A COMPARATIVE STUDY OF PROTEOLYSIS IN CHEDDAR CHEESE AND YEAST-INOCULATED CHEDDAR CHEESE DURING RIPENING.

By

MARYNA BOTHMA

B.Sc. Hons. (UOFS)

Dissertation submitted in fulfilment of the degree

MAGISTER SCIENTIAE

In the Department of Food Science,
Faculty of Agricultural and Natural Science, at the
University of the Orange Free State,
Bloemfontein,
South Africa

May, 2000

Supervisor: Prof. G. Osthoff
D.Sc. (PU for CHE)

Co-supervisor: Prof. B.C. Viljoen
Ph.D. (UOFS)

To my parents, Faan and Ina,

and to Alphons

TABLE OF CONTENTS

Acknowledgements

I wish to express my sincere thanks to the following persons and institutions, who made it possible for me to complete this study.

* My supervisor, Prof. G. Osthoff, for his persistant guidance, useful advice and support.

* My co-supervisor, Prof. B.C. Viljoen, for his useful advice.

* The University of the Orange Free State, especially the Departments of Food Science and Microbiology and Biochemistry, for providing me with the opportunity and the facilities to conduct this study.

* The NRF for their financial contribution to the project.

* Clover SA, Ladybrand, for manufacturing and sampling of the cheese and providing useful advice and information.

* Mr. Piet Botes, for his help, advice and patience with the HPLC-analysis.

* Mrs. Carina Bothma, for her help with the sensory analysis in the Department of Food Science, UOFS.

* Mr. Stephen Collett, for his help and patience with the presentation of the results.

* My family and in particular my parents, to whom I dedicate this dissertation, for their love, support and encouragement and for always believing in me.

* My fiancè, Alphons, for his love, support, encouragement and for believing in me.

* All my friends and colleagues, in particular Nadene, Eileen, Celia, Carina, Elaine and Rosalie, for their constant support, help, encouragement and friendship.

* For my Heavenly Father who made it possible to conduct this study.

Chapter 1

Literature review

1.1 Introduction

Cheese is one of the most diverse food groups: 500 varieties produced from cow's milk and 500 more produced from sheep's and/or goats' milks. In spite of its long history (cheese production is one of the oldest forms of biotechnology, dating perhaps from 6 000 B.C.), cheese still has a very vibrant image and enjoys consistent growth of about 4% p.a. (Fox *et al.*, 1993).

World cheese production is 14 x 10^6 tons per annum, approximately 75% of which is ripened (matured) for periods ranging from 3 weeks (e.g. Mozzarella) to more than 2 years (e.g. Parmesan, extra mature Cheddar) (Fox and McSweeney, 1996). Clover SA (NCD) produces 4 984 594 kg Cheddar and 1 469 028 kg extra mature Cheddar cheese p.a. which represents ~50-60% of the South African Cheddar cheese production. Clover SA produces ~41% of the total cheese production in South Africa (Laubscher, 2000).

1.2 Cheese production and manufacture

Cheese manufacture is essentially a dehydration process in which the fat and casein of milk are concentrated 6-to-10-fold, depending on the cheese variety. Dehydration is tradionally achieved by coagulating the casein enzymatically, isoelectrically or by a combination of heat and acid. If present, the fat is included in the coagulum. Most ripened cheeses are produced by rennet coagulation. Proteolysis is a *sine qua non* in the manufacture of these cheeses and also plays a major role during ripening. Some proteolysis also occurs in the milk before coagulation. Thus, proteolysis can be divided into three phases: premanufacture, coagulation and ripening (Fox, 1989).

The first step in cheese manufacture essentially involves coagulating the principle group of milk proteins, the caseins, by one of three methods:

1. Limited proteolysis by a crude proteinase (rennet): this method is employed for the vast majority of ripened and some fresh cheeses.

2. Isoelectric precipitation at ~pH 4.6, used mainly for fresh cheeses, usually by *in situ* production of lactic acid by a starter culture and less frequently by direct acidification with acid, usually HCl, or acidogen, usually gluconic acid-δ-lactone.
3. Acid plus heat, i.e. acidification to ~pH 5.2 with acid whey, citrus juice, vinegar or acetic acid at 80-90^{0}C, e.g. Ricotta, Queso Blanco.

Rennet-coagulated cheeses include all the ripened varieties; their great diversity is facilitated by the syneretic properties of rennet-coagulated casein gels, which enables the production of cheese ranging in moisture content from ~30 to ~50% and the numerous biochemical reactions that occur during ripening, catalyzed by a great diversity of enzymes (Fox *et al.*, 1993).

The traditional manufacture of Cheddar cheese consists of: a) coagulating milk, containing a starter culture, with rennet, b) cutting the resulting coagulum into small cubes, c) heating and stirring the cubes with the concomitant production of a required amount of acid, d) whey removal; e) fusing the cubes of curd into slabs by cheddaring; f) cutting (milling) the cheddared curd; g) salting; h) pressing and i) packaging and ripening (Fox *et al.*, 1993).

Paracasein, which constitutes the cheese matrix, represents about 99% of the proteins in most cheese. Whey proteins, which may represent up to 18% of the proteins in cheese made from ultra-filtered milk accounts for only 1% in traditional cheese. Based upon composition and sequence of amino acids and genotypes, caseins are classified in four different fractions: α_{s1}, α_{s2}, β and κ-caseins, which occur roughly in the proportion 3:1:3:1. In addition to their genetic variants, these caseins are differentiated within each fraction according to their degree of phosphorilation or sugar content (Grappin *et al.*, 1985).

The α_{s1}-group (199 amino acid residues) is a mixture of α_{s0} and α_{s1} (the latter being predominant) with 9 and 8 PO_4 groups, respectively. The α_{s2} group (207 amino acid residues) is composed of 5 proteins (α_{s2}, α_{s3}, α_{s4}, α_{s6} and α_{s5} ; the latter is a dimer of α_{s3} and α_{s4}) with PO_4 contents ranging from 10 to 13 per molecule (Grappin *et al.*, 1985).

The β-casein (209 amino acid residues) contains 5 PO_4 residues, and under the action of proteases, especially plasmin, yields three components called γ-caseins, which were identified as the C-terminal fractions of β-casein; γ_1 (29-209), γ_2 (106-209) and γ_3 (108-208). These three β-casein fragments were formerly named γ, TS (A_2, A_3)or S, and R or TS-B. Within each group of γ-caseins, several fractions can be identified that evolved from particular genetic variants of β-casein. These caseins are sometimes considered as a fourth group of the casein complex because they are already present in freshly drawn milk, representing about 3% of the proteins in normal milk and up to 10% in late lactation milk (Grappin *et al.*, 1985).

The κ-caseins (169 amino acid residues) with only 1 PO_4 residue, exist as seven different forms (κ_1-κ_7) according to their glycoside content. After the primary action of chymosin, which cleaves the Phe_{105}-Met_{106} bond, only the hydrophobic 1-105 fragment of κ-casein, called para-κ-casein, remains in the cheese curd. The so-called macropeptide is drained away with the whey. A common feature of κ-casein and α_{s2}-caseins is the presence of 2 cysteine residues, which α_{s1}- and β-caseins lack (Grappin, *et al.*, 1985).

1.3 Proteolysis in cheese

1.3.1 Proteolysis in milk premanufacture:

There are two main causes of proteolysis in cheese milk premanufacture: microbial and indigenous milk proteinases. Psychrotrophic bacteria dominate the microflora of milk cooled on-farm prior to collection, during transportation and during storage at the factory (Jooste and Britz, 1986). Storage of raw milk at low temperatures for 4 or 5 days is common in developed dairy countries. Apparently, psychrotrophic bacteria are not significant as far as proteolysis is concerned unless the population exceeds about 10^6 cfu/ml. Higher psychrotroph populations are likely to cause reduced recovery of the milk solids as cheese, higher moisture contents, pasty texture and off-flavours in the cheese (Fox, 1989). Due to differences in proteolytic specificity, proteases of psychrotrophs; some being heat stable and therefore passing heat treatments such as pasteurization, may cause off-flavours (Law, 1979 a, b and Venter *et al.*, 1999).

Lipases produced by psychrotrophs are probably more important in the development of flavour defects in cheese than proteinases because the proteinases are water-soluble and are lost in the whey, whereas the lipases are adsorbed onto the fat globules and are concentrated in the cheese.

As it comes from the cow, milk contains many proteinases, the principle one being plasmin, i.e. alkaline milk proteinase, which hydrolyses β-casein to γ-caseins and proteose-peptones. It also hydrolyses α_{s2}-casein rapidly, but the products have not been identified (Fox, 1989). The α_{s1}-casein is hydrolyzed slowly and λ-casein (consists predominantly of fragments of α_{s1}-caseins derived by plasmin) may be one of the products (Aimutis and Eigel, 1982). Plasmin has little effect on κ-casein, which is fortunate in view of the importance of this constituent for milk stability (Fox, 1989).

The classical proteose-peptone fraction consists of 38 peptides, 52% of which may originate from β-casein by plasmin cleavage, 29% from α_{s1}-casein, 9% from α_{s2}-casein and 4% from κ-casein. The γ-caseins normally represent approximately 3% of the casein nitrogen (N) in milk but may be as high as 10% in late lactation or in milk from mastitic cows. The proteose-peptone fraction represents approximately 3% of total N (Schaar, 1985).

Most of the proteose-peptone fraction is lost in acid or rennet whey and consequently a reduction in cheese yield can be assumed as a consequence of plasmin action, although definite information on this is lacking. The rennetability of milk and the syneresis properties of the resulting gel deteriorate with advancing lactation and cheese made from such milk has a high moisture content (Block, 1951). Grufferty and Fox (1988) showed that very significant plasmin activity had little effect on the rennet coagubility of milk, because most of the action of plasmin in milk occurs within the mammary gland with relatively little after milking.

Proteinases from leucocytes are a further potential cause of proteolysis in milk, especially from cows suffering from clinical or sub-clinical mastitis. Plasmin in milk was shown to be 2 to 8 times more proteolytic than leucocytes added to milk at 10^6 cfu/ml. However, leucocyte proteinases are capable of causing proteolysis in milk, which may adversely affect cheese yield and quality (Fox, 1989).

1.3.2 Enzymatic coagulation of milk:

Rennet coagulation of milk involves proteolysis with the formation of para-casein and nonprotein nitrogen (NPN). It is well known that rennet coagulation is a two-stage process, the first involves the enzymatic formation of para-casein and peptides, the second, the precipitation of para-casein by Ca^{2+} at temperatures >20^{0}C (Fox, 1989).

The primary phase of rennet action occurs as specific proteolysis during the primary phase of rennet action and is complete before the onset of coagulation. More than one peptide is produced and α-casein, rather than β-casein is the substrate for this specific proteolysis (Fox, 1989).

With the isolation of κ-casein and the demonstration that it is responsible for micelle stability and that its micelle-stabilizing properties are lost on renneting, it became possible to define the primary phase of rennet action, the cleavage site being Phe_{105}-Met_{106}. This particular bond is many times more susceptible to hydrolysis by acid proteinase (most commercial rennets are acid proteinases) than any other proteinase in the milk protein system (Fox, 1989).

The unique sensitivity of the Phe-Met bond has aroused interest. Both the length of the peptide and the sequence around the susceptible bond are important determinants of enzyme-substrate interaction (Fox, 1989). The sequence of κ-casein around the chymosin-susceptible bond increases the efficiency with which the Phe-Met bond is hydrolysed by chymosin. The sequence His_{98}-Lys_{111} appears to contain the necessary determinants for rapid cleavage of the Phe-Met bond by chymosin and presumably by other acid proteinases. Thus, the sequence around Phe-Met bond, rather the bond itself, contains the important determinants of hydrolysis (Fox, 1989). The particular important residues are Ser_{104}, the hydrophobic residues Leu_{103} and Ile_{108}, at least one of the three histidines (residues 98, 100 or 102, as indicated by the inhibitory effect of photooxidation), some or all of the four prolines (residues 99, 101, 109, 110) and Lys_{111}, Lys_{112} or Lys_{111} plus Lys_{112} (Visser *et al.*, 1987).

The sequence Leu_{103} -Ile_{108}, which probably exists as an extended β-structure fits into the active site cleft of acid proteinases. The conformation is stabilized by Pro residues at positions 99, 101,109 and 110. The three His residues, His_{98}, His_{100}, His_{102} and Lys_{111} are probably involved in electrostatic bonding between enzyme and substrate (Visser *et al.*, 1987). The significance of electrostatic interactions in chymosin-substrate complex formation is indicated by the effect of low levels of NaCl on the hydrolysis of κ-casein: addition of NaCl to milk up to 3mM reduces the rennet coagulation time, but high concentrations of NaCl have an inhibitory effect. It is claimed that the effect of NaCl is on the primary enzymatic phase (Visser *et al.*, 1983).

Factors affecting hydrolysis of κ-casein:

- pH
- organic acids
- ionic strength
- temperature
- degree of glycosylation
- other proteins
- heat treatment of milk (Fox, 1989).

The secondary (non enzymatic) phase of coagulation is marked by proteolysis of κ-casein which reduces the zeta potential and steric stabilization of the casein micelles. When approximately 85% of the κ-casein has been hydrolyzed, the casein micelles begin to aggregate (Fox, 1989).

1.3.3 Proteolysis during ripening:

Some cheese varieties, especially acid-coagulated cheese, are consumed fresh, but the majority of rennet-coagulated cheeses are ripened (matured) for periods ranging from 4 weeks to more than 2 years (Fox, 1989). The duration of ripening is more or less inversely proportional to the moisture content of the cheese and to the intensity of flavour desired (Fox *et al.*, 1993).

During ripening a multitude of chemical and biochemical changes occur in which the principle constituents of the cheese, namely proteins, lipids and residual lactose are degraded to primary products. Among the principle compounds that have been isolated from several cheese varieties are peptides, amino acids, amines, acids, thiols, thioesters (from proteins), fatty acids, methyl ketones, lactones and esters (from lipids), organic acids, especially lactic acid but also acetic acid and propionic acids, carbon dioxide, esters and alcohols (from lactose). In the right combinations these compounds are responsible for the characteristic flavour of various cheeses (Fox, 1989).

Perhaps the main consequence of proteolysis is the conversion of the rubbery texture of green curd into the smooth-bodied finished cheese (O'Keeffe, *et al.*, 1975).

Ripening involves 3 primary biochemical events: 1) glycolysis of the residual lactose and its constituent monosaccharides, glucose and galactose; 2) lipolysis and 3) proteolysis. Depending on the variety, the products of these primary reactions are modified to a greater or lesser extent. Lactic acid may be isomerized (in most varieties), or may be converted to acetic (many varieties), propionic (Swiss) or butyric (Swiss, Dutch, etc.) acid, CO_2 (Swiss, Camembert), H_2O or H_2 (Swiss, Dutch). Citric acid is converted to diacetyl and CO_2, especially in Dutch varieties, or formic acid. Fatty acids may be oxidized to 2-alkanones, which may be reduced to 2-alkanols (especially in Blue cheeses). Amino acids may be deaminated, transaminated, decarboxylated, desulfurated, or converted to alcohols, carbonyls or hydrocarbons; these transformations occur to some extent in all varieties, but especially in varieties with a surface smear containing *Brevibacterium* and yeasts (Fox and McSweeney, 1996).

Although the ripening of some varieties e.g. Blue, Romano and Parmesan is dominated by the consequences of lipolysis, proteolysis is more or less important in all varieties. In the case of Cheddar cheese and some other varieties, many authors are of the opinion that proteolysis is the major biochemical event during ripening (Fox, 1989). A high correlation exists between the intensity of Cheddar cheese flavour and the concentration of free amino acids (Aston *et al.*, 1983).

Proteolysis contributes to cheese ripening in at least four major ways:

1. a direct contribution to flavour via amino acids and peptides, some of which may cause off-flavours such as bitterness (due to hydrophobic peptides) or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters, etc.
2. greater release of sapid compounds from the cheese matrix during mastication
3. liberation of substrates (amino acids) for other flavour-generating reactions (e.g. deamination, decarboxylation and desulfuration)
4. textural changes via
   a) breakdown of the protein network
   b) increase in pH due to the production of NH_3 by deamination of free amino acids (particularly in surface mould varieties)
   c) decrease in a_w through greater water binding by the newly formed (liberated) amino and carboxyl groups (Fox, 1989).

Fat has no direct influence on proteolysis in cheese. The small differences found could be explained instead by the adjustments in the manufacturing procedure necessary because of variations in fat content. The casein is found to be hydrolyzed to a rather greater extent in the normal fat cheeses compared with reduced-fat cheeses during the first 3 weeks. In the ripened cheeses, significant differences in the total amounts of very small peptides and amino acids are found between cheese varieties with different contents of moisture in non-fat solids (MNFS). The differences in the peptide profiles between the normal fat cheeses and reduced-fat cheeses can be attributed to different enzymatic activities in the cheeses (Ardö and Gripon, 1995).

Four, to five agents are involved in the ripening of cheese: 1) rennet or rennet substitute (i.e. chymosin, pepsin or microbial proteinases) 2) indigenous milk enzymes, which are particularly important in raw milk cheeses but are also important in pasteurized milk cheese, especially those subjected to high cooking temperatures 3) starter bacteria and their enzymes, which are released after the cells have lyzed 4) enzymes from secondary starters (e.g. propionic acid bacteria, *Brevibacterium linens,* yeasts and moulds, such as *Penicillium roqueforti* and *Penicillium candidum*) are of major importance in some varieties 5) nonstarter bacteria e.g. organisms that either survive pasteurization of the cheese milk or gain access to the pasteurized milk or curd during manufacture. On death, these cells lyze

and release enzymes. The contribution of enzymes from nonstarter bacteria to cheese quality is controversial – there is a commonly held view that lactobacilli (in the case of Cheddar), pediococci and micrococci probably have negative effects on cheese quality, although they most certainly contribute to the intensity of cheese flavour. Nonstarter bacteria can be excluded by an aseptic milking technique and the use of aseptic vats. Antibiotics are usually included, and are probably necessary, especially for starter-free cheese. Starter bacteria produce a range of antibiotics, which very effectively inhibit the growth of nonstarter bacteria (Fox and McSweeney, 1996).

1.4 Proteolytic systems / agents in cheese

Proteolysis in cheese during ripening is catalyzed by enzymes from:

1. Coagulant (chymosin, pepsin or fungal acid proteinase)
2. Milk (plasmin and perhaps cathepsin D and other somatic cell proteinases) indigenous proteinases
3. Starter bacteria
4. Nonstarter, adventitious microflora
5. Secondary inoculum (in some varieties), e.g. *P. roqueforti, P. camemberti, Br. linens, Lactobacillus* spp. (a recent development in Cheddar)
6. Exogenous proteinases and/or peptidases or attenuated bacterial cells have been investigated recently as a means of accelerating ripening or accentuating flavour (Fox and McSweeney, 1996).

The relative contribution of proteolytic enzymes from these sources depends on the variety and has been estimated in cheeses manufactured under controlled microbiological conditions (Fox and McSweeney, 1996).

The progress of proteolysis in most ripened cheeses can be summarized as follows: initial hydrolysis of caseins is catalyzed primarily by residual coagulant, and to a lesser extent, by plasmin and perhaps cathepsin D and other somatic cell proteinases, resulting in the formation of large and intermediate-sized peptides which are then subsequently degraded by the coagulant and enzymes from the starter and nonstarter flora of the cheese. The production of small peptides and free amino acids results from the action of bacterial

proteinases and peptidases (Fox and McSweeney, 1996). This general outline of proteolysis can vary substantially between varieties due to differences in manufacturing practices.

1.5 Action and specificity of the principle proteinases during cheese ripening

The rate, extent and nature of proteolysis during cheese ripening as well as the amount and nature of the degradation products, vary according to the enzyme involved, the type of cheese and the environmental conditions of ripening. Primary proteolysis in cheese may be defined as the degradation of various individual proteins that constitute the cheese matrix, that is, the changes in (β)-, (γ)-, (α)-caseins, peptides and other minor bands that can be detected by polyacrylamide gel electrophoresis (Grappin *et al.*, 1985).

1.5.1 Coagulant

1.5.1.1 Chymosin

Chymosin (EC 3.4.23.4), which is the principle proteinase in traditional rennets used for cheesemaking, is an aspartyl proteinase from the gastric secretion by young mammals. The principle role of chymosin in cheesemaking is to specifically hydrolyze the Phe_{105}-Met_{106} bond of the κ-casein (the micelle-stabilizing protein) as a result of which the colloidal stability of micelles is destroyed, leading to gelation at temperatures ≥20^{0}C. Most of the rennet added to cheesemilk is removed in the whey, but some is retained in the curd and plays a major role in the initial proteolysis of caseins in many cheese varieties. More or less 6% of the chymosin added to the cheesemilk is retained in the curd, but the amount increases as the pH of whey drainage decreases (Creamer *et al.*, 1985). Pepsins, especially porcine pepsin, are more pH sensitive than chymosin and hence the amount of these coagulants retained in the cheese curd is very strongly dependent on the pH of milk at setting and shortly thereafter; in fact, increasing the pH of the curd whey to ~ 7 after coagulation of milk by porcine pepsin is one of the methods used to produce rennet-free cheese curd (O'Keeffe *et al.*, 1977, 1978). Only 2-3% of *Mucor* rennets are retained in the curd and appear to be independent of pH (Creamer *et al.*, 1985). In high-cooked cheeses, e.g. Emmental, chymosin is extensively denatured and makes little or no contribution to ripening (Singh and Creamer, 1990).

According to Fox and McSweeney (1996), the action of chymosin on the B-chain of insulin indicates that it is specific for hydrophobic and aromatic amino acid residues. Chymosin is weakly proteolytic; indeed, limited proteolysis is one of the characteristics to be considered when selecting proteinases for use as rennet substitutes. A coagulant is necessary to produce a rennet cheese. Porcine pepsin may be used as coagulant since it is more sensitive to high pH and is rapidly denatured at pH 7. Piglet chymosin hydrolyzes bovine κ-casein but is incapable of hydrolyzing α_s- or β-casein in cheese curd. Immobilized rennet hydrolyzes κ-casein without being incorporated in the curd (Fox, 1989).

The primary chymosin cleavage site in the milk protein system is the Phe_{105}-Met_{106} bond in κ-casein which is more susceptible to chymosin than any other bond in milk proteins. Cleavage of κ-casein Phe_{105}-Met_{106} yields *para*-κ-casein (κ-CNf1-105) and glycomacropeptides (κ-CNf 106-169). Most of the glycomacropeptides are lost in the whey but the *para*-κ-casein remains attached to the casein micelles and is incorporated into the cheese. α_{s1}-, α_{s2}- and β-caseins are not hydrolyzed during milk coagulation, but may be hydrolyzed in cheese during ripening (Fox and McSweeney, 1997).

It is generally accepted that rennet plays the major role in the initial breakdown of α_{s1}- casein, giving rise to the peptide α_{s1}-I. This peptide is present at least in the early stages of ripening in all types of cheeses (Grappin *et al.*, 1985).

The primary site of chymosin action on α_{s1}-casein is Phe_{23}-Phe_{24} (McSweeney *et al.*, 1993a). Cleavage of this bond is believed to be responsible for the softening of cheese texture (de Jong, 1976) and the small peptide (α_{s1}-CNf 1-23) is rapidly hydrolyzed by starter proteinases. Chymosin cleaves α_{s1}-casein in solution at Phe_{23}-Phe_{24}, Phe_{28}-Pro_{29}, Leu_{40}-Ser_{41}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156} -Asp_{157}, Tyr_{159}-Pro_{160} and Trp_{164}-Tyr_{165}. These bonds are also hydrolyzed at pH 5,2 in the presence of 5% NaCl (e.g.conditions in cheese) together with Leu_{11}-Pro_{12}, Phe_{32}-Gly_{33}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143} and Phe_{179}-Ser_{180} are also cleaved by chymosin (McSweeney *et al.*, 1993a).

The hydrolysis of α_{s1}-casein by chymosin is influenced by pH and ionic strength (Mulvihill and Fox, 1977, 1980). Although the amount of α_{s1}-I peptide increases during aging of cheese,

with a concomitant decrease of the native α_{s1}-casein, the relationship between the amounts of α_{s1}- casein and α_{s1}-I peptide in cheese is very weak, probably because α_{s1}-casein can be hydrolyzed to other products of low molecular weight (less than 11 000 Da) that are not detected by PAGE and also because α_{s1}-I undergoes further degradation by either rennet or by other proteases. The α_{s1}-I peptide can be almost entirely degraded during ripening (Grappin *et al.*, 1985).

The β-casein is hydrolyzed by chymosin in solution (0,05 M Na acetate buffer, pH 5.4) at 7 sites, but NaCl inhibits the hydrolysis of β-casein by chymosin to an extent dependent on pH; hydrolysis is strongly inhibited by 5% NaCl and completely by 10% NaCl (McSweeney *et al.*, 1993a).

The β-casein may be expected to undergo two types of hydrolysis during the first stages of ripening, one by the action of rennet to form β-I, β-II and β-III peptides, and one by plasmin, giving γ-caseins (Grappin *et al.*, 1985).

The α_{s2}-casein appears to be relatively resistant to proteolysis by chymosin (Richardson and Creamer, 1973). Cleavage sites are restricted to the hydrophobic regions of the molecule (sequence 90-120 and 160-207), i.e., Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Tyr_{98}, Tyr_{98}-Leu_{99}, Phe_{163}-Leu_{164}, Phe_{174}-Ala_{175}, Tyr_{179}-Leu_{180} (McSweeney *et al.*, 1994).

Although *para*-κ-casein has several potential chymosin cleavage sites, it does not appear to be hydrolyzed either in solution or in cheese. Presumably, this reflects the relatively high level of secondary structure in κ-casein compared to the other caseins (Green and Foster, 1974; Swaisgood, 1992).

1.5.1.2 Pepsins

Calf rennet contains about 10% bovine pepsin (EC 3.4.23.1) and many calf rennet preparations contain up to 50% bovine pepsin. The proteolytic products produced from Na-caseinate by bovine pepsin are similar to those produced by chymosin, although the specificity of bovine or porcine pepsins on bovine caseins has not been determined. The

hydrolysis of bovine, ovine, caprine and porcin β-caseins by chymosins and pepsins from these species, suggested generally similar specificities for chymosins and pepsin on the large peptides produced, although differences are apparent in the short peptides. Pepsins are more proteolytic than the corresponding chymosins (Fox and McSweeney, 1997).

Recombinant calf chymosins, expressed in *Aspergillus niger* var. *awamori, Kluyveromyces marxianus* var. *lactis*, or *Escherichia coli*, were recently introduced and they are now widely used for cheesemaking. Cheesemaking trials on several cheese varieties have shown only small differences between cheese made using calf rennet or recombinant chymosin. Recombinant chymosins may contain only one genetic variant of this enzyme, while calf rennet may contain three chymosin variants (A, B and C) as well as some bovine pepsin (Green *et al.*, 1985; O'Sullivan and Fox, 1991; Nuñez *et al.*, 1992).

1.5.1.3 Fungal rennets

The supply of calf rennet has been insufficient to meet demand and much effort has been expended on searching for suitable rennet substitutes for cheesemaking (Green, 1977). Several proteinases have been assessed but only bovine pepsin and proteinases from *Rhizomucor pussillus, R. miehei* and *Cryphonectria parasitica* have been used extensively in commercial practice (Phelan *et al.*, 1973). The original *R. miehei* proteinase is considerably more heat stable than chymosin, which caused problems when whey containing it was used as a food ingredient. The current *Rhizomucor* rennet has been rendered heat labile by chemical modification due to oxidation of methionine. The gene for *R. miehei* proteinase has been cloned in *Aspergillus cryzae,* resulting in a rennet containing less contaminating proteolytic enzymes. This coagulant is found to be very suitable for the manufacture of Cheddar cheese. *C. parasitica* proteinase is considerably more proteolytic than chymosin, especially on β-casein and is used for cheesemaking in high-cooked varieties, in which the proteinase is extensively denatured by the high cooking temperature (Fox and McSweeney, 1997).

Hydrolyzates of sodium caseinate by chymosin, *R. miehei* proteinase and *C. parasitica* proteinase were compared and the specificities were clearly very different (*C. parasitica* is

particularly active on β-casein). However, the bonds cleaved by fungal proteinase have not been reported (except that the primary cleavage of κ-casein by *C. parasitica* proteinase is at Ser_{104}-Phe_{105} rather than Phe_{105}-Met_{106}, which is cleaved by chymosin and *R. miehei* proteinase) (Fox and McSweeney, 1997).

The secondary proteolytic action of the coagulant influences flavour in three ways:

1. Some rennet-produced peptides are small enough to influence flavour. Unfortunately, some of these peptides are bitter and excessive proteolysis, e.g. due to too much or excessively proteolytic rennet or unsuitable environmental conditions, e.g. too much moisture or too little NaCl, leads to bitterness.
2. Rennet-produced peptides serve as substrate for microbial proteinases and peptidases which produce small peptides and amino acids. These contribute at least to background flavour, and perhaps, unfortunately, to bitterness if the activity of such enzymes is excessive. Catabolism of amino acids by microbial enzymes, and perhaps alterations via chemical mechanisms, lead to a range of sapid compounds (amines, acids, NH_3, thiols), which are major contributors to characteristic cheese flavours.
3. Alterations in cheese texture appear to influence the release of flavourous and aromatic compounds, arising from proteolysis, lipolysis, glycolysis and secondary metabolic changes, from cheese during mastication which may be the most significant contribution to cheese flavour (Fox *et al.*, 1993).

1.5.2 Indigenous milk proteinases

1.5.2.1 Plasmin

Plasmin (fibrinolysin, EC 3.4.21.7) is a trypsin-like serine proteinase, which is optimally active at about pH 7.5 and 37^{0}C. It is highly specific for peptide bonds to which lysine, and to a lesser extent, arginine, contribute the carboxyl group. It is active on all caseins, but especially on α_{s2} - and β- casein. Proteinases other than milk-clotting enzymes contribute very little to the formation of large peptides from α_{s1}-casein (Thomas and Pearce, 1981).

The primary plasmin cleavage sites in β-casein are: Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}, with the formation of the polypeptides, β-CNf29-209 (γ_1-CN), f106-209 (γ_2-CN) and f108-209 (γ_3-CN), β-CN-f1-105 and f1-107 (proteose peptone 5), β-CN-f29-105 and f29-107 (proteose peptone 8-slow), and β-CN-f1-28 (proteose peptone 8-fast). Additional cleavage sites are Lys_{113}-Tyr_{114}, and Arg_{183}-Asp_{184} (Fox *et al.*, 1994).

The presence of γ-caseins is noted in almost all kinds of cheese, which indicates that the alkaline milk proteinase plays the major role in the hydrolysis of β-casein (Creamer, 1979).

There is a good relationship between plasmin activity in Cheddar cheese and the amount of β-casein degraded (Richardson and Pearce, 1981). Salt content and pH also play an important role (Creamer, 1975). The overall breakdown of β-casein in cheese is clearly affected by the salt concentration.

The α_{s2}-casein in solution at pH 7.8 is completely degraded by plasmin with concomitant appearance of faint bands of high electrophoretic mobilities and diffuse bands in the negative direction. The extent of degradation of α_{s2}-casein in Cheddar is related to the plasmin content in cheese (Richardson and Pearce, 1981). Plasmin cleaves α_{s2}-casein in solution at 8 sites: Lys_{21}-Gln_{22}, Lys_{24}-Asn_{25}, Arg_{114}-Asn_{115}, Lys_{149}-Lys_{150}, Lys_{150}-Thr_{151}, Lys_{181}- Thr_{182}, Lys_{188}-Ala_{189} and Lys_{197}-Thr_{198}, producing about 4 peptides, 3 of which are potentially bitter (α_{s2}-CNf198-207, f182-207 and f189-207) (Le Bars and Gripon, 1989).

Although plasmin is less active on α_{s1}-casein than on α_{s2}- and β- caseins, the formation of λ-casein, a minor casein component, has been attributed to its action on α_{s1}-casein (Aimutis and Eigel, 1982). The specificity of plasmin on α_{s1}-casein cleavage sites in solution are Arg_{22}-Phe_{23}, Arg_{90}-Tyr_{91}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}- Val_{106}, Lys_{124}-Glu_{125} and Arg_{151}-Gln_{152} (McSweeney *et al.*, 1993b). Plasmin has very low activity on κ-casein although it contains several lysine residues (Fox and McSweeney, 1996).

1.5.2.2 Cathepsin D

Cathepsin D (EC 3.4.23.5), an indigenous acid proteinase in milk is relatively heat labile and has a pH optimum of 4.0 (Kaminogawa and Yamauchi, 1972). The specificity of cathepsin D

on the caseins has not been determined, although electrophoretograms of caseins incubated with milk acid proteinase (cathepsin D) indicate a specificity very similar to that of chymosin. Surprisingly, cathepsin D has very poor milk clotting activity (McSweeney *et al.*, 1995).

1.5.2.3 Other indigenous milk proteinases

The presence of other minor proteolytic enzymes in milk, including thrombin and a lysin aminopeptidase, has been reported. In addition to cathepsin D, other proteolytic enzymes from somatic cells may also contribute to proteolysis in cheese. Somatic cell proteinases and plasmin produce distinctly different peptides and the plasmin inhibitor 6-aminohexanoic acid is suitable for studying the action of somatic cell proteinases without interference from plasmin. Somatic cell proteinases are capable of activating plasminogen and this may influence proteolysis in cheese by elevating plasmin levels (Verdi and Barbano, 1991).

Although leucocyte proteinases are more active on β-casein at pH 6.6 than at pH 5.2, their activity at the lower pH is such as to suggest that they may be active in cheese during ripening. Leucocyte extracts hydrolyzed the caseins in the order $\alpha_{s1} > \beta >> \kappa$. Gouda cheeses made from milks with the same total somatic cell count but different levels of polymorphonuclear leucocytes had more rapid production of α_{s1}-I-casein (α_{s1}-CNf24-199) and total free amino acids compared to cheese made from milk with high polymorphonuclear levels (Verdi and Barbano, 1991).

Two cysteine proteinases are found in milk (45kDa and > 150 kDa) and it is considered that these two proteinases originated in somatic cells and their level increases during mastitic infection (Suzuki and Katoh, 1990).

1.5.3 Proteolytic enzymes from starter cultures

Although lactic acid bacteria (LAB) are weakly proteolytic, the cultures do possess a proteinase and a wide range of peptidases which are principally responsible for the formation of small peptides and amino acids in cheese. The genus most widely used as a cheese starter is *Lactococcus*. Its proteolytic system is capable of hydrolyzing casein completely to

free amino acids. The proteinase in LAB is anchored to the cell membrane and protrudes through the cell wall, giving it ready access to extracellular proteins. All the peptidases are intracellular although some (e.g., PepX) appear to be orientated toward the outer surface of the cell membrane. The oligopeptides produced by the proteinase are actively transported into the cell where they are hydrolyzed further by the battery of peptidases (Fox and McSweeney, 1996).

1.5.3.1 Proteinases from *Lactococcus*

Cell wall associated proteinases of *Lactococcus* have been classified into 3 groups, P_1-, P_{111}- and mixed $P_{1/111}$- types; P_1 type proteinase degrades β- but not α_{s1}- casein at a significant rate, while P_{111}- type proteinase rapidly degrades both α- and β- caseins (Visser *et al.*, 1986).

Lactococcus spp. possess intracellular proteinases. Three such enzymes were demonstrated in the cytoplasmic fraction of the cell wall associated proteinase-negative mutant, *L. lactis* spp. *cremoris* MG 1363. These proteinases (P_1 dimeric, Mr = 124 kDa; P_2, monomeric, Mr = 64 kDa; and P_3, monomeric, Mr = 47 kDa) are optimally active at pH 7.0 and 35^{0}C (P_1) or pH 7.5 and 45 ^{0}C (P_2 and P_3). P_1 is a serine proteinase while P_2 and P_3 are metalloenzymes. An intracellular proteinase from *L. lactis* subsp. *lactis* NCDO 763 was isolated. This metalloenzyme (93 kDa) is optimally active at pH 7.5 and 45^{0}C and exhibits thermolysin-like specificity. These enzymes are probably involved in intracellular protein processing and are not involved in producing peptides for growth of the cells in milk, however, they are presumably released on cell lysis and may therefore contribute to proteolysis in cheese (Fox and McSweeney, 1996).

There are four intracellular proteinases with caseinolytic activity and eight enzymes with activity on CBZ-Phe–Phe-Arg-benzyloxycarbonyl-L-Phe-Arg-7-(4-methyl)coumarylamide. The caseinolytic enzymes ranged from 12-160 kDa and have a pH optima from 5.5-7.0. Two of these enzymes are metalloproteinases, one a serine proteinase and one a thiol enzyme (Fox and McSweeney, 1996).

1.5.3.2 Peptidases of *Lactococcus*

The peptidases produced by the lactococcal cell envelope proteinase (CEP) are too large to be transported into the cell, which possess a battery of endo-and exopeptidases capable of degrading peptides to amino acids, as well as a number of independent peptide and amino acid transport systems.

Lactococcus spp possess at least 2 endopeptidases [variously called *Lactococcus* endopeptidase (LEP), neutral oligopeptidase (NOP) or metalloendopeptidase (MEP), PepO or PepF] which are incapable of hydrolyzing intact caseins but can hydrolyze casein-derived peptides containing up to at least 34 residues, e.g., α_{s1}-CNf165-199. A second oligopeptidase (PepF) from *Lactococcus* was characterized to be similar to an enzyme previously referred to as LEP 1 (Fox and McSweeney, 1996).

Lactococcus spp possess 3 aminopeptidases [a general aminopeptidase (PepN), a glutamyl/aspartyl aminopeptidase (PepA), and a thiol aminopeptidase (PepC)], an iminopeptidase, which releases N-terminal proline; and a postproline dipeptidyl aminopeptidase (PepX), which releases X-Pro dipeptides from the N-terminal of peptides. They possess a tripeptidase and 3 dipeptidases: a general dipeptidase and 2 proline-specific dipeptidases [prolinase (ProX) and prolidase (Xpro)] (Fox and McSweeney, 1996).

Thus, *Lactococcus* spp. are well equipped to metabolize proline-rich peptides, which is very important for their growth in milk since the caseins are very rich in proline e.g., 35 of the 209 residues in β-casein are proline (Fox and McSweeney, 1996).

Lactococcus spp. appear not to possess a carboxypeptidase (Fox and McSweeney, 1996).

1.5.3.3 Proteinases and peptidases of thermophilic *Lactobacillus* spp.

Thermophilic lactobacilli are widely used in dairy fermentations, e.g., *Lb. delbrueckii* subsp *bulgaricus* in yoghurt; *Lb. helveticus, Lb. acidophilus, Lb. bulgaricus* and *Lb. lactis* in Swiss and Italian cheeses, including Mozzarella. The proteolytic system of *Lactobacillus* spp.

appears to be generally similar to that of *Lactococcus*. The principle proteinase in *Lactobacillus* is associated with the cell wall/membrane (Fox and McSweeney, 1996).

There are 9 peptidases from *Lactobacillus* characterized. The cell-wall proteinase of *Lb. helveticus* CNRZ 303, a serine enzyme, is optimally active at pH 7.5-8.0 and 42^{0}C. It hydrolyses β-casein at Leu_6 -Asn_7, Lys_{105}-His_{106}, Phe_{119}-Thr_{120}, Gln_{175}-Lys_{176}, Gln_{182}-Arg_{183}, Phe_{190}-Leu_{191} and Leu_{192}-Tyr_{193} and α_{s1}-casein at Ile_6-Lys_7 and Gln_9-Gly_{10} (Fox and McSweeney, 1996).

1.5.3.4 Proteinases and peptidases of *Streptococcus salivarius* subsp. *thermophilus*

Str. salivarius subsp. *thermophilus* is a component in the cultures used for yoghurt, Swiss-type cheeses and Mozzarella. It is less proteolytic than the thermophilic lactobacilli and usually grows in milk in symbiosis with a *Lactobacillus*, which provides peptides for the *Streptococcus*. The dipeptidase and aminopeptidase activities of 7 strains of *Str. salivarius* subsp. *thermophilus* have been studied. All strains of *Str. salivarius* subsp. *thermophilus* have leucine aminopeptidases and PepX activities and some have arginine aminopeptidases. The proteinase of most strains is able to hydrolyze β- and κ-caseins. The peptidases are intracellular and the proteinases have a peripheral location (Meyer *et al.*, 1989). The cell envelope associated proteinases (CEP) of *Str. salivarius* subsp. *thermophilus* CNRZ 385 and CNRZ 703, are serine enzymes, optimally active at ~pH 7.0 and 37-45^{0}C (Fox and McSweeney, 1996).

An intracellular aminopeptidase appears to be generally similar to the PepN of other LAB, e.g. it is a metalloenzyme with a pH and temperature optima of 7 and 36^{0}C, respectively; it has a molecular weight of 97kDa and appears to exist as a monomer (Fox and McSweeney, 1996).

1.5.3.5 Proteolytic enzymes of *Leuconostoc*

Leuconostoc spp. are included in starters for Dutch type and Blue Cheeses. *Leuconostoc* is heterofermentative and produces lactic acid, ethanol and CO_2. Its principle function in

cheese cultures is to produce CO_2 for eye development in Dutch-type cheeses and the open texture of importance for prolific mold growth in Blue Cheeses. However, its proteolytic system presumably contributes to proteolysis, although this may be negligible in comparison with that of other proteinases and peptidases, especially in Blue Cheeses where the mold, *P. roqueforti* is the most important proteolytic agent.

The cell wall associated proteinase of *Leu. mesenteroides* subsp. *mesenteroides* CNRZ 1019 is optimally active at pH 7.0 and 40^0C. Cell wall associated peptidase activity was also found (Fox and McSweeney, 1996).

1.5.4 Proteolytic enzymes of Nonstarter lactic acid bacteria

The starter organism *Lactococcus* reaches maximum numbers (~10^9 cfu/g) in Cheddar and Dutch-type cheeses during curd manufacture or shortly thereafter (within 24h). Numbers then decline at rates characteristic of the strain due to the pH, lack of a fermentable sugar and a high NaCl concentration. Although initially present at low numbers (< 50 cfu/g in Cheddar made from pasteurized milk in modern plants) (Folkertsma *et al.*,1996), adventitious nonstarter lactic acid bacteria (NSLAB) grow rapidly to reach ~10^7 cfu/g within about 4 weeks and remain relatively constant thereafter. Thus, depending on the rate of lysis of the starter, NSLAB can dominate the viable microflora of Cheddar and extramature Dutch cheeses throughout the ripening period. However, in spite of this, the proteolytic system of NSLAB has received little attention compared to that of *Lactococcus*. The proteolytic specificity of proteinases from NSLAB on the caseins has not been determined (Fox and McSweeney, 1996). The predominant NSLAB in Cheddar and Dutch-type cheeses are mesophilic *Lactobacillus* spp. *Lb. casei* subsp. *casei* is the principle mesophilic *Lactobacillus* in Irish Cheddar, with lesser numbers of *Lb. plantarum*, *Lb. pseudoplantarum* and *Lb. curvatus*. Raw-milk Cheddar cheeses contain more *Lactobacillus* species than pasteurized-milk cheeses (McSweeney *et al.*, 1993c).

Mesophilic lactobacilli enzymes are generally similar to the proteinases of *Lactococcus* and thermophilic *Lactobacillus*. The β-casein is preferentially hydrolyzed by a number of strains of *Lb. plantarum* and *Lb. casei* but some strains hydrolyzed α_{s1}-casein as well. The

proteinase of *Lb. plantarum* DPC 2739 hydrolyzes α_{s1}- and β-caseins at more or less equal rates, apparently with broad specificity. The specificity of any proteinase from mesophilic *Lactobacillus* spp on the individual caseins has not been established (Fox and McSweeney, 1996).

Many mesophilic lactobacilli grow very poorly in milk (they require supplementation with a source of small peptides and amino acids, e.g. yeast extract), suggesting low CEP activity compared to *Lactococcus* and thermophilic lactobacilli. Since the NSLAB in Cheddar appear not to lyse as readily as the starter (at least their numbers do not decline sharply during ripening) and they have low CEP activity, they may contribute little to proteolysis in cheese (Fox and McSweeney, 1996).

Lactobacillus CEP degrades chymosin-produced peptides in a similar manner to lactococcal CEP but is much less active and in normal Cheddar probably makes little contribution to proteolysis at this level. Perhaps this is due to the relatively low number (10^7 cfu/g) of NSLAB compared to starter organisms (10^9/g) (Lane and Fox, 1996). A range of intracellular peptidases, including dipeptidase, aminopeptidase and endopeptidases have been identified in mesophilic *Lactobacillus*. Interestingly, carboxypeptidase activity, which has not been found in lactococci, has been identified in *Lb. casei*. These peptidases are generally similar to their lactococcal counterparts. Since NSLAB do not appear to lyze in Cheddar, the question arises as to how much these intracellular peptidases contribute to proteolysis in cheese. However, since the NSLAB cells are viable, even if not multiplying, they may transport small peptides into the cells. Certainly, *Lactobacillus* contributes to the formation of free amino acids in cheese, e.g. the concentration of free amino acids in raw- milk Cheddar (10^8 NSLAB/g) was about twice that in a pasteurized-milk counterpart (10^7 NSLAB/g) and was higher in a controlled microflora cheese containing *Lactobacillus* than in a control cheese without a *Lactobacillus* adjunct (McSweeney *et al.*, 1993c; Fox and McSweeney, 1996).

NSLAB also include *Micrococcus* and *Pediococcus*. All *Micrococcus* spp appear to produce intracellular proteinases and some also produce extracellular proteinases. Three strains of *Micrococcus* studied produced extracellular proteinases which preferentially hydrolyzed α_{s1}-casein while intracellular proteinases preferentially hydrolyze β-casein. Two extracellular

metalloproteinases (23,5 and 42,5 kDa) from *Micrococcus* sp. GF are optimally active at ~ 45^{0}C and pH 8.5 to 11. One proteinase preferentially hydrolyzes β-casein, while the other hydrolyzes both α_{s1}-casein and β-caseins at approximately the same rate. *Micrococcus* spp also possess membrane-associated - and intracellular proteinases (Fox and McSweeney, 1996).

There is proteolytic activity in *Pediococcus*, e.g. Leu and Val aminopeptidase activities in *P. pentosaceus* as well as aminopeptidase, dipeptidase and cell wall associated proteinase activity in *Pediococcus* spp LR (Fox and McSweeney, 1996).

Enterococci are components of the microflora of some cheese varieties. Extracellular proteinase of *Enterococcus faecalis* subsp *liquefaciens* L61 (isolated from Monchego cheese) accelerated proteolysis in Monchego cheese (Fox and McSweeney, 1996).

1.5.5 Proteinases from secondary starter cultures

The NSLAB are adventitious and no measures are taken to promote their growth, in fact, precautions are taken, perhaps inadvertently, to prevent or at least control their growth. However, many cheese varieties have a secondary microflora which is added intentionally and/or encouraged to grow by environmental conditions. We refer to these microorganisms as *secondary starters*. While the principle function of the primary starter (*Lactococcus, Lactobacillus* or *Streptococcus*) is to produce the correct amount of acid at an appropriate rate and time, the secondary starter performs a diverse range of functions, depending on the organisms used. *Lc. lactis* subsp *lactis* biovar *diacetylactis* and *Leuconostoc* spp are used as secondary starters in Dutch-type cheeses, primarily to produce CO_2. A secondary starter is not used in Cheddar-type cheeses, although as discussed previously, it has a significant adventitious secondary flora. However, in recent years there has been increasing interest in inoculating milk for Cheddar with selected strains of *Lactobacillus*, with the objectives of accelerating ripening and accentuating flavour. Such cultures, which have been referred to as adjunct starters, are usually mesophilic lactobacilli, the proteolytic system of which was discussed before (Fox and McSweeney, 1996).

1.6 Texture, flavour and aroma of Cheddar cheese

The objective of cheese manufacture is to produce a product with the flavour, aroma and texture of the intended variety, free of defects and in the shortest time possible. Textural changes, although very complex, are probably the least complex of these three changes. The texture of cheese is determined initially by the composition of the cheesemilk, especially by the fat:casein ratio, by the manufacturing operations which control the extent of syneresis and hence the moisture content of the cheese, and the rate of acidification which controls the extent of demineralization of the curd and which in turn has a major influence on the textural parameters of the cheese (Fox *et al.*, 1993). The texture of the cheese changes during ripening due to proteolysis, especially of the α_{s1}-casein by the rennet, the decrease in a_w due to the liberation of water-binding ionic groups, redistribution of salt, and, in many cases, evaporation of water and to changes in pH due to proteolysis and catabolism of lactic acid, which is most marked in surface mould-ripened cheeses (Lawrence *et al.*, 1987). Undoubtedly, cheese aroma and flavour are influenced by cheese texture, e.g. by consumer perception and release of sapid and aromatic compounds from the cheese mass during mastication (McGugan *et al.*, 1979).

Research on cheese flavour and aroma expanded greatly during the 1960's and 1970's and indicated the presence of hundreds of compounds that could contribute to cheese flavour and aroma. Most of these are present at very low concentrations, many below their flavour thresholds, but which may still affect cheese quality. Thus, cheese flavour is due to the correct balance of a mixture of compounds (Aston *et al.*, 1983).

In internally bacterially ripened cheese such as Cheddar, there appears to be fairly good agreement that the water-insoluble fraction (consisting mainly of proteins and large peptides) is devoid of flavour and aroma, that the water-soluble non-volatile fraction (small peptides, amino acids, organic acids) contains most of the compounds responsible for flavour while the aroma is principally in the volatile fraction. There appears to be strong support for the view that products of proteolysis are the principal contributors to cheese flavour (McGugan *et al.*,1979; Aston *et al.*, 1983).

The principal contributors to aroma are less clear, but sulphur-containing compounds and carbonyl compounds are probably important. It has long been considered that sulphur compounds are major contributors to the flavour of Cheddar cheese (Kristofferson, 1985).

Dunn and Lindsay (1985) described the presence of several Strecker-derived aldehydes and alcohols in Cheddar cheese. The principal compounds found were phenylacetaldehyde and phenethanol (from phenylalanine), p-cresol, phenol (from tyrosine), 3-methylbutanal (from leucine), 2-methyl butanal (from isoleucine) and 2-methyl pentanal (from valine). All of these, except phenol, had distinctly unclean flavours. Interestingly, Phe, Tyr, Leu, Ile and Val are participants in peptide bonds hydrolyzed by chymosin early during ripening and hence are accessible for release by bacterial aminopeptidases.

There has been considerably more progress on elucidating off-flavours than desirable flavours in cheese - this is probably not too surprising since each off-flavour usually has a specific cause. Examples include fruity (ethyl hexanoate and ethyl octanoate, resulting from high concentrations of ethanol), butyric acid flavour in Swiss cheese and bitterness which is common in many cheeses. It is generally agreed that bitterness is due to the accumulation of hydrophobic peptides but there is disagreement on the cause of the problem, whether it is due to a deficiency of peptidase activity or too much proteinase activity in certain starters. There has been a certain shift from the analysis of cheese volatiles by GC to analysis and characterization of the non-volatile fraction by HPLC. The non-volatile water-soluble compounds, especially peptides and amino acids, are important in cheese flavour, and in view of this, the role of starter proteinases and peptidases in cheese quality (Fox *et al.*, 1993).

Several researchers have reported the contribution of nonvolatile water-soluble fractions to cheese flavour. These components give an essential background flavour, while volatile components contribute to more characteristic "cheesy" qualities (McGugan *et al.*, 1979). Biede (1974) reported that burned flavour correlated with proteolysis and Barlow *et al.* (1989) found that cheese flavour correlated well with water-soluble nitrogen, lactic acid and H_2S. Langler *et al.* (1967) showed that amino acids (mostly proline) were involved in sweet flavour and peptides could influence cheese flavour. Biede and Hammond (1979) reported that

amino acids and dipeptides were responsible for brothy-nutty flavour, whereas burned and bitter flavours were due to tri-, tetra-, penta- and hexapeptides. Kowaleska *et al.* (1985) asserted that the water-soluble nonvolatile fraction, including amino acids, peptides, salts, lactic acid, Ca^{2+} and Mg^{2+} ions, have a very intense flavour.

Aston and Creamer (1986) ascribed the taste intensity of a Cheddar cheese with bitter and brothy notes, to the water-soluble peptide-containing fraction. Hydrophobic peptides are bitter and responsible for the bitter defect in cheese. However, di- or tripeptides containing N-terminal L-glutamic acid have a umami taste. Free amino acids contribute to the characteristic taste of food. Monosodium glutamate (MSG) plays an important role because of its brothy, umami taste and its enhancing properties. "Umami" is a Japanese word meaning delicious. Umami taste is complex and typical of MSG. Glu and glutamyl peptides are important for the flavour of cheese, especially γ-glutamyl peptides (Roudot-Algaron *et al.*, 1994).

Cheese flavour components in the water-soluble fraction are of low molecular weight (Mr) (<1000); the flavour intensity is greatest in the fraction relatively rich in the free amino acids, Met and Leu. Because in this fraction the NaCl concentration is also highest, the latter is considered to have an additional effect on the total flavour intensity (Visser, 1993).

As already discussed above, various proteinases and peptidases in cheese hydrolyze caseins to peptides and free amino acids. Small peptides and free amino acids contribute directly to the background flavour of cheese (McGugan *et al.*, 1979; Aston and Creamer, 1986; Cliffe *et al.*, 1993; Singh *et al.*, 1994). Most of the savory cheesy taste of the water-soluble extract of cheese is in the ultra-filtration permeate (10 kDa. nominal molecular weight cut-off), which contains small peptides, free amino acids, salts, and other low molecular weight compounds, whereas the retentate, which contains intermediate size peptides, is bland (Singh *et al.*, 1994). Engels and Visser (1994) concluded that, with the exception of Edam, the ultra-filtration permeate with a molecular weight (MW)<500, of Cheddar, Gouda, Gruyère, Maasdam, Parmesan and Proosdij cheeses contained the components responsible for flavour. The permeate of these cheeses contained low molecular weight peptides (probably not larger than tetrapeptides), amino acids, breakdown products of amino acids

(e.g. γ-aminobutyric acid and ornithine), and short-chain fatty acids (<C_9). The dia-filtration permeate of the water-soluble extract is dominated by two peptides, α_{s1}-CN f1-9 and α_{s1}-CN f1-13, which accumulate during ripening (Singh *et al.*, 1994). Kaminogawa *et al.* (1986) reported that α_{s1}-CN f1-9, α_{s1}-CN f1-13 and α_{s1}-CN f1-14 also accumulate in Gouda cheese. These peptides are formed from α_{s1}-CN f1-23, produced on cleavage of the bond Phe_{23}-Phe_{24} of α_{s1}-casein by chymosin during the early stages of ripening, by the starter cell-envelope proteinase (CEP). Exterkate and Alting (1995) showed that incubation of α_{s1}-CN f1-23 with lactococcal proteinases and peptidases failed to generate cheeselike flavour. This observation indicates that flavour development, insofar as it is based on proteolysis, is apparently initiated relatively late in Cheddar or Gouda cheese and is dependent on further proteolysis of other casein fragments.

The majority of the peptides characterized in the water-soluble dia-filtrate permeate originate from α_{s1}-casein. The bond Phe_{23}-Phe_{24} is hydrolyzed rapidly by chymosin during the early stages of ripening, resulting in the formation of small, α_{s1}-CN f1-23 and large, α_{s1}- CN f124-199 fragments. The α_{s1}-CN f1-23 is hydrolyzed rapidly by starter CEP. Hydrolysis of bonds Gln_9-Gln_{10} and Gln_{13}-Glu_{14} by CEP results in the formation of peptides α_{s1}-CN f1-9 and 1-13 (Singh *et al.*, 1994). These two peptides accumulate in cheese during ripening. Four peptides originate from peptide 1-23 of α_{s1}-CN by cleavage of CEP and four peptides originate from the large peptides α_{s1}- CN f24-199 (produced by chymosin) by an aminopeptidase. Five peptides are produced from α_{s2}-casein by chymosin and CEP.

Plasmin is mainly responsible for the primary hydrolysis of β-casein in cheese, resulting in the formation of γ-caseins and proteose-peptones. Most of the peptides in the retentate of water-soluble extract of Cheddar cheese originate from the N-terminal half of β-casein. The concentration of γ-caseins increases during ripening of Cheddar cheese, but the proteose-peptones are further hydrolyzed by CEP (Singh *et al.*, 1995). Only one β-casein derived peptide was identified in the water-soluble permeate fraction.

The principal free amino acids in water-soluble dia-filtrate permeate extract are Glu, Leu, Val, Pro and Lys (Fernandez *et al.*, 1998). It has been reported by Law *et al.* (1976) that the

most abundant amino acids in Cheddar cheese are Glu, Leu, Val, Ile, Lys and Phe. Many of the bonds hydrolyzed by CEP expose Glu/Gln residues; the presence of high amounts of Glu indicates easy removal of N-terminal Glu residues from peptides by an aminopeptidase (PepA). Activity of the general aminopeptidase (PepN) can explain the release of amino acids such as Leu, Val and Lys. A considerable amount of Pro was present, which in the apparent lack of PepX activity, suggests considerable proline aminopeptidase activity in Cheddar cheese. Thus: starter *Lactococcus* spp. CEP and peptidases play an important role in the degradation of the larger peptides produced by chymosin and plasmin from caseins (Fernandez *et al.*, 1998).

1.7 Uses of yeasts in the dairy products

Most fermented dairy products are produced using bacteria rather than yeasts. Kefir is an exception and is the product of a mixed fermentation with yeasts and bacteria (Bottazzi, 1983).

Yeasts are not the most dominant microorganisms in dairy products. Bacteria, especially the psychrotrophs are causing spoilage, and bacterial starter cultures contribute most to the final product adding to the aroma and taste. Despite the fact that yeasts play an essential role in the preparation of certain fermented dairy products and in the ripening of certain cheeses, they also contribute substantially to the final product due to interactions between the yeasts and bacteria, which also include the starter cultures (Viljoen, 1999).

The occurrence of yeasts in dairy products is significant because they can cause spoilage, affect desirable biochemical changes and they may adversely affect public health (Fleet and Mian, 1987).

The yeast interactions either contribute to the fermentation by supporting the starter cultures, inhibiting undesired microorganisms causing quality defects, or adding to the final product by the production of aromatic compounds, proteolytic activities, etc; or the interactions may be detrimental in causing spoilage, inhibit the growth of the starter cultures and consequently also the final outcome of the product or lead to excessive gas formation, off-flavours or discolouration (Viljoen, 1999).

There is increasing evidence that some yeast species contribute to flavour and texture development during the ripening of certain cheeses (Marth, 1978). However, over-ripening can be interpreted as a form of cheese spoilage by yeasts (Ingram, 1958; Walker and Ayres, 1970).

Since yeasts are not added under normal circumstances as part of the starter cultures, the yeast strains are derived as contaminants from the environment, since the environment and the products allow growth of a vast range of yeasts. However, only part of this primary microflora will survive under the selective pressures exerted by the internal and external environments of dairy products and the presence of the rest is regarded as purely accidental. Those strains capable to respond, will develop in a dominant yeast community and eventually play the major role in interactions between the yeasts and starter cultures (Viljoen, 1999).

The high number of yeasts in dairy products may be attributed to the following: growth at low temperatures, assimilation/fermentation of lactose, assimilation of organic acids like succinic acid, lactic acid, lipolytic and proteolytic activities, low water activities, resistance against high salt concentrations and resistance against cleaning compounds and sanitizers which all add to the survival and progression of the yeasts (Viljoen,1999).

The occurrence of yeasts in cheese is not unexpected because of the low pH, low moisture content, elevated salt concentration and low storage temperatures. The significance of this presence depends on the type of cheese. Yeast populations exceeding 10^7 were reported and counts of 10^4 to 10^6 cfu/g are frequently found. Especially in soft cheeses, the yeasts contribute to the cheese flavour and texture (Viljoen, 1999).

Of the six groups of dairy products (milk, cream, butter, yogurt, cheese and ice-cream) examined by Fleet and Mian (1987), yogurt and cheeses exhibited the highest incidence and level of yeast contamination. This finding is probably related to the fact that they are both low pH products and would form a selective environment for yeast growth. Yeast counts in the range 10^4- 10^7 cells per g are not uncommon. It appears that such populations can occur in all types of cheeses, whether they be hard, semi-hard or soft varieties. Highest populations probably occur in the softer cheeses such as Camembert, Saint-Nectaire, blue

cheese, limburger and brick where counts of 10^8-10^9 cells per g may develop at surface locations and counts of 10^5-10^7 cells per g may occur in the interior zones. In these cases, it is believed that yeasts make a positive contribution to the development of cheese flavour and texture during the ripening stage through their metabolism of protein, fat, lactose and lactic acid. However, over-ripening by yeast activity can lead to flavour and textural defects as well as the development of surface discolouration. None of the Cheddar cheeses examined in the study were considered spoiled despite the occurrence of 10^5-10^6 yeasts per g in some samples. Unripened, cottage cheese can also contain significant populations of yeasts, which cause swelling and bulging of the product container if they grow to levels exceeding 10^6 cells per g. They are also responsible for the development of gassiness and off-flavours in such products (Fleet and Mian, 1987).

The predominance of *Candida famata* and its sporulating counterpart *Debaryomyces hansenii*, *Kluyveromyces marxianus, Candida diffluens* and to a lesser extent, *Rhodotorula glutinis* predominate in dairy products and this could be related to their ability to produce extracellular proteases and lipases and growth abilities at 5^0C. These properties could be more important than lactose fermentation in determining the occurrence of yeasts in dairy products (Fleet and Mian, 1987).

All of the yeasts present during processing and the ripening of harder cheeses originated as contaminating yeasts from the equipment, air, hands, and aprons etc. During the ripening of cheeses, yeasts continue to increase at a faster rate than the starter cultures, but most important, there are no inhibition of either of the populations. Therefore, the mutualistic interaction may contribute to the final product due to the production of flavour compounds, support the starter culture during maturation by excreting lipolytic or proteolytic enzymes, etc. (Viljoen, 1999).

The significance of the presence of yeasts depends on the particular type of cheese. In some cheeses, yeasts contribute to spoilage or make a positive contribution to flavour development during the maturation stages. Yeast spoilage is recognized as a problem in cheese causing typical defects such as excessive gas production, fruit flavours, increased acidity, changes in texture and the production of bitter and rancid flavours. However, in some cases yeasts may contribute positively to the fermentation and maturation process of

cheeses by inhibiting undesired microorganisms present, supporting the function of the starter culture by proteolytic activity and lipolytic activity, metabolising the lactic acid leading to an increase in pH, excrete growth factors and gas production leading to curd openness (Welthagen and Viljoen, 1998).

It is still not widely appreciated that yeasts can be an important component of the microflora of many, if not all, cheese varieties. Yeasts in cheese are considered as insignificant at the earlier stages of cheese production, but play a significant role in the later stages, being present as natural contaminants in the curd during maturation (Welthagen and Viljoen, 1999).

The yeasts found in the inner part of the Blue-veined cheeses, are mainly *Candida* (formerly *Torulopsis)* species, while the yeasts isolated from the surface of the cheese belonged to the genera *Pichia, Debaryomyces* and *Rhodotorula.* Contamination with yeasts usually occurs during manufacturing, ripening and storage of the cheeses. However, contamination of the *P. roqueforti* culture of cheese cannot be excluded (De Boer and Kuik, 1987).

Yeasts probably have an important influence on the organoleptic and microbiological quality of the end product. It was suggested that *P. roqueforti* and yeasts may act in a complimentary manner in the production of flavouring compounds. Lactose-fermenting yeast species producing ethanol and thus allowing the production of esters and fatty acids, have a positive effect on the taste of the cheese. The addition of yeast species like *D. hansenii* to the starter culture possibly will have a positive effect on the quality of mould-ripened cheeses, as was found for dry sausages. Yeast species growing on the surface of the cheese metabolize lactic acid, making the growth of acid-sensitive bacterial species such as micrococci and *Brevibacterium* species possible which may result in slime formation on the surface (De Boer and Kuik, 1987).

Proteolysis throughout ripening of La Serena cheese was influenced by pH, NaCl-in-moisture (NaCl) concentration and, to a lesser degree, by moisture content. Lactobacilli and yeasts were the only microbial groups in the interior of La Serena cheese with significant proteolytic activity. The α_{s1}- and β-casein breakdown is enhanced by yeasts owing to endoproteinase

activity (Szumski and Cone, 1962) and only yeasts affected β-casein hydrolysis. Yeasts and lactobacilli on the cheese surface significantly influenced breakdown of α_{s1}- casein, but not of β-casein (Del Pozo *et al.*, 1988).

High numbers of yeasts were found in Brie and Camembert cheeses in a rather high percentage of the rind samples of the Brie and Camembert cheeses (>75% $>10^5$ cfu/g) (Nooitgedagt and Hartog, 1988). During ripening, yeasts are growing in the outer part of the cheeses where the supply of oxygen is most favourable (Lenoir, 1984). *Yarrowia lipolytica, Debaryomyces hansenii* and *Kluyveromyces marxianus* were the species most frequently isolated. The use of yeast species in starters for the manufacture of Brie and Camembert is still the exception rather than the rule. However, yeast contamination can easily occur during the cheesemaking process. Yeasts, growing on the surface, increase the pH, in particular by metabolizing lactic acid, thus rendering the rind fit for growth of *Geotrichum candidum* and *Penicillium camemberti.* (Nooitgedagt and Hartog, 1988).

Yeast species found in brines are more or less the same as reported for the yeast flora of cheese rind. However, there is one exception. In brines, *C. versatilis* dominates quite often. This apparently does not apply to the cheese rind. Other yeasts typical in brines are *D. hansenii, C. rugosa, C. beigelii* and *C. polymorpha.* The yeast flora in brines of different dairies seems to be quite spesific (Seiler and Busse, 1990).

Some manufacturers market starter cultures containing *K. marxianus* or *D. hansenii,* either to be sprayed on the cheese or added to the solutions used as inoculum. It remains to be clarified whether this might promote uniformity in the cheese aroma (Seiler and Busse, 1990).

Yeasts are frequently found within the microflora of Camembert and Blue-veined cheeses. They originate as natural contaminants of the cheesemaking process and, depending on the species, growing to populations as high as 10^6-10^9 cfu/g during the maturation phase of production. The main species found during maturation and retailing include *Debaryomyces hansenii, Kluyveromyces marxianus, Yarrowia lipolytica* and various species of *Candida.* These yeasts impact on cheese quality through their production of lipolytic and proteolytic

enzymes, fermentation of residual lactose, utilization of lactic acid and their autolysis (Roostita and Fleet, 1996).

According to Welthagen and Viljoen (1999), the dominant yeast species found during maturation and at retail outlets included *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* and various species of *Candida*. These yeasts have an impact on cheese quality, due to their production of lypolytic and proteolytic enzymes, fermentation of residual lactose, utilization of lactic acid and autolysis. Yeast levels in cheese as high as 10^5 cfu g^{-1} might be present without any deleterious effect on the quality of the product. The initial progress in yeast numbers during maturation is much slower compared to the bacterial numbers. Yeasts and lactic acid bacteria occur simultaneously in many natural food habitats because they share common ecological determinants. Yeasts presumably utilize by-products of bacterial metabolism as energy sources, while bacteria depend on several growth factors supplied by yeasts. *D. hansenii* and *C. albidus* are the predominant species frequently associated with Cheddar cheese making. Based on isolates obtained during the ripening of Cheddar cheese, *D. hansenii* was clearly dominant being present from days 1 to 51.

Laubscher and Viljoen (1999) reported that yeast species islolated from matured Cheddar cheese comprised of *Trichosporon beigelii, Debaryomyces hansenii, Dekkera custersiana, Candida versatilis, Rhodotorula minuta, Rhodotorula mucilaginosa, Candida rugosa, Dekkera bruxellensis, Dekkera anomala, Torulaspora delbrueckii, Rhodotorula glutinis* and *Debaromyces vanrijae*. *Thichosporon beigelii* and *Debaryomyces hansenii,* the perfect state of *Candida famata*, predominated in most studies of yeasts associated with dairy products.

The possibility of using *Debaryomyces hansenii* and *Yarrowia lipolytica* as starter cultures for Cheddar cheese production were proposed, since both species stimulate ripening of cheese by means of lipolytic and proteolytic activities. The inclusion of *Debaryomyces hansenii* as a starter culture was supported by Yamauchi *et al.*, (1976), based on the specie's proteolytic activity encouraging the survival and growth of lactic acid bacteria. The inclusion of *Debaryomyces hansenii* as part of the starter culture, has a dual role by also inhibiting the germination of undesired microorganisms, like *Clostridium butyricum* and *Clostridium tyrobutyricum* in cheese brines (Fatichenti *et al.*, 1983). Based on its inhibitory effect on the

growth of spoilage microorganisms and the specie's proteolytic activity, Deiana *et al.*, (1984) and Fatichenti *et al.*, (1983) proposed the incorporation of *Debaryomyces hansenii* as part of the starter culture for the making of Romano cheese. Welthagen and Viljoen (1998) and Laubscher and Viljoen (1999) suggested further research on the possibility of including *Debaryomyces hansenii* as part of cheese starter cultures, due to its great resistance against high salt concentrations, low temperatures and proliferating activities.

Some manufacturers of starter cultures, however, market cultures containing *Kluyveromyces marxianus* or *Debaryomyces hansenii* to promote uniformity in the cheese aroma, but it seems conceivable that each cheese factory might harvest its own specific population of yeast species which contributes essentially to the sensory variety of the different cheeses (Viljoen and Greyling, 1995).

1.7.1 Proteolytic enzymes of yeasts

Secondary microflora, which grow and produce enzymes in cheese, include non-starter lactic acid bacteria, *Brevibacterium linens* and the *Penicillium* moulds. Other bacteria and some yeasts are also present but their proteinases are not well defined. The contribution of secondary lactic acid bacteria to proteolysis is qualitatively similar to that of the starter strains, though quantitatively, probably less important (Law *et al.*, 1993).

Two biochemical activities of the yeasts isolated from Camembert cheese that might play an important role during ripening are esterase and aminopeptidase activity. The proteolytic activity is intracellular and has a pH optimum of 5.5-6.0. There is only a slight difference in the level of activity between the species and between strains within one species. Species are: *Kl. lactis, T. sphaerica, Kl. fragilis, C. pseudotropicalis, Z. rouxii, C. versatilis, Kl. bulgaricus, S. cerevisiae, S. italicus, D. hansenii.* A study of the proteolytic system showed that two endopeptidases are present, with an optimum pH of 5.5. Intracellular aminopeptidase activity has a pH optimum of 7.5-8.0 and the production level varies, depending on the species. The nature of the system can also differ with the species.

Carboxypeptidase activity was estimated at three levels of pH (4.0-5.5-7.0) and on 2 different substrates (2-glu-tyr and 2-gly-val). There was relatively small difference in levels of activity

at pH 4.0 between species (*K. lactis, C. sphaerica, K. marxianus, C. pseudotropicalis, D. hansenii* and *S. cerevisiae*) and variations from 1-2 were observed between strains. The enzyme system would appear to be complex since there were differences in activity at pH 7.0 on both substrates (Lenoir, 1984).

Amongst the roles that can be attributed to yeasts during ripening are the following:

- The fermentation of lactose that influences the formation of aroma in various ways: by the formation of ethanol, acetaldehyde etc., by limiting acidification by lactic acid and thus affecting the texture of the cheese and by the formation of CO_2 and hence, eyes in the cheese. It may be noted that there is a risk of too much openness and the development of a yeasty flavour if the number of yeasts at the centre exceeds 10^6 cfu/g.

- The assimilation of lactic acid is a key property of the various species isolated from Camembert contributing to the neutralization of the cheese.

- The formation of components or precursors of aroma (amino acids, fatty acids, etc.) due to their proteolytic, lipolytic and esterifying processes.

- The effects of stimulation on the growth of other microorganisms, in particular moulds (*Penicillium; Geotrichum*) and bacteria (micrococci and corynebacteria) due to the excretion of growth factors (group B vitamins and amino acids). It is also possible that these effects could constitute one of the main roles of yeast during ripening (Lenoir, 1984).

1.8 Assessment of proteolysis in cheese

Assessment of the extent and pattern of proteolysis in cheese is of interest as an index of cheese maturity and quality (Fox and McSweeney, 1996).

1.8.1 Extraction and fractionation of cheese nitrogen

It is desirable and necessary to separate the various proteins, peptides and amino acids which result from the action of the proteolytic enzymes from the milk, rennet, starter and non-starter bacteria. Various approaches have been used to extract and fractionate cheese nitrogen, as an aspect of the study of proteolysis in general and to prepare extracts for the identification and quantification of peptides and free amino acids (McSweeney and Fox, 1993).

Several approaches have been adapted to monitor quantitatively proteolysis in cheese during ripening: 1) Solubility of peptides in various solvents or precipitants, 2) liberation of reactive functional groups, 3) various forms of chromatography and, 4) various forms of electrophoresis (Grappin *et al.*, 1985; Rank *et al.*, 1985; Fox, 1989).

1.8.1.1 Solubility

Several protein solvents and precipitants have been used to extract or fractionate cheese nitrogen, including water; buffers near pH 4.6; $CaCl_2$; 5% NaCl; 2, 5, 10 or 12% trichloroacetic acid (TCA); 5% phosphotungstic acid (PTA); 2,5% sulphosalicilic acid (SSA); picric acid; ethanol; ethanol-acetone; chloroform-methanol; butanol; $Ba(OH)_2/ZnSO_4$; ethylenediaminetetraacetic acid (EDTA); trifluoroacetic acid (TFA)/formic acid; dialysis and ultrafiltration (Fox, 1989).

1.8.1.1.1 Primary extraction methods

1.8.1.1.1.1 Water soluble extracts

Quantification and characterization of nitrogen in a water extract of cheese are commonly used indexes of cheese ripening (Christensen *et al.*, 1991). Water-soluble extracts are also frequently used for the isolation of peptides and amino acids. The preparation of a water-soluble extract is an efficient procedure for separating the small peptides in cheese (McSweeney and Fox, 1993). This initial step is usually combined with or followed by another fractionation procedure further to select peptides and then make correlations to

cheese flavour development (Rank *et al.*, 1985). This method extracts 70% of the soluble nitrogen and higher yields can be achieved by repeating the procedure on the precipitate (Rank *et al.*, 1985). Several researchers have used different techniques to obtain water-soluble extracts of cheese.

1.8.1.1.1.2 Extraction at pH 4.6

The pH 4.6-soluble N is also widely used as an index of cheese ripening (Christensen *et al.*, 1991). The pH of a water extract of internal bacterially ripened cheeses, e.g. Cheddar, is approximately 5.2. Thus, for these cheeses there is little difference between levels of N soluble in water or in buffers at pH 4.6. Fractionation at pH 4.6 is the most suitable extraction method for young cheeses (McSweeney and Fox, 1993). Sodium acetate buffer is used to isolate small and medium peptides. 95% of the nitrogenous materials in the first aqueous extract of cheese remains soluble when it is acidified to 4.6 with HCl (Rank *et al.*, 1985).

1.8.1.1.2 Fractionation

1.8.1.1.2.1 Fractionation with $CaCl_2$

Only 40% of the water-soluble nitrogen are soluble in 0,1M $CaCl_2$. The increase in $CaCl_2$ – soluble N correlates with the age of the cheese and the extracts containing whey proteins, peptides and amino acids, while the $CaCl_2$-insoluble fraction contains caseins and high molecular weight peptides, similar to those in the water-insoluble fraction, (McSweeney and Fox, 1993).

Summarizing the results indicates that the same categories of products are separated by the three fractionation methods (fractionation with water, fractionation at pH 4.6, fractionation in $CaCl_2$) in the soluble as well as the non-soluble fraction (Christensen *et al.*, 1991).

1.8.1.1.2.2 Fractionation with NaCl

Fractionation with NaCl is suitable for very young cheeses and fractionation with 5% NaCl is not as discriminating for the fractionation of cheese N as water (McSweeney and Fox, 1993). Thus it is not sufficiently discriminating for the assay of proteolysis (Christensen *et al.*, 1991).

1.8.1.1.2.3 Fractionation with chloroform/methanol

The chloroform/methanol procedure described by Harwalkar and Elliot (1971) consistently extracts more N than water, which is understandable considering the relatively hydrophobic nature of many casein-derived peptides, but both extracts yield identical chromatographs on Sephadex G-25. Extraction with chloroform/methanol is useful for isolating the more hydrophobic peptides in cheese (McSweeney and Fox, 1993).

1.8.1.1.2.4 Fractionation with butanol

Butanol has been used to extract bitter peptides from casein hydrolysates but has not been applied to the fractionation of cheese (McSweeney and Fox, 1993).

1.8.1.1.2.5 Fractionation with trichloroacetic acid (TCA)

Different concentrations of TCA have been applied, varying from 2% (Harwalkar and Elliot, 1971) and 2.5% to 12% (Reville and Fox, 1978). These values refer to the % of TCA in the final solution (Christensen *et al.*, 1991).

TCA precipitates medium and small peptides in water- and/or pH 4.5-soluble extracts of cheese, according to Fox (1989). According to Rank *et al.*, (1985), 2% TCA precipitates approximately 10% of pH 4.6 soluble nitrogen fraction, whereas 12% TCA precipitates 50%. Ninety percent of the water soluble N in Cheddar cheese is soluble in 2% TCA and 50%-60% of a UF-retentate (10 000 Da membranes) is soluble in this solvent. A disadvantage of TCA for peptide fractionation is the necessity to remove it prior to further analysis of the fractions, for example, by chromatography or electrophoresis. Small peptides and free amino acids will be lost on dialysis (McSweeney and Fox, 1993).

1.8.1.1.2.6 Fractionation with ethanol

A variety of concentrations have been used for cheese (Christensen *et al.*, 1991). According to Rank, *et al.* (1985), there is a preference to use 70% ethanol instead of 12% TCA. Both give approximately the same degree of precipitation, but not necessarily the same peptides. Another advantage of ethanol is that it gives very clean fractionation of peptides between supernatant and precipitate. With TCA, many peptides appear to partition between soluble and insoluble fractions (McSweeney and Fox, 1993).

The soluble fraction contains low molecular weight peptides and amino acids, whereas the non-soluble fraction contains proteins and medium peptides (Christensen *et al.*, 1991).

Fractionation of a UF retentate (10 000 Da) of a water-soluble extract of Cheddar using increasing concentrations of ethanol (30-80%) shows that most of the precipitable peptides are precipitated by 30% ethanol at pH 5.2. Thus, fractionation of UF retentate with 30% ethanol at pH 6.5; followed by adjustment of the filtrate to pH 5.5 is quite effective (McSweeney and Fox, 1993).

Consequently, ethanol precipitation is a useful method for fractionating cheese and for sub-fractionating the water-soluble fraction (Christensen *et al.*, 1991).

1.8.1.1.2.7 Fractionation with phosphotungstic acid (PTA)

Only low molecular weight peptides (< 600 Daltons) are extracted from cheese with PTA. The 5% PTA-soluble fraction contains small peptides and free amino acids, and the fraction non-soluble in 5% PTA contains proteins and peptides with MW > 600 Da (Christensen *et al.*, 1991).

PTA-H_2SO_4 is a very discriminating protein precipitant and yields a fraction that has been completely characterized (Fox, 1989). Free amino acids, except dibasic amino acids, are soluble in 5% PTA but peptides greater than about 600 Da are precipitated. PTA-soluble nitrogen is used as an index of free amino acids in cheese (McSweeney and Fox, 1993).

the resolution of small peptides (Fox, 1989).

1.8.3.2 Gel permeation chromatography

Gel filtration on various types of Sephadex has been widely used. Gel permeation chromatography is too slow to be suitable for large numbers of samples. Gel filtration is an effective method for resolving the water-soluble fraction, although homogenous fractions are not obtained, probably due to the aggregation of peptides. Amino acids can be resolved by chromatography on Sephadex G-15 (Kuchroo and Fox, 1983a,b). Sephadex G-10 will resolve molecules with molecular weights of 700 to 1500 Da; G-25, 1 000 to 5 000 Da; G-50. 1 500 to 30 000 Da and G-75, 3 000 to 80 000 Da (Rank *et al.*, 1985).

Chromatograms are generally quantified by spectrophotometry at UV wavelengths; 280 nm is generally used and is suitable for fractions containing large peptides, but for fractions containing smaller peptides absorbance of the carbonyl group in the peptide bond at a lower wavelength, e.g. 220 nm is preferable since smaller peptides may not contain aromatic residues, which are relatively scarce in caseins (McSweeney and Fox, 1993).

1.8.3.3 Cellulose derivatives

DEAE cellulose (a weak anion exchanger) gives good resolution of 70% ethanol-soluble and insoluble fractions of the water-soluble N. It is also quite effective for the 2% TCA soluble and insoluble fractions of the UF retentate of the water-soluble fraction. A major attraction of chromatography on DEAE cellulose is the possibility of fractionating relatively large samples compared with most other chromatographic methods (Fox, 1989).

Ion-exchange chromatography on DEAE cellulose has considerable potential for fractionating water-insoluble peptides in cheese (McSweeney and Fox, 1993).

1.8.3.4 Hydrophobic chromatography

Kuchroo and Fox (1983a,b) fractionated the water-soluble-N into two well-resolved fractions by hydrophobic chromatography on phenyl or octyl Sepharose CL-4B. Bitter peptides can be isolated by chromatography on Sephadex LH 20 (Visser *et al.*,1983).

1.8.3.5 High performance liquid chromatography (HPLC)

HPLC appears to be suitable for the quantitative method for the large water-insoluble peptides in cheese, e.g., residual α_{s1}- and β- caseins (Fox, 1989).

HPLC analysis of cheese peptides is becoming increasingly popular, especially on reversed-phase columns. Gradient elution with water/acetonitrile and trifluoroacetic acid (TFA) as an ion-pair reagent are most commonly used. Detection is generally by UV spectrophotometry, usually at wavelengths in the region of 200 – 230 nm (which measures the carbonyl in the peptide bond), although 280 nm has been used in cases where larger peptides, which are more likely to contain aromatic residues, are expected (McSweeney and Fox, 1993).

1.8.3.6 Ion – exchange chromatography

A few authors have used ion-exchange chromatography on Dowex 50 resins to fractionate cheese peptides (McSweeney and Fox, 1993).

Huber and Klostermeyer (1974) purified peptides in a fraction of water-soluble N obtained by gelfiltration by chromatography on Dowex 50W x 2. Mabbitt (1955) fractionated the amino acids in a cheese extract by chromatography on Dowex 50.

1.8.3.7 Column chromatography on silica gel

Column chromatography on silica gel G with n-propanol/water as eluent can be used to fractionate bitter peptides from rennet–treated casein and monitored at 206 and 280 nm. The technique has potential, but it is not widely used (Visser *et al.*,1983).

1.8.3.8 Metal chelate (ligand exchange) chromatography

Metal chelate (ligand exchange) chromatography on Cu-Sephadex is used to isolate peptides from Cheddar, as well as separation of peptides from amino acids.

1.8.3.9 Duolite S-571

Duolite-S-571 has been used to remove bitter peptides, which are presumably hydrophobic, from casein hydrolyzates (Roland *et al.*, 1978).

Hydrophobic chromatography on Duolite S-571 is unsuccessful since the peptides in the water-soluble fraction of cheese do not adsorb onto this medium (Kuchroo and Fox, 1983 a,b).

1.8.4 Electrophoresis

Electrophoresis has been applied widely to study primary proteolysis in cheese (Fox, 1989). Since only proteins and large peptides can be visualized by staining, the technique is limited to the assessment of casein loss and the formation and subsequent hydrolysis of the primary products of casein proteolysis. However, it is a powerful technique for studying proteolysis during the early stages of ripening (Creamer, 1991). The peptides in a 10 000 Da UF permeate do not stain on urea-PAGE, but the retentate water-soluble-N contains several detectable peptides (O'Sullivan and Fox, 1990).

1.9 Characterization of proteolysis in cheese

The extent of proteolysis in cheese varies from very limited (e.g., Mozzarella), to very extensive (e.g., blue-mold varieties) (Fox and McSweeney, 1996). The pattern and extent of proteolysis can be characterized by polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC). Both the PAGE and HPLC patterns change and become more complex as the cheese matures and are very useful indices of cheese maturity and to a lesser extent of its quality. They have potential in the objective assessment of quality (O'Shea *et al.*, 1996). Complete characterization requires isolation and

Chapter 2

Cheese manufacture and sensory analysis

2.1 Introduction

As discussed in Chapter 1, cheese manufacture essentially involves concentrating the fat and casein of milk 6-12 fold by coagulating the casein, enzymatically or isoelectrically, and inducing syneresis of the coagulum which can be controlled by various combinations of time, temperature, pH, agitation and pressure. At the end of the manufacturing phase, all the rennet-coagulated cheeses are essentially very similar, consisting of a matrix of calcium para-caseinate in which various proportions of lipids are dispersed and with moisture contents typically in the range 35-50%. Depending on the cooking temperature used during manufacture and the moisture content, fresh rennet cheeses are more or less "rubbery" and are essentially flavourless. Although they may be consumed in this state, this is not usually done. Instead, they are matured (ripened) for periods ranging from about three weeks (e.g. Mozzarella) to two or more years, depending on the moisture content of the cheese and the intensity of flavour desired (Fox *et al.*, 1993). Matured Cheddar cheese is typically ripened for 6-9 months (Wilkinson, 1993).

The basic composition and structure of cheese are determined by the curd manufacturing operations, but it is during ripening that the individuality and unique characteristics of each cheese variety develop, as influenced by the composition of the curd and other factors, e.g. the microflora established during manufacture. Some bacterial growth does occur in cheese during ripening, especially of the non-starter lactic acid bacteria, and of moulds in the case of the mould-ripened varieties. Although the actual growth of these microorganisms contributes to cheese ripening, perhaps very significantly in some varieties, cheese ripening is essentially an enzymatic process (Fox *et al.*, 1993).

Three primary events occur during cheese ripening, i.e. glycolysis, proteolysis and lipolysis. These primary reactions are mainly responsible for the basic textural changes that occur in the cheese curd during ripening and are also largely responsible for the basic flavour of cheese. However, numerous secondary changes occur concomitantly and it is these secondary transformations that are mainly responsible for the finer aspects of cheese flavour

Cheese ripening is essentially the controlled slow decomposition of a rennet coagulum of the constituents of milk. Hydrolysis of the casein network, specifically α_{s1}-casein, by the coagulant, appears to be responsible for the initial changes in the coagulum matrix (Creamer and Olson, 1982). The role of plasmin in Cheddar cheese flavour has been reported that the rate and extent of characteristic flavour development in Cheddar cheese slurries appeared to be related directly only to the degradation of β-casein (Harper *et al.*, 1971). Plasmin may well therefore prove to be an enzyme of considerable importance in the development of cheese flavour. As the original casein network is broken down, the desired balance of flavour and aroma compounds is formed. Any organism that grows in the cheese, whether starter or non-starter, and any active enzyme that may be present such as chymosin or plasmin, must have an influence on the subsequent cheese flavour. There appears to be only two "facts" on which all research workers on Cheddar cheese flavour seem to agree: firstly, that milk fat has to be present for the perception of flavour and secondly that lactic starters must be used for the development of a typical balanced Cheddar flavour (Lawrence *et al.*, 1993). In Fig. 2.1 the complex development of flavour in Cheddar cheese is demonstrated schematically.

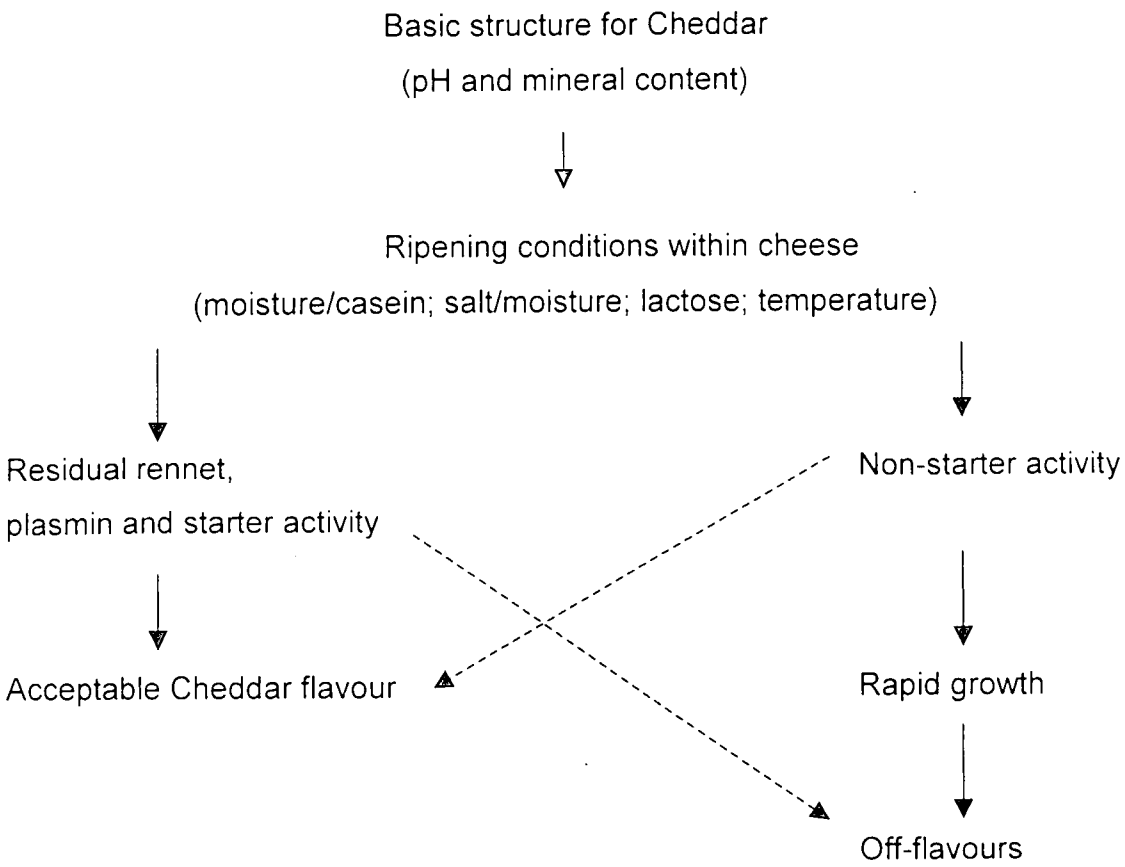

Fig 2.1 Main factors that determine the development of flavour in Cheddar cheese (taken from Lawrence *et al.*, 1993).

In this chapter the manufacture and a comparative sensory evaluation of the standard Cheddar cheese and the yeast-inoculated Cheddar is discussed.

2.2 Materials and Methods

2.2.1 Cheddar cheese manufacture

Cheddar cheese was manufactured at a commercial cheese factory of Clover SA in the Free State Province of South Africa. The cheese was made in an open vat with HTST-pasteurized (72^0C; 15s), 4% fat-standardized milk. When cooled to the inoculating temperature of 32-35^0C, the starter culture was added (RST-743 phage control mesophilic homofermentative culture, 250 units/ 5 000 l freeze-dried Lactic Culture for direct vat set (DVS)). Calcium chloride (45%) (4 000 ml/ 16 000 l) and annatto dye (600 ml/ 16 000 l) were added just before

the rennet (750 ml/ 16 000 l). The milk was set for ~35 min at a temperature of ~31.5^{0}C. whereafter the curd was cut into 1 cm^3 cubes and held for 15 min without stirring. The temperature of the curd-whey mixture was raised to 36^{0}C over 30 min with gentle stirring and the mixture was then held at 36^{0}C until the pH was 6.2 and enough whey expelled from the curd. The whey was drained and the curd cheddared until the pH declined to 5.4 (~60 min). The blocks of curd were milled into cubes that were dry-salted (44 kg/ 16 000 l), mellowed for 15 min, placed in molds and pressed overnight. Following pressing, the cheeses were vacuum-packed and ripened at 8-12^{0}C.

A yeast-inoculated Cheddar cheese was prepared by adding *Debaryomyces hansenii* (LAF 3 non-fermentative yeast with aminopeptidase activity, 1unit/ 100 l DVS) together with the RST-743 culture. Otherwise, the whole process was the same as for the standard Cheddar cheese.

2.2.2 Sensory analysis

It should be noted that only one standard Cheddar cheese was evaluated sensorically against the yeast-inoculated Cheddar cheese. The chemical analysis in Chapter 3 showed that the three batches analyzed did not differ much. Sensorically this was verified by the expert grading panel (data not shown). It was thus assumed that a high constant standard regarding manufacture and quality prevails in the mentioned factory, so that one Cheddar cheese may act as a standard.

2.2.2.1 Sensory analysis by consumer panel

Taste quality of cheese (three months of age) was evaluated using the duo-trio test and preference ranking (Basker, 1988) and was carried out in a sensory analysis laboratory of the Department of Food Science of the University of the Orange Free State. For the duo-trio test, a consumer panel comprising sixty panelists (19-65 years of age) were recruited from students (31) and staff (29) of the University of the Orange Free State (42 females; 18 males). Fifty-eight panelists (19-65 years of age) were used for the preference ranking, also from the students (31) and staff (27) of the University of the Orange Free State (39 females; 19 males).

The cheese was cut into cubes of 12,5 x 10 x 7,5mm. A balanced reference duo-trio test was done in which the judges received three samples simultaneously. One sample was marked reference and was the same as one of the two coded samples. The judges had to pick the coded sample that was most similar to the reference.

For the preference ranking, the panelists assigned ranks to the two coded samples by using a scale of 1 = most preferred sample and 2 = least preferred sample. Tap water at room temperature was provided for rinsing the palates between samples during taste sessions. Thinly sliced apples were also available to clean the palate from excessive mouth coating.

Two cheese samples (standard, yeast) were evaluated by panelists. Samples were coded using three-digit numbers picked from a table of random numbers. Evaluations were performed at room temperature (20-22^{0}C) in individual testing booths under red lights in the Sensory Evaluation Laboratory, Department of Food Science, University of the Orange Free State.

2.2.2.2 Sensory analysis by expert graders

The yeast-inoculated Cheddar cheese was graded by four official graders from Clover SA. The method for grading cheese developed by Clover SA was used.

2.3 Results and discussion

2.3.1 Sensory analysis by consumer panel

In the duo-trio test three cheese samples were evaluated by a panel of 60 assessors. The observed number of correct identifications was 27 and the observed number of incorrect identifications was 33. From the Roessler table for paired and duo-trio tests (one-tailed), the minimum number of correct judgements to establish significance at probability level p = 0.05, is 37. To further determine the significant difference between the two products, the adjusted χ^2 (chi-square) test was done:

$$\chi^2 = \frac{(0.5-|O_1-E_1|)^2}{E_1} + \frac{(0.5+|O_2-E_2|)^2}{E_2}$$

O_1 = observed number of correct identifications

O_2 = observed number of incorrect identifications

E_1 = expected number of correct identifications

E_2 = expected number of incorrect identifications

$E_1 = E_2$ = ½ of total number of observations

From the calculations it was found that $\chi^2 = 0.4167$, which is smaller than the value of 2.706 at $p = 0.05$ which was obtained from the χ^2 tables. These tables were calculated for a two-tailed test, therefore twice the probability level ($p = 0.01$) was used for the duo-trio test, which is one-tailed. The test was thus not significant, meaning that the differences between the samples were too small to be recognized sensorically.

In the same session two cheese samples (standard, yeast) were ranked by a panel of 58 assessors and the following results were obtained:

Table 2.1 Ranking test of a standard Cheddar and a yeast-inoculated Cheddar cheese

Sample	**A (standard)**	**B (yeast)**
Rank sum	90	84
Difference vs. A		6

From Table 1 (Basker, 1988), the $p = 0.05$ significance level is attained when the rank sum differences are greater than or equal to 14.9 and from Table 2 (Basker, 1988), the $p = 0.01$ significance level is attained when the rank sum differences are greater or equal to 19.6.

Table 2.2 Significance level of differences obtained by the ranking test

Significance level	$p = 0.05$	$p = 0.01$
Critical difference	14.9	19.6
Sample B	A	A
A	a_b	a_b

Product B was not significantly preferred (p = 0.05 and 0.01) over product A. An overlapping range of preference had been identified.

2.3.2 Sensory analysis by expert graders

Grading results of the yeast-inoculated Cheddar by expert graders (Clover SA) are given in Table 3.3.

Results obtained by expert graders indicate that the yeast-inoculated Cheddar received full marks for body and texture, colour as well as appearance, but was judged to be bitter. The bitter taste identified by the expert graders could have been the result of excessive proteolysis, which is known to lead to bitterness in cheese (Fox *et al.*, 1993). It is generally agreed that bitterness is due to the accumulation of hydrophobic peptides but there is disagreement on the cause of the problem, whether it is due to a deficiency of peptidase activity or too much proteinase activity in certain starters (Fox *et al.*, 1993). Biede and Hammond (1979) reported that bitter flavours were due to tri-, tetra-, penta- and hexapeptides. Aston and Creamer (1986) ascribed the taste intensity of a Cheddar cheese with bitter and brothy notes to the water-soluble peptide containing fraction.

The non-volatile, water-soluble compounds, especially peptides and amino acids are important in cheese flavour, and in view of this, the role of starter proteinases and peptidases in cheese quality (Fox *et al.*, 1993). While the principal function of the primary starter (*Lactobacillus, Lactococcus* or *Streptococcus*) is to produce the correct amount of acid at an appropriate rate and time, the secondary starter performs a range of functions, depending on the organism used (Fox and McSweeney, 1996) and this assumingly influences proteolysis differently and may cause bitterness.

Table 2.3 Sensory evaluation of the yeast-inoculated Cheddar by expert graders (Clover SA)

Defects	Intensity E	Intensity D	Intensity S	Results
1. Flavour and aroma				
Non-characteristic	9	7	5	
Undeveloped	9	7	5	
Acid	9	7	4	
Bitter	9	7	4	4
Fruity	7	4	1	
Unclean	7	4	1	
Rancid	7	4	1	
Whey	8	6	4	
Yeasty	7	4	1	
Stale	5	3	0	
Sulfide	8	5	2	
Metal/ oxidated	8	5	2	
Feed	9	7	4	
Weed	8	5	2	
Salt/ too less salt	9	7	5	
Unknown	5	3	0	
Marks		**10**		**4**
2. Body and texture				
Rubbery	9	7	4	
Curdy	8	5	2	
Dry	9	7	4	
Crumbly	7	5	3	
Soft-doughy	9	6	3	
Short	9	7	4	
Floury	38	5	2	
Fissures	8	5	2	
Open (gassy)	8	5	2	
Open (mechanical)	9	7	4	
Marks		**10**		**10**
3. Colour				
Too less colourant	4	3	2	
Too much colourant	4	3	2	
Not uniform	3	2	1	
Atypical	3	2	1	
Marks		**5**		**5**
4. Appearance				
Moulds	3	1	0	
Faulty rind	4	3	1	
Rough	4	3	2	
Dirty	3	2	1	
Faulty wax	4	3	1	
Faulty packaging	3	2	1	
Marks		**5**		**5**
Total marks		**30**		**24**

E= slightly

D= clearly

S= strong

2.4 Conclusion

No significant difference between the standard Cheddar and the yeast-inoculated Cheddar was found sensorically by the consumer panel of the UOFS, neither were any of the two cheeses significantly preferred. This, however, does not indicate that the two cheeses are chemically the same. The expert panel judged the yeast-inoculated Cheddar to be bitter. In the following chapters the investigation of differences in primary and secondary proteolysis between the two types of Cheddar cheese will be discussed by means of electrophoretical- and chromatographic analysis in an attempt to explain the bitter defect in the yeast-inoculated Cheddar.

As proteins and peptides in cheese are very different with respect to size, solubility and conformation, it is often necessary to separate the nitrogen components of cheese into more homogenous fractions prior to analysis. The oldest and probably still most used technique for separation of the nitrogen components of cheese is based on selective precipitation. With different precipitation chemicals it is possible to separate the nitrogen components in cheese into differently soluble fractions (Table 3.1). Normally, the content of nitrogen components in the soluble fractions is then determined by Kjeldahl analysis (Christensen *et al.*, 1991).

Table 3.1 Composition of soluble nitrogen fractions obtained by different precipitation techniques (taken from Christensen *et al.*, 1991).

Fractionation chemicals	**N-components in the soluble fraction**
Water	Proteins, peptides and amino acids
Acid (to pH 4,6)	Proteins, peptides and amino acids
$CaCl_2$	Proteins, peptides and amino acids
NaCl	Proteins, peptides and amino acids
Chloroform-methanol	Hydrophobic peptides, amino acids
TCA	Peptides, amino acids
Ethanol	Peptides, amino acids
SSA	Peptides, amino acids
PTA	Very small peptides, amino acids

Kuchroo and Fox (1982 a, b) compared the influence of several operational parameters such as temperature, equipment and duration of homogenization. Based on their results, they recommended a standard procedure: 20 g of cheese are homogenized in 40 g of water at 20°C for 10 min and kept at 40°C for 1hr. After centrifugation, the water-soluble fraction is collected; it contains about 70% of the soluble N. Higher yields can be achieved by repeating the procedure on the precipitate (Rank *et al.*, 1985). The pH of a water extract of Cheddar cheese is approximately 5.2 and there is little difference between the levels of N soluble in water or in buffers at pH 4.6 (Christensen *et al.*, 1991). Fractionation at pH 4.6 was reported to be the most suitable extraction method for young cheeses (McSweeney and Fox, 1993).

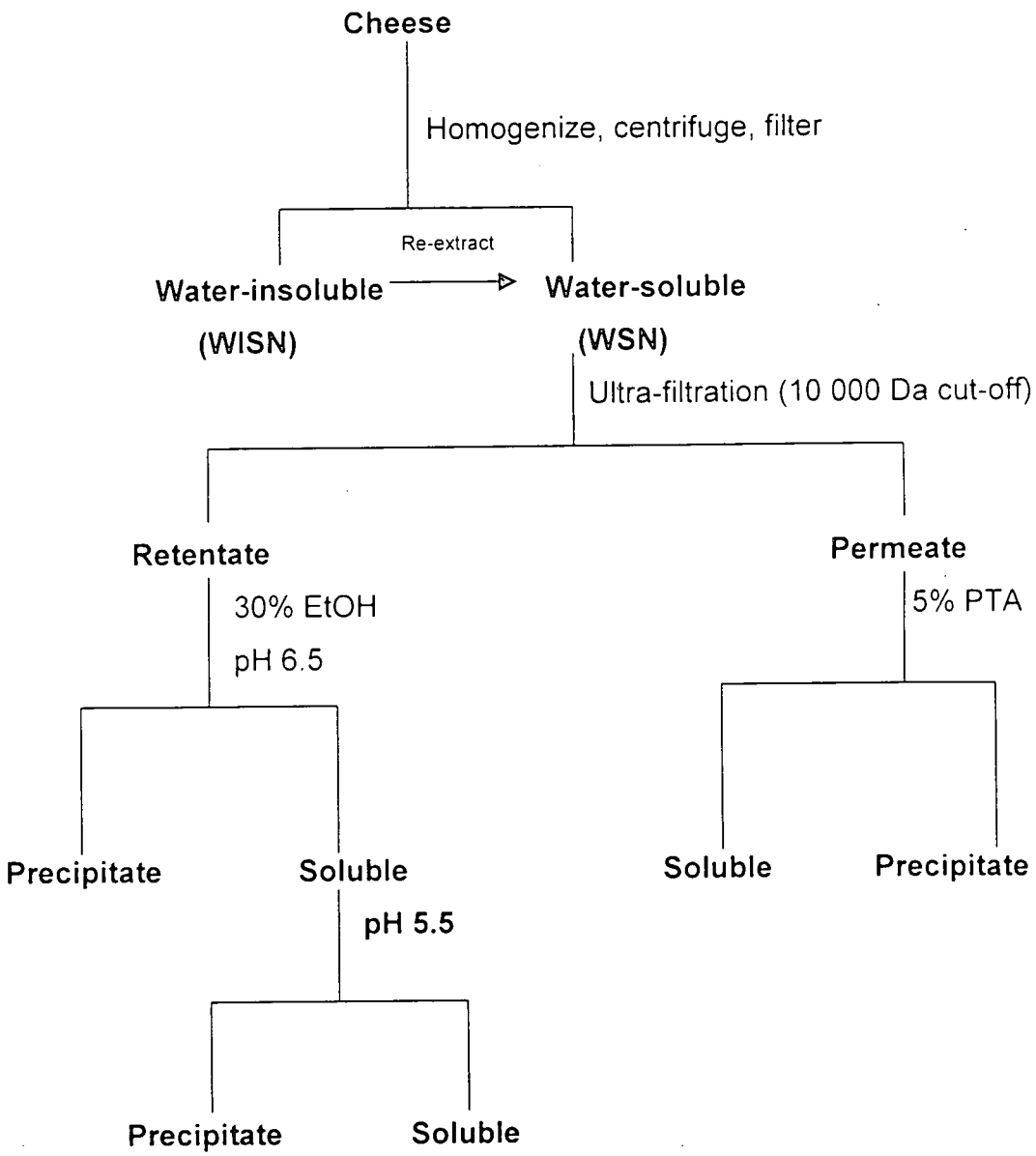

Fig. 3.1 Scheme for extraction and fractionation of WSN (taken from McSweeney and Fox, 1993).

The water-insoluble fraction was frozen at –20 ^{0}C until later analysis (not longer than three months). Extraction was followed by fractionation of cheese water-soluble peptides. The first step of fractionation of WSN was done by means of ultra-filtration through a regenerated cellulose membrane with a molecular cut-off of 10 000 Daltons (Millipore) at a trans-membrane pressure of 2kg/cm^2. The permeate obtained was further fractionated by precipitation with 5% PTA, (Merck). The retentate was fractionated with 30% EtOH (Illovo, SA) at pH 6.5, followed by adjustment of the filtrate to pH 5.5 with HCl (BDH).

3.2.4 Nitrogen (N) determination

The nitrogen in cheese and WSN was determined by the macro Kjeldahl procedure according to the Büchi manual (slight variation on the AOAC method, 1970), and the WSN expressed as a percentage of total N.

Free amino acids should be mainly water-soluble and permeable through 10 000 Da membranes on ultra filtration and although some amino acids, especially lysine, are insoluble in PTA, it is expected that the free amino acids will be primarily in the PTA-soluble fraction.

3.2.5 Analysis by Urea-PAGE

The WISN and WSN-retentate, insoluble in 30% EtOH samples were analyzed by alkaline urea-polyacrylamide gel electrophoreses using the method of Andrews (1983). Samples were prepared as described by Shalabi and Fox (1987). Staining and de-staining were by the method of Blakesley and Boezi (1977).

Cheese sample sub-fractions were dispersed in sample buffer, pH 7.6 (Shalabi and Fox, 1987) and aliquots of 5-20 µl were run on the gels consisting of a resolving gel (12,5%T and 4%C, pH 8.8) and a stacking gel (12,5%T and 4%C, pH 8.4) according to the method of Andrews (1983). Electrophoresis was done on a Mighty Small miniature slab gel electrophoreses unit, SE 260 (Hoefer Scientific Instuments) using a Hoefer Scientific Instrument, model PS 500X DC Power supply set at 210 volts (constant) (set for 2 gels running simultaneously).

Staining and de-staining of the peptide bands was done according to the method of direct staining by Blakesley and Boezi (1977) with Coomassie Blue G250 (Fluka). No de-staining was necessary and the gels were stored in distilled water where the protein bands became more intense. Thus, the background was not stained and the protein zones were visualized directly. The electrophoretograms were photographed.

3.3 Results and discussion

3.3.1 Nitrogen (N) analysis

The nitrogen analyses and calculations of N-contents for the three standard Cheddar cheeses and the yeast-inoculated Cheddar cheese are given in Table 3.2 and graphically in Fig. 3.2. The WSN, expressed as a percentage of total N, is shown.

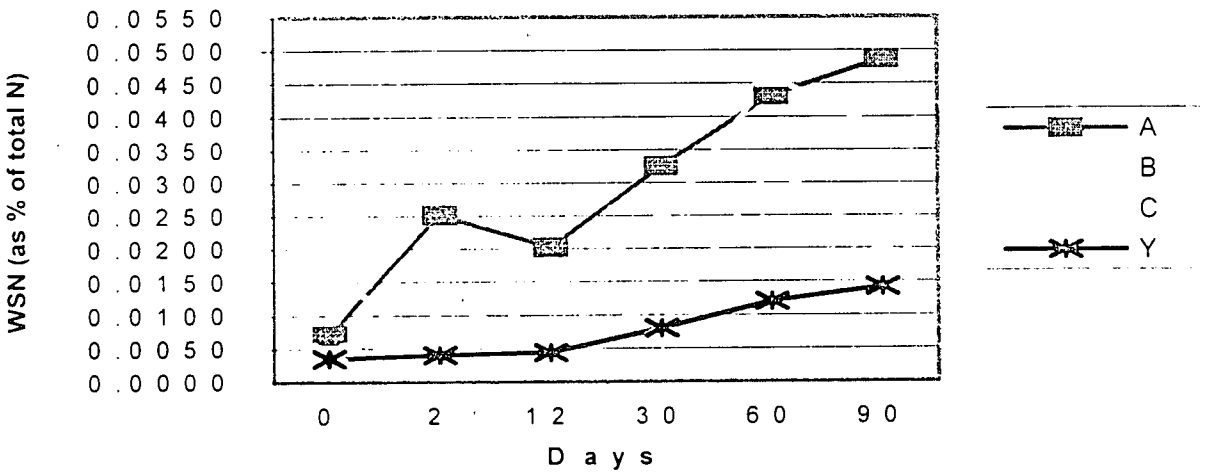

Fig. 3.2 Water-soluble nitrogen (WSN) as a percentage of total N during ripening of standard Cheddar cheeses (batches A,B and C) and a yeast-inoculated Cheddar cheese (Y).

An attempt was made to evaluate proteolysis by comparing the propensity of WSN, as percentage of total N, to increase during ripening (Farkye and Fox, 1990; Law *et al.*, 1992, 1993). A definite pattern was observed for both the standard cheese as well as the yeast-inoculated cheese. For the standard cheese an increase in WSN was observed from age 0 days to 90 days. The sharp variation in water-soluble nitrogen at age 0 and 2 days observed

for all the cheeses may be explained by uncertainty about the sampling of the cheeses, as it was done while the curd was still in the cheese vat, during whey drainage or after the cheddaring process (Table 3.2 and Fig. 3.2). The amount of whey and whey proteins present may differ, resulting in the differing amounts of WS protein. The WSN/Total N ratio of all four cheeses showed a tendency to increase during ripening, which is in agreement with Farkye and Fox (1990); Law *et al.*, (1992) and Law *et al.*, (1993). When comparing the batches of standard Cheddar cheese, batch C was found to show a slower increase in WSN during ripening. This batch was also shown to be slower in development during ripening analyses.

The levels of water-soluble N in the three standard batches of the same age were not significantly different, compared with the much lower level of soluble N observed for the yeast-inoculated Cheddar. This low level of WSN is a clear indication of the difference in proteolysis between the two different types of Cheddar cheese. In Chapter 2 it was shown that flavour development was different between the standard Cheddar and the yeast-inoculated Cheddar, with the yeast-inoculated Cheddar judged by an expert panel to be bitter. From the literature (Chapter 1), it is known that peptides of WSN are responsible for the bitter defect in Cheddar cheese, therefore it could be expected that a higher level of WSN in the yeast-inoculated Cheddar will be found (Biede and Hammond, 1979; Aston and Creamer, 1986 and Law *et al.*, 1992, 1993).

Furthermore, the lower level of WSN, as % of total N, observed for the bitter, yeast-inoculated Cheddar, is in agreement with results obtained by Kristiansen *et al.*, (1996). These authors showed that higher amounts of WSN, as % of total N, were obtained for cheeses containing starters with high proteolytic acitivity (indicating that proteolysis had been more extensive), as well as no bitter off-flavour, while the amount of WSN, as % of total N, was much lower in the cheeses containing starters with low proteolytic activity and which had a bitter off-flavour.

Table 3.2: N-analysis at different time intervals during ripening

	% Moisture	% Dry Matter	% WSN in wet cheese	% WSN in dry matter	% Total N in dry matter	% WSN/Total N
Cheese vat A	55.420	44.580	0.653	0.073	10.460	0.007
Cheese vat B	51.200	48.800	0.978	0.100	5.675	0.018
Cheese vat C	50.200	49.800	0.374	0.037	8.140	0.005
Cheese vat Y	37.740	62.260	0.262	0.020	5.774	0.004
Drainage A	47.740	52.260	0.917	0.088	5.753	0.015
Drainage B	48.830	54.170	0.164	0.015	6.703	0.002
Drainage C	51.860	48.140	0.463	0.046	7.991	0.006
Drainage Y	39.560	60.440	0.328	0.025	5.289	0.005
48 hr (2days) A	22.940	77.060	1.894	0.123	4.869	0.025
48 hr (2 days) B	31.870	68.130	0.197	0.014	6.067	0.002
48 hr (2 days) C	26.620	73.380	0.542	0.037	4.750	0.008
48 hr (2 days) Y	33.850	66.150	0.256	0.019	4.686	0.004
12 days A	36.690	63.310	1.253	0.152	7.455	0.020
12 days B	36.690	63.310	0.919	0.093	6.700	0.014
12 days C	36.690	63.310	0.727	0.088	6.150	0.014
12 days Y	36.690	63.310	0.312	0.024	5.542	0.004
30 days A	34.720	65.280	2.687	0.230	7.067	0.033
30 days B	34.720	65.280	1.740	0.158	6.646	0.024
30 days C	34.720	65.280	0.526	0.058	7.074	0.008
30 days Y	34.720	65.280	0.557	0.041	5.467	0.008
60 days A	31.370	68.630	3.704	0.268	6.193	0.043
60 days B	35.050	64.940	3.200	0.246	5.383	0.046
60 days C	33.620	66.380	2.803	0.211	6.434	0.033
60 days Y	35.480	64.520	0.928	0.070	5.998	0.012
90 days A	33.460	66.540	4.443	0.319	6.543	0.049
90 days B	34.050	65.950	3.358	0.247	6.526	0.038
90 days C	33.460	66.540	1.656	0.123	6.222	0.020
90 days Y	36.690	63.310	1.211	0.091	6.432	0.014

A, B & C = standard Cheddar cheese
Y = Yeast-inoculated Cheddar cheese

3.3.2 Urea-PAGE

For each group of caseins the differences in mobilities may occur as influenced by the PO_4 content and genetic variants of the different groups of caseins (Grappin *et al.* 1985).

Primary proteolysis in Cheddar cheese results from the actions of chymosin (EC 3.4.23.4) and plasmin (EC 3.4.21.7) (Fox *et al.*, 1994). Both rennet and alkaline protease are mainly responsible for the breakdown of casein to large peptides which are then hydrolyzed primarily by starter enzymes to smaller peptides and amino acids (Marcos *et al.*, 1979).

The protein breakdown of the major casein fractions and the development of relatively large peptides in one batch of standard Cheddar cheese is shown in Fig. 3.3. Peptides were identified according to work by Marcos *et al.* (1979); Creamer (1991) and McSweeney *et al.* (1994). The development of α_{s1}-casein (CN) degradation products in the pre-α_s-region (see Fig. 3.3) during ripening are clearly indicated (lanes 3-9). The intensity of the α_{s1}-CN degradation bands also increased, especially from 30 days onwards. The α_{s1}-I (α_{s1}-CNf24-199) increased during the first two months of ripening (lanes 3-8), but decreased thereafter (90 days, lane 9). Peptides α_{s1}-CN(f24-•) and α_{s1}-CN(f110-•) are formed later during ripening, that is after one month (lanes 7-9). The intensity of α_{s1}-CN(f60-•) increased during ripening (lanes 3-9). A different peptide pattern can be seen for the commercial Cheddar (lane 10) where α_{s1}-CN(f110-•) and α_{s1}-CN(f24-•) are absent. The results also differ somewhat from those of other researchers (Creamer and Richardson, 1974; McSweeney *et al.*, 1994 and Fox *et al.*, 1994). These differences can be ascribed to the cheeses in this study differing in age of ripening, but also differences in manufacturing practices materials, e.g. rennet of different activity and starter cultures consisting of different strains, as was explained in Chapter 1.5.2 and 1.5.3.

Generally these results are in agreement with work by others such as Creamer and Richardson (1974) who indicated that rennet plays the major role in the initial breakdown of α_{s1}-casein, giving rise to the peptide α_{s1}-I, which is present at least in the early stages of ripening, in all types of cheeses (Marcos *et al.*, 1979). Reville and Fox (1978) found very low amounts α_{s1}-casein in old Cheddar cheeses, whereas Phelan *et al.* (1973) reported that a

large amount of α_{s1}-casein (not quantified) remained unattacked in a 7-month-old Cheddar cheese. The α_{s1}-I peptide can be almost entirely degraded during ripening (Creamer, 1975).

O'Keefe *et al.* (1975) found that the rate of proteolysis of α_{s1}-casein was accelerated during manufacturing and the early stages of ripening when high levels of starters were used, as compared to cheese made with less starters. However, the differences in proteolysis between cheeses declined after 7 weeks of ripening at 4^0C. Except for the action of milk acid proteinase and surface flora on certain type of cheeses, proteinases other than milk-clotting enzymes contribute very little to the formation of large peptides from α_{s1}-casein (O'Keefe *et al.*, 1975; Visser, 1977).

Furthermore, rennet is completely responsible for the observed degradation of α_{s1}-casein and for the decomposition of β-casein during the first month of ripening. In the longer term starter bacterial enzymes also appear to contribute, especially in the further degradation of β-casein. Starter bacteria are also able to degrade part of the α_{s1}- and β-casein in these cheeses after some time of ripening. This activity is relatively low in the first month of ripening (Visser and De Groot-Mostert, 1977).

This correlates with the results shown in Fig. 3.3 where the γ-peptides (γ_2 and γ_3) were the first to be formed (lanes 3-5) and of which the intensity increased further during ripening (lanes 5-9). Further degradation of β-CN later during ripening was also observed. The peptide marked as "m" was identified by Creamer (1991) as α_{s2}-CN, while McSweeney *et al.* (1994) refer to it as β-CN degradation products. For this study, it will be named β-CN(f1-•) according to McSweeney *et al.*, (1994). The α_{s2}-CN stayed unattacked during the ripening period (lanes 3-9). The β-CN was degraded by plasmin to form the γ-peptides. The γ_2 and γ_3 were already present in the early stages of ripening (lane 3) while γ_1 formed later during ripening (lane 5). The intensity of the γ-bands increased as ripening proceeded (lanes 3-9). The degradation of β-CN by chymosin (rennet) from the N-terminal was indicated by the presence of the β-CN products with a higher electrophoretic mobility than β-CN, i.e. β-CN(f1-•). The intensity of "m" (β-CNf1-•) increased during ripening (lanes 3-9) and was more clearly indicated by the commercial Cheddar marker (lane 10).

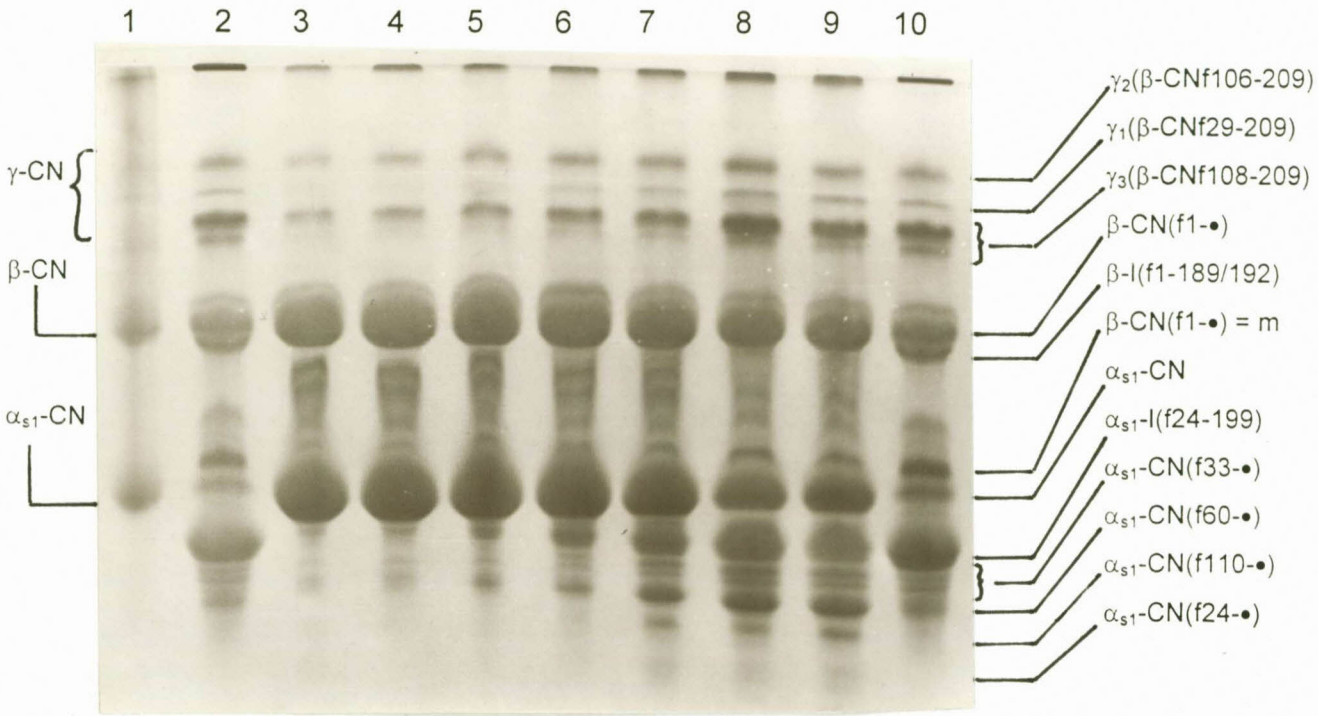

Fig. 3.3 Urea-Page of the WISF of standard Cheddar cheese, batch B at different time intervals during ripening. Lane 1: marker, Na-caseinate. Lanes 2,10: commercial Cheddar. Lane 3: cheese vat. Lane 4: drainage. Lane 5: 48 h. Lane 6: 12 days. Lane 7: 30 days. Lane 8: 60 days. Lane 9: 90 days.

There is a basic electrophoretic pattern, common to most cheeses made from cow's milk in the number and relative mobility of the main components. The basic pattern is those of fast moving components, α_s-casein, β-casein and the slow moving regions of γ_1-, γ_2-, and γ_3-casein. The α_{s1}-casein was formed earlier and is the more intense band of the three α_s-casein found in the electrophoretic region of α_s-casein. Two bands of fast electrophoretic mobility that appear in position pre-α_s are degradation products from α_s-caseins. Of these, the slower and major one is the peptide named α_{s1}-I, the first product formed by the action of rennet on α_{s1}-casein. The α_{s1}-casein has many bonds susceptible to the action of rennin. The most susceptible is the bond between residues 23-24 or 24-25. Hydrolysis of this leads to the formation of a basic peptide containing 23 or 24 residues from the N-terminus and the high molecular weight polypeptide α_{s1}-I. This peptide is more acidic than the α_{s1}-casein and has a slightly greater electrophoretic mobility. The other band, of maximal electrophoretic mobility may be a degradation byproduct of α_{s1}-I caused by the endopeptidase activity of lactic bacteria (Marcos *et al.*, 1979).

The α_{s2}-casein (band m, also named β-CN(f1-∘) is unique, because it is completely resistant to the action of chymosin (Richardson and Pearce, 1981). These authors also reported that the extent of degradation of α_{s2}-casein in Cheddar cheeses was related to the plasmin content in the cheese. Le Bars and Gripon (1989) showed that plasmin cleaved α_{s2} casein at 8 sites in solution, but it has not found to be cleaved in cheese.

In the region of β-casein there is a major central band of β-casein between two minor bands, usually masked by the major one. The minor band that migrated slightly faster than the accompanying major protein probably represents a breakdown product named β-I. Rennin acts on β-casein to give three N-terminal peptides designated β-I (f1-189/192), β-II (f1-164/166) and β-III (f1-139) in the order of appearance and of increasing electrophoretic mobility in polyacrylamide gels at alkaline pH. They are faster moving than β-casein because the N-terminal sequences are more acidic than the apolar peptides released from the C-terminal end. Three β-casein bands, cleaved by rennin, are between residues 164-165, 189-190 and 192-193. The peptides β-I, β-II and β-III have been identified as the sequences 1-189, 1-164 or 166 and 1-139 of β-casein. The three major peptides γ_1-,γ_2- and γ_3-casein are

residues 29-209, 106-209 and 108-209 of β-casein (found in the most negative charged region of the molecule) (Marcos *et al.*, 1979) (Fig.3.4).

Para-κ-casein is not degraded during cheese ripening (Foster and Green, 1977; Grappin *et al.*, 1985). Whey proteins such as α-lactalbumin, β-lactoglobulin and blood serum albumin are completely resistant to the action of various proteinases in Cheddar cheese (O'Keefe *et al.*, 1978) and do not appear to play a role in proteolysis.

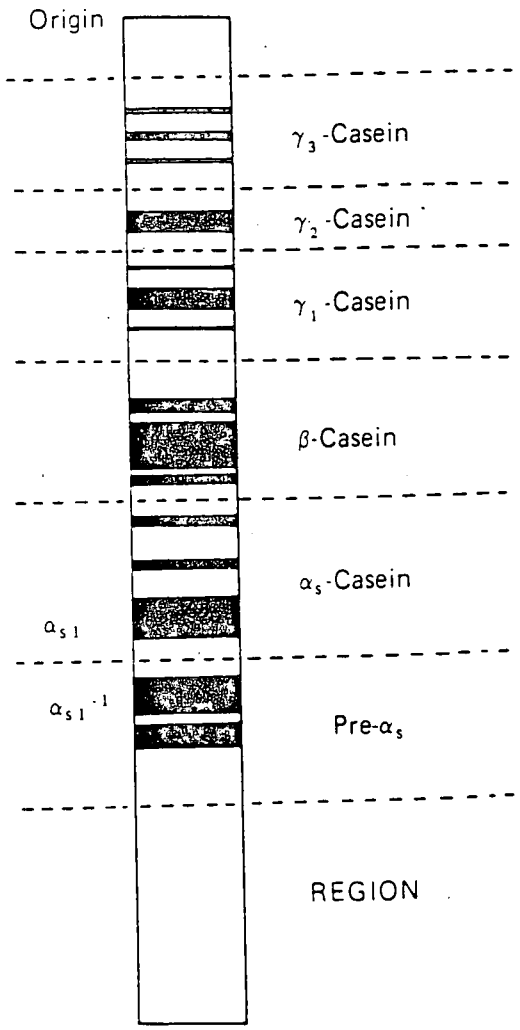

Fig. 3.4 Schematic electrophoretic patterns showing the relative position of typical components found in cheeses made from cow's milk, clotted by rennet (teken from Marcos *et al.*, 1979).

The identity of the γ-casein (CN) bands is generally recognized, but the order in which they migrate (γ_3, γ_1 and γ_2) has been misinterpreted (Farkye and Fox, 1990) and correspond to those of γ-CN produced from isolated β-casein by plasmin (Farkye and Fox, 1990).

Some of the variability in products may be explained by the importance of the salt content and pH (Thomas and Pearce, 1981). This is illustrated in Figure 3.5, where differences among the three batches of standard Cheddar cheeses during ripening are indicated. Differences can also be noted between the commercial Cheddar (age and manufacturing process unknown) and the Cheddar cheeses under study.

The degradation of β-casein among the three batches differs during ripening. This is indicated by the slow formation of γ_1 in batch C (absent at 30 days) (lane 7). The formation of the α_{s1}-degradation peptides is also slower for batch C (lanes 5-7), especially α_{s1}-CN(f110-•) and α_{s1}-CN(f24-•) which are absent at 30 days (lane 7), while the intensity of α_{s1}-CN(f60-•) and α_{s1}-CN(f33-•) are much lower than those in batches A and B (lanes 5-6). No differences among the three batches were however observed at 90 days, indicating that a high constant standard regarding manufacture and quality prevails in the factory of production. These data therefore verify the use of only one batch of Cheddar cheese for sensory analysis, as was carried out in Chapter 2.

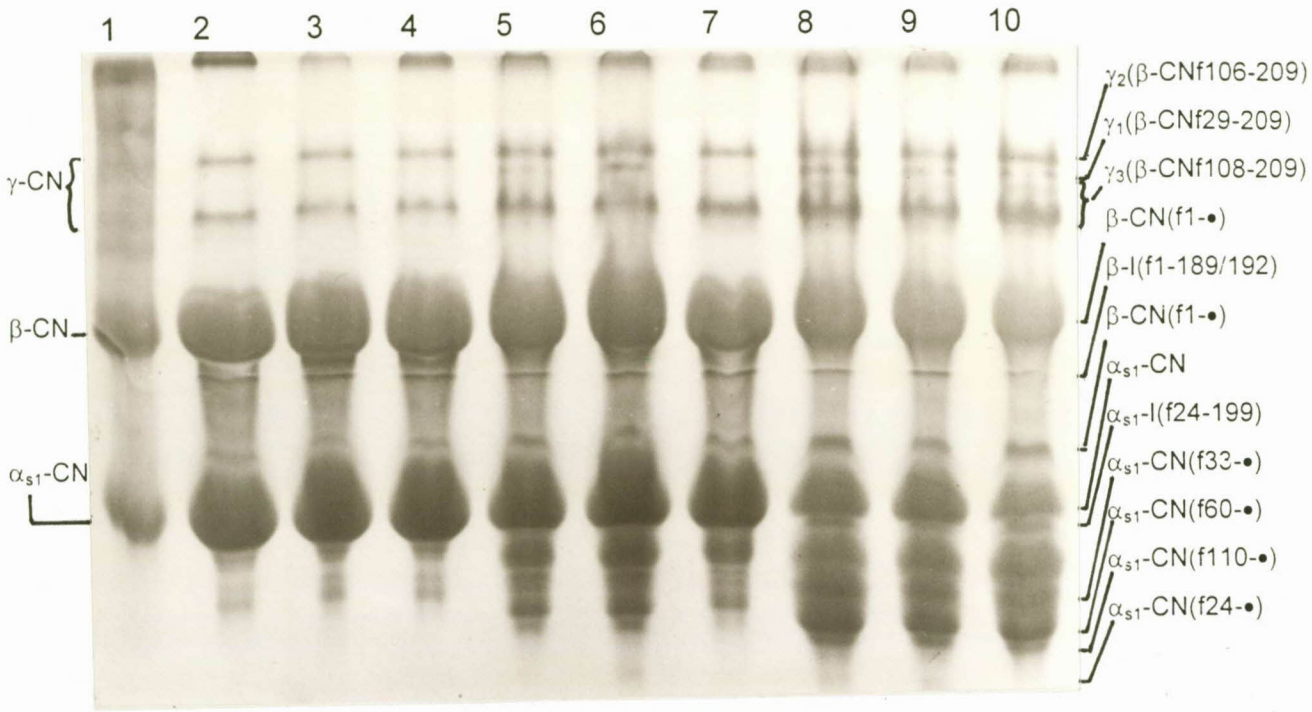

Fig. 3.5 Comparison of the WISF of the three standard Cheddar cheeses during ripening. Lane 1: Marker, Na-caseinate. Lane 2: A, cheese vat. Lane 3: B, cheese vat. Lane 4: C, cheese vat. Lane 5: A, 30 days. Lane 6: B, 30 days. Lane 7: C, 30 days. Lane 8: A, 90 days. Lane 9: B, 90 days. Lane 10: C, 90 days.

Peptides in the retentate ppt of WSN were identified according to the results obtained from McSweeney *et al.* (1994) and Singh *et al.* (1995) and analysis of a standard Cheddar, batch B is shown in Fig. 3.6.

The development of β-derived peptides during ripening are indicated in lanes 4-9, especially from 30 days onwards (lanes 7-9). The intensity of the bands clearly increased from 60-90 days (lanes 8-9). The formation of β-CN(57-?), β-CN(58-?) and its subsequent degradation are indicated in lanes 6-9. The development of β-CN(43-?) from 60-90 days is indicated in lanes 8-9. The development of the β-CN(1-?) peptides from 30 days are clearly indicated in lanes 7-9, with the intensity of the β-CN(7-?) at its highest at 90 days (lane 9). The unidentified peptides, marked m-x also showed differences in development and degradation. They seem to be unique for the South-African Cheddar cheese, perhaps for the reasons of differences in manufacture already explained. Peptide m developed during the last month of ripening and is visible only at 90 days (lane 9). Peptides n and o developed from 30 days onwards (lanes 7-9), while peptide p developed from 48 h onwards (lanes 5-9). Peptide q was only present at 48 h (lane 5). Peptide r developed at drainage stage (lane 4) with the intensity increasing during maturation (lanes 4-9). Peptide s developed at 60 days (lane 8) and the intensity increased much towards 90 days (lane 9). Peptide t developed from drainage onwards (lane 4) with a maximum intensity observed at 12 days (lane 6). Peptide u was observed from 12 days, with its intensity decreasing towards the end of ripening (lanes 6-9). Peptide v developed from drainage and the intensity increased towards 90 days (lanes 4-9). Peptide w developed from drainage to 30 days (lanes 4-7) after which it was degraded and not visible thereafter. Peptide x was present throughout maturation and its intensity increased towards 90 days (lanes 3-9).

The same pattern of proteolysis can be seen in Fig.3.7 where the WSN-retentate-ppt of batch C was investigated for comparison.

The formation of β-CN-derived fragments during ripening are indicated. Peptide β-CN(f57-?),(f58-?) developed from 12 days throughout ripening (lanes 6-9). The peptide β-CN(f43-?) formed after 60 days (lanes 8,9). Peptide β-CN(f1-?), developed at 30 days, with increasing intensity from 30 days (lanes 6-9). Peptides marked m-x are unidentified but differences in

their development and degradation did occur. Peptide m developed at 12 days and the intensity increased towards 90 days (lanes 6-9). Peptide n faintly developed at 12 days and was clearly present throughout ripening. Peptide o developed at 48 h and the intensity increased towards the end of ripening. Peptides p, q and r developed at drainage and were present during maturation. Peptide s only developed after 60 days (lanes 8,9). Peptide t was present since drainage, with peptide t_1 developing from 30 days and t_2 from drainage. Peptides v, w and x were present from the beginning (cheese vat) of ripening and the intensity increased as the cheese matured (lanes 3-9). Thus, the formation and development of bands is clearly indicated from 30 days (lane 7) with a correlated increase in intensity of this degradation products.

According to McSweeney *et al.* (1994), most (45) of the total (51) peptides in the dia-filtrate retentate originate from β-casein, especially from a short region in the N-terminal half of the molecule. Only six peptides originated from α_{s1}-casein.

Comparison of the WSN-retentate-ppt peptides among the 3 standard batches is shown in Fig. 3.8.

The development of β-CN(f43-?), β-CN(f57-?), β-CN(f58-?) and β-CN(f1-?)' are much faster in batch C than in batches A and B (lanes 6,9), with β-CN(f43-?) only present in batch C at 90 days (lane 9). The development of peptides m-x is faster for batches B and C at 30 days (lanes 5-7) than batch A with peptides s-w absent in batch A. Thus, the development of peptides is much faster in batch C than in the other two batches at 30 days and 90 days (lanes 5-10).

The results indicate that different batches of Cheddar cheese may ripen at different tempos, as indicated by the slower or faster development of casein peptides, however, with controlled conditions, these differences are limited indicating that a high constant standard regarding manufacture and quality prevails in the factory of production. These data therefore verify the use of only one batch of Cheddar cheese for sensory analysis, as was carried out in Chapter 2.

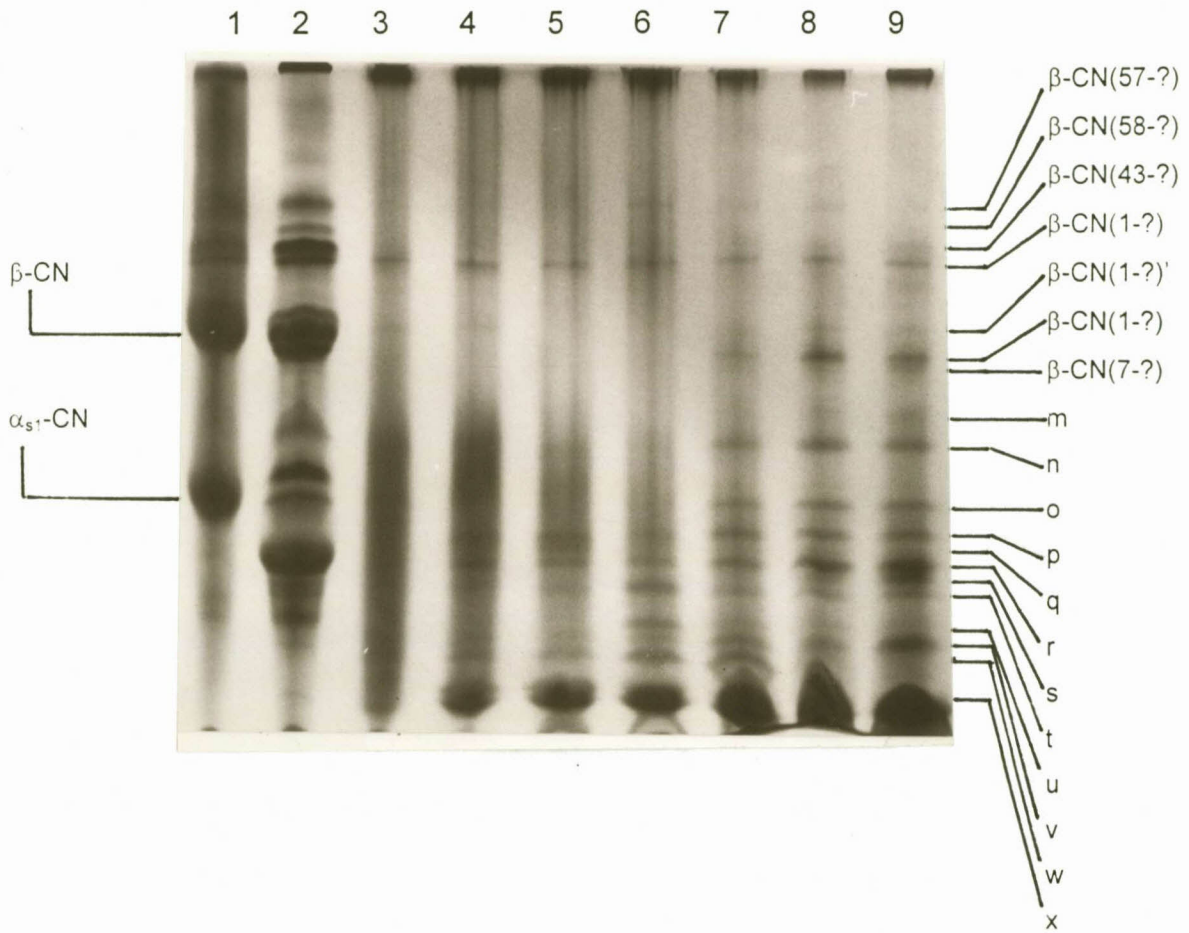

Fig.3.6 Water-soluble, ethanol-insoluble fraction (retentate-ppt) of a standard Cheddar cheese, batch B, during ripening. Lane 1: marker, Na-caseinate. Lane 2: WISF, commercial Cheddar. Lane 3: cheese vat. Lane 4: drainage. Lane 5: 48 h. Lane 6: 12 days. Lane 7: 30 days. Lane 8: 60 days. Lane 9: 90 days.

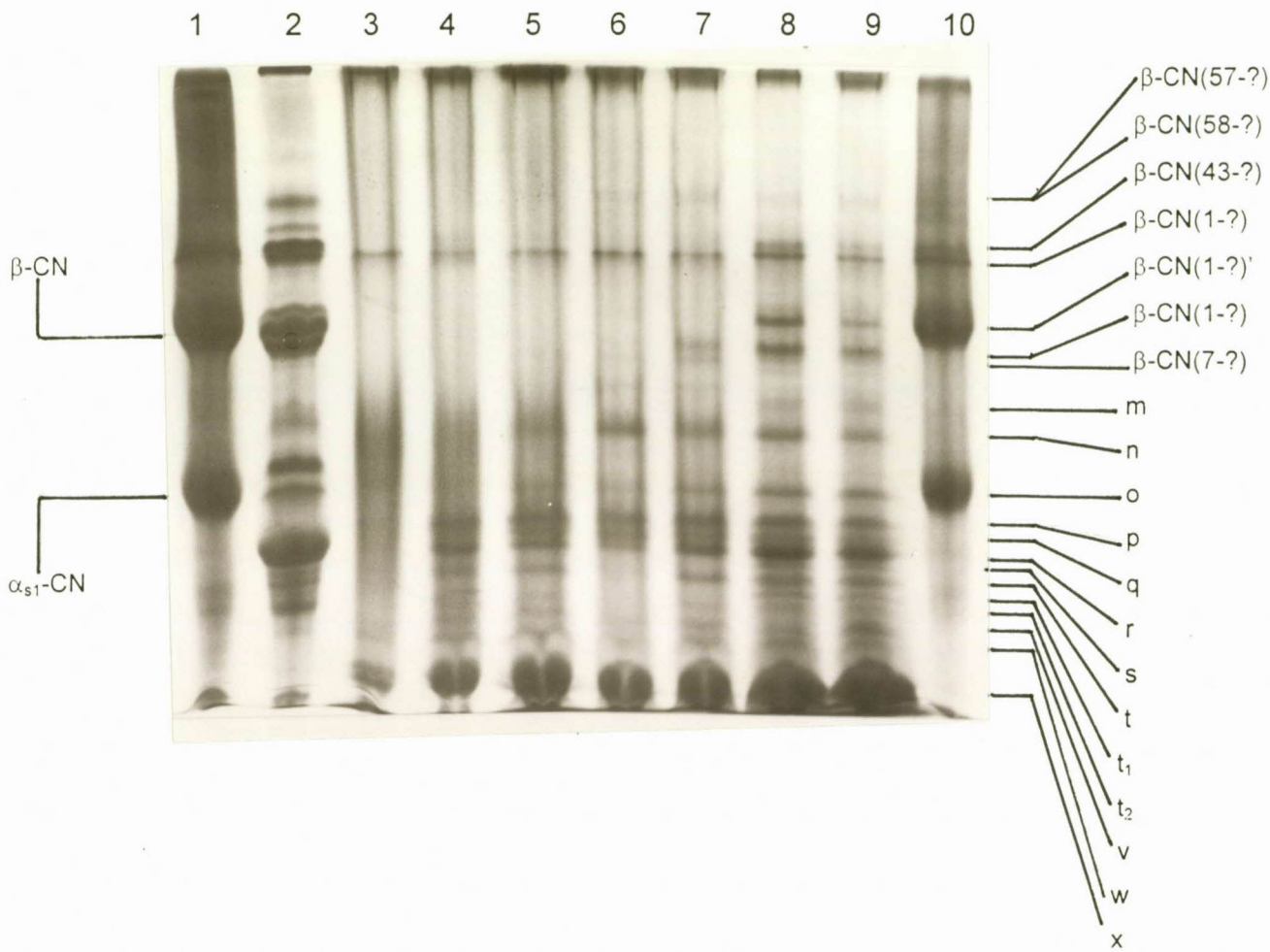

Fig.3.7 Retentate-ppt. of standard Cheddar cheese during ripening, batch C. Lane 1: marker, Na-caseinate. Lane 2: WISF, commercial Cheddar. Lane 3: cheese vat. Lane 4: drainage. Lane 5: 48 h. Lane 6: 12 days. Lane 7: 30 days. Lane 8: 60 days Lane 9: 90 days. Lane 10: marker, Na-caseinate.

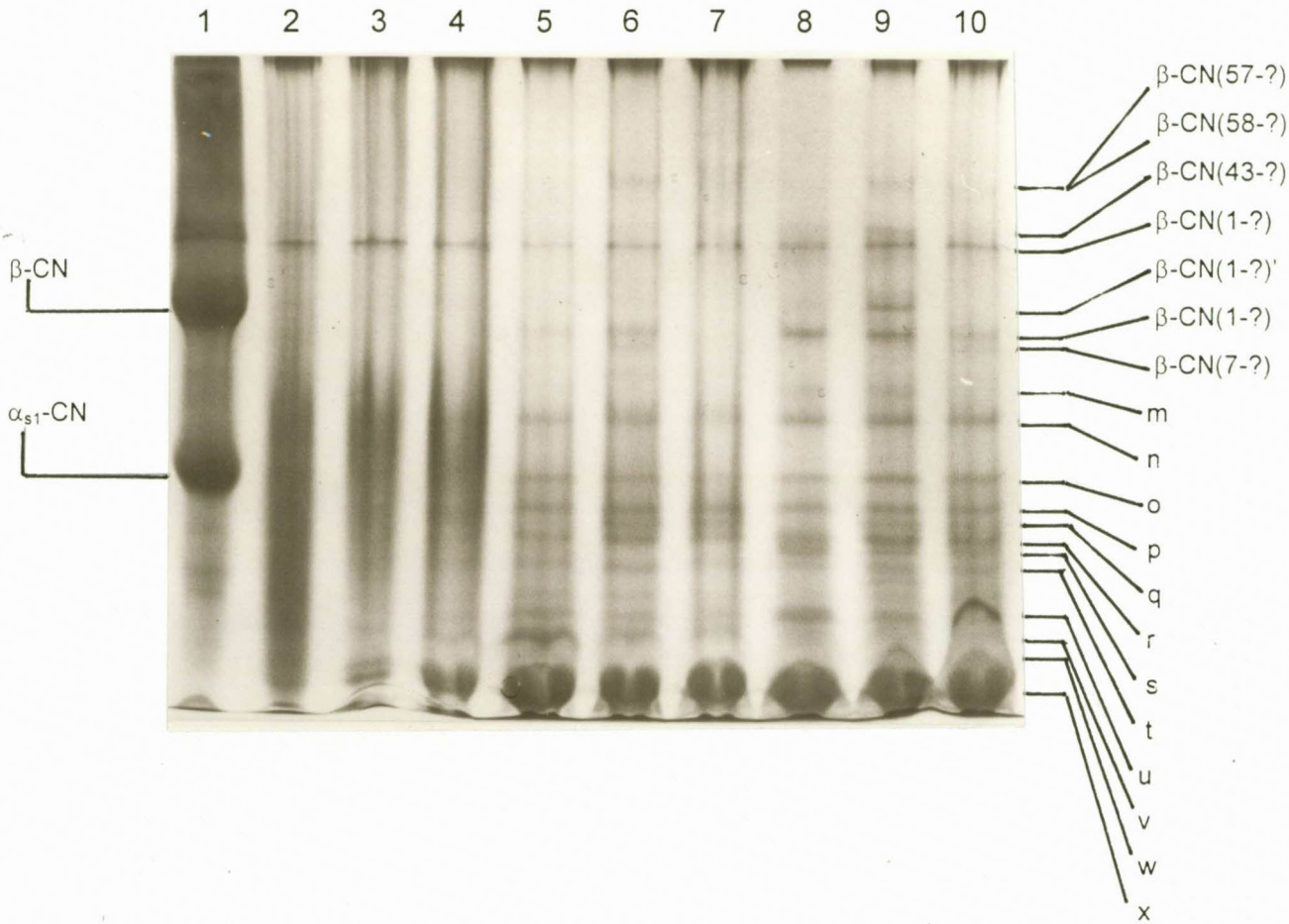

Fig. 3.8 Comparison among the three standard cheese batches during ripening. Lane 1: marker, Na-caseinate. Lane 2: B, cheese vat. Lane 3: C, cheese vat. Lane 4: A, cheese vat. Lane 5: B, 30 days. Lane 6: C, 30 days. Lane 7: A, 30 days. Lane 8: B, 90 days. Lane 9: C, 90 days. Lane 10: A, 90 days.

Protein degradation and peptide formation in the WISF of the yeast-inoculated Cheddar cheese during ripening are indicated in Figure 3.9.

The intensity of γ_3 increased from 30 days onwards (lanes 5-7), while the intensity of β-CN peptides decreased during the first month of ripening (lanes 2-6). The β-CN is degraded with the subsequent formation of β-I (β-CNf1-189/192) of which the intensity increases from 12 days (lanes 5-8). The p-, q- and r- β-CN degradation peptides decreased until absent during ripening (lanes 2-7).

Concerning the degradation of α_{s1}-casein, the formation of α_{s1}-peptides is much faster and more intense in the yeast-inoculated cheese. The α_{s1}-CN is degraded with the formation of the α_{s1}-I peptide clearly indicated. The presence of the other α_{s1}-CN degradation products is indicated throughout ripening, with α_{s1}-CN(f102-•) and α_{s1}-CN(f24-•) developing after one month of ripening (lanes 6-8).

When compared to the standard Cheddar cheeses, it seemed that the degradation of β-casein of the yeast-inoculated Cheddar correlated with the "slower" standard batch, namely C, in terms of peptide development. These findings are indicated in figures 3.10 and 3.11, where the development of yeast-inoculated cheese is compared with batches B and C of standard Cheddar cheese respectively.

Degradation of α_{s1}-CN and the subsequent formation of peptides are clearly indicated for both types of cheeses during ripening. The formation and intensity of the α_{s1}-CN degradation products derived from α_{s1}-I and α_{s1}-CN(F102-•) is faster in the standard Cheddar than observed in the yeast-inoculated Cheddar at 30 and 90 days (lanes 5-10). The α_{s1}-I peptide was formed faster.

The development of γ_3 peptides is faster in the standard Cheddar (batch B) than in the yeast-inoculated Cheddar at 30 days (lanes 5-7) and 90 days (lanes 8-9). The intensity of the other β-CN degradation products (peptides p, q and r) decreased during ripening for both cheeses. The γ_3-peptide formation in batch C correlated with that in the yeast-inoculated Cheddar, with only a higher intensity at 30 and 90 days. The pattern of proteolysis (development and

degradation of peptides) is almost the same for the two types of cheeses, except for the intensity of the different bands. The α_{s1}-degradation products, namely α_{s1}-CN(f33-∘), α_{s1}-CN(f60-∘) and α_{s1}-CN(f110-∘) formed faster in the standard Cheddar at 60 days and 90 days at a higher intensity. As explained above, it seems as if yeasts are not able to degrade α_{s1}-CN further and therefore, these peptides cannot be formed.

According to Creamer and Richardson (1974) rennet plays the major role in the initial breakdown of α_{s1}-I. Visser and De Groot-Mostert (1977) concluded that rennet is completely responsible for the degradation of α_{s1}- and β-CN during the first month of ripening, with enzymes from lactic acid bacteria contributing to proteolysis in the longer term, especially on β-CN. Fox *et al.*, (1994) concluded that primary proteolysis results from the action of chymosin and plasmin, with Marcos *et al.*, (1979) stating that rennet and alkaline proteinases break casein into large peptides which are cleaved into small peptides by starter proteinases and peptidases. Therefore, conditions in the two types of Cheddar were the same (rennet/chymosin, plasmin, temperatures during manufacture and ripening as well as salt content), except the starter cultures added, which will in turn lead to the formation of different smaller peptides from the rennet-derived peptides (refer to specificity of enzymes from starter culture, Chapter 1.5.3). The α_{s1}-casein is degraded faster in the yeast-inoculated Cheddar cheese, however, with less peptides formed (only α_{s1}-CN(f102-∘) and α_{s1}-CN(f24-∘). It can therefore be concluded that yeasts are not capable to hydrolyze these peptides further. The yeast enzymes seem to have the same activity and specificity as the rennet, resulting in faster degradation of α_{s1}-CN, since rennet is completely responsible for the degradation of α_{s1}-CN (Visser and De Groot-Mostert, 1977). It can thus be concluded that if it was only the role of rennet, the peptides formed should be the same (see the formation of α_{s1}-I from α_{s1}-CN). Therefore, starter enzymes help to defold the caseins. Furthermore, if it only was the role of rennet, then peptides p, q and r would not be formed that early in maturation in the yeast-inoculated Cheddar. Alternatively it may be concluded that they are slower hydrolyzed in the yeast-inoculated Cheddar.

Electrophoretogram of the WSN-retentate-ppt of yeast-inoculated Cheddar is shown in Fig. 3.12.

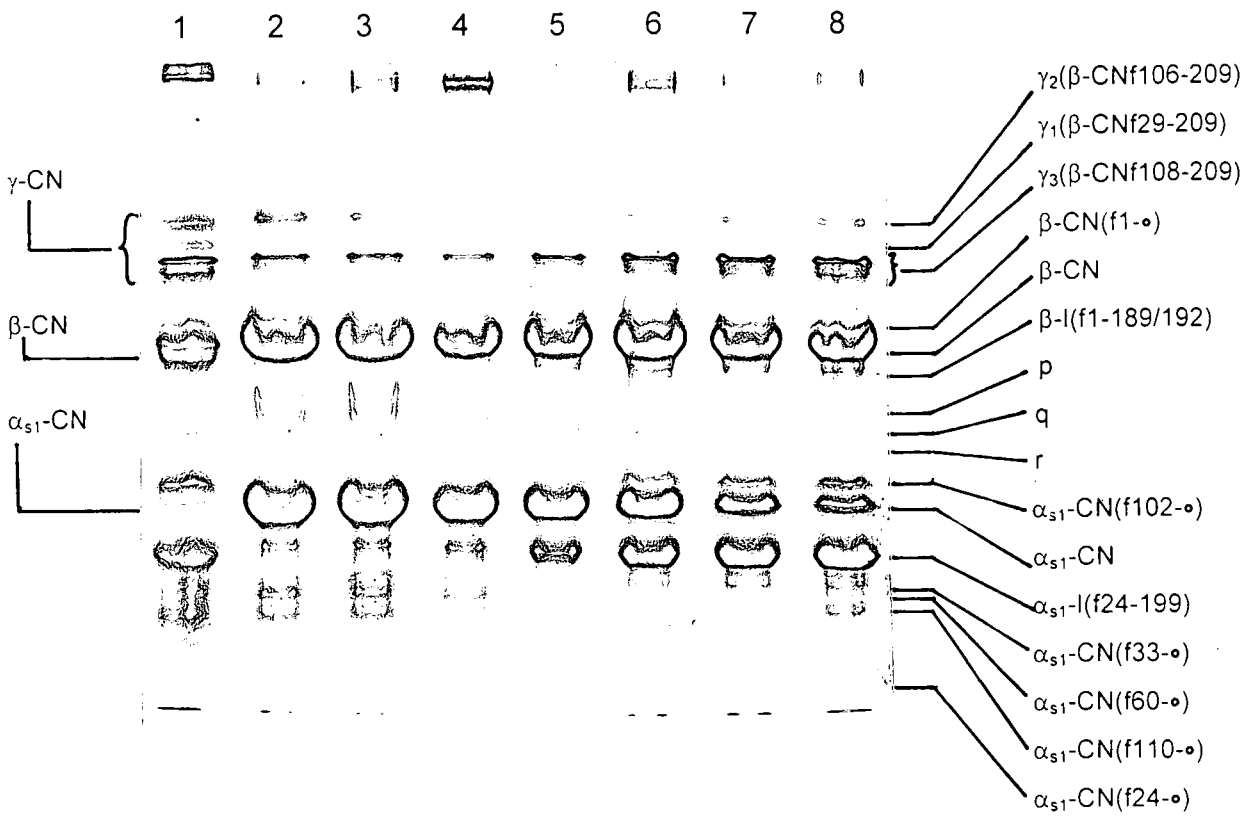

Fig. 3.9 WISF of the yeast-inoculated Cheddar cheese throughout ripening compared with a commercial Cheddar cheese marker. Lane 1: marker, commercial Cheddar. Lane 2: before salt. Lane 3: after salt. Lane 4; 48 h. Lane 5: 12 days. Lane 6: 30 days. Lane 7: 60 days. Lane 8: 90 days.

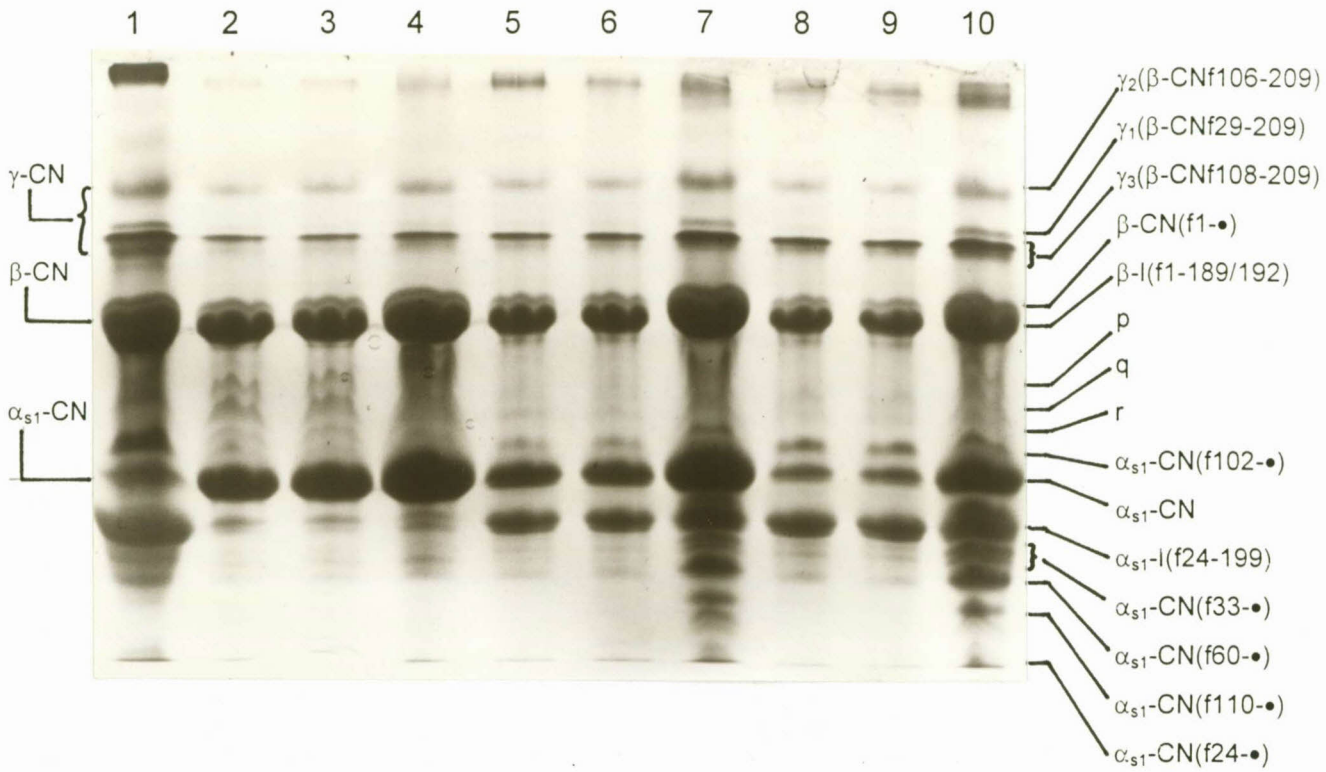

Fig.3.10 Comparison of the WISF between yeast-inoculated Cheddar and standard Cheddar, batch B. Lane 1: commercial Cheddar. Lanes 2,3 yeast-inoculated Cheddar, before salt. Lane 4: B, cheese vat. Lanes 5,6: yeast-inoculated Cheddar, 30 days. Lane 7: B, 30 days. Lanes 8,9 yeast-inoculated Cheddar, 90 days. Lane 10: B, 90 days.

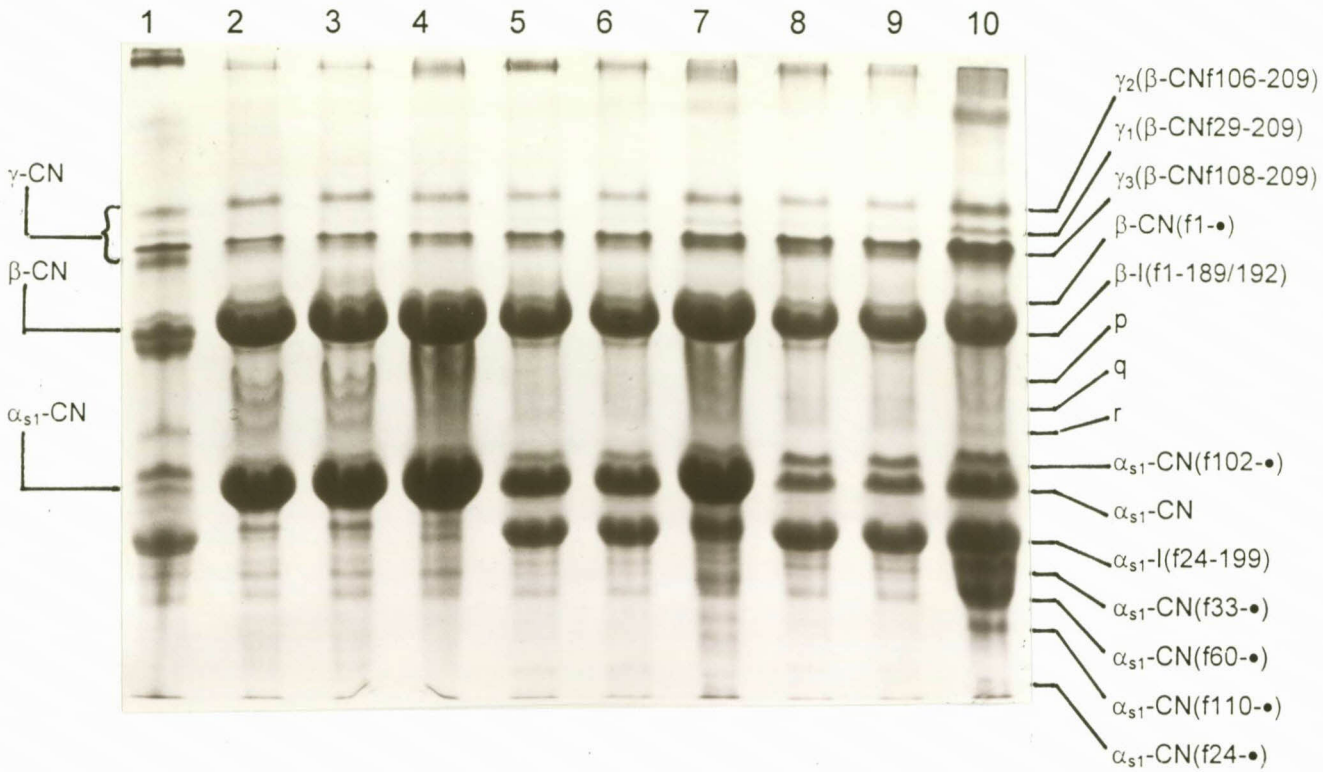

Fig.3.11 Comparison of the WISF between the "slower" standard Cheddar, batch C, and the yeast-inoculated Cheddar cheese. Lane 1: commercial Cheddar. Lanes 2,3: yeast-inoculated Cheddar, before salt. Lane 4: C, cheese vat. Lanes 5,6: yeast-inoculated Cheddar, 30 days. Lane 7: C, 30 days. Lanes 8,9: yeast-inoculated Cheddar, 90 days. Lane 10: C, 90 days.

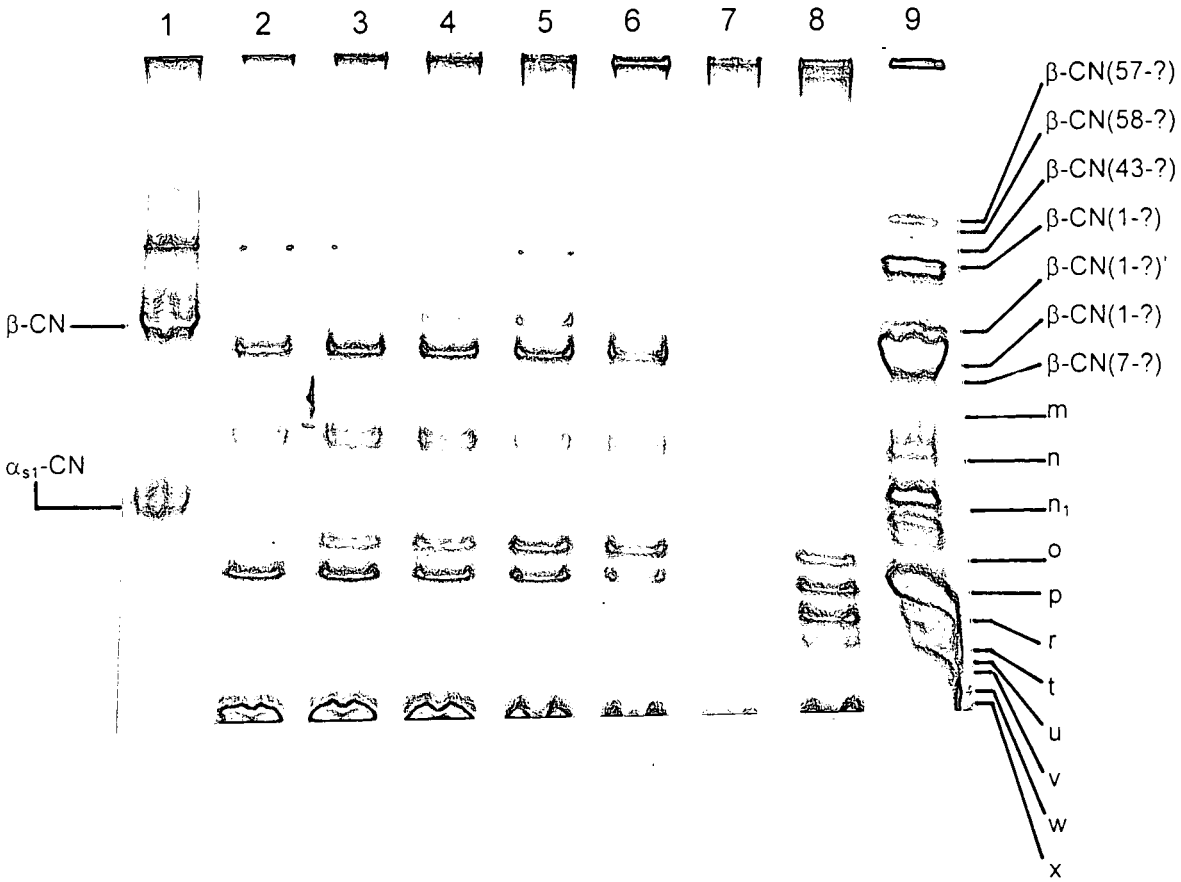

Fig. 3.12 Retentate ppt of the yeast-inoculated Cheddar cheese during ripening. Lane 1: marker, Na-caseinate. Lane 2: before salt. Lane 3: after salt. Lane 4: 48hrs. Lane 5: 12 days. Lane 6: 30 days. Lane 7: 60 days. Lane 8 : 90 days. Lane 9: commercial Cheddar (WISF).

The β-CN degradation products, β-CN(58-?), β-CN(57-?) and β-CN(43-?) intensity decreased during ripening and were thus almost entirely degraded. The intensity of β-CN(1-?)' increased from after salting until 12 days, whereafter it decreased. The intensity of β-CN(7-?) increased during ripening. No further changes in degradation of β-CN (peptides m-x) were observed during ripening.

Comparison between the retentate-ppt. fraction of the standard Cheddar cheeses and the yeast-inoculated Cheddar cheese also indicated different peptide development and protein degradation during ripening and are indicated in Fig. 3.13.

Peptides n, p, r and t developed faster in the yeast-inoculated Cheddar cheese. Just as for the α_{s1}-casein, the yeasts' proteases are not able to degrade the β-CN in the same way as the normal starter cultures. They do, however, degrade β-CN. Instead of forming the β-CN and β-I in the WISN (Fig. 3.13), they form other β-derived peptides (n-x) visible in the WS, ethanol-insoluble (retentate ppt) fraction. Therefore, the yeast-inoculated Cheddar cheese did not develop faster than the standard Cheddar, but developed completely different.

Peptides β-CN(xx-?) identified from the work done by McSweeney *et al.*, (1995) are not visible in the standard Cheddar, but are present in the yeast-inoculated Cheddar. This may be attributed assumingly to hydrolysis of the peptides by starter enzymes in the standard Cheddar, while the peptides stay intact in the yeast-inoculated Cheddar after being formed by rennet.

According to McSweeney *et al.* (1994) the WISF is flavourless, but peptides in the water-soluble fraction is important to cheese flavour. Thus, during primary proteolysis in the WISF, relatively large peptides were formed, that is during the early stages of proteolysis. The yeast-inoculated Cheddar cheese was evaluated bitter during the sensorical evaluation and could therefore be explained by the formation of the smaller, bitter peptides later during the ripening process. The production of small peptides and free amino acids is due primarily to the activity of starter and non-starter proteinases and peptidases (McSweeney *et al.*, 1994).

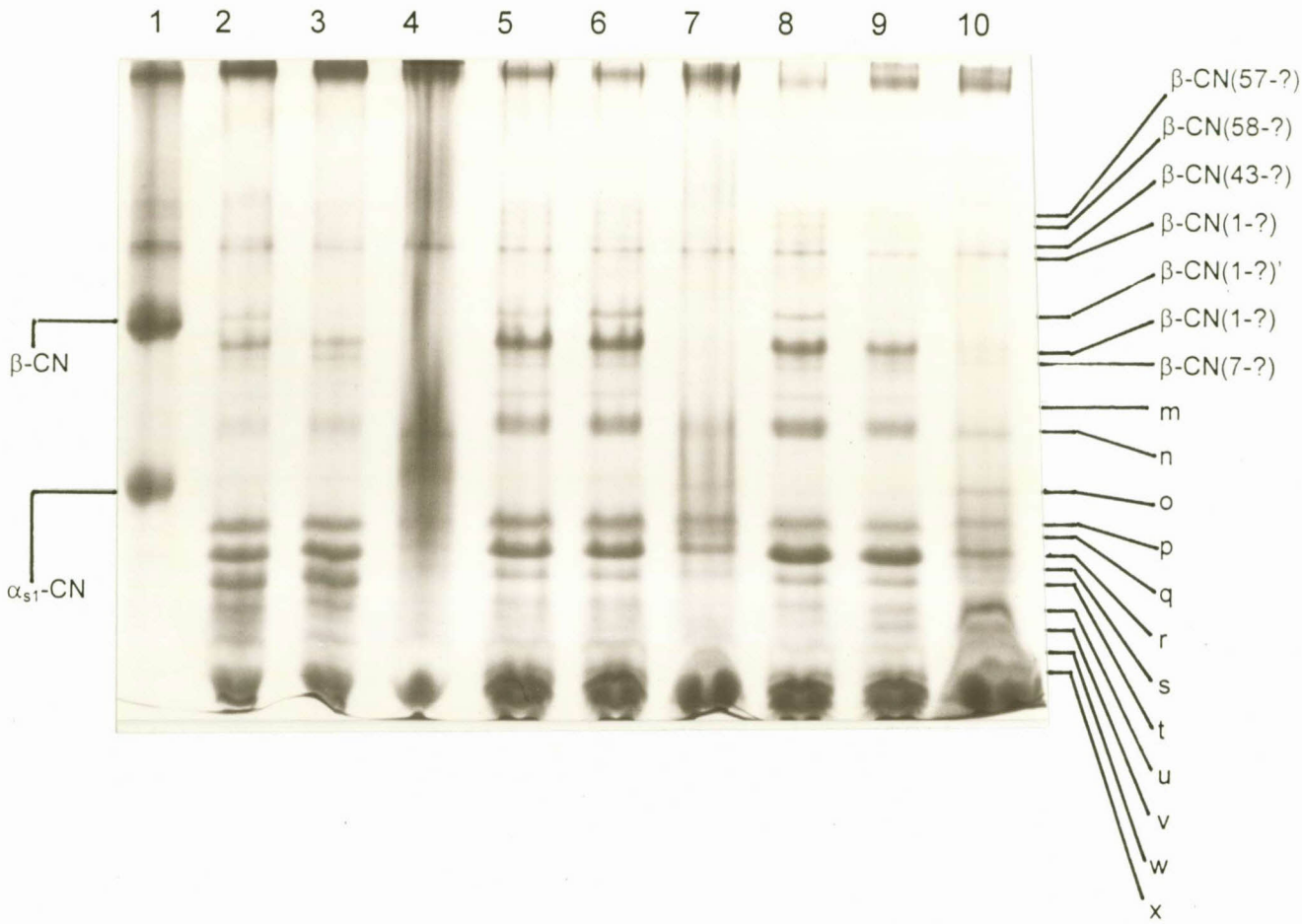

Fig. 3. 13 Water-soluble, ethanol-insoluble (retentate ppt) fraction of the yeast-inoculated Cheddar cheese and the standard Cheddar cheese, batch B. Lane 1: marker, Na-caseinate. Lanes 2,3: yeast-inoculated, before salt. Lane 4: B, cheese vat. Lanes 5,6: yeast-inoculated, 30 days. Lane 7: B, 30 days. Lanes 8,9: yeast-inoculated, 90 days. Lane 10: B, 90 days.

3.4 Conclusion

When comparing the WISF of the standard Cheddar with that of the yeast-inoculated Cheddar, marked differences in peptide development and -degradation were observed. These results can be attributed to the roles of the different starter activities in the two different types of cheeses, with the yeast-enzymes showing almost the same enzymatic activity and specificity as the rennet.

From the results (N-analysis and Urea-PAGE) obtained for the WSNF it appears that the yeast-inoculated cheese contains higher amounts of peptides. Macro Kjeldahl analyses showed lower levels of WSN in the yeast-inoculated Cheddar cheese. This is also visible in the electrophoretograms where the yeast-inoculated Cheddar showed more peptides, especially in the WS, ethanol-insoluble fraction (retentate ppt). Both results indicate that caseins are not fully hydrolyzed by the yeast-enzymes.

As discussed earlier, the different way of proteolysis may be the reason for the bitter (Weir, 1992) defect of the cheese analyzed sensorically by expert graders. Electrophoresis provided, however, not enough evidence to prove the bitter defect because the WISF consists mainly of larger peptides with higher molecular weights, which can be detected by Urea-PAGE (Singh *et al.*, 1995; McSweeney *et al.*, 1994 and Breen *et al.*, 1995). Therefore, the subsequent analysis of peptides by means of HPLC is necessary for three reasons: to determine hydrophobic characteristics of peptides, to determine the additional bands of the yeast-inoculated cheese in more detail and because the permeable peptides (<10 000 Da) do not stain on Urea-PAGE gels, need analysis by means of HPLC and gelfiltration. Certain bands on the electrophoretograms could not be identified from the work done by Singh *et al.* (1995), McSweeney *et al.* (1994) and Breen *et al.* (1995). A recommendation for further research will include isolation and identification of these individual peptides by electrospray mass spectrometry.

Chapter 4

Chromatographic analysis of cheese peptides and free amino acids

4.1 Introduction

Products of proteolysis in cheese range from polypeptides, comparable in size to intact caseins, to free amino acids and their degradation products. In order to establish the contribution of peptides to cheese flavour and to identify the proteolytic agent responsible for their formation, it is necessary to resolve peptides to homogeneity (Singh *et al.*, 1994).

While cheese flavour development is positively affected through the production of peptides and amino acids, excessive or incorrect proteolysis may cause certain flavour defects such as bitterness (Fox *et al.*, 1995b) (discussed in Chapter 1). Amino acids may be degraded by decarboxylation, deamination, desulphuration and demethylation to yield products which contribute to the overall flavour of cheese (Law, 1987).

Several methods have been developed for quantifying the degree of ripening, identifying the key compounds responsible for cheese flavour and off-flavours and characterizing the role of the numerous proteinases and peptidases in cheese maturation (Gripon *et al.*, 1977; Grappin *et al.*, 1985; Rank *et al.*, 1985; Fox, 1989; IDF, 1991; McSweeney and Fox, 1993 a, b; Fox *et al.*, 1995a). Electrophoretic- and chromatographic methods are used to characterize the peptides formed during proteolysis (O'Shea *et al.*, 1996).

It has long been established that the bitter components in cheese reside in the peptide fraction. Emmans *et al.*, (1962) showed that the strain of bacterial starter used for making the cheese could be linked with the bitterness of the final cheese, a finding that has since been confirmed and utilized commercially (discussed in Chapter 2). Rank *et al.*, (1985) suggested that rennet produced a series of large peptides, mainly non-bitter and that starter proteases produce bitter peptides from these larger peptides, a finding which was confirmed by results discussed in Chapter 3). According to Richardson and Creamer (1973) bitterness is the result of starter protease action on rennin produced peptides and they suggested that α_{s1}-casein is the likely precursor. These authors also predicted that bitter cheese would contain a great

variety of low molecular weight (MW) peptide material as a result of more extensive starter proteinase activity.

No apparent differences in protein breakdown examined by means of gel electrophoresis could be found by Richardson and Creamer (1973) but proteolysis in cheese can, however be distinguished by gel chromatography on Sephadex. The bitter cheese has a greater variety of peptides. Chromatography by gel filtration partially separate peptides / amino acids on the basis of the size of the molecular species present in the solution. The technique allows the estimation of molecular weights, e.g. Sephadex G-10 will resolve molecules with MW of 700-1500 Da (Fox, 1989).

Gel filtration and high performance liquid chromatography (HPLC) are used to characterize key peptides. Cheeses give characteristic peptide patterns that elucidate the effects of various cultures or rennets (Rank *et al.*, 1985). Reversed-phase HPLC (RP-HPLC), associated with statistical data will probably allow cheeses to be classified according to the age of maturation and provides indices of ripening. This method was found to be an excellent tool for the separation (and for the identification) of the first main peptides appearing during maturation (Singh *et al.*, 1994). Therefore, objective assessment of Cheddar cheeses quality could be performed successfully if a suitable "model" is designed, i.e. using equal numbers of samples based on uniform graders' data. This model could be used to categorize test samples, i.e. to determine (predict) the quality of such samples without employing sensory analysis (O'Shea *et al.*, 1996).

Although the volatile fraction of cheese contributes mainly to its aroma, the water-soluble fraction (WSF) is mainly responsible for its taste (Aston *et al.*, 1983). Most of the nitrogenous compounds that contribute to cheese flavour are soluble in aqueous solvents. PTA-soluble N was found to correlated significantly with the age and flavour intensity (Aston and Dulley, 1982) and was found by Jarrett *et al.*, (1982) to contain small peptides and free amino acids. Proteolysis influences flavour since free amino acids formed have typical flavour characteristics and probably contribute to background flavour (Mabbitt, 1955). O'Keeffe *et al.*, (1976) suggested that free amino acids in Cheddar cheese are mainly the result of microbial peptidase activity. The differences in peptide profiles and in the content of amino acids can be attributed to different enzymatic activities in the cheese (Ardö and Gripon, 1995).

The WSF can be resolved effectively by HPLC and it is interesting to group its constituents according to some specific property such as molecular weight or hydrophobicity, to study peptides that either occur at high concentrations or are significant for cheese flavour or to use HPLC chromatograms to identify the cheese variety or to establish its age (O'Shea *et al.*, 1996).

The formation and degradation of the large WISF-peptides are usually monitored by polyacrylamide gel electrophoresis (PAGE) which is difficult to quantify (Fox and McSweeney, 1996). PAGE is the most widely used technique for monitoring primary proteolysis in cheese (Fox, 1989). Bacterial proteinases, from either starter or NSLAB, contribute very little to the formation of large peptides (Fox and McSweeney, 1996).

In this chapter: gel filtration chromatography and HPLC was used to monitor the secondary proteolysis in the standard Cheddar and the yeast-inoculated Cheddar cheeses, that is the formation of small peptides and free amino acids and attempts were made to identify differences in the peptide profiles of the two types of Cheddar cheese and to attribute the differences to the different enzymatic activities in the two cheeses and explain taste differences (bitterness).

4.2 Materials and Methods

4.2.1 Manufacture of Cheddar cheese

The manufacture of cheese was discussed in Chapter 2.2.1. Three batches of standard Cheddar cheeses were manufactured. In this chapter only one will be analyzed as a representative, e.g. batch B, since it was established in Chapter 3 that they do not differ much in chemical composition.

4.2.2 Sampling of cheese

The sampling procedures were discussed in 3.2.2

4.2.3 Extraction and fractionation of water-soluble nitrogen

The extraction and fractionation of the proteins was discussed in 3.2.3 (see Fig. 3.1).

4.2.4 Peptide analysis by high-performance liquid chromatography

Lyophilized samples of the water-soluble, 5%PTA-soluble and -insoluble fractions (UF-permeate) were dissolved in deionized water (Milli-Q water) (1mg/500µl), and filtered through a 0,45µm Cameo (Separations) pre-filter and stored frozen. Samples of the water-soluble, EtOH-soluble fraction (UF retentate) were directly injected into the HPLC chromatograph.

HPLC was carried out on a HPLC system, which comprised a Hewlett Packard 1100 sample injector and quartenary gradient pump solvent delivery system, and a Shimadzu Variable Wavelength detector operated at 230 nm. Samples were chromatographed on a 5µm Jupiter (Separations) RP18 250mm x 4,6mm reversed-phase column preceded by a Jour-Guard RP/C18 - 5µm guard column (Separations) at ambient temperature. A linear gradient of 0,15% TFA (Trifluoroacetic acid),(ion-pairing agent) in water (eluant A) to 0,1% TFA (Merck) in 80:20 (v/v) acetonitrile (Burdick & Jackson) in water (eluant B) was increased to 70% B over 70 min at 1ml/min flow rate.

Solvents were degassed under vacuum and samples were filtered through 0,45µm filters before use.

4.2.5 Gel filtration chromatography of peptides and amino acids

Lyophilized samples of the water-soluble, 5% PTA-soluble (UF permeate) fraction were dissolved in distilled water (8mg/100µl). Aliquots of 8mg of water-soluble, 5% PTA-soluble material, dissolved in 100µl, were loaded.

Gel filtration was performed on Sephadex G-10 fine (Pharmacia Fine Chemicals) using distilled water as eluant. A glass column (64,5cm x 0,75cm) was used to fractionate the peptides at a flow rate of 7,2 $ml.h^{-1}$ (provided by a LKB Bromma 2132 Microperpex peristaltic pump). Fractions of 1,2 ml were collected and protein content was determined by

absorbances at 280nm and 230 nm.

Detection at 280 nm is generally suitable for fractions containing large peptides, which are more likely to contain aromatic residues (McSweeney and Fox, 1993). Absorbance at this wavelength gives limited information about where smaller peptides and amino acids are eluated. The amino acids Trp and Tyr absorb the light at 280 nm. At wavelengths 254 and 220 nm, more amino acids and peptide bonds absorb light (Ardö and Gripon, 1991). Detection at 200-230 nm measures the carbonyl group in the peptide bond, since smaller peptides may not contain aromatic residues (McSweeney and Fox, 1993).

4.3 Results and discussion

4.3.1 Peptide analysis by high performance liquid chromatography

Results of RP-HPLC done on the WS, EtOH-soluble retentate fraction of a standard Cheddar, batch B, are shown in Fig. 4.1(a). Indicated is the development of the group of peptides eluted at 20-35 min from 48 h towards 90 days of ripening. Another important feature is the development of peptide x, eluted at 3.96 min, after 30 days of ripening. A different pattern of development of peptides, eluted at 20-35 min, can be seen for the yeast-inoculated Cheddar as is indicated in Fig. 4.1(b). A distinctive peptide, peptide y, eluting at 15.35 min is also found in the yeast-inoculated Cheddar, developing only after 60 days of ripening.

The chromatograms from the cheese before drainage of whey seem to show no definite pattern of development, which can be attributed to the sampling of the cheese, as the samples may differ in whey content, thus resulting in differing amounts of WSN.

The basic peptide pattern observed here for the standard Cheddar cheese, more specifically the group eluting between 20-35 min and group z, eluting at 37.25min (Fig. 4.2 a) correlate with the chromatographic results of Fox and McSweeney (1996), who indicated that the HPLC pattern of the WSF of cheese is characteristic of the variety. A slight variation occurs between the results shown in Fig 4.1 and the results of Fox and McSweeney (1996), in terms of peptides eluted, the principal peptides (x and z) correlated with their results. The variation can be explained by the fact that the results of Fox and McSweeney (1996) were obtained for

a commercial Irish Cheddar, which can vary slightly from the standard Cheddar used in this study, as well as the fact that the age and manufacturing process is unknown.

Futhermore, the RP-HPLC patterns in Fig. 4.1 not only changed (developed) but became more complex as the cheese matured. This is also in agreement with Fox and McSweeney (1996) who indicated that the HPLC pattern of the WS, EtOH-soluble and -insoluble fractions of Cheddar cheese change and become more complex as the cheese matures. The yeast-inoculated cheese shows an elution profile closely resembling the mentioned Cheddars, but with a distinct peak at 15.35 min and the somewhat different pattern of peaks between 20-35 min. The difference in the latter group may also be due to the lower amount of total WSN, resulting from limited hydrolysis of α_{s1}- and β-caseins. The peaks z, eluting at 37.25 min are, however, the same.

Although the retentate is bland, according to Singh *et al.* (1994), differences in the RP-HPLC patterns were observed for the standard Cheddar and the yeast-inoculated Cheddar (Fig. 4.1), as well as in the formation of more peptides in this fraction analyzed by PAGE (Chapter 3, Fig. 3.13).

No relative information exists in the literature regarding specific identification of HPLC fractions of WS, EtOH-soluble retentate. The distinctive peptides found in yeast-inoculated Cheddar cheese are also not identified in this study, but should be purified and identified in a subsequent study.

Investigation of the WS, 5% PTA-insoluble permeate fraction is shown in Fig. 4.2. The development of the group of peptides, eluted at 20-36 min, in standard Cheddar differs totally from that of the yeast-inoculated Cheddar (Fig. 4.2b) in terms of the amounts of peptides formed as well as with the formation of specific peptides (peaks). Although some peaks are common for both, distinct peptide peaks are shown as n (eluted at 23.9 min) and o (eluted at 30.1 min) in the yeast-inoculated Cheddar. Although these peptides absorb to a C_{18} column, they are eluted fairly early indicating a hydrophilic nature. It can, however, be possible that they may be responsible for the difference in taste between the two cheeses. Peptide p (hydrophobic) (eluted at 41.9 min, Fig. 4.2a) is present in both cheeses, and seems not to

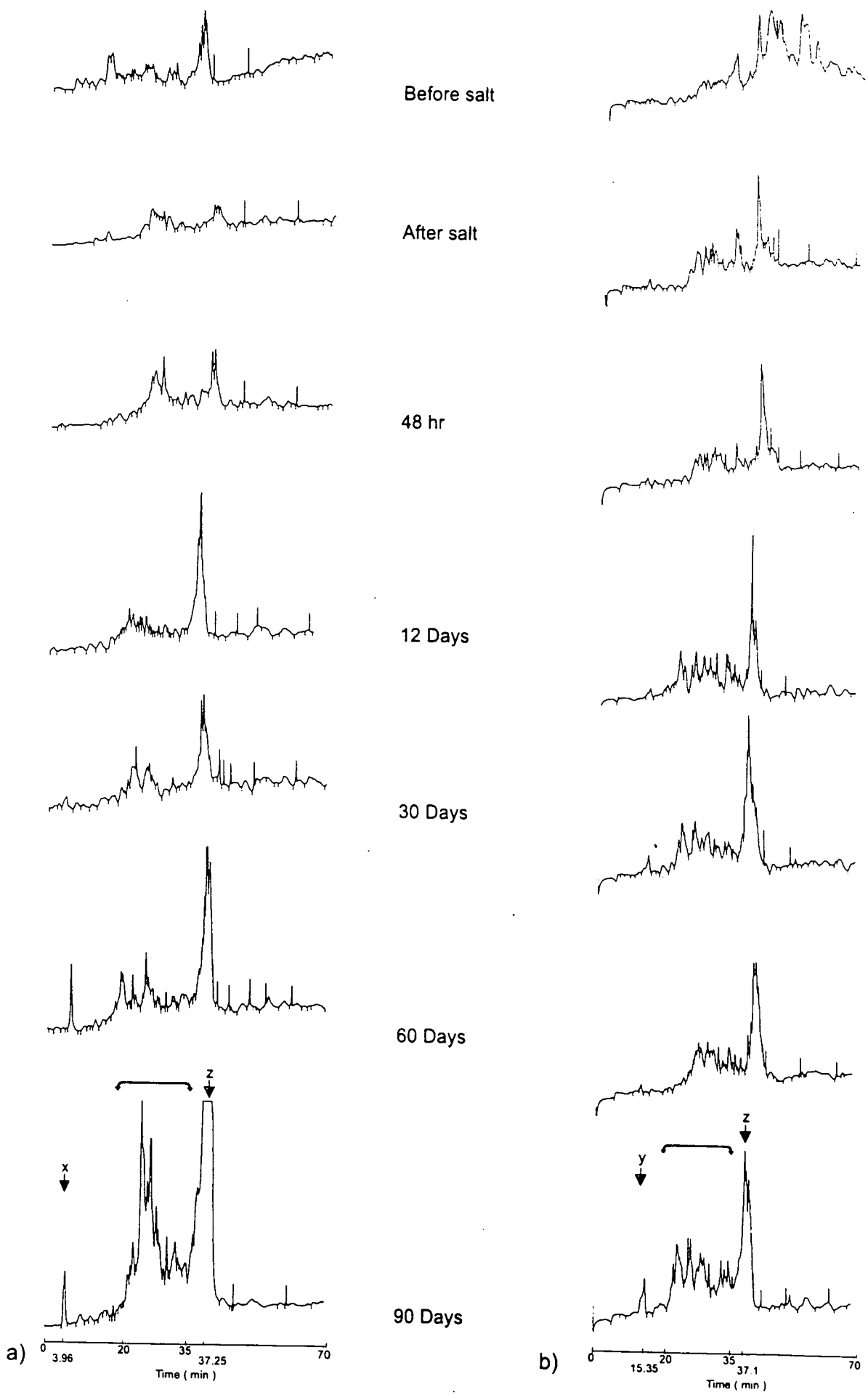

Fig 4.1 RP-HPLC of the WS, EtOH-soluble retentate fraction of a) standard Cheddar, batch B and b) yeast-inoculated Cheddar

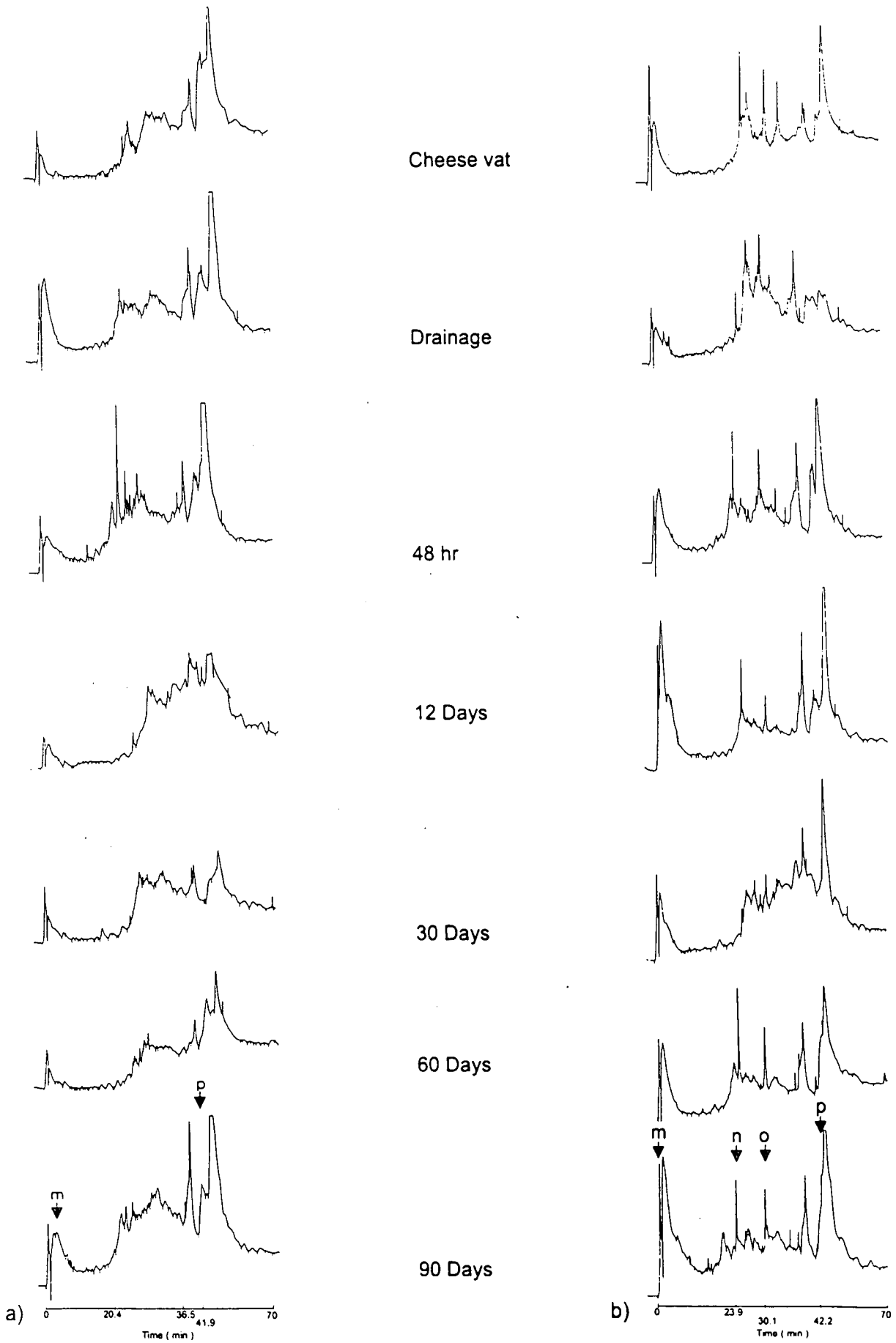

Fig 4.2 RP-HPLC of the WS, 5%PTA-insoluble permeate fraction of a) standard Cheddar, batch B and b) yeast-inoculated Cheddar

differ in development between the two cheeses. The level of peptide m (which eluted in the hydrophilic region) was much higher in the yeast-inoculated Cheddar (Fig. 4.2b).

Engels and Visser (1994) concluded that, with the exception of Edam, the ultra-filtration permeate with a MW<500 Da of Cheddar, Gouda, Gruyère, Maasdam, Parmesan and Proosdij cheeses contained the components responsible for flavour. The permeate of these cheeses contained low MW peptides (probably not larger than tetrapeptides), amino acids (e.g. γ-aminobutyric acid and ornithine) and short-chain fatty acids ($<C_9$). RP-HPLC of the permeate, (5%PTA-insoluble)- and retentate fractions indicated most of the differences between the standard Cheddar and the yeast-inoculated Cheddar (Figure 4.3) with the development of distinctly different peptides (a, b and c, Fig. 4.2b) in the 5%PTA-insoluble permeate fraction of the yeast-inoculated Cheddar, which is in agreement with the results obtained by Singh *et al.* (1994).

Marked differences in the RP-HPLC patterns of the WS, 5%PTA-soluble permeate fraction were observed between the two types of Cheddar cheese and are indicated in Fig. 4.3. The development of the group of peptides eluted from 20-40 min in the standard Cheddar (Fig. 4.3a) differs from that of the yeast-inoculated Cheddar (Fig. 4.3b). Two distinct peptide peaks (r and s) were also found in the yeast-inoculated cheese). Similar to the peptide peaks o and p in Fig. 4.2b, they are fairly hydrophilic, but may also have hydrophobic characteristics, which cause them to absorb to a C_{18} column. These peptides therefore could also be responsible for a difference in taste, perhaps bitterness, between the cheeses, as was found in Chapter 2.

Various proteinases and peptidases in cheese hydrolyze caseins to peptides and free amino acids. Small peptides and free amino acids contribute directly to the background flavour of cheese (McGugan *et al.*, 1979; Aston and Creamer, 1986; Cliffe *et al.*, 1993; Singh *et al.*, 1994). Most of the savoury, cheesy taste of the water-soluble (WS) extract of cheese is in the ultra-filtration permeate (10 kDa nominal molecular weight cut-off), which contains small peptides, free amino acids, salts and other low (MW) compounds, whereas the retentate, which contains intermediate size peptides, is bland (Singh *et al.*, 1994).

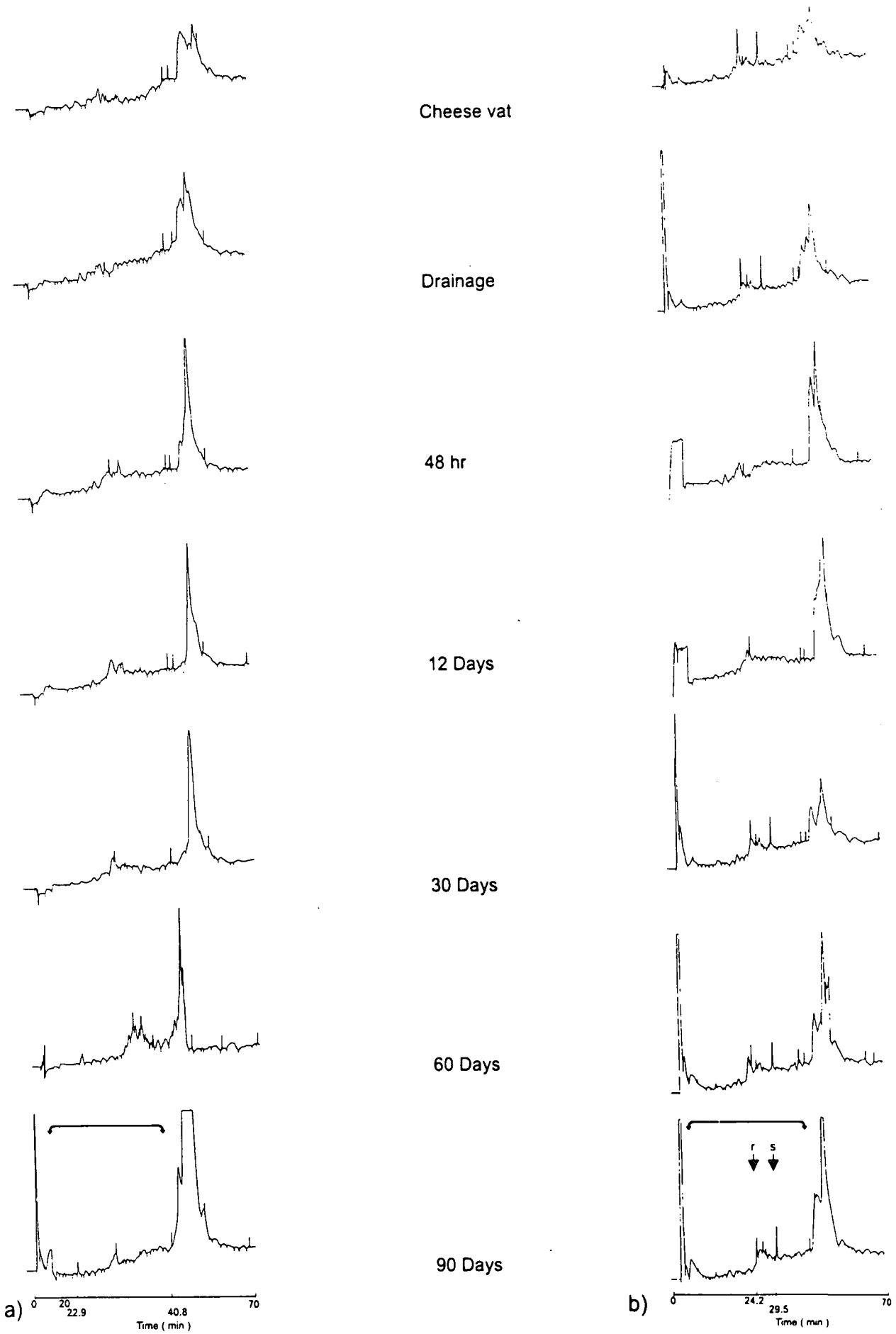

Fig 4.3 RP-HPLC of the WS, 5%PTA-soluble permeate fraction of a) standard Cheddar, batch B and b) yeast-inoculated Cheddar

4.3.2 Gel filtration chromatography of peptides and amino acids

No differences in chromatograms were obtained when absorbance was read at 230 or 280nm. This would indicate that the amount of aromatic amino acids in this fraction would be low. Chromatograms of absorbances read at 280 nm are shown in Fig. 4.4.

Two distinct peaks (a and b) and a shoulder (c), as well as a third less conspicuous peak (d) were observed in both the standard Cheddar, batch B and the yeast-inoculated Cheddar (Fig. 4.4). Variation in the development of a, b and c did occur during ripening between the cheeses. Peak d was mainly found in cheese before drainage and seemed to disappear or become less distinct thereafter. A different pattern was observed for the yeast-inoculated Cheddar (Fig. 4.4b) where peak a seems to diminish, and peak c becoming more distinct when compared to the elution profile from the standard Cheddar cheese. This may suggest a different mix of small peptides, for which evidence is provided by HPLC of these fractions in Fig. 4.3.

4.4 Conclusions

The bitter taste of the yeast-inoculated Cheddar cannot solely be attributed to the peptides observed by RP-HPLC and gel-filtration chromatography. Where differences in peptide hydrolysis in cheese ripening was monitored in the WISF by PAGE in Chapter 3, HPLC was used to do the same for WSF. Clear differences were obtained in terms of peptides formed during ripening in the standard Cheddar and the yeast-inoculated Cheddar in the fractions. Specific peptides were identified named x and y in WS, ethanol-soluble retentate, n and o in the WS, 5% PTA-insoluble permeate and r and s in the WS, 5% PTA-soluble permeate fraction. These are however, not the only peptides that could result in differences in taste between the yeast-inoculated Cheddar and the standard Cheddar, since types and amounts of peptides also differ as is evidenced by the rest of the chromatographic profiles. Gel filtration chromatography of the WS, 5% PTA-soluble fraction did not contribute much to the data already obtained from HPLC.

The data obtained here indicate that part of the difference in taste may be caused by the WSN fraction, but it is premature to ascribe it to the specific peptides identified. In future work these peptides should be purified and characterized.

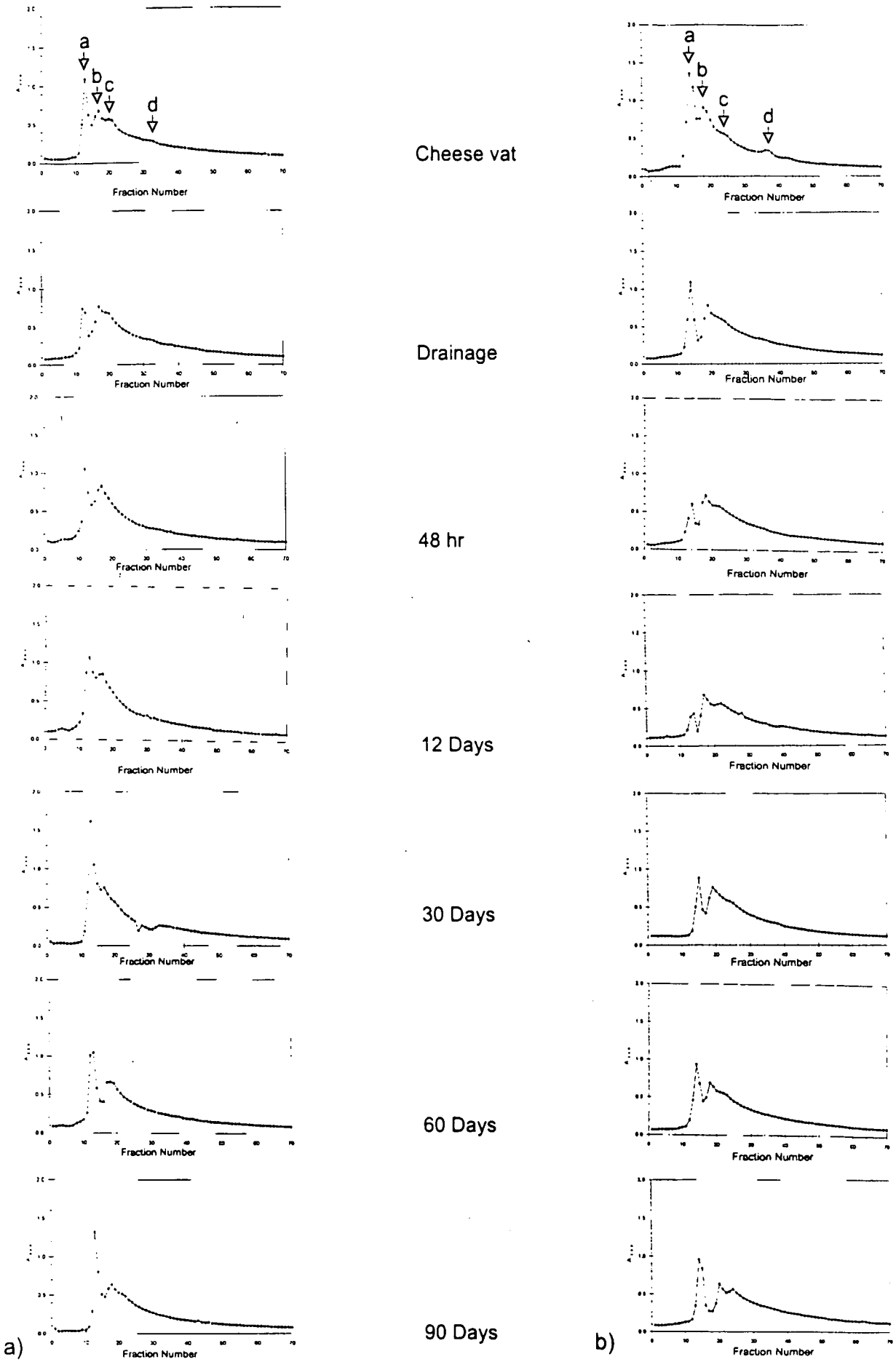

Fig 4.4 Gel-filtration chromatography of WS, 5%PTA-soluble permeate fraction of a) standard Cheddar, batch B and b) yeast-inoculated Cheddar, detected at 280 nm

Chapter 5

General discussion and conclusion

Production of Cheddar cheese enjoys a consistent growth of 4% p.a. and represents ~50-60% of the South-African cheese production. The majority of rennet-coagulated cheeses are ripened (matured) for periods ranging from 4 weeks to more than 2 years. Matured Cheddar cheese is typically ripened for 6-9 months. The duration of ripening is more or less proportional to the intensity of the flavour desired. The objective of cheese manufacture is to produce a product with the flavour, aroma and texture of the intended variety, free of any defects and in the shortest time possible.

The objective of cheese manufacture is to produce a product with the flavour, aroma and texture of the intended variety, free of any defects and in the shortest time possible. In the case of Cheddar cheese, proteolysis is the major biochemical event during ripening. Not only is proteolysis responsible for the conversion of the rubbery texture of green curd into the smooth-bodied finished cheese, but also for the characteristic flavour of the cheese.

The occurrence of yeasts in cheese is not unexpected because of the low pH, low moisture content, elevated salt concentration and low storage temperatures. Yeasts are not added as part of the starter culture under normal circumstances, but derived as contaminants from the environment. There is increasing evidence that some yeast species contribute to flavour and texture during ripening of cheeses and over-ripening may be a form of cheese spoilage by yeasts.

It is still not widely appreciated that yeasts can be an important component of the microflora of many, if not all, cheese varieties. Some manufacturers market starter cultures containing *K. marxianus* or *D. hansenii*, but it still remains to be clarified whether this might promote uniformity in the cheese aroma. Based on its inhibitory effect on the growth of spoilage microorganisms, its proteolytic activity encouraging the survival and growth of lactic acid bacteria, its great resistance against high salt concentrations, low temperatures and proliferating activities, the inclusion of *D. hansenii* to Cheddar cheese as part of the starter culture was suggested.

The aim of the study was to determine the effect of an inoculated yeast, *Debaryomyces hansenii* and its enzymes on proteolysis in Cheddar cheese by means of sensorical- (consumer and expert panel), electrophoretical-, chromatographical- and WSN analysis. Since the three batches of standard Cheddar did not differ much, as verified by the expert grading panel as well as by means of Urea-PAGE, the standard Cheddar cheeses were reduced to one batch used in subsequent analysis. In a sensorical comparative study between the standard Cheddar and the yeast-inoculated Cheddar, no significant difference was found between the standard Cheddar and the yeast-inoculated Cheddar by the consumer panel, neither was any of the two significantly preferred. This, however, did not indicate that the two cheeses are chemically the same. An expert panel judged the yeast-inoculated cheese to be bitter and since bitterness in cheese is attributed to excessive proteolysis and to the formation of small, hydrophobic peptides, a comparative chemical study was conducted.

Although the volatile fraction of cheese contributes mainly to its aroma, the water-soluble fraction (WSF) is mainly responsible for its taste. Most of the savoury, cheesy taste of the water soluble (WS) extract of cheese is in the ultra-filtration permeate which contains small peptides, free amino acids, salts and other low molecular weight (MW) compounds, whereas the retentate, which contains intermediate size peptides, is bland.

The levels of water soluble nitrogen (WSN) (determined by the macro Kjeldahl method) in the three standard batches of the same age were not significantly different compared with the much lower level of soluble nitrogen (N) observed for the yeast-inoculated Cheddar. This low level is a clear indication of the difference in proteolysis between the two different types of Cheddar cheese, since the starter organisms differ, and therefore contribute differently to proteolysis. Furthermore, the bitter defect in the yeast-inoculated Cheddar may be an indication of low proteolytic activity of the starter culture. Polyacrylamide gel electrophoresis (PAGE) was used to monitor primary proteolysis. Results obtained indicated that different batches of Cheddar cheese may ripen at different tempos, but, under the controlled manufacturing conditions, these differences are limited. These data therefore verified the use of only one batch of Cheddar cheese for sensory analysis as well as RP-HPLC and gel filtration chromatography. When comparing the WISF of the standard Cheddar with that of the yeast-inoculated Cheddar, marked differences in peptide development and –degradation were observed. The degradation of α_{s1}-CN and the formation of α_{s1}-peptides are much faster

in yeast-inoculated cheese, showing that the yeast exhibited the same specificity and activity as rennet. The yeast-inoculated Cheddar showed more peptides, especially in the WS, ethanol-insoluble (retentate ppt) fraction. The results of both fractions indicated that caseins were not fully hydrolyzed by yeast-enzymes. Thus, these results could be attributed to the roles of the different starter activities in the two different types of cheeses.

The β-CN-derived peptides developed faster in the yeast-inoculated Cheddar cheese. Just as for the α_{s1}-casein, the yeasts' proteases were not able to degrade the β-CN the same way as the normal starter cultures. They did, however, degrade β-CN. Instead of forming the β-CN and β-I in the WISN (Chapter 3), they formed other β-derived peptides (named peptides n-x) visible in the WS, ethanol-insoluble (retentate ppt) fraction.

PAGE, however, did not provide enough evidence to prove the bitter defect in the yeast-inoculated Cheddar cheese.

The reversed-phase high performance liquid chromatography (RP-HPLC) patterns of the two types of cheese not only changed, but became more complex as the cheese matured. The bitter taste of the yeast-inoculated Cheddar could not solely be attributed to the results obtained by RP-HPLC and gel-filtration chromatography. Where differences in primary proteolysis was monitored in cheese ripening in the WISF by PAGE in Chapter 3, HPLC was used to monitor secondary proteolysis in the WSF. Clear differences were obtained in terms of peptides formed during ripening in the standard Cheddar and the yeast-inoculated Cheddar in the fractions. Specific peptides were identified in the WS, ethanol-soluble retentate (peptides), the WS, 5% PTA-insoluble permeate (2 peptides) and the WS, 5% PTA-soluble permeate fraction (2 peptides). The peptides in the latter two fractions are however, not the only peptides that could result in differences in taste between the yeast-inoculated Cheddar and the standard Cheddar, since types and amounts of peptides also differ as is evidenced by the rest of the chromatographic profiles. Gel filtration chromatography of the WS, 5% PTA-soluble fraction did not contribute much to the data already obtained from HPLC.

In future research the isolation and identification of the small peptides, found to be unique in the yeast-inoculated Cheddar cheese, should be attempted in order to identify their properties

and origin, as well as the analysis of free amino acids. Data obtained may shed more light on the specific activity of the yeast-proteases, as well as flavour development.

References

Aimutus, W.R. and Eigel, W.N. 1982. Identification of λ-casein as plasmin-derived fragments of bovine αs1-casein. *J. Dairy. Sci.* 65: 175-181.

Andrews, A.T. 1983. Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* 50:45-55.

Ardö, Y and Gripon, J. 1995. Comparative study of proteolysis in some semi-hard round-eyed cheese varieties with different fat contents. *J. Dairy. Res.* 62:543-547.

Ardö, Y. and Gripon, J.C. 1991. Cromatographic methods used to measure proteolysis in cheese. *Bull of IDF* 261/1991: 29-34. Brussels, Belgium.

Association of Official Analytical Chemists. 1970. Official Methods of Analysis. 11th edition. Washington, DC.

Aston, J.W. and Creamer, L.K. 1986. Contribution of the components of the water-soluble fraction to the flavour of Cheddar cheese. *N.Z. J. Dairy Sci. Technol.* 21:229-248.

Aston, J.W. and Dulley, J.R. 1982. Cheddar cheese flavour. *Austr. J. Dairy Technol.* 37: 59-64.

Aston, J.W., Durward, I.G. and Dulley, J.R. 1983. Proteolysis and flavour development in Cheddar cheese. *Aust. J. Dairy Technol.* 38: 55-59.

Barlow, I., Lloyd, G.T. and Ramshaw, E.H. 1989. Correlations and changes in flavour and chemical parameters of Cheddar cheese during maturation. *Aust. J. Dairy Technol.* 5:7-18.

Basker, D. 1988. Critical values of differences among rank sums for multiple comparisons. *Food Technol.* 42(**2**):79.

Biede, S.L. 1974. The influence of *Lactobacillus bulgaricus* on Swiss cheese flavour. M.Sc. Thesis. Iowa State University.

Biede, S.L. and Hammond, E.G. 1979. Swiss cheese: 1. Chemical analysis. *J. Dairy Sci.* 62:227-237.

Blakesley, R.W. and Boezi, J.A. 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G-250. *Anal. Biochem.* 82:580-582.

Block, R.J. 1951. Some amino acids, peptides and amines in milk, concentrated milks and cheese. *J. Dairy Sci.* 34:1-10.

Bottazzi, V. 1983. Other fermented dairy products. In *Biotechnology, Food and feed production with micro-organisms* (Ed:G.Reed). Volume 5. Verlag Chemie, Weinheim. pp.315-363.

Breen, E.D., Fox, P.F. and McSweeney, P.L.H. 1995. Fractionation of peptides in a 10kDa ultrafiltration retentate of a water-soluble extract of Cheddar cheese. *Ital. J. Food Sci.* 3:211-220.

Christensen, T.M.I.E., Bech, A. and Werner, H. 1991. Methods for crude fractionation (extraction and precipitation) of nitrogen components in cheese. *Bull. of IDF 261/1991.* pp.4-9.

Cliffe, A.J. and Law, B.A. 1990. Peptide composition of enzyme-treated Cheddar cheese slurries, determined by reverse-phase high performance liquid chromatography. *Food Chem.* 36:73-80.

Cliffe, A.J., Marks, J.D. and Mullholland, F. 1993. Isolation and characterization of non-volatile flavours from cheese: Peptide profile of flavour fractions from Cheddar cheese determined by reverse-phase high performance liquid chromatography. *Int. Dairy J.* 3:379-387.

Cliffe, A.J., Revell, D. and Law, B.A. 1989. A method for the reverse phase high performance liquid chromatography of peptides from Cheddar cheese. *Food Chem.* 34:147-160.

Creamer, L.K. 1975. β-casein degradation in Gouda and Cheddar cheese. *J. Dairy. Sci.* 58:287-292.

Creamer, L.K. 1976. A further study of the action of rennin on β-casein. *N.Z. J. Dairy Sci. Technol.* 11:30-39.

Creamer, L.K. 1979. Degradation of casein components during cheese maturation. Proc. 5th Int. Congr. Food Sci. Technol. In H. Chiba, M. Fujimaki, K. Iwai, H. Mitsuda and Y. Morita (Eds). Elsevier Publishers, New York. Pp 340-346.

Creamer, L.K. 1991. Electrophoresis of cheese. *Bull of IDF 261/1991*:14-28. Brussels, Belgium.

Creamer, L.K. and Olson, N.F. 1982. Rheological evaluation of maturing Cheddar cheese. *J. Food Sci.* 41:631-636.

Creamer, L.K. and Richardson, B.C. 1974. Identification of the primary degradation product of α_{s1}-casein in Cheddar cheese. *N.Z. J. Dairy Sci. Technol.* 9:9-13.

Creamer, L.K., Lawrence, R.C. and Gilles, J. 1985. Effect of acidification of cheese milk on the resultant Cheddar cheese. *N.Z. J. Dairy Sci. Technol.* 20:185-203.

De Boer, E. and Kuik, D. 1987. A survey of the microbiological quality of blue-veined cheeses. *Neth. Milk Dairy J.* 41:227-237.

De Jong, L. 1976. Protein breakdown in soft cheese and its relation to consistency. 1. Proteolysis and consistency of "Noordhollandse Meshanger" cheese. *Neth. Milk Dairy J.* 30:242-253.

Deiana, P., Fatichenti, F., Farris, G.A., Mocqaut, G., Lodi, R., Todesco, R. and Cecchi, L. 1984. Metabolization of lactic and acetic acids in Perconino Romano cheese made with a combined starter of lactic acid bacteria and yeast. *Le Lait* 64:380-394.

Del Pozo, B.F., Gaya, P., Medina, M., Rodrìguez-Marin, M. A. and Nunez, M. 1988. Changes in chemical and rheological characteristics of La Serena ewe's milk cheese during ripening. *J. Dairy Res.* 55: 457-464.

Dunn, H.C. and Lindsay, R.C. 1984. Evaluation of the role of microbial Strecker-derived aroma compounds in unclean-type flavours of Cheddar cheese. *J. Dairy Sci.* 68: 2859-2874.

Emmans, D.B., McGugon, W.A., Elliot, J.A and Morse, P.M. 1962. Effect of combining single strain cultures as cheese starters on bitterness in cheese at six months of age. *J. Dairy Sci.* 45:595-600.

Engels, W.J.M. and Visser, S. 1994. Isolation and comparative characterization of compounds that contribute to the flavour of different cheese types. *Neth. Milk Dairy J.* 48:127-140.

Exterkate, F.A. and Alting, A.C. 1995. The role of starter peptidases in the initial proteolytic events leading to amino acids in Gouda cheese. *Int. Dairy J.* 5:15-28.

Farkye, N.Y. and Fox, P.F. 1990. Preliminary study on the contribution of plasmin to proteolysis in Cheddar cheese: cheese containing plasmin inhibitors, 6-amino hexanoic aid. *J. Agric. Food Chem.* 39:786-788.

Fatichenti, F., Bergere, J.L., Deiona, P. and Fams, G.A., 1983. Antagonistic activity of *Debaryomyces hansenii* towards *Clostridium tyrobutyricum* and *Clostridium butyricum*. *J. Dairy Res.* 50: 399-457.

Fernandez, M., Singh, T.K. and Fox, P.F. 1998. Isolation and identification of peptides from the diafiltration permeate of the water-soluble fraction of Cheddar cheese. *J. Agric. Food Chem.* 46:4512-4517.

Fleet, G. and Mian, M.A. 1987. The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiol.* 4:145-155.

Folkertsma, B. and Fox, P.F. 1992. Use of Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. *J. Dairy Res.* 59:217-224.

Folkertsma, B., Fox, P.F. and McSweeney, P.L.H. 1996. Accelaration of Cheddar cheese ripening at elevated temperatures. Int. Dairy J. 6:1117-1134.

Fox, P.F. 1989. Proteolysis during cheese manufacture and ripening. *J. Dairy Sci.* 72: 1379-1400.

Fox, P.F. and McSweeney, P.L.H. 1996. Proteolysis in cheese during ripening. *Food. Rev. Int.* 12(**4**): 457-509.

Fox, P.F. and McSweeney, P.L.H. 1997. Rennets: their role in milk coagulation and cheese ripening. In *Microbiology and Biochemistry of Cheese and fermented Milk.* (Ed: B.A. Law). Blackie Academic and professional (An imprint of Chapman & Hall). pp.1-49

Fox, P.F., Law, J., McSweeney, P.L.H. and Wallace, J. 1993. Biochemistry of cheese ripening. In *Cheese: Chemistry, Physics and Microbiology.* Volume 1(Ed: P.F. Fox). London, Chapman & Hall. pp. 389-437.

Fox, P.F., McSweeney, P.L.H. and Singh, T.K. 1995a. Method for assessing proteolysis in cheese during ripening. In *Chemistry of Structure/Function Relationships in Cheese*. (Eds: E.L. Malin and M.H. Tunick). Plenum Publishing Corp. New York. p.161-194.

Fox, P.F., Singh, T.K. and McSweeney, P.L.H. 1994. Proteolysis in cheese during ripening. In *Biochemistry of milk products*. (Eds: A.T. Andrews and J. Varley). Royal Society of Chemistry, Cambridge. pp. 1-31.

Fox, P.F., Singh, T.K. and McSweeney, P.L.H. 1995b. Biogenesis of flavour compounds in cheese. In *Chemistry of Structure/Function Relationships in Cheese*. (Eds: E.L. Malin and M.H. Tunick). Plenum Publishing Corp. New York. p.59-89.

Grappin, R., Ronk, T.C. and Olson, N.F. 1985. Primary Proteolysis of Cheese Proteins During Ripening. A Review. *J. Dairy Sci.* 68: 531-540.

Green, M.L. 1977. Reviews on the progress of dairy science: Milk coagulants. *J. Dairy Res.* 44:159-188.

Green, M.L. and Foster, P.D.M. 1974. Comparison of the rates of proteolysis during ripening of Cheddar-cheeses made with calf rennet and swine pepsin as coagulants. *J. Dairy Res.* 41:269-282.

Green, M.L., Angal, S., Lowe, P.A. and Morston, F.A.O. 1985. Cheddar cheese making with recombinant calf chymosin synthesized in *Escherichia coli*. *J. Dairy Res.* 52:281-286.

Gripon, J.C., Desmazeaud, M.J., Le Bars, D. and Bergere G.L. 1977. Role of proteolytic enzymes of *Streptococcus lactis*, *Penicillium roqueforti* and *Penicillium casiecolu* during cheese ripening. *J. Dairy Sci.* 60:1532-1538.

Grufferty, M.B. and Fox, P.F. 1988. Milk alkaline proteinase: a review. *J. Dairy Res.* 55:609-630.

Harper, W.J., Carmona, A. and Kristoffersen, T. 1971. Protein degradation in Cheddar cheese slurries. *J. Food Sci.* 36:503-506.

Harwalkar, V.R. and Elliot, J.C. 1971. Isolation of bitter and astringent fraction from Cheddar cheese. *J. Dairy Sci.* 54:8-11.

Huber, L. and Klostermeyer, H. 1974. Isolierung und identifiziering eines Bitterstoffes aus Butterkäse. *Milchwissenschaff* 29:449-455.

Ingram, M. 1958. Yeasts in food spoilage. In *The Chemistry and Biology of Yeasts* (Ed. A. H. Cook) pp. 603-633. New York, Academic Press.

Jarrett, W.D., Aston, J.W. and Dulley, J.R. 1982. A simple method for estimating free amino acids in Cheddar cheese. *Aust. J. Dairy Technol.* 37:55-58.

Jooste, P.J. and Britz, T.J. 1986. The significance of flavobacteria as proteolytic psychrotrophs in milk. *Milchwissenschaft* 41: 618-621.

Kaminogawa, S. and Yamauchi, K. 1972. Acid protease of bovine milk. *Agric. Biol. Chem.* 36:2351-2356.

Kaminogawa, S., Yan, T.R., Azuma, N. and Yamauchi, K. 1986. Identification of low molecular weight peptides in Gouda-type cheese and evidence for the formation of these peptides from 23 N-terminal residues of α_{s1}-casein by proteinases of *Streptococcus cremoris* H61. *J. Food Sci.* 51:1253-1256.

Kowaleska, J., Zelakowska, H., Babochowski, A., Hammond, E.G. and Ross, F. 1985. Isolation and aroma-bearing material from *Lactobacillus helveticus* culture and cheese. *J. Dairy Sci.* 68: 2165-2171.

Kristiansen, K.R., Otte, J., Sørensen, N.K. and Qrist, K.B. 1996. Fractionation and characterization of water soluble peptides from cheese. Poster no.10. IDF Symposium on ripening and quality of cheeses. Unpublished data. Besancon, France. 26-28 Febr.

Kristofferson, T. 1985. Development of flavour in cheese. *Milchwissenschaft* 40: 197-199.

Kuchroo, C.N. and Fox P.F. 1982a. Fractionation of the water-soluble nitrogen from Cheddar cheese: Chemical methods. *Milchwissenschaft* 37: 651-653.

Kuchroo, C.N. and Fox, P.F. 1982b. Soluble nitrogen in Cheddar cheese:comparison of extraction procedures. *Milchwissenschaft* 37: 331-335.

Kuchroo, C.N. and Fox, P.F. 1983a. A fractionation scheme for the water-soluble nitrogen in Cheddar cheese. *Milchwissenschaft* 38:389-391.

Kuchroo, C.N. and Fox, P.F. 1983b. Fractionation of the water-soluble nitrogen from Cheddar cheese: Chromatographic methods. *Milchwissenschaft* 38: 76-79.

Lane, C.N. and Fox, P.F. 1996. Contribution of starter and added lactobacilli to proteolysis in Cheddar cheese during ripening. *Int. Dairy J* 6(**7**):715-728.

Langler, J.E., Libbey, L.M. and Day, S. 1967. Identification and evaluation of selected compounds in Swiss cheese flavour. *J. Agr. Food Chem.* 15:386-391.

Laubscher, P.J. 2000. Personal communication.

Laubscher, P.J. and Viljoen, B.C. 1999. The resistance of dairy yeast against commercial compounds and sanitizers. *Food Technol. Biol.* 37:281-286.

Law, B.A. 1979. Review of the progress of dairy science;enzymes of psycrotrophic bacteria and their effects on milk and milk products. *J. Dairy Res.* 46:573-588.

Law, B.A. 1987. Proteolysis in relation to normal and accelerated cheese ripening. In *Cheese Chemistry, Physics and Microbiology.* (Ed: P.F.Fox). Volume 1. Chapman & Hall, London. pp.365-392.

Law, B.A. and Wigmore, A.S. 1983. Accelerated ripening of Cheddar cheese with commercial proteinase and intracellular enzymes from starter *Streptococci. J. Dairy. Res.* 50:519-525.

Law, B.A., Andrews, A.T., Cliffe, A.J., Sharpe, M.E. and Chapman, H.R. 1979. Effect of proteolytic raw milk psychrotrophs on Cheddar cheese making with stored milk. *J. Dairy Res.* 46:497.

Law, B.A., Castanon, M.J. and Sharpe, M.E. 1976. The contribution of starter *Streptococci* to flavour development in Cheddar cheese. *J. Dairy Res.* 43:301-311.

Law, J., Fitzgerald, G.F., Daly, C., Fox, P.F. and Farkye, N.Y. 1992. Proteolysis and flavour development in Cheddar cheese made with the single starter strains *Lactococcus lactis* ssp. *Lactis UC317* or *Lactococcus lactis* ssp. *cremoris* HP. J. Dairy Sci. 75:1173-1185.

Law, J., Fitzgerald, G.F., Uniacke-Lowe, T., Daly, C. and Fox, P.F. 1993. The contribution of *Lactococcal* starter proteinases to proteolysis in Cheddar cheese. *J. Dairy Sci.* 76:2455-2467.

Lawrence, R.C., Creamer, L.K. and Gilles, J. 1987. Texture development during cheese ripening. *J. Dairy Sci.* 70:1748-1760.

Lawrence, R.C., Gilles, J. and Creamer, L.K. 1993. Cheddar cheese and related dry-salted varieties. In *Cheese: Chemistry, Physics and Microbiology.* Second edition. Volume 2. (Ed: P.F. Fox). Chapman & Hall, London. pp.1-39.

Le Bars, D. and Gripon, J.C. 1981. Role of *Penicillin roqueforti* proteinases during blue cheese ripening. *J. Dairy Res.* 48: 479-487.

Le Bars, D. and Gripon, J.C. 1989. Specificity of plasmin towards bovine α_{s2}-casein. *J. Dairy Res.* 56:817-821.

Lenoir, J. 1984. The surface flora and its role in the ripening of cheese. *Bull of IDF* 171: 3-20.

Mabbitt, L.A. 1955. Quantitative estimation of the amino acids in Cheddar cheese and their importance in flavour. *J. Dairy Res.* 22:224.

Marcos, A., Esteban, M.A., Leon, F. and Fernandez-Salguero, J. 1979. Electrophoretic patterns of European cheeses: Comparison and quantitation. *J. Dairy Sci.* 62:892-900.

Marth, E. H. 1978. Dairy products. In *Food and Beverage Mycology* (Ed. L.R. Beuchat) pp. 145-172. Westport, Connecticut, AVI Publishing.

McGugan. W.A., Emmons, D.B. and Larmand, E. 1979. Influence of volatile and nonvolatile fractions on intensity of Cheddar flavour. *J. Dairy Sci.* 62:398-403.

McSweeney, P.L.H. and Fox. P.F. 1993. Cheese:Methods of Chemical Analysis. In *Cheese Chemistry, Physics and Microbiology.* (Ed: P.F.Fox). Volume 1. Chapman & Hall, London. pp 341-387.

McSweeney, P.L.H., Fox, P.F. and Olson, N.F. 1995. Proteolysis of bovine caseins by cathepsin D: Preliminary observations and comparison with chymosin. *Int. Dairy J.* 5: 321-336.

McSweeney, P.L.H., Fox, P.F., Lucey, J.A., Jordon, K.N. and Cogan, T.M. 1993c. Contribution of the indigenous microflora to the maturation of Cheddar cheese. *Int. Dairy J.* 3:613-634.

McSweeney, P.L.H., Olson, N.F., Fox, P.F. and Højrup, P. 1993a. Proteolytic specificity of chymosin on bovine α_{s1}-casein. *J. Dairy Res.* 60:401-412.

McSweeney, P.L.H., Olson, N.F., Fox, P.F., Healy, A. and Højrup, P. 1993b. Proteolytic specificity of plasmin on bovine α_{s1}-casein. *Food Biotechnol.* 7: 143-158.

McSweeney, P.L.H., Pochet, S., Fox, P.F. and Healy A. 1994. Partial identification of peptides from the water-insoluble fraction of Cheddar cheese. *J. Dairy Res.* 61:587-590.

Mulvihill, D.M. and Fox, P.F. 1977. Proteolysis of α_{s1}-casein by chymosin: Influence of pH and urea. *J.Dairy Res.* 44:533-540.

Mulvihill, D.M. and Fox, P.F. 1980. Proteolysis of α_{s1}-casein by chymosin in dilute NaCl solutions and in Cheddar cheese. *Ir. J.Food Sci. Technol.* 4:13-23.

Nooitgedagt, A.J. and Hartog, B.J. 1988. A survey of the microbiological quality of brie and camembert cheese. *Neth. Milk Dairy J.* 42:57-72.

Nuñez, M., Medina, M., Gaye, P., Guillen, A.M. and Rodriquez-Marin, M.A. 1992. Effect of recombinant chymosin on ewes milk coagulation and Manchego cheese characteristics. *J. Dairy Res.* 59: 81-87.

O'Keeffe, A.M., Fox, P.F. and Daly, C. 1977. Denaturation of porcine pepsin during Cheddar cheese manufacture. *J. Dairy Res.* 44:335-343.

O'Keeffe, A.M., Fox, P.F. and Daly, C. 1978. Proteolysis in Cheddar cheese: role of coagulant and starter bacteria. *J. Dairy Res.* 45: 465-477.

O'Keeffe, K.B., Fox, P.F. and Daly, C. 1975. Proteolysis in Cheddar cheese: influence of the rate of acid production during manufacture. *J. Dairy Res.* 42:111-122.

O'Keeffe, K.B., Fox, P.F. and Daly, C. 1976. Contribution of rennet and starter proteases to proteolysis in Cheddar cheese. *J. Dairy Res.* 43:97-107.

O'Shea, B.A., Uniacke-Lowe, T. and Fox, P.F. 1996. Objective assessment of Cheddar cheese quality. *Int. Dairy J.* 6: 1135-1147.

O'Sullivan, M. and Fox, P.F. 1990. A scheme for the partial fractionation of cheese peptides. *J. Dairy Res.* 57:135-139.

O'Sullivan, M. and Fox, P.F. 1991. Evaluation of microbial chymosin from genetically engineered *Kluyveromyces lactis*. *Food Biotechnol.* 5:19-32.

Phelan, J.A., Guiney, J. and Fox, P.F. 1973. Proteolysis of β-casein in Cheddar cheese. *J. Dairy Res.* 40:105.

Rank, T.C., Grappin, R. and Olson, N.F. 1985. Secondary proteolysis of cheese during ripening. *J. Dairy Sci.* 68: 801-805.

Reville, W.J. and Fox, P.F. 1978. Soluble protein in Cheddar cheese: A comparison of analytical methods. *Ir. J. of Food Sci. Technol.* 2: 67-76.

Richardson, B.C. and Creamer, L.K. 1973. Casein proteolysis and bitter peptides in Cheddar cheese. *N.Z. J. Dairy Sci. Technol.* 8:46-51.

Richardson, B.C. and Pearce, K.N. 1981. The determination of plasmin in dairy products. *N.Z. J. Dairy Sci. Technol.* 16:209-220.

Roland, J.F., Mattis, D.L., Kiang, S and Alan, W.L. 1978. Hydrophobic chromatography: debittering protein hydrolysates. J. Food Sci. 43:1491-1493.

Roostita, R. and Fleet, G.H. 1996. The occurrence and growth of yeasts in Camembert and blue-veined cheeses. *Int. J. Food Microbiol.* 28: 393-404.

Roudot-Algaron, F., Le Bars, D., Kerhoas, L., Einhorn, J. and Gripon, J.C. 1994. Phosphopeptides from Comté cheese. Nature and origin. *J. Food Sci.* 59(**3**):544-547.

Schaar, J. 1985. Plasmin activity and proteose-peptone content of individual milks. *J. Dairy Res.* 52(**3**): 369-378.

Seiler, H and Busse, M. 1990. The yeasts of cheese brines. *Int. J. Food Microbiol.* 11:289-304.

Shalabi, S.I. and Fox, P.F. 1987. Electrophoretic analysis of cheese: comparison of methods. *Ir. J. Food Sci. Technol.* 11:135-151.

Singh, H. and Creamer, L.K. 1990. A sensitive quantitative assay for milk coagulants in cheese and whey products. *J. Dairy Sci.* 73:1158-1165.

Singh, T.K., Fox, P.F. and Healy, A. 1995. Water soluble peptides in Cheddar cheese: Isolation and identification of peptides in the UF-retentate of water-soluble fractions. *J. Dairy Res.* 62:629-640.

Singh, T.K., Fox, P.F. Højrup, P. and Healy, A. 1994. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. *Int. Dairy J.* 4:111-122.

Suzuki, J. and Katoh, N. 1990. Cysteine protease in bovine milk capable of hydrolyzing casein as the substrate and elevation of the activity during the course of mastitis. *Japan. J. Vet. Sci.* 52:947-954.

Swaisgood, H.E. 1992. Chemistry of the caseins. In *Advanced Dairy Chemistry - 1: Proteins* (Ed: P.F. Fox). Elsevier Applied Science, London. pp.63-110.

Swaisgood, H.E. and Bruner, J.R. 1961. The isolation of λ casein from concentrated TCA-urea solutions. *J. Dairy Sci.* 44:163.

Szumski, S.A. and Cone, J.F. 1962. Possible role of yeast endoproteinases in ripening of surface-ripened cheeses. *J. Dairy Sci.* 45: 349-353.

Thomas, T.D. and Pearce, K.N. 1981. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. *N.Z. J. Dairy Sci. Technol.* 16:253-289.

Thompson, M.P., Kiddy, C.A., Johnston, J.O. and Weinberg, R.M. 1964. Genetic polymorphism in caseins of cows' milk. II. Conformation of the genetic control of β-casein variation. *J. Dairy Sci.* 47:378-381.

Venter, H., Osthoff, G. and Litthauer, D. 1999. Purification and characterization of a metalloprotease from *Chryseobacterium indologenes* Ix9a and determination of the amino acid specificity with electrospray mass spectrometry. *Prot. Expression and purification* 15: 282-295.

Verdi, R.J. and Barbano, D.M. 1991. Effect of coagualnts, somatic cell enzymes and extra-cellular bacterial enzymes on plasminogen activation. *J. Dairy Sci.* 74:772-782.

Viljoen, B.C. and Greyling, T. 1995. Yeasts associated with Cheddar and Gouda making. Int. J. Food Microbiol. 28:79-88.

Viljoen. B.C. 1999. Interaction between yeasts and bacteria in "restricted" dairy environments. Presented at Ann. Mtg., SASDT, South Africa, March 2-4.

Visser, F.M.W. 1977. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino acid fractions. *Neth. Milk. Dairy J.* 31:210.

Visser, F.M.W. and de Groot-Mostert, A.E.A. 1977. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 4. Protein breakdown: A gel electrophoretic study. *Neth. Milk Dairy J.* 31:247-264.

Visser, S. 1993. Proteolytic enzymes and their relation to cheese ripening and flavour: An overview. *J. Dairy. Sci.* 76:329-350.

Visser, S. and Slangen, K.J. 1977. On the specificity of chymosin (rennin) in its action on bovine β-casein. *Neth. Milk Dairy J.* 31(**1**):16-30.

Visser, S., Exterkate, F.A., Slangen, C.J. and de Veer, G.J.C.M. 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{s1}-, β- and κ-casein. *Appl. Environ. Microbiol.* 52:1162-1166.

Visser, S., Slangen, C.J. and Van Rooijen, P.J. 1987. Peptide substrated for chymosin (rennin). Interaction sites in κ-casein-related sequences located outside the (103-108)-hexapeptide region that fits into the enzyme active-site cleft. *Biochem. J.* 224(**3**):553-558.

Visser, S., Slangen, K.J., Hup, G. and Stadhouders, J. 1983. Bitter flavour in cheese. 3. Comparative gel-chromatographic analysis of hydrophobic peptide fractions from twelve Gouda-type cheeses and identification of bitter peptides isolated from a cheese made with *Streptococcus cremoris* strain HP. *Neth. Milk Dairy J.* 37:181-191.

Walker, H.W. and Ayres, J.C. 1970. Yeasts as spoilage organisms. In *The yeasts.* Vol. III (Eds: A.H. Rose and J.S. Harrison) pp. 463-527. Academic Press, London.

Weir, G.S.D. 1992. Proteins as a source of flavour. In *Biochemistry of Food Proteins.* (Ed: I.Hudson). Elsevier Science Publishers, England. pp.363-377.

Welthagen, J.J. and Viljoen, B.C. 1998. Yeast profile in Gouda cheese during processing and ripening. *Int. J. Food Microbiol.* 41:185-194.

Welthagen, J.J. and Viljoen, B.C. 1999. The isolation of yeasts obtained during the manufacture and ripening of Cheddar cheese. *Food Microbiol.* 16:63-73.

Wilkinson, M.G. 1993. Accelaration of cheese ripening. In *Cheese Chemistry, Physics and Microbiology.* (Ed: P.F.Fox). Volume 1. Chapman & Hall, London. pp.523-555.

Yamauchi, K., Kong, K.H. and Kaminogowa, S. 1976. Proteolysis by *Deboromyces Hansenii* and lactic starters in skim milk culture. *Japanese Journal of Zootechnology Science* 47:12-17.

Summary

Proteolysis is regarded as the most important event in the ripening of Cheddar cheese to contribute to the development of flavour. The characteristic proteolysis of each type of cheese is brought about by the enzymes used in the manufacturing process, e.g. rennet, as well as enzymes from the specific microbial cultures used in each cheese type. The effect of an inoculated yeast, *Debaryomyces hansenii*, and its enzymes on proteolysis in Cheddar cheese was investigated. Proteolysis and development of peptides of the yeast-inoculated Cheddar cheese was followed throughout the ripening process and compared to the proteolysis in a standard Cheddar cheese. In a sensorical comparative study, no difference was found by a consumer panel between the two types of cheese, nor was any of the two significantly preferred. An expert panel however, judged the yeast-inoculated Cheddar cheese to be bitter. The proteins and peptides from the cheeses were extracted and fractionated by virtue of differences in solubility and molecular size. Yeast-inoculated Cheddar cheese contained less water-soluble nitrogen indicating a difference in proteolysis between the two types of cheese. Urea-polyacrylamide gel electrophoresis of the water-insoluble fraction indicated that in the yeast-inoculated cheese rennet hydrolysis of α_{s1}-casein was increased with faster formation of one primary peptide, α_{s1}-I as well as (α_{s1}-CN(f102-•)), with little further hydrolysis. The β-casein was hydrolyzed slowly but with several additional peptides occurring. Reversed-phase high performance liquid chromatography of the water-soluble fraction indicated that a different peptide profile was formed, with at least five unique peptides at high amounts.

Keywords: Proteolysis, yeast-inoculated, *Debaryomyces hansenii*, sensorical, HPLC, gel-filtration chromatography, Urea-PAGE, chemical, flavour

Opsomming

Proteolise word gereken as die belangrikste gebeurtenis in die rypwording van Cheddarkaas om by te dra tot die ontwikkeling van geur. Die karakteristieke proteolise in elke tipe kaas word veroorsaak deur ensieme betrokke in die vervaardigingsproses, nl. rennien, asook die ensieme afkomstig vanaf die spesifieke mikrobiese kultuur gebruik in elke kaas variëteit. Die effek van 'n geïnokuleerde gisspesie, *Debaryomyces hansenii*, en sy ensieme, op proteolise in Cheddarkaas is bepaal. Proteolise en die ontwikkeling van peptiede in die gis-geïnokuleerde Cheddarkaas is gevolg tydens die rypwordingsproses en vergelyk met die proteolise in 'n standaard Cheddarkaas. Tydens 'n sensoriese, vergelykende studie deur 'n verbruikerspaneel is geen verskil gevind tussen die twee tipes kaas nie en is geen kaas bo die ander verkies nie. 'n Opgeleide paneel het egter die gis-geïnokuleerde kaas as bitter beoordeel. Die proteïene en peptiede is geekstraheer en gefraksioneer vanuit die kaas op grond van verskille in oplosbaarheid en molekulêre grootte. Gis-geïnokuleerde Cheddarkaas het 'n laer inhoud water-oplosbare stikstof bevat en dien as 'n indikasie van die verskil in proteolise tussen die twee tipes kaas. Ureum-poliakrielamied gel elektroforese gedoen op die water-onoplosbare fraksie het gewys dat rennien-hidrolise van α_{s1}-kaseïn versterk is in die gis-geïnokuleerde kaas deur die vinnige vorming van die primêre peptiede α_{s1}-I asook α_{s1}-CN(f102-$\circ$), met 'n geringe verdere hidrolise. Die β-kaseïne is stadig gehidroliseer, maar met verskeie addisionele peptiede gevorm. Tru-fase hoë druk vloeistof chromatografie van die water-oplosbare fraksie het 'n ander peptiedprofiel getoon, met ten minste vyf unieke peptiede teenwoordig in groot hoeveelhede.