Coagulation Properties of Milk

Association with Milk Protein Composition and Genetic Polymorphism

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Abstract

Concentrations of the different proteins in milk are important for the outcome of the coagulation processes which yield our dairy products, whereas total milk protein content is a poor indicator of coagulation properties of milk. In order to design the milk protein composition to meet dairy processing requirements, selection for genetic variants of milk proteins have been proposed. This work aimed to study genetic milk protein polymorphism and its association with the detailed milk protein composition, and effects on milk coagulation. Both chymosin-induced (cheese) and acid-induced (fermented milk products) coagulation was considered.

An association of the κ-casein B allele with improved chymosin-induced milk coagulation properties, and a corresponding unfavourable effect of the E allele, was found, probably an effect of the κ-casein concentration. In a cheese-making model it was shown that concentration of κ-casein in milk was important during the initial stages of cheese making, and also to reduce casein losses into whey. After pressing of the curd, a high ratio of casein to total protein and total casein content of milk were important for casein retention in curd and fresh curd yield. Increased casein retention in curd was also associated with the β-lactoglobulin BB genotype, possibly due to the association of this genotype with higher proportion of casein to total protein. A low κ-casein concentration in milk was associated with an increased risk of non-coagulation, a phenomenon that has recently been highlighted.

The concentration of β-lactoglobulin in milk showed a positive influence on curd firmness in acid-induced gels. As the AA and AB genotypes of β-lactoglobulin were associated with increased β-lactoglobulin concentration in milk, milk from cows carrying these genotypes resulted in firmer acid gels compared to BB. There was an effect of β-lactoglobulin genotype also at equal β-lactoglobulin concentrations, possibly due to an improved structure of acid gels of milk from β-lactoglobulin BB cows.

Keywords: milk proteins, genetic polymorphism, milk coagulation, rheological properties, chymosin, acid gel, curd, casein retention, κ-casein, β-lactoglobulin

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Inför Berzelius staty

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Hallén, E., Wedholm, A., Andrén, A. & Lundén, A. (2008). Effect of β-casein, κ-casein and β-lactoglobulin genotypes on concentration of milk protein variants. *Journal of Animal Breeding and Genetics* 125(2), 119-129.

II Hallén, E., Allmere, T., Näslund, J., Andrén, A. & Lundén, A. (2007). Effect of genetic milk protein polymorphism on rheology of chymosin-induced milk gels. *International Dairy Journal* 17(7), 791-799.

III Hallén, E., Lundén, A., Allmere, T. & Andrén, A. Casein retention in curd and loss of casein into whey at chymosin-induced coagulation of milk (submitted).

IV Hallén, E., Lundén A., Westerlind, M. & Andrén, A. Composition of poorly and non-coagulating milk and effect of calcium addition (submitted).

V Hallén, E., Allmere, T., Lundén, A. & Andrén, A. Effect of genetic milk protein polymorphism on rheology of acid-induced milk gels. *International Dairy Journal* (accepted).

Elin Hallén's contribution to the papers:

I Participated in planning of the study and milk sample pre-treatment, extracted DNA from blood samples for genotyping, analysed milk protein composition by RP-HPLC, participated in the statistical analysis and evaluation of results, prepared the manuscript including tables and figures.

II Participated in planning of the study and milk sample pre-treatment, extracted DNA from blood samples for genotyping, determined phenotype by FPLC, performed rheological analysis of milk gels on a Bohlin VOR, participated in the statistical analysis and evaluation of results, prepared the manuscript including tables and figures.

III Participated in planning of the study and milk sample pre-treatment, extracted DNA from blood samples for genotyping, produced micro-cheeses, analysed milk protein composition by RP-HPLC, participated in the statistical analysis and evaluation of results, prepared the manuscript including tables and figures.

IV Participated in planning of the study, extracted DNA from blood samples for genotyping, analysis of milk protein composition by RP-HPLC, measured the casein micelle size by PCS, participated in the statistical analysis and evaluation of results, prepared the manuscript including tables and figures.

V Participated in planning of the study and milk sample pre-treatment, extracted DNA from blood samples for genotyping, performed rheological analysis of milk gels on a Bohlin VOR, analysis of milk protein composition on by RP-HPLC, participated in the statistical analysis and evaluation of results, prepared the manuscript including tables and figures.

Abbreviations

CCP	colloidal calcium phosphate
CMP	caseinomacropeptide
CN	casein
CN ratio	ratio of total casein to total protein
CT	coagulation time
FPLC	fast protein liquid chromatography
G'	elastic modulus, curd firmness
GDL	glucono-δ-lactone
IMCU	international milk clotting units
NC	non-coagulating
RP-HPLC	reversed phase high performance liquid chromatography
SLB	Swedish Holstein breed
SRB H	Swedish Red breed, selection line for high milk fat percentage
SRB L	Swedish Red breed, selection line for low milk fat percentage

Introduction

1.1 Use and composition of milk

Dairy products have been a part of the human diet for thousands of years, with evidence of cheese being made as long as 7,500 years ago.

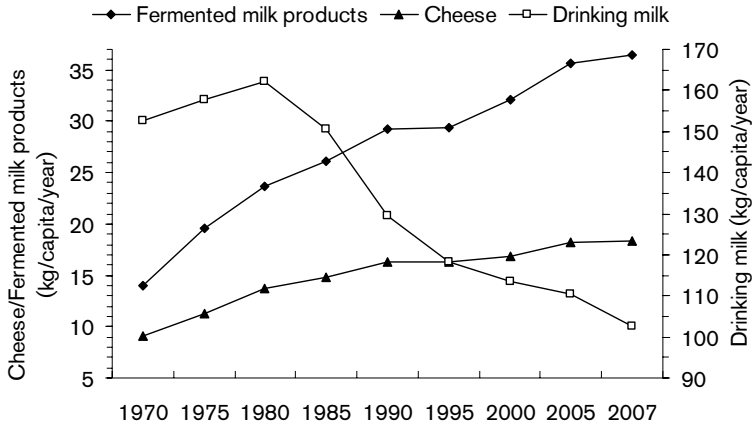

Figure 1. Consumption of dairy products in Sweden (Swedish Dairy Association, 2008)

The Swedish consumption of cheese and fermented milk products (e.g. yoghurt, sour milk 'filmjölk') is showing a positive trend, whereas the consumption of drinking milk, despite a certain boost thanks to the caffè latte trend of recent years, has been decreasing steadily during the past decades (Fig. 1). Properties and yield of dairy products are influenced to a great extent by the amounts and relative proportions of each of the milk constituents. Consequently, as an increasing part of the milk produced is utilised for processed dairy products (Swedish Dairy Association, 2008) milk

constituents such as protein and fat have achieved higher economic significance.

The general process of coagulating liquid milk into dairy products such as cheese and yoghurt/filmjölk is based on the formation of an aggregated protein network, which mainly consists of a certain group of proteins known as caseins. In this network water, fat, and other milk constituents are entrapped. The biochemical processes differ between cheese and fermented milk products, where cheese making involves the separation of casein from whey, whereas in fermented milk products the whole milk is included in the final product.

1.1.1 Milk is milk is milk, all the same?

During the past decades the focus of milk production has been kg's of milk protein, whereas the protein composition, i.e. relative proportions of the different proteins, has not been addressed. A comparison of the Swedish dairy milk 1970 and 1996 (Lindmark-Månsson *et al.*, 2003), showed that although there was no difference in total protein concentration, the proportion of casein in total protein was significantly decreased 1996. A direct effect of a decreased casein level is that a larger quantity of milk is required to make a set amount of cheese. Stagnating cheese yields despite increased total protein concentration of milk has been reported in France (Coulon *et al.*, 2001), accentuating the aspect of milk protein composition in order to provide dairies with milk well suited for dairy products manufacture.

1.1.2 Options for improvement

All of the above indicate that exploring possibilities for improving the protein composition of dairy milk is justified. This has long been a subject of interest for dairy researchers world wide, although examples of practical implementations are scarce. Lack of simple routine analyses to measure e.g. casein content in milk, is one major factor limiting progress in this direction. Genetic variants of milk proteins have been shown to be associated with the protein composition and thereby with the technological properties of milk (Jakob & Puhan, 1992; Martin *et al.*, 2002; Ng-Kwai-Hang, 1998). It is reasonable to assume that the set of milk proteins in today's dairy cows has been somewhat altered due to the efficient selection for milk production and that this may be one part in explaining the changed milk protein composition observed over the past decades. Differences in protein genotype frequencies between native and modern dairy cattle breeds (Lien *et al.*, 1999) may illustrate this development. Information on milk protein genotype

could be utilised in marker assisted selection to improve milk protein composition without having to phenotype large progeny groups. Considering such an option, it would be desirable to gain further knowledge about effects of milk protein genetic variants on milk protein composition and on the coagulation processes of milk which yield our common dairy products.

1.2 Protein composition of milk

Milk is a highly diverse fluid consisting of a vast number of substances, the main ones being water, lactose, fat, protein, organic acids, and minerals. Throughout this thesis focus will be on the protein fraction of milk, a heterogeneous group of molecules where over 200 different types have been characterised (Ng-Kwai-Hang, 2002), of which the six major ones will be considered. Milk proteins are traditionally defined by their solubility at pH 4.6. The precipitate formed when adjusting milk to pH 4.6 is casein, whereas the protein remaining in solution is whey protein, or serum protein. Bovine milk generally contains about 3.5 % protein, of which approximately 80 % are caseins and 20 % are whey proteins (Table 1).

Table 1. *Characteristics of bovine milk proteins*

Protein	Molecular weight (kD)	Conc in milk (g/l)	Amino acid residues			No of PO_4	CH_2O
			Total	Pro	Cys		
α_{S1}-CN	23.6	10.0	199	17	0	8-9	0
α_{S2}-CN	25.2	2.6	207	10	2	10-13	0
β-CN	24.0	9.3	209	35	0	5	0
κ-CN	19.0	3.3	169	20	2	1-3	+
β-LG	18.0	3.2	162	8	5	0	0
α-LA	14.2	1.2	123	2	8	0	0
BSA	66.3	0.4	582	28	35	0	0
Ig	< 1,000.0	0.7		8.4 %	2.3 %	-	+
Others		0.8					

1.2.1 Caseins and the casein micelle

The first method to separate casein was described by Berzelius in 1814. For a long time believed to be one protein, heterogeneity of the casein fraction was first demonstrated in the 1920's and then confirmed using electrophoresis by Mellander in 1939. The three casein components found were called α-CN, β-CN, and γ-CN in order of decreasing electrophoretic mobility. Waugh & von Hippel (1956) used $CaCl_2$ to find that α-CN could be further divided in two fractions named α_s-CN (calcium sensitive) and κ-CN (calcium insensitive). It was later shown that α_s-CN consists of two proteins, α_{s1}- and α_{s2}-CN (Annan & Manson, 1969), and that γ-CN represents the C-terminal segment of β-CN after protelytic cleavage by plasmin (Groves, 1969). Consequently, casein consists of α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN in approximate proportions 4:1:4:1. Synthesised in the mammary gland, post-translational modifications such as phosphorylation, glycosylation, disulphide bonding, proteolysis, and the existence of genetic variants, cause further diversity within the casein group (Ng-Kwai-Hang, 2002).

Caseins show little tertiary or organised secondary structure due to a high proline content (Fox & McSweeney, 1998). This accounts for the stability of caseins against heat denaturation, as there is very little structure to unfold. It also means that they are susceptible to proteolysis without prior denaturation, e.g. by heat or acid. Polar and apolar regions on the casein peptide chains are not uniformly distributed, giving them an amphiphilic structure. This, in addition to their proline and phosphate content, constitutes the basis for the ability of caseins to form micelles. Phosphate groups are esterified to the caseins via the hydroxyl group of serine. These phosphoserine residues bind calcium, which in turn binds colloidal calcium phosphate (CCP). These bonds contribute to linking the caseins together to form micelles. Of the very high calcium content in milk (~1200 mg/l), about half is bound to the casein fraction via CCP. The concentrations of protein and calcium generally found in milk would cause precipitation of α_{s1}-CN, α_{s2}-CN, and β-CN by calcium binding to their phosphoserine residues. The κ-CN, however, is not only soluble in calcium, it interacts with and stabilises the other caseins to initiate formation of micelles and a stable colloidal state (Farrell *et al.*, 2006). Whereas the phosphorylation level in κ-CN is low, the protein is found in several glycosylated forms where the C-terminal part (the caseinomacropeptide; CMP) contains varying numbers of *O*-glucosidic linked residues (Farrell *et al.*, 2004). Further, κ-CN and the other minor casein, α_{s2}-CN, contain two cysteine residues each, which in α_{s2}-CN exist as intermolecular disulphide bonds (Walstra *et al.*, 2006).

In milk about 95 % of the caseins are aggregated in colloidal structures, casein micelles, whose major function is to fluidise the casein molecules and solubilise calcium and phosphate (Farrell *et al.*, 2006). Since the pioneering work of Waugh (1958) several theories of the casein micelle structure have been proposed. Although there is no unanimously accepted model, there are some general properties that are commonly established. These include the notion of partly hydrophobic caseins being stabilised by κ-CN predominantly located near the micelle surface and the integral role of CCP in micelle structure (Fig. 2).

The sub-micelle model (Schmidt, 1982) evolved over several decades and implied the inclusion of either κ-CN rich or κ-CN depleted sub-micellar structures within the micelles (Fig. 2a). CCP clusters and hydrophobic interactions link the sub-micelles together with those rich in κ-CN located at the surface. The hydrophilic and negatively charged C-terminal end of κ-CN protrudes from the micelle, which is open and porous, to form a hairy layer that by steric and electrostatic repulsion prevents any further sub-micelle aggregation and also micelle flocculation (Walstra, 1999).

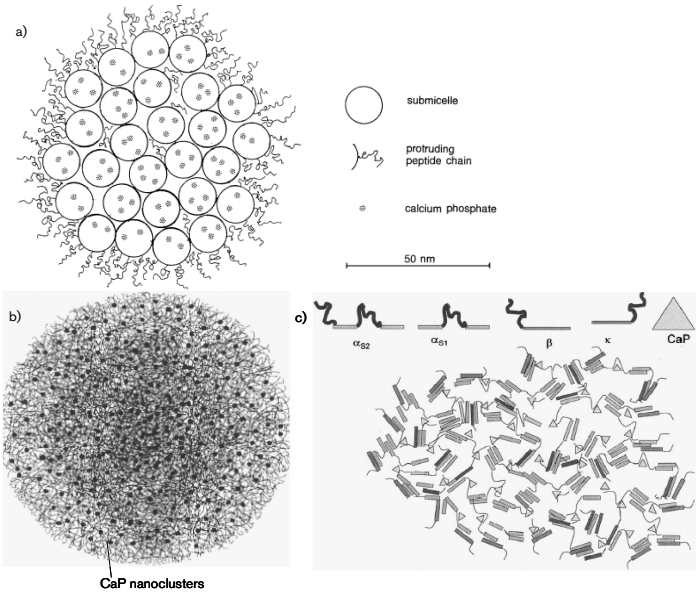

Figure 2. Models of the casein micelle; a) The submicelle model (Walstra, 1999), b) The hairy model (Holt & Horne, 1996), c) The dual-binding model (Horne, 1998).

Although the existence of a stabilising κ-CN layer and the cementing role of CCP are accepted in the model proposed by Holt (1992), the concept of sub-micelles is not. In this model the micelle is regarded as a mineralised, cross-linked protein gel of tangled, flexible casein molecules (Fig. 2b). CCP nanoclusters interact with the phosphoserine residues of the casein molecules and cross-linking and network formation give a micro-gel particle (Holt & Horne, 1996). There is, however, no mechanism suggested to limit micelle growth in this model and there is no role for κ-CN, which does not have a phosphate cluster, nor is there any explanation for the observed surface location of κ-CN (Horne, 2002). In the dual-binding model suggested by Horne (1998), the concept of Holt (1992) is further developed to resolve these issues. Only considering gross hydrophobic interactions of the caseins, micelle stability is suggested to be maintained by excess of hydrophobic attraction over electrostatic repulsion (Farrell *et al.*, 2006). The amphiphilic casein molecules act as block co-polymers that are crosslinked through hydrophobic regions and bridged across CCP nanoclusters (Fig. 2c). Micelle formation yields an internal gel-like structure with nanoclusters of calcium and phosphate. Containing only one phosphoserine residue, micelle growth is limited by κ-CN acting as a dead end capping unit, which becomes part of the micelle surface structure (Horne, 1998). From electron micrographs of individual casein micelles, showing an uneven surface with no coating, Dalgleish *et al.* (2004) suggested that micelles are organised in tubular structures with gaps in between, κ-CN positioned at the ends near the surface.

Average diameter of casein micelles is approximately 120 nm, ranging from 50 to 500 nm (Fox & Brodkorb, 2008). The stabilising function of κ-CN and its role in micelle growth makes it a key protein in determining micelle size and also some functional properties. It has been shown that milk with a high concentration of κ-CN contains smaller micelles compared to milk with lower concentration (Dalgleish *et al.*, 1989; Donnelly *et al.*, 1984; Risso *et al.*, 2007).

1.2.2 Whey proteins

Whey proteins, or serum proteins, share few common characteristics other than being soluble at pH 4.6. The three main proteins are β-LG, α-LA and blood serum albumin (BSA), representing approximately 50, 20 and 10 % of total whey proteins, respectively. The remaining part comprises immunoglobulins (Ig) and trace amounts of several other proteins, including enzymes. Most whey proteins are globular with organised secondary and tertiary structures, which in contrast to the caseins make them sensitive to

heat denaturation at temperatures above 60°C (DeWit & Klarenbeek, 1984). Whey proteins contain a relatively large number of cysteine residues as internal disulphide bonds (Fox & McSweeney, 1998). A reactive thiol group is exposed at heat denaturation of β-LG, which forms disulphide-thiol interchanges with other β-LG molecules as well as with κ-CN (Creamer *et al.*, 2004; Lowe *et al.*, 2004; Sawyer, 1969). In the biosynthesis of lactose α-LA is important as a sub-component in the lactose synthetase complex (Ng-Kwai-Hang, 2002). Whereas α-LA and β-LG are synthesised in the mammary gland, BSA is a major component of blood serum and gains entrance to milk via leakage from the blood. Immunoglobulins (Ig) are complex proteins whose function is to provide various types of immune defence to the organism. The concentration of Ig in milk immediately after parturition can be up to 100 g/l with levels quickly decreasing to about 1 g/l within one week.

1.3 Coagulation of milk

The ability of casein micelles to stay in solution at natural milk pH (~6.7) relies on the net negative charge and hydrophilic character of the C-terminal end of κ-CN at the micelle surface. There are two approaches to induce micelle aggregation; by enzymatic action (cheese) or by acidification (fermented milk products). The outcome of these reactions is to a large extent determined by amounts and proportions of the various components in milk, with the protein composition contributing significantly in this regard. To determine the coagulation properties of a given milk, different traits to describe the process are measured. Coagulation time (CT), defined as the time from addition of coagulant until coagulation starts, and curd firmness at a given time after addition of coagulant, will be used throughout this work.

1.3.1 Enzyme-induced coagulation

Enzymatic coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation (Fox & McSweeney, 1998). Rennet is traditionally extracted from calf abomasa and is a mixture of the two gastric proteases chymosin and pepsin (Andrén, 2002). Chymosin is the major and the most active component, specifically cleaving the peptide bond Phe_{105}-Met_{106} of κ-CN. Chymosin-induced coagulation of milk may be described by three phases. During the primary phase the enzymatic hydrolysis of κ-CN into para-κ-CN and CMP takes place, with the hydrophilic CMP part being released

into the whey. This causes loss of a negatively charged group and decreased steric stabilisation (Senge *et al.*, 1997). When approximately 70 % of the κ-CN is hydrolysed (Walstra *et al.*, 2006), colloidal stability of the micelles is reduced enough for the spontaneous, secondary aggregation phase to start. A gel forms as molecular chains connect through hydrophobic bonds to form a three-dimensional network, followed by further solidification through calcium cross-linking. Finally in the third phase whey is expelled from the casein network by syneresis (more contraction through cross-links). Coagulation is enhanced by decreasing pH, increasing calcium concentration and temperature (no aggregation below 20°C). Syneresis is augmented by increasing temperature, pH and applied pressure, e.g. stirring.

1.3.2 Acid-induced coagulation

At acid coagulation of milk, casein micelle properties are altered by a lowered milk pH (Lucey & Singh, 1997). This causes CCP to dissociate from the micelles and the negative charges in the casein micelles are neutralised, with aggregation occuring as the isoelectric point of the casein micelle (pH 4.6) is approached. A porous network of loosely linked aggregates is formed.

Milk used in manufacture of fermented milk products is generally subjected to a quite severe heat treatment (90°C, 5-10 min), with a marked effect on the end product. Temperatures above 60°C cause denaturation of whey proteins (mainly β-LG), which via disulphide bonds either associate with κ-CN on the casein micelles (McKenzie *et al.*, 1971; Sawyer, 1969) or form soluble aggregates (Guyomarc'h *et al.*, 2003a; Haque & Kinsella, 1988). This results in increased curd firmess (Dannenberg & Kessler, 1988a) due to an increased number and strength of bonds of the acid gel, as denatured whey proteins associated with casein micelles interact with each other (Lucey & Singh, 1997). Further, the concentration of protein in the gel network will be increased because of the active participation of denatured whey protein in structure formation.

1.3.3 Factors influencing coagulation of milk

Coagulation of milk is a complex process, influenced by many different factors. The most obvious are pH, calcium content and temperature. Decreasing the pH and increasing the temperature will decrease the coagulation time. Regarding calcium, the coagulation reaction is favoured both by increased levels of bound calcium (CCP) and free calcium ions. Adding calcium to the milk will increase these levels in addition to lowering pH. Many factors are intertwined and the milk protein fraction, which has

different effects on the coagulation properties and will be discussed more below, can vary with the presence of different genetic variants, but there are also effects of breed (Auldist *et al.*, 2004; Chiofalo *et al.*, 2000; Malossini *et al.*, 1996), stage of lactation (Ostersen *et al.*, 1997), parity (Lindström *et al.*, 1984; Schaar, 1984), season and feeding (Christian *et al.*, 1995; Macheboeuf *et al.*, 1993), and cow health (Grandison & Ford, 1986). Milk from cows with mastitis is associated with a high pH and low levels of casein (Barbano *et al.*, 1991; Larsen *et al.*, 2004; Urech *et al.*, 1999) and has been suggested to have negative effects e.g. for the manufacture of cheese (Barbano *et al.*, 1991; Leitner *et al.*, 2006).

Milk that does not coagulate in the presence of chymosin has puzzled researchers at least since the 1920's (Cassandro *et al.*, 2008; Claesson, 1965; Comin *et al.*, 2008; Ikonen *et al.*, 1999a; Ikonen *et al.*, 2004; Jõudu *et al.*, 2007; Koestler, 1925; Losi *et al.*, 1982; Okigbo *et al.*, 1985c; Tervala & Antila, 1985; Wedholm *et al.*, 2006b). The causes of non-coagulating (NC) milk are not fully understood, largely due to the still elusive structure of the casein micelle and the complexity of the milk coagulation process with its numerous controlling factors. However, it has been recognised that in addition to NC milk that can be explained by cows being in very late lactation (Flüeler, 1978; Okigbo *et al.*, 1985c) or having mastitis (Koestler, 1925; Okigbo *et al.*, 1985a), the phenomenon cannot be fully explained by environmental factors as it is prevalent also in healthy cows in mid lactation (Ikonen *et al.*, 2004; Tyrisevä *et al.*, 2003). Ikonen *et al.* (1999a) observed large differences between breeding bulls in their proportion of daughters producing NC milk and suggested that the underlying cause was partly genetic. Recently, two candidate genes associated with NC milk were identified (Tyrisevä *et al.*, 2008). A genetic disposition to produce NC milk does not exclude significant influences of environmental factors. It has been shown that addition of calcium will restore coagulation of NC milk (van Hooydonk *et al.*, 1986), although not to the level of well coagulating milk (Okigbo *et al.*, 1985b).

1.4 Genetic polymorphism of milk proteins

Genetic polymorphism can be defined as the existence, in a population, of two or more alternative nucleotides at a given position in the genome. Single nucleotide substitutions in regulatory sequences of a gene may give rise to quantitative differences in gene product (Ehrmann *et al.*, 1997a; Lum *et al.*, 1997; Robitaille *et al.*, 2005; Szymanowska *et al.*, 2004), whereas if substitutions take place in coding sequences of a gene this may give rise to

amino acid shifts. Structural variants of a protein are caused by mutations leading to substitution or deletion of one or several amino acids along the polypeptide chain. First discovered in 1955 for β-LG (Aschaffenburg & Drewry), polymorphism has since then been established in all major milk proteins (Aschaffenburg, 1961; Blumber & Tombs, 1958; Grosclaude *et al.*, 1976; Neelin, 1964; Thompson *et al.*, 1962). Amino acid substitutions in common polymorphs (variants) of α_{s1}-CN, β-CN, κ-CN and β-LG are presented in Table 2. Variant B of α_{s1}-CN carries one more negative charge than variant C via the substitution of Gly for Glu. With two positively charged amino acid residues (Arg and His), the B variant of β-CN has one and two more net positive charges than A^1 and A^2, respectively. The substitutions between variants A, B and E of κ-CN are located around midway along the CMP, where two polar residues in κ-CN A and E, Thr_{136} and Asp_{148}, are replaced by the hydrophobic Ile and Ala, respectively, in κ-CN B. The E variant also has Ser at position 155 substituted for Gly. Presence of Asp in β-LG A means that this variant is more negatively charged than β-LG B. The α_{s2}-CN and α-LA proteins have previously been shown to be essentially monomorphic in Western dairy breeds (Aschaffenburg, 1968; Farrell *et al.*, 2004), and variation at the α_{s1}-CN locus is found only in the SLB breed with high frequencies (0.855) of the allele coding for the B variant of the protein (Lundén *et al.*, 1997). Hence, genetic variants of α_{s1}-CN, α_{s2}-CN or α-LA were not considered in this work.

Table 2. *Amino acid substitutions in milk protein genetic variants found in Swedish dairy cattle*

Protein	Variant	Position and amino acid in the protein variant		
α_{s1}-CN	B	192 Glu		
	C	192 Gly		
β-CN	A^1	67 His	106 His	122 Ser
	A^2	67 Pro	106 His	122 Ser
	A^3	67 Pro	106 Gln	122 Ser
	B	67 His	106 His	122 Arg
κ-CN	A	136 Thr	148 Asp	155 Ser
	B	136 Ile	148 Ala	155 Ser
	E	136 Thr	148 Asp	155 Gly
β-LG	A	64 Asp		118 Val
	B	64 Gly		118 Ala

In addition to differences in the phenotypic expression of the genetic mutation, the physico-chemical properties may differ between the protein polymorphs. It has been suggested that the repulsive forces between casein micelles containing variants such as α_{s1}-CN C, β-CN B and κ-CN B, in which amino acid substitution results in lower net negative charge, are decreased compared to micelles containing more negatively charged protein variants (McLean, 1986). This would thus facilitate aggregation. Since the variant substitutions in κ-CN are all found in the CMP (C-terminal) part of the protein, which is split off during the enzymatic coagulation phase, these mutations can be of no importance during the aggregation process, i.e. have no impact on curd firming. Coagulation time, i.e. cleavage of the Phe_{105}-Met_{106} bond in κ-CN by chymosin, can however be affected by the charge differences between variants as mentioned above. Milk containing different genetic variants have also been shown to yield gels with an altered structure, due to different bonding and cross-linking patterns (Nuyts-Petit *et al.*, 1997; Walsh *et al.*, 1998).

Apart from effects of polymorphism in the coding part of the gene on the resulting protein structure, polymorphism in the non-coding regions of milk protein genes is believed to affect protein transcription (Ehrmann *et al.*, 1997a; Lum *et al.*, 1997; Robitaille *et al.*, 2005; Szymanowska *et al.*, 2004). Because of the close linkage of the casein loci on bovine chromosome VI (Ferretti *et al.*, 1990; Threadgill & Womack, 1990), the alleles of the different caseins are in linkage disequilibrium indicating shared DNA regions controlling protein synthesis. An epistatic effect of primarily the β-CN locus on κ-CN content was indicated by Graml & Pirchner (2003). Also, due to the linkage disequilibrium the different casein alleles are not expressed against a random combination of alleles at the linked loci. Consequently, aggregate casein genotypes should be considered when estimating genotypic effects.

1.4.1 Protein polymorphism and protein composition of milk

In general, the B allele of κ-CN has been associated with a higher κ-CN concentration in milk compared to A (Aaltonen & Antila, 1987; Bobe *et al.*, 1999; Ehrmann *et al.*, 1997b; Graml & Pirchner, 2003; Ikonen *et al.*, 1997; Lodes *et al.*, 1997; Mayer *et al.*, 1997; McLean *et al.*, 1984; van Eenennaam & Medrano, 1991), and also with higher total protein and CN ratio. The E allele has been associated with a lower κ-CN content compared to B, possibly also to A (Ikonen *et al.*, 1997; Oloffs *et al.*, 1992). Cows carrying the β-CN B allele have been reported to produce milk with increased total protein and β-CN concentrations (McLean *et al.*, 1984; Ng-Kwai-Hang *et*

al., 1986) and it has been shown that the amount of protein and casein decrease in the order $A^1A^1 > A^1A^2 > A^2A^2$ (Jakob & Puhan, 1994; Ng-Kwai-Hang *et al.*, 1986). The B variant of β-LG has been shown to be expressed at a markedly lower level in milk compared to the A variant, with a concomitant increase in CN ratio (Braunschweig & Leeb, 2006; Ehrmann *et al.*, 1997b; Ford *et al.*, 1993; Lodes *et al.*, 1997; Lundén *et al.*, 1997; Mayer *et al.*, 1997; Ng-Kwai-Hang & Kim, 1996).

1.4.2 Protein polymorphism and coagulation properties of milk

The influence of milk protein variants on coagulation properties of milk is often due to their association with an altered protein composition. Consequently, regarding chymosin-induced coagulation has the κ-CN B allele in numerous studies been associated with the most favourable properties (Davoli *et al.*, 1990; Ikonen *et al.*, 1997; Mayer *et al.*, 1997; Pagnacco & Caroli, 1987; Schaar, 1984; van den Berg *et al.*, 1992), whereas κ-CN A has been associated with longer coagulation times and softer curds. Poorest milk coagulation properties has been ascribed to the κ-CN E allele (Caroli *et al.*, 2000; Ikonen *et al.*, 1999a; Lodes *et al.*, 1996; Oloffs *et al.*, 1992). These effects of the different variants with coagulation properties of milk are also found regarding cheese yield (Rahali & Ménard, 1991; Schaar *et al.*, 1985; Walsh *et al.*, 1995; Walsh *et al.*, 1998; van den Berg *et al.*, 1992).

Also at the β-CN locus has the B allele been linked to an improved coagulation compared to the A variants. Higher protein recovery in cheese has been reported for β-CN A^1A^1 compared to A^1A^2 (Marziali & Ng-Kwai-Hang, 1986), and for β-CN A^2B compared to β-casein A^2A^2 (Mayer *et al.*, 1997).

Although β-LG itself is not involved in the enzymatic process of coagulation of unheated milk, it has been shown that the genetic variants of β-LG may be affecting coagulation properties of raw milk (Ng-Kwai-Hang *et al.*, 2002). Furthermore, β-LG B has been reported to be associated with a higher cheese yield than β-LG A (Lodes *et al.*, 1996; Schaar *et al.*, 1985; van den Berg *et al.*, 1992). This may partly be due to the association of the β-LG genotype with casein content of milk.

At acid-induced coagulation association rates for the heat-induced reaction between β-LG and κ-CN have been determined for different genetic variants, where it was more rapid in milk from cows homozygous for the B alleles of both proteins compared to in milk from cows carrying the A alleles (Allmere *et al.*, 1998b). The B allele of β-LG was also associated with acid gels with a higher firmness, whereas the A and B alleles of κ-CN

showed no significant effect (Allmere *et al.*, 1998a). A higher heat stability has been shown for β-LG A compared to B (Dannenberg & Kessler, 1988b; Manderson *et al.*, 1999; Manderson *et al.*, 1998; McKenzie *et al.*, 1971), and the B variant has been shown to form firmer acid gels with a more cross-linked network compared to A (Graveland-Bikker & Anema, 2003).

1.5 Considerations for improved coagulation of milk

Factors influencing the composition of cow's milk and thus the manufacturing properties can be of environmental nature, e.g. feeding, stage of lactation, parity, but also of genetic origin, e.g. milk protein variants. Since genetic variants show a Mendelian mode of inheritance, it is possible to select dairy cattle regardless of sex for desired milk protein variants.

Genetic improvement of coagulating properties of milk by direct selection for these traits has been suggested (Bittante *et al.*, 2002; Caroli *et al.*, 1990; Cassandro *et al.*, 2008; Ikonen *et al.*, 1999a; Tyrisevä, 2008). This would mean that the coagulation of milk for each cow needs to be measured perhaps three times during one lactation (Tyrisevä, 2008), for a proper evaluation to be made. Although new and more automated measuring techniques for milk coagulation are emerging (Dal Zotto *et al.*, 2008; Klandar *et al.*, 2007), this is still a very laborious task. Therefore, the lack of an appropriate high-throughput analysis for routine determination of milk coagulation is limiting this possibility at present. Indirect selection via milk protein genetic variants associated with increased levels of desirable proteins or protein fractions can however readily be applied by genotyping. Selection on the κ-CN locus to improve coagulation properties of milk is a viable option, e.g. selection against the E allele (Ojala *et al.*, 2005).

The rather frequent occurrence of NC milk among Finnish Ayshire cows (about 10 %) has driven research in this area in Finland (Ikonen, 2000; Tyrisevä, 2008). It has been suggested that selection on the κ-CN locus would probably not reduce the prevalence of NC milk (Ikonen *et al.*, 1999a). However, the finding of candidate genes for non-coagulation of milk (Tyrisevä *et al.*, 2008) may present new possibilities for genetic selection regarding milk coagulation.

The coagulation ability of milk is essential for the manufacture of both cheese and fermented products. However, as different parts of the protein fraction are important at enzyme and acid induced coagulation, it is possible that the ideal protein composition differ depending on which product the milk is intended for.

2 Aims

The overall aim of the work presented in this thesis was to gain further knowledge about genetic milk protein polymorphism and its association with detailed milk protein composition, and the effect this may have on milk quality in terms of coagulation. Both chymosin-induced coagulation, which is the base for cheese production, and acid-induced coagulation, which is utilised for fermented milk products manufacture was considered.

Specific aims were to:

Study associations of genetic polymorphism of β-CN, κ-CN and β-LG with detailed protein composition of milk (paper I).

Study effects of genetic polymorphism of β-CN, κ-CN and β-LG on chymosin-induced coagulation of milk (paper II).

Study how milk protein composition and the genetic polymorphism of milk proteins were associated with retention of casein in curd at chymosin-induced coagulation (paper III).

Characterise the composition of milk with poor coagulating properties compared to well coagulating milk, and study the effect of calcium addition on poor coagulating milk (paper IV).

Study effects of milk composition and genetic polymorphism of β-CN, κ-CN and β-LG on acid-induced coagulation of milk (paper V).

3 Materials & Methods

3.1 Cows and milk samples

Individual morning milk samples were collected from cows of Swedish Holstein (SLB) and Swedish Red (SRB) breeds belonging to Jälla experimental dairy herd of the Swedish University of Agricultural Sciences (Uppsala, Sweden). SRB cows belonged either to a selection line for high milk fat percentage (SRB H) or to a selection line for low milk fat percentage (SRB L), but with equivalent total milk energy production in both lines. For paper IV, samples were also collected from SRB cows belonging to Kungsängen experimental dairy herd at the Swedish University of Agricultural Sciences (Uppsala, Sweden). To reduce potential effects of lactation stage or mastitis, samples were collected from cows in mid-lactation (lactation week 9-35) and an upper limit of $300 \star 10^3$ was set for SCC (somatic cell count). Milk samples were treated with 2 μl/ml of a 17 % (w/v) Bronopol solution (Boots Microcheck, Nottingham, England) at sampling (paper II, IV, V), cooled and kept at 4°C. Samples were defatted prior to all further analyses by pre-warming (30°C, 30 min) followed by centrifugation (2465 x **g**, 3°C, 25 min) (Centrifuge 5810R, Eppendorf AG, Hamburg, Germany). Information regarding morning milk yield, parity number, lactation week, and time of sampling was known for each sample.

3.1.1 Typing of milk protein variants

Typing for variants of the β-CN (A^1, A^2, A^3, B) and κ-CN (A, B, E) genes was carried out as described in detail in paper II. Briefly, Pyrosequencing™ (Biotage AB, Uppsala, Sweden) was used, a PCR method based on real-time sequencing where a detection primer is hybridised onto an amplified fragment containing the polymorphism of interest. Genetic variants of the β-

LG gene (A, B) were derived from chromatograms of RP-HPLC (reversed phase high performance liquid chromatography) analysis. In paper II, ion exchange FPLC (fast protein liquid chromatography; ÄKTA*FPLC*™, GE Healthcare, Uppsala, Sweden) of whey was used to phenotype cows for β-LG variants, as the RP-HPLC method was not set up at that time. A genotype analysis for β-LG variant was performed on a few samples to verify the FPLC/HPLC phenotyping results.

3.2 Milk analyses

All analyses were performed on fresh milk stored at 4°C, except milk protein composition, calcium concentration and casein micelle size, which were analysed in samples stored at -80°C.

3.2.1 Gross composition

Protein, fat and lactose concentrations of milk were analysed by MIR (mid-infrared) spectroscopy (MilkoScan FT120; A/S Foss Electric, Hillerød, Denmark), and SCC by electronic fluorescence based cell counting (Fossomatic 5200; A/S Foss Electric) (paper I-V). Total calcium concentration of milk (paper IV) was analysed using inductively coupled plasma spectroscopy at Steins Laboratorium AB (Löddeköpinge, Sweden).

3.2.2 Milk protein composition

Concentrations of the six major proteins in milk (α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, β-LG, α-LA) were analysed by RP-HPLC (paper I, III-V) with a method modified from Bordin *et al.* (2001). The chromatographic system used was D-7000 from Merck-Hitachi (Darmstadt, Germany) fitted with pump L-7100, auto injector L-7200, UV-detector L-7400 and the software D-7000 HPLC System Manager (HSM v 4.0). Buffer A and buffer B were composed of water, acetonitrile and TFA (triflouro acetic acid) in proportions 900:100:1 (v/v/v) and 100:900:1 (v/v/v), respectively. Separations were carried out at ambient temperature and flow rate 0.300 ml/min on a BioBasic-4 C_4 column (Thermo Electron Corporation, Runcorn, UK) with 150 x 3 mm I.D., 300 Å pore diameter and 5 μm particle size. Solutions of purified protein standards (Sigma-Aldrich, Steinheim, Germany) were used for peak identification and determination of absorption coefficient of each protein. The eluting gradient was: 26 to 36 % buffer B in 10 min, isocratic elution at 36 % B for 10 min, 36 to 43 % B in 13 min, isocratic elution at 43 % B for 6 min, 43 to 50 % B in 11 min, isocratic elution at 50 % B for 10 min, 50 to 28 % B in 1 min and finally

column equilibration at 28 % B for 14 min. Each chromatographic sample run was 75 min and milk/whey samples were prepared daily. Samples were diluted 1:5 in a reducing buffer (8 M urea, 20 mM DTT; dithiotreitol) and left to stand one hour in room temperature before dilution 1:3 in buffer A containing 6 M urea. Injection volume was 20 µl for milk samples and 40 µl for whey samples.

Concentration of major proteins was calculated as the sum of concentrations of the individual proteins (α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, β-LG and α-LA). Total casein concentration was calculated as the sum of concentrations of the individual caseins (α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN). Casein (CN) ratio was calculated as total casein concentration divided by concentration of major proteins.

3.2.3 Casein micelle size

Mean size of casein micelles (paper IV) was determined by PCS (photon correlation spectroscopy) (ZetaSizer 3000HS; Malvern Instruments Ltd, Malvern, UK). Samples were defrosted 40 min at room temperature, followed by incubation in a water bath (25°C, 20 min). After dilution 1:1000 in SMUF (simulated milk ultra filtrate) (Jenness & Koops, 1962), samples were further incubated (25°C, 20 min) before analysis. Unimodal mode was used and analyses were performed at room temperature, scattering angle 90° and wave length λ=633 nm. After a delay of 90 s, each sample was run in triplicate, sample time was set auto.

3.2.4 Coagulating agents

Chromatographically pure chymosin was used in paper II and III, prepared as described by Andrén *et al.* (1980) with a total milk clotting activity of 174,000 IMCU/g (international milk clotting units) (IDF, 1997). Working solutions of 0.4 mg/ml (paper II) and 1.5 mg/ml (paper III) were prepared in a 0.1 M phosphate buffer (pH 5.7). Chymax Plus (Christian Hansen A/S, Hørsholm, Denmark), 200 IMCU/ml, was used in paper IV.

The gradual pH decrease of a starter culture was imitated by adding 1.5 % GDL (glucono-δ-lactone) to the milk samples in paper V.

3.2.5 Rheological measurements

Coagulating properties of milk were analysed (paper II, IV, V) using a Bohlin VOR Rheometer (Malvern Instruments Nordic AB, Uppsala, Sweden) fitted with a C25 measuring system and a 2 g*cm torsion bar. Oscillation mode with a frequency of 1 Hz and a constant strain of 0.0412 was applied at a constant temperature of 30°C. Milk samples (12 ml) were

pre-warmed (30°C, 30 min) before analysis. G' (elastic modulus, curd firmness) of the developing gel was plotted against time and coagulation time (CT) was recorded as the time from coagulant addition until an increase in G' was detected by the instrument.

3.2.6 Cheese-making model

In paper III, milk samples (10 ml) in test tubes were pre-warmed (30°C, 30 min). Chymosin solution (25 µl, 1.5 mg/ml) was added to each sample, after which they were vortexed gently and incubated for another 30 minutes in a shaking water bath. The coagulum was vertically cut in four equally sized sections, using a four-edged knife specifically made to fit the tubes. After another 30 minutes of incubation the tubes were removed from the water bath and a 300 µl sample of the expelled whey was withdrawn by pipette (Whey1). Pressing of the curd was simulated by centrifugation at room temperature (1258 x **g**, 25 min) (Centrifuge 5810R, Eppendorf AG). Expelled whey was decanted and measured by weighing (Whey2). Samples of defatted milk, Whey1 and Whey2 were stored at -80°C pending analysis of protein composition by RP-HPLC.

3.3 Statistical analysis

Effects of analysed variables on the various traits were performed in the statistical software SAS (SAS Institute Inc, Cary, USA) using the GLM procedure (paper I, II, V), the MIXED procedure (paper III), and the GLIMMIX procedure (paper III, IV).

Aggregate β-/κ-CN genotypes were considered. Values for SCC, CT and G' were transformed to their natural logarithms (ln) in order to improve the linear relationship between these and the milk protein components. Cows were grouped into three breed groups according to breed and selection line; SRB H, SRB L and SLB. For further details of the statistical analyses, see paper I-V.

3.4 Summary of studies

3.4.1 Paper I

Effect of genetic variants of β-CN, κ-CN, and β-LG on protein composition, and allele specific protein expression in milk samples from individual cows, analysed by RP-HPLC (n=116).

3.4.2 Paper II

Effect of genetic variants of β-CN, κ-CN and β-LG on chymosin-induced coagulation of milk samples from individual cows (n=121). Chymosin (100 µl, 0.4 mg/ml) was added to 12 ml milk and rheological properties were measured in a Bohlin VOR Rheometer, registering CT and G' at 25 minutes after chymosin addition (G'_{25}).

3.4.3 Paper III

A cheese making model was used to study effects of milk protein composition and genetic variants of β-CN, κ-CN and β-LG on casein retention in curd and casein losses in whey. Chymosin (25 µl, 1.5 ml/mg) was added to individual milk samples (10 ml), which were subjected to cutting and simulated pressing (n=110). Fresh curd yield (Yf) was calculated as the weight difference between the initial milk sample and the expelled Whey2, and expressed as grams of curd per 100 grams of milk. The initial milk, Whey1 (whey after cutting) and Whey2 (whey after simulated pressing) were analysed for milk protein composition by RP-HPLC, obtaining casein content of Whey1 (CNwhey1) and Whey2 (CNwhey2), and casein retention in curd (retCN).

3.4.4 Paper IV

NC and poorly coagulating milk was compared with well coagulating milk (chymosin induced), regarding milk composition and effect of calcium addition (0.05 %) on coagulating properties. Thirty-seven cows were sampled 1-7 times (99 samples). Milk protein composition was analysed by RP-HPLC, rheological properties were measured in a Bohlin VOR Rheometer, registering CT, and G' at 30 min after chymosin addition (G'_{30}). Milk coagulation was treated as a categorical trait with four response levels; 1=good, 2=normal, 3=poor, 4=NC. In the statistical analysis only the most extreme response levels (1 and 4) were included. An independent sample set of 18 individual milk samples, obtained from Dr A-M Tyrisevä (University

of Helsinki, Finland), were also analysed, belonging to either of the two most extreme response levels; 1=good, 4=NC. For details on response levels see Table 1, paper IV.

3.4.5 Paper V

Effect of milk protein composition and genetic variants of β-CN, κ-CN and β-LG on acid-induced coagulation of milk samples from individual cows (n=80). GDL (1.5 %) was added to 12 ml milk and rheological properties were measured in a Bohlin VOR Rheometer, registering CT, and G' at two, eight and ten hours after GDL addition (G'_{4h}, G'_{8h}, G'_{10h}).

4 Results

4.1 General results

4.1.1 Allele and genotype frequencies

The gene counting method was used to calculate allele frequencies at the β-LG, β-CN and κ-CN loci for the total number of cows included in this work (Table 3). There were no significant differences between the different breed groups, although it was noted that the κ-CN B allele was less frequent and the E allele more frequent among SRB H cows compared to SLB L. At the β-LG locus there was an approximately even distribution of β-LG A and B alleles. The A^2 allele was most frequent within the β-CN locus, and the A allele within the κ-CN locus.

Table 3. *Allele frequencies of β-LG, β-CN and κ-CN in Swedish Holstein (SLB) breed and in two selection lines of the Swedish Red (SRB) breed*

Locus	Allele	Frequency		
		SRB H[a] (n=57)	SRB L[b] (n=48)	SLB (n=71)
β-LG	A	0.33	0.47	0.36
	B	0.67	0.53	0.64
β-CN	A^1	0.51	0.33	0.30
	A^2	0.47	0.66	0.66
	A^3	0	0	0
	B	0.02	0.01	0.04
κ-CN	A	0.70	0.68	0.80
	B	0.11	0.24	0.14
	E	0.18	0.08	0.06

[a, b] Cows from selection lines for high milk fat percentage (H) or low milk fat percentage (L), but with similar total milk energy production

Frequencies of β-LG and aggregate β-/κ-CN genotypes of the same cows are presented in Table 4. Genotype frequencies were in Hardy-Weinberg equilibrium for all breed groups. The most common genotypes for the individual milk proteins were β-LG AB, β-CN A^2A^2 and κ-CN AA for the SRB L and SLB cows, whereas β-LG BB, β-CN A^1A^2 and κ-CN AA was the most common among SRB H (data not shown). Aggregate β-/κ-CN genotype A^1A^2/AA was the most frequent among the SRB H cows, A^2A^2/AA among the SLB cows, whereas frequencies were more even in the SRB L group with A^2A^2/AB being the most frequent. Frequency of A^2A^2/AA was higher among the SLB cows compared to the two groups of SRB cows. The A^1A^1/AE and A^1A^2/AE genotypes were more common in the SRB H group than in the other two groups.

Table 4. *Genotype frequencies in cows of the Swedish Holstein (SLB) breed and in two selection lines of the Swedish Red (SRB) breed*

Locus	Genotype	n	Frequency		
			SRB H[a] (n=57)	SRB L[b] (n=48)	SLB (n=71)
β-LG	AA	26	0.14	0.21	0.10
	AB	83	0.39	0.52	0.52
	BB	66	0.47	0.27	0.38
β-/κ-CN	A^1A^1/AA	10	0.05	0.08	0.04
	A^1A^1/AB	4	0.04	0	0.03
	A^1A^1/AE	11	0.12	0.04	0.03
	A^1A^1/EE	2	0.02	0	0.01
	A^1A^2/AA	40	0.26	0.15	0.25
	A^1A^2/AB	16	0.09	0.17	0.04
	A^1A^2/AE	21	0.21	0.08	0.06
	A^1A^2/BE	1	0	0.02	0
	A^1B/AB	1	0	0	0.01
	A^2A^2/AA	37	0.11	0.17	0.32
	A^2A^2/AB	20	0.05	0.21	0.10
	A^2A^2/AE	2	0	0.02	0.01
	A^2A^2/BB	4	0.02	0.04	0.01
	A^2B/AA	2	0	0.02	0
	A^2B/AB	6	0.02	0	0.07

[a, b] Cows from selection lines for high milk fat percentage (H) or low milk fat percentage (L), but with similar total milk energy production

4.1.2 RP-HPLC of milk proteins and their genetic variants

The major proteins in milk (α_{s1}-CN, α_{s2}-CN, β-CN, κ-CN, β-LG, α-LA) were successfully separated and quantified by the RP-HPLC method described in this work (paper I, III-V). A linear relationship between peak surface area and protein quantity was found for the analysed proteins. In addition, most of the genetic variants present in the material (κ-CN A, B, E; β-CN A^1, A^2, B; β-LG A, B) were resolved and quantified (paper I), the exception being κ-CN variants A and E which co-eluted. Consequently, κ-CN A and E could not be quantified in milk from heterozygous AE cows. There was a partial overlap of the β-CN A^1 and A^2 variant peaks, their respective peak areas were therefore calculated by the peak deconvolution function in the chromatography software using EMG (exponentially modified Gaussian) functions.

4.1.3 Protein composition of milk (paper I)

Aggregate β-/κ-CN genotype was associated with concentration of κ-CN in milk. Lowest concentration was found in milk from cows with genotypes including κ-CN E (A^1A^2/AE, A^1A^1/AE) and also A^2A^2/AA milk, whereas highest levels were associated with the five genotypes including κ-CN B (Table 4, paper I).

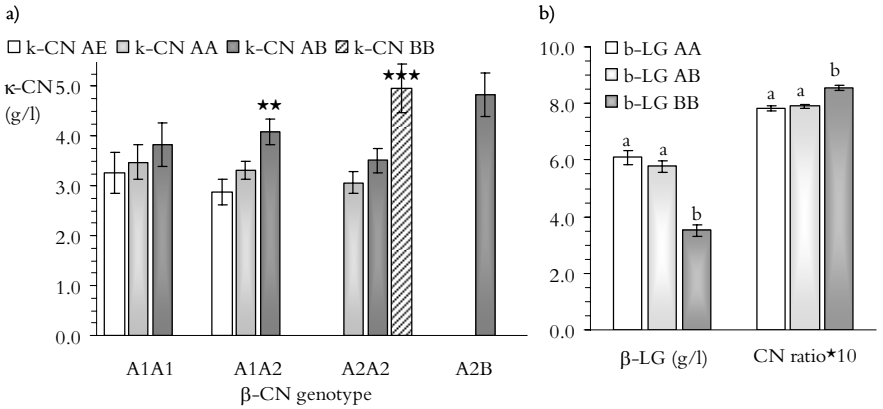

Figure 3. Least squares means (± SE); a) Effect of aggregate β-/κ-CN genotype on κ-CN concentration in milk samples from individual cows (**$P < 0.01$; ***$P < 0.001$), b) Effect of β-LG genotype on β-LG concentration and CN ratio in milk samples from individual cows. Bars with different letters are statistically different ($P < 0.001$).

Within β-CN genotype, there was a trend of increasing κ-CN concentration when replacing a κ-CN A allele with a B allele, whereas a corresponding exchange with an E allele had a decreasing effect (Fig. 3a). These genotype differences were, however, not statistically significant within the β-CN A^1A^1 genotype. Highest β-CN concentration was found in milk from cows carrying the β-CN B allele. CN ratio was positively and β-LG concentration negatively associated with the β-LG BB genotype (Fig. 3b).

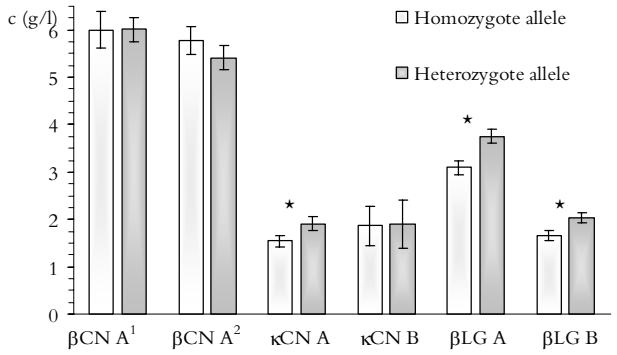

Figure 4. Least squares means (± SE) for expression of β-CN, κ-CN and β-LG protein by their respective alleles in milk samples from individual cows ($\star P < 0.05$).

Some differences in expression between individual alleles were found (Fig. 4). Expression of the two β-LG variants in milk differed significantly between all genotypic combinations; A in genotype AB > A in genotype AA > B in genotype AB > B in genotype BB. At the β-CN locus the A^2 protein variant was found at a higher concentration in milk of A^2B heterozygote cows than in combinations with A^1 or A^2. As regards κ-CN, only expression of the A and B protein variants could be compared, because of co-elution of the E and A variants in the HPLC analysis. The κ-CN A allele was expressed at a higher level in milk in heterozygous combination with the B allele than in homozygous form (AA), whereas no such trend was found for the B variant. In heterozygote cows, β-CN A^1 and β-LG A proteins were found at higher concentrations in milk compared to the protein variant encoded by the alternative allele at these loci (β-CN A^1 to A^2 ratio 1.1; β-LG A to B ratio 1.7), whereas κ-CN A and B variants were found at similar concentrations in heterozygote AB cows (Fig. 5).

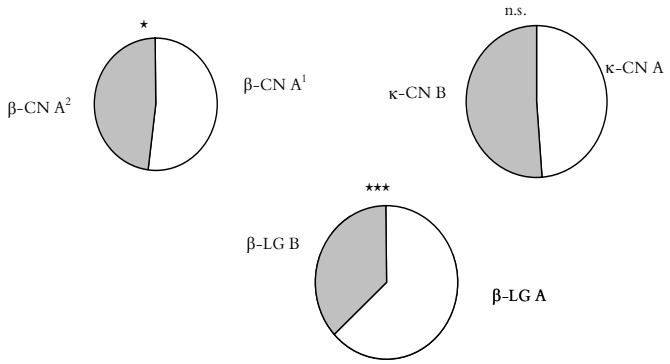

Figure 5. Proportions of the two alternative protein variants in milk from cows heterozygous for β-CN A^1A^2, κ-CN AB and β-LG AB (*$P < 0.05$; ***$P < 0.001$; n.s.=not significant).

4.2 Chymosin-induced coagulation of milk

4.2.1 Rheological properties (paper II)

CT and G' of the most frequent casein genotypes are presented in Fig. 6. The κ-CN BB genotype was associated with shorter CT as compared to both AA and AB (Table 7, paper II), and there were indications of increased CT of milk from cows carrying κ-CN AE compared to κ-CN AB ($P < 0.1$). CT values of milk from cows of κ-CN AA and AB genotypes did not differ. Milk from cows carrying the κ-CN AE genotype showed significantly lower values of G'_{25} than both AA and AB (Fig. 6b). When comparing the estimates of G'_{25} within the β-CN A^2A^2 genotype, a positive linear effect of copy number of κ-CN B alleles was noted (Table 8, paper II). The β-CN A^1A^2 genotype was associated with decreased CT and increased G'_{25} compared to β-CN A^2A^2 (Fig. 6a). Total protein concentration of milk was positively associated with G'_{25}, but showed no association with CT.

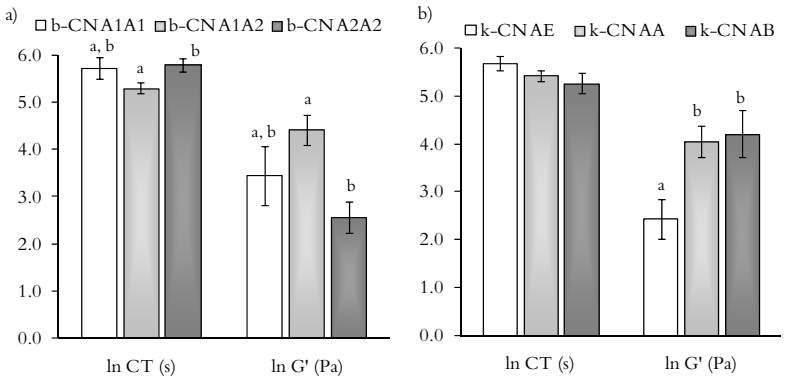

Figure 6. Least squares means (± SE) of coagulation time (CT) and curd firmness (G') at chymosin-induced coagulation of individual milk samples; a) Effect of β-CN genotype, analysed within the κ-CN AA and AB genotypes, b) Effect of κ-CN genotype, analysed within the β-CN A^1A^1 and A^1A^2 genotypes. Values are transformed to the natural logarithm scale. Bars with differing letters are statistically different ($P < 0.01$).

4.2.2 Casein retention in curd (paper III)

A higher concentration of κ-CN in milk was associated to lower levels of CNwhey1, whereas CNwhey2 was negatively associated with CN ratio and positively associated with levels of major proteins and α-LA in milk (Table 2, paper III). Milk samples with no measurable loss of casein in whey were characterised by increased κ-CN concentration, compared to milk samples with more casein lost in whey (Fig. 7).

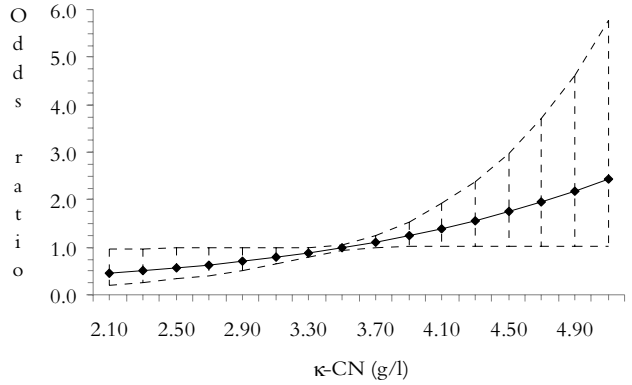

Figure 7. Association of milk κ-CN concentration with casein retention in curd during chymosin-induced coagulation, cutting and simulated pressing of individual milk samples. A higher odds ratio is the increased chance of no casein loss into whey (95 % CI, $P < 0.05$).

Yf was positively associated with concentrations of major proteins, total casein, α_{s1}-CN and β-CN in milk (Table 2, paper III), and showed a strong correlation with retCN (R^2=0.60). No effect of protein genotype on Yf or CNwhey was found. The β-LG BB genotype was associated with increased retCN.

4.2.3 Poorly and non-coagulating milk (paper IV)

A low κ-CN concentration of milk was associated with non-coagulation (Fig. 8). Addition of $CaCl_2$ (0.05 %) improved the coagulation properties (CT, G'_{30}) of milk, eliminating differences in G' between poorly/non-coagulating and well coagulating milk samples.

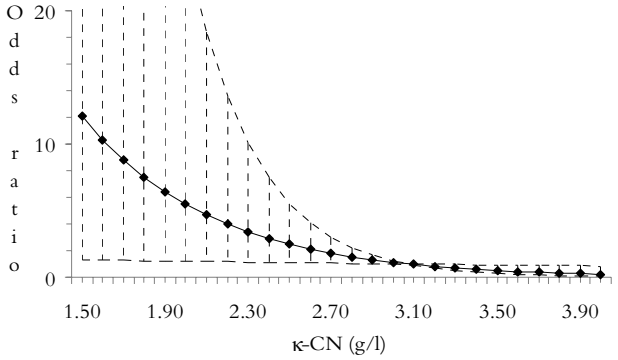

Figure 8. Association of milk κ-CN concentration with non-coagulation of milk samples from individual cows. A higher odds ratio is the increased risk of non-coagulation (95 % CI, $P < 0.05$).

4.3 Acid-induced coagulation of milk (paper V)

Genotype of β-LG was associated with acid coagulation properties, β-LG concentration and CN ratio of milk. When no adjustment for β-LG concentration was made, milk from cows carrying the AA genotype were superior to AB and BB regarding CT and milk from cows carrying AA and AB genotypes were superior compared to BB regarding G' (Fig. 9). Although not showing an overall significance, this pattern was reversed at equal β-LG concentrations, with β-LG BB milk exhibiting significantly higher values regarding G' compared to milk from cows with the AB genotype. No significant effect of β-/κ-CN genotype on acid coagulation was observed.

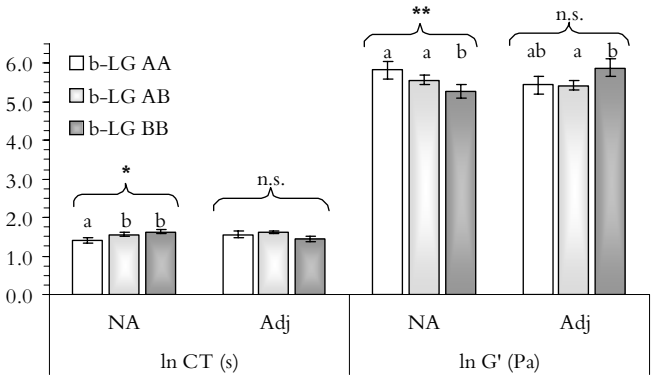

Figure 9. Least squares means (± SE) for effect of β-LG genotype on coagulation time (CT) and curd firmness (G'_{10}) at acid-induced coagulation of individual milk samples, before (NA) and after (Adj) adjustment for β-LG concentration. Values are transformed to the natural logarithm scale. Bars with differing letters are statistically different ($P < 0.05$). *$P < 0.05$; ** $P < 0.01$; n.s.=not significant.

Concentrations of β-LG, α-LA, and lactose in milk were negatively (thus favourably) associated with CT, whereas a higher CN ratio was associated with a longer CT (Table 4, paper V). Concentrations in milk of major proteins, β-LG, and lactose were positively associated with G' during the whole coagulation process, whereas a positive association was observed for α-LA only up to 4 h. Lactose concentration was shown to improve G' in milk with low β-LG concentrations (Fig. 4, paper V).

5 Discussion

5.1 Protein composition of milk

Our finding regarding the association of aggregate β-/κ-CN genotype with κ-CN concentration in milk (paper I) was in agreement with Ikonen *et al.* (1997) and Jõudu *et al.* (2007). The B allele of κ-CN was associated with increasing concentration κ-CN in milk, whereas the opposite was observed for the E allele (Fig. 3a). Aggregate α_{s1}-/β-/κ-CN genotype has previously been reported to significantly influence percentages of α_{s1}-CN, β-CN, κ-CN and β-LG (Cardak *et al.*, 2003; Ehrmann *et al.*, 1997b). In these two studies, however, information on the κ-CN E allele was not included. In most studies the effect of κ-CN genotype has been estimated separately and in the majority were only the A and B variants considered. A positive effect of κ-CN B on κ-CN concentration in milk, and in some cases total protein and casein concentration, is generally reported.

Similar concentrations of the κ-CN A and B variants in milk from heterozygous cows were found (Fig. 5), which is in agreement with the results by Ehrmann *et al.* (1997b). In contrast, others have reported an elevated expression level of the B allele compared to the A allele when analysing milk from cows heterozygous for κ-CN AB (Robitaille & Petitclerc, 2000; Vachon *et al.*, 2004; van Eenennaam & Medrano, 1991).

The positive association of β-LG B with CN ratio and its negative association with β-LG concentration in milk (Fig. 3b) were in agreement with previous studies (Bobe *et al.*, 1999; Cardak *et al.*, 2003; Ehrmann *et al.*, 1997a; Ehrmann *et al.*, 1997b; Ikonen *et al.*, 1997; Lundén *et al.*, 1997; McLean *et al.*, 1984). Similar to results by Ng-Kwai-Hang & Kim (1996), Kim *et al.* (1996) and Graml *et al.* (1989), there was approximately 1.5 times higher expression of the β-LG A variant than the B variant in milk from

heterozygote AB cows (Fig. 5). The A allele promoter region has been found to have higher affinity for transcription factors and, consequently, it was suggested that the β-LG A allele was expressed more efficiently (Lum et al. 1997). Interestingly, both the β-LG A and B variants, and also the κ-CN A variant, was found in higher concentrations when present in heterozygous cows compared to homozygous cows (Fig. 4), the β-LG results being supported by Graml & Pirchner (2003).

5.2 Chymosin-induced coagulation of milk

We found concentration of total milk protein to be positively associated with milk coagulation as it increased curd firmness (paper II). This is in accordance with the results by Okigbo *et al.* (1985c) and Lindström *et al.* (1984), whereas others have reported contrasting results (Ikonen *et al.*, 1999a; Ikonen *et al.*, 2004). Although Pagnacco & Caroli (1987) found similar effects with improved curd firmness, interestingly this was associated with prolonged coagulation times. These conflicting reports reflect the limitation of using total protein content as a quality parameter as milk coagulation is concerned.

Results in paper II-IV showed that the protein composition of milk was important in chymosin-induced coagulation, whereas total protein or total casein content was not always indicative of coagulation properties. The association of aggregate β-/κ-CN genotype with κ-CN concentration in milk (paper I), was probably the underlying reason for the effect of κ-CN genotype on milk coagulation properties (paper II). An association of the κ-CN B allele with increased κ-CN concentration in milk compared to the E allele (paper I), concurred with a higher curd firmness associated with κ-CN B compared to E (paper II). A favourable association of κ-CN B and a unfavourable association of κ-CN E with coagulation properties of milk have been shown previously (Caroli *et al.*, 2000; Comin *et al.*, 2008; Ikonen *et al.*, 1999a; Ikonen *et al.*, 1997; Lodes *et al.*, 1996). The fact that milk from κ-CN AA and AB cows showed similar G', differing from the AE genotype (paper II), supports the statement by Schaar (1984) that the charge difference between the AA and AB variants is not important for curd firmness. Instead our results indicate that the κ-CN protein concentration plays a major role for curd firmness, similar to previous reports (Ikonen *et al.*, 1997; Jõudu *et al.*, 2008; McLean, 1986; van den Berg *et al.*, 1992). The positive association of β-CN A^1A^2 with milk coagulation properties (paper II) was in agreement with results by Ikonen *et al.* (1997) and Jõudu *et al.* (2007), and concurred with a higher concentration of κ-CN in milk from cows with aggregate β-

/κ-CN genotype A^1A^2/AB compared to A^2A^2/AB (paper I). Despite a strong association of the β-LG BB genotype with CN ratio (paper I), no influence of β-LG genotype was found on the coagulating properties of milk in paper II. Previous studies have found an effect of β-LG genotype on milk coagulation properties (Ikonen *et al.*, 1999a; Kübarsepp *et al.*, 2005; Lodes *et al.*, 1996; Ng-Kwai-Hang *et al.*, 2002; van den Berg *et al.*, 1992), whereas others found no effect (Ikonen *et al.*, 1997; Pagnacco & Caroli, 1987). These ambiguous results indicate that the caseins as a group are not always a good indicator of milk coagulation.

Analysis of rheological properties, as in paper II, reflects the cheese-making process up till the point of cutting the curd. Although a high curd firmness at cutting has been associated with increased cheese yield (Aleandri *et al.*, 1990; Bynum & Olson, 1982; Ng-Kwai-Hang *et al.*, 1989; Riddell-Lawrence & Hicks, 1989), it has been suggested that as long as conditions are kept relatively consistent, curd firmness will have only minor consequences for cheese yield (Mayes & Sutherland, 1984; Mayes & Sutherland, 1989). Subsequent stages of cheese making, such as cutting and pressing of the curd, were studied on a laboratory scale in paper III. The results can be considered to highlight two different aspects of cheese making. There is the aspect of yield, a quantitative trait which depends on the concentration of casein in milk available for curd formation, and the aspect of casein loss, that depends on the qualitative curd forming properties of milk. These two traits should ideally be combined for efficient cheese making, which would require milk with a high total casein concentration to ensure cheese yield potential, and a high κ-CN concentration to improve the milk coagulation properties.

The effect of κ-CN concentration in milk on casein losses into whey (Fig. 7) may partly be explained by the negative association of κ-CN concentration with casein micelle size (Dalgleish *et al.*, 1989; Donnelly *et al.*, 1984; Risso *et al.*, 2007). Milk containing smaller micelles has been shown to form gels with an improved structure, which may increase the ability to entrap milk constituents (Niki *et al.*, 1994; Nuyts-Petit *et al.*, 1997; Walsh *et al.*, 1998). Milk with high κ-CN concentration results in a short coagulation time (Nuyts-Petit *et al.*, 1997; van den Berg *et al.*, 1992), which would leave more time for curd firming and consequently a higher curd firmness at cutting, possibly reducing the casein losses into whey (Fagan *et al.*, 2007; Ng-Kwai-Hang *et al.*, 1989).

The impact of κ-CN concentration during the initial stages of cheese making was observed both in paper II and paper III. However, after further agitation and syneresis of the curd, only the caseins as a group had a

significant effect. Fresh curd yield (Yf) was dependent on amount of casein available for curd formation, reflecting the milk casein content, whereas there was no association between casein content of milk and casein content of whey. Concentration of casein in whey showed only a weak association with fresh curd yield, as milk with a high initial casein concentration tolerated a larger loss without compromising fresh curd yield, compared to a milk with low casein concentration.

No effect of protein genotype on fresh curd yield or casein losses into whey was found in paper III. However, we observed an association between β-/κ-CN genotype and κ-CN concentration in milk, which in turn influenced casein in whey. Less curd fines in whey from milk containing κ-CN AB compared to AA (van den Berg *et al.*, 1992) supports the assumption of a genotype effect and there are numerous reports of a positive association of the κ-CN B allele with cheese yield (Marziali & Ng-Kwai-Hang, 1986; Mayer *et al.*, 1997; Nuyts-Petit *et al.*, 1997; Schaar *et al.*, 1985; Walsh *et al.*, 1995; Walsh *et al.*, 1998; van den Berg *et al.*, 1992). The κ-CN E allele has been included in several studies on coagulation properties of milk (Caroli *et al.*, 2000; Comin *et al.*, 2008; Ikonen *et al.*, 1999a; Jõudu *et al.*, 2007; Kübarsepp *et al.*, 2005; Lodes *et al.*, 1996; Matejickova *et al.*, 2008; Oloffs *et al.*, 1992), which all report a negative association, at least for the AE genotype. This is in accordance with our results on the effect of κ-CN E on κ-CN concentration (paper I) and coagulating properties (paper II) of milk. However, as there are no studies available on κ-CN E and cheese yield more research is needed. Some authors have suggested that the protein genotype effect on cheese yield should mainly be ascribed to an increased fat retention in curd (Nuyts-Petit *et al.*, 1997; Walsh *et al.*, 1995; Walsh *et al.*, 1998). This may explain the lack of genotype effect in paper III, as we used skimmed milk samples. The association of β-LG genotype with casein retention in curd (retCN) was probably resulting from a direct effect on CN ratio. The β-LG genotype has also been associated with cheese yield (Aleandri *et al.*, 1990; Boland & Hill, 2001; Marziali & Ng-Kwai-Hang, 1986; Rahali & Ménard, 1991; van den Berg *et al.*, 1992; Wedholm *et al.*, 2006b), possibly via an altered CN ratio.

Characterisation of NC milk samples in paper IV showed that they contained low levels of κ-CN compared to well coagulating milk (Table 2, paper IV) and that a low κ-CN concentration in milk significantly increased the risk of non-coagulation (Fig. 8). A similar association of κ-CN concentration with poorly and non-coagulating milk was also reported by Wedholm *et al.* (2006a) and Jõudu *et al.* (2008). RP-HPLC of NC samples after addition of chymosin demonstrated the formation of para-κ-CN,

confirming previous reports that the primary phase of milk coagulation proceeds in a normal way in these samples (Tervala & Antila, 1985; van Hooydonk *et al.*, 1986). The addition of $CaCl_2$ (0.05 %) improved all non-coagulating milk samples (NCs) to the level of well coagulating milk, particularly regarding curd firmness. Although calcium addition has previously been shown to improve the coagulation time of poorly coagulating milk (van Hooydonk *et al.*, 1986), curd firmness was still inferiour compared to normal milk (Okigbo *et al.*, 1985b) and resulted in lower cheese yield (Nsofor, 1989). In the latter two studies, however, milk was sampled from cows in late lactation where part of the casein may have been degraded due to increased plasmin activity. Although coagulation properties of poorly/non-coagulating milk are improved by the addition of calcium and cheese yield is not affected (Ikonen *et al.*, 1999b; Wedholm *et al.*, 2006b), this milk may still be unsuitable for cheese manufacture as it is likely to result in cheese with higher moisture content (Ikonen *et al.*, 1999b; Nsofor, 1989), which for some cheese varieties is negative for the product quality.

If direct selection for milk coagulation was to be implemented, a simple and fast analytical method to screen large numbers of milk samples would be needed. Considering the results in this work, using a cheese-making model might be an option. If the main purpose is to identify cows producing poorly/non-coagulating milk, this may be a practical alternative to rheological measurements. The particular format used in paper III would however not be suitable for large scale analysis. Instead a micro-scale cheese model, as recently described by Bachmann *et al.* (2008), might be a useful alternative for screening individual milk samples for cheese-making properties. This method allows for simultaneous manufacturing of up to 600 cheeses in an individual, miniaturised micro-titer format. It could also be used to assess implications of NC milk in cheese-making. Another alternative is the prediction of milk coagulation properties by mid-infrared spectroscopy (Cecchinato *et al.*, 2008; Dal Zotto *et al.*, 2008). Although not very accurate, the analysis can be used for selection purposes, integrated with the routine milk recording.

5.3 Acid-induced coagulation of milk

Characteristics such as thickness and water holding capacity of the acid gel at manufacture of fermented milk products are vastly improved by the heat pre-treatment (90°C, 4-5 min), during which whey proteins become part of the coagulum network through denaturation and partial association with the

casein micelles. Paper V showed a major influence of protein composition on the acid coagulation properties of heated milk, and it also pointed to a significant contribution of β-LG genotype. Increased curd firmness was found for milk samples with higher concentration of whey protein and lower CN ratio (Table 3, paper V), traits associated with β-LG AA and AB milk (Fig. 3b). In accordance with these results, Puvanenthiran *et al.* (2002) showed that decreasing the proportion of casein in milk while maintaining a constant total protein concentration, caused an increase in gel strength and elastic response of yoghurt. Milk with a higher proportion of whey protein has been shown to yield larger aggregates of higher whey protein/κ-CN ratio (Guyomarc'h *et al.*, 2003b), which also may have contributed to the increased curd firmness. There were also indications of a β-LG genotype effect beyond its association with β-LG concentration in milk, as β-LG BB was the superior genotype as regards curd firmness at equal β-LG concentrations (Fig. 9). This was also found by Allmere *et al.* (1998a), adjusting for CN ratio, and was suggested to be due to a difference in reaction time of β-LG A and B during the heat induced aggregation with the casein micelle. It is questionable whether this was the case in the present study, however, as samples were acidified when 100 % of the available β-LG and 90 % of α-LA was expected to have been denatured (Dannenberg & Kessler, 1988b). Differences in aggregation behaviour may, however, explain the differences in curd firmness observed between the A and B variants at equal β-LG concentrations. It has been suggested that the β-LG A variant forms smaller aggregates, which are less efficient at forming a cross-linked network during acidification, both in the serum and associated with κ-CN at the micelle surface (Manderson *et al.*, 1998). Bikker *et al.* (2000) showed that acid gels containing the B variant of β-LG resulted in gels with markedly higher G' and a more dense, cross-linked structure compared to A.

The significant effect of α-LA concentration (Table 4, paper V) might be explained by the relatively slow heating of the milk samples (5 min to reach 90-95°C) and the association behaviour of α-LA and β-LG. Containing four disulphide bonds, α-LA is incorporated into aggregate structures by first forming heat-induced complexes with β-LG, which thereafter associate with the casein micelle (Elfagm & Wheelock, 1978). In this study, α-LA and β-LG might have denaturated and formed aggregates during heating >80°C, subsequently associating with the micelle as the temperature was raised to 90-95°C (Oldfield *et al.*, 1998).

A positive association of lactose concentration with acid coagulation (Table 4, paper V) was in line with suggestions by Niki & Motoshima

(2006) that lactose improves acid gelation by strengthening hydrophobic interactions between casein micelles.

In agreement with Allmere *et al.* (1998a), no effect of the κ-CN A and B alleles were found on acid coagulation. We showed this to be true also for κ-CN E (paper V). Nor was there any effect of the casein concentration of milk on acid coagulation. Nonetheless, the casein fraction constitutes an essential part of the basic structure of acidified milk gels (Harwalkar & Kaláb, 1988), whereas the denatured whey proteins are responsible for the increase in curd firmness by increasing the number and strength of bonds in the acid gel (Lucey *et al.*, 1998; Lucey & Singh, 1997).

5.4 All for one, one for all?

In order to improve the cheese-making properties of milk, it might be more efficient to increase the proportion of κ-CN, rather than the total casein concentration in milk. The results in paper I indicate that selection for fat content, and thereby indirectly for protein content, will not change the proportion of individual proteins in milk (Table 3, paper I). Our results, supporting previous literature reports (Ikonen, 2000; Ojala *et al.*, 2005), suggest selection for κ-CN B as a practicable means to achieve more favourable processing characteristics of milk. This would also provide an option to decrease the frequency of the E allele (Ikonen, 2000), given its negative association with chymosin-induced coagulation and rather high frequency in SRB cows. However, according to Tyrisevä (2008) selection on the κ-CN locus is not likely to solve the problem with NC milk. In paper II and III, 4-5 % of the milk samples were NC, which may not reflect the true prevalence of NC milk. A screening of the Swedish cow population regarding the presence of NC milk is warranted.

Based on the observations in paper V, both alleles at the β-LG locus could benefit acid coagulation. As β-LG B was associated with firm acid curds but lower β-LG concentration, and β-LG A was associated with higher β-LG concentration, which was associated with firm acid curds, it seems preferable to aim for equal frequencies of the β-LG A and B alleles in the dairy cattle population. Increasing the frequency of the β-LG B allele would, however, also positively influence chymosin-induced coagulation given its association with increased CN ratio, which improved casein retention in curd (paper III).

The work presented in this thesis follows two different tracks; chymosin-induced and acid-induced coagulation of milk. One could speculate if it is along these two tracks that dairy cattle breeding should move in the future;

cows producing milk genetically designed to fulfil the criteria of an ideal milk protein composition for a specific dairy product.

6 Main findings & Conclusions

- Effect of milk protein genotypes on protein composition:
  - aggregate β-/κ-CN genotype was associated with concentration of κ-CN in milk. Lowest concentration was found in milk from cows with genotypes including the κ-CN E allele and also the A^2A^2/AA genotype, whereas the highest κ-CN concentrations were associated with genotypes including the κ-CN B allele
  - both the β-LG A and B protein variants, and also the κ-CN A variant, was found in higher concentrations when present in heterozygous cows compared to homozygous cows
  - in heterozygote cows, β-CN A^1 and β-LG A protein variants were found at higher concentrations in milk compared to the protein variant encoded by the alternative allele at these loci, whereas κ-CN A and B protein variants were found at similar concentrations in heterozygote AB cows
  - the β-LG BB genotype was negatively associated with β-LG concentration and positively associated with CN ratio
- At chymosin-induced coagulation of milk:
  - the κ-CN B allele showed a positive effect on curd firmness, whereas the κ-CN AE genotype showed a negative effect, particularly compared to κ-CN AB
  - the A^2A^2 genotype of β-CN was associated with poor coagulating properties compared to β-CN A^1A^2
  - a low κ-CN concentration in milk increased the risk of non-coagulation

- In a cheese making model:
  - a higher concentration of κ-CN in milk was associated with a lower risk of casein being lost into whey, both after cutting and simulated pressing
  - casein in whey after cutting was negatively associated with κ-CN concentration in milk
  - casein in whey after simulated pressing was negatively associated with CN ratio in milk
  - fresh curd yield was positively associated with concentrations of major proteins, total casein, α_{s1}-CN and β-CN in milk
  - the β-LG BB genotype was associated with increased casein retention in curd
- At acid-induced coagulation of milk:
  - the AA and AB genotypes of β-LG were associated with higher curd firmness compared to BB, possibly due to an increased concentration of β-LG in milk
  - milk from cows with the β-LG BB genotype produced a firmer curd at equal β-LG concentrations

7 Future research

Our studies showed a low κ-CN concentration and poor coagulation properties of milk to be associated with the κ-CN E allele. The assessment of κ-CN E milk in cheese manufacture would be an area for further studies.

It is urgent to establish the prevalence of NC milk in Swedish dairy cows by screening a larger group of cows. We found calcium addition to improve coagulation of NC milk to the level of well coagulating milk. However, it remains to be proven that the quality of the resulting cheese is not compromised. Further, the compensatory mechanism of calcium in the aggregation phase, and the underlying mechanisms of association with low κ-CN of NC milk need to be elucidated.

The upper limit of SCC applied in this work is relatively high for a cow composite milk sample. Thus, the possibility that some milk samples were affected by an udder infection causing proteolysis of casein can not be excluded. The SCC of a composite milk sample does not necessarily reflect the level of proteolysis in the milk. At the same SCC, an udder with three healthy quarters and one infected could be expected to show a higher degree of proteolysis than a composite sample where the SCC is evenly distributed over the four quarters. In order to elucidate the factors behind occurrence of NC milk it is instrumental to identify when this is caused by udder infection.

Based on the observed favourable effect of the β-LG B protein variant on acid coagulation it would be interesting to evaluate sensory properties (mouth feel, taste) of fermented milk products containing different β-LG genotypes, in pilot plant scale trials using conventional starter cultures.

Further investigations are needed to clarify the mechanisms behind the observed phenomenon where both the β-LG A and B protein variants, and also the κ-CN A variant, were found in higher concentrations when present in heterozygous cows compared to homozygous cows.

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