Variation in Milk Protein Composition and its Importance for the Quality of Cheese Milk

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Abstract

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Variations within the bovine milk protein profile can have both positive and negative impacts on the quality of cheese milk. As main contributors to the cheese structure, the caseins are important for the cheese yield. During the last decades, the proportion of casein in relation to the total protein amount (the casein number) has decreased in Swedish bulk milk. This decline is unexplained and the reason for this deteriorating trend is unknown. Therefore, more knowledge in how the protein composition varies is needed. The main objective of this thesis was to increase the understanding of how the cheese yield is affected by variation in the detailed milk protein profile.

Milk was sampled from a total of 134 individual cows of Swedish red and white (SRB), Swedish Holstein (SLB) and Danish Holstein (SDM). The milk was analysed for major milk components, urea, calcium, somatic cell count, pH, level of protein degradation, the detailed milk protein composition and low-molecular-weight milk peptides. Low-fat model cheeses were made from four litres of pasteurised skimmed milk and cheese yield was recorded. Variations in the milk protein profile between breeds or herds, before and after pasteurisation and the effect of protein composition on cheese yield were studied. Finally, low-molecular-weight peptides identified in the acid whey fraction from milk samples with different levels of somatic cell count were related to earlier publications on identified cleavage specificities of milk proteases.

Concentrations of total protein, total casein, β-casein and κ-casein were higher in SRB milk compared to SLB milk. As expected, pasteurisation did not influence the milk protein markedly and only small changes in concentration of α_{S1}-casein and proteose peptone component 3 were seen. The milk proteins α_{S1}-, β-, and κ-casein and β-lactoglobulin B had significantly positive effects on the cheese yield whereas the casein number was significant for the transfer of protein from milk to cheese. Moreover, several forms or proteolytic products of α_{S1}-, α_{S2}-, and β-casein positively affected this trait. About 30% of the milk samples were poorly coagulating or non-coagulating with a significantly lower concentration of κ-casein. Finally, peptide cleavage sites corresponding to earlier identified specificities of elastase and cathepsing B were identified in milk with high somatic cell count.

Keywords: Milk protein composition, casein, whey protein, dairy cow, pasteurisation, cheese yield, milk coagulation properties, protein fragment, peptide, proteolysis, plasmin, cathepsin, elastase.

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Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Wedholm, A., Hallén, E., Larsen, L.B., Lindmark-Månsson, H., Karlsson, A.H. & Allmere, T. 2006. Comparison of milk protein composition in a Swedish and a Danish dairy herd using reversed phase HPLC. *Acta Agricultuarae Scandinavia Section A, 56:8-15*

II. Larsen, L.B., Wedholm, A., Møller, H.S., Lindmark-Månsson H. & Andrén, A. 2008. Proteome analysis of chymosin separated casein and whey from raw and pasteurised milk. *Manuscript to be submitted*

III. Wedholm, A., Larsen, L.B., Lindmark-Månsson, H., Karlsson, A.H. & Andrén, A. 2006. Effect of protein composition on the cheese making properties of milk from individual dairy cows. *Journal of Dairy Science, 89:3296-3305*

IV. Wedholm, A., Møller, H.S., Stensballe, A., Lindmark-Månsson, H., Karlsson, A.H., Andersson, R., Andrén, A. & Larsen, L.B. 2007. Effect of milk protein composition on cheese yield and quality as determined by proteomics and multivariate data analysis. *Submitted to Journal of Dairy Science*

V. Wedholm, A., Møller, H.S., Lindmark-Månsson, H., Rasmussen, M.D., Andrén, A. & Larsen, L.B. 2008. Identification of peptides in milk as a result of proteolyses at different levels of somatic cell counts using LC MALDI MS/MS detection. *Journal of Dairy Research, 75 (1):76-83*

Anna Wedholm´s contribution to the papers

I. Planned and performed the sampling of milk in Sweden, pasteurisation of milk samples, analysis of milk protein composition on RP-HPLC, statistical analysis and evaluation of results, writing the report.

II. Pasteurisation of milk samples, multivariate data analysis, statistical analysis and evaluation of the results, writing parts of the report.

III. Planned and performed the sampling of milk in Sweden, pasteurisation of milk, cheese making and rheological measurements, analysis of milk protein composition on HPLC, multivariate data analysis, evaluation of results and writing the report.

IV. Planned and performed the cheese making, analysis of protein composition by 2-DGE in some of the milk samples, multivariate data analysis, evaluation of results and writing the report.

V. Sample preparation and separation of peptides by capillary RP-HPLC, identification of peptides by MALDI-TOF MS/MS, evaluation of results and writing the report.

Introduction

Background

The world cheese production has increased by millions of tons since the beginning of this decade and in EU an increasing fraction of all the milk produced is used for cheese making (USDA, 2007). In Sweden, approximately one-third of the milk produced is used for this purpose (SCB, 2007) which implies that cheese is one of the most important products within the dairy industry. Recently, milk prices on the world market have increased drastically due to a limited milk supply. More than ever, it would thus be an economic advantage if the cheese yield could be increased. The most important of all milk components for the manufacture of cheese and other fermented dairy products are the milk proteins. In most countries, the milk payment systems are based on concentrations of total protein and milk fat. However, the milk protein composition is not considered in the milk grading even though it is well known that the proportion of casein in relation to total protein (casein number) is important for the cheese yield. Unfortunately, the casein number in Swedish bulk milk has decreased during the last decades (Lindmark-Månsson, Fonden & Pettersson, 2003). This decline might be because breeding programmes earlier were focused on high milk yield instead of high casein concentration.

The two dominating dairy breeds in Sweden are the Swedish Red and White (SRB) and the Swedish Holstein breed (SLB) (Swedish Dairy Association, 2006). Milk from SRB cows is expected to contain higher concentration of total protein and milk fat compared to SLB (Bergsten *et al.*, 1997), but little is known about the differences in milk protein composition. However, the differences in milk protein composition between breeds are most probably related to frequencies of different genetic variants of milk proteins (Ng-Kwai-Hang & Grosclaude, 2003).

The milk protein composition also varies due to several "non-genetic" factors such as stage of lactation (Auldist, Walsh & Thomson, 1998), lactation number (Ng-Kwai-Hang, Hayes & Moxley, 1987) and udder health of cow (Ng-Kwai-Hang *et al.*, 1984b). The udder health status of dairy cows is determined by measuring the number of somatic cells (white blood cells) in milk. In Sweden, the milk farmers are paid less for milk exceeding 200,000 cells/mL. This is not only because of cow health concerns, but also because high somatic cell counts (SCC) are known to be associated with deteriorated milk quality, e.g. increased proteolytic degradation of caseins (Verdi *et al.*, 1987). The milk protein degradation is a continuous process but it can be reduced by pasteurisation (72º C for 15 s), because the protelytic enzymes are partly inactivated.

More knowledge is needed about milk composition, especially the milk proteins, and how different components interact so that the use of milk for cheese manufacturing can be optimised. The main objective of this doctoral thesis was to increase the understanding in how the detailed protein composition varies between individual cows and to evaluate its effect on the cheese yield.

Milk proteins

Milk is a complex fluid consisting of an enormous number of components. The major constituents of milk are water, lactose, fat, protein, organic acids and minerals in the approximate concentrations as presented in Figure 1 (Walstra, Wouters & Geurts, 2006). Several other minor constituents are often present only in trace amounts, e.g. vitamins and enzymes. Bovine milk contains about 3.3% milk proteins, excluding non-protein nitrogen, e.g. urea, ammonia and free amino acids (Walstra, Wouters & Geurts, 2006). About 80% of the total protein amount consists of caseins, which are defined chemically as the milk proteins that precipitate at pH 4.6. The remaining 20% are soluble at this pH and referred to as whey proteins or milk serum proteins.

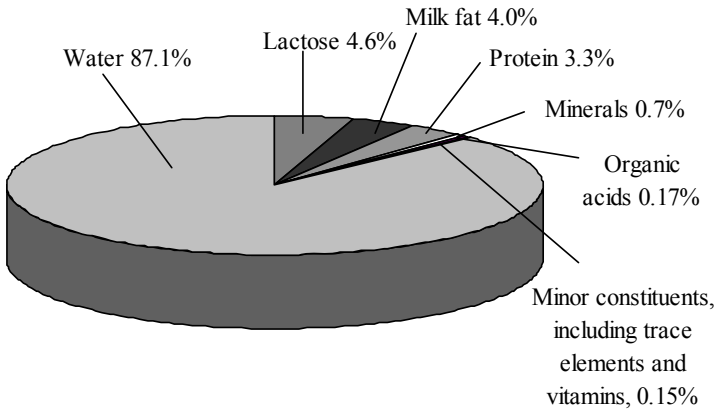


Fig 1. Approximate composition of milk (Walstra, Wouters & Geurts, 2006)

Caseins

Both caseins and whey proteins show a high degree of heterogeneity, which means that they consist of several proteins that vary considerably in composition and functions. Heterogeneity of the casein group was first demonstrated in the 1920's by Linderström-Lang & Kodama (1925). Later on, in 1939, Mellander confirmed this finding when he separated three casein components by electrophoresis.

The separated fragments were determined and named α, β and γ-casein (α-CN, β-CN and γ-CN). The fraction called α-CN was later shown to contain two proteins, one sensitive and one insensitive to precipitation by Ca^{2+}, named α_S-CN and κ-CN respectively (Waugh & Von Hippel, 1956). Today it is known that α-CN contains two proteins called α_{S1}- and α_{S2}-CN and that γ-CN is a proteolytic product after

cleavage of β-CN by plasmin. Hence, the bovine casein group contains four individual gene products known as α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN in approximate proportions of 4:1:4:1 (w/w). Each of the four main casein components show further variability due to different degree of phosphorylation, glycosylation, disulphide bonding, proteolysis and genetic polymorphism (Ng-Kwai-Hang, 2002). Phosphorylation occurs in all casein molecules, but with highest degree in α_{S2}-, α_{S1}- and β-CN. The degree of phosphorylation in κ-CN is very low, instead this molecule can be glycosylated on up to 6 amino acid sites (Pisano *et al.*, 1994). Disulphide bonds can only occur between cystein or cystin residues, which do not exist in α_{S1}-CN or β-CN. However, α_{S2}-CN contains two cystein residues forming one intermolecular disulfide bridge whereas κ-CN contains two free cystein residues (Walstra, Wouters & Geurts, 2006).

Caseins have a relatively high prolin content and therefore very little secondary or tertiary structure that can be denaturated (Fox & McSweeney, 1998). However, at higher temperatures (>140º C) the caseins will heat coagulate (Singh, 1995) either through colloidal aggregation or chemical cross-linking. The colloidal aggregation, which involves calcium bridges, is not only dependent on temperature but also on calcium concentration and pH (Walstra, Wouters & Geurts, 2006). Less is known about the reactions and the actual cross-links involved in chemical cross-linking.

The low content of α-helical structure makes the caseins open and flexible. This facilitates the accessibility for the proteolytic enzymes on the casein molecules and makes them easy to degrade (Ng-Kwai-Hang, 2002). The caseins are able to associate with each other (e.g α-CN with β-CN) or self-associate (e.g α-CN with α-CN). These reactions are dependent on pH, ionic strength and temperature (Swaisgood, 1997). Depending on the number of phosphoserine residues in the molecule, the various casein components show different calcium binding ability. Kappa-CN has a very low, if any, calcium binding capacity with only one phosphoserine whereas α_{S1}-, α_{S2}- and β-CN, with several phosphoserines, have high binding capacities (Ng-Kwai-Hang, 2002).

The casein micelle

The majority of the caseins in bovine milk (about 95%) are incorporated into large colloidal structures, known as micelles. It is still not fully understood how the caseins are arranged in the micelles but several models have been suggested. The three most referred to and discussed models are “the submicelle model”, “the hairy model” and the “dual binding model”. The submicelle model (Slattery & Evard, 1973; Schmidt & Both, 1982) suggests that casein submicelles are linked together with colloidal calcium phosphate, Figure 2a. The κ-CN rich submicelles are concentrated at the surface, whereas submicelles with poor κ-CN content form the micellear core. This structure is suggested to be open and porous.

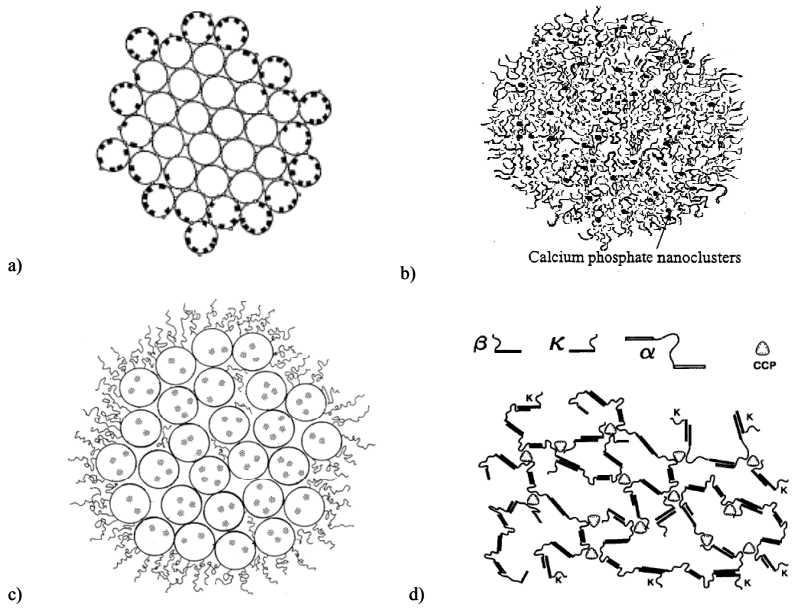


Fig 2. Four proposed casein models: a) The submicelle model (Schmidt, 1982), b) The hairy model (Holt & Horne, 1996), c) The modified submicelle model (Walstra, 1999), and d) The dual-binding model (Horne, 1998).

The hairy model (Holt, 1992; Holt & Horne, 1996), in Figure 2b, is an alternative that proposes that casein molecules are held together by calcium phosphate nanoclusters and that κ-CN forms a hydrophilic, negatively charged, hairy layer on the micelle surface. The structure suggested by Holt is believed to be flexible and gel-like. Inspired by this hairy model, Walstra (1999) suggested a modified submicelle model (Figure 2c) with a hairy layer and with calcium phosphate nanoclusters incorporated into the submicelles. In this modified model, the submicelles are believed to be held together by hydrophobic forces. A fourth model, the dual binding model (Figure 2d) was proposed by Horne (1998) and (2002) and suggests that the casein micelle is stabilised by two distinct types of bonds. The first is a crosslink between hydrophobic casein regions and the second is a bridge across calcium phosphate nanoclusters. In this model, κ-CN functions as a chain terminator. This means that κ-CN can be linked to the growing α- and β-CN chains through its N-terminal whereas the C-terminal, with no phosphoserines, cannot form any nanocluster links. In spite of differences between the three proposed casein micelle models, they all have two important aspects in common; the inclusion of calcium phosphate and the κ-CN rich surface. In 2004 Dalgleish, Spagnuolo & Goff were able to present a detailed micellar surface structure by electron microscopy (Figure 3). They suggested that the caseins are organised into tubular substructures with gaps in between and that κ-CN is grouped at the end of these tubules, on the outer part of the micelle.

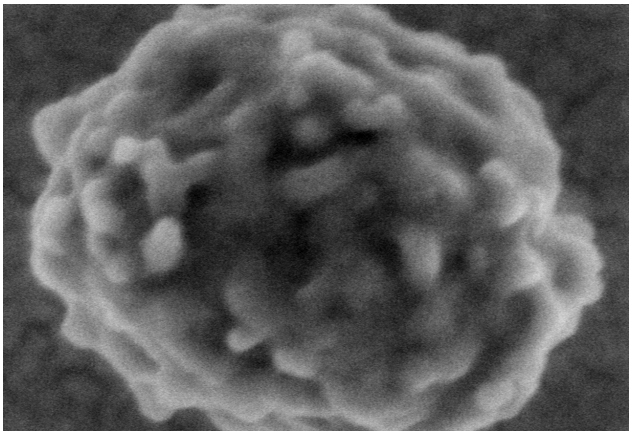


Figure 3. Electron micrograph of a single casein micelle (By kind permission of Douglas G Dalgleish)

Whey proteins

The whey protein fraction mainly consists of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), in a ratio of 3:1 (w:w), as well as of smaller amounts of serum albumin, immunoglobulins, small casein proteolyse products and several enzymes. Compared to the caseins, the whey proteins have a more organised secondary and tertiary structure and are commonly globular proteins. They are also more heat sensitive with initiation of denaturation/unfolding at temperatures around 60º C. It has been suggested that α-LA is unfolded already at 63-67º C and β-LG at 74-77º C (de Wit, Klarenbeek & Hontelez-Backx, 1983; Paulsson & Dejmek, 1990). The whey proteins are more hydrophobic than the caseins and most of them contain high amounts of α-helical structure (Ng-Kwai-Hang, 2002).

The crystallographic structures of the major whey proteins α-LA (Chrysina, Brews & Acharya, 2000) and β-LG (Papiz *et al.*, 1986) have been determined. Alpha-LA consists of one larger subdomain with three α-helixes and one smaller subdomain containing a three-stranded antiparallel β-sheet (Chrysina, Brews & Acharya, 2000). Alpha-LA has an important biological function in the synthesis of lactose from UDP-D-galactose and β-glucose. As a key component in the enzyme system lactose synthetase, α-LA constitutes one of two subunits (Ebner, Denton & Brod-Beck, 1966), the other is UDP-galactosyltransferase (Brew, Vanaman & Hill, 1968). It is α-LA that contributes to the specificity of lactose synthetase, i.e. UDP-galactosyltransferase is able to catalyse lactose alone but very inefficiently. The concentration of lactose in milk is directly related to the concentration of α-LA (Brew, Vanaman & Hill, 1968).

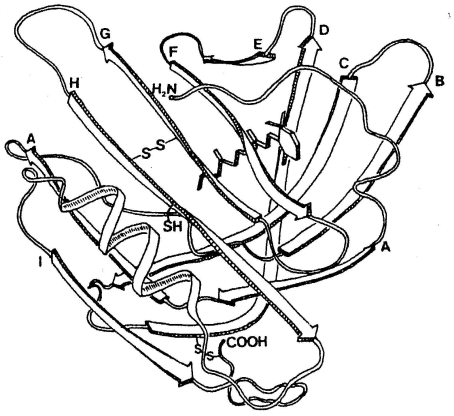


Fig 4. Structure of β-lactoglobulin (Papiz*, et al.*, 1986)

The β-LG structure (Figure 4) is similar to that of the plasma retinol binding protein, with an eight stranded antiparallel β-barrel constituting the molecular core (Papiz, *et al.,* 1986). Probably, also β-LG fulfils a biological role by transporting retinol (vitamin A) to the small intestine of newborn (Perez & Calvo, 1995). It has been suggested that β-LG binds retinol in a hydrophobic pocket, located on the surface of the molecule, during the transport (Monaco *et al.,* 1987) and thereby protecting it from oxidation. Beta-LG contains 5 cystein residues, resulting in two disulphide linkages and one very reactive free sulfhydryl group, which in the native state is hidden within the molecule. At natural milk pH, about 6.7, β-LG exists as a dimer of two monomeric subunits. When pH decreases to below pH 5.2, an octamer is formed, whereas the protein exists as a monomer at pH above 7.5 (Fox & McSweeney, 1998).

Genetic polymorphism of milk proteins

All major milk proteins occur in different genetic variants. Genetic polymorphism of milk proteins arise when amino acids are either substituted to or deleted from the amino acid chain. These alterations are the consequence of mutations causing changes in the gene base sequences (Ng-Kwai-Hang & Grosclaude, 2003). Aschaffenburg & Drewry (1955) were the first to discover two forms of β-LG, termed β_1 and β_2. When they examined milk from Shorthorn, Friesian, Ayrshire and Guernsey breeds, they also found that β_2-LG was the most common variant and that the amount of β_1 was highest in milk containing both variants. Today, the β_1-LG is known as β-LG A and the β_2-LG as β-LG B. The two variants both contain 164 amino acids but the A variant exhibits an aspartic acid residue at position 64 and a valine residue at position 118, whereas the B variant has a glycine residue and an alanine residue at these positions, respectively (Piez *et al.*,1961; Presnell *et al.*, 1990). Until 2003, a total of 44 genetic variants of milk proteins have been identified (Ng-Kwai-Hang & Grosclaude, 2003), see Table 1. The genes encoding for the major whey proteins, α-LA and β-LG, have been mapped on chromosomes 5 (Threadgill & Womack, 1990) and 11 (Hayes & Petit,

1993), respectively. Ferretti, Leone & Sgaramella (1990) found the four casein loci to be located close together within a region of 300 kb in the following order: κ-CN, α_{S2}-CN, β-CN and α_{S1}-CN. It is, however, uncertain whether the casein genes are located on chromosome 4 (Hayes *et al.*, 1993) or on chromosome 6 (Threadgill & Womack, 1990).

Table 1. *Detected genetic variants of major milk proteins*

Milk protein	Detected genetic variants
α_{S1}-CN	A, B, C, D, E, F, G, H
α_{S2}-CN	A, B, C, D
β- CN	A^1, A^2, A^3, B,C, D, E, F,G
κ-CN	A, B, C, E, F^S, F^I, G^S, G^E, H, I, J
β-lactoglobulin	A, B, C, D, E, F, H, I, J
α-lactalbumin	A, B, C

(Ng-Kwai-Hang & Grosclaude, 2003)

α-LA

Two genetic variants of α-LA, A and B, have been found to dominate in bovine milk (Bhattacharya, Roychoudhury & Sinha, 1963). An arginine residue at position 10 instead of a glutamine differentiates the B from the A variant (Gordon *et al.*, 1968). In northern European cattle breeds, however, α-LA appears monomorphic with the B-allele existing only (Ng-Kwai-Hang & Grosclaude, 2003)

β-LG

Beta-LG A and B are the most frequent of all identified β-LG variants and are associated with variations in milk protein yield and composition. Beta-LG A has more negative net charge, compared to β-LG B, due to an extra residue of aspartic acid. Therefore, β-LG A associates more strongly in the octamere form at pH below 5.2 (Fox & McSweeney, 1998). Concentration of β-LG is higher in milk with the AA-genotype compared to AB or BB (McLean, Bruce Graham & Ponzoni, 1984; Ng-Kwai-Hang, Hayes & Moxley, 1987; Graml *et al.,* 1989; Hallén *et al.,* 2008). Graml *et al.,* (1989) showed that in heterozygote animals (β-LG AB), the production of β-LG A is about 50% higher than the production of β-LG B. McLean, Bruce Graham & Ponzoni (1984) found higher concentrations of dry matter (dm), fat and casein in β-LG AB and BB milk than in β-LG AA milk. Several workers have also reported that β-LG B genotype is significantly correlated with the casein number (Schaar, Hansson & Pettersson, 1985; van den Berg *et al.,* 1992; Lundén, Nilsson & Janson, 1997; Hallén *et al.,* 2008).

α -CN

In Holstein dairy breeds, the α_{S1}-CN B variant predominates and the much rarer A and C variants only exist as heterozygous AB and AC (Ng-Kwai-Hang *et al.*, 1984a). Due to exon skipping, variant A is 13 amino acids shorter than variant B and C (Grosclaude *et al.*, 1970). The α_{S1}-CN B together with β-CN A loci have been associated with higher milk and fat yields (Ng-Kwai-Hang*, et al.*, 1984a).

Among genetic variants of α_{S2}-CN, the A-variant is most frequently observed in western dairy breeds. Variant D was found in Vosgienne and Montbéliarde breeds in France and is the second example of a casein variant with an amino acid deletion (Grosclaude, Joudrrier & Mahe, 1979). Bouniol, Printz & Mercier (1993) suggested that the deletion is caused by the skipping of exon VIII, encoding amino acid 51 to 59 of α_{S2}-CN A. However, no reports exist on associations between genetic variants of α_{S2}-CN, resulting from differences in the coding region of the gene, and milk composition.

β-CN

The A^1 variant of β-CN differs from the A^2 variant by a substitution of a proline residue for a histidine residue at position 67. Variant B arises due to two substitutions, one at position 67 and one at position 122, with a histidine residue for a proline residue and an arginine residue for a serine residue, respectively (Eigel *et al.*, 1984). The A variants are common polymorphs to all dairy cattle breeds (Thompson, 1971). McLean, Bruce Graham & Ponzoni (1984) concluded that β-CN AB (A^1B, A^2B) heterozygote cows produced milk higher in both concentration and proportion of β-CN compared to cows with other genotypes (A^1A^1, A^1A^2, A^2 A^2, BB). This is also in agreement with the results of Hallén *et al.* (2008).

κ-CN

In bovine milk, two major genetic variants of κ-CN have been identified, A and B. In the A variant, a threonine residue is located at position 136 and an aspargine residue at position 148, whereas variant B shows an ileonine residue and alanin residue (Grosclaude *et al.,* 1972). Milk from Holstein cows with the κ-CN BB genotype has been shown to contain 0.13% more protein than milk from κ-CN AA cows (Ng-Kwai-Hang, *et al.,* 1984a) and several workers have found the B allele of κ-CN to be associated with significant higher concentration of κ-CN (McLean, Bruce Graham & Ponzoni, 1984; Aaltonen & Antila, 1987; van den Berg, *et al.,* 1992; Bobe *et al.,* 1999; Hallén, *et al.,* 2008). In SRB (related to Ayrshire) and Finnish Ayrshire breed the frequency of the E-variant of κ-CN has been reported to be high (Hallén *et al.,* 2007; Ikonen *et al.,* 1996). The E-variant of κ-CN is associated with unfavourable coagulation properties (Ikonen, Ojala & Syvaoja, 1997; Hallén, *et al.,* 2007), whereas the B-variant has a positive effect (Schaar, 1984; Ikonen *et al.,* 2004; Hallén, *et al.,* 2007).

Variation in milk protein composition

In addition to genetic polymorphism of milk proteins, the total concentration and composition of milk protein vary due to several other factors. These include breed (Auldist *et al.*, 2004), stage of lactation (Ostersen, Foldager & Hermansen, 1997), lactation number (Ng-Kwai-Hang *et al.*, 1987), season (O'Brien *et al.*, 1999), feeding (Mackle *et al.*, 1999) and health of the cow (Verdi*, et al.*, 1987).

Differences in concentration of total protein between breeds have been reported earlier (Cerbulis & Farrell, 1975; Custer, 1979; Milk Marketing Board of England and Wales, 1984). Common to these reports is that milk from Ayrshire contains higher concentration of total protein than Holstein cows. This was also supported by Tyrisevä *et al.* (2004) when comparing the milk composition in Finnish Ayrshire and Holstein Friesian cows. SRBis related to Ayrshire and it is therefore expected that the protein concentration is higher in SRB compared to SLB. In the textbook of Bergsten *et al.* (1997), SRB milk was reported to contain in average 3.44 % of protein compared to 3.33% in SLB milk.

Several workers have reported that concentrations of total milk protein and casein are higher in late stage of lactation than in early stage of lactation (Auldist, Walsh & Thomson, 1998; Coulon *et al.*, 1998; Ostersen, Foldager & Hermansen, 1997). Moreover the lactation number, which is related to age of cow, has been shown to influence the protein composition. Ng-Kwai-Hang, Hayes & Moxley (1987) reported that when the cow aged the major casein constituents (α-CN, β-CN and κ-CN) declined, with a corresponding increase in the serum protein concentration. On the other hand, Schaar (1984) found no correlation between lactation number and total casein concentration as analysed by infrared (IR) spectroscopy. Most probably, the lower concentration of individual caseins observed by Ng-Kwai-Hang*, et al.* (1987) was related to an increased milk proteolysis, which not was observed by Schaar (1984) when analysing total casein concentration by IR spectroscopy. A higher casein proteolysis has been observed in milk from older cows because plasmin activity increases with cow age (Schaar, 1985; Bastian, Brown & Ernstrom, 1991). It has also been suggested that a higher serum protein concentration in milk from older cows is related to increased permeability through the mammary epithelia caused by permanent damage from previous mastitis infections (Mackle*, et al.*, 1999).

It has been documented that concentrations of casein and total protein in Swedish bulk milk show seasonal variations due to the outdoor grazing in summer (Lindmark-Månsson, Fondén & Pettersson, 2003). Seasonal variation in protein concentration has also been observed in Irish retail milk as well as manufactured milk (O'Brien*, et al.*, 1999). The protein concentration reached its maximum in the end of the outdoor season (in October) and its minimum in the end of the indoor season (in March). These variations were due to differences in feeding in combination with stage of lactation, i.e. a larger number of the cows were in early lactation in the beginning of the year. This implies that seasonal variations in milk protein concentration can be due to a combination of several factors.

Milk Proteolysis

Milk proteins, especially the caseins, are susceptible to degradation by proteolytic enzymes (Fox, 1992). The most important proteolytic enzyme in milk is plasmin with its inactive form; plasminogen (Bastian & Brown, 1996), which is transferred from blood to milk (Eigel *et al.,* 1979). There is strong evidence for analogy between plasminogen existing in blood and in milk. It has been shown that parts of the sequences in the milk type are identical to the corresponding sequences of plasminogen isolated from bovine blood (Benfeldt *et al.*, 1995). Of the major caseins, β-CN and α_{S2}-CN are more susceptible than α_{S1}-CN to hydrolysis by plasmin (Bastian & Brown, 1996; Grufferty & Fox, 1988). Furthermore, it has been suggested that κ-CN is resistant to plasmin (Eigel, 1977) or that hydrolysis occurs, but only to some extent (Andrews & Alichanidis, 1983; Grufferty & Fox, 1988).

The plasmin hydrolysis of Lys^{28}- Lys^{29}, Lys^{105}-Lys^{106} and Lys^{107}-Gly^{108} in β-CN results in the formation of six peptides: γ1-casein (f29-209), γ2-casein (f106-209), γ3-casein (f108-209), proteose peptone component 8-fast (f1-28)**,** β-casein (f29-105 and f29-107) and proteose peptone component 5 (f1-105 and f1-107; Bastian & Brown, 1996). On α_{S1}-CN, a total of 17 plasmin cleavages sites after a Lys- or Arg-residue has been identified. Le Bars & Gripon (1993; 1998) and McSweeney *et al.* (1993) found 8 cleavage sites on α_{S2}-CN, 1 cleavage after an Arg-residue and 7 cleavages after Lys-residues. However, these results were obtained from model studies and it has not been determined whether all the peptides released from α_{S1}- or α_{S2}-CN exist naturally in milk (Bastian & Brown, 1996).

Several other active proteinases in milk originate from somatic cells (Verdi & Barbano, 1988). The lysosomes of most cells contain both cystein and acid proteinases (Owen & Campbell, 1999). Activity derived from the cystein proteinase cathepsin B and the acid proteinase cathepsin D has been identified in milk by Magboul *et al*. (2001) and Kaminogawa (1972) respectively.

When the cow suffers from mastitis the SCC, which in milk from healthy cows mainly consists of macrophages, will increase and consequently the proteolysis. In high SCC milk both somatic cell derived enzymes, e.g. cathepsin B and D, as well as plasmin are responsible for the proteolysis (Larsen *et al.,* 2004 and 2006). The polymorphonuclear leucocytes (PMN) is a somatic cell type that dominates in milk during mastitis (Azzara & Dimick, 1985) and contains several enzymes including cathepsin G and elastase (Kirschke & Barrett, 1987; Owen & Campbell, 1999; Travis, 1988). However, it has not been determined whether these enzymes are secreted, or released when the cells are damaged, into milk (Kelly, O´Flaherty & Fox, 2006) but their specificities towards α_{S1}- and β-CN has been demonstrated in model systems (Considine *et al.,* 1999, 2000 and 2002)

Milk analysis

Milk protein analysis

Simultaneous separation and quantification of major caseins and whey proteins can be obtained using RP-HPLC (Visser, Slangen & Rollema, 1991; Bobe *et al.*, 1998; Bordin *et al.*, 2001) or capillary zone electrophoresis (de Jong, Olieman & Oileman, 1993; Recio & Oileman, 1996). However, each of these methods have advantages and disadvantages. The capillary electrophoresis method by de Jong, Olieman & Oileman (1993) gave a better separation of some of the genetic variants compared to an RP-HPLC reference method (Visser & Slangen, 1992), i.e. better separation between β-CN A^1, A^2 and A^3. However, to overcome adsorption of milk proteins to the capillary fused-surface (which is negatively charged) a buffer system with high ionic strength and which contains modified cellulose is needed (de Jong, Olieman & Oileman, 1993). These buffers are both more expensive and time consuming to prepare compared to buffers for RP-HPLC, simply containing acetonitrile and water.

In order to separate all the milk proteins, including different genetic variants and all post-translational modification products, two-dimensional gel electrophoresis (2-DGE) is a useful tool (Galvani, Hamdan & Righetti, 2000; Galvani, Hamdan & Righetti, 2001; Holland, Deeth & Alewood, 2004). This technique in combination with mass spectrometric analysis, for identification of separated proteins, is one of the most frequently used methods within proteomics, i.e. the systematic separation, identification and characterisation of proteins from a common source (O´Donell *et al.*, 2004). The 2-DGE technique facilitates separation of proteins in two dimensions, one according to molecular mass and one to isoelectric point (pI). This enables separation of a wide range of milk proteins with similar molecular masses or similar pI, i.e different products of phosphorylation , glycosylation or proteolysis and genetic variants.

Different staining methods have been applied for the visualization of the separated protein spots, including colloidal Coomassie Brilliant Blue (CBB), silver staining and more recent fluorescence staining methods such as Sypro Ruby or Flamingo Pink. The sensitivity of fluorescent dyes is comparable with silver staining, whereas CBB is less sensitive (Harris *et al.,* 2007).

Several mass spectrometric techniques are suitable for characterisation and identification of proteins and peptides. A mass analyser consists of an ionisation device which is coupled to a mass detector. The ionisation process is necessary for conversion of the sample into ions because only the masses of charged atoms or molecules can be determined by mass spectrometry (Willoughby, Sheehan & Mitrovich, 2002). Two ionisation techniques are appropriate for large biomolecules, such as proteins and peptides, i.e. the electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) techniques (Alomirah, Alli & Konishi, 2000). Both these devices are compatible with a range of mass detectors of which the time of flight mass spectrometer (TOF-MS) is preferable when analysing high-molecular-weight compounds (Willoughby, Sheehan &

Mitrovich, 2002). In a TOF-MS analyser, the ions “fly” through a drift tube with the detector positioned in the end. The greater the mass of the ion, the slower it will travel towards the detector (Wiley & McLaren, 1955). There is no practical mass upper limit for this type of analysis, which enables separation of ions within a wide mass spectrum (Willoughby, Sheehan & Mitrovich, 2002). Two mass detectors can also be coupled in series into a tandem mass spectrometer, MS/MS. The second analyzer in MS/MS enables high efficiency scanning of the ions and a more detailed sequence characterisation (Willoughby, Sheehan & Mitrovich, 2002).

The use of 2-DGE in combination with MALDI-TOF/MS has been applied by several workers for the characterisation and identification of milk proteins, including their iso-forms (Galvani, Hamdan & Righetti, 2000; 2001; Holland, Deeth & Alewood, 2004; Yamada *et al.*, 2002).

Rheological measurements

In the following section the basic rheological terminology and principles elaborated in the books of Barnes, Hutton & Walters (1989) and Macosco (1994) have been summarised.

Milk clotting properties during renneting can be determined by rheological measurements. Rheology means *the study of the deformation and flow of matter.* All material behaves in viscous or elastic ways or both. It is the stress applied and the duration time of the application that decide the dominating property. Viscosity is a measure of “resistance to flow” and is a common term when defining the rheological behaviour of liquids. Viscosity is equal to the force per unit area required to produce a motion (which also is called shear stress). A “shear stress” results in “flow”. For some liquids the flow persists as long as the stress is applied; these are called Newtonian viscous liquids, e.g. water and oil.

Robert Hooke developed his ‘true theory of elasticity’ in 1678. His idea was that the extension is doubled with doubled tension. A Hookean (elastic) solid shows the opposite behaviour compared to a Newtonian liquid. When a shear stress is applied to the surface the result is an instantaneous deformation. A material often consists of both elastic and viscous properties and is then referred to as viscoelastic. The elastic and viscous behaviour in terms of the laws of Hooke and Newton are both linear. Within the viscoelastic behaviour also non-linearity is possible. Examples are shear thinning, where an increasing rate of shear in a steady flow results in a reduction of the viscosity, and shear thickening which is the opposite.

The gel resulting after aggregation of particles in renneting milk shows viscoelastic behaviour (Walstra, Wouters & Geurts, 2006). This behaviour is characterised by the elastic or storage modulus (G’) and the viscous or loss modulus (G’’). These two parameters describe whether the elastic or viscous properties prevail. If G’ is much greater than G’’ the material is more elastic and if G’’ is much greater than G’ it is more viscous.

Several instruments are available for rheological measurements of milk gels. The formagraph was evaluated by McMahon & Brown (1982) to record coagulating properties of cheese milk. Equivalent to the formagraph for this purpose is the gelograph (Najera, de Renobales & Barron, 2003). Earlier, the viscometer was commonly used to study the coagulation properties of milk-enzyme systems (Scott-Blair & Oosthuizen, 1961; Kopelman & Cogan, 1976; Korolczuk & Maubois, 1987). Nowadays it is common to use a rheometer for viscoelastic measurements of milk gels with an oscillating cup and bob measuring system. The Bohlin VOR rheometer is an instrument that controls strain, when the stress is measured. With this technique it is possible to measure the deformation of a gel over a strain gradient at constant oscillating frequency. This makes it very suitable for the measurement of milk gelling properties and curd strength.

Milk processing

Pasteurisation of milk

Pasteurisation was first introduced in the 1860's by Louis Pasteur for treatment of wine and was soon applied also on milk to prevent epidemics of tuberculosis (Nationalencyklopedin, 2008). The pasteurisation process is either accomplished at low temperature for a long time, 63º C for 30 min, or at a higher temperature for a shorter time, 72º C for 15 s (O´Connell & Fox, 2003). Both treatments will inactivate the milk enzyme alkaline phosphatase and almost all pathogens present in milk are killed (Walstra, Wouters & Geurts, 2006). At higher heating temperatures, from about 85-90º C, a high proportion of the whey proteins will denaturate and attach to the casein micelles surfaces. These interactions involve disulphide bindings between β-LG and β-LG as well as between β-LG and κ-CN (Sawyer, 1969). However, β-LG is not the only whey protein involved in these heat induced aggregates. Disulfide-linked aggregates are also formed between β-LG and α-LA when a mixture of these whey proteins are heated at 75-80° C (Hines & Foegeding, 1993; Dalgleish, Senaratne & Francois, 1997; Havea, Singh & Creamer, 2001). There seems to be a synergistic reaction between α-LA and β-LG because α-LA only reacts in the presence of a high β-LG concentration (Dalgleish, Senaratne & Francois, 1997). At pasteurisation temperatures, however, only about 7% of the whey proteins in milk will be denaturated (Lawrence, 1991). As mention earlier, the caseins cannot be denaturated but they undergo heat dependent aggregations that increase with temperature until a maximum at 140° C (Singh, 1995). Pasteurisation positively affects the casein micelles and the calcium phosphate equilibrium, which reduces the rennet coagulation time of milk (Fox, 1969).

Cheese making

After an archaeological survey of the area between the rivers Euphrates and Tigris in Iraq, it was concluded that cheese was made there, from goat and cow milk about 6-7000 years ago (Woolley & Hall, 1931). Nowadays a wide range of cheese varieties are produced worldwide. Due to all these varieties a simple definition of cheese is difficult to obtain. According to the Food and Agricultural

Organization (FAO) codex general standard for cheese (CODEX STAN A-6-1978, Rev.1-1999, Amended 2003): “Cheese is the ripened or unripened soft or semi-hard, hard and extra hard product, which may be coated, and in which the whey protein/casein ration does not exceed that of milk, obtained by; (a) coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from such coagulation and/or (b) processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a)”.

To coagulate milk for cheese making, extracts of the abomasum (the “fourth stomach”) of young calves containing the milk-clotting enzymes chymosin and pepsin, have been used since ancient times. The extract of ruminant abomasa is called “rennet”, of which chymosin constitutes the most important clotting enzyme. In the abomasa of milk fed calves the proportion of chymosin to pepsin is about 90:10, but the concentration of pepsin increases as the calves becomes older and are weaned (Andrén, 2002). In the 1950’s it appeared that the supply of calf stomachs would not fulfill the future requirements of rennet. The increasing cheese production in Western Europe required calf rennet that corresponded to approximately 5 million calf stomachs per year (Crawford, 1985). In Sweden it was necessary to import calf stomachs for the production of rennet because the number of suckling calves slaughtered was insufficient (Crawford, 1985). Today, pure chymosin produced by gene technology is the main clotting agent used in many countries (Walstra, Wouters & Geurts, 2006), whereas bovine rennets are still the main coagulants used in Sweden. Besides chymosin, a range of alternative microbial coagulants exists, of which *Rhizomucor miehei* proteinaseis is the most common used (Andrén, 2002).

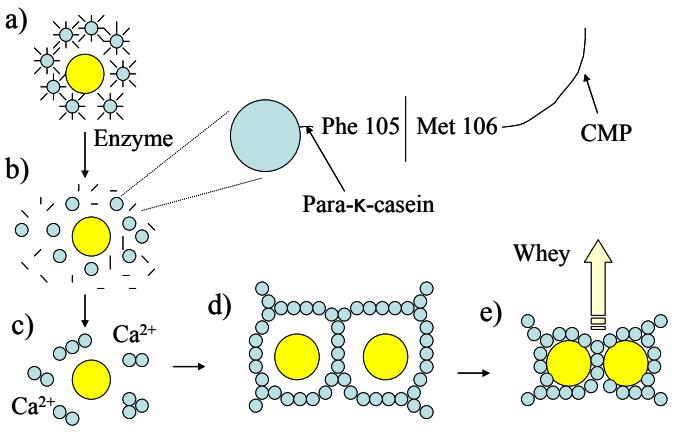


Fig 5. The renneting process

The biochemistry of cheese making is described by Fox & McSweeney (1998); Hyslop (2006); Walstra, Wouters & Geurts (2006) and summarized in the following paragraph and in Figure 5. Casein micelles, with the hydrophilic tail of κ-CN and a negative net charge on the surface, are freely distributed and repelling each other in the milk serum, which also contains milk fat globules (Figure 5a). During renneting, chymosin splits the Phe-Met bond of κ-CN into para-κ-CN and a caseinomacropeptide (CMP), which ends up in the whey fraction (Figure 5b). The para-κ-CN micelles are now hydrophobic, neutral net charge, and will aggregate spontaneously with each other (Figure 5c). The curd is formed in two stages. First the paracasein micelles form small aggregates with an irregular or elongated shape. In the next stage a three dimensional network is created which is stabilised by hydrophobic bonds and electrostatic interactions between positive and negative regions of the micelle surface and also by calcium phosphate linkages between the micelles. Fat globules are entrapped in the network which now has become a gel or curd (Figure 5d). The clotting reaction is enhanced by increasing the calcium concentration, decreasing the pH and by keeping an optimal temperature, around 30º C. As more bonds are formed between casein micelles the curd becomes firmer. When the gel is cut, liquid (whey) is expelled from the curd (Figure 5e) and this reaction is called syneresis. By increasing the pressure applied to the curd, e.g. by stirring, syneresis is enhanced. In this way some bonds are broken and new ones formed more easily. Other factors that influence syneresis are increased temperature and reduced pH of milk.

Objectives

The main objective of this thesis was to study the variation in the detailed protein profile in milk from individual cows and to identify markers with significance for the cheese making properties of milk.

I) The milk protein composition varies due to several reasons, which can both positively and negatively affect the quality of cheese milk. In Paper I the effect of different factors on the milk protein composition were examined, including breed of cow, genetic polymorphism of milk proteins, stage of lactation, lactation number, season, and herd variations. The objective of this work was to compare the concentrations of individual milk proteins, as separated by RP-HPLC, between milk samples of SRB and SLB cows and between herds of Swedish (SLB) and Danish Holstein cows.

II) The influence of high temperature heat treatment (>90º C) on the milk protein composition is well documented whereas more knowledge is needed in the context of the effect of pasteurisation. In Paper II, the milk protein profile, as determined by proteomics, was evaluated in pasteurised and in unpasteurised milk samples. The objective of this work was to determine whether pasteurisation influenced the milk protein composition, e.g. denaturation of whey proteins and aggregation with caseins.

III) Variations in milk protein composition and concentration are crucial for the cheese making properties. The effect of variations in major milk protein composition between individual cows on the cheese making properties, i.e. coagulation properties and cheese yield, was investigated in Paper III. The objective of this work was to find markers within the milk protein profile significant for the cheese making properties.

IV) Cheese making is a complex process and it is important to increase the knowledge about how the proteins are distributed within the different fractions after separation by chymosin. In Paper IV, the effect of minor proteins or peptides, as analysed by 2-DGE and MS detection in chymosin-separated whey and casein, on cheese yield was evaluated. The objective of this work was to find markers within the minor milk protein profile, in the chymosin-separated whey and casein fractions, significant for the cheese making properties.

V) The milk protein composition, and consequently the cheese making properties, is also influenced by proteolysis in milk. Some of the proteolytic enzymes are only present in milk with high content of somatic cells. The final work of this thesis, Paper V, aimed to develop an RP-HPLC MS/MS method for identification of milk peptides produced from the most important proteolytic milk enzymes.

Materials and methods

Milk sampling and experimental design

Evening whole milk (the total milk volume) was sampled from cows of Swedish red and white breed (SRB) and Swedish Holstein breed (SLB) at the experimental dairy herd at Jälla (Swedish University of Agricultural Sciences) and from Danish Holstein (SDM) cows at the Research Center Foulum (Danish Institute of Agricultural Sciences, University of Aarhus). Sampling took place at three occasions; in September 2003, January 2004 and in May 2004. The number of milk samples from SRB, SLB or SDM, respectively, at each sampling occasion is presented in Table 2.

Table 2. *Number of Swedish red and white breed (SRB), Swedish Holstein breed (SLB) and Danish Holstein breed (SDM) at each sampling occasion*

	September	January	May
SRB	16	22	20
SLB	15	8	9
SDM	14	15	15

In total, 134 dairy cows were included in the study and no cow was sampled twice. The intention was to select cows within mid lactation and with a maximum milk SCC of 200,000 cells/ml. However, because of a limited number of available cows at the two dairy herds, some of the cows selected did not fulfil these criteria. Feeding and management were carried out according to standard practices at the two experimental herds. All cows were considered clinically healthy and milked twice a day. The cows were grouped into four classes according to stage of lactation, as presented in Table 3, and also according to lactation number, Table 4.

Table 3. *Number of Swedish red and white breed (SRB), Swedish Holstein breed (SLB) and Danish Holstein breed (SDM) within each stage of lactation class*

	Early lactation w 6-15	Mid lactation w 16-30	Late lactation w 31-45	Very late lactation w 46-
SRB	10	36	11	1
SLB	9	15	7	1
SDM	9	18	11	6

Table 4. *Number of Swedish red and white breed (SRB), Swedish Holstein breed (SLB) and Danish Holstein breed (SDM) in each lactation number class*

	First lactation	Second lactation	Third lactation	Fourth or higher lactation
SRB	23	19	10	7
SLB	10	10	8	3
SDM	17	13	4	10

In the first study (Paper I) the protein composition in milk from SRB (58 cows) was compared with SLB (32 cows) to evaluate differences between breeds within the Swedish herd. SLB (32 cows) and SDM (44 cows) were considered to have more similar genetic background (both Holstein) and therefore, a comparison between these breeds reflected differences between the Swedish and the Danish herd. The effect of heat treatment on the total milk protein composition, as determined by 2-DGE-analysis (Paper II), was evaluated in milk from 6 SDM cows. However, milk samples from all individual 134 cows were included when examining the effect of variations in major milk protein composition on the cheese making properties (Paper III). When analysing the effect of the milk proteins or peptides in chymosin-separated whey or casein, as determined by 2-DGE-analysis, on the cheese making properties (Paper IV) one-third of the milk samples (all SDM samples) were used. In the final study (Paper V), three milk samples with varying somatic cell count were used to optimise a method for separation of indigenous proteinase produced peptides in milk. Two milk samples with 50,000 and 500,000 cells/ml, respectively, were selected from the sample material in Paper V, whereas the third sample, representing acute clinical *Streptococcus uberis* mastitis, was taken from another trial (Larsen *et al.*, 2004).

Pasteurisation of milk samples

Milk samples (à 10 litres) collected in this study were stored at 4° C for two days before further treatment. A part of each milk sample (approximately 1 litre) was kept untreated for evaluation of milk protein composition in raw milk. Prior to the separation of milk fat, the milk samples were preheated to 40° C to make the fat fraction more soluble. The milk was then defatted using a farm separator. The skimmed milk samples were further pasteurised (72° C for 15 s) in a pilot plate heating equipment as described by Allmere *et al.* (1998).

Cheese making and rheological measurements

Low fat model cheeses were made from 4 litres of skimmed milk as described in Paper III. Immediately after addition of chymosin to cheese milk, a sample (12 ml) was taken for rheological evaluation in a rheometer, also as described in Paper III. The milk clotting properties were evaluated by recording the elastic modulus (G´), in Pa, over time. The milk clotting time (MCT), i.e. the time in min from chymosin addition until the G´ starting point (G'_{start}), and G´ at 15 min (G'_{15}) was recorded (Figure 6). G´ was only recorded during 15 min due to a

limited time schedule at the cheese making procedure. Further evaluation of the curd firmness at 30 min after chymosin addition, the moment for cutting the curd, was evaluated subjectively. If curd was firm enough to form cubes it was denoted firm; otherwise it was denoted weak. However, even though the curd was not firm enough at 30 min to form cubes, it was stirred and treated as the rest of the samples. The values of G'_{15} and G'_{start} were not normally distributed and therefore logarithmically transformed before statistical analysis. G'_{15} values of 0 was treated as 0.05 to be included.

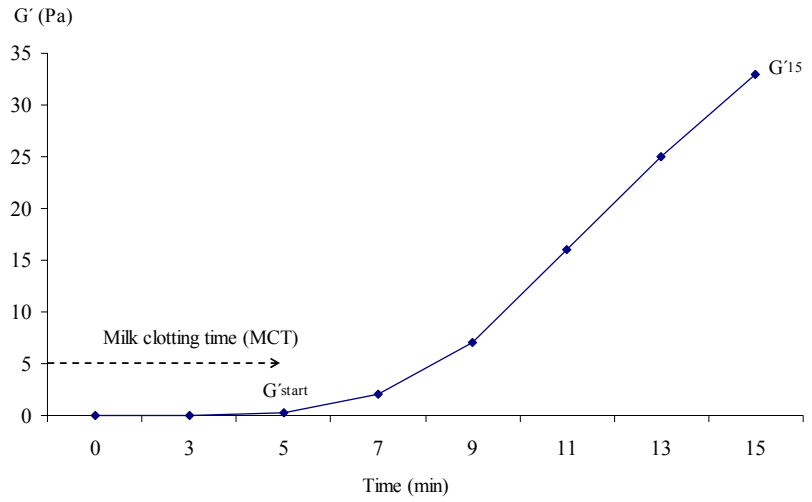


Fig 6. A rheogram illustrating the elastic modulus G´ in pascals over time in minutes

The amount of cheese obtained from a certain amount of milk or milk protein was expressed in three different ways; as g of cheese per 100 g of milk (cheese yield), as g of dry cheese solids per 100 g of milk (moisture free cheese yield) or as g of dry cheese solids per 100 g of milk protein (a sort of protein transition number) as recommended by Emmons (1993). The water holding capacity (WHC) of cheese was expressed as the difference between g of cheese per 100 g of milk and g of dry cheese solids per 100 g of milk.

Analysis of milk components

Whole milk samples were analysed for concentration of total milk protein, milk fat, lactose, urea and pH by a Milkoscan FT 6000 (A/S N. Foss Electric, Hillerød, Denmark) and for somatic cell count by a Fossomatic 5000 (A/S N. Foss Electric) at Steins laboratory (Holstebro, Denmark). Calcium concentration was analysed by absorption spectrophotometry as described in Paper I. Proteolysis was determined in pasteurised milk samples as the level of free amino terminals by the fluorescamine method modified for milk samples (Wiking *et al.,* 2002; Larsen *et al.,* 2004). Concentrations of major milk proteins (α_{S1}-, β-, κ-CN, α-LA

and β-LG), in pasteurised milk samples, was determined by RP-HPLC as described in Paper I. Lactoferrin in pasteurised whey samples was quantified using a commercial enzyme-linked immunosorbent assay (ELISA), Bovine Lactoferrin ELISA Quantification kit (Bethyl Laboratories, Inc, Montgomery, TX, USA). The complete milk protein composition was analysed by 2-DGE in pasteurised and raw milk as described in Paper II and IV. The milk samples were chymosin separated prior to 2-DGE analysis and the casein and whey fractions were analysed on separate gels. The major milk proteins were separated and identified by MS or MS/MS in reduced and unreduced milk samples (Paper II and IV). Protein spots with significant effect on the cheese making properties and those present in concentrations high enough for extraction from 2-DGE gels were identified by MALDI TOF MS or MS/MS (Paper IV). Low-molecular-weight peptides in acid whey was separated by capillary RP-HPLC and identified by MALDI TOF MS/MS as described in Paper V.

Genotyping for β- and κ-casein genetic variants

SRB and SLB cows were genotyped for genetic variants of β- and κ -casein by Pyrosequencing™ (Biotage AB, Uppsala Sweden), as described in detail by Hallén *et al.* (2007). Briefly, a DNA lysate was prepared from whole blood samples and mixed with a PCR buffer in combination with a number of PCR primers (six for β-CN and four for κ-CN). A binding buffer was used to immobilise the PCR products onto streptavidin-coated separose beads. The immobilised PCR products were further washed in NaOH and a washing buffer to obtain single stranded DNA. Three and two detection primers were used to identify allelic variants of β- and κ-CN, respectively. Pyrosequencing was accomplished on a Pyrosequencing instrument using a reagent kit (Biotage AB, Uppsala Sweden). Three single nucleotide polymorphisms (SNP; sites 8101, 8219 and 8267) were analysed to discriminate between the four β-CN variants A^1, A^2, A^3 and B. By combining the results from these three positions the allelic variants could be identified. In the same way, the allelic variants of κ-CN (A, B and E) were identified by analysing two SNPs (sites 5365 and 5345).

Statistical analysis

Analysis of variance (ANOVA) and the general linear model (GLM) procedure in SAS (Ver. 9.1. SAS Institute Inc., Cary, NC, USA) was used to analyse the effect of breed or herd on the major milk protein composition (α_{S1}-CN, β-CN, κ-CN, α-LA and β-LG), milk fat, urea, lactose, calcium, lactoferrin, free amino terminals, pH, SCC and evening milk yield. The SCC was logarithmically transformed to obtain normally distributed data. For further description of these analyses, see Paper I. The GLM was also used to study the effect of κ-CN AA, AB or AE on the κ-CN concentration (Paper III) and the effect of β-CN A^1A^1, A^1A^2, A^2A^2 or A^2B on the β-CN concentration (Paper I). Finally, the GLM was used to test the overall differences in cheese yield between poorly coagulating and normal coagulating milk, as described in Paper III. A student t-test was used in Paper II to compare

the intensity of each protein spot on the 2-DGE gels before and after pasteurisation.

Multivariate data analysis

Principal component analysis

Principal component analysis (PCA) was carried out using the software Unscrambler (version 9.0, CAMO ASA, Oslo, Norway) to test correlations between all variables in Paper I and III. The PCA model contained the continuous x-variables; cheese yield (g of cheese per 100 g of milk), moisture free cheese yield (g of dry cheese solids per 100 g of milk), water holding capacity (WHC), protein transition number, elastic modulus G´ at 15 min (G´15), elastic modulus starting point (G´start), poorly coagulating and non-coagulating milk, free amino terminals (mM Leucine), casein number, pH, somatic cell count (SCC), total protein concentration as analysed by HPLC or by IR, concentrations of α_{S1}-CN, β-CN, κ-CN, total casein, whey protein, α-LA, total β-LG, β-LG A, β-LG B, lactoferrin, calcium, milk fat, lactose, urea, evening milk yield and the descriptive x-variables breed (Swedish red and white; SRB, Swedish Holstein; SLB or Danish Holstein; SDM) stage of lactation (early; E, medium; M, late; L or very late; VL), lactation number (one; L1, two; L2, three; L3, four or higher; L4) and sampling occasion (September; S1, January; S2 or May; S3). The number of principal components (PCs) used were 7, explaining 62% of the total variance for the PCA model. Standardised (centred: $\mu=0$ and normalized: 1/SD) and full cross validation were used. PCA was also used in Paper II to analyse the effect of heat treatment on the milk protein composition as determined by 2-DGE.

Partial least square regression analysis

Partial least square regression-1 (PLS-1) analysis in Unscrambler (version 9.0, CAMO ASA, Oslo, Norway) was used to test the effect of major milk protein composition on the cheese making properties, as described in Paper III. The same model was used in Paper IV to test the effect of minor proteins or peptides in chymosin-separated casein and whey, as determined by 2-DGE, on the cheese making properties.

Results

The PCA-plot in Figure 7 presents correlations between all variables that were analysed in the individual milk samples from 134 individual cows (Paper I and III).

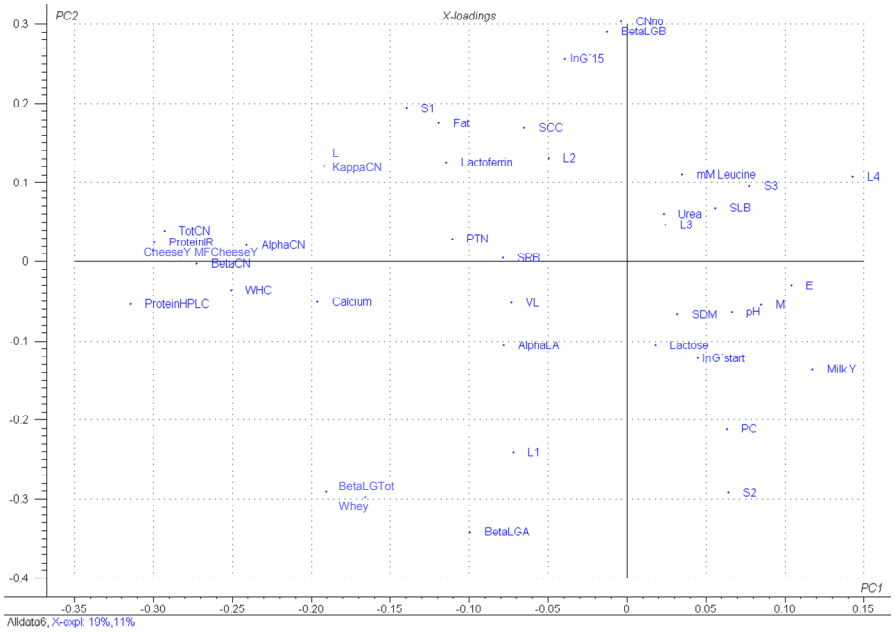


Fig 7. Principal component analysis (PCA) loading plot including the continuous x-variables; cheese yield (Cheese Y), moisture free cheese yield (MFCheeseY), water holding capacity (WHC), protein transition number (PTN), the logarithmically transferred elastic modulus G´ at 15 min (lnG´15), the logarithmically transferred elastic modulus starting point (lnG´start), poorly coagulating milk (PC), free amino terminals (mM Leucine), casein number (CNno), pH, somatic cell count (SCC), evening milk yield (MilkY) total protein concentration as analysed by HPLC (ProteinHPLC) or by IR (ProteinIR), concentrations of, α_{S1}-casein (AlphaCN), β-casein (BetaCN) κ-casein (KappaCN), total casein (TotCN), whey protein (Whey), α-lactalbumin (AlphaLA), total β-lactoglobulin (BetaLG), β-lactoglobulin A (BetaLGA), β-lactoglobulin B (BetaLGB), lactoferrin, calcium, milk fat (Fat), lactose, urea and the descriptive variables breed (Swedish red and white; SRB, Swedish Holstein; SLB or Danish Holstein; SDM) stage of lactation (early; E, medium; M, late; L or very late; VL), lactation number (one; L1, two; L2, three; L3, four or more; L4) and sampling occasion (September; S1, January; S2 or May; S3). The number of principal components (PCs) used were 7, explaining 63% of the total variance for the PCA model.

Concentration of total protein, (as analysed on HPLC or by IR), total casein, α_{S1}-CN, β-CN, κ-CN and calcium were all positively correlated with cheese yield, moisture free cheese yield and the WHC of cheese. Concentrations of κ-CN and milk fat correlated with late stage of lactation and sampling number one. Of the

whey proteins, β-LG A correlated with total whey and total β-LG concentrations. β-LG A correlated negatively with the casein number whereas β-LG B correlated positively with casein number. The casein number was further correlated with the elastic modulus G´, measured 15 min after chymosin addition ($G´_{15}$). It can also be noted that a high G´ value was correlated with an early G´starting point ($G´_{start}$). Poorly coagulating and non-coagulating milk (see definition in Paper III) correlated with sampling number two, in January.

Variables in the centre of the plot contributed less to the total variation of the PCA model. However, SDM was related to higher lactose concentration and pH, whereas SLB was more related to high concentration of urea and level of free amino terminals. SRB correlated with concentration of α-LA and protein transition number. SCC correlated with concentration of lactoferrin, milk fat and lactation number 2.

Variation in milk composition between breeds or herds

SRB milk had significantly higher concentrations of total protein, total casein, β-CN and κ-CN ($p<0.01$, $p<0.05$, $p<0.05$ and $p<0.05$, respectively) compared to SLB milk (Table I, Paper I). When including the effect of β-CN genotype (A^1A^1, A^1A^2, A^2A^2 or A^2B) in the statistical analysis the significant difference between the two breeds remained (Table IVb, Paper I). Milk fat concentration was also significantly higher in SRB milk ($p<0.05$), whereas SLB milk had higher concentration of lactose, level of free amino terminals and pH ($p<0.001$, $p<0.05$ and $p<0.001$, respectively). Of the κ-CN genotypes, AB and AE were more frequent within SRB, whereas AA was overrepresented within SLB in this particular herd (Table IVa, Paper I).

Of the milk proteins analysed, only concentration of α-LA differed significantly between the two herds, i.e. α-LA concentration was higher in SDM milk ($p<0.05$). Since no other differences were found in milk protein composition, these results were not shown in Paper I. Of other milk variables analysed, concentration of urea and level of free amino terminals were significantly higher ($p<0.001$ and $p<0.001$, respectively) in the Swedish compared to the Danish herd. Calcium concentration, on the other hand, was significantly higher ($p<0.01$) in the Danish herd (Table III, Paper I).

Determination of milk proteins by proteomics and effect of pasteurisation

In the rennet casein fraction the major caseins; α_{S1}-CN, α_{S2}-CN, β-CN and remnants of κ-casein were all separated and identified by 2-DGE and MS or MS/MS (Paper II and IV). Most of the κ-casein was cleaved due to the addition of chymosin but the para-κ-CN or caseinomacropeptide were not identified. In Paper II, the dimer of α_{S2}-CN was detected when non reduced casein was applied to the 2-DGE-gels. However, the sensitivity was improved at reduced conditions and some minor spots containing α_{S1}-CN cleavage products could be identified. The

major whey proteins α-LA and β-LG were identified in the sweet whey fraction in addition to some minor proteins: lactoferrin, BSA, immunoglobulin G-λ-light chain, vitamin D-binding protein and lactoforrin/PP3.

No major differences were observed when comparing the milk protein profile before and after pasteurisation. This is shown in the PCA plots for the casein and whey proteins, respectively (Figure 1b and 3b, Paper II), where there were no clear groupings between pasteurised and raw milk samples. When comparing the relative spot volume in the casein fraction before pasteurisation with the corresponding spot volume after pasteurisation, it was shown that four spots containing α_{S1}-CN and one spot containing α_{S2}-CN increased significantly (Table 1, Paper II). The molecular weights of these α_{S1}-CN forms (spot IDs 35, 37, 45 and 48) were higher than the expected mass for α_{S1}-CN (Figure 1a and 2, Paper II). In the whey protein fraction the intensity of six spots were found to increase, whereas seven spots decreased after pasteurisation (Table 3, Paper II). Some of the proteins that decreased in concentration after pasteurisation were identified by MS to contain proteose peptone component 3 (PP3) or lactoforrin. Among the proteins that increased in intensity as a consequence of pasteurisation, one was identified as an α_{S1}-CN fragment (spot ID 93, Table 3 and 4, Paper II).

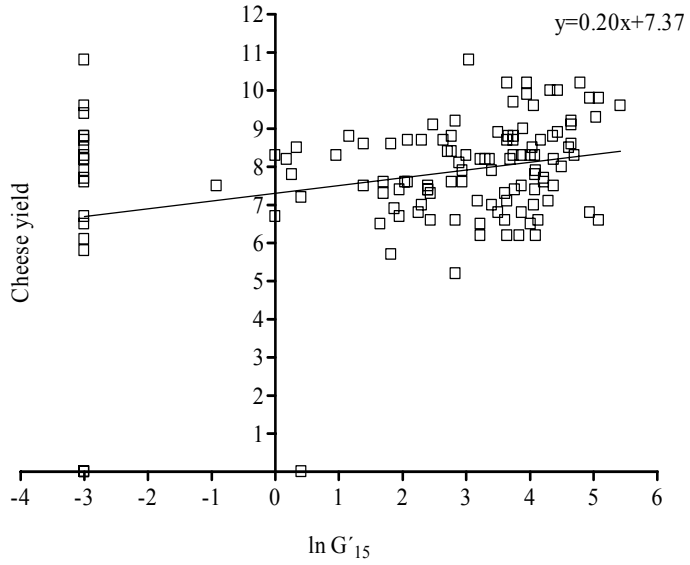
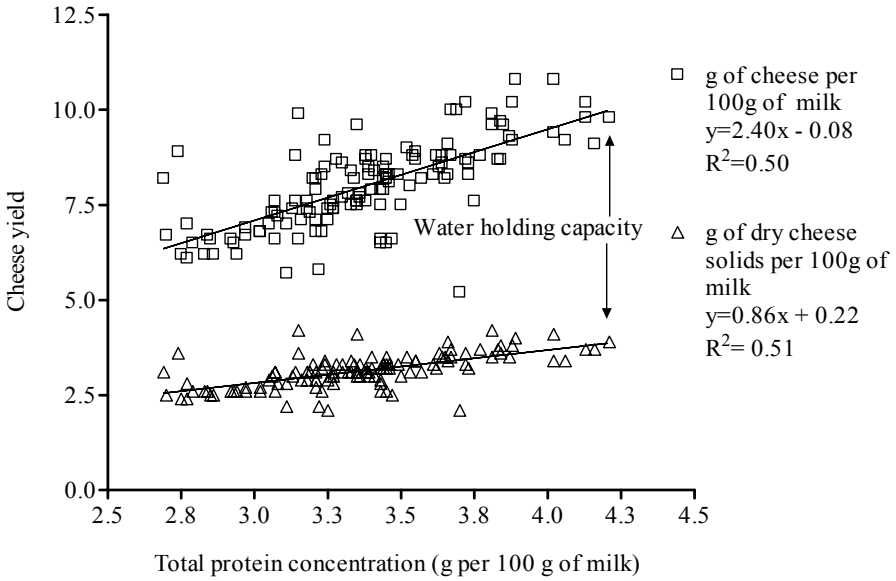


Fig 8. Regression between ln G'_{15} and cheese yield expressed as g of cheese per 100 g of milk

Coagulation properties and cheese yield

lnG'_{15} was significantly correlated with cheese yield ($p<0.001$, results not shown) and cheese yield increased by 0.20 units for each unit increase in lnG'_{15} (Figure 8). However, when comparing average cheese yield obtained from poorly coagulating milk with that from well coagulating milk, only a tendency to significantly higher yield was obtained from well coagulating milk (Table 2, Paper III). For each unit increase in concentration of total protein, cheese yield increased by 2.40 and moisture free cheese yield increased by 0.86 units (Figure 9a). The WHC, referred to as the difference between cheese yield and moisture free cheese yield, increased by 1.49 units per each unit increase in protein concentration (Figure 9b). However, the protein transition number was not significantly correlated with concentration of total protein (results not shown). The coagulation properties varied considerably among the milk samples. As much as 28% of all the samples were poorly coagulating and 3% were non-coagulating. Another 8% had a low clotting rate but developed a firm curd within 30 min.



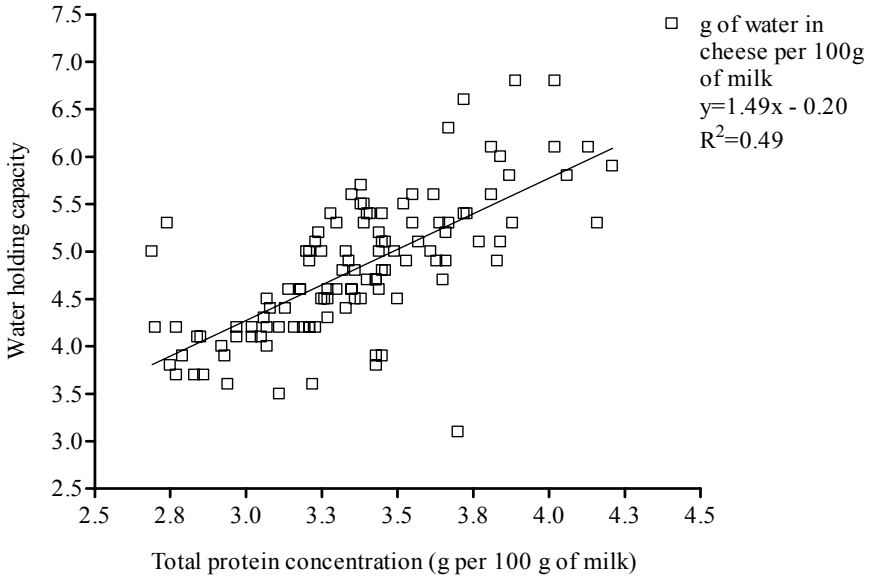


Fig 9 a-b. Regressions between concentration of total protein and cheese yield expressed as g of cheese per 100 g of milk or as g of dry cheese solids per 100 g of milk (a) and as g of water in cheese per 100 g of milk (b).

Effect of major milk protein composition on milk coagulation and cheese yield

All of the individual caseins analysed (α_{S1}-CN, β-CN, κ-CN), as well as total casein and protein concentrations, significantly affected cheese yield and moisture free cheese yield. Of the whey proteins analysed, total β-LG and β-LG B concentrations significantly and positively cheese yield and moisture free cheese yield. In this particular study, the highest cheese yield was obtained from milk sampled in September (sampling number 1), from cows in late lactation and lactation number 1, whereas early lactation and sampling number 4 were associated with lower cheese yield (Figure 1a, Paper III). The protein transition number was improved at increasing concentrations of β-LG B, casein number, β-LG B to total whey protein ratio and α-LA to total whey protein ratio, whereas β-LG A and β-LG A to total whey protein ratio negatively affected this trait (Figure 1b, Paper III). The effect of major milk protein composition on WHC did not differ from that on cheese yield or moisture reduced yield except for the effect of β-LG B to total whey protein ratio, which was not significant for WHC (results not shown). Concentration of κ-CN and κ-CN to total casein ratio were lower in poorly coagulating and non-coagulating milk compared to well coagulating milk (Figure 2, Paper III). Poorly coagulating and non-coagulating milk was also associated with lactation number 1. The κ-CN AE genotype was more frequent within poorly coagulating and non-coagulating milk compared to well coagulating milk (Table 5, Paper III). However, κ-CN concentration did not differ significantly between the AE genotype and the other κ-CN genotypes; AA or AB (Table 5, Paper III). Finally, there were no significant differences in cheese yield, moisture free cheese yield, protein transition number or coagulating properties between the investigated breeds or herds.

Effect of minor proteins or peptides in chymosin-separated whey and casein on cheese yield and protein transition number

The influence of milk protein composition in chymosin-separated whey and casein as determined by 2-DGE and MS detection on cheese yield and protein transition number was evaluated in Paper IV. Identified proteins in the casein fraction with positively significant effects on cheese yield or protein transition number corresponded to minor proteins or protein fragments; e.g. four different forms of α_{S2}-CN, four proteolysis products of α_{S1}-CN, three β-CN forms, a β-CN fragment and BSA (Table 2, 4 and 5, Paper IV). In the whey protein fraction the following proteins were identified to influence cheese yield or protein transition number positively; lactoferrin, a β-LG variant, a β-LG complex and an α-LA form. Several proteins were also identified in the whey protein fraction with a negative effect on cheese yield or protein transition number; a β-LG complex, an α-LA form, BSA, vitamin D-binding protein and immunoglobulin G λ-light chain (Table 3, 4 and 5, Paper IV).

Identification of peptides in acid whey

In the final paper (Paper V), a method for separation of milk peptides in acid whey was optimised on capillary RP-HPLC. Furthermore, as many as possible of the separated peptides, monitored at 280 nm, were identified by MALDI TOF-MS/MS (Table 1, Paper V). The identified peptides, most of them containing aromatic residues, corresponded to fragments of α_{S1}-, α_{S2}- and β-casein. Most of the identified peptides were derived from either the C- or N-terminal regions of the protein molecules. Some of the identified cleavage sites could also be related to earlier identified enzyme specificities to casein substrate demonstrated in model studies (Table 2, Paper V). Suggested enzymes responsible for these cleavages were plasmin, cathepsin G, cathepsin B, cathepsin D and elastase. By use of the capillary RP-HPLC method optimised for separation of peptides it was possible to compare the peptide composition and concentrations in milk with different levels of SCC (Figure 1a-c, Paper V). In milk with normal SCC, i.e. < 50,000 cells per ml, only two peptides were identified whereas the number and concentration of peptides increased considerably in milk with ≥ 500,000 cells per ml.

Discussion

The results from this thesis suggest that there are several variables within the milk protein profile, before and after chymosin separation, with significance for the cheese yielding capacity. It is evident from these results that cheese milk quality cannot be predicted by a very few markers only since the cheese making process is very complex. In the present thesis the protein composition in milk was related to the yield of low fat model cheeses. Instead of homogenising the milk prior to cheese making, the fat was removed, which also reduced the number of variables influencing cheese yield. The model cheeses were of Emmental type and the external factors influencing cheese yield were reduced as much as possible, e.g. pH was controlled by addition of a fixed amount of starter culture, the temperatures were controlled and the time between each step in the cheese making procedure was exactly the same for each cheese. Previously, only a few studies have been made on the milk protein composition in relation to cheese yield from a substantial number of individual milk samples, including the work of Ng Kwai Hang *et al.* (1989) in which 53 individual cows were included. It can therefore be stated that the originality with this thesis is the substantial number of model cheeses (134) that were made from individual milk samples.

The principal component analysis (PCA) gave a general overview of all milk variables analysed in Paper I and III and was a good tool in the initial stage of data evaluation. The PCA score plot (Figure 7) shows the correlations between all milk variables and if the correlations are strong or weak, positive or negative. Significantly correlated variables are oriented in the outer parts of the PCA plot whereas less important variables are concentrated to the plot centre. From the PCA plot in Figure 7 it was clear that lactose, free amino terminals (mM Leucine), pH, urea and SCC were of less importance for the coagulation properties and cheese yield. These variables were therefore not included in the PLS model in Paper III.

The main finding when comparing protein composition in milk from the two most common Swedish dairy breeds, SRB and SLB (Paper I) was the significant difference in casein composition, i.e. the higher concentration of β- and κ-CN in SRB milk. These discrepancies were accompanied with a significantly higher degree of proteolysis in SLB milk, which could explain the lower β-casein concentration in this milk. This assumption was supported by the results from the ANOVA model including the effect of β-CN genotype, i.e. the β-CN concentration differed between the two breeds even though the β-CN genotype was included. The κ-CN concentration, on the other hand, was most probably influenced by different frequencies of κ-CN genotypes among SRB and SLB cows in the current herd, e.g. the κ-CN B allele is associated with a higher κ-CN concentration (Paper III; van den Berg*, et al.*, 1992; Bobe*, et al.*, 1999; Hallén*, et al.*, 2008). In the present investigated herd, the AB genotype was more frequent among SRB compared to SLB cows.

It was expected that protein composition in milk from SLB would be similar to that in SDM milk since they are genetically related, i.e. both are of Holstein

breed. What was surprisingly, though, is that the higher level of protein degradation found in SLB compared to SDM milk did not correspond to a lower β-CN concentration. This may be due to the transport of milk from Sweden to Denmark prior to analysis of free amino terminals. Although the storage period was the same the temperature was unfortunately not fully controlled during the transport from Sweden to Denmark and hence no further conclusions were made from these results. Variations in milk composition between SLB and SDM that were assumed to be herd related were the higher urea concentration in SLB milk and the higher calcium concentration in SDM milk. The higher urea concentration in SLB milk is most probably related to a higher protein to carbohydrate balance given to the Swedish cows (Gustafsson & Carlsson, 1993) whereas the reason for differences in calcium concentration could not be explained.

The major milk proteins and a range of minor proteins and peptides were successfully separated and identified by 2-DGE and MS or MS/MS detection (Paper II and IV). In addition to the major α_{S1}-CN, a range of α_{S1}-CN fragments were identified in Paper II. These were suggested to correspond to cleavage products from chymosin, which is known to cleave α_{S1}-CN at several sites (McSweeney *et al.,* 1993).

Two different staining methods for 2-DGE-gels were used in Paper II and IV, colloidal CBB and silver staining, respectively. The silver staining was more sensitive compared to the colloidal CBB and therefore more suitable for analysis of minor proteins, as was the purpose in Paper IV. On the other hand, CBB is a better choice when analysing major proteins and protein complexes as in Paper II. Furthermore, CBB has a better dynamic range (protein content in relation to spot intensity) compared to silver staining. The lower dynamic range of silver staining complicated the quantification of proteins in the rennet casein fraction in Paper IV. For that reason, it could only be determined whether the presence of a particular protein significantly affected cheese yield or not, i.e. it was not possible to compare relative spot volumes.

Although expected, it was good to confirm that pasteurisation did not influence the milk protein profile considerably. The changes were too small to be observed by the PCA (Figure 1b and 3b, Paper II) and probably the variations between samples were larger than between raw and pasteurised milk. The small changes that were observed within the casein fraction, by statistical t-test, included the increased intensity of some protein spots containing α_{S1}-CN after pasteurisation (Table 1 and 2, Paper II). It is suggested that these changes corresponded to some kind of complex bindings between two α_{S1}-CN molecules since the molecular masses of these forms were higher compared to the major α_{S1}-CN spot (Figure 1a and 2, Paper II). However, the actual cross-links and bonds involved in these complexes could not be explained. Moreover, very small changes in the protein profile occurred within the sweet whey fraction. It is suggested that the decrease in lactophorin/PP3 concentration is due to an increasing association with this protein component to the milk fat globule membrane since it is known to associate with membrane phospholipids (Campagna *et al.,* 2001). However, it is not known

whether these interactions are enhanced due to pasteurisation. Nor is it fully understood why the concentration of a α_{S1}-CN fragment increased in the whey fraction as a consequence of pasteurisation. A hypothesis is that the availability of α_{S1}-CN as a substrate for chymosin increases due to pasteurisation. It has been shown that the mobility of casein molecules located in the micelle core increases at temperatures above 60º C (Rollema & Brinkhuis, 1989). No changes in concentrations of β-LG and κ-casein, as a consequence of heat denaturation of β-LG and aggregation with κ-casein, were observed in this study. This was expected since it earlier has been reported that the whey protein denaturation in pasteurised milk was only about 3-7% (Rynne *et al.*, 2004; Lawrence, 1991).

Measurement of curd firmness is commonly used to predict cheese yield because several workers have found that a firm curd at cutting is significantly correlated with higher cheese yield (Aleandri, Schneider & Buttazzoni, 1989; Riddell-Lawrence & Hicks, 1989; Martin *et al.*, 1997). In this thesis, there was a significant correlation ($p<0.001$) between curd firmness, as measured at 15 min after chymosin addition, and cheese yield. However, there was only a tendency to significant higher average cheese yield ($p<0.055$) obtained from well coagulating than from poorly coagulating milk (Table 2, Paper III). Several of the poorly coagulating milk samples resulted in medium to high cheese yield, see Figure 10. However, it is possible that cheeses obtained from poorly coagulating milk would have obtained poorer texture and sensory properties, which was not examined in this thesis. Johnson, Chen & Jaeggi (2001) found that a weak curd at cutting resulted in harder cheddar cheeses due to a lower water content.

The number of poorly coagulating milk samples found, 28%, was surprisingly high. However, similar results has been reported by Tyrisevä*, et al.* (2004) who found that about 30% of the cows in a Finnish Ayrshire herd produced poorly coagulating milk. In this thesis, the non-coagulating milk samples, 3%, had a lower total calcium concentration (0.10 mg/100g) compared to the average (0.12 mg/100g). This was not statistically verified due to the small number of non-coagulating milk samples found, but it is possible that the calcium concentration will have an impact (Okigbo, 1985), perhaps in combination with a lower κ-CN concentration (see further discussion later). High heritability estimates has been reported for the milk coagulation properties and it has been suggested that genetic parameters might be involved (Ikonen *et al.*, 1999). Recently, a chromosomal region common to several cows that produced non-coagulating milk was identified in Finnish Ayrshire (Elo *et al.,* 2007).

Many results from Paper III are in line with previously reports e.g. the significance of concentration of total casein (Babcock, 1895; McDowall, 1936; Lundstedt, 1978; Gilles & Lawrence, 1985) and the individual caseins (Marziali & Ng-Kwai-Hang, 1986) for the cheese yield, the significance of κ-casein concentration for the coagulation properties (Marziali & Ng-Kwai-Hang, 1985; Auldist *et al.*, 2002), the effect of κ-CN B on κ-CN concentration (McLean, Bruce Graham & Ponzoni, 1984; van den Berg*, et al.*, 1992; Bobe*, et al.*, 1999; Hallén*, et al.*, 2008) and the correlation between β-LG B and casein number (Schaar, Hansson & Pettersson, 1985; van den Berg, *et al.,* 1992; Lundén, Nilsson & Janson, 1997). What is

remarkable though is that the casein number did not affect cheese yield significantly (expressed as g of cheese per 100 g of milk) but did affect the transfer of protein from milk to cheese. Probably, this discrepancy was due to the influence of a number of factors during the cheese making procedure that also affected the water content of cheeses, e.g. variations in the pressure applied at stirring, small pH and temperature fluctuations. However, the effects of each individual casein on cheese yield was higher than the effect of β-LG indicating that the caseins were more important than the whey proteins. Moreover the significant effect of β-LG B, which correlated with the casein number (Figure 9), supported this finding. Newertheless, the impact of casein number on cheese yield is indirectly shown by the significant correlation between casein number and curd firmness (Ikonen, Ojala & Syvaoja, 1997), which in turn is positively correlated with higher cheese yield (Aleandri, Schneider & Buttazzoni, 1989). Decreasing cheese yield has also been related to low casein to true protein ratios as a result of increasing somatic cell count and milk proteolysis (Barbano, Rasmussen & Lynch, 1991; Barbano, 1994).

The milk composition explained only a small part of the total variation in coagulation properties, which is supported by Auldist*, et al.* (2002 and 2004). This indicated that several factors influence the coagulation properties that were not measured in Paper III. One important variable could be the proportion of colloidal calcium phosphate (undissolved) to free calcium in milk since a higher amount of micelle bond calcium is favourable for the milk coagulation (Walstra, Wouters & Geurts, 2006). The total calcium concentration was measured but did not influence the coagulation properties significantly (Figure 2, Paper IV). However, the calcium concentration significantly affected the cheese yield (Figure 1, Paper IV). In Paper IV it is stated: "the significant effect of calcium concentration on cheese yield was very low", which is wrong. Instead, it should have been stated that: "the calcium concentration had a high effect on cheese yield compared to the other milk variables". The reason for this misinterpretation of the results was due to an error in the PLS analysis; the calcium concentration was not standardised (centred: $\mu=0$ and normalized: 1/SD) as the rest of the variables.

The concentration of κ-CN in relation to total casein was significantly lower in poorly and non-coagulating milk. This ratio was one of the most important variables analysed for the coagulating properties in Paper III and the findings were supported by the results of St-Gelais & Hache (2005). Furthermore, the κ-CN concentration was higher in milk from AB than from AA cows, whereas no differences were observed between AA and AE or between AE and AB cows (Table 5, Paper III). However, the frequency of κ-CN AE was higher in poorly coagulating milk compared to well coagulating milk (Table 5, Paper III), suggesting that the E-allele of κ-CN influenced the coagulation properties in some other way than solely through the κ-CN concentration. A significant negative effect of κ-CN E on the milk coagulation properties has earlier been demonstrated by Ikonen *et al*. (1999) and Hallén *et al*. (2007).

Minor proteins or protein fragments identified in the chymosin-separated whey and casein fractions of individual milk samples had low individual effects on

cheese yield or protein transition number. There were additional protein spots on the 2-DGE-gels with significant effects that could not be verified by MS or MS/MS. However, it has to be stressed that these spots also contributed to the total variation in cheese yield or protein transition number. For the casein fraction it could only be stated whether the presence of a particular protein was important or not, which was due to the discriminant PLS model used, as described in Paper IV. Hence, all of the proteins identified with significant effect on cheese yield or protein transition number corresponded to minor proteins, protein variants (different phosphorylated forms or genetic variants) or protein fragments. The responsible proteolytic enzymes for the different casein cleavage products could not be verified. Possibly some of the α_{S1}- or α_{S2}-CN fragments were derived from cleavage by chymosin. Chymosin is known to initially cleave α_{S1}-CN to α_{S1}-I (f25-199) and later to α_{S1}-II (f25-169) and further products (Fox, 1988). McSweeney *et al.* (1994) showed that chymosin was able to cleave α_{S2}-CN at 8 sites in the hydrophobic regions of the molecule.

The β-CN identified in spot number 40 (Table 5, Paper IV) probably corresponded to a genetic variant of β-CN or a different phosphorylated form. This is because it had a different pI but a similar molecular mass compared to the major β-casein spot (Figure 1, Paper IV). Beta-CN identified in spot IDs 51 and 57 (Figure 1 and Table 5, Paper VI) both had a somewhat lower molecular weights than the major β-CN spot. However, spot ID 51 was located in a different pI region (around pI 6.3) compared to spot ID 57 (around pI 4.9) indicating that their degree in phosphorylation and/or amino acid sequences differed in some way. This could, however, not be verified from the MS analysis because neither the complete amino acid sequence nor all the phosphorylated residues were identified. Earlier, it has been concluded that milk from β-CN AB (A^1B or A^2B) cows produced milk with higher concentrations of β-CN and total casein (McLean, Bruce Graham & Ponzoni, 1984; Ng-Kwai-Hang *et al.*, 1984a; Hallén *et al.*, 2008), which will improve the cheese milk quality. Therefore, possibly the different β-CN variants identified in Paper IV corresponded to A^1B or A^2B.

The β-LG variant identified in the sweet whey fraction, spot ID 359 (Table 3 and figure 2, Paper IV), with a positive effect on cheese yield was located in a higher pI region on the 2-DGE gel and thereby exhibited a more positively net charge. Most probably, this spot corresponded to the B-variant of β-LG because the A-variant, due to an extra aspartic acid residue at position number 64 (Presnell *et al.*, 1990) is more negative charged. The positive effect of β-LG B on cheese yield was also reported in Paper III and is indirectly due to a higher casein number (Schaar, Hansson & Pettersson, 1985; van den Berg *et al.*, 1992; Lundén, Nilsson & Janson, 1997).

The significant effects of BSA, α-LA, lactoferrin, immunoglobulin G λ-light chain or vitamin D-binding protein on cheese yield or protein transition number were most probably indirectly due to variations in concentration of other whey proteins. Earlier, it has been suggested that α-LA influences cheese yield indirectly through the concentration of β-LG; i.e. a lower concentration of β-LG, which is associated

with a higher casein concentration, is compensated by a higher α-LA concentration (Marziali & Ng-Kwai-Hang, 1986; Kroeker *et al.*, 1985).

The final paper of this thesis (Paper V) aimed to establish a method for separation and identification of small milk peptides (> 10 kDa), i.e. peptides that were not retained on the 2-DGE gels. Initially, the aim was to relate these peptides to the cheese yield in the same way as in Paper III and IV. However, concentrations of peptides in milk with normal SCC (< 100,000 cells per ml) were found to be very low and the variations between individual milk samples were difficult to observe. Instead, a qualitative study on the variation in peptide profile between milk samples with different SCC, normal to high, was carried out. The peptides, as separated by capillary RP-HPLC, were monitored at 280 nm since the chromatogram obtained at 214 nm was too complex and some of the peaks were found by MS/MS to contain non-protein components.

In Paper V the suggested enzyme specificities to the identified peptides derived from α_{S1}-, α_{S2}-, and β-CN (Table 2, Paper 5) were based on previously published specificities to these cleavage sites (Le Bars & Gripon, 1989; 1993; McSweeney, Fox & Olson, 1995; Larsen *et al.*, 1996; Considine *et al.*, 1999; 2000; 2002; 2004). The studies referred to here were all based on model studies, i.e. the enzyme specificities were not studied in milk but in fractionated casein (α_{S1}-, α_{S2}-, and β-CN) solutions. It has to be stressed that the hydrolysis pattern in model systems and in milk can differ to some degree (Fox & McSweeney, 1998) e.g. due to differences in pH and salt concentration (Fox & Guiney, 1973; Lane & Fox, 1999). These differences are most probably one of the reasons why the cleavage pattern observed in Paper V was much narrower compared to the model studies. Another reason was that the fractionation monitored at 280 nm almost solely results in a selection of peptides containing aromatic residues (Trp or Phe), which limits the number of fractionated peptides.

The results from this final study (Paper V) showed that both the concentration and number of detected peptides in raw milk increased with SCC. The increasing number of peptides further indicated that more protease types with different specificities became involved in the proteolysis occurring at increased cell count. This was also demonstrated by the MS detection of the separated peptides and the assignment of possible responsible proteases for the different cleavage sites, including plasmin, elastase and cathepsins B, D and G.

One important result from Paper V was that some of the identified milk peptides could be linked to the enzyme activity of cathepsin B and elastase (Table 2, Paper V), which earlier mainly has been studied in model systems (Considine *et al.*, 1999; 2000; 2004). It has been found to be difficult to detect elastase activity in milk, possibly due to the complex binding between elastase and its inhibitor (Christensen & Sottrup-Jensen, 1994; Kelly, O´Flaherty & Fox, 2006). One of the cleavage sites identified in high SCC milk, > 500,000 cells per ml, agreed with an earlier identified cleavage site for cathepsin D. This was the only site that could be assigned cathepsin D activity. However, since cathepsin B and elastase could also be responsible for this cleavage (Table 2, Paper V) it was not established. As

expected, peptides potentially derived from the activity of elastase or cathepsin B were only detected in high SCC milk (Figure 1 and Table 2, Paper V), whereas the peptides identified in normal SCC milk (50,000 cells/ml most probably resulted after cleavage by plasmin (Le Bars & Gripon, 1993; Weinstein & Doolittle, 1972). One of the cleavage sites identified in milk with normal SCC milk agreed with an earlier identified cleavage site of cathepsin G (Figure 1 and Table 2, Paper V). However, it is unlikely that cathepsin G would be active in milk with normal SCC and this was therefore suggested to be a plasmin cleavage site.

The occurrence of somatic cell proteases (e.g. cysteine proteases, cathepsin D and elastase) in sub-clinical milk (e.g. milk containing 500,000 cells/ml) could be a problem for the quality of bulk milk after mixing good quality milk with sub-clinical milk at tank and silo levels (Leitner *et al.,* 2008). Even though the sub-clinical milk is diluted in the tank, the proteases can contribute to break-down of the valuable milk protein, especially during storage. It would thus be an advantage if sub-clinical milk could be identified before it reaches the milk tank, which demands further research to evaluate the actual contribution of sub-clinical milk to deterioration of tank milk quality.

Conclusions

- Milk from SRB cows contained higher concentrations of total protein, total casein, β-casein and κ-casein compared to SLB cows in the investigated herd.

- Concentration of urea and degree of protein degradation were significantly higher in milk from the Swedish compared to the Danish herd, whereas α-LA was higher in the Danish milk.

- There was no pronounced effect of pasteurisation on the milk protein profiles in the rennet casein or sweet whey fractions.

- As much as 28% of all the milk samples were poorly coagulating and 3% did not form any curd at all within 30 minutes from rennet addition.

- Poorly coagulating milk was associated with low κ-CN concentration and a low proportion of κ-CN in relation to total casein.

- The κ-CN concentration was higher in milk from AB heterozygote than from AA homozygote cows. No difference in κ-CN concentration was found between the AE- and AA-genotype or between AE and AB.

- The casein number, and indirectly β-LG B, had a positive effect on the transfer of proteins from milk to cheese, whereas β-LG A had a negative effect.

- A 2-DGE-MS method was optimised and the following milk proteins were successfully separated and identified: α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, PP3, α-LA, β-LG, lactoferrin, BSA, immunoglobulin G-λ-light chain and 12 proteolysis products or forms/variants of α_{S2}-CN, α_{S1}-CN, β-CN, β-LG and α-LA.

- One α_{S2}-CN form, three β-CN forms, three proteolysis products from α_{S2}-CN and four proteolysis products from α_{S1}-CN, as identified in the rennet casein fraction, significantly affected the protein transition number.

- A RP-HPLC MS/MS method was optimised for the identification of peptides in milk with different levels of SCC.

- Peptide cleavage sites that corresponded to earlier documented specificities of elastase and cathepsin B were identified in acid whey from high SCC milk, i.e. > 500,000 cells per ml.

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