Effect of genetic variants on hydrolysis of bovine κ-casein by chymosin and pepsin

by

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Abstract

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Caseins are present in milk in the form of large spherical complexes called micelles that are stabilized by κ-casein found on the surface. This stabilizing effect is lost when milk clotting enzymes hydrolyze κ-casein thereby initiating the coagulation process. The objective of this study was to analyze the effect of κ-casein polymorphism upon hydrolysis by proteases. The A and B forms are the most common genetic variants of κ-casein in Canadian Holstein dairy herds, representing three phenotypes AA, AB and BB. Whole casein from Holstein milk samples was fractionated into its four major components by ion-exchange chromatography using Express Ion Exchanger Q Anion Exchanger and Macro-Prep High Q Anion Exchange Support columns. The κ-casein fraction was isolated, dialyzed, and assessed for its phenotype and purity by PAGE. The pure forms of the three different phenotypes of κ-casein (AA, AB, BB) at a final concentration of 0.5% were then hydrolyzed by calf chymosin (1:500) and porcine pepsin (1:1000) at a pH of 5.8 at 37°C. Aliquots were collected at 0,5,15,30,60 and 90 min and the reaction stopped by using 24 % NH_4OH. The rate of hydrolysis of the three phenotypes was analyzed by comparing the disappearance of the substrate with time from the RP-HPLC chromatograms using Waters MAXIMA software. SDS-PAGE was used to calculate the approximate molecular weight of the hydrolytic products. Analysis of the hydrolysate profiles indicated that there was significant difference ($P<0.05$) in the rate of hydrolysis between the phenotypes. Under the present conditions at a pH of 5.8, the AA phenotype showed a significantly slower rate of hydrolysis by both chymosin and pepsin, than the other two phenotypes. There was no significant difference in the rate of hydrolysis between the phenotypes AB and BB during chymosin hydrolysis. The BB phenotype is hydrolyzed more extensively and the AB phenotype is intermediate between the two variants in the pepsin digest.

Résumé

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Effet des variants génétiques de la κ-caséine sur son hydrolyse par la chymosine et la pepsine

Les caséines sont présentes de lait sous forme de larges complexes sphériques appelés micelles. Ces micelles sont stabilisées par la presence de κ-caséine en surface. Cet effet stabilisant est perdu lorsque les enzymes coagulantes utilisées en production fromagére hydrolysent la κ-caséine et ainsi initient le processus de coagulation. L' objectif de cette etude a été l'analyse de l'effet du polymorphisme de la κ-caséine sur sa propre hydrolyse par les proteases. Les formes A et B de la κ-caséine sont les variants génétiques les plus répandues dans les troupeaux laitiers de la race Holstein du Canada, représentant trois phenotypes AA, AB et BB. La quantité totale de caséine contenue dans des échantillons de lait de vache de race Holtstein, a été fractionnée en ces quatre composants majeurs par chromatographie échangeuse d'ions, utilisant l' <Express Ion Exchanger Q Anion Exchanger> et les <Macro-Prep High Q Anion Exchange Support> columns. La fraction correspondant à la κ-caséine a été isolée, dialysée, et son phénotype ainsi que sa pureté ont été déterminés par la technique PAGE. La forme pure de chaque phénotype (AA, AB, BB) de la κ-caséine, à la concentration finale de 0.5 %, a été hydrolysée par de la chymosine de veau (1:500) et de la pepsine porcine (1:1000) à un pH de 5.8 à 37° C. Des aliquots ont été collectés à 0,5,15,30,60 et 90 minutes et la reaction à été stoppée utilisant 24 % NH_4OH. La vitesse de la reaction d'hydrolyse pour les trois phenotypes a été analysée en comparant, la concentration du substrat en fonction du temps, calculée à partir des chromatogrammes RP-HPLC utilisant le logiciel Waters Maxima. La SDS-PAGE a été utilisée pour estimer le poids moléculaire du produit de chaque hydrolyse. L'analyse de profils de réaction d'hydrolyse indique qu'il existe une différence significative ($P < 0.05$) de vitesse de réaction d'hydrolyse entre les différents phénotypes. Pour ces conditions d'expérience (37°C à un pH de 5.8), la vitesse d'hydrolyse de la κ-caséine de phenotype AA est significativement inférieure aux vitesses de réaction pour les deux autres phénotypes, aussi bien par la chymosine que par la pepsine. Il n'y a pas de difference significative de vitesse d'hydrolyse par la chymosin entre les phénotypes AB et BB. La vitesse d'hydrolyse de la κ-caséine de phénotype BB est supérieure à celle de la κ-caséine de phenotype AB qui est elle même supérieure à celle de la variante génétique de phénotype AA.

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1. Introduction

Cheese is one of the widely consumed dairy products in the world. In North America and Europe, commercial production of cheese is around 40% and 50% of the total milk produced respectively, which is significantly higher than the world average of around 30% (Fox 1993). Cheese is also the most diverse group of dairy products with many different varieties and presumably, the most interesting academically. An important aspect in the production of good quality cheese involves a basic knowledge of the chemistry and biochemistry of milk constituents and the enzymology of cheese manufacture. With the gradual accumulation of scientific and technological literature on milk and cheese over the past few decades, it became possible to direct the changes involved in cheese making in a more controlled fashion. Cheese production is largely characterized by a series of biochemical events that ultimately lead to products with highly desirable aromas.

Enzymatic (rennet) coagulation of milk is the primary step in the manufacture of most cheese varieties. Proteases, predominantly, chymosin, pepsin and certain fungal proteases are widely used for the coagulation of milk in the cheese industry. Milk protein, caseins form the chief substrate for these enzymes. Casein occurs in milk in the form of large spherical complexes called micelles. κ-Casein is one of the main components of bovine casein since it stabilizes casein micelles and prevents other calcium sensitive caseins from precipitating in the presence of calcium ions present in milk. κ-Casein is distributed throughout the casein micelles in milk, predominantly on their surfaces. The primary stage of milk coagulation involves the attack of κ-casein present on the surface of the casein micelles. This proteolytic reaction destabilizes the casein micelles and triggers their aggregation, eventually forming curds, which are processed to make cheese. Casein aggregation plays an important role in forming the curd used for making cheese (Pearce, 1976)

It has been observed in general that the existence of different genetic variants of milk proteins may influence some of the technological properties of milk. In particular, genetic polymorphism of κ-casein has been shown to influence the composition of milk, physico-chemical properties of milk, cheese yield and cheese composition. Interestingly,

milk containing the BB phenotype of κ-casein were associated with the following properties as compared to the AA and the AB phenotypes: highest fat and casein content (Buchberger et al., 1982; McLean et al., 1984; Ng-Kwai-Hang et al., 1984, 1986), increased heat stability (McLean et al., 1987; Robitaille, 1995) higher cheese yield during cheese manufacture (Aleandri et al., 1990; Marziali and Ng-Kwai-Hang, 1986a, Ng-Kwai-Hang, 1990; Walsh et al., 1995), higher velocity of the enzymatic reaction during the renneting process (Hooydonk et al., 1986), shorter rennet coagulation time (Schaar et al., 1984, 1985; Aaltonen and Antila, 1987; Pagnacco and Caroli, 1987; Rampilli et al., 1988; Walsh et al., 1995), desirable properties during curd formation (Schaar et al., 1985; Ng-Kwai-Hang et al., 1987; Marziali and Ng-Kwai-Hang, 1986b; Walsh et al., 1995), better incorporation of casein and fat into cheese (McLean and Schaar, 1989; Ng-Kwai-Hang, 1990). Higher κ-casein contents were observed in milk containing smaller sized micelles. The presence of smaller micelles leads to a shorter rennet coagulation time and a firmer curd (Dalgleish et al., 1981; Donnelly et al., 1984; Ekstrand et al., 1980; Ford and Grandison, 1986). It was also observed that the addition of κ-casein accelerates renneting as measured by a decreased rennet coagulation time (Ekstrand et al., 1980). Thus, κ-casein appears to be very important for the renneting behavior of milk.

The A and B variant of κ-casein are the most common variants observed in Canadian Holstein dairy herds (Kim et al., 1996). Although much research have been done, as shown above, on the effect of genetic polymorphism of κ-casein on certain properties of milk and milk products, there are less reports on the direct effect of κ-casein genetic variants on hydrolysis by enzymes commercially used in the cheese industry.

In the present study, κ-casein corresponding to the three different phenotypes was isolated and purified in sufficient quantities using Ion-Exchange Chromatography. Milk samples from Holstein dairy herds were used as a source of κ-casein. The purified samples were then subjected to limited proteolysis by chymosin and pepsin. The hydrolysates were subsequently analyzed by Reverse-Phase HPLC and SDS-PAGE to observe any differences in the pattern of hydrolysis between the phenotypes AA, AB and BB. The results of this study could pave the way for further research on the effect of genetic variants of κ-casein on the renneting properties of milk.

2. Review of Literature

2.1 Milk

Milk is a secretion characteristic of all species of mammals and primarily intended by nature for the growth and nourishment of the newborn. The composition of milk is therefore thought to be unique to each species depending on the nutrient requirements and the growth rate of the young animal. Water forms the bulk of milk in all species in addition to proteins, lipids, carbohydrates, salts and other minor components such as vitamins and enzymes. The proteins of milk are one of the most widely characterized food proteins and also the subject of intensive research for several years. The lipids found in milk, usually referred to as fat are mostly triglycerides with little quantities of free fatty acids, mono- and diglycerides, phospholipids, sterols and hydrocarbons. Milk fat exists mostly in the form of globules and is associated with certain proteins and vitamins. Lactose is the chief carbohydrate in the milk of all species studied so far and responsible for the slight sweet taste. Lactose is a disaccharide composed of glucose and galactose and milk forms the richest source of lactose found in nature. Milk salts include both organic and inorganic substances.

Bovine milk is composed of approximately 87% water, 3.9 % fat, 3.3 % protein, 4.6 % lactose, 0.65 % minerals and other minor components (Walstra and Jenness, 1984). There are several factors that significantly affect the composition of milk including breed, feed composition and stage of lactation. Much of the discussion in this text pertains to milk of bovine origin unless otherwise specifically mentioned.

2.2 Milk Proteins

The proteins in milk are traditionally classified into two main categories: the caseins and whey proteins. There are several other minor proteins found in milk, which may have biological significance, and these include various enzymes and enzyme inhibitors, binding or carrier proteins, growth factors, antibacterial agents and proteolytic products of milk protein themselves. The exact physiological role of many of these proteins and peptides remains largely unknown due to lack of adequate research in these areas and also difficulty in their isolation and purification.

2.2.1. Caseins

Caseins are a heterogeneous group of phospho-proteins found exclusively in milk, which precipitate from raw skim milk upon acidification to pH 4.6 at 20 ° C. The whole casein fraction contains four different polypeptide components encoded by separate genes, designated as α_{s1}-,α_{s2}-,β- and κ- casein including their breakdown products. The α_{s1}-,α_{s2}- and the β- caseins are generally referred to as the calcium sensitive caseins because they were found to precipitate in the presence of low concentration of calcium and are maintained in a micelle suspension in milk as a result of their interaction with κ- casein. Colloidal calcium phosphate, which accounts for approximately 5 % of the weight of casein micelles, is sequestered by the micelles through interactions with the clustered serine phosphate residues of the calcium sensitive caseins (Sleigh et al., 1979). The individual families were initially identified by alkaline urea gel electrophoresis (Whitney et al., 1976). Polymorphism is observed in all these casein fractions, more commonly using electrophoretic techniques. In addition to this, post-translational modifications add further to the heterogeneous nature of the caseins. (Eigel et al., 1984; Whitney, 1988).

2.2.1.1. α_{s1}-Casein

α_{s1}-Casein is a polypeptide of 199 amino acids residues with a molecular weight of 23,600 (Eigel et al., 1984) and accounts for 40 % of the caseins in milk (Swaisgood, 1982). Eight genetic variants have been identified so far and denoted as A, B, C, D, E, F, G and H (Eigel et al., 1984; Prinzenberg et al., 1998; Ng-Kwai-Hang and Grosclaude, 2002). The variants arise mostly due to amino acid substitutions in their primary sequence except in the case of variant A and H. Variant A contains a 13 amino acid residue deletion from position 14-26 (Grosclaude et al., 1973; Dewan et al., 1974; Whitney et al., 1976). It is postulated that this deletion arose as a result of abnormal mRNA splicing to one exon of the gene (McKnight et al., 1989). Variant H is characterized by deletion of 8 amino acid residues in exon 8 and this deletion probably arose by exon skipping (Mahé et al., 1999).

2.2.1.2. α_{s2} – Casein

α_{s2} – Casein is a polypeptide of 207 amino acid residues with a molecular weight of 25,230 (Brignon et al., 1977). There are four genetic variants designated as A, B, C and D and all except one arise due to amino acid substitutions of the primary sequence

(Grosclaude et al., 1979; Swaisgood, 1982; Eigel et al., 1984). Variant D results from a deletion of amino acids from position 50-58 in the polypeptide chain.

2.2.1.3. β-Casein

β-Casein is polypeptide of 209 amino acid residues and a molecular weight of 23,983 (Eigel et al., 1984). There are ten genetic variants of β-casein identified so far (A_1, A_2, A_3, B, C, D, E, F, and G) and all of them involve amino acid substitutions at positions of the primary sequence (Grosclaude et al., 1972a; Visser et al., 1995; Dong and Ng-Kwai-Hang, 1998; Han et al., 2000).

2.2.1.4. κ-Casein

κ-Casein is polypeptide containing 169 amino acids with a molecular weight of 19,007 (Wong, 1988). The structure is amphipathic with a N-terminal hydrophobic domain and a C-terminal polar domain. The major component of κ-casein contains only one phosphoseryl residue but some evidence indicates a minor amount of doubly phosphorylated protein (Vreeman et al., 1977). The primary structure shows different degrees of glycosylation and sites of phosphorylation (Fournet et al., 1979; van Halbeek et al., 1980).

Polymorphism of κ- caseins involve amino acid substitutions at positions 10, 97, 104, 135, 136, 148 and 155 of the primary sequence (Mercier et al., (1973); Seibert, et al., 1987;Di Stasio and Merlin, 1979) producing 11 variants as shown in Table 1. Grosclaude et al., (1972b) and Mercier et al., (1973) established the primary structures of the two genetic variants A and B of κ-casein. Seven new variants were recently identified. Ikonen et al., (1996) demonstrated a variant termed κ- casein F, in 2 cows (a dam and its daughter) in a study comprising 20,990 Finnish Ayrshire cows phenotyped for the major milk proteins by isoelectric focusing in polyacrylamide gel. Sulimova et al., (1996) identified two variants (F^S, G^S) with a substitution of valine and alanine at position 148 respectively instead of aspartate when compared to variant A at the same position. Another variant κ-casein G^E with an allele frequency of 0.003 was demonstrated in Pinzgauer cattle from Austria and Bavaria, Germany by isoelectric focusing in polyacrylamide gels and by alkaline polyacrylamide gel electrophoresis (Erhardt, 1996) and it had an arginine to cysteine substitution at amino acid position 97

Table 1.Position and amino acid differences of genetic variants of κ-casein (adapted from Ng-Kwai-Hang and Grosclaude, 2002)

Variant	Position and amino acid differences						
	10	97	104	135	136	148	155
A	Arg	Arg	Ser	Thr	Thr	Asp	Ser
B					Ile	Ala	
C		His					
E							Gly
F^S						Val	
F^I	His				Ile	Ala	
G^S						Ala	
G^E		Cys			Ile	Ala	
H				Ile			
I			Ala				
J							Arg

when compared to variant B (Prinzenberg et al., 1996). Variant H differs from variant A by a threonine to isoleucine substitution at position 135 (Prinzenberg and Erhardt, 1998). The DNA sequence of variant I revealed a substitution of serine to alanine at position 104 (Prinzenberg et al., 1999). The A and the J variant differed at position 155 with the replacement of serine by arginine (Mahé et al., 1999).

The A and the B variants can be resolved by polyacrylamide gel electrophoresis under alkaline conditions in the presence of a reducing agent in order to break the disulphide bonds (Mackinlay et al., 1966).

2.2.2. Whey Proteins

Whey proteins are the fraction that remain after isoelectric precipitation of casein from milk at pH 4.6 and constitute around 20 % of the total proteins in milk. There are four major whey proteins: β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulin. β-Lactoglobulin is polypeptide of 162 amino acid residues. All the ten genetic variants of β-lactoglobulin discovered so far involve amino acid substitutions (McKenzie et al., 1972; Bell et al., 1981; Eigel et al., 1984; Ng-Kwai-Hang and Grosclaude, 2002). The minor components of whey include several other proteins such as lactoferrin, transferrin, β_2 –microglobulin and proteose peptone fractions. The proteose peptone fraction represents 10 % of the total whey proteins (Sorensen and Peterson, 1993) and contains a complex mixture of more than 38 kinds of glycoproteins, phosphoproteins and proteolysis peptides from casein by indigenous proteinases present naturally in milk (Andrews, 1978a, 1978 b; Andrews and Alichanidis, 1983; Nejjar et al., 1986; Paquet et al., 1988; Paquet 1989).

2.3. Genetic polymorphism of milk proteins

Aschaffenburg and Drewry (1955) first reported the occurrence of genetic polymorphisms of milk proteins in the whey protein β-lactoglobulin. They observed that when milk samples were subject to paper electrophoresis under suitable conditions, it produced distinct bands that were denoted as β_1- and β_2 - lactoglobulin in the order of decreasing mobility. Since then, several polymorphisms were identified using various methods (Aschaffenburg, 1961;Thompson et al., 1962; Grosclaude et al., 1976;Brunner, 1981) and nomenclature for these variants is reviewed in detail (Eigel et al., 1984). The most recent account of milk protein polymorphism identified so far has been published

this year (Ng-Kwai-Hang and Grosclaude, 2002). Most of the work in this area is done in cattle due to obvious economic reasons. More genetic polymorphism of milk proteins is likely to be identified in the future with use of more sensitive methods and large scale screening of diverse populations. There are several reports on the identification of polymorphism at the level of DNA that may or may not be reflected in the primary structure of the protein due to the degenerate nature of the genetic code.

2.4. Molecular basis of milk protein polymorphism

Genetic polymorphism in the major milk proteins arises mostly due to amino acid substitutions and in certain cases, amino acid deletions. The exact location of the mutation can be ascertained only by sequencing and the complete sequence of α-lactalbumin (Brew et al., 1970), β-lactoglobulin (Braunitzer and Chen, 1972), α_{s1}-casein (Mercrier et al., 1971), α_{s2}-casein (Brignon el al., 1977), β-casein (Ribadeau Dumas et al., 1972), and κ-casein (Grosclaude et al., 1972b) has been identified. It must be noted here that genetic polymorphism arise only due to change in the amino acid sequence of the protein. Polymorphism due to post-translational modifications such as phosphorylation and glycosylation do occur in milk proteins and generally not considered genetic in nature. The term "polymorphism" is therefore confined only to genetic polymorphism throughout this text. Electrophoresis is the method widely employed to identify the genetic variants of milk proteins due to its simplicity and ease of application to a large number of samples. In certain cases, polymorphism due to amino acid substitution may not result in a change in the net electric charge of the protein and such type of variants will not be resolved under standard electrophoretic conditions. The term "silent variant" is referred to those variants and other methods have to be used for their identification (Carles, 1986; Dong and Ng-Kwai-Hang, 1998).

2.5. Frequency distribution of milk protein polymorphism

The frequency distribution of milk protein polymorphism in a population as a whole is closely associated with the breed and the species. Although, there is much information available on the occurrence and frequencies of milk protein variants in three major species of the genus Bos – *Bos Taurus*, *Bos indicus* and *Bos grunniens*, the data are incomplete and important gaps exists (Ng-Kwai-Hang and Grosclaude, 2002).

The A and the B variants of β-lactoglobulin are widely distributed in bovine and zebu herds and the B variant was more frequent in zebu as well as in most of the Western breeds such as Holstein and Jersey (Ng-Kwai-Hang et al., 1984, 1990; Kim, 1994). Variant C and D is usually considered as a rare variant. The B and the C variant of α-$_{s1}$ casein are universally distributed in all zebu and taurine breeds. The C variant is the most frequent in all Zebu herds, while the B variant is commonly found in most of the Western breeds. Variant A and D are rare variants found in a few European breeds (Ng-Kwai-Hang and Grosclaude, 2002). The A variant of α-$_{s2}$ casein is found to be the most common variant and the other two variants, B and C were observed specifically in the Yak populations (Grosclaude et al., 1982). α-$_{s1}$ caseins have not attracted much attention and several genetic variants in cattle still remains to be discovered. The A^1 , A^2 and the B variants are the common β-casein variants present in nearly all cattle populations in the world. The variant A^3 and variant C are relatively rare in the population.

2.5.1. κ-Casein variants

The A and the B variants of κ-casein were commonly observed in all the populations studied so far (Ng-Kwai-Hang and Grosclaude, 2002). The B variant was observed at a higher frequency in the Jersey breeds (Ng-Kwai-Hang et al., 1984; Kim, 1994), the Normande (Balland, 1987) and several other Italian breeds (Russo and Mariani, 1974). The variant D is identified only in German Simmental cattle so far (Seibert et al., 1987) and variant E was observed in Holstein-Friesian, German Red cattle and Angler with a low frequency (Erhardt, 1989; Buchberger, 1995). The two recently identified variants designated κ- casein F and G was observed only in Finnish Ayrshire cows (Ikonen et al., 1996) and in Pinzgauer cattle from Austria and Bavaria, Germany (Erhardt, 1996) respectively. All the variants excluding the A and the B variant are considered to be rare and their relative frequency is found to be very low in the population.

2.6. Significance of milk protein polymorphism

There is extensive literature on the possible relationships between genetic polymorphism of milk proteins and certain economic traits in the dairy industry. Several studies indicate statistical correlation between the occurrence of a milk protein variant in the population and traits like milk production, milk composition, reproductive

performance, susceptibility to mastitis, and growth rate and body weight. Since milk proteins are inherited in accordance with Mendelian mode of inheritance and coded by autosomal genes, it is possible to breed for desirable genetic variants. But, it is emphasized that in most cases little is known about the cause and effect relationship and any effect of a certain genetic variant on production traits are in the strictest sense statistical effects only (Ng-Kwai-Hang and Grosclaude, 2002).

Studies also indicate that milk protein variants influence milk composition. The properties of milk and dairy products largely depend on the amounts and relative proportions of the individual milk components. Polymorphism in a milk protein could have an effect on its level of expression, physico-chemical and functional properties and could be thereby exploited to our own advantage by appropriate breeding strategies.

2.6.1. Milk production:

Several reports indicate a relationship between genetic variants of milk proteins and milk production. It is a well-known fact that milk production is influenced by several factors. Consequently, the reports are conflicting with regard to the association between genetic variants and milk production due to possible contribution from other factors.

In case of β-lactoglobulin, some studies have reported no relationship with milk production (McLean et al., 1984; Ng-Kwai-Hang et al., 1984, 1990; Lin et al., 1986; Gonyon et al., 1987; Haenlein et al., 1987; De-Lange et al., 1991) whereas others reported higher production with phenotype BB (Janicki, 1978; Jairam and Nair, 1983). Few other reports show that the A variant cows are higher producers (Aleandri et al., 1990; Bovenhuis et al., 1992). A significant effect of β-lactoglobulin AA genotype on higher milk production is reported in Holstein Friesian cows (Ng-Kwai-Hang et al., 1986). Hetergozygous AB phenotypes were associated with a higher milk production in Estonian Black Pied cows (Pupkova, 1980).

No significant associations were observed between milk yield and genetic variants of α_{s1},β- and κ-casein (McLean et al., 1984, Gonyon et al., 1987). There were no significant differences in milk production between the different genetic variants of α_{s1} – casein in Aryshires, Brown Swiss and Jersey for three lactations (Kim et al., 1996) and Holsteins in the first and third lactation (Ng-Kwai-Hang et al., 1990) among Canadian dairy herds. Many other studies have reported a significant association. α_{s1}- casein B

variant has a higher milk production when compared with the other variants in some studies (Aleandri et al., 1990; Ng-Kwai-Hang et al., 1984, 1986,1990; Lin et al., 1986; Pupkova, 1980). β-Casein variant A^2 and A^3 were associated with a superior milk yield (Ng-Kwai-Hang et al., 1984,1986; Lin et al., 1986; Babukov et al., 1982;Kim et al., 1996). Some studies indicate that β-casein variants have no association with milk production (McLean et al., 1984; Ng-Kwai-Hang et al., 1984, 1990), while other studies have shown the there is some association with respect to the AA variant (Gonyon et al., 1987; Bovenhuis et al., 1992). The heterozygous AB phenotype cows were higher milk producers in another study (Ng-Kwai-Hang et al., 1986; Kim, 1994).

2.6.2. Milk composition

The composition of milk is an important criterion in determining the market value, nutrition and technological properties of milk. There have been several reports on the association of genetic variants of milk proteins with milk composition. It should be noted here that the relationship between milk protein variants and milk composition has yielded fairly consistent results in several studies, unlike that of milk production discussed in the previous section.

2.6.2.1. Milk proteins

The genetic variants of milk proteins were associated with casein and whey protein contents in several studies. β-Lactoglobulin was the first protein to be associated with milk composition (Aschaffenburg and Drewry, 1957). β-Lactoglobulin AA milk contained more protein than the AB or the BB milks (Morini et al., 1979; Ng-Kwai-Hang et al., 1984, 1986,1990; McLean et al., 1984; Graml et al., 1986) The milk of BB type cows had higher total protein content than AA cows (Coulon et al., 1998) and in another study suggested a positive additive effect on casein content and on the ratio of casein: total protein (Lunden et al., 1997). But, no relationships were found in other studies (Gonyon et al., 1987; Haenlein et al., 1987; Hill, 1993). Ng-Kwai-Hang and Kim (1996) reported that the concentrations of β-lactoglobulin in milk are associated with the genetic variants of the protein and are in the following decreasing order of magnitude, according to phenotypes: AA>AB>BB. In their study, the concentrations of total protein, casein and whey protein were determined in milk samples derived from 1278 Ayrshire, 718 Jersey, 396 Brown Swiss and 271 Canadienne cows which were all heterozygous for β-

lactoglobulin AB. PAGE, followed by densitometric scanning, were used to quantify the relative proportions of β-lactoglobulin A and B in the whey protein fraction. For the 4 breeds studied, a higher proportion of β-lactoglobulin was produced in the A variant than the B variant. In the heterozygous β-lactoglobulin AB, the A form was present in higher amounts of 49, 41, 40 and 31% when compared to the B form of the protein in Jersey, Canadienne, Brown Swiss and Ayrshire respectively. These results supported previous observations.

Phenotype BC of α_{s1}- casein is associated with higher contents of α_{s1}- casein, total casein and total protein, but lower β-lactoglobulin and whey protein than the BB or AB types (McLean et al., 1984; Kroeker et al., 1985; Ng-Kwai-Hang et al., 1986, 1987; Aleandri et al., 1990; Bovenhuis, 1992). Others reported no association between the genetic variants of α_{s1}- casein and protein in milk (Kim, 1994; Gonyon et al., 1987; Ng-Kwai-Hang et al., 1990).

β-Casein A^2A^2 phenotype were associated with higher protein concentration than A^1A^1 and A^1A^2 which are intermediate during three lactations in the Holstein breeds (Ng-Kwai-Hang et al., 1986; 1990). Other studies indicated no association in Ayrshires, Jerseys and Friesans (Mc Lean et al., 1984; Aleandri et al., 1990; Kim, 1994). It was observed that the A^1 variant was associated with a higher milk protein content than the A^2 variant (Gonyon et al., 1987; Ng-Kwai-Hang et al., 1986) while it was the A^2 variant in another study among Finnish Ayrshire cows (Ikonen et al., 1999).

The B variant of κ-casein is commonly associated with a higher amount of total protein and κ-casein in milk (McLean et al., 1984; Kroeker et al., 1985; Ng-Kwai-Hang et al., 1986; Gonyon et al., 1987; Rampilli et al., 1988; Aleandri et al., 1990; Bovenhuis et al., 1992; Coulon et al., 1998; Ikonen et al., 1999). In one study, it was shown that BB κ-casein is associated with a higher α_s-casein, κ-casein, serum albumin and immunoglobulins, but lower β-casein, β-lactoglobulin and α-lactalbumin content than κ-casein AA milk (Ng-Kwai-Hang et al., 1987). Ojala et al., (1997) reported certain interesting observations in a study of production traits comprising 916 Holstein and 116 Jersey cows using data on first-lactation milk yield, fat and protein percentages, and fat and protein yields. They demonstrated that differences between the β-lactoglobulin

genotypes for production traits were not statistically significant in either breed. Neither the β-casein A^2 allele nor the κ-casein B allele alone had a positive effect on milk and protein yield, but the joint effect was strongly positive. It was suggested that the results could be explained by the epistatic effects between the κ-casein and β-casein loci.

It is observed that the κ-casein variants A and B are synthesized differentially in the lactating mammary gland of heterozygous animals (van Eenennaam and Medrano, 1991). In a recent study, on an average, 13.4% more allele B specific than A specific mRNA transcripts were found in mammary epithelial cells using RT-PCR and capillary electrophoresis (Debeljak et al., 2000). DNA sequencing of the proximal promoter region in several homozygous animals (κ-casein AA, BB, EE) did not reveal any allele specific polymorphisms. Sequence analysis of the 3'-UTR (untranslated region) of the κ-casein gene revealed seven allele specific sites. Two of these allelic differences were close to previously identified 3' end regulatory sequences. In addition, allele specific differences in length between mRNAs of both the variants were found. The two later findings suggest a possible post-translational control determining the differences in the content of κ-casein in milk. Robitaille and Petitclerc (2000) observed that in a group of 18 lactating Holstein cows, all heterozygous AB for κ-casein, there was more messenger RNA transcribed from allele B than from A in 8 cows. This discrepancy is thought to arise as a result of a polymorphism in the non-coding region of the gene on one allele. They suggested that for heterozygous κ-casein AB cows, the content of allele A- and B-specific messenger RNA will either be similar when the 2 alleles had the same DNA sequence within the polymorphic region or different when 1 of the 2 alleles carried the mutation that caused the differential expression of the κ-casein gene.

2.6.2.2. Milk Fat

There are conflicting reports on the association of genetic variants of milk proteins with milk fat content. Some studies indicate no significant correlation between the genetic variants of β-lactoglobulin with milk fat content (Lin et al., 1986; Kim, 1994), while others show positive association (McLean et al., 1984; Ng-Kwai-Hang et al., 1984, 1986; Graml et al., 1986). Highest milk fat is associated with the BC variant of α_{s1}-casein (Munro, 1978; Ng-Kwai-Hang et al., 1986), B variant of β-casein (McLean et al.,

1984; Ng-Kwai-Hang et al., 1986) and the B variant of κ-casein (Ng-Kwai-Hang et al., 1986). For the different possible phenotypes of -βcasein that were observed, the fat content of milk was in the following descending order according to the genetic variants: A^1B, A^1A^1, A^1A^2, A^2A^2 , A^1A^3 , A^2A^3 , A^2B (Ng-Kwai-Hang et al., 1986).

2.6.2.3. Physico-chemical properties

The genetic variants of milk proteins have been clearly demonstrated to influence the behavior and functional properties of milk. This has important implications in the dairy industry during the manufacture of different dairy products especially cheese.

The coagulation properties of milk are greatly influenced by the genetic variants. Sherbon et al., (1967) observed that milks from cows of κ-casein AA phenotype showed both significantly longer rennet clotting times and lower curd tension that κ-casein AB and particularly κ-casein BB phenotypes. κ-Casein containing milk is associated with shorter coagulation time, higher rate of curd formation and higher curd firmness (Davoli et al., 1990; Ikonen et al., 1999). Other reports suggested that the κ-casein B milk had better synergetic properties and shorter coagulation time (Scar, 1984; Pancake and Carole, 1987; Alexandra et al., 1990). β-Lacto globulin AA milk had a better clotting time and curd firmness when compared to AB and BB phenotype (Martial and Ng-Kuwait-Hang, 1986b). The B variant of κ-casein and β-lacto globulin was associated with increased heat stability (McLean et al., 1987). The phenotype combination β-lactoglobulin AA + κ-casein BB is associated with the highest maximum coagulation time in milk in a recent study (Paterson et al., 1999). But, it is suggested that the heat stability of different β-lactoglobulin phenotypes was variable and dependent on the pH and composition of buffer (Imafidon and Ng-Kwai-Hang, 1991).

Milk protein polymorphism had significant effect in cheese yield. Milk containing of β-casein A^1, κ-casein B and β-lactoglobulin B variant were found to be more favorable for cheese yield (Ng-Kwai-Hang, 1990, 1993; Tong et al., 1993). Milk containing the BB phenotype of κ-casein and β-lactoglobulin had higher cheese yield and better rennet coagulation characteristics (Marziali and Ng-Kwai-Hang, 1986a; Ng-Kwai-Hang, 1990, 1993; Horne et al., 1993; Puhan and Jakob, 1993; van den Berg, 1993). Choi and Ng-Kwai-Hang (1998) investigated the effect of genetic variants of κ-casein and β-

lactoglobulin on cheese yielding capacity of laboratory-scale Cheddar cheeses using 834 samples of preheated milk with different phenotypes of κ-casein and β-lactoglobulin and preheating temperatures of 30, 70, 75 and 80°C. Least squares analysis of the data indicated that 37%-moisture-adjusted cheese yield was highest for milk with phenotypes BB for κ-casein and β-lactoglobulin and lowest for phenotypes AA for both proteins. Increasing the preheating temperature of cheese milk resulted in higher cheese yield. α_{s1}-Casein BB and β-casein A^1A^1 were also associated with a higher cheese yield (Aleandri et al., 1990). Ng-Kwai-Hang (1994) reported that the milk from cows with β-casein AA, κ-casein BB and β-lactoglobulin BB were associated with higher cheese yielding capacity and suggested that differences due to fat and casein in milk associated with the genetic variants only partially explained the observed differences in cheese yield according to milk protein types.

2.7. Phenotyping of milk proteins

The identification of genetic variants of the whey protein β-lactoglobulin by Aschaffenburg and Drewry (1955) using paper electrophoresis followed by the correlation of milk protein variants with various economic traits in the dairy industry led to the development of several methods of phenotyping milk proteins over the years. The important techniques used to characterize the proteins in milk include electrophoresis, liquid column chromatography and immunochemical methods. Among these techniques, electrophoresis is the most widely used and rapid method for determining the genetic variants of the major milk proteins in raw milk.

2.7.1. Electrophoresis

There are numerous electrophoretic methods employed to identify the milk protein variants with several modifications to improve the efficiency and reduce the time for large scale screening of populations. Electrophoresis is mainly carried out in paper, starch, polyacrylamide and agarose usually in the presence of a reducing agent to enable the dissociation of certain caseins held together by disulphide bridges. Paper electrophoresis is largely replaced by starch gel and polyacrylamide electrophoresis in recent days.

The principle of electrophoretic separation involves differential migration of proteins due to differences in their net electric charge determined by the amino-acid

composition. Milk protein polymorphisms arise mostly due to amino-acid substitutions and in certain cases deletions in the primary structure of the protein. These modifications can be detected in electrophoresis if the amino acid that is involved contains a net electric charge. Other modifications involving neutral amino acids cannot be resolved by electrophoresis and different techniques have to be used for their identification.

2.7.1.1. Starch gel electrophoresis

Starch gel urea electrophoresis under alkaline conditions was first introduced by Thompson et al., (1962) for the identification of the three α-$_{s1\ casein}$ variants in Holstein, Brown Swiss and Ayrshire breeds. Since then, attempts were made to identify the polymorphs of all the other milk proteins. Bell (1962) used this method without urea to detect the three variants of β-lactoglobulin in Australian Droughtmaster beef cattle. The A and the B variants of α-lactalbumin can be identified used starch gel electrophoresis (Blumberg and Tombs, 1958; Bhattacharya et al., 1963). Thompson et al., (1964) and Michalak (1967) used this electrophoresis for the detection of genetic variants of β-casein A, B and C. Further separation of the β-casein A variants was accomplished under acidic conditions using starch gels (Kiddy et al., 1966; Peterson and Kopfler, 1966; Arave, 1967). It was not until the inclusion of sufficient quantities of a reducing agent such as mercaptoethanol either in the sample buffer or the electrophoretic gel that the genetic variants of κ-casein and α_{s1}-casein were identified. κ-Casein was resolved into two bands by this minor modification (Neelin, 1964; Schmidt, 1964; Woychik, 1964) and later confirmed by genetic analysis to represent the two genetic variants of κ-casein (Grosclaude et al., 1972b). The three variants of α_{s1}- casein were identified by electrophoresis in starch gels containing 2-mercaptoethanol and 7 M urea under alkaline conditions (Grosclaude et al., 1972a).

2.7.1.2. Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels is widely used for phenotyping caseins and whey proteins (Raymond and Wang; 1960; Aschaffenburg, 1964; Thompson et al., 1964; Aschaffenburg and Thymann, 1965) and generally replaced the use of starch gels due to ease of preparation without heat treatment enabling easy manipulation. These gels are prepared by polymerizing monomers of acrylamide by cross-linking with N, N'-methylene-bisacrylamide. The polymerization is initiated by the addition of ammonium

persulfate and the reaction is enhanced by N,N,N'-tetramethylethylenediamine, which catalyzes the formation of free radicals from ammonium persulfate. Care should be taken in the laboratory during the preparation of gels since the monomeric acrylamide is a potential neurotoxic agent. Apart from the safety precautions, polyacrylamide gels are the most efficient and rapid method for large-scale phenotyping of the major milk proteins.

Under alkaline conditions of PAGE with urea, the relative order of migration of the major caseins from the fastest to the slowest are: α_{s1}- casein > α_{s2}- casein > β-casein > κ-casein due to the decreasing net negative charge from α_{s1}- casein to κ-casein (Kiddy 1975; Ng-Kwai-Hang et al., 1984). According to Ng-Kwai-Hang et al., (1984), the separation of the caseins can be performed with a larger pore size gel containing 8 % acrylamide and 4 M urea, and the whey proteins β-lactoglobulin and α-lactalbumin could be accomplished with 12% polyacrylamide in the absence of urea. As in the case of starch gels, resolution of β-casein A variants is not successful under alkaline conditions. PAGE under acidic conditions is required for further resolution of the A variant of β-casein (Peterson and Kopfler, 1966; Ng-Kwai-Hang et al., 1984; Medrano and Sharrow, 1989). More recently, a rapid capillary zone electrophoresis (CZE) method was established for separating and quantifying major casein and whey proteins in milk (Vallejo-Cordoba, 1997). Optimum sample preparation and electrophoretic conditions in a coated capillary maintained at 40° C allowed accurate and reproducible quantification of milk proteins in a single analysis. The accuracy and reproducibility of the technique permitted the quantitation of individual protein concentrations in milk samples, which agreed with ranges reported in the literature. CZE might be well suited for routine use by dairies and regulatory agencies, since it allowed the determination of milk proteins in less than 60 min. There are several methods and modifications of PAGE to phenotype milk proteins and to discuss each one of them in detail would be beyond the scope of this text.

2.7.1.3. Isoelectric focussing (IEF)

This technique was applied to phenotype milk proteins much earlier (Peterson, 1969; Finlayson and Chrambach, 1971; Kaplan and Foster, 1971; Josephson et al., 1971; 1972) although proper identification of the various components of whole casein came only later (Trieu-Cuot and Gripon, 1981). IEF requires long running times than PAGE since the mobility of the proteins decrease as they approach the isoelectric point. But this

technique has a better resolution capable of separating proteins differing by only 0.02 pH units in their isoelectric points (Peterson, 1969). This property was exploited for phenotyping the genetic variants of milk proteins. κ-Casein variant E was identified by this method (Erhardt, 1989) and also the existence of genetic variants of α_{s1}- casein, α_{s2}-casein and β-lactoglobulin (Krause et al., 1998).

2.7.1.4. Two-dimensional electrophoresis

This technique has a very high resolution in separating complex mixture of proteins since electrophoresis is carried out in two dimensions using different criteria usually the isoelectric pH and molecular weight. Trieu-Cuot and Gripon (1981) using IEF in the first dimension and SDS-PAGE in the second to fractionate caseins. Several others have successfully used this technique and achieved better resolution of milk protein variants (Klose, 1987; Basha, 1988; Holt and Zeece, 1988; Addeo et al., 1988; Tutta et al., 1991; Girardet et al., 1991). The much higher resolution of this method can be exploited to study the nature of complex milk protein hydrolysates such as those in conditions of cheese ripening, which is difficult to ascertain using conventional techniques.

2.7.1.5. Immuno-chemical techniques

The antigenic nature of proteins is exploited in these methods and used occasionally to detect the genetic variants of milk proteins (Addeo et al., 1995; Chianese et al., 1992; 1995; Lopez-Galvez et al., 1995). The combination of electrophoresis and immuno-chemical techniques makes it a highly sensitive technique with no ambiguities in the interpretation of results quite commonly observed while using conventional techniques. The limitations of this method are the relatively poor antigenicity of caseins and aggregation behaviour in solution.

2.7.1.6. Chromatographic techniques

Chromatography, although much laborious and time consuming than electrophoresis, can be used for phenotyping milk proteins. Thompson and Pepper (1964) separated β-casein A and C in a DEAE-cellulose system containing urea. Later, Ng-Kwai-Hang and Pelissier (1989) resolved the B and the C variant of β-casein using QAE-cellulose ion exchange chromatography. κ-Casein A and B variants were resolved as separated components using DEAE-cellulose column containing imidazole-HCl-urea at

pH 7.0 (Thompson, 1966). Ion-exchange chromatography is inefficient in phenotyping milk proteins due to lack of discrimination between the variants based on their net electrical charge.

The time limitations of this technique were dramatically reduced and the resolution improved with the advent of Fast Protein Liquid Chromatography (FPLC) and High Performance Liquid Chromatography (HPLC). HPLC was used to separate the A and the B variants of β-lactoglobulin by several authors (Pearce, 1983; Humphrey and Newsome, 1984; Andrews et al., 1985) and later shown to resolve the A and the B variant of κ-casein by others (Dalgleish, 1986; Visser et al., 1986; Guillou et al., 1987; Dong, 1992).

2.8. Fractionation of milk proteins

There are several methods described in literature pertaining to the fractionation and purification of milk proteins. The use of salt and solvent fractionation techniques during earlier periods (McKenzie, 1971) is largely superseded by the advancement in chromatographic techniques over the years. Various forms of liquid column chromatography is the single most important method for the separation and characterization of the major proteins.

2.8.1. Gel Filtration

This method is based on the molecular size of the proteins and first attempted by Yaguchi and Tarassuk, (1967) for direct analysis of skim milk on Sephadex G 200 column packing. The resolution was poor and casein separated into three fractions. Since then several attempts were made to separate skim milk proteins on high performance gel filtration columns (Dimenna and Segall, 1981; Shimazaki and Sukegawa, 1982; Gupta, 1983). Gel filtration is inefficient in the fractionation of skim milk and whole caseins since caseins exist in the form of micelles and form large aggregates in the absence of calcium. The molecular weight of different caseins is also too close to each other to permit fractionation by gel filtration.

The fractionation of whey proteins using this method produced much better results and used for their characterization (Dimenna and Segall, 1981; Shimazaki and Sukegawa, 1982; Gupta, 1983; Humphrey, 1984; Andrews et al., 1985).

2.8.2. Ion exchange

Ion-exchange chromatography can be successfully used for the fractionation of whole casein and whey proteins. The net electric charge of the various casein and whey proteins are significantly different and is fully exploited by ion-exchange methods. Earlier attempts were made for the use of cation exchange columns for the separation of milk proteins. α_{s1}-Casein was purified on a Sulphoethyl Sephadex C-50 column (Annan and Manson, 1969). Snoeren et al., (1977) resolved α_s- caseins and κ-casein on a cation exchange Amberlite C650 column with 3M urea and NaCl gradient at pH 6.0. Andrews et al., (1985) and St-Martin and Paquin (1990) fractionated caseins on a cationic Mono S ChR/5/5 column in a pH of 3.2-3.8 buffers. Purification of certain whey proteins can also be achieved by Sulphopropyl cation-exchange chromatography (Yoshida and Ye-Xiuyun, 1991).

By and large, anion-exchange columns are more frequently employed than cation exchangers. DEAE-cellulose is the most commonly used anion-exchange resin for the fractionation of caseins. There are several classical methods described: Thompson (1966); Davies and Law (1977); Andrews and Alichanidis (1983). Most of these methods used urea to disintegrate the casein micelles and mercaptoethanol to dissociate the disulphide linkages in κ- and α_{s2}- caseins. The major differences among them are the NaCl gradients used to elute the caseins. An increasing gradient of salt concentration aided in the separation of α_{s1}- casein, β-casein and κ-casein (Gordin et al., 1972; Davies and Law, 1977; Donnelly, 1977; McGann et al., 1979). Humphrey and Newsome (1984) used a TSK gel DEAE-5PW and Mono Q HR5/5 anion-exchange column with increasing ionic strength buffer gradient for the compositional analysis of caseins. Vreeman et al., (1977; 1986) purified κ-casein on a DEAE column by decreasing pH gradient from 6.0 to 4.5. A recent advancement in this technique is the use of DEAE-Sepharose that allowed higher flow rates than DEAE-cellulose. Ng-Kwai-Hang and Pelissier (1989) fractionated bovine caseins by mass ion exchange chromatography on a QAE Zeta Prep 250 cartridge using urea containing 0.2 M imidazole and 0.03 M 2-mercaptoethanol. Purified forms of the four major caseins were obtained using a salt gradient in a Protein-Pak DEAE 15HR anion-exchanger in Tris-urea buffer (Ng-Kwai-Hang and Dong, 1994). Whey proteins were also subject to fractionation by anion-exchange method on a DEAE-cellulose

column with a step-wise increase in salt concentration (Yaguchi and Rose, 1971; Swaisgood, 1982). Imafidon and Ng-Kwai-Hang (1992) employed a QAE Zeta Prep cartridge for the isolation and purification of β-lactoglobulin by eluting with 0.05 M phosphate buffer containing 0.3 M NaCl.

2.8.3. Hydroxyapatite

The whole caseins are fractionated in this method according to their phosphate content. Addeo et al., (1977) reported separation of caseins using hydroxyapatite columns. Visser et al., (1986) obtained separation of caseins on a Bio-Gel HPHT column with KH_2PO_4 and $CaCl_2$ containing mobile phase. κ-Casein A and B variants could be partially separated but α_{s1}-casein and α_{s2}-casein were poorly resolved. Kawasaki et al., (1986) also made similar observations.

2.8.4. Reverse phase HPLC

This method is based on hydrophobic interactions between the stationary phase and the proteins to be separated. The fixation of proteins on the phase is carried out in aqueous solution of low ionic strength and increasing the hydrophobicity of the mobile phase results in elution of the proteins. The column packing material used as stationary phase in RP-HPLC are usually made of silica derivatized with non-polar groups. RP-HPLC has greatly reduced the time and achieved a better resolution enabling separation of various proteins and peptides. Large pore C-4 (Parris et al., 1990), C-8 (Strange et al., 1991; Mikkelsen et al., 1987) and C-18 (Visser et al., 1986; 1991; Carles, 1986) columns have produced excellent analytical separations of whole caseins. The solvent system usually used in TFA-water-acetonitrile (0.1:70:30).

Whey proteins can also be separated with reversed phase columns. Diosady et al., (1980) separated whey proteins into its three major fractions using a C-8 column and an isopropanol gradient. Similarly, a C-6 column can be used to fractionate whey proteins in an acetonitrile gradient (Pearce, 1983).

2.9. Enzymatic hydrolysis of caseins

2.9.1. Rennet

The hydrolysis of caseins by enzymes is widely employed in the food industry for the manufacture of diverse products especially cheese. Traditionally, salt extracts from the stomach of young animals containing various proportions of chymosin and pepsin,

referred to as rennet were used for the initial coagulation of milk during the manufacture of cheese. Calf chymosin is still the prevailing milk coagulant used widely in the cheese industry. But due to shortage of this source of rennet due to increase in the production and demand for cheese, pepsin and other sources of rennet from microbes and plants have been increasingly used as rennet substitutes either alone or in combination with chymosin (Foltmann, 1993).

The proteases in animal and fungal rennet all belong to the group referred to as aspartic proteases, also called acid proteases. The names ascribed indicate that the carboxyl groups of two aspartic acid residues are the catalytic groups in the active sites of the enzyme and also the pH optima are around pH 2-4. These proteases are inhibited by pepstatin and pepsin is the best-studied enzyme of this group (Whitaker, 1994).

2.9.1.1. Properties of rennet

All the acid proteinases have the ability to hydrolyze caseins at approximately the required position, but they are not identical in their properties. The effects of pH and temperature on the activity and stability for general proteolytic activity for the various enzymes have been studied in many laboratories worldwide. Although, the values are within a narrow range in different studies, it has to be recognized that the observed values depend on the experimental conditions such as denaturation of substrates, ionic strength of the solutions, duration and temperature of the experiments and the methods used to follow the progress of the reaction (Dalgleish, 1992). Under the following conditions with hemoglobin as substrate, at optimum pH, and solutions of equal milk clotting activity against bovine milk, the ratios of proteolytic activities of different enzymes were as follows: Calf chymosin/bovine pepsin/porcine pepsin/: 1/3/6 (Foltmann, 1966).

Autolysis is observed at pH optima for chymosin and pepsin, whereas the fungal proteases were stable down to pH 2.5 (Whitaker, 1970; Arima et al., 1970). Porcine pepsin and calf chymosin lose activity at pH values above 6.5, but the rate of inactivation is more for porcine pepsin, which shows a sharp drop in stability at pH 7 (Foltmann, 1966). Bovine and chicken pepsins are stable at pH 7 but are inactivated at pH 8 (Bohak, 1970). The stability of various mammalian gastric proteases in urea is different, with pepsin being the most stable and this can be used to differentiate among bovine enzymes (Raap et al., 1983).

When chymosin is compared with almost all the other rennet substitutes in terms of its proteolytic activity, none of them is found to be favorable. In a study using synthetic substrates, chymosin had less proteolytic activity than Mucor proteinases and bovine pepsin, which had the highest proteolytic activity among the three (Martin et al., 1980). Of all the available enzymes, porcine pepsin is the only one which is potentially less proteolytic than chymosin, since it is readily denatured in the cheese making process (Green and Foster, 1974).

Since milk is a complex biological secretion containing many components, the properties of rennet in aqueous solutions may not truly reflect the conditions present in milk. Therefore, several studies were undertaken to understand the properties of rennet in conditions closely resembling milk. The stability of rennet in whey around pH 5.2-7.0 and between 68.3-73.9°C was studied (Thunell et al., 1979). Among the six rennet's, the protease from *M.meihei* is the most heat stable followed in order by *M.pusillus* protease, calf rennet, bovine pepsin, *E.parasitica* protease and porcine pepsin. With decreasing pH, the heat stability of all enzymes increased with the exception of *E.parasitica* protease.

2.9.1.2 Recombinant rennet

Rennets represent the single largest industrial use of enzymes with a world market of approximately 25 x 10 ^{6} L of standard rennet amounting to approximately £100 million (Fox, 1986). Consequently, rennet is attractive to industrial enzymologists and biotechnologists.

The gene for calf prochymosin has been cloned in several laboratories and its structure and properties analyzed (Hidaka et al., 1986; Nishimori et al., 1981; Harris et al., 1982; Moir et al., 1982; Emtage et al., 1983; Marston et al., 1984; McCaman et al., 1985; Kawaguchi et al., 1987). It is observed that the enzymic properties of recombinant E.coli chymosin are indistinguishable from those of native calf chymosin (Kawaguchi et al., 1987; Meisel 1987, 1988).

Saccharomyces cerevisiae or baker's yeast can be employed to clone the gene for prochymosin and the level of expression is 0.5 –0.2 % of total yeast protein (Mellor et al., 1983; Goff et al., 1984). In yeast, 80 % is still associated with cell debris and only about 20 % of the prochymosin can be released in soluble form that can be activated directly.

One of the primary objectives of the production of recombinant rennet is that the microorganisms secrete larger quantities of the protein into the culture medium with a reasonable milk clotting activity. Filamentous fungi were found to be advantageous in these aspects when compared to yeast. Prochymosin can be expressed in *Aspergillus nidulans* (Cullen et al., 1987), *Aspergillus niger* (Ward et al., 1990) and *Tricoderma reesei* (Harkki et al., 1989).

Recombinant chymosin has been used in several cheese making experiments (Hicks et al., 1988; Green et al., 1985; Lieske and Konrad, 1996) and the general aspects of recombinant chymosin is reviewed in detail (Teuber, 1991; Mohanty et al., 1999). The current opinion is that in cheese making experiments no major difference could be detected between cheeses made with cloned chymosin or the natural enzyme.

2.9.2. Coagulation of milk

Coagulation of milk is an essential characteristic step in the manufacture of all cheese varieties. The vast majority of ripened and some fresh cheeses are produced by enzymatic rennet coagulation that involves limited proteolysis by a crude proteinase (rennet). Isoelectric precipitation by in-situ production of lactic acid by a starter culture or direct acidification with pre-formed acid, usually HCl, or acidogen, like gluconic acid β--lactone, is used mainly for fresh cheeses. A combination of acid and heat, usually by acidification to a pH value above the isoelectric point with acid whey, acid milk, citrus juice, vinegar or acetic acid at elevated temperatures is also used for the coagulation of milk in production of certain cheese varieties.

The suitability of a protease for milk clotting is largely determined by a high ratio of milk clotting activity to general proteolysis and inability to form bitter peptides even after several months of storage of cheese. This specific criterion excludes the use of several proteases and confines the milk clotting agents in commercial cheese industry to a select few. Only six rennet substitutes have been found to be more or less acceptable for one or more cheese varieties: bovine, porcine and chicken pepsins and fungal proteases from Mucor meihei, M. pusillus and Endothio parasitica (Fox, 1986).

2.9.2.1. Phases of enzymatic coagulation

The enzymatic coagulation of milk occurs in two phases: enzyme dependent primary phase followed by a secondary non-enzymatic phase (Dalgleish, 1992). Primary

phase involves the attack on κ-casein by the proteolytic enzymes added to milk followed by a secondary stage in which the aggregation of the caseins takes place due to the de-stabilization caused by the enzymatic attack. Much of the following discussion will pertain to the primary phase of milk coagulation. It should be noted however that the coagulation of milk is a two-stage process and any variation in the conditions could affect these two stages differently. For instance, no clotting occurs below 15°C, which is not due to loss of enzyme activity, but by the low coagulation of renneted micelles around this temperature scale (Berridge, 1942). Since κ-casein form an essential substrate in the primary stage, knowledge of the nature of κ-casein and their behavior in milk would facilitate better understanding of the coagulation process itself and provide clues to potentially exploit the process to our own advantage.

2.9.2.2. Casein micelles:

Caseins occur in milk in the form of large spherical complexes called micelles. Several models have been proposed to explain the structure of the micelles. According to the widely accepted model proposed by Walstra (Walstra and Jenness, 1984), the micelles are constituted by numerous small sub-micelles (Figure.1). The sub-micelles found in the interior of the native micelle is relatively rich in the calcium-sensitive caseins and those found on the surface are relatively rich in κ-casein. The calcium-phosphate salt bridges help in cross-linking the sub micelles to form the final native micelle.

2.9.2.3. Structure and role of κ-casein:

The structure and properties of κ-casein is different from other milk proteins due to its unique role in milk. The primary structure of κ-casein consists of 169 amino acids (Mercier et al., 1973). Residues 1-105 (para-kappa casein) are hydrophobic and the residues 106-169 (glyco-macropeptide) are extremely hydrophilic in nature. So, the primary structure is extremely amphipathic in nature (Hill, 1968) and its physical

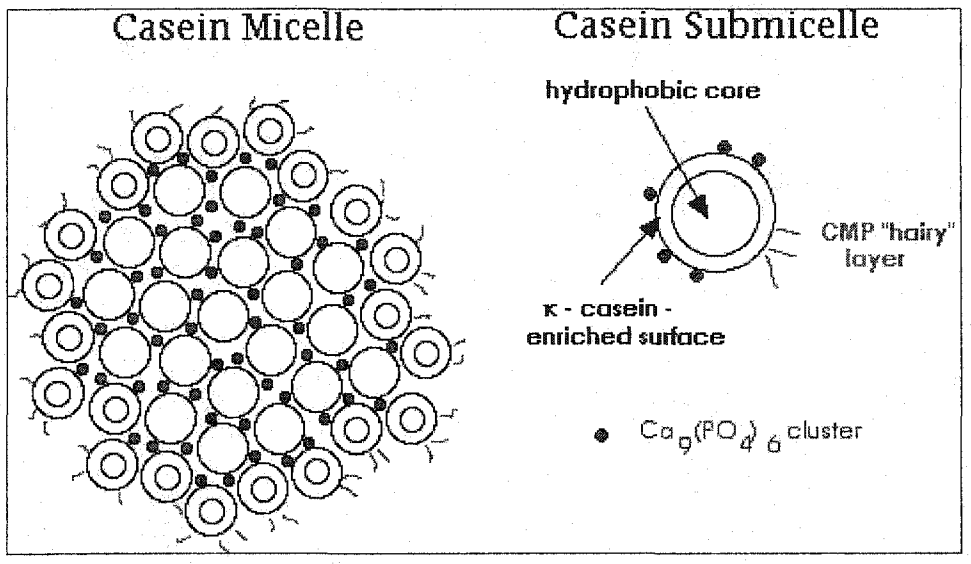

Figure1. Sub-Micelle Model Of Casein Micelles

(Adapted from Waltstra and Jenness, 1984)

properties insensitive to the presence of calcium. It is soluble at a concentration of calcium found in milk whereas other caseins are insoluble in those conditions. There is also glycosylation of threonyl residues and a marked absence of phosphoseryl clusters, and only one or two sites of phosphorylation in the whole structure (Swaisgood, 1993). The amphipathic nature of the κ-casein has a stabilizing effect on the structure of the micelles. κ-Casein is located mainly on the surface of the native micelles (Donnelly et al., 1984; Rollema et al., 1988) with the hydrophilic macro peptide protruding in the surrounding medium interacting with the solvent. This hydrophilic moiety is considered to stabilize the micelle (Holt 1975; Holt and Dalgleish 1986; Horne 1984, 1986; Walstra, 1979). The stability of the casein micelle is dependent on κ-casein present on the surface of the micelle as already discussed above. It functions as an interface between the hydrophobic calcium sensitive caseins of the micelle interior and the aqueous environment of the milk serum. The three-dimensional structure of κ-casein plays a key role in regulating the function of the protein on the surface of the micelle. κ-Casein needs to bind to the calcium-casein complexes of the micelle core and must be able to prevent premature micellar coagulation. It has been speculated that individual κ-casein molecules cross-link into disulfide-bonded polymers with a structure such that the hydrophilic tails project into the milk serum and the hydrophobic regions attach to the micelle core. Since, κ-casein is found to be strongly self-associating in both oxidized (S-S-) and reduced (SH-) forms (Talbot and Waugh, 1970; Slattery and Evard, 1973) and never been crystallized, the usual means of determining structure are unavailable. Instead, indirect methods such as algorithms, circular dichroism spectroscopy (Plowman et al., 1997) and more recently NMR spectroscopy (Plowman et al., 1999) are used to predict selected secondary structures.

2.9.2.4. Primary stage of coagulation

Milk clotting enzymes cleave κ-casein present on the surface of the micelle at the junction between the para-kappa casein and macropeptide moieties (Jolles et al., 1968). This results in the loss of the macro peptide into the whey and absence of its stabilizing effect on the native structure of the micelle. Release of the polar glycomacropeptide domain from κ-casein eliminates the polar steric stabilization of the micelle surface, with an increase in surface hydrophobicity allowing micelles to associate, leading to clot

formation (Schmidt, 1980). The micelles then begin to aggregate once enough of κ-casein has been hydrolyzed. The coagulation process is initiated and it is understood that the milk may begin to clot even before the enzymatic cleavage of κ-casein is complete.

During the process of renneting by the action of acid proteinases, the casein micelle-protective capacity of κ-casein is destroyed (Waugh and von Hippel, 1956). κ-Casein is also the only milk protein hydrolyzed during the primary phase of rennet action and only one peptide bond; Phe_{105}-Met_{106} is hydrolyzed, releasing the C-terminal region (the glycomacropeptide) (Delfour et al., 1965). The unique sensitivity of the Phe-Met bond of κ-casein that is cleaved optimally at pH 5.1-5.5 (Humme, 1972) captured the attention of several workers in the cheese industry. Subsequently, cleavages at other points occur but it is considered that any general proteolysis of the caseins during the clotting reaction is a disadvantage, leading to increased losses of soluble peptides.

2.9.3. Specificity of κ-casein hydrolysis

All the acid proteinases used in commercial cheese making are highly specific for the Phe_{105}-Met_{106} bond of κ-casein and only this results in a highly effective milk clotting reaction (Jolles et al., 1968). Any non-specific hydrolysis generally produces a weak curd, bitterness of cheese and loss of peptides in whey. This excludes the use of many proteases found in nature for the coagulation of milk. As already mentioned, the suitability of a protease to be used for milk coagulation includes a high ratio of milk clotting activity to general proteolysis and inability to form bitter peptides even after long time storage of cheese.

2.9.3.1. Hydrolysis of the chymosin sensitive bond of κ-casein

The specificity of the Phe_{105}-Met_{106} bond of κ-casein towards attack by chymosin and other rennet have been analyzed in detail to devise possible mechanisms to improve the rate of hydrolysis and produce good quality cheese. The bond specificity is thought to arise from various factors such as the primary structure around the bond, the conformation of κ-casein and possibly its state of aggregation.

Residues 97 – 129 of the primary structure appears to be important and changes in this region affected the reactivity of the bond (Beeby, 1979; Hill and Hocking, 1978). Chymosin does not cleave Phe-Met dipeptide (Voynick and Futon, 1971) or a tri- or tetra- peptide (Hill, 1968; Schattenkerk and Keerling, 1973). Hill (1969) demonstrated

that the rate could be enhanced by incorporation of the bond into a pentapeptide. Further extending this bond at either end influences the reactivity of the bond (Raymond et al., 1973; Schattenkerk and Keerling, 1973; Visser et al., 1977). The peptides used in these types of studies were identical to that of κ-casein. Genetically engineered κ-casein cDNA mutated to change the chymosin sensitive site from a Phe_{105}-Met_{106} bond to a Phe_{105}-Phe_{106} bond were hydrolyzed with chymosin at 30°C and the mutant κ-casein (Phe_{105}-Phe_{106}) is hydrolyzed approximately 80% faster than the wild-type (Phe_{105}-Met_{106}) κ-casein as determined using Western blots, followed by immunochemical staining and laser gel scanning (Oh and Richardson, 1991). Visser et al., (1980) observed that the residues 98-112 undergo hydrolysis at a similar rate to intact κ-casein. This observation suggested that the histidine residues, which are present only in this region, participate in enzyme-substrate binding. The hydroxyl group of Ser_{104} is also strongly involved in both binding and catalysis, and the proline residues at positions 109 and 110 are also important (Visser, 1981). Hence, the residues in the immediate vicinity of the sensitive bond appear to play a role in the reaction.

The strategic location of the bond on κ-casein molecule so that it is readily accessible to the enzyme is also a factor in governing the reaction mechanism. So far, determining the conformation of κ-casein was unsuccessful. But models based on calculations predict that the Phe-Met bond is situated in a projecting beta structure (Raap et al., 1983) and according to another model could also interact with the enzyme by a β-sheet formation (Jenkins et al., 1977). Another prediction suggested that the bond is located in β-turn rendering it prone to enzymatic attack (Loucheux-Lefebvre et al., 1978).

The size of the micelles upon which the κ-casein is the third major factor to influence the reaction. The clotting time of medium sized micelles were significantly shorter that either larger or smaller micelles (Ekstrand et al., 1980). It was therefore, suggested that κ-casein in micelles of different sizes were hydrolyzed differently, since it is known that the rate of aggregation of fully renneted micelles does not depend on micellar size (Dalgleish, 1980). Effects of the genetic and non-genetic variants of κ-caseins on the renneting reaction were also studied and the effect of glycosylation of κ-casein on the reaction rate is contradicting, some showing no effect (Chaplin and Green,

1980; Dalgleish, 1986) and others indicating that glycosylation had a significant effect (Addeo et al., 1984; Sinkinson and Wheelock, 1970; Hooydonk and Walstra, 1987; Vreeman et al., 1986). The most common genetic variants of κ-casein, the A and the B variant seen in Holstein dairy herds, did not show any significant variation on the rate of hydrolysis (Dalgleish, 1986), though it is generally agreed that milk containing the B variant clots more rapidly than milk containing κ-casein A (Aaltonen and Antila, 1987; Pagnacco and Caroli, 1987; Schaar, 1984).

2.9.3.2. Reaction kinetics

Milk clotting reaction is thought to involve a single-step enzymatic hydrolysis of the κ-casein substrate and assumed to follow the standard Michaelis-Menten kinetics. Several studies have been done to understand the kinetics of hydrolysis in this way (Azuma et al., 1984; Carles and Martin, 1985; Castle and Wheelock, 1972; Chaplin and Green, 1980; Dalgleish, 1979; Hooydonk et al., 1984). The hydrolysis of synthetic peptides by chymosin obeyed Michaelis-Menten behavior in aqueous systems (Visser et al., 1980). But, there is always a reasonable amount of speculation as to whether the Michaelis-Menten mechanism is the correct formulation to use to describe the reaction (Dalgleish, 1992). This is because of the implicit assumption that the enzyme and the substrate are able to equilibrate at all times and also mobile so as to freely interact with each other during the reaction process. The conditions are quite different during the actual coagulation process. The proteases, relatively small in size compared with the casein micelles, freely move through the solution. The chief substrate, κ-casein confined mainly to the surface of the micelles, in turn is constrained to move through the solution as the micelles move and this could be relatively slow as compared to the enzyme (Hooydonk and Walstra, 1987). Brinkhuis and Payens (1985) suggested that the enzyme molecules bind to the micelles and create patches of para kappa casein by attacking adjacent κ-casein molecules one after the other by a "catch and razor" mechanism. Another hypothesis based on the aggregation behavior of the micelles predicted that the enzymes produce randomly distributed individual molecules of para kappa casein (Dalgleish, 1992).

The kinetics of the proteolysis reaction has a K_m value ranging from 3×10^{-6} to about 5×10^{-4} mole liter $^{-1}$ (Dalgleish, 1992). This relatively wide range of values were

observed possibly due to the different nature of κ-casein used as a substrate, which can be either in a soluble or micellar form and also due to differing experimental conditions. The lower values of K_m were observed when isolated κ-casein or peptides were used (Azuma et al., 1984; Visser et al., 1980; Vreeman et al., 1986; Turhan and Mutlu, 1997) while the higher values were noticed when casein micelles in dispersion or in milk were used (Chaplin and Green, 1980; Dalgleish, 1979; Hooydonk et al., 1984; Turhan and Mutlu, 1997). The aggregation of κ-casein also increased the K_m in another study. (Vreeman et al., 1986). These reports suggested that there might be significant differences between the kinetics of chymosin action in milk and in model systems. Therefore, care has to be taken to extrapolate results before findings are applied on a large scale.

The comparison of rate constants from several studies can be further hampered by the differing experimental conditions. Although, it is well known that the pH optimum for chymosin is in the range 5.0-5.5 (Humme, 1972), it is different in milk, with a pH optimum of 6 (Hooydonk et al., 1986). The ionic strength of the medium is also very important in this regard. The activity of the enzyme goes through a maximum as the ionic strength is increased, since it enables efficient interaction with the substrate at the optimum ionic concentration (Payens and Both, 1980; Payens and Visser, 1981). It is clearly established that the addition of calcium accelerates the clotting reaction, mostly due to increase in the rate of aggregation (Dalgleish, 1983).

2.9.3.3. Extent of proteolysis and changes during coagulation

In milk, κ-casein is distributed throughout casein micelles, predominantly on their surfaces, and proteolysis of one κ-casein molecule within the micelle is not sufficient to bring about aggregation (Dalgleish, 1980). By the time coagulation is observed physically, proteolysis of the κ-casein in milk has been at least 90 %, and proteolysis of κ-casein by chymosin is essentially complete at 80 % of the visually observed coagulation time (Chaplin and Green, 1980; Foltmann, 1959). There are two schools of thought regarding extent of proteolysis of κ-casein required before coagulation occurs. According to one group, κ-casein needs enzymatic hydrolysis for coagulation to occur but only sufficient to initiate aggregation of micelles (Cheryan et al., 1975). Later, it was

suggested that micelles remain uncoagulable until extensive conversion of surface of κ-casein has occurred (Dalgleish, 1979).

2.9.3.4. Rate of the reaction

Estimation of the rate and extent of hydrolysis of κ-casein during the primary stage of the clotting process can be done by using more than one method. One way is to measure the disappearance of κ-casein, the substrate of the reaction. Limitations to this method are caused by the naturally occurring genetic variants of κ-casein and the different degrees of glycosylation of the primary structure (Armstrong et al., 1967; Raap et al., 1983; Schmidt et al., 1966). A number of fractions are produced on column chromatography or electrophoresis complicating the estimation (Vreeman et al., 1986). Hence, it is much easier to study the kinetics of the reaction by analyzing the products viz., the macro peptide or the para κ-casein.

Para κ-casein produces a single band on electrophoresis and also positively charged at neutral pH, whereas other caseins are negatively charged at this pH. Hence, its estimation is simple by electrophoretic techniques (Chaplin and Green, 1980). Some other physico-chemical properties including insolubility in the absence of calcium (Lawrence and Creamer, 1969) and its higher isoelectric point compared to that of the whole κ-casein can be exploited for its quantification (Bingham, 1975).

The quantification of the macro peptide released during clotting is a bit complex due to its loss in the serum portion, different degree of glycosylation and its lack of aromatic amino acids. Precipitation of renneted milk with 2% tricholoroacetic acid produces a supernatant containing largely the macro peptide along with β-lactoglobulin (Vreeman et al., 1986). The degree of glycosylation affects the solubility of the macro peptide in TCA holding a direct relationship with the concentration of TCA used (Armstrong et al., 1967). The quantity of macro peptide that is left then depends on the concentration of TCA used and relative amounts of the macro peptide in the mixture with different degrees of glycosylation. But this difference in solubility can be exploited to differentiate glycosylated and non-glycosylated fractions by comparing the solubility in 2% and 12% TCA (Hindle and Wheelock, 1970). Since there is no absorbance at 280 nm due to lack of aromatic amino acids, absorbance at 215 nm or measuring the N content of the TCA filtrate requires further purification. Rapid methods of estimation include HPLC

(Humphrey and Newsome, 1984; van Hooydonk et al., 1984) , FPLC (Dalgleish, 1986) and fluorescence techniques (Udenfriend et al., 1972).

3. Hypothesis and objectives

The hydrolysis of κ-casein present on the surface of the casein micelles is the primary step in the manufacture of most cheese varieties. It is well established that the most susceptible bond for the hydrolysis of κ-casein by chymosin and pepsin is the Phe_{105}-Met_{106} bond, producing two products upon limited hydrolysis, the para-κ-casein and the macro peptide (Dalgleish, 1992). This crucial process eventually leads to the coagulation of milk and is controlled by several factors. The para-κ-casein forms the curd that is incorporated into the cheese and the macro-peptide is lost in the whey.

Chymosin and pepsin belong to the group of enzymes referred to as aspartic or acid proteases. The proteases are active at an acidic pH and the carboxylic groups of the two aspartic residues in their primary structure are responsible for the catalytic activity. The catalytic functional groups present in the enzyme can interact transiently with a substrate and activate it for the reaction. Several multiple non-covalent bonds between the enzyme and the substrate facilitate this interaction. The three-dimensional structure of κ-casein specifies the exact nature of these bonds and thereby influences the rate of the reaction.

The higher order structures of κ-casein may be altered due to genetic polymorphism. This could influence the rate of hydrolysis between the genetic variants of κ-casein. Significant differences in the rate of hydrolysis between the various genetic variants could be exploited in the manufacture of cheese. Indirect approaches are followed to understand the structure-function relationship of κ-casein to explain the coagulation properties of milk for the lack of detailed three-dimensional structures of caseins.

The advent of recombinant DNA techniques has enables us to obtain nucleotide sequence specific information regarding the caseins. cDNA sequences are now available for the four caseins found in bovine milk. (Stewart et al., 1984, 1987). The nucleotide sequences that encode the caseins show evidence of high point mutation rate and major insertion/deletion events and of other sequential arrangements except in the case of κ-casein (Stewart et al., 1984; Bonsing and MacKinlay, 1987). Dot matrix comparison of κ-casein cDNA sequences of the bovine and the rat shows the overall architecture of the

molecule has been preserved and major rearrangements have not occurred (Bonsing and MacKinlay, 1987). The authors concluded that there are functional restraints on κ-casein structure, which includes the necessity to interact with other caseins in order to stabilize them against precipitation by calcium and maintenance of the sequence that determines the sensitivity of κ-casein to limited proteolysis by chymosin (Bonsing and MacKinlay, 1987). An interesting feature of the plot is that the conservation of the sequence is much more pronounced downstream of the chymosin sensitive bond than it is upstream. It is in this downstream region where the mutation that causes the amino acid substitution between the A and the B variant is found to occur. This could possible influence the interaction of κ-casein with the enzyme. Plowman and Creamer (1995) using molecular dynamic methods, observed that the conformational and electrostatic charge effects were important in the binding of the κ-casein peptide 98-111 which lies close to the chymosin cleavage site of κ-casein with bovine chymosin and porcine pepsin. This could presumably apply to other sequences as well necessary for interaction with the enzyme, although the exact regions of κ-casein that interact with the enzyme still remain unclear.

The objective of this study was to monitor the pattern of hydrolysis of the genetic variants of κ-casein A and B since milk containing a specific variant differed in certain functional properties during various stages of cheese manufacture. Isolated pure form of three phenotypes of κ-casein AA, AB and BB were employed to observe any difference in the rate of hydrolysis at an acidic pH similar to those used in the manufacture of cheese.

4. Materials and methods

4.1. Source of milk samples

From a previous study, cows from 15 different Holstein dairy herds comprising around 600 cows distributed in different regions of Quebec were phenotyped by polyacrylamide gel electrophoresis for the genetic variants of α_{s1}- casein,β-casein, κ-casein, and β-lactoglobulin. In the present study, milk samples from identified cows were selected to provide representatives of the different genetic variants of κ-casein present in the region. It was possible to obtain the A and the B variant of κ-casein in either the homozygous or heterozygous form.

4.2. Preparation of whole casein

Approximately 4 L of fresh milk samples were collected from the selected cows and subject to centrifugation twice at 3000 x g for 15 min at 4 ° C and the fat portion was carefully removed. The resultant skim milk was adjusted to pH 4.6, the isolectric point of casein by the addition of 1 M HCl at room temperature. The precipitated whole casein was subject to centrifugation at 3000 x g and the supernatant containing whey proteins were discarded. The precipitated whole casein was subsequently washed with deionized water and stored at -20° C until further analysis. Electrophoresis was performed to ascertain the phenotypes of κ-casein prior to fractionation of the caseins.

4.3. Phenotyping of caseins

Polyacrylamide gel electrophoresis was used to determine the genetic variants of casein components. The electrophoresis was performed in a vertical Bio-Rad minigel system (Bio-Rad Laboratories, Richmond, CA). The system consists of two gel slabs with the dimension of 10 x 8 x 0.1 cm with a maximum capacity of ten loading wells each. PAGE was carried out under alkaline conditions (pH 8.3) according to the method described by Kiddy (1975) and modified by Ng-Kwai-Hang et al., (1984). The resolving gel consisted of 4.5 M urea; 12 % acrylamide; 0.05M Tris base; 0.132 M glycine and 0.006M EDTA. The polymerization of the gel was initiated by the addition 0.6 % of freshly prepared 10 % ammonium persulfate and 0.06 % of N,N,N–tetramethylethylenediamine and it took approximately 30 min for complete polymerization. The whole casein samples were dissolved in sample buffer at the concentration of 50 mg/ml. The composition of the sample buffer was as follows: 4.5 M

urea; 0.05 M Tris base; 0.132 M glycine; 0.006M EDTA and 2% β-mercaptoethanol. Two drops of 0.1 % bromophenol blue were used as a tracking dye and mixed with every 10 ml of the sample buffer. Five µl of solubilized whole casein sample were loaded into each well of the gel slab for electrophoresis. Tris-barbitol solution that had the same concentration of Tris, glycine and EDTA as the polyacrylamide gel, was used as the electrode buffer. Electrophoresis was carried out at a constant voltage of 100 V for 100 min. The gels were then transferred into staining solution containing 35% methanol, 7 % acetic acid and 0.1% Coomassie brilliant blue R-250 in distilled water, for 10 min. The destaining of the gels was carried out by diffusion in a solution containing 35% methanol and 7% acetic acid. After sufficient destaining, the phenotypes of the casein components were assessed by visualization of the corresponding bands using transmitted light. Under the above conditions, κ-casein A had a faster electrophoretic mobility than κ-casein B.

4.4. Preparation of casein samples

Sufficient quantity of wet casein, depending on the column capacity, was dissolved in the appropriate running buffer used and the pH adjusted to 7 by the drop wise addition of 1M NaOH prior to loading. At neutral pH and above, all the caseins are negatively charged which could be exploited during the purification process. Approximately 8 mg of dithiothreitol/g of wet casein was added to the final mixture and left for 15 min. This was to ensure the breakage of the interlinking disulphide bonds present in caseins that enables efficient purification. The column packing material obtained commercially contained positively charged particles that bound to the negatively charged casein. The relative strength of binding depends on the charge of the protein and different salt concentrations were used to elute the caseins starting from the one that had the least negative charge.

4.5. Fractionation of casein components

Ion-Exchange Liquid Column Chromatography method was used for the large-scale fractionation of whole casein into its major components: α_s-, β- and κ-casein fractions. Caseins exist in milk in the form of large spherical complexes called micelles. Hence, it was necessary to use a denaturating agent to disrupt the tertiary structure before attempting fractionation. A 4.5 M urea solution was used for that purpose in this study. The urea solution was passed through a glass column containing 500 g of mixing bed AG

501-X8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA) to remove ionic contaminants from commercial preparations. The solution was again filtered through Durapore ® Membrane Filters (Milipore, Bedford, MA) with a pore size of 0.45 μm to remove physical contaminants.

The following three Chromatographic Systems were used for the large-scale fractionation of caseins in this study:

4.5.1. Automated econo system (Bio-Rad Laboratories, Richmond, CA): The ES-1 Econo System Controller forms the primary unit that integrates the individual components into a low-pressure chromatography system. The components include an EP-1 Econo Pump, EM-1 Econo UV Monitor, EG-1 Econo Gradient Monitor, EV-1 Econo Buffer Selector and 1326 Econo (Dual-Pen) Recorder. This system allows gradient proportioning, mixing and peak detection with a programmed control of up to 5 solutions. Buffer A consisted of 20 mM Tris in 4.5 M urea solution and the eluting buffers had NaCl in addition to Tris-Urea. A sequential stepwise elution procedure for the different casein components was carried out with the following NaCl concentrations: 0.10 M, 0.15 M, 0.175 M, 0.25 M and finally 1.0 M NaCl as a clean up procedure prior to the next run. Approximately 6 g of wet casein were dissolved in 20 mM Tris-Urea buffer and the pH adjusted to 7. The reconstituted casein solution was loaded onto a glass column (25 x 2.5 cm) packed with Express Ion Exchanger Q Anion Exchanger (Whatman International Ltd., Maidstone, England). The flow rate was maintained at 6 ml/min and a stepwise elution with each salt concentration lasted for 30 min and extended if necessary.

4.5.2. Con Sep LC 100 (Millipore Corp., Bedford, MA): A high flow gear pump combined with a four-buffer inlet system and a high performance Model CR112 stable UV detector is the main operating unit of this system. The program used to fractionate the caseins is shown in Table 2. Buffer A consisted of 20 mM Tris in 4.5 M urea solution of pH 8 and buffer B consisted of 0.5 M NaCl in buffer A. Approximately 8 g of wet casein were dissolved in 20mM Tris-Urea buffer and the pH adjusted to 7 by the addition of 1M NaOH. The reconstituted casein solution was loaded onto a glass column (20 x 5 cm) packed with the same packing material as above. The flow rate was maintained at 10 ml/min and a stepwise elution with each salt concentration lasted for 30 min and extended if necessary.

Table 2. Program gradient for a single run of casein fractionation using Con Sep LC 100

Percentage of Buffers		Time (min)
A	B	
100	0	0
100	0	40
45	55	90
55	45	140
40	60	180
20	80	230
0	100	290
100	0	365

Table 3. Program gradient for a single run of casein fractionation using Biologic HR

Percentage of Buffers		Time (min)
A	B	
100	0	0
100	0	40
80	20	90
65	35	140
40	60	190
0	100	265
100	0	365

4.5.3. Biologic HR gradient chromatography system (Bio-Rad Laboratories, Richmond, CA): This integrated chromatography system is microprocessor controlled, with a graphic interface and menu-driven software for manual operation, system set-up, method editing and run operations. Buffer A consisted of 100 mM Tris in 4.5 M urea solution of pH 8.5 and buffer B consisted of 0.5 M NaCl in buffer A. The program followed to fractionate the casein is shown in Table 3. Approximately 10 g of wet casein were dissolved in 100 mM Tris-Urea buffer and the pH adjusted to 7 by the addition of 1M NaOH. The reconstituted casein solution was loaded onto a glass column (30 x 5 cm) packed with Macro-Prep High Q Anion Exchange Support (Biorad Laboratories, Richmond, CA). The flow rate was maintained at 10 ml/min.

4.6. Isolation and purification of κ-casein

The fractions corresponding to the different casein components were collected in accordance with their chromatographic peaks and elution time intervals. In this study, the κ-casein fractions from multiple runs of the same sample derived from 40-50 g of wet casein were collected and purified for further analysis. A model 8400 ultra filtration cell (Amicon Division, Danvers, MA) fitted with an YM 10 membrane with a molecular weight cut-off of 10,000 Daltons (Whatmann, Maidstone, England) was used for desalting and concentrating the collected fractions. Filtration was carried out under pressure by using nitrogen gas at 40 psi with constant stirring. Each fraction was concentrated to a final volume of approximately less than 50 ml. The concentrated samples were then re-purified under similar conditions of chromatography and subject to ultra filtration using the same procedure described above. The remaining traces of salt and urea were removed by overnight dialysis using regenerated cellulose tubular membrane (Fisher Scientific, PA) with a pore size of 1.8 nm and a molecular weight cut-off of 6000-8000. The dialyzed samples were then frozen, freeze-dried and stored at –20° C pending further analysis. The protein concentration of the samples were measured spectrophoretically at 280 nm (Swaisgood, 1982)

The purity of the final κ-casein samples obtained by ion-exchange chromatography was ascertained by polyacrylamide gel electrophoresis under alkaline conditions (pH 8.3) as previously described. Whole casein sample diluted with an equal volume of sample buffer was used as standard to compare the electrophoretic bands.

Fifteen µl of the sample was loaded in each well and PAGE was run at a constant voltage of 100 V for 100 min. The gels were then stained, destained and observed in the usual manner.

4.7. Effect of genetic variants of κ-casein on hydrolysis by different enzymes

4.7. 1. Hydrolysis of κ-casein variants by chymosin

The stock solution of chymosin from calf stomach (Sigma Chemical Co., St Louis, MO) was prepared by dissolving 10 mg of the crystallized and lyophilized powder in 1 ml of phosphate buffer of pH 5.8 containing 0.06 M Na_2HPO_4 and 0.06 M KH2PO4. The stock solution of the enzyme was diluted five fold with the phosphate buffer buffer in the reaction mixture. The stock solution of κ-casein was prepared in the same buffer (pH 5.8) containing 0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4 and adjusted to a final concentration of 0.5 %. The pure casein fractions corresponding to the three phenotypes of κ-casein were chosen according to their electrophoretic pattern. The stock solutions of the enzyme and κ-casein were then stored at – 20° C as 200 µl and 1 ml aliquots respectively and thawed prior to use. The κ-casein solution was primed at 37 ° C for 5 min before the start of the reaction. The hydrolytic reaction was carried out by adding the enzyme solution to a proper volume of desalted casein. In a typical experiment, the ratio of enzyme to the substrate was 1:500. The reaction mixture was then thoroughly agitated in a vortex mixer. The conditions were optimized to allow for a slow and a steady rate of hydrolysis by repeated digestions at different enzyme-substrate ratios (1:100, 1:200, 1:400).

The digestion was performed in a water bath at 37 ° C. Aliquots were collected at 0,5,15,30,60 and 90 min and the reaction was terminated by quickly transferring 80 µl of the reaction mixture into a tube containing 20 µl of 24 % NH_4OH. Ammonium hydroxide solution can stop the enzymatic reaction by increasing the pH of the reaction mixture. After mixing in a vortex mixer, the reaction mixture was centrifuged at 3000 rpm for 5 min and the supernatant transferred to glass auto sampler vials with polypropylene inserts for further analysis by HPLC. A small portion of the supernatant was used for analysis by SDS-PAGE. The centrifugation ensured that only the soluble components were subject to further analysis.

4.7.2. Hydrolysis of κ-casein variants by pepsin

Pepsin from porcine stomach mucosa (Sigma Chemical Co., St Louis, MO) was used for the hydrolysis reaction. The stock solution of chymosin was prepared by dissolving 10 mg of the crystallized and lyophilized powder in 1 ml of phosphate buffer of pH 5.8 containing 0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4. The stock solution of the enzyme was diluted five folds with the phosphate buffer in the reaction mixture. The stock solution of κ-casein was prepared by dissolving the purified, desalted casein fraction with the phosphate buffer (pH 5.8) and adjusting the final concentration to 0.5 %. The pure casein fractions corresponding to the three phenotypes of κ-casein were selected according to their electrophoretic pattern. The stock solution of chymosin and κ-casein were stored as 200 µl and 1 ml aliquots respectively at –20° C and thawed prior at room temperature to use. The κ-casein solution was then primed at 37° C for 5 min before the start of the reaction. The hydrolysis was carried out by adding the enzyme solution to a proper volume of desalted casein. In a typical experiment, the ratio of the enzyme to substrate was 1:1000. The reaction mixture was then thoroughly agitated in a vortex mixer. The conditions were optimized for a slow and a steady rate of hydrolysis by comparing different enzyme substrate ratios (1:100; 1:200; 1:400). The digestion was performed in a water bath maintained at 37° C and aliquots were collected at 0,5,15,30,60 and 90 min following the addition of the enzyme. The reaction was terminated immediately by quickly transferring 80 µl of the reaction mixture into a tube containing 20 µl of 24 % NH_4OH. Ammonium hydroxide solution stops the enzymatic reaction by increasing the pH of the reaction mixture. Aliquots containing the reaction mixture terminated at different time intervals were vortexed and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to glass auto sampler vials with polypropylene inserts for further analysis by HPLC. A small portion of the supernatant was used for analysis by SDS-PAGE. The centrifugation ensured that only the soluble components were subject to further analysis.

4.7.3. RP-HPLC of κ-casein hydrolysates

The κ-casein hydrolysates obtained from enzymatic hydrolysis using chymosin and pepsin after different time intervals were analyzed by a reversed-phase HPLC system

(Waters, Milford, CA). The HPLC system consisted of a Waters 600 E Multisolvent delivery system, a multiwavelength 490 E UV detector and Maxima software to integrate the chromatograms. A Vydac 214 TP C4 HPLC Column (Sigma-Aldrich Corp., PA) with a dimension of 250 x 4.6 mm and a particle size of 5 μm was used for the separation process. The whole system was automated via a Varian 9090 auto sampler (Walnut Creek Division, CA).

The composition of solvent A used was 5% acetonitrile, 0.1 % trifluoroacetic acid (TFA), 95% distilled water and that of solvent B was 60 % acetonitrile in 0.1% TFA, 40% distilled water. Both the solvents were filtered through 0.2 μm pore size nylon membrane filters (Whatman International Ltd., Maidstone, England) and degassed with helium at a flow rate of 100 ml/min for a minimum of 15 min prior to the chromatographic run. The flow of helium was then maintained at 10 ml/min throughout the run to ensure reproducibility of the running conditions. All the solvents used in this study were prepared using double distilled water and deionized using NANO Ultrapure water system (Barnstead Thermolyne Corp., Dubuque, IW).

Sixty μl of the κ-casein hydrolysate was injected via the auto sampler into the reverse-phase column. The flow rate was maintained at 1.0 ml/min throughout the run with a backpressure ranging from 450-500 psi and a temperature of 40 ° C. The running conditions were optimized according to the maximum resolution of the peptides from the hydrolysates. The eluting peptides were monitored at a wavelength of 280 nm. The program gradient for the separation of the hydrolysate is shown in Table 4.

4.7.4. SDS-PAGE of κ-casein hydrolysate

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was also used to monitor the pattern of hydrolytic products of κ-casein. SDS is a strong anionic detergent, which is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides become negatively charged upon binding to SDS and the amount of binding is proportional to the molecular weight of the polypeptide. As a result, the migration of the polypeptide is in accordance with the size of the polypeptide in contrast to the charge of the protein in case of PAGE under alkaline conditions.

Table 4. Program gradient for a single run of RP-HPLC

Percentage of Buffers		Time (min)
A	B	
100	0	0
100	0	5
57	43	7
47	53	37
0	100	40
0	100	50
100	0	52
100	0	62

SDS-PAGE was carried out in a discontinuous buffer system consisting of a 5 % stacking gel and a 15 % resolving gel. The solution for preparing resolving gels for Tris-glycine SDS-PAGE contained the following components: 30% acrylamide mix, 1.5 M Tris (pH 6.8) 10 % SDS, 10 % ammonium sulphate and 0.04 % N,N,N',N'-tetramethylethylenediamine (TEMED). The solution for preparing 5 % stacking gels contained the following components: 30 % Acrylamide mix, 1.0 M Tris (pH 6.8), 10 % SDS, 10 % ammonium sulphate and 0.1% TEMED.

After polymerization was complete, the gels were mounted in the electrophoresis apparatus and the electrophoresis buffer was added. The electrophoresis was then performed in a vertical Bio-Rad minigel system (Bio-Rad Laboratories, Richmond, CA). The system consisted of two gel slabs with the dimension of 10 x 8 x 0.1 cm with a maximum capacity of ten loading wells each. The buffer used for electrophoresis consisted of 25 mM Tris, 250 mM glycine and 0.1 % SDS. The samples were prepared by heating them to 100 ° C for 3 min in a 1 x SDS gel-loading buffer to denature the proteins. The loading buffer consists of 50 mM Tris.HCl (pH 6.8), 100mM dithiothreitol, 2 % SDS, 0.1% bromophenol blue and 10 % glycerol. Fifteen µl of each of the samples was loaded in a predetermined order into the bottom of the wells. The electrophoresis was carried out under a constant voltage of 100 V and allowed to run until the dye reaches the bottom of the resolving gel.

After the run was complete, the gels were carefully removed from the glass plates and stained, destained and observed in the same manner as described for PAGE in the previous section. The solutions used for staining and destaining the gels were the same as those used for PAGE. Pre-cast Tris-Tricine polyacrylamide gels (BioRad Laboratories, Richmond, CA) were also used to analyze the hydrolysates. The Tris-tricine gels provide better resolution of peptides and had the following gel concentration: 4% stacking gel and 10-20 % linear gradient of running gel. The electrode buffer composition was as follows: 100 mM Tris, 100 mM Tricine, 0.1% SDS, and pH 8.3. The sample buffer consisted of 200 mM Tris-HCl pH 6.8, 2% SDS, 40% glycerol and 0.04 % Coomasie blue. The staining solution was prepared using 0.25 g Coomasie Blue G-250 in 10% acetic acid. The destaining solution was prepared similarly without the dye. Polypeptide SDS-PAGE molecular weight standards ranging from 1.4-26.6 kD (BioRad Laboratories, Richmond,

CA) were used to arrive at an approximate value of the molecular weight of the peptide breakdown products. Both the hydrolysates and the standard were denatured for 5 min at 95 ° C with the sample buffer prior to loading and run till the dye reached the bottom of the gel. The gels were then placed in a fixative solution (40 % methanol and 10% acetic acid) for 30 min. The fixed gels were stained for one hour and then placed in a destaining solution for 3 x 15 min washes until the desired stain is reached.

4.7.5. Statistical analysis of the results

The effect of genetic variants on hydrolysis of κ-casein by chymosin and pepsin were examined. The experiments were replicated thrice for all the three phenotypes to ensure reproducibility of the results. The area of the peaks from RP-HPLC chromatograms representing the substrate concentrations was studied as a function of time. The data obtained was analyzed by least squares methods using the PROC GLM procedures (SAS Institute Inc., Cary, NC). The statistical model used was:

$Y_{ijk} = \mu + time_i + phenotype_j + e_{ijk}$

Where Y_{ijk} is the area of the chromatogram of the Kth sample

μ is the overall mean

i is the effect of the ith time interval (0,5,15,30,60 and 90 min)

j is the effect of the jth phenotype (AA, AB, BB)

e_{ijk} is the random error associated with kth sample

Type III sum of squares were used to evaluate the effect of time intervals and protein genotypes. The statistical significance was tested by the Scheffe's multiple comparison test. The differences in estimates of the area of the chromatograms (dependent variable) of κ-casein phenotypes were examined.

5. Results and discussion

5.1. Phenotyping of κ-casein by electrophoresis

The phenotypes of the major casein components can be determined by polyacrylamide gel electrophoresis (PAGE) under alkaline conditions (Kiddy, 1975; Ng-Kwai Hang et al., 1984). The isoelectric points of caseins are around pH 4.6 and are therefore negatively charged at an alkaline pH. The rate of migration during PAGE depends on the charge of the protein. The protein with the highest negative charge migrates faster followed by the next and so on. The phenotypes of κ-casein were determined by PAGE and the relative migration order of the casein components were found to be the following: α_{s1}->α_{s2}->β->κ-casein (Figure 2). It is evident from the migration rate that α_{s1} – casein has the highest and κ-casein has the lowest negative charge among all the major casein components.

The genetic variants of κ-casein could easily be distinguished by their relative mobility in the gels. This could be explained by observing the primary structure of κ-casein A and B. The A and the B variant differ due to an amino acid substitution at position 136 and 148 of the primary sequence. At these two positions, variant A has a threonine and aspartate residue and variant B has an isoleucine residue and an alanine residue respectively. Since aspartate is an acidic amino acid and alanine is a neutral amino acid, the second substitution imparts a more negative charge on variant A and therefore migrates faster compared to the B variant under the said electrophoretic conditions (Dong, 1998). The homozygote AA (lanes 1,4,5) and BB (lanes 2,3,7) contains only a single band corresponding to the particular variant, whereas the heterozygote AB (lanes 6,8) contains both the bands. Within the heterozygote, the B band appears to be more intense than the A band. This has been attributed to higher expression B than the A gene of κ-casein (van Eenennman and Medrano, 1991). Apart from the two major bands, A and B, several other distinct bands do appear in the region close to them in PAGE (Figure 2), in all the casein samples used in this study. This heterogeneity could possibly arise due to different degrees of post-translational modification such as phosphorylation and glycosylation of the primary structure (Vreeman et al., 1977, 1986).

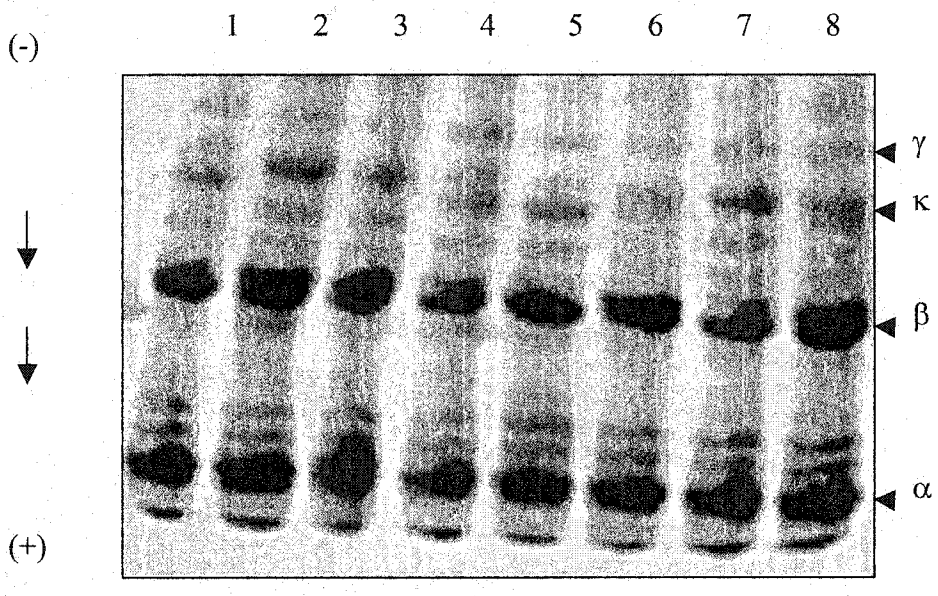

Figure 2. PAGE of whole casein showing the different phenotypes of κ-casein κ-casein AA (lanes 1, 4, 5) κ-casein BB (lanes 6, 8) κ-casein BB (lanes 2, 3, 7)

5.2. Fractionation of whole casein by anion-exchange chromatography

Three Liquid Chromatography systems were used to fractionate the whole casein into its major components. The systems differed in the column capacity and/or packing material used for separating the casein components. A higher column capacity enables large-scale isolation and purification of casein components in less time.

Bio-Rad Econo LC System equipped with an Express Ion Exchanger Q Anion Exchanger (Whatman International Ltd., Maidstone, England) packed inside a glass column was used to fractionate approximately 6.0 g of precipitated casein in a single run. The fractionation was achieved by an elution procedure with increasing molarity of NaCl in Tris-urea buffer at a pH of 7, starting from a salt free mobile phase to 0.10 M, 0.15 M, 0.175 M, 0.2 M and finally 1.0 M NaCl as a clean-up procedure. The chromatogram of a typical elution procedure is shown in Figure 3. The whole casein was separated into five fractions denoted as 1 to 5, during a 300 min elution period and extended if necessary. The identity and purity of each casein fraction was ascertained by electrophoresis. The first peak on the elution profile, denoted as 1, corresponded to the breakdown products of β-casein and referred to γ-casein. The major casein components started to elute at approximately 45 min and the last peak eluted around 270 min of the gradient program. κ-Casein eluted in peak 2 at 0.10 M NaCl/Tris-urea buffer. β-Casein, α_{s2}-casein and α_{s1}-casein were eluted at 0.15 M, 0.175 M and 0.20 M NaCl/Tris-urea buffer respectively. Each casein fraction was collected in separate containers and stored in refrigeration conditions for further analysis. This method was efficient in fractionating whole casein into its major components of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein in electrophoretically pure forms with little cross-contamination between these components. Hence, this method was performed on a routine basis to fractionate precipitated whole casein in gram quantities.

Millipore Con Sep ^{TM} LC 100 equipped with the same column packing as above was used to fractionate approximately 8 g of wet casein in a single run with a flow rate of 10 ml/min. The representative chromatogram of a program gradient is shown in Figure 4. The fractionation was achieved by increasing the molarity of NaCl from 0.0 M to 1.0 M and equilibrating the column with salt free buffer before starting the next run. The whole casein separated into five peaks denoted 1 to 5 in a total run time of around 4 hours. The

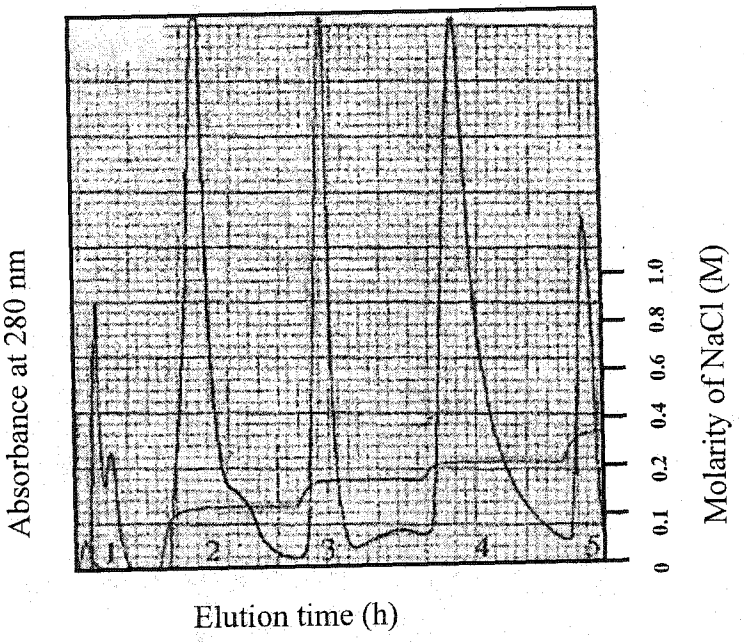

Figure 3. Millipore chromatogram of a typical whole casein fractionation Procedure with an Express Q Anion exchange column.
Peaks 1-5: γ-, κ-, β- and α_s- casein respectively

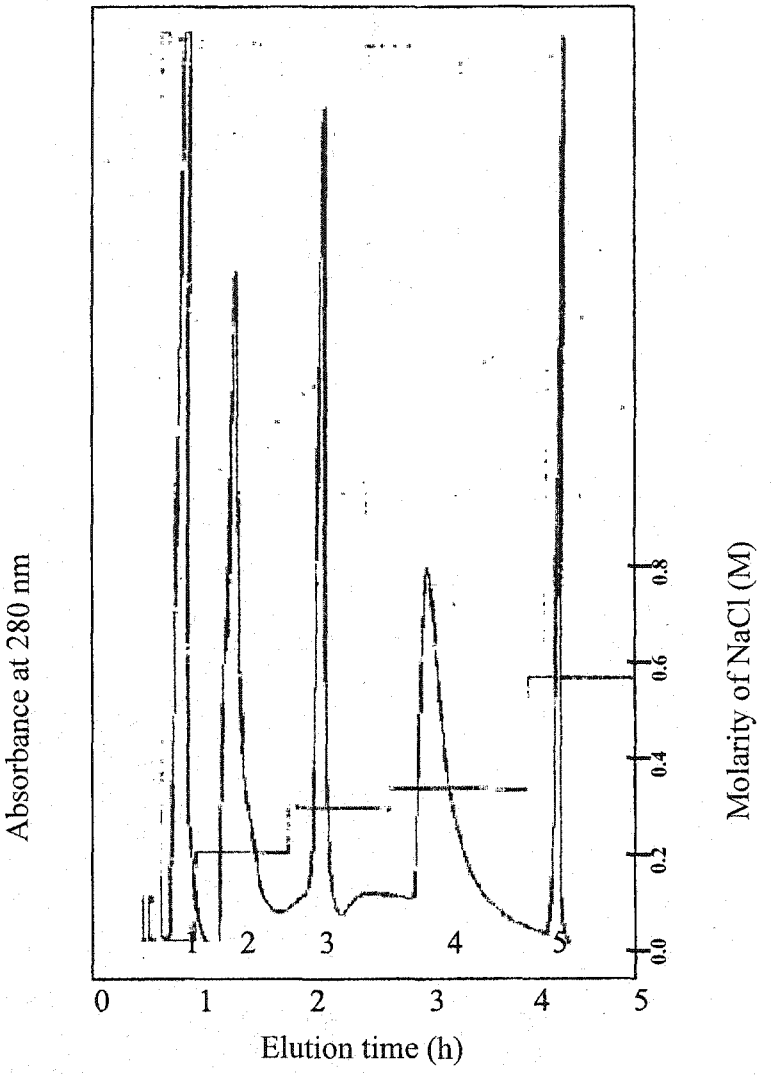

Figure 4. ConSep LC 100 chromatogram of a typical fractionation of whole casein with an Express Ion Exchanger Q anion exchange column with increasing salt concentration

Peaks 1-5: γ-, κ-, β-, α_{s1} and α_{s2}- casein respectively

run time was extended if necessary till the peak level reached a fairly lower level to prevent any cross-contamination between any two eluting fractions. κ-Casein eluted in peak 2 at a salt concentration of 0.2 M NaCl. Each fraction was collected separately and stored at 4 ° C. A larger amount of wet casein can be fractionated using this instrument due to more column capacity than the previous system, although a longer run time is required.

Bio-Rad Biologic HR Gradient Chromatography System equipped with Macro-Prep High Q Anion Exchange Support (Biorad Laboratories, Richmond, CA) was used to fractionate approximately 10 g of wet casein with a flow rate of 10 ml/min. The chromatogram of a typical fractionation procedure is shown in Figure 5. Whole casein fractionated into four major components denoted as 1 to 4. The κ-casein fraction eluted in the second fraction at a salt concentration of 0.1 M NaCl. It is evident from the chromatogram that fraction 2 consists of several small peaks arising possibly due to various degrees of post-translational modification of κ-casein each eluting at a slightly different salt gradient. This system could handle a larger column capacity under a relatively high pressure and could facilitate large-scale fractionation of caseins than the other two systems. However, α_{s2}-casein could not be isolated separately from α_{s1}-casein under the existing conditions. Isolation of κ-casein and β-casein was found to be more satisfactory using this method.

The identification of the different genetic variants of milk proteins using ion-exchange chromatography was not attempted, although this has been done for κ-casein in a previous study (Dong, 1998) using different anion exchange column packing. The purification of proteins using an ion-exchange chromatography method involves the formation of several electrostatic bonds between the charged groups on the surface of the exchanger and opposite charges on the protein. This is followed by a selective breakage of these bonds by an alteration in the concentration of chloride ions or the pH of the eluting buffers (Yaguchi and Rose, 1971). Under the aforementioned conditions, the charge difference between the genetic variants of κ-casein was not significant to achieve efficient separation.

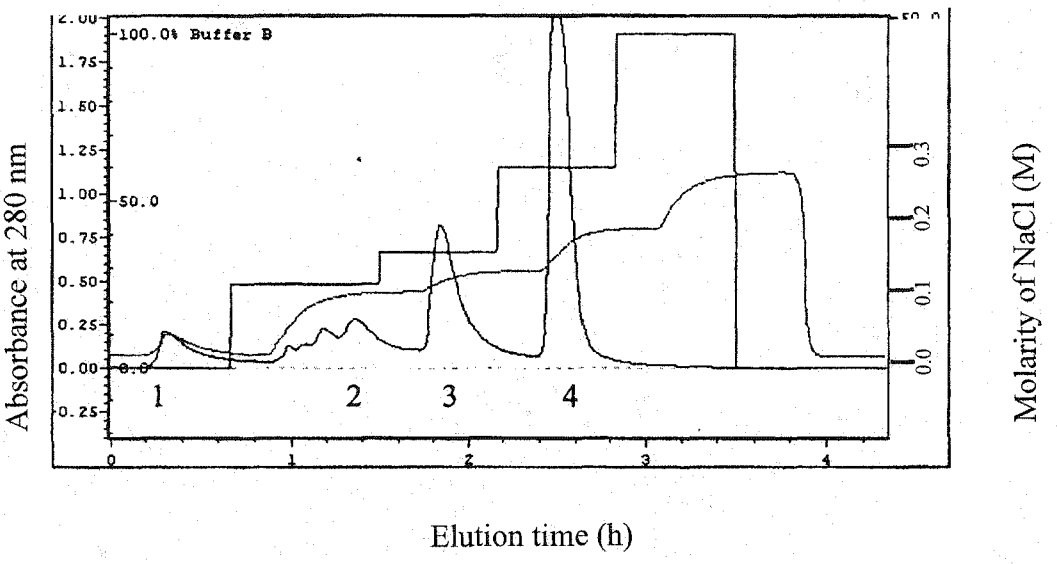

Figure 5. Biologic HR workstation chromatogram of whole casein separation with Macro-Prep High Q anion exchange column showing the major caseins
Peaks 1-4: γ-, κ-, β- and α_{s1}- casein respectively

5.3. Purity of κ-casein

Pure samples of the different phenotypes of κ-casein without any significant amount of contaminating peptides are essential for further analysis to obtain meaningful results. The different samples of κ-casein were relatively pure based on the elution profiles of the chromatographic separation of whole casein and the results of electrophoresis of the collected fractions (Figure 6). All the casein fractions in the chromatographic system were detected by UV absorbance at a wavelength of 280 nm and the eluting peaks corresponded to the differences in absorbance of the individual caseins at the particular wavelength. The absorbance values are 0.95 cm^2/mg for κ-casein, 0.46 cm^2/mg for β-casein, 1.46 cm^2/mg for αs_1- casein and 1.11 cm^2/mg for αs_2- casein (Swaisgood, 1982). The absorbance values at 280 nm depend on the number of aromatic amino acids in the primary sequence and the relative proportion of each component in whole casein. The κ-casein fraction collected for each sample was concentrated by ultra filtration and repurified by repeating the chromatographic process using Bio-Rad Biologic HR Gradient Chromatography System under the same eluting conditions. It is evident from the re-run chromatogram (Figure 7) that the absorbance of the κ-casein fraction is proportionately higher than the other fractions. This final fraction was again concentrated, dialyzed, freeze-dried and stored at –20 ° C pending further analysis. The ion-exchange chromatographic methods used for fractionation of caseins with a step-wise gradient of NaCl were successful in obtaining a relatively pure form of κ-casein (Figure 6) and in sufficient quantity for further experimentation.

5.4. Enzymatic hydrolysis

It will be interesting to observe if the three different phenotypes of κ-casein, AA, AB and BB show any variation in the rate of hydrolysis by enzymes. Also the frequency of the B variant of κ-casein is lower than that of the A variant in Canadian Holstein dairy herds (Ng-Kwai-Hang et al., 1984; Ng-Kwai-Hang et al., 1990) which forms the predominant cattle population in Canada. This apparent discrimination against the B allele is in spite of the fact that the B variant is associated with several important traits in the dairy industry especially cheese manufacture. These include better renneting properties, firmer curd, higher fat and protein content in cheese and cheese yield. In any

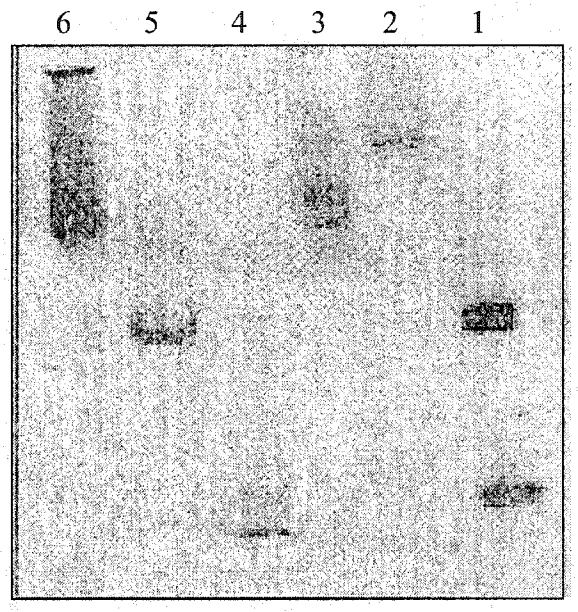

Figure 6. PAGE of casein components after purification by ion-exchange chromatography
Lanes: 1- Whole casein standard; 2-6: γ-, κ-, α_{s1}-, β- and pure κ casein respectively

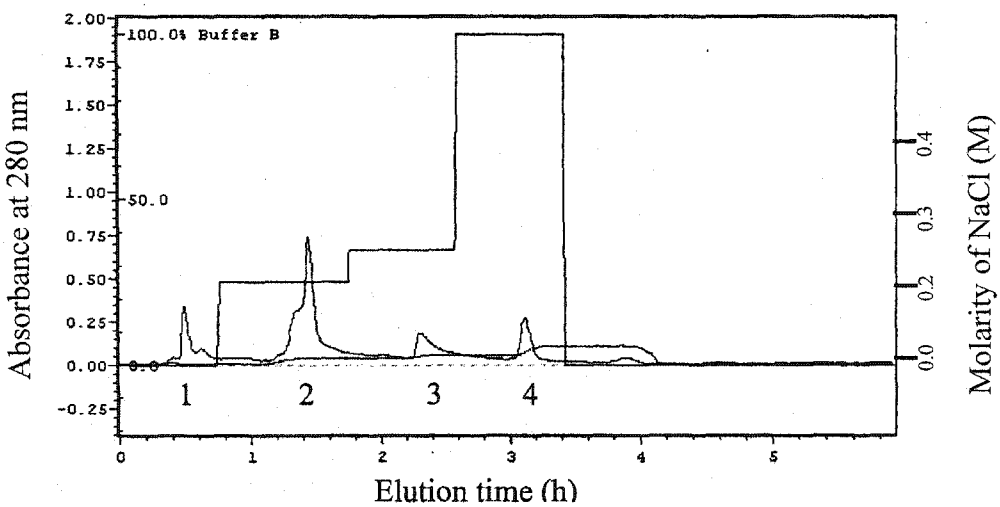

Figure 7. Re-run chromatogram indicating a higher proportion of κ-casein (peak 2)

enzyme-catalyzed reaction, interaction of the enzyme with the substrate to form the enzyme-substrate (ES) complex determines the rate of the reaction. The multiple weak bonds such as hydrogen, hydrophobic, ionic and van der Waals interactions that occur between the enzyme and the substrate enable the formation of the ES complex. These non-covalent interactions are specified by the conformation of both the enzyme and the substrate. The amino acid substitutions that occur between the genetic variants could possibly alter the three-dimensional structure of κ-casein and thereby influence the rate of the reaction. This could have some potential beneficial effects in the cheese industry if any significant difference could be observed on the rate of hydrolysis between the genetic variants of κ-casein. Since the exact three-dimensional structures of caseins are not available, indirect approaches are followed to understand the structure-function relationship of κ-casein to explain the renneting properties of milk.

In this study, purified samples of the different genetic variants of κ-casein were subject to hydrolysis by chymosin and pepsin under acidic conditions (pH 5.8) at 37 $^{\circ}$ C. There is an advantage in using purified κ-casein over that of whole milk containing the variant of interest for this type of study. Although, several striking reports have been published on the better rennet coagulation properties of κ-casein B containing milk (Mariani et al., 1976; Aaltonen and Antila, 1987; Berg et al., 1990), certain others have reported no significant differences in coagulation time between the genetic variants (Politis and Ng-Kwai-Hang, 1988; Schaar, 1984). Marziali and Ng-Kwai Hang (1986b) did not observe any effect of κ-casein polymorphism on renneting properties of milk. Schaar et al., (1985) observed only minor effects of κ-casein variants on cheese yield, composition and recovery of milk constituents except the fat content of ripened cheese. Graham et al., (1984) did not observe a higher dry matter content of cheese produced from κ-casein BB type milk, as reported by others (Morini et al., 1979; Marziali and Ng-Kwai-Hang, 1986c; Berg et al., 1990). Milk has several other constituents that make the analysis of the results rather complicated. The interaction between milk constituents could possibly influence certain functional properties of milk. For instance, with respect to β-lactoglobulin phenotype in cheese making experiments by Mariani et al., (1976) the cows in the κ-casein BB milk group consisted of β-lactoglobulin AA, AB and BB,

whereas all the cows in the κ-casein AA group were of the β-lactoglobulin AA type. The significance of the results obtained becomes limited since attention was focused on the effect of a single locus. In many studies, relevant information on other casein loci was not taken into consideration. The close linkage among the casein loci and interaction between the different components found in milk should be taken into account to obtain meaningful results. Imafidon and Faryke (1994) observed that κ-casein B was most extensively hydrolysed at pH 5.3 and 5.6, but at pH 6.0-6.7 the hydrolysis of the three variants were identical. But, they also observed that κ-casein A was most susceptible to hydrolysis (at pH 5.3) in heated solutions containing different genetic combinations of κ-casein plus β-lactoglobulin. A more direct approach in understanding the effect of a genetic variant on certain properties of milk would be the use of isolated pure forms of proteins to obtain a more valid comparison of the inherent properties among the genetic variants. Hence, pure samples of κ-casein with phenotypes AA, AB and BB were used in this study.

Chymosin and pepsin are highly specific for the Phe_{105}-Met_{106} bond of κ-casein resulting in two major fragments, although further proteolysis was reported under extreme conditions (Coolbear et al., 1996). The enzyme-substrate ratio (1:500 w/w) was optimized to allow for a slow and steady rate of hydrolysis as observed by a gradual disappearance of the substrate by chromatography. Several enzyme substrate ratios (1:100 w/w; 1:200 w/w; 1:400 w/w) were first attempted to determine an optimal hydrolysis over an incubation period of 90 min. A higher enzyme-substrate ratio resulted in almost complete disappearance of the κ-casein band even after one minute of hydrolysis. Either monitoring the appearance of the products or the disappearance of the substrate can be followed to monitor the course of the reaction. The substrate profile was monitored in this study, as it is more suitable for comparison without any interference from minor contaminating peptides. Hydrolysis of κ-casein has been observed in several ways including RP-HPLC of the hydrolysate (Shammet et al., 1992), measuring the sialic acid content of TCA filtrates (Wheelock and Knight, 1969), measuring the turbidity of TCA filtrates after adding phosphotungstic acid (Pearce, 1979), disappearance of κ-casein by electrophoresis (El Negoumy, 1968), separating milk protein using HPLC (Davies and Law, 1977) or by reaction with 2,4,6-trinitrobenzenesulphonic acid (Imafidon and Farkye, 1994).

SDS-PAGE can be used to monitor the pattern of hydrolysis of the genetic variants of κ-casein by chymosin and pepsin at various time intervals for an incubation period of 90 min. SDS-PAGE is preferred to ordinary PAGE previously used for phenotyping of caseins described above since the property that determines the rate of migration of proteins is different in these two electrophoretic conditions. Since κ-casein is subject to various degrees of post-translational modification, each contributing to an increased charge density, multiple bands are produced under ordinary PAGE. In SDS-PAGE, the detergent SDS imputes a negative charge irrespective of the intrinsic charge of the protein and the migration is solely dependent on the mass of the protein. Thus, κ-casein produces a single band and ensures an unambiguous observation of the total amount of κ-casein present in the corresponding sample.

RP-HPLC is an efficient tool to study the hydrolysates produced by enzymatic hydrolysis. The retention of proteins/peptide in an RP-HPLC column is based on the interactions between the hydrophobic domains of the protein/peptide and the hydrocarbon coating on the column support matrix. Thus, the greater the hydrophobicity of the protein/peptide, the more strongly it will bind to the column. For κ-casein, this occurs predominantly in the para-κ-casein moeity, and so both κ-casein and para κ-casein elute at the same retention time. In contrast, the κ-casein-macro peptide is less hydrophobic interacting weakly with the column and elutes earlier.

The hydrolysates of both chymosin and pepsin were both subject to the same run conditions. The products of hydrolysis eluted first due to low degree of hydrophobicity and weaker interaction with the column packing. The different hydrolytic peptides therefore elute depending on the content of hydrophobic amino acids with increasing gradient of the solvent buffer. Most of the products increased with the time course of the reaction seen by increase in the peak height and area. The products that are hydrolyzed further show a decrease in peak height and area after a period of increase. κ-Casein eluted at time interval of approximately 34 min in a total run time of 60 min. κ-Casein is also the last component to elute since it is the most hydrophobic component in the reaction mixture. There may be other peptides that remain unresolved under the present condition of elution and co-eluted with the major peaks. Although the elution conditions can be made more efficient by using a shallow gradient and varying the flow rate, maximum

resolution in a shorter running time and the shape of the eluting peak was taken into consideration before a final choice was made. Several trial runs were carried out to achieve the maximum resolution in a shorter period of time. In general, the nature of the peaks was consistent and behaved predictably as expected for a hydrolytic reaction. The exact sites of cleavage were not determined in this study. Hence, knowledge of the composition and the nature of the peptides produced by hydrolysis require further analysis.

5.4.1. Hydrolysis by chymosin

According to the primary structure of κ-casein (Figure 8), there are 13 chymosin-susceptible bonds in the 169 amino acid polypeptide chain. The two most common variants of κ-casein in Holstein, termed A and B variants is also shown in figure and arise due to amino acid substitutions of threonine by isoleucine at position 136 and aspartic acid by alanine at position 148 for the A variant. The most susceptible bond of chymosin hydrolysis is between positions 105-106 of the primary structure. It is evident from Fig. 8 that the hydrolytic sites are not present in the region where there is a difference between the κ-casein variants A and B. However, the amino acid substitutions may cause a change in the three dimensional structure of the whole protein. This may influence the interaction of the κ-casein variants with chymosin with subsequent effects on the rate and extent of hydrolysis. If all the potential specific cleavage sites by chymosin were split, 14 intermediate products with different size of peptide fragments could be expected. However, the hydrolysis was carried out under mild conditions and not allowed to completion. Hence, the number of peptides would be less than the number of potential cleavage sites in the primary structure. The reaction was carried out under acidic conditions (pH 5.8) at 37 ° C and terminated after an incubation period of 90 min. Under the reaction conditions only the most susceptible bonds are expected to be cleaved by chymosin.

5.4.1.1 RP-HPLC profile

Figure 9 represents a typical peptide profile of chymosin digests of κ-casein at different time intervals of incubation. There are five peaks seen in the HPLC profile, both large and small ones with relatively clear separation in an incubation period of 90 min. κ-Casein elutes at approximately 34 min (0 min of incubation) and the peak decreases with

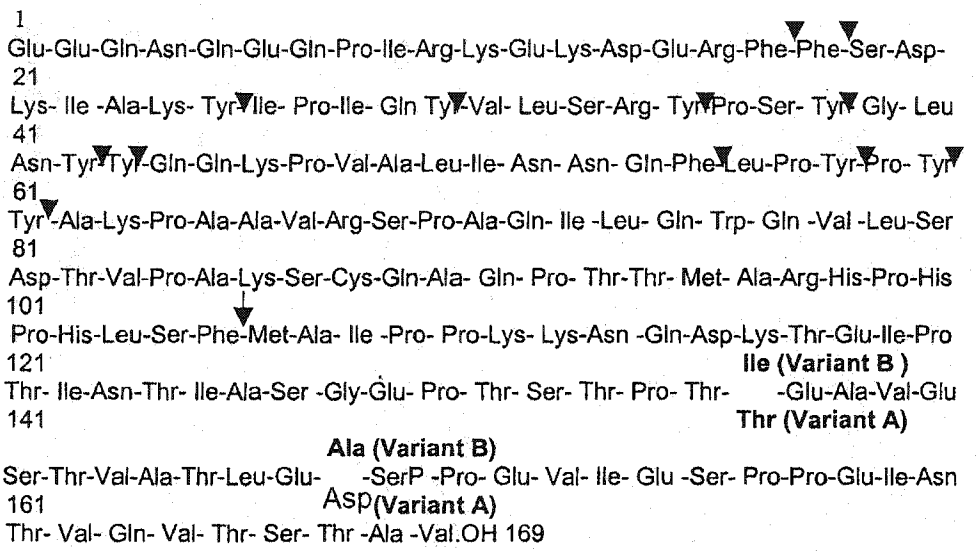

Figure 8. Primary structure of bovine κ-casein indicating all the possible chymosin and pepsin cleavage sites (arrow indicates the most susceptible bond) [Adapted from Eigel et al., 1984]

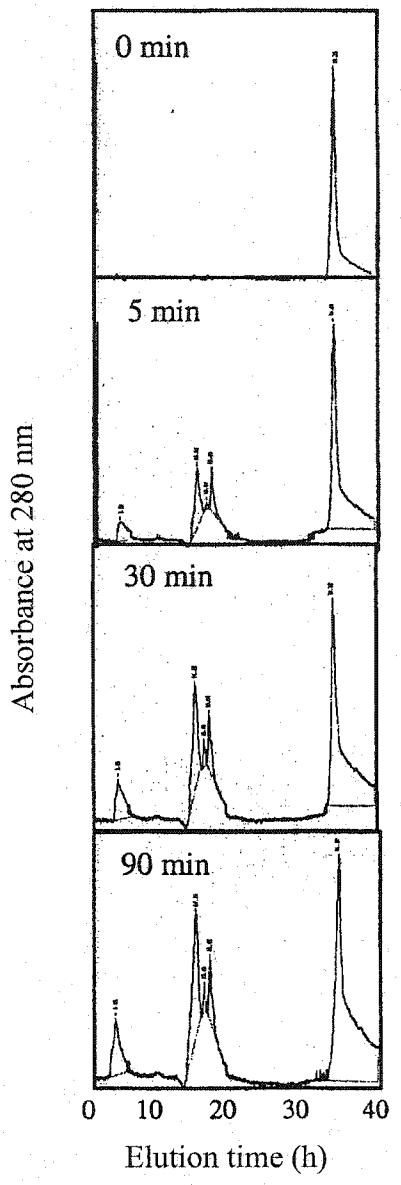

Figure 9. RP-HPLC chromatogram of hydrolysates from κ-casein by chymosin at different times of incubation

the progress of the reaction indicating the hydrolysis of κ-casein by chymosin. The κ-casein peak completely disappears after 3 h of incubation (data not shown). The products of hydrolysis are represented by four major peaks and appear within 5 min of incubation. The peptide eluting at approximately 3 min is presumably the least hydrophobic and eluted at 3-4% acetonitrile gradient. The other three major peaks eluted at approximately 14, 15 and 16 min respectively due to differences in the degree of hydrophobicity between the peptides. It is evident from the chromatogram that κ-casein is cleaved at more than one site apart from the most susceptible Phe_{105}-Met_{106} bond in the primary structure. It is possible that more than one peptide elute under the same acetonitrile gradient producing a single large peak. Since the objective was to observe the decrease in the area of the κ-casein peak over time, the run conditions were optimized for maximum resolution of the peptides from the hydrolysate in a given period. Peptides within a close range of hydrophobicity therefore remain indistinguishable without disturbing the outcome of the final analysis.

5.4.1.2. Analysis by SDS-PAGE

SDS-PAGE of the hydrolysate is shown in Figure 10. The average molecular weight of the peptides can be ascertained by this method, since the rate of migration is in proportion to the size. Pure κ-casein forms a band in the molecular weight range of 19 kD. Several bands with a faster rate of migration, due to lower molecular weight, appear after 5 min of hydrolysis as a result of the breakdown of κ-casein by chymosin. The peptides that get hydrolyzed further disappear after 5 min of incubation with the enzyme, followed by the appearance of their breakdown products in the later time course of the reaction. By examining all the cleavage sites from the primary structure, the potential peptides that are possibly produced by hydrolysis can be speculated by assigning approximate molecular weight to the peptides depending on the number of amino acids and comparing them to those seen in the gel. Two peptides with a molecular weight in the range of 14-17 kD are seen in all the lanes. These peptides could represent the major breakdown products of κ-casein viz., the para κ-casein (residues 1-105) and the macro-peptide moiety (residues 106-169). The peptide band close to 17 kD, possibly the para-κ-casein moiety, was further hydrolysed with increasing time intervals, as evident by the reduction of the band intensity in the gel. This could be possibly due to the presence of

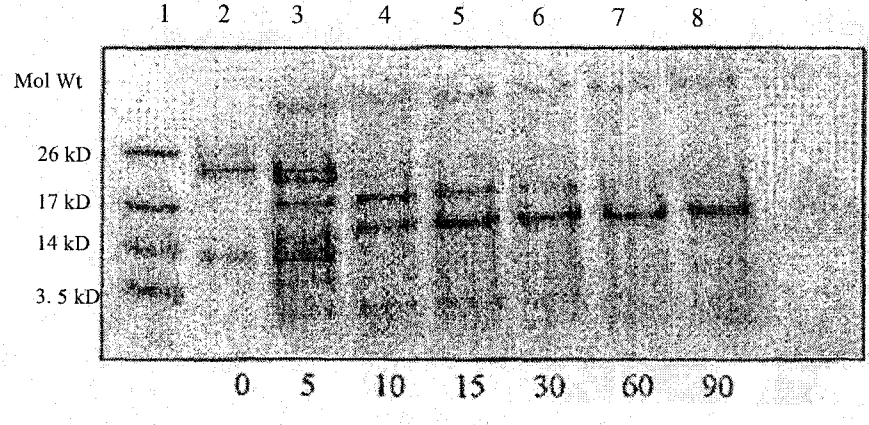

Figure 10. SDS-PAGE of chymosin hydrolysis with increasing time intervals
Lanes: 1- MW std; 2- κ-casein; 3-8 (5, 10, 15, 30, 60 and 90 min of incubation time respectively)

multiple cleavage sites between the residues 1-105 (Figure 8). The molecular weight of the putative para-κ-casein band is larger than expected which is probably due to the presence of post-translational modification such as phosphorylation that may occur within this region. The peptide band close to 14 kD, probably representing the macro peptide, is produced at a faster rate than it is hydrolyzed, as can be seen by the increase of band intensity with time. This is consistent with the fact that there are no potential chymosin cleavage sites between residues 106-169 (Figure 8). The putative macro peptide band migrates close to 14 kD, which is larger than what would be expected for a peptide of 64 amino acids. This is possibly due to the presence of several carbohydrate moieties in this region that may contribute to the overall molecular weight resulting in a lower migration rate. This fragment is also referred as the glyco-macropeptide due to the presence of sugar groups (Dalgleish, 1992). Peptides with the least molecular weight migrate faster and the smallest peptide as seen from the gel is less than 3.5 kD suggestive of a peptide of less than 25-30 amino acids. The putative peptides can be deduced from the potential chymosin cleavage sites present in the primary structure (Figure 8). These could include the peptides fragments from position 1-17, 19- 30, 31-42 and 43-58. Further analysis is required to ascertain the exact nature and composition of the peptides produced by the hydrolysis of κ-casein by chymosin.

5.4.1.3. Rate of hydrolysis

The extent of hydrolysis was monitored by the disappearance of κ-casein peak and the appearance of the peptide peaks derived from κ-casein as the incubation time increased. The effect of the genetic variants of κ-casein on the rate of hydrolysis was estimated by plotting the decrease in the amount of the protein as determined by the area of the κ-casein peak in the RP-HPLC chromatogram as a function of time (Figure 11). κ-Casein is hydrolyzed rapidly during the first 5 min and thereafter reaches a plateau throughout the course of the reaction for all the three phenotypes. It is evident from the figure that the rate of hydrolysis is similar for the AB and BB phenotype and is higher than the AA phenotype. Statistical analysis (Table 5) of the data did not show any significant difference between the phenotypes AB and BB. However, the AA phenotype is hydrolyzed slower than the other two variants and the difference was statistically significant ($P < 0.05$).

Time (min)	Phenotypes of κ-casein		
	AA	AB	BB
	Mean ± S.D	Mean ± S.D	Mean ± S.D
0	75.81 ± 0.41	58.01 ± 0.04	63.81 ± 0.72
1	54.33 ± 0.01	36.97 ± 0.01	40.11 ± 0.50
5	47.14 ± 0.88	30.19 ± 1.76	26.91 ± 1.63
10	38.95 ± 0.01	27.07 ± 0.05	23.72 ± 0.30
15	38.32 ± 0.01	24.04 ± 1.14	23.13 ± 1.13
30	36.17 ± 0.05	22.47 ± 0.37	22.33 ± 3.34
60	35.98 ± 0.30	20.24 ± 0.04	21.35 ± 2.13
90	34.06 ± 0.03	19.98 ± 0.71	21.46 ± 2.41

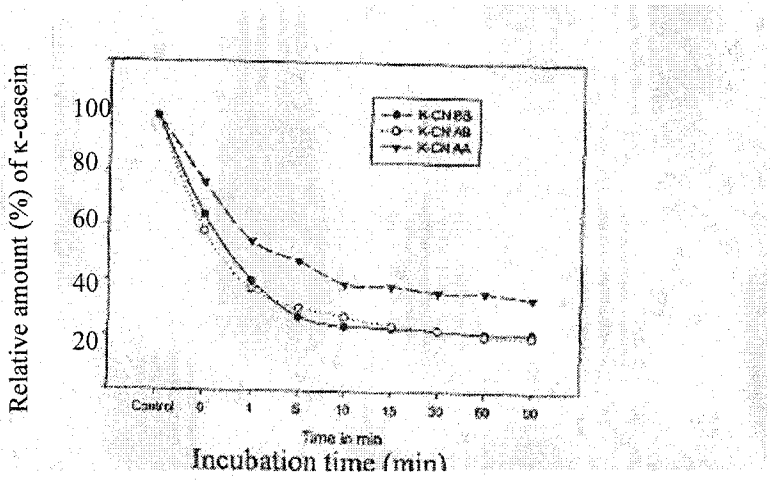

Figure 11. Rate of hydrolysis of κ-casein phenotypes by chymosin

Dependent Variable: Area of κ-casein peak

Source	DF	Type III Sum of Squares	Mean Square	F-Value	Pr > F
Phenotype	2	1180.77	590.38	112.79	0.0001
Time Interval	7	4632.65	661.8	126.44	0.0001

A. ANOVA for rate of hydrolysis of κ-casein

Phenotype	LS Mean	Std. Err. of LS Mean	Pr > |T |
BB	30.35	0.80	0.0001
AB	30.08	0.80	0.0001
AA	45.09	0.80	0.0001

B. Least square means for κ-casein phenotypes

Time	LS Mean	Std. Err. of LS Mean	Pr > |T |
0	69.14	1.32	0.0001
1	42.91	1.32	0.0001
5	33.23	1.32	0.0001
10	29.22	1.32	0.0001
15	28.53	1.32	0.0001
30	26.95	1.32	0.0001
60	26.13	1.32	0.0001
90	25.27	1.32	0.0001

C. Least square means for time intervals of hydrolysis

Table 5. Statistical analysis for the hydrolysis of κ-casein phenotypes by chymosin

5.4.2. Hydrolysis by pepsin

The potential cleavage sites of pepsin are similar to that of chymosin but the bonds that are more susceptible to hydrolysis may vary between the two enzymes. This arises possibly due to differences in the catalytic activity of the enzymes. The hydrolysis was carried out under acidic conditions (pH 5.8) at 37 ° C for an incubation period of 90 min and aliquots were collected at 0, 5, 15, 30, 60, and 90 min. It can be deduced from the primary structure that there are 13 cleavage sites (17-18, 18-19, 25-26, 30-31, 35-36, 38-39, 42-43, 43-44, 55-56, 58-59, 60-61, 61-62 and 105-106) that can result in 14 hydrolytic peptide fragments. Since the hydrolytic reaction was carried out under conditions of limited proteolysis, the number of peptides is expected to be less than the number of potential cleavage sites.

5.4.2.1. RP-HPLC profile

The peptide digests of κ-casein by pepsin at different incubation periods are shown in Figure 12. There are six peaks with different retention times seen in the representative chromatograms. The products of hydrolysis are indicated by 5 peaks that elute at approximately 3, 14, 16, 17 and 18 min elution time respectively. The peaks are clearly separated from each other and therefore differ significantly in their degree of hydrophobicity. Four peaks appear within 5 min of the reaction and all the peaks except one increase in area and height with longer incubation periods. This suggests that the peptides are produced faster than the rate of hydrolysis. The peak eluting at 16 min decrease in height and area after 30 min of incubation indicating that that the peptide is further broken down by pepsin. It is evident that κ-casein is cleaved at multiple points in addition to the most susceptible bond resulting in several peaks. As in the case of chymosin hydrolysis, several peptides can elute in a single large peak. Carles and Martin (1985) analyzed the hydrolysis of κ-casein by chymosin and pepsin by RP-HPLC to determine the Michaelian parameters. Their results are similar to those obtained using the fragment 98-111 of κ-casein (Visser et al., 1980). They concluded that neither the micellar state nor the presence of the whole peptide chain of κ-casein significantly affect the action of chymosin on fragment 98-111, which presumably contains all the information that makes bond 105-106 highly sensitive to chymosin. In a similar study, Vreeman et al., (1986) determined the release of macropeptide (fragment 106-169) from

κ-casein after chymosin hydrolysis by high-performance gel-permeation chromatography. Their results suggest that the carbohydrate-free and carbohydrate-containing fractions differ in the kinetics of the reaction.

5.4.2.2. Analysis by SDS-PAGE

The approximate molecular weights of the peptides were determined by using SDS-PAGE (Figure 13). This information can be used to determine the possible sites of hydrolysis from the knowledge of known cleavage sites from the primary structure of κ-casein. Two peptides of molecular weight near 17 kD and 14 kD appear after 5 min of incubation and remain intact even after 90 min of the reaction. A small peptide close to 3 kD is seen earlier in the reaction and disappears after 15 min of hydrolysis. This may be due to further hydrolysis of the peptide into fragments of insufficient concentration to be observed in the gels. The smallest peptide less than 3.5 kD produced a faint band at the bottom of the gel. The identity of the peptides can be assessed tentatively by examining the cleavage sites on the primary structure and comparing the bands seen in the gel. Peptides of less than 3.5 kD can be produced by residues 1-17 or 43-58. Peptides of more than 10 kD can be produced by residues 25-61, 1-105, 62-169 or 106-169. Amino acid profile and sequencing is required to elucidate the exact nature and location of these peptides in the primary structure of κ-casein.

5.4.2.3. Rate of hydrolysis

The difference in the rate of hydrolysis between the three different phenotypes was monitored by calculating the decrease in the amount of the κ-casein over time as in the case of chymosin. Statistical analysis of the data was performed to identify any significant difference in the rate of hydrolysis between the phenotypes. A plot of the amount of κ-casein as measured by the substrate area percentage, as a function of time is shown in Fig.14. The rate of hydrolysis is rapid during the initial time course of the reaction and a steady state is achieved at longer incubation periods. The extent of hydrolysis is maximum during the initial 5 min of the reaction. The rate of the reaction remains largely unchanged thereafter within a time course of 90 min of the reaction. A significant difference in the hydrolytic rate can be observed between all the three phenotypes (Table 6). The phenotype BB is hydrolyzed more extensively than the other

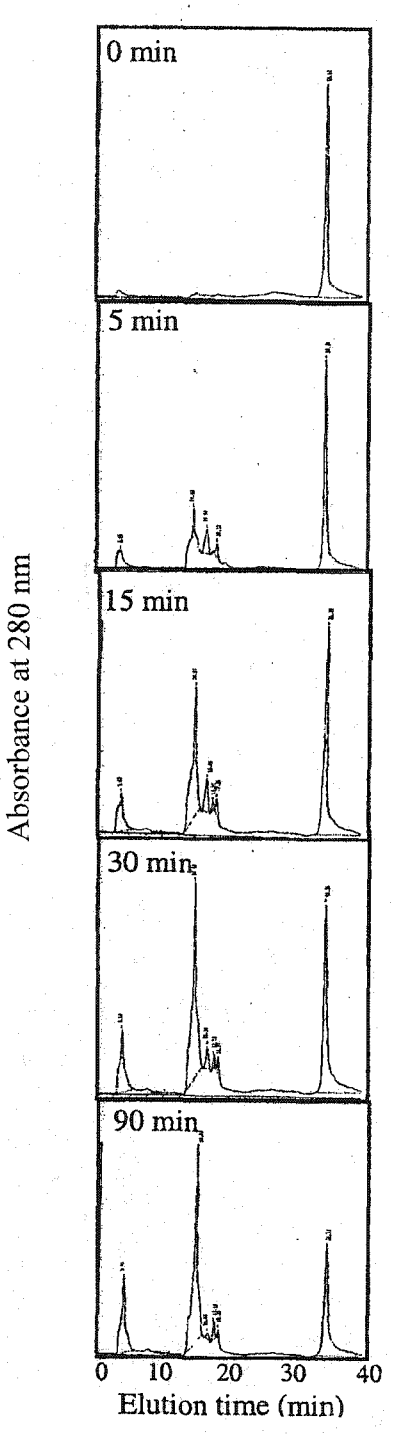

Figure 12. RP-HPLC chromatogram of hydrolysates from κ-casein by pepsin at different times of incubation

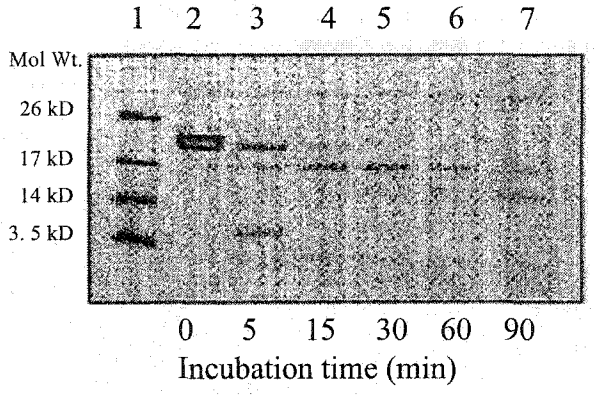

Figure 13. SDS-PAGE of pepsin hydrolysis at increasing time intervals
Lanes: 1- MW Std.; 2- κ-casein ; 3-7 (5, 15, 30, 60 and 90 min of incubation time respectively)

Time (min)	Phenotypes of κ-casein		
	AA	AB	BB
	Mean ± S.D	Mean ± S.D	Mean ± S.D
0	69.31 ± 2.5	76.33 ± 3.82	72.27 ± 2.93
1	54.09 ± 0.04	41.06 ± 3.16	41.33 ± 1.34
5	50.93 ± 2.07	32.37 ± 1.87	21.56 ± 2.26
10	45.77 ± 1.74	30.34 ± 1.28	18.90 ± 1.04
15	45.71 ± 2.23	29.74 ± 1.76	18.09 ± 0.05
30	45.47 ± 0.70	28.05 ± 1.38	18.01 ± 0.05
60	42.93 ± 0.78	27.35 ± 2.64	17.96 ± 0.42
90	39.76 ± 0.50	26.66 ± 1.64	17.76 ± 0.16

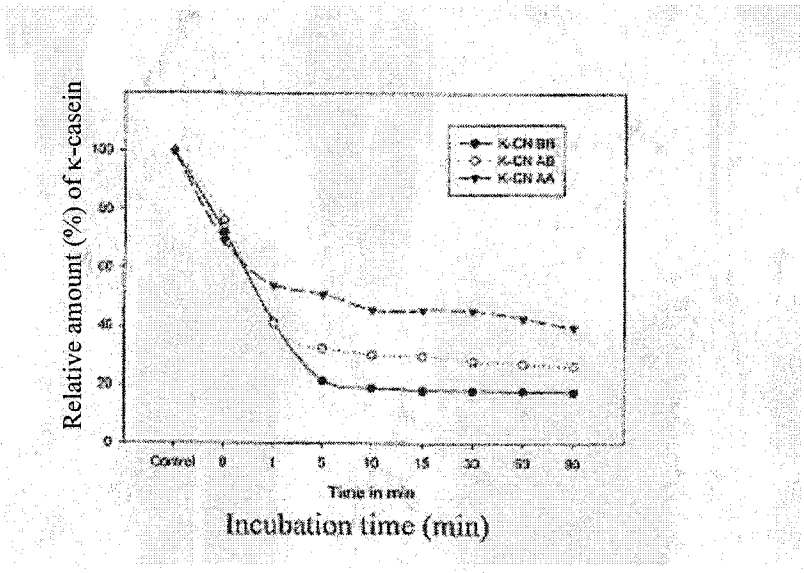

Figure 14. Rate of hydrolysis of κ-casein phenotypes by pepsin

Dependent Variable: Area of κ-casein peak

Source	DF	Type III Sum of Squares	Mean Square	F-Value	Pr > F
Phenotype	2	1792.66	896.33	26.08	0.0001
Time Interval	7	4740.43	677.20	19.70	0.0001

A. ANOVA for rate of hydrolysis of κ-casein

Phenotype	LS Mean	Std. Err. of LS Mean	Pr > |T |
BB	28.23	2.07	0.0001
AB	36.50	2.07	0.0001
AA	49.24	2.07	0.0001

B. Least square means for κ-casein phenotypes

Time	LS Mean	Std. Err. of LS Mean	Pr > |T |
0	72.63	3.38	0.0001
1	45.49	3.38	0.0001
5	34.98	3.38	0.0001
10	31.67	3.38	0.0001
15	31.18	3.38	0.0001
30	30.51	3.38	0.0001
60	29.41	3.38	0.0001
90	28.06	3.38	0.0001

C. Least square means for time intervals of hydrolysis

Table 6. Statistical analysis for the hydrolysis of κ-casein phenotypes by pepsin

two phenotypes. As in the case of chymosin, AA phenotype shows a significant slower rate of hydrolysis but the phenotype AB is intermediate between the two phenotypes.

The difference in the extent of hydrolysis between the two variants could be explained by the fact that the amino acid substitutions may alter the three-dimensional conformation of the whole protein. Since the nature of the hydrolysis in any enzymatic reaction depends on the interaction of the enzyme with the substrate, any change in substrate conformation could potentially alter this interaction. The complete three-dimensional structure of the κ-casein variants has to be solved to confirm this hypothesis. κ-Casein has not been crystallized so far but the nature of the interaction between κ-casein and proteases has been studied using analogous peptides. Plowman et al., (1995) used molecular modelling and energy minimization techniques to study the interaction of a κ-casein derived peptide (amino acid residues 98-111) with calf chymosin and porcine pepsin. They observed that electrostatic binding at either end of the active site cleft of chymosin is important for the positioning of residues 103-108 in the cleft. They also identified an acidic region in porcine pepsin that is in a position to form strong electrostatic interactions with the histidines at the N-terminus of the peptide. There were no significant differences in the extent of hydrolysis between chymosin and pepsin under conditions used in this study.

6. Summary and conclusion

Three different phenotypes of κ-casein were identified using polyacrylamide gel electrophoresis under alkaline conditions. The different components of casein were then isolated and purified using ion-exchange liquid chromatography system. The four major casein fractions were identified according to their electrophoretic mobilities in alkaline polyacrylamide gels. In the present study, all the major casein components were separated into electrophoretically pure forms and κ-casein was purified in relatively large quantities for further analysis.

The A and the B variant of κ-casein differ in their net charge due to amino acid substitutions in their primary structure and hence may produce different properties in milk, especially hydrolysis by milk coagulation enzymes like those used in the cheese industry. This study demonstrated that there is a significant difference in the rate of hydrolysis between the different phenotypes of κ-casein.

The three different phenotypes of κ-casein (AA, AB, BB) were subject to enzymatic hydrolysis by calf chymosin and porcine pepsin. The hydrolysates were analyzed by RP-HPLC and SDS-PAGE to observe any difference in the rate of hydrolysis. In the case of chymosin, there was no significant difference in the rate of hydrolysis between the phenotypes AB and BB. But, the AA phenotype is hydrolyzed slower than the other two phenotypes ($P<0.05$). Pepsin hydrolysis indicated a significant difference ($P<0.05$) in the rate of hydrolysis between the three phenotypes. The BB phenotype is hydrolyzed more extensively than the other two phenotypes. The AA phenotype shows a significantly slower rate of hydrolysis and the AB phenotype is intermediate between the two variants.

This study suggests that the AA phenotype of κ-casein is hydrolyzed more slowly than the other two phenotypes (AB, BB) under the present conditions. The experiments were performed using purified preparations of κ-casein and do not simulate the conditions seen in whole milk. Further analysis would be required to observe the interaction of κ-casein genetic variants with components found in whole milk especially the other types of casein and the whey proteins. This will provide a greater understanding of the properties of casein micelles with different κ-casein phenotypes and how this could

influence further processing of milk as in enzyme hydrolysis. Such studies could have possible implications in the cheese industry.

7. References

Aaltonen, M.L. and V. Antila (1987). Milk renneting properties and the genetic variants of proteins. Milchwissenschaft 42:490-492.

Addeo, F., J.M. Chobert and B. Ribadeau-Dumas (1977). Fractionation of whole casein on hydroxyapatite: Application to a study of buffalo κ-casein. J. Dairy Res. 44:63-68.

Addeo, F., G. Garro, N. Intorcia, L. Pellegrino, P. Resmini and L. Chianese (1995). Gel electrophoresis and immunoblotting for the detection of casein proteolysis in cheese. J. Dairy Res. 62:297-309.

Addeo, F., R. Mauriello and A. Di Luccia (1988). A gel electrophoretic study of caprine casein. J. Dairy Res. 55:413-421.

Addeo, F., P.Martin and B. Ribadeau-Dumas (1984) Susceptibility of buffalo and cow α-caseins to chymosin action [in milk]. Milchwissenschaft 39:202-205.

Aleandri, R., L. Chianese, A. Di Leccia, P. Petelli, R. Mauriello and G. Davoli (1990). The effects of milk protein polymorphism on milk components and cheese producing ability. J. Dairy Sci. 73: 241-255.

Andrews, A. T. (1978a). The composition, structure and origin of proteose-peptone component 5 of bovine milk. Eur. J. Biochem. 90:59-65.

Andrews, A. T. (1978b). The composition, structure and origin of proteose-peptone component 8F of bovine milk. Eur. J. Biochem. 90:67-71.

Andrews, A. T. and E. Alichanidis (1983). Proteolysis of caseins and the proteose-peptone fraction of bovine milk. J. Dairy Res. 50:275-290.

Andrews, A. T., M.D.Taylor and A. J. Owen (1985). Rapid analysis of bovine milk proteins by fast protein liquid chromatography. J. Chromatography 348: 177-185.

Annan, W. D. and W. Manson (1969). A fractionation of α_s-casein complex of bovine milk. J. Dairy Res. 36:259-268.

Arave, C.W. (1967). Procedure for simultaneous phenotyping of β-casein and β-lactoglobulin variants in cow's milk. J. Dairy Sci. 50:1320-1322.

Arima, K., J.Yu and S.Iwasaki (1970). Milk clotting enzyme from *Mucor pusillus var.lindt.* In Methods in Enzymology. Vol.19 Ed., Perlmann, G.E. and L. Lorand. pp.347-358.

Armstrong, C.E., A.G. MacKinlay, R.J. Hill and R.G.Wake (1967). The action of rennin on κ-casein: The heterogeneity and origin of the soluble product. Biochim.Biophys. Acta.140:123-131.

Aschaffenburg, R. (1961). Inherited casein variants in cow's milk. Nature 192:431-432.

Aschaffenburg, R. (1964). Protein phenotyping by direct polyacrylamide gel electrophoresis of whole milk. Biochim. Biophys. Acta. 82:188-191.

Aschaffenburg, R. and J. Drewry (1955). Occurrence of different β-lactoglobulins in cow's milk. Nature 176:218-219.

Ashaffenburg, R. and J. Drewry (1957). Genetics of β-lactoglobulins of cow's milk. Nature 180:376-378.

Ashaffenburg, R. and M.Thyman (1965). Simultaneous phenotyping procedure for the principal proteins of cow's milk. J.Dairy Sci. 48:1524-1526.

Azuma, N., S. Kaminogawa and K.Yamamuchi (1984). Properties of glycomacropeptide and para-κ-casein derived from human κ-casein and comparison of human and bovine κ-caseins susceptibility to chymosin and pepsin. Agri. Biol.Chem. 48:2025-2031.

Babukov, A.V., V.E. Mityutko and A.D. Komissarenko (1982). Use of genotypes of polymorphic milk proteins for improvement of Black and White cattle. Proc. XXI Intern. Dairy Congr. Moscow, Vol.1. Book 1, p39.

Balland, O. (1987). Analyse et évolution du polymorphisme électrophoretique des lactoprotéines des races bovines Montbéliarde et Normande. (Mimeographed report).

Basha, S.M. (1988). Two-dimensional gel electrophoretic separation of proteins. In Methods for Protein Analysis. Ed: Cherry, J.P. and R.A. Barford. pp70-87. American Oil Chemists' Society, Champaign, Illinois.

Beeby, R. (1979). The proteolysis of casein by immobilized preparation of α-chymotrypsin, chymosin and a fungal protease. N.Z.J. Dairy Sci. Technol.14:1-11.

Bell, K. (1962). One-dimensional starch-gel electrophoresis of bovine skim milk. Nature 195:705-706.

Bell, K., H.E. McKenzie and D.C.Shaw (1981). Bovine β-lactoglobulin E, F and G of Bali (Bentang) cattle, Bos (Bilbos) javanicus. Aust. J. Biol. Sci. 34:113.

Berg., J.A., K.J.van den Laken, A.J.J.van den Ooyen, T.C.H.M. Renniers, K.Rietveld, A.Schaap, A.J. Brake, R.J. Bishop, K.Schulz and D. Moyer (1990). *Kluveromyces* as a host for heterologous gene expression: expression and secretion of prochymosin. Biotechnology 81:135-139.

Berridge, N.J. (1942). The second phase of rennet coagulation. Nature 149:194-195.

Bhattacharya, B. S., A. K. Roychoudhury, N. K. Sinha and A.Sen (1963). Inherited α-lactalbumin and β-lactoglobulin polymorphism in India Zebu cattle. Comparison of Zebu and buffalo α-lactalbumin. Nature 181:447-451.

Bingham, E.W. (1975). Action of rennin on κ-casein . J.Dairy Sci. 58:1-13.

Blumberg, B. S. and M. P. Tombs (1958). Possible polymorphism of bovine α-lactalbumin. Nature 181:683-684.

Bohak, Z. (1970). Chicken pepsiongen and chicken pepsin. In Methods in Enzymology. Vol.19 Ed. Perlmann, G.E. and L.Lorand. Pp347-358.

Bonsing, J. and A.G. Mackinlay (1987). Recent studies on nucleotide sequences encoding the caseins. J. Dairy Res. 54:447-467.

Bovenhuis, H., A. M. van Arendonk and S. Korver (1992). Associations between milk protein polymorphisms and milk production traits. J. Dairy Sci. 75:2549-2559.

Braunitzer, G. and Chen. R (1972). Die Spaltung des β-lactoglobulins AA mit Bromcyan. Hoppe-Seyler's Z.Physio. Chem. 353:674-676.

Brew, K., F. J. Castellino, T. C. Vanaman and R. L. Hill (1970). The complete amino acid sequence of bovine α-lactalbumin. J. Biol. Chem. 245:4570-4582.

Brignon, G., B. Ribadeau-Dumas, J.C. Mercier, J. P. Pelissier and B.C.Das (1977). Complete amino acid sequence of bovine α_{s2}-caseins. FEBS Lett. 76:274.

Brinkhuis, J. and T.A.Payens (1985). The rennet induced clotting of para-κ-casein revisited: inhibition experiments with pepstatin A. Biochim. Biophy. Acta 832:331-336.

Brunner, J.R. (1981). Cow milk proteins: Twenty five years of progress. J.Dairy Sci. 64:1038-1054.

Buchberger, J. (1995). Genetic polymorphism of milk proteins: differences between breeds. Internat. Dairy Federation Bulletin 304, Brussels, Belgium. Pp 5-6

Buchberger, J., O.Kiermeier, R.Graml and F. Pirchner (1982). Effect of genetic variants of milk proteins on milk composition. Proc. XXI Internat. Dairy Congr. Moscow, Vol.1 Book 1, pp40-41.

Carles, C. (1986). Fractionation of bovine caseins by reverse phase high performance liquid chromatography: Identification of a genetic variant. J. Dairy Res. 53:35-41.

Carles, C. and P. Martin (1985). Kinetic study of the action of bovine chymosin and pepsin A on bovine κ-casein. Arch. Biochem. Biophys. 242:411-416.

Castle, A.V. and J.V. Wheelock (1972). Effect of varying enzyme concentration on the action of rennin on whole milk. J.Dairy Res. 39:15-22.

Chaplin, B. and M.L. Green (1980). Determination of the proportion of κ-casein hydrolyzed by rennet on whole milk. J. Dairy Res. 47: 351-358.

Cheryan, M., P.J.Wyk, N.F.van Olson, and T.Richardson (1975). Secondary phase and mechanism of enzymic milk coagulation. J.Dairy Sci. 58:477-481.

Chianese, L., G. Garro, P. Ferranti, A. Malorni, F. Addeo, A. Rabasco and P. M. Pons (1995) Discrete phosphorylation generates the electrophoretic heterogeneity of ovine β-casein. J. Dairy Res. 62:89-100.

Chianese, L., R. Mauriello, L. Moio, N. Iunziatina and F.Addeo (1992). Determination of ovine casein heterogeneity using gel electrophoresis and immunochemical techniques. J. Dairy Res. 59:39-47.

Choi, J.W. and K.F. Ng-Kwai-Hang (1998). Effect of genetic variants of κ-casein and β-lactoglobulin on cheese yielding capacity of milk preheated at different temperatures. Korean J. Dairy Sci.20: 113-122.

Coolbear, K.P., D.F. Elgar, T.Coolbear, and J.S. Ayers (1996). Comparitive study of methods of isolation and purification of bovine κ-casein and its hydrolysis by chymosin. J. Dairy Res. 63:61-71.

Coulon, J.B., C. Hurtaud, B. Remond and R. Verite (1998). Factors contributing to variation in the proportion of casein in cow's milk true protein: a review of recent INRA experiments. J. Dairy Res. 65:375-387.

Cullen, D., G.L.Gray, L.J.Wilson, K.J. Hayenga, M.H.Lamsa and R.M. Berka (1987). Controlled expression and secretion of bovine chymosin in *Aspergillus nidulans*. Biotechnology 5:369-376.

Dalgleish, D.G. (1979). Proteolysis and aggregation of casein micelles treated with immobilized or soluble chymosin. J. Dairy Res. 46:643-661.

Dalgleish, D.G. (1980). A mechanism for the chymosin-induced flocculation of casein micelles. Biophys. Chem.11: 147-155.

Dalgleish, D.G. (1983). Coagulation of renneted bovine casein micelles: dependence on temperature, calcium ion concentration and ionic strength. J. Dairy Res. 50:331-340.

Dalgleish, D.G. (1986). Analysis by fast protein liquid chromatography of variants of κ-casein and their relevance to micellar structure and renneting. J. Dairy Res. 53:43-51.

Dalgleish, D.G. (1992). The enzymatic coagulation of milk. In Advanced Dairy Chemistry. Vol. 1 Proteins. Ed. P.F. Fox. Elsevier Applied Science, New York. Pp579-619.

Dalgleish, D.G., J.Brinkhuis and T.A.J. Payens (1981). The coagulation of differently sized casein micelles by rennet. Eur. J. Biochem.119: 257-261.

Davies, D. T. and A. J. R. Law (1977). An improved method for the quantitative fractionation of casein mixtures using ion-exchange chromatography. J. Dairy Res. 44:213-221.

Davoli, R., S.Dall'Olio and V. Russo (1990). Effect of κ-casein genotype on the coagulation properties of milk. J. Anim. Breed. Genet.107: 458-464.

Debeljak, M., S. Susnik, R. Marinsek-Logar, J.F.Medrano and P. Dovc (2000). Allelic differences in bovine κ-casein gene which may regulate gene expression. Pfluegers Archiv. Euro. J. Physiol. 439: (Suppl.3) R4-R 6

De-Lange, D. J., E.H.H.Meyer, L. Van Rensburg and A. Swanepoel (1991). The polymorphic protein system in cow's milk: 1. The association with the production of milk protein and fat during lactation. South African J. Dairy Sci. 57:394-398.

Delfour, A., J.Jolles, C.Alais and P. Jolles (1965). Caseino-glycopeptide: Characterization of a methionine residue and of the N-terminal sequence. Biochem. Biophys. Res. Comm. 19:425-461.

Dewan, R.K., A.Chudgar, V.A. Bloomfield and C.V. Morr (1974). Size distribution and solvation of casein micelles in milk containing α_{s1}-casein A. J. Dairy Sci. 57:394-398.

Dimenna, G.P. and H.J. Segall (1981). High-performance gel-permeation chromatography of bovine skim milk proteins. J. Liquid Chromatography. 4:639-649.

Diosady, L.L., L.Bergen and V.R. Harwalkar (1980). High performance liquid chromatography of whey proteins. Milchwissenschaft 35: 671-674.

Distasio, L. and P. Merlin (1979). Polimorfismi bichimici del latte nella razza bovina Grigio Alpina. Riv. Zootec. Vet. 2: 64-67.

Dong, C. (1992). Existence of silent variants of milk proteins. M. Sc. Thesis, McGill University, Montreal, Canada.

Dong, C. (1998). Characterization of genetic variants of milk proteins that are not identifiable by electrophoresis. Ph.D., Thesis, McGill University, Montreal, Canada.

Dong, C. and K.F. Ng-Kwai-Hang (1998). Characterization of a non-electrophoretic genetic variant of β-casein by peptide mapping and mass spectrometric analysis. Internat. Dairy J. 8:967-972.

Donnelly, W. J., D.M. O'Callaghan, D.P.Caroll, D.J. McConnell, and F. Gannon (1984). Multiple forms of calf prochymosin and chymosin. Trans. – Biochem. Soc. 12:440-441.

Eigel, W. N., J.E.Butler, C.A.Ernstorm, H.M. Farrell Jr., V.Harwalker, R. Jenness and R. McL. Whitney (1984). Nomenclature of proteins of cow's milk: Fifth revision. J.Dairy Sci. 67:1599-1631.

Ekstrand, B., M.Larsson-Radnikiewicz and C. Perlmann. (1980). Casein micelle size and composition related to the enzymatic coagulation process. Biochim. et Biophys. Acta 630:361-366.

El Negoumy, A.M. (1968). Starch gel electrophoresis of products of action of crystalline rennin on casein and its components. J. Dairy Sci. 51:1013-1017.

Emtage, J.S., S.Angal, M.T.Doel, T.J.R. Harris, B.Jenkins, G.Lilley, and P.A. Lowe (1983). Synthesis of calf prochymosin (prorennin) in *Escherichia coli*. Proc. Nat. Acad. Sci. 80: 3671-3675.

Erhardt, G. (1989). κ-casein in dairy cows: evidence of another allele (κ-CnE) in different breeds. J. Anim. Breed. Genet. 106:225-231.

Erhardt, G. (1996). Detection of a new κ-casein variant in milk of Pinzgauer cattle. Anim. Genet. 27:105-107.

Finlayson, G. R. and A. Chrambach (1971). Isoelectric focusing in polyacrylamide gel and its preparative application. Anal. Biochem. 40:292-311.

Foltmann, B. (1959). On the enzymatic and the coagulation stages of the renneting process. In Proc. XV Internat. Dairy Congr. (London), Vol.2, pp651-661.

Foltmann, B. (1966). A review on prorennin and rennin. C.R. Trav. Lab Carlsberg 35:143-231.

Foltmann, B. (1993). General and molecular aspects of rennet. In Cheese: Chemistry, Physics and Microbiology. Vol. 1. General Aspects. Chapmann and Hall Ltd., London, UK. Ed. 2 pp 37-68.

Ford, G.D. and A.S. Grandison (1986). Effect of size of casein micelles on coagulation properties of skim milk. J. Dairy Res. 53:129-133.

Fournet, B., A.M. Fiat, C.Alias and P. Jolles (1979). Cow κ-casein structure of the carbohydrate portion. Biochim. Biophys. Acta 576:339-346.

Fox, P. F. (1986). Coagulants and their action. In Milk: The Vital Force. Proc. XXIV Internat. Dairy Congr. The Hague. Sep.29-Oct.3. D.Reidel Publishing Company. Pp 37-68.

Fox, P. F. (1993). Cheese: An overview. In Cheese: Chemistry, Physics and Microbiology. II Edn. Vol. 1. General Aspects. Chapmann and Hall Ltd., London, UK.

Girardet, J. M., A.Mati, T. Sanogo, L. Etienne and G. Linden (1991). Fast protein liquid chromatography purification of hydrophobic fraction of bovine milk proteose-peptone and characterization by bidimensional electrophoresis. J. Dairy Res. 58:85-98.

Goff, C.G., D.T. Moir, T. Kohno, T.C. Gravius, R.A. Smith, E. Yamasaki and A. Taunton-Rigby (1984). Expression of calf prochymosin in *Saccharomyces cerevisiae.* Gene 27:35-46.

Gonyon, D. S., R.E. Mather, H.C. Hines, G.F.W. Haenlein, C.W. Arave and S. N. Grant (1987). Association of bovine blood and milk polymorphisms with lactation traits: Holsteins. J. Dairy Sci. 70:2585-2598.

Gordin, S., Y.Birk and R. Volcani (1972). Fractionation and characterization of milk proteins by column chromatogrphy and electrophoresis. J. Dairy Sci. 55:1544-1549.

Graham, E.R.B., D.M. McLean and P.Zviedrans (1984). The effect of milk protein genotypes on the cheese making properties of milk and on the yield of cheese. 4th Conf. Aust. Ass. Anim. Breed. Genet. Adelaide, 4-6 June, pp136-137.

Graml, R., G.Ohmayer, F. Pirchner, L. Erhardt, J.Buchberger and A. Mostageer (1986). Biochemical polymorphism in Egyptian Baladi cattle and their relationship with other breeds. Anim. Genet. 17:61-76.

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

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

Grosclaude, F., P.Joudrier and M.F. Mahé (1979). A genetic and biochemical analysis of a polymorphism of bovine α_{s2}-casein. J. Dairy Res. 46:211-213.

Grosclaude, F., M.F.Mahé and J. P. Accolas (1982). Note sur le polymorphisme génétique des lactoprotéines de bovins et de yaks Mongols. Ann. Génét. Sél. Anim. 14:545-550.

Grosclaude, F., M.F.Mahé, J.C.Mercier and B. Ribadeau-Dumas (1972a). Charactérisation des variants génétiques des caséines α_{s1}- et β bovines. Eur. J. Biochem. 26:328-337.

Grosclaude, F., M.F. Mahé, J.C.Mercier and B. Ribadeau-Dumas (1972b). Localization des substitutions d'acides amines differenciant les variant A et B de la caséine κ bovine. Ann. Génét. Sél. Anim. 4:515-521.

Grosclaude, F., M.F.Mahé, J.C. Mercier, J. Bonnemaire and J.H. Teissier (1976). Polymorphisme des lactoproteines de bovines Nepalais.I. Mise en evidence, chez le Yak, et biochimique de deux nouveaux variants: β-lactoglobuline D (Yak) et caseine α_{s1}-E. Ann. Génét. Sél. Anim. 8:461-479.

Grosclaude, F., J.C. Mercier and B.Ribadeau-Dumas (1973). Genetic aspects of cattle casein research. Neth. Milk Dairy J.27:328-340.

Guillou, H., G. Miranda and J. P. Pelisser (1987). Analyse quantitative des caséines dans le lait de vache par chromatographie liquide rapide d'échange d'ions. Lait 67:135148.

Gupta, B.B. (1983). Determination of native and denatured milk proteins by high performance size exclusion chromatography. J. Chromatogr. 282:463-475.

Han, S. K., Y.C.Shin and H.D. Byun (2000). Biochemical, molecular and physiological characterization of a new β-casein variant detected in Korean cattle. Animal Genetics 31:49-51.

Haenlein, G.F.W., D.S.Gonyon, R.E. Mather and H.C. Hines (1987). Associations of bovine blood and milk polymorphism with lactation traits: Guernseys. J.Dairy Sci.70: 2599-2609.

Harkki, A., J.Uusitala, M.Bailey, M. Penttila and J.K.C. Knowles (1989). A novel fungal expression system: secretion of active calf chymosin from the filamentous fungus Trichoderma reesi. Biotechnology 7:596-603.

Harris, T.J., P.A.Lowe, A. Lyons, P.G.Thomas, M.A.Eaton, T.A. Millican, T.P. Patel, C.C. Bose, N.H. Carey and M.T. Doel (1982). Molecular cloning and nucleotide sequence of cDNA coding for calf preprochymosin. Nucleic Acids Res. 10:2177-2187.

Hicks, C.L., J.O'Leary and J. Bucy (1988). Use of recombinant chymosin in the manufacture of Cheddar and Colby cheese. J. Dairy Sci.71: 1127-1131.

Hidaka, M., K.Sasaki, T.Uozumi and T. Beppu (1986). Cloning and structural analysis of the calf prochymosin gene. Gene 43:197-203.

Hill, J. P. (1993). The relationship between β-lactoglobulin phenotypes and milk composition in New Zealand Dairy Cattle. J.Dairy Sci.76: 281-286.

Hill, R.D. (1968). The nature of the rennin-sensitive bond in casein and its probable relation to sensitive bonds in other proteins. Biochem. Biophys. Res. Commun. 33:659-663.

Hill, R.D. (1969). Synthetic peptide and ester substrates for rennin. J.Dairy Res. 36:409-415.

Hill, R.D. and V.M. Hocking (1978). A study of the structural factors that affect the reactivity of the chymosin sensitive bond in κ-casein. N.Z.J. Dairy Sci. Technol. 13:195-201.

Hindle, E.J. and J.V. Wheelock (1970). The primary phase of rennin action in heat sterilized milk. J. Dairy Res. 37:389-396.

Holt, C. (1975). The stability of bovine casein micelles. In proceedings of the Conference on Colloid and Surface Science, Budapest, Ed. E.Wolfram. Akademiai Kiado, Budapest, pp.641-644.

Holt, C. and D. G. Dalgleish (1986). Electrophoretic and hydrodynamic properties of bovine casein micelles interpreted in terms of particles with an outer hairy layer. J. Colloid and Interf. Sci. 114:513-524.

Holt, D. L. and M. G. Zeece (1988). Two-dimensional electrophoresis of bovine milk proteins. J. Dairy Sci. 71:2044-2050.

Hooydonk, A.C.M. van., I.J.Boerrigter and H.G. Hagedoorn (1986). pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk. Neth. Milk. and Dairy J. 40:297-313.

Hooydonk, A.C.M. van., C.Olieman and H.G. Hagedoorn (1984). Kinetics of chymosin-catalyzed proteolysis of κ-casein in milk. Neth. Milk. and Dairy J. 38:207-222.

Hooydonk, A.C.M. van. and P.Walstra (1987). A consistent model for the clotting of milk by rennet; a reply to a comment. Neth. Milk and Dairy J.41: 293-295.

Horne, D. S. (1984). Steric effects in the coagulation of casein micelles by ethanol. Biopolymers 23:989-993.

Horne, D. S. (1986). Steric stabilization and casein micelle stability. J. Colloid and Interf. Sci. 111:250-260.

Horne, D. S., D.D.Muir, J. Leaver, T.A. McCreight, J.M. Banks and A. J. R. Law (1993). Influence of κ-casein genotype on rennet coagulation of bovine milk. IDF Seminar, Cork. Ppl42-150.

Humme, H.E. (1972). The optimum pH for the limited specific proteolysis of κ-casein by rennin (primary phase of milk clotting). Neth. Milk Dairy J. 26:180-185.

Humphrey, R. S. (1984). Whey proteins. In Handbook of HPLC for the separation of amino acids, peptides and proteins. Vol.2. Ed., W.S.Hancock. CRC Press, Boca Raton. Pp471-478.

Humphrey, R. S. and L. J. Newsome (1984). High performance ion exchange chromatography of the major bovine milk proteins. N. Z. J. Dairy Sci. Technol. 19:197-204.

Ikonen, T., M.Ojala and O. Ruottinen (1999). Associations between milk protein polymorphism and first lactation milk production traits in Finnish Ayrshire cows. J. Dairy Sci. 82:1026-1033.

Ikonen, T., O.Ruottinen, G.Erhardt and M. Ojala (1996). Allele frequencies of the major milk proteins in the Finish Ayrshire and the detection of a new κ-casein variant. Anim.Genet. 27:179-181.

Imafidon, G. I. and N.Y. Farkye (1994). Influence of pH on chymosin action in solutions of different κ-casein variants. J. Agri. and Food Chem. 42:1598-1601.

Imafidon, G. I. and K. F. Ng-Kwai-Hang (1991). Differential scanning calorimetric study of different genetic variants of lactoglobulin. J. Dairy Sci. 74:2416-2422.

Imafidon, G.I. and K. F. Ng-Kwai-Hang (1992). Isolation and purification of β-lactoglobulin. J. Dairy Res. 59:101-104.

Jairam, B.T. and P.G. Nair (1983). Genetic variants of milk proteins in different breeds of cattle. Ind. J.Dairy Sci. 36:5-11.

Janicki, C. (1978). β-lactoglobulin and some milk production characteristics of Holstein-Friesan cows in Poland imported from USA and Canada. Rocz. Akad. Roln. Poz.25: 169-175.

Jenkins, J.A., I.J.Tickle, T. Sewell, L. Ungaretti, A. Wollmer and T.L.Blundell (1977). X-ray analysis and circular dichroism of the acid protease from *Endothia parasitica* and chymosin. Adv. Exp. Med. Biol. 95:43-60.

Jolles, J., C.Alais and P. Jolles (1968). The tryptic peptide with the rennin sensitive linkage of cow's κ-casein. Biochim. Biophys. Acta 168:591-593.

Josephson, R. V., S.K. Maheswaran, C.V. Morr, R. Jenness and R. K. Lindorfer (1971). Effect of urea on pI19 of ampholytes and casein in isoelectric focusing. Anal. Biochem. 40:476-482.

Josephson, R. V., E.M.Mikolajcik and D. P. Sinha (1972). Gel isoelectric focusing of selected bovine immunoglobulins. J. Dairy Sci. 55:1508-1510.

Kaplan, L. J. and J. F. Foster (1971). Isoelectric focusing behavior of bovine plasma albumin, mercaptalbumin, and lactoglobulins A and B. Biochemistry 10:630-636.

Kawaguchi, Y., S.Kosugi, K. Sasaki, T.Uozumi and T. Beppu (1987). Production of chymosin in *Escherichia coli* cells and its enzymatic properties – purification and characterization of recombinant enzyme. Agri. Biol. Chem. 51:1871-1877.

Kawasaki, T., K.Ikeda, S.Takahashi and Y. Kuboki (1986). Further study of hydroxyapatite high-performance liquid chromatography using both proteins and nucleic acids, and a new technique to increase chromatographic efficiency. Eur. J. Biochem.155: 249-257.

Kiddy, C. A. (1975). Gel electrophoresis in vertical polyacrylamide beds. Procedures I and II. In: Methods of Gel Electrophoresis of Milk Proteins. ed: H. E. Swaisgood, B. L. Lawson, E. B. Kalan, J. R. Brunner, C. V. Morr and P. M. T. Hansen. Amer. Dairy Sci. Assoc. Champaign, IL. Pp14.

Kiddy, C. A., R.F.Peterson and F. C. Kopfler (1966). Genetic control of the variants of β-casein A. J. Dairy Sci. 49:742.

Kim, S. (1994). Genetic polymorphism of milk proteins and their association with production traits in Ayrshire, Jersey, Brown and Canadienne. M. Sc. Thesis, McGill University, Montreal, Canada.

Kim, S., K.F.Ng-Kwai-Hang and J. F. Hayes (1996). The relationship between milk protein phenotypes and lactation traits in Ayrshires and Jerseys. Asian Austral. J. Anim. Sci. 6:685-693.

Klose, J. (1987). New electrophoresis techniques used in studying protein polymorphism. Anim. Genet. 18 (suppl. 1): 37-40.

Lawrence, R.C. and L.K.Creamer (1969). The action of calf rennet and other proteolytic enzymes on κ-casein. J.Dairy Res. 36:11-20.

Lieske, B. and G. Konrad (1996). Selected biochemical and functional characteristics as criteria for comparing recombinant and natural chymosin. Part II. Proteolytic specificity and activity. Deutsch Milchwirtschaft 47:24, 1136, 1138-1141.

Lin, C.Y., A.J.McAllister, K.F. Ng-Kwai-Hang and J.F.Hayes (1986). Effects of milk protein loci on first lactation production in dairy cattle. J. Dairy Sci. 69:704-712.

Lopez-Galvez, G., M.Juarez and M. Ramos (1995). Two dimensional electrophoresis and irmunoblotting f or the study of ovine whey protein polymorphism. J. Dairy Res. 62:311-320.

Loucheux-Lefebvre, M.H., J.P.Aubert and P. Jolles (1978). Prediction of the conformation of cow and sheep κ-caseins. Biophys. J. 23:323-336.

Lunden, A., M. Nilsson and L. Janson (1997). Marked effect of β-lactoglobulin polymorphism on the ratio of casein to total protein in milk. J. Dairy Sci. 80:2996-3005.

Mackinlay, A.G., R.J.Hill and R. G. Wake (1966). The action of rennin on κ-casein: The heterogeneity and origin of the insoluble products. Biochim. Biophys. Acta 115:103-112.

Mahé, M.F., G.Miranda, R. Queval, A.Bado, P.Souvenir Zafindrajaona and F. Grosclaude (1999). Genetic polymorphism of milk proteins in African *Bos taurus* and *Bos inducus* populations. Characterization of variants α_{S1}-Cn H and κ-Cn J. Génét. Sél. Evol. 31:239-253.

Mariani, P., G.Losi, V.Russo, G.B.Castagnetti, L.Grazia, D.Morini and E. Fossa (1976). Caseification tests made with milk characterized by variants A and B of κ-casein in the production of Parmigiano-Reggiano cheese. Scien. Tecn. Latt.-Casear. 27: 208-227.

Marston, F.A.O., P.A.Lowe, M.T. Doel, J.M. Schoemaker, S.White and S.Angal (1984). Purification of calf pro-chymosin(prorennin) synthesized in *Escherichia coli*. Biotechnology 2:800-804.

Martin, P., M.N.Raymond, E. Bricas and B.Ribadeau Dumas (1980). Kinetic studies on the action of *Mucor pusillus, Mucor meihei* [fungi] acid proteases and chymosins A and B on a synthetic chromophoric hexapeptide. Biochim. Biophys. Acta 612:410-420.

Marziali, A. S. and K. F. Ng-Kwai-Hang (1986a). Relationship between milk protein polymorphisms and cheese yielding capacity. J. Dairy Sci. 69:1193-1201.

Marziali, A. S. and K. F. Ng-Kwai-Hang (1986b). Effects of milk composition and genetic polymorphism on the coagulation properties of milk. J. Dairy Sci. 69:1793-1798.

Marziali, A. S. and K. F. Ng-Kwai-Hang (1986c). Effects of milk composition and genetic polymorphism on cheese composition. J. Dairy Sci. 69:2533-2542.

McCaman, M.T., W.H.Andrews and J.G. Files (1985). Enzymatic properties and processing of bovine prochymosin synthesized from *Escherichia coli*. J. Biotechnology 2: 177-190.

McGann, T. C. A., R. D. Kearney and W. J. Donnelly (1979). Developments in column chromatography for the separation and characterization of casein micelles. J. Dairy Res. 46:307-311.

McKenzie, H. A. (1971). β-Lactoglobulin. In: Milk Proteins. Ed: H. A. McKenzie. Academic Press, New York. Pp257-330.

McKenzie, H. A., G.B. Ralston and D. C. Shaw (1972). Location of sulfhydryl and disulfide groups in bovine β-lactoglobulin and effect of urea. Biochemistry 11:4539-4547.

McKnight, R. A., R.Jimenes-Flores, Y.Kang, L.K.Creamer and T. Richardson (1989). Cloning and sequencing of a complementary deoxyribonucleic acid coding for a bovine α_{s1}-casein A from mammary tissue of a homozygous B variant cow. J. Dairy Sci.72: 2464-2473.

McLean, D. M., E.R.B.Graham, R.W. Ponzoni and H. A. McKenzie (1984). Effects of milk protein genetic variants on milk yield and composition. J. Dairy Res. 51:531-546.

McLean, D. M., E.R.B Graham, R.W. Ponzoni and H. A. McKenzie (1987). Effects of milk protein genetic variants and composition on heat stability of milk. J. Dairy Res. 54:219-235.

McLean, D. M. and J. Schaar (1989). Effects of β-lactoglobulin and κ-casein genetic variants and concentrations on syneresis of gels from renneted heated milk. J. Dairy Res.56: 297-301.

Medrano, J. F. and L. Sharrow (1989). Milk protein typing of bovine mammary gland tissue used to generate a complementary deoxyribonucleic acid library. J. Dairy Sci.72: 3190-3196.

Meisel, H. (1987). Characterization of rennet preparations produced by genetic engineering in comparison with calf rennet. 1. Materials and Methods. Milchwissenschaft 42:787-789.

Meisel, H. (1988). Characterization of rennet preparations produced by genetic engineering in comparison with calf rennet. 1. Results. Milchwissenschaft 43:71-75.

Mellor, J., M.J.Dobson, N.A. Roberts, M.F.Tuite, J.S.Emtage, S.White, P.A. Lowe, T. Patel, A.J.Kingsman and S.M. Kingsman (1983). Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. Gene 24:1-14.

Mercier, J.C., G.Brignon and B. Ribadeau-Dumas (1973). Structure primaire de la caséine κ B bovine. Séquence compléte Eur. J. Biochem. 35:222-235.

Mercier,J.C., F.Grosclaude and B. Ribadeau-Dumas (1971). Structure primaire de la caséine α_{s1}- bovine. Eur. J. Biochem. 35:41-51.

Michalak, W. (1967). Anomalous electrophoresis pattern of milk proteins. J. Dairy Sci. 50:1319-1320.

Mikkelsen, J., P.HΦjrup and J.Knudsen (1987). Purification of goats' milk casein by reversed-phase high-performance liquid chromatography and identification of α_{s1}-casein. J. Dairy Res. 54:361-367.

Mohanty, A.K., U.K.Mukhopadhyay, S.Grover and V.K.Batish (1999). Bovine chymosin: production by rDNA technology and application in cheese manufacture. Biotech. Adv. 17:205-217.

Moir, D., J.Mao, J.W.Schuum, G.F.Vovis, B.L.Alford and A. Taunton-Rigby (1982). Molecular cloning and characterization of double stranded cDNA coding for bovine chymosin. Gene 19:127-138.

Morini, D., G.Losi, G.B.Castagnetti and P. Mariani (1979). Cheesemaking experiments with milk characterized by κ-casein variants A and B: characteristics of ripened cheese. Scien. Tecn. Latt-Casear. 30:243-262.

Munro, G. L. (1978). Effects of genetic variants of milk proteins on yield and composition of milk. Proc. XX Internat. Dairy Congr. Paris. p10.

Neelin, J. M. (1964). Variants of κ-casein revealed by improved starch gel electrophoresis. J. Dairy Sci. 47:506-509.

Nejjar, Y., D.Paquet, G.Godbillon and J.Y. Le Deaut (1986). Immunological relationship between the hydrophobic fraction of proteose-peptone and the milk fat globule membrane of bovine milk. Inter. J. Biochem. 18:893-900.

Ng-Kwai-Hang, K. F. (1990). Protein composition of milk and cheese making. Modern Dairy February. Ppl4-15.

Ng-Kwai-Hang, K. F. (1993). Genetic variants of milk proteins and cheese yield. IDF Seminar, April 93, Cork, Ireland, ppl60-166.

Ng-Kwai-Hang, K. F. (1994). Genetic variants of milk proteins and cheese yield. Cheese yield and factors affecting its control. IDF, Brussels, Belgium 160-166.

Ng-Kwai-Hang, K. F. and C. Dong (1994). Semi preparative isolation of bovine casein components by high performance liquid chromatography. Internat. Dairy J. 4:99-110.

Ng-Kwai-Hang, K. F. and F. Grosclaude (2002). Genetic polymorphism of milk proteins. In: Advanced Dairy Chemistry 1. Ed: P. F. Fox and P.L.H. McSweeney. Kluwer Academic/Plenum Publishers New York, NY.

Ng-Kwai-Hang, K. F. and S. Kim (1996). Different amounts of β-lactoglobulin A and B in milk from heterozygous AB cows. Internat. Dairy J. 6:689-695.

Ng-Kwai-Hang, K. F., J.F.Hayes, J.E.Moxley and H. G. Monardes (1984). Association of genetic variants of casein and milk serum proteins with milk, fat, and protein production by dairy cattle. J. Dairy Sci. 67:835-840.

Ng-Kwai-Hang, K. F., J.F.Hayes, J.E.Moxley and H. G. Monardes (1986). Relationship between milk protein polymorphism and major milk constituents in Holstein-Friesian cows. J. Dairy Sci. 69:22-26.

Ng-Kwai-Hang, K. F., J.F.Hayes, J.E.Moxley and H. G. Monardes (1987). Variation in milk protein concentration associated with genetic polymorphism and environmental factors. J. Dairy Sci. 70:563-570.

Ng-Kwai-Hang, K. F., H.G.Monardes and J. F. Hayes (1990). Association between genetic polymorphism of milk proteins and production traits during three lactations. J.Dairy Sci. 73:3414-3420.

Ng-Kwai-Hang, K. F. and J.P. Pelissier (1989). Rapid separation of bovine caseins by mass ion exchange chromatography. J. Dairy Res. 56:391-397.

Nishimori, K., Y.Kawaguchi, M.Hidaka, T.Uozumi and T. Beppu (1981). Cloning in *Escherichia coli* of the structural gene of prorennin, the precursor of calf milk-clotting enzyme rennin [Protease essential for cheese making]. J. Biochem. 90:901-904.

Oh, S. and Richardson, T. (1991). Genetic engineering of bovine κ-casein to enhance proteolysis by chymosin. Acs Symposium Series – Amer. Chem. Soc. 454:195-211.

Ojala, M., T.R.Famula and J.F. Medrano (1997). Effect of milk protein genotypes on the variation for milk production traits of Holstein and Jersey cows in California. J. Dairy Sci. 80:1776-1785.

Pagnacco, G. and A. Caroli (1987). Effect of casein and lactoglobulin genotypes on renneting properties of milks. J. Dairy Res.54: 479-485.

Paquet, D. (1989). Review: The proteose peptone fraction of milk. Lait 69:1-21.

Paquet, D., Y.Nejjar and G. Linden (1988). Study of hydrophobic protein fraction isolated from milk proteose-peptone. J. Dairy Sci. 71:1464-1471.

Parris, N., E.A.White and H. M. Farrell (1990). Identification of altered proteins in nonfat dry milk powder prepared from heat-treated skim milk. J. Agri. Food Chem. 38:824.

Paterson, G. R., A.K.H. MacGibbon and J.P. Hill (1999). Influence of κ-casein and β-lactoglobulin phenotype on the heat stability of milk. Internat. Dairy J. 9:375-376.

Payens, T.A.J. and P. Both (1980). Enzymatic clotting processes. IV. The chymosin-triggered clotting of para-κ-casein complex. In Biochemistry: Ions, Surfaces And Membranes. Ed. M.Blank.ACS Adv. Chem. Ser.188: 129-141.

Payens, T.A.J. and S. Visser (1981). What determines the specificity of chymosin towards κ-casein? Neth. Milk and Dairy J. 35:387-389.

Pearce, R. J. (1976). Moving boundary electrophoresis of native and rennet-treated caseins micelles. J.Dairy Res. 43:27-36.

Pearce, R. J. (1979). Use of fluorescamine to determine the rate of release of the caseino-macropeptide in rennet treated milk. N.Z.J.Dairy Sci. Techno. 14:233-237.

Pearce, R. J. (1983). Analysis of whey proteins by high performance liquid chromatography. Aust. J. Dairy Techno. 38:114-117.

Peterson, R. F. (1969). Electrofocusing of milk proteins. J. Dairy Sci.52:903.

Peterson, R. F. and F. C. Kopfler (1966). Detection of new types of β-casein by polyacrylamide gel electrophoresis at acidic pH: a proposed nomenclature. Biochem. Biophys. Res. Comm. 22:388-392.

Plowman, J.E. and L.K. Creamer (1995). Restrained molecular dynamics study of the interaction between bovine κ-casein peptide 98-111 and bovine chymosin and porcine pepsin. J. Dairy Res. 62: 451-467.

Plowman, J.E., L.K.Creamer, M.H.Smith and J.P.Hill (1997). Restrained molecular dynamics investigation of the differences in the association of chymosin to κ-casein A and C. J. Dairy Res. 64:299-304.

Plowman, J.E., L.K.Creamer, M.J.Liddell and J.J.Cross (1999). Structural features of a peptide corresponding to human κ-casein residues 84-101 by (1) H-nuclear magnetic resonance spectroscopy. J. Dairy Res.66: 53-63.

Politis, I. and K.F. Ng-Kwai-Hang (1988). Effect of somatic cell count and milk composition on cheese composition and cheese making efficiency. J.Dairy Sci.71: 1711-1719.

Prinzenberg, E.M., P.Anglade, B.Ribadeau-Dumas and G. Erhardt (1998). Biochemical characterization of bovine α_{s1}-casein F and genotyping with sequence specific primers. J.Dairy Res. 65: 223-231.

Prinzenberg, E.M., S.Heindleder, T.Ikonen and G. Erhardt (1996). Molecular genetic characterization of new bovine κ-casein alleles CSN3F and CSN3G and genotyping by PCR-RFLP. Anim. Genet. 27:347-349.

Prinzenberg, E.M., I. Krause and G. Erhardt (1999). SSCP analysis of the bovine CSN3 locus discriminates six alleles corresponding to known protein variants (A, B, C, E, F, G) and three new DNA polymorphisms (H, I, A1). Anima. Biotech. 10:49-62.

Pham, Z. and E. Jacob (1993). Genetic variants of milk proteins and cheese yield. In: Proceedings of the IDF Seminar on Cheese Yield and Factors Affecting its Control. IDF Seminar, April 93, Cork, Ireland. Pp111-122

Pupkova, G.V. (1980). Milk protein polymorphism and milk production of Estonian Black Pied cows. Dairy Sci. Abstr. 45:6620

Raap, J., K.E.T.Kerling, H.J.Vreeman and S.Visser (1983). Peptide substrates for chymosin (rennin): conformational studies of κ-casein and some κ-casein related oligopeptides by circular dichroism and secondary structure prediction. Arch. Biochem. Biophys. 221:117-124.

Rampilli, M., A.Caroli, P.Bolla and G. Pirlo (1988). Relationships of milk protein genotypes with casein composition and rennetability of milk during lactation. Scien. Tecn. Latt-Casear. 39:262-279.

Raymond, M.N., E.Bricas and J.C. Mercier (1973). Synthesis of new oligopeptide substrates of chymosin (rennin) and kinetic parameters of their hydrolysis. Neth. Milk and Dairy J. 27:298-303.

Raymond, S. and Y.J. Wang (1960). Preparation and properties of acrylamide gel for use in electrophoresis. Anal. Biochem. 1:391-396.

Ribadeau-Dumas, B., G.Brignon, F.Grosclaude and J.C. Mercier (1972). Structure primarie de la caseine β bovine. Séquence complete. Eur. J. Biochem. 25:505-514.

Robitaille, G. (1995). Influence of κ-casein and β-lactoglobulin genetic variants on the heat stability of milk. J. Dairy Res. 62:593-600.

Robitaille, G. and D. Peticlerc (2000). Expression polymorphism of κ-casein gene in Holstein cows. J. Dairy Res. 67:107-111.

Rollema, H.S., J.A.Brinkhuis and H.J.Vreeman (1988). H-NMR studies of bovine κ-casein and casein micelles. Neth.Milk and Dairy J. 42:233-248.

Russo, V. and O.Mariani (1974). Polymorfismo delle proteine del latte nelle razze bovine da carne. III. Chianina. Riv. Zootec. Vet. 2:139-143

Schaar, J. (1984). Effects of κ-casein genetic variants on renneting properties of individual milks. J. Dairy Res. 51:397-406.

Scharr, J., B.Hansson and H. E. Peterson (1985). Effect of genetic variants of κ-casein and β-lactoglobulin on cheese making. J. Dairy Res. 52:429-437.

Schattenkerk, C. and K.E.T.Keerling (1973). Relation between structure and capacity to function as a rennin substrate. Neth. Milk and Dairy J. 27:286-287.

Schmidt, D. G. (1964). Starch gel electrophoresis of κ-casein. Biochim. Biophys. Acta 90:411-414.

Schmidt, D. G. (1980). Colloidal aspects of casein. Neth. Milk and Dairy J., 34:42-64.

Schmidt, D. G., P.Both and P.J. de Koning (1966). Fractionation and some properties of κ-casein variants. J. Dairy Sci. 49: 776-782.

Seibert, B., G.Erhardt and B. Senft (1987). Detection of a new κ-casein variant in cows milk. Anim. Genet. 18:269-272.

Shammet, K.M., R.J.Brown and D.J. McMahon (1992). Proteolytic activity of some milk clotting enzymes on κ-casein. J. Dairy Sci. 75:1373-1379.

Sherbon, J. W., R. A. Ledford and J. Regenstein (1967). Variants of milk proteins and their possible relation to milk proteins. J. Dairy Sci. 50:951.

Shimazaki, K.I. and K.Sukegawa (1982). Chromatographic profiles of bovine milk whey components by gel filtration on Fractogel TSK HW55F column. J. Dairy Sci. 65:2055-2062.

Sinkinson, G. and J.V. Wheelock (1970). Carbohydrates of the glycopeptides released by the action of rennin on whole milk. Biochim.Biophys. Acta 215:517-521.

Slattery, C.W. and R. Evard (1973). A model for the formation and structure of casein micelles from subunits of variable composition. Biochim. Biophys. Acta 317:529-538.

Sleigh, R.W., T.B.Sculley and A.G. MacKinlay (1979). The binding of β-casein to hydroxyapatite: the effect of phosphate content and location. J. Dairy Res. 46:337-342.

Snoeren, T. H. M., C.A.Van Der Spek and T. A. J. Payens (1977). Preparation of κ- and α_{s1}-casein by electrostatic affinity chromatography. Biochim. Biophys. Acta 490:255-259.

Sorensen, E. S. and T. E. Peterson (1993). Purification and characterization of three proteins isolated from the proteose peptone fraction of bovine milk. J. Dairy Res. 60:189-197.

Stewart A, F., J.Bonsing, C.W.Beattie, F.Shah, I.M.Willis and A.G. Mackinlay (1987). Complete nucleotide sequences of bovine alpha S2- and beta-casein cDNAs: comparisons with related sequences in other species. Mol. Bio. Evol. 4:231-41.

Stewart A. F., I.M.Willis and A.G. Mackinlay (1984). Nucleotide sequences of bovine α_{S1}- and κ-casein cDNAs. Nucleic Acids Res. 12:3895-3907.

St-Martin, M. and P.Paquin (1990). Ion-exchange fast protein liquid chromatography: Optimization of the purification of caseins using a non-denaturing detergent. J. Dairy Res. 57:63-68.

Strange, E. D., D.van Hekken and M. P. Thompson (1991). Qualitative and quantitative determination of caseins with reverse-phase and anion-exchange HPLC. J. Food Sci. 56:14151420.

Sulimova, G.E., Y.N.Badagueva and I.G. Udina (1996). Polymorphism of the κ-casein gene in subfamilies of the Bovidae. *Genetika* (*Moskva*) 32:1576-1582.

Swaisgood, H. (1982). Chemistry of milk proteins. In: Developments in Dairy Chemistry Vol. I. Proteins. Ed: P. F. Fox. Appl. Sci. Publishers, London. Pp l-59.

Swaisgood, H. (1993). Review and update of casein chemistry. J. Dairy Sci. 76:3054-3061.

Talbot, B. and D.F. Waugh (1970). Micelle-forming characteristics of monomeric and covalent polymeric κ-caseins. Biochemistry 9:2807-2813.

Teuber, M. (1991). Production of chymosin by microorganisms and its use for cheese making. IDF Bulletin No. 251. Pp 3-15.

Thompson, M.P. (1966). DEAE-cellulose-urea-chromatography of casein in the presence of β-mercaptoethanol. J. Dairy Sci. 49:792-795.

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

Thompson, M. P., C.A.Kiddy, L.Pepper and C. A.Zittle (1962). Variations in the κ-casein fraction of individual cow's milk. Nature 195:1001-1002.

Thompson, M. P. and L. Pepper (1964). Genetic polymorphism in caseins of cow's milk. IV. Isolation and properties of β-casein A, B and C. J. Dairy Sci. 47:633-636.

Thunell, R.K., J.W.Duersch and C.A. Ernstrom (1979). Thermal inactivation of residual milk clotting enzymes in [Cheddar cheese] whey. J. Dairy Sci. 62:373-377.

Tong, P.S., S.Vink, N.Y.Faryke and J.F. Medrano (1993). Effect of genetic variants of milk proteins on the yield of Cheddar cheese. IDF Seminar, Apl.'93, Cork, Ireland. Pp179-187.

Trieu-Cuot, P. and J.C. Gripon (1981). Electrofocusing and two-dimensional electrophoresis of bovine caseins. J. Dairy Res. 48:303-310.

Turhan, M. and M. Mutlu (1997). Kinetics of κ-casein/chymosin hydrolysis. Milchwissenschaft 52:559-563.

Tutta, C., A.Curioni and A.D.B. Peruffo (1991). A two-dimensional electrophoretic method for the study of caprine α_{s1}- and α_{s2}- casein polymorphism. J. Dairy Res.58: 247-250.

Udenfriend, S., S.Stein, P.Bohlen, W.Dairman, W.Leimgruber and M. Weigele (1972). Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomolar range. Science 178:871-872.

Vallejo-Corboda, B. (1997). Rapid separation and quantification of major caseins and whey proteins of bovine milk by capillary electrophoresis. J. Cap. Electro. 4:219-224.

van den Berg, G. (1993). Genetic polymorphism of κ-casein and β-lactoglobulin in relation to milk composition and cheesemaking properties. IDF Seminar, April 93, Cork, Ireland, ppl23-133.

van Eenennaam, A. L. and J. F. Medrano (1991). Differences in allelic protein expression in the milk of heterozygous κ-casein cows. J. Dairy Sci. 74:1491-1496.

van Halbeek, H., L. Dorland, J. F. G. Vliegenthart, A. M. Fiat and P. Jolles (1980). A 360-Mhz 1H-NMR study of three oligosaccharides isolated from cow κ-casein. Biochim. Biophys. Acta 623:295-300.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. Neth. Milk and Dairy J. 35:65-88.

Visser, S., C.J.Slangen and H. S. Rollema (1986). High performance liquid chromatography of bovine caseins with the application of various stationary phases. Milchwissenschaft 41:559-562.

Visser, S., C.J.Slangen and H. S. Rollema (1991). Phenotyping of bovine milk proteins by reversed phase high performance liquid chromatography. J. Chromatography 548:361-370.

Visser, S., C.J.Slangen, F.M.Langerwerf, W.D.van Dongen and J. Haverkamp (1995). Identification of a new genetic variant of bovine β-casein using reverse-phase high

performance liquid chromatography and mass spectrometric analysis. J. Chromatography 711:141-150.

Visser, S., P.J.Rooije, C.van Schattenkerk, and K.E.T. Kerling (1977). Peptide substrates for chymosin (rennin): Kinetic studies with bovine κ-casein (103-108) hexapeptide analogues. Biochim. Biophys. Acta 481:171-176.

Visser, S., P.J.Rooije and C.J. Slangen (1980). Peptide substrates for chymosin (rennin): isolation and substrate behaviour of two tryptic fragments of bovine κ-casein. Eur. J. Biochem. 108:415-421.

Voynick, I.M. and J.S. Futon (1971). The comparative specificity of acid proteinases. Proc. Nat. Acad. Sci. U.S.A. 68:257-259.

Vreeman, H.J., P.Both, J.A.Brinkhuis and C. Van der Speck (1977). Purification and some physicochemical properties of bovine κ-casein. Biochim. Biophys. Acta 491:93-103.

Vreeman, H. J., S.Visser, C.J.Slangen and J. A. M. van Riel (1986). Characterization of bovine κ-casein fractions and the kinetics of chymosin-induced macro peptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance liquid gel-permeation chromatography. Biochem. J. 240:87-97.

Walsh, C.D., T.Guinee, D.Harrington, R.Mehra, J.Murphy, J.F.Connolly and R.J. Fitzgerald (1995). Cheddar cheese making and rennet coagulation characteristics of bovine milks containing κ-casein AA or BB genetic variants. Milchwissenschaft 50:492-496.

Walstra, P. (1979). The voluminosity of bovine casein micelles and some of its implications. J. Dairy Res. 46:317-323.

Walstra, P., and R. Jenness (1984). Dairy chemistry and physics. John Wiley and Sons Ltd. pp98-122.

Ward, M., L.J.Wilson, K.H. Kodama, M.W.Rey and R.M. Berka (1990). Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Biotechnology 8:435-440.

Waugh, D.F. and P.H. von Hippel (1956). κ-Casein and the stabilization of casein micelles. J. Amer. Chem. Soc. 78:4576-4582.

Wheelock, J.V. and D.J. Knight (1969). The action of rennin on whole milk. J. Dairy Res. 36:183-189.

Whitaker, J.R. (1970). Protease of *Endothia parasitica*. In Methods in Enzymology. Vol.19. Ed., Perlmann, G.E., and L, Lorand. Pp436-445.

Whitaker, J.R. (1994). Principles of Enzymology for Food Sciences, 2nd Ed. Marcel Dekker Inc., New York. Pp 469-516

Whitney, R. M. (1988). Proteins of milk. In: Fundamentals of Dairy Chemistry. Ed: N. P. Wong, R. Jenness, M. Keeney and E. H. Marth. 3rd ed. Van Nostrand Reinhold Co., New York. Pp8l-170.

Whitney, R.M., J.R.Brunner, K.E.Ebner, H.M.Farell Jr., R.V.Josephson, C.V.Morr and H.E. Swaisgood (1976). Nomenclature of the proteins of cow's milk: fourth revision. J. Dairy Sci. 59:795-815.

Wong, P.N. (1988). Fundamentals of Dairy Chemistry. III Edn. Pp 81-169.Van Nostrand Reinhold Company, New York.

Woychick, J. H. (1964). Polymorphism of κ-casein of cow's milk. Biochem. Biophys. Res. Comm. 16:267-271.

Yaguchi, M. and D. Rose (1971). Chromatographic separation of milk proteins: A review. J. Dairy Sci. 54:1725-1743.

Yaguchi, M. and N.P. Tarassuk (1967). Gel filtration of acid casein and skim milk on sephadex. J. Dairy Sci. 50:1985-1988.

Yoshida, S. and Ye-Xiuyun (1991). Isolation of lactoperoxidase and lactoferrin from bovine milk rennet whey and acid whey by sulphopropyl cation-exchange chromatography. Neth. Milk Dairy J.45: 273-280.