have been responsible for the differences observed. However, preliminary results of a further study involving the manufacture of Cheddar from raw skim milk and pasteurized cream show that little differences in flavour are detectable between this cheese and raw milk Cheddar, indicating that the fat globule enzymes may not have been responsible for the flavour differences observed between the raw and pasteurized milk cheeses found in this study.

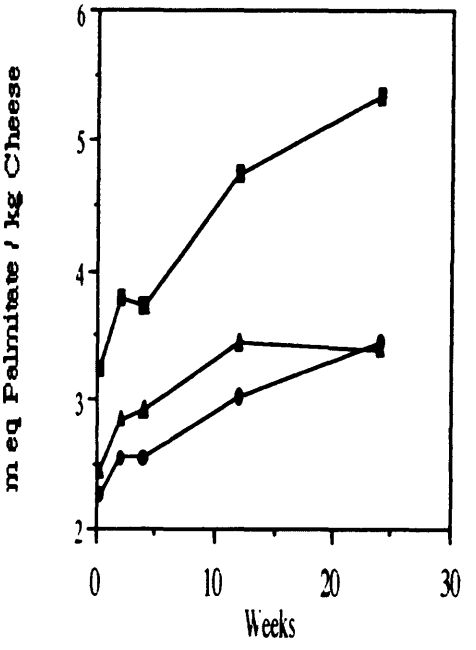

Fig. 10.8: Liberation of free fatty acids in Cheddar cheese made from raw (■), pasteurized (●) or microfiltered (▲) milk as determined by the Cu-soaps method.

CONCLUSIONS

The importance of the non-starter microflora to proteolysis, lipolysis and flavour development was demonstrated in this study. Differences observed between the raw and pasteurized milk cheeses was attributed to the destruction of the non-starter microflora rather than heat-induced changes in indigenous enzymes. Differences in proteolysis observed were due to peptidases of the non-starter lactobacilli. The microflora of the raw and pasteurized milk cheeses differed but all strains identified were lactobacilli. Presumably the lactobacilli in the pasteurized milk cheese resulted from post-process contamination while those in the raw milk cheese were from the milk as well as cheesemaking equipment. Cheddar cheeses made from pasteurized and microfiltered milks were similar in all respects studied.

ACKNOWLEDGEMENTS

The authors wish to thank Mr Finbarr Drinan and Mr Eddie Mulholland for technical assistance.

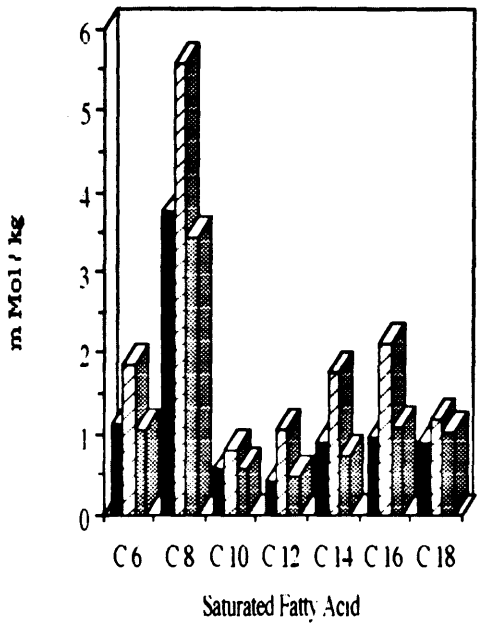

Fig. 10.9: Partial free fatty acid profile of Cheddar cheeses made from raw (▨), pasteurized (▩) or microfiltered (■) milk.

REFERENCES

Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* **50**, 45-55.

Blakesley, R. W. & Boezi, J. A. (1987). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Anal. Biochem.* **82**, 580-581.

Bullock, D. H. & Irvine, O. R. (1956). A chromatographic study of Cheddar cheese ripening. *J. Dairy Sci.* **39**, 1229-1235.

Bynum, D. G., Senyk, G. F. & Barbano, D. M. (1984). Determination of free fatty acid content of Cheddar cheese by a copper soaps method. *J. Dairy Sci.* **67**, 1521-1524.

deMan, J.C.,Rogosa, M. & Sharpe, M.E. (1960). A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23**, 130-135.

Folkertsma, B. & Fox, P. F. (1992). Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. *J. Dairy Res.* **59**, 217-224.

Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *J. Dairy Sci.* **72**, 1379-1400.

Kandler, O. & Weiss, N. (1984). Regular, non-sporing Gram-positive rods. In *Bergey's Manual of Systematic Bacteriology*, 1st ed., Vol 2, eds. P. H. A. Sneath, N. S. Mair, M. E. Sharpe, J. G. Holt, Williams & Williams, Baltimore, MA, USA. pp 1208-1243.

Khalid, N. M. & Marth, E. H. (1990a). Lactobacilli- their enzymes and role in ripening and spoilage of cheese: a review. *J. Dairy Sci.* **73**, 2669-2684.

Khalid, N. M. & Marth, E. H. (1990b). Proteolytic activity by strains of *Lactobacillus plantrum* and *Lactobacillus casei*. *J. Dairy Sci.* **73**, 3068-3076.

Kitchen, B. J. (1985). Indigenous milk enzymes. In *Developments in Dairy Chemistry-3*, ed. P. F. Fox, Elsevier Applied Science Publishers, Barking, pp. 239-279.

Kuchroo, C. N. & Fox, P. F. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft* **37**, 331-335.

Lau, K. Y., Barbano, D. M. & Rasmussen, R. R. (1990). Influence of pasteurization on fat and nitrogen recoveries and Cheddar cheese yield. *J. Dairy Sci.* **73**, 561-570.

Lau, K. Y., Barbano, D. M. & Rasmussen, R. R. (1991). Influence of pasteurization of milk on protein breakdown in Cheddar cheese during ripening. *J. Dairy Sci.* **74**, 727-740.

Martley, F. G. & Lawrence, R. C. (1972). Cheddar cheese flavour. II: characteristics of single strain starters associated with good or poor flavour development. *N. Z. J. Dairy Sci. Technol.* **7**, 38-50.

Martinez-Castro, I., Alanso, L. & Juarez, M. (1986). Gas chromatographic analysis of free fatty acids and glycerides of milk fat using tetramethylammonium hydroxide as catalyst. *Chromatographia* **21**, 37-40.

Maubois, J.-L. (1989). Applications of membrane techniques in the dairy industry-proposals for a new group of IDF experts. *Bulletin 244*. pp. 26-29; International Dairy Federation, Brussels.

O'Sullivan, M. & Fox, P. F. (1990). A scheme for the partial fractionation of cheese peptides. *J. Dairy Res.* **57**: 135-139.

Peterson, S. D. & Marshall, R. T. (1990). Nonstarter lactobacilli in Cheddar cheese: a review. *J. Dairy Sci.* **73**, 1395-1410.

Peterson, S. D., Marshall, R. T. & Heymann, H. (1990). Peptidase profiling of lactobacilli associated with Cheddar cheese and its application to identification and selection of strains for cheese-ripening studies. *J. Dairy Sci.* **73**, 1454-1464.

Price, W. V. & Call, A. O. (1969). Cheddar cheese: comparison of effects of raw and heated milk on quality and ripening. *J. Milk Food Technol.* **32**, 304-311.

Scarpellino, R. & Kosikowski, F. V. (1962). Evolution of volatile compounds in ripening raw and pasteurized milk Cheddar cheese observed by gas chromatography. *J. Dairy Sci.* **45**, 343-348.

Sharpe, M. A. (1981). The genus Lactobacillus. In *The Prokaryotes, a Handbook on Habitats, Isolation and Identification of Bacteria*. Vol 2. eds. M. P. Starr, H. Stolp, H. G. Truper, A. Balows, H. G. Schlegel. Springer-Verlag, Berlin and Heidelberg. pp 1653-1679.

Sherwood, I. R. (1936). Observations on the ripening of cheeses made from raw and pasteurized milk. *J. Dairy Res.* **7**, 271-283.

Terzaghi, B.E.& Sandine, W.E. (1975). Improved media for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29**, 807-813.

Visser, F. M. W. (1977). Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 1. Description of cheese and aseptic cheesemaking techniques. *Neth. Milk Dairy J.* **31**, 120-133.

11

GENERAL DISCUSSION

As discussed in Chapter 2, proteolysis is a major biochemical event during the ripening of Cheddar cheese. The initial stages of proteolysis are of particular importance in the development of cheese texture and the production of small peptides. These peptides are subsequently degraded further to smaller peptides, free amino acids and products of amino acid catabolism, many of which may contribute to cheese flavour.

Proteolysis in cheese during ripening has been studied for many years, although much of the early research was done using rather non-specific methods to assess proteolysis (see Chapter 3). Methods included measurement of the rate of cleavage of peptide bonds by reaction of liberarated amino groups with colorimetric or flurometric reagents, measurement of the rate of apperence of peptides soluble in various reagents or peptide profiling by electrophoresis or chromatography. These studies provided important information about the relative importance of proteolytic agents and the rates of proteolysis at various levels. However, few peptides have been identified in cheese and thus relatively little is known about which peptide bonds are cleaved during ripening.

During cheese ripening, caseins (M_r ~20-25 kDa) are hydolyzed to a wide variety of peptides, ranging in size from values similar to the intact caseins to free amino acids. Consequently, the direct isolation of individual peptides is rarely possible and some form of fractionation is necessary before peptides can be isolated and identified.

Identification of peptides from cheese permits their location in the casein sequence to be established and the peptide bonds cleaved during cheese ripening to be determined. However, this information is of little benefit as it does not permit identification of which proteolytic agent is responsible for peptide bond cleavage, thus limiting the posibility of controlling proteolysis. Studies on the specificity of the proteinases and peptidases involved in cheese ripening on casein substrates complement those involving the isolation and identification of cheese peptides, since, if the specificity of proteinases on the caseins is known, it is possible to identify the agent(s) responsible for the production of peptides. However, the specificity of proteinases in model systems is of little benefit *per se* as it is not known to what extent the results of such studies can be extrapolated to cheese, in which the environment may be very different

Both approaches were used in this study. The results described in Chapters 4 to 7 involved determination of the specificity on casein substrates of proteinases (chymosin, plasmin and cathepsin D) which are thought to be important in the initial stages of proteolysis during cheese ripening. Attempts were made in Chapters 8 and 9 to establish to what extent the results of these (and other) studies can be extrapolated to Cheddar cheese. The rôle of the non-starter lactic acid bacteria (NSLAB) in Cheddar cheese ripening was assessed in Chapter 10.

Chymosin is the principal active constituent in rennet preparations used in the manufacture of Cheddar cheese. Although only ~6% of the chymosin activity added to milk is retained in the cheese curd, chymosin is considered to be the agent responsible for much of the initial proteolysis in Cheddar cheese (see Chapter 2). The optimum pH of chymosin action is closer to the pH for cheese than that of the principal indigenous proteinase, plasmin.

The bond-type in α_{s1}- and α_{s2}-caseins found to be susceptible to chymosin (Chapetrs 4 and 5) was in good agreement with the specificity of chymosin on β- and κ-caseins (Visser and Slangen, 1977; Vreeman *et al.*, 1986) and the B-chain of insulin (Fish, 1957). Cleavage sites generally contained hydrophobic or aromatic

residues at the P_1 or P'_1 postion. The primary chymosin cleavage sites on α_{s1}-casein (Phe_{23}-Phe_{24} and Trp_{164}-Tyr_{165}) and a major site in α_{s2}-casein (Phe_{88}-Tyr_{99}) contain aromatic residues at both the P_1 and P'_1 positions. There is considerable sequence homology (Table 1.1) and similarity between the 3-dimensional structures of the aspartyl proteinases from sources as dissimilar as retroviruses (e.g., HIV), fungi (e.g., *Rhizomucor miehei*) or mammals (e.g., pig) (Fig. 11.1); the molecules have dimensions of ~65 x 30 x 30 Å and consist of 2 domains. The active site of the aspartyl proteinases is contained in a cleft which extends for about 30 Å between the two domains of the molecule. The cleft can accomodate at least 7 residues. Aspartyl proteinases preferentially hydrolyse peptide bonds with a large, bulky residue (e.g., Leu or Phe) at the P_1 position, but interactions between enzyme and substrate in regions adjacent to the scissile bond are not without influence (Kay and Dunn, 1992). This general specificity was observed for chymosin (Chapters 4 and 5) and cathepsin D (Chapter 7)

The presence of indigenous acid proteinases in milk has been recognized (Kamiogawa and Yamauchi, 1972) and they probably originate from lysosomes in somatic cells in milk. The principal indigenous acid proteinase in milk is probably cathepsin D. The action of this enzyme on the caseins was investigated in Chapter 7. Cathepsin D and chymosin exhibited very similar specificities on α_{s1}- and κ-caseins and somewhat similar specificities on α_{s2}- and β-caseins. These findings are consistent with those of Kaminogawa *et al.* (1980). Cathepsin D and chymosin are both aspartyl proteinases (E.C. 3.4.23.X) and have generally similar structure and catalytic apparatus (Szecsi, 1992). Furthermore, there is considerable sequence homology between chymosin and cathepsin D (Table 11.1). It is not surprising, therefore, that the two enzymes appear to exhibit similar substrate specificity and appear to differ primarily with respect to the relative rate of peptide bond clevage. This difference in rates of bond cleavage is illustrated in Fig. 7.8, where β-II was produced from β-casein by both enzymes but appeared not to accumulate in the cathepsin D hydrolysate, presumably because it is more rapidly hydrolysed by cathepsin D than by chymosin. Likewise, the apparent inability of cathepsin D to coagulate milk and its relatively slow action on isolated κ-casein points to differing rates of cleavage of peptide bonds. A similar peptide (presumably the glycomacropeptide) was produced from κ-casein by chymosin and cathepsin D (Fig. 7.9) but the proteolytic activity necessary to produce the degree of hydrolysis apparent in Fig. 7.9 differed for the two enzymes. Expressed as a precentage of the proteolytic activity used to hydrolyse α_{s1}-casein (which resulted in approximately equal levels of degradation of this protein, Fig. 7.3), hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein by chymosin required only 25% of the activity used to hydrolyse α_{s1}-casein, while cathepsin D required 250%; therefore, cathepsin D appears to be substantially less active on the Phe_{105}-Met_{106} bond of κ-casein than chymosin. Kaminogawa *et al.* (1978) found that κ-casein was hydrolysed more slowly than α_{s1}- or β-casein by the acid proteinase isolated from milk. Presumably, the apparent inability of cathepsin D to coagulate milk is as a result of the enzyme preferentially hydrolyzing α_{s1}- and β-caseins and thus damaging the coagulability of the micelles.

Table 11.1. Homologies between the active sites of some aspartyl proteinases (from Kay and Dunn, 1992)

Enzyme	*Sequence homology (%)*
Human cathepsin E	100
Porcine pepsin	74
Human cathepsin D	66
Calf chymosin	53
Human renin	49
HIV proteinase	14

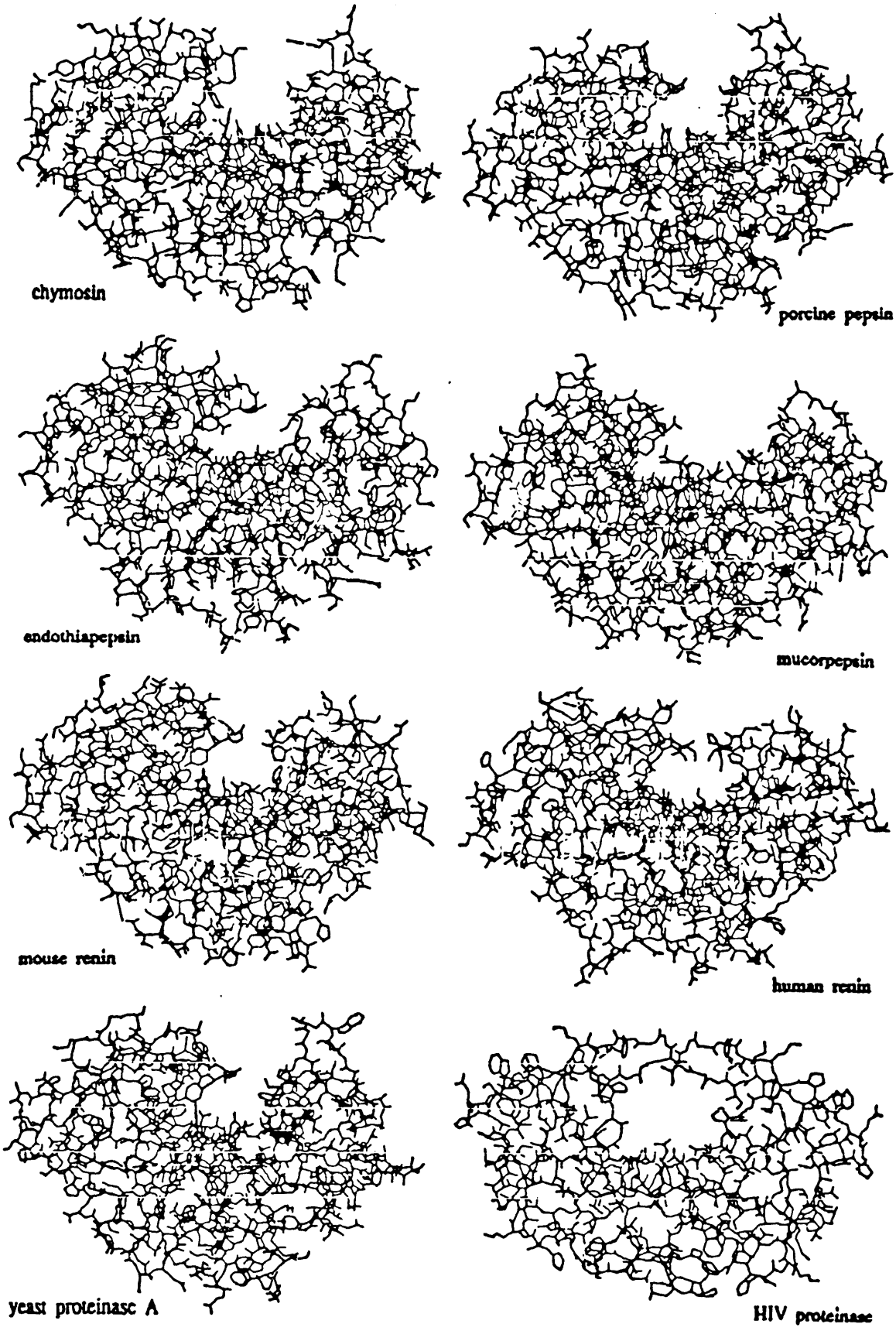

Fig. 11.1. Three-dimensional structures of aspartyl proteinases from different sources determined by X-ray crystallography (from Pitts et al., *1992)*

Plasmin, the principal indigenous proteinase in milk, is incorporated into the curd with the casein micelles, with which it is associated. Plasmin is responsible for proteolysis in milk prior to cheese manufacture (forming γ- and λ-caseins from β- and α_{s1}-caseins, respectively). The optimum pH for plasmin action is ~7.5 and the pH of cheese is unfavourable for its action. Plasmin is specific for bonds of the type Lys-X and, to a lesser extent, Arg-X (Weinstein and Doolittle, 1972). The bond-type cleaved by plasmin in α_{s1}-casein (Chapter 6) is in agreement with the specificity of this enzyme on α_{s2}- (Visser *et al.*, 1989; Le Bars and Gripon, 1989) and β-caseins (Gordon *et al.*, 1972; Eigel *et al.*, 1984).

α_{s1}-Casein is hydrolyzed initially by chymosin towards its terminals, i.e. at Phe_{23}-Phe_{24} and Trp_{164}-Tyr_{165} and at pH 6.5 further proteolysis continues from the extemities of the molecule towards its centre (Chapter 4). The primary sites of chymoin action on β-casein [Leu_{192}-Tyr_{193}, Ala_{189}-Phe_{190}, Gln_{167}-Ser_{168}, Leu_{165}-Ser_{166}, Leu_{163}-Ser_{164} (Visser and Slangen, 1977)] are in its C-terminal region and proteolysis progresses in the direction of the N-terminus. However, the primary sites of chymosin on α_{s2}-casein (Phe_{88}-Tyr_{89} and Phe_{174}-Ala_{175}, Chapter 5) and κ-casein (Phe_{105}-Met_{106}) are towards the centre of those molecules. Likewise, the primary sites of plasmin action on α_{s1}- and β-caseins are generally towards the centre of the molecules (Chapter 6; Eigel *et al.*, 1984). However, the primary sites of plasmin action on α_{s2}-casein (Le Bars and Gripon, 1989; Visser *et al.*, 1989) are towards the N- and C-terminii of the molecule. Plasmin and, to a lesser extent chymosin, are strongly influenced by residues adjacent to the scissile bond and thus the bonds at which these enzymes cleave a substrate are determined largely by the amino acid squence of the protein. This is clearly seen in the hydrolysis of α_{s2}-casein by chymosin; cleavage sites were resticted to the two hydrophobic regions of the molecule (residues 90 to 120 and 160-207). Steric factors probably influence the susceptibility of individual peptide bonds to cleavage (e.g. Chapter 4, Discussion). The order in which peptides are released by chymosin and cathepsin D from the N- and C-terminal regions of α_{s1}-casein towards its centre (Chapters 4 and 7) suggests that this molecule posesses a certain degree of tertiary structure; however, there is insufficient information available concerning the three-dimensional structures of the caseins to reach firm conclusions.

The bond Phe_{23}-Phe_{24} of α_{s1}-casein is cleaved rapidly by chymosin (Chapter 4) and the neighbouring bond Arg_{22}-Phe_{23} is amongst the major plasmin cleavage sites on this protein. In addition to these enzymes, it has been shown that bonds in the region of residues 22-24 are cleaved by cathepsin D (Chapter 7) and the proteinases of *Cynara cardunculus* (Sousa, 1993), *Penicillium* spp. (Trieu-Cout *et al.*, 1982) and *Lactocccus* spp. (Reid *et al.*, 1991). Present models of α_{s1}-casein structure (e.g. Kumosinski *et al.*, 1991) are unable to explain the susceptibility of this region of the molecule to proteolysis. Presumably, this region of α_{s1}-casein must possess some structural features which render peptide bonds in the region susceptible to hydrolysis by proteinases of such dissimilar specificities as chymosin and plasmin.

The caseins are relatively rich in hydrophobic residues and are potential sources of bitter peptides (Chapter 2, Section 5.6). The mean hydrophobicities of most of the peptides released from α_{s1}-casein by chymosin suggest that they should not be bitter, although the peptides α_{s1}-CN f24-28 and f24-32 are relatively hydrophobic (1.92 and 1.80 kcal mol^{-1}, respectively) and thus may be bitter; the peptide α_{s1}-CN f24-34 has been identifed as being bitter (see Sullivan and Jago, 1972). The action of LEP III-I (Stepaniak and Fox, 1993) on the peptide α_{s1}-CN f165-199 releases the bitter dipeptide, α_{s1}-CN f198-199 (Leu-Trp), which was isolated from Alpkäse cheese by Guigoz and Solms (1974). The primary sites of chymosin action on β-casein (Visser and Slangen, 1977) are in its hydrophobic C-terminal region and result in the formation of bitter peptides.

The action of plasmin on β-casein probably does not result in the formation of bitter peptides, although plasmin action on α_{s2}-casein produces 3 pepides from its C-terminus (α_{s1}-CN f182-207, f189-207 and f198-207; Le Bars and Gripon, 1989) which have high mean hydrophobicities and are potentially bitter. Tryptic digests of

α_{s1}-casein are bitter (Hill and van Leeuwen, 1974; Pélissier and Manchon, 1976). As plasmin and trypsin have similar specificities, it is possible that the hydrolysis of α_{s1}-casein by plasmin (Chapter 6) produces bitter peptides (e.g. α_{s1}-CN f23-34 and f91-100, also identified by Hill and van Leeuwen, 1974). However, the extent to which plasmin hydrolyses α_{s1}-casein in Cheddar cheese appears to be limited.

Angiotensin I converting enzyme (ACE) is a peptidyl dipeptide hydrolase (E.C. 3.4.15.1) which cleaves a dipeptide from the C-terminus of peptides. The enzyme acts on angiotensin I producing the potent vasoconstrictor angiotensin II and on the vasodilator, bradykinin, producing vasoinactive peptides. Maruyama and Suzuki (1982) isolated an ACE inhibitor from a tryptic digest of casein and identified it as as α_{s1}-CN f22-34. This peptide is also produced by plasmin (Chapter 6) and a very similar peptide (α_{s1}-CN f23-32) is produced by chymosin (Chapter 4). Maruyama *et al.* (1989) found that a hexapeptide from a tryptic digest of α_{s1}-casein (α_{s1}-CN f194-199) was inhibitory to ACE activity. This peptide is also produced by plasmin (Chapter 6). Exorphins have been isolated from peptic hydrolysates of α-casein and identified as α_{s1}-CN f90-95/6 (Zioudrou *et al.*, 1979). A similar peptide (α_{s1}-CN f90-100) is produced as a major product of the action of plasmin on α_{s1}-casein (Chapter 6).

The extent to which the results of hydrolysis studies on isolated indivdual caseins can be extrapolated to cheese is not always clear. The hydrolysis of β-casein by cell wall-associated proteinases of *Lactococcus* has been the object of a number of studies (see Chapter 2, Section 3.2.3.1). β-Casein in solution is readily hydrolysed by these proteinases, but it remains substantially intact in Cheddar cheese. Electrophoretograms of β-casein hydrolysed with cell wall-associated proteinases of 3 strains of *Lactococcus* were compared with Cheddar cheese made using these strains as starters (Chapter 8). The results suggest that the cell wall-proteinases of *Lactococcus* is not important in the primary proteolysis of β-casein in Cheddar cheese.

The apparent resistance of β-casein to proteolysis in Cheddar is of interest as its Leu_{192}-Tyr_{193} bond is the second most susceptible bond to chymosin action (after Phe_{105}-Met_{106} of κ-casein) and is more susceptible to chymosin than Phe_{23}-Phe_{24} of α_{s1}-casein (which is hydrolysed rapidly in cheese, see Chapter 2, Section 3.2.1.). β-Casein in isolation is rapidly hydrolysed by chymosin to β-I (β-CN f1-189/192) (e.g. Fig. 7.8) and in milk, but not in Cheddar cheese. The limited hydrolysis of β-casein which does occur in Cheddar appears to be due to plasmin action (Chapter 9). The primary cleavage sites of chymosin and lactococcal cell wall proteinase on β-casein are in its hydrophobic C-terminal region (see Chapter 2). It is possible that the inability of chymosin or cell wall-associated proteinases to hydrolyse β-casein to a significant extent in Cheddar is due to hydrophobic interactions between the C-terminal regions of the molecule which would render the potential cleavage sites inaccessible to these proteinases.

Identification of the primary degradation products of caseins present in Cheddar cheese (Chapter 9) indicated that β-casein was partially hydrolysed while α_{s1}-casein was extensively degraded. The majority of the hydrolysis products of β-casein identified (Fig. 9.4) could be accounted for in terms of plasmin action, although one, relatively minor, peptide (Fig. 9.4) probably corresponds to β-I (β-CN f1-189/192), the primary product of chymosin on β-casein. Likewise, all of the peptides which were identifed as being derived from α_{s1}-casein could be explained in terms of chymosin action. Thus, the primary sites for plasmin on β-casein and for chymosin on α_{s1}-casein in solution can be extrapolated to Cheddar. Chymosin action on α_{s2}- or β-caseins or plasmin action on α_{s1}-casein do not appear to be important in the primary proteolysis of these proteins in Cheddar, at least during the early stages of ripening.

No peptides originating from α_{s2}- or para-κ-caseins were identified in Cheddar. The fate of these proteins during cheese ripening remains unclear. Results of the few studies in which the susceptibility of para-κ-casein to proteolysis *in vitro* was investigated suggest that it is resistant to proteolysis (see Chapter 2, Section 3.2). As this protein migrates towards the cathode in urea-polyacrylamide gel

electrophoretograms (PAGE), which are commonly used to assess the initial stages of proteolysis during cheese ripening, it is often overlooked in cheese ripening studies. Likewise, because of its variable degree of post-translational phosphorylation, α_{s2}-casein migrates as multiple bands on urea-PAGE gels and in Cheddar cheese a proteose-peptone migrates in the same region as α_{s2}-casein (Chapter 9), making it difficult to determine the fate of α_{s2}-casein in Cheddar by urea-PAGE. However, α_{s2}-casein was clearly visible in starch gel electrophoretograms of Gouda presented by van den Berg and de Koning (1989) and decreased in concentration over time. α_{s2}-Casein is susceptible to hydrolysis by starter cell wall-associated proteinases (Monnet *et al.*, 1992) and by chymosin (Chapter 5), but is more readily hydrolysed by plasmin (Grufferty and Fox, 1988). Farkye and Fox (1991), who compared proteolysis in Cheddar cheeses made with and without the plasmin inhibitor, 6-aminohexanoic acid (AHA), found that bands migrating in the region of α_{s2}-casein was more intense in cheeses containing AHA, suggesting that plasmin hydrolyses α_{s2}-casein in Cheddar cheese.

Despite the rapid cleavage of Trp_{164}-Tyr_{165} in α_{s1}-casein by chymosin under the ionic conditions present in Cheddar (Chapter 4), the peptide α_{s1}-CN f165-199 has not been identified in cheese. Indeed, the only reported isolation of a peptide originating from this region of α_{s1}-casein is that of Guigoz and Solms (1974) who found the dipeptide, α_{s1}-CN f198-199 (Leu-Trp), in Alpkäse. Small peptides originating from α_{s1}-CN f1-23 have been identified in Gouda (Kaminogawa *et al.*, 1986) and Cheddar (Singh *et al.*, 1993). Further work remains to be done to establish the extent to which Trp_{164}-Tyr_{165} is hydrolysed in Cheddar.

Although non-starter lactic acid bacteria (NSLAB) often dominate the microflora of Cheddar for much of its ripening period, their rôle in proteolysis is often overlooked. Proteolysis in Cheddar cheese with different NSLAB microflora were compared in Chapter 10. Despite both qualitative and quantitative differences in their NSLAB microflora, cheeses and water-soluble extracts therefrom, were essentially indistinguishable by urea-PAGE (Figs. 10.2 and 10.3). Cheeses also contained essentially similar levels of water-soluble N (Fig. 10.4), but differed in respect of the total concentrations of free amino acids (Fig. 10.5), free glutamate (Fig. 10.6), peptide profiles by reversed-phase HPLC (Fig. 10.7) and free fatty acids (Figs. 8 and 9). These results suggest that NSLAB are not important in the primary proteolysis of caseins in Cheddar, but appear to be important in the hydrolysis of small and intermediate-sized peptides.

OPPORTUNITIES FOR FURTHER RESEARCH

Some of the studies described in this thesis may lend themselves to further investigations. As discussed above, the region of α_{s1}-casein from residues 22 to 24 appears to be particularly susceptible to the action of a number of proteinases, including plasmin and chymosin. The influence of primary structure on the proteolysis of the bonds in this region (Arg_{22}-Phe_{23} and Phe_{23}-Phe_{24}) could be determined by investigating the kinetics of the proteolysis of synthetic peptides approximating the α_{s1}-casein sequence in this region. Molecular models of any such synthetic peptides may be valuable in understanding the action of chymosin on this region of α_{s1}-casein.

Data on the influence of pH and NaCl on the kinetics of chymosin action on synthetic peptides containing its primary cleavage sites of α_{s1}- and β-caseins would be very valuable in understanding the proteolysis of these proteins in cheese.

The pathways of proteolysis of α_{s1}-casein presented in Figs. 4.6 and 4.7 are based on the relative rates of appearence of the small (pH 4.6-soluble) peptides. It would be of interest to identify the large (pH 4.6-insoluble) peptides visible in electrophoretograms of the hydrolysates (Fig. 4.5) and thus confirm the pathways proposed in this thesis. Identification of these large peptides may lead to a better understanding of the proteolysis of this protein in cheese.

The observation that cathepsin D is apparently unable to coagulate milk was unexpected and is of interest. Cathepsin D appears to be able to hydrolyse the Phe_{105}-Met_{106} bond of κ-casein in solution (Fig. 7.9). It is not known whether cathepsin D is able to cleave this bond in milk and thus measurement of the release of glycomacropeptide (by HPLC or measurement of the increase of pH 4.6-soluble N-acetylneuraminic acid) from milk treated with cathepsin D appears to be warranted. Kinetics of the action of cathepsin D on a synthetic peptide containing the sequence of κ-casein in the region of its Phe_{105}-Met_{106} bond (e.g. κ-CN f98-111) may also provide information on its action on this bond in milk.

The results presented in this and other studies permit a good understanding of the biochemistry of the initial stages of Cheddar cheese ripening. It should be possible, therefore, to model the initial stages of cheese ripening in solution. Previous attempts (e.g. Gou and Fox, unpublished) have had only limited success due to excessive hydrolysis of β-casein by chymosin. To successfully model the proteolysis of caseins in solution it will be necessary to inhibit the action of chymosin and lactococcal proteinases on the hydrophobic C-terminal region of β-casein. It may be possible to achieve this by the use of cyclodextrins which interact with this region of β-casein and have been shown to inhibit its aggregation in solution (Lee and Fennema, 1991).

The action of proteinases of importance during cheese ripening on intact caseins has been the subject of numerous studies (see Chapter 2). However, the action of chymosin and plasmin on partially hydrolyzed caseins have not been determined. Partial hydrolysis of caseins may render bonds accessible to the action of other proteinases which may not easily hydrolyse these bonds in intact caseins. Studies could include the action of chymosin on the γ-caseins or plasmin and lactococcal proteinases on α_{s1}-CN f24-199, or other products of chymosin action on this protein. Such studies may also be closer to the stiuation in cheese where many of the potential substrates for proteinases are partially hydrolyzed rather than intact caseins.

The contribution of individual proteolytic agents in cheese has been determined in aseptic model systems (see Chapter 2, Section 2.1). The majority of these studies were performed before the developent of modern anayltical techniques which allow investigation of the biochemistry of cheese ripening. Studies in which the action of various proteolytic agents are eliminated could provide further evidence for the extent to which chymosin and plasmin act in Cheddar cheese.

CONCLUSIONS

The results presented in this thesis are consistent with the hypothesis that the primary proteolysis of caseins in Cheddar cheese is due mainly to the action of chymosin and plasmin. α_{s1}-Casein is hydrolysed primarily by chymosin and β-casein by plasmin, and to a much lesser extent, chymosin. The results of studies of chymosin action on α_{s1}- and of plasmin on β-casein in solution can be extrapolated to cheese, while plasmin action on intact α_{s1}- and chymosin action on α_{s2}- and β-casein are probably limited in Cheddar cheese. Proteinases from *Lactococcus* and NSALB probably act mainly on short and intermediate-sized peptides produced as a result of the primary proteolysis of the caseins by chymosin and/or plasmin.

FINIS

REFERENCES

Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M., Jr., Harwalkar, V. R., Jenness, R. and Whitney, R. McL., 1984, *J. Dairy Sci.* **67**, 1599.

Farkye, N.Y. and Fox, P.F., 1991, *J. Agric. Food Chem.* **39**, 786.

Fish, J. S., 1957, *Nature* **180**, 345.

Gordon, W. G., Groves, M. L., Greenberg, R., Jones, S. B., Kalan, E. B., Peterson, R. F. and Townend, R. E., 1972, *J. Dairy Sci.* **55**, 261.

Grufferty, M.B. and Fox, P.F., 1988, *J. Dairy Res.* **55**, 609.

Guigoz, Y. and Solms, J., 1974, *Lebensm. Wiss. Technol.* **7**, 356.

Hill, R. D. and van Leeuwen, H., 1974, *Aust. J. Dairy Technol.*, **29**, 33.

Kaminogawa, S. and Yamauchi, K, 1972, *Agric. Biol. Chem.* **36**, 2351.

Kaminogawa, S. and Yamauchi, K., 1978 Proc. 20th Intern. Dairy Congr. (Paris), **E**, 313.

Kaminogawa, S., Yamauchi, K., Miyazawa, S. and Koga, Y., 1980, *J. Dairy Sci.* **63**, 710.

Kamogawa, S., Yan, T. R., Azuma, N. and Yamauchi, K., 1986, *J. Food Sci.* **51**, 5.

Kay, J. and Dunn, B. M., 1992, *Scand. J Clin. Lab. Invest.*, **52** (Suppl. 210), 23.

Kumosinski, T. F, Brown, E. M. and Farrell, H. M., Jr., 1991, *J. Dairy Sci.* **74**, 2889.

LeBars, D. and Gripon, J.-C., 1989, *J. Dairy Res.* **56**, 817.

Lee, M.-J. and Fennema, O. R., 1991, *J. Agric. Food Chem.* **39**, 17.

Maruyama, S. and Suzuki, H., 1982, *Agric. Biol. Chem.* **46**, 1393.

Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizuka, A. and Suzuki, H., 1989, *Agric. Biol. Chem.* **51**, 2557.

Monnet, V., Ley, J. P. and Gonzàlez, S., 1992, *Int. J. Biochem.* **24**, 707.

Pélissier, J.-P. and Manchon, P., 1976, *J. Food Sci.* **41**, 231.

Pitts, J. E., Dhanaaj, V., Dealwis, C. G., Mantafounis, D., Nugent, P., Orprayoon, P., Cooper, J. B., Neuman, M and Blundell, T. L., 1992, *Scand. J Clin. Lab. Invest.*, **52** (Suppl. 210), 39.

Reid, J. R., Ng, K. H, Moore, C. H., Coolbear, T. and Prichard, G. G., 1991 *Appl. Microbiol. Biotechnol.* **36**, 344.

Singh, T. K., Fox, P. F., Højrup, P. and Healy, A., 1993, *Intern. Dairy J.* (in press).

Sousa, M.-J., 1993, *Plant Rennet Subsitiute from Flowers of* Cynara Cardunculus, M.Sc. Thesis, National University of Ireland, Cork.

Stepaniak, L. and Fox, P. F., 1993, Proc. 29th Norwegian Biochemical Soc. Meeting, Beito, Jan. 21-24, 1993, p. 97.

Sullivan, J. J., Jago, G. R., 1972, *Aust. J. Dairy Technol.* **27**, 98.

Szecsi, P., 1992, *Scand. J Clin. Lab. Invest.*, **52** (Suppl. 210), 5.

Trieu-Cout, P., Archieri-Haze, M. J. and Gripon, J.-C., 1987, *J. Dairy Res.* **49**, 487.

van den Berg, G. and de Koning, P. J., 1990, *Neth. Milk Dairy J.* **44**, 189.

Visser, S. and Slangen, K.J., 1977, *Neth. Milk Dairy J.* **31**, 16.

Visser, S., Slangen, K.J., Alting, A.C. and Vreeman, H.J., 1989, *Milchwissenschaft* **44**, 335.

Vreeman, H. J., Visser, S., Slangen, C. J. and van Reel, J. A. M., 1986, *Biochem. J.* **240**, 87.

Weinstein, M. J. and Doolittle, R. F., 1972, *Biochim. Biophys. Acta* **258**, 577.

... and finally!

Do not imagine that [scientific] research is accomplished in a fine poetic rapture; before the dawn appears one must drudge along labyrinthine ways, or roll the stone like Sisyphus, endlessly uphill.

A. J. Cronin

Many's the long night I've dreamed of cheese ...

Robert Louis Stevenson