NATIONAL UNIVERSITY OF IRELAND
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Variations in total and differential milk somatic cell counts and plasmin levels and their role in proteolysis and quality of milk and cheese.

THESIS

presented by

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For the degree of

DOCTOR OF PHILOSOPHY

In Food Technology

Supervisor: Professor John Foley, M.Sc., Ph.D.

July 1995.

This thesis is dedicated to my mother and father, for their encouragement and support, and above all, their belief in me, and to Brenda, for keeping me sane and happy.

"- I think it was 'blessed are the cheesemakers'
- What's so special about the cheesemakers ?
- It's not meant to be taken literally - obviously it refers to all manufacturers of dairy products "

Monty Python, The Life of Brian, 1979

ACKNOWLEDGEMENTS

I would firstly like to thank Professor John Foley for giving me the opportunity to undertake this study, and for his constant support and encouragement, and for allowing me the freedom to take this work down whatever peculiar avenues beckoned me. I am also extremely grateful for the great encouragement he showed with regard to fostering my academic career, and for the many opportunities in this and every regard he has given me in my time under his supervision.

I would like to thank Professor Charles Daly for the financial and moral backing of this work, and for his advice and encouragement. I am also grateful to the staff of the NFBC for all their help and organisation, particularly Mary Hough. I would also like to extend my gratitude to Professor Joe Buckley, Dr Elke Arendt and Mr Matt Murphy for their suggestions and help. I also greatly appreciate the assistance and contributions of academic and technical staff of all the other departments of the Food Science and Technology Faculty.

In the Food Technology Department, the cheesemaking and photographic skills of Nicholas Bergin have been essential, as has the help of Maurice Conway, and I must thank Mr Pat McCarthy, now retired, for always finding me a mastitic cow at short notice. I also wish to thank Conor Nyhan, Noel O'Shea, Jim McNamara, Ann Collins, Eddie Beatty, Billy Walsh, Mick Kearney, Jimmy Byrnes, Mick Kelly, Tom Hannon and Donal Humphreys for their help over the years. The assistance of a number of farmers and co-operatives in locating and supplying various milks was also vital in this work.

I wish to thank Dr Paddy Joyce of the Zoology Department, University College Dublin, for introducing me to the mysterious ways of the polymorphonuclear leucocyte, and for his frequent advice, cooperation and stimulating discussion in our ongoing collaboration. Also at U.C.D., I would like to thank Dr Alan Shattock and Denise and Susan for their considerable inputs into this project. At Teagasc, Moorepark, I am grateful for the collaborations, suggestions and comments of Drs Raj Mehra and Barry Connolly, and Mr Billy Meaney. I am also grateful for the technical assistance of Des Eason and Joanne Hayes in completing the Formagraph analysis.

And so to the 'A-team', those foolhardy individuals who apparently trusted me enough to endure many a long day and strange idea. Diarmuid P.M. Sheehan gets a medal for enduring not only a fourth year project but an MSc too under my dubious tutelage, but whose help, encouragement, concern and friendship have been very important to me over the years. Likewise, I must thank Mary M.G. O'Connor for her help, commitment and support over her MSc work. I also want to thank Anna and Anna-Marie for their hard work and enthusiasm, and to acknowledge all the fourth years who have worked with me; John, Colm, Mary, Fiona, Mark F., Mark H., Maurice, Diarmiud (again!), Aindrias, Karen, with honourable mentions for Barry and Ian.

I would also like to thank all the other postgraduates of the Food Technology Department over the years, too numerous to mention, for making it a very wierd place

to work. Particular mention must go, however, to Joanna Earle, for her good humour, friendship, crosswords and eleventh-hour proofreading skills, and John F., who I'll probably insult greatly by calling almost an honourary A-team member.

I am beginning to wonder if this may set a record for the longest acknowlegements in history..... but I would also like to thank Tanoj and Paul Mc for teaching a technologist some of the finer points of dairy chemistry, and Margaret Cole for her advice and patience while giving me a greatly appreciated crash course in statistics and data reporting.

The support and friendship of my friends from the Biotechnology class of 1990 must be recognised. In particular Denis gets a little gold star for himself for sharing the cleanest tidiest house in Cork with me for four years, and I would also like to thank Hugh, Conor and Martin for being themselves, basically. To David and Steven at home I say good luck and God help on their new marriages, and to Enda, I say, good luck on becoming the second Dr Kelly on the road.

Again, I wish to thank my parents for their trust in my judgement, their faith in my ability and for always being there when I need them, and my brothers and sisters and extended family for the bemused and bewildered support and encouragement of their 'strange' little brother.

Finally, to Brenda, I feel that this would not have been possible without your support, patience, encouragement, love and unswerving belief in me, and I owe you more than I could ever express. Thanks for being there through the brightest days and darkest hours, and for coming home.

OK ramblers, lets get rambling.....

ABSTRACT

Increased plasmin and plasminogen levels and elevated somatic cell counts (SCC) and polymorphonuclear leucocyte levels (PMN) were evident in late lactation milk. Compositional changes in these milks were associated with increased SCC. The quality of late lactation milks was related to nutritional status of herds, with milks from herds on a high plane of nutrition having composition and clotting properties similar to, or superior to, early-mid lactation milks. Nutritionally-deficient cows had elevated numbers of polymorphonuclear leucocytes (PMNs) in their milk, elevated plasmin levels and increased overall proteolytic activity. The dominant effect of plasmin on proteolysis in milks of low SCC was established. When present in elevated numbers, somatic cells and PMNs in particular had a more significant influence on the proteolysis of both raw and pasteurised milks than plasmin. PMN protease action on the caseins showed proteolysis products of two specific enzymes, cathepsin B and elastase, which were also shown in high SCC milk. Crude extracts of somatic cells had a high specificity on α_{s1}-casein. Cheeses made from late lactation milks had increased breakdown of α_{s1}-casein, suggestive of the action of somatic cell proteinases, which may be linked to textural defects in cheese. Late lactation cheeses also showed decreased production of small peptides and amino acids, the reason for which is unknown. Plasmin, which is elevated in activity in late lactation milk, accelerated the ripening of Gouda-type cheese, but was not associated with defects of texture or flavour. The retention of somatic cell enzymes in cheese curd was confirmed, and a potential role in production of bitter peptides identified. Cheeses made from milks containing high levels of PMNs had accelerated α_{s1}-casein breakdown relative to cheeses made from low PMN milk of the same total SCC, consistent with the demonstrated action of PMN proteinases. The two types of cheese were determined significantly different by blind triangle testing.

CONTENTS

INTRODUCTION AND OBJECTIVES

In the Food Technology Department at University College Cork, attempts to develop a semi-soft surface ripened cheese consistently met with difficulties in cheese quality when milk from college creamery suppliers was used after mid-September of each year. This corresponded to the time when most suppliers milk consisted of late lactation milk. Cheese defects encountered included a bitter or sharp flavour and soft pasty texture, arising from elevated cheese moisture content and excessive proteolysis during ripening. Preliminary work showed that plasmin levels and somatic cell counts (SCCs) were elevated in the milk at this time and some experimental cheeses were made from milks to which both components had been added separately. Elevated milk plasmin levels did not produce defects in the cheese but addition of somatic cells, recovered centrifugally from mastitic milk, gave extremely poor quality cheese. The purpose of the project described in this thesis was to explore the relationships between somatic cells and plasmin in milk, particularly in late lactation milk, and to evaluate the role of these two sources of proteolytic activity in milk in cheese manufacture and ripening.

Seasonal variation in the quality of milk and cheese places a major constraint on the Irish dairy industry, and has arisen due to a tradition of cows being calved in spring to take advantage of the summer grass growing season for low-cost milk production. This has resulted in a peak-to-trough ratio of 15:1 between summer and winter milk production, with winter milk going mainly for liquid consumption. This situation has led to a near complete dependence on long shelf-life cheese varieties, such as Cheddar, and the needs of the Irish dairy industry to compete in European markets are hampered by the poor quality of late lactation milk. This, along with EC regulations concerning maximum SCC levels which are being regularly revised, means that the issue of quality of late lactation milks and the influence of somatic cells on quality is of great interest to Irish dairy producers and farmers alike.

Milk plasmin activity is elevated in late lactation and high somatic cell count milk (Politis *et al.*, 1989a,b; Benslimane *et al.*, 1990) and has been associated with decreased syneretic properties of milk (Donnelly *et al.*, 1984; Okigbo *et al.*, 1985). However, it has more recently been shown that elevated plasmin activity does not impair milk clotting properties (Pearse *et al.*, 1986; Bastian *et al.*, 1991) and does not lead to quality defects in Cheddar cheese (Farkye and Fox, 1991; Farkye and Landkammer, 1991). This suggests that the source of undesirable proteolytic activity in late lactation milk and cheese may be the lysosomal proteinases of somatic cells.

The presence of more than one proteinase in normal milk was suggested by Andrews (1983a). The presence of a number of proteinases in mastitic, high SCC milk, was shown by Andrews (1983b) and although the proportionate roles of plasmin and non-plasmin proteinases in these milks was disputed (Barry and Donnelly, 1981), it is accepted that as SCC increases, the relative contributions of somatic cell enzymes to proteolysis will increase. An acid proteinase similar to cathepsin D has been isolated from normal milk (Kaminagowa and Yamauchi, 1972b, Larsen *et al.*, 1993) and two

cysteine proteinases have been isolated from mastitic milk (Suzuki and Katoh, 1990). The origin of both these enzymes is believed to be somatic cells, which are also known to be the source of acid phosphatase in milk (Andrews, 1976).

Leucocytes from bovine blood were shown to have a limited action on caseins by Grieve and Kitchen (1985), but further work by Verdi and Barbano (1988, 1991b) has shown firstly that milk somatic cells have higher proteolytic activity than their blood counterparts and secondly that milk leucocytes have considerable action on caseins under milk and cheese-like conditions. These proteinases were shown to be resistant to pasteurisation (Senyk *et al.*, 1985; Verdi and Barbano, 1988) and DeRham and Andrews (1982) stated that proteinases will survive cheesemaking conditions and continue to act during cheese maturation, almost certainly with harmful effects on cheese flavour and texture. The lysosomal acid proteinase, cathepsin D, has a specificity on α_{s1}-casein similar to that of chymosin and may act synergistically with chymosin (Kaminagowa *et al.*, 1980; McSweeney *et al.*, 1995). However, the interactions and roles of somatic cell proteinases and plasmin in milk proteolysis have not been fully elucidated and the exact actions of specific somatic cell proteinases, other than cathepsin D, on milk and cheese proteolysis are unknown.

Somatic cell counts in bovine milk are known to be elevated in late lactation (Emanuelson *et al.*, 1989) and such milk is associated with increased rennet clotting times, decreased syneretic properties and high cheese moisture (O'Keeffe *et al.*, 1979; O'Keeffe, 1984; Lucey and Fox, 1992). High SCC milks are likewise associated with poor cheesemaking properties of milk (Brus and Jaartsveld, 1971; Grandison and Ford, 1986; Politis and Ng-Kwai-Hang, 1988). However it has been recently hypothesised that the cheesemaking functionality of late lactation milk may be improved by selecting herds on a high plane of nutrition (Lucey and Fox, 1992; Kefford *et al.*, 1992), but the effects of such improved husbandry practices on milk SCC and enzymology have not been described.

Andrews (1983b) suggested that the identity of the cells making up the total somatic cell population in milk was important in determining the proteolytic activity in the milk. Normal milk contains approximately 12% polymorphonuclear leucocytes (PMNs), 28% lymphocytes and 60% macrophages (Burvenich *et al.*, 1995) but on mastitic infection, there is an influx of PMNs and these cells soon represent 95% of the total population (Saad and Östensson, 1990). It is known that the different cell types possess different proteolytic enzymes (Verdi and Barbano, 1991b) and thus the differential cell count may play a role in determining the nature of the proteolytic activity in milks. O'Sullivan *et al.* (1992) developed an enzyme-linked immunosorbent assay (ELISA) technique for measurement of PMN antigen in milk, for use in early detection of subclinical mastitis, and found a high correlation between milk SCC and PMN level (0.94). However, when applied to a large study of bulk tanks (Joyce, personal communication) a low correlation (0.50) was found with bulk tanks of similar total SCCs showing apparently greatly different levels of PMNs. With different cell types possessing different proteolytic activities, it was thought possible that these milks may give cheeses of different ripening properties.

The objectives of this work, based on the above background, were as follows.

1. To examine the compositional and enzymatic properties (somatic cell count, PMN levels and plasmin activity) of normal, mastitic and late lactation milks and to determine the influences of these factors on proteolysis in raw and pasteurised milk. There are three parts to this work

(i) Examination of the effects of seasonal and lactational factors on SCC in a range of milks from individual cows

(ii) Examination of the influence of these two factors plus SCC on PMNs, plasmin and plasminogen in the same milks

(iii) A study of the relationships between seasonality, SCC, PMN level and plasmin activity, and milk composition and proteolysis

2. To examine the levels and products of proteolysis in milks of a range of SCCs. To investigate the proteolytic specificity of purified and crude somatic cell proteinase preparations on caseins and compare the products to those found in milk.

3. To study the effect of plane of nutrition on somatic cells, PMNs, plasmin and proteolysis in milk in a joint study with Teagasc, Moorepark, and to investigate the influence of dietary restrictions and poor husbandry practices on the quality and functionality of late lactation milk, with reference to proteolytic activity.

4. To examine the coagulation and ripening properties of Dutch cheeses made from milks of various SCCs, from herds at various stages of lactations This area was to be examined in three ways

(i) Examination of the composition and coagulation properties of bulk milk samples from herds at different times of the year

(ii) Preparation and analysis of small-scale cheese batches made from milks of different SCCs and stages of lactation

(iii) Examination of the ripening properties (chemical and organoleptic) of Gouda cheeses made on a pilot scale from early, mid and late lactation milks

5. To establish the effect of elevated cheesemilk plasmin levels on ripening properties of Gouda cheeses. Cheeses were also to be made in which the only active proteolytic agents were the indigenous milk enzymes (somatic cell enzymes and plasmin). Normal and mastitic milks were to be used, and the activities of the milk enzymes on proteolysis in this system, at a range of pHs, determined.

6. The ripening characteristics of Gouda-type cheeses made from milks of the same total SCC but different levels of PMNs were to be compared.

Much of the proteinase activity (*in mastitic milk*) will survive cheesemaking conditions and continue to act during cheese maturation, almost certainly with harmful effects on cheese quality in terms of texture and flavour, but these aspects remain to be investigated in detail.

deRham and Andrews (1982)

Proteases associated with elevated somatic cell counts will damage raw milk quality upon storage, pasteurised fluid milk over shelf-life, and milk during cheesemaking.

Senyk *et al.* (1985)

Protease activity at low pH by enzymes present in cells isolated from milk may cause casein proteolysis during cheese ageing if these proteases are retained in cheese during cheese manufacturing.

Verdi and Barbano (1991)

However, unlike small molecules or enzymes other than protease, protease is an essential, but not additive, indicator of mastitis, because proteolytic action on tissues is fundamentally important in the pathogenesis of inflammation.

Suzuki and Katoh (1992)

Chapter 1

Seasonality and cheese manufacture

1.1. Seasonality and the Irish Dairy Industry

1.1.1. Introduction

The history of Irish cheesemaking can be traced back as far as the earliest available documentary sources, with a reported attempt on the life of Saint Patrick with poisoned curd, and Queen Maeve reputedly having been killed by a piece of pressed curd hurled from a sling (Foley, 1993). As well as uses as a weapon, cheese also developed as a foodstuff, and was regarded as a part of the everyday diet of the Irish through the middle ages, with exportation beginning in the 15th century. In 1902, training courses and trials were carried out in the manufacture of Cheddar and Caerphilly cheeses, with two hundred creameries being involved in cheese manufacture by 1919, mostly for export to England. The next 40 years were relatively dormant for the industry, with manufacture dominated by Cheddar, and small amounts of Gouda, Gruyere, Caerphilly and processed cheese. In 1960 a growth and development phase began, involving amalgamation of small co-operatives into dairy companies and establishment of large factories. In 1992 Irish cheese manufacture, primarily Cheddar, stood at 77,000 tonnes (Foley, 1993).

One of the main problems facing the Irish cheese industry is seasonality, with the vast majority of cows being calved in the spring and most of the milk is thus produced during the summer months. This is to take advantage of the plentiful grass supply for least cost milk production, resulting in a peak-to-trough ratio of milk production of 9:1 for manufacturing milk and 2:1 for liquid milk (Keane, 1986). This is in contrast with most countries where calving occurs throughout the year, resulting in a relatively constant supply of milk, with the possible exception of New Zealand (Phelan *et al.*, 1982). The principal disadvantage of the Irish system with regard to dairy product manufacture is that, while suitable for the production of long shelf-life products such as Cheddar cheese, increasing consumer demand for short shelf-life products, such as yoghurts, flavoured milks and soft cheeses, and a need for diversification in cheese product mix means that seasonality places a considerable constraint on the development of the Irish dairy industry. As a result of the predominance of spring-calving cows, most Irish winter milk comprises of late lactation milk, which is associated with poor quality of dairy products (Lucey and Fox, 1992). Thus, manufacturing milk is hugely influenced by seasonality while bulk liquid milk has a composition intermediate between that of spring and autumn-calving herds, reflecting the even calving pattern of those herds supplying the liquid milk trade (Phelan *et al.* 1982).

1.1.2. The composition of late lactation milk

1.1.2.1. CHANGES IN PROTEIN COMPOSITION

Barry and Donnelly (1980) examined the compositional parameters of milk from spring calving and autumn calving herds of Friesian cattle, and found that overall protein composition was similar for both herds, as were changes in late lactation. These changes were characterised by an increase in γ-caseins and unidentified components and a decrease in ß- and α_s- casein. Fluctuations in κ-casein proportions were small, and slightly higher levels were recorded in early and late lactation. These findings were broadly in agreement with the findings of Davies and Law (1977b) and these changes were attributed to the action of plasmin and differences in the relative rates of protein biosynthesis. There was also a difference in α_s- casein levels between autumn- and spring-calving herd milks, which was ascribed to environmental influences on biosynthesis, such as temperature. These trends were confirmed by Donnelly and Barry (1983) who examined the composition of Irish milk for manufacturing throughout the year (see Fig. 1.1).

Phelan *et al.* (1982) found that late lactation milk had higher total protein, whey protein and non-protein nitrogen (NPN) than mid-lactation milk, and lower urea levels.

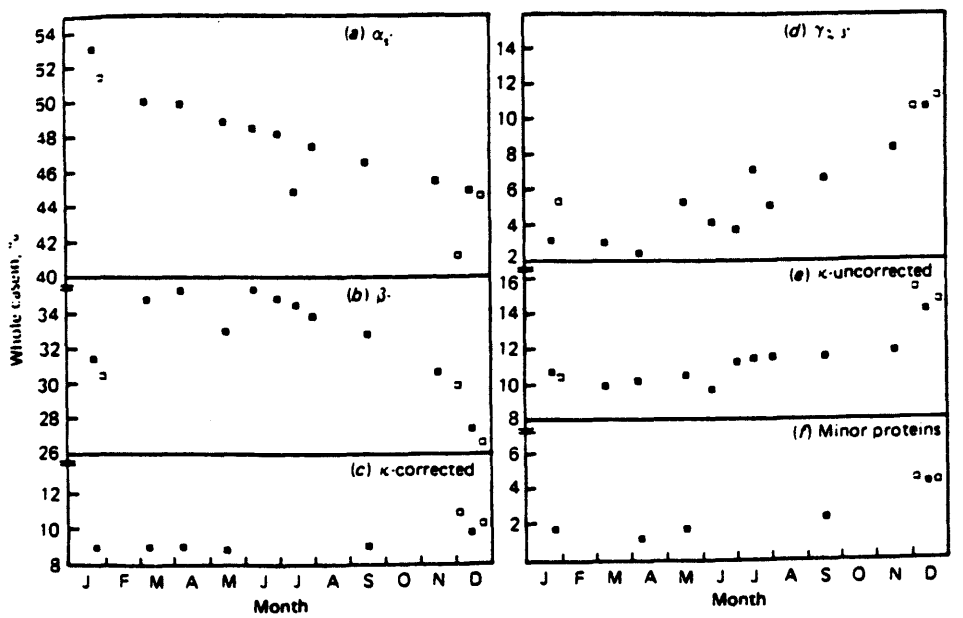


Fig. 1.1 *Variations throughout the year in proportions of individual casein components in Irish milk for manufacturing. Data are for a single 12-month period except for open symbols which refer to repeat analysis in a subsequent year (from Donnelly and Barry, 1983)*

The changes were of a greater magnitude in spring-calved and manufacturing milk than in liquid milk or autumn-calved milks. The casein/total protein ratio also dropped in late lactation, due to proteolysis by plasmin. Both seasonal and lactational factors influenced total protein level, with predominance of the former, while proteose peptone levels, indicative of plasmin action, showed a clearly lactational trend. Increased total protein content and lower casein numbers in late lactation milk were also reported by Lucey and Fox (1992) and Barbano *et al.* (1991). Donnelly *et al.* (1984) isolated casein micelles from milk and observed that those recovered from late lactation milk were smaller, richer in Ca, Mg and P, and more hydrated than those from mid-lactation milks. The former milks were also higher in soluble casein, again possibly due to the increased action of plasmin.

1.1.2.2. CHANGES IN NON-NITROGENOUS COMPONENTS

Increases in fat and decreased lactose in late lactation milk were reported by Dawson and Rook (1974), Phelan *et al.* (1982) and Lucey and Fox (1992), while the former authors found clear seasonal trends in free fatty acid (FFA) levels with the highest levels in January. Keogh *et al.* (1982) found marked seasonal trends in mineral levels in Irish manufacturing milk, with increased levels of Na, colloidal P, Ca (colloidal, caseinate and ionic) and both colloidal and soluble Mg and decreased levels of soluble Ca, K, soluble P and citrate. These changes were less pronounced for autumn calving herds, reflecting the higher plane of nutrition (summer grass) available to these cows in late lactation, compared to the poor feeding regimes encountered by spring calving cows in late lactation, and thus it was suggested that the processability of late lactation milk might be improved by raising the level of nutrition of the relevant herds at this time. The increase in sodium and chloride in late lactation milk results in the drop in lactose levels reported by Keogh *et al.* (1982) and Phelan *et al.* (1982) as this system regulates the milk osmotic pressure. Late lactation milk is also linked to low titratable acidities and high pH (Phelan *et al.*, 1982; Lucey and Fox, 1992; O'Keeffe *et al.*, 1982). Organoleptically, stage of lactation has been linked to flavour score and the incidence of feed, salty and metallic flavours, with these being most pronounced in early lactation (Kratzer *et al.*, 1967).

1.1.2.3. CHANGES IN PLASMIN LEVEL AND SOMATIC CELL COUNT

As described above, many authors ascribe the changes in casein composition in late lactation milk to elevated activity of the alkaline milk proteinase, plasmin (Barry and Donnelly, 1980; Donnelly and Barry, 1983). The latter study showed that treatment of milk with porcine plasmin induced changes similar to those found in late-lactation milk, and by measuring the breakdown of radiolabelled ß-casein, they estimated that the activity of plasmin in late lactation milk was almost double that in mid-lactation milk. Richardson (1983b) used a synthetic substrate to measure plasmin levels in milk and found plasmin levels of 0.15, 0.29 and 0.37 mg plasmin /L milk in early, mid and late

lactation milks respectively, and that the level of the inactive precursor of plasmin, plasminogen, was also highest in late lactation milk. Increased plasmin activity in late lactation has also been reported by Scharr (1985), Politis *et al.* (1989) and Benslimane *et al.* (1990). At the other end of the season, plasmin and plasminogen levels have been shown to drop rapidly after parturition, with plasminogen levels quickly starting to rise in early lactation, while plasmin levels remained low (Kaartinen and Pyörälä, 1989).

The role of the zymogen, plasminogen, in late lactation must also be considered as Korycka-Dahl *et al.* (1983) reported that the level of plasmin in fresh milk was the same in mid and late lactation milk, but the levels of plasminogen in the latter were twice that in the former, and thus on storage activation of plasminogen could lead to increased plasmin levels. Politis *et al.* (1989) found that the plasminogen:plasmin ratio halved in late lactation, supporting this theory of increased plasminogen activation in late lactation leading to increased plasmin levels, as opposed to transfer of more plasmin across the mammary epithelium. However, Benslimane *et al.* (1990) found a slight increase in plasminogen:plasmin ratio in late lactation, and the exact mechanism of activation and its seasonal variation remains unclear.

Somatic cell count (SCC) has also been shown to rise in late lactation milk by, among others, Johnson and Trudel (1933), Blackburn (1966), Emanuelson *et al.* (1988), Wever and Emanuelson (1989) and Barbano *et al.* (1992). Emanuelson *et al.* (1988) also showed that milk antitrypsin, a proteinase inhibitor, increased with advancing lactation. The influence of increased levels of somatic cells and plasmin on dairy products are discussed in chapters 2 and 3.

1.1.3. Cheesemaking and late lactation milk

Late lactation milk from autumn-calving herds was reported to yield high moisture cheese by O'Keeffe *et al.* (1979). In 1982 O'Keeffe *et al.* investigated the factors influencing seasonal variation in milk functionality for cheesemaking and found that late lactation milk had increased rennet clotting time (R.C.T) and decreased curd strength and syneresis. This was most pronounced in spring calving herds. This was also found by Lucey and Fox (1992). Poor syneresis of curd was attributed to proteolysis by plasmin by Donnelly *et al.* (1984), who treated milk with trypsin and saw subsequently higher moisture contents in Cheddar cheese made from this milk. O'Keeffe (1984) found that spring-calving herd milk was more likely to give a high moisture cheese than autumn-calving milk, and that cheese moisture was inversely related to the nutritional state and milk yield of the herd. The factors they associated with high moisture in cheese were high natural milk pH, reduced casein proportion and elevated soluble casein content, all of which are factors associated with late lactation milk. Elevated milk pH reduces the effect of the acid protease chymosin in clotting milk, and the low soluble casein levels found in late lactation milk will effect the second, non-enzymatic, phase of rennet coagulation (Lucey and Fox, 1992). ß-casein, which is hydrolysed by plasmin in late lactation milk, has been associated with proper

syneresis of curd (Ali *et al.*, 1980), possibly through hydrophobic interactions (Lucey and Fox, 1992), and this proteolysis may also lead to increased moisture levels in late lactation cheeses. Okigbo *et al.* (1985a) found that late lactation milk had poor clotting properties, which they ascribed to high pH, plasmin action, increased presence of monovalent cations (such as sodium) and increased presence of blood constituents. They also found decreased κ-casein content, which will affect micelle aggregation but this has not always been found in late lactation milk (Barry and Donnelly, 1982).

Improvement of the quality of cheese made from late lactation milk, and thus extension of the cheesemaking season in Ireland, is vital for the expansion of the dairy industry. O'Keeffe (1984) proposed that this could be achieved by selecting milk of herds with high average yields and normal pH and composition, with pH being proposed as a parameter for selection of suitable cheesemilks. Addition of calcium had little effect on rennet clotting time, but if 15% sodium caseinate (as proportion of total casein) was added and provided there is sufficient available calcium, normal R.C.T.s may be achieved (O'Keeffe *et al.*, 1982). Rennet clotting times may also be decreased by incorporation of denatured whey protein following high heat treatment of milk, but inferior quality cheese results (O'Keeffe *et al.*, 1982). Late lactation milk R.C.T.s have been improved by calcium chloride addition, pH adjustment, increasing rennet concentrations and blending with mid-lactation milk (Lucey and Fox, 1992), but these treatments did not improve curd firmness in early-spring calved herds which were on a poor quality nutritional plane in late lactation. By contrast, these authors found that late lactation clotting properties were far superior in cows from a herd on a higher plane of nutrition, which were dried off on a yield basis and had lactose levels consistently above 4%. The poor gel firmness in the low nutritional-grade herd appeared to be due to protein damage by plasmin. Addition of calcium chloride improves clotting properties by increasing the soluble calcium and reducing the pH, but excessive addition will retard coagulation. In contrast, O'Keeffe *et al.* (1982) found that in late lactation milk addition of calcium chloride did not improve R.C.T, unless casein was also supplemented, whereas in early and mid-lactation milk the effect of the calcium was to reduce R.C.T. This may be due to an enzymatic as well as a chemical component to casein coagulation, with plasmin action having rendered the casein unsuitable for good clotting, even if supplemented with calcium. However, Pearse *et al*, (1986) found that extensive hydrolysis with plasmin did not alter gel formation and syneresis and thus this area requires further elucidation. Late lactation cheeses are also associated with high initial free fatty acid levels (O'Keeffe, 1984), which may be associated with off-flavours in cheese.

In summary, Lucey and Fox (1992) suggested that not all late lactation milk was unsuitable for cheesemaking, as herds on a high nutritional plane, with improved milking practices can produce milk which is suitable for processing. This is in agreement with the findings of Kefford *et al.* (1992) who reported that diet quality, rather than stage of lactation, was the main factor influencing the functional capacity of milk in Cheddar cheese manufacture, with milk from cows on low-quality diets yielding higher moisture cheese than milk from cows on high-quality diets. Efficiency of

conversion of milk solids to cheese was greatest in late lactation cows offered high quality diets. Thus it is possible that improvement in the functionality of cheesemilk can be addressed by improving the quality of the cow diet at certain times of the year, and thus seasonal calving patterns may not need to be changed. This has significant consequences for the economics of milk production.

1.2. The manufacture and ripening of Dutch-type cheese

1.2.1. Introduction

Dutch-type cheeses constitute one of the most important classes of cheese produced in the world, with a tonnage comparable to that of Cheddar (Walstra *et al.*, 1987). Traditionally, there are two main types of cheese made in the Netherlands; Gouda (which was first referred to by name in 1697 (Scott, 1986)) and Edam, although there are a number of related minor varieties such as Meshanger, Tilsiter, Maasdammer and Limburger. Edam and Gouda are named after the villages in which they originated (Kosikowski, 1982). Dutch type varieties are generally defined by the following characteristics (Walstra *et al.*, 1987):

- Made from fresh, partially skimmed milk (leading to at least 40% fat in the dry matter (FDM) of the cheese, ranging in practice from 40-50%)
- Clotted by rennet
- Use starters consisting of mesophilic Lactococci and Leuconostocs which generally produce CO_2
- Have a water content in fat-free cheese below 63% (typical range 53-63%)
- Are pressed to obtain a closed rind
- Generally brine salted (to a final level from 2-7% in moisture) and have no essential surface flora
- Are at least somewhat matured, and thus have undergone significant proteolysis

These cheeses thus have a semi-hard to hard consistency, a smooth texture, usually with small holes, and a flavour intensity which may vary widely (due to differences in starters, degree of acidification, maturation conditions etc.). The flavour is generally described as mild and slightly nutty (Kosikowski, 1982). pH in the cheese may be anywhere from 5.0 to 5.6. The difference between Edam and Gouda lies predominantly in shape and size, with Gouda traditionally coming in large, flat cylindrical loaves (4-12 kg) while Edam is an oblate sphere of about 2 kg. Edam is generally matured for 6 weeks, and has a lower fat content and a shorter texture than Gouda, which may be ripened for 6-60 weeks. Traditionally Edam is coated with red wax, while Gouda is covered in a yellow or sienna coloured plastic coat.

The basic principle of manufacture of these cheeses is cooking and frequently washing rennet curds, allowing little acid development, with curds being generally

matted under warm whey. Following drainage, the curds are poured into characteristic moulds, pressed, brine salted and ripened under a range of conditions, which are described in section 1.2.2.2. Typical composition of Gouda cheese is >48 % fat in dry matter, 25-28 % protein, 1.5-2.2 % salt and 42-45 % moisture. However, moisture content may vary from 41.5% for block Gouda to 47% for small loaves and 53.0 % for Gouda 20+ (Lolkema, 1993).

1.2.2. Manufacture of Dutch-type cheeses

1.2.2.1. MILK SELECTION

Milk quality must be excellent for the manufacture of Dutch-type cheeses, as the high pH of ripening is outside the optimum growth restriction action of lactic acid against spoilage bacteria (Kosikowski, 1982; Scott, 1986). Seasonal and lactational variations in clotting properties and suitability of milk for cheesemaking are dealt with elsewhere in this review. Genetic variants of milk protein will influence cheesemaking properties of milk, with the κ-casein variant AA giving a Gouda curd which is more susceptible to mechanical damage and increased fat losses in the whey than that prepared from milk containing variant AB or BB (van der Berg *et al.*, 1992). These authors also reported that ß-lactoglobulin variant B correlated with increased conversion of milk protein to cheese, and thus these factors may be considered when selecting cheesemilks. Milks to be stored for long periods before cheesemaking are frequently given a mild heat treatment (thermisation, around 10-15 sec at 63-65°C) to kill psychrotrophic organisms without greatly altering the milk. Prior to cheesemaking the milk is then HTST pasteurised (72.5°C for 15 sec). A third treatment sometimes found in Dutch cheese factories is bactofugation, where high speed centrifugation removes bacteria (including spores) from the milk in a sediment which is UHT treated and returned to the milk, which enhances the microbiological quality of the milk even further. The milk is frequently standardised to yield the desired FDM in the cheese, with 1.6% milkfat yielding a FDM of 30% and 3.0% milkfat giving a FDM of 45% (Scott, 1986). Standardisation on a casein/fat basis to a final ratio of 0.65-0.75 has also been reported (Kanawjia *et al.*, 1991).

Permitted additives to the milk may include calcium chloride (to speed up syneresis and reduce variability in milk renneting characteristics), colouring agents and sodium nitrate (to prevent blowing by coliforms and growth of *Clostridium tyrobacterium*). Nitrate may sometimes be added to the curd-whey mixture after removal of about half of the whey, for reasons of economy and reducing nitrate levels in whey (Kanawjia *et al.*, 1991). It has been shown that nitrate addition to Gouda cheese does not result in nitrosamine formation during ripening (Goodhead *et al.*, 1976). Whey protein concentrate (WPC) has been added to Gouda cheesemilk in order to reduce fat content in experimental trials, but has been reported to lead to textural problems (Abrahamsen, 1979). Buttermilk has also been added to Edam cheesemilk

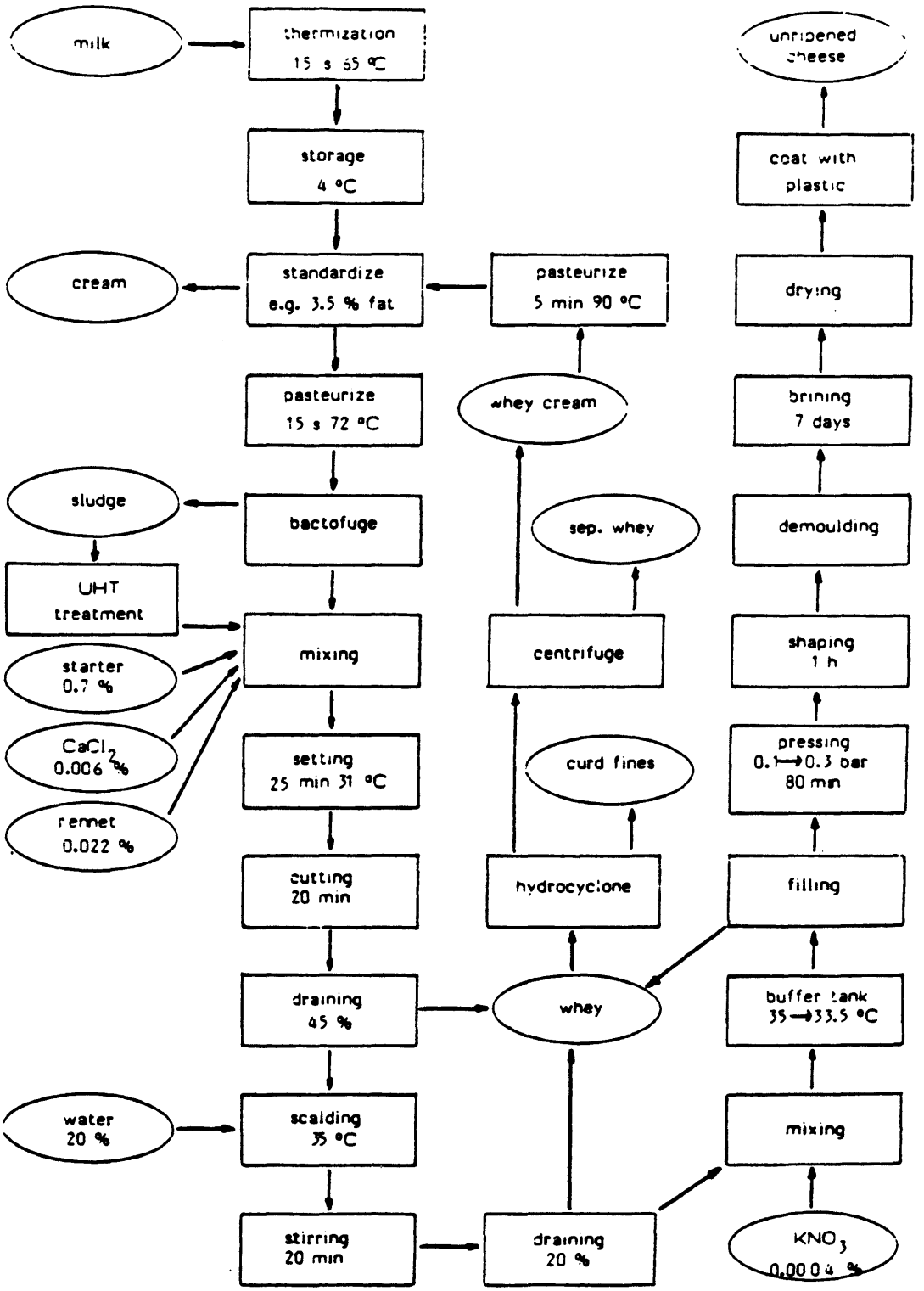


Fig. 1.2 *Example of a flow-sheet for manufacture of 12 kg blocks of Gouda cheese. Time from cutting to start of filling is 60 min. NaCl content of brine, 14%, brine temperature, 14°C. If bactofugation is omitted, 0.0016% KNO_3 is added. (From Walstra et al, 1987)*

and has been reported to accelerate rennet coagulation and lactic acid production, with reduced cheese yield (Kanawjia *et al.*, 1991).

1.2.2.2. CHEESEMAKING PROCESS

A typical process flow sheet for the manufacture of Gouda cheese is shown in Fig. 1.2. The main points to be considered in relation to final cheese composition are final moisture content, cheese pH and the retention of calcium phosphate and rennet in the curd. The syneresis rate is vital in regulation of the process and subsequent cheese composition.

Starter cultures are adventitious micro-organisms which are utilised to bring about desirable changes in the curd during preparation and ripening. These are usually added before renneting, to produce acid and hasten the coagulation of milk. In Gouda cheesemaking, starter is generally added at 30°C, and allowed ripen for 20-30 mins. The level and activity of starter will effect curd pH and the retention of calcium phosphate, which, if low, leads to low yields, low buffering capacity and subsequent soft cheese texture. Low curd pH leads to increased rennet retention in the curd. Typical starters consist of strains of *Lactococcus lactis* subsp. *cremoris* and/or *Lactococcus lactis* subsp. *lactis* as acid producers and *Leuconostoc lactis* and/or *Leuc. cremoris* as citric acid fermenters (B-type starters). Alternatively both the leuconostocs plus *L. lactis* biovar. *diacetylactis* may be used, in which case there is more rapid citric acid fermentation and CO_2 production (known as BD starters). Direct vat starters (DVS, or direct vat inocculum, DVI) which are supplied lyophilised or deep frozen offer great practical, hygienic and economical advantages over older culture preparation methods. Gouda cheese made with DVS starters, when compared to cheese made with conventional starter propagation procedures, gave comparable yields, acid development, improved eye formation and preferable organoleptic scores (Delbeke, 1985). "Practice (P)" starters, which are used in Netherlands cheese factories, have been selected for their taste and flavour attributes, rate of acidification, eye formation and phage resistance characteristics (Kanawjia *et al.*, 1991).

In the next step, rennet is added (20mls/100L) and the curd cut after 30 minutes, a time judged to give a curd which can be cut easily, does reduces fines in whey and synereses rapidly. The curd cubes are of 8-15 mm size and are stirred gently at first to minimise creation of fines, and then, as the curd firms, more vigorously. Microbial rennets (produced by a genetically engineered strain of *Kluyveromyces fragilis*, van den Berg and deKoning, 1990) and immobilised chymosin (immobilised using paraffin wax, Shindo *et al.*, 1980) have been used experimentally to coagulate milk for Gouda cheese manufacture, and both were concluded to allow a good quality Gouda, similar to that obtained with calf rennet, to be produced. Microbial rennets, although cheap and easily produced, have been reported to differ from calf rennet in terms of proteolysis of casein, particularly ß-casein (Kanawjia *et al.*, 1991).

After some time (15-30 min.), the curds are allowed settle and part of the whey (generally about a third) is removed to make stirring more effective by increasing forces

on curd particles and thus syneresis. Warm water (50-60°C) is added to the mix gradually (over 10-20 min, to avoid plasticising the curd), by spraying, until the original volume is reached again. The final temperature reached may be 33°C (Edam, Walstra *et al.*, 1987), 35°C (Gouda, Walstra *et al.*, 1987) or 36-37°C (Edam and Gouda, with reported range of 32-40°C, Scott, 1986). Cooking has the effect of speeding up syneresis, removing lactose and buffering substances and resulting in a higher cheese pH. This occurs because lactose diffuses from the grains of curd to the diluted whey to equalise the lactose concentrations, leaving less lactose for lactic acid production. However, excess water addition results in yield losses. The pH of the cheese is best controlled by adjustment of the washing step, while final cheese moisture content is very much dependant on curd cutting and stirring.

After a certain time (pH, for Gouda cheese, down to 6.5, typically a further 20-30 min.) stirring is stopped and the grains allowed to sediment. Curd expulsion at this stage is enhanced by placing perforated metal plates on top of the curd and applying pressure (pre-pressing). This promotes curd fusion and removes any trapped air to yield a flexible homogeneous texture. The curds are then cut to size, fitted into shaped moulds and pressed for several hours, for example, at 5-10 psi for 8-12 hours (Kanawjia *et al.*, 1991). The cheeses are then removed from the press, and if sufficient lactose to lactic acid conversion has taken place, they are placed in brine (around 18% salt is usual) to cool the cheeses and ensure a final salt content of 1.5-2.2% salt in cheese after a suitable brining time. Following removal from the brine, the cheeses are waxed (in the case of traditional Edam cheese) or more recently, covered in a dispersion of polymer molecules, which when dry forms a semi-permeable plastic coat on the cheese surface. Linseed oil or paraffin may also be used to package these cheeses (Kanawjia *et al.*, 1991). Ripening conditions vary and may comprise 12-16°C and 85-90% relative humidity (RH) (Walstra *et al.*, 1987), 3-4 weeks at 12-14°C (Edam, Scott, 1986), 3 months at 15°C (Kosikowski, 1982) or 2-6 weeks at 15°C (Gouda, Scott, 1986). The reactions involved in the maturation of this type of cheese will be discussed separately.

A number of recent advances have been made in the automation of manufacture of these cheeses. In particular the Casomatic system allows continuous prepressing of cheese curds (Alfa-Laval, Tebel Mkt, Holland). These are towers which are initially filled with whey, before curds and whey are pumped in, and the curds on descending the tower are pressed under their own weight, under whey. On arrival at the bottom of the tower, a guillotine system cuts blocks of predetermined size and shape. Horizontal presses where the curds are prepressed under whey between perforated steel plates on a continuously moving conveyor are also used, with the residence time on the belt being chosen to give the required degree of pressing. APV Anhydro Ltd. have developed a system whereby milk is ultra- and dia-filtrated to the desired solids level (55-60%) and filled directly into moulds, whereupon rennet and starter are added and the cheeses formed in the mould. The cheeses are then salted, packaged and ripened.

1.2.3. The ripening of Dutch-type cheese

Development of the properties of Dutch-type cheese varieties is due particularly to the conversion of lactose, protein, fat and citric acid during the ripening, or maturation period, leading to changes in the structure, composition and organoleptic properties of the cheese (Walstra *et al.*, 1987). Biochemical, chemical, physical and microbiological aspects are involved.

1.2.3.1. FERMENTATION OF LACTOSE AND CITRIC ACID

The starter bacteria ferment lactose quickly and almost completely to L-lactate, preserving the cheese by reducing pH and available carbohydrate. This is generally complete within 24-48 hours of manufacture. The lactate may be further broken down to acetate (Fox *et al.*, 1993). *L. lactis* biovar. *diacetylactis* and *Leuconostoc* species in Dutch-type cheese starters metabolise milk citrate rapidly to CO_2 and a number of by-products such as pyruvate, diacetyl, acetoin, 2,3-butyleneglycol and possibly lactate. Production of CO_2 is one of the main factors responsible for eye formation in these cheeses. Starters high in leuconostocs plus). *L. lactis* biovar. *diacetylactis* (known as BD starters) may be selected for more rapid CO_2 production, and hence more extensive eye formation (Walstra *et al.*, 1987).

1.2.3.2. LIPOLYSIS

In Dutch type cheese varieties lipolysis occurs during ripening, but only to a limited extent (Walstra *et al.*, 1987), and even a moderate level of free fatty acids in these cheeses can lead to their being rejected as rancid. Pasteurisation largely inactivates native milk lipase, and thermisation prevents production of psychrotrophic lipases on cold storage of cheesemilk. Thus, any lipolysis which occurs during ripening is due to the action of residual milk lipase, heat-resistant psychrotrophic lipases or lipases of starter bacteria. These latter enzymes cannot degrade triglycerides easily, but can produce free fatty acids from mono- or di-glycerides which have been produced by the action of other lipases (Stadhouders and Veringa, 1973). These may be supplied by Gram-negative bacteria (Driessen and Stadhouders, 1975). Milk lipase activity is reduced by the NaCl content of cheese, but increases at higher temperatures, and so has always been estimated to have a limited role in cheese ripening. The role of pH is unclear, as in substrates other than cheese, lipase activity decreases with pH, but in cheese the acidity of fat has been reported to increase in cheeses of low pH, possibly due to altered distribution of short-chain fatty acids in the fat phase. Lipoprotein lipase (LPL), the predominant milk lipase, has been shown to increase free fatty acid levels in cheese if added to the cheesemilk, with high levels of short-chain fatty acids being produced (Vlaemynck, 1992).

1.2.3.3. PROTEOLYSIS - METHODS FOR DETERMINATION

The three main proteolytic agents active in Dutch-type cheeses during ripening are rennet, starter and milk proteinase. Each of these components will be discussed in detail later, but first it is worth considering the techniques which have been developed for the examination of the separate roles of these agents, and quantitative and qualitative examination of proteolysis in cheese.

(a) Production of cheeses in which the actions of various proteinases are separated

Kleter (1975) described an apparatus for making Gouda cheese under strictly aseptic conditions, in sterilised enclosed vats, which when combined with an aseptic milking technique (Kleter and deVries, 1974), provided an early system for attempting these type of experiments. To examine the action of rennet and milk proteinases, but not bacterial enzymes, glucono-delta-lactone (GDL) was used to lower pH. GDL has the advantage of gradual hydrolysis to gluconic acid, with an accompanying decrease in curd pH to the desired levels, without acid-shock. This method was used in 1977 to examine starter action in Gouda cheese (Kleter, 1977). These techniques were also used by Visser (1977a-d) in a similar but extended study. Aseptic rennet-free (ARF) cheese was made by a process of inactivation of the enzyme (by pasteurisation) after completing its primary action in chilled milk from which the calcium had been removed by ion-exchange, prior to coagulation (Visser, 1976). Starter and calcium chloride are then added and the mix warmed to 30°C, whereupon a rennet free coagulum forms. This method involves a longer make time due to the slower syneresis of the curd. Aseptic starter free (ASF) cheese was acidulated by GDL and lactic acid addition, and a combination of these techniques was used to manufacture aseptic starter and rennet free cheese (ASRF).

The above method of preparation of rennet-free curd was used by Noomen (1978), who also included thimerosal - ((carboxyphenyl)thio) ethylmercury sodium salt - as an antibacterial preservative. Curd was frozen, homogenised and mixed with water to make simulated Noordhollandaise Meshanger cheese (a soft Dutch variety). Noomen (1978) also prepared a calcium paracaseinate-calcium phosphate complex for model cheese ripening experiments.

(b) Analysis of cheese proteolysis

The subject of assessment of proteolysis in milk is dealt with in detail in chapter 3. In this section, however, I will briefly introduce the methods used to examine proteolysis in Gouda-type cheeses. For a comprehensive review of methods of assessing proteolysis in cheese see MacSweeney and Fox (1993).

1. Quantitative methods Gupta *et al.* (1974) extracted brine-soluble protein (BSP) from Gouda cheese and quantified the total and noncasein nitrogen (N) therein, as an index of proteolysis during ripening. Visser (1977c) fractionated the soluble N (SN) fractions in Gouda cheese for quantification by Kjeldahl as follows. Cheese was

dissolved in a hydrochloric acid solution for determination of total N (TN), while SN was estimated in an extract of cheese made with a calcium chloride/sodium chloride solution, and amino acid N (AN) was prepared by filtration of a phosphotungstic acid (PTA) extract of the SN extract. Individual free amino acids were determined after acid hydrolysis. Similar methods were used by Exterkate *et al.* (1987) and Bartels *et al.* (1987a and b). A method for determining the ripening time of Edam and Gouda cheese by monitoring the water-soluble N/TN ratio and 12% TCA soluble N/TN ratio was reported by Venema *et al.* (1987).

2. Qualitative methods Urea polyacrylamide gel electrophoresis (urea-PAGE) was used by Creamer (1975), Gupta *et al.* (1974), Visser (1977c) and Visser and DeGrout-Mostert (1977) among others to follow the breakdown of α_{s1}- and ß-casein in Gouda cheese during ripening. Conventional column chromatography techniques were used to examine the range of proteolysis products in Gouda cheese by Creamer (1975, ion exchange chromatography on DEAE cellulose), Visser (1977b, gel filtration on Sephadex G50) and Visser (1977, molecular sieving of hydrophobic peptides on Sephadex LH20). High performance liquid chromatography (HPLC) on reversed phase supports (RP-HPLC) was used by Kaminagowa *et al.* (1986) to identify low molecular weight peptides in Gouda cheese. Haasnoot *et al.* (1989) compared RP-HPLC to anion-exchange- cation-exchange- and gel filtration-HPLC columns for determination of the extent of proteolysis in Gouda cheese and concluded that the ratio of γ-casein peaks to ß-casein peaks can serve an indicator of cheese maturity. RP-HPLC was concluded to be the most suitable method for separation of water-soluble breakdown products, but had the disadvantage that classification of cheeses is based on unidentified peaks. The method of Harwalker and Elliott (1971) for extraction of bitter peptides from cheese was used by Visser (1977d) and Bartels *et al.* (1987), followed by chromatography on Sephadex LH-20 and Sephadex G-25 respectively to examine bitter peptide production in Gouda cheese. Igoshi *et al.* (1986) used extraction in various buffers followed by ion-exchange chromatography to extract and characterise proteinases from ripening Gouda-type cheese and isolated two serine- and two acid-proteinases.

1.2.3.4. THE CONTRIBUTION OF VARIOUS RIPENING AGENTS TO PROTEOLYSIS IN GOUDA CHEESE

Visser (1977 a-d) manufactured Gouda cheese in which the contributions of each of the enzymatic components of cheese to protein breakdown were isolated and measured, using the methods described above. A summary of the results of this study is presented in Table 1.1, and a graph of the relative contributions of the different components is shown in Fig. 1.3. The contributions of the individual ripening agents as determined in this and other studies are described below.

Table 1.1 *Quantity of soluble N compounds as produced in aseptically made Gouda cheese by the combined and separate actions of rennet, starter bacteria and milk proteinase (Visser, 1977c, Walstra et al., 1987)*

Ripening Time (mo.)	Proteolytic System	Soluble N as % of total N				
		Total	As peptides of MW			Amino acids
			>14,000	14,000-1400	<1400	
1	Rennet	6.7	2.7	2.7	1.2	0.1
	Starter	2.5	0.2	0.6	0.4	1.3
	Milk proteinase	2.0	0.2	0.4	1.3	0.1
	All (cheese)	12.2	1.8	2.3	6.1	2.0
3	Rennet	12.7	3.6	5.2	3.7	0.2
	Starter	4.7	0.3	0.7	1.4	2.3
	Milk proteinase	3.3	0.4	0.7	1.9	0.3
	All (cheese)	19.5	2.3	3.3	9.1	4.8
6	Rennet	17.3	4.4	4.1	8.4	0.3
	Starter	7.6	0.9	0.3	2.4	4.0
	Milk proteinase	4.7	0.5	1.0	2.7	0.5
	All (cheese)	26.0	5.5	2.3	10.8	7.4

1. Rennet The single most important enzymatic reaction in cheese biochemistry, the hydrolysis of the sensitive Phe (105) - Met (106) bond of κ-casein, is due to the action of chymosin on calf rennet. This reaction leads to the destabilisation of the casein micelle, leading to aggregation under the influence of calcium ions which gives the curd at the start of cheesemaking (Visser, 1981; Fox, 1989). Any rennet substitute must fulfil this function. The rate of this reaction is reduced at elevated milk pHs and in high heat treated milk (Fox, 1989).

The action of rennet depends greatly on the quantity retained in the curd, which is determined by the quantity of rennet added to, and heat treatment of the cheesemilk, the cheese cooking temperature, moisture and pH (Walstra *et al.*, 1987). Typically 6% of added rennet is retained in the curd (Fox, 1989). Increased heat treatment of the milk, greater pH drops during cheesemaking and lower cooking temperatures, all result in increased rennet retention. Rennet is responsible for the formation of the largest part of the soluble nitrogen by direct hydrolysis of paracasein, and the liberation of high and low molecular weight peptides, but not amino acids (Table 1.1). Microbial rennets from *Mucor miehei, Mucor pusillis* and *Endothia parasitica* were found by Vanderpoorten and Weckx (1972) to liberate more non-protein nitrogen (NPN) than

calf rennet in Gouda cheese, with elevated proteolysis of both α_{s1}-casein and ß-casein. This was also reported by Fox *et al.* (1993).

Studies on isolated α_{s1} and ß-casein show that the most sensitive bonds of α_{s1}-casein to chymosin action are the Phe (23) - Phe (24) and Phe (24) - Val (25) bonds, cleavage of which leads to the formation of α_{s1}-I casein (Visser, 1981). Hydrolysis of ß-casein towards the C-terminal end at the Leu (192) - Tyr (193) and Ala (189) - Phe (190) bonds leads to the formation of ß-I, ß-II and ß-III caseins. Stadhouders *et al.* (1983) found that purified chymosin produced bitter peptides from all caseins irrespective of salt concentration.

Visser and de Groot-Mostert (1977) found that in normal aseptic cheeses α_{s1}-casein is attacked more rapidly than ß-casein during ripening, with the former being nearly degraded after one month of ripening and 50% of the latter remaining after 6 months ripening. The degradation of α_{s1}-casein, and some hydrolysis of ß-casein was due primarily to rennet action in starter free cheeses. The conditions of young Gouda cheese (pH near 5, 4% salt in moisture) provide near optimal conditions for the action of chymosin, but hydrophobic intramolecular interactions protect ß-casein to a great degree from attack by chymosin. Rennet has also been implicated in the formation of bitter peptides in Gouda cheese (Visser, 1977b), and this author concluded that increasing the quantity of rennet causes this defect to appear earlier and to be more intensive.

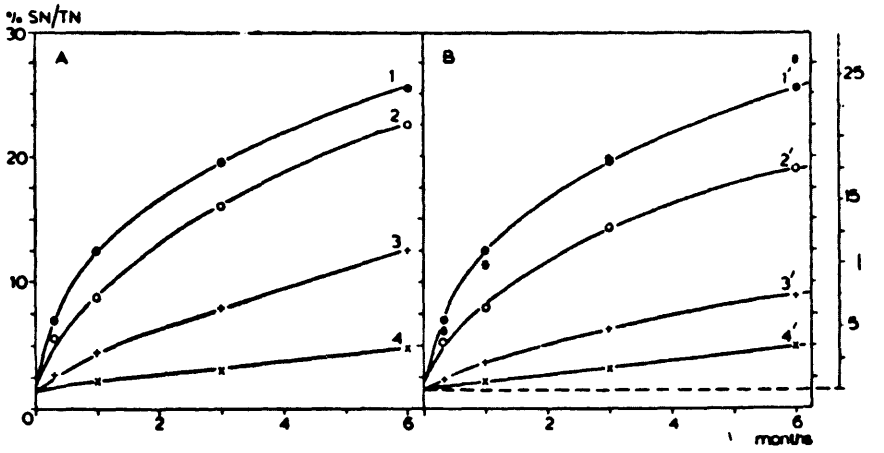


Fig. 1.3 *(A) Survey of the average soluble N values developed during the ripening of normal aseptic cheeses (1), Aseptic starter free cheeses containing the normal amount of rennet (2), aseptic rennet free cheeses (3) and Aseptic starter and rennet free cheeses (4). (B) Net contribution of rennet (2'), starter (3') and milk protease (4') to soluble-N production as compared to normal aseptic cheese (1'*) (Visser, 1977b).*

2. *Starter* The lactic acid bacteria used as cheese starters are only weakly proteolytic, but contain a wide range of proteinases and peptidases capable of hydrolysing casein (Fox, 1989). It is thought that starter bacteria attain maximum numbers in Cheddar and Dutch cheeses towards the end of manufacture, and then decline quickly, lysing after death and releasing their intracellular enzymes into the cheese but this has to be confirmed (Fox, 1989).

When acting alone, starter bacterial enzymes hydrolyse intact caseins, but relatively slowly (Walstra *et al.*, 1987; Visser, 1977b; Visser and deGroot-Mostert, 1977). From Table 1.1 it can be seen that the enzymes of starter bacteria are the only ones capable of producing amino acids, due to the action of intracellular peptidases released during cheese ripening. In normal aseptic cheeses Visser (1977b) found a higher level of free amino acids than in aseptic rennet free cheeses, indicating that rennet produces suitable substrate polypeptides for the action of starter bacteria in liberating amino acids. Gel filtration fractionation of proteolysis products in this study showed that the proteolytic systems of starter bacteria progressively convert high- and medium-MW soluble products of rennin action to low-MW peptides and amino acids.

Starter strains may be divided into bitter and non-bitter strains based on their tendency to produce bitter off-flavours (due to hydrophobic peptides) in Gouda cheese. Kleter (1977) found a remarkable difference between *L. lactis* subsp. *cremoris* strains in their ability to produce free amino acids in aseptic Gouda cheese, with bitterness being associated with lower degrees of production of these components. Visser (1977a-d) and Visser and deGroot-Mostert (1977) used bitter and non-bitter strains of *L. lactis* subsp. *cremoris* in their examination of the contribution of the various enzymes to proteolysis in Gouda cheese. Distinct bitterness was observed in cheeses made from bitter strains from one month of ripening onwards. It was shown that non-bitter strains accumulate far higher levels of free amino acids in normal aseptic and aseptic rennet free cheeses than bitter strains, although the strains did not differ appreciably in their ability to liberate certain amino acids, and showed similar electrophoretic patterns of casein breakdown. It was concluded that non-bitter strains may possess a more powerful peptidase system and degrade bitter peptides in cheese more intensively than bitter starters. Hydrophobic extracts of bitter peptides from these cheeses showed that non-bitter strains of starter do produce bitter peptides, but that these are broken down by peptidases before reaching a threshold organoleptic level (Visser, 1977d).

Stadhouders *et al.* (1983) confirmed that non-bitter starters are more capable of degrading bitter peptides than bitter starters, possibly due to a different, less restricted location of intracellular enzymes. Membrane proteinases were particularly associated with bitter peptide breakdown. This was further investigated and supported by work in model casein systems by Visser *et al.* (1983a), who found cell wall proteinases to produce bitter peptides and membrane associated proteinases plus cytoplasmic proteinases to degrade these. It was found in this study that salt decreases the permeability of the cell envelope and causes hydrophobic association of bitter peptides (which are mainly produced from the C-terminal region of ß-casein (Visser *et al.*, 1983b)), which in combination result in preventing these peptides from reaching the

debittering peptidase system. This was proposed to be particularly true for bitter strains of starter, although both types of starters produce bitter peptides in cheese, and it is only the efficiency of degradation of these which determines whether a bitter off-flavour will occur or not.

In solution, the cell-wall proteinase of *L. lactis* subsp. *lactis* NCD0763 has been shown to hydrolyse 5 bonds in the very hydrophobic region of ß-casein, but its action in cheese is unknown (Fox, 1989). Kaminagowa *et al.* (1986) identified several low molecular weight peptides from α_{s1} -casein hydrolysed by cellular proteases of *L. lactis* subsp. *cremoris* H61 and concluded that this represented one of the principal means of α_{s1} -casein degradation during Gouda-type cheese ripening. All peptides were produced by hydrolysis of Glu-Gln bonds, of which there are many in caseins, so this enzyme may make a major contribution to casein breakdown during the ripening of cheese. Balaiah and Joshi (1980a, b) showed that Gouda cheese made from a 50/50 *L. lactis* subsp. *lactis*/ subsp. *cremoris* starter had a higher flavour score and degree of proteolysis than cheeses made with either organism alone. When *S. lactis* subsp. *cremoris* was also included (at 40% of total starter) the cheese had a bitter flavour and received a low score.

3. Milk proteinase Visser (1977c) showed that in aseptic starter and rennet free cheeses (ASRF) milk proteinase, on it's own, contributed to a minor, but measurable degree, to soluble-N production (see Table 1). The action of plasmin was evident in the accumulation of lysine over ripening, at which residues plasmin preferentially attacks. In these cheeses ß-casein degradation was visible by urea-PAGE, as was a slight decrease in α_{s1} -casein (Visser and deGroot-Mostert, 1977). Milk protease action on ß-casein has been identified as a source of bitterness in milk, but never in Gouda cheese (Visser, 1982). Evidence of milk acid protease action during Meshanger cheese ripening was reported by Noomen (1978). The role of plasmin and other milk proteinases in cheese ripening is described in detail in Chapter 2.

1.2.3.5. NON-STARTER BACTERIA IN GOUDA CHEESE

Non-starter, or adventitious, bacteria in Gouda cheese must also be considered. Sources of micro-organisms in the manufacture of cheese are summarised in Fig. 1.4. The contaminating bacteria may be classified as (Walstra *et al.*, 1987);

- coliforms, which will grow during the early stages of cheesemaking at high pH and temperatures, and may lead to off-flavours and 'blowing' of cheese;
- butyric acid bacteria (such as *Clostridium tyrobutyricum*), which degrade lactic acid to butyric acid, CO_2 and H_2, and which lead also to 'blowing' during later stages of ripening (late blowing);
- thermoresistant streptococci;
- propionic acid bacteria;
- lactobacilli;

The use of nitrate in cheese manufacture prevents blowing by coliforms, propionic acid bacteria and butyric acid bacteria. Butyric acid bacteria are anaerobic spore formers, and thus will be removed by bactofugation of the cheesemilk, allowing nitrate utilisation to be reduced. Also, milk and plant hygiene must be considered as contamination by increased numbers of coliforms in cheese will cause nitrate to disappear too quickly from the cheese, increasing the possibility of butyric acid fermentation. Kleter *et al.* (1984) suggested that butyric acid bacteria growth could be controlled by increasing the lactic acid (adding calcium lactate to milk) content in combination with use of a certain initial salt content, without inhibiting the lactic acid bacteria unacceptably. Propionic acid bacteria keep down cheese pH by production of propionic acid and acetic acid from lactates (which can lead to a sweet taste), and cause a very open texture due to CO_2 production. The growth of these bacteria is inhibited by nitrate and high salt concentrations.

Mesophilic normal or salt-tolerant lactobacilli growing in cheese may cause flavour and texture defects, and enter the cheese generally through contamination after pasteurisation of the milk (Kleter, 1977). Growth may be controlled by use of acidified brines (pH ≤ 4.6) kept at low temperatures (13°C) (Walstra *et al.*, 1987). The role and activity of these bacteria during cheese ripening has been the source of much interest. Kleter (1977) stated that they have negligible effect on proteolysis and may lead to undesirable off-flavours and Nieuwenhof *et al.* (1969) considered that their growth stimulated the growth of propionic acid bacteria, and thus excessively open cheese texture. Laleye *et al.* (1987) showed that heterofermentative lactobacilli were more

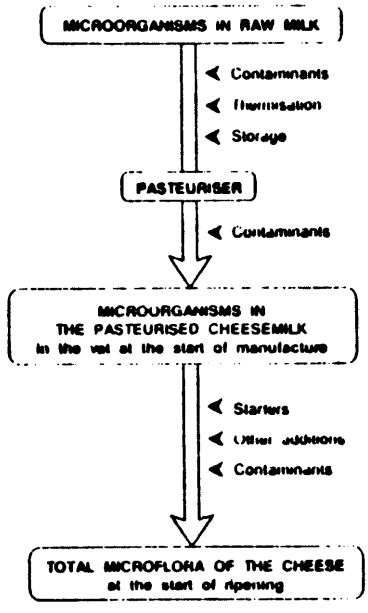


Fig. 1.4 *Potential sources of micro-organisms in the manufacture of cheese (Martley and Crow, 1993).*

numerous in a range of defective cheeses (including Gouda) than high quality products, and, if present, increased in numbers in aged cheese to become a major part of the dominant microflora of the cheese. They concluded that these bacteria were more important in gas blowing than coliforms, yeasts, moulds and spore-formers. Narvhus *et al.*, (1993) investigated the presence of lactobacilli in Gouda-type cheese made from commercial DI starters (not containing lactobacilli), and found these organisms to be present in cheeses from ten out of eleven Norwegian dairies tested. Predominant were *Lactobacillus casei* subsp. *casei* and *pseudoplantarum*, with numerous other types also detected. Non-starter leuconostocs were also detected in 35% of cheeses and were associated with abnormal eye formation. Neither species nor number of lactobacilli isolated were correlated with sensory score, degree of proteolysis or volatiles in cheese.

Martley and Crow (1993) studied the role of non-starter micro-organisms in cheese manufacture and ripening, and suggested that pasteurisation efficiency should be measured by comparison of bacterial counts rather than reliance on the alkaline phosphatase test. They also concluded that since adventitious non-starter lactic acid bacteria (NSLAB) are always likely to gain access to cheese in commercial factories, deliberate addition of selected adjunct cultures along with the normal lactic culture, which will inhibit undesirable NSLAB growth, may become a practical means of controlling cheese flavour development. This inhibition may be direct or indirect, where competition for substrate controls bacterial growth. In the case of Gouda cheese, leuconostocs fulfil this role to an extent, as well as contributing to eye formation.

1.2.3.6. ACCELERATED RIPENING OF GOUDA CHEESE

There has been an increased interest in speeding up the process of flavour generation in, and texture modification of, cheeses in the past few years, principally due to the costs of storage and ripening for long periods. The four main methods which have been used are; control of temperature, use of added enzymes, addition of attenuated or mutant starters and the use of cheese slurries (Exterkate *et al.*, 1987). Extreme care must be taken to avoid unbalanced flavour developments and textural defects. For example, when adding increased starter numbers, the problem of accelerated acid production during cheesemaking must be considered. This was addressed by Exterkate *et al.* (1987a) who added heat-treated mixed strain starter cells to the cheesemilk, which could be added in increased numbers (two to four times more than controls) without risk of uncontrolled acidification due to partial or complete inactivation of lactose degrading enzymes. This effect can also be obtained by treating cells with butanol, in which case peptidase activity and production may actually increase (Stadhouders *et al.*, 1983). The cheeses produced by the accelerated process of Exterkate *et al.* (1987) had a better taste, moderately increased Gouda flavour, but only a moderate increase in the extent of proteolysis, particularly in the case of amino acid nitrogen, which was concluded to be produced independently of primary casein conversion by starter. Overall, ripening time was reduced by at least 25%.

Exterkate (1987) reviewed the area of accelerated ripening of Gouda cheese and suggested two pathways for accelerating Gouda cheese ripening. These depend on whether or not starter cells enzymes play a major role in primary proteolysis of caseins. Firstly, if starter enzymes are involved in primary proteolysis of caseins, amino acid nitrogen (AN) production could be increased by using, for example, Lac^- $Prot^+$ cultures (which can produce proteinases but cannot metabolise lactose) in increased numbers, to maximise the level and distribution of proteolytic activity in cheese, without the need for heat-shocking, which may inactivate some enzymes. However, this may lead to adverse effects on cheese consistency, and thus further knowledge is required to assess if proteinase or peptidase activity determines the rate of AN production. Alternatively, if primary proteolysis of caseins is dominated by chymosin, and effect of treated starter cells will only affect amino acid production, this fact should be exploited in designing methods to accelerate amino acid production in cheese without excessive gross hydrolysis of casein. For example, a blend of $Prot^-/Prot^+$ (which will have overall increased peptidase activity but still possess a degree of proteinase activity) cultures may work best for the breakdown of chymosin proteolysis products at a controlled rate, followed by maximal utilisation of these products for amino acid production. Addition of increased levels of chymosin itself is unsuitable, as it generally leads to bitterness (Visser, 1977b).

Bartels *et al.* (1987a) used sub-lethally heat shocked Lactobacilli and Streptococci to accelerate ripening of Gouda cheese and found that some *Lactobacillus* strains substantially increased proteolysis and release of amino acids (particularly *Lb. helveticus*). They also reduced bitterness (and the production of bitter peptides, as monitored by chromatography) but increased in some cases acetaldehyde and peppery flavours. Overall, accelerated cheeses were preferred to control cheeses, however, and it was recommended that control of addition levels could control bitterness and accelerate flavour development. The use of freeze-shocked *Lb. helveticus* was investigated (Bartels *et al.*, 1987b) and it was found that large numbers of cells could be added to the milk without detrimental effect to cheesemaking procedures and cheese quality, and that increased water soluble nitrogen and amino acid production resulted, with increased flavour intensities and reduced bitterness, while avoiding development of any off-flavours. The use of fungal proteases and freeze-shocked *Lb. helveticus* to accelerate the ripening of Gouda cheese made from normal and UF-concentrated milk was investigated by Kim *et al.* (1994), but this study merely examined effects of these treatments on cheese composition,, which was found to be unaffected by the enzymes or cells.

Addition of enzymes such as crude protease preparations (from *Micrococcus caseolyticus, Aspergillus oryzae* or *Mucor pucillus*), purified proteases, lipases and catalase to accelerate the ripening of Gouda cheese have also been described (Kanawjia *et al.*, 1991). Addition of enzymes is reported to shorten ripening times considerably, but the levels and combinations used must be carefully controlled to retain the characteristic flavour of the cheese.

1.2.3.7. THE FLAVOUR OF GOUDA CHEESE

Flavour is defined as the complex sensation comprising aroma, taste and texture. The parameters of taste, saltiness and texture are strongly related to the maturity of Gouda and Edam cheese, and maturity is often judged on these parameters (Venema *et al.*, 1987). In Dutch-type cheeses flavour components are principally the breakdown products of lactose and citric acid (lactic acid, diacetyl, CO_2 etc.), lipids (free fatty acids) and paracasein (peptides and amino acids). Little is known about aroma components in these cheeses (Walstra *et al.*, 1987). A correct balance must be maintained between all flavour components for suitable organoleptic properties. Lactic acid causes the refreshing acid taste, and a shortage of this component renders cheese insipid, while an excess leads to sourness. Large changes in flavour and aroma develop during maturation due to fermentation of lactose and transformation of the by-products of this pathway (such as aldehydes, ketones, esters, alcohols, organic acids).

Proteolysis also affects flavour directly, as tasteless paracasein is degraded to a range of peptides and amino acids which may contribute bitter, sweet, nutty or brothlike notes to the taste of the cheese. Higher temperatures, higher pH, higher moisture content and lower salt levels all favour proteolysis and particularly the formation of amino acids. The subject of water soluble flavour components in Cheddar cheese was extensively studied by Aston and Creamer (1986), who found that free amino acids make a significant contribution to the flavour but that other materials, probably peptides, are necessary for full flavour. Bartels *et al.* (1987), studying Gouda cheese, stated that proline and amino acids associated with calcium and magnesium ions are associated with sweet flavour. Amino acids may also be further degraded to volatile components such as ammonia, amines and hydrogen sulphide. Visser (1977b) studied the contributions of various ripening agents to development of cheese flavour in Gouda cheese and found that non-bitter starters, in rennet-free cheeses, led to the formation of characteristic cheese flavour during ripening, while bitter strains developed considerably less flavour. Very little flavour was detected in cheeses made without starter, and it was concluded that rennet played only a minor role in cheese flavour development.

Bitterness is one of the principal flavour defects affecting Gouda cheese, and is attributable to the formation and accumulation of bitter tasting peptides liberated from casein components by proteolytic action. The contributions of rennet, starter and milk enzymes to bitterness was studied by Visser (1977b) who found that rennet and starter, but not milk enzymes, were capable of producing bitter flavour in cheese. The findings of this study with regard to proteolysis have been discussed earlier in this review, and may be summarised in Fig. 1.5. Bitter starters liberate important amounts of bitter peptides directly from paracasein while non-bitter starters, while also producing bitter peptides, have the capacity to degrade them to non-bitter products. Rennet produces bitter peptides non-specifically via non-bitter intermediate polypeptides, and these are also broken down by the peptidases of non-bitter starters.

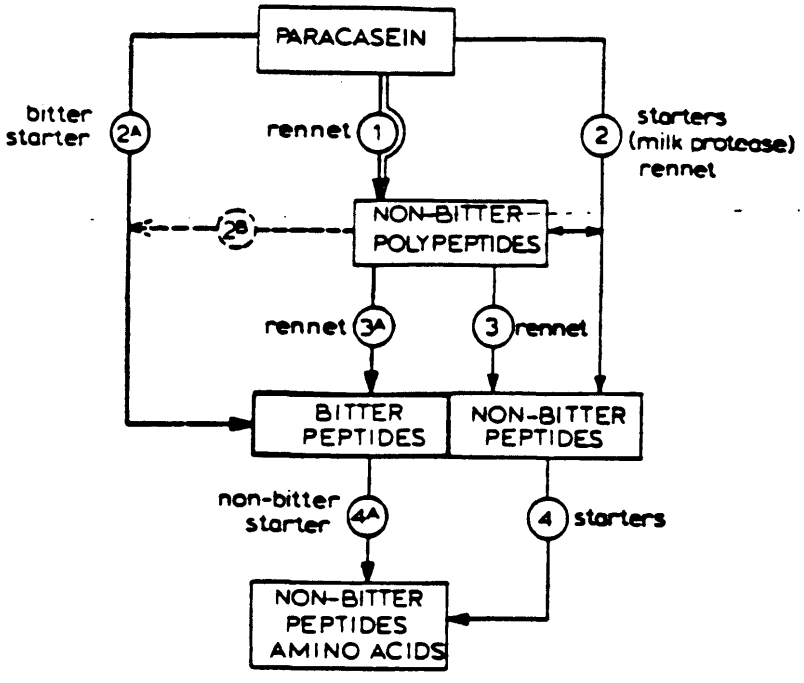


Fig. 1.5 *Mechanism for the development of bitterness in Gouda-type cheese. Thin lines: paths of general proteolysis in cheese; thick lines: paths important for bitterness development (Visser, 1977b).*

Salt accentuates cheese flavour and CO_2 also appears to influence retention of flavour. Venema *et al.*, (1987) found that texture and saltiness strongly influenced perception of maturity of Gouda and Edam cheese, as drier and saltier samples from the rind of a cheese will always be perceived as older than samples from the centre of the same cheese. Salt also inhibits formation of bitter peptides, rather than simply masking the taste, by affecting aggregation of peptides and permeability of starter cell membranes (Stadhouders *et al.*, 1983). Free fatty acids render mature cheeses piquant, but in excess will lead to rancidity.

1.2.3.7. THE TEXTURE OF GOUDA CHEESE

The main factors affecting consistency in Dutch-type cheeses are moisture content, extent of proteolysis, pH, NaCl and fat contents, inhomogeneity in composition and temperature (Walstra *et al.*, 1987). During ripening the cheese structure becomes more uniform due to fusion of curd grains, the cheese loses moisture due to evaporation and syneresis due to proteolysis, the paracaseinate network is broken down, pH increases and gas is formed. Thus overall, during maturation the elastic modulus of the cheese increases, the fracturability increases and the fracture stress decreases at first and then increases. Kalab (1977) compared the structure of

Gouda cheese to that of Cheddar and found no orientation of curd granules or protein and finer junctions between particles than those found in Cheddar cheese. This is presumably due at least in part to the prepressing stage of Dutch-type cheeses, which presses curd gently into a homogeneous mass before full pressing, compared to the pressing together of milled curd particles which occurs during the manufacture of Cheddar cheese. Also, the lack of a cheddaring step leads to less structured, orientated cheese. This results in a more elastic, less fracturable green curd.

Luyten *et al.* (1991a) described Gouda cheese as a viscoelastic material, which behaved in a viscous-like manner in young cheeses with low acid, whereas mature cheeses, or young cheeses with a low pH, showed more brittleness. The viscous properties were due to the properties of the protein matrix and friction with other components (such as fat particles) and interactions between the aqueous solution and the protein matrix. Short consistency of old Gouda cheese leads to fracture problems when attempting to slice and when biting this cheese, only small pressure is required to break a piece of cheese into smaller pieces, compared to younger cheese which requires far more effort to split completely. Shortness of cheese can be measured by judging how far a long cylinder of cheese must be bent before it fractures.

The number, size and shape of holes in the curd is considered an important texture characteristic in Dutch type cheeses. Holes are formed by CO_2 production by starter bacteria and may be excessive in case of spoilage by undesirable bacteria. Nuclei for hole formation are tiny air bubbles trapped in the curd, and holes form if gas pressure is sufficiently high (sufficiently fast gas production without loss from the curd). If the rate of gas production is sufficient and the cheese texture allows for slow viscous flow of the cheese material eyes (spherical holes) develop. If the consistency is short, due to low pH or calcium phosphate content, or there has been considerable proteolysis, or gas production is too fast, slits may develop in the vicinity of the holes (Walstra *et al.*, 1987; Luyten *et al.*,1991a).

Chapter 2

Plasmin and somatic cells in bovine milk

2.1. Plasmin, its nature and significance

2.1.1. OCCURRENCE, ISOLATION AND MEASUREMENT

Normal bovine milk is known to contain a number of different proteolytic enzymes, of which the best studied and characterised is the alkaline proteinase, plasmin. The existence of this enzyme has been known since the late 19th century, and the presence of its inactive precursor, plasminogen, was confirmed in 1979 (Eigel *et al.*, 1979). Plasmin is a serine proteinase whose primary physiological function is the solubilisation of fibrin clots in blood. It has a high specificity for peptide bonds containing lysine and it is optimally active at pH 7.5 and 37°C. The molecular weight of bovine plasminogen is approximately 90,000, and cleavage by urokinase, the best-characterised plasminogen activator, gives plasmin, a two chain molecule with a heavy (A) chain (mol. wt. 60,000) and a light (B) chain (mol. wt. 25,000) connected by a disulphide bond (for review, see Grufferty and Fox, 1988c). Milk plasmin has been shown to be identical to blood plasmin (Kaminagowa and Yamauchi, 1972).

Plasmin in milk is associated with casein in milk, as also is plasminogen, and it may be dissociated from the micelles by long exposure to ethanol (Warner and Polis, 1945), stirring at low pH (<4.6, Grufferty and Fox, 1988c) or addition of lysine or an analogue of lysine, 6-aminohexanoic acid (Richardson, 1983a), suggesting that plasmin is associated with casein micelles via lysine residues. The first approaches to purification of plasmin from bovine milk were by ammonium sulphate precipitation at low pH, (Warner and Polis, 1945; Zittle, 1965) and later approaches used chromatographic techniques to further purify these fractions (Kaminagowa *et al*, 1971; Reimerdes and Klostermeyer, 1974), including affinity chromatography to exploit the role of lysine in associating plasmin to casein (Halpaap *et al*, 1977, Rollema *et al.*, 1981).

Snoeren and Van Riel (1979) purified plasmin from crude α_{s2}-casein by ion-exchange chromatography with a NaCl elution gradient, a technique which was also used by Reimerdes (1981) to isolate plasmin and a thrombin-like enzyme from milk. Recently, Humbert *et al* (1990) used lysine-sepharose chromatography and immunoaffinity chromatography to purify milk plasmin and confirm that it is immunologically identical to blood plasminogen (or plasmin). Politis *et al*, in 1992, examined the relative amounts of immunoreactive plasmin and plasminogen in different fractions of bovine milk, and showed plasminogen to exist in several forms, with 82% of the total amount associated with the casein fraction, but lower concentrations found

in serum, cream and milk fat globule membrane (MFGM) fractions. Active plasmin was present in casein, serum and cream, and the low concentrations of both enzyme and zymogen found in serum and cream were associated with low casein contents found in these fractions.

Noomen (1975) described a method using rennet, calcium chloride and pH adjustment to coagulate protein, and used the increase in soluble protein, under these conditions, on incubation of preserved milk as a measure of total milk proteinase activity, which at the time he assumed to comprise exclusively of plasmin. More recently, however, two principal, more rapid, sensitive and convenient methods for the measurement of plasmin in milk or purified preparations of milk have been used. Both methods exploit the fact that plasmin has a high affinity for lysine residues and cleaves Lys-x bonds by utilising synthetic substrates made by linking lysine residues to chromogenic or fluorigenic labels. The first, chromogenic, method uses release of p-nitroaniline, which can be measured at a wavelength of 405 nm, from the synthetic peptide H-D-valyl-leucyl-lysyl-p-nitroanilide (S2251, Snoeren and Van Riel, 1979). Increase in absorbance at 405 nm is linear for up to 3 hours and is proportional to plasmin activity. Politis et al (1993) proposed a modification to this method, due to inhibition of plasmin plus plasminogen activity by ß-lactoglobulin and bovine serum albumin (BSA), which they suggested leads to underestimation of real plasmin activity in milk under current assay protocols. Their modified method used high speed (100,000g) centrifugation to remove casein micelles from milk (and inhibiting factors). The micelles were then resuspended in Tris buffer containing 50mM amino hexanoic acid, incubated to dissociate the plasmin and plasminogen, and recentrifuged to give a casein free supernatant which was then assayed as before.

The second method, described by Richardson (1981), uses measurement of the fluorescent product 7-amino-4-methyl coumarin (AMC), which is cleaved by plasmin from the non-fluorescent synthetic tripeptide, N-succinyl-L-alynyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl-coumarin ('coumarin peptide'). The rate of increase in fluorescence at excitation and emission wavelengths of 380 nm and 460 nm respectively is proportional to plasmin activity, with one AMC unit of plasmin activity defined as releasing 1 nanomol AMC per minute under standard assay conditions (Tris buffer, 0.2mM coumarin peptide, assay volume 0.80 ml, pH 7.5, 25°C). This method was used by Richardson (1981) to measure plasmin and plasminogen in Swiss-type and Cheddar cheese, sodium caseinate, acid casein raw and pasteurised milks. Both methods utilise an incubation step in the presence of urokinase (typically 60 minutes at 37°C) to activate plasminogen to plasmin, allowing determination of total plasmin plus plasminogen activity, and by subtraction of the measured plasmin activity, plasminogen -derived activity.

In the case of the coumarin peptide assay, samples are prepared by dispersion in 0.1M Trisodium citrate and centrifugation at 27,000g to recover supernatant, which is assayed. In the case of the first assay, samples are either centrifuged at 100,000g to recover a serum fraction, which will contain the plasmin if amino-hexanoic acid is

Table 2.1 *Use of chromogenic substrate (CS) and coumarin peptide (CP) methods to determine plasmin (PL) and plasminogen in milk (PG)*

Authors	Samples	Method	Units
Snoeren *et al.* (1979)	Milk fractions	c.s.	Δ Abs 405 nm
Richardson and Pearce (1981) and Richardson (1983 a,b)	Milk, cheese, casein	c.p.	AMCunits/ml, μg/ml, μg/g PL,
Korycka Dahl *et al* (1983)	Milk	c.s.	units/ml PL,PG
Rollema *et al* (1983b)	Milk	c.s.	Δ Abs 405 nm
Scharr (1985)	Milk	c.p.	AMC units/ml
Ollikainen and Nyberg (1988)	Swiss cheese	c.s.	nmol p-Na units/g
Politis *et al* (1989a)	Milk	c.s.	abs units/ml PL
Politis *et al* (1989b)	Milk	c.s.	mg/L PL, PG
Benslimane *et al* (1990)	Milk	c.p.	AMC units/ml, μg/ml
Politis *et al* (1992)	Milk fractions	c.s.	mg/L PL, abs. units/ml PG
Farkye and Fox (1992)	Cheddar cheese	c.p.	AMC units/g
Politis *et al* (1993)	Milk	c.s.	abs units/ml Pl,PG

added, and a casein fraction where the plasmin will be found otherwise (Politis *et al*, 1993), or dispersed with trisodium citrate as described above (Politis and NgKwai-Hang, 1989b) and then assayed. Both assays may be carried out on microtitre plates, and so choice of method may be a question of sample preparation required and equipment available, as no information on relative sensitivity has been published.

The widespread use of two different methods leads to difficulties in comparing plasmin levels reported by different groups, depending on the method used, with results being variously expressed as AMC units/ml, units based on increase in abs 405 nm (for the first method) and concentration of enzyme (see Table 2.1). Thus there is a need for standardisation, if not of methodology, then of units, of measurement of plasmin activity. There have also been occasionally reported some other methods of measurement of plasmin activity such as ELISA assays (Collin *et al*, 1988).

2.1.2. ACTIVATION, INHIBITION AND INHIBITION OF ACTIVATION OF PLASMIN

The relative levels of plasminogen and plasmin in milk are controlled by a complex system of inhibitors and activators, the full nature and interactions of which are not fully understood as yet. Plasminogen activators, which convert plasminogen to plasmin, are found in bovine milk and have been shown to be associated with somatic cells (Verdi and Barbano, 1991; Zachos *et al*, 1992) and casein micelles (Korycka-Dahl *et al*, 1983; Richardson, 1983b). Milk plasminogen activators are heat and acid stable

(Richardson, 1983a). Storage of milk leads to increased plasmin levels and decreased plasminogen levels (deRham and Andrews, 1982a; Richardson, 1983a), due to plasminogen activation.

Plasminogen activators (PAs), which are serine proteinases and are assumed to have passed into milk from blood, occur in at least two main types, tissue type (t-PA) and urokinase type (uPA). Lu and Nielsen (1993) isolated five plasminogen activators from skim milk, with molecular weights of 93, 57, 42, 35 and 27 kDa, all of which were urokinase type activators. Mastitis, or inflammation of the udder, results in 8 - fold increase in milk somatic cell plasminogen activator activity per cell, and this activity was shown to be tissue type (Zachos *et al* ,1992). Verdi and Barbano (1991) also showed that somatic cells could convert plasminogen to plasmin, while blood leucocytes, coagulants and extracellular bacterial enzymes could not. This may cause the elevated plasmin levels associated with mastitic milk. Politis *et al*, (1989) however, proposed that somatic cell plasminogen activation occurs mainly in late lactation, and that in mastitis, damage to the mammary epithelium results in the increased plasmin levels found.

With regard to inhibitors, Honkanen-Buzalski and Sandholm (1981) showed elevated levels of α1-antitrypsin and α2-macroglobulin in mastitic milk. The former is a trypsin inhibitor of molecular weight 60,000, also known as α1-antiproteinase, which is associated with defence of tissue, such as the lung, from proteolytic attack (Travis and Salvesen, 1983), while the latter is a massive molecule (mol. wt. 725,000) which physically entraps a broad spectrum of proteinases, but still allowing their activity against small substrates. α1-antitrypsin was also isolated more recently from good quality raw bovine milk, and shown to be active against trypsin and elastase but not plasmin (Weber and Neilsen, 1991). There is also evidence (Grufferty and Fox, 1988) of α2-antiplasmin activity in milk, which is believed to control one or more forms of plasminogen activator. ß-lactoglobulin and BSA in milk are also known to inhibit plasmin action (Politis *et al*, 1993), with both native and denatured ß-lactoglobulin being active in this regard.

Grufferty and Fox (1986) showed that this reaction occurs in the case of denatured ß-lg via thiol-disulphide interchange, which is inhibited in the presence of potassium iodate, which thus stimulates proteolysis in milk. Potassium iodate was concluded to oxidise the SH group of ß-lg, preventing interaction with the plasmin enzyme. Increased proteolysis in skim milk in the presence of oxidising agents was also shown by Igarashi (1990), using ascorbic acid and hydrogen peroxide, but they raised the possibility that, as well as a sulphydryl interaction, there could be oxidative inactivation of inhibitors in the plasmin system. Bovine plasminogen activator inhibitor, α1-antitrypsin and human plasma α2-antiplasmin are all inactivated by oxidation (Travis and Salvesen, 1983).

Plasminogen activators in milk can be assayed by a number of means. Verdi and Barbano (1991) used a two step assay in which firstly, at pH 6.6, plasminogen is converted to plasmin in the absence of ß-casein, and then a second step is used to detect conversion of plasminogen to plasmin, based on plasmin's ability to breakdown

ß-casein at pH 8.45 (measured by SDS-PAGE and densitometry). Zachos *et al* (1992) used an incubation in the presence of plasminogen, fibrin and H-D-valyl-leucyl-lysyl-p-nitroanilide to measure PA activity of isolated milk somatic cells. Finally, Lu and Neilsen (1993) used the same substrate in a microtitre plate assay to measure PA activity in non-micellar casein prepared from skim milk. The bovine milk proteinase inhibitors isolated by Lu and Neilsen (1993) were inhibited by α2-antiplasmin but not by α1-antitrypsin.

Thus, in summary, the system of activation and inhibition, and inhibition of activation of plasminogen and plasmin is a complex one, and one which varies with external factors such as mastitic infection and storage and heat treatment of the milk.

2.1.3. HEAT STABILITY OF THE PLASMIN SYSTEM IN MILK

Isolated plasmin was completely inactivated in 10 min at 80°C at pH 7.0 (Kaminagowa *et al.*, 1972). Noomen (1975) reported that milk proteolytic activity was increased by 30-40% when milk was pasteurised for 15 sec at 72°C (HTST pasteurisation), and by 8-24% when heated to 63°C for 30 min (LTLT pasteurisation). Richardson (1983a) found that pasteurisation of milk (72°C for 15 sec) decreased plasmin activity in milk by 17% (a figure also obtained by Korycka-Dahl *et al*, 1983) but that plasmin activity in incubated pasteurised milks increased three fold compared to raw milks on incubation at 37°C for 3 days, with concomitant decrease in plasminogen levels. This suggested increased plasminogen activator activity in pasteurised milks, due to the inactivation of an inhibitor of a plasminogen activator, as opposed to the denaturation of a plasmin inhibitor, as these inhibitors were shown to have similar heat stabilities to plasmin and plasminogen. Richardson calculated the heat stabilities of plasmin, plasminogen and trypsin inhibitor in milk to be equal. Plasmin is also widely accepted as at least partially surviving ultra-high temperature treatment (UHT) conditions (Driessen and van der Waals, 1978), and being responsible for problems such as gelation. These last authors calculated that it is necessary to heat milk to 142°C for 18 secs to completely inactivate plasmin.

Grufferty and Fox (1988a) found that the heat stability of the plasmin system was lower in milk than in serum free micellar casein dispersions, and that the presence of ß-lactoglobulin, when heat denatured, inhibited plasmin/plasminogen activity, as described earlier. They also suggested that the micelle structure of casein protects the enzymes from denaturation and that this effect is reduced at higher pHs, and that addition of lysine (0.1M) increases the heat stability of isolated plasmin by permitting binding onto casein micelles.

Tissue type plasminogen activator from somatic cells was found to be inactivated by heating to 63°C for 30 min by Verdi and Barbano (1991), and they concluded that the milk which Richardson (1983a) examined may have had a more heat stable PA present (one of the uPas perhaps) or that LTLT treatment is more efficient at inactivating the PAs then HTST treatment. It is likely that the uPAs and tPAs measured by the separate groups accounted for the discrepancy, also the complexity of the

activator-inhibitor system makes it quite possible that such differences will occur. This system may be in part summarised in Fig. 2.1.

2.1.4. SPECIFICITY OF PLASMIN ON MILK PROTEINS

Plasmin is a serine proteinase, with a pH optimum of 7.5 and a high specificity for bonds of the type Lys-X, followed by Arg-X bonds. It readily degrades ß-casein to γ-caseins and certain proteose peptones in milk and cheese (Andrews and Alichanidis, 1983; Fox, 1989). The γ-caseins in milk, γ_1, γ_2 and γ_3 correspond to residues 29-209, 105-209 and 107-209 of ß-casein respectively. The proteose peptones ascribed to plasmin action are PP5 (ß-casein f1-105 and f1-107), PP8-slow (ß-casein f29-105 and f29-107) and PP8-fast (ß-casein f1-28) (Eigel *et al.*, 1984).

Plasmin was shown by McSweeney *et al* (1993) to hydrolyse α_{s1} -casein at least 18 bonds, 13 of the type Lys-X and 5 of the type Arg-X. Aimutis and Eigel (1982) ascribed the formation of λ-casein to the proteolysis of α_{s1} -casein by plasmin.

α_{s2} -casein was shown by LeBars and Gripon (1989) to be hydrolysed in solution at pH 8.4 by plasmin to 14 peptides (7 Lys-X sites and one Arg-X site identified), three of which were from the hydrophobic C-terminal of the protein, and, due to their high average hydrophobicities, were probably bitter.

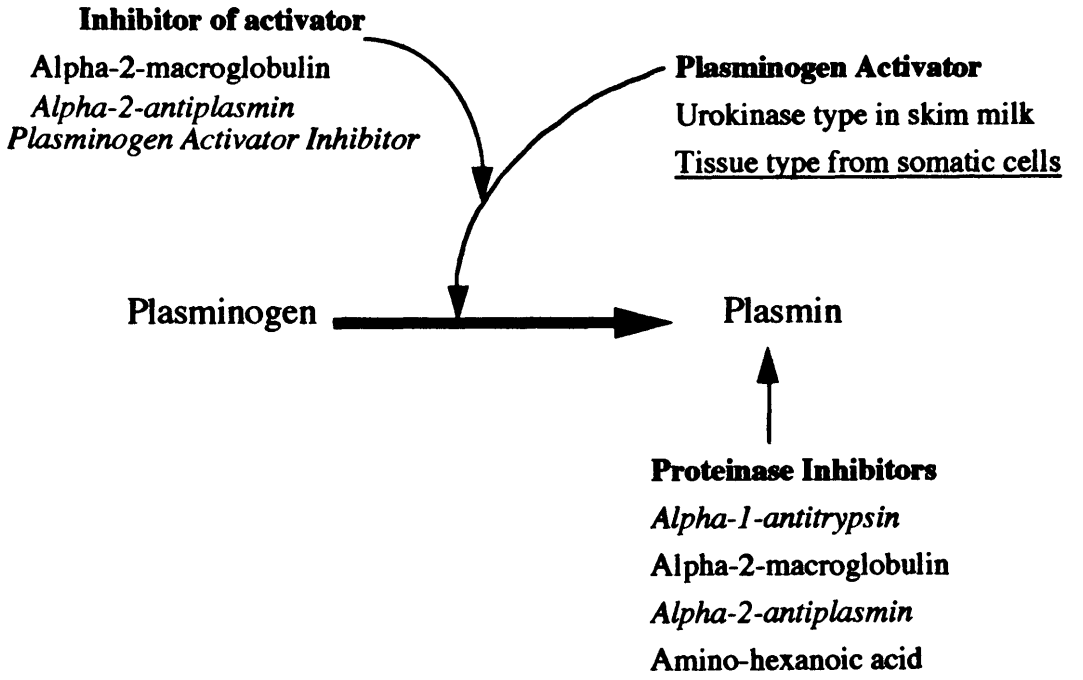


Fig. 2.1. *Plasminogen activation cascade in bovine milk, with oxidatively inactivated components in italics, and heat labile components underlined. (After Richardson, 1983a; Christman et al, 1979)*

The activity on κ-casein of plasmin is very low, with Andrews and Alichanidis (1983) reporting that 4% of peptides produced by the action of plasmin in pasteurised milk for 7 days at 37°C originated from this source, but no hydrolysis sites have been as yet confirmed.

2.1.5. FACTORS AFFECTING PLASMIN ACTIVITY IN MILK

Increasing stage of lactation has been associated with reduced levels of ß-casein and increased levels of γ-caseins, thought to be due to elevated plasmin activity (Donnelly and Barry, 1983). Increased levels of plasmin and plasminogen in late lactation milk were confirmed by Richardson (1983b), Scharr (1985), Politis *et al.*, (1989a) and Benslimane *et al.*, (1990). Politis *et al.*, (1989b) ascribed a drop in plasminogen:plasmin ratio from 6.55 in early lactation to 3.29 in late lactation to increased activity of plasminogen activators.

Age of cow (and increasing lactation number) has been shown by Davies and Law (1977b), Scharr (1985) and Politis *et al.*, (1989) to be positively correlated to plasmin activity. Richardson (1983b) found higher plasmin activity in Friesian milks than Jersey milks, but Scharr (1985) found that statistically, this effect was removed by making adjustments for the difference in casein contents of milks from different breeds. Plasmin levels reported in milks in various studies are compared in Table 2.2, and from this can be seen again the problem in comparing results due to different methods of reporting activities.

Mastitic infection is known to increase plasmin activity (Barry and Donnelly, 1981; deRham and Andrews, 1982b; Andrews, 1983b), and while Barry and Donnelly ascribed most of the proteolytic activity in such milks to plasmin, deRham and Andrews showed that two-thirds of the activity was non-plasmin derived. Somatic cells, which are present in greatly elevated numbers in mastitic milk, possess plasminogen activators (Verdi and Barbano, 1991) and Politis *et al.* (1989a) found that somatic cell counts (SCC) in milk were significantly positively correlated to plasmin activity ($r=0.62$). In another study, Politis *et al.*, (1989b) concluded that increased plasmin activity in mastitis was due to influx of the enzyme, with other blood components, across the damaged mammary epithelium, rather than by somatic cell activation, as there was no significant drop in plasminogen:plasmin ratio. However, Zachos *et al*, (1992) found an 8-fold increase in plasminogen activator activity per somatic cell in cells from mastitic milk compared to cells isolated from good quality milk. They concluded that caution must be taken in relating the findings of cell PA activity to the milk enzyme system, because of the obvious interplay of a number of disparate factors in the plasminogen-plasmin system in mastitis.

Table 2.2. *Plasmin and plasminogen-derived activities reported in various milk studies.*

Authors	Milk	Plasmin	Plasminogen
		mg/L or (AMC units/ml)	
Richardson (1983b)	Early Lactation Jersey	0.15 (0.042)	1.43(0.38)
	Mid Lactation Jersey	0.29 (0.078)	2.15(0.57)
	Late Lactation Jersey	0.37 (0.098)	3.00(0.80)
	Early Lactation Friesian	0.27 (0.073)	-
	Late Lactation Friesian	0.53 (0.141)	-
Scharr (1985)	Swedish Friesians	(0.112)	-
	Swedish Jersey	(0.084)	-
	Swedish red and White	(0.091)	-
	1st lactation cows	(0.079)	-
	2nd lactation cows	(0.086)	-
	5th lactation cows	(0.134)	-
	<100d in lactation	(0.055)	-
	>200d in lactation	(0.146)	-
Politis *et al.* (1989b)	Milk SCC 100,000/ml	100 units	-
	Milk SCC 1,300,000/ml	230 units	-
	Early lactation milk	85 units	-
	Late lactation milk	170 units	-
Politis *et al.* (1989a)	Milk SCC 250,000/ml	0.18	0.85
	Milk SCC 750,000/ml	0.28	-
	Milk SCC >1,000,000/ml	0.37	1.48
	Early lactation	0.15	0.94
	Late Lactation	0.38	1.25
Benslimane *et al.* (1990)	Early lactation	0.25 (0.25)	1.07(1.07)
	Late Lactation	0.38 (0.38)	2.01(2.01)
	Overall range	0.15-0.42	0.83-2.01
		(0.15-0.42)	(0.83-2.01)

2.1.6. SIGNIFICANCE OF PLASMIN IN DAIRY PRODUCTS

Proteolysis by plasmin is associated with a number of physicochemical changes in dairy products, such as decreased viscosity of high pH caseinates and UHT products such as custards, increase in pH 4.6 soluble products in milk and casein solutions and gelation on storage of UHT milk products (this will be dealt with in greater detail in Chapter 3).

With regard to the cheesemaking characteristics of milk, Grufferty (1986) showed that very extensive hydrolysis of micellar casein was required to increase the

rennet clotting time (RCT), and Pearse *et al.* (1986) found that up to 50% of total micellar casein in a milk system can be degraded by plasmin without increasing RCT, with RCT decreasing in fact with limited hydrolysis. Grufferty and Fox (1988) concluded that the cheesemaking problems associated with late lactation milk were not directly attributable to the elevated plasmin therein.

2.1.7. THE ROLE OF PLASMIN IN CHEESE RIPENING

Visser (1977a-d) and Visser and deGroot-Mostert (1977), in an extensive study of the contribution of individual proteolytic agents to proteolysis and flavour development in Gouda cheese, found that cheeses in which milk protease (assumed at the time to be plasmin) was the only proteolytic agent present, showed a gradual increase in soluble nitrogen fractions over ripening, with ß-casein breakdown and γ-casein production. There was also some slight degradation of α_{s1} -casein and production of α_{s1} -I casein, and at no stage was bitterness detected in the cheeses. These findings indicated that the milk enzyme system had been incorporated into cheese and was stable in such conditions. The cheeses showed a low level of amino acids and low-MW peptides produced during ripening, and it was concluded that the low pH of the cheese was unsuitable for plasmin action. Noomen (1978) made simulated soft cheeses of the Meshanger type with milk enzymes as the sole ripening agent at a range of pHs, and found high ß-casein breakdown at pH 6.2, but more rapid breakdown of α_{s1} -casein at pH 5.2. Breakdown was optimal at 2% salt in cheese moisture. In this and the above study, the presence and activity of other proteinases of lower pH optima than plasmin was indicated.

Richardson (1981) measured the plasmin activity in a range of cheeses and found higher activity in Swiss type cheese (1.5-3.4 AMC units/g cheese) compared to Cheddar (1.1 AMC units/g cheese), corresponding to a higher breakdown of ß-casein and α_{s2} -casein and slower breakdown of α_{s1} -casein in Swiss type cheese. These differences he attributed to the relative pH, moisture and salt contents of the two cheeses. Ollikainen and Kivelä (1989) showed that in Swiss type cheese, because rennet is inactivated by the high cooking temperatures, plasmin is the principal agent responsible for breakdown of ß-casein and provision of substrate polypeptides for the starter bacteria. Farkye and Fox (1990) compared plasmin activities in various experimental and commercial cheeses, and their results are summarised in Table 2.3.

Farkye and Fox (1990) found that increased cooking temperatures increase plasmin activity in cheese, rennet curd and micellar casein dispersions, possibly due to conversion of plasminogen or inactivation of inhibitors. The role of salting is unclear as Fox (1989) suggested that since plasmin can be dissociated from casein micelles by NaCl, salting before pressing would result in loss of plasmin in the press whey compared to brine salted cheeses, but Farkye and Fox (1990) found no effect of method of salting on plasmin activity.

Table 2.3. *Plasmin activities in various commercial and experimental cheeses (from Farkye and Fox, 1990)*

Cheese Type	Plasmin activity (AMC units/g)	
	Commercial	*Experimental*
Emmental	2.68	1.71
Cheddar	1.07	1.26
Cheshire	0.76	0.86
Gouda	1.85	
Blarney	2.59	
Leicester	1.40	
Wensleydale	1.35	
Romano-Type	2.91	

Addition of a plasmin inhibitor, 6-aminohexanoic acid, to Cheddar cheese curd, led to a reduced production of γ-caseins and water soluble nitrogen (Farkye and Fox, 1991) while addition of porcine plasmin to milk had exactly the reverse effects, and added plasmin cheeses had accelerated ripening and were judged organoleptically superior. Addition of plasmin did not affect phosphotungstic acid-soluble nitrogen. This was also verified using bovine, as opposed to porcine, plasmin, by Farkye and Lankdammer (1992) who concluded that three fold increase in cheese plasmin resulted in superior Cheddar cheese sensory quality.

Overall it would appear that plasmin is involved in the primary proteolysis of caseins and large polypeptides, rather than production of small amino acids and peptides, and does not result in bitterness or unsuitable organoleptic qualities during cheese ripening.

2.1.8. NON-PLASMIN PROTEINASES IN MILK

Andrews (1983a), in a study of proteinases in normal bovine milk, found differences in milk enzyme inhibition patterns and slight differences between electrophoretic patterns of proteolysis induced by storage and by added plasmin which led him to conclude that, although the principal proteinase in milk was plasmin, at least one other enzyme was also present.

As described in the section on plasmin activity in cheese ripening, Noomen (1978) and Visser (1977a-d) described the action of a second milk proteinase with an acid pH optimum in aseptic, starter and rennet free cheeses. This is most likely the acid proteinase isolated by Kaminagowa and Yamauchi (1972b), which has a mol. wt. of 36,000 and a pH optimum of 4.0, and which was concluded from examination of inhibition patterns to be identical to Cathepsin D. Kaminagowa *et al.* (1980) examined the proteolytic specificity of this enzyme, purified from milk, and found it to have a specificity similar to chymosin, producing α_{s1} -I casein from α_{s1} -casein and ß-I and ß-II casein from ß-casein. κ-casein was found to be hydrolysed to a para-κ-casein like

protein, but much more slowly than by chymosin, as was the rate of hydrolysis of ß-casein, while α_{s1}-casein was hydrolysed more rapidly than for chymosin. McSweeney at al (1995) compared the proteolysis patterns of caseins hydrolysed by Cathepsin D to those produced by chymosin, and found that while the hydrolysis of α_{s1}-casein was similar, the specificities on α_{s12}-casein differed somewhat. Cathepsin D was more active on α_{s1}-casein than ß-casein, which it hydrolysed primarily in a manner similar to chymosin. Cathepsin D hydrolysed κ-casein in solution, giving HPLC profiles similar to chymosin, but was unable to coagulate milk. Thus, Cathepsin D, which appears to be incorporated into cheese curd, probably has an action which in most normal cheese systems is masked by that of chymosin, but acts synergistically with this enzyme.

Reimerdes (1983) isolated two serine proteinases from milk and although one was identified as identical to blood plasmin, the other showed inhibition and specificity patterns which led him to tentatively identify it as thrombin (also Reimerdes, 1981). This enzyme shows a high specificity for Arg-X bonds, but has never been reported elsewhere in milk or milk products. In the same study, the author, through the use of free amino acid-p-nitroanilide substrates, reported the presence in milk of aminopeptidases, which were alleged by comparison with blood sera from the same animal, to have originated in blood. The same author also suggested in an earlier paper the identification of a leucine-aminopeptidase activity with a pH optimum of 7.0 associated with casein micelles.

In 1990, Suzuki and Katoh found at least two types of cysteine protease in bovine milk, with molecular weights of 45,000 and 150,000 respectively, and pH optimum for activity on casein of 6.0. The activity was elevated in mastitic milk and was correlated well with California Mastitis Test, SCC and protein concentration, suggesting that the enzyme is involved in the pathogenesis of mastitis. Milk proteolytic activity associated with elevated somatic cell counts in milk will be discussed in detail in Chapter 3.

2.2. Somatic cells in bovine milk - function and significance

2.2.1. INTRODUCTION: CAUSES OF VARIATION IN MILK SCC

Somatic cells in milk are a combination of epithelial cells from the secretory tissue of the udder and leucocytes (white blood cells) from the bloodstream. A value of 500,000 cells/ml milk from cows at normal milking times in normal lactation was generally accepted as an index of non-specific mastitis, and somatic cell count (SCC) is generally regarded as a sensitive indicator of mastitis and milk quality. However, these levels have been constantly revised and EU directive 92/46 reduces the maximum acceptable SCC from 500,000 cells/ml to 400,000 cells/ml.

In 1932, Johnson and Trudel studied herds of cows with and without mastitis, and concluded that the SCC in milk rises during the latter stages of the lactation cycle,

and in mastitis, with the change preceding the appearance of *Streptococci* or *Staphylococci* in the milk. Blackburn (1966) also recorded rising SCC with stage of lactation, and found that the average total cell count increased from one lactation to the next, as was also reported by Emanuelson and Persson (1984). The fraction of milk taken was shown to effect SCC by Paape and Tucker (1966), who showed that if 20 ml fractions were removed from the udder, the SCC did not differ significantly from milk taken subsequently from all quarters by a milking machine, but that final hand-stripped milk and milk exuded on oxytocin injection, had significantly higher SCC. Jain and Jasper (1967) found more viable cells in milk containing bacteria, and that cell viability decreased when milk was refrigerated for several days. Mean SCCs of 1,120,000/ml for infected and 414,000/ml for non-infected quarters were reported by Ward and Schultz (1972), who also found a loss in milk yield in infected quarters.

Newbould (1978) sampled approximately milks from 1000 bulk tanks over 1 year and reported mean SCCs of 283,000 and 1,118,000 cells/ml for uninfected and infected cows respectively. These counts for uninfected cows are in close agreement with the 265,000/ml reported by Hogan *et al.* (1988). Brolund (1985), in an extensive study, concluded that milk SCC was affected primarily by bacteriological status of the udder (accounting for 40% of variation), with the next most significant source of variation being lactation number, with SCC increasing with lactation number. Other significant effects were those of milk yield, morning/evening sampling, stage of lactation and sampling period, but he concluded that stage of lactation effect may be due to reduction of daily yield in late lactation. This was, however, disputed by Wever and Emanuelson (1989), who found that SCC was clearly affected by stage of lactation, with a probable major effect of variation in infection rates throughout lactation, as also found by Kennedy *et al.* (1982). Environmental-heat-stress and injections of corticotropin have also been shown to increase SCC in milk (Wegner *et al.*, 1976).

Mastitis is defined as an inflammatory reaction of the mammary gland, and is classified according to the cause or principal cause of the inflammation. Pathogens principally enter via the teat duct canal, which provides the first line of defence against infection, and then meet the second line of defence, the resident cell population of the healthy gland. These cells initiate the inflammatory response necessary to eliminate the invasive bacteria. This is characterised primarily by a massive influx of polymorphonuclear leucocytes (PMN cells). PMN cells comprise eosinophils, basophils and neutrophils, the latter being the only type found in milk. These cells phagocytose the bacteria and provide the most effective defence against bacterial colonisation of the mammary gland (SCC (Wever and Emanuelson ,1989; Burvenich *et al.*, 1995). Subsequent changes in the mammary gland and increased permeability of the inflamed tissue results in changes in the levels of enzymes, salts and serum proteins. In subclinical mastitis, these changes can only be detected by laboratory tests, such as measuring somatic cell count, whereas with clinical mastitis, changes in the udder and milk are visible. In acute mastitis, onset is sudden, while chronic mastitis is characterised by it's long duration. Mastitis occurs most commonly in older cows and in the early part of lactation (Bramley and Dodd, 1984).

Over 100 different micro-organisms have been reported as a cause of mastitis in cows. The primary bacteria associated with mastitis are *Staphylococcus aureus* (the most common cause) *Streptococci* spp. (principally *S. agalactiae, S. dysgalactiae, S. uberis*) and least commonly, *E. coli*. Mastitic infections may also be associated with *Pseudomonas aeruginosa, Mycoplasma bovis* and corynaebacteria (Bramley and Hood, 1984). Three major pathogen classifications have been identified, contagious pathogens, environmental pathogens and coagulase-negative staphylococci (CNS), occasionally referred to as skin flora opportunists. The contagious pathogens were traditionally responsible for the majority of infections, but are being controlled, at least partially, by post-milking teat disinfection (teat-dipping) and dry cow therapy. However, these measures control CNS pathogens only slightly and environmental pathogens not at all (Smith and Hogan, 1995). Contagious pathogens (*Staph. aureus, Strep. agalactiae, Strep. dysgalactiae, Mycoplasma bovis* and *Corynaebacteria bovis*) grow on the teat skin and within the teat duct and spread primarily at milking time through poor hygienic practices. Contagious pathogen infections result in subclinical mastitis with 40% eventually leading to clinical symptoms, with infections being of long duration in the absence of antibiotic intervention. Environmental pathogens (*Streptococci, Strep. uberis, Enterococci, E. coli, Bacilli* and yeasts, moulds and algae) are present in the environment of cows (manure, dirt, pools of standing water, feeds and bedding materials) and can directly infect cows or contaminate products such as teat dips or multiple-use antibiotic containers. Infections due to these micro-organisms are generally of short duration, are more likely to result in clinical mastitis, and thus are less likely to result in problems such as elevated bulk tank SCC because milk from mastitic animals can be withheld. CNS organisms are considered to be part of the normal flora of animals and have low pathogenicity, resulting in SCCs generally below 500,000 in infected quarters, thus contributing to bulk milk SCCs. It is felt that the importance of CNS pathogens is increasing as the legal upper threshold for SCCs continues to be reduced.

2.2.2. BIOLOGY AND FUNCTION OF MILK SOMATIC CELLS

2.2.2.1. The different types of somatic cell present in milk

Milk contains three main types of somatic cell, lymphocytes, polymorphonuclear granulocytes (PMN cells or neutrophils) and macrophages (or monocytes) (O'Sullivan *et al.*, 1992). Milk may also contain epithelial cells shed from the udder. Mastitic inflammation of the udder results in an influx of PMN cells. Burvenich *et al.* (1995), in a review of defence systems of the mammary gland, reported that cell counts from non-infected mammary glands consisted of ±12% PMN, ±60% macrophages and ±28% lymphocytes. These figures represent a consensus of various studies and the findings of various other studies into the differential cell counts of non-mastitic milk are summarised in Table 2.4. The higher proportions of PMNs reported by Dulin *et al.* (1982) were attributed to the cows being fitted with

Table 2.4 *Differential SCCs in non-mastitic milk as reported in the literature*

Authors	Milk	% PMNs	% Lymphocytes	% Macrophages
Blackburn (1955)	*Early lactation*			
	<100,000 cells/ml	44	-	-
	100,000-500,000 cell/ml	51	-	-
	>500,000 cells/ml	55	-	-
	Late lactation			
	<100,000 cells/ml	33	-	-
	100,000-500,000 cell/ml	40	-	-
	>500,000 cells/ml	45	-	-
Lee *et al.* (1980)	Colostrum	0-11	3-8	81-93
	Mid lactation milk	0-11	10-27	66-88
Jensen and Eberhart (1981)	Dry Period	14-24	25-39	44-56
	Early Lactation	30-37	20-27	37-50
Dulin *et al.* (1982)	<70,000 cells/ml	71	0.4	29
	70,000-150,000 cells/ml	63	0.4	36
	>150,000 cells/ml	65	0.4	33
Azzara and Dimick (1985)	Healthy cows	21-29	14-20	51-63
Östensson *et al.* (1988)	<100,000 cells/ml	36	52	12
	100,000-570,000 cells/ml	40	31	29
Wever and Emanuelson (1989)	Healthy cows	36	15	49
Saad and Ostensson (1990)	Mid-late lactation	18	8	74
Östensson (1993)				

polyethylene intramammary devices (IMD), which cause relative increase in PMNs relative to monocytes or macrophages. Otherwise, the relative proportions of the 3 main cell types are in broad agreement, but there is still a range of 18-71% in the proportion of PMNs, as a percentage of total cell count, reported in healthy milk.

Blackburn *et al.* (1955) and Blackburn (1966) concluded that in late lactation both the numbers of PMNs and non-PMN cell types rose, and that as number of cow lactations increased, the numbers of PMNs increased and the counts of cells other than PMNs remained relatively constant. It is now known that during late lactation, the percentage of PMNs tends to increase while the percentage of lymphocytes decreases and PMNs are the predominant cell type for 4 weeks after cessation of milking but towards parturition lymphocytes predominate in milk (Burvenich*et al.*, 1995). Lymphocytes respond to foreign antigens and function in humoral and cell-mediated immunity and constitute 47% T-cells and 20-28% B-cells, with increased B-cell levels in dry-gland secretions (Concha *et al.*, 1978; Concha *et al.*, 1980). Macrophages, like PMNs, are phagocytic cells which ingest and kill mastitis pathogens and secrete inflammatory mediators.

2.2.2.2. Methods for determining differential cell counts in milk

Total somatic cell counts in milk may be determined by use of a haemacytometer, microscope and methylene blue staining, coulter counter (which counts particles of a certain size), or by electronic counting of ethidium bromide stained nuclei of cells using a Fossomatic, which is the most common routine test to determine SCC (IDF bulletin 132, 1981). Fossomatic instruments are generally calibrated by direct microscopic counts. Cell viability is routinely estimated using trypan blue staining (Mishell and Shiigi, 1980; Concha *et al.*, 1980; Lohuis *et al.*, 1990).

Many studies of differential cell counts in milk used specific differential cell stains and microscope counting to determine the total cell counts, and relative counts of each cell type. Stains such as Sudan black-Leishmans stain (Blackburn *et al.*, 1955), Wrights stain (Dulin *et al.*, 1982; Jensen and Eberhart, 1981; Miller *et al.*, 1990; Miller *et al.*, 1991) or pyronin-Y-methyl green (Guidry *et al.* , 1975) are commonly used. Morphological studies were also carried out using electron microscopy (Paape *et al.*, 1977; Harmon and Heald, 1982). A cytospin centrifuge has been developed for concentration of cells from milk onto microscope slides, to improve differential staining techniques (Dulin *et al*, 1982). Esterase staining is also used to estimate differential cell counts (Zecconi *et al.*, 1994). Staining using a monoclonal antibody to cytokeratin, coupled with a chromogenic reaction, was used by Miller *et al.*, (1991) to perform differential counts on milk smears.

An early electronic cell counting protocol was described by Meek *et al.* (1980) who used a Coulter counter which sized the counted particles into 16 groups or channels, and it was found that milk from infected quarters showed the greatest difference in one specific channel, and it was proposed that the cells in this channel

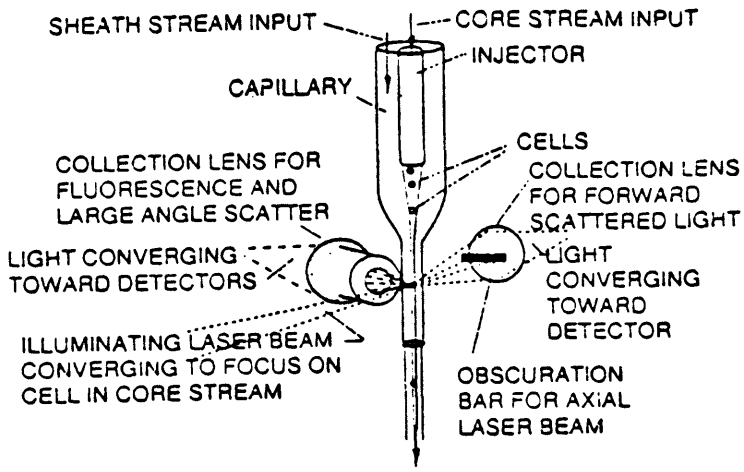


Fig. 2.2 *Schematic of a typical flow cytometer (Shapiro, 1988)*

were PMNs, and this count was shown to have potential in detecting infected herds, cows and quarters. The most significant advance of recent years in the area of differential somatic cell counting, however, has been the development of flow cytometry.

Flow cytometry is defined as a process in which measurements are made while the cells or particles pass, preferably in single file, through the measuring apparatus in a fluid stream (Shapiro, 1988). Cells can be counted and classified on the basis of size, shape, granularity, pigmentation, fluorescence (natural or due to added markers), nucleic acid content or antigens. A schematic of a typical flow cytometer is shown in Fig. 2.2. This instrument is set up to measure fluorescence emission from, and light scattered at large and small angles by, each cell which passes through it. The cells are passed through the injector, which is coaxial with a larger tube through cell-free sheath fluid is passing, and scattered light and fluorescence are measured by suitably placed detectors. Regions of cells of interest can be selected and these cells collected by charging and deflection of droplets of interest (cell sorting).

Hageltorn and Saad (1986) published the first use of flow cytometry of bovine blood and milk leucocytes, which were prestained with the nucleic acid stain, acridine orange, sorted, and identified by staining and microscopy. Acridine orange stains RNA red and DNA green, and thus each type of cell will have a distinct red/green fluorescence pattern, and can be distinguished on this basis. They showed that monocytes, macrophages, lymphocytes and PMNs could be easily distinguished and counted in blood and milk. A typical cytofluorogram of cells in a mastitic milk sample is shown in Fig. 2.3. Cells in each group are selected and counted by computer and expressed as a % of total cells counted. This method was subsequently used by Saad (1987), Östensson *et al.* (1988), Wever and Emanuelson (1989), Saad and Östensson

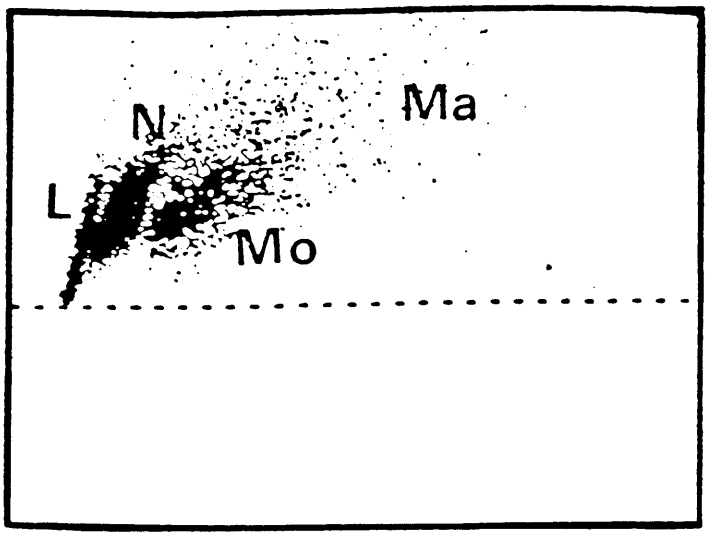


Fig. 2.3. *Cytofluorogram of cells in a mastitic milk sample, with each leucocyte represented as a point, with its co-ordinates (red and green fluorescence on horizontal and vertical axes). L=lymphocytes, N=PMNs, Mo=monocytes, Ma=Macrophages (Hageltorn and Saad, 1986).*

(1990) and Östensson (1993) to determine differential milk somatic cell counts. Milk samples were typically prepared by dilution 1:200 with phosphate buffered saline (PBS) containing 4mg/L acridine orange and filtered through a 50μm nylon filter before measurement (Östensson *et al.* (1988)). Sordillo *et al.*, (1991) used monoclonal antibodies specific for bovine leucocyte surface antigens to enumerate bactericidal activity of isolated lymphocytes using flow cytometry. Similar monoclonal antibody labelling techniques were used by Leitner *et al.* (1995) and Concha *et al.* (1995) to estimate differential milk cell counts.

The labelling by chemical conjugation of an enzyme to either antibody or antigen allows detection of immune complexes formed on a solid phase as the fixed enzyme, once washed free of interfering substances, can be used to catalyse a colorimetric reaction which can be used to quantify the antigen measured. These assays are referred to as enzyme-linked immunosorbent assays (Catty and Raykundalia, 1989). O'Sullivan *et al.* (1992) described a capture ELISA assay for diagnosis of bovine mastitis using a monoclonal antibody specific to bovine PMNs, as an alternative to cumbersome and expensive flow cytofluorimetric methods. The potential use for this test was as a cow side test for infected quarters or cows, to prevent milk from cows with subclinical mastitis from entering the bulk tank, keeping milk quality high. The assay was highly correlated to total SCC (r=0.94) and had high sensitivity and specificity and it was concluded that the test had excellent potential for determination of bovine mastitis.

With regard to separation of somatic cells from blood, the method of Carlson and Kaneko (1974) is widely used to recover poly (PMN) and mono (non-PMN cells) fractions from blood. Milk cells are routinely recovered from milk by centrifugation and resuspension in phosphate buffered saline (PBS) (Paape *et al.*, 1977; Verdi and Barbano, 1988; Verdi and Barbano, 1991). Methods using the differential densities of different cell types to separate milk cells on Ficoll (Concha *et al.*, 1978; Zecconi *et al.*, 1994), Percoll (Grommers *et al.*, 1989; Sordillo *et al.*, 1991; Lohuis *et al.*, 1990) or discontinuous metrazamide gradients (Hallen-Sandgren and Björk, 1988; Hallen-Sandgren *et al.*, 1991) have been described. Lymphocytes have been recovered from milk using filtration through loosely packed cotton wool (Concha *et al.*, 1980). Paape *et al.* (1977) stated that isolation of PMNs from residual milk 11 hours after intramammary infusion of saline containing 0.1% glycogen will provide large numbers of PMNs for in vitro studies of the cellular defence of the mammary gland.

2.2.2.3. Effects of mastitis on differential somatic cell count of milk

It has long been recognised that high SCC is an indicator of mastitis, and that the proportion of polymorphonuclear leucocytes (PMNs) rises on bacterial invasion of the udder (Blackburn *et al.*, 1955; Meek *et al.* , 1980; Jensen and Eberhart, 1981; Azzara and Dimick, 1985b; Grommers *et al.* , 1989; Saad and Östensson, 1990). Paape *et al.* (1977) showed that chemotaxis or recruitment of PMNs to the mammary gland caused a four-fold increase in PMNs 11 hours after infusion of saline or saline containing glycogen (both chemotactic agents). These PMNs had a high phagocytic activity against *Staphylococcus aureus* (98% of bacteria killed within 1 hr at 37°C). PMNs were also found to phagocytose fat globules in milk. Harmon and Heald (1982) found that in experimentally induced *Staphylococcus aureus* mastitis, infected tissue showed progressive tissue degeneration and PMNs were seen under electron microscopy to adhere to the capillary walls and migrate across the blood-milk barrier through gaps in the epithelial layer left by lysis of epithelial cells. Grommers *et al.* (1989) showed that cows that responded to a low infusion of *E. coli* endotoxin with a rise in SCC had enhanced PMN chemotactic activity, while in cows which did not develop a high SCC this activity was diminished. This suggested that some cows may be better able to mobilise PMNs and quickly eliminate an infection (responders) than others (non-responders).

Wever and Emanuelson (1989) found that differential SCC was affected by the presence or absence of pathogens in infected quarters of the udder, but not in adjacent quarters, with 1.8 fold higher total SCC, increased PMNs, slightly increased lymphocytes and decreased macrophages. Increasing SCC from <100,000 cells/ml to >800,000 cells/ml led to an increase in %PMNs as proportion of total cells from 32% to 49%. However, Emmanuelson and Wever (1989), investigating the potential of differential cell counts as an indicator of mastitis in milk samples found that log (SCC) was a better means for differentiating infected and pathogen-free quarters than

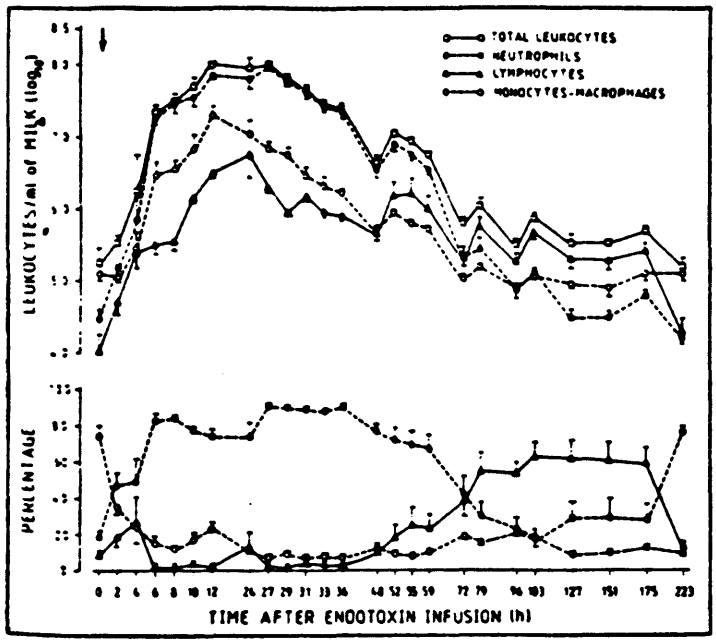


Fig. 2.4. *Total and Differential milk leucocyte counts after infusion of S. typhimurium endotoxin (arrow) into mammary glands. Values are mean ± SEM of 5 quarters from 3 cows (Saad and Östensson, 1990)*

differential cell counts, but suggested that functionality, rather than quantity of cells such as PMNs should be considered in establishing their role in mastitic infection.

In the most detailed study found on the influence of mastitis on differential cell counts, Saad and Östensson (1990) used *Salmonella typhimurium* endotoxin to induce mastitis in cows and examined subsequent differential cell counts in blood, lymph and milk. This was different to the approaches used above which correlated bacteriological status of large numbers of milk samples with total and differential SCCs, and was the first study of the sequential changes in proportions and changes in cell populations during the course of experimentally induced mastitis. There was a huge increase in total milk SCC, accompanied by a blood cell population decrease, due to massive mobilisation of cells to udder compartments and milk. PMNs were seen to appear at the beginning of the infection, and to be the predominant cell type in milk (up to 95% of total cells) for up to 59 hours after endotoxin infusion. Both monocytes-macrophages and lymphocytes increased also, but when total SCC increase was taken into account, the proportions of those cells had dropped. Three days post-infusion total SCCs began to drop, reaching preinfection levels during the fourth day, while at this time lymphocyte levels remained high, and lymphocytes were the predominant cell type between post infusion days 4 and 8, due to a drop in PMN and monocyte-macrophage numbers to preinfection levels. The role of lymphocytes was suggested to be immunological, as these cells do not phagocytically destroy bacteria. A graph of the total and differential leucocyte counts during the course of this experiment is shown in Fig. 2.4. Zecconi *et al.* (1995), however, found that in mastitic animals found in herd

situations, as opposed to cases of experimentally induced mastitis, PMN percentage was never higher than 65%.

2.2.2.4. The function of the PMN cell in the udder

PMN cell function may be estimated by examining a number of factors. Phagocytosis of an opsonised stimulus (zymosan), quantified by chemiluminescence, was used for this purpose by Zecconi *et al.* (1994) and Lohuis *et al.* (1990). Binding and ingestion assays using *E. coli* as a target organism and migration assays to investigate chemotaxis were also used by Lohuis *et al.* (1990). *Listeria monocytogenes* was similarly used by Kelly Bunning *et al.* (1988). Phagocytic and bactericidal assays for PMNs were described by Paape *et al.* (1977). A method for measuring chemotaxis and spontaneous migration of human PMNs was described by Nelson *et al.* (1975) and used by Grommers *et al.* (1989). An assay for superoxide anion generation was described by Grommers *et al.* (1989). Assays for hydrogen peroxide release and intracellular killing were used to study PMN function in normal and mastitic milks by Zecconi *et al.* (1995). Tests for intracellular survival of *Staph. aureus* and *Listeria monocytogenes* in PMNs were described by Williams *et al.* (1985) and Kelly Bunning *et al.* (1988).

The role of the PMN cell in non-infected, healthy udders was studied by Hallén-Sandgren (1991). Macrophages and PMNs had high phagocytic activity but macrophages showed a low chemiluminescent activity, which is a measure of oxygen-dependant killing capacity, as PMNs, when activated, emit light as a consequence of production of reactive oxygen metabolites, which have a bacteriostatic function. PMN chemiluminescent activity was depressed, with selectively decreased extracellular release of oxygen radicals, and hence protective capacity, in Friesian cows and in early lactation milk. This was linked to the higher incidence of mastitis in early lactation, and suggested that later in lactation PMNs have an enhanced protective function.

Functional depression of milk PMNs in cows with subclinical mastitis was also observed and higher SCCs were associated with higher PMN activity. This latter finding suggests that selection for cows with low SCCs may diminish the protective function of PMNs in the udder, which may be connected to the fact that low SCCs in bulk milk are associated with higher rates of clinical mastitis, and indicating the protective role of PMNs. It was also found that the presence of bacteria in the udder was associated with depressed PMN function, suggesting that the presence of bacteria is due to the depression in function of the milk PMNs, or perhaps that the bacteria are depressing PMN function. Saad (1987) reported a lower proportion of active, phagocytic PMNs in non-mastitic milk compared to cells isolated from blood from the same cows, but in late lactation and high SCC milk similar or higher activity of milk PMNs was shown by Hallén-Sandgren (1991). It is possible that phagocytosis of casein and fat in milk exhausts the neutrophil, resulting in decreased microbicidal function. Milking removes compromised PMNs, which are replaced by healthy PMNs, thus enhancing defence against bacterial infection. This may explain why cows milked

four times a day have a reduced incidence of clinical mastitis as compared to cows milked twice a day (Burvenich *et al.*, 1995). The possibility that immunocompromisation of mammary gland defences, through low phagocytic and chemiluminescent activity of PMNs, could predispose to clinical mastitis, was supported by Zecconi *et al.* (1994).

During intramammary challenge PMNs are recruited by chemoattractant molecules released during the initial steps of the inflammatory reaction, such as complement components, lipopolysaccharides or endotoxins. PMNs thus recruited by endotoxin to the udder produce oxygen radicals, such as superoxide anions and hydrogen peroxide, on binding of complement and immunoglobulins. During phagocytosis, PMN cytosolic granules fuse with the invasive micro-organisms plasma membrane to form the phagolysosome, which is a highly toxic microenvironment. Release of the oxygen-dependant killing agents to this cavity is very effective at eliminating Gram-negative bacteria such as *E. coli*. *S. aureus*, however, has cunningly adapted to survive these conditions by producing catalase which disarms the hydrogen peroxide mechanism. Williams *et al.* (1985) showed that PMNs from cows with a high rate of phagocytosis generally killed internalised *S. aureus* more efficiently, and that intracellular survival of this organism was linked to reduced chemiluminescent activity, or oxidative pathways.

The oxygen independent killing mechanisms of PMNs consist of the release of bactericidal enzymes such as the proteolytic enzymes Elastase and Cathepsin G, which may also be injurious to host tissue (Travis,1988; Travis; 1990; Henson and Johnston, 1987). It is theorised that the decreased activity of the milk PMNs described above means that disproportionate levels of PMNs are required to prevent infection, and that means should be explored to increase the phagocytic activity of PMNs (Burvenich *et al.*, 1995). In this area, the work of Sordillo (1995) showed that specific cytokines (naturally produced proteins which function in immunoregulation, inflammation and immunity) can enhance PMN chemotactic, bactericidal and antibody-dependant cytotoxic activity, and can reduce rate, severity and duration of infections in certain mastitis model systems.

Thus it is possible that the recruited PMNs while serving to destroy the invading bacteria, actually cause a large part of the inflammatory reaction in the udder, and that if tissue damage results in loss of feedback control of oxygen-radical tension, progressive tissue damage will continue, possibly leading to the development of chronic mastitis. When considering possible PMN proteinase activity in milks of high PMN levels, it is worth noting that α1-antitrypsin (α1-antiproteinase) and α2-macroglobulin, two inhibitors associated with mastitic milk, are involved in the biological control of Elastase and Cathepsin G (Travis, 1988) and so their presence and activities may be linked, implying presence of these enzymes also in mastitic milk.

In summary the primary role of the PMN cell in the udder is in mastitis, where production of oxygen radicals and proteinases destroy invasive bacteria, but possibly injuring the udder tissue in the process. Functional depression of milk PMNs, as happens in early lactation, can predispose to mastitis, which can also be caused by

selection for cows of low SCC. The primary role of the PMN cell in healthy milk is protective, and phagocytosis of milk components may lead to depressed PMN function, which may also increase possibility of successful bacterial colonisation of the udder.

2.2.3. INFLUENCE OF MILK SCC ON DAIRY PRODUCT QUALITY

2.2.3.1. Milk composition and quality

Mastitis and high SCCs are associated with a number of changes in the compositional and enzymatic properties of milk and this area has been extensively reviewed (see Mitchell *et al.*, 1986; Munro *et al.*, 1984; Politis and Ng-Kwai-Hang, 1988a-c; Kitchen, 1981). A summary of the principal effects of SCC on milk composition is presented in Table 2.5. The relationship between elevated SCCs and proteolysis in milk is discussed in Chapter 3, but also of interest is the relationship between mastitis and lipoprotein lipase activity and lipolysis in milk (Azzara and Dimick, 1985a,b; Murphy *et al.* , 1989), which may have implications for high-fat dairy products such as soft cheeses, which rely on controlled lipolysis for flavour development. This lipolytic activity was shown to be due to macrophages producing lipolytic enzymes by Azzara and Dimick (1985a), and leads to higher free fatty acid (FFA) concentrations in raw milk at milking and these increase on subsequent storage of mastitic milk (Murphy *et al.*, 1989).

Janzen (1972) noted a significant correlation between SCC and flavour score of pasteurised milk, and when Rogers and Mitchell (1989) studied the correlation between log (SCC) and organoleptic grade of pasteurised milk samples from two farms, stored over 14 days at 4°C, the same significant relationship was found ($P<0.05$) for one farm, but not for the other. When the SCC data were divided into bands of 250,000 cell/ml, there was further indication of decreasing grade with increasing SCC. The latter authors also studied the effect of log (SCC) on skim milk powder manufacture and concluded that powders made from high SCC milk had lower titratable acidity and ferrocyanide reducing value, but that solubility and heat stability were not affected. Munro *et al.* (1984) reported that the altered heat stability of mastitic milk could be important in determining the quality of recombined and sweetened condensed milk, and that mastitic milk was less resistant to high temperatures than normal milk. Needs *et al.* (1988) showed that increasing SCC decreased the whipping time and stiffness of 38% fat creams.

2.2.3.2. Effects on cheesemaking

Coagulation properties of milk samples, measured as rennet clotting time, rate of curd formation and final curd firmness, as determined by formagraph, were correlated to milk SCC by Politis and Ng-Kwai-Hang (1988c). Elevated SCCs were associated with significant increase in rennet clotting time and decreased curd firmness. These clotting properties are also correlated adversely with milk pH, which is increased

Table 2.5 *Influence of bovine mastitis on milk properties (from (1) Munro et al. (1984), (2) Grandison and Ford (1986), (3) Kitchen (1983), (4) Haenlein et al. (1972), (5) Barry and Donnelly, 1981,(6) Mitchell et al (1986 a-e) and Murphy et al (1989)).*

Constituent	Influence of increasing SCC	References
Total solids	Decreases	(1), (6)
Fat	Decreases at very high SCC	(1)
Lactose	Decreases	(1),(2), (6)
Titratable acidity	Decreases	(2)
pH	Increases	(1),(3), (4)
Calcium	Decreases	(1),(2), (3)
Potassium	Decreases	(6)
Sodium/Chloride	Increase	(1),(2),(3), (6)
Total casein	Decreases	(1),(2), (3), (4)
α_{s1} casein	Decreases	(1),(3),(5),(6)
ß-casein	Decreases	(1),(2),(3),(5), (6)
κ-casein	Increases (?)	(1),(3), (5), (6)
para-κ-casein	Increases	(3)
γ-caseins	Increase	(1), (5)
Whey proteins	Increase	(1), (3), (4)
α-lactalbumin	Decreases	(1), (3), (6)
ß-lactoglobulin	Decreases	(1), (3), (6)
Immunoglobulins	Increase	(1), (3), (6)
Serum Albumin	Increase	(1), (3)
Proteose peptones	Increase	(3)
Short Chain fatty acids	Increase	(1)
Long chain fatty acids	Decrease	(1)
Enzymes	Increase in general	(1)
N-acetyl-ß-D glucosominadase	Increase	(1)
Catalase	Increase	(1)
Xanthine oxidase	Increase	(1)
Lipase	Increase	(1), (7)
Proteolytic enzymes	Increase	(7)

in mastitic milk (Munro *et al.*, 1984). Decreased coagulum strength and poor coagulum forming properties with increasing SCC were also reported by Grandison and Ford (1986), Mitchell *et al.*, (1989b), Ali *et al* (1980) and Munro *et al.* (1984). Mastitis is also associated with an increase in casein in the soluble phase, as opposed to micelles, most of which is contributed by ß-casein (Ali *et al.*, 1980) as well as losses in total casein due to proteolysis. Thus less casein is available for incorporation into the casein network of the cheese curd. Calcium and phosphate in the soluble phase of milk also increase on short term refrigerated storage of high SCC milk and both this and the change in protein will obviously affect the milk clotting properties.

Brus and Jaartsveld (1971) compared batches of Gouda cheese made from bulk milks with low and high SCC and found that in general, preference was given to cheese made from milk of a low SCC, with regard to consistency, flavour and appearance, and testers could pick out the high SCC milk cheese in triangle tests ($P<0.05$). Grandison and Ford (1986) made Cheddar cheese from low SCC milks, mixed with high SCC milk to create a range of SCCs from 39,000-2,587,000 cells/ml and found that moisture content increased with increasing milk SCC and cheese fat and protein levels decreased while protein losses in whey increased. Lactose in whey decreased, and cheese lactic acid increased. The compression strength of the cheese was decreased, as was cheese springiness and firmness, with increasing SCC, while stickiness and total off-flavours were increased. The authors concluded from statistical interrelations of cheese and milk parameters, that good quality milk with a high casein content, unlike high SCC milk, will produce a firm coagulum and require less time to produce a firm, elastic cheese with low moisture content, and less protein will be lost in the whey in the process. Similarly, Politis and Ng-Kwai-Hang (1988a) and Mitchell *et al.*, (1986b) found that milk SCCs were negatively related to fat, protein, total solids and fat-in-dry-matter in Cheddar cheese, and positively related to protein losses in whey, cheese moisture, and cheese protein-in-dry-matter. High SCC milk was characterised by it's inability to incorporate protein into the curd. Politis and Ng-Kwai-Hang (1988a,b) recommended that to improve cheese quality, milk SCC should be maintained below 500,000/ml and possibly below 300,000/ml. Munro *et al.* (1984) reported that high SCCs were also related to poor cheesemaking properties in Parmesan, Grana, Kostroma, Emmental, Tilsit, Gouda and Camembert cheese.

Cheese yield appeared to be unaffected by SCC, according to Grandison and Ford (1986) but this is in conflict with the work of most authors, who found up to 5% decrease in Cheddar cheese yield from mastitic milk (Lawrence, 1991). Politis and Ng-Kwai-Hang (1988b) found that milk SCCs were inversely related to moisture-adjusted cheese yield and efficiency of cheese yield and that increases in SCC from 100,000/ml to 500,000/ml and further to 1,000,000/ml reduced adjusted yield by 5% and 8.7% respectively and yield efficiency by 11% and 13% respectively. The influence of milk SCC and milk age on cheese yield was investigated by Barbano *et al.* (1991), who found that storage of high SCC milk for 5 days at 4°C reduced casein as a percentage of true protein and cheese yield efficiency, and increased milk SCC and age increased fat and protein losses in whey. Cheese moisture increased with milk SCC and decreased

with milk age, due to negative effect of milk age on starter activity leading to longer make times (also reported by Randolph, 1969), and it was concluded that any increase in SCC above 100,000/ml could have a negative impact on cheese yield efficiency for milks from groups of cows of similar SCC, but that this effect would be diluted down in commingled milks.

2.2.3.3. Properties of proteases from isolated somatic cells

Since the acid phosphatase of bovine milk was shown to be of somatic cell origin in healthy and mastitic milk (Andrews and Alichanidis, 1975; Andrews, 1976), it has been considered likely that the large complements of proteinases associated with these cells might also be present in milk, and thus be significant in casein breakdown in dairy products.

The procedure of Carlson and Kaneko (1973) was used by Grieve and Kitchen (1985) to recover two fractions, rich in PMN cells and non-PMN cells respectively, and these cells were incubated into casein solutions at pH 6.8 to determine caseinolytic activity. Refrigerated storage did not result in protein breakdown, even after cell disruption by homogenisation, and pasteurisation resulted in loss of cell vitality.

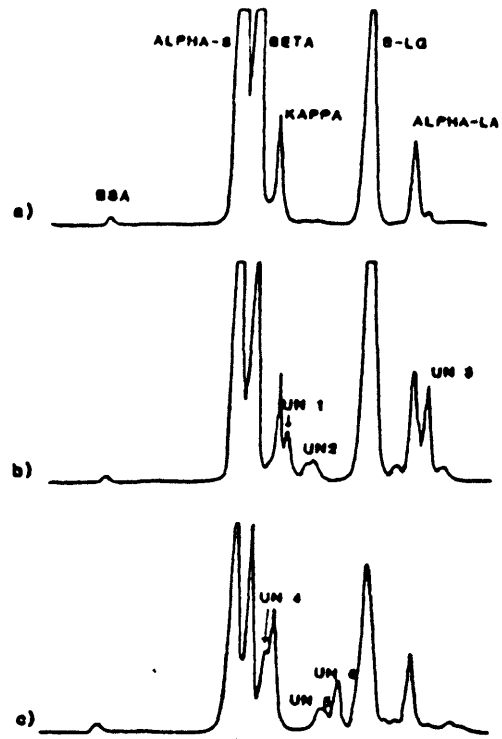


Fig. 2.5 *Densitometric scans of milk samples. (a) unincubated fresh milk, (b) preserved high quality milk with added urokinase (to activate plasminogen to plasmin) incubated for 12 hr at 37°c, (c) preserved high quality milk plus 6-aminohexanoic acid plus urokinase plus somatic cell isolate (equivalent to 2,300,000 cells/ml) incubated for 12 hr at 37°C. (Verdi and Barbano, 1988)*

Table 2.6. *Proteinases in milk somatic cells (from Verdi and Barbano (1991b) and Barrett(1979))*

Cell type	Proteinase	Type	pH optimum
PMNs	Elastase	Serine-	7-8
	Cathepsin G	Serine-	5-7
	Proteinase 3	?	
	Collagenase	Metallo-	Neutral pH
	Cathepsin B	Cysteine-	6.0-6.3
	Cathepsin D	Aspartic-	3.5-4.0
Macrophages	Cathepsin D	Aspartic	3.5-4.0
	Elastase	Serine	7-8
	Neutral cathepsin	?	
	Pepsin	Aspartic	2-4
	Chymotrypsin	Serine	7.5-8.5

Casein proteolysis by PMN and non-PMN leucocyte extracts at 37°C were shown to produce similar polypeptide maps, but these were different to those produced by bacterial proteinases and plasmin. The rate of proteolysis of casein appeared to be in the order α_{s1}> ß >> κ -casein, and overall activity of cells at 1,000,000 cells/ml was less than the levels of natural milk proteinase activity found in milk from healthy cows. A different approach was taken by Verdi and Barbano (1988), who recovered cells from mastitic milk, as opposed to blood, and found that proteolysis products of casein, liberated by these cells, were different to those produced by plasmin and that up to 67% of somatic cell proteolytic activity from mastitic milks survived pasteurisation at 63°C for 30 minutes. Non-plasmin proteolysis was examined using the plasmin inhibitor 6-aminohexanoic acid. Densitometer scans of SDS-PAGE separation of proteolysis products in milks incubated with plasmin and somatic cell isolates are shown in Fig. 2.5.

The differences between proteolytic activity of milk and blood somatic cells was investigated by Verdi and Barbano (1991) in a system of ß-casein in pH 6.6 and 5.2 buffers. Bovine milk somatic cells from cows with mastitis degraded 27.5 and 13.6% of ß-casein after 24 hours at 37°C at pH 6.6 and 5.2 respectively, but these activities were 16.0 and 8.4% for blood cells respectively. No reason for the higher activity of milk cells compared to blood cells was offered, but this may explain the low importance of such proteases concluded by Grieve and Kitchen (1985). PMNs and macrophages contain a number of very active proteases (Table 2.6). Verdi and Barbano(1991) suggested on the basis of studies of inflammation in human tissues that macrophages may be the predominant cell type in chronic infections, and that the levels of their proteases would thus be higher in mastitic milk than blood.

However, this macrophage-dominated somatic cell complement in mastitis is thought unlikely due to studies such as that of Saad and Ostensson (1990) on the somatic cell ecology dynamics of a mastitic infection. Verdi and Barbano (1991b) also suggested that macrophages contain more intracellular proteases at certain stages of development and activity than PMNs (see also Guyton, 1986), but this is based on studies of rabbit and mouse, and is known to vary from species to species so the role in bovine cells is relatively unknown (Cohn, 1975). It is likely that macrophages do however contain higher quantities of cathepsin D than PMNs (Cohn, 1975), which may explain why this, of all leucocyte proteinases, has been the only one isolated from normal milk. From Table 2.6 it can be seen that both cell types contain similar enzymes and that pH may also play a role in the activity of cell-derived proteinases. The role of proteases from PMNs and macrophages in milk and the contributions of the proteases from different cell types in mastitic and normal milk requires further elucidation. It is recognised that the acid protease of milk is of lysosomal, leucocyte, origin (McSweeney *et al.*, 1995) and the cysteine proteases discovered in mastitic milk by Suzuki and Katoh (1990) and shown to be of somatic cell origin, are probably at least partially due to cathepsin B action. It can only be hypothesised that the greatly elevated non-plasmin protease activity associated with mastitic milk, which is known to contain greatly elevated numbers of PMNs, suggests that it is the enzymes of this cell type which are of most interest to dairy science. In macrophage-dominated normal milk, plasmin activity far overshadows any contribution of proteases from these cells, including the contribution of cathepsin D, unless the pH is lowered, as in the case of cheesemaking, and the conditions are more optimal for cathepsin D and less optimal for plasmin action. The levels and action of cathepsin D in mastitic milk are unknown.

Chapter 3

Indigenous proteinases and proteolysis in bovine milk

3.1. Introduction

Proteolysis in milk is a subject which has been intensively studied, as control of the extent and nature of milk proteolysis is the basis of production of a wide range of dairy products, such as cheeses, yoghurts and cultured foods, while undesirable proteolysis can lead to loss of functionality and spoilage. Proteinases of interest to the dairy industry can be classified into three groups, based on their origin (Fox, 1981). The first group are indigenous, or native to the milk, and the primary and most widely studied enzyme in this class is plasmin. Endogenous enzymes are secreted by micro-organisms in cheese and psychrotrophs in milk. The third group consists of exogenous enzymes, which are added to milk or dairy products in semi-purified states during processing, and the most widely familiar example is rennet. Other exogenous enzymes are used in acceleration of cheese ripening and production of protein hydrolysates with modified functional or organoleptic properties. For the purposes of this review, I will deal with the first group exclusively, those proteinases associated with the milk and specifically their role in proteolytic degradation of raw, heat-treated and mastitic milk.

3.2. Methods of assessing proteolysis in milk

3.2.1. METHODS BASED ON NITROGEN ESTIMATION BY KJELDAHL

Bengtsson *et al* (1973) examined the influence of heat resistant proteases on gelation of UHT milk and determined total nitrogen (N), decrease in casein N and increase in non-protein N over long term storage, by Kjeldahl. Noomen (1975), examining proteolytic activity of milk protease in raw and pasteurised cow's milk, used the Kjeldahl method to determine proteolysis by estimating total N in milk samples and the N in the soluble fraction extracted from incubated, rennet-clotted milk using a calcium chloride solution. Soluble N (SN) was then expressed as a % of total N (%SN/TN), and its change on incubation observed.

Aschaffenberg and Drewry (1959) described a method of determining proteolysis in milk, which was later modified as described below by Kang and Frank (1988), who examined proteolysis in raw milk during storage. Milk was acidified to pH 4.65 with acetic acid-sodium acetate buffer at 20°C, then centrifuged and filtered. The casein pellet was washed and washings and filtrate made up to standard volume in which N was determined in duplicate by Kjeldahl. The method of Aschaffenberg and Drewry was also used to assess proteolysis of UHT milk by Guthy *et al.* (1983).

Andrews (1983c) and Scharr (1985) measured proteose peptones in milk by heating milk at 95°C for 20 minutes, cooling to 20°C, acidifying as above and centrifuging out the whey protein-casein coprecipitate. The filtered supernatant was mixed with an equal volume of 24% TCA, recentrifuged and the N content of the proteose peptone pellet determined by Kjeldahl.

3.2.2. SPECTROPHOTOMETRIC AND FLUORIMETRIC METHODS

3.2.2.1. Introduction

The relationship between proteolysis of milk by native-milk or psychrotrophic proteinases and spoilage and loss of functionality has led to the development of numerous assays to monitor proteolysis in milk, which are easier and faster for large numbers of samples than Kjeldahl or electrophoretic methods. The main problem with such methods is the background interference due to the turbid nature of milk, and most methods require an initial step to remove this factor, yielding in the process a clarified extract in which the nitrogenous products produced by proteolysis may be quantified. The most common procedure for this is precipitation of undigested large protein molecules by addition of trichloroacetic acid (TCA) followed by filtration, or centrifugation, and estimation of proteolysis products in the soluble phase. The final concentration in the TCA determines the range of products remaining soluble, with 2 and 2.5% TCA soluble extracts containing small and medium sized peptides and amino acids and 12% TCA soluble extracts containing small peptides and amino acids only. Levels of 0.8% (Haemoglobin method of Nakai *et al.*, 1964), 2% (Hide powder assay method, McKellar, 1984), 7.8% (TNBS method of McKellar, 1981), 10% (Lowry method, Juffs, 1973; OPA method, Church *et al.*, 1983), 12% (A_{280}, Lowry, TNBS and Flourescamine methods, Kwan *et al.*, 1983) and 25% (fluorescamine assay, Chism *et al.*, 1979) have been used. Thus, it is difficult to compare the results and findings of methods using different concentrations of TCA. Some methods do not require the use of TCA (e.g., original HPA method), and some methods use clearing agents or solution of detergents to remove background turbidity of samples (modified TNBS method of Humbert *et al.* 1990; Stead, 1987; Owen and Andrews, 1984), and thus avoid inconsistencies in reporting what breakdown products are actually being measured.

The second factor to be considered in comparing methods is what exactly is being quantified. In the case of most assays, free amino groups, which obviously increase on proteolytic hydrolysis of peptide bonds, are measured by labelling with a colorimetric or fluorimetric group, while in the Lowry assay, only those TCA-soluble peptides containing tryptophan or tyrosine are subsequently measured, and some methods measure breakdown of an added substrate, such as hide powder azure or haemoglobin, rather than the milk protein itself.

3.2.2.2. Absorbance at 280 nm

This is the simplest method of estimating proteolysis in TCA soluble extracts of proteinase hydrolysed milk, based on absorbance of aromatic amino acid residues. Samples are generally diluted 1 in 5 before reading (Kwan *et al.*, 1983). Richardson and TeWhaiti (1978) used absorbance at 280 nm (A_{280}) to assay psychrotrophic proteases in milk.

3.2.2.3. Haemoglobin substrate method

This method was used by Nakai *et al.* in 1964 to examine proteolysis in sterile milks. Milk was mixed with a solution of sterile haemoglobin in borate buffer and incubated at 37°C for various times and the reaction stopped by precipitation of protein with TCA. Hemin-containing split products were then determined by absorbance at 400 nm.

3.2.2.4. The 'tyrosine value'

The oldest, and possibly most commonly used, method of assessing proteolysis in milk is the assay of Lowry *et al.* (1951), as modified by Juffs (1973), also known as the 'tyrosine value' method. In this method, amino acids and peptides, liberated by proteolysis, are extracted from milk by precipitation of larger proteins and fat with trichloroacetic acid (TCA), and then quantified by use of a Lowry-Folin phenol reagent (also known as Folin-Ciocalteu reagent), which reacts with aromatic amino acid residues such as tyrosine and tryptophan. Results are expressed in terms of a standard curve of known tyrosine concentrations, hence 'tyrosine value'. Juffs (1973) found no relationship between tyrosine value and bacterial counts, but in a later paper (1975) he showed the procedure to have application as an index of organoleptic and bacteriological quality of raw milk, and to have applications for monitoring proteolysis in pasteurised milk and cream. Skudder (1981) used the Lowry assay to monitor proteolysis in UHT milk, as did Richter *et al.* (1979) for UHT cream. Senyk *et al.* (1985) found the tyrosine value, used as an index of milk proteolysis, to correlate significantly with somatic cell count, both before and after incubation at 37°C. Murphy *et al.* (1989) used the test to follow proteolytic activity in milk on mastitic infection.

3.2.2.5. Colorimetric measurement of ammonia

Koops *et al.*, (1975) digested small samples of milk with sulphuric acid and hydrogen peroxide, using a potassium sulphate/mercuric oxide catalyst. The ammonia liberated was then determined colorimetrically by reaction with an alkaline mixture of sodium salicytate and sodium dichloro-isocyanurate. Comparison to Kjeldahl analyses for samples of milk, milk powders, whey, cheese, milk nitrogen fractions and dairy

effluent was favourable, with high repeatability. Snoeren *et al.* (1979, 1981) examined proteolysis in UHT milks during storage by fractionation of total milk N into non-casein N, non-protein N and 2% TCA soluble N and determined N by the above method as did deKoening *et al.* (1985), who examined the role of plasmin in age-thinning and gelation in UHT sterilised skim milk.

3.2.2.6. The TNBS method

Fields (1971) developed a technique for the measurement of amino groups in proteins and peptides using 2,4,6-trinitrobenzene sulponic acid (TNBS) to form sulphite complexes with trinitrophenylated amino groups, with measurement of the resulting absorbance at 420 nm. McKellar (1981) applied this method to TCA filtrates of milk, adding a borate buffer (pH 9.2 to counteract the acid) and an aqueous solution of TNBS, allowing colour development to proceed for 30 min in the dark at 25°C and stopping the reaction by addition of sodium phosphate/sodium sulphite buffer, before reading absorbances at 420 nm against a standard curve of glycine. He claimed that this method could be used to detect proteolysis before spoilage or significant electrophoretic change had taken place. deRham and Andrews (1982b) also used this reagent to assay levels of proteolytic activity in raw and pasteurised milks.

Humbert *et al.*, (1990) used TNBS in combination with a new dissolving agent to measure proteolysis in milk. Their method involved taking a diluted sample of milk (1/25 or 1/40) and adding carbonate buffer and an aqueous solution of solution of TNBS, shaking for 1 hr at 37°C and adding 1.8M acetic acid and a patented alkaline dissolving agent, comprised of an alkaline mixture of organic solvents and detergents (hence the addition of acid at the same time, to prevent alkaline hydrolysis of TNBS at very high pHs). This reduced background interference and allowed estimation of proteolysis in whole milk, as opposed to TCA filtrates, and the method correlated very strongly (r=0.995) with the ninhydrin procedure (Reimerdes and Klostermeyer, 1975).

The TNBS method of McKellar (1981) was also used by Harwalker *et al.*, (1993) to monitor proteolysis of milk by psychrotrophic proteinases and plasmin, in relation to astringency and off-flavours, and Manjai *et al.*, (1986) to monitor proteolysis of stored UHT milk. Church *et al.*, (1981) used a variation of the method to monitor hydrolysis of milk proteins by immobilised Pronase in a glass bead column

3.2.2.7 The OPA method

An o-phthaldialdehyde (OPA) reagent, in a buffer containing ß-mercaptoethanol and SDS, was used to determine proteolysis in milk and isolated milk proteins, based on the formation of an adduct which absorbs strongly at 340 nm between the OPA and ß-mercaptoethanol and α-amino groups released by proteolysis (Church *et al.*, 1983). The use of SDS reduces background due to contaminating protein, stops the reaction, and ensures exposure of all amino groups. Compared to the Lowry reaction the method is more sensitive, due to detection of all hydrolytic products. The assay was also

reported to be more rapid and convenient than ninhydrin, TNBS or fluorescamine methods. Again, 10% TCA soluble extracts of milk were used to isolate proteolysis products for measurement.

3.2.2.8. The Hide Powder Azure method

Hide powder azure is a general proteinase substrate prepared from collagen (Rinderkecht *et al.*, 1968), by denaturation with a covalently bound dye which makes it more susceptible to proteolytic attack. Cliffe and Law (1982) developed a method to use this substrate to determine proteolysis by bacterial proteinases in milk, with the solid substrate kept in suspension in the milk or enzyme solution to be tested by constant inversion for various incubation periods at 37°C. The mixture was then skimmed and mixed with diethyl ether, recentrifuged and casein removed from the lower layer by centrifugation at 150,000g. Release of dye labelled peptides into the mid-natant after this step was then measured at 595 nm. They concluded this assay to be quicker, more precise and 3.2 times more sensitive than assays of soluble N released from casein, although HPA is expensive. Kalogridou-Vassiliadou and Law (1982), compared sensitivity of this method to direct observation of proteolysis by electrophoresis, and found it able to detect the presence of proteinases before their effects became visible using electrophoretic techniques. McKellar (1984) modified and simplified the method, using TCA precipitation instead of ultracentrifugation. Owen and Andrews (1984), developed a method to clarify milk based on addition of Triton-X 100 and EDTA, and Stead (1987) used this approach to clear milks after digestion of added HPA, allowing direct measurement of light absorbance, after a short incubation period, removing the need for TCA precipitation or centrifugation.

3.2.2.9. Azocoll method

Rollema *et al.* (1989) reported the use of collagen labelled with azo dye groups (azocoll) to detect proteolysis by bacterial cultures in milk. Casein was removed from the milks by centrifugation at pH 3.8, pH readjusted to 6.7, azocoll added and the mixtures incubated at 37°C for various times in a shaking water bath. TCA was added to a final concentration of 1.8% and the absorbance of the resulting supernatant measured at 520 nm relative to a blank (milk without added enzyme incubated with azocoll). This increase in absorbance was taken as a measure of proteolytic activity.

3.2.2.10. Fluorescent assays for proteolysis

Chism *et al.*, (1979) reported a sensitive assay for proteases in sterile milk, based on the reaction of primary amino groups of TCA-soluble peptides and amino acids with fluorescamine, which possesses no fluorescence itself, but fluoresces at excitation and emission wavelengths of 390 and 475 nm respectively when tagged to these groups at pH 9.0 (Schwabe, 1973). Chism *et al.* (1979) used the method to

monitor proteolysis in casein digested with papain and TCA filtrates of UHT-sterilised milk. Slight modifications of this method were reported by Rollema *et al.* (1989).

3.2.2.11. Comparative studies of methods

Kwan *et al.*, (1983) compared the A_{280}, fluorescamine, Lowry and TNBS methods for detection of proteolysis in the 12%-TCA soluble fraction of milk protein which had been digested with trypsin, comparing assay methods by a dimensionless detectability factor. They found that, of the four methods, the fluorescamine method was the most reliable and sensitive, and that the TNBS method was the next most reliable, followed by A_{280} and the Lowry method. The poor performance of the Lowry assay may be due to the fact that it alone measures specific proteolysis products (aromatic amino acids) as opposed to total products, and significant proteolysis may occur without production of TCA-soluble peptides containing these amino acids. McKellar (1984) compared the hide powder azure (HPA) and TNBS methods, and concluded that the methods did not differ significantly in reliability. The HPA method is simpler and requires less reagents than the TNBS method, making it more attractive for routine use. However, HPA is relatively expensive.

Samples *et al.* (1984) compared the Lowry and TNBS methods for measuring proteolysis in Cheddar cheese slurries, and found that again, the TNBS assay was more suitable, detecting a linear increase in TCA soluble nitrogen over 4 days incubation, while the Lowry method only detected a significant increase on the third day.

Rollema *et al.* (1989) carried out an inter-laboratory study of 2 fluorescamine assays, a TNBS assay, an azocoll assay, a HPA assay, a thin layer casein diffusion assay and an ELISA method with regard to detection of proteolytic enzymes from psychrotrophic bacteria. They found the fluorescamine, TNBS and azocoll methods to be comparable with respect to sensitivity. The HPA assay showed poor sensitivity and no linear relationship between proteolytic activity and proteinase concentration. Also the Lowry assay and thin-layer caseinate diffusion assays were judged too insensitive for use in quality control of dairy products, but were thought to be useful as a measure of the rate of casein degradation. With regard to quality control of dairy products and assessing keeping quality of products, the fluorescamine, TNBS and azo-coll assays were all judged to be reasonably satisfactory.

3.2.3. ELECTROPHORETIC METHODS OF EXAMINING MILK PROTEOLYSIS

3.2.3.1. Urea based electrophoresis methods

In 1969 Morr used vertical zonal electrophoresis in 8% acrylamide gels (pH 8.6) to examine protein aggregation in normal and UHT skim milk, and in 1971 he compared starch gel and polyacrylamide gel electrophoresis (SGE and PAGE) for examining casein degradation products in cheese (Morr, 1969; Morr, 1971). The PAGE method involved 7% acrylamide gels, pH 9.2, 4.5 M urea, with mercaptoethanol and

urea used to dissociate samples. In the later study he found that while both gave similar numbers and locations of protein zones, SGE gave more symmetrical, better defined zones and better resolution, and allowed simultaneous resolution of cationic and anionic proteins.

With regard to starch gel electrophoresis (SGE), heating a relatively large concentration of starch granules in an aqueous buffer, followed by cooling, leads to a gel forming, which under electrophoretic conditions, provides a excellent medium for protein separations. However, the composition of natural starch can vary with regard to proportions of polymer forming subunits, and so reproducibility is hard to achieve, and SGE gels are hard to analyse by densitometry (Andrews, 1986). Thus, despite the favourable comparisons reported by Morr (1971) polyacrylamide gel electrophoresis has become the preferred medium.

Peterson (1963) and Thomson *et al.* (1964) described a system using continuous, 5% gels with a tris-chlordide-EDTA buffer system without urea which was supplied by some manufacturers of electrophoresis equipment, and thus became standard protocols in many laboratories (Creamer, 1991). This method was used by Harwalker (1972a,b) to examine astringent flavour fractions extracted from milk and cheese. Randolph *et al.* (1974) used the continuous PAGE method of Morr (1969) to examine protein distribution in milk proteins from mastitic cows. A later continuous system using 4.5% acrylamide, 4.5M urea containing gels (tris-EDTA-barbitone buffer system, pH 7.9) was described by Davies and Law (1977a) and used by Barry and Donnelly (1980) to examine casein composition in Friesian herd milks.

These were all continuous systems, where the same buffer ions are present throughout the sample, gel and electrode vessel reservoirs, at constant pH. The protein sample is loaded directly onto the gel in which separation is to take place, which has pores sufficiently small to allow a size fractionation of the sample components during electrophoresis (Hames, 1990). In 1972, Kiddy and Rollins described the use of discontinuous PAGE for immunoglobulin typing of cow's milk, with 7% Cyanogum acrylamide, pH 8.9 gels, with no urea or mercaptoethanol present. Discontinuous systems use a two stage gel, with the proteins entering a stacking gel, of 4% acrylamide, pH 6.7 before entering the larger, main separating gel described above. The reservoir buffer surrounding the gel was also of different composition, being a Tris-Glycine buffer, pH 8.3 as opposed to Tris-HCl buffers used in the gel solutions. The advantage of discontinuous systems over continuous systems is that large volumes of dilute samples can be applied to the gel and good resolution obtained, because of the concentration of proteins into narrow zones or stacks in the large-pore (small acrylamide concentration) stacking gel prior to resolution in the small-pore separating (or resolving) gel. A change in the mobility and behaviour of the buffer ions on entering the separating gel, combined with the sieving effect of the small-pore gel, causes the proteins to be unstacked and resolve.

In 1975, Andrews used a variant of the discontinuous system of Kiddy and Rollins (1972) discussed above to examine protein polymerisation in UHT milk, incorporating urea into the buffers, and applying samples in buffer with urea and

mercaptoethanol. They used cylindrical gels run in quartz tubes (5 mm x 75 mm), with one sample applied per tube, as opposed to the slab gels previously used, and quantified the separated proteins by densitometry at 280 nm (Andrews, 1975). They used slab-gel SGE to qualitatively compare the proteins in different samples. Anderson and Andrews (1977) used the method of Kiddy and Rollins (1972), omitting spacer (stacking) gels and including internal standards to correct for gel-to-gel variation, to quantify separation of whey proteins and caseins in high somatic cell count milk, and this method was also used to observe casein proteolysis by bacterial enzymes by Kalogridou-Vassiliadou and Law (1982).

deRham and Andrews used the method of Kiddy and Rollins (1972) to examine proteolysis in normal and mastitic milks (deRham and Andrews, 1982 a,b), but a year later, Andrews published a new method for examination of proteolysis products in milk (Andrews, 1983a). This used discontinuous 12.5% acrylamide gels (stacking gel 4.2% acrylamide), with no urea, buffered with Tris-HCl to pH 8.9 and 7.6 respectively, to perform qualitative electrophoresis in slab gels. In the case of quantitative casein measurement in tube gels, urea was incorporated into the gel buffers, and mercaptoethanol was included in the stacking gel buffer, both of which measures will ensure that no proteins are aggregated, which would cause anomalous comigration. While urea is always included in cheese gel buffers to break up the protein structure, it is frequently omitted from milk gel recipes, where often better resolution of whey proteins is obtained in this case. The reason for this is that denatured whey proteins (as would be present in urea-containing gels) behave quite like caseins in their solubility behaviour and thus will not resolve effectively. This principle is evident in the methods of Anderson and Andrews (1977), who isoelectrically removed whey proteins and casein from milk and separated them on gels, omitting and containing urea respectively. Comparison of electrophoretograms obtained using the two methods (Kiddy and Rollins (1972) and Andrews (1983a)) shows that the 12.5% gels gave clearer, better resolved gels, as more acrylamide leads to a closer-textured gel, with a higher degree of separation of closely related proteins or polypeptides.

Andrews also used his new method to examine the role of somatic cell proteinases in casein breakdown in mastitic milks (Andrews, 1983b) again using both tube and slab gels, and in a study of proteose peptones in milk (Andrews and Alichanidis, 1983) this same strategy was used. In 1990, Igarashi used a variation of the 1970s method of Kiddy, with tris-Hcl-urea buffers to examine γ-caseins in milk.

The most recent developments of urea-based electrophoresis systems have been the use of the integrated Pharmacia Phast-System electrophoresis unit, with tris-EDTA-urea- mercaptoethanol buffer systems and gradient polyacrylamide gels to separate astringent off-flavour components from milk (Harwalker *et al.*, 1989, 1993).

3.2.3.2. SDS-PAGE techniques

During electrophoresis of proteins in a polyacrylamide gel, separation takes place according to both size and charge differences of the molecules, due to the electric field applied across the gel causing more negatively charged proteins to move faster towards the positive electrode at the opposite end of the gel, while the pore structure of the gel impedes the progress of larger molecules. The proteins are either not dissociated, or if urea is included, they are denatured by disruption of hydrogen bonds without affecting their intrinsic charge.

If the proteins are heated to 100°C in the presence of the ionic detergent sodium dodecyl sulphate (SDS) and a thiol reagent such as mercaptoethanol (to cleave disulphide bonds), however, the intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound detergent. Thus the SDS-polypeptide complexes migrate in polyacrylamide gels of appropriate porosities strictly on the basis of size, allowing the determination of the molecular mass of polypeptides by comparison to the migration distances of standard proteins of known molecular weight run on the same gel.

The classic SDS-PAGE method, on which most procedures are based, was developed by Laemmli (1970) to compare bacteriophage head proteins. This involved 8 or 10% acrylamide gels, containing tris-HCl buffers (pH 6.8) and 0.1% SDS, with 3% acrylamide stacking gels, run in glass tubes. Samples were prepared in buffer containing mercaptoethanol and 2% SDS, and immersed in boiling water for 1.5 min to ensure complete dissociation. A similar method was described by Swank and Munkres (1971) for separation and molecular weight estimation of peptides, using 10 or 12.5% acrylamide gels and incorporating 0.1 to 1% SDS and 8 M urea into all buffers.

Church *et al.* (1981) used this latter method to examine hydrolysis of milk proteins by pronase, and McPherson and Kitchen, in the same year, separated proteins from the aqueous phase of butter on Laemmli gels of 7.5% or 15% acrylamide. Grieve and Kitchen (1985) used the method of Laemmli (1970) (15% acrylamide gels) to separate proteolysis products of natural milk and bacterial proteinases on caseins.

Verdi *et al.* (1987) used SDS-PAGE to separate proteolysis products in low and high SCC milk, but prepared separating gels with a gradient of acrylamide concentration from 10% to 20%, with 6% stacking gels. Milk samples were mixed 1:10 with buffer and boiled, and it was found that the presence or absence of dithiothreitol (a cysteine reducing agent) would affect separation, with some proteose peptone components migrating to the same position as ß-lactoglobulin in its presence, but resolving well if it is omitted. The advantages of gradient gels over conventional, single concentration, gels is that a wider range of molecular weight components may be fractionated, and much sharper protein bands are produced (Hames, 1990). This method was also used in analysis of proteolytic activity in mastitic milk by Saeman *et al.* (1988), and to examine the effect of the milk proteinase system on UHT milk (Kohlmann *et al.*, 1988).

3.2.3.3. Detection and quantification of proteins in gels

Randolph *et al.* (1974) used a 1% solution of amido black in a methanol:acetic acid:water mix to stain gels after electrophoresis, with electrolytic destaining. This basic procedure was used by most authors for urea based and SDS gels, (Kiddy and Rollins, 1972; Barry and Donnelly, 1980) with minor variations such as first fixing the proteins with a TCA containing solution or including TCA in the stain (Church *et al.* ,1981; deRham and Andrews, 1982b; Andrews, 1983a; Laemmli, 1970; McKellar, 1981), substituting coomassie blue for amido black (Church *et al.* ,1981; Anderson and Andrews, 1977; McPherson and Kitchen, 1981), and destaining in a methanol:acetic acid:water solution (Church *et al.* ,1981; McPherson and Kitchen, 1981). Harwalker *et al.*, (1989, 1993) used the far more sensitive silver stain to detect proteins after electrophoresis.

The second major approach to analysing the breakdown of caseins is quantification by densitometry of tube or slab gels, either by scanning unstained gels at 280 (Andrews, 1975) or by staining and then scanning at a wavelength suitable for the colour of the stained proteins (Anderson and Andrews, 1977; Verdi *et al.* , 1987).

3.2.4. ALTERNATIVE METHODS FOR ASSESSING PROTEOLYSIS IN MILK

An enzyme linked immunosorbent assay (ELISA) technique was described by Rollema *et al.* (1989) using antibodies specific for a proteinase of *Pseudomonas fluorescens* P1, which was highly sensitive and rapid, but still in a developmental stage with regard to quality control applications. Picard *et al.* (1994) developed an ELISA method for determination of caseinomacropeptide in order to estimate the proteolysis of κ-casein by psychrotrophic bacteria in bulk raw milk.

Rollema *et al.* (1989) also described a thin layer caseinate diffusion assay, using petri dishes full of casein-agar with wells, into which the sample to be tested is placed, and width of hydrolysis zones measured after 24 hr at 30°C, but this was found to be too insensitive for use in quality control of dairy products. Igarashi (1990) described a method for determination of γ-caseins in milk due to plasmin action, involving extracting them from milk with an ethanol-sodium thiocyanate-calcium chloride buffer and separating and measuring them on a DEAE-cellulose column.

Reversed phase high performance liquid chromatography (RP-HPLC) is a powerful method for the separation and quantification of proteolysis products in cheese (Ardo and Gripon, 1991), but very few authors have applied it to the study of milk proteolysis. Lopez-Fandino *et al.* (1993) used this technique to investigate protein breakdown during storage of UHT milk, and compared chromatograms of the pH 4.6 soluble portion of milk to those obtained by digesting casein with plasmin and psychrotrophic bacterial enzymes, and concluded that it was a reliable method with high resolving power.

Harwalker *et al.*, (1989,1993) used fast protein liquid chromatography (FPLC) with Mono-Q anion-exchange columns and a stepwise NaCl gradient to separate

astringent flavour components from milk, and Girardet *et al.* (1991) used hydrophobic interaction FPLC to purify hydrophobic components of milk proteose peptone, which they then characterised by two-dimensional electrophoresis.

3.3. Proteolysis in good quality, raw milk

In 1975, Noomen investigated the proteolytic activity of milk protease in raw and pasteurised cow's milk and found that α_s -casein and ß-casein were both attacked by milk protease (at the time, it was only becoming widely recognised that milk contains even one proteolytic enzyme of non-bacterial origin). His approach was to take raw milks of high bacteriological quality (colony counts <30/ml), add a preservative to prevent bacterial growth (thimerosal), and incubate them at 37°C, anaerobically, for up to 3 days. ß-casein was attacked two to three times faster than α_s -casein, and he concluded that the contribution of this enzyme to cheese could be of greater interest than generally expected (Noomen, 1975). Reimerdes and Herlitz (1979) showed that proteolysis of ß-casein to γ-caseins in raw milk was greater at 4°C than 26°C, due to a reversible dissociation of ß-casein and 'trypsin-like enzymes' (plasmin, like trypsin, is a serine proteinase) from the micelles and subsequent irreversible proteolytic degradation of the protein. They concluded that cold storage of milk for up to 48 hours would not affect the flavour development or processing properties, but longer storage may cause problems with the cheesemaking properties of milk.

The proteose peptone fraction of milk can be prepared by heating bulk raw milk at 95 °C for 30 min, cooling, adjusting the pH to 4.6, removing the denatured whey proteins and caseins by centrifugation, and precipitating the proteose peptones by adding TCA to 12%. This fraction contains at least 38 components and is very heterogeneous (Andrews and Alichanidis, 1983). Andrews (1979), investigating proteolysis of ß-casein during storage of raw milk, showed that proteose peptone components PP5 and PP8-fast represent residues 1-105, 1-107 and 1-28 of ß-casein produced by the proteolytic cleavages which form the γ1, γ2 and γ3 caseins (residues 29-209, 106-209, and 108-209 respectively, all commencing at lysine residues), which are ascribed to the action of plasmin on the C-terminal end of ß-casein (for a review of early work in this area see Humbert and Alais, 1979). Eigel *et al.* (1979) studied autoproteolysis of preserved milk at 37°C, and found that aminohexanoic acid and diisopropyl fluorophosphate, inhibitors of serine proteinases, inhibited the breakdown of casein, while urokinase accelerated the process, and they concluded that bovine plasmin occurs in milk and is identical to alkaline milk protease. Barry and Donnelly (1980), investigating casein composition in Friesian Herd Milks, found increased levels of γ-caseins and decreased levels of α_s -casein and ß-casein in late lactation milk, which they ascribed at least partly to action of milk proteinase.

deRham and Andrews (1982a) examined the role of plasmin and plasminogen during proteolysis in normal bovine milk over 24 hours at 37°C, and found significant urokinase-activated plasmin activity, most of which was micelle-associated and inhibited by soya bean trypsin inhibitor (SBTI, a serine proteinase inhibitor). The

remaining activity was in the serum, was not inhibited by SBTI, not activated with urokinase and gave a poorly defined proteolytic pattern, with few clearly defined products. Longer incubations demonstrated spontaneous activation of plasminogen in milk, and the main proteolysis products of the breakdown of ß-casein, which occurred preferentially, were γ-caseins, PP5 and PP8-fast. Unidentified α_s -casein breakdown products were also visible. This study also concluded that far more realistic information on the roles of milk proteinase(s) could be obtained from the study of milk as opposed to systems of caseins in buffer, as previously widely used. Andrews and Alichanidis (1983) however, used exactly such experiments with highly purified caseins to probe the origins of the 38 or more proteose peptone components in milk, and identified 90% of these in this way, including a fragment corresponding to ß-casein residues 29-105. They concluded that the composition of the proteose peptone mixture varied over time and temperature of the milk, and could not thus be defined unless the history of the milk was also defined. The findings of the last two studies mentioned were essentially reconfirmed by Andrews (1983a), who reported that PP5 was an intermediate product in the proteolytic pathway, but that most proteose peptones increased during storage, due to the action of proteolytic enzymes. Inhibition studies revealed that the major proteinase was plasmin, but differences in the proteolytic pattern induced by storage and by added plasmin, and use of SBTI, showed clearly at least one other enzyme to be present in normal milk.

Verdi *et al.* (1987), showed that, in low SCC milk, incubation at 37°C for 24 h led to a decrease by 21% of total casein, and that whey proteins were unaffected by milk proteolytic activity. The ß-casein, α_s -casein and κ -casein were all attacked, in that order, but the breakdown of κ -casein, not elsewhere reported, was thought to be an anomaly. Kang and Frank (1988) used the same incubation conditions as the above researchers and found proteose peptone N to be a good index of relative plasmin activity, which accounted for most of the proteolytic activity in raw milk, but that there was only low proteolytic activity in milk at 4°C, in contrast with the results of Reimerdes and Herlitz (1979). The former authors also found that plasmin activity still only accounted for 33% of cold-storage change in proteose peptone nitrogen, in contrast with Scharr (1985) who found highly significant ($P<0.001$) correlation between plasmin activity and proteose peptones, and concluded that either there may be some non-proteolytic release of proteose peptones from casein, or non-plasmin proteinases were responsible for the rest of the activity seen.

Igarashi (1990), investigating γ-casein formation in raw milk, showed that this reaction increases with incubation time and temperature, and is accompanied by a time lag, the length of which increases with decreasing temperature (again by contrast with Reimerdes and Herlitz (1979)), being 1hr at 37°C, 6 hr at 11°C and much longer at 4°C. This, he proposed, was due to activation of plasminogen, which, it has been reported, is the form in which most or all plasmin in freshly secreted milk is found (deRham and Andrews, 1982a), and possibly the interaction of inhibitors. Igarashi (1990) also suggested that two separate proteolytic activities with pH optima at 7.2-7.5 and 8.0

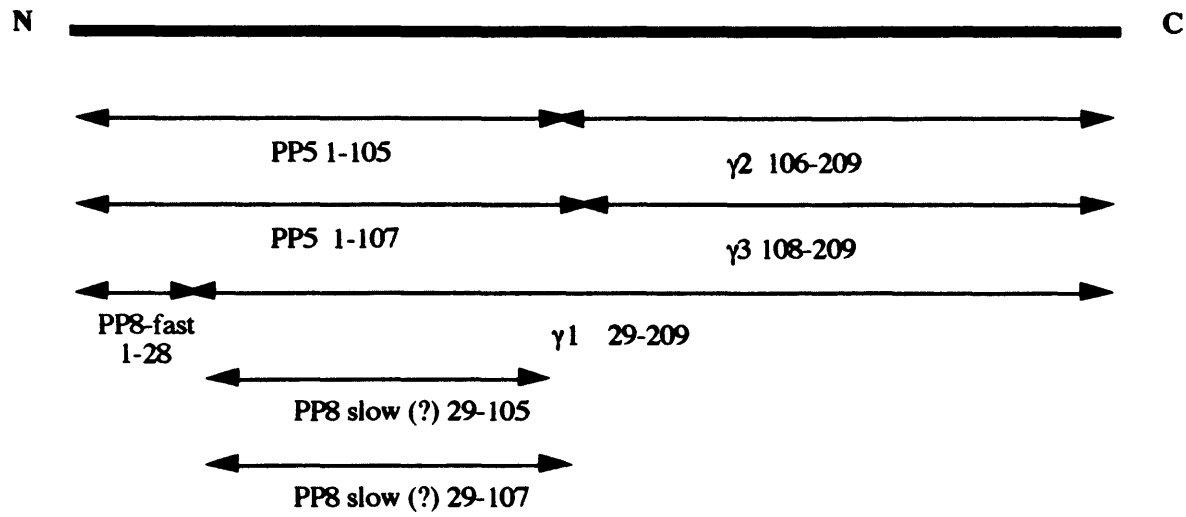


Fig. 3.1 *Cleavage sites on bovine ß-casein known to be acted on by plasmin in milk, and the products arising from this action. The identity of PP8slow has been called into question by Andrews and Alichanidis (1983), but is still frequently represented as shown.*

were responsible for production of γ-caseins. The proteose peptones were separated on hydrophobic interaction-FPLC by Girardet *et al.* (1991), who found that the hydrophobic proteose peptone fractions had molecular masses of 28-30,0000, 19,000 and 11,000, consisting of 13, 4 and 2 glycoprotein fragments respectively. The γ-caseins were shown by Harwalker *et al.* (1993) to be responsible for a strongly astringent off-flavour in milk, if over-produced by plasmin.

The principal plasmin-derived breakdown products of ß-casein in milk are shown in Fig. 3.1. In summary, work on good quality, raw milk showed that the principal proteinase in this case was plasmin, and the action of this enzyme on ß-casein has been well studied, but there are definite indications that at least one other enzyme is present.

3.4. Proteolysis in high somatic cell count milk

Mastitis, or inflammation of the udder, due to bacterial infection, is associated with elevation of somatic cell count (SCC) and influx of blood components, including plasmin and plasminogen, to the milk, resulting in alterations to the proteolytic activity therein. The distribution of milk proteins in cows testing positive with the Wisconsin Mastitis Test (WMT) was examined by Randolph *et al.* (1974), who found that proteose peptone, serum albumin, immunoglobulins, α_s-casein, κ-casein and a number of unidentified electrophoretically mobile products were all present in higher quantities

than in WMT negative milks, indicating overall altered protein synthesis and higher proteolytic activity in these milks. ß-lactoglobulin and ß-casein were present in higher quantities in mastitic milks and α-lactalbumin levels were unaffected. They concluded that changes in the protein distribution could greatly influence functional properties, such as heat stability and micelle formation.

Anderson and Andrews (1977) induced mastitis in cows by infusion of *Str. agalactiae* or *Escherichia coli* endotoxin, and followed the subsequent changes in concentrations of milk protein components, with both treatments giving similar increases in SCC and changes in milk proteins, but with the endotoxin infusion causing shorter-term effects. The treatments had little effect on α-lactalbumin or ß-lactoglobulin levels, but serum albumin and immunoglobulins levels rose significantly. The α_{s1} -casein and ß-casein levels were reduced and inversely related to SCC, with appearance of para-κ-casein, not normally present in milk (Figs. 3.2 and 3.3). Casein concentrations returned to pre-infection levels 2d and 5d after endotoxin and bacterial infusions respectively. The reduction in α_{s1} -casein and ß-casein levels was also found by Andrews (1983b), Barry and Donnelly (1981) and Verdi *et al.* (1987).

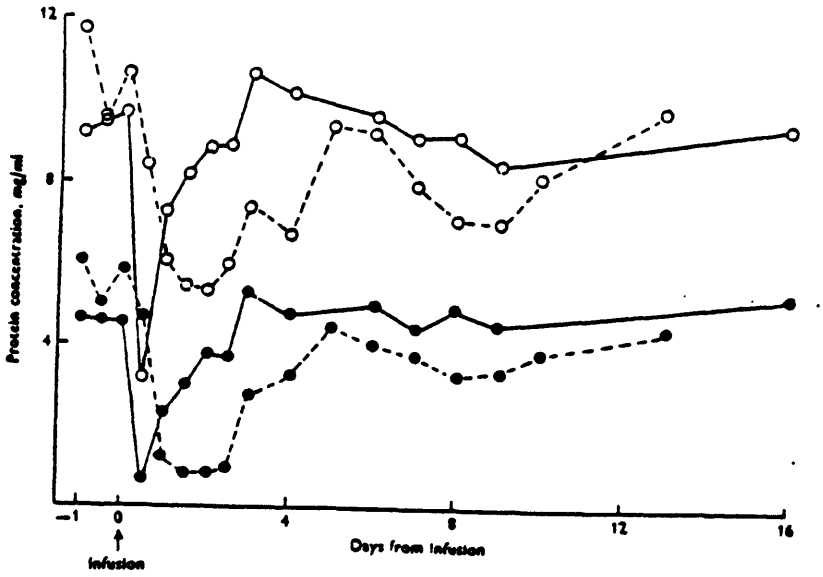


Fig. 3.2 *Progressive changes in the skim-milk concentrations of α_{s1} -casein (open circles) and ß-casein (closed circles), following the intramammary infusion of 1 µg of E. coli endotoxin (full line) or a culture of Str. agalactiae (dashed line) (from Anderson and Andrews, 1977)*

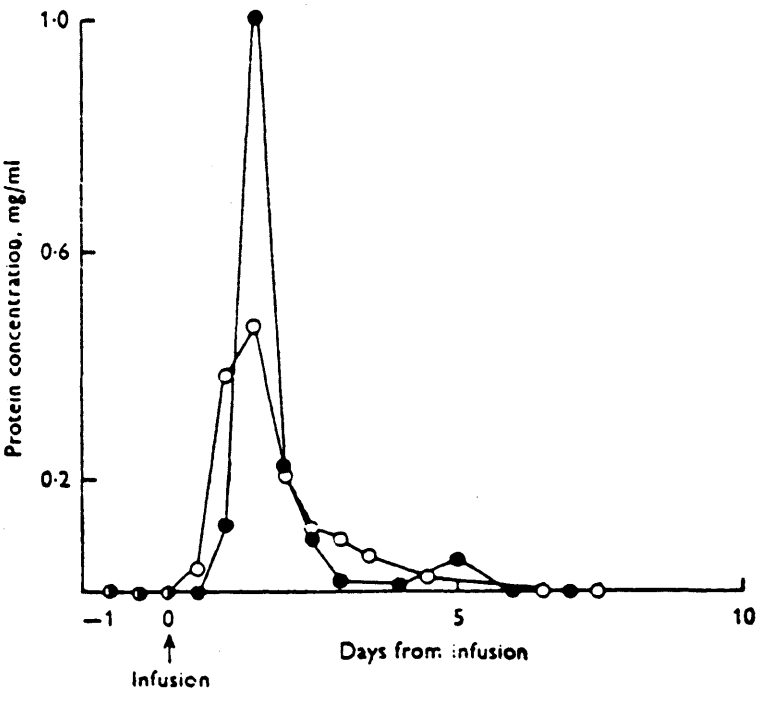


Fig. 3.3 *The effect of intramammary infusions of E. coli endotoxin (open circles) or Str. agalactiae (closed circles) on detection of para-κ-casein in the skim-milk (from Anderson and Andrews, 1977).*

These results were in conflict with those of Randolph *et al.* (1974), who suggested that some individual casein levels may rise, but this was thought to be in terms of the reporting of levels in terms of total output of milk components, absolute concentrations (mg/ml) or relative levels. Anderson and Andrews (1977) concluded that elevated proteolytic activity, possibly of somatic cell origin, is present in mastitic milk. Barry and Donnelly (1981) found that when milks from infected quarters were incubated with SBTI (soya bean trypsin inhibitor), most of the proteolytic activity was inhibited, leading them to conclude that elevated plasmin activity was sufficient to account for most of the changes seen. However, it must be considered that the milk studied by Barry and Donnelly (1981) had up to ten-fold lower SCCs then those milks studied in many reports of somatic cell proteinase activity in milk, presumably due to the severity of the infection (Grufferty and Fox, 1988c).

A 5-10 fold increase in proteolytic activity in mastitic milk compared to normal milk was reported by deRham and Andrews (1982b), with plasmin estimated as accounting for merely 33% of this activity (table 3.1). Another third was micelle associated, not-inhibited by SBTI and gave three characteristic protein fragments on PAGE, while the remainder was in the serum, and gave no well defined electrophoretic bands. Electrophoretic analysis showed that α_{s1} -casein and ß-casein were degraded at approximately the same rate, and although typical plasmin products were visible,

Table 3.1: *Contributions of plasmin and somatic cell enzymes to proteolysis in milk (adapted from deRham and Andrews, 1982b). Proteolysis is expressed in arbitrary units.*

Samples	A	B	C	D	E
SCC (,000s/ml)	34	23	26,000	20,000	30,000
Control	2.0	1.0	17.5	18.0	15.0
+ SBTI	0.0	0.0	10.5	13.0	10.0
+ urokinase	8.0	8.5	23.0	30.0	-
Plasmin activity as % of total	100%	100%	40%	28%	33%
Micellar proteinase activity	1.0	1.0	14.0	15.0	10.5
+ SBTI	0.0	0.0	5.0	8.0	4.0
Serum proteinase activity	0.0	0.0	7.0	7.0	6.5
Freeze thawed skim	-	-	-	19.0	20.0

(they estimated that there was a doubling of plasmin activity) and a higher rate of plasminogen activation in mastitic milk, there were atypical changes in the γ-casein region. The proteolytic activity was concentrated by centrifugation into the cream phase and was enhanced by freezing and thawing. They concluded that there was very efficient hydrolysis of the caseins in high SCC milk to a large range of peptides, many quite small, and that this proteolytic activity could well play a role during cheese maturation, as well as affecting cheesemaking properties of the milk, and harmfully affect cheese texture and flavour.

The role of somatic cell proteinases in mastitic milk was again investigated by Andrews (1983b) who found that some of the proteinase activity originated in the somatic cells, and suggested that the identity of the cells making up the total population was important, due to differences in relative proteinase activity and length of elevation of this activity depending on whether endotoxin or bacterial induction of mastitis was examined. The proteolytic activity had a very broad temperature and pH optima curve (Figs. 3.4 and 3.5), and this and inhibition studies suggested that several different enzymes were present, of which one was clearly plasmin.

A more qualitative approach to relating milk proteolysis to SCC was taken by Senyk *et al.* (1985), who found high correlations between SCC and initial milk tyrosine value ($r = 0.60$) and tyrosine value after incubation ($r=0.79$), for samples ranging from <50,000 to >2,000,000 somatic cells/ml). Refrigeration of milks of high SCC (>1,000,000 cells/ml) led to 1.5 times higher proteolysis than in milks with <60,000 cells/ml, which could have implications for cheese yields and quality. Verdi *et al.*

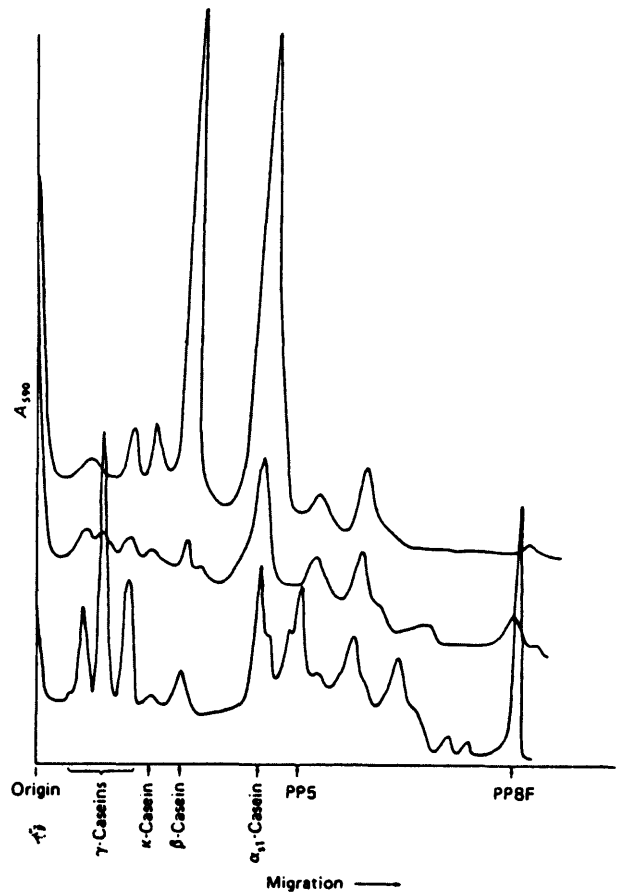


Fig. 3.4 *Densitometer scans of PAGE gel rods comparing proteolytic activity on incubation at 37°C in mastitic milk to that found in normal milk. Upper trace, fresh raw milk. Middle trace, milk from same animal after intramammary infusion with Str. agalactiae (SCC 3,000,000/ml). Lower trace, control milk + porcine plasmin (Andrews, 1983b).*

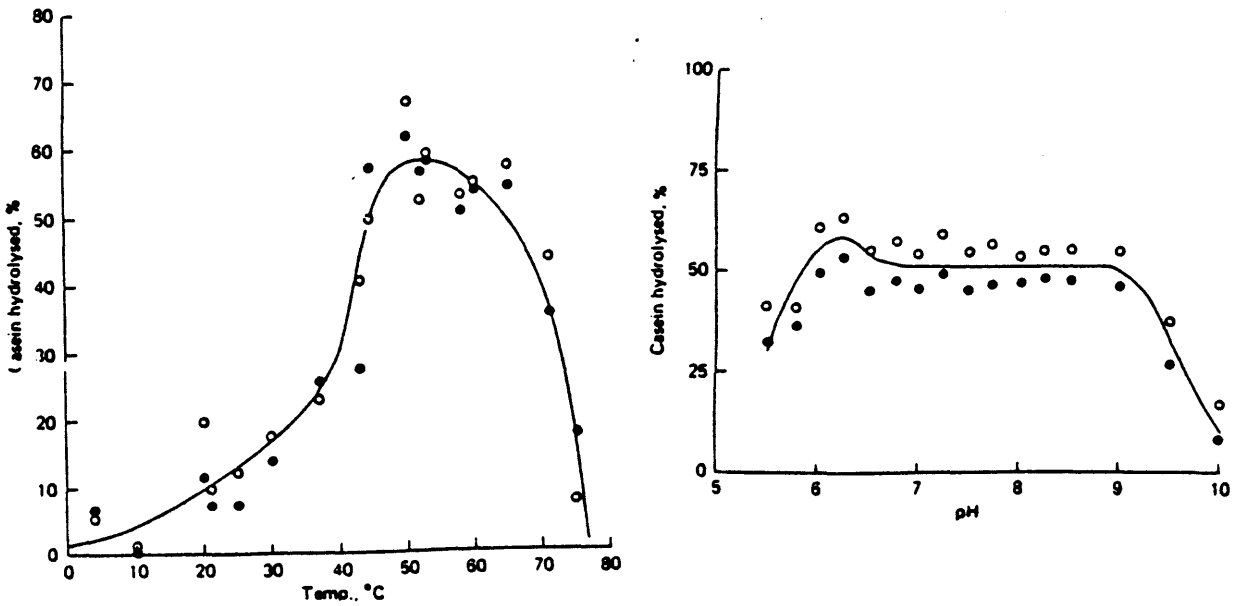


Fig. 3.5. *Influence of incubation temperature (left) and pH (right) on proteolytic breakdown of α_{s1}-casein (open circles) and β-casein (closed circles) in high SCC milk (21,700,000/ml) from an animal infused with E. coli endotoxin (Andrews, 1983b).*

(1987) found similar increases in proteolysis for high SCC compared to low SCC milk, and showed that casein as a percentage of total nitrogen was reduced in high SCC milks. Fresh high SCC milk appeared to have already undergone very significant proteolysis, equivalent to that obtained by low SCC milk only after 24 hours incubation at 37°C, and a number of non-plasmin derived but unidentified proteolysis products were seen in high SCC milk, which could be due to action of somatic cell proteases. This was also concluded by Saeman *et al.* (1988) who experimentally induced *Str. agalactiae* mastitis into cows and followed proteolytic activity during the course of an infection. They found that SCC, total milk proteolytic activity and nonplasmin proteolytic activity (using the inhibitor 6-aminohexanoic acid) was elevated in infected quarters relative to preinfection levels. Post-infection, SCC and non-plasmin proteinase levels dropped to preinfection levels, but total proteolytic activity remained high, probably due to plasmin which had been converted from plasminogen. This was also shown by Murphy *et al.* (1989). Thus the detrimental effects of mastitis on milk quality, including possible predisposition to bitterness in dairy products, may continue even after infection has been eliminated. Barbano *et al.* (1991) found increased levels of proteolysis in fresh milk and increased relative milk protease activity with increasing SCC, but ascribed most of this action to increased plasmin activity.

The role of plasminogen activators (PAs), associated with somatic cells, in proteolysis in mastitic milks was addressed by Verdi and Barbano (1991a), who showed that milk somatic cell enzymes, unlike those from coagulants and bacteria, can convert plasminogen to plasmin and Zachos *et al.* (1992) who found 8-fold higher PA activity per cell in somatic cells recovered from mastitic milk, as compared to control milk. Mastitic milk has also been shown to contain elevated levels of the proteinase inhibitors α2-macroglobulin (Rantamaki and Möller, 1992) and α1-antitrypsin (Honkanen-Buzalski and Sandholm, 1981; Sandholm *et al.* , 1984), which may be due to increased permeability between blood and milk, and which may also affect the proteolytic pathways in milk.

Suzuki and Katoh (1990) isolated at least two cysteine proteases which could hydrolyse casein from mastitic milk, and found cysteine protease activity to be well correlated with stage of mastitis (California mastitis test), SCC and protein concentration. They also suggested that the cysteine protease(s) were of somatic cell origin, and were involved in the pathogenesis of inflammation.

SUMMARY

In summary, whereas in good quality raw milk the dominance of plasmin to almost a complete extent over proteolysis in milk was apparent, on increasing SCC either linearly over a large number of samples or by mastitic infusion, the roles of other proteinases becomes apparent, and may even overshadow the action of plasmin. A number of classifications and preliminary investigations of the specificities of these enzymes have been carried out, but the only proteinases to have been conclusively identified have been cysteine proteinases. The detection of para-κ-casein by Anderson

and Andrews (1977) may provide another clue, as the acid protease of milk, Cathepsin D, which may be of somatic cell origin, produces this peptide in solution (Kaminagowa *et al.*, 1980; McSweeney *et al.*, 1995). The cysteine proteases of Suzuki and Katoh (1990), with pH optima around 6.0, may also be responsible for the bump in the pH-proteolytic curve of Andrews (1983b, see Fig. 3.5), seen at this pH. Finally, the roles of elevated plasminogen activator and proteinase inhibitor levels in mastitic milk may provide further factors affecting the actions and interactions of plasmin and other enzymes, of somatic cell or other origin, in mastitic milk.

3.4. The effect of heat treatment on native milk proteinases and their actions in pasteurised and UHT treated milks

3.4.1. INFLUENCE OF PASTEURISATION ON MILK PROTEOLYSIS

Proteolysis in milk due to plasmin is known to be increased after pasteurisation (for review see Chapter 2). Noomen (1975) found 30-40% increase in milk proteolytic activity after heating to 72°C for 15 s, due, according to Richardson (1983a) to increased activation of plasminogen to plasmin, following inactivation of an inhibitor of this action. Andrews and Alichanidis (1983) confirmed higher proteolysis in pasteurised milks, with electrophoretic patterns of proteose peptone produced in pasteurised milk over 2-3 days incubation at 37°C being similar to those produced under the same conditions in 6 days in raw milk. Heat denaturation of whey protein may also influence the accessibility of caseins to proteolytic attack (Lau *et al.*, 1991). Harwalker *et al.* (1993) isolated plasmin-derived astringent fractions from pasteurised milk.

With regard to survival of proteolytic activity in high SCC milk, when milks of varying SCC were pasteurised, cooled and refrigerated, proteolysis was higher in high SCC milks, as was the case when subsequent incubations to simulate cheesemaking operations were carried out (Senyk *et al.*, 1985). Kosikowski (1988) proposed that the use of bactofugation to remove and biologically destroy somatic cells from high SCC milk without impairing the milk for cheesemaking may be preferable to pasteurisation, as it has been suggested that somatic cells may harbour micro-organisms such as *Listeria monocytogenes* and protect them during milk pasteurisation.

3.4.2. NATIVE PROTEASES IN UHT MILK

3.4.2.1. Introduction

Milk intended for long shelf-life markets is frequently treated to temperatures of 130-150°C, for a couple of seconds in Ultra-High Temperature sterilisation (UHT). The main bacteriological effect of this treatment is the thermal death of bacterial spores, and chemically, the Maillard browning reaction, whey protein denaturation, destruction of many vitamins and inactivation of most of the enzymatic activity of milk result

(Burton, 1984). However, it has long been accepted that the very heat-stable milk proteinase, plasmin, may survive UHT treatments, and contribute to problems on subsequent storage of the milk, which shall be the subject of this section of the review.

3.4.2.2. Gelation of UHT milk

The problem most frequently associated with UHT milks is gelation, which Samel *et al.* (1971) and Kocak and Zadow (1985) showed to occur within 13 months of storage of treated milks at 4, 20 and 30°C, but not at 37°C or higher. Protein breakdown, however, according to both these studies, was negligible at 4 and 20°C, but substantial at the higher temperatures, and was not related to time of onset of gelation. This was also reported by Nakai *et al.* (1964). It was concluded that the interplay of a number of independent factors including proteolysis and aggregation of casein micelles led to gelation. pH does not affect gelation, but severe preheat treatments of raw milk can delay gelation (Zadow and Chituta, 1975). Gelation of UHT milk, studied by electron microscopy, shows that at 4°C, casein micelles are bridged by tendrils of protein into a network, while at 30°C this phenomenon was absent and the casein micelle size was considerably greater (Andrews *et al.*, 1977; deKoning *et al.*, 1985). Kocak and Zadow (1985) proposed a two stage mechanism for age-gelation, where initially limited proteolysis of proteins is followed by aggregation of destabilised micelles due to storage induced physicochemical changes in the milk.

Snoeren *et al.* (1979) and Snoeren and Both (1981) showed that in good bacteriological quality milk (*Pseudomonas fluorescence* proteinase being very heat resistant) indirect UHT treatment (142°C for 4 s) led to negligible protease activity. Direct UHT treatment, at the same temperature and for the same time, however, led to considerable attack of α_{s2} -casein and ß-casein, and slower attack of α_{s1} -casein over storage at 28°C, a classical plasmin pattern, and the milk coagulated after 90 days, without formation of a typical gel structure. The difference between the processes was ascribed to the intensity of indirect heating causing more denaturation of the enzyme (Guthy *et al.*, 1983), or more denaturation of ß-lactoglobulin, which then inhibits the action of the plasmin (Grufferty and Fox, 1986). This second hypothesis was supported by Skudder (1981), who showed that potassium iodate induces proteolysis in UHT milk, which Grufferty and Fox (1986) showed to be due to iodate inhibiting inhibitory linkage of plasmin and denatured ß-lactoglobulin. Möller *et al.* (1977) showed that caseins from stored UHT milks were more resistant to proteolysis than those from unheated milks, but suggested that this was due to the Maillard reaction between lactose and proteins, perhaps stearically hindering proteolytic attack.

Indirect and direct UHT systems were again compared by Manji *et al.* (1986), who found indirectly heated milks to have no residual plasmin activity, but 19% of the original plasminogen activity, after processing, while directly heated milks had 9 and 19% residual plasmin and plasminogen respectively. Plasminogen activation was observed in both milks on storage, and gelation was only observed in directly heated milks stored at 22-25°C, and was inhibited by low and high temperatures. Again, no

relationship was found between the extent of proteolysis and gelation time. It has been suggested that α_{s1} -casein is more involved in polymerisation reactions than ß-casein (Andrews, 1975), and this provides a possible explanation of why plasmin action on storage is not directly related to gelation. These findings are in agreement with those of Snoeren *et al.* (1979) and Snoeren and Both (1981), but show that long term storage of indirectly heated milks will increase proteolytic activity due to the action of heat-stable plasminogen activators.

deKoning *et al.* (1985) examined age-thinning and gelation in UHT milks using plasmin inhibitors and showed that plasmin activity correlated well with casein breakdown and gelation, but that on addition of inhibitors, these phenomena virtually ceased. No plasmin activity or proteolysis were observed in UHT milk, preconcentrated to 23% w/v total solids, but gelation did occur. Age-thinning, or decrease in viscosity, was seen to be caused exclusively by heat treatment, and to be a purely physicochemical effect. The role of the plasmin-plasminogen system was examined by Kohlmann *et al.* (1988), who found that their control milks (mixture of directly and indirectly heated samples) did not gel, nor did milks with added plasmin or trypsin, but milks with added plasminogen did gel after 5 months at 25°C, suggesting that plasminogen derived activity promotes gelation of UHT milk, and again the existence of heat-stable plasminogen activators. The lack of gelation in proteinase-added milks suggests that rapid proteolysis of caseins does not allow for association of enzyme-modified proteins into a gel network, and a clear yellow serum forms upon prolonged storage.

3.4.2.3. Off-flavours in UHT milk

Bitterness in UHT creams was examined by Richter *et al.* (1979), and suggested to be due to a range of enzymes of varying pH and temperature optima, and Mottar (1981) showed that taste loss in UHT milk was more of a proteolytic than a lipolytic effect. McKellar (1981) demonstrated the effect of psychrotrophic enzymes on UHT milk off-flavours, but Harwalker *et al.* (1989) isolated astringent off-flavour components from directly heated UHT milk and showed them to be γ-casein-like breakdown products of casein, probably arising from plasmin action.

Chapter 4

General Materials and Methods

CONTENTS

4.1. CHEESEMAKING

4.1.1 Manufacture of model Dutch cheeses in viscubator

Small experimental batches of Dutch-type cheese were made, in duplicate, using approximately 12.5 kg of milk per batch, in a Viscubator (Type 4/4, Laboratorium, Wiesby). The milk was pasteurised at 72.5°C for 15 s, cooled to 32°C and calcium chloride (0.02% in milk), sodium nitrate (0.016%) and Flora Danica DVI starter (Chr. Hansens Laboratory) , at an inoculum level of 0.2g per 12 kg milk, were added. The milk was stirred for 10 minutes and allowed to ripen for 30 minutes, at the end of which time 3.4 ml of standard strength calf rennet (Chr. Hansen's Laboratory) (diluted in approximately 100 mls distilled water) was added per vat. The milk and rennet mixture was stirred for 3 min and allowed set for 60 minutes. At the end of set, the coagulum was cut with vertical and horizontal wire knives to cubes of 1cm size, allowed a 5 minute heal time and stirred for 30 minutes. One third of the milk weight in whey was then removed and this amount added as 60°C hot water, in three increments over 20 minutes, to cook the curds to 37°C, with continuous gentle stirring during cooking. After a further 5 min stirring, the amount of water added was removed as whey, and the curds stirred for a further 30 min. The curds were then pre-pressed under whey for 60 min, and pressed at 2.5 Bar overnight. The cheeses were then brine salted for 7.5 h in an 18% NaCl brine, allowed to dry, plastic coated and ripened for 15 weeks at 11°C. While a Gouda-type cheese recipe was followed, the high moisture in the cheeses made in the Viscubator, and the loss of moisture through the plastic coat during ripening made these cheeses quite similar to a cross between a Meshanger and a Gouda type (Noomen and Mulder, 1976; Noomen 1977a, b). Meshanger cheese, which has a moisture content of 57-60%, was recommended as an ideal model system to investigate the contribution of milk enzymes to cheese ripening (deJong, 1976). The manufacture process of this cheese differs from that of Gouda principally in that it is not cooked during manufacture, and the stirring time is greatly reduced (Noomen, 1977b). The ripening conditions are typically 13°C for 3-5 weeks whereas here a more typical Gouda ripening profile was utilised.

4.2.2 Large Scale manufacture of Gouda cheese

Gouda cheese was made in large stainless steel vats from 100-gallon batches of milk, which was pasteurised in the U.C.C. creamery at 72.5°C for 15 s, and standardised to a fat/protein ratio of 1.0. The milk was then cooled to 30°C, and filled into a 1200L circular stainless steel vat (MKT Tehtart 1975) with a steam heating jacket. Calcium chloride and sodium nitrate were added at the levels given above. Frozen starter B11 (Chr. Hansens Laboratory, Little Island, Cork) was added to the milk at a level of 45g/100 gallons, stirred in and allowed ripen for 25 minutes. Hansens normal strength rennet was added at 1ml/gallon (diluted in distilled water) and stirred in for 2 minutes, and the mixture allowed set for 45 minutes, or until judged sufficiently firm to cut.

The coagulum was then cut using the vat knives, allowing two rotations of the knives, a heal time of 2 minutes, repeating this and then starting to stir slowly for 10 minutes further. Then 40% of the milk volume (40 gallons) of whey was removed by siphon and 35 gallons

added back as hot water (45-50°C), sprinkling gently to avoid heat shocking the curds, over 7-8 minutes. The curds and whey were then stirred for 45-60 minutes, or until the pH was below 6.35. The mix was pitched into a horizontal prepress vat where the curds were pressed hydraulically at 1.5 Bar under whey for 20 minutes, whereupon the whey was removed and the curds filled into circular moulds and pressed at 3 Bar overnight.

The cheeses were transferred to an 18% NaCl brine bath for a time calculated to give a final 1.5-2.0% salt level in cheese (2-3 days with regular turning), then removed to a 5°C room for 2 days to dry and cool before vacuum packing. A two stage ripening was used for these Gouda cheeses with the cheeses being first held at 13°C for 6 weeks before being transferred to a 5°C room for a further 9 weeks maturation. These cheeses were sampled at 12 days and 5, 10 and 15 weeks for analysis and organoleptic evaluation.

4.2. COMPOSITIONAL ANALYSES AND CHEESE EXTRACTIONS

4.2.1 Milk analysis

Milk samples for cheese making or enzymatic analysis, were tested for fat, protein, lactose and total solids using a Milkoscan (Foss Electric, Hillerod, Denmark). Milk pH was determined at room temperature using a Radiometer type M82 pH meter. Somatic cell counts were determined using a Fossomatic 90 cell counter (Foss Electric, Hillerod, Denmark), calibrated using direct microscopic cell counts. Chloride in milk was determined by the British Standard method (1963). PMN antigen in milk was assayed using the method of O'Sullivan et al. (1992), at University College Dublin, on milk samples stored on collection at -20 °C.

4.2.2 Cheese analysis

Wedges of cheese were taken at 12 d of age, stripped of plastic coat if necessary and grated. Cheese moisture (duplicate) was determined gravimetrically by drying a 2-g sample for 24 h in an oven at 105°C. Cheese fat was determined by the Gerber method and protein by the standard macro-Kjeldahl method (AOAC, 1984), both in duplicate. Cheese pH was measured at room temperature and cheese salt was determined in duplicate by the potentiometric method of Fox (1963).

4.2.3 Fractionation of cheese proteolysis products

Samples for analysis of cheese proteolysis during ripening were taken at 12d and 5, 8, 11 and 15 wk age and analysed as follows.

Water-soluble nitrogen (WSN) was extracted by the method of Kuchroo and Fox (1982). 50g of grated cheese was homogenised with 100ml distilled water for 5 minutes using a Colworth Stomacher 500 and the homogenate held in a waterbath at 40°C for 1 hour. The mixture was then centrifuged at 3000g for 30 minutes at 4°C to remove insoluble material and fat. The supernatant, or WSN was filtered through glass wool and Whatman No. 113 filter paper, and portions taken for analysis, further fractionation or lyophilisation. The water-insoluble nitrogen (WISN) precipitate was washed in warm distilled water to remove residual fat, grated and lyophilised for further analysis.

Portions of fresh WSN were ultrafiltered using Centricon-100 ultracentrifugal microconcentrators (Amicon Ltd., Beverly, Ma., USA), with a molecular weight cutoff of 10,000 daltons, in a Funke-Gerber centrifuge at 3000-5000 g until 1ml of UF permeate had been passed (30-45 minutes), of which 500μl was lyophilised for examination by RP-HPLC .

From fresh WSN, pH 4.6 soluble extracts were prepared by addition of 150 μl of 30% Acetic acid to 5ml of WSN extract, holding at room temperature for 10 minutes, and adding of 150 μl of 3.33 N Sodium Acetate. The mixture was centrifuged at 14,000g for 30 minutes and the supernatant recovered, recentrifuged and lyophilised.

The 12% TCA soluble N was extracted from cheese by the method of Bynum and Barbano (1985) and determined by Kjeldahl. The 12% TCA-soluble N was expressed as a percentage of total protein in the cheese to obtain a measure of the increase in soluble proteins during aging.

4.3. BIOCHEMICAL ANALYSES

Polyacrylamide Gel Electrophoresis

4.3.1 Urea Polyacrylamide Gel Electrophoresis (Urea-PAGE)

Electrophoresis in polyacrylamide gels (12.5% C, 4%T, pH 8.9) was preformed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts, UK), according to a modification of the method of Andrews (1983). The following stock solutions were prepared and stored at 4°C.

1. Acrylamide Solution 40% w/v Acrylamide in distilled water.
2. Stacking Gel Buffer 8.3g tris (hydroxymethyl) methyl amine + 300g Urea + 2 g N, N, N,', N', methylamine bisacrylamide, dissolved in and made up to 1 L with distilled water, with pH being adjusted to 7.6 with HCl.
3. Separating Gel Buffer 64.3g tris (hydroxymethyl) methyl amine + 385.7g Urea + 5g N, N, N,', N', methylamine bisacrylamide, dissolved in and made up to 1L with, distilled water, with pH being adjusted to 8.4 with HCl.
4. Electrode Buffer 6g tris (hydroxymethyl) methyl amine + 29.2g glycine dissolved in and made up to 2L with, distilled water.
5. Sample Buffer 0.75g tris (hydroxymethyl) methyl amine + 49g Urea + 0.4 ml conc. HCl + 0.7ml 2-mercaptoethanol + 0.15g Bromophenol Blue, dissolved in and made up to 100 ml with, distilled water.
6. Double Strength Sample Buffer (McSweeney et. al., 1994) 1.5g Tris + 49g Urea + 1.4ml 2-mercaptoethanol + 0.15g Bromophenol Blue, dissolved in and made up to 100 ml with distilled water, with pH being adjusted to 7.6 with HCl.
7. Ammonium persulphate 10% w/v in distilled water, frozen in 1 ml aliquots in eppendorfs at -20°C until needed.
8. Staining Solution (Blakesley & Boezi, 1977) 1L of 0.2% w/v Coomassie Brilliant Blue G250 in distilled water was added to 1L 1M H_2SO_4 and held overnight. The solution was then filtered through Whatman No. 1 filter paper and the filtrate mixed 9:1 with 10M

KOH. Trichloroacetic acid was then added to a final concentration of 12% w/v TCA in the stain. The stain was stored in a dark bottle.

The following gel recipes were then prepared immediately before pouring the respective gels (sufficient for 2 gels).

	Separating Gel	Stacking Gel
Acrylamide Solution	18 ml	2.5 ml
Separating Gel Buffer	45 ml	–
Stacking gel Buffer	–	22.5 ml
TEMED*	30 µl	12.5 µl
Ammonium Persulphate	226 µl	150 µl

* N, N, N', N', tetramethylene bisacrylamine.

Samples for electrophoresis were prepared as follows, being heated to 50°C for a few minutes if necessary to help dissolve the samples.

Sample	Concentration (mg/ml)	µl applied
Cheese	15	10
Water Soluble Nitrogen [1]	15	12
Milk (1:4 with sample buffer)	–	15
Casein Hydrolysate	2.5 [2]	12

[1] Lyophilised.

[2] 5mg/ml hydrolysate mixed 1:1 with double strength sample buffer.

The electrophoresis unit was assembled according to the manufacturer's instructions, The separating gel was poured to within 2 cm of the top of the glass plates, and overlaid with a layer of water. On polymerisation (about 60 min) the water was poured off and the stacking gel prepared and poured, a slot-former placed in position and polymerisation allowed 60 minutes to complete. The slot-former was then removed and the gel unit assembled and filled with electrode buffer.

The gels were pre-run at 280V for 30-45 minutes and the samples applied. The samples were then run at 280V through the stacking gel and at 300V through the separating gel until the tracking dye is within 1 cm of the bottom of the gel (4-5 hours). The gels were stained overnight in staining solution and destained in several changes of distilled water, until the background becomes clear.

4.3.2 SDS-PAGE electrophoresis

SDS-PAGE was carried out in 10-20% precast gradient gels (Bio-Rad Laboratories Ltd., Watford, Herts, UK) following the manufacturer's instructions, using a Bio-Rad Mini-Protean II cell. Electrode buffer used contained 25 mM Tris-glycine pH 8.3, 0.1% SDS. Sample buffer contained 10mM Tris-HCl pH 6.8, 1.0% SDS, 20% glycerol and 0.02% bromophenol blue. Milk samples for analysis were diluted accurately 1:10 (vol/vol) in sample

buffer and immersed in boiling water for 2 min. Sample loading was 10 μl per well, and gels were run at 200V constant voltage until the tracking dye approached the end of the gel (30-45 min). Gels were stained with 0.1% Coomassie Brilliant Blue R250 in a methanol:acetic acid:water solution (40:10:50) for 1 hour and destained in 40% methanol/10% acetic acid. Gels were preserved by washing in 7% acetic acid and placing in air-tight bags.

High Performance Liquid Chromatography

4.3.3 Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

Reversed-phase HPLC was performed using a Beckman HPLC system (Beckman, San Ramon, Ca., USA) ,consisting of a model 506 autosampler, a model 126 programmable solvent module and a programmable detector (interfaced with a personal computer using Beckman System Gold software). A nucleosil C_8 column (250 x 1.5 mm, 250μm pore size, Jones Chromatography) was used for all separations.

Samples
(i) Cheese water-soluble nitrogen (WSN)
(ii) Ultrafiltration permeate of cheese WSN (UFP)
(iii) pH 4.6 soluble extracts of casein hydrolysates and milk

Lyophilised samples were reconstituted as follows. All samples were filtered through 0.45 μm filters (Millipore Ltd,) before injection.

WSN and UFP	5 mg/ml in Solvent A (20 μl injected)
WISN	5 mg/ml in 50mM Tris-HCl, pH 7.2 with 20% Acetonitrile, 540 mg/ml Urea and 7% 2-mercaptoethanol (10 μl injected)

The pH 4.6 soluble fraction from milk and casein hydrolysates was isolated by addition of 30 μl of 33.3% w/v acetic acid to 1ml of sample, which was vortexed and held at room temperature for 10 minutes. 30 μl of 3.33M sodium acetate was then added and the mixture centrifuged at 16,000g for 10 minutes. The supernatant was then filtered and 50 μl injected onto the column. The injected samples were eluted at 0.75 ml min^{-1} using a 75 minute water/acetonitrile/trifluoroacetic acid (TFA) gradient. Solvent A consisted of 0.1% TFA in water and solvent B of 0.1% TFA in acetonitrile. The elution profile consisted of initially running at 100% A for 5 min, increasing to 50% B over 55 min, holding at 50% B for 5 min, increasing to 60% B over 5 min, and finally holding at this level for 5 min. The column was then washed by increasing to 95% B for 5 min and returning to 100% A and allowing re-equilibrate for 15 min, before injection of the next sample.

4.3.4 Kjeldahl Method

The macro-Kjeldahl method (AOAC, 1990) was used to determine total N in samples of cheese (0.5g), WSN (5 ml) or 12% TCA-soluble N extracts (as per Bynum and Barbano, 1985).

4.3.5 Lowry Method

A modification of the Lowry assay for protein (Lowry et al., 1951) was used to quantify N in TCA-soluble extracts of milk and casein hydrolysed by somatic cell extracts. The method used was as follows.

Lowry Reagent A: 1% Copper sulphate
Lowry Reagent B: 1% w/v sodium potassium tartarate
Lowry Reagent C: 2% w/v sodium carbonate in 0.1M NaOH
Copper alkali solution (reagent D): (prepared fresh daily)
1 ml of A and 1 ml B were mixed, and 98 mls C added.
BSA Standard: 0.25% bovine serum albumin in water

To 200 μl sample was added 2.1 ml Lowry Reagent D, mixed, and the mixture equilibrated at room temperature for 10 min. 250 μl of diluted Folin-Ciocalteau reagent (1:1 with distilled water) was added and colour development allowed proceed for 30 min at room temperature, whereupon the absorbance at 750 nm was measured and results expressed as mg BSA/ml from a standard curve.

4.3.6 Fluorescamine Method

The fluorescamine method of Chism et al. (1979), as reported by Kwan et al. (1983) was used as a general method to determine proteolysis in milk and casein solutions by reaction of the fluorescamine reagent with free amino groups liberated during proteolysis. Commonly, the solution to be assayed was mixed with an equal volume of 24% Trichloroacetic Acid (TCA), vortexed and centrifuged (10,000g, 5 min). To 100 μl of filtrate was added 300μl 3M dipotassium hydrogen orthophosphate, followed by 150 μl of 0.03% (w/v) fluorescamine in acetone. The assay mixture was then immediately vortexed, due to the short stability of the fluorescamine reagent under the assay conditions. Distilled water (3ml) was added to dilute and bring the sample to cuvette volume. Fluorescence ($\lambda_{excitation}$= 395 nm, $\lambda_{emmission}$ = 480 nm) was determined using a Corning-EEL spectrofluorimeter. Results were expressed as nmols Leu-Leu via a standard curve.

4.3.7 Cadmium Ninhydrin Method for Determining Free Amino Acids

Free amino acids in cheese WSN were estimated using the method of Folkertsma and Fox (1992). Cadmium Ninhydrin reagent was prepared by dissolving 0.8g Ninhydrin in a mixture of ethanol (80ml) and acetic acid (10ml), and adding 1g Cadmium Chloride dissolved in 1 ml distilled water, and the assay performed as follows.

An aliquot of fresh cheese WSN (10-100 μl depending on concentration of free amino acids, which increases with cheese age) was diluted with distilled water to 1 ml and 2 ml of reagent added. The mixture was heated to 84°C for 5 min, cooled and the absorbance at 507 nm determined against a blank containing no WSN. Results may be expressed as mg Leucine/g cheese when read off a suitable standard curve.

Determination of plasmin and plasminogen in milk and cheese

The method of Richardson (1981) was used to determine plasmin activity in milk and cheese, and plasminogen levels in milk. This assay employs the non-fluorescent substrate N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin ('coumarin peptide', Sigma Chemical Co.) which is hydrolysed by plasmin to give a fluorescent product. The rate of increase in fluorescence intensity is proportional to the quantity of plasmin present.

Milk samples (30ml) were diluted with 10ml 0.4M trisodium citrate solution and centrifuged (27,000g, 4°C, 10 min), and plasmin content of the supernatant determined. Cheese plasmin was extracted by dispersing 10 g of finely grated cheese in 90 ml of 2% (w/v) trisodium citrate, followed by equilibration at 37°C for 15 min, homogenisation in a Colworth stomacher for 10 minutes and centrifugation as before. Samples were frozen at -20°C until assayed. Plasminogen in citrate-treated milk samples was determined as the increase in plasmin content induced by the addition of the plasminogen activator, urokinase. In this case, 0.25 ml citrate-treated milk was mixed with 0.05 ml urokinase solution (1000 Ploug units/ml) and 0.2 ml 50mM Tris-HCl buffer pH8.5 containing 20 mM lysine, 0.14M NaCl and 50% (w/v) glycerol and incubated at 37°C for 1 hr before assaying for total plasmin activity.

For the assay, 70μl of the solution to be assayed was equilibrated for 5 min in a cuvette with 826 μl 50 mM Tris-HCl buffer, pH 7.5, and 224 μl coumarin peptide solution (25 mg coumarin peptide dissolved in 6.67 ml dimethyl sulphoxide, and 26.59 ml 50mM Tris-HCl, pH 7.5 added to give a final peptide concentration of 1mM) added to start the reaction. Fluorescence readings were taken using a Shimadzu RF-551 fluorescence detector at regular intervals over a 10-30 minute period, and the rate of increase in fluorescence per minute calculated (the slope of a fluorescence units against time plot). A standard curve of 0 - 1.0 nmol 7-amino-4-methyl coumarin (AMC) in assay buffer is included with each batch of assays (a 0.1 mM AMC solution contains 1 nmol/100μl, with standards thus comprising of 0-100 μl of this solution, made up to 1ml with assay buffer before reading fluorescence. The inverse slope of the standard curve (nmol AMC/fluorescence unit) can be used to calculate the plasmin content of the sample in AMC units/ml (for milk) or AMC units/g (for cheese), once the dilution of the initial cheese or milk is taken into account. One AMC unit of plasmin releases 1 nmol AMC per minute under the assay conditions specified.

Chapter 5

Variability in individual cows' milk somatic cell count, polymorphonuclear leucocyte level, plasmin activity, gross composition and proteolysis as affected by season and stage of lactation

SUMMARY

A total of 103 milk samples from Holstein Friesian cows, calved at different times of the year, were taken at various stages of the lactation cycle. Somatic cell counts (SCC) were found to vary with calving season, cow age and stage of lactation, and were influenced by interactions between these factors. Milk polymorphonuclear leucocyte (PMN) levels, as a proportion of total SCC, varied with calving season, with there being more PMN cells on increasing SCC in spring calving than autumn calving milks. There was an overall linear correlation coefficient of 0.88 between SCC and PMN level. Plasmin and plasminogen levels were significantly higher in late lactation than early/mid lactation milks, and there appeared to be increased plasminogen activator activity in milks from older cows. Milk SCCs were significantly positively correlated with milk fat, protein, pH and total solids level, and significantly negatively correlated with lactose level in milk. Plasmin activity was positively correlated to milk protein, SCC and plasminogen level. Late lactation milks examined had mean SCCs twice that of early-mid lactation milks and had compositional changes reflecting this. Relative proteolytic activity was more highly correlated with plasmin activity than SCC or PMN level, but initial proteolysis levels in fresh milks were most highly correlated with SCC. This may have been due to the overall low SCC of the herd studied. Electrophoretic proteolysis patterns of incubated milks verified high plasmin activity in late lactation samples, but there was clear evidence of non-plasmin proteolytic action in high SCC milks.

5.1 INTRODUCTION

The imminent introduction of EU directive 92/46 concerning reduction of maximum permissible milk somatic cell count (SCC) from 500,000 cells/ml to 400,000 cells/ml has led to increased interest in the causes of variation in milk somatic cell count and its relationship with milk quality, particularly in countries with very seasonal calving patterns. Bovine mastitis, or inflammation of the mammary gland, the most important cause of elevated SCC in milk, causes an altered protein distribution in milk and a decrease in casein and lactose level (Munro *et al.*, 1984; Mitchell *et al.*, 1986a) and in cheesemaking, high SCC milk is associated with increased rennet clotting time, losses in whey and cheese moisture and reduced curd rigidity and yield (Verdi *et al.*, 1987).

Milk somatic cells consist of polymorphonuclear leucocytes (PMN cells or neutrophils), macrophages and lymphocytes. Healthy milk has been reported to contain approximately 12% PMNs, 60% macrophages and 28% lymphocytes (Burvenich *et al.*, 1995). On exposure to bacterial endotoxin, however, within 6 hours PMNs comprise greater than 90% of total leucocytes, and remain so for up to 59 hours after infusion (Saad and Ostensson, 1990). As an alternative to cumbersome flow cytofluorimetric or differential cell staining techniques, O'Sullivan *et al.* (1992) developed a capture ELISA assay for PMN antigen in milk from individual cows, which was very highly correlated with total SCC (r=0.94). However, preliminary investigation of bulk tank correlations showed a wide variation in PMN levels for milks of the same total SCC, particularly at the 400,000-500,000 cells/ml level (Joyce, personal communication). PMNs are known to possess a different proteolytic enzyme complement to other cell types (Verdi and Barbano, 1991b) and preliminary investigations revealed probable differences in ripening characteristics of cheeses made from milks with similar total SCC but different PMN level (see chapter 10).

Levels of the alkaline proteinase plasmin, the principal proteinase in healthy milk, and of its inactive precursor, plasminogen, are known to be increased in late lactation and high SCC milks (Richardson, 1983b; Benslimane *et al.*, 1990; Politis *et al.*, 1989a), due to damaged mammary epithelium or increased plasminogen activation by somatic cells. Plasmin is extremely heat resistant (Richardson, 1983a). Proteolysis in milk has also been shown to correlate with SCC (Senyk *et al.*, 1985), and elevated non-plasmin proteolytic activity has been reported in mastitic milks (Andrews, 1983 a-c, Saeman *et al.*, 1988), but in all these studies, either plasmin action or effect of elevated SCC were considered separately and the two have not been correlated in milks.

The objectives of the study were

(1) to examine the effects of seasonal and lactational factors on SCC in a range of milks from individual cows

(2) to examine the influence of these two factors plus SCC on PMNs, plasmin and plasminogen in the same milks

(3) to study the relationships between seasonality, SCC, PMN level and plasmin activity and milk composition and proteolysis

5.2 MATERIALS AND METHODS

5.2.1. Milk Sample collection and analysis

A total of 103 milk samples were collected from the individual milk recording jars of Holstein Friesian cows at a local farm during the afternoon milking session. Batches of approximately 35 samples were taken at 3-monthly intervals so as to get a combination of spring and autumn calving cows, at various stages of lactation. The same cows were not necessarily sampled each time. Data concerning lactation date and number were supplied by the farm. Milks were returned to the laboratory and % fat, protein, lactose and total solids (by Milkoscan) and pH determined, as described in section 4.2.1. Somatic cell count (SCC) was determined by Fossomatic and portions (1.5 ml) of milk frozen at -20°C. When all batches of samples had been collected, these were dispatched to University College Dublin for determination of PMN antigen by the method of O'Sullivan *et al.* (1992). On the day of collection, trisodium citrate dissociation of plasmin and plasminogen, and measurement of these parameters by the coumarin peptide assay method, as described in section 4.3.7, was also carried out. Chloride in milk was determined by the British Standard method (1963).

Further portions of the milks were incubated at 37° C for 24 hours in the presence of 0.05% Sodium Azide, and proteolysis examined in two ways. Firstly, 12% TCA-soluble proteolysis products were quantified in the initial milk and the same milk after incubation using the fluorescamine reagent, with results being expressed as initial level of free amino groups and increase in free amino groups under the conditions specified, and secondly caseins and polypeptides present in the milks before and after incubation were compared by urea-PAGE. Both these techniques were as described in sections 4.3.1 and 4.3.6 respectively.

5.2.2. Statistical Analysis

The frequency distributions for SCC, PMN level, plasmin activity, plasminogen concentration and plasminogen/plasmin ratio were skewed and thus log transformations were applied to these variables prior to analysis. Three grouping variables or factors were considered in this analysis; calving season, stage of lactation and number of lactations. Season had two categories (spring and autumn calved), stage of lactation was categosised into two subclasses (early and late (<200 and >200 days in lactation)), and number of lactations were divided into three categories (1-2,3-4 and 5 or more lactations). Group means were compared by t and F tests on log transformed data and results presented as original, untransformed data. Pearsons correlation coefficients and linear regression were used to investigate relationships between variables. Also, for some analyses, to examine the behaviour of milk from cows free of mastitic infection samples of greater than 500,000 somatic cells/ml milk were removed.

Effect of season and lactation stage and number on SCC, PMN level and milk plasmin and plasminogen activities and ratios were analysed using the General Linear Models (GLM) procedure, available in the Minitab statistical analysis package. In this type

of modelling a hierarchical model for multi-parameter analysis of variance is constructed, taking into account all possible interactions between factors which will influence the variable of interest. Least significant interactions are then removed one by one in decreasing order, until the simplest model is obtained, in which all terms are significant, or non-significant terms cannot be removed because they are involved in significant higher-order interactions. Models were also checked to verify the assumptions of normal distribution of residuals and random distributions in plots of residuals against predicted values. The models were used to calculate means (adjusted for all other terms in the model) for each level of the categories which were found to be significant. Log_{10}SCC was treated as a covariate, or continuous variable, where appropriate.

5.3. RESULTS

5.3.1. SCC and PMN levels in trial milks

The mean SCC and PMN levels in the individual milks sampled were calculated on the basis of season, number of lactations and stage of lactation (Table 5.1). Spring

Table 5.1 *Influence of season, stage of lactation and number of lactations on mean SCC and PMN levels in milk ([A] t-tests and F-tests were performed on log transformed data). All values for individual milks were means of triplicate determinations.*

Group	*SCC (,000s ± SD)*			*PMN (A_{507} ± SD)*		
	n	Mean	SD	n	Mean	SD
Calving category						
Spring Calving	53	249	315	53	0.411	.335
Autumn Calving	49	201	286	49	0.342	.240
t-value [A]:		1.60			1.08	
p:		0.110			0.280	
Stage of lactation						
Early/Mid Lactation	63	177	234	63	0.339	.291
Late Lactation	38	308	378	38	0.442	.292
t-value [A]:		2.85			2.65	
p:		0.006			0.010	
Number of lactations						
1-2	47	132	147	47	0.305	.242
3-4	23	302	396	23	0.430	.327
≥ 5	32	309	356	32	0.448	.232
F-value [A]:		6.16			4.25	
p:		0.003			0.017	

calving cows were found to have higher SCCs and PMN levels on average than autumn calving cows, although these differences were not significant. There was a substantial rise ($p<0.01$) in both SCC and PMN levels at the end of lactation. Cows of 3 or more lactations had higher mean SCCs than cows of 1 or 2 lactations ($p<0.01$) and mean PMN levels rose in cows of increasing lactations ($p<0.05$).

5.3.2. GLM modelling of SCC in trial milks

The GLM procedure was employed to carry out an estimation of the simultaneous effects of the three factors (calving season, stage of lactation and lactation number) on $\log_{10}$SCC values. The following model was fitted to the data.

$$\text{Log}_{10}\text{SCC} = \text{Calving season} + \text{Stage of Lactation} + \text{No of Lactations} + \text{two and three way interactions thereof} \quad (1)$$

After deleting non-significant terms from the model as outlined in section 5.2.2, the final model consisted of five terms (Table 5.2). The two way interaction terms, season by lactation number and lactation number by stage of lactation were significant. The terms in the model explained 30% of the variation in $\log_{10}$SCC seen in the samples taken.

Table 5.2 *GLM analysis of factors affecting Log_{10}SCC in milk*

Factor	**d.f.**	**Adjusted means squares**	**F-value**	**P**
Calving season	1	1.748	12.73	0.001
Lactation number	2	0.786	5.72	0.005
Stage of lactation	1	2.209	16.08	0.000
Season * Lactation number	2	0.681	4.96	0.009
Lactation number * Stage of lactation	2	0.595	4.33	0.01
Error	93	0.137		
Multiple R^2 value 30.0%				

The pattern of variation in SCC due to lactation number was found to differ with season and stage of lactation. In Table 5.3 SCC is summarised for the season by lactation number and lactation number by stage of lactation combinations. It can be seen from these data why the interaction terms were present in the model. In spring calving milk, the highest cell counts (mean 385,000) were found in cows of 3-4 lactations, while in autumn calving milk the reverse was the case (mean 78,000). In the second half of the table the highest SCCs (mean 400,000) in late lactation milk were seen in cows of 3-4 lactations, while cows of this number of lactations had the lowest mean early lactation SCCs (74,000).

Table 5.3 *Influence of season, stage of lactation and number of lactations on milk SCC (in ,000s, means ± SD calculated from GLM model, adjusted for all other terms in the model and transformed to original scale)*

Lactation number	*1-2*			*3-4*			*≥5*			*All*		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
Season												
Spring calving	26	103	97	9	385	15	18	241	47	18	53	212	61
Autumn calving	21	96	77	14	78	30	14	158	26	14	49	106	56
Stage of lactation												
Early lactation	35	87	156	11	74	17	18	160	47		64	101	72
Late lactation	12	114	20	12	400	30	14	244	26		38	223	44
All	47	99	54	23	173	28	32	195	37				

5.3.3. GLM modelling of PMN levels in trial milks

The effect of seasonal and lactational factors on the variation in log_{10}PMN levels was also examined using GLM analysis. In this analysis, the model included Log_{10}SCC as a covariate along with the other factors (calving season, stage of lactation and number of lactations).

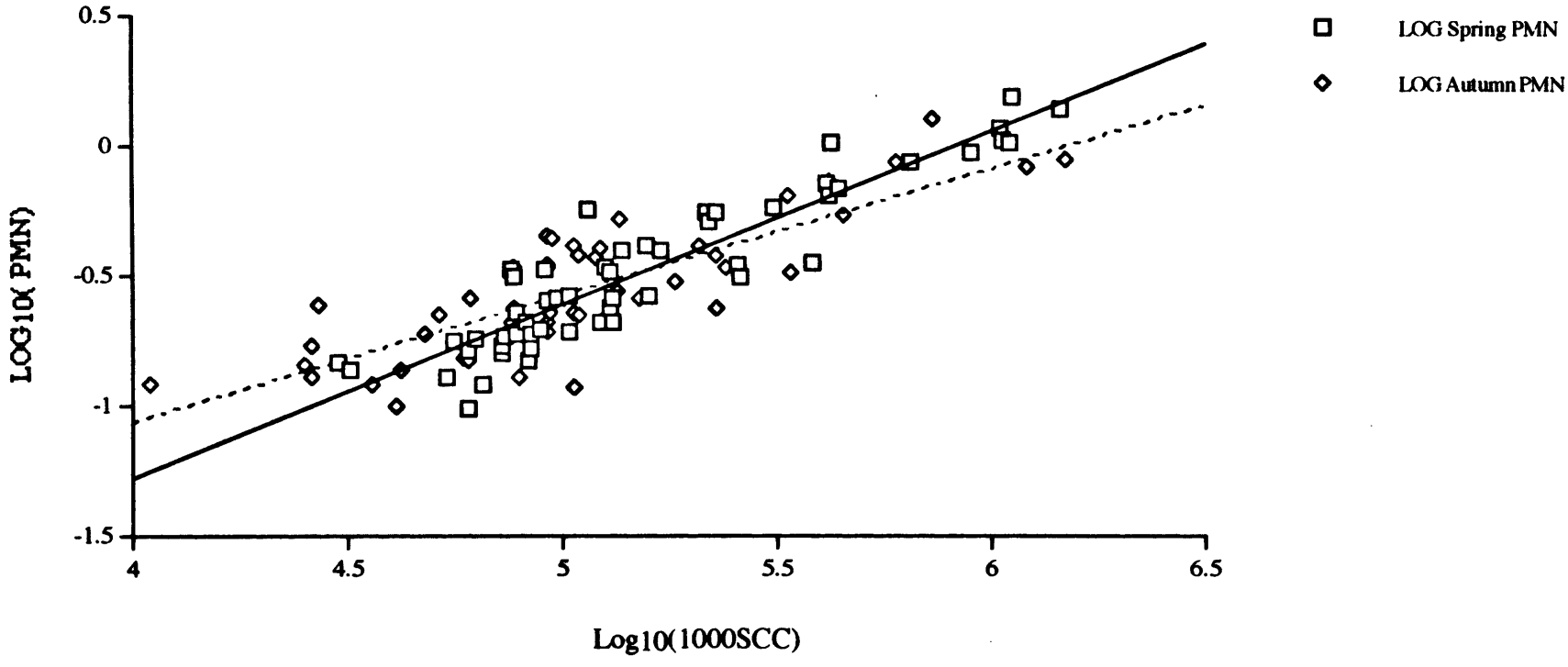
$$\begin{aligned} Log_{10}PMN = {} & Log_{10}SCC + \text{Calving season} + \text{No of Lactations} \\ & + \text{Stage of lactation} \\ & + \text{all possible 2-way interactions of the above (4 terms)} \\ & + \text{all possible 3-way interactions of the above (4 terms)} \\ & + \text{the one 4-way interaction of the factors} \end{aligned} \qquad (2)$$

After deleting non-significant terms as described in section 5.2.2, the final model consisted of three terms, log_{10}SCC, calving season and the interaction between log_{10}SCC and calving season (Table 5.4). Approximately 80% of the variation in log_{10}PMN was explained by this model. This model suggested that stage of lactation and lactation number are not required when log_{10}SCC and calving season are present in the model. A graphical illustration of the final model is shown in Fig. 5.1, where it is seen that the slopes of plots of log_{10}SCC versus PMN level for autumn calving milk samples are significantly lower than those of plots for spring calving milks. Thus the rate of increase of PMN cells with increasing SCC was higher in spring calving milk than autumn calving milk. It can also be seen from Fig. 5.1 that at low SCCs (below approximately 160,000 cells/ml) there are higher levels of PMNs per cell in the autumn calving milk, but above this level the milk with the higher proportion of PMNs per somatic cell is from spring calving cows.

Table 5.4 *GLM analysis of factors affecting log $_{10}$PMN level in milk*

Factor	**d.f.**	**Adjusted means squares**	**F-value**	**P**
Log_{10}SCC	1	6.533	321.11	0.000
Calving season	1	0.448	22.02	0.000
Log_{10}SCC * calving season	1	0.451	22.16	0.000
Error	99	0.020		
Multiple R^2	78.77%			

Fig. 5.1. *Effect of calving season on relationship between log_{10}PMN antigen and log_{10}SCC in individual milks as influenced by season (Autumn and Spring calving cows) (comparison of regression lines).*



5.3.4. Correlation coefficients relating SCC and PMN levels

O'Sullivan *et al.* (1992) examined the relationship between SCC and PMN level in milks from individual cows and found a correlation coefficient of 0.94 between these parameters. A plot of SCC versus PMN level for the milks in this trial is shown in Fig. 5.2. There was an overall Pearsons correlation coefficient of 0.88 between PMN level and SCC, which compared well with the correlation coefficient of O'Sullivan *et al* . (1992) When this data was divided on the basis of stage of lactation and season of calving, comparable correlations were found in the subgroups (Table 5.5). When samples of SCC>500,000/ml were removed, winter milks (spring-calving late lactation and autumn-calving early lactation milks) had the lowest correlation coefficients (0.69 and 0.63).

Fig. 5.2 *Regression plot of PMN level in milk versus milk SCC.*

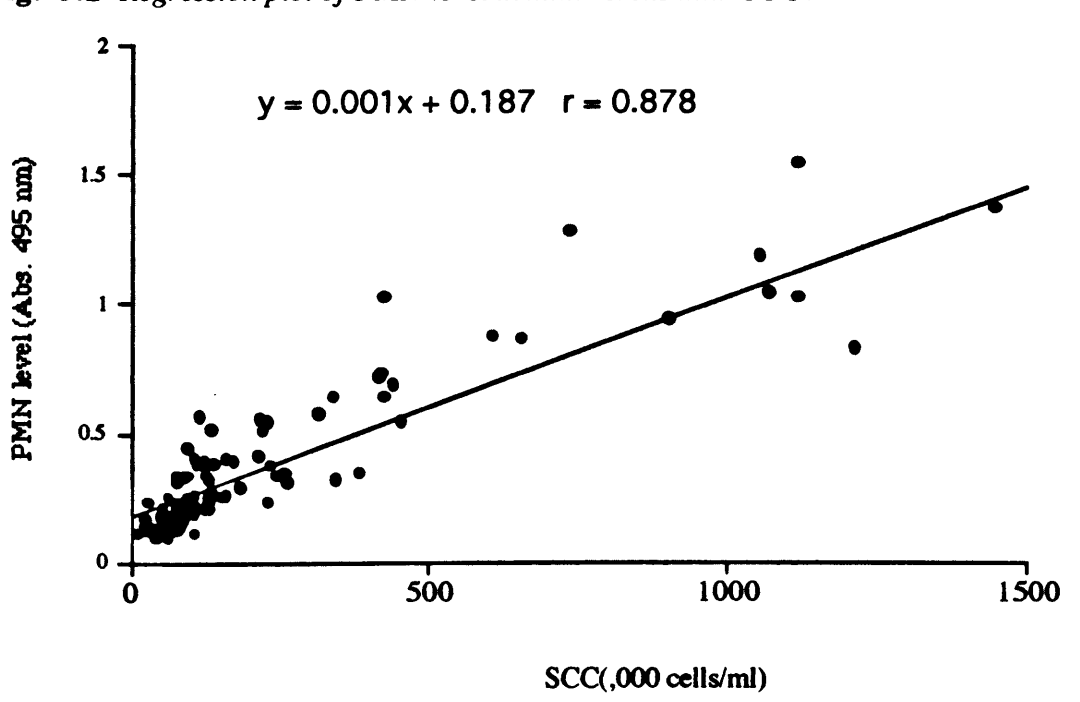


Table 5.5. *Pearsons correlation coefficients for SCC and PMN antigen level, divided on the basis of calving season and stage of lactation and for SCC levels above and below 500,000 cells/ml (number of samples in brackets). All correlations were significant (p<0.001), except ** (P<0.05)..*

Season	Stage of Lactation	Correlation Coefficient, r	
		All samples	Samples <500,000 cells/ml
Spring	Early/Mid	0.964 (38)	0.925 (34)
Spring	Late	0.900 (17)	0.691 (14) **
Autumn	Early/Mid	0.873 (26)	0.625 (25)
Autumn	Late	0.856 (23)	0.791 (20)

5.3.3. Plasmin and plasminogen levels and plasminogen/plasmin ratios in milks.

A table of mean plasmin, plasminogen and plasminogen/plasmin ratios for the trial samples is presented in Table 5.6. Elevated plasmin ($p<0.001$) and plasminogen ($p<0.01$) levels were associated with late lactation milk but there was no evidence to suggest that overall these quantities were affected by either calving season or number of lactations.

Table 5.6 *Influence of seasonal and lactational factors on mean plasmin and plasminogen levels and plasminogen/plasmin ratio in milk (standard deviation in brackets)* [B] *t-tests performed on log transformed data.*

Group	*Plasmin (AMC units/ml)*		*Plasminogen (AMC units/ml)*		*Plasminogen/Plasmin ratio*	
	n	Mean	n	Mean	n	Mean
Calving category						
Spring Calving	50	0.148 (.158)	39	0.965 (.691)	39	10.43 (9.86)
Autumn Calving	37	0.141 (.067)	31	0.790 (.704)	31	5.92 (5.78)
t-value [B]:		1.61		1.62		2.85
p :		0.110		0.110		0.006
Stage of lactation						
Early/Mid Lactation	55	0.098 (.058)	45	0.735 (.687)	45	9.38 (9.00)
Late Lactation	32	0.225 (.169)	25	1.162 (.640)	25	6.71 (7.55)
t-value [B]:		5.70		3.31		1.49
p:		0.000		0.002		0.140
Number of lactations						
1-2	40	0.128 (.103)	33	0.919 (.712)	33	10.78 (10.86)
3-4	21	0.158 (.161)	18	0.895 (.558)	18	7.35 (5.70)
≥ 5	26	0.159 (.133)	19	0.826 (.814)	19	5.38 (4.35)
F-value [B]:		1.16		0.42		1.94
p:		0.319		0.659		0.151

Plasminogen/plasmin ratios were decreased in autumn calving milk compared to spring calving milk ($p<0.01$). There was a tendancy for lower ratios in late lactation milk compared to early lactation milk and milk from cows of increasing numbers of lactations, but these changes were not significant. The decreased ratio in autumn calving as opposed to spring calving, without a concomitant increase in plasmin level is linked to the lower level of plasminogen in the former milk, and suggests that plasminogen transport to this milk is impaired. The decrease in late lactation ratio may be due to increased transport of

both components or increased activation of plasminogen, or a combination of these factors. There is evidence of increased plasminogen activator activity in milks from cows of advancing number of lactations because the plasmin level increases while the level of plasminogen decreases in such milks.

5.3.4. GLM analysis of factors influencing plasmin activity in milk

The same GLM model as used to determine the variables affecting the PMN levels (model (2)) in the milks was applied to the plasmin and plasminogen data. However, due to the non-normally distributed (skewed) nature of these data, a $\log_{10}$ transformation was applied to both sets of data before analysis. Approximately 42% of the variation in $\log_{10}$ (plasmin activity) was explained by the model. The model for $\log_{10}$(plasmin activity), treating $\log_{10}$SCC as a covariate, is as shown in table 5.7. It can be seen that the only terms significantly affecting milk plasmin activity were $\log_{10}$SCC and the interaction between season and stage of lactation.

Table 5.7 *GLM analysis of factors affecting $\log_{10}$plasmin activity in milk*

Factor	d.f.	Adjusted means squares	F-value	P
Log_{10}SCC	1	0.581	8.98	0.004
Calving season	1	0.040	0.62	0.435
Stage of lactation	1	1.529	23.63	0.000
Calving season * stage of lactation	1	0.718	11.09	0.001
Error	99	0.065		
Multiple R^2 value	42.2%			

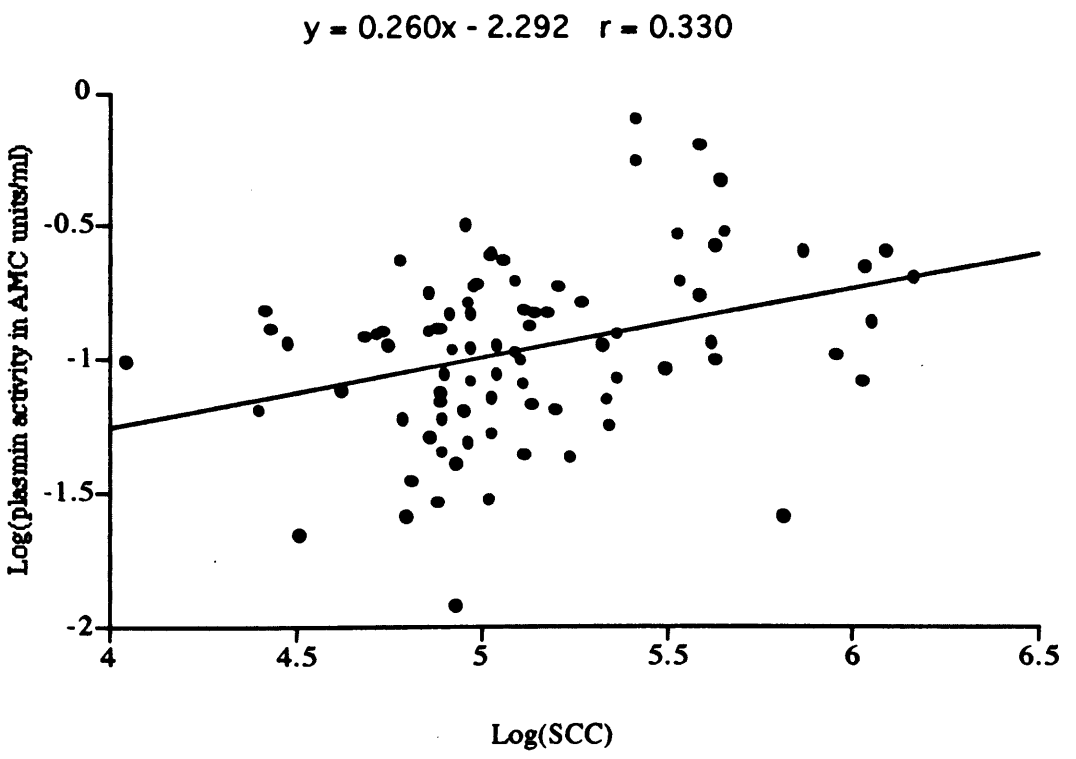
The mean plasmin activities by season and stage of lactation are given in table 5.8.

Table 5.8 *Influence of season and stage of lactation on plasmin activity in milk (AMC units/ml ± SD) calculated from GLM model, adjusted for all other terms in the model and transformed to original scale.*

	Plasmin Activity (AMC units/ml)								
	Spring calving			Autumn calving			All seasons		
	n	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*	*Mean*	*SD*
Stage of lactation									
Early/Mid	35	0.069	.03	20	0.119	.07	55	0.090	.01
Late	15	0.207	.09	17	0.148	.09	32	0.175	.03
All stages	50	0.119	.01	37	0.133	.03			

It can be seen that spring calving late lactation milk has nearly three times the plasmin level of early/mid lactation spring calving milk (0.207 compared to 0.069 AMC units/ml milk).This finding was not reproduced in autumn calving milks. The weak but positive relationship between $\log_{10}$(plasmin activity) and $\log_{10}$(SCC), found by the model to be significant ($P<0.01$) is shown in Fig. 5.3.

Fig. 5.3 *Regression plot of the relationship between $\log_{10}$(plasmin activity) and $\log_{10}$(SCC) (correlation coefficient significance <0.01)*



5.3.5. GLM analysis of factors influencing plasminogen level in milk

The plasminogen model derived using GLM model (2) showed that the only factor affecting plasminogen level in milks was stage of lactation, with early/mid lactation milks having higher plasminogen levels than late lactation milks, as seen from Table 5.6. Thus there is a substantial rise in plasminogen levels in milk from late lactation cows, and when this is taken into account, variations due to season, SCC or stage of lactation did not contribute significantly to the model.

5.3.6. GLM analysis of factors influencing plasminogen/plasmin ratio in milk

Finally $\log_{10}$(plasminogen/plasmin ratio) was also analysed using GLM model 2 and the model collapsed to a two term equation as shown in Table 5.9. Approximately 19% of variation in $\log_{10}$(plasminogen/plasmin ratio) was explained by this model. From this model it was calculated that the plasminogen/plasmin ratio is higher in milk from spring

Table 5.9 *GLM analysis of factors affecting log_{10}(plasminogen/plasmin ratio) in milk*

Factor	d.f.	Adjusted means squares	F-value	P
Calving season	1	1.244	10.53	0.002
Number of lactations	2	0.750	3.17	0.048
Error	99	0.118		
Multiple R^2 18.5%				

calving (7.33) than autumn calving cows (3.89). The ratio decreases in older cows (6.44, 6.36 and 3.73 for cows of 1-2, 3-4 or 5 or more lactations respectively), suggesting that increasing plasminogen activator activity occurs in milks from older cows.

The fact that autumn calving cows show a lower ratio overall than spring calving cows is difficult to explain. However, from Table 5.6 it appears that the mean plasmin activity in spring and autumn calving milks are equivalent, but the latter contains less plasminogen for some reason, which would cause a decreased plasminogen/plasmin ratio, without increased plasminogen activator activity (no increase in plasmin level). In summary, the levels of plasmin and plasminogen are far more stable in autumn calving milk, and less subject to lactational variability, but the ratio is significantly different, and there is definite evidence of increased plasminogen activator activity in milks from older cows.

5.3.7 Statistical analysis of gross composition of trial milks

Late lactation milk is associated with a change in the levels of many components in milk, with accompanying changes in milk processability (Phelan *et al.*, 1982; Keogh *et al.*, 1982; Lucey and Fox, 1992). The mean compositional analyses for a number of the milks sampled in this trial, as divided by stage of lactation, were determined. These are summarised in Table 5.10. Higher levels of somatic cells, total solids, fat and chloride and lower levels of lactose were found in late lactation milks ($p<0.01$). Protein levels were also elevated in late lactation milk, although not significantly. Levels of constituent proteins (caseins and whey proteins) were not measured. Milk pH was elevated in late lactation, and parameters relating to milk proteolysis, such as plasmin, plasminogen and total relative protease activity were also elevated in this milk ($p<0.05$). The level of initial proteolytic damage to milk protein, representative of proteolysis in the udder prior to milking, was unaffected by stage of lactation.

Correlation coefficients between log_{10}SCC, PMN level as measured by ELISA and milk plasmin activity and milk composition are presented in table 5.11. Log_{10}SCC correlated significantly with all the compositional parameters measured, with the exception of milk fat and pH, while PMN levels in general showed a lower degree of correlation with all parameters. Plasmin activity was more strongly correlated with the

Table 5.10. *Levels of milk constituents and proteolysis parameters in early and mid lactation milks (*, P<0.05; **, P<0.01; ***, p<0.001; n.s., not significant)*

Constituent	Units	Early Lactation Milks			Late Lactation Milks			
		n	Range	Mean±SE	n	Range	Mean±SE	Significance
SCC	cells/ml	68	11-1117	175±28	39	48-1503	329±43	*
Total solids	%	21	11.33-15.53	12.80±.24	11	11.6-14.5	13.49±.32	n.s.
Fat	%	21	3.25-5.25	4.12±.15	10	3.39-5.62	4.64±.23	*
Protein	%	21	2.52-4.88	3.33±.13	11	2.72-4.16	3.73±.14	n.s.
Lactose	%	21	4.42-5.01	4.76±.04	12	3.59-4.88	4.47±.11	**
pH	-	55	6.35-6.73	6.60±.01	31	6.54-6.85	6.65±.01	**
Plasmin	AMC units/ml	55	0.01-0.29	0.10±.01	32	0.05-0.80	0.23±.03	***
Plasminogen	AMC units/ml	42	0.12-3.07	0.78±.11	25	0.22-2.64	1.23±.13	**
Chloride	%	12	0.03-0.11	0.07±0.01	6	0.09-0.15	0.11±.01	**
Lactose/Chloride ratio x 100	-	10	0.63-2.49	1.57±.19	6	1.99-4.21	2.69±.33	**
Proteolytic Activity	Arbitrary units	23	0-32.75	11.7±1.71	12	4-56	20.17±4.0	*
Initial proteolytic damage	Arbitrary units	24	4.5-23	10.02±.82	12	4.5-19.75	10.25±1.28	n.s.

Table 5.11. *Correlation coefficients between log (SCC), PMN level, plasmin activity and various milk constituents and proteolysis parameters for milks from individual cows (*, P<0.05, **; P<0.01, ***; P<0.001; n.s., not significant).*

Constituent		Log (SCC)		PMN			Plasmin	
	n	Correlation Coefficient	Level of Significance	Correlation Coefficient	Level of Significance	n	Correlation Coefficient	Level of Significance
Total Solids	31	0.426	*	0.343	n.s	n.d.	n.d.	n.d
Fat	31	0.327	n.s.	0.310	n.s.	n.d.	n.d.	n.d.
Protein	31	0.646	***	0.395	*	27	0.502	**
Lactose	31	-0.475	**	-0.252	n.s.	27	-0.741	***
Chloride	18	0.581	*	0.465	n.s.	15	0.648	**
Lactose/ chloride ratio	18	0.544	*	0.398	n.s.	15	0.770	***
pH	93	0.191	n.s.	0.146	n.s.	93	0.306	n.s.
Plasmin	93	0.332	**	0.173	n.s.	-	-	-
Plasminogen	72	0.262	*	0.162	n.s.	72	0.516	***
Total Proteolytic Activity	36	0.225	n.s.	0.189	n.s.	30	0.587	***
Initial Proteolysis	36	0.477	*	0.119	n.s.	30	0.275	n.s.

lactose/chloride system parameters, which are associated with regulation of milk osmotic pressure, than SCC or PMN level.

Milk protein was correlated strongly with SCC, PMN and plasmin, most significantly with $\log_{10}$SCC, while proteolytic activity in milk was correlated more strongly with plasmin activity than with either cell measurement. However, the initial degree of protein breakdown of milk appears to be more related to total SCC than either of the other factors mentioned. Protein level in milk was correlated to initial protein breakdown level ($r=0.523$, $P<0.05$), as might be expected, but not subsequent proteolysis on incubation. Apart from the relationship of plasmin, total SCC and PMN level with lactose and lactose/chloride ratio, all significant correlations were positive. Due to small sample sizes, subdivision on the basis of season, or any of the other classifications used hereforeto, was not implemented. However, the influence of these factors on the correlations requires further investigation, to determine for example if the same relations hold in spring and autumn calved cows.

5.3.4. Examination of proteolysis products in milk

A preliminary electrophoretic examination of proteolysis products in milk is shown in Fig. 5.4 (see also Table 5.12). Although the milks examined were limited to spring calving-late lactation and autumn calving-early lactation milks, it can be seen that early lactation low SCC milk had the lowest level of proteolytic damage on incubation for 24 hours at 37°C, with a slight increase in γ -caseins being the only visible change. By comparison, in early lactation, high SCC milk, a far higher increase in proteolysis products is seen after incubation, with both α_{s1}- and β -casein being apparently attacked, with higher degradation of the latter in 24 hours. Late lactation, high SCC milk is seen to contain elevated levels of γ -caseins and a number of the fastest moving proteolysis products even before incubation, and to have the highest degree of α_{s1}- and β -casein breakdown after incubation. The remainder of the late lactation samples, of lower SCCs, have less apparent proteolytic activity, but still a greater level than that seen in early lactation, low SCC samples. Particularly marked is the higher level of γ-caseins present, in sample G the extensive breakdown of β-casein correlating to the extremely high level of plasmin measured in this milk. α_{s1} casein is also extensively attacked on incubation of this milk.

It should also be noted that the highest recorded levels of plasmin and 12% TCA-soluble products liberated on incubation in this study were found in sample G. Also the early lactation samples had the lower proteolysis quantified in low SCC milks as opposed to high SCC milks, while this was not the case for late lactation milks. The reason for this is unknown.

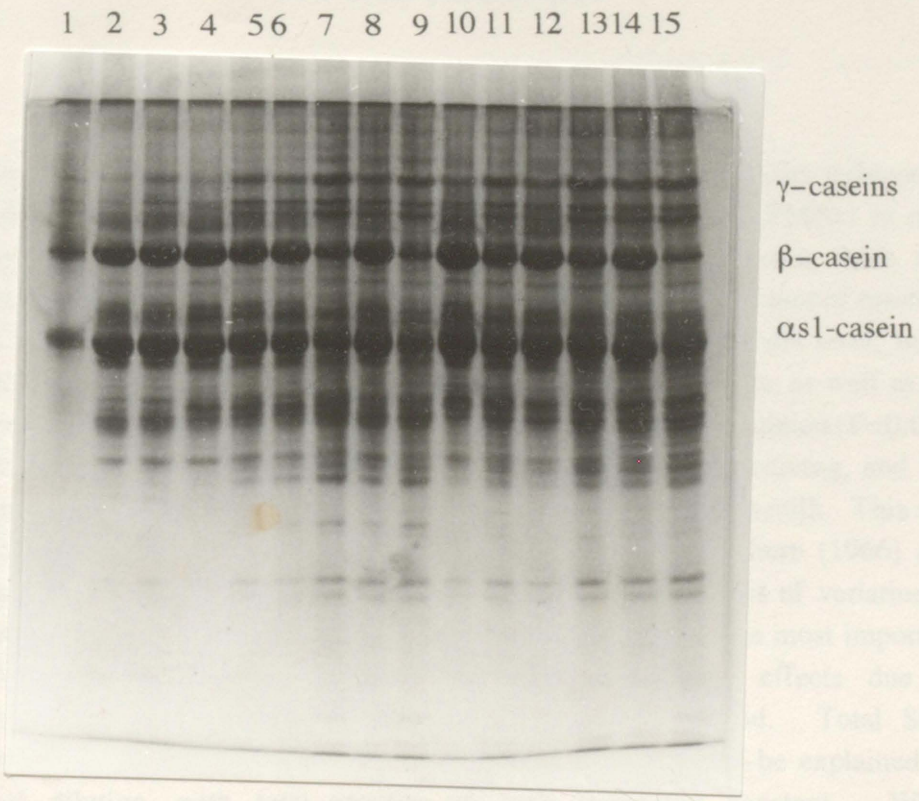


Fig. 5.4. *Urea-PAGE (12% T, 4%C) analysis of proteolysis products in milks before and after incubation at 37°C for 24 hours in the presence of 0.05% NaN_3. Lane 1, sodium caseinate. Lanes 2,3 A, before and after incubation (as for all following pairs of samples). Lanes 4,5 B. Lanes 6,7 C. Lanes 8,9 D. Lanes 10,11 E. Lanes 12,13 F. Lanes 14, 15 G. For keys to samples see table 9.*

Table 5.12. *Samples incubated for electrophoretic analysis*

Sample	Season	Stage of Lactation	SCC (,000s)	Plasmin (AMC units/ml)	Proteolysis (arbitrary units)
A	Autumn	Early	11	0.097	7
B	Autumn	Early	61	0.060	4
C	Autumn	Early	734	0.252	19.5
D	Spring	Late	1070	0.221	4
E	Spring	Late	91	0.314	22
F	Spring	Late	104	0.245	10.75
G	Spring	Late	259	0.797	56

5.4 DISCUSSION

5.4.1. Individual milk SCC and PMN levels

The aim of this section of the work was to elucidate some factors influencing somatic cell count (SCC) and level of polymorphonuclear leucocytes (PMNs) in milk from individual cows, and also to examine the relationship between these two parameters, as PMNs, in elevated numbers, may be regarded as an indicator of mastitis. When SCC was examined, it was found to be far more variable than PMN level, being significantly affected by season, lactation number and stage of lactation, as well as the interactions between lactation number and both season and stage of lactation ($P<0.05$). Spring calving cows were found to have higher SCCs than autumn calving, and late lactation milk was found to have nearly twice the SCC of early lactation milk. This rise in late lactation SCC was in accordance with the findings of Blackburn (1966) and Emanuelson *et al.* (1988). Brolund (1985), in a large study of causes of variation in milk somatic cell counts, found that bacteriological status was the single most important source, followed by lactation number and minor significant effects due to morning/evening sampling, stage of lactation and sampling period. Total SCC increased with lactation number and effect of daily milk yield could be explained by proportional dilution, with total number of cells remaining constant. While bacteriological analyses were not performed on milks in the present trial, the influence of lactation number on SCC was apparent, with generally higher SCCs in cows of 3 or more calvings compared to cows of 1 or 2 calvings. The increases were considerably more dramatic in spring calving than autumn calving cows, whose SCCs remained relatively low at all ages. Emanuelson *et al.* (1988) found that SCC rose at the end of lactation in cows determined free of mastitis, but suggested that this may be due to decreased milk yield.

With regard to differential, as opposed to total, SCCs, good quality, low SCC milk is expected to contain up to 90% macrophages, but on infusion of endotoxin this level drops rapidly and is replaced within 1 hour by PMNs as the dominant cell type (Saad and Ostensson, 1990). However, proportions of PMNs in healthy milk have also been reported as 70% (Dulin et al (1982) in milks<70,000 cells/ml), 33-44% (mid and late lactation milks respectively, < 100,000/ml, Blackburn et al (1954)), 8-30% (depending on milking fraction, Ostensson *et al.*, 1988, milks < 100,000/ml), and 36% (milks with no infective pathogens present, Wever and Emanuelson, 1989). Ostensson (1993) found that PMNs accounted for 34% and 55% of cells in foremilk and residual milk respectively, that the proportion of macrophages decreases after 6 months of lactation and that the correlation between proportion of PMNs and SCC was 0.25 in residual milk, and negligible in foremilk, for all cows tested. In the present study PMN levels were found to be highest overall in milks from spring calving as opposed to autumn calving cows, and to increase in late lactation milk and milks from cows of three or more lactations, as opposed to younger animals. The major effect was that of calving season, as opposed to stage of lactations or number of lactations, as

shown by GLM analysis, where, along with $\log_{10}$SCC, season was the only factor to influence PMN level.

Plots of PMN level against $\log_{10}$SCC (fig. 5.1) showed that the increase in PMN levels for an equivalent increase in SCC was less in autumn calving than spring calving milk. With regard to linear regression coefficients for SCC versus PMN plots as determined by O'Sullivan *et al.* (1992), it was found that, while a high overall correlation coefficient was seen, there were differences in coefficients between summer and winter milks. This phenomenon, seen clearly when the influence of mastitic samples (>500,000 cells/ml) were removed, suggested that environmental and nutritional factors may also influence this relationship. The dominant effect of season shown here suggests that feeding and husbandry practices may be the determining factors influencing PMN level. In winter, lactating (autumn-calved) cows are fed concentrates due to lack of available grass and this promotes health and keeps PMN levels low, while non-lactating spring calving cows have poor diets and health during the winter, and are perhaps more susceptible to mastitis in early lactation, when taken outdoors, as reported by Ostensson (1993). The practice of feeding concentrates after parturition does not appear to immediately rectify this problem, and mean SCC and PMN levels will be consequently high in spring-calving cows. It could be proposed that in autumn calving milks are found generally lower levels of PMNs per cell, due to nutritional influences (as indicated by the slopes of the log plots), but that a separate set of influences, perhaps environmental, cause a less predictable relationship between total SCC and PMN level at this time (as indicated by the linear regression correlation coefficients). The question of whether a low level of PMN cells in milk is actually beneficial is contentious, with the possibility existing that selection for cows of low SCCs, and hence low PMN levels, may diminish the protective function of the PMN cell in the udder, predisposing to infection (Saad, 1987), while among the problems associated with high PMN levels is tissue damage due to PMN proteases (Travis, 1988).

O'Sullivan *et al.* (1992) used a capture ELISA immunoassay to measure polymorphonuclear leucocyte (PMN) antigen in milk from individual cows, and found a correlation of 0.94 between the assay and total somatic cell count, higher than that previously reported for a number of physiological and enzymatic parameters used to characterise mastitic milk. The reason for the poor correlations found, by comparison, by Ostensson (1993) is unknown, but the differential cell counting in that study was carried out by flow cytometer, as opposed to ELISA assay which may be assumed to be a more specific means of counting PMN cells. The former authors concluded that the test, once adapted to a single-step dip-stick format, could be used as a cow-side diagnostic test for mastitis, enabling rapid identification and isolation of infected cows and quarters. This would allow elimination of mastitic milk from the bulk tank, thus keeping farm SCC down and milk quality high. Preliminary surveys of bulk tanks indicated lower correlations (as low as 0.40) between SCC and PMN level, which may be partially attributable to commingling of mastitic milks with non-mastitic milks of various lower SCCs (see Chapter 10). Regression equations for SCC against PMN

levels were determined in this study, and the only correlation coefficient approaching that found by O'Sullivan *et al.* (1992) was found in spring calving, early lactation milk. This was also seen when samples likely to possess mastitic character (i.e. high SCC) were deleted and correlations recalculated. It can be postulated that any samples of individual cows milks obtained by these authors, from the majority of Irish farms, for most of the year, would consist of this type of milk. However, the lower correlations in the other groups suggest that the use of PMN level to predict SCC, for example in a cow-side ELISA test, is dubious when all possible milk types are considered, and furthermore that there are unknown factors governing the proportion of PMNs out of the total cell count in these milks, probably concerning season, and hence diet and plane of nutrition. The wide range of published values for PMNs as a percentage of total SCC in non-mastitic milk may be attributable to the variability in correlations, and hence predictive relationship between these two quantities.

With regard to variation in total SCC, if late lactation increases in total milk SCC can be explained by reference to milk yield, it must be considered that the PMN proportion, and its change between spring and autumn calving cows, is independent of milk yield, as fig. 5.1 shows the increase in PMN level per cell, which is independent on total cell concentration, and hence milk volume. A study is currently being undertaken by the authors to compare PMN, lymphocyte and macrophage levels in a range of milks, as affected by a number of environmental and seasonal factors.

5.4.2 Plasmin activity in milk

A range of plasmin activities of 0.012 to 0.80 AMC units/ml was found for milk in this study, with a range of plasminogen concentrations from 0.094-3.073 AMC units/ml, as compared to published ranges of 0.023-0.127 and 0.383-0.802 (Richardson, 1983b) and 0.15-0.32 and 0.83-1.82 (Benslimane *et al.*, 1990). Thus the values found in our study are generally in a typical range, although three cows had plasmin activities of 0.55, 0.64 and 0.80 respectively, among the highest values reported in any study published. All three cows were spring calving, late lactation and had SCCs in the range 258-383,000 cells/ml. Benslimane *et al.* (1990) reported that individual cows had higher plasmin activities than herd milks, which were still higher than bulk milks, so this must be taken into account when considering levels reported.

Plasmin and plasminogen activity in milks in this study were found to double in late lactation as compared to early/mid lactation milk (Table 5.8). Richardson (1983b) found mean levels of 0.073 AMC units/ml milk and 0.141 AMC units/ml milk in early and late lactation Friesian milks respectively, with corresponding plasminogen levels of 0.457 (early lactation Friesian milk) and 0.802 (late lactation Jersey milk) AMC units/ml milk. These milks were all taken from autumn (August-September) calving bulk supplies, and were all pasteurised. These levels are only slightly lower than the mean plasmin levels found in this study (0.090 and 0.175 AMC units/ml for early and late lactation). Korycka-Dahl *et al.* (1983) found only a slight increase in plasmin activity between early and late lactation milks from individual cows, but that the

concentration of plasminogen was doubled between these times. However, Davies and Law (1977b), Barry and Donnelly (1983), Politis *et al.* (1989a) and Benslimane *et al.* (1990) found that from early to late lactation plasmin and plasminogen both increased as shown in this study, and the latter authors concluded that stage of lactation was the principal factor influencing plasmin activity.

The interaction of calving season with stage of lactation and their effect on plasmin activity shows a higher mean plasmin activity in autumn calving milk, but that this type of milk is far less subject to seasonal variation. Benslimane *et al.* (1990) examined plasmin levels in herd milks throughout the year, but since each monthly result contains a mix of the two types of milk, comparisons are difficult. However, a mix of the levels as found here would suggest considerably increased plasmin activity in bulk milks in August-November (mix of spring calving late lactation and autumn calving early lactation) compared to February - June (mix of spring calving early lactation and autumn calving late lactation). This would agree broadly with the findings of Phelan *et al.* (1982) and Donnelly and Barry (1983) on milk casein patterns. The slight (non-significant) increase in late lactation milk plasmin levels from cows of increasing age was also reported by Bastian *et al.* (1991).

Richardson (1983b) found a plasminogen/plasmin ratio of 9, which did not vary appreciably over lactation, but unlike the other studies referred to, this author examined pasteurised bulk skim milks, as opposed to raw milks from individual cows. This probably accounts for the lower plasmin levels reported (Richardson (1983a) calculated a 17% decrease in plasmin activity on pasteurisation) and in bulk milks variations in the relative levels of the enzyme and zymogen may be evened out. In the present study it was found that plasminogen/plasmin ratio was higher in milk from spring calving compared to autumn calving cows and in early/mid lactation milks than late lactation milks. The decrease in ratio in late lactation (although non-significant) was also found by Politis *et al.* (1989a) and Bastian *et al.* (1991). Plasminogen activation is one mechanism for increased plasmin activity, which can also be caused by increased plasmin and plasminogen transport from blood, evidenced by the significant rise in levels of both these components in late lactation observed in our study and that of Politis *et al.* (1989b). However, if no increased activation was involved, and both components were transported at the same rates, the ratio would remain constant. Benslimane *et al.* (1990) found that the ratio actually increased slightly with increasing stage of lactation in Montbeliard cows' milk, which were predominately autumn calved, and so season may be considered to play a role in this mechanism. The ratios found for spring calving herds here are far higher than those found for autumn calving herds (7.33 compared to 3.89), because although the plasmin levels were similar in both groups (0.119±.01 compared to 0.133±.03) the plasminogen levels in autumn calving milks were lower than in spring calving milk (0.790±.70 for autumn calving milk compared to 0.965±.69 for spring calving milk). Also, the plasmin level in autumn calving milk was considerably less affected by stage of lactation than that of spring calving milk (table 5.8). This implies, in summary, an environmental influence on plasmin and plasminogen levels and possibly plasminogen activator activity, and hence

permeability of the blood-milk barrier, with far more variability in spring calving cows, kept outdoors and fed grass, than autumn calved cows.

The role of somatic cell plasminogen activators (PAs), shown by Verdi and Barbano (1991), is questionable, as in both this study and that of Politis *et al.* (1989b) elevated SCCs did not affect the ratio significantly. Zachos *et al.* (1992) found that somatic cell PA activity was not related to stage of lactation, but was associated with somatic cells in mastitis, but the mean SCC of the milks studied herein was probably too low to allow such a mechanism to be detected. However, these mastitic cells would comprise predominantly PMNs, which are present in reduced numbers in autumn calving milk, irrespective of stage of lactation, and this would suggest that the plasminogen/plasmin ratio should be lower in spring calving cows' milk, due to increased plasminogen activation. The fact that this is not seen here may be accounted for by the possibility that the decreased plasminogen concentration in the former milk is masking this effect. The decrease in ratio observed in older cows is in agreement with the results of Bastian *et al.* (1991).

Politis *et al.* (1989b) derived correlation coefficients between plasmin activity and some milk components and found a correlation of 0.62 (least squares analysis) against SCC and a correlation of 0.19 against pH, which compare to correlation coefficients derived in this study of 0.33 against log SCC (see Fig. 5.3) and 0.31 against pH (table 5.10). The correlation of SCC against pH was not significant. The relationship between plasmin and SCC is expected due to the role of plasminogen activators described above, and the correlation with pH is explained by the high pH optima (7.5-8.0) of both plasmin and milk plasminogen activators. Plasmin was also found to be significantly correlated to plasminogen ($r = 0.52$), exactly equal to the correlation found by Benslimane (1990), and total milk protein ($r = 0.50$), as expected due to its association with the casein micelle in milk (Grufferty and Fox, 1988c).

5.4.3. Milk composition

The correlations of log (SCC) with other milk compositional parameters are in good agreement with those reported by Mitchell *et al.* (1986a) for farm bulk milks, using similar numbers of samples, but in general the correlation coefficients herein are higher (protein, total solids, lactose, chloride) with some slightly lower (fat, pH) (table 5.10). The correlations might be higher because in individual milks more cow-to-cow variation will be detected, while some of this variability may be diluted out in bulk milks. Milk fat is the least affected by SCC, and the small degree of correlation found may be due to reduced total milk yield with increasing SCC, and a rise in fat content in late lactation milk independent of rising SCC. Munro *et al.* (1984) in a review of the influence of mastitis on milk composition, reported that while evidence is mixed, milk fat probably increases with increasing SCC until very high SCCs are reached, when the fat content is reduced. The low proportion of milks in this study of SCC>1,000,000/ml (7%) probably masked this trend. The correlation between SCC and pH is in

accordance with the trends reported by Haenlein *et al.* (1973) and Barry and Donnelly (1981).

Protein was found here to be significantly positively correlated to $\log_{10}$SCC. Haenlein *et al.* (1973) and Verdi et al (1987) found no significant difference in total protein between high and low SCC farms, but significant changes in individual protein components. Mitchell *et al.* (1986a) and Ng-Kwai-Hang *et al.* (1982) found an increase in total nitrogen with increasing SCC, mainly from changes in the serum protein fraction. Munro *et al.* (1984) concluded that since caseins are reduced and whey proteins elevated in mastitic milk the overall effect on total protein depends on the degree of infection, and this, combined with seasonal and lactational variations leads to the wide range of values and trends reported. While the levels of the individual proteins were not determined in this study, one would thus expect an increase in secreted proteins at the expense of casein.

Lactose:chloride ratio was significantly related (correlation of -0.54) to log (SCC). This correlation accords with the findings of Mitchell (1986d). Lactose synthesis decreases with mastitic damage of mammary gland secretory cells, and accompanying increased permeability of blood capillaries leads to an influx of sodium and chloride ions from extracellular fluid to maintain osmotic pressure, and thus the ratio of lactose:chloride decreases with increasing SCC (Kitchen, 1981).

Mitchell *et al.* (1986a) attempted to use a threshold value of SCC>500,000 cells/ml as an indicator of abnormal (mastitic) milk but found that the two groups (above and below threshold) overlapped considerably, and thus the effect of mastitis on gross milk composition was unclear, which might account for the poorer correlations with gross composition found for PMN antigen than total SCC. This suggests that the factors relating to total SCC, which have been shown herein to be in many cases unrelated to PMN level in milk, are more deterministic of milk composition. The chemical and physiological factors resulting in influx of PMNs to the udder must therefore be different. However, again it must be stressed that these correlations were derived for a milk sample set of low mean SCC and PMN levels, and that in a more typical set of milks, of a wider range of SCCs, a more predominant role for PMNs may be apparent.

The mean compositional data for late lactation milks in Table 5.9 show higher SCC, fat, total solids, protein, chloride and pH and lower lactose content and lactose/chloride ratio in late lactation milk than early/mid lactation milks. Phelan *et al.* (1982), in a study of seasonal changes in composition of Irish milk, found that for milk from creamery and liquid milk suppliers and spring- and autumn-calving herds there was a maximum protein level reached between August and November, and that at this time the spring milk protein (late lactation) was considerably higher than that in early lactation autumn milk (4.16% as compared to 3.78%), with a mean creamery and liquid milk levels at this time of 4.27 and 3.45% respectively. These trends agree closely with the range of values found in this study, although mean values were lower. Phelan *et al.* (1982) concluded that late lactation milk has elevated fat, protein, pH and reduced lactose compared to mid-lactation milks, as found herein. This was also

reported by Lucey and Fox (1992). Verdi *et al.* (1987) found higher total protein levels in farm milks between October and November, and both this and the Irish study probably reflect the predominance of late lactation milk at this time of the year. Keogh *et al.* (1982) studying the seasonal variation in mineral levels of Irish milk, found a 50% rise in sodium levels in late lactation milk, and since sodium and chloride are transported into milk concomitantly, the 57% rise in chloride level reported here appears in good agreement with this trend.

With regard to cheesemaking properties of late lactation milk, the elevated fat and increased proteolysis in late lactation milk will lead to poor syneretic properties and elevated cheese moisture levels, as reported by O'Keeffe (1984). It is also interesting to note that plasmin is more highly related to chloride (0.65) and lactose (-0.74) than log (SCC), and both these parameters have been suggested as indices of abnormal or mastitic milk (Mitchell, 1984a), and so this begs the question of whether plasmin is more directly and significantly affected by mastitic infection than SCC, presumably through the 60-fold increased plasminogen activator activity reported for somatic cells in mastitic milk (Zachos *et al.*, 1992). A second possible explanation for this is that while fat, protein and most other milk components are synthesised in the udder, chloride levels in milk are regulated by entry from blood, from which plasmin and plasminogen also derive. Thus, mechanisms which control the permeability of the blood-milk barrier, in response, say, to a drop in lactose concentration due to tissue damage, would cause migration of both these components, resulting in the high degree of correlation seen. However, it is not known if these mechanisms are the same as those which allow somatic cells to cross into the milk, which remains to be addressed.

5.3.4. Proteolysis in milk

Relative proteolytic activity in late lactation milk was approximately twice that in early/mid lactation milks, but initial level of proteolytic damage to caseins was not affected by season. Relative proteolytic activity was more strongly correlated with plasmin activity in milk ($r = 0.59$) than log (SCC) ($r=0.23$), PMN level ($r = 0.19$) or days in lactation ($r=0.38$). The initial level of proteolysis measured (before incubation), however, was more strongly correlated with log (SCC) ($r = 0.48$) than PMN level ($r = 0.12$), days in lactation ($r = 0.01$) or plasmin activity ($r = 0.28$). It was most strongly correlated with total protein ($r = 0.52$) as may be expected, as higher protein levels have been associated with high SCC milks and late lactation milks, which will also contain relatively high levels of protein breakdown products and non-protein nitrogen, and hence free amino groups to react with the fluorigenic reagent used. Relative proteolytic activity was not significantly correlated to % total protein.

High SCC milks are known to contain lower levels of casein, presumably due to proteolytic damage (Haenlein *et al.*, 1972; Ng-Kwai-Hang *et al.*, 1982). Senyk *et al.*(1985) found very good correlations between SCC and initial milk tyrosine value and tyrosine value after incubation under similar conditions to those used in our study (0.60 and 0.79 respectively). Elevated proteolytic action in mastitic milk was also reported by

Murphy *et al.* (1989) and Verdi *et al.* (1987). However, in neither of these studies was plasmin activity in the milks measured, and the proteolysis in any milk must be considered as plasmin plus non-plasmin proteolysis, including that contributed by proteases associated with somatic cells. These proteases include cathepsin D, which is known to hydrolyse α_{s1}-casein in a manner similar to chymosin (McSweeney *et al.*, 1995), cathepsin B and neutrophil elastase, both of which hydrolyse both α_{s1}- and β -casein readily under milk conditions (see Chapter 6).

Andrews (1982, 1983a-c) showed that the principal proteinase in normal milk was plasmin, but that inhibition patterns suggested the presence of another enzyme(s), and that high SCC milk proteolytic activity had a broad temperature and pH optimum curve, suggesting the presence of several different enzymes, of which plasmin represented one third of total proteinase activity. Saeman *et al.* (1988) estimated non-plasmin proteolytic activity to be 17% in normal milk and 46% in mastitic milk, with a significant correlation with SCC. Grieve and Kitchen (1985) estimated that caseinolytic activity of somatic cell preparations at 1,000,000 cells/ml in casein solutions was less that that present in normal milk, but their cells were recovered from bovine blood, which have been shown to be far less proteolytically active that milk somatic cells (Verdi and Barbano, 1991). Thus it is difficult to say which factor influences proteolysis in isolation from the contribution of the other, as for example in late lactation milk, while relative proteolytic activity is doubled compared to early/mid lactation milk, so also are both total SCC and plasmin activity. It appears from this study, however, that damage to protein due to elevated SCC takes place in the udder, prior to milking, as the initial proteolysis level is very closely correlated to log (SCC). This may be due to the factors which increase non-casein nitrogen in milk, such as infection, which increase SCC and thus presence of somatic cell proteinases and both of these factors lead to increased protein damage in milk, which will indirectly influence cheese quality and yield.

Further information on the proteolysis taking place in the milks was obtained from the electrophoretogram of incubated early and late lactation milks (Fig. 5.4). Early lactation milks are seen to have slight proteolysis of ß-casein to γ -caseins on incubation, but in high SCC early lactation milk, a higher level of initial proteolysis products, including γ -caseins, is seen. Subsequent incubation of the latter milks causes significant proteolysis of both α_{s1}- and β -caseins, giving a range of products including very slow moving peptides, visible as three bands above the γ -caseins, which have been associated by this group with somatic cell protease action in milk and aseptic, starter and rennet free cheeses. High SCC late lactation milk, by comparison, yields a similar pattern before and after incubation, with perhaps slightly faster degradation of α_{s1}-casein. Lower SCC late lactation milks show much higher γ -casein levels, before and after incubation than early lactation samples, correlating with a high plasmin level in these milks, although plasmin level did not correlate well with initial proteolysis level, suggesting an accompanying, greater level of proteolysis by somatic cell proteinases, which have not been shown to produce γ -caseins. The final milk sample contained the highest level of plasmin yet reported for a milk sample, and the

degradation of β -casein is almost complete after 24 hours incubation. However, there were a number of proteolysis products, such as the three topmost bands, which are not produced at all, or only at low levels, in this milk, which has a low SCC, compared to higher SCC milks. These may either have been produced or degraded within the 24 hours or are more likely due to the action of somatic cell proteases.

By comparison, Andrews (1983 a,b) found degradation of both α_{s1}- and β -caseins on incubation at 37°C of normal raw milk, with proteose peptones and γ -caseins the predominant products and on similar incubation of high SCC, mastitic milks, found low yields of typical plasmin induced breakdown products, which he believed to have been broken down by other proteinases. He also refers to changes in pattern in the γ -casein region which may be the same as noted above. Verdi *et al.* (1987) compared high SCC milk proteolysis patterns low those from low SCC milk by SDS-PAGE and found that the former milk had higher initial levels of casein proteolysis products, as we found, and that the low SCC milk after 24 hours at 37°C was at the same level of proteolysis as the initial high SCC milk, and that high SCC milk showed substantial breakdown of both α_{s1}- and β -caseins on incubation, again exactly as we found.

In conclusion, the proteolysis in our milks appears to be plasmin dominated, but the influence of somatic cell proteinases is apparent both in the initial level of damage, and in the electrophoretic patterns of proteolysis products in high SCC milks. Further work is necessary to elucidate the relative roles of the two types of enzymes in milk proteolysis. However, it must also be stated that the herd examined was a pedigree herd of excellent quality, with a mean year-round SCC of approximately 200,000 cells/ml, which is exceptionally low. Thus it may be expected that plasmin activity will dominate the proteolytic activity found, moreso than in most typical Irish herds, and thus a wider study of farm milk proteolysis levels is required.

Chapter 6

The role of somatic cells and plasmin in proteolysis of raw and pasteurised milks of various somatic cell counts and the proteolytic specificity of somatic cell proteinases on casein.

SUMMARY

Levels of initial proteolysis, and proteolysis on incubation at 37°C for 24 hours, in raw and pasteurised milks over a range of SCCs were significantly correlated to $\log_{10}$SCC and milk polymorphonuclear leucocyte (PMN) level ($P<0.001$). Significant correlations were also found for incubated raw and pasteurised samples containing the plasmin inhibitor 6-aminohexanoic acid, and plasmin was overall less well correlated with proteolysis parameters than SCC or PMN level. This suggested a more significant contribution of somatic cell proteases to milk proteolysis than was previously believed. Urea-PAGE gel electrophoresis showed increasing breakdown of ß- and α_{s1}-casein with increasing SCC, and significant somatic-cell derived proteolysis after pasteurisation. Crude somatic cell extracts from milk and blood were shown to hydrolyse α_{s1}-casein strongly at pH 5.2 to α_{s1}-I casein, which may play a role in cheese ripening by affecting texture. This hydrolysis was accelerated for extracts from mastitic milk, which contained large numbers of PMNs. Milk somatic cells were more proteolytically active than blood leucocytes. Both ß- and α_{s1}-casein were hydrolysed by milk somatic cell proteinases at pH 6.5, and this activity was only slightly decreased by pasteurisation. PMN elastase was found to have a broad specificity on micellar casein and κ-casein, and PMN cathepsin B was found to hydrolyse α_{s1}-casein to α_{s1}-I casein. The importance of somatic cell proteinases in milk and cheese quality is apparent, and the implications of this work are discussed.

6.1 INTRODUCTION

Indigenous proteolytic activity in good quality milk is generally dominated by the action of the alkaline milk proteinase plasmin, which preferentially hydrolyses ß-casein, with a slower action on α_{s1}-casein (Noomen, 1975; Eigel *et al.*, 1979). Principal breakdown products of ß-casein due to plasmin action in milk include the proteose peptones and the γ-caseins (Humbert and Alais, 1979). Plasmin action is known to be elevated in late lactation milk (Barry and Donnelly, 1980; Politis *et al.*, 1989a; Benslimane *et al.*, 1990). Mastitis, or inflammation of the udder, is associated with elevated somatic cell counts (SCC) in milk with a concomitant increase in proteolytic activity. Increased plasmin activity in such milks may be due to elevated plasminogen activator activity of somatic cells isolated from such milks (Zachos *et al.*, 1992; Verdi and Barbano, 1991). A 5-10 fold increase in proteolytic activity in mastitic milk compared to normal milk was reported by deRham and Andrews (1982b). However, only 33% of this activity was attributable to plasmin and the origin of the increased activity was shown to be the somatic cells (Andrews, 1983b). These enzymes degraded α_{s1}-casein and ß-casein at approximately the same rate to yield a large range of peptides, many of which were quite small, and the authors concluded that these enzymes could well play a role during cheese ripening, possibly affecting cheese texture and flavour. Para-κ-casein has also been identified in high somatic cell count milk (Anderson and Andrews, 1977). High correlations between both initial milk tyrosine value and milk tyrosine value after incubation with SCC were found by Senyk *et al.* (1985).

The first non-plasmin proteinase in milk to be identified was the acid protease isolated by Kaminagowa and Yamauchi (1972b), which was later shown to have a specificity similar to chymosin, producing α_{s1}-I casein from α_{s1}-casein and ß-I and ß-II casein from ß-casein (Kaminagowa *et al.*, 1980). κ-casein was hydrolysed to para-κ-casein, but more slowly than by chymosin, as was the rate of breakdown of ß-casein, while α_{s1}-casein was hydrolysed more rapidly than by chymosin. The acid proteinase was later accepted to be cathepsin D, a somatic cell lysosomal proteinase associated with macrophages, and it's specificity on the caseins was determined (McSweeney *et al.*, 1995). It was suggested by the latter authors that this enzyme could be incorporated into cheese curd and act synergistically with chymosin during ripening. Two cysteine proteases have been isolated from mastitic milk, and been shown to be highly correlated with somatic cell count (Suzuki and Katoh, 1992).

The action of somatic cells, isolated from bovine blood, on caseins was examined by Grieve and Kitchen (1985) and found to be very low, with the caseins being attacked in the order α_{s1}>ß>>κ-casein. Verdi and Barbano (1991), however, found that cells isolated from blood had much lower proteolytic activity than cells isolated from milk and that milk cells were highly active on ß-casein. Somatic cells in milk may be divided into macrophages, epithelial cells and polymorphonuclear leucocytes (PMN cells) (O'Sullivan *et al.*, 1992). Macrophages contain cathepsin D,

pepsin and chymotrypsin, while the principal PMN proteases are the cathepsins B, D and G and elastase and collagenase (Verdi and Barbano, 1991).

To be able to consider fully the consequences of proteinases from somatic cells, and polymorphonuclear leucocytes (PMN cells), the action of these proteinases on caseins in milk and blood systems must be known. Hydrolysis of casein forms the basis for the manufacture of many dairy products, particularly cheese, and excessive or uncontrolled hydrolysis can lead to functional and organoleptic problems in many products, such as UHT milks, cheese and yoghurt. If the action of somatic cell proteinases in milk leads to extensive degradation of caseins, then the role of these enzymes in product quality must not be underestimated. This is particularly relevant for milks of elevated SCCs, such as mastitic and late lactation milks.

The aim of this series of experiments was twofold. Firstly, the connection between milk SCC and plasmin activity and individual cows' milk composition and proteolysis, shown to an extent in Chapter 5, was to be further examined, particularly with regard to milk proteolysis products. The objective of the second part of this study was to isolate crude somatic cell proteinase preparations from blood and milk, from both healthy and mastitic animals, and examine electrophoretically the breakdown of caseins induced by incubation of these enzymes with casein in a suitable buffer for various periods of time. Two of the principal PMN proteinases, cathepsin B and elastase, were also purchased in a pure form and their action on the caseins examined.

6.2 MATERIALS AND METHODS

6.2.1 Milk sample collection and analysis

For the milk proteolysis trial, 40 samples were obtained from the milk recording jars of individual Holstein Friesian cows, which were 2-4 months in lactation. No record of parity was taken as it was desired to examine the milks in terms of random samples, as would be received by dairy plants such as cheese factories. Milk gross composition, SCC, plasmin and plasminogen levels and PMN levels were determined as described in sections 5.2.1 and 4.2.1. In measurement of PMN levels, the samples above 1,500,000 cells/ml gave off-scale optical density (OD) values in the ELISA test and their OD values were interpolated, for purposes of correlations, from the regression equation for the remaining samples. For assessment of proteolytic activity, proteolysis products as shown by Urea-PAGE analysis and 12% TCA-soluble products were examined in all 40 samples, treated in the following ways (0.05 % sodium azide (NaN_3 was included in all incubations).

1. I Initial milk, with TCA added to sample on day of collection
2. R Milk, incubated for 24 hours at 37°C (raw)
3. R+A Milk incubated as for R, with 120 mM 6-Amino-hexanoic acid (6-AHA) added (15.7 mg/ml) to inhibit plasmin action

4. P Milk heated to 72.5°C for 15 secs in thin walled glass test tube in a water bath (<10 sec come-up) and then incubated as for R (pasteurised).
5. P+A Milk with 120 mM 6-AHA added, heated to 72.5°C for 15 secs in thin walled glass test tube in a water bath and incubated as before

0.75 ml Samples were taken from R, R+A, P and P+A after 24 hours at 37°C and 0.75 ml 24% TCA added. Samples were then mixed, centrifuged and proteolysis products in the supernatant quantified using the Lowry method described in section 4.3.5. The Lowry method was used as opposed to the more sensitive fluorescamine method due to the unacceptably high background levels given by the fluorescamine reagent in the presence of 6-AHA. Proteolysis levels were expressed as mg bovine serum albumin (BSA)/L milk by reference to a standard curve. Initially and after 1, 3, 6 and 9 days incubation 100μl samples were taken, mixed with 400μl electrophoresis buffer (single strength) and frozen for electrophoretic analysis by the method of Andrews (1983a) as described in section 4.3.1.

6.2.2 Preparation of enzyme extracts and casein digests

Bovine blood (1 L), obtained at a local slaughterhouse, was taken into 100 mls of 1.5% EDTA in 0.0132M phosphate buffered 0.7% NaCl (pH 6.8) (equimolar mixture of Na_2HPO_4 and KH_2PO_4). Blood polymorphonuclear leucocyte (PMN) and lymphocyte/macrophage-monocyte (non-PMN) fractions were isolated from the blood by the method of Carlson and Kaneko (1973). Cell counts in buffer were counted by the Fossomatic 90 and haemacytometer counts and viability estimated using the trypan blue staining method (Mishell and Shiigi, 1980). A high degree of agreement was found between haemacytometer and Fossomatic cell counts, once viability was taken into account. Washed cell fractions were frozen at -20°C until needed.

Due to difficulties in obtaining pure fractions of different cell types from milk, it was decided to compare the proteolytic activities of somatic cells isolated from milks from healthy and mastitic animals, on the assumption that the former milk cells would be enriched in macrophages and lymphocytes, while the latter milks should comprise greater than 90% PMNs (Saad and Ostensson, 1990). These assumptions were supported by the low and high ELISA results obtained for the control and mastitic cell preparations respectively using the PMN immunoassay of O'Sullivan *et al.* (1992). Cells were isolated from milk by centrifugation at 1000g and 4°C for 20 minutes and resuspending and washing the pellets in PBS (see Verdi and Barbano, 1988). Sufficient milk volumes were taken to obtain a concentrated cell suspension of 10-20 x 10^6 cells/ml. Cells were divided into aliquots and frozen at -20°C until used.

The proteolysis patterns arising from the digestion of casein by somatic cell preparations were examined using micellar casein and sodium caseinate substrates. Micellar casein substrate was prepared by diluting pasteurised skim milk 1:6 with distilled water and this substrate was used both at the natural pH and after adjustment to pH 5.2 with 1M HCl to simulate the pH of ripening cheese. For examination of the

activity of the cell preparations on caseins in milk and cheese like substrate systems, sodium caseinate was dissolved in 100 mM phosphate buffer (pH 6.6) and 100 mM phosphate buffer (containing 5% NaCl, pH 5.2). Digests were carried out by adding the cell suspensions to the substrate at a level calculated to give a set concentration of somatic cells/ml of substrate (typically 500,000 cells/ml), and incubating at 37°C for various periods of time. At certain time intervals, 100μl of sample were taken, added to 100μl of double-strength electrophoresis sample buffer (see section 4.3.1) and boiled. When all samples were taken, they were examined by alkaline urea-PAGE gel electrophoresis (section 4.3.1). For determination of survival of pasteurisation of enzymatic activity, cells + substrate mixtures were heated to 73°C in thin-walled glass test tubes in a water bath, held at this temperature for 15 sec. and then cooled rapidly by immersion in iced-water.

Purified bovine cathepsin B (EC 3.4.22.1) and porcine elastase (EC 3.4.21.36) were obtained from the Sigma Chemical Co., St. Louis, MO). These enzymes were selected as two major PMN proteases (Verdi and Barbano, 1991). Micellar casein and sodium caseinate were prepared as described above and Cathepsin B and Elastase added at 1.4 units/ml (where 1 unit hydrolyses 1 μmole of Nα-CBZ-lysine-p-nitrophenyl ester per min at pH 5.0 at 25°C) and 0.88 units/ml (where one unit will solubilise 1 mg of elastin in 20 min at pH 8.8 at 37°C) substrate respectively. Cathepsin B digests contained 2.7 mM cysteine as a thiol activator.

6.2.3 Statistical analysis of data

Frequency distributions for SCC and plasmin data were skewed and a log transformation was carried out on these data before analysis. Pearsons correlation coefficients and linear regression were used to examine the relationships between variables. Multiple regression was also used to examine the effects of treatments on milk proteolysis, where proteolysis was correlated simultaneously against $\log_{10}$SCC, $\log_{10}$(plasmin activity) and PMN level, and least significant terms removed in a stepwise manner. The influence of milk treatment on proteolysis levels was examined using GLM analysis (using the Minitab statistical analysis package), where there were 40 sets of values for samples from individual cows and five treatment subclasses (initial milk, raw milk after incubation, raw milk + 6-aminohexanoic acid after incubation, pasteurised milk after incubation and pasteurised milk + 6-aminohexanoic acid after incubation). This model was also used to calculate mean proteolysis levels (in mg BSA/L milk) in each treatment group.

6.3 RESULTS

6.3.1. Milk composition.

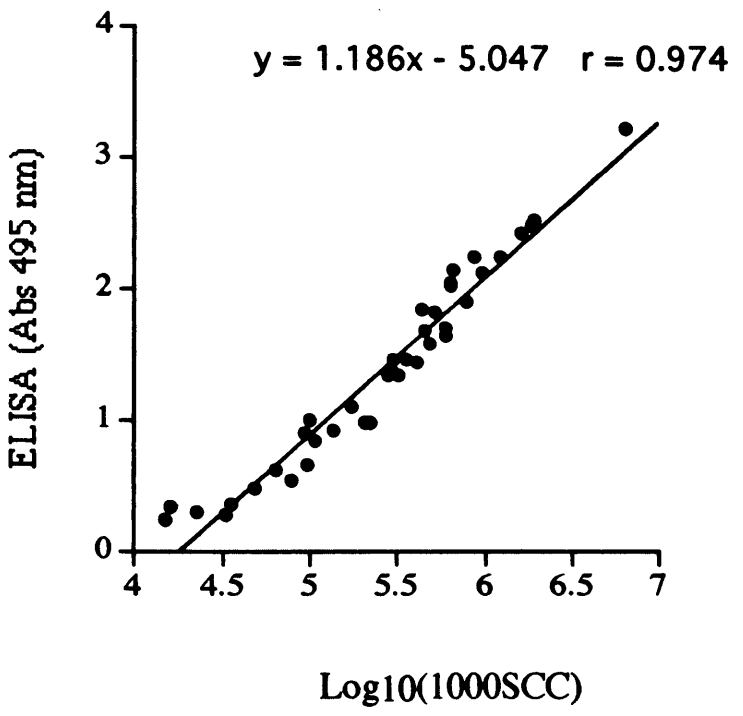
The 40 milk samples were divided into 4 groups on the basis of SCC (I: <100,000 SCC/ml; II: 100,000-400,000 SCC/ml; III: 400,000-800,000 SCC/ml and IV: >800,000 SCC/ml) and mean compositional parameters calculated (Table 6.1). It can be seen that increasing SCC is associated with steady increases in fat and decreases in lactose levels, that protein is relatively unaffected by SCC and that changes in solids-non-fat, total solids and pH are slight and only really apparent at high SCCs. Investigation of correlation coefficients showed that the only significant correlations were between $\log_{10}$SCC and fat (0.32, $P<0.05$) and lactose (-0.63, $P<0.001$).

The correlation between $\log_{10}$(SCC) and PMN level, as measured by the ELISA method of O'Sullivan *et al.* (1992) is shown in Fig. 6.1. Log plotting was used to avoid biasing the curves by the small number of samples of very high SCC. The correlation coefficient estimated from a non-log plot was 0.95, and so this transformation did not alter the behaviour of the data. From this graph it can be seen that there appears to be a tailing off effect at the start of the graph, where increasing $\log_{10}$SCCs up to 4.5 did not appear to influence PMN content of the milk, and thus the cells in the milk must not be PMNs. This log value corresponds to a linear somatic cell count of 31,000 cells/ml.

Table 6.1 *Compositional data for trial milks (mean % ± SE)*

Group	**I**	**II**	**III**	**IV**
SCC	**<100,000/ml**	**100,000-400,000/ml**	**400,000-800,000/ml**	**>800,000/ml**
n	10	12	10	8
Fat	3.54 ± .33	4.04 ± .37	4.22 ± .24	4.49 ± .37
Protein	3.07 ± .06	3.22 ± .07	3.15 ± .08	3.21 ± .08
Lactose	4.89 ± .03	4.78 ± .03	4.66 ± .04	4.54 ± .07
Solids-non-fat	8.56 ± .08	8.60 ± .07	8.41 ± .09	8.35 ± .11
Total solids	12.10 ± .28	12.65 ± .43	12.46 ± .28	12.80 ± .42
pH	6.54 ± .02	6.56 ± .01	6.55 ± .01	6.60 ± .02

Fig 6.1. *Regression plot of log_{10}SCC versus PMN level as measured by ELISA.*



The plasmin and plasminogen levels and the plasminogen/plasmin ratio in the milks, divided on the basis of SCC as before, are given in Table 6.2. To provide further information concerning the interactions of these factors, Pearsons correlation coefficients relating milk plasmin activity, plasminogen level and plasminogen/plasmin ratio were determined. Plasmin was seen to increase with increasing SCC (correlation coefficient between log_{10}SCC and plasmin activity was 0.530, $P>0.001$) but plasminogen level remained relatively unaffected by SCC, with no significant difference between levels in groups I and IV and a slight (non-significant) negative correlation coefficient relating the two factors. The plasminogen/plasmin ratio decreased with increasing SCC (correlation coefficient between log_{10}SCC and ratio was -0.375, $P>0.05$), but appeared to be unaffected by further increasing SCC above 400,000 cells/ml. Plasminogen and plasmin levels in milk were highly correlated ($r = 0.736$, $P<0.001$). There was no correlation between log_{10}SCC and total plasminogen plus plasmin level. This implies that plasminogen activation is increased at elevated SCCs, without increased transport of enzyme or zymogen from blood. Alternatively, it is possible that there is some transport at low SCCs, as evidenced by the higher plasminogen level in group II compared to group I, but that at higher SCCs this transport is overshadowed by activation of the plasminogen to plasmin.

One-way ANOVA showed that only plasmin activity was significantly affected by SCC group ($F = 4.65$, $P<0.01$). Plasmin activity in milk was significantly correlated to PMN level ($r = 0.501$, $P<0.001$), % lactose ($r = -0.532$, $P<0.001$) and

Table 6.2 *Plasmin activities, plasminogen levels and plasminogen/plasmin ratios in trial milks (enzyme levels in AMC units/ml milk, mean ±SE)*

Group	I	II	III	IV
SCC	<100,000/ml	100,000-400,000/ml	400,000-800,000/ml	>800,000/ml
n	10	12	10	8
Plasmin	0.17 ± .02	0.26 ± .03	0.33 ± .04	0.42 ± .09
Plasminogen	0.586 ± .09	0.672 ± .13	0.557 ± .13	0.572 ± .13
Plasminogen/ plasmin ratio	3.84 ± .83	3.40 ± .79	1.79 ± .58	2.23 ± .58

pH (r = 0.439, P<0.01) and plasminogen level was significantly correlated to % protein (r = 0.372, P<0.05).

6.3.2. Quantitative assessment of proteolysis in trial milks

The mean levels of initial TCA-soluble proteolysis products and the levels attained over 24 hours incubation at 37°C for raw and pasteurised milk samples, with or without 6-aminohexanoic acid, were measured as an estimate of milk proteolytic activity. GLM analysis of the variation in TCA-soluble proteolysis product levels in milk as affected by cow-cow variation and treatment (initial and raw and pasteurised samples, with and without 6-aminohexanoic acid) is shown in Table 6.3. It can be

Table 6.3. *GLM analysis of proteolysis levels in 40 milks measured after 5 treatments (initial level as 1 treatment plus incubations of raw and pasteurised milk portions, ± 6-aminohexanoic acid)*

Source	d.f.	Adjusted mean squares	F-value	p
Individual cow	39	8466	13.94	0.000
Treatment	4	59133	97.35	0.000
Error	150	607		

Treatment means (mg BSA/L milk), SE in brackets

Initial	157.1 (3.90)
Raw	249.5 (3.90)
Raw + 6-aminohexanoic acid	158.1 (4.10)
Pasteurised	211.7 (4.03)
Pasteurised + 6-aminohexanoic acid	194.6 (3.96)

seen that all incubations increased proteolysis levels significantly relative to the initial samples, with the exception of the raw samples incubated with 6-aminohexanoic acid. Treatment means differing by ±2SE were considered to be different. The initial proteolysis levels had, as expected, the lowest means, and the treatments increased in the order; raw + 6-AHA, pasteurised + 6-AHA, pasteurised and, with the highest levels, raw.

The levels of TCA soluble proteolysis products before and after treatments, as divided on the basis of SCC group as before, are shown in Table 6.4. Initial proteolysis levels increased with increasing SCC group, as did the levels of proteolysis reached on incubation of raw and pasteurised samples. Estimation of the percentage of total proteolytic activity remaining after pasteurisation gives 74.1%, 52.7%, 59.3% and 62.4% survival of activity for milks from groups I, II, III and IV respectively. In the case of addition of 6-aminohexanoic acid it can be seen that the non-plasmin activity is highest after pasteurisation. It can be estimated that the % of proteolytic activity due to non-plasmin proteases is 12-15% in raw milk and 60-80% in the pasteurised samples (lowest in the higher SCC samples). The decrease in TCA-soluble products in the group II samples incubated raw with 6-AHA is thought to be an anomaly, as the results are not significantly different from the initial results for that group ($P>0.1$).

However, it must be considered that in the milks of increasing SCC, plasmin activity and PMN level were also increasing. Therefore, to estimate the relative contribution of somatic cells, PMNs and plasmin to proteolysis, the Pearsons correlation coefficients relating proteolysis parameters and SCC, PMN levels and plasmin concentrations were determined (Table 6.5). It can be seen that, apart from the initial level, proteolysis parameters were more highly correlated with PMN level than $\log_{10}$SCC or $\log_{10}$ (plasmin activity). These data sets were log transformed prior to deriving correlations due to the skewed nature of the data. Log_{10}SCC and PMN level are more highly correlated with proteolysis levels than is $\log_{10}$(plasmin activity).

Table 6.5. *Correlation coefficients between log_{10}SCC, PMN level and plasmin activity and proteolysis before and after incubation of raw and pasteurised milk samples, with and without aminohexanoic acid. n=40 for all correlations.* ** *$P<0.01$,* *** *$P<0.001$.*

TCA-soluble products	Log_{10}SCC	PMN (Abs 495 nm)	Log_{10}(Plasmin Activity) (AMC units/ml)
Initial	0.697 ***	0.661 ***	0.610 ***
Incubated			
Raw	0.802 ***	0.813 ***	0.634 ***
Raw + 6-AHA	0.645 ***	0.706 ***	0.497 **
Past	0.739 ***	0.778 ***	0.635 ***
Past + 6-AHA	0.703 ***	0.712 ***	0.560 **

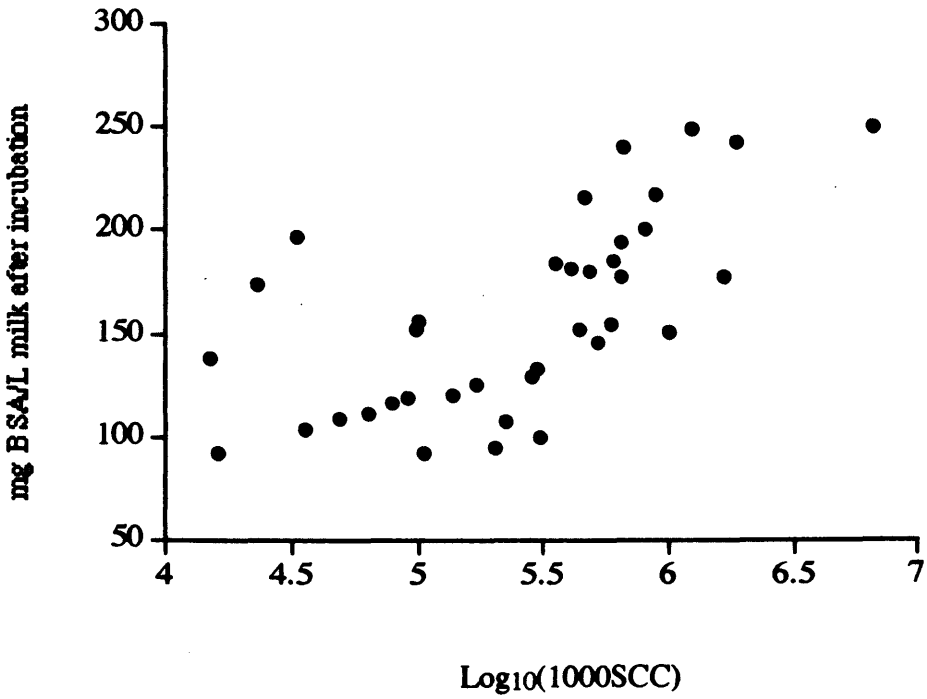
Table 6.4. *Proteolysis in initial and incubated milk samples (mg BSA/ L milk ± SE) Figures in brackets are Δ proteolysis (incubated value - initial value).*

Group	SCC/ml	n	mg BSA initial	mg BSA after incubation at 37°C for 24 hours			
				Raw	*Raw + 6-AHA*	*Past*	*Past + AHA*
I	<100,000	10	123.03 ± 8.78	191.02 ± 12.10 (68.0)	131.32 ± 10.53 (8.3)	169.70 ± 10.32 (46.7)	159.53 ± 10.97 (36.5)
II	100,000 - 400,000	12	150.75 ± 6.48	232.66 ± 8.62 (81.9)	124.3 ± 9.07 (-26.5)	193.95 ± 8.30 (43.2)	179.56 ± 5.07 (28.8)
III	400,000 - 800,000	10	169.27 ± 11.22	273.45 ± 8.57 (104.2)	182.55 ± 9.21 (13.3)	231.06 ± 8.41 (61.8)	212.09 ± 9.73 (42.8)
IV	>800,000	8	194.15 ± 10.91	318.14 ± 13.63 (124.0)	212.3 ± 14.56 (18.15)	271.49 ± 21.44 (77.34)	241.10 ± 15.91 (46.95)

As would be expected, proteolysis levels in the milk samples where the plasmin inhibitor was present are the least correlated with plasmin activity. The fact that there is still a significant correlation is attributable to the correlation between $\log_{10}$SCC and plasmin activity (0.530).

Examination of the plot of final proteolysis levels in the raw + aminohexanoic acid group shows that, barring outriders, there appears to be a trend towards a sudden increase in non-plasmin proteolytic activity above 300,000 cells/ml, which in most cases should be caused by an influx of PMNs due to mastitic infections (Fig. 6.2). Thus, this may constitute further evidence that PMNs are specifically responsible for increased proteolytic activity in raw milk. The shape of this graph may also explain why there was overall no increase in proteolysis in the raw samples containing 6-aminohexanoic acid according to the GLM analysis (Table 6.3).

Fig. 6.2 *Plot of $\log_{10}$SCC versus mg BSA/L raw milk after incubation at 37°C for 24 hours in the presence of 6-aminohexanoic acid.*



The relative contributions of plasmin, PMN levels and SCC to milk proteolysis were further broken down by multiple regression analysis of the factors affecting proteolysis in milk before and after incubation, for each of the treatments examined. For the initial proteolysis level, the following regression equation was used to model the behaviour of the data:

$$\text{mg BSA initial} = \beta_0 + \beta_1(\text{Log}_{10}\text{SCC}) + \beta_2(\text{Log}_{10}\text{Plasmin}) + \beta_3\,(\text{PMN level})$$

where β_0 = constant

β_1 = regression coefficient for $\log_{10}$(SCC)
β_2 = regression coefficient for $\log_{10}$(plasmin activity)
β_3 = regression coefficient for PMN level

Plasmin activities and PMN levels were as measured in AMC units/ml milk and Abs. 495 nm, respectively. Equations for proteolysis levels after incubation included a term to take into account the initial levels of proteolysis in the milks. In all cases, models were simplified by stepwise removal of least significant terms until all terms remaining were significant ($p<0.05$). These models are summarised in Table 6.6.

Initial proteolysis level was affected by a combination of plasmin activity and somatic cell count. Proteolysis levels attained in all milk samples on incubation were significantly correlated to PMN level, while $\log_{10}$SCC did not appear in any of these regression equations. The significant influence of PMN level on non-plasmin proteolysis in raw milk is as predicted from Fig. 6.2. Log_{10}(plasmin) influenced the level of proteolysis after incubation of the raw samples, but was not involved in any other regression equation. The initial level of proteolysis in milk was a significant factor in determining the final level of proteolysis attained in pasteurised milk samples.

Table 6.6. *Multiple regression analysis of the influence of milk $\log_{10}$SCC, $\log_{10}$(plasmin activity) and PMN level on levels of proteolysis in fresh milks and milks after 24 hours incubation at 37°C, raw and pasteurised, with or without the plasmin inhibitor 6-aminohexanoic acid. SD, standard deviation.*

Factor	Coefficient	SD	t-value	p	Multiple R^2
(i) Initial proteolysis level					
Constant	111.49	30.94	3.60	0.001	0.524
Log_{10}(SCC)	32.07	8.44	2.80	0.001	
Log_{10}(plasmin)	53.52	23.03	2.32	0.026	
(ii) Proteolysis level in incubated raw milk					
Constant	217.51	25.28	8.60	0.000	0.484
Log_{10}(plasmin)	63.89	27.38	2.33	0.025	
PMN level	50.85	8.23	6.18	0.000	
(iii) Proteolysis level in incubated raw milk containing 6-aminohexanoic acid					
Constant	97.83	11.68	8.37	0.000	0.484
PMN level	44.68	7.57	5.90	0.000	
(iv) Proteolysis level in incubated pasteurised milk					
Constant	91.95	22.72	4.05	0.000	0..638
Initial proteolysis	0.434	0.189	2.29	0.028	
PMN level	37.72	9.64	4.91	0.000	
(v) Proteolysis level in incubated pasteurised milk, containing 6-aminohexanoic acid					
Constant	65.09	18.16	3.58	0.001	0.666
Initial proteolysis	0.646	0.144	4.48	0.000	
PMN level	20.44	7.398	2.76	0.009	

6.3.3. Electrophoretic examination of proteolysis products in trial milks

Urea-PAGE electrophoretograms of proteolysis products in low, medium and high SCC milks (64,000, 655,000 and 6,566,000 cells/ml respectively) are shown in Fig. 6.3 (3d) and Fig. 6.4 (9d). Similar trends were seen in gels of samples after 24 hours incubation (results not shown). From Fig. 6.3, it can be seen that proteolysis is obviously related to SCC, and is of the order pasteurised>raw>raw + aminohexanoic acid > past + 6-aminohexanoic acid. The 6-AHA can be seen to reduce proteolysis of ß-casein significantly, with no further γ-casein formation relative to the initial samples. In the low SCC sample, there is negligible proteolysis in its presence. The pasteurised low SCC sample shows accelerated proteolysis relative to the raw low SCC sample, due presumably to post-pasteurisation plasminogen activation. The 655,000 SCC/ml sample shows accelerated proteolysis of both α_{s1}- and ß-casein and a number of proteolysis products just ahead of the α_{s1}-casein band, even in the presence of 6-AHA.

In the absence of 6-AHA there are three bands (M1, M2 and M3) forming just above the γ-caseins while in the plasmin-inhibited samples, it appears that only M1 and M2 are forming, and more slowly. The high SCC sample shows increased production of M1 and M2, with 6-AHA having little effect on its appearance. Plasmin action results primarily in appearance of γ-2 casein (ß-casein f106-209) and at high SCCs γ-1

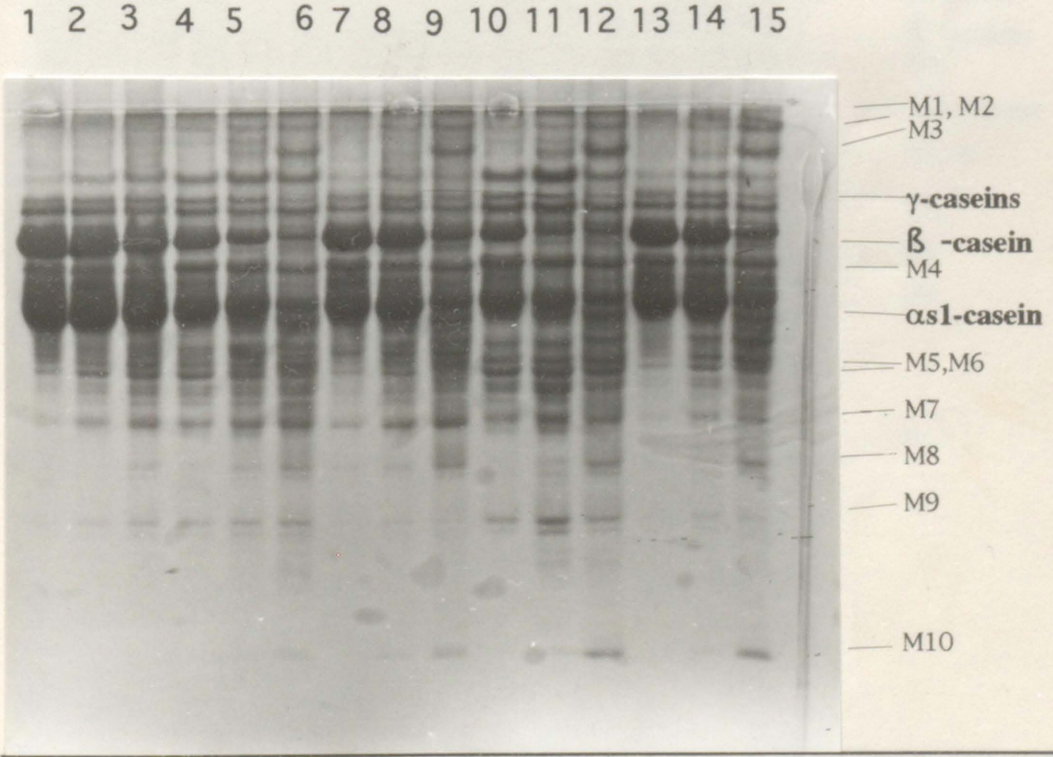


Fig. 6.3. *Urea-PAGE electrophoretogram of proteolysis products produced in milks of 64,000, 655,000 and 6,566,000 cells/ml (A,B and C) on incubation at 37°C for 3d. Lanes 1-3 A, B and C initial. Lanes 4-6, raw after incubation. Lanes 7-9, raw + 6-AHA after incubation. Lanes 10-12, pasteurised after incubation. Lanes 13-15, pasteurised + 6-AHA after incubation.*

casein (ß-casein f29-209) is further broken down, but this may be due to the elevated plasmin levels in this milk. There is a band situated between the α_{s1}- and ß-casein bands (M4) which is either not as strongly formed or further broken down at high SCCs. Below α_{s1}- casein a doublet of bands (M5 and M6) appear to be formed only after pasteurisation. Production of band M7 is increased with increasing SCC and band M8 is formed only at high SCCs, and both of these actions are independent of pasteurisation. Band M9 is only produced significantly in systems where plasmin is active. Band M10 appears only in the high SCC pasteurised sample, irrespective of plasmin action.

From Fig. 6.4 it can be seen that after 9 days incubation at 37°C there is still relatively little proteolysis in the low SCC samples, particularly in the absence of plasmin action. In the raw samples and pasteurised samples with elevated SCCs there is very little residual casein, particularly in raw sample B. The γ-caseins have been further broken down in high SCC samples and band M2 has accumulated, as have

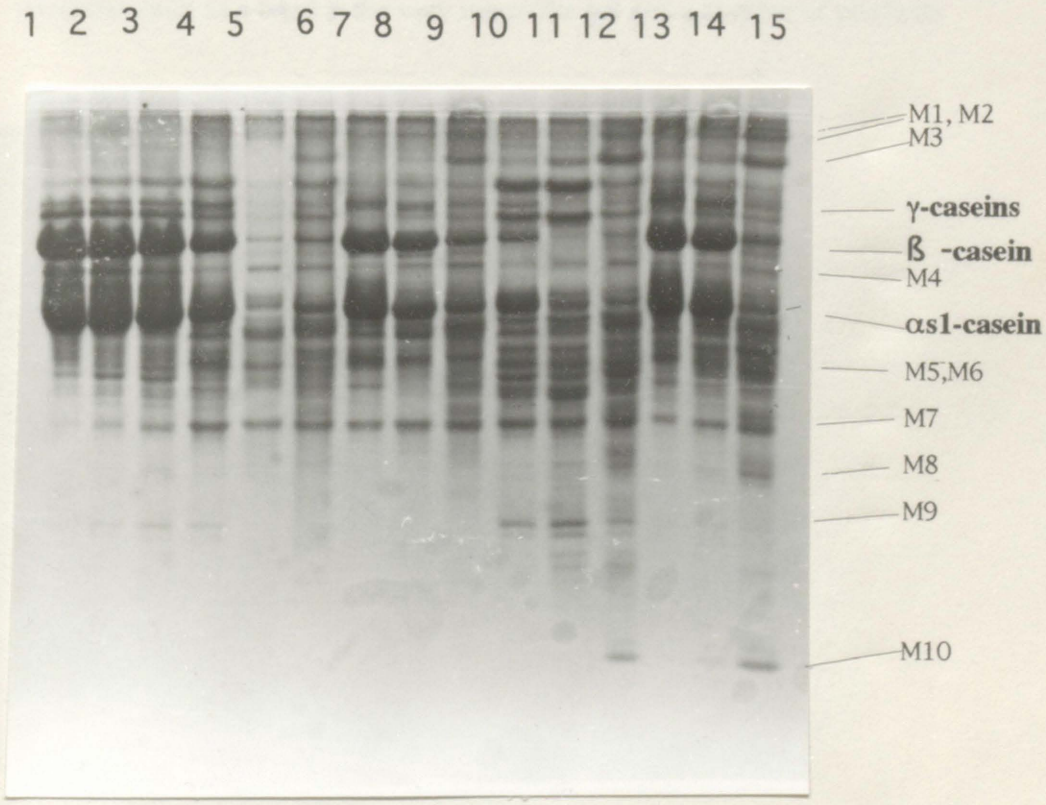


Fig. 6.4. *Urea-PAGE electrophoretogram of proteolysis products produced in milks of 64,000, 655,000 and 6,566,000 cells/ml (A,B and C) on incubation at 37°C for 9d. Lanes 1-3 A, B and C initial. Lanes 4-6, raw after incubation. Lanes 7-9, raw + 6-AHA after incubation. Lanes 10-12, pasteurised after incubation. Lanes 13-15, pasteurised + 6-AHA after incubation.*

bands M7, M9 and M10. Bands M4, M5, M6 and M8 appear to have been degraded. In the samples containing aminohexanoic acid there is a faint band just ahead of the ß-casein, which may be ß-I casein (ß-casein f1-189/192). Again the order of overall proteolysis is raw slightly greater than pasteurised, but there appears to be marginally more breakdown in the pasteurised + AHA than the raw + AHA.

6.3.4. Digestion of micellar casein and sodium caseinate by crude somatic cell preparations.

Urea-PAGE electrophoretograms of micellar casein digested by PMNs isolated from bovine peripheral blood are shown in Fig. 6.5. It can be seen that the activity is higher at pH 5.2 than 6.5 and that α_{s1}-casein is hydrolysed far faster than ß-casein. The principal proteolysis products have electophoretic mobilities similar to ß-I (ß-casein f 1-189/192) and α_{s1}-I casein (α_{s1}-casein f 24-199), although this was not verified by isolation and sequencing of the peptides. There are a number of other faint proteolysis products such as a band at the very top of the gel and a number of products

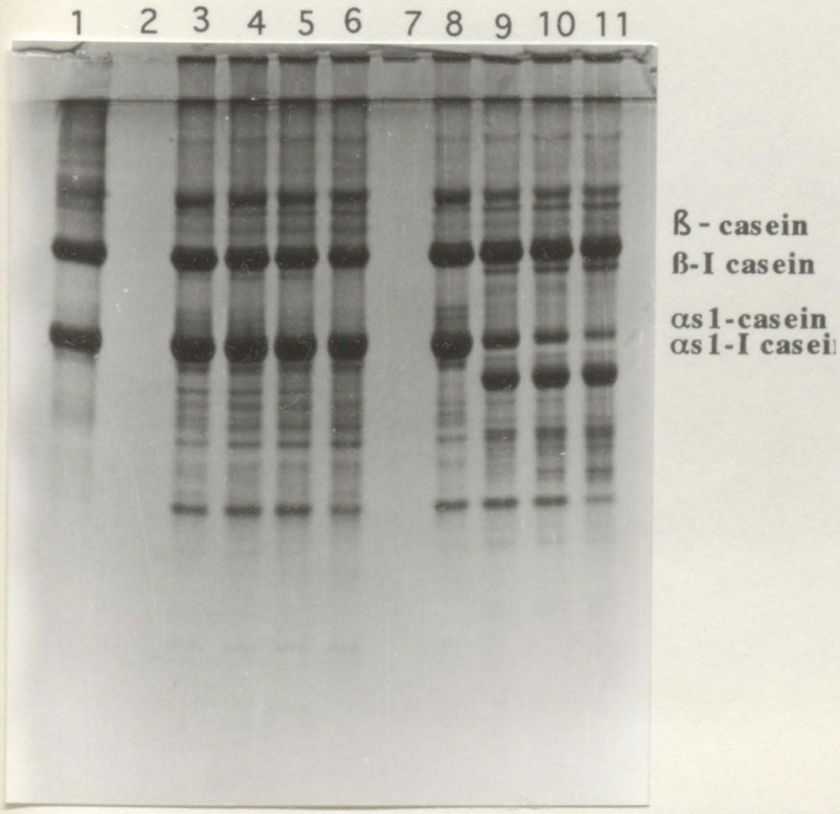


Fig. 6.5. *Urea-PAGE electrophoretogram of sodium caseinate (lane 1) and micellar casein digested by 500,000 PMN cells/ml isolated from bovine blood at 37°C and pH 6.6 for 0, 24, 48 and 72 hours (lanes 3-6) and pH 5.2 for 0, 24, 48, 72 hours (lanes 7-10)*

migrating ahead of the α_{s1}-I casein band. Similar digestion of micellar casein by the non-PMN fraction isolated from the blood showed only very minor proteolysis of α_{s1}-casein under the same conditions (gel not shown).

Digests of sodium caseinate by milk somatic cell isolates are shown in Fig. 6.6. and 6.7 (raw and pasteurised systems respectively). It is immediately apparent that the proteolytic activity of milk leucocytes is far higher than that of blood leucocytes, with faster degradation and a wider spectrum of proteolysis products produced. In both systems cells from mastitic milk are more active than those isolated from good quality milk, and the order of proteolysis is again α_{s1}- casein faster than ß-casein. At pH 5.2, in the presence of 5% NaCl, there is a smaller range of proteolysis products than at pH 6.5 and the primary product appears to be α_{s1}-I casein, which is produced faster by mastitic milk cells (PMNs presumably). Under these conditions ß-casein appears to be hardly attacked at all. At pH 6.5 both cell types produce 2 or 3 very slow moving bands, and there is no evidence of γ-casein formation. The proteolysis patterns at pH

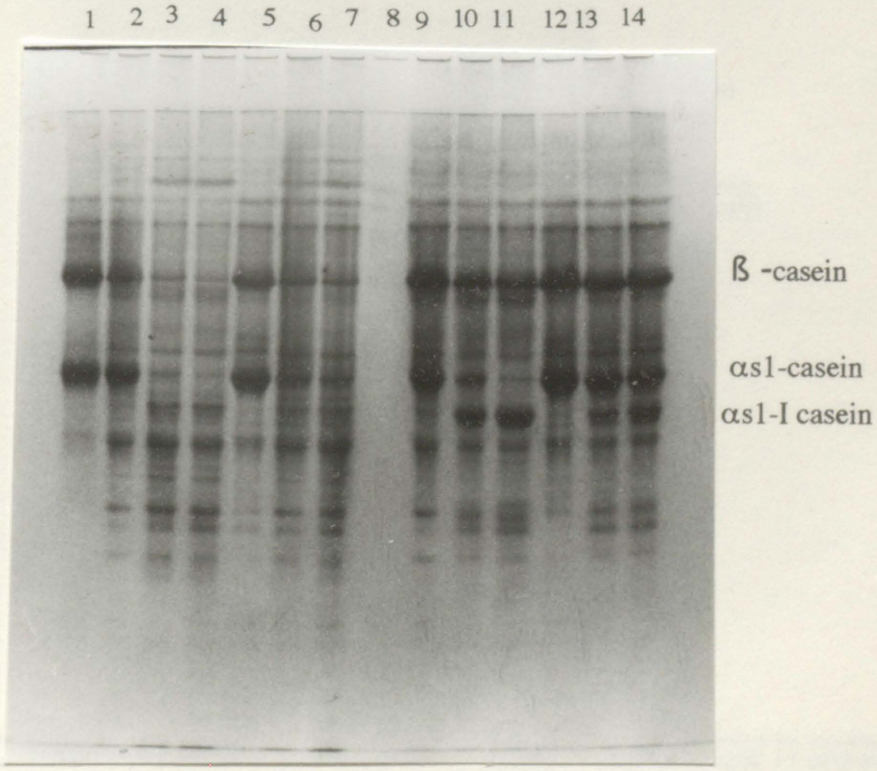


Fig. 6.6. *Urea-PAGE electrophoretogram of sodium caseinate (lane 1) and sodium caseinate digested by 500,000 cells/ml from mastitic (M) and healthy (H) cows milk at 37°C. Lanes 2-4, M, pH 6.5, 0,24,48 hours. Lanes 5-7, H, pH 6.5, 0, 24, 48 hours. Lanes 9-11, M, pH 5.2, 5% NaCl, 0, 24, 48 hours. Lanes 12-14, H, pH 5.2, 5% NaCl, 0, 24, 48 hours.*

6.5 for the two types of cells are quite similar, with there being quantitative rather than quantitative differences between them. At pH 5.2, there are some products migrating just behind α_{s1}- casein in the healthy cell digests which appear to be absent from the mastitic digests.

Post-pasteurisation it is apparent that breakdown of α_{s1}-I casein is considerable in mastitic digests under cheese-like conditions and minimal in the healthy cell digests, which has considerable implications for cheese manufacture (Fig. 6.7). It appears that pasteurisation has only a minor effect on proteolysis of caseins by milk somatic cells.

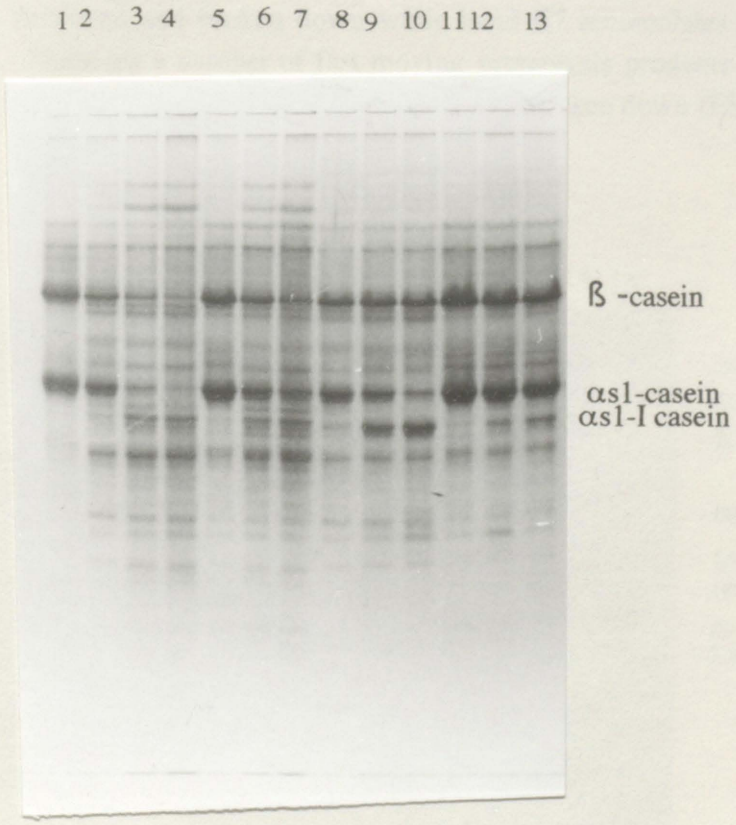


Fig. 6.7. *Urea-PAGE electrophoretogram of sodium caseinate (lane 1) and sodium caseinate digested by 500,000 cells/ml from mastitic (M) and healthy (H) cows milk at 37°C post pasteurisation. Lanes 2-4, M, pH 6.5, 0,24,48 hours. Lanes 5-7, H, pH 6.5, 0, 24, 48 hours. Lanes 8-10, M, pH 5.2, 5% NaCl, 0, 24, 48 hours. Lanes 11-13, H, pH 5.2, 5% NaCl, 0, 24, 48 hours.*

6.3.5. Digestion of micellar casein and sodium caseinate by purified elastase and cathepsin B

Electrophoretograms of micellar casein digested by porcine pancreatic elastase at pH 6.5 and pH 5.2 are shown in Fig. 6.8. It can be seen that elastase hydrolyses ß-casein faster than α_{s1}- casein and α_{s1}-casein degradation is marginally faster at pH 5.2 than 6.5. A number of distinctive slow moving products are formed rapidly (E1, E2, E3 and E4). Of these, bands E1 and E3 are broken down on prolonged incubation while bands E2 and E4 accumulate more slowly. There are three products (E5, E6 and E7) which are formed only transiently at pH 6.5 whereas at pH 5.2, bands E5 and E6 are slowly produced and broken down while band E7 accumulates up to 12 hours incubation. There are a number of fast moving proteolysis products formed, two of which (E9 and E11) accumulate while two are further broken down (E8 and E10).

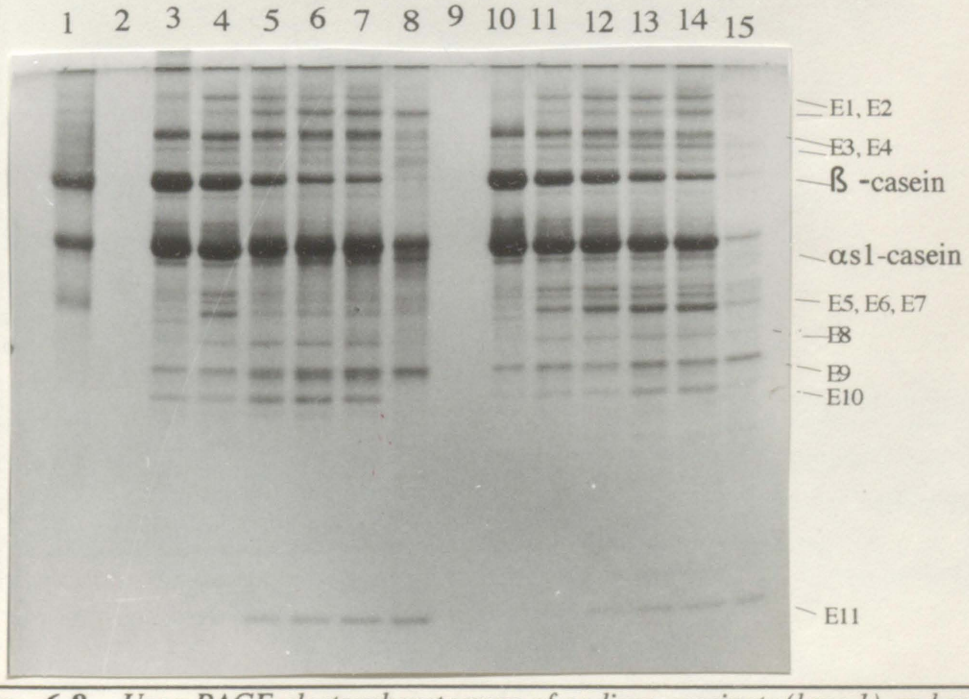


Fig. 6.8. *Urea-PAGE electrophoretogram of sodium caseinate (lane 1) and micellar casein digested with 1.4 units/ml Elastase at 37°C for 0,1, 3, 6, 9 and 24 hours (lanes 3-8) at pH 6.6 and for 0, 1, 3, 6, 9 and 24 hours at pH 5.2 (lanes 10-15)*

A urea-PAGE electrophoretogram of digestion of sodium caseinate by cathepsin B in the presence of cysteine is shown in Fig. 6.9. It can be seen here that proteolysis was marginally faster at pH 6.5 than pH 5.2 (in the presence of 5% NaCl) and that the main proteolysis products are a slow moving band (CB1), a band of electrophoretic mobility similar to that of α_{s1}- I casein (CB2) and two fast moving products (CB3 and CB4), the first of which is produced transiently and the second of which accumulates.

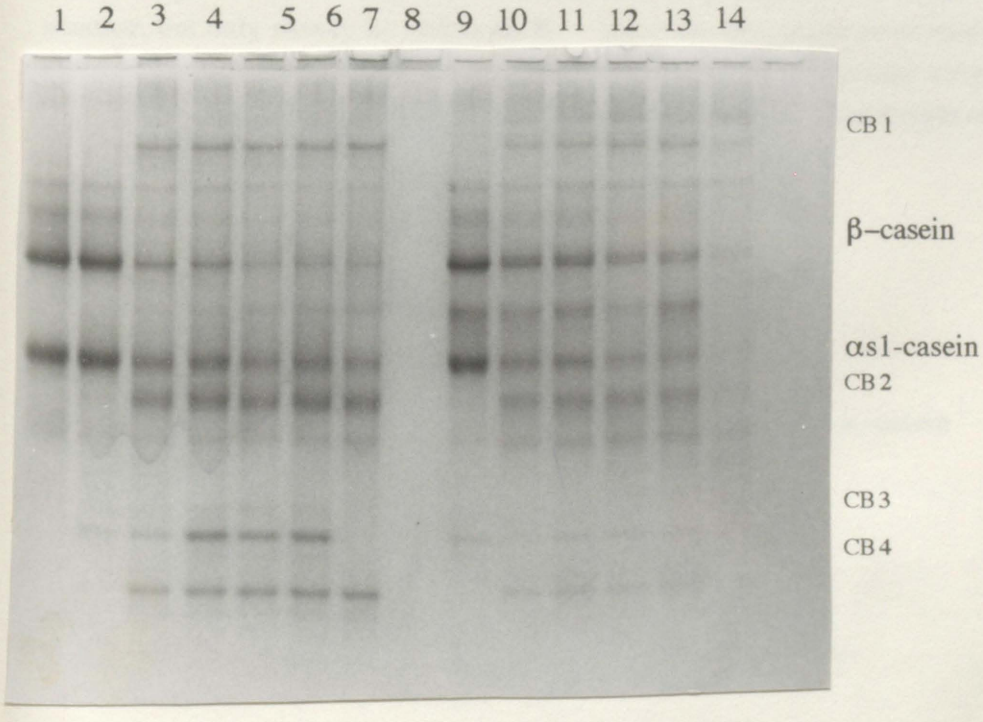


Fig. 6.9. *Urea-PAGE electrophoretogram of sodium caseinate (lane 1) and sodium caseinate digested with 0.88 units/ml Cathepsin B at 37°C in 100 mM phosphate buffer, pH 6.6 for 0,1, 3, 6, 9 and 24 hours (lanes 3-8) at pH 6.6 and in 100 mM phosphate buffer, pH 5.2 with 5% NaCl for 0, 1, 3, 6, 9 and 24 hours (lanes 10-15)*

6.3.6. Digestion of κ-casein by PMN cell preparations and purified Elastase and Cathepsin B

Digestion of κ-casein by proteolytic enzymes is critical in determining the clotting properties of milk and the action of somatic cell enzymes on this protein was examined using SDS-PAGE (Fig. 6.10). SDS-PAGE was used as opposed to urea-PAGE to overcome the anomalous migration behaviour of κ-casein in the latter gels due to its charge. It can be seen that κ-casein is readily degraded by blood PMNs and elastase, but only slowly by cathepsin B. There are two major proteolysis products, one produced by the crude cell preparation of approximate molecular weight 16,000-17,000 and the other, produced by both enzymes, with a molecular weight of 14,000.

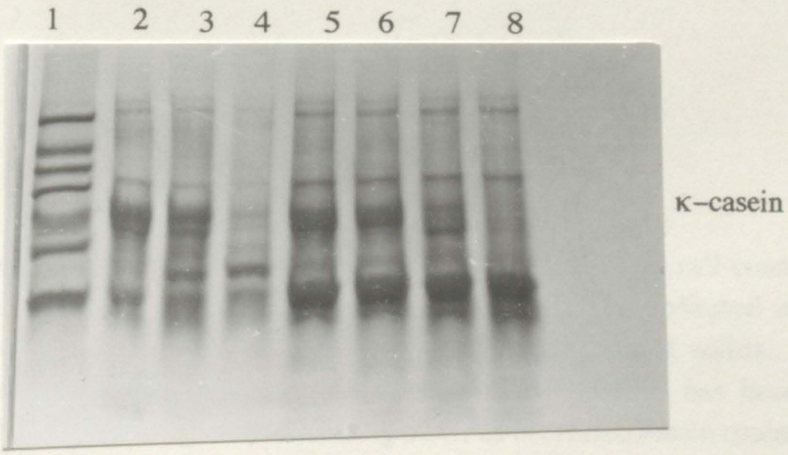


Fig. 6.10. *SDS-PAGE electrophoretogram showing digestion of κ-casein by crude and purified somatic cell proteinases. Lane 1, molecular weight standards, 66,000 (bovine serum albumin), 45,000 (egg albumin), 36,000 (rabbit muscle glyceraldehyde-3-phosphate dehydrogenase), 29,000 (bovine erythrocyte carbonic anhydrase), 24,000 (trypsinogen), 14,,200 (soya bean trypsin inhibitor). Lane 2, initial κ-casein . Lanes 3-4, κ-casein digested by PMN extract from bovine blood (as in Section 6.3.4) at 500,000 cells/ml after 24 and 48 hours at 37°C. Lanes 5-6, κ-casein digested by 0.88 U/ml cathepsin B after 6 and 24 hours at 37°C. Lanes 7-8, κ-casein digested by 1.4 U/ml elastase after 6 and 24 hours at 37°C.*

6.3.7. Comparison of electrophoretic patterns of incubated milk samples with proteolysis profiles arising from digestion of casein by crude and purified somatic cell enzymes.

A final urea-PAGE gel was run to compare proteolysis products visible in gels shown in Figs. 6.3, 6.6, 6.8 and 6.9. (Fig. 6.11). It can be seen that proteolysis products arising from the incubation of milks in section 6.3.3 have similar electrophoretic mobilities to products arising from digestion of sodium caseinate by crude preparations of milk somatic cells. Also, many of these products can be identified as products of the action of elastase, particularly in the very slow and very fast moving regions. Far more peptides in milk are visible in the crude enzyme digest, suggesting that there are a number of other enzymes in the crude extracts than cathepsin B and elastase which are active in high SCC milk.

6.4 DISCUSSION

6.4.1. Milk composition

Bovine mastitis, and concomitant increases in somatic cell count (SCC), are associated with a number of changes in milk composition. Mitchell *et al.* (1986a) found that $\log_{10}$SCC correlated significantly with milk total solids, fat (positive correlations) and lactose (negative correlation). Milk protein has been reported to remain relatively unchanged by increasing SCC, as decreased casein content is balanced by increasing levels of blood-derived whey proteins such as the immunoglobulins and bovine serum albumin (BSA) (Kitchen, 1981). The composition of the milks in the present study, when examined by SCC group, are in agreement with these trends. Milk pH, while quite low, was found to be increased in those milks of SCC > 800,000 cells/ml, which would be expected for mastitic milk samples.

Polymorphonuclear leucocyte (PMN) levels in milk were highly correlated to $\log_{10}$SCC, but showed a slight tail in the graph, with very little change in milk PMN level until a SCC of 31,000 is exceeded. The graph of O'Sullivan *et al.* (1992) does not show this trend but in that case a log plot was not used, which was employed in this study to eliminate the influence of small numbers of samples of very high SCCs on correlations. This 'tailing-off' effect may represent a steady state in very healthy udders, where PMNs are the minor cell type by comparison to lymphocytes and macrophages (Burvenich *et al.*, 1995), but implies that even tiny increases in SCC may be due to an influx of PMNs. This, along with the steady changes of milk composition found even at low SCCs, suggests that there is no clear 'cut-off' SCC beyond which milk composition and quality may begin to change, but that changes are occurring far below the current EU maximum SCC of 400,000 cells/ml.

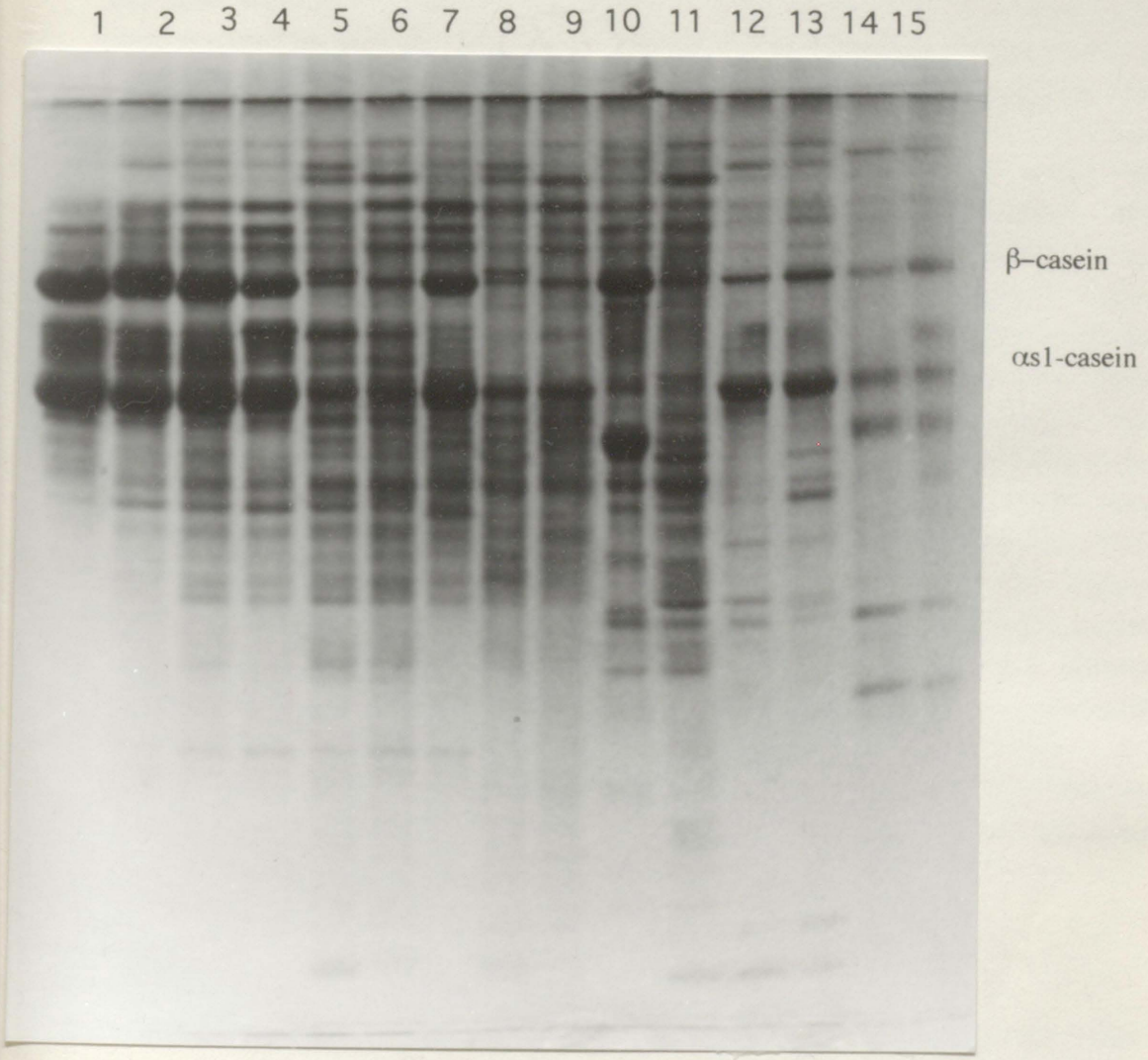


Fig. 6.10. *Comparison of urea-PAGE chromatograms of milk and sodium caseinate digested by crude and purified somatic cell proteinases. Lanes 1-3, milks of 64,000, 655,000 and 6,566,000 cells/ml respectively, initial (fresh milks). Lanes 4-6, same milks after 3d incubation (raw) at 37°C. Lanes 7-9, same milks, after 6d incubation, (raw) at 37°C. Lane 10, sodium caseinate digested by cells recovered from mastitic milk at 500,000 cells/ml for 24 hours at 37°C at pH 5.2, in the presence of 5% NaCl. Lane 11, as lane 10, at pH 6.5. Lane 12, micellar casein incubated with 1.4 U/ml elastase at 37°C for 6 hours, at pH 6.5. Lane 13, as lane 12, at pH 5.2, in the presence of 5% NaCl. Lane 14, sodium caseinate incubated with 0.88 U/ml cathepsin B at 37°C for 6 hours, at pH 6.5. Lane 15, as lane 14, at pH 5.2, in the presence of 5% NaCl.*

The plasmin and plasminogen levels measured in the milk samples were higher than those reported for healthy milk samples by Richardson (1983) but were in the range reported by Benslimane *et al.* (1990) in a larger study of plasmin levels in Montbeliard cows' milk, which they found to have only a slightly higher plasmin level than the Friesian cows in this study. The rise in plasmin level with SCC found here was in agreement with that found by Politis *et al.* (1989b), who found an 2.3-fold increase in plasmin activity on increasing SCC from 100,000 to 1,300,000/ml. The increase in plasmin level between groups I (<100,000/ml) and IV (>800,000/ml) in this study was 2.5 fold. The relative stability of plasminogen levels over the SCC range, with only a slight increase between groups I and II, however, is in contrast with the large increase in plasminogen on increasing SCC found by Politis *et al.* (1989a). These authors found a similar decrease in plasminogen/plasmin ratios as was seen in this study, from 4.7 at 250,000 SCC/ml to 4.0 at 1,000,000/ml. This was attributed to the increased plasminogen activator (PA) activity of somatic cells from mastitic milk (Zachos *et al.*, 1992). The results of the present study suggest that, since $\log_{10}$SCC was not correlated to plasmin plus plasminogen levels and the former was slightly negatively correlated with plasminogen level, there was little or no increased transport of the enzyme or its zymogen from the blood to the milk, and the increased plasmin activity is due to increased PA activity associated with increased SCC. Elevated milk pH may also lead to increased activity of plasminogen activators in milk (Politis *et al.*, 1989b). Thus, overall, it appears that in high SCC milk, the secretion of PAs by somatic cells is responsible for most of the increased plasmin activity, in agreement with the conclusions of Grufferty and Fox (1988) in this regard.

6.4.2 Milk proteolysis

deRham and Andrews (1982b) found that proteolytic activity in mastitic skim-milk was elevated compared to normal milk from healthy cows, its level being related to SCC but not precisely correlated with it. Senyk *et al.* (1985) studied milks of a range of SCCs and found correlation coefficients of 0.60 between SCC and initial tyrosine value and 0.79 between SCC and tyrosine value after incubation at 37°C for 24 hours ($P<0.001$ for both correlations). Similar influences of SCC on milk proteolysis were reported by Murphy *et al.* (1989) and Verdi *et al.* (1987). In the present study significant correlations were found between $\log_{10}$SCC and initial milk proteolysis level, and between $\log_{10}$SCC and proteolysis on incubation at 37°C for 24 hours, for both raw and pasteurised milk samples.

Kang and Frank (1988) showed that plasmin activity was responsible for much of the observed proteolytic activity in high quality raw milk (SCC less than 500,000/ml) during storage. Barry and Donnelly (1981) argued that most of the proteolytic activity in mastitic milk was due to elevated plasmin levels in this milk, but studies of milks of higher SCCs, such as the studies mentioned above and that of Andrews (1983b) have consistently pointed to a role for somatic cell-associated proteinases. Thus, there is a need to elucidate the relative contributions of the two

enzyme sources at various SCC levels. Verdi and Barbano (1988) used 6-aminohexanoic acid, at the levels used here (120 mM) to inhibit plasmin and showed that it did not inhibit somatic cell proteases. This molecule causes a conformational change in plasminogen which prevents its activation to plasmin and interacts with the active site of plasmin (Christman *et al.*, 1979). In this study, use of this inhibitor reduced the proteolytic activity considerably in both raw and pasteurised milks, but there was still significant activity. This was confirmed as non-plasmin in origin by urea-PAGE, which showed no increase in γ-caseins in these samples, which would be characteristic of plasmin activity. The % non-plasmin activity was 12-15% in raw milk samples, increasing with SCC, and 78, 67 and 69% in pasteurised samples from groups I, II and III. The activity dropped to 60% in group IV, presumably due to elevated plasmin activation in these samples, which were most probably mastitic. The raw milk estimates are lower than those of Saeman *et al.* (1988) who found that 23% of total proteolytic activity in milks of less than 2,000,000 cells/ml was of nonplasmin origin, ranging from 17% in healthy quarters to 46% in mastitic quarters during infection. However, these authors studied milks of far higher SCCs than those studied here.

Multiple regression analysis showed that the level of PMNs appears to greatly influence proteolysis in milks, to a greater extent than plasmin activity or SCC. In particular, non-plasmin proteolysis in raw milk can be described by an equation dependant on PMN level alone. The fact that plasmin activity is not present in the models for pasteurised milk samples is as predicted by the high level of non-plasmin activity in these milks, but remains in contrast with published work on plasminogen activation in pasteurised milks. The relationship between increasing PMN level and SCC and non-plasmin proteolysis is clear from the milk sample electrophoretograms, where raw and pasteurised sample proteolysis is increased at both normal and elevated SCCs.

deRham and Andrews (1982b) stated that PAGE patterns of high SCC milk reflected three types of change, *i.e.* those due to the influence of the disease (such as increased membrane permeability leading to increased levels of blood constituents in milk), those resulting from proteolysis in the udder itself before milking and those resulting from proteolysis during storage or incubation. From the electrophoretograms shown here it can be seen that the first two types of changes (seen in the initial milk samples) are clearly affected by SCC with a number of minor proteolysis products present in the fresh high SCC milk which were not present in the good quality milk. The decrease in ß-casein in samples of increasing SCC without AHA (plasmin acting) on incubation was as reported by Politis *et al.* (1989b) and Kroeker *et al.* (1985), and is most likely due to the increased plasmin activity in these milks. However, ß-casein is also broken down in the presence of the plasmin inhibitor. Politis *et al.* (1989b) found a slight negative correlation between SCC and levels of α_{s1}- casein in milk, while in this trial this association appeared quite strong. deRham and Andrews (1982b) found that in mastitic milks α_{s1}- and ß-casein were degraded at about the same overall rate, which is in agreement with the electrophoretic patterns of protein breakdown in very high

SCC samples, except in raw milk where ß-casein was hydrolysed marginally more after 3 days incubation. This was also reported by Andrews (1983), who showed that breakdown of both proteins was increased when freezing and thawing was used to rupture cell membranes, and decreased when cells were centrifugally removed, showing that somatic cells are a major source of proteolytic activity in high SCC milks. In contrast, Verdi *et al.* (1987) found that while α_{s1}- casein was substantially degraded before milking in milk from high SCC herds compared to low SCC milk, subsequent incubation did not lead to any further breakdown, but the low and high SCC herds in this study only differed by 138,000 cells/ml over a year, and thus there may not have been a large influence of mastitis.

Verdi and Barbano (1988) found that in two cows of SCCs 11,000,000 and 7,000,000 cells/ml there were 67% and 32% respectively of somatic cell proteolytic activity remaining after pasteurisation. In the present study, while these levels were typical of total milk proteolytic activity surviving after pasteurisation, there was an increase in non-plasmin activity after pasteurisation relative to raw milk, and PMN level was shown to predict 80% of proteolysis of pasteurised milks. deRham and Andrews (1982a), Noomen (1975) and Richardson (1983a) all found that milk plasmin activity increased on storage after pasteurisation, which was thought to be due to inactivation of an inhibitor of a plasminogen activator. This was seen in this study where γ-caseins were produced more in pasteurised than raw samples and were even further degraded on long incubation of high SCC milk. However, the multiple regression analysis implies that this increased plasmin activity is overshadowed by the action of PMN proteases, which must be increased to a greater extent. In the case of plasmin-inhibited samples, electrophoretic examination of milk samples in the present study showed considerable proteolysis with elevated SCC, but very little difference between raw and pasteurised samples. The fact that there was approximately three fold higher proteolytic activity in pasteurised milks with 6-aminohexanoic acid than raw milks with the same inhibitor added but that there was little difference in PAGE band patterns between these two sets of samples may be explained by the presence of serum-phase exo- or endo-peptidases hypothesised by deRham and Andrews (1982b). The activity of these enzymes could be elevated in milk without significant changes in gel patterns. These may include the leucine-aminopeptidase with a pH optimum of 7.0 whose presence in milk was suggested by Reimerdes *et al.* (1975). However, this implies that these enzymes are very heat stable, and may even be more active in pasteurised milk, possibly due to the inactivation of an inhibitor of their activity. This area requires further investigation.

Janzen (1972) and Rogers and Mitchell (1989) found that SCC was negatively correlated with flavour score in pasteurised milk, and that flavour scores decreased on storage of high SCC milks and Harwalker *et al.* (1993) showed that proteolysis of pasteurised milk could lead to astringent off-flavours. Senyk *et al.* (1985) stated that proteases associated with elevated SCCs will damage raw milk quality upon storage, pasteurised fluid milk over shelf-life, and milk during cheese manufacture and recommended an SCC limit for milk supplies of under 200,000/ml. Barbano *et al.*

(1991) found that an increase in SCC above 100,000 cells/ml would have a negative impact on cheese yield efficiency. Thus, the high correlations between $\log_{10}$SCC and total and non-plasmin proteolysis in milks in this study show that the impact of even quite low SCCs on liquid milk quality must not be underestimated and require further examination.

6.4.3. Casein digestion by somatic cell proteinases.

Grieve and Kitchen (1985) stated that the caseinolytic activity of PMN and macrophage-lymphocyte fractions extracted from bovine blood and added to casein substrate at 1,000,000 cells/ml was less than the levels of milk proteinase activity found in milk from healthy cows. However, Verdi and Barbano (1991) found that blood leucocytes had a lower proteolytic activity than milk somatic cells, as was found here. However, they suggested that this difference may be due to a higher proportion of macrophages in milk isolated from mastitic cows than in the blood which they isolated from healthy cows. This idea of increased levels of macrophages, as opposed to PMNs, in mastitic milk, was derived from studies of human inflammatory reactions. In the bovine, however, it is known that in mastitic infections PMNs are the predominant cell type, for at least 58 hours after infusion of endotoxin in model systems (Saad and Ostensson, 1990). Zecconi *et al.* (1995) found that at least 60% of cells in mastitic cows milks were PMNs, and that the proportion of macrophages was actually decreased relative to uninfected controls. Thus the macrophage-dominated proteolysis proposed by Verdi and Barbano (1992) is unlikely, which leads to the opposite conclusion, that the increased proteolysis in milk is actually due to PMNs, which are present in elevated numbers in mastitic milk as opposed to healthy circulating blood. This is supported by the cell ratios of 12% PMNs, 60% macrophages and 28% lymphocytes reported by Burvenich *et al.* (1995) for healthy milk. Thus, the elevated proteolytic activity found in cell preparations recovered from mastitic as compared to healthy cows milk is probably due to the action of PMN proteases.

The overall faster degradation of α_{s1}- casein than ß-casein by both milk and blood cells is in agreement with the findings of Grieve and Kitchen (1985). It was found that both milk and blood cell proteolytic activity on ß-casein were higher at pH 6.6 than 5.2, which is agreement with the findings of Verdi and Barbano (1991). However, the accumulation of α_{s1}- I casein was considerably higher at pH 5.2, and was considerably faster for the cells from the mastitic milk, which had a higher proportion of PMNs. It is not known whether this is due to an alternative pathway at pH 6.5 which does not produce large quantities of this polypeptide, or that this polypeptide is broken down as it is produced and does not accumulate. The wider diversity of proteolysis products at pH 6.5 shows that either of these possibilities may be the case. Similar incubation experiments by Andrews (1983) showed that breakdown of caseins by proteinases from high SCC milk was due to a number of proteinases, with different pH and temperature optima and reaction to a range of proteinase inhibitors, which makes interpretation of crude proteolysis patterns difficult.

It has long been recognised that there is an acid protease in bovine milk (Kaminagowa *et al.*, 1971; Kaminagowa and Yamauchi, 1972) which hydrolysed all caseins at a pH optimum of 4.0, with a specificity somewhat similar to that of chymosin (Kaminagowa *et al.*, 1980). This gradually became accepted as being identical to cathepsin D, and inactive procathepsin D has been isolated from milk (Larsen *et al.*, 1993). This precursor could not autolytically be activated to cathepsin D, but could form at pseudocathepsin D at acidic pHs, which is most likely the enzyme previously isolated and shown to hydrolyse caseins. Cathepsin D is an aspartic proteinase associated with the lysosomes of somatic cells, and is more associated with macrophages than PMN cells (Cohn, 1975). Proteolysis of α_{s1}- casein by cathepsin D has been shown to produce α_{s1}- I casein as an intermediate, which does not accumulate (McSweeney *et al.*, 1995). In that study, digestion of micellar casein by this enzyme at pH 5.5 was shown to produce a number of bands closely stacked around the α_{s1}- I casein region, which appear to be present in the pH 6.5 digests of sodium caseinate with added somatic cells, both raw and pasteurised (Figs. 6.6 and 6.7). These were most likely ß-casein degradation products by comparison with the electrophoretograms of McSweeney *et al.* (1995), and thus are not produced at pH 5.2. They may also been have produced by elastase.

Reimerdes (1981; 1983) has consistently hypothesised the presence of a thrombin-like enzyme in milk with a high specificity for arginyl bonds and a pH optimum of 7.0. Thrombin is a blood serum serine proteinase with a pH optimum of 9.0 and a molecular weight of 40,000, which would be expected to be in milk by the same reasoning as explains the presence of the other serum serine proteinase, plasmin. The low molecular weight substrates Reimerdes used to assay for this activity were benzoyl-arginyl-p-nitroanilides. Assays for the thiol (cysteine) proteinase cathepsin B frequently use these substrates (Barrett, 1979a). Cathepsin B is a somatic cell protease associated with PMNs (Verdi and Barbano, 1991) which has a pH optimum of 6.0 (closer to Reimerdes enzyme than that of thrombin), a high specificity for arginyl and lysyl residues and a molecular weight of 27,000-52,000 (Boyer, 1971; Barrett, 1979). It may be possible that the thrombin-like enzyme isolated by Remierdes (1981) may in fact be cathepsin B, or a mixture of thrombin and cathepsin B, as the author comments that contamination of his preparation made further purification impossible. It is unlikely that the preparation contained cathepsin D as this enzyme would not have an affinity for the substrate used and furthermore Kaminagowa *et al.* (1972b) reported that in their first purification step the acid protease activity was unadsorbed on the DEAE-cellulose column while Reimerdes (1981) had to elute his enzyme from a similar column with a NaCl gradient. However, insufficient experimental data with regard to inhibition patterns or any other details about the activity of the thrombin-like enzyme make precise conclusions impossible. Thrombin is a highly selective proteinase, with practically an exclusive action on fibrinogen, and thus would probably not be of major importance in casein proteolysis, even if present.

Suzuki and Katoh (1990) isolated at least two types of cysteine protease from mastitic milk and showed that their activity was well correlated with SCC and localised

within milk somatic cells. These enzymes were definitely not thrombin as the mercaptoethanol used in the assay buffer would have inactivated this enzyme by reduction of its disulphide bonds. The protease activities had pH optima of 6.0 and molecular weights estimated as 45,000 and 150,000 daltons, and were inhibited by monoiodoacetic acid and PMSF. Cathepsin B2, which is a lysosomal thiol carboxypeptidase (Barrett, 1979a), has a molecular weight of 50,000 and is possibly the smaller enzyme, but the identity of the larger enzyme is unknown. Thus it is possible that cathepsin B is a second milk acid proteinase derived from somatic cells, and PMNs specifically, which may have been previously mistakenly identified as thrombin. Barrett (1979) refers to PMN thiol proteinases which release from bovine IgM a factor chemotactic for lymphocytes, which are recruited in the later stages of a mastitic infection, thus suggesting a valid physiological reason for the presence of cathepsin B in milk.

Cathepsin B was seen in this study to produce a number of defined polypeptides from sodium caseinate at pH 6.5 and 5.2 (with 5% NaCl) including a peptide with an electrophoretic mobility similar to that of α_{s1}- I casein, and possibly ß-I casein at the lower pH, as seen to be produced by blood cell proteinase action in Fig. 6.5. ß-casein was attacked faster than α_{s1}- casein at pH 6.5, but this order was reversed at pH 5.2.

Elastase is a serine proteinase with a molecular weight in the region 22-36,000, a pH optimum of 8.0-9.0 and a very broad specificity against protein substrates, with a preferred specificity for bonds involving uncharged, non-aromatic amino acids (e.g. Ala, Val, Leu, Ile, Gly, Ser) (Starkey, 1979; Bond, 1989). Plasmin, by comparison, has a high specificity for bonds involving lysine and arginine residues. Barry and Donnelly (1981) stated that elastase would not have the required specificity to contribute significantly to proteolysis patterns of caseins seen in milk from clinically infected quarters, but this was in regard to milks of SCCs less than 1,500,000/ml, which were very much dominated by plasmin activity, which they therefore felt was sufficient to account for the extra proteolysis seen. Bovine caseins contain a high proportion of the bonds hydrolysed by elastase, and so its role, as the major PMN alkaline proteinase, in proteolysis of caseins was examined.

It was found, as expected, that elastase had a broad specificity on the caseins. ß-casein was attacked marginally faster than α_{s1}- casein at both pH 6.5 and 5.2 (in the presence of 5% NaCl). There were 5 peptides with electrophoretic mobilities slower than that of ß-casein and a triplet of bands migrating in approximately the expected position of α_{s1}- I casein, which did not appear to be produced in significant quantities. The action of elastase in milk is probably controlled by inhibitors such as α1-antitrypsin, which does not inhibit plasmin (Travis and Salvesen, 1982; Weber and Neilsen, 1991).

The comparison of electrophoretograms (Fig. 6.10) shows that many of the proteolysis products seen in high SCC milks probably arise from the specific action of somatic cell proteinases. Three bands, migrating just below the position of α_{s1}- I casein seem to appear in both elastase digests and milk incubations, and a number of the

elastase derived products of faster mobility may be similar to those produced by milk leucocytes on incubation with sodium caseinate. The furthest moving peptide, which accumulates, appears to be the peptide of very fast mobility in very high SCC milk and in the proteolytic degradation patterns of micellar casein by blood PMNs at pH 6.5 (Fig. 6.5). The slow-moving proteolysis products in incubated milk samples, as suggested earlier, appear to be products of elastase action, and thus the role of this enzyme in milk proteolysis requires further evaluation. It is difficult to see any of the main cathepsin B products in milk, but the role of this enzyme in degrading α_{s1}- casein to α_{s1}- I casein remains clear, and thus the role of this enzyme may be principally during cheese ripening.

A major question arising from these digests is why ß-casein in micellar casein and sodium caseinate solutions at pH 5.2 is hardly attacked by the somatic cell preparations from either blood or milk, despite the fact that cathepsin B, cathepsin D and elastase can all hydrolyse this protein under these conditions. This is an area which requires further investigation.

Politis *et al.* (1989b) found that plasmin activity was not significantly correlated with κ-casein in milk, which was due to the fact that plasmin did not attack this protein (Eigel *et al.*, 1977). However, Barry and Donnelly (1981) and Anderson and Andrews (1977) associated para-κ-casein formation with somatic cell proteinases in mastitic milk, and this has been supported by the findings presented here, and by Kaminogawa *et al.* (1980) and McSweeney *et al.* (1995) that somatic cell proteinases can cleave κ-casein. The role of cathepsin B and elastase in milk clotting pathways has yet to be elucidated.

With regard to the possible role of these proteinases in cheese ripening, Noomen (1978) found that α_{s1}- casein breakdown to α_{s1}- I casein was increased at low pHs in aseptic starter and rennet free cheeses made from good quality milk, but there appeared to be a maximum production of α_{s1}- I casein at pH 5.4, which is inconsistent with the only acid proteinase being cathepsin D with a pH optimum of 4.0 (Kaminagowa and Yamauchi, 1972) but would be explained by the presence of a protease such as cathepsin B with a high specificity for α_{s1}- casein at this pH. Breakdown of α_{s1}- casein to α_{s1}- I casein is an important step in softening of cheese (Farkye and Landkammer, 1992).

The proteolytic activity versus pH curve of Andrews (1983) for high SCC milk is likewise consistent with cathepsin B being the predominant acid proteinase in such milk, as there is an activity maximum at pH 6.0. The alkaline activity has a broad plateau of activity to beyond pH 9.0 and this may be a mix of alkaline cathepsins, elastase and, of course, plasmin. The sharp tail off of the graph below pH 6.0 leads to the question of where the cathepsin D activity is (optimum 4.0), but this is explicable if cathepsin D is assumed to be primarily a macrophage proteinase. In this case, on mastitic infection, the macrophages are swamped by PMNs, carrying, among other enzymes, cathepsin B which becomes the dominant proteinase active at cheese pHs. This explains why, except for the proteinases identified by Reimerdes (1981; 1983), the only other proteinase to be isolated from normal milk was cathepsin D and why the

first attempt to isolate a nonplasmin enzyme from mastitic milk yielded a cathepsin B-like thiol proteinase.

Chapter 7

Influence of plane of nutrition on somatic cells, the plasmin system and proteolysis in milk

SUMMARY

Milk plasmin, plasminogen, somatic cell counts (SCC), polymorphonuclear leucocytes (PMNs) and proteolysis products in milks from three groups of cows, on restricted (A), standard (B) and supplemented (C) diets were measured over the course of a lactation. Herds A and C both had elevated SCCs as compared to group B, but group A showed a greater increase in late lactation SCC than control or supplemented diets. PMN levels were elevated in the group A milk and this was linked to increased conversion of plasminogen to plasmin in the milk. Control of the defence mechanisms of the udder, as manifest as total and differential somatic cell counts and PMN activity, appeared to be different in herds A and C. Total proteolytic activity was greatest in milk A and increased steadily over the lactation, and qualitative examination of proteolysis products showed that several unidentified non-plasmin products were present. Elevated degradation of both ß- and α_{s1}-casein was seen in milks from group A. Diet appears to be a major factor influencing the proteolytic system in milk, and this may be linked to processing and product quality problems encountered when using late lactation milk.

7.1 INTRODUCTION

One of the principal difficulties facing Irish dairy product manufacturers is maintaining a consistently high level of product quality throughout the year. The main cause of variation in product quality is the large seasonal and lactational variation in milk composition and functionality due to the predominantly spring-calving nature of the Irish dairy herd. In particular, late lactation milk is associated with changes in protein composition, such as increased γ-caseins and decreased ß- and α_{s1}-casein levels (Donnelly and Barry, 1983) and higher levels of whey protein and non-protein nitrogen (Phelan *et al.*, 1982). Decreased casein numbers are linked to increased levels of plasmin in the milk at this time (Barry and Donnelly, 1980; Politis *et al.*, 1989a,b; Benslimane *et al.*, 1990). Late lactation milk also has increased fat and decreased lactose levels (Dawson and Rook, 1974; Lucey and Fox, 1992) and altered mineral composition (Keogh *et al.*, 1982). Phelan *et al.* (1982) found that milk fat, protein, non-protein nitrogen, urea, and lactose levels were most seasonally variable in creamery milk and spring calving herd supplies, and least variable in autumn calving herd milks. Free fatty acid levels were found to be highest in winter creamery milk and late lactation herd milks.

These changes result in a marked decrease in the quality of Cheddar cheese made from Irish winter milk. Functional defects include high cheese moisture (O'Keeffe *et al.*, 1979), increased milk rennet clotting time (R.C.T.) and decreased curd syneresis (O'Keeffe *et al.*, 1982). High moisture in cheese and poor milk clotting properties have been linked to high milk pH, plasmin action, increased presence of monovalent cations, reduced casein proportion and increased soluble casein content (O'Keeffe *et al.*, 1982; Okigbo *et al.*, 1985b).

Strategies for improving the cheesemaking properties of late lactation milk, without particular reference to cheese quality, include selection of milks with high natural pH, addition of sodium caseinate, high milk heat treatment, calcium chloride addition and blending with mid-lactation milk (O'Keeffe *et al.*, 1982; Lucey and Fox, 1992). However, Lucey and Fox (1992) found that late lactation milk clotting properties were far superior in milk from cows on a higher plane of nutrition at this time, which were dried off on a yield basis and had lactose levels consistently above 4%. They concluded that not all milk was unsuitable for cheesemaking, as herds on a high nutritional plane, with improved milking practices, can produce milk which is suitable for processing. Low-grade diets of cows were also linked to poor cheesemaking characteristics by O'Keeffe (1984). Similarly, Kefford *et al.*, (1992) reported that diet quality, rather than stage of lactation, was the main factor influencing the functional capacity of milk for Cheddar manufacture. They found that low-nutrition herd milk yielded higher moisture cheese, decreased yield, altered milk protein interactions and lower milk solids conversion to cheese than milk from cows on a high-quality diet. Late lactation spring-calving cows are generally grazing during a time of declining grass growth and quality, and colder, wetter weather, and it was thought that these factors led to nutritional deficiencies and poor milk quality. By contrast, late

lactation autumn calving cows are fed on high quality spring grass, and were similarly on a higher plane of nutrition during the dried-off period, which may lead to the increased functionality of this milk and decreased seasonal variability in composition.

Emery (1978) reported that although the influence of diet composition on milk protein is small compared to environmental and genetic influences, changes in nutritional strategy will provide quicker response. The relationships between feed constituents and milk composition are complex, with the greatest observable changes being brought about in milk fat concentration (Sutton, 1989). Milk protein has historically not been as closely monitored as milk fat, because firstly, pricing systems tended to be based on milk fat and secondly, the magnitude of changes in milk protein were less than those observed for milk fat, but it has been generally found that increasing levels of concentrates in the feed, and hence energy intake, increased milk protein levels (dePeters and Cant, 1992).

As well as increased levels of plasmin, late lactation milks are associated with high somatic cell counts (SCCs) (Blackburn, 1966; Emanuelson *et al.*, 1988; Barbano *et al.*, 1991), which are also related to decreased milk functionality for cheesemaking (Poilitis and Ng-Kwai-Hang, 1988a,b,c; Grandison and Ford, 1986; Munro *et al.*, 1984). The results of the trials described in Chapter 5 of this thesis suggest that plane of nutrition influences SCC and that the SCC is highly correlated to milk composition and initial proteolytic damage to milk protein. This, and other studies, have shown that seasonality and stage of lactation both influence plasmin level in milk, which has been linked to decreased casein content and cheesemaking problems in late lactation milk. This latter area also requires further investigation however, as Pearse *et al.*, (1986) found that extensive hydrolysis by plasmin did not impair subsequent curd formation and syneresis.

The work presented in this chapter arose out of a study at Teagasc, Moorepark, into the effect of plane of nutrition on the processability of milk, with specific regard to milk composition, Cheddar cheese quality and lipid oxidation in milk powders made from such milks. Three diets were to be used: a control grassland management system, a restricted diet (reflecting situations of high stocking rates, poor grass growth conditions or periods of drought) and a grassland system with concentrate supplementation, which would represent a higher plane of nutrition. As an additional aspect of this work, it was decided that the SCCs, polymorphonuclear leucocyte (PMNs or neutrophils) and plasmin and plasminogen levels would be monitored in the milks of the three groups of cows for the duration of the trial and compared, and proteolysis products therein compared quantitatively and qualitatively. Thus the objective was to see how the enzymatic components of milk processability could be influenced by plane of nutrition, and to determine whether improved nutrition could affect the plasmin activity and elevated somatic cell counts associated with late lactation milk.

7.2. MATERIALS AND METHODS

7.2.1. Experimental Design

Three herds of spring calving Friesian cows, with n=16 cows in each herd, were each allocated a nutritional regime as shown in Table 7.1. Samples from the bulked milks of the 16 cows in each herd were taken at regular intervals over a 28 week trial from 20 April to 27 October, 1994, somatic cell count measured at Moorepark, and a portion (200 ml) frozen and sent to U.C.C. for enzymatic analysis.

Table 7.1 *Herd Management and nutrition scheme.*

Herd	**A**	**B**	**C**
Treatment	**Restricted Herd**	**Standard Grassland Management System**	**Standard Management + Supplementation**
Stocking Rates (acres/cow)			
Apr 1-June 20	0.40	0.45	0.45
June 21-Aug 20	0.50	0.55	0.55
Aug 21-Oct 31	0.80	0.90	0.90
Land Allocation (acres)			
Apr 1-June 20	6.4	7.2	7.2
June 21-Aug 20	8.0	8.8	8.8
Aug 21-Oct 31	12.8	14.4	14.4
Concentrate Supplementation (kg/day)			
Apr 1-Apr 30	0	2	4
May 1-Sept 14	0	0	3
Sept 15-Oct 31	0	2	4
Target post-grazing sward height (cm)			
All year	<5.0	6.0	6.0-8.0

7.2.2. Milk Analysis

Milk plasmin and plasminogen activities were determined as in section 4.3.7. PMN antigen in milk was assayed by the method of O'Sullivan *et al.* (1992). Proteolysis on incubation at 37°C in the presence of 0.05% sodium azide was quantified using the fluorescamine reagent and proteolysis products examined using

urea-PAGE and RP-HPLC, all as described in Chapter 4.

A standard plasmin digest of skim milk was prepared by incubation of skim milk (9% w/v reconstituted skim milk powder) with 0.0025 Units/ml Sigma Porcine Plasmin at 37°C for 24 hours, and pH4.6-soluble products therein examined by RP-HPLC.

7.2.3. Statistical Analysis

Means of analyses within feeding groups were compared by t-test. For two-way ANOVA (analysis of variance) there were three feeding subclasses (cow groups A, B and C) and three time (stage of lactation) subclasses (weeks 1-10, 11-22 and 23-28).

7.3 RESULTS

7.3.1 Plasmin, plasminogen, somatic cell levels and proteolysis levels in trial milks

The plasmin and plasminogen levels in milk from each of the herds during the trial are shown in Fig. 7.1. It can be seen that in herd A the level of plasmin is higher throughout the trial, and the level of plasminogen therein is concomitantly lowered, due presumably to activation of plasminogen to plasmin without an increase in total (inactive + active) plasmin level. It can also be seen that the plasmin level decreased slightly over the course of the lactation, with possibly a slight increase at the very end of the trial, while the plasminogen level is relatively constant.

The polymorphonuclear leucocyte (PMN or neutrophil) level, as measured by the ELISA test of O'Sullivan *et al.* (1992) for PMN cell surface antigen, was generally highest in herd A, and lowest in herd C (Fig. 7.2). PMN numbers increased gradually over the course of the lactation, with a pronounced peak between weeks 10 and 15. The level of total proteolysis, measured as the increase in 12% TCA-soluble products on incubation, also follows this trend, over the limited number of weeks when this analysis was carried out (Fig. 7.3), and there is a marked gradual increase in proteolysis in the milk from herd A as the lactation proceeds.

When somatic cell counts, carried out at Teagasc, Moorepark, were plotted for comparison (Fig. 7.4), it was seen that herd A had the consistently highest SCCs but that, for much of the season, the differences between herds A and C were generally less than those between either of these milks and that from herd B. At the end of the lactation, however, the highest SCCs were predominantly found in herd A. There was, as for PMNs, an unexplained rise in SCCs in all herds between weeks 10 and 15.

When means of plasmin, plasminogen, PMN antigen levels and proteolysis levels are compared by t-test (Table 7.2) it can be seen that, over the trial, herd A has significantly higher levels of 12% TCA-soluble proteolysis products and plasmin, and the highest PMN levels while SCC is not significantly different in herds A and C.

Fig. 7.1. *Plasmin and plasminogen levels in experimental milks during the trial*

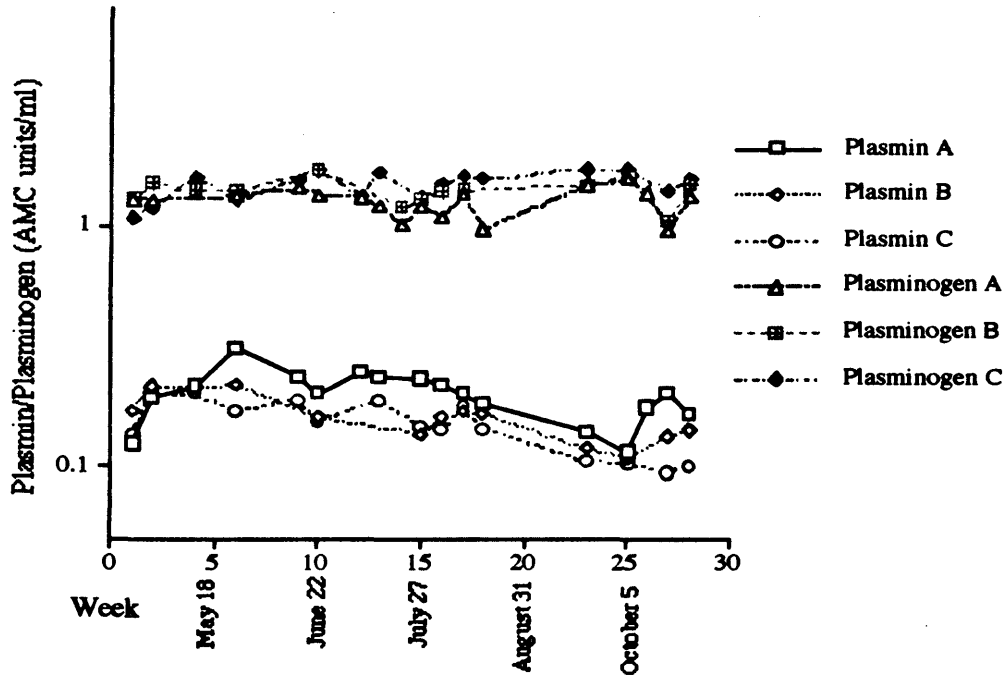


Fig. 7.2. *Polymorphonuclear (PMN) leucocyte levels in experimental herd milks over the duration of the trial as measured by enzyme immunoassay (ELISA)*

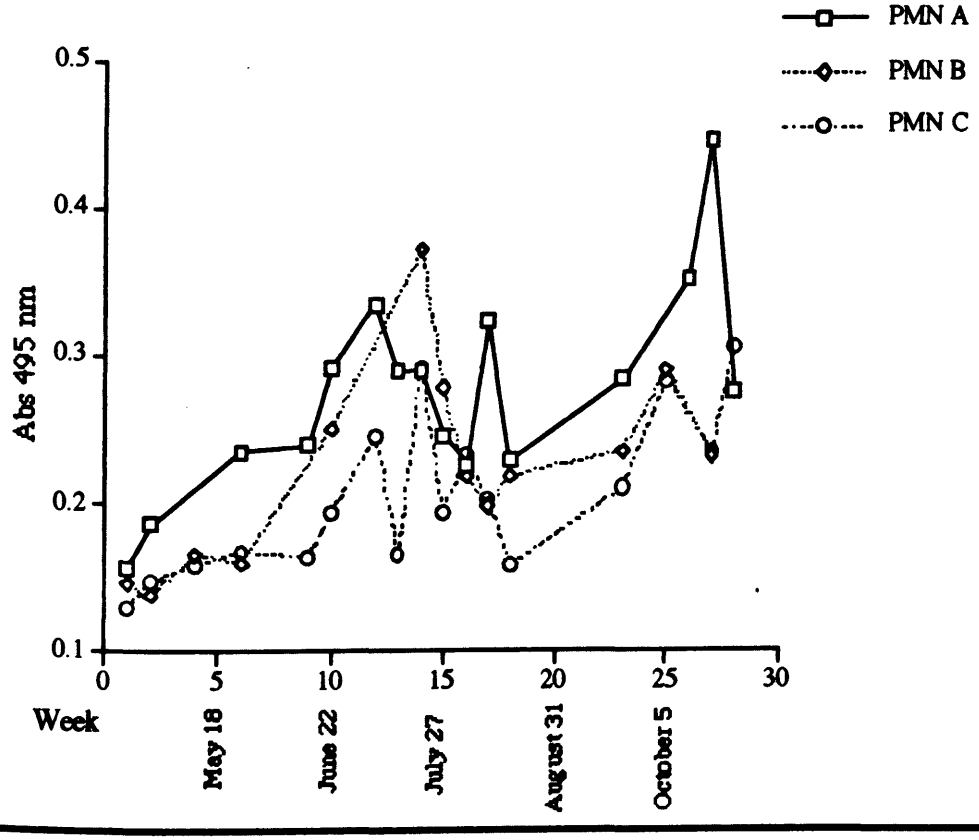


Fig.7.3. *Total proteolysis levels in experimental milks from herds A, B and C over the duration of the trial (nmol Leu-Leu liberated on incubation at 37°C for 24 hours).*

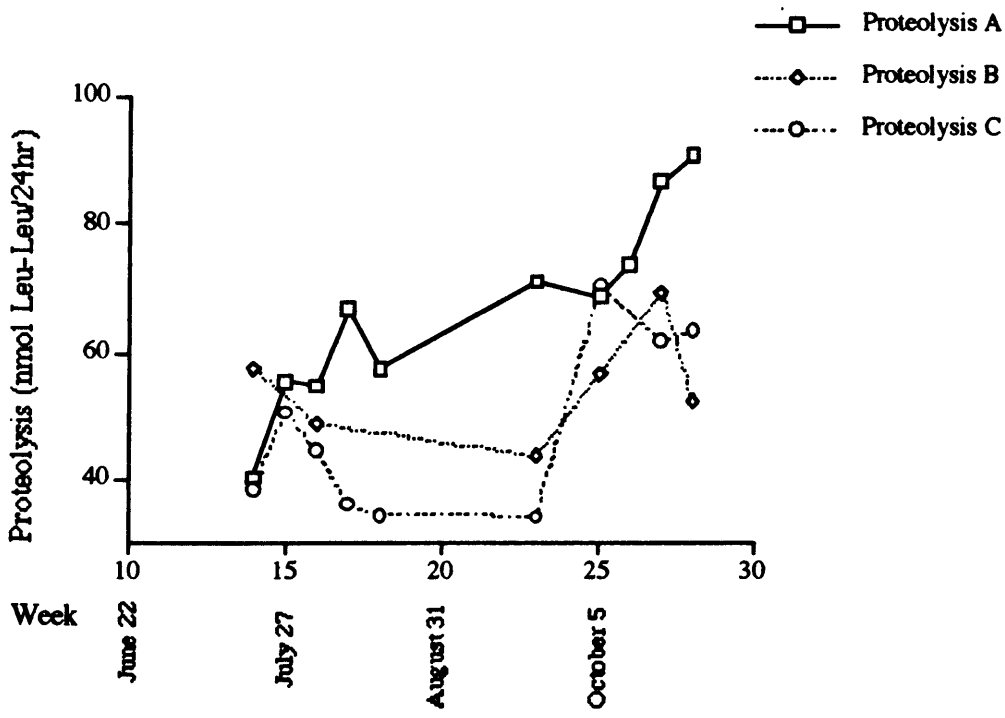


Fig. 7.4. *Somatic cell counts in experimental herd milks over the duration of the trial*

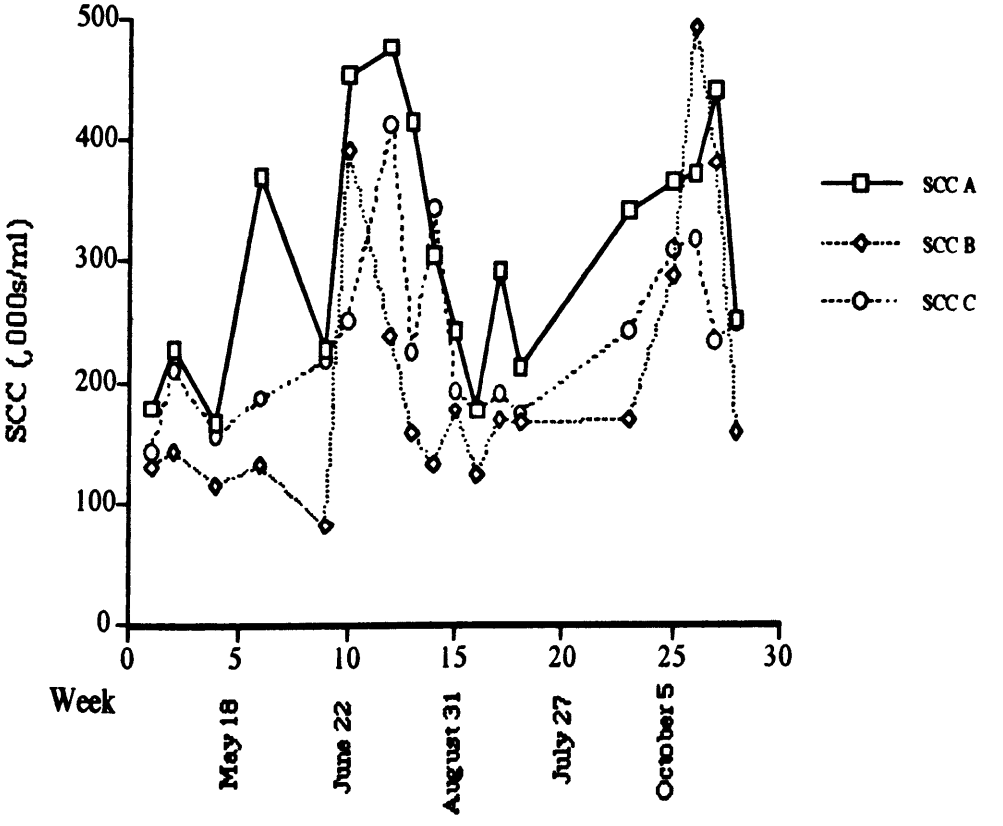


Table 7.2. *Comparison of means within feeding groups using Students' t-test. Within a row, means followed by a different letter are significantly different (P<0.05).*

	A	B	C
Proteolysis products			
	66.6 ± 15.4a	54.8 ± 8.8b	48.4 ± 13.9b
Plasmin	0.192 ± 0.04a	0.163 ± 0.04b	0.151 ± 0.04b
Plasminogen	1.28 ± 0.18	1.41 ± 0.18a	1.50 ± 0.20b
SCC (,000s)	311 ± 110a	202 ± 113b	332 ± 406a
ELISA	0.29 ± 0.08a	0.22 ± 0.07b	0.20 ± 0.05b

ANOVA (Table 7.3) shows that the only factor to be significantly affected by the interaction between feed and season is plasmin, and that while SCC was not significantly affected by stage of lactation, PMN levels, plasmin activity and proteolysis levels were (P<0.005). All parameters were significantly affected by feeding regime.

7.3.2 Qualitative assessment of proteolysis products

Urea-PAGE electrophoretograms of proteolysis products in milks from herds A, B and C are shown in Fig. 7.5. It can be clearly seen that milks from herd A, in late lactation, when the proteolysis levels are known to be highest, have a considerably advanced breakdown of ß-casein, and a greater diversity of fast moving proteolysis products, as compared to milks from herds B and C incubated under the same conditions (37°C for 24 hours). There is at least one proteolysis product (visible just ahead of the α_{s1}-casein band) which is present in higher concentrations in milks from herd C. It can be seen that the milks from herd A also have initially reduced ß-casein levels and concomitantly greater levels of γ-caseins, indicative of plasmin proteolysis in the milk prior to incubation. Prolonged incubation of these milks over 8 days at 37°C showed clearly higher levels of TCA-soluble proteolysis products in milks from herd A (Fig. 7.6), and electrophoretograms (Fig. 7.7) show both α_{s1}- and ß-casein breakdown to be of the order A>B>C, and that long term incubation produces a large variety of unidentified polypeptides and peptides, which vary in a principally quantitative manner between herds.

Table 7.3 *ANOVA analysis of relationships between enzymatic factors in milk, feeding regimen and stage of lactation (subclasses as described in the text)*

Factor and Sources	Sum of Squares	Deg. of Freedom	Mean Squares	F-ratio	P
Plasmin					
Feed	0.01	2	0.005	6.39	0.005
Time	0.02	2	0.01	16.26	0.000
Interaction	0.01	4	0.003	2.64	0.053
Error	0.02	30	0.000		
TOTAL	**0.06**	**38**			
PMN					
Feed	0.03	2	0.02	6.80	0.005
Time	0.03	2	0.02	7.61	0.003
Interaction	0.01	4	0.00	1.03	0.413
Error	0.05	21	0.00		
TOTAL	**0.12**	**29**			
SCC					
Feed	73828	2	36914	4.56	0.019
Time	34683	2	17341	2.14	0.100
Interaction	11673	4	2918	0.36	0.835
Error	243026	30	8101		
TOTAL	**363210**	**38**			
Proteolysis					
Feed	1604	2	802	6.92	0.007
Time	1305	1	1305	11.27	0.004
Interaction	116	2	58.2	0.50	0.615
Error	1737	15	116		
TOTAL	**4761**	**20**			

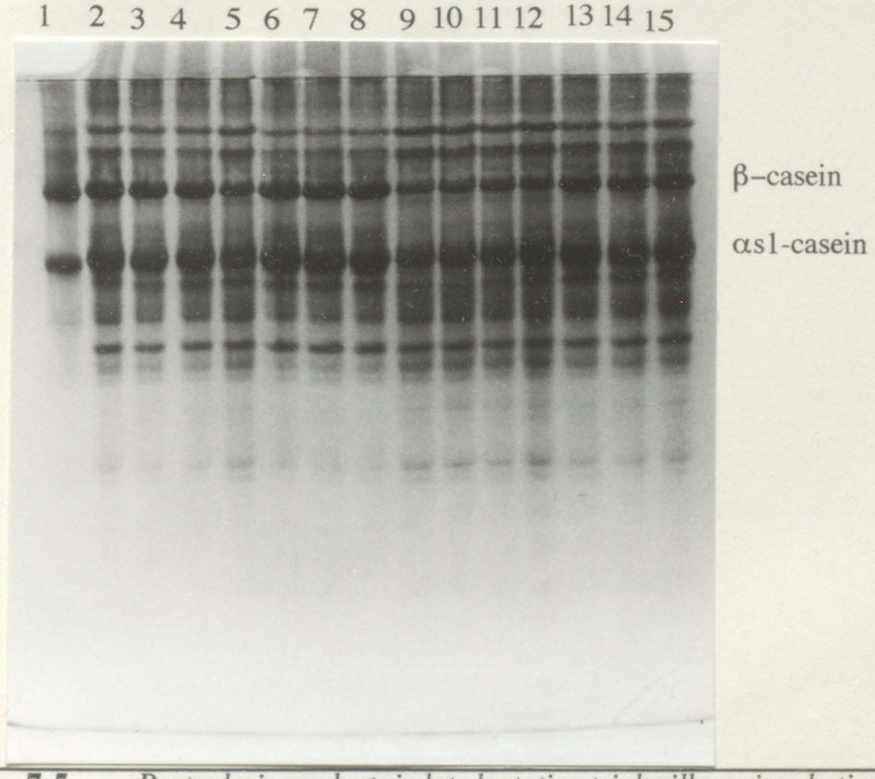
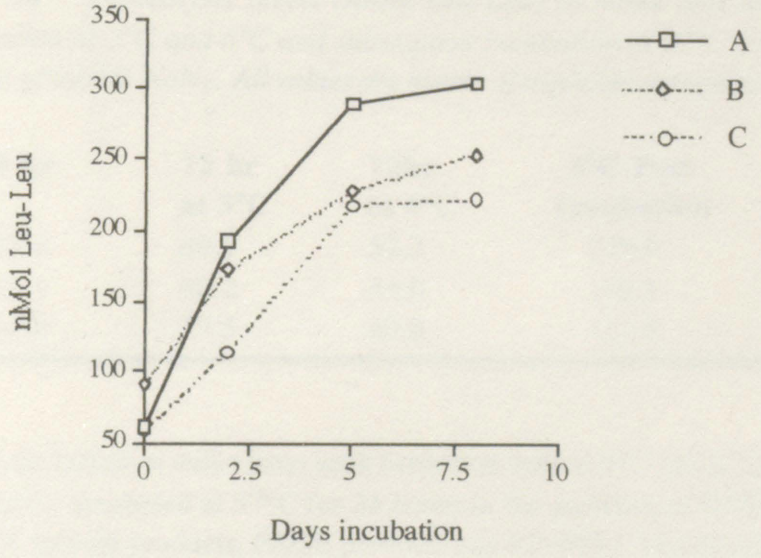


Fig 7.5 *Proteolysis products in late lactation trial milks on incubation at 37°C*

Lane 1	Sodium Caseinate			
Lane 2	A	27/10	251,000 SCC/ml	Initial
Lane 3	A	19/10	440,000 SCC/ml	Initial
Lane 4	A	12/10	371,000 SCC/ml	Initial
Lane 5	B	19/10	381,000 SCC/ml	Initial
Lane 6	C	21/9	242,000 SCC/ml	Initial
Lane 7	C	19/10	234,000 SCC/ml	Initial
Lane 8	C	27/10	247,000 SCC/ml	Initial

Lanes 9-15 As above, in order, after 24 hours incubation at 37°C

Fig. 7.6 *Proteolysis in milks from herds A, B and C on prolonged incubation at 37°C*



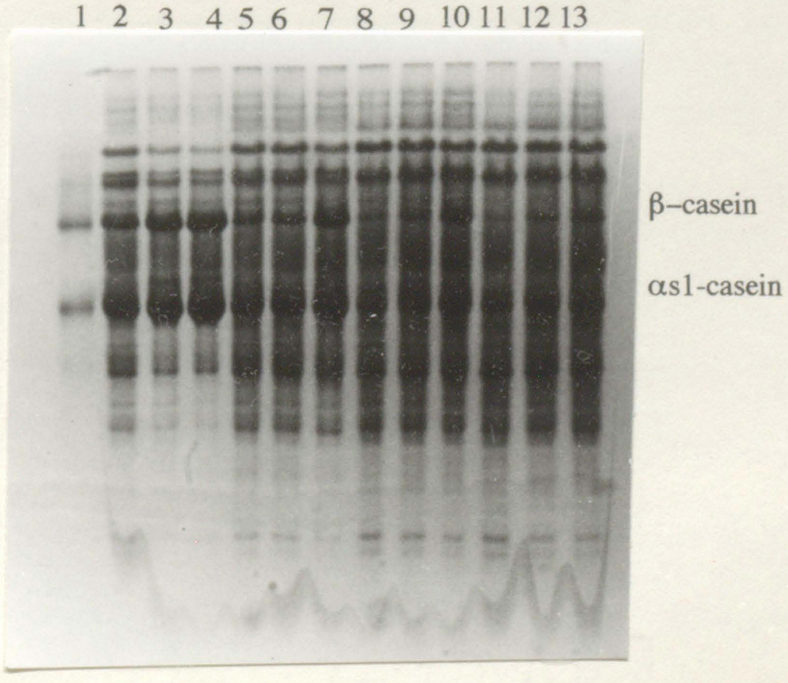


Fig. 7.7 *Proteolysis in milk on prolonged incubation at 37°C*
Lane 1 Sodium Caseinate. Lane 2 A (27/10 SCC 251,000 SCC/ml) Initial. Lane 3, B (19/10 SCC 381,000 SCC/ml) Initial. Lane 4, C (19/10 234,000 SCC/ml) Initial. Lanes 5, 6, 7 as above, in order, after 48 hours incubation at 37°C. Lanes 8, 9, 10 as above, in order, after 5 days incubation at 37°C. Lanes 11, 12, 13 as above, in order, after 8 days incubation at 37°C

7.3.3 *Effect of refrigeration on proteolysis in milk*

Table 7.4 *Proteolysis levels (nMol Leu-Leu) in milks and levels attained on refrigeration at 3°C and 6°C and subsequent incubation at 37°C for 24 hours in the presence of 0.05% NaN_3. All values are means of triplicate determinations.*

Milk	0 hr	72 hr at 3°C	72hr at 6°C	3°C Post Incubation	6°C Post Incubation
A	47.6	49.3	52.3	229.9	219.8
B	37.1	40.2	44.0	192.3	184.1
C	34.8	37.5	40.8	131.9	131.9

Late lactation milks from each herd were held at 3°C and 6°C for 72 hours and subsequently incubated at 37°C for 24 hours in the presence of 0.05% sodium azide. 12% TCA soluble products, PAGE products and RP-HPLC chromatograms of pH 4.6-soluble products compared, both after the refrigeration and incubation periods. Levels of 12% TCA-soluble proteolysis products attained are shown in Table 7.4. It can be seen that only slight proteolysis occurred on refrigerated storage, but that when the milks were subsequently incubated, considerable proteolysis took place, to a higher extent than that found on direct incubation of milks (see Fig. 7.3). The order was, as before, A>B>C. There appeared to be no difference between the two refrigeration temperatures as regards proteolysis in milk. Plasmin levels in the trial milks are shown in Table 7.5.

It appears that refrigeration has no effect on plasminogen activation, but that a small amount of activation occurs on subsequent incubation. Proteolysis products in the refrigerated milks were compared by PAGE (results not shown) and as before, it was found that overall levels of proteolysis products and breakdown of both α_{s1}- and β-casein is greatest in milk A, followed by B then C. Negligible changes in protein levels were seen between initial and post -refrigeration samples. The pH 4.6-soluble peptides in post-incubation samples were examined by RP-HPLC (Fig. 7.8), and,

Table 7.5. *Plasmin activities (AMC units/ml) in milks following refrigeration at 3°C and 6°C and subsequent incubation at 37°C for 24 hours in the presence of 0.05% NaN_3. All values are means of duplicate determinations. n.d. missing data*

Milk	0 hr	72 hr at 3°C	72hr at 6°C	3°C Post Incubation	6°C Post Incubation
A	0.141	0.146	0.143	n.d.	0.152
B	0.101	0.104	0.101	0.129	0.114
C	0.107	0.107	0.111	0.115	n.d.

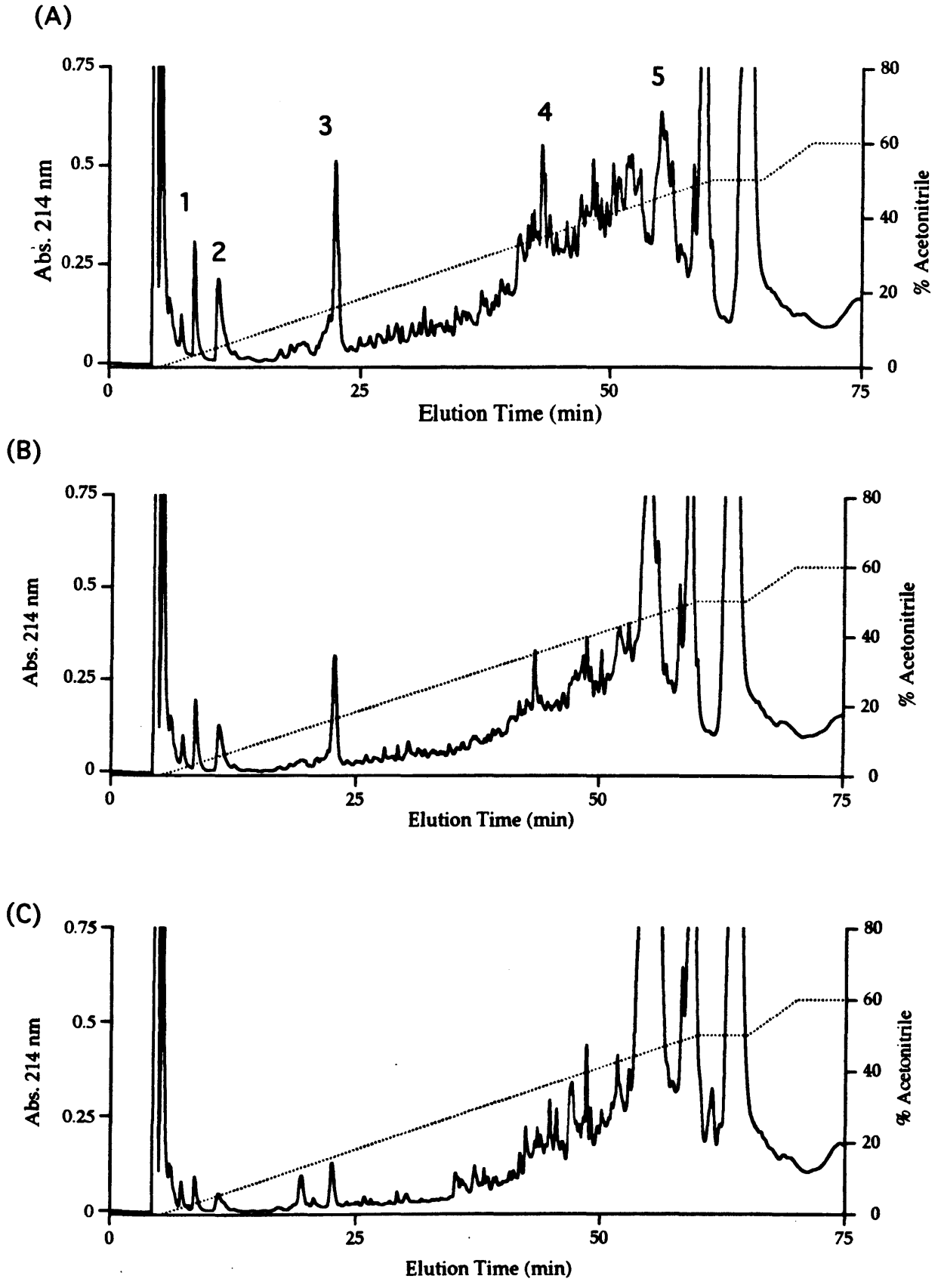


Fig. 7.8 *RP-HPLC chromatograms of proteolysis products in milks A (top), B (middle) and C (bottom) after refrigeration at 6°C for 72 hours followed by incubation at 37°C for 24 hours in the presence of 0.05% sodium azide.*

predictably, milk A shows the greatest diversity of proteolysis products, with higher quantities of unidentified components 1, 2, 3 and 4 while milks B and C show higher quantities of what appears to be a group of peptides (area 5).

Comparison to the chromatogram of a sample of skim milk digested with added porcine plasmin (Fig. 7.9) shows that at least two components appear not to be plasmin-derived products (peaks 1 and 2) while one is only produced very slowly by plasmin (peak 3). Whether or not this is related to other parameters, shown to be elevated in milk A, specifically PMN levels, requires further investigation. Milks B and C show more clearly plasmin-dominated proteolysis patterns.

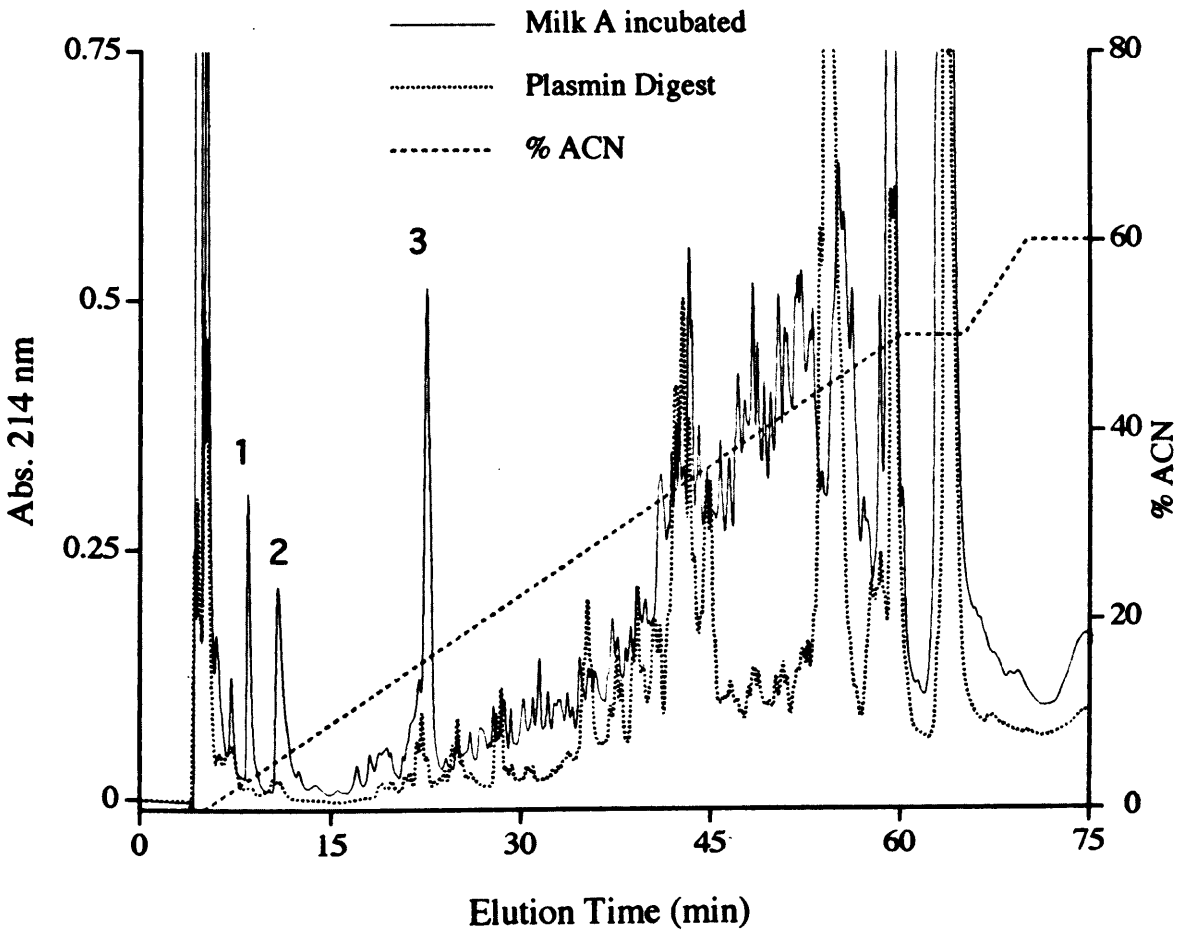


Fig. 7.9 *RP-HPLC chromatograms of milk from herd A (incubated as described in the text (whole line) and skim milk digested with porcine plasmin (dotted line). Dashed line is acetonitrile gradient.*

7.4 DISCUSSION

7.4.1. Plasmin and plasminogen activity in milks

The plasmin and plasminogen levels found are in general agreement with those reported by Scharr (1985) and Benslimane *et al.* (1990). There were found to be significant differences in plasmin and plasminogen levels between the three herds, with significantly higher plasmin levels found in the nutritionally restricted herd (herd A) compared to the control and supplemented herds (herds B and C respectively), which had similar mean plasmin levels (Table 7.2). Politis *et al.* (1989b) found that winter milks, which may be similarly environmentally challenged, had elevated plasmin levels, but this was believed to be insignificant when adjusted for milk yield, SCC, lactation stage and parity. However, although these authors did not study plasminogen levels, the fact that in this trial the plasmin and plasminogen followed opposite trends suggests that the influence of yield is not sufficient to explain the differences, while parities were matched for all herds and the trend was apparent at all stages of lactation. Mean SCCs taken over the lactation were also, similar in herds A and C, although there were differences at many points of the lactation. Benslimane *et al.* (1990) considered, as a part of a larger study, the effect of feeding on plasmin activity by considering herds fed silage, hay and hay with fodder beets during the winter, and found that although there was no clear effect on plasmin and plasminogen contents, the last regime had a slight negative effect. This may suggest that diet supplementation may decrease plasmin level, but this is the only comparable published study.

The plasmin levels showed a slight downward trend throughout the trial, with an increase at the very end, most markedly in herd A, corresponding to the end of the lactation. The plasminogen levels were relatively unchanged throughout the trial. The decrease during mid lactation is in contrast to the published data concerning lactational trends, such as those published by Richardson (1983b) and Politis *et al.* (1989b), and this trend remains unexplained. Refrigerated storage of milks at 3°C or 6°C was seen to have little effect on plasminogen activation to plasmin or formation of γ-caseins. Although milks from herd A had poorer curd forming and firmness properties (B. O'Brien, Teagasc, Moorepark, personal communication), Bastian *et al.* (1991) found that plasmin activity was unconnected to milk clotting properties and Pearse *et al.* (1986) found that extensive degradation of casein by plasmin did not impede subsequent curd formation and syneresis, so these factors are unlikely to be related to the increased plasmin activity in milk A.

The plasminogen levels were found to be concomitantly lower in herd A over the period of the trial, suggesting an increase in conversion of plasminogen to plasmin as opposed to an increased transport of one or both components over the blood-milk barrier. This was confirmed by examination of the plasminogen:plasmin ratios for the herds over the lactation, which were 7.28±.64, 9.19±.76 and 10.35±.95 for herds A, B and C respectively (A was significantly lower than C, $P<0.05$). The range of figures

found is in broad agreement with the plasminogen ratios reported by Richardson (1983b) and Benslimane *et al.* (1990), but higher than those of Politis *et al.* (1989a) and those found in a study of Irish individual cows milks (see Chapter 5). However, the first two studies mentioned, as in the current study, examined bulk milks as opposed to milk from individual cows. This may be due to the fact that any secretory disturbances leading to elevated somatic cell counts (SCC) are linked to decreased plasminogen levels and increased plasmin levels, due to increased somatic cell plasminogen activator activity, and thus decreased ratio (Chapter 6; Zachos *et al.* 1992), but these milks also have decreased milk yield and so are diluted out in the bulk tank, where there is thus an overall higher ratio. Bastian *et al.* (1991a) stated that plasminogen activation was highest in fall and winter milks, and this may be interpreted as showing a link with diet and environmental stress. In summary, the increased plasmin activity appears to be due to increased plasminogen activator activity in the milks from cows on restricted diets, as opposed to increased transport of plasmin into the milk due to altered permeability of the mammary epithelium.

7.4.2 . Somatic cells and PMNs in trial milks

Somatic cell counts (SCCs) were found to be elevated in both herds A and C relative to the control herd, herd B, and to increase over lactation, with a general increase in SCCs in all herds around weeks 10-15 (Fig. 7.4). Brolund (1985) found that bacteriological status of the udder was the single most important factor determining SCC, followed by lactation number. In this study cows showing signs of infection were removed from the study, and thus the effect of bacteriological status was minimised. Also, cows were selected on the basis of level parity and thus both these factors should be controlled. The next most important factor examined by Brolund (1985) was daily milk yield, but in this case, while milk yield in herd A was significantly lower than that in herd C (B. O'Brien, Teagasc, Moorepark, personal communication), the SCCs were relatively similar, indicating that there was considerably lower total numbers of somatic cells in milks from herd A. However, it is unclear whether the total level of somatic cells or the concentration (taking yield into account) is more representative of the udder status. For example, mastitis will result in a decrease in milk yield but also an influx of somatic cells (PMNs) and the rise in SCC is obviously far more related to the latter than the former effect.

The increase in late lactation somatic cell count is as reported by Blackburn (1966) and Emanuelson *et al.* (1988), among others. Blackburn *et al.* (1955) and Ostensson (1993) observed that PMN levels as a proportion of total somatic cells remained relatively unchanged over the course of a lactation, and here the increases in SCC and PMN level over the lactation seem to show such a relationship. In herd A, however, the rise in SCC is more dramatic than in herd C, suggesting that supplementation of diet in late lactation may help to reduce the SCC of milk at this time and thus increase acceptability of late lactation milk. It is interesting to note that the PMN levels rise relatively constantly over the lactation and that the large increase in SCC observed between weeks 10 and 15 is less manifest from the PMN results.

The levels of polymorphonuclear neutrophils (PMNs) in the trial milks were elevated in milks from herd A compared to those in herds B and C, which had similar levels. The PMN levels also rose more significantly in late lactation in herd A than in the control or supplemented herds. Comparison with the SCC means shows that there were more PMNs per somatic cell in herd A, irrespective of milk yield. PMNs are the cell type predominantly associated with mastitis and the defence systems of the udder in general, being recruited in large numbers upon bacterial invasion. The somatic cells, particularly PMNs, in milk provide the second line of defence against infection after the teat canal itself and depletion of PMNs can result in increased susceptibility to mastitic infection (Burvenich *et al.*, 1995). Two mechanisms control PMN influx into milk. Firstly, milking stimulates PMN influx to the udder and replaces PMNs which have ingested fat and casein with fresh cells. Secondly, components of the immune system, such as cytokines, act as chemoattractants, triggering PMN recruitment to sites of infection. The results presented in Chapter 5, however, showed that PMN numbers are elevated in milks from individual spring-calving cows compared to milks from autumn-calving cows, irrespective of stage of lactation. Spring calving cows are fed in early-mid lactation on spring-summer grass and as the grass growing season ends, enter late lactation. They are kept indoors during the infection-prone dry period, feeding generally on silage. Autumn-calving cows, however, are kept indoors and fed good quality feeds during the milk producing period, and are feeding on summer grass during the late lactation and dry periods, and thus could be said to be on an overall higher plane of nutrition. Thus, both these sets of results suggest that PMN levels, as well as being influenced by infection status of the udder, are also very much linked to the nutritional status of the cow.

With regard to interaction of the somatic cell system with plasmin and plasminogen activation, the plasminogen/plasmin ratio was negatively correlated against both PMN level, as measured by ELISA, and SCC in herd A and positively correlated with both these parameters in herds B and C. Thus, it appears that in herds B and C, plasminogen and plasmin behave as components which are transported across the mammary epithelium due to increased permeability, as Politis *et al.* (1989a) described the effect of elevated SCC on plasmin level. This could be interpreted as implying that an unchanged or elevated ratio with elevated SCC or influx of PMNs across the compromised epithelium. In herd A, however, the ratio decreases with elevated PMN levels, suggesting that the PMNs in this case are actively converting plasminogen to plasmin, and thus have a different physiological and enzymatic activity to the cells in milks B and C. Politis *et al.* (1989a) found that somatic cells had high plasminogen activator (PA) activity, but only when freeze-thawed, but it is not stated whether these cells came from normal or mastitic milk. Zachos *et al.* (1992) found that somatic cells from mastitic milk had greatly increased plasminogen activator (PA) activity per cell, and that this may be due to increased somatic cell PA activity, secretion of PAs by epithelial cells or transport of PAs from blood. Verdi and Barbano (1991a) found that milk somatic cells had PA activity but blood leucocytes did not, and suggested that PAs are produced only when the cells are immunologically stimulated. Both these studies may suggest that conditions which result in increased PMN presence in milk are linked

to increased cell PA activity, as appears to be the case in the present study.

7.4.3. Proteolysis in trial milks

Levels of proteolysis in milks from herd A were higher than in groups B or C and rose towards the end of lactation. The elevated plasmin levels in milks from herd A were confirmed by the electrophoretogram of milk proteolysis products (Fig. 7.5), which evidences decreased ß-casein and increased γ -caseins in initial and incubated milks. Increased α_{s1}-casein breakdown in herd A milks was also visible. If Fig. 7.6 is considered, showing proteolysis on prolonged incubation of late lactation milks from each of the three herds, these trends are clearly seen and the order of increasing proteolysis is obviously in the direction of poorer nutritional status. This corresponds to elevated PMN levels and plasmin levels in milk A at this time. Proteolysis over 72 hours refrigeration was seen to be minimal in all milks, but on incubation of refrigerated milks, huge increases in proteolysis were seen, which would not appear to be due to plasmin activation, as the increase in plasmin levels was seen to be minor. The explanation for this is unknown. Kang and Frank (1988) similarly found that 4 days storage of raw milk at 4°C resulted in only slight proteolysis compared to milks held at 11°C, 21°C and 37°C. Senyk *et al.* (1985) showed that milk SCC was positively correlated to initial and incubated milk tyrosine values and the increase in tyrosine value on refrigerated storage. While this was over a far wider range of SCCs than examined in the current study, it may be hypothesised that, since there is a high correlation between SCC and PMN level in milks from individual cows (O'Sullivan *et al.*, 1992), these effects are due to PMNs and milks with higher levels of PMNs per cell should show higher proteolysis than milks of similar total SCC but lower levels of PMNs. Comparison of the results of the present study with published studies is difficult because this is the first case where differential, as opposed to total, SCC must be considered with regard to proteolysis. Nonetheless, Verdi *et al.* (1987) found that milk from farms with consistently high SCC, on the basis of the Wisconsin Mastitis Test, had significantly higher proteolysis levels and breakdown of α_{s1}- and ß-casein, than milks from low SCC farms, which would seem to be in agreement with the current results.

The relationship between proteolysis and milk clotting properties remains to be conclusively elucidated, but Okigbo *et al.* (1985b) found that poor chymosin coagulating milk had a higher content of γ -caseins and degraded caseins than good-coagulating milk. Although α_{s1}-casein levels were not found to be very variable, low levels of this protein were linked to poor clotting properties.

Verdi and Barbano (1988) showed that plasmin and somatic cell proteases produce distinctly different proteolysis products and suggested that the presence of specific casein proteolysis products in milk could provide information on the source of major proteases present in milk. RP-HPLC analysis of the milks in the present study showed that the milks from herd A had a wider diversity of proteolysis products, but less accumulation of products in a region which we have tentatively identified with large proteolysis products such as α_{s1}-I casein (O'Connor, 1995), but I believe that

this is due to further breakdown of these products preventing accumulation, as α_{s1}-I casein has been shown to be produced by PMNs and purified PMN enzymes (see Chapter 6). Andrews (1983b) also found that somatic-cell derived enzymes in milk from a cow with clinical mastitis hydrolysed α_{s1}-casein. The early-eluting hydrophillic peptides in incubated milks from herd A remain to be identified, but comparison with skim milk digested by plasmin shows them not to be plasmin products. Their possible production by somatic cell, or PMN, proteases can only be suggested at this point, and requires further investigation.

7.4.4. Conclusions and suggestions for further work

Kefford *et al.* (1992) stated that diet quality, not stage of lactation, is the dominant factor influencing the functional capacity of milk in Cheddar cheese manufacture, with higher moisture cheese being associated with milk from cows on low-quality diets. In this study, it was found that diet had significant effects on proteolysis in milk, with particular regard to the levels and activities of plasmin and PMN cells in the milk. In particular, factors which have been associated with problems in milk and milk product quality such as somatic cell count and plasmin levels are reduced in levels by diet control and supplementation. This is in agreement with the findings of Kefford *et al.* (1992) and Lucey and Fox (1992), who both stated that late lactation milk, if chosen from herds on sufficiently good planes of nutrition, may not show the processing difficulties associated with such milks. Further work is necessary to link these changes to cheese quality, or indeed quality of any heat treated milk products. Also, this study has raised interesting points concerning the physiological role of somatic cells and the mechanisms controlling their levels in milk, with particular implications for mastitis research. It would be important to determine the defensive capacities of the milks from cows on restricted and supplemented diets.

The link between nutritional status and immunosuppression or activation of mammary gland defence systems remains to be elucidated. In this regard, it must also be considered that the mean SCCs in herd C over the lactation were higher than those from herd B, but herd C milks had marginally lower PMN levels, suggesting that supplementation, as well as starvation, influences the immune system of the cows, with apparently less PMNs per cell in milk C than the control milk.

The question of the exact function of somatic cells in the non-infected udder or quarter must be addressed at this point. If it is assumed that the function of somatic cells is to act as a defensive system to initiate an immune reaction on bacterial invasion, and the cells removed by milking are automatically replaced during the period between milkings, are the cells replaced at a constant rate, irrespective of yield? This would appear to be the case if it is believed that elevated SCCs in late lactation milk are a result of decreased milk yield alone, leading to a concentration effect. In this case the cows in herd C would appear to have an elevated transport of somatic cells into the udder by comparison to herds A and B, while the cows in herd A have a different response, leading to increased levels of PMNs migrating to the udder, while total cell transport remains lower than in herd C. This may imply that a different physiological approach

to udder defence is being seen in these two herds, with an elevated level of non-PMN cells in milk from herd C, whether macrophages or lymphocytes, while in herd A there is a PMN-led immunoprotective system. This may be related to nutritional deficiencies in herd A causing an alteration of the chemotactic regulation which controls the number of PMNs in milk. This is confounded, however, by the fact that herd A milks had lower levels of whey proteins, which would be expected to be elevated with increased permeability of the mammary epithelium, due to increased transport of serum albumin and immunoglobulins (Kitchen, 1981). Levels of lactose in trial milks, in contrast, were decreased in herd A compared to herds B and C, suggesting physiological changes in the udder tissue, which may be related to the influx of PMNs. In normal milk the PMNs are the minor cell fraction compared to macrophages and lymphocytes, and are recruited only as needed, through the epithelium which is thus altered. However, in herd A it may be that due to altered immunoregulatory mechanisms, and possibly also physiological changes in the udder, the cell proportions are altered, but differential cell counts are needed to verify this result.

Also, the major question, I believe, is which cows, starved or supplemented, are more immunologically favoured and able to resist a bacterial or endotoxin challenge. Wegner *et al.* (1976) showed that cows with evidence of current mammary infection could not mobilise blood leucocytes to the same extent as cows with healthy udders, suggesting that stressed cows were making constant demands on the emopoietic centres and thus may not have had the capacity to mobilise leucocytes on stress-demand. This implies that stressed cows with high PMN levels may be less able to resist bacterial challenges and this may be linked to seasonal patterns in mastitis incidence, with increased cases of infection and elevated SCCs associated with early lactation and cows going to pasture (Kennedy *et al.*, 1982). This is an area which requires further investigation.

Chapter 8

Composition and clotting properties of bulk milk and ripening characteristics of Gouda-type cheeses as affected by somatic cell count and stage of lactation

SUMMARY

The clotting properties of late lactation milks from spring-calving herds were found to be superior to those of early lactation spring calving milks, which was linked to increased fat and protein levels and decreased lactose levels in the former milks. Autumn calving early-lactation milk had clotting properties superior to spring calving early lactation milk. Milk fat significantly influenced rennet clotting time while curd formation and curd firmness were affected by milk pH. Milk clotting properties were found to be significantly correlated. Small-scale model cheeses made from high SCC milks had increased losses of fat and protein in whey leading to decreased levels of these components and increased moisture in cheese. Proteolysis, in particular the breakdown of α_{s1}-casein, was accelerated in these cheeses. Gouda cheeses made on a pilot scale from late lactation milks had increased moisture compared to those made from early and mid lactation cheeses. The former cheeses, while having accelerated breakdown of α_{s1}-casein showed lower rates of small peptide and free amino acid development during ripening, which may have been related to poorer flavour scores attributed to these cheeses. Biochemical explanations for the textural and flavour problems frequently encountered in cheese made from late lactation milk are presented.

8.1 INTRODUCTION

Cheesemaking patterns in Ireland are greatly influenced by seasonality, with cows being traditionally calved during the spring months to take advantage of summer grass for feeding. This has resulted in a peak-to-trough ratio of 9:1 between summer and winter milk production (Keane, 1986). Thus, Irish dairy companies have tended to produce long-ripening cheeses, exemplified by Cheddar, which could be made from February to October and stored to allow continued supply over the winter months.

At the beginning of the winter however, much Irish manufacturing milk comprises late lactation milk, which is associated with poor quality of dairy products (Lucey and Fox, 1992). Changes in late lactation milk include reduced levels of α_{s1}- and ß-casein and increased total protein due to increased whey protein and non-protein nitrogen (Barry and Donnelly, 1980: Barbano *et al.*, 1991; Phelan *et al.* 1982), decreased lactose and elevated pH (Lucey and Fox, 1992) and increased sodium and chloride levels (Keogh *et al.*, 1982). Late lactation milks are also associated with increased levels of the alkaline milk proteinase, plasmin (Donnelly and Barry, 1983; Politis *et al.*, 1989a,b) and increased somatic cell counts (Emanuelson *et al.*, 1988; Barbano *et al.*, 1991). With regard to cheesemaking properties, late lactation milk has been shown to have increased rennet clotting time (R.C.T.) and decreased syneretic properties and to yield high moisture cheese (O'Keeffe *et al.*, 1979; O'Keeffe *et al.*, 1982; Lucey and Fox, 1992). The poor clotting properties of this milk have been associated with ß-casein hydrolysis by plasmin, high pH, increased presence of sodium and other monovalent ions and increased presence of blood constituents (Okigbo *et al.*, 1985a; Lucey and Fox, 1992). Proposed means to improve the cheesemaking properties of late lactation milk, and thus extend the Irish cheesemaking season, have included calcium chloride addition, milk pH adjustment, use of increased rennet concentrations and selection of herds on high nutritional planes with improved milking practices (Lucey and Fox, 1992; Kefford *et al.*, 1992).

The elevated somatic cell counts (SCC) associated with late lactation milk (Emanuelson *et al.*, 1988) have also been associated with impaired milk functionality for cheesemaking. Principally, high SCC milk has been associated with increased rennet clotting time and decreased curd firmness leading to decreased coagulum strength and syneretic properties and increased losses of fat and protein in whey (Politis and Ng-Kwai-Hang, 1988a,b,c; Grandison and Ford, 1986; Mitchell *et al.*, 1986b). High SCCs are also associated with increased cheese moisture and decreased cheese yield (Lawrence, 1993; Barbano *et al.*, 1991) and decreased organoleptic properties of Gouda cheese made from such milks (Brus and Jaartsveld, 1971).

The series of trials described herein arose from the decrease in semi-soft cheese quality, consistently observed at U.C.C. when milk supplies contained high proportions of late lactation milk. Specific problems encountered include bitter or sour off-flavours and short, pasty texture. In the first part of the trial, bulk winter milks from both autumn and spring calving herds, and spring calving early lactation milks were to be studied with regard to composition, SCC, polymorphonuclear leucocyte

(PMN) level and plasmin levels and these correlated to the compositional and clotting properties of the milks, as measured by Formagraph. This was required to provide information concerning possible milk-related causes for poor late lactation cheese quality. The second part of the trial comprised cheesemaking trials to examine the separate and confounded effects of late lactation and high SCC milk on cheese quality, and consisted of

(1) Small scale model Gouda-Meshanger type cheeses to examine the effects of stage of lactation and SCC on cheese composition, yield and proteolysis during ripening.

(2) Pilot-scale Gouda cheese manufacture to examine the functionality of mid and late lactation milks with reference to cheese composition, ripening and organoleptic properties.

8.2 MATERIALS AND METHODS

8.2.1 Bulk tank milk collection and analysis

94 bulk tank samples were collected over the months September-April. In the first part of the trial 70 samples from a total of 14 farm bulk tanks were taken over a number of weeks from September to November, consisting of 35 spring calving samples and 35 autumn calving samples. Preliminary statistical analysis showed that there was no significant trends associated with changes in composition or clotting properties in individual bulk tanks over the sampling period and so all results were pooled on the basis of season and stage of lactation. In the second part 24 spring calving (early lactation) tanks were sampled on one day in April and examined in the same way. 300 ml samples were taken from the tanks in each case and composition, SCC, plasmin and plasminogen concentrations and PMN levels measured as described previously (section 5.2.1). A portion (80 mls) was sent to Teagasc, Moorepark for Formagraph analysis and a further sample, adjusted to pH 6.55, also sent for analysis.

8.2.2 Cheesemilk selection and cheese manufacture

Bulk milks were obtained from a local co-operative. Some samples of high somatic cell count (SCC) were selected by examining SCCs for a number of farms for a number of weeks leading up to cheesemaking. The sets of trials are divided into two batches, with most of the cheeses being made over a fifteen month period. The cheeses made in trial A were similar to high moisture Gouda-type cheeses, and may be regarded as similar in many respects of the Noordhollandaise-Meshanger cheese whose manufacture was described by Noomen (1977), which was described as a model for research on Dutch-type cheese ripening. Each batch was made in the viscubator from 12L of pasteurised milk. These were high moisture cheeses, which were plastic coated

during ripening and which lost moisture at a rate of 0.5-1% per week during this time, to give a final moisture content at 15 weeks of 32-36%. The manufacturing procedures used for both large and small batches of cheese are described in the Materials and Methods section (4.1.1 and 4.1.2.). Trial A cheesemilks were unstandardised. Trial B cheesemilks were standardised to a 1:1 or 1:0.97 fat:protein ratio and 454L (100 gallons) pasteurised milk taken for cheesemaking. Trial B cheeses were vacuum packed during ripening.

8.2.3. Cheese analysis

Milks taken for cheesemaking were analysed for composition using a Milkoscan, somatic cell count (SCC) using a Fossomatic 90 and PMN antigen (if measured) at University College Dublin using the method of O'Sullivan *et al.* (1992). At 12 days of age all cheeses were sampled and analysed for composition as described in section 4.. Samples were taken at various intervals over a 15 week ripening for examination of proteolysis by measurement of soluble nitrogen fractions, measurement of free amino acids and HPLC and gel electrophoretic analysis, also as described in Section 4. Taste panels were also carried out as described therein.

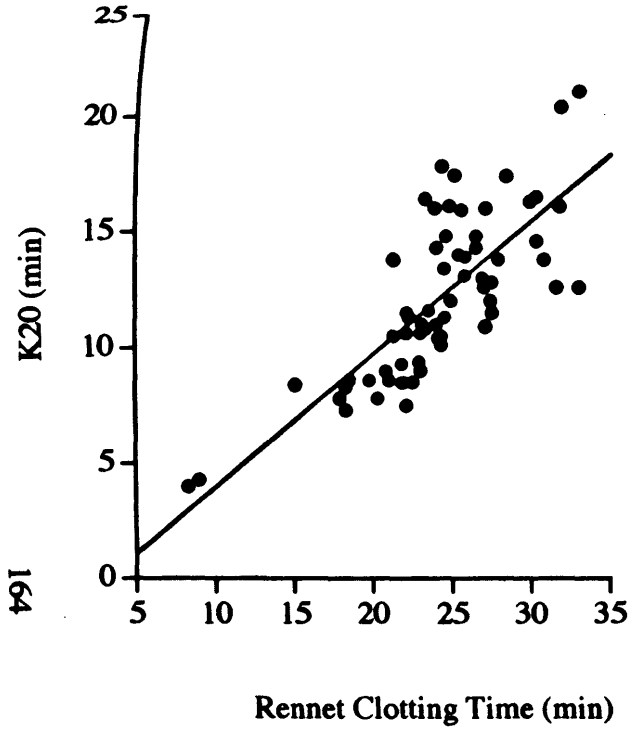
8.2.4. Statistical analysis of data

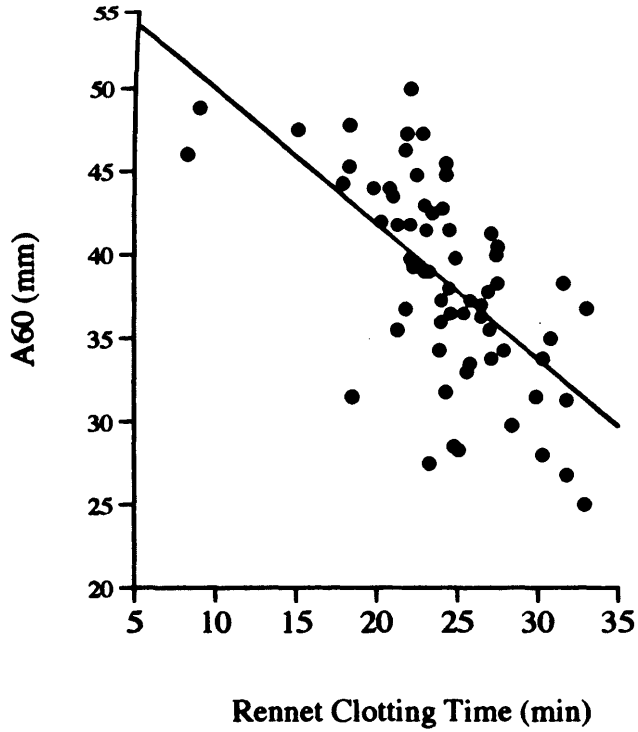
Relationships between pairs of variables were examined using Pearsons correlation coefficients. Multivariate analysis of variance using the GLM function of the MINITAB statistics package was used to estimate effects of calving season, stage of lactation, milk SCC, milk pH and milk composition on milk clotting parameters. The only factors included as categorised variables were season, which had two categories (spring calving and autumn calving), and stage of lactation, which also had two categories (early and late, depending on the time of collection). All other variables (milk pH, lactose, fat, protein and $\log_{10}$SCC) were included in the model as covariates. The SCC was linearised by using the logarithm to the base 10. Differences in cheese ripening parameters in different batches of cheese were compared by students t-test.

8.3 RESULTS

8.3.1. Clotting properties, SCC, enzymology and composition of bulk milks

The Formagraph is an instrument developed to measure clotting properties by assessing three parameters, taken from the movement of pendulums immersed in linearly oscillating samples of renneted milk, as measured optically and recorded on photographic paper (Aleandri *et al.*, 1989). Fig. 8.1 shows a typical Formagraph trace. The rennet clotting time (RCT) is measured when the arms begin to separate. The measure of curd firming rate (K20) is the time taken for the arms of the graph to achieve a separation of 20 mm. Curd firmness, A60, is the final separation (in mm) of the arms





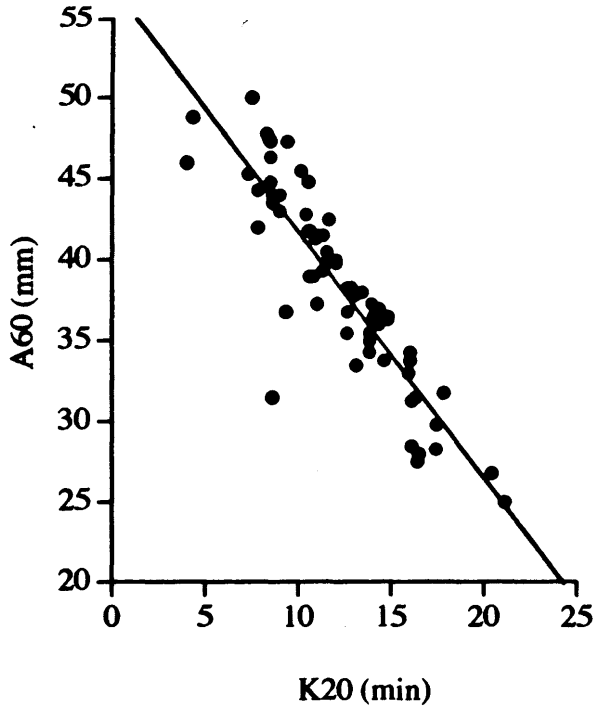


Fig. 8.2 *Graph of correlations between Formagraph parameters (RCT (in min), K20 (in min) and A60 (in mm)), with regression equations and Pearsons Correlation Coefficients.*

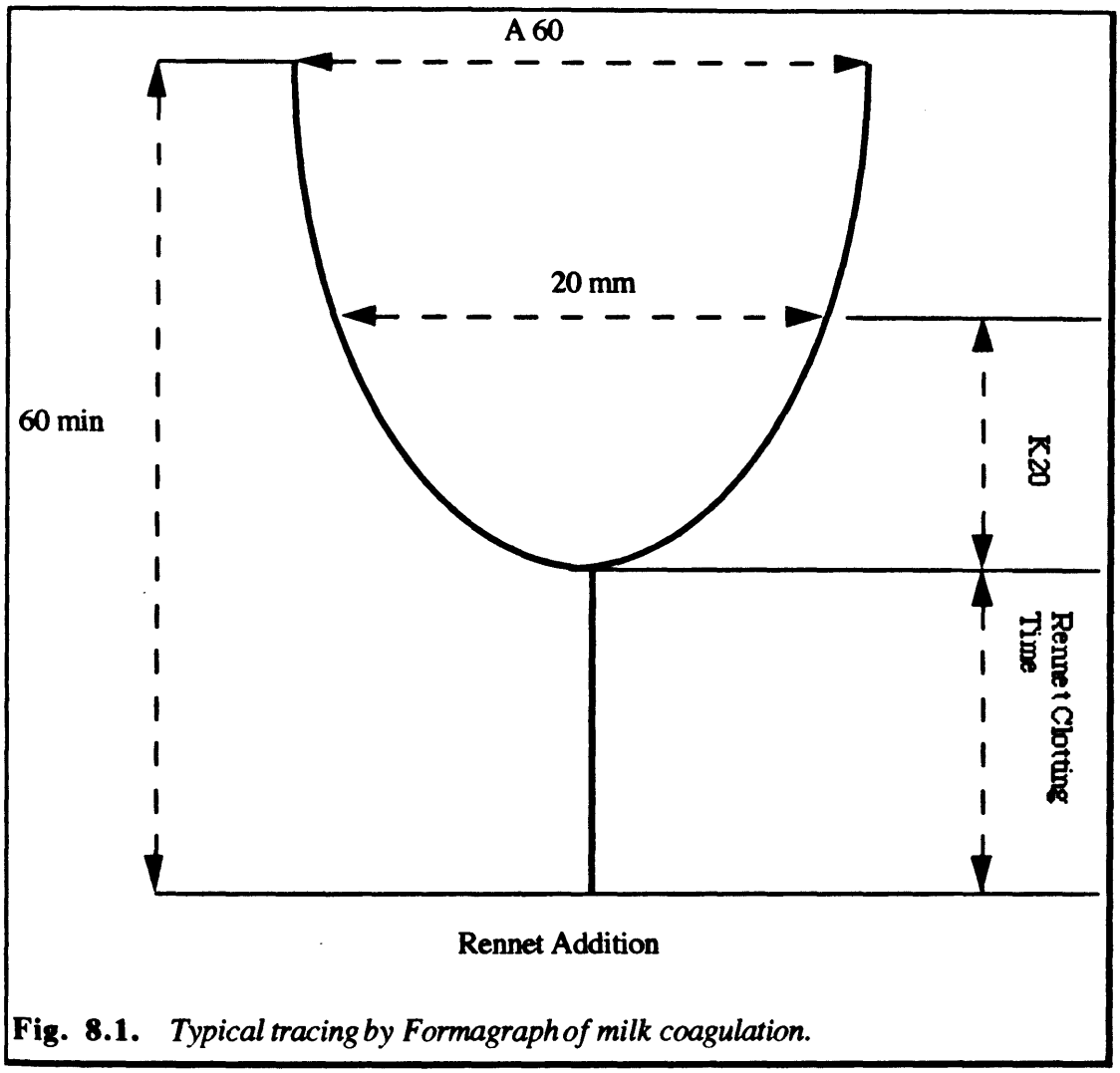


Fig. 8.1. *Typical tracing by Formagraph of milk coagulation.*

at the end of the evaluation. Clotting properties were measured for the original milk sample, and for a portion of each sample, the pH of which had been adjusted to 6.55, to remove the effect of pH variability on chymosin activity during the measurement.

Clotting parameters in 94 bulk milk samples over an 8-month period were taken and their compositional and clotting properties measured. Plots of the three variables against one another (Fig. 8.2) shows that significant ($P<0.001$) relationships exist between all three. Samples with low RCT will have fast curd formation rate, with less time being required to give a firm curd, and a high final curd firmness. Rate of curd formation and final curd firmness are highly correlated. When values for all three parameters, measured on the same milks after pH adjustment to pH 6.55, were correlated, all correlations remained significant ($P<0.001$).

Milk clotting properties were correlated with measured compositional and enzymatic parameters (Table 8.1). It can be seen that while milk fat and protein did not affect RCT, they were both significantly correlated with faster curd formation after initial rennet clotting and subsequent curd firmness. Lactose, while giving a firmer clot at higher levels, was correlated with poor curd formation rates. $Log_{10}SCC$ was not significantly correlated with RCT, but high SCCs were connected to fast curd formation

Table 8.1 *Correlation coefficients of compositional and enzymological parameters with coagulation properties of milk and somatic cell count (SCC). (number of samples in brackets, significance levels, n.s., not significant; *, P<0.05; **, P<0.01; ***, P<0.001)*

Parameter	RCT	K20	A60	Log_{10}SCC	Plasmin
Log_{10}SCC	-0.206 (65) n.s.	-0.449 (65) ***	0.284 (65) *	-	0.366 (51) **
PMN level (ELISA)	0.010 (65) n.s.	-0.027 (65) n.s.	-0.057 (65) n.s.	0.819 (94) ***	0.169 (51) n.s.
Milk composition					
Fat	0.043 (65) n.s.	-0.360 (65) **	0.364 (65) **	0.593 (65) ***	0.046 (51) n.s.
Protein	-0.115 (65) n.s.	-0.579 (65) ***	0.513 (65) ***	0.593 (90) ***	0.165 (51) n.s.
Lactose	0.132 (65) n.s.	0.456 (65) ***	0.364 (65) **	-0.716 (94) ***	-0.206 (51) n.s.
pH	-0.158 (65) n.s.	-0.391 (65) **	0.175 (65) n.s.	0.552 (94) ***	0.430 (51) **
Milk enzymology					
Plasmin	-0.068 (41) n.s.	0.092 (41) n.s.	-0.046 (41) n.s.	0.366 (80) ***	-
Plasminogen					
Initial proteolysis	0.072 (26) n.s.	0.095 (26) n.s.	-0.046 (26) n.s.	0.337 (26) n.s.	0.507 (26) **
Proteolysis after incubation	-0.183 (26)	-0.319 (26)	0.106 (26) n.s.	0.359 (26) n.s.	0.549 (26) **

and increased curd firmness. This is unexpected but may be explained by the highly positive correlations of $\log_{10}$SCC with milk fat and protein and the negative correlation with lactose, all of which will also affect curd formation and firmness. PMN, plasmin activity and plasminogen levels in milk appear unrelated to milk clotting properties. Proteolysis in milk was more highly correlated with milk plasmin level than $\log_{10}$SCC or PMN level (correlations of PMN level with initial and final proteolysis levels were 0.352 and 0.207 respectively, neither correlation significant) and had no effect on milk clotting. Milk plasminogen level was significantly correlated to milk plasmin activity (0.601, $P<0.001$), but not $\log_{10}$SCC (0.156, $n = 51$). It can be seen that similar trends are generally followed by A60 and K20, as would be expected due to the high correlation between the two parameters.

Clotting and compositional parameters were divided on the basis of the predominant calving pattern and stage of lactation of the bulk tanks sampled (Table 8.2). It can be seen that spring calving, early-mid lactation (SE) milk has lower fat and protein and high lactose levels than milk from spring calving late lactation (SL) milk or autumn calving early lactation (AE) milk. As all these parameters are correlated to decreased K20s, it is as expected that the K20 in the SL and AE milks are lower than those in the SE milk. The RCTs were lowest in the AE milk and highest in the SE milks, but as no measured factors were found to significantly influence this parameter, it is difficult to explain this result. Mean curd firmness, A60, was highest in the SL and

Table 8.2 *Clotting and composition parameters of bulk milks divided on the basis of calving season and stage of lactation.*

Season	Spring Calving			Spring Calving			Autumn Calving		
Stage of lactation	Early-mid lactation			Late lactation			Early-mid lactation		
Parameter	n	Mean	SD	n	Mean	SD	n	Mean	SD
Protein	24	3.01	.02	31	3.48	.03	32	3.42	.04
Fat	24	3.82	.13	31	4.37	.10	32	4.28	.05
Lactose	24	4.84	.01	31	4.61	.02	32	4.63	.02
pH	24	6.60	.00	30	6.68	.01	34	6.68	.01
RCT	23	26.12	.54	21	24.4	.89	21	21.8	1.3
K20	23	15.29	.52	21	10.6	.43	21	9.85	.69
A60	23	34.08	1.00	21	41.1	.87	21	41.4	1.2
$RCT_{pH\ 6.55}$	23	20.50	.41	21	17.4	.97	21	17.4	1.4
$K20_{pH\ 6.55}$	23	11.78	.39	21	7.43	.52	21	7.12	.77
$A60_{pH\ 6.55}$	23	40.83	.74	21	48.08	.85	21	47.1	1.7
SCC	23	126	13	31	438	53	31	366	28

Table 8.3 *Analysis of variance for coagulation parameters (RCT, rennet clotting time; K20, curd formation rate; A60, curd firmness; SS, sum of squares; F, F-value) (*, P<0.05; **, P<0.01; ***, P<0.001).*

Source	d.f.	RCT		K20		A60	
		SS	F	SS	F	SS	F
Season	1	143.6	15.35 ***	53.65	20.18 ***	49.77	15.85 ***
Stage of lactation	1	175.6	9.78 **	97.23	14.19 ***	327.7	14.47 ***
$Log_{10}SCC$	1	3.68	0.93	0.32	0.000	18.39	0.44
Protein	1	11.75	2.15	108.1	0.51	313.1	2.6
Fat	1	19.26	5.40 *	35.88	0.88	165.7	0.09
Lactose	1	39.63	2.29	3.69	0.57	5.27	0.23
pH	1	35.37	1.26	120.7	4.29 *	117.5	8.73 **
Error	57	987.6		368.3		1320.1	

AE milks and this may be related to the increased fat and protein levels in these milks relative to the SE milk. The SL milks had the highest SCC and the lowest SCCs were seen in the SE milks. Clotting parameters measured for pH adjusted milks showed the same trends as for the unadjusted milks (data not shown), implying that the seasonal variation in pH was not a major factor in influencing milk coagulation.

Analysis of variance of compositional factors influencing milk clotting parameters was performed (Table 8.3). It was thereby confirmed that RCT, K20 and A60 were significantly affected by both calving season and stage of lactation. pH significantly affected K20 and A60 and fat significantly influenced RCT. No other influences of composition on milk clotting in this model were significant. Milk enzymological data were not included in this analysis because of the smaller number of data points for these parameters.

8.3.2. Trial A - Small scale Gouda-Meshanger type cheese batches

8.3.2.1. Cheese compositional analysis

A number of batches of small scale cheeses were made from milks from herds at different stages of lactation and mean SCCs. It can be seen from the compositional data presented in Table 8.4 that cheese fat, protein and moisture levels were only slightly affected by stage of lactation, but were greatly affected by SCC. Fat and protein levels were decreased in high SCC batches and cheese moisture was increased. Mean milk fat levels for the low SCC-mid lactation, low SCC-late lactation and high SCC-late lactation milks were 3.75, 4.38 and 3.68% respectively, with concomitant protein levels of 3.08, 3.72 and 3.58% and lactose levels of 4.55, 4.37 and 4.13%

Table 8.4 *Compositional, yield and proteolysis data for small-scale Gouda-Meshanger type cheeses made in the Viscubator from mid- and late-lactation milk of low and high SCC.*

Stage of lactation	*Mid*		*Late*		*Late*	
SCC	*<150,000/ml*		*<200,000/ml*		*>300,000/ml*	
n	10		6		4	
	Mean	SD	Mean	SD	Mean	SD
Cheese						
Protein	20.6	2.0	19.6	.75	16.7	.83
Fat	25.3	2.5	26.4	1.3	20.4	1.4
Moisture	49.1	2.5	49.5	1.2	55.0	2.9
Salt	1.50	.21	1.81	.25	1.90	.39
pH_{12d}	5.10	.04	5.10	.07	5.08	.05
Second whey						
Protein	0.66	.16	0.68	.03	0.76	.12
Fat	0.41	.12	0.42	.07	0.55	.46
Total solids	3.52	.36	4.23	.45	4.55	.44
Yield						
Y_{actual}	12.45	.41	12.78	1.49	13.6	.88
$Y_{moisture\ adjusted}$	10.28	.43	10.60	1.18	10.06	.67
Proteolysis (%WSN/TN)						
12d	7.36	.31	9.36	.80	9.96	1.2
35 d	11.29	.72	12.26	.34	13.01	1.6
70 d	16.59	1.2	16.36	2.0	21.66	.75
105 d	19.91	2.2	19.82	2.2	25.65	.72

respectively. The pH of the cheeses after 12 days maturation was unaffected by season or SCC. The lower fat and protein levels in cheese made from high SCC milk were related to increased losses of both these components in the whey. The actual yield of cheese (kg cheese/100 kg milk) was increased in late lactation and high SCC milks. However, when the effect of increasing moisture was removed by adjusting all yields on the basis of a 40% moisture cheese, it was seen that while there was increased cheese yield (in terms of milk solids converted to cheese) in the late lactation cheese, there was a lower solids yield in cheese made from high SCC milk.

From the proteolysis data presented, it can be seen that cheese made from high SCC milk appeared to give greatly increased levels of water soluble nitrogen over

ripening. The late lactation, low SCC cheeses had slightly increased levels of WSN compared to the mid-lactation control cheeses during the early stages of ripening.

8.3.2.2. Electrophoretic analysis of cheeses during ripening

Urea-PAGE examination of cheese proteolysis products shows that in high SCC cheeses there is increased α-$_{s1}$ casein breakdown and accelerated breakdown of ß-casein, with a number of proteolysis products appearing in this region (Fig. 8.2). In the low SCC cheeses there are higher levels of certain high mobility proteolysis products, but there appears to be a greater diversity of proteolysis products in the high SCC cheese. The levels of γ-caseins in all cheeses was similar, suggesting that the differences were not due to plasmin action. In the 15 week high SCC sample there was evidence of a number of very slow moving proteolysis products, previously associated with somatic cell proteinase action (see Chapters 7 and 9).

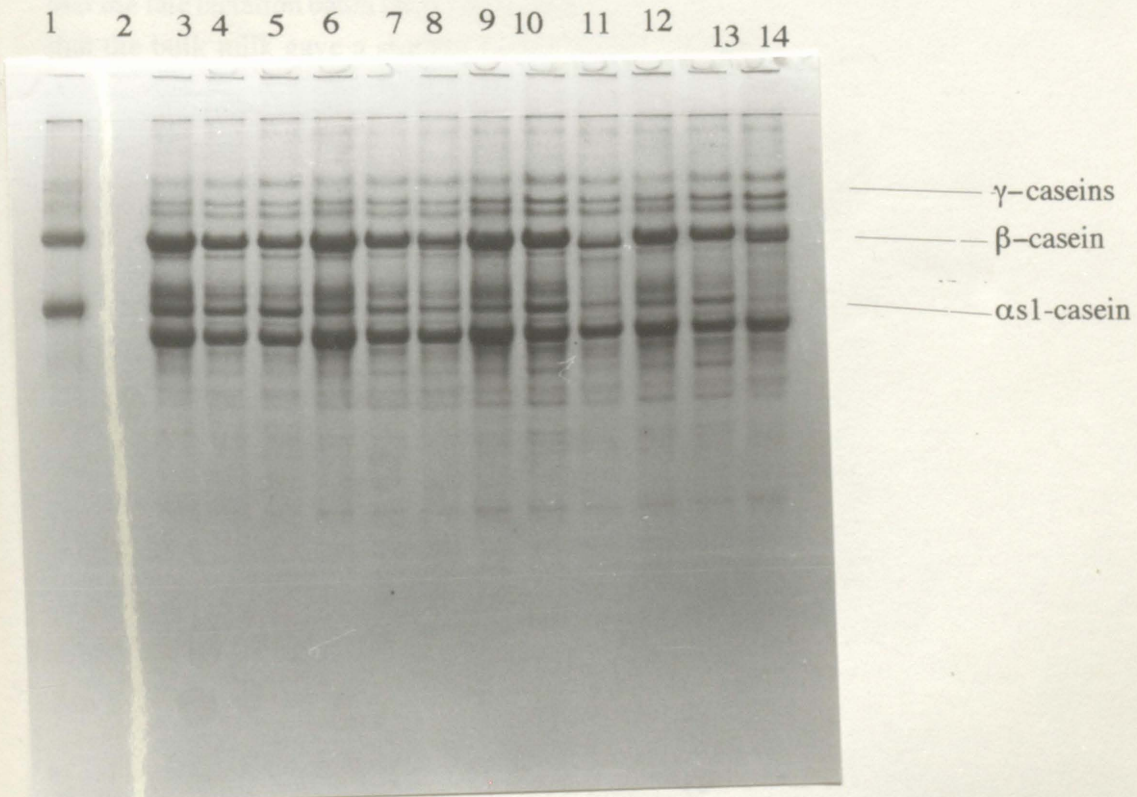


Fig. 8.2. *Urea-PAGE electrophoretograms of protein breakdown in small scale cheeses during ripening. Lane 1, sodium caseinate. Lanes 3-5, mid lactation, low SCC cheese; late lactation, low SCC cheese; late lactation, high SCC cheese, all after 12 d ripening. Lanes 6-8, as 3-5, after 5 weeks ripening. Lanes 9-11, as 3-5, after 10 weeks ripening. Lanes 12-14, as 3-5, after 15 weeks ripening.*

8.3.3 Trial B - Large scale Gouda cheese batches

8.3.3.1. Cheese compositional analysis

Three sets of cheeses were made, divided on the basis of the season and stage of lactation of the milk used. Batch B1 was made in June-July 1994 from non-seasonal creamery milk and comprised primarily early-mid lactation milk (3 vats of cheese). Batch B2 was made in August-September from mid-lactation milk from one farm whose cows were solely spring-calving (2 vats of cheese). Batch B3 was made from late lactation milk from the same farm which supplied the milk for batch B2, and comprised of 3 vats of cheese made over the period October-November. The standardised milk compositions for these milks were all similar (3.35% fat, 3.22% protein, 4.60% lactose, with lactose dropping to 4.40% in Batch B3) and the mean SCCs were 120,000 cells/ml (B1) 177,000 (B2) and 190,000 cells/ml (B3). The mean compositional analyses for these cheeses were as shown in Table 8.5. It can be seen that the late lactation batch (B3) had a higher moisture content, as may be expected, and that the bulk milk gave a slightly higher protein content in cheese, but otherwise the compositional data were similar for all batches. The moisture contents for all batches were quite high but fell into the range of Gouda moistures in Netherlands cheese factories reported by Lolkema (1992), with the exception of the late lactation cheeses.

Table 8.5 *Compositional analysis of Trial B cheeses at twelve days of age (mean ± SD of duplicate analysis on two (B2) or three (B1 and B3) cheeses)*

Batch	Moisture, %	Fat, %	Protein, %	Salt, %	pH
B1	44.5 ± 2.3	25.6 ± .6	24.0 ± 1.1	1.93 ± .2	5.35 ± .05
B2	46.3 ± 1.6	25.1 ± .9	22.2 ± .5	2.15 ± .1	5.40 ± .11
B3	48.3 ± 2.0	24.7 ± 1.1	22.7 ± .99	2.20 ± .1	5.33 ± .14

8.3.3.2. Quantitative measurement of proteolysis during cheese ripening

Levels of water soluble nitrogen (WSN), as expressed as % of total N (%WSN/TN) were measured, as were levels of free amino acids as measured by the Cadmium Ninhydrin method (expressed as $Abs_{507}nm$, converted to take account of the dilution required to get extracts from older cheeses on an equivalent scale to those from young cheeses). These results are tabulated in Table 8.6, along with the cheese pHs during ripening. It can be seen that while there was very little difference in the rates of water-soluble N production between the batches, there appeared to be a considerably lower level of free amino acids produced in the late lactation cheeses than in either batch B2 or B3, except perhaps, anomolously, at 70 days of ripening when batch B3 has a higher level of free amino acids than batch B2. Overall, the bulk milk cheeses have the highest levels of free amino acids throughout ripening.

Table 8.6 *Production of water-soluble N (% WSN/TN) and free amino acids (A507) and change in cheese pH in trial B cheeses during ripening (means ±SD for duplicate (%WSN/TN and pH) or triplicate (abs_{507}) analyses on two (B2) or three (B1, B3) cheeses). n.d. not determined.*

Age of cheese (days)		12	35	70	105
Batch/Parameter					
B1	%WSN/TN	8.97 ± .95	14.09 ± .57	19.81 ± 1.3	20.64 ± .93
	Abs_{507}	n.d.	4.21 ± .20	6.26 ± 1.4	8.36 ± 1.79
	pH	5.35 ± .05	5.42 ± .03	5.53 ± .03	5.62 ± .08
B2	%WSN/TN	10.70 ± .26	15.19 ± .66	20.23 ± 2.8	20.65 ± 1.2
	Abs_{507}	1.49 ± .06	3.14 ± .33	4.70 ± 1.37	6.82 ± 1.08
	pH	5.40 ± .11	5.49 ± .11	5.56 ± .18	5.70 ± .02
B3	%WSN/TN	9.75 ± .99	13.22 ± .53	18.98 ± 1.9	20.48 ± .66
	Abs_{507}	1.32 ± .16	2.34 ± .28	5.00 ± .37	4.81 ± .57
	pH	5.33 ± .14	5.49 ± .14	5.57 ± .14	5.50 ± .16

8.3.3.3. Organoleptic assessment of cheeses during ripening

Table 8.7 *Taste panel scores for trial B cheeses during ripening (means ±SD for 6-10 scores for two (B2) or three (B1, B3) cheeses). A score of 1 is least desirable, 5 is most desirable.*

Age of cheese (days)		35	70	105
Batch	*Characteristic*			
B1	Colour	4.30 ± .67	4.12 ± .38	3.97 ± .09
	Flavour	3.75 ± .78	3.62 ± .76	3.62 ± .67
	Texture	3.60 ± .44	3.59 ± .45	3.81 ± .46
	Overall score	3.78 ± .58	3.48 ± .59	3.67 ± .61
B2	Colour	3.88 ± .25	3.88 ± .23	3.75 ± .56
	Flavour	3.50 ± .41	3.44 ± .49	3.60 ± .42
	Texture	3.31 ± .24	3.40 ± .43	3.32 ± .51
	Overall score	3.75 ± .25	3.56 ± .33	3.19 ± .96
B3	Colour	3.90 ± .21	3.65 ± .58	3.38 ± .83
	Flavour	3.06 ± .53	3.04 ± 1.03	3.42 ± .67
	Texture	3.20 ± .86	3.36 ± .80	3.31 ± .93
	Overall score	3.25 ± .68	3.35 ± .69	3.10 ± .77

The cheeses were sampled by a panel of 6-10 experienced graders after 35, 70 and 105 days of ripening and ranked on a 1-5 point scale for organoleptic characteristics. The results of these taste panels are shown in Table 8.7. The late lactation cheeses had considerably poorer flavour and texture than the other two groups of cheeses, while, in general, the mid lactation cheeses (B2) were intermediate in score between the early and late lactation batches. The flavour of the late lactation cheeses was significantly poorer ($p<0.05$) than the early lactation cheeses at 35 days of ripening. In overall desirability scores groups B1 and B2 were ranked closely while group B3 (late lactation) were consistently deemed less desirable. Ranking of bitterness scores for the cheeses (data not shown) showed that none of the cheeses were deemed significantly bitter, but there was a slight tendency towards bitterness in the late lactation cheeses after 105 days of ripening. Colour scores were marginally lower in the late lactation cheeses in general. There was a high consistency in scoring throughout the ripening period, with, perhaps surprisingly, little change in mean flavour scores as the cheeses matured.

8.3.3.4. Electrophoretic analysis of protein breakdown during cheese ripening

Urea-PAGE electrophoretic analysis of protein breakdown in the cheeses during ripening showed that breakdown of α-$_{s1}$ casein was greatest in cheeses from batch B3, those made from late lactation milk (Fig. 8.3). Breakdown of ß-casein was

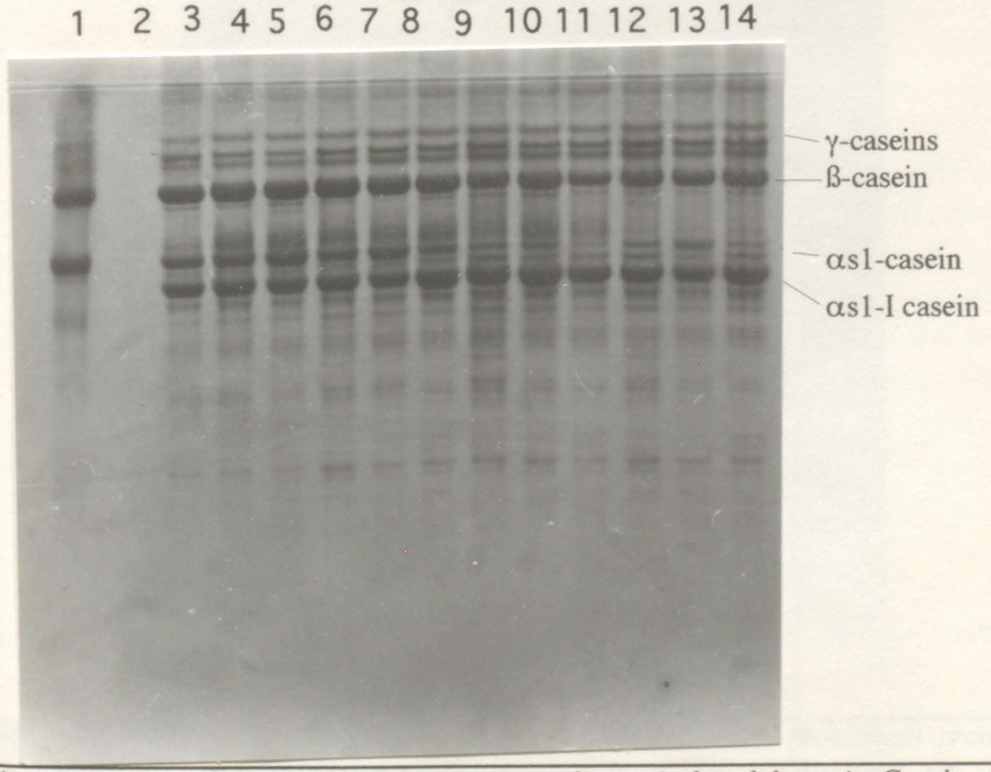


Fig. 8.3 *Urea-PAGE electrophoretogram of protein breakdown in Gouda cheeses made from early, mid and late lactation cheesemilks (B1, B2 and B3). Lane 1, sodium caseinate. Lanes 3-5, B1, B2, B3, all at 12 d ripening. Lanes 6-8, 35 d ripening. Lanes 9-11, 70 d ripening. Lanes 12-14, 105 d ripening.*

approximately equal in all cheeses, but there was a band migrating ahead of ß-casein which may be ß-I casein, which appears to be produced faster initially in the late lactation cheeses. A doublet of bands just behind the α-$_{s1}$ casein band appears to be produced faster in late lactation cheeses and subsequently degraded later in ripening. A number of fast moving proteolysis products appear to be produced faster in the early lactation cheeses (B1) at all stages of ripening, with there being quantitative and qualitative differences in the products produced.

Urea-PAGE electrophoretograms of the water-soluble nitrogen fraction (WSN) of the cheeses are shown in Fig. 8.4. From this gel it appears that there are marginally higher levels of water-soluble proteolysis products in cheeses at increasing stages of lactation at all stages of ripening. There are three bands above the location of ß-casein which are present in higher quantities in the later lactation cheeses. The principal differences are quantitative rather than qualitative. This could indicate that there was an accumulation of certain larger peptides in the late lactation cheeses which were further broken down in the early lactation cheeses to small peptides and amino acids, which would not be visible on an electrophoresis gel. This could result in overall similar measured levels of WSN, but higher free amino acid levels, as detected by the cadmium ninhydrin reagent, in the early lactation cheeses.

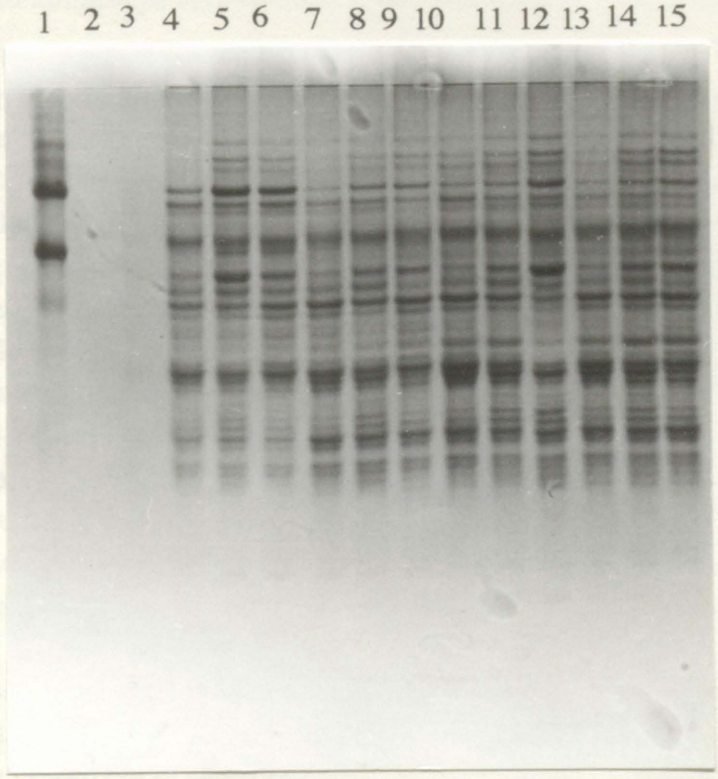


Fig. 8.4 *Urea-PAGE electrophoretogram of water-soluble proteolysis products in Gouda cheeses made from early, mid and late lactation cheesemilks (B1, B2 and B3). Lane 1, sodium caseinate. Lanes 4-6, B1, B2, B3, all at 12 d ripening. Lanes 7-9, 35 d ripening. Lanes 10-12, 70 d ripening. Lanes 13-15, 105 d ripening.*

8.3.3.5. HPLC analysis of proteolysis products in cheeses

Reversed phase HPLC (RP-HPLC) analyses of UF-permeable fractions of the water soluble extracts of all cheeses were prepared. This was in order to examine the production of small proteolysis products (small peptides and amino acids), which would not be visible on electrophoretograms or HPLCs of WSN. RP-HPLC chromatograms of batches B1 and B3 cheese UF-permeable nitrogen fractions at 10 and 15 weeks of ripening are presented in Fig. 8.5. It can be seen that at both 10 or 15 weeks there was an overall lower level and less diversity of small proteolysis products in the late lactation cheeses compared to the early-mid lactation cheeses. In the early-mid lactation cheeses there is an increase in complexity between 10 and 15 weeks, indicating continued production and accumulation of these products, while less change is apparent in the late lactation chromatograms. This would be in close agreement with the measurements of free amino acid production in the cheeses at this time, assessed using the cadmium ninhydrin reagent (see Table 8.6).

8.4 DISCUSSION

8.4.1. Relationships between milk composition and clotting properties

The Formagraph results for milk clotting properties showed high correlations between the three clotting properties measured, rennet clotting time (RCT), rate of curd formation (K20) and curd firmness (A60), suggesting that milks of poor coagulation properties should show clotting difficulties visible from any of the three measurements. The relationship between RCT and K20 is as expected as K20 depends on clotting time, with samples taking longer times to clot requiring longer times to reach the prescribed curd firmness (Bastian *et al.*, 1991a). The strongly negative correlation between RCT and A60 was as reported by Okigbo *et al.* (1985a). The relationship between A60 and K20 was visible from the correlations determined for milk composition, but the RCT was not influenced to the same degree as the other two parameters by any measured milk constituent or property, which is unexpected given the high degrees of correlation between RCT and both K20 and A60. Thus, in practice, whether a milk has a high or low rennet clotting time may not necessarily be predictive of subsequent curd formation and firmness.

The positive influence of milk fat on curd formation rate and curd firmness were in contrast with the findings of Politis and Ng-Kwai-Hang (1988c) and Marziali and Ng-Kwai-Hang (1986) but in agreement with those of Bastian *et al.* (1991a). Aleandri *et al.* (1989) found that fat level in milk was closely related to curd firmness and was important in determining cheese yielding capacity of milk. Increased lactose levels in milk were found to increase RCT slightly, decrease K20 (improved curd formation) and increase final curd firmness. Politis and NgKwai-Hang (1988c) found that lactose was

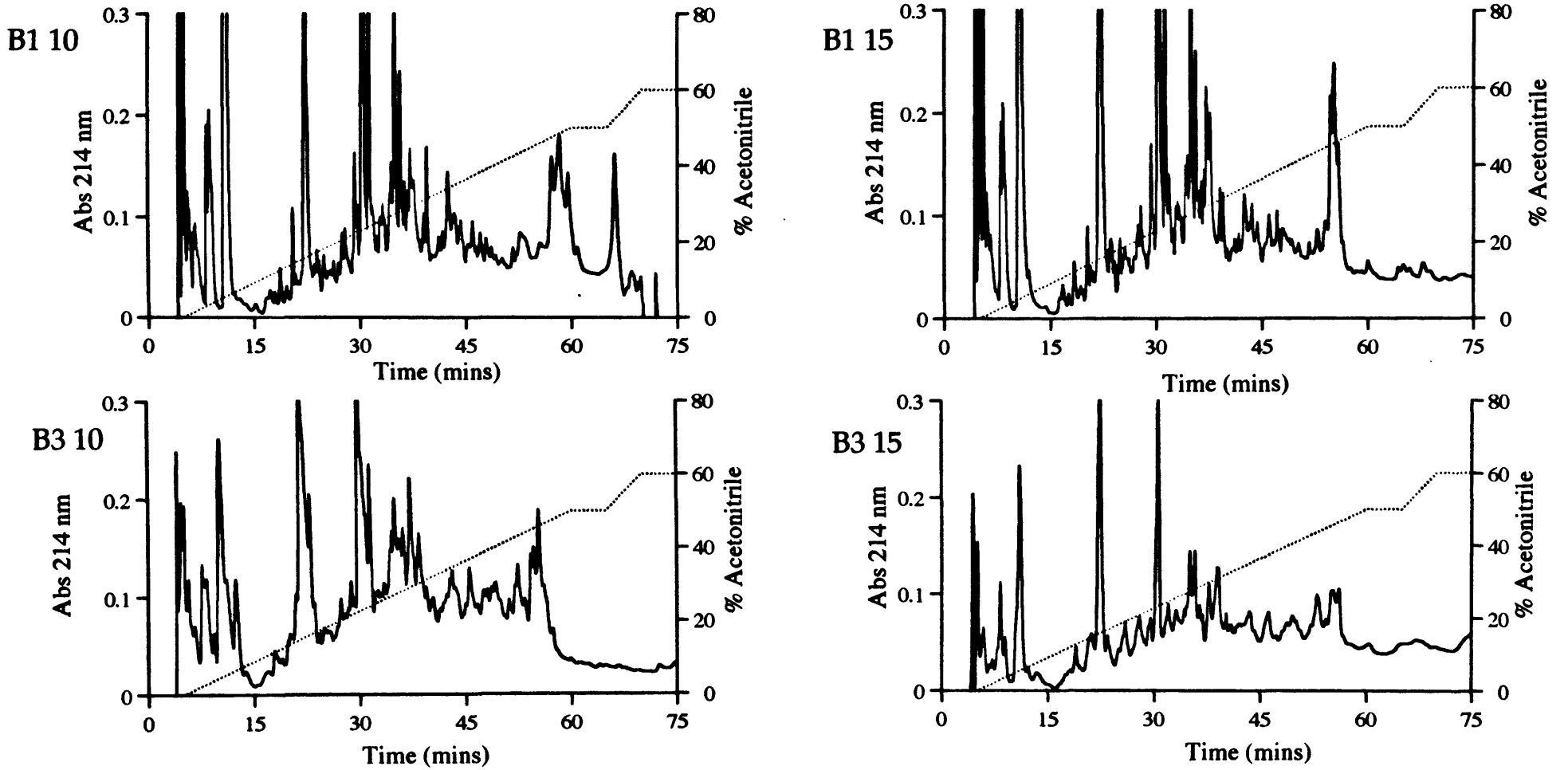


Fig. 8.6 *RP-HPLC chromatograms of UF-permeable water soluble material from cheeses B1 and B3 at 10 and 15 weeks of ripening. Dotted line is acetonitrile gradient*

slightly inversely related to RCT, and found non-significant relationships between lactose and K20 and A60.

Total protein was found to have a slight negative effect on RCT and to be significantly correlated to K20 (negative correlation) and A60 (positive correlation). Bastian *et al.* (1991a) found that increasing milk protein decreased RCT and K20 and increased A60, exactly as found here. The protein most likely to affect RCT was found by Marziali and Ng-Kwai-Hang (1986) to be α-$_{s1}$ casein, while all the caseins and α-lactalbumin significantly affected K20 and A60. Negative correlations of total casein with K20 and positive correlations with A60 were reported by Okigbo *et al.* (1985a) and Politis and Ng-Kwai-Hang (1988c). RCT was found to be less significantly affected by any measured parameter than K20 or A60. Factors previously found to affect RCT were pH (inverse relationship, Bastian *et al.*, 1991a; Okigbo *et al.*, 1985c; Grandison *et al.*, 1985), lactose (inverse relationship, Politis and Ng-Kwai-Hang, 1988c) and stage of lactation (direct relationship, Okigbo *et al.*, 1985a). The non-significant negative correlation between pH and RCT is thus as expected, but the effect of lactose on RCT may have been confounded by lactational variability within the samples tested.

Increasing somatic cell count (SCC) was found to lower RCT and K20 and increase A60. RCT has been found to be positively correlated with SCC in high SCC milks (Mitchell *et al.*, 1986b) but to have little effect in surveys of milks under 500,000 cells/ml (Politis and Ng-Kwai-Hang, 1988c; Ali *et al.* ,1980; Marziali and Ng-Kwai-Hang, 1986; Okigbo *et al.*, 1985a). Thus, the slight decrease in RCT with increasing SCC is of no significance. K20 was lowered in samples of increasing SCC and the effect of SCC on A60 was not significant. This concurs with the findings of Politis and Ng-Kwai-Hang (1988c), Bastian *et al.* (1991a) and Marziali and Ng-Kwai-Hang (1986) who found SCC to have no significant effect on K20 or A60 at SCCs below 500,000 cells/ml. Overall, it appears that at the mean SCC of the samples examined (321 ± 25 (SE)) the increased SCCs were associated with increased fat and protein levels which would improve the clotting properties, and there was little indication of negative influences on clotting associated with very high SCCs. These would include decreased casein content, increased blood components and accumulation of plasmin degradation products (Politis and Ng-Kwai-Hang, 1988c). It is possible that at high SCC ranges there would have been a greater effect of SCC on milk clotting. The steady decrease in lactose with increasing SCCs appears to actually help curd formation, as measured by K20. It appears that the increases in milk pH were not sufficient to adversely affect milk coagulation properties. It appeared that PMN levels in milk were not significantly associated with any of the milk clotting properties measured.

An influence of plasmin activity on rennet coagulation was suggested by Okigbo *et al.* (1985b), who found decreased levels of ß-casein and increased levels of γ-caseins in poor coagulating milk samples, suggestive of increased plasmin activity. α-$_{s1}$ casein levels were also found to be related to milk clotting properties. The effect of plasmin activity on milk coagulation was also thought to be detrimental by Politis and Ng-Kwai-Hang (1988c), due to accumulation of proteolysis products and hydrolysis of ß-casein,

which is essential for curd firming. In the present study, increasing milk plasmin activity was not significantly correlated with any milk clotting parameters, which is more in agreement with the findings of Bastian *et al.* (1991a) and Pearse *et al.* (1986) who found that extensive degradation of casein by plasmin did not impede curd formation. The former authors postulated that higher plasmin activities than those seen may have influenced clotting properties. The significant positive correlation between $\log_{10}$SCC and plasmin activity was as found by Politis *et al.* (1989a). Initial protein breakdown was found to have no significant effect on clotting properties, but milk proteolytic activity, as measured by the free amino group level attained on subsequent incubation, was seen to have apparently (non-significant) beneficial effects on milk clotting. This proteolysis was found to be more highly correlated with plasmin activity than $\log_{10}$SCC, as has been found previously for samples of mean SCCs lower than 500,000 cells/ml (Chapter 5). However, the relationship between proteolysis and clotting was only determined for a small number of samples of relatively low SCC and plasmin activity and thus this requires further investigation, to resolve the role of plasmin in affecting milk suitability for cheesemaking.

8.4.2 The influence of seasonality on compositional and clotting properties of bulk milks

When the data were collated on the basis of herd calving season and stage of lactation, it was seen that winter milks, whether from spring or autumn calving herds, had higher fat and protein and lower lactose levels than spring calving early lactation milks. Lucey and Fox (1992) found that the differences in composition between mid and late lactation milks were not as pronounced for late lactation milks on a high plane of nutrition. Phelan *et al.* (1982) found that fat and lactose levels were affected by stage of lactation more than season, while total protein was affected more by season, being consistently higher in autumn calving herds than in spring calving herds. The results presented here show the expected seasonal trends for spring calving herds, but show autumn calving early lactation milk to have a composition closer to spring calving late lactation milk. The late lactation fat and protein levels reported here are lower than those reported by Lucey and Fox (1992) for two spring calving late lactation herds (4.81% and 3.98% respectively) and closer to those reported for those authors for late lactation herds on a high plane of nutrition, implying that the nutritional status and milk quality of the herd bulk tanks sampled was high, probably accounting for the small differences found between these milks and the autumn calving early-lactation milks. These milks were taken at the same time as the autumn calving early lactation milk, so there was parity in environmental and seasonal factors, so relative planes of nutrition must account for the similarity in results. The spring calving early lactation milks were taken in April and had compositions similar to those reported by Lucey and Fox (1992) for mid-lactation milk, and were sampled close to the months for minimum fat and protein content as reported for Irish milks by Phelan *et al.* (1982), and thus, were as , expected. The pHs were in the range expected for good quality milks and the generally

higher pH levels in winter milk were similar to those reported by Phelan *et al.* (1982). In summary, it must be concluded that the late lactation herds were on a high plane of nutrition, and did not show exaggerated late lactation characteristics as regards gross composition. The idea that high planes of nutrition can favourably improve the quality of late lactation milk was proposed by Kefford *et al.* (1992) and Lucey and Fox (1992).

Winter milks were found to have shorter RCTs, lower K20s and higher curd firmness than spring milks and shortest RCTs were in autumn calving early lactation milks. Okigbo *et al.* (1985a) found that late lactation RCTs were increased and the same held for winter milks in general, which is in contrast with the findings here. Bastian *et al.* (1991a) found that winter milks had increased RCTs, K20s and A60s relative to spring milks, which, except for the values for A60, is also in contrast with the results found here. The same authors also found that in late lactation milks, while RCT was decreased relative to mid lactation milks, K20 and A60 were unaffected. This was found to be true here relative to autumn calving milks but not the case for spring calving early-mid lactation milks. The findings here that winter milks had higher fat and protein levels, which would decrease K20 and increase A60 were obviously confounding factors in these figures, and it is difficult to say what the true effect of season and stage of lactation is from this data. However, the analysis of variance model employed (Table 8.3) showed that while season and stage of lactation influenced clotting properties significantly, only fat affected RCT significantly, and thus the meaning of these results remains unclear. It can only be surmised that, as the compositions of the milks were not as expected for late lactation milk, and the herds were concluded to have a good nutritional status and husbandry practices, the alterations in clotting properties were also not as seen in previous studies of late lactation milk.

The late lactation SCCs were considerably elevated relative to spring calving early lactation milk, but were close to those of the autumn calving early lactation milk. There was no comparable study which showed the effects of nutritional status on SCCs, and the significance of this with regard to clotting properties is unknown, except that for the total group of cows sampled here, SCC appeared to be favourably related to clotting properties. Thus, it would appear that the range of total SCCs was not enough to cause detectable clotting defects.

However, it must be stated that there were a number of factors associated with late lactation milk which may influence the clotting properties of such milks, which were not measured in this study. These include the levels of soluble and colloidal calcium and the levels of whey proteins and caseins in the milk. In summary, there may be a balance in effects of increasing SCCs, with increasing SCCs to a certain point being sufficiently related to fat and protein in the milk to actually improve clotting characteristics. Above this point, however, decreased casein content, increased pH and other factors outweigh the compositional changes and the cheesemaking quality of the milk drops. Further work is required to establish this cut-off level.

8.4.3. Seasonality, SCC and small scale cheese manufacture and quality

Noordhollandaise-Meshanger cheese is a variety which, while similar to Gouda, is characterised by changing physical and chemical conditions during ripening, principally caused by loss of moisture from the cheese. Thus local conditions in the cheese may be more favourable to milk protease activity than those found in typical Gouda or Cheddar cheese. Cheeses made using this recipe show high proteolytic degradation during ripening, and were thus proposed as a model for studying milk protease activity in Dutch-type cheeses (Noomen, 1978). The cheeses prepared in the Viscubator, while made following a Gouda recipe, had a high moisture, and dried out somewhat during ripening, and thus were similar to a cross between a Gouda-type and Meshanger-type cheese. The batches prepared and described in Table 8.4 were a preliminary examination of the effects of SCC and seasonality on cheese quality and proteolysis.

It can be seen that cheeses prepared from high SCC milks had higher moisture levels and lower fat and protein contents concomitant with increased losses of both of these components in the whey. This is in agreement with the findings of Ali *et al.* (1980), Grandison and Ford (1986), Politis and Ng-Kwai-Hang (1988 a,b) and Barbano *et al.* (1991), regarding the influence of SCC on the composition of Cheddar cheese. Politis and Ng-Kwai-Hang (1988a) found that an increase in SCC from 100,000 to above 1,000,000 cells/ml resulted in a cheese containing 6.8 and 3.6% less fat and protein respectively and 4.9% more moisture, while those differences found here between the late lactation low and high SCC cheese batches were 22.7, 14.8 and 11.1% respectively. These are also higher than the figures quoted by Barbano *et al.* (1991) for milks of increasing SCC but this may be due to the confounding effect of stage of lactation, in that the cheesemaking functionality of the milk was already impaired by the late lactation nature of the milk (O'Keeffe, 1984). The milk composition was also altered as would be expected in late lactation milk, with elevated levels of protein and decreased levels of lactose, which will obviously further influence the composition of the cheese. The increased salt content of the high SCC cheeses is as found by Grandison and Ford (1986).

The actual cheese yield was increased in high SCC cheesemilks, as found by Grandison and Ford (1986) and Aleandri *et al.* (1989), which is due to increased fat and protein in high SCC milks. However, the moisture content is also very much increased, and when this was corrected for by adjustment of yields to constant moisture, it is found that the high SCC milks gave a lower conversion of fat and casein to cheese than the low SCC milks, as shown by increased losses of both in whey. This was also found by Barbano *et al.* (1991). It is recognised that the relationship between SCC and cheese yield is complex, as differences between individual cows and herds and the influences of stress and mastitis on SCC make prediction of cheese yield based on this parameter alone difficult (Lawrence, 1993). However, there is definite evidence that cheese yield may be increased by improving herd health and reducing SCC. The decreased cheese yielding capacity of late lactation milk is associated with poor

syneresis due to proteolysis of ß-casein by plasmin (Barbano *et al.*, 1991), but contested the findings of Pearse *et al.* (1986) and others. Thus the combination of SCC and plasmin activity in the different milks may have affected conversion of solids to cheese but this requires further investigation. Aleandri *et al.* (1989) stated that the formagraph measure of curd firmness was related to cheese yield as an indicator of casein aggregation properties of the milk, and the increased A60 in the spring calving late lactation milk may be reflective of this.

The rate of formation of water-soluble nitrogen (WSN) in the cheese appeared to be independent of stage of lactation in the low SCC samples, but appeared considerably faster in the high SCC late lactation sample. This is assumed to be due to the increased plasmin content of the milk and the increased moisture content of the cheese. However, electophoretic analysis suggested that the action of proteolytic enzymes in the high SCC cheeses, while greater than in the low SCC cheeses, was not necessarily due to plasmin action, with evidence of somatic-cell associated proteolysis products. The order of proteolysis during ripening in all cheeses, α_{s1}-casein faster than ß-casein, with formation of α_{s1}-I casein, is in agreement with the findings of deJong (1976) for this type of cheese. The results concerning degree of proteolysis are generally in agreement with those of Rogers and Mitchell (1994), who found that SCC was positively correlated to TCA- and PTA-soluble nitrogen in Cheddar cheese at 3 months of age. These authors also found negative correlation between $\log_{10}$SCC and textural properties of cheese, such as texture grade, fracturability, cohesiveness and springiness. If cheese texture is highly dependant on α_{s1} -casein integrity, as postulated by Creamer and Olson (1982), breakdown of this protein in cheese made from high SCC milk would adversely affect cheese texture and quality.

Noomen (1977) suggested that in this type of cheese, the degree and nature of protein breakdown in the cheese could allow the softening of cheese texture of cheese, but that this softening would only occur if suitable chemical and physical conditions existed (such as pH and moisture). The same author also stated that cheese moisture was critical in determining the retention of rennet in the cheese, and this must be taken into account when comparing the proteolytic breakdown of these cheeses, made from low and high SCCs. However, the presence of at least one non-rennet derived product (above the γ-caseins), which is associated with somatic cell proteinases and the possible synergistic effect of many somatic cell proteinases with chymosin in breakdown of α_{s1}-casein (e.g., cathepsin D, McSweeney *et al.*, 1995) leads one to conclude that the role of somatic cell proteinases in the ripening of this type of cheese cannot be neglected and requires further investigation.

8.4.4. A comparison of the ripening properties of Gouda cheeses made from early-mid, mid and late lactation bulk cheesemilks.

Due to the small scale nature of the cheeses made in Trial A, and the fact that the effects of SCC and stage of lactation were not separated, a further trial on large scale batches of normal Gouda cheese was carried out, to assess the role of stage of lactation on cheesemaking, proteolysis during ripening and organoleptic properties. All batches of cheesemilks were from spring calving herds.

The batches of cheese, made each from 100 gallons of milk, had slightly higher moisture than normal Gouda cheeses, and the moisture was seen to rise in late lactation cheeses. The latter observation is in agreement with the findings of O'Keeffe (1984) who compared cheeses made from late lactation milk from spring calving herds to cheeses made from autumn calving late lactation milk, retail and manufacturing milks at the same time. The changes in milk composition which this author associated with high cheese moisture were high natural milk pH, reduced casein as a percentage of total casein (due to proteolysis and/or increased whey protein content) and elevated serum casein content. Milk SCC was shown to influence both milk proteolytic activity and Cheddar cheese moisture by Barbano *et al.* (1991), with increased moisture for cheese made from milk of >127,000 cells/ml, so the influence of the minor increases in SCC seen in this study, on cheese moisture cannot be ruled out.

Donnelly *et al.* (1984) found that Cheddar cheese made from milk which had been treated with trypsin had higher moisture content than cheese made from untreated milk and concluded that proteolysis of casein by native milk proteinase (plasmin) was an important factor determining moisture levels and quality of late lactation cheese. They postulated, however, that this difference may only be of significance where there was a very pronounced stage-of-lactation effect on milk composition. Although plasmin levels were not measured in the milks from which these cheeses were made, late lactation spring calving milks of relatively low SCCs have been found to have greatly increased levels of plasmin (Chapter 5). However, the degradation of ß-casein was not significantly faster in the late lactation cheeses made in this study, and any effects of plasmin on cheese quality and moisture were not apparent from this trial.

Overall, the changes in composition in cheese made from late lactation milk were as expected from the work of Politis and Ng-Kwai-Hang (1988a) on cheese composition as affected by SCC. The pHs at 12 days were marginally high, according to the figure of 5.15-5.20 quoted by Lolkema (1993), and rose steadily over ripening.

Water soluble nitrogen levels in the three batches of cheese all increased over the ripening period but there was very little differences between batches. This was as found for the low SCC mid and late lactation cheeses described earlier. The activity of milk proteinases, which are elevated in late lactation milk and cheese, in producing soluble nitrogen (SN), was shown by Visser (1977c) to be minor compared to that of starter and rennet. Thus, it may be expected that the overall rate of protein breakdown would be relatively unaffected by changing plasmin levels and moderate increases in SCC in late lactation milk. The levels of free amino acids, however, were consistently

lower in the batch B3 cheeses than those in B1, with batch B2 being somewhat intermediate at most stages of ripening. This reduced production of small late products of proteolysis was confirmed by HPLC. In normal Gouda cheese, rennet action stimulates starter bacterial enzymes to accumulate amino acids and low MW peptides (Visser, 1977c). The starter bacterial enzymes are the only agents capable of liberating important amounts of amino acids, with 'bitter' strains being less capable of degrading bitter peptides to non-bitter peptides and free amino acids.

Thus it appears that this degradation pathway is being in some way impaired in late lactation cheeses, and this may be linked to the defect of bitterness frequently associated with such cheeses. One possible explanation for this would be increased presence of proteinase inhibitors such as α_1-antitrypsin in late lactation milk (Sandholm *et al.*, 1984, Emanuelson *et al.*, 1988) which although generally lost in the whey, may be trapped in high moisture cheeses to a sufficient extent to inhibit the breakdown of polypeptides by starter enzymes. Protein breakdown in Cheddar cheese, for example, was impaired when the plasmin inhibitor 6-aminohexanoic acid was added to curds (Farkye and Fox, 1991). Alternatively, it may be that enzymes, such as those from somatic cells or specific types of somatic cells, which are present in greater quantities in late lactation milk, may produce polypeptides or peptides which cannot be degraded by starter enzymes and accumulate in the cheese during ripening (Chapters 6 and 9). The finding of Rogers and Mitchell (1994) that SCC was positively correlated to cheese PTA-soluble N content suggests that this may be a seasonal rather than directly cell-associated effect.

Urea-PAGE examination of protein breakdown in cheeses during ripening shows higher quantities of a number of polypeptides being produced in batch B1 relative to batch B3, while overall α_{s1}-casein breakdown is accelerated in late lactation cheeses. This may suggest that alternative pathways are being followed in late lactation cheese, and as α_{s1}-casein is regarded as the source of SN proteolysis precursors in Gouda cheese (Exterkate, 1987), this may have a significant effect on later proteolysis and free amino acid production. The overall faster breakdown of α_{s1}-casein than ß-casein is as described for Gouda cheese by Visser and deGroot-Mostert (1977). The accelerated breakdown of α_{s1}-casein was seen in the previously described cheese trial and possible reasons for the breakdown are discussed in Section 8.4.3. The WSN gel also suggests that certain peptides are accumulating in the late lactation cheeses, but differences are relatively minor. Detailed examination of the breakdown products of late lactation milk is required to resolve this issue.

With regard to organoleptic properties of cheeses, accelerated breakdown of α_{s1}-casein may be linked to the decreased texture scores accorded to late lactation cheeses during ripening (Creamer and Olson, 1982), and may be linked to the textural defects associated with late lactation milk, such as short or pasty texture. While significant bitterness was not detected in any of the cheeses during ripening, it may be possible that impaired breakdown of peptides by starter enzymes may lead to accumulation of bitter peptides, and lead to the bitterness often linked to late lactation cheeses. Despite the lack of bitterness, flavour scores decreased slightly as the lactation proceeded, which

may be linked to the contribution of certain free amino acids to the taste of cheese (Aston and Creamer, 1986), and the importance of certain water soluble peptides in this respect. The quantitative differences in WSN electrophoretical patterns may thus be linked to differences in flavour scores. Overall, late lactation cheeses were less desirable than those in either of batches B1 or B2.

Finally, Grandison and Ford (1986) made cheese from milks to which different proportions of high SCC milk had been added to give modestly elevated SCC. They found that both flavour intensity and total off-flavours were positively correlated to SCC, and suggested that the quality of cheese could be impaired by the inclusion of even small quantities of high SCC milk in bulk milk for cheesemaking. In this experiment, although the late lactation cheesemilk SCC was not significantly elevated, it is probable that there was a definite contribution of high SCC milk, which occurs most frequently in late lactation, which may have thus influenced quality and ripening of the cheeses, and giving differences greater than those which would be expected for milks of apparently relatively small differences in SCC.

CHAPTER 9

Effect of plasmin and somatic cell enzymes on the ripening of normal and aseptic starter and rennet free cheeses

SUMMARY

Gouda-type cheeses were made from milk to which porcine plasmin had been added to give 2.6 and 4.7 times the normal plasmin level in the cheese. Cheeses with added plasmin had accelerated production of water soluble N (WSN), with faster degradation of ß-casein and production of γ-caseins. Amino acid production was not affected overall by plasmin addition. There were also quantitative differences in urea-PAGE electrophoretograms of cheese and WSN during ripening and it appeared that many products which were produced at the 2.6 fold level were further broken down at the 4.7 fold level. Therefore, there may be an optimum level of plasmin addition for improvement of flavour characteristics and acceleration of ripening. Organoleptically, the plasmin added cheeses were judged to have a superior flavour, texture and overall desirability during ripening. In a separate experiment aseptic, rennet and starter free (ASRF) cheeses were prepared from mastitic and good quality milks. Mastitic curds had higher proteolysis than controls over a range of pHs from 6.4 to 4.8 and had accelerated breakdown of ß- and α_{s1}-caseins, with the appearance of certain proteolysis products associated with somatic cell proteinases. The mastitic curds also had an accumulation of hydrophobic bitter material over 10 weeks ripening at 11°C, which, together with the accelerated breakdown of α_{s1}-casein may indicate a role for somatic cell proteinases in influencing cheese ripening. There was evidence of more than one acid proteinase active in the curds, and it is proposed that Cathepsin B is a proteinase in mastitic milks.

9.1 INTRODUCTION

The role of native milk proteinases in cheese ripening is ill-defined. Plasmin, the principal indigenous milk proteinase, is associated with the casein micelle and is thus incorporated into the rennet cheese curd (Grufferty and Fox, 1988c). Plasmin hydrolyses all the major caseins in milk, with the exception of κ-casein, producing hydrophobic peptides such as the γ-caseins, and plasmin has been hypothesised to contribute to production of hydrophobic bitter peptides in cheese (Le Bars and Gripon, 1989). Ollikainen and Kivelä (1989), showed that in the case of Swiss-type cheese, where the rennet is inactivated by the high cooking temperature, plasmin is the principal agent responsible for breakdown of ß-casein, and provision for substrate polypeptides for starter peptidases. Increased cooking temperatures increase plasmin activity in cheese, rennet curd and micellar casein dispersions, possibly due to conversion of plasminogen (Farkye and Fox, 1990). Since plasmin is dissociated from the casein micelles by NaCl, salting before pressing, as in the case of Cheddar cheese, may lead to loss of plasmin in press whey, accounting for the lower plasmin level found in this variety as compared to brine-salted varieties such as Swiss and Dutch cheeses (Fox, 1989).

Addition of plasmin to milk for Cheddar cheese manufacture has been shown to increase ß-casein breakdown, with a concomitant increase in γ-casein formation, and to increase water soluble-N but not phosphotungstic acid-soluble N (Farkye and Fox, 1992). These authors also found that plasmin-enriched cheeses were judged organoleptically superior to control cheeses, and that ripening was considerably accelerated. Addition of a plasmin inhibitor, 6-amino-hexanoic acid to Cheddar curd led to slower production of γ-caseins and a slower rate of increase in water-soluble N (Farkye and Fox, 1991).

In Gouda cheese, Visser *et al.* (1977 a-d) concluded that milk proteases are responsible for the production of 'minor caseins', but that their contribution to the breakdown of α_{s1}- and ß-casein was small compared to those of rennet and starter bacteria, and that, on their own, they liberate only small amounts of amino acids and low-MW peptides. Noomen (1978), examined the role of milk proteases in simulated Noordhollandse Meshanger type soft cheese, by production of rennet free cheese curd, acidified using gluconic acid-∂-lactone to a range of pHs. He found that at a high pH (e.g. 6.2), ß-casein was much more quickly degraded than α_{s1}-casein, but that at low pH (e.g. 5.4) α_{s1}-casein was more quickly degraded, which he concluded to be due to the action of an acid milk proteinase. Alkaline milk proteinase activity was found to increase with increasing ripening temperature, but was concluded to be of little importance to ripening of normal cheese, with the exception of soft cheeses with a surface flora, which would have ripening conditions which would favour the activity of alkaline milk protease.

The acid proteinase in milk is now recognised as Cathepsin D, and its specificity on α_{s1}-casein has been shown to be similar to that of chymosin (McSweeney *et al.*, 1995). It's role in cheese ripening has yet to be established. Proteolytic activity in milk is known to increase with increasing somatic cell count

(SCC) (Senyk et al, 1985: Verdi *et al.*, 1987), but the role of proteinases from somatic cells in cheese ripening also has yet to be elucidated.

The object of this study was to examine the role of added plasmin in ripening of Gouda-type cheese, and also to compare the ripening patterns of Gouda type cheeses made from milks with low and high SCC, where the milk contributed the only proteolytic agents present during ripening. This was achieved by making aseptic starter and rennet free cheeses where the coagulant, pepsin, was inactivated as part of the manufacturing process and acidulation was achieved by addition of glucono-∂-lactone.

9.2. MATERIALS AND METHODS

9.2.1 Milk source and cheesemaking

9.2.1.1. Manufacture of plasmin-added cheeses

Porcine plasmin (Fibrinolysin EC 3.4.21.7) was obtained from the Sigma Chemical Co. (Poole, U.K.) and 12.5 mg (50 units) dissolved in 90 ml distilled water. Three batches of Gouda-type cheese were manufactured in a Viscubator as outlined in section 4.1.1 with the following plasmin additions. All milks used were good quality mid-lactation milks from the college creamery.

Batch A	Five control vats
Batch B	Two vats with 15 ml Plasmin solution added to 12.5L milk
Batch C	Two vats with 30 ml Plasmin solution added to 12.5L milk

Plasmin activity in milk and cheese and cheese composition were measured as outlined in Section 4.3.7. The cheeses were allowed to ripen for 15 weeks at 11°C and proteolysis measured as described in section 4.2.3. Organoleptic evaluation was by a panel of 6-10 experienced tasters at each stage of ripening.

9.2.1.2. Manufacture of aseptic, starter and rennet-free (ASRF) cheeses

Two batches of milk were obtained for this experiment, the first being good quality creamery milk and the second being milk from a cow with clinical mastitis, identified by a local farmer. The somatic cell counts of the milks, determined by Fossomatic at U.C.C., were 210,000/ml and 2,042,000/ml respectively. ASRF cheeses were made by the following modification of the method of Noomen (1978). The method of Visser (1976) was attempted in preliminary trials, but it was found impossible to produce a suitable coagulum with mastitic milk, and thus the following protocol was developed.

To 12.5L pasteurised milk (72.5°C for 15 secs), cooled to 33°C, was added 2.4g calcium chloride and 1.25g thimerosal (sodium ethylmercurithiosalicylate, an antimicrobial agent). A 10% solution of porcine pepsin in water (11.4 mls) was added, stirred in and a clot allowed form for 45 minutes, whereupon the curd was cut, and the

pH adjusted to 6.44 using conc. lactic acid. After 15 minutes stirring, one third of the whey was drained and the curds and whey cooked to 40°C in 20 min by the addition of 60°C water (an amount equal to that removed as whey). At this point, the pH was raised to 6.70 by addition of 10N sodium hydroxide and maintained at this level and temperature by titration for 10 min. The whey was then removed, pH lowered to 6.31 with lactic acid, and the mix stirred for 30 min. The curds were then prepressed under whey for 1 hour, pressed overnight at 2.5 Bar and frozen at -20°C until use.

On defrosting the curds were ground and their moisture determined (45.1±.27% for control and 46.6±.25% for mastitic milk). Small batches (45g) of curd were then mixed with 0.675g NaCl and various amounts of gluconic acid-∂-lactone (GDL), and pressed together in 50ml plastic beakers. The amount of GDL added was calculated to obtain a range of final curd pHs and had been determined from preliminary trials, where the regression equation relating GDL and pH was determined (pH = 6.12 - 0.499 (gGDL/g curd)). A total of 28 small curds were prepared, vacuum packed and ripened at 11°C for 10 weeks (see table 9.1).

Table 9.1 *Recipes for ASRF cheeses made from good quality and mastitic milk* [a]

Cheese	Milk	Salt (g)	GDL (g)	pH
1	Control	0.0	0.0	6.43
2	Control	0.675	0.0	6.24
3	Control	0.675	0.45	5.63
4	Control	0.675	0.9	5.28
5	Control	0.675	1.5	4.98
6	Control	0.675	1.8	4.75
7	Control	0.675	2.25	4.72
8	Mastitic	0.0	0.0	6.45
9	Mastitic	0.675	0.0	6.32
10	Mastitic	0.675	0.45	5.77
11	Mastitic	0.675	0.9	5.54
12	Mastitic	0.675	1.5	5.09
13	Mastitic	0.675	1.8	5.00
14	Mastitic	0.675	2.25	4.86

[a] Each cheese represents a duplicate pair, and pHs are means of duplicate analyses on these curds. Curd quantity used in each recipe was 45g.

9.2.2 Analysis of ASRF cheeses

At 12d, 5 weeks and 10 weeks, samples of the small ASRF curds were taken and assessed for proteolysis. Small-scale water soluble nitrogen (WSN) extractions were performed and cheese samples and corresponding WSNs compared using Urea-PAGE as described in section 4.3.1. RP-HPLC was used to examine peptide profiles of cheese WSN (4.3.3).

Owing to the small scale of sampling, proteolysis was quantified in freeze dried WSN using a modification of the o-phthaldialdehyde assay for proteolysis as described by Church *et al.* (1983). To 50μl of a 50mg/ml solution of lyophilised WSN was added 2 ml of o-phthaldialdehyde (OPA) reagent (prepared daily by mixing 400mg o-phthaldialdehyde in 8 ml methanol, 250 ml 0.1 M sodium tetraborate, 25 ml 20% SDS and 100 μl mercaptoethanol, with total volume made to 500 ml with distilled water). Absorbance at 340 nm was measured after 2 min incubation at room temperature and compared to a standard curve of glycine (0-120mM).

In the case of cheeses 2, 5, 9 and 13 (mastitic and control at pH 6.3 and 5.0) a portion of one of each pair of duplicates was extracted by the method of Harwalker and Elliott (1971) to quantify hydrophobic, bitter material produced, in order to examine the role of milk enzymes in causing bitterness in cheese. 10g grated, freeze-dried (lyophilised) cheese was blended with 50 ml chloroform:methanol (2:1) in a high speed mixer, and the resulting mixture filtered. The residue was re-extracted with 30 ml solvent and the filtrates were combined and made biphasic by addition of 0.2 vols distilled water. After overnight separation, the upper methanolic layer was recovered, methanol removed by evaporation under reduced pressure, and freeze dried in weighed containers. The recovery of bitter peptides (g/g of original cheese) was calculated.

9.3 RESULTS

9.3.1 Ripening of plasmin added cheeses

9.3.1.1. Cheese Composition

The trial cheeses had compositions as shown in Table 9.2. The moistures were high for Gouda type cheese, being more like that of a Meshanger cheese, but this was a consequence of the small scale manufacture procedure followed. The milks were unstandardised, and this accounted for the relatively minor differences in fat and protein levels between batches. Average plasmin activities in milk and cheese following plasmin addition are shown in table 9.3. The cheesemilks in group B and C had 2.3 and 3.4 times more plasmin than the initial milk after the additions described leading to 2.6 and 4.7 fold increases in cheese plasmin, respectively.

Table 9.2 *Compositional analysis of control and plasmin-added Gouda-type cheeses*

Cheese	Fat, %	Protein, %	Moisture, %	Salt, %	pH
A †	25.60±1.75	21.67±2.14	50.24±1.57	1.50±.21	5.10±.06
B ‡	19.0±.65	22.80±.47	52.73±.51	1.51±.05	5.23±.03
C ‡	19.5±.5	23.24±.76	51.97±.48	1.98±.11	5.20±.02

† Means ± SD of duplicate determination on each of five cheeses

‡ Means ± SD of duplicate determination on each of two cheeses

Table 9.3 *Plasmin activity in cheesemilks and Gouda type cheeses.†*

Batch	Cheesemilk	Cheese
A	0.148	0.901
B	0.345	2.370
C	0.507	4.229

† Milk plasmin levels are means of duplicate determinations and are expressed as AMC units/ml milk. Cheese plasmin levels are means of duplicate determinations and are expressed as AMC units/g cheese.

9.3.1.2 Proteolysis

Levels of WSN in the control and plasmin added cheeses during ripening are given in Fig. 9.1, and it is apparent that addition of plasmin to cheesemilk leads to elevated levels of WSN in the later stages of ripening, but that after 70 days, while both the 2.6 and 4.7-fold increases in plasmin give higher WSN levels than the controls, the difference between the experimental cheeses B and C was less than the difference between these cheeses and cheeses in group A. Free amino acid production in the cheeses as shown in Table 9.4 was apparently unaffected generally by addition of plasmin to cheesemilk, although it appears that at the 2.6-fold level of plasmin increase,

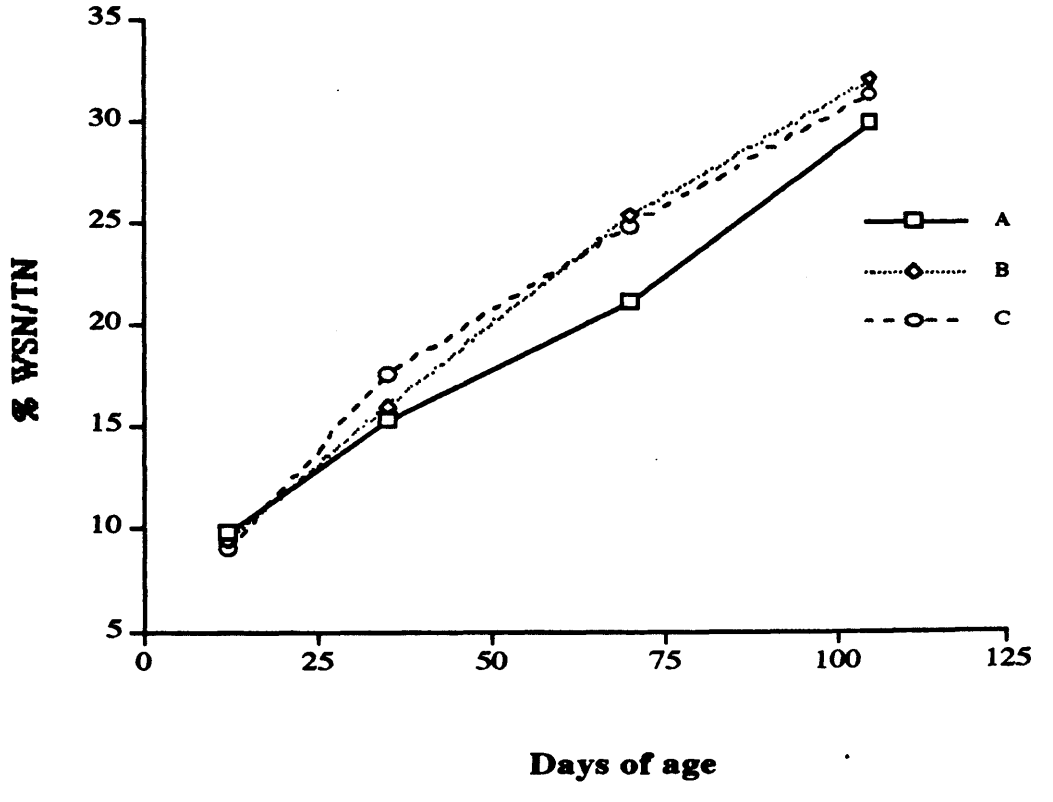


Fig. 9.1 *Levels of water soluble nitrogen as % of total nitrogen (%WSN/TN) in control (A) and plasmin added cheeses (B and C) during ripening*

Table 9.4. *Free amino acid levels in cheeses made from milks with added plasmin, and control cheeses during ripening, determined as A507nm using the Cadmium Ninhydrin reagent.*

Cheese	Days after manufacture			
	12	35	70	105
A †	0.94±.18	2.25±.45	4.95±.59	7.54±.66
B ‡	1.01±.24	2.50±.60	5.79±1.15	8.52±1.06
C ‡	0.84±.44	2.18±.23	4.59±.97	7.79±1.14

† Mean± SD of triplicate determinations on each of five cheeses

‡ Mean± SD of triplicate determinations on each of two cheeses

the levels of free amino acids are marginally higher at all stages of ripening. The fact that the group C cheeses have a level closer to that of the control batch suggests this may be unrelated to plasmin activity, or that the amino acids produced have been further degraded in these cheeses, due to the accelerated ripening induced by the addition of plasmin. This is a result which requires further verification.

Typical urea-PAGE electrophoretograms of the cheeses during ripening are shown in Fig. 9.2. It can be clearly seen that elevated plasmin levels led to greater

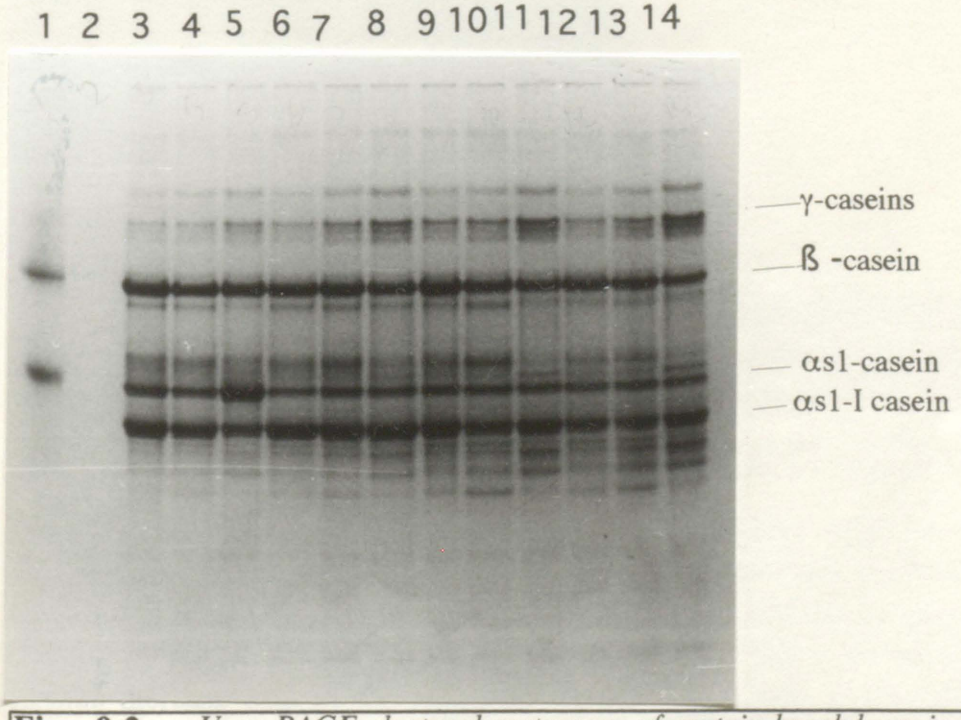


Fig. 9.2. *Urea-PAGE electrophoretogram of protein breakdown in control and plasmin added cheeses during ripening. Lane 1, sodium caseinate. Lanes 3-5, cheeses A, B, C at 12 days. Lanes 6-8, cheeses A, B, C at 5 weeks. Lanes 9-11, cheeses A, B, C at 10 weeks. Lanes 12-14, cheeses A, B, C at 15 weeks.*

degradation of ß-casein and production of γ-caseins at all stages of ripening. Overall, breakdown of α_{s1}-casein appears to be unaffected by plasmin level. At the later stages of ripening, it appears that a number of peptides accumulate in cheese B but not in cheese C, suggesting further breakdown of these products in the most highly plasmin-enriched cheeses. Other peptides are produced in increasing quantities with increasing plasmin level (e.g. the γ-caseins). Electrophoretograms of cheese WSN are shown in Fig. 9.3. It can be seen that at each stage cheese C contains the greatest diversity of proteolysis products, and has the highest levels of many products. However, again in cheese B there are a number of polypeptides produced which accumulate to greater levels than in cheese C.

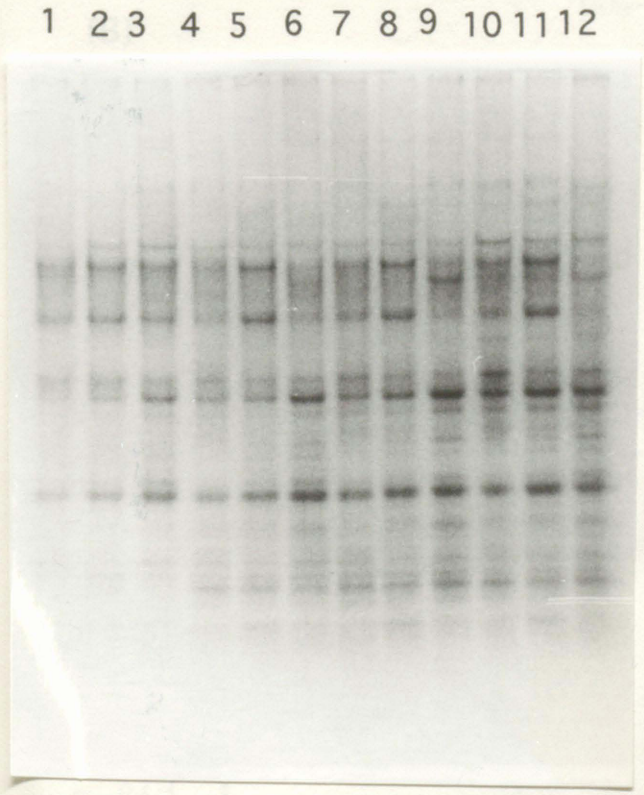


Fig. 9.3. *Urea-PAGE electrophoretogram of proteolysis products in water-soluble nitrogen (WSN) fraction of control and plasmin added cheeses during ripening. Lanes 1-3, cheeses A, B, C at 12 days. Lanes 4-6, cheeses A, B, C at 5 weeks. Lanes 7-9, cheeses A, B, C at 10 weeks. Lanes 10-12, cheeses A, B, C at 15 weeks.*

Representative RP-HPLC chromatograms of cheeses from batches A, B and C at 10 weeks of age are shown in Fig. 9.4. Once again, there are a number of products which are present in greater quantities in the control cheeses in batch A (peak 2 decreases with increasing plasmin concentration at all stages of ripening), products which increase with greater plasmin concentration (peak 3) and products which although present in greater quantities in the plasmin-enriched cheeses, are either lower in cheese C or present in similar quantities in cheeses B and C (peak 1). A number of other unlabelled peaks can be seen to vary in these ways between the three cheese types.

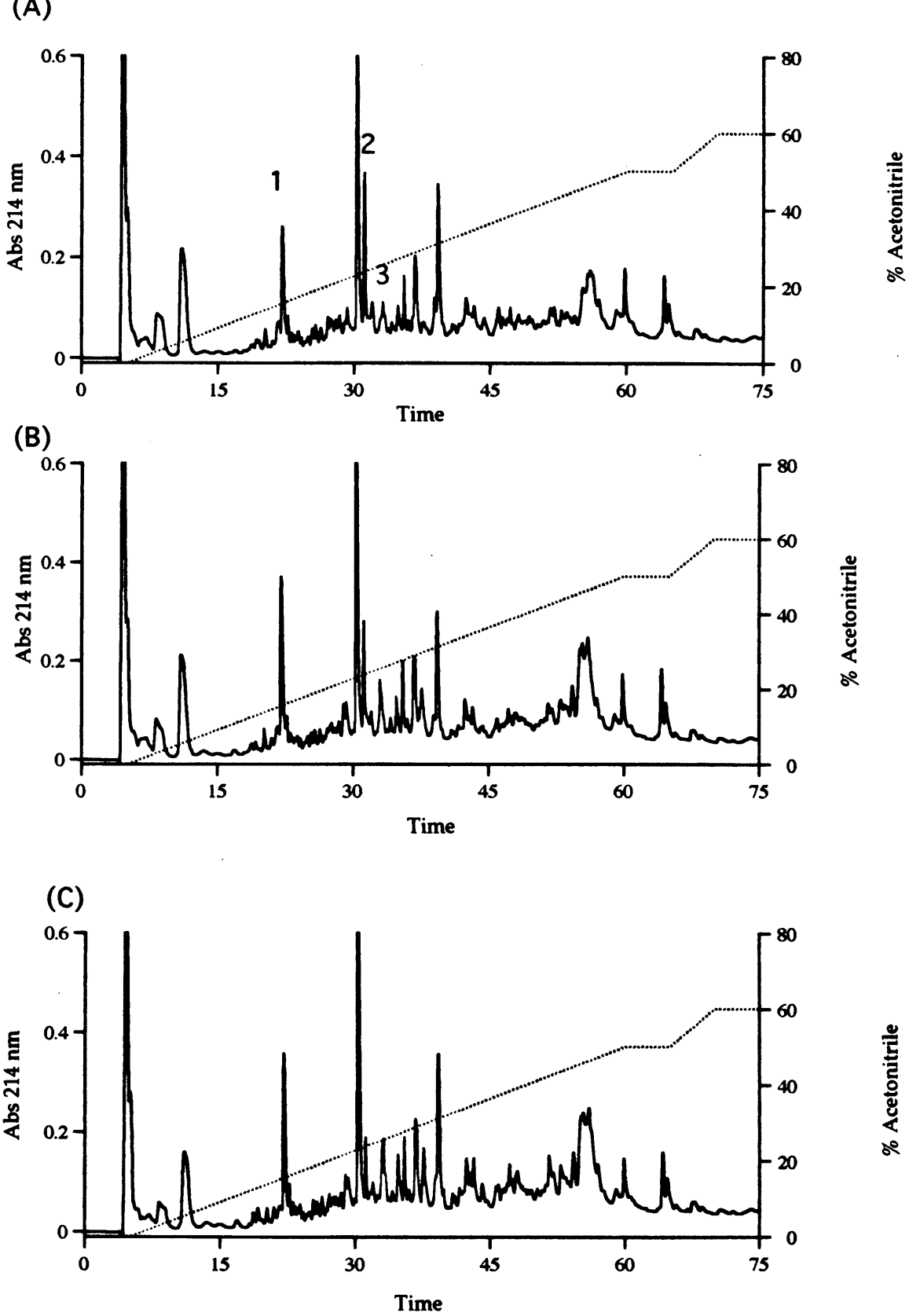


Fig. 9.4. *C8 RP-HPLC chromatograms of water-soluble N fraction of plasmin added cheeses after 10 weeks of ripening at 11°C. (A) control batch cheese (B) medium level of plasmin addition (C) high level of plasmin addition.*

Table 9.5. *Organoleptic evaluation of plasmin-added cheeses and controls during ripening (1 is least desirable score, 5 is most desirable)*

Cheese	Flavour	Texture	Overall Score
10 weeks age			
A	2.94±.86	2.66±.67	2.75±.74
B	3.42±.52	3.60±.44	3.38±.39
C	3.71±.43	3.73±.43	3.80±.33
15 weeks age			
A	3.15±.60	3.38±.47	3.21±.68
B	3.44±.9	4.00±.65	3.38±.70
C	3.84±.52	4.12±.58	3.88±.21

9.3.1.3. Organoleptic evaluation

Organoleptic evaluation of the cheeses at various stages of ripening gave the flavour, texture and overall desirability scores shown in Table 9.5. On the two occasions when taste panels were conducted, increasing plasmin level gave improved scores for all characteristics. Bitterness was not detected in any of the plasmin-enriched cheeses.

9.3.2. Aseptic starter and rennet free cheese ripening

9.3.2.1. Proteolysis during ripening

The levels of free amino groups in water soluble N in the model cheeses over ripening are plotted in Fig. 9.5. It can be seen that the proteolysis levels were higher in the cheeses prepared from high SCC, mastitic milk at all pHs, at each stage of ripening, except at pH 5.8 at 1 week of age. At 5 and 10 weeks of age, it appeared that proteolytic activity in curds prepared from good quality milk increased with increasing pH, but with a possible rise in activity at the lower end of the pH scale. This would be indicative of plasmin dominated proteolysis with trace activity of acid milk proteinase. In the mastitic curds at these times, however, there appears to be a peak of activity at around pH 5.8-6.0.

The plasmin levels in the cheeses are given in table 9.6, and are, as expected, higher in the cheeses made from mastitic milk, and lower in cheeses with a lower pH.

Table 9.6. *Plasmin levels in aseptic, starter and rennet free cheeses (AMC units/g)*

Mastitic Milk pH 5.0	0.801
Mastitic Milk pH 6.3	0.836
Control Milk pH 5.0	0.631
Control Milk pH 6.3	0.705

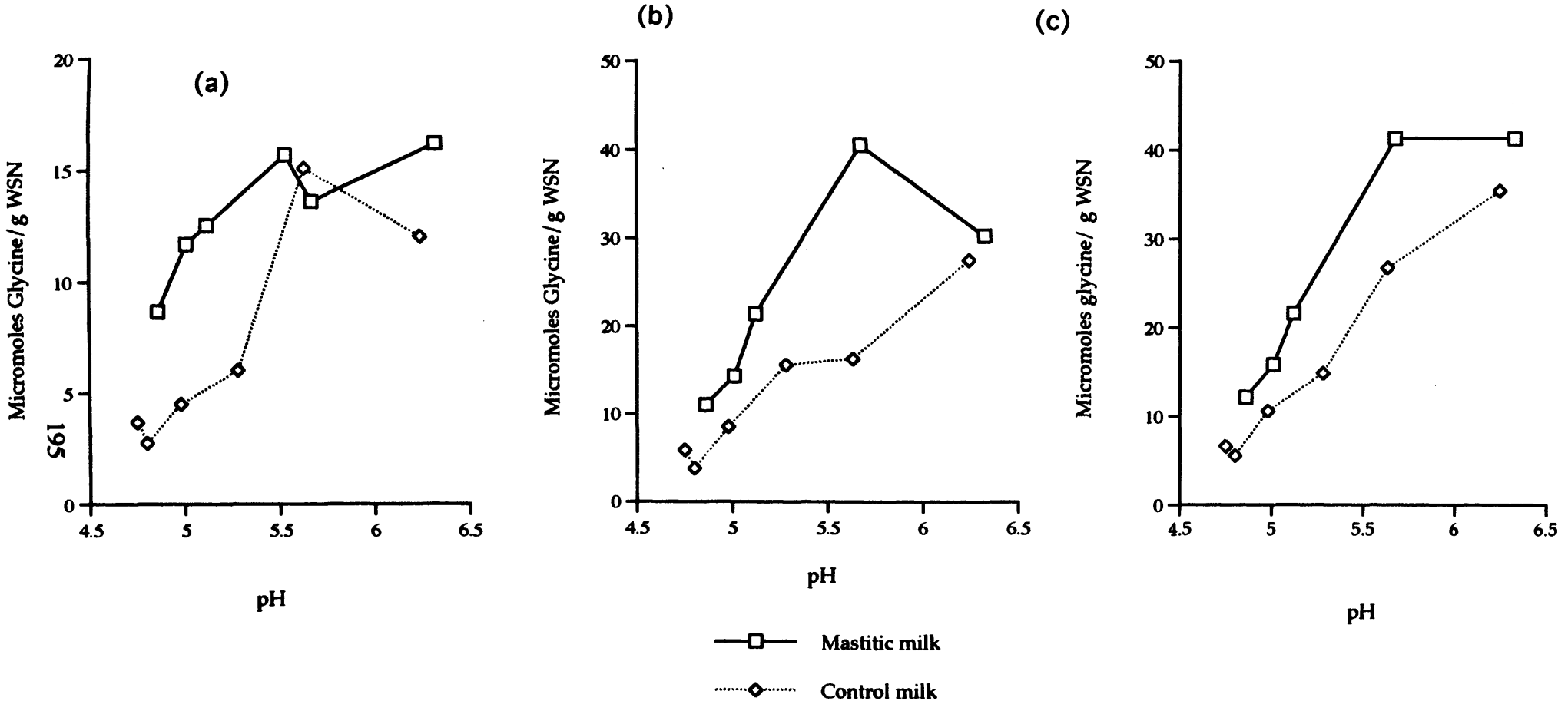


Fig. 9.5 *Levels of free amino groups (expressed as µmol Glycine/g WSN) in aseptic starter and rennet-free cheeses at 1 (a), 5 (b) and 10(c) weeks of ripening at 11°C*

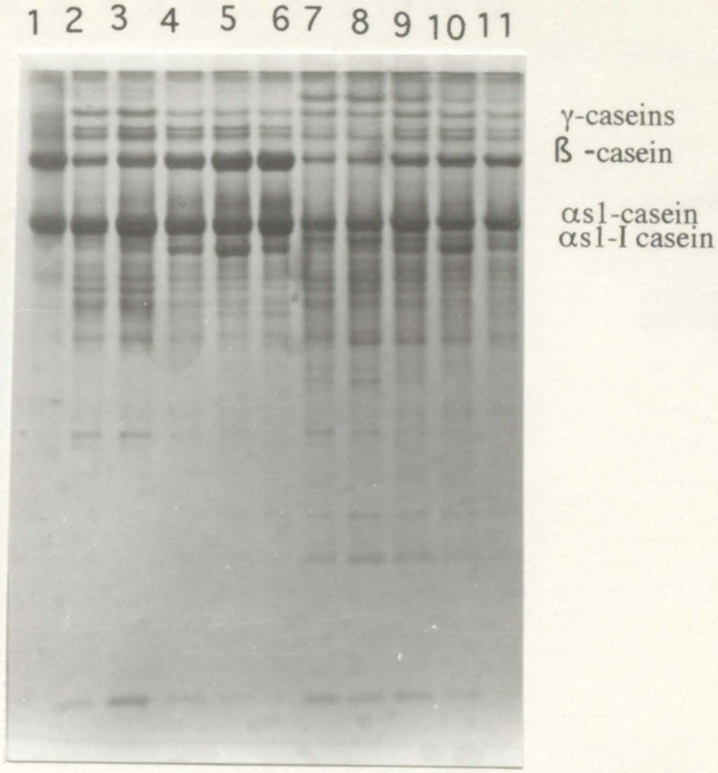


Fig. 9.6 *Urea-PAGE electrophoretograms of ASRF cheeses prepared from control and mastitic milks at 5 weeks of ripening. Lane 1, sodium caseinate. Lane 2. Control curd at pH 6.4 without NaCl added. Lanes 3-6, control curds with 1.5% NaCl at pHs 6.2, 5.6, 5.3, 4.8. Lane 7, mastitic curd at pH 6.5 without NaCl added. Lanes 8-11, mastitic curds with 1.5% NaCl at pHs 6.3, 5.8, 5.1, 4.8.*

From the electrophoretogram of the cheese protein breakdown after 5 weeks of ripening at different pHs, shown in fig. 9.6, it can be seen that there was far greater gross proteolysis in the curds prepared from mastitic milk. In the control curds γ-caseins are the principal proteolysis products, and their production decreases with pH, consistent with plasmin dominated proteolysis. α_{s1}-casein breakdown to α_{s1}-I casein is more apparent at lower pHs, and appears to be greatest at pH 5.7. In mastitic curds production of α_{s1}-I casein appears to be retarded, but overall α_{s1}- and ß-casein breakdown is considerably faster than in the controls. The α_{s1}-I casein band is definitely less pronounced below pH 5.0 than at pH 5.6, suggesting either that it is further broken down at lower pHs, or that optimum pH for it's production is

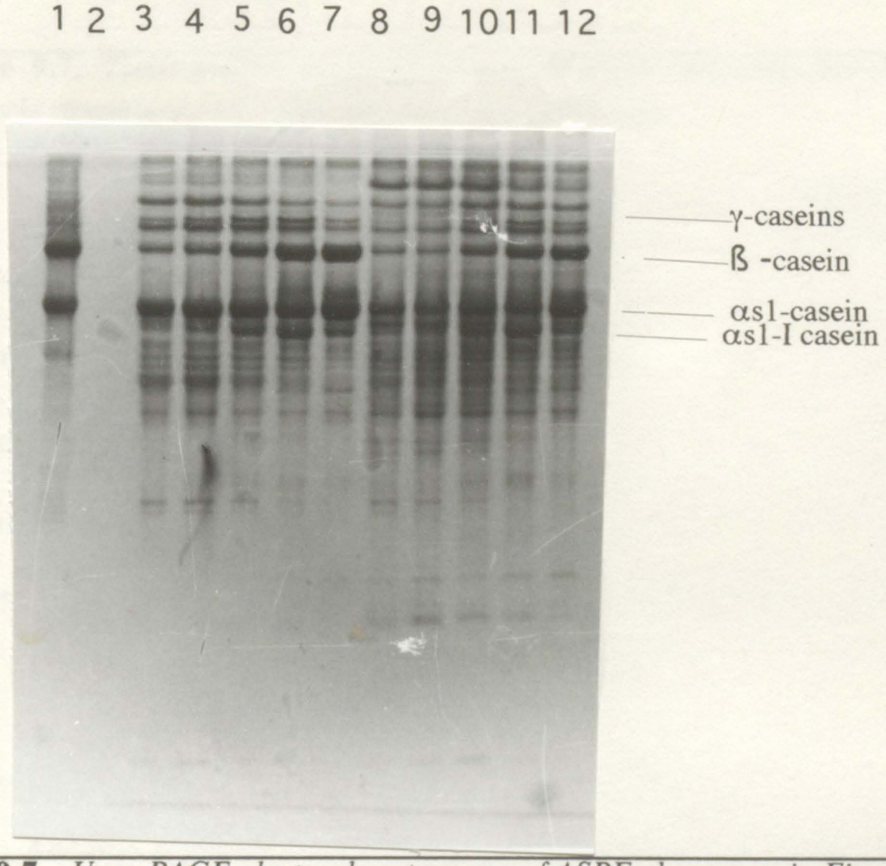


Fig. 9.7. *Urea-PAGE electrophoretograms of ASRF cheeses as in Fig. 9.6, at 10 weeks ripening. Lane 1, sodium caseinate. Lanes 3-12 as in Fig. 9.6, in order*

above 5.0. The second possibility seems most likely since there is overall less proteolysis products in the lower pH curds. There is also a distinct band above the three γ-caseins which is present in the mastitic curds only, and which is produced at the higher end of the pH gradient. Comparison of the curds prepared with or without NaCl shows that there is relatively little difference in the control cheeses but that there is slightly greater proteolysis in the presence of 5% NaCl in the curds prepared from mastitic milk. The 10 week cheese electrophoretograms (Fig. 9.7) show essentially the same picture. The γ-caseins have been further degraded in the high pH curds and the band above these products has continued to accumulate. There is also a band above the latter band which appears to be produced in the mastitic cheeses at decreasing pHs, and possibly a band between the two lower γ-caseins, produced at higher pHs. Water soluble N electrophoretograms and RP-HPLC examination of proteolysis products in these cheeses showed only a small number of products, which were present in elevated quantities in the mastitic curds (results not shown).

Production of hydrophobic, typically bitter peptide material was estimated in duplicate ripened curds, at pH 6.3 and 4.9 for both control and mastitic cheesemilks (Table 9.7). It can be seen that at 5 weeks production in all cheeses was quite low, but

approximately equal, but that by 10 weeks the peptides appeared to have broken down in the curds prepared from the control milk, while the levels had risen sharply in those prepared from mastitic milk.

Table 9.7. *Yields of bitter material from aseptic starter and rennet free cheeses over 10 weeks ripening at 11°C (calculated on a dry curd basis).*

Milk	Cheese pH	% Bitter Peptides (5 weeks)		% Bitter Peptides (10 weeks)
Control	6.3	I	1.5	0.41
		II	0.99	0.17
Control	4.9	I	0.65	0.13
		II	0.39	0.12
Mastitic	6.3	I	0.87	4.50
		II	1.12	2.87
Mastitic	4.9	I	0.39	3.56
		II	0.47	4.27

9.4 DISCUSSION

9.4.1 Plasmin added cheese ripening

Addition of plasmin to cheesemilk is known to accelerate the ripening of Cheddar cheese, increasing breakdown of ß-casein and total N in the water-soluble extract (Farkye and Fox, 1991; Farkye and Landkammer, 1992). The results of the first part of this study show similar alteration of ripening characteristics in maturation of Gouda cheese. Addition of plasmin to cheesemilk gave a greater yield of plasmin in cheese than predicted, which may be due to activation of plasminogen in the milk, initiated by addition of porcine plasmin. This requires further investigation. Farkye and Fox (1991) reported that a 30 minute incubation should be significant to ensure full binding of plasmin to casein, which was used here, but the kinetics of the binding reaction are unknown and this incubation at 30°C may have promoted plasminogen activation. Since large variations in plasmin activity has not been found to influence milk clotting properties (Pearse *et al.*, 1986; Bastian *et al.*, 1991) it is unlikely that this incubation step should alter the cheesemaking characteristics of the milk. However, the lower levels of total protein found in the plasmin-added cheese may be due to some loss of proteose peptones due to hydrolysis of ß-casein during this time, as suggested by Farkye and Fox (1992). The levels of plasmin in the cheeses (in AMC units/g cheese) were higher than those found in commercial Swiss-type cheeses wherein, due to inactivation of rennet, it represents the main primary proteolytic agent present during ripening (Richardson and Pearce, 1981; Farkye and Fox, 1990).

Plasmin has a broad spectrum of activity on milk proteins, hydrolysing α_{s1}-, α_{s2}- and ß-caseins, but not κ-casein under normal conditions (Grufferty and Fox,

1988c). It was found here that in normal cheese made from milk to which plasmin had been added ß-casein hydrolysis was accelerated to a greater degree than that of α_{s1}-casein, supporting the theory of Farkye and Fox (1991) that ß-casein is the protein more readily hydrolysed by plasmin in cheese. Residual α_{s1}-casein levels were lower than those of ß-casein, as found by Visser and deGroot-Mostert (1977) for Gouda cheeses. Richardson and Pearce (1981) and Ollikainen and Kivelä (1989) also found that in Swiss-type cheeses breakdown of ß-casein and production of γ-caseins was correlated to cheese plasmin content.

Water-soluble nitrogen (WSN) levels were found to be increased by between 5 and 20% at various stages of ripening, and electrophoretic patterns of both WSN and cheese and RP-HPLC analysis of cheese WSN showed that added plasmin increased overall proteolysis in the cheese over ripening. This was in general agreement with the findings of Farkye and Fox (1992) and Farkye and Landkammer (1992). The contribution of plasmin to production of water-soluble peptides in Gouda cheese was shown by Visser (1977c) to be significant, with some low production of free amino acids. Plasmin is also responsible for production of water-insoluble polypeptides such as the γ-caseins in cheese. The results also showed the role of plasmin in the dynamic ripening of cheese, as some of the proteolysis products are more pronounced in cheese B than C, suggesting that plasmin itself further degrades these polypeptides, preventing accumulation. Also, it appears that there is less of a difference between the higher levels of plasmin addition, as compared to the difference between either cheeses B or C and the control, suggesting an optimum level of plasmin addition for acceleration of ripening. Free amino acid levels in the cheeses were overall unaffected by plasmin addition, presumably because of the relatively large size of peptides produced directly by plasmin, but this also implies that starter peptidases do not break down plasmin products at an accelerated rate relative to that found in control cheeses. Alternatively, as in batch B elevated levels of free amino acids appeared to be present at all stages of ripening, it could be that they are indeed being produced, but are being subsequently degraded to products not reactive with the cadmium ninhydrin reagent.

Organoleptic evaluation showed that, as in Cheddar (Farkye and Fox, 1992), extensive proteolysis during Gouda cheese ripening improves cheese flavour and body scores, and somewhat surprisingly, does not lead to bitterness, despite the fact that on hydrolysis of both α_{s2}- and ß-caseins, hydrophobic, potentially bitter peptides are liberated (Le Bars and Gripon, 1989). Also, plasmin has been linked to the production of astringent off-flavour components in milk which may be the γ-caseins (Harwalker *et al.*, 1993), but this does not appear to contribute to cheese flavour. As pointed out by Farkye and Fox (1992), the fact that no off-flavours occur suggests a role for plasmin as a cheese ripening accelerator, with the advantages of easy and complete incorporation into cheese curd by adhesion to casein micelles, but the remaining disadvantage of expense.

The acceleration of WSN production without significantly increased amino acid production may be linked to the improvement of flavour scores, as Aston and Creamer (1986) found that the WSN fraction made the greatest contribution to total flavour intensity of aged Cheddar cheese. Amino acids alone lacked full flavour, which was

thus attributed to peptides. The amino acids leucine, lysine, glutamic acid and serine were produced by milk proteases in aseptic starter and rennet free cheeses made by Visser (1977c), although the total amino acid levels were very small. Glutamic acid, leucine, lysine and serine were also shown to be produced by ß-casein autodegradation (which was presumably due to the action of plasmin) by Moreno and Kosikowski (1972). Aston and Creamer (1986) stated that the first two of these amino acids were important cheese flavour determinants. As Visser (1977b) found that milk proteases on their own did not contribute significantly to cheese flavour development, it must be concluded that in cheese the principal role of plasmin is in accelerated production of substrate polypeptides for production of flavour components by starter peptidases, but there may be a small but important contribution to cheese flavour due to specific amino acid production without significant alteration of total free amino acid levels. This is supported by the fact that there is some elevated amino acid production in cheeses from batch B.

However, it is implied by the work of Visser (1977d) that production of these amino acids a required that a number of intermediate hydrophobic bitter peptides must be formed. Also, Champion and Stanley (1982) found that bitter extracts from Cheddar cheese contained a high proportion of lysine residues and thus in theory may be plasmin degradation products, and so the role of plasmin in cheese flavour requires further investigation. With regard to acceleration of cheese ripening using plasmin, Exterkate (1987) reasoned that breakdown of α_{s1}-casein degradation products was more important than ß-casein in reaching significantly higher levels of amino acid-N in cheese, and thus a distinction may be necessary between increasing total amino acid levels or targeting specific flavour components, and it is in the latter regard that plasmin use appears promising. More detailed peptide and free amino acid profiling of plasmin-added cheese extracts would be necessary to resolve the contribution of plasmin in these cheeses to flavour.

The results shown here also lead to the conclusion that the elevated levels of plasmin found in late lactation milk are not responsible for the poor cheesemaking properties and flavour defects described in cheeses made from such milks. For instance, plasmin wouldn't be expected to lead to the textural problems associated with such cheeses. Also, the action of plasmin does not affect curd formation or syneresis (Pearse *et al.*, 1986) and plasmin does not hydrolyse α_{s1}-casein to α_{s1}-I casein, a reaction thought to be responsible for the softening of cheese (Farkye and Landkammer, 1992).

9.4.2. Aseptic starter and rennet free cheese ripening

The use of model cheese systems in which one or more of the proteolytic agents acting in cheese ripening are isolated has been frequently used to study proteolysis and bitterness development in cheese during ripening (Noomen, 1978; Visser, 1977a-d; Stadhouders et al. 1983 a-c). High somatic cell count milk has been shown to contain elevated levels of proteolysis (Andrews, 1983b; Verdi *et al.*, 1989) and the poor cheesemaking properties of high SCC milks are known. Rogers and Mitchell (1994)

found that proteolysis in Cheddar cheese (TCA soluble tyrosine and PTA soluble amino acid N) was significantly correlated to SCC and that cheeses made from high SCC milks gave lower flavour and body scores than cheeses made from low SCC milk. The qualitative effect of proteinases in such milk on cheese ripening has not been elucidated. Visser and deGroot Mostert (1977) found that in cheeses in which plasmin was the only ripening agent present, ß-casein was gradually broken down, with accompanying production of γ-caseins and some slight degradation of α_{s1}-casein. In the experiments outlined herein, it was shown that elevated milk somatic cell counts lead to elevated levels of proteolysis during cheese ripening in aseptic, starter and rennet free cheeses. ß-casein hydrolysis was elevated overall, as would be expected as the curds prepared from mastitic milk were shown to have higher levels of plasmin. ß-casein was also hydrolysed faster at higher pHs, as expected for an alkaline proteinase, to γ-caseins which were further broken down on prolonged incubation (at 11°C) of high pH curds, presumably by plasmin.

There appeared to be a peak of gross proteolytic activity at pH 5.0-5.5 for curds prepared from mastitic milks. The finding that α_{s1}-I casein production was higher at pH 5.0 than below this pH in both control and mastitic curds is unexpected, and contradicts the belief that production of this polypeptide in aseptic, starter and rennet free cheeses is due solely to the acid milk proteinase, cathepsin D which has a pH optimum of 4.0 (Kaminagowa *et al.*, 1980; Fox, 1989; McSweeney *etal.*, 1995). In the work of Noomen (1978) it was found that α_{s1}-casein breakdown was maximal at pH 5.3, which again supports the presence of an enzyme of higher pH optimum than cathepsin D. Noomen (1978) suggested in his work that this may be an artefact due to residual rennet in the cheese, but in the current study pepsin was used as coagulant and the low pH optimum of this enzyme (2-4) argues against the type of pattern seen, even if some pepsin was not inactivated by heat treatment during manufacture. The alternative possibility is a physico-chemical change in the proteins in the cheese at different pHs which may result in altered susceptibility of the caseins to proteolytic attack.

The presence of another proteinase is thus suggested, and it is possible that this is a cysteine proteinase, probably the polymorphonuclear leucocyte (PMN) lysosomal proteinase cathepsin B, which has a pH optimum of 6.0 (Barrett, 1979a). This enzyme has been shown to produce α_{s1}-I casein in solution (Chapter 6) at pH 6.5 and pH 5.2 in the presence of 5% NaCl. Cysteine protease activity in milk has been shown to correlate closely with SCC (Suzuki and Katoh, 1990). PMN cells are the cell type recruited in large numbers in cases of mastitic infection and thus their enzymes would be expected to be present in large quantities in mastitic milk. It appears from these results that somatic cell enzymes may be incorporated into the curd during cheesemaking. The enzymes of different cell types in the two milks may explain the finding that more α_{s1}-I casein is present in the normal curds at the lowest pH (4.8), which may be due to cathepsin D action being contributed by macrophages, which are the principal cell type present in normal milk (Burvenich *et al.*, 1995). Andrews (1983b), in his investigation of the properties of proteinases in high SCC bovine milks, found that while the pH optimum range of such activity was broad, there was a

perceptible peak at pH 6.0-6.25, which I believe to be due to the contribution of cathepsin B, despite the fact that this is slightly higher than the pH of maximum α_{s1}-casein proteolysis in these cheeses. There are also 2-3 very slow moving proteolysis products at the very top of the mastitic cheese lanes, particularly at elevated pHs, in a region where similar peptides have been shown to be produced by somatic cell proteinases (Chapter 6).

The finding that ASRF cheeses made from high SCC milk had a higher accumulation of hydrophobic, bitter material during ripening is interesting. The accumulation over the 10 week ripening period suggests that these peptides could not be broken down by the milk proteinases themselves, as appeared to happen in the control curds. Further work is needed to identify whether these peptides arise from the action of plasmin or somatic cell proteinases, as plasmin, although associated with production of bitterness on digestion of caseins in solution, is not associated with bitterness in cheese (see section 9.4.1). On the other hand elevated SCCs were positively correlated with cheese off-flavours by Grandison and Ford (1986). The accelerated α_{s1}-casein breakdown found in this study in the cheese made from high SCC milk may also be linked to the negative correlations between SCC and cheese elasticity, firmness and body and texture scores found by Grandison and Ford (1986) and Rogers and Mitchell (1994) as breakdown of this protein is thought to be responsible for softening of cheese (Creamer and Olsen, 1982).

In the ASRF cheeses, in summary, there appears to be two acid proteases acting on the caseins, probably cathepsins B and D, which alter in proportion and activity in milks from healthy and mastitic cows. This is in agreement with the suggestion of Andrews (1983b) that the identity of cells making up the total somatic cell population was important in determining the proteolysis of caseins in milk. These enzymes appear to play a role in both bitterness and textural properties of cheese and may be linked to similar problems in late lactation milks, which may contain elevated SCCs.

Chapter 10

Bovine Milk Polymorphonuclear Leucocyte (PMN) Content and its Effects on Cheese Quality

SUMMARY

A poor correlation was found between bulk tank total SCC and polymorphonuclear leucocyte (PMN) cell content, as measured by an ELISA assay for PMN antigen. Milks which had a total SCC of 500,000/ml had a wide variation in PMN cell levels, which are the predominant cell type found in mastitic milk. Commingling of mastitic and good quality milks was shown to contribute to this variability. Semi-soft Dutch cheeses manufactured from milks having an SCC of 500,000/ml but with high and low levels of PMNs as judged by ELISA showed differences in proteolysis during ripening and differed in organoleptic properties. The cheeses made from milks with high levels of PMNs had a faster breakdown of α_{s1}-casein during ripening as measured by PAGE and different RP-HPLC peptide profiles. Also, the cheeses made from milks of a high PMN level had inferior texture, and were judged to be significantly different on blind tasting. The results indicate that measurement of PMNs levels may to be a more discerning method of selecting milk for cheese manufacture than total SCC.

10.1 INTRODUCTION

Proteolytic activity in milk has a broad temperature and pH optimum range, suggesting that a number of enzymes with different properties and specificities contribute to overall casein hydrolysis (Andrews, 1983 b). The principal proteinase in milk is the alkaline proteinase, plasmin, but the presence of other proteinases has long been recognised (Andrews, 1983a; deRham and Andrews, 1982b). The acid proteinase in milk has been identified as cathepsin D, and its specificity on bovine caseins has been determined (McSweeney *et al.*, 1995). A thrombin-like enzyme (Reimerdes, 1981) and two cysteine proteases in milk (Suzuki and Katoh, 1990) have also been reported.

Increased somatic cell counts (SCC) in milk, and mastitic milk in particular, have been associated with increased milk proteolytic activity (Andrews, 1983b; Murphy *et al.*, 1989; Saemen *et al.*, 1988; Senyk *et al.*, 1985; Suzuki and Katoh, 1990). Elevated SCCs have also been linked to poor cheesemaking properties. The curd retains moisture and fat and protein losses increase, leading to a lower cheese yield (Barbano *et al.* 1991). Longer rennet clotting time and decreased curd rigidity have also been demonstrated (Ali *et al.* 1980; Politis and Ng-Kwai-Hang, 1988a,b,c). Gouda cheese made from low SCC milk were preferred during ripening, by graders, to batches made with milk of a high SCC (Brus and Jaartsvelt, 1971). Somatic cells can convert plasminogen (the inactive form of plasmin) to plasmin in milk, which can lead to increased proteolytic damage to casein (Kaartinen *et al.*, 1988; Verdi and Barbano, 1991; Zachos *et al.* 1992).

Milk somatic cells consist of lymphocytes, macrophages and neutrophils (polymorphonuclear granulocytes or PMN cells) (O'Sullivan *et al.* 1992). PMN cells, which are the predominant cell type in mastitic milk contain a number of neutral proteinases such as the serine proteinases elastase and cathepsin G and the acid proteases cathepsin B and cathepsin D (Verdi and Barbano, 1991). Macrophages contain the acid proteinases cathepsin D and pepsin. Suzuki and Katoh (1990) have suggested that somatic cells are the source of cysteine proteinase activity in milk. Somatic cells in milk are enumerated by a number of direct and indirect methods, but the most common method in commercial factories and dairies is total cell counting using a Fossomatic cell counter. However, an ELISA test has been developed to measure PMN cells (the cell type predominantly elevated in mastitis) in milk, as a more sensitive means of detecting early subclinical mastitis than total SCC. The correlation between total SCC and the ELISA assay for 95 samples from individual cows was shown to be 0.94 (O'Sullivan *et al.*, 1992).

This study investigated the correlation between total SCC and the ELISA assay of O'Sullivan *et al.* (1992) for PMN cell antigen in milk samples taken from farm bulk tanks. Since different cell types have different proteolytic complements, milks with different quantities of PMN cells were used for cheese manufacture and the quality examined and proteolysis during ripening compared.

10.2 MATERIALS AND METHODS

10.2.1. ELISA Screening of Milk Samples

Bulk tank milk samples were collected from 17 suppliers in the Cork area on three occasions within one month. The milks were divided for somatic cell counting (stored overnight at 4°C) and for assay for PMN antigen by ELISA (stored at -20°C). Somatic cell counts were determined using a Fossomatic 90 cell counter (Foss Electric, Hillerod, Denmark), calibrated using direct microscopic cell counts. PMN antigen was assayed using the method of O'Sullivan *et al.* (1992). Milks for cheesemaking were selected as outlined in section 10.3.1. Milk samples, taken immediately prior to cheese making, were tested for fat, protein, lactose and total solids as in Section 4.2.1.

10.2.2. Cheesemaking and analysis

Duplicate Gouda-Meshanger type cheeses were made from each trial milk in the viscubator as described in the materials and methods (section 4.1.1). Cheese samples were taken at 12 days of age and cheese fat, protein, pH and salt determined in duplicate as described in section 4.2.1. Samples for analysis of proteolysis during ripening were taken at 12d and 5, 8, 11 and 15 wk age, as described above. Water-soluble nitrogen (WSN), pH 4.6 soluble extracts and 12% TCA soluble N were prepared as described in section 4.2.3. 12% TCA-soluble N was expressed as a percentage of total protein in the cheese to obtain a measure of the increase in soluble proteins during aging. Two further portions of the WSN were (a) lyophilised and (b) frozen at -20°C for determination of free amino acids by the Cadmium Ninhydrin method (Folkertsma and Fox, 1992). Urea polyacrylamide gel electrophoresis of cheese and pH 4.6-soluble peptides and reversed-phase HPLC of water-soluble cheese peptides was carried out as described in the materials and methods (sections 4.3.1 and 4.3.3).

10.2.3. Organoleptic Assessment and Statistical Analysis.

A panel of 8-10 persons within the department, who were experienced in grading cheese, was used to compare the cheeses at 5, 8, 11 and 15 wk. Two methods were used to determine the effect of PMN population in milk on cheese quality. Firstly, the cheeses were ranked on a score of 1 to 5 (1 being least acceptable and 5 being most acceptable) on the characteristics of colour, flavour, texture and overall desirability. Secondly, a triangle test (British Standards Institute) was used to determine if the cheeses differed significantly after 11 and 15 weeks of ripening, as judged by a panel of 15 to 19 tasters. This panel consisted primarily of experienced tasters, and some laboratory staff. The results were analysed as described in the procedure for the test. Results of cheese ripening analyses and organoleptic assessments were compared using the Students t-test.

10.3. RESULTS

10.3.1. Bulk Tank Sampling

The ELISA method of O'Sullivan *et al.* (1992) measures polymorphonuclear granulocyte (PMN) antigen in bovine milk, and preliminary results indicated that a wide range of PMN populations existed in milk bulk tanks of similar total SCC. To identify tanks of similar total SCC, but different PMN content, 17 farm bulk tanks were sampled on 3 occasions. ELISA scores were found to vary greatly for samples of the same total SCC as measured by Fossomatic (Table 10.1), with an overall correlation of 0.54. For example within the range of SCCs from 500,000 - 550,000 ml^{-1}, a range of Abs_{492} from 0.248 - 0.526 was found. This is in contrast with the results of

TABLE 10.1 *Somatic cell counts and ELISA scores of bulk tanks sampled.*

	Sample 1		Sample 2		Sample 3	
Bulk Tank	SCC[1]	Abs_{492}[1]	SCC	Abs_{492}	SCC	Abs_{492}
1	445	0.304	387	0.228	269	0.213
2	786	0.351	326	0.200	424	0.197
3	797	0.418	426	0.254	423	0.275
4	540	0.302	599	0.342	394	0.353
5	718	0.291	356	0.267	281	0.266
6	401	0.384	244	0.282	189	0.179
7	340	0.167	525	0.281	94	0.159
8	410	0.380	734	0.380	425	0.330
9	507	0.340	248	0.210	147	0.220
10	488	0.316	374	0.320	403	0.515
11	517	0.453	549	0.526	467	0.275
12	486	0.304	369	0.210	332	0.147
13	610	0.380	474	0.440	246	0.240
14	496	0.297	366	0.268	239	0.255
15	380	0.234	332	0.273	347	0.174
16	421	0.245	394	0.226	269	0.271
17	536	0.248	362	0.252	609	0.205

[1] Means of triplicate analysis

O'Sullivan *et al.* (1992) who found a correlation factor of 0.94 between Abs_{492} and SCC for milk samples taken from individual cows. This was investigated by preparation of test mixtures of bovine milk samples from healthy and mastitic cows (Table 11.2). The results showed that, depending on the presence of mastitic cows, two bulk tanks may have the same total SCC but different PMN populations as measured by ELISA. This may contribute to the large variation in milk tank cell compositions seen in Table 10.1.

There was a large variation in daily SCC and ELISA score, and thus 25 litre samples were obtained from tanks 3, 4, 10, 11, and 17 (Table 10.1), on a fourth occasion, and were again analysed for ELISA and SCC. These tanks were selected on the basis of consistently high SCCs. Tank 11 (SCC 470,000 ml^{-1}, Abs_{492} of 0.71) and a blend of tanks 4 and 10 (SCC 470,000 ml^{-1}, Abs_{492} of 0.41) were selected and cheese made immediately from these milks. These cheeses were coded HN (High ELISA, tank 11) and LN (Low ELISA, tanks 4/10).

TABLE 10.2 *ELISA scores of experimentally mixed mastitic and control milks.*

Mix	Individual SCCs (cells ml^{-1})	Bulk SCC	Bulk ELISA score
A	1,922,000M	584,000	0.82
	1,244,000M		
	1,034,000M		
	6,000		
B	1,034,000M	520,000	0.78
	6,000		
C	526,000	508,000	0.43
	659,000		
	384,000		
	460,000		
	674,000		
	346,000		

M Refers to milks taken from mastitic cows. All other milks are from non-mastitic, healthy animals.

10.3.3. Cheese Compositional Analysis

Both HN and LN cheeses had a far higher moisture content than desired (Table 10.3). The small scale of cheesemaking used may explain this, and the final composition may be compared to that of a Meshanger cheese (Noomen, 1978). This is a high moisture Dutch type cheese which was recommended as a model system for examining ripening in Dutch cheeses. In this trial, the milks taken were not

Table 10.3. *Composition of cheeses made from milks with high (HN) and low (LN) proportions of PMNs after 12 days of ripening.*

(Values are mean percentages ± SE for duplicate cheeses)

	HN cheeses	LN cheeses
Fat	21.00 ± .71	18.25 ± .96
Protein	16.68 ± .74	17.95 ± 1.0
Moisture	53.99 ± 1.0	55.32 ± 1.4
Salt	2.03 ± .23	2.12 ± .12
pH	5.12 ± .05	5.30 ± .07

standardised, and this accounts for the differences in fat levels between the cheeses. The original milks had fat contents of 4.36 and 3.86%, protein contents of 3.64 and 3.69% and lactose contents of 4.20 and 4.38% (HN and LN respectively). This suggests that although the milks may have been taken in early November, there may have been a certain amount of late lactation milk in the tanks, to account for the slightly low lactose levels.

10.3.4. Extent of Proteolysis

High and medium molecular weight peptides and caseins are gradually broken down during ripening to lower molecular weight peptides and amino acids which are soluble in 12% TCA (Christensen *et al.*, 1991), and thus the amount of 12% TCA-soluble protein increases with cheese age. From the data presented in Table 10.4, it can be seen that the LN cheese appears to have a higher amount of 12% TCA-soluble nitrogen during the early stages of ripening, ($P<0.05$ at 8 weeks) but beyond 11 weeks of ripening, no significant differences were apparent. The cadmium-ninhydrin reagent reacts strongly with free amino acids and is thus suitable for estimation of the later

Table 10.4. *Proteolysis characteristics† of cheeses made from milks with high (HN) and low (LN) levels of PMNs.*

		Days after Manufacture				
Cheesemilk	Characteristic	12	35	56	77	105
HN	12% TCA-soluble N‡	ND	6.16	9.83	10.2	10.54
	Free Amino Acids	0.60	1.76	2.53	4.55	6.84
LN	12% TCA-soluble N‡	ND	7.08	11.39	10.55	10.56
	Free Amino Acids	0.51	1.43	2.23	3.67	6.97

† Average values for two cheesemaking trials.

‡ Expressed as % of total N.

§ Expressed as A_{507nm} measured on reaction with cadmium-ninhydrin reagent.

TCA: trichloroacetic acid; ND: not determined.

stages of proteolysis in ripening cheese (Folkertsma and Fox, 1992). The amount of cadmium ninhydrin-reactive groups in the test cheeses increased, as expected, on maturation (Table 10.4). The HN cheeses had a significantly ($p<0.05$) higher level of free amino acids at 5 and 11 weeks, but the levels were similar at 15 weeks, indicating a more rapid initial rate of proteolysis in HN cheeses.

10.3.5. Characteristics of Proteolysis

Cheese samples, taken during ripening, were analysed to allow estimation of casein degradation by the proteolytic agents present, using Urea-PAGE (Figure 10.1). It can be seen clearly that in the HN cheese a far faster breakdown of α_{s1}-casein occurs during ripening, with very little remaining after 5 weeks of age as compared with the LN cheese. The major breakdown product of this protein, α_{s1}-I casein (α_{s1}-casein f24-199), is also seen to be broken down during ripening, without accumulating to the levels seen in the LN cheese. ß-casein appears to be degraded at approximately the same rate in both cheeses, with perhaps a marginally faster production of ß-I casein in the HN cheeses.

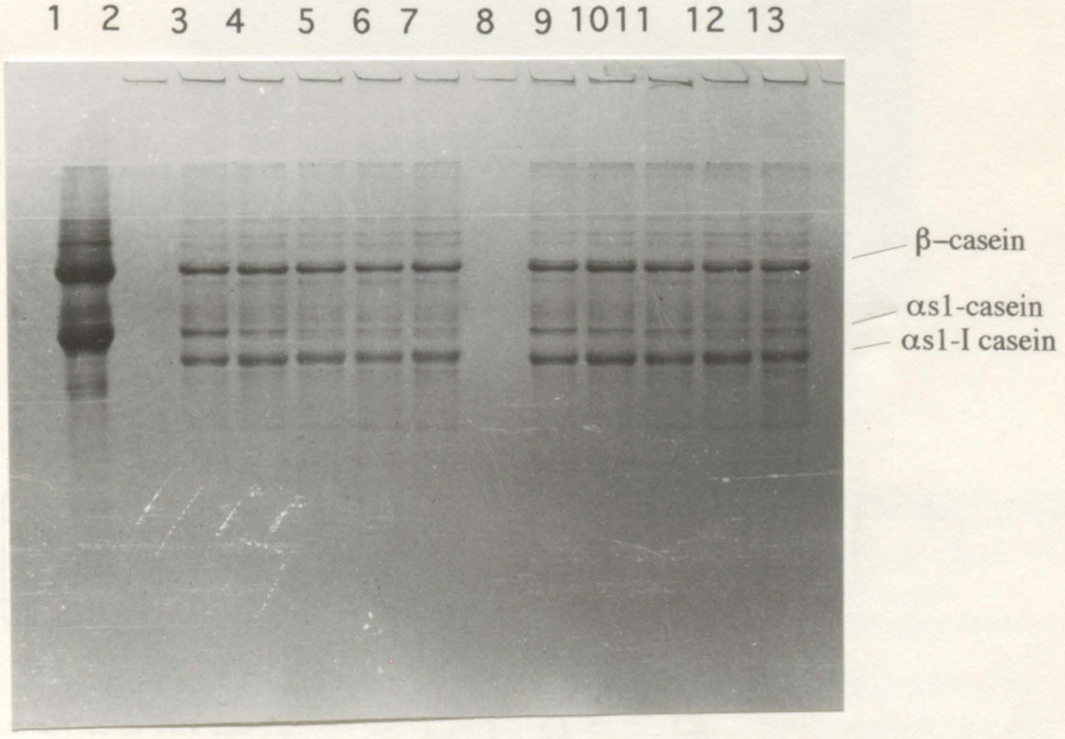


Fig. 10.1 *Urea-PAGE 12.5% polyacrylamide gel of protein breakdown in cheeses during ripening. Lane 1 Sodium Caseinate. Lanes 3-7 HN cheese at 12d ,5, 8, 11 and 15 weeks. Lanes 9-13 LN cheese at 12d, 5, 8, 11 and 15 weeks.*

The cheese nitrogen fraction soluble at pH 4.6 is very heterogeneous (Christensen *et al.*, 1991). Many of the components are produced by the action of chymosin, and include whey proteins, proteose peptones, low molecular weight peptides derived from casein hydrolysis and free amino acids. The components soluble and non-soluble at pH 4.6 are almost identical to those obtained by fractionation of cheese nitrogen with water. As can be seen from Figure 10.2, the HN cheeses develop a quantitatively and qualitatively different range of pH 4.6-soluble products, with many components being produced faster than in the LN cheeses, and some components being produced which are not present in the latter cheeses.

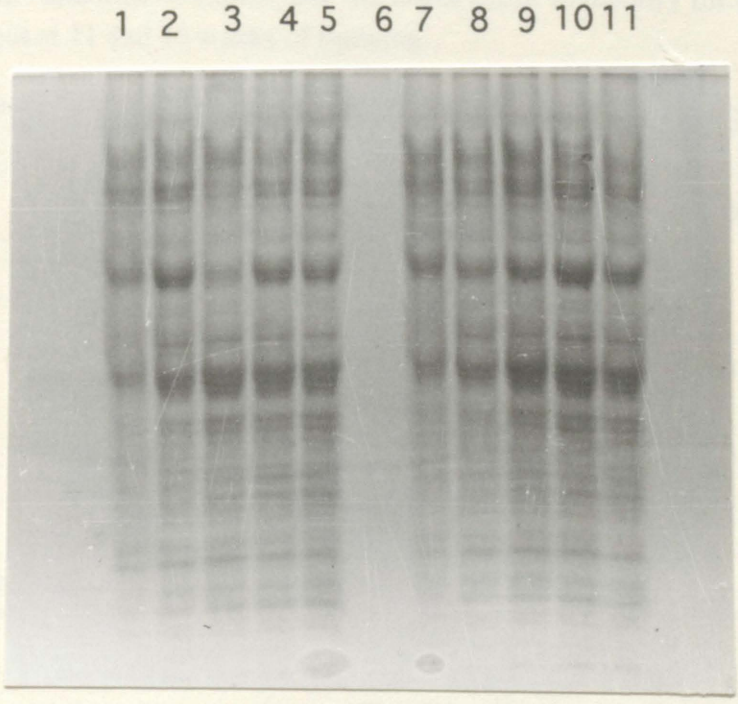


Fig. 10.2 *Urea-PAGE 12.5% polyacrylamide gel of proteolysis products in water soluble nitrogen fraction of cheeses during ripening. Lanes 1-5 HN cheese at 12d ,5, 8, 11 and 15 weeks. Lanes 7-11 LN cheese at 12d, 5, 8, 11 and 15 weeks.*

Reversed-phase HPLC separates peptides on the basis of hydrophobicity, and analysis of HN and LN pH 4.6-soluble fractions, at different ages, show a number of principally quantitative differences. Examination of the profiles at 8 weeks (Figure 10.3) shows lower levels of component 1 in HN cheeses relative to LN but lower levels of peaks 2, 3, 4 and 5, and this trend was confirmed at other stages of ripening (data not shown). This further supports the trend of accelerated proteolysis in cheeses made from high PMN milk.

10.3.6. Organoleptic Assessment of Cheeses

At 5, 8, 11 and 15 weeks of ripening the cheeses were compared by a panel of tasters and the data presented in Table 10.5. The textures of the cheeses improved over ripening, due to loss of moisture through the plastic coat. The LN cheeses consistently ranked as having a more desirable texture, ($P<0.05$ at 8 weeks) with the HN being weaker and pastier. With regard to flavour scores, it can be seen that while the differences were not significant, the LN cheese ranked higher during the later stages of ripening than the HN. Triangle tests in which three samples were presented to a number of research staff in the laboratory, in combinations of either 2 HN and 1 LN sample, or 1 HN and 2 LN samples, and the taster asked to identify the single sample, were carried out at 11 and 15 weeks of ripening.

Table 10.5. *Organoleptic assessment †of cheeses made from milks with high (HN) and low (LN) levels of PMNs.*

		Days after manufacture			
Cheesemilk	Characteristic	35	56	77	105
HN	Flavour	2.71	3.0	2.85	2.93
	Texture	2.32	2.75	2.91	3.11
LN	Flavour	2.65	3.17	3.06	3.29
	Texture	2.64	3.25	3.50	3.79

† Average value for two cheesemaking trials.

At 11 weeks 11 out of 19 people correctly identified the odd sample ($P<0.05$) and at 15 weeks, 10 out of 15 tasters were correct ($P<0.01$). During the 11 week panel, there were a sufficient level of experienced cheese tasters present to assess these as a separate group, within which 10 out of 10 panellists identified the difference ($P<0.001$ significance).

10.4. DISCUSSION

Proteolytic activity in milk due the action of somatic cell proteinases has been shown (Andrews, 1983b, Senyk *et al.*, 1985), but their role in cheese ripening has never been conclusively demonstrated. The work of Visser (1977a-d) and Noomen (1978) has suggested the activity of an acid proteinase in cheese ripening, whose origin is possibly lysosomal. This enzyme is thought to be cathepsin D. In this work we show the relative importance of different somatic cell proteinases, and particularly the role of proteinases of polymorphonuclear leucocytes (PMNs), which are the principal cell type found in mastitic milk. In a survey of bulk tanks we found a wide variation in PMN levels, which may arise from incidence of sub-clinical mastitis, where an influx

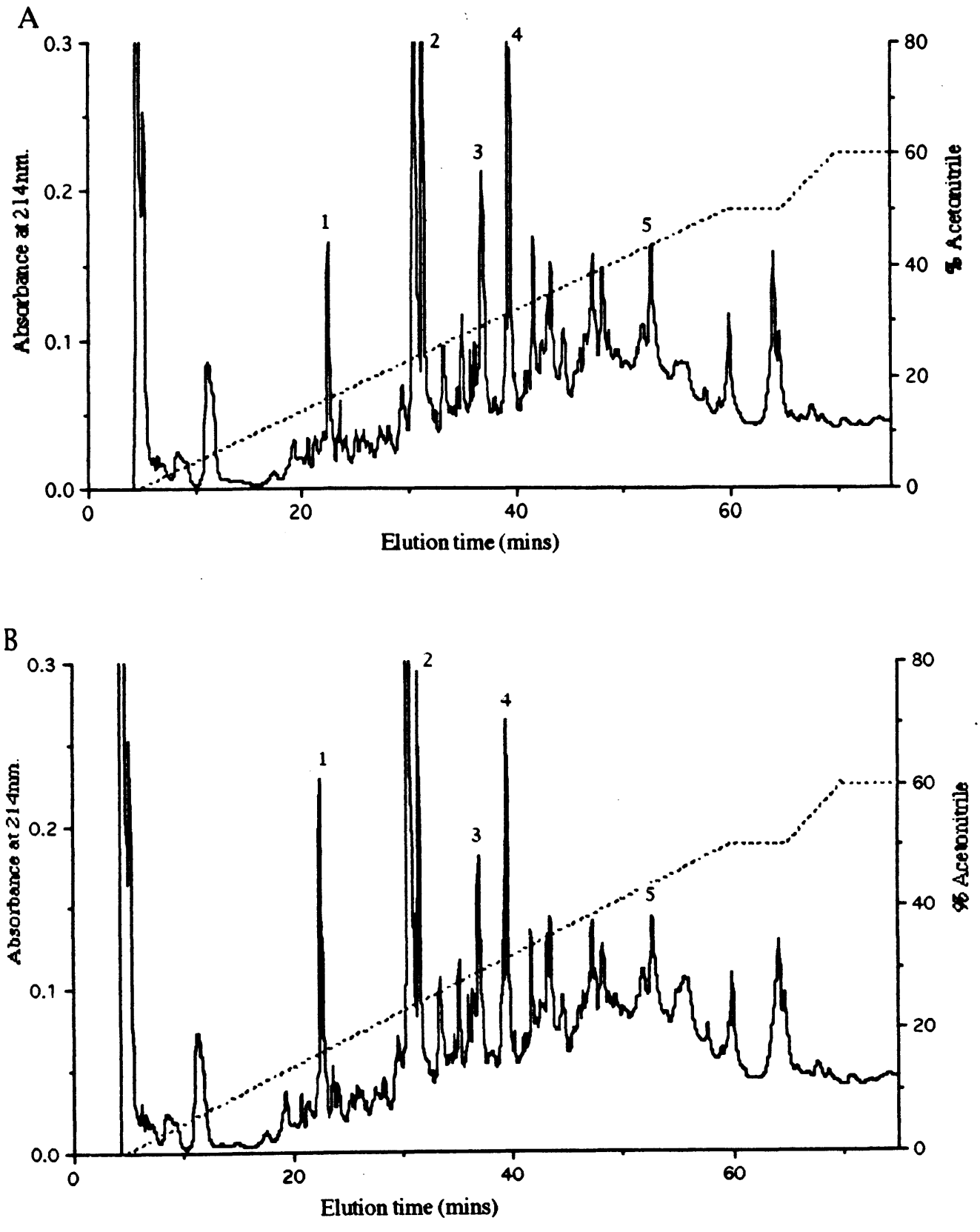


Fig. 10.3 *Reversed phase HPLC chromatograms of pH 4.6-soluble peptides from HN (A) and LN (B) cheeses at 8 weeks of ripening. Dotted line represents acetonitrile gradient.*

of such cells to the udder occurs (Saad and Östensson, 1990). The original purpose of the ELISA test was to detect sub-clinical mastitis, where there are no obvious clinical signs of mastitis. In this regard, it appears that the test has promising applications in identifying tanks with apparently low SCCs which have a sizeable contribution of high SCC milk. The effects of addition of even quite low levels of high SCC to bulk tanks on cheese quality were stressed by Grandison and Ford (1986). From the results presented in Chapter 5 and Chapter 7 it can be seen that there are also seasonal, nutritional and lactational factors influencing PMN levels in milk.

It has been shown that PMNs have a protective role in the healthy udder in the later part of a lactation and in milk with higher somatic cell counts, and that activated, primed neutrophils are present in colostrum and late lactation milk (Hallén Sandgren, 1991). The proteinase elastase is a constituent of PMN granules and its release from these cells is associated with tissue damage in the udder (Hensen and Johnston, 1987). PMNs recruited by bacterial endotoxin to the teat on mastitic infection release primary granule contents during phagocytosis (Hallén Sandgren, 1991). Thus, the presence of these cells in large numbers in milk implies presence of their constituent enzymes in such milk, which may play a role in proteolysis in milk and cheese. An analogous situation is the origin of acid phosphatase in milk, which has been known for some time to be of somatic cell origin in healthy and mastitic milk (Andrews and Alichanidis, 1975; Andrews, 1976). Further work to examine the changing levels and activity of neutrophils in milks as the lactation proceeds, with reference to non-plasmin proteolytic activity in milk and plasminogen activator activity, which has also been demonstrated for somatic cells (Verdi and Barbano, 1991a), is described elsewhere in this thesis. Plasminogen activator activity has been found to be elevated in late lactation somatic cells (Zachos *et al.* 1992), and thus may be related to PMN function.

During ripening of the cheeses it is seen that high PMN cheesemilks lead to accelerated breakdown of α_{s1}-casein and production of free amino acids and pH 4.6-soluble peptides. This may be due to at least two proteases associated with somatic cells. Cathepsin D has been shown by McSweeney *et al.* (1995) to have a specificity similar to that of chymosin on α_{s1}-casein, yielding α_{s1}-I casein, as seen here to be produced faster in the HN cheese. However, this is a proteinase associated more with macrophages, the predominant cell type in normal milk (Burvenich *et al.* 1995; Cohn, 1975). It has been shown (Chapter 6) that Cathepsin B, a thiol protease with a pH optimum of 6.0 associated with PMNs (Barrett, 1979a), also produces this peptide in solution and in aseptic starter and possibly rennet free cheese made from high SCC milks, at a range of pHs (Chapter 9). Thus the role of PMN proteases in cheese ripening may be expected due to the specificities of these enzymes, but it appears that the overall α_{s1}-casein degrading activity in the cheese made from the milk with a high level of PMNs (high levels of cathepsin B) is considerably higher than that in the low-PMN milk (macrophage-and cathepsin D dominated) milk. A knowledge of the levels of individual enzymes, such as elastase and cathepsins B and D, in milks of low and high SCCs, and different levels of PMNs, both raw and post-pasteurisation, is vital in deciphering the role of differential SCCs in milk and cheese proteolysis. In Chapter 6 it was shown that cell extracts from milks with high levels of PMNs (mastitic milks)

caused considerably faster degradation of α_{s1}-casein under cheese conditions, post-pasteurisation, than cells from normal milks. This is very much in agreement with the degradation patterns of cheese in the present study.

Andrews (1983b) in his study of proteinases in high cell count milk (21.7x 10^6 cells/ml) found a broad pH optimum of proteolytic activity, with a possible peak at pH 6.0-6.25, which may correspond to the cysteine proteinase activity reported by Suzuki and Katoh (1990), and linked to possible cheese proteolysis here. A study of high SCC milk proteinase activity by Andrews (1983b), using freeze-thaw cycles to rupture cell membranes and proteinase inhibitors to examine the contributions of individual enzymes showed several different enzymes to be present, but with no clear-cut inhibition patterns. Hydrolysis of ß-casein was slightly faster than that of α_{s1}-casein. He concluded that total milk proteinase activity increased with increasing SCC and that milk storage and handling, which would rupture membranes, would increase the contribution of leucocyte enzymes. The survival of somatic cell proteinase activity in pasteurised milk is documented (Senyk *et al.*, 1985; Verdi and Barbano, 1988; Chapter 6), and examination of high and low somatic cell milk has indicated substantial proteolytic breakdown of α_{s1}-casein and ß-casein by proteinases associated with elevated somatic cell counts. (Verdi *et al.*, 1987; Anderson and Andrews, 1977). The proportionate roles of concomitantly elevated or activated plasmin and somatic cell proteinases have to be determined, but the presence of unidentified non-plasmin derived proteolysis products in such milks is accepted.

Accelerated breakdown of α_{s1}-casein, the principal structural component of cheese, should lead to rapid texture breakdown during ripening and this is seen here (Creamer and Olson, 1982). This evidence supports the role of high SCC milk in cheese manufacture problems such as fat and protein losses in whey, long rennet clotting time and increased cheese moisture (Politis and Ng-Kwai-Hang, 1988a,b,c; Barbano *et al.* 1991). The role of somatic cell proteinases in cheese flavour requires further investigation, but differences in triangle tests indicate that their role in enzymatic alteration of ripening characteristics significantly affects cheese flavour. The interaction between PMNs and plasmin in milk also requires further investigation, and the putative role of plasmin in cheese flavour development has been discussed in Chapter 9. Impaired production in late lactation milk of amino acids was shown in Chapter 8, but the increased production of amino acids in cheeses made from high PMN milks supports the theory that this was a seasonal, rather than cell associated, phenomenon. As stated before, further investigation into the relationships between milk enzymes, amino acid production and cheese flavour development is necessary.

The influence of differential, as opposed to total, somatic cell counts on proteolysis and cheese quality must be addressed, as the variations shown here in PMN levels in bulk milks, and their effects on cheese ripening, suggest that suppliers of milk of relatively high SCC but low levels of PMNs may be penalised unfairly under schemes which use total SCC as a determinant of milk quality. The idea that dilution of high SCC milk into bulk tanks (giving a range of PMN levels) leading to impaired quality of cheese made from this milk, which was raised by Grandison and Ford (1986), was supported by this work.

Chapter 11

Overall discussion and conclusions

CONTENTS

11.1. INTRODUCTION

The Irish dairy industry is highly constrained by seasonal patterns of milk production, with there being 15 times as much milk produced in summer, taking advantage of the grass growing season, than in winter. Most winter milk, produced by autumn calving herds, is used for liquid consumption. Late lactation milk, as comprises much of Irish winter milk, is associated with altered composition, high somatic cell counts (SCC) and plasmin levels and poor cheesemaking properties. This was also found in the U.C.C. cheese development program, and this study was initiated to investigate the effect of elevated SCCs and plasmin levels on milk and cheese quality, with particular regard to proteolysis.

11.2 SOMATIC CELL COUNTS AND MILK COMPOSITION AS AFFECTED BY SEASONALITY AND STAGE OF LACTATION.

11.2.1. Factors influencing levels of somatic cells and polymorphonuclear leucocytes (PMNs) in milks

Total somatic cell counts (SCCs) were elevated in individual cows milk samples from spring-calved relative to autumn-calved cows, late lactation milk relative to early-mid lactation milk and cows of increasing numbers of lactations. Older cows were also more likely to have elevated SCCs in late lactation. With regard to creamery milk taken in the winter for cheesemaking, high SCCs would thus be expected from milks with a high proportion of spring-calving late lactation milk. Examination of bulk tanks in the Cork area showed this trend, but also showed increased SCCs in autumn calving herd milks taken at this time, compared to spring calving early lactation milk, although GLM analysis of individual cows milk samples showed that the interaction of season and stage of lactation on SCC was not significant. Thus early-winter milk in general appears to have elevated SCCs. Increasing nutritional status of spring-calved cows in late lactation reduced the rise in SCC at this time, and restriction of diet exaggerated the SCC rise.

Improvement of the quality of late lactation milk by selection of herds on a high plane of nutrition was suggested by Kefford *et al.* (1992) and Lucey and Fox (1992), who did not refer to SCCs, but it appears that this may be reflected in the results seen here. The milks studied in Chapter 5 were from a liquid milk herd, which had relatively low SCCs overall during the periods studied, while the bulk tanks sampled in Chapter 8 were from manufacturing milk suppliers, and had probably more exaggerated seasonal SCC and composition changes, so the models shown in the former study may not reflect accurately the changes in milk SCC, particularly in the autumn calved cows. The fact that the winter herd SCCs were higher than the relative individual cow samples from the good quality herd studied supports this conclusion. However, the composition of the bulk tank milks was still not typical of poor-quality late-lactation milk (Lucey and Fox, 1992), and these herds must be assumed to be on a relatively

high plane of nutrition, which would also explain the good coagulation properties measured in these milks. A study of the seasonal SCC behaviour in a wider range of manufacturing milk herds and individual cows would be required to explain further SCC patterns in typical Irish milk for cheese manufacture.

Polymorphonuclear leucocyte (PMN) levels in milk were found to be elevated in late lactation milk relative to early-mid lactation milk and to be elevated in spring calving relative to autumn calving cows. Herds on a restricted plane of nutrition had elevated PMN levels throughout a lactation. The relationship between PMN level and nutritional plane appears fundamental, as the autumn-calved cows would generally be on an overall higher plane of nutrition throughout the year (spring-summer grass during late lactation and dry period, concentrates in winter) than spring calved herds. Above an SCC of 160,000 cells/ml spring calving cows, irrespective of season, had higher PMN levels than autumn calving cows (Fig. 5.1). The SCCs in the nutrition trial (Chapter 7) had SCCs consistently above this level and the PMN levels reflected this.

PMN levels were highly correlated to SCC, with a range of Pearsons correlation coefficients from 0.63 to 0.95 for SCC versus PMN level being found in this study, which compare to the value of 0.94 published by O'Sullivan *et al.* (1992). It was found that this correlation was seasonally affected, being lowest in autumn calving and spring calving late lactation milks. The correlation was consistently found to be reduced by elimination of samples of SCC greater than 500,000 cells/ml (samples which were probably from cows with subclinical mastitis), which implies that at low SCCs, increases in SCC may not necessarily be due to PMN influx. This was reinforced by examination of the relationship using $\log_{10}$SCC as a co-ordinate, rather than SCC, to normalise the sample distribution and prevent biasing of the data by small numbers of high SCC samples. In these graphs (for example Fig. 6.1) there appeared to be a tail at very low SCCs where PMN level is static and unchanging. On increasing SCCs beyond this point, the increase in SCC is relatively linear. Overall, caution must be expressed in use of a PMN test as an absolute indicator of mastitis, because the combination of seasonal influences on PMN level and the mixing factors in bulk tanks (Chapter 10) can lead to a range of PMN levels in milks of similar SCCs, as evidenced by the low correlations (0.4-0.6) frequently found between bulk tank SCC and PMN level. However, as will be discussed later, this may lead to a better application for such a test, in discriminating milks for cheese manufacture.

11.2.2. SCC, seasonality and milk composition

It was found consistently that in individual and bulk milk samples elevated SCCs were associated with increased fat, protein and chloride levels in milk and decreased lactose levels. pHs were generally slightly elevated in very high SCC milks. These trends were generally in agreement with those of Mitchell *et al.* (1986a) and Munro *et al.* (1984). PMN level in general was less well correlated with milk compositional parameters than $\log_{10}$SCC.

Late lactation milk was found to have similar characteristics as high SCC milk, which is to be expected as this milk had generally higher SCC than early lactation milk. Further statistical analysis is required to separate the confounding effects of stage of lactation and SCC on milk composition, which was not possible with the data presented here. In mastitic milk, decreased lactose levels are due to damage to mammary gland secretory cells, and the increased permeability of capillaries allows increased chloride influx to milk to control the osmotic pressure (Kitchen, 1981). Increased protein levels are due to a decrease in casein content with a larger concomitant increase in whey protein level and fat levels are increased, generally due to milk yield being decreased to a greater degree than milk fat is synthesised. Little information is available on the physiological changes occurring in the late lactation udder, but the finding by Lucey and Fox (1992) that improved husbandry practices reduced the magnitude of compositional changes in late lactation milks suggests that any physiological changes brought on are not necessarily an integral part of the lactation process, and can be avoided. However, it was found that mean SCCs over lactation were not different in herds on restricted and supplemented planes of nutrition (Chapter 7), corresponding to poor and good husbandry practices, while some changes usually associated with physiological changes in cows with a high SCC, such as decreased lactose levels, were seen in the starved herd. This suggests that the compositional changes associated with poor husbandry and nutritional conditions are not necessarily due solely to increased SCCs, but whether this is exactly analogous to the situation in late lactation is unknown.

The improvement of clotting properties of late lactation milk seen here suggests, in agreement with Kefford *et al.* (1992) and Lucey and Fox (1992) that late lactation milk may not all be unsuitable for cheesemaking, and may actually be more suitable than spring calving early lactation milks.

11.3. THE BIOLOGICAL ACTIVITY OF SOMATIC CELLS IN MILK

11.3.1. The function of the PMN cell in milk

PMN cells levels, as measured by ELISA, were found to be elevated in milks from cows who were stressed, specifically by poor nutritional status. The role of the PMN cell in the healthy uninfected udder is believed to be protective, with high phagocytic and bacteriostatic activity being associated with these cells, particularly in late lactation (Hallén-Sandgren, 1991). The question thus arises whether cows of a poor nutritional status have an enhanced protective capacity against bacterial infection, and this is a promising area for further investigation. The presence of bacteria in the udder was also associated with decreased PMN function by the above author. It may be possible that improved PMN function in late lactation milk is related to the compositional changes in this milk, with decreased casein levels reducing compromisation of the cells by non-specific phagocytosis, but increased fat levels at this time make clear conclusions in this regard difficult. The evidence that there may be an alteration of the immune defences in the restricted and supplemented herds in

Chapter 7, with far more non-PMN cells (presumably macrophages and lymphocytes) in the latter herd, is a further complicating factor in this area.

11.3.2. The proteolytic enzymes of somatic cells and their role in milk proteolysis.

There was considerable evidence of a role for somatic cell enzymes in milk proteolysis to a degree not previously described. In two studies of proteolysis in milks from individual cows (Chapters 5 and 6) it was found that in a group of samples of low mean SCC, plasmin dominated milk proteolysis, but in the group of samples of mean SCCs more akin to those seen in late lactation bulk milks, proteolysis, both in raw and pasteurised samples, was more highly correlated with SCC and PMN level than plasmin activity. Electrophoretic examination showed clear evidence of non-plasmin activity (not inhibited by 6-aminohexanoic acid). The high PMN nutritionally-restricted herd in Chapter 7 had high levels of proteolysis relative to the control and supplemented herds, despite having similar mean SCCs to the latter herd.

All these pieces of evidence point to an important role for PMN proteinases in milk proteolysis, and examination of two of these enzymes, cathepsin B and elastase, showed them to have a broad specificity on the caseins. The proposal of Andrews (1983b) that while proteinase activity in milk could originate from somatic cells, the identity of cells making up the total cellular population was important, was supported by the different breakdown patterns obtained when cell populations from healthy or mastitic milks were incubated with buffered sodium caseinate at the same total cell levels. The breakdown patterns obtained with cells from high SCC milk were consistent with the combined activities of cathepsin B, elastase and a number of other proteinases, probably including cathepsin D.

Electrophoretic patterns of milks of different SCCs incubated at 37°C showed clear evidence of somatic cell proteinase action. Fig. 5.3 showed considerable ß-casein breakdown in a sample of extremely high plasmin activity, but much less considerable α_{s1}-casein breakdown compared to the electrophoretogram of high SCC milk in Chapter 6, indicating that somatic cell proteinases, as opposed to plasmin, are responsible for much of the α_{s1}-casein proteolysis in high SCC milk. This would give the higher correlations for proteolytic activity against SCC and PMN level than plasmin activity seen in Chapter 6. Proteolysis was very much related to plane of nutrition in Chapter 7, which again suggests a role for PMN proteinases which are elevated in nutritionally-stressed cows' milk.

The overall inference regarding the identity of the somatic cell proteinases which degrade protein at cheese pHs is that in high SCC (PMN) milk, cathepsin B is the principal proteinase, while in cheese made from normal milk, the principal proteinase present is cathepsin D. At alkaline and milk pHs there are a number of enzymes active, including obviously plasmin, and probably elastase.

11.4. PLASMIN, PLASMINOGEN AND PLASMINOGEN ACTIVATION IN MILK

11.4.1. Factors affecting plasmin activity in milk

Plasmin activity in milk was elevated in late lactation milk and milk from cows of high SCC, but as before the relative contributions of these confounding factors remain to be elucidated. The mean plasmin activities for early-mid and late lactation milks in Chapter 5 were as predicted from the SCCs of these milks, according to the data in Table 6.2. The plasmin levels in the herd milks measured in Chapter 7 were somewhat lower, but this may be due to dilution of high SCC, high plasmin, low milk yield samples in the bulk tank with samples having low plasmin activity (Benslimane *et al.*, 1990). The findings regarding increased plasmin activity in late lactation and high SCC milks were as found by Barry and Donnelly (1983) and Politis *et al.* (1989 a,b), among others. The decrease in plasmin activity over the lactation seen in Chapter 7 remains unexplained. Plasmin activity was found to be consistently correlated to $\log_{10}$SCC, milk protein level, milk pH and plasminogen level. Plasminogen was similarly seen to be increased in high SCC and late lactation milks, suggesting that increased transport of both plasmin and plasminogen from blood occurs in these milks.

11.4.2. Plasminogen activation in milk

The ratio of plasminogen to plasmin was decreased in late lactation milks compared to early-mid lactation milks, suggesting increased activation of plasminogen to plasmin as well as increased transport of both components from blood described above. There appears to be a balance of the two mechanisms, with the influence of plasminogen activators (PAs) being greater than that of increased transport in the high SCC milks studied in Chapter 6. The role of somatic cells (and PMNs specifically) in plasminogen activation was apparent in Chapter 7, where increased PMN levels in herd A were associated with decreased plasminogen/plasmin ratio, suggesting that these cells were responsible for increased PA activity. The supplemented cows in herd C had similar SCCs but increased plasminogen/plasmin ratios, suggesting that in this case the influence of transport was greater than that of PA activity. Zachos *et al.* (1992) found that PA activity per somatic cell was greatly increased in mastitic milk, which would contain high levels of PMNs, and thus milks with elevated PMN levels should be expected to show high plasminogen activation. This supports the decreased ratios found in Chapter 6 at high SCC (PMN) levels, with correlation coefficients of -0.375 and -0.476 between plasminogen/plasmin ratio and $\log_{10}$SCC and PMN level respectively ($p<0.05$ and $p<0.01$ respectively). Thus PMNs appear to be associated with increased PA activity in milk and in milks where PMN level is elevated, such as spring calving milks and late lactation milks, increased plasmin levels will be found. Season and number of lactations were also found to significantly affect plasminogen/plasmin ratios.

Spring calving milks had similar plasmin levels to autumn calving milks, but elevated plasminogen levels, and hence elevated plasminogen/plasmin ratios, but this appeared to be more related to transport from the blood, which would result in the elevated SCCs and PMN levels seen in these milks, than PA activity. This effect of season (and nutritional status) on PMN PA activity was also seen in Chapter 7, where it appeared that PMNs from nutritionally deficient cows had an elevated physiological activity, as evidenced by increased PA activity.

11.5. SOMATIC CELLS, PLASMIN AND CHEESE RIPENING.

11.5.1. The roles of plasmin and somatic cell proteinases in cheese ripening

Elevated milk plasmin levels were found to have no undesirable effects on cheese ripening, as reported by Farkye and Fox (1992) and Farkye and Landkammer (1992). The potential use of plasmin as an accelerator of cheese ripening was again raised, with particular reference to production of specific amino acids important to cheese flavour. Elevated levels of somatic cells, in contrast, were found to lead to accelerated breakdown of α_{s1}-casein and higher levels of overall proteolysis, which may influence cheese texture and flavour. The increased activity of somatic cell proteinases on α_{s1}-casein, as opposed to ß-casein, was as expected from studies of milk and somatic cell digests of casein (Chapters 5-7), and a number of proteolysis products, known to arise from somatic cell proteinase action in milk, appeared to be present in cheeses made from high SCC milk. The inference that cathepsin B may be have a significant influence on the ripening of cheese made from milk with elevated levels of somatic cells was supported by the aseptic starter and rennet free cheese study (ASRF). The inclusion of the cells and/or their enzymes in the cheese curd and their action during ripening, as proposed by Verdi and Barbano (1991) was confirmed. The action of milk proteinases in ASRF curds made from late lactation milk was associated with accumulation of bitter peptides, and may be linked to the bitter defect frequently associated with late lactation milks of elevated SCCs. Cheeses prepared from milks containing high levels of PMNs and their proteinases showed the elevated breakdown of α_{s1}-casein already noted from the study of the proteolytic specificity of somatic cells from mastitic milk (PMNs) on sodium caseinate in cheese-like buffer systems (Chapter 6), and showed the expected concomitant textural defects arising from accelerated breakdown of α_{s1}-casein (Creamer and Olson, 1982).

11.5.2. Seasonality and cheese quality

Seasonal changes in the cheesemaking properties of milk suggested that two main alterations in late lactation milk, which act in opposition to each other, are important in cheesemaking. Firstly, increased solids levels (fat and protein) in the milk associated with both late lactation and high SCC milk improve the clotting properties of late lactation milk. Also, increased plasmin levels are found, but these would not

appear to be related to quality defects in cheese. Secondly, increased SCCs in milk, while being associated with the changes mentioned, are also associated with increased proteolytic activity, which may affect the moisture content of the cheeses and will play a specific role during ripening by accelerating breakdown of α_{s1}-casein, which will affect cheese texture. Hydrolysis of this protein prior to cheese manufacture may be involved in the retention of moisture of late lactation cheeses, which seems logical as both PMN level and SCC are highly correlated to initial protein damage in milk, which represents proteolysis in the udder. These two influences are balanced, presumably until a certain SCC, which remains to be determined, is reached.

It was for some time believed that hydrolysis of ß-casein by plasmin was responsible for rennet gels of poor syneretic properties giving high moisture cheeses (Donnelly *et al.*, 1984; Barbano *et al.*, 1991), but Pearse *et al.* (1986) showed that extensive hydrolysis of casein by plasmin did not impede subsequent curd formation and syneresis. Thus it may be that other enzymes, such as those from somatic cells, may be responsible for the hydrolysis which impairs the coagulation of late lactation and high SCC milks and results in increased losses of fat and protein in whey. The influence of high late lactation milk pHs on clotting must also be considered due to its effect on chymosin action, soluble calcium content and casein micelle structure (Lucey and Fox, 1992), but in this study, pHs greater than 6.70 were not seen in late lactation milks.

During ripening, late lactation milks of elevated SCCs had increased production of water soluble nitrogen (WSN) during ripening, which was shown electrophoretically to be due to increased breakdown of both α_{s1}- and ß-casein. Cheeses made from late lactation milks of low SCCs, however, had similar WSN production rates to those made from early-mid lactation cheeses, but showed increased α_{s1}-casein breakdown, suggestive of increased levels of PMN proteinases in the milk, which led to decreased cheese texture scores during ripening. Production of small peptides and free amino acids was also decreased in late lactation cheeses during ripening, and this may have been linked to poorer flavour characteristics of late lactation cheeses.

11.5. THE USE OF PMN LEVELS AS A DETERMINANT OF MILK SUITABILITY FOR CHEESEMAKING

PMN levels in milk were generally not as well correlated to milk compositional properties as was $\log_{10}$SCC, but were highly related to non-plasmin milk proteolysis, particularly at SCCs above 300,000/ml. This proteolytic activity was verified both quantitatively and qualitatively, with evidence of typical PMN proteinase action in milk and cheese. This implies that while PMN level in milk may not be an absolute indicator of milk compositional changes, it will reflect proteolytic activity in milk and probably milk suitability for cheesemaking. The potential use of a test for PMNs is further supported by the poor correlations between PMN level and total SCC in winter and bulk tank milks. It must be considered that although total SCC is well correlated to total proteolysis in milk, if PMN proteinase action is more specifically related to proteolysis

of α_{s1}-casein, and concomitant textural defects in cheese, then this may indeed be a better indicator of milk suitability for cheesemaking than SCC.

SUGGESTIONS FOR FURTHER RESEARCH

Milk somatic cells and udder defence mechanisms

Further exploration of the factors influencing differential counts in milk is required, with regard to the other types of somatic cell (macrophages and lymphocytes) and their levels and significance in milk. The ELISA test for PMNs in milk requires calibration, so that neutrophil numbers/ml can be expressed, as opposed to an optical density. This may be done by differential staining or flow cytometry. With regard to mastitis research, I feel that the role of PMNs, particularly in milks of similar total SCCs but different PMN levels, in udder defence requires elucidation. The apparent alteration of physiological and enzymatic activity of PMNs on entry into the milk from blood also remains unexplained.

Milk enzymology

The levels and activities of specific enzymes, such as elastase and the cathepsins, in normal, late lactation and mastitic milks remain to be determined. Problems to be overcome include the measurement of proteinases of acid pH optima without interference from precipitation of caseins or whey proteins at their isolelectric points. The lysosomal enzymes in milk, arising from somatic cells, remain to be conclusively isolated and identified, and their actions characterised. As well as the proteolytic enzymes examined in this study, somatic cell derived enzymes whose actions are poorly characterised include lipases and oxidoreductases. Further work on the influence of lysosomal enzymes on cheese ripening may be carried out using cheese slurry systems to allow modelling of the interactions of ripening agents in cheeses of constant composition.

Milk and cheese quality

Detailed examination of the free amino acid and small peptide profiles of plasmin added and late lactation cheeses are necessary to elucidate the alteration of proteolysis pathways apparent in these cheeses during ripening, with particular reference to the role played by these components in cheese flavour. The confounding effects of the late stages of lactation and elevated somatic cell counts need to be examined separately, to determine the alterations in udder physiology and compositional changes which occur independently of the rising SCC at this time. Work is needed also to establish recommended 'safe' levels of SCC and PMNs which would not interfere adversely with milk composition and cheese quality. Further study of the potential role of PMN measurement as a determinant of milk suitability for cheesemaking is needed, with particular regard to seasonal and nutritional influences on milk PMN levels. The influence of PMNs and the plasminogen activation on other products, such as UHT milks, should also be addressed.

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