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CHARACTERISATION OF GENETIC VARIANTS OF MILK PROTEINS THAT ARE NOT IDENTIFIABLE BY ELECTROPHORESIS

by

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Abstract

Doctor of Philosophy Chin Dong Animal Science

CHARACTERISATION OF GENETIC VARIANTS OF MILK PROTEINS THAT ARE NOT IDENTIFIABLE BY ELECTROPHORESIS

Genetic variants of milk proteins result from amino acid substitutions or small fragment deletion in the polypeptide chain. It is well documented that certain variants are closely related to milk production, milk composition and physico-chemical properties of milk such as heat stability and coagulation properties during cheesemaking. So far, all the genetic variants have been characterized by various electrophoretic methods. Therefore, only variants involving differences in net charges could be identified. Silent variants are the results of amino acid substitutions or deletions which do not accompany charge differences and hence remain undetected by conventional electrophoretic methods. The objective of the present project is to develop proper methodology to identify and characterize silent variants of milk proteins based on hydrophobic properties of amino acids. Individual caseins were isolated from 635 milk samples by anion-exchange chromatography and their electrophoretic phenotype was determined by polyacrylamide gel electrophoresis under alkaline and acidic conditions. Trypsin hydrolysis of α_{s1}-casein, β-casein and κ-casein followed by reversed-phase HPLC was performed to identify possible mutations causing changes in hydrophobicities of amino acids. Among 627 α_{s1}-casein BB, 415 κ-casein AA, 158 β-casein A^1A^1 and 128 β-casein A^2A^2 according to electrophoresis, it was possible to find 25, 11, 16 and 7 samples respectively as potential silent variants. Further analysis of the aberrant peptides from α_{s1}-casein BB, κ-casein AA and β-casein A^2A^2 by mass spectrometry did not confirm the existence of silent variants; whereas analysis of aberrant peptide from β-casein A^1A^1 revealed a mutation resulting in an increase of 16 Da. Analysis of amino acid composition of this aberrant β-casein A^1A^1 peptide 114-169

showed a Leu replacing a Pro residue. Results from amino acid sequencing confirmed this mutation to be located at position 137 of β-casein A^1. A frequency of 4.4% within β-casein A^1 was observed for this novel silent variant.

Résumé

Docteur en Philosphie Chin Dong Sciences Animales

LA CARACTERISATION DES VARIANTES GENETIQUES DES PROTEINS DU LAIT NON IDENTIFIABLE PAR L'ELECTROPHORESE

Les variantes génétiques des protéines du lait viennent de substitution du groupement amino-acide ou de la perte de petit fragment sur la chaîne polypeptitique. Il est bien connu que certaines variantes sont reliées à la chaleur , à la composition ou aux propriétés physico-chimiques du lait comme la stabilité à la chaleur et les propriétés de coagulation pendant la frabrication du fromage. Jusqu'à maintenant, toutes les protéines variantes génétiques sont caractérisées par des méthodes électrophorétiques. Cependant, seul les protéines avec des différences de charges nettes peuvent être identifiées. Les variations silencieuses sont le résultat de substitutions des groupements amino-acide ou de perte de fragment qui donne lieu à une protéine n'ayant pas de différence de charge et qui ne sont donc pas détectable par électrophorèse. Le but de ce projet est de développer une méthodologie convenable pour identifier et caractériser ces protéines portant des modifications silencieuses. La méthodologie sera basée sur les propriétés hydrophobiques des amino-acides. Des caséines individuels sont isolés de 635 échantillons de lait par chromatographie d'échange d'anion. Leur phénotype électrophorétique est déterminé par électrophorèse sur gel polyacrylamide sous conditions alkaline et acide. L'hydrolyse de α_{s1}-casein, β-casein et κ-casein par la trypsine est suivie par CLHP de phase renversé pour identifier les mutations pouvant changer les propriétés hydrophobiques des amino-acides. Parmi les 627 α_{s1}-casein BB, 415 κ-casein AA, 158 β-casein A^1A^1 et 128 β-casein A^2A^2 , il est possible de trouver 25, 11, 16 et 7 échantillons respectivement comme des variantes silencieuses. L'analyse de peptides aberrant de α_{s1}-casein BB, κ-casein AA et β-casein A^2A^2 par le spectroscopie de masse n'a pas confirmé l'existence des variantes silencieuses. Cependant

l'analyse du peptide aberrant venant de β-casein A^1A^1 a montré une mutation qui cause une augmentation de en poid moléculaire de 16 Da. L'analyse de la composition de l'amino-acides de cet aberrant β-casein A^1A^1 peptide 114-169 a montré une substitution d'un groupement Pro par un groupement Leu. Les résultats de l'analyse séquencielle des amino-acide ont confirmés que la mutation doit être en position 137 de β-casein A^1. Une frequence de 4.4% pour ce nouveau variante est observé dans β-casein A^1.

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1. Introduction

Bovine milk is an important food source and has been investigated for decades. Studies on milk protein polymorphism and their relationship with production traits have been well documented since the first discovery of genetic variants in β-lactoglobulin (Aschaffenburg and Drewry, 1955). Most of the studies indicate that genetic variants of milk proteins are associated with milk production, milk composition and milk quality (Kroeker *et al.*, 1985a, 1985b; Lin *et al.*, 1986; van Eenennaam and Medrano, 1991; Ng-Kwai-Hang and Grosclaude, 1992). It is well accepted that α_{s1}-casein B, β-casein A^2, β-casein A^3 and κ-casein A variants are associated with higher milk yield when compared with other variants (Lin *et al.*, 1986; Ng-Kwai-Hang *et al.*, 1984, 1986, 1990; Aleandri *et al.*, 1990; De-Lange *et al.*, 1991). It is generally agreed that κ-casein B and β-lactoglobulin B milk contains more casein and fat (Ng-Kwai-Hang *et al.*, 1986, 1990; McLean, 1987; De-Lange *et al.*, 1991; van den Berg *et al.*, 1992; Tong *et al.*, 1993; Kim *et al.*, 1996). Milk with κ-casein BB and β-lactoglobulin BB give higher cheese yield and provides better coagulation properties (Marziali and Ng-Kwai-Hang, 1986a; Pagnacco and Caroli, 1987; Aleandri *et al.*, 1990; Ng-Kwai-Hang, 1990; 1993; Tong *et al.*, 1993).

Identification of milk protein variants provides an important tool for improving the yield and quality of milk and dairy products. Theoretically, genetic polymorphism of milk

proteins is due to the results of amino acid substitutions or small peptide deletions along the polypeptide chains. The variation caused by post-translational modification is not considered as genetic variant. All the genetic variants so far described have been identified by different electrophoretic techniques. Therefore, they could be referred as "electrophoretic variants". Although electrophoresis is a fast and simple procedure for determination of genetic variants of milk proteins, it has limitation because it could not be applied to detect mutations which do not result in changes of net charge of the protein. Non-electrophoretic variants are sometimes referred to as "Silent variant" and they result from amino acid substitutions or deletions which do not generate a difference in net charges and hence cannot be identified by electrophoresis.

It is of interests to point out that only five out of the twenty amino acid residues carry either positive (Arg, His, Lys) or negative (Glu, Asp) charges, whereas, the remaining fifteen have uncharged side chain. Based on the random occurrence of mutation, one would expect three times as many non-electrophoretic variants as there are electrophoretic ones. Like the electrophoretic genetic variants, the silent variants are most likely associated with milk yield, milk composition and other functionality properties of milk. The limitation of electrophoresis for the identification of all the possible genetic variants has prompted interests for searching of procedures to identify the non-electrophoretic variants.

Therefore, it is necessary to develop appropriate methodology for detecting silent variants of milk proteins.

The objectives of the present study are:

1. To develop a suitable method for fractionation of pure forms of α_{s1}-casein, β-casein and κ-casein in enough quantities for identification of silent genetic variants.
2. To develop appropriate reversed-phase HPLC methods for the resolution of tryptic hydrolysates derived from caseins.
3. To develop sensitive methods capable of detecting changes in hydrophobicities of casein peptides due to mutations causing substitutions of amino acids.
4. To identify the existence of silent variants.
5. To obtain the frequency distribution of silent variants.
6. To characterize the identities and positions of amino acids involved in the silent variant.

2. Review of Literature

2.1. The nature of milk

Milk is a complex biological fluid and is widely used for the nutrition of the young mammals. The milk from certain species could be used as a raw material for the making of a wide variety of food products. On the average, bovine milk consists of water (87.3%), fat (3.9%), proteins (3.3%), carbohydrates (4.6%) and many other components such as minerals (0.65%), vitamins and enzymes (Walstra and Jenness, 1984). Although milk fat consists of numerous types of lipids, 98% of this fraction is made up of triglycerides. Lactose is the distinctive sugar of milk and contributes to a slight sweet taste. It is a disaccharide composed of glucose and galactose which are linked through a β-1,4 bond. The minerals in milk are mainly inorganic salts, and together with other fat-soluble and water-soluble components, are mainly derived from blood plasma.

2.2. Milk proteins

The total amount and relative proportions of milk protein components directly influence the nutritional values and manufacturing properties of the milk. There are two main groups of milk proteins which are classified as caseins and whey or serum proteins. Bovine caseins comprise approximately 80% of the total milk proteins which are phosphoproteins and precipitate out upon acidification to pH 4.6 at 20°C. There are four genes responsible for the expression of caseins: α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. The relative proportion of these four caseins are 40,

10, 35 and 12% respectively. Whey proteins, consisting of α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin, occur in the ratio of 20:60:7:13 (Davies and Law, 1980; Swaisgood, 1982). Most of the major milk proteins are known to exhibit genetic polymorphism. Milk also contains numerous "minor proteins" such as proteose-peptone fraction and enzymes including lipase, phosphatase, oxidase and plasmin.

In their native state, caseins are associated with calcium, citrate, phosphate and other inorganic ions and exist as colloidal particles ranging from 20 to 300 μm in diameter (Schmidt, 1982). Those intricate particles, known as casein micelles, are comprised of 20 to 150,000 casein molecules and are responsible for the high turbidity of skimmed milk. The casein micelle is similar to globular proteins by having a hydrophobic core to bury the most hydrophobic parts of the casein molecules. Colloidal calcium phosphate has been proposed to act as a "cementing agent" which maintains the integrity of the micellar structure (Richardson and Creamer, 1974; Walstra and Jenness, 1984).

2.3. Genetic polymorphism of milk proteins

The occurrence of genetic polymorphism of the milk proteins was first demonstrated in β-lactoglobulin by Ashaffenburg and Drewry (1955). Since then, hundreds of papers have been published on the existence of genetic variants of major milk proteins. All the genetic variants of milk proteins described so far have been revealed by different electrophoretic methods. Therefore, only

mutations giving rise to differences in electrical charges due to amino acid substitutions or deletions would be detectable. Mutations which do not result in differences of net charges will remain undetectable and the term "silent variants" will be used throughout this text to differentiate these variants from the known electrophoretic variants.

2.3.1. Caseins

Caseins which account for 76 to 86% of total bovine milk proteins are a group of phosphate-containing milk-specific proteins (Ng-Kwai-Hang and Kroeker, 1984). Four kinds of polypeptide chains, designated as α_{s1}-, α_{s2}-, β- and κ-casein together with their proteolytic products, are included in the whole casein fraction. Non-genetic variants also occur in some of the milk proteins because of post-translational modifications which include phosphorylation and glycosylation.

2.3.1.1. α_{s1}-Casein

The primary structure of α_{s1}-casein consists of 199 amino acid residues with a calculated molecular weight of 23,600 (Eigel *et al.*, 1984). It contains a calcium-sensitive region between position 41 and 80 in the amino acid sequence, a cluster of phosphoseryl residues and three hydrophobic regions between amino acid sequence 1 to 44, 90 to 113 and 132 to 199 (Walstra and Jenness, 1984).

Genetic polymorphism of α_{s1}-casein was first reported by Thompson *et al.*, (1962). Since then, five genetic variants have been characterized according to their relative electrophoretic mobilities and are denoted as A, D, B, C and E (Eigel *et al.*, 1984). Compared to the most frequent variant of α_{s1}-casein B, variant A contains a 13 amino acid residue deletion from position 14 to 26. It could be the result of mRNA abnormal splicing to one exon of the gene (McKnight, *et al.*, 1989). The C variant results from a single amino acid substitution at position 192 where Glu is replaced by Gly. Owing to another substitution of Ala for Thr at position 53, α_{s1}-casein D variant could be differentiated from B variant. The E variant consists of two amino acid substitutions: Gln to Lys at position 59 and Glu to Gly at position 192. α_{s1}-Casein is highly sensitive to Ca^{2+} which can form an ester with its phosphate groups. Therefore, it aggregates and precipitates at very low concentration of Ca^{2+} (7mM Ca^{2+}, 28mM NaCl). In milk, there are small amounts of peptides known as λ-caseins which have been considered as originating from proteolysis of α_{s1}-casein.

2.3.1.2. α_{s2}-Casein

α_{s2}-Casein, the most hydrophilic of the caseins contains 207 amino acid residues with a calculated molecular weight of 25,230 (Brignon *et al.*, 1977). Since it contains 11 phosphoseryl residues, it is highly sensitive to precipitation by Ca^{2+}. Furthermore, α_{s2}-casein has a remarkable dipolar structure with a

concentration of negative charges near the N-terminus and positive charges near the C-terminus. Bovine α_{s2}-casein contains intermolecular disulphide bonds (Annan and Manson, 1969).

Genetic polymorphism of α_{s2}-casein was first discovered in the milk of Yaks and Zebus (Grosclaude *et al.*, 1976a). Up to now, four genetic variants of α_{s2}-casein have been detected and are designated as A, B, C, and D. Like α_{s1}-casein, one of the genetic variants of this protein, α_{s2}-casein D, results from a deletion in the sequence involved in residues 50-58, 51-59 or 51-60 which remove one cluster of phosphoseryl residues in the structural sequence. The amino acid replacements of Glu, Ala and Thr by Gly, Thr and Ile at the positions 33, 47 and 130, gives rise to variant C when compared with variant A of α_{s2}-casein (Grosclaude *et al.*, 1979; Swaisgood, 1982; Eigel *et al.*, 1984).

2.3.1.3. β-Casein

β-Casein is the most hydrophobic of all major caseins. The β-casein A^2-5P consists of a single polypeptide chain with 209 amino acid residues and a calculated molecular weight of 23,983 (Eigel *et al.*, 1984). Ribadeau-Dumas *et al.* (1972) was the first to report the primary structure of β-casein. Seven genetic variants for β-casein have been revealed so far, all involving various amino acid substitutions at the different positions. When compared with the primary structure of β-casein A^1 variant, A^2 is the result of a single amino acid mutation of His to Pro at position 67. Another

amino acid replacement of His to Gln of variant A^2 at position 106 gives β-casein A^3. Mutation of Ser to Arg at position 122 gives the B variant. As rarely reported variants, D and E are the result of amino acid substitutions of Ser to Lys at position 18 and Glu to Lys at position 36 respectively. The major genetic variants of caseins were separated by alkaline urea gel electrophoresis. Electrophoresis under acidic conditions was also required to differentiate A^1, A^2 and A^3 variants for β-casein (Peterson and Kopfler, 1966).

The family of γ-caseins found even in fresh raw milk have been identified as proteolytic product of C-terminal portions of β-casein sequence. γ_1, γ_2, and γ_3-Caseins contain the peptide sequences of 29 to 209, 106 to 209 and 108 to 209 respectively (Tripathi and Gehrke, 1969; Gordon *et al.*, 1972; Groves *et al.*, 1972, 1973). Proteose-peptone components 5, 8-slow and 8-fast are formed during proteolysis of N-terminal of β-casein by plasmin (Eigel *et al.*, 1984).

2.3.1.4. κ-Casein

The primary structure of κ-casein which contained 169 amino acids was elucidated by Mercier *et al.*(1973). In common with other caseins, the structure of κ-casein has an N-terminal hydrophobic domain and a C-terminal polar domain. κ-Casein is the only member in the casein family which contains both phosphorylation site

(SerP-149) and heterogeneous glycosylation component (N-acetyl neuraminic acid) (Fournet *et al.*, 1979; van Halbeek *et al.*, 1980).

κ-Casein is an important milk protein in food industry because it stabilises the dispersion of casein micelles in milk and is involved the milk-clotting process. κ-Casein is rapidly hydrolysed at the Phe(105)-Met(106) bond by enzyme chymosin or other proteases, yielding para-κ-casein which contains an N-terminal fragment, and C-terminal macropeptide. κ-Casein macropeptide contains all of the post-translational groups as well as genetic substitutions of the known A and B variants. The difference between A and B variants are due to two amino acid mutations of Thr to Ile at position 136 and Asp to Ala at position 148. Compared to the primary structure of A variant, C variant consists of a replacement of His by Arg at position 97 while D variant comprises a Gly instead of Ser at position 155. In the native form, bovine κ-casein occurs as polymer through -S-S- linkages which are formed from two Cys residues in para-κ-casein segment.

The separation of κ-casein A and B variants can be accomplished by polyacrylamide gel electrophoresis under alkaline condition in the presence of reducing agent in order to break the disulfide bonds (Mackinlay *et al.*, 1966). In most of the Western breeds such as Holstein and Ayrshire, the A variant of κ-casein is predominant. However, κ-casein B variant appears to be more frequent in Jersey herds. A comprehensive review on all genetic

variants of κ-casein was given by Ng-Kwai-Hang and Grosclaude (1992).

2.3.2. Whey proteins

Whey proteins which account for 20% of total milk proteins remain in solution during acidic precipitation of casein at pH 4.6 at 20°C. They are composed of four major proteins: β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulin with a relative proportion of 60:20:7:13 (Ng-Kwai-Hang and Kroeker, 1984). Whey proteins also contain other minor fractions such as β_2-microglobulin, lactoferrin, transferrin and proteose-peptone fractions. The propteose peptone fraction is a complex mixture of more than 38 kinds of glycoproteins, phosphoproteins and proteolysis peptides from casein by indigenous proteinases (Andrews, 1978a, 1978b; Andrews and Alichanidis, 1983; Nejjar *et al.*, 1986; Paquet *et al.*, 1988; Paquet, 1989). It represents approximately 10% of total whey proteins (Sorensen and Peterson, 1993).

2.3.2.1. β-Lactoglobulin

The primary structure of β-lactoglobulin consists of 162 amino acid residues and it has some similarities with plasma retinol-binding protein (Papiz *et al.*, 1986). An important feature of β-lactoglobulin is the presence of both disulfides and a thiol group in its polypeptide. During the heat treatment, the existence of this thiol group which is equally distributed between Cys-119

and Cys-121, is of great importance for the reaction with other proteins, notably κ-casein and α-lactalbumin.

β-Lactoglobulin undergoes conformational changes with varying pH. It exists naturally as a dimer of two noncovalently-linked subunits. Seven genetic variants of β-lactoglobulin have been described so far and all of them are due to different amino acid substitutions (McKenzie *et al.*, 1972; Bell *et al.*, 1981a; Eigel *et al.*, 1984; Hambling *et al.*, 1992; Ng-Kwai-Hang and Grosclaude, 1992). Compared to A variant, B variant has two amino acid substitutions: Asp to Gly at position 64 and Val to Ala at position 118. The replacement of Gln by His at position 59 gives C variant. The replacement of Glu in variant B by Gln at position 45 results in D variant (Grosclaude *et al.*, 1976b). When compared to B variant, E variant has a Gly instead of Glu at position 158. Variants F and G are restricted to Bali cows (Bell *et al.*, 1981a). Both variant F and G contain a mutation at position 158 with a Gly instead of Glu. In addition, F variant contains two mutations: Pro to Ser at position 50 and Asp to Tyr at position 130. G variant is the result of replacement of Ile in B variant by Met at position 78.

2.3.2.2. α-Lactalbumin

Bovine α-lactalbumin is a nearly spherical, compact globular protein which is made up of 123 amino acid residues with a calculated molecular weight of 14,175 (Brew *et al.*, 1970; Eigel *et al.*, 1984). It has a particularly interesting feature because of

its homology with lysozyme. α-Lactalbumin is also a modifier protein of the enzyme galactosyl transferase which promotes the transfer of galactose from UDP-galactose to glucose in lactose biosynthesis.

Amino acid substitutions give rise to three genetic variants: A, B and C in α-lactalbumin. Variant A differs from B by a amino acid substitution of Gln for Arg at position 10. Variant C involves most probably a substitution of Asn for Asp. Major components of bovine α-lactalbumin are devoid of carbohydrate whilst minor α-lactalbumin (<10%) is reported to contain covalently bound carbohydrate moiety (Gordon, 1971; Hopper and McKenzie, 1973; Bell *et al.*, 1981b).

2.3.2.3. Bovine serum albumin

As the major component of blood serum albumin, bovine serum albumin is synthesised in the liver and secreted into milk by mammary epithelium. It comprises only about 1.2% of the total milk proteins. The single polypeptide of serum albumin consists of 582 amino acid residues. It also contains different kinds of heterogeneity due to polymer formation, sulfur linkages and microheterogeneity. Although, no genetic variant has been described so far, its heterogeneity appears as several bands by isoelectric focusing separation (Spencer and King, 1971).

2.3.2.4. Immunoglobulins

The immunoglobulin are the most heat-sensitive protein in whey protein group. Among the five classes of immunoglobulins in mammals, four (IgG, IgA, IgM and IgE) have been found in bovine milk (Butler, 1969; Butler *et al.*, 1971). Like bovine serum albumin, immunoglobulins are lacteal secretions from blood serum and are antibodies synthesised in response to stimulation by foreign antigens. The monomeric form of all immunoglobulins consists of two identical heavy chains and light chains which are covalently linked by disulfide bonds. No genetic variant has been described for immunoglobulins even through they exhibit numerous specific binding sites for exogenous antigens.

2.4. Genetic aspect of milk protein polymorphism

All the major milk proteins are the products from autosomal genes transmitted from parents according to Mendelian inheritance (Aschaffenburg, 1968). They are expressed in female-limited trait during lactation. Detection of genetic polymorphism of milk protein can be approached at both DNA and protein levels. With the development of technology in molecular genetics, the methods involved in polymerase chain reaction and restriction fragment length polymorphism are applicable to detect some genetic variant of milk protein genes (Levéziel *et al.*, 1988, 1994; Zadworny *et al.*, 1990; Schlieben *et al.*, 1991; Schlee and Rottmann, 1992; Lien and Rogne, 1993).

Genetic variants of milk proteins are caused by either amino acid substitutions or a small peptide deletion. Up to now, only

two genetic variants give rise to small fragment deletions. The one reported in α_{s1}-casein A is due to 13 amino acid deletion between positions 14 and 26 and that of α_{s2}-casein D is the result of 9 amino acid deletion from position 50 to 58. The remaining of 28 variants so far described in the milk proteins are due to substitutions of amino acid residues at different positions in the polypeptide chain. The post-translational modifications which give rise to the multiple bands during electrophoresis of milk proteins are not considered as genetic variants. These modifications include different degrees of phosphorylation, glycosylation and polymerization.

Each milk protein is expressed from one or more alleles of a gene (Grosclaude, *et al.*, 1973). In this case, genetic variants of the same protein will represent the identical density bands on electrophoresis gel. However, it is of interest to point out that differences exist in allelic protein expression in the milk of heterozygous κ-casein (B>A) and β-lactoglobulin (A>B)(Imafidon and Ng-Kwai-Hang, 1991; Ng-Kwai-Hang and Kim, 1996).

Another important aspect of milk protein is the close linkage of four casein genes. The model proposed by Grosclaude (1979) suggests that α_{s1}-casein and β-casein loci are located close to each other whilst α_{s2}-casein locus is situated between β-casein and κ-casein loci. The phenomenon of close linkage of certain genetic variants of milk proteins have also been reported. For example, β-casein B and κ-casein B combination was frequently observed in Holstein breed (Brignon *et al.*, 1989).

2.5. Frequency distribution of genetic variants in milk proteins

It is well known that genetic variants, as gene products, reflect transmission of autosomal genes from parents to offsprings. The occurrence of genetic polymorphism of milk proteins are closely associated with breeds and species. At the DNA level, four major casein genes are clustered on 200 kb. Therefore, it is of interest to study the possible linkage between milk protein genes as well as exploration of the ancestral variants.

2.5.1. α_s-Casein

As a major component of casein, α_{s1}-casein B variant is the predominant variant in most western breeds. In contrast, C variant was reported as having a frequency higher than 0.25 in Jersey, Guernsey and Normande (Ng-Kwai-Hang *et al.*, 1984; Ng-Kwai-Hang and Grosclaude, 1992). The most recent survey within Quebec population revealed that Jersey contained a frequency of 0.431 of α_{s1}-casein C variant (Kim, 1994). Also C variant is found predominantly in the Yak which lacks of B variant (Grosclaude, 1979). Being derived from B, A variant, is the result of abnormal mRNA splicing, was only found with very low frequencies in Holstein (0.003) and Red Danish (<0.01) (Ng-Kwai-Hang *et al.*, 1984; Bech and Kristiansen, 1990). As a rare variant, α_{s1}-casein E was initially found in Flamande, and also occurs in 21 other breeds distributed in

French, Dutch, Italian, Polish and Canadian population (Grosclaude, 1979; Ng-Kwai-Hang and Grosclaude, 1992).

In α_{s2}-casein, A variant appear as the most common variant. The other two variants, B and C were found specifically in yak with a frequency around 0.10 (Grosclaude *et al.*, 1982). Moreover, α_{s2}-casein D variant, with frequencies of 0.01 and 0.09 were observed in Montbéliarde and Vosgienne respectively (Grosclaude *et al.*, 1978).

2.5.2. β-Casein

β-Casein A^1, A^2 and B variants are the common variants present in western breeds. During the studies by Ng-Kwai-Hang *et al.*(1984) and Kim (1994), A^1 was found predominantly in Holstein (0.561), Ayrshire (0.601) and Canadienne (0.585) while A^2 has a frequency of 0.499 and 0.518 in Jersey and Brown Swiss. For β-casein B, it has a frequency of 0.007 in Holstein, 0.002 in Ayrshire, 0.308 in Jersey, 0.161 in Brown Swiss and 0.079 in Canadienne. With respect to a rare β-casein variant A^3, it was reported to have a frequency of 0.011 (Holstein), 0.161 (Brown Swiss) and less than 0.001 in Ayrshire, Jersey, Canadienne. As a rare variant, C is absent in Jersey (Aschaffenburg, 1968), Zebu and possibly *Bos taurus*. However, it was observed in Mongolian (Grosclaude *et al.*, 1965), Korean (Han *et al.*, 1983) as well as Egyptian breeds (Graml *et al.*, 1986).

2.5.3. κ-Casein

As two common variants, A and B were found in most of the western breeds. Variant A is predominant in Holstein (0.744), Ayrshire (0.881), Brown Swiss (0.704) and Canadienne (0.789). B variant represented higher frequency in Jersey (0.74) (Ng-Kwai-Hang *et al.*, 1984; Kim 1994). Seibert *et al.* (1987) detected a new κ-casein variant, denoted as D, only in German Simmental cattle. Later, κ-Casein E with the low frequency was demonstrated in Holstein-Friesian, German Red cattle and Angler with a frequency of less than to 0.05 (Erhardt, 1989; Buchberger, 1995).

2.5.4. β-Lactoglobulin

As the major component of whey proteins, β-lactoglobulin was found to have seven genetic variants. A and B variants are commonly distributed within bovine and Zebu breeds. It was found that B variant was more frequent than A variant in Zebu as well as most of the western breeds such as Holstein, Ayrshire, Jersey, Brown Swiss and Canadienne (Ng-Kwai-Hang *et al.*, 1984, 1990; Kim, 1994). Variant C was first observed in Australian Jersey by Bell (1962). Variant D with a frequency less than 0.04 was reported in Simmental cattle as well as Danish Jersey (Baker and Manwell, 1980).

2.6. Significance of genetic polymorphism of milk proteins

Studies on variation of major milk proteins and their relationship with dairy related traits have been initiated for

decades. Although earlier results are controversial, most of researchers agree that genetic factors such as breed and genetic polymorphism of milk proteins and environmental factors such as season, age of cow, stage of lactation, udder infection, nutrition and ambient temperature have close relationship with milk production, milk composition and milk quality (Anderson and Andrews, 1977; Ng-Kwai-Hang *et al.*, 1982, 1984, 1990; Kroeker *et al.*, 1985a; Lin *et al.*, 1986; van Eenennaam and Medrano, 1991; Bovenhuis *et al.*, 1992; Lawrence, 1993; Kim, 1994). Among the six major milk proteins, α_{s2}-casein and α-lactalbumin are considered to be relatively monomorphic in most of the dairy breeds, therefore, most of the interests on the implication of genetic polymorphism of milk proteins were focused on α_{s1}-casein, β-casein, κ-casein and β-lactoglobulin.

2.6.1. Milk production

Genetic variants of milk protein was extensively studied during the past decade since it could be used as genetic marker for economic production trait. However, there is no significant trends with regard to genetic variants and milk production. Controversial reports on the relationship between genetic variants and milk production have been published due to various reasons including population size, breeds, method of measurement, frequency of genetic variants under consideration as well as statistical model being used. In some studies, it is well accepted that α_{s1}-casein B variant has higher milk yields when compared with

other variants (Ng-Kwai-Hang *et al.*, 1984, 1986, 1990; Lin *et al.*, 1986; Aleandri *et al.*, 1990). In β-casein, superior milk yield was associated with A variant especially with A^2 and A^3 (Matyukov, 1983; Ng-Kwai-Hang *et al.*, 1984, 1986; Lin *et al.*, 1986). It is commonly accepted that κ-casein BB is associated with higher protein yield but lower milk yield when compared with AA phenotype, while κ-casein AB is intermediate in term of protein and milk yields (Ng-Kwai-Hang *et al.*, 1984, 1986). However, other groups reported that no significant effect was observed between genetic variant of caseins and whey proteins with respect to milk yield over a complete lactation in Jersey, Friesian and Holstein herds (McLean *et al.*, 1984; Gonyon *et al.*, 1987).

A significant effect of β-lactoglobulin AA genotype on the higher milk yield has been frequently reported in Holstein Friesian cows (Ng-Kwai-Hang *et al.*, 1986). However, many reports show that there is no significant correlation between β-lactoglobulin genetic variants and milk production (Ng-Kwai-Hang *et al.*, 1984; McLean *et al.*, 1984; Lin *et al.*, 1986; Gonyon *et al.*, 1987; De-Lange *et al.*, 1991). More recently, Meyer *et al.* (1990a) reported that heifers with β-lactoglobulin AA had significantly lower milk yield than cows having AB or BB phenotype. At the same time, their paper indicated that the cow carrying α_{s1}-casein BB, β-casein A^1A^2 and κ-casein AB in combination with β-lactoglobulin AB or BB had superior milk production than other combinations.

2.6.2. Milk composition

Milk composition as well as the relative proportions of components will influence the overall value of milk. The effects of genetic variants on milk composition are more consistent than that on milk yield.

2.6.2.1. Milk proteins

Many reports have consistently shown that genetic variants of milk protein have close relationship with casein and whey protein contents. McLean (1987) summarized that the concentrations of α_{s1}-casein, β-casein and κ-casein in milk were significantly influenced by α_{s1}-casein (C>B), β-casein (B>A) and κ-casein (B>A). Among various phenotype of milk proteins, α_{s1}-casein BC, β-casein A^1B and κ-casein BB are correlated with casein contents (McLean *et al.*, 1984; Ng-Kwai-Hang *et al.*, 1984, 1986; Scharr *et al.*, 1985). In the β-casein system, A^2A^2 phenotype is associated with higher protein concentration than A^1A^1 and A^1A^2 being intermediate during three lactation in Holstein (Ng-Kwai-Hang *et al.*, 1986, 1990). Meyer *et al.*(1990b) reported that for heifers in the first lactation, α_{s1}-casein BB, β-casein A^2A^2 and κ-casein BB are more favourable for protein yields than BC, A^1A^1 and AA respectively.

Among the whey proteins, β-lactoglobulin makes up about 55% of the amount of whey proteins. Genetic variants of β-lactoglobulin has the most visible effect on its content in milk.

It is agreed that β-lactoglobulin BB milk has higher casein than AA with the intermediate being AB (Kroeker *et al.*, 1985b; Schaar *et al.*, 1985; Ng-Kwai-Hang *et al.*, 1987; Meyer *et al.*, 1990b). The opposite result was reported by De-Lange *et al.* (1991) who suggested that β-lactoglobulin A variant has a superiority in protein contents. McLean et al (1984) reported that β-lactoglobulin BB was associated with lower protein concentration than other types in Australian Jersey and Friesian breeds. Most recent study indicates that selection of β-lactoglobulin A and κ-casein B will result in higher protein content (Kim, 1994; Jakob and Puhan, 1995).

Apart from total protein concentration in milk, genetic polymorphism of milk protein is also associated with relative proportion of major individual milk proteins. Milk with α_{s1}-casein BC phenotype have higher α_s-casein and lower β-lactoglobulin content than the other phenotypes (Ng-Kwai-Hang *et al.*, 1987). Meanwhile, β-casein B variant has lower α_s-casein, β-lactoglobulin but higher β-casein and α-lactalbumin concentration when compared to β-casein A^1, A^2, and A^3 variants. In κ-casein, it is generally agreed that BB phenotype is more desirable because of its higher casein (α_{s1}-casein, κ-casein) content and lower whey protein contents (McLean *et al.*, 1984; Kroeker *et al.*, 1985b; Ng-Kwai-Hang *et al.*, 1987). More recently, several papers have confirmed that κ-casein B and β-lactoglobulin B are related with higher casein content.

2.6.2.2. Milk fat

Regarding the association of genetic variant with milk fat content, conflicting results are observed. Although it appears that genetic variants has no consistent effect on fat yield (Kim, 1994). Some reports show that α_{s1}-casein BC provides higher fat content in milk (Munro, 1978; Ng-Kwai-Hang *et al.*, 1986). It is commonly agreed that highest milk fat is associated with β-casein B variant as well as κ-casein B variant (McLean *et al.*, 1984; Ng-Kwai-Hang *et al.*, 1986). For whey protein, in most case, β-lactoglobulin B variant was considered to be superior in fat percentage (van den Berg, 1993; Tong *et al.*, 1993).

2.6.3. Milk quality

Manufacturing properties of milk have been extensively investigated for years. Genetic variants of milk protein have been clearly demonstrated to influence physico-chemical behaviour of milk. The influence of genetic variants on coagulation properties of milk was first observed by Sherbon *et al.* (1967). During cheesemaking, κ-casein phenotype exerts the greatest effect. It is reported that κ-casein B and β-lactoglobulin B are associated with increased heat stability (McLean *et al.*, 1987). Other workers suggested that heat stability of different β-lactoglobulin phenotypes was variable and depended on pH and composition of buffer (Imafidon and Ng-Kwai-Hang, 1991).

During cheesemaking, α_{s1}-casein BB as well as β-casein A^1A^1 are associated with higher cheese yield (Aleandri *et al.*, 1990). Milk with κ-casein BB and β-lactoglobulin BB has 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). Milk containing β-casein A^1, κ-casein B and β-lactoglobulin B variant are more favourable for cheese yield (Ng-Kwai-Hang, 1990, 1993; Tong *et al.*, 1993).

Genetic polymorphism of milk proteins can affect the coagulation properties of milk. Davoli *et al.* (1990) reported that κ-casein A was associated with shorter coagulation time, higher rate of curd formation and higher curd firmness. While others suggested that κ-casein B allele led to better syneresis properties, shorter coagulation time (Schaar, 1984; Pagnacco and Caroli, 1987; Aleandri *et al.*, 1990). β-Lactoglobulin AA milk gave better clotting time and curd firmness when compared to AB and BB phenotype (Marziali and Ng-Kwai-Hang, 1986b). More recently, it was demonstrated that the nature of caprine milk associated with no β-casein had longer coagulation time and softer curd during comparative assays on the aptitude of individual milk to coagulate (Chianese *et al.*, 1993).

During yogurt manufacture, it was suggested that milk with κ-casein AA and β-lactoglobulin BB were more preferred due to their lower syneresis (McLean and Schaar, 1989).

So far, a considerable amount of information on genetic polymorphism is available and it can be used to select for production trait. In spite of extensive research on genetic polymorphism of milk proteins, efforts to promote genetic improvement intended for certain selection have become more and more important in order to optimize protein composition and processing properties of milk.

2.7. Methods for detection of genetic variants

Prior to the 1950s, the separation of milk proteins was only limited to the so-called whole casein and the classical lactalbumin and lactoglobulin factions of whey proteins (Walstra and Jenness, 1984). The most commonly used method is based upon acid precipitation at the isoelectric pH of casein in order to minimize solubility. Later on, fractionation of casein has been achieved by high-speed centrifugation and salt precipitation (Wake and Baldwin, 1961; McKenzie, 1971). Proteins in milk were considered to be homogeneous until the discovery of genetic variants of β-lactoglobulin (Aschaffenburg and Drewry, 1955). Consequently, genetic polymorphism of casein was first evidenced by Blumberg and Tombs (1958) and Aschaffenburg (1961) who used urea in order to dissociate casein micellar forms. During the past decades, a number of procedures were developed to identify the genetic variants of individual milk proteins, and they would be mainly classified as electrophoretic and chromatographic methods.

2.7.1. Electrophoretic methods

The basis of electrophoretic separation of genetic variants of milk proteins depends on the difference of their net charges. With the exception of β-casein A variants, most of the common variants of casein as well as whey proteins were achieved at alkaline pH (Peterson and Kopfler, 1966; Kiddy, 1975; Grosclaude *et al.*, 1979; Ng-Kwai-Hang and Kroeker, 1984; Medrano and Sharrow, 1989). In most cases, urea was used as dissociation reagent to disaggregate casein micelle while β-mercaptoethanol was applied for cleavage disulfide bonds in α_{s2}-casein and κ-casein. More recently, separation of bovine β-lactoglobulin A, B and C variants was also achieved by capillary zone electrophoresis (Paterson *et al.*, 1995).

2.7.1.1. Paper electrophoresis

Different electrophoretic techniques have been applied to the study of genetic polymorphism of milk protein. Paper electrophoresis was the first to be used for the resolution of different variants of casein and whey proteins. After the separation of A and B variants of β-lactoglobulin by Aschaffenburg and Drewey in 1955, this method has been applied for the detection of genetic variants A, B, and C of β-casein in the presence of 6.0M urea (Blumberg and Tombs, 1958; Aschaffenburg, 1961). However, the attempt to identify genetic variants of α_{s1}-casein and κ-casein was unsuccessful by paper electrophoresis.

2.7.1.2. Starch gel electrophoresis

Since paper electrophoresis was not applicable for the resolution of genetic variant of α_s-casein and κ-casein, starch gel electrophoresis was introduced and the separation of three polymorphs of α_{s1}-casein was reported by Wake and Baldwin (1961) and Thompson *et al.*(1962).

The electrophoretic heterogeneity of κ-casein remained undetectable since it has the strongest tendency to aggregate among of the caseins. It was only after the reduction of the disulfide bonds between the two polypeptides by reducing agent such as β-mercaptoethanol and dithioerythritol that the heterozygous κ-casein could be separated into two distinct bands, denoted A and B with different mobility on alkaline gel electrophoresis (Swaisgood and Brunner, 1962; Neelin, 1964; Schmidt, 1964; Woychik, 1964). Further resolution of β-casein A variant family was only accomplished by starch gel electrophoresis under acidic conditions (Kiddy *et al.*, 1966; Peterson and Kofler, 1966; Arave, 1967).

In addition to the two variants of β-lactoglobulin resolved by paper electrophoresis, Bell (1962) separated another variant, termed as β-lactoglobulin Dr in Australian droughtmaster beef cattle. For α-lactalbumin, starch gel electrophoresis was also used to reveal A and B variants (Blumberg and Tombs, 1958; Bhattacharya *et al.*, 1963). As an alternative method, agar gel was

used to successfully separate A, B, C and presumably D variant in β-lactoglobulin (Aschaffenburg, 1965).

2.7.1.3. Polyacrylamide gel electrophoresis

Polyacrylamide gels form after polymerization of monomeric acrylamide by cross-linking of with N,N'-methylene-bisacrylamide. The polymerization reaction is initiated by the addition of ammonium persulfate and the reaction is accelerated by N,N,N'-tetramethylethylenediamine, which catalyzes the formation of free radicals from ammonium persulfate. After its introduction, polyacrylamide gel was explored by Raymond and Wang (1960) as a means of identifying genetic variants of milk proteins. Since then, it had become the standard technique for studying genetic variability of proteins because it could be prepared easily without heat treatment and could be stained without slicing, as well as conveniently stored (Raymond and Wang, 1960; Ashaffenburg, 1964).

The procedure of alkaline PAGE developed by Kiddy (1975) and modified by Ng-Kwai-Hang *et al.* (1984) provided sufficient separation of most major caseins and whey proteins. According to Ng-Kwai-Hang *et al.* (1984), major casein separation can be accomplished with larger pore size gel containing 8% polyacrylamide and 4M urea, while resolution of major whey proteins especially β-Lg and α-La were performed on the 12% polyacrylamide in the absence of urea. However, electrophoresis in alkaline gels does not resolve β-casein A family into its three

components A^1, A^2 and A^3. An alternative electrophoretic run under acidic pH 3.0 is required for further resolution (Aschaffenburg, 1966, 1968; Ng-Kwai-Hang *et al.*, 1984; Medrano and Sharrow, 1989).

Over the last decades, considerable development has been achieved for detection of protein polymorphism in bovine, ovine, caprine, and porcine milk (Ng-Kwai-Hang and Grosclaude, 1992). Zonal electrophoresis with paper, starch, agarose and polyacrylamide supports are commonly used for milk protein phenotyping. Genetic variants of milk proteins were classified according to the chronological order they are being identified and their different migration on the supports (Thompson and Pepper, 1964; Eigel *et al.*, 1984). Under alkaline condition, α_s-casein contains more negative charges than β-casein and κ-casein, and the relative mobilities of major caseins are: α_{s1}-casein> α_{s2}-casein> β-casein> κ-casein. Similarly, according to their difference in net charges, among the five known genetic polymorphs of α_{s1}-casein, the order of electrophoretic mobilities is A>D>B>C>E (Eigel *et al.*, 1984; Whitney, 1988). The resolution of seven genetic variants of β-casein needs to be performed by coupling alkaline PAGE with acidic PAGE. Under alkaline conditions, variants A^1, A^2, A^3 carry the same net charges and have similar electrophoretic mobility band denoted as A; whereas, the migration order of genetic variants of β-casein is in the order of: A>B>D; E>C (Eigel *et al.*, 1984). With a supplemental low pH run, the following order of migration is observed: $C>B>D>A^1=E>A^2>A^3$ (Aschaffenburg, 1966; Eigel *et al.*, 1984). In the native state, κ-casein locus is generally

considered as diallelic, consisting of the variants A and B which had been discovered simultaneous by Neelin (1964), Schmidt (1964) and Woychik (1965). Monomers of κ-casein are usually linked together through intermolecular disulfide bond and other components attached to κ-casein molecule also contribute to the difference in net charges (Woychik, 1965; Fox, 1982; Walstra and Jenness, 1984). As a good example for electrophoretic separation under alkaline conditions, A variant contains Asp at position 148 while Ala is found in B variant which results in one more negative charge in A variant. Therefore, A moves faster than B under alkaline condition. Both variants exhibit multiple bands which are due to the variation in different levels of post-translational modification, mainly glycosylation by attachment of sialic acid. Fox (1989) reported that the probability of phosphorylation may, somehow, also have influence on the migration of genetic variants of κ-casein.

2.7.1.4. Isoelectric focusing (IEF)

Although electrofocusing was introduced in 1960s, the application of this technique only started in 1970s for the purpose of phenotyping milk proteins. This is due to the requirements of longer running times and higher voltages (Peterson, 1969, 1971; Finlayson and Chrambach, 1971; Kaplan and Foster, 1971; Josephson *et al.*, 1971, 1972; Josephson, 1972). Isoelectric focusing is a powerful technique which provides reliable phenotyping according to its high resolution. Peterson

(1969) reported the resolution of peptide with differences as little as 0.02 pH unit in their isoelectric point. The isoelectric points of major caseins ranged from pH 4.9 to 5.9 with the following increasing order: α_{s1}-casein> α_{s2}-casein> β-casein> κ-casein (Swaisgood, 1982). The effective resolution of genetic variants of milk protein has also been reported by this method in polyacrylamide medium with sucrose gradient (Spencer and King, 1971, Josephson, 1972; Pearce and Zadow, 1978).

After 1980s, substantial technical improvement in isoelectric focusing explored its application for demonstration of genetic polymorphism of milk protein (Trièu-Cuot and Gripon, 1981; Seibert *et al.*, 1985, 1987; Erhardt and Senft, 1987; Klose, 1987; Addeo *et al.*, 1988; Bovenhuis and Verstege, 1989). Trièu-Cuot and Gripon (1981) identified the bovine caseins and some of their degradation products by IEF carried out in a gel containing 5% acrylamide for 3 hr. Improved method for simultaneous phenotyping of genetic variants in cow's milk by isoelectric focusing has been reported by Seibert *et al.* (1985). Isoelectric focusing employed on ultrathin-layer polyacrylamide gels can resolve all polymorphic protein fractions within a 45 min single run. A new variant of κ-casein, termed as κ-casein D with a frequency of less than 0.01% has been identified by the same group (Seibert *et al.*, 1987).

In addition, a modified IEF procedure using a vertical polyacrylamide minigel system has been described by Robertson *et al.* (1987). Later, phenotyping of milk protein variants was achieved in one single run by Bovenhuis and Verstege (1989) and

this procedure have the advantage of standardization, short separation time and high resolution.

2.7.1.5. Two-dimensional gel electrophoresis

High resolution two dimensional gel electrophoresis is capable of separating more than a thousand proteins on a single gel. In most of the cases, it couples the first dimensional isoelectric focusing with the second dimensional SDS-PAGE or the reverse (Chianese *et al.*, 1992). However, two-dimensional gel separations often suffer the difficulty of reproducibility and interpretation. More recently, instead of SGE, PAGE or ordinary IEF, two-dimensional electrophoresis with use of immobilized pH gradient for the first dimension have improved reproducibility.

Trièu-Cuot and Gripon (1981) first described the application of two-dimensional electrophoresis for detection of the genetic variants of milk protein. Later, this technique was applied on an immobilized pH gradient and accomplished higher resolution of milk protein variants (Klose, 1987; Basha, 1988; Holt and Zeece, 1988). Recently, Addeo *et al.* (1988) and Tutta *et al.* (1991) reported studies of two-dimensional electrophoresis for caprine milk protein polymorphism. Girardet *et al.* (1991) reported the achievement of a proteose-peptone cartograph and Addeo *et al.* (1992) confirmed the occurrence of Welsh variants in ovine α_{s1}-casein which had the mobility half way between α_s-casein and β-casein using two-dimensional electrophoresis containing PAGE vs isoelectric focusing.

2.7.1.6. Immunoblotting

The most recent development in electrophoresis is to combine electrophoretic and immunochemical techniques in order to detect genetic polymorphism of milk proteins (Addeo *et al.*, 1992; Chianese *et al.*, 1992; 1995; Addeo *et al.*, 1995; Lopez-Galvez *et al.*, 1995). When compared with the traditional Coomassie staining procedures, immunoblotting is more sensitive and make the interpretation of results more specific and easier. Therefore, it reveals a bewildering array of milk proteins and their derivatives in dairy products such as cheese (Addeo *et al.*, 1992, 1995). Chianese *et al.* (1992) described the application of electrophoretic techniques coupled with immunoblotting with specific polyclonal antibodies against casein fractions to identify ovine casein variants. Lopez-Galvez *et al.* (1995) reported the resolution of a major and a minor "satellite" band in ovine β-lactoglobulin and the application of immunoblotting confirmed that the "band" belongs to β-lactoglobulin and α-lactalbumin fractions by using specific immunosera against them.

2.7.2. Application of column chromatography

2.7.2.1. Fractionation of milk proteins

Prior to 1980s, a number of techniques have been used with respect to separation and purification of milk proteins. Such methods mainly used salt and solvent fractionation (McKenzie, 1971). Isolation and purification of different milk proteins were

traditionally achieved by ion-exchange chromatography and gel filtration due to net charge and molecular size (Thompson, 1966; Rose *et al.*, 1969; Tripathi and Gehrke , 1969; McKenzie, 1971; Davies and Law, 1977; Yoshida, 1990). With the development of technology, the application of FPLC and HPLC made it possible to achieve efficient separation of milk protein in relatively short time and with larger quantity as have been well reviewed (Pearce, 1983; Humphrey and Newsome, 1984; Andrews *et al.*, 1985; Barrefors *et al.*, 1985; Visser *et al.*, 1986; Davies and Law, 1987; Christensen and Munksgaard, 1989; Gonzalez-Llano *et al.*, 1990; Law, 1993; Swaisgood, 1993).

2.7.2.1.1. Anion-exchange chromatography

Proteins are amphoteric and therefore their overall charge is very dependent on pH. Ion-exchange columns discriminate between proteins with regard to their ionic character and selectivity inherent to the protein could be altered (Kopaciewicz and Regnier, 1983). Generally, salt gradient are used to increase the ionic strength of the mobile phase, to reduce the strength of electrostatic interactions, and to selectively displace the charged residues of the retained proteins from ionic sites on the support surface. α_{s1}-, α_{s2}-, β- and κ-Caseins are the four main components which constitute whole casein of milk. These phosphoryl-proteins tend to form large micelles. Anion-exchange chromatography with DEAE-cellulose column was introduced by Ribadeau-Dumas (1961) and Thompson (1966) and is now widely used

for casein fractionation. In most cases, denaturing agent such as urea was added to counteract casein micelle, and reducing agent such as β-mercaptoethanol or DTT was used to dissociate the disulfide bond between κ-casein molecules. A gradient of sodium chloride concentration ascertain the moderate fractionation of α_s-casein, β-casein and κ-casein (Rose *et al.*, 1969; Yaguchi and Rose, 1971; Gordin *et al.*, 1972; Davies and Law, 1977; Donnelly, 1977; McGann *et al.*, 1979; Manji *et al.*, 1985). More recently, the compositional analysis has been achieved by TSK gel DEAE-5PW and Mono Q HR5/5 anion-exchange column with increasing ionic strength buffer gradient (Humphrey and Newsome, 1984). Wei and Whitney (1985) described the batch fractionation method on DEAE-cellulose and step-wise salt extraction by using imidazole-NaCl (pH 7.0) buffer system.

Different physico-chemical characteristic has also been used for fractionation of different caseins. Vreeman *et al.* (1977; 1986) purified κ-casein on a DEAE-cellulose column by decreasing pH gradient from 6.0 to 4.5. Sango *et al.* (1989) used $CaCl_2$ as eluant in an acetate buffer to purify α_{s1}-casein since it is more phosphorylated than any other caseins.

During recent years, development in FPLC and HPLC gave rise to more efficient analytical scale fractionation and purification of caseins. They provide several advantages in having high rapidity and great reproducibility. Fractionation of milk proteins has been ascertained on TSK gel DEAE-5PW and Mono Q HR5/5 anion-exchange column by salt elution gradient (Humphrey and

Newsome, 1984). For the separation and purification of minor caseins, Léonil and Mollé (1990) reported the purification of carbohydrate free caseinmacropeptide (CMP) from rennet-hydrolysed caseinnate by trichloroacetic acid precipitation and DEAE-TSK Fractogel-650 ion-exchange chromatography with salt elution in 20 mM Tris-HCl buffer (pH 7.5). A rapid separation of bovine caseins was obtained by mass ion-exchange chromatography on a QAE Zeta Prep 250 cartridge using urea solution containing 0.02M imidazole and 0.03M 2-mercaptoethanol (Ng-Kwai-Hang and Pélissier, 1989). Later, four electrophoretically pure casein forms were obtained from Protein-Pak DEAE 15HR anion-exchanger using NaCl gradient in Tris-urea buffer (Ng-Kwai-Hang and Dong, 1994).

The major whey proteins such as α-La and β-Lg are also amenable to be isolated and purified on a DEAE-cellulose column with step-wise sodium chloride concentration (Yaguchi and Rose, 1971; Swaisgood, 1982). Later, isolation and purification of β-lactoglobulin was employed on a QAE Zeta Prep cartridge by eluting with 0.05M phosphate buffer containing 0.3M NaCl. By this method, the fractionation could be performed at the gram level. (Imafidon and Ng-Kwai-Hang, 1992).

2.7.2.1.2. Cation-exchange chromatography

Utilisation of cation-exchange columns explored another option for casein separation. Annan and Manson (1969) first described the procedure to purify α_s-casein on a Sulphoethyl Sephadex C-50 column. Later on, further resolution of minor α_s-

casein (α_{s2}-, α_{s3}-, α_{s4}- and α_{s6}-casein), as well as κ-casein were performed on cation-exchange Amberlite C650 column with 3M urea and NaCl gradient at pH 6.0 (Snoeren *et al.*, 1977). The separation of casein was also achieved on a cationic Mono S ChR/5/5 column with pH 3.2-3.8 buffers as reported by Andrews *et al.* (1985) and St-Martin and Paquin (1990).

As an ingredient for dietetic foods, GMP attracted lots of interests recently. Satisfactory separation of macro-peptide of κ-casein had been accomplished by Léonil and Mollé (1991) using a Mono S column with pH 2.0 buffer. Purification of some whey protein components were achieved by Sulphopropyl cation-exchange chromatography (Yoshida and Ye-Xiuyun, 1991).

2.7.2.1.3. Hydroxyapatite

Whole casein separation was achieved on various stationary phase. Fractionation of whole casein on hydroxyapatite was obtained by Addeo *et al.* (1977). Later, Visser *et al.* (1986) reported that casein fractionation was performed on a Bio-Gel HPHT column with KH_2PO_4 and $CaCl_2$ containing mobile phase. From the result, κ-casein A and B variants are partly distinguishable. However, α_{s1}-casein and α_{s2}-casein are co-eluted.

2.7.2.1.4. Affinity chromatography

The review of affinity chromatography by Wilchek *et al.* (1984) discusses available methods for activation of solid supports, coupling of ligands, absorption of proteins, and elution

of protein from affinity columns. The fractionation of casein was also performed by affinity chromatography in bovine (Nijhuis and Klostermeyer, 1975), porcine (Brignon *et al.*, 1977; Cerning-Beroard and Zevaco, 1984), caprine (Razanajatova and Alais, 1977) and ovine (Dall'Olio *et al.*, 1990) milk. Recently, sheep milk casein was fractionated into two components composed of α_{s1}-casein, β-casein and α_{s2}-casein, κ-casein on an activated thiol-sepharose 4B affinity column (Dall'Olio *et al.*, 1990). However, no successful fractionation of different genetic variants of milk proteins has been reported so far.

2.7.2.1.5. Hydrophobic interaction

Hydrophobic interaction chromatography was first introduced by Creamer and Richardson (1981) and then modified by Chaplin (1986) for bovine whey protein and casein separation on a phenyl-superose column. In this study, different casein components show the relative migration order of: β<α, α_{s2}-<κ-<α_{s1}-casein. Later, a rapid reproducible and less denaturation method to separate proteose-peptone was described by Girardet *et al.* (1991) on a TSK-Phenyl 5PW column with 1M to 0M ionic strength of NaH_2PO_4 at pH 6.8. Hydrophobic interaction chromatography was also adapted to purify α_{s1}-casein with a semipreparative spherogel TSK-G Phenyl 5PW using 0.05M sodium phosphate urea buffer (Sanogo *et al.*, 1989). An improved method for isolation of disulphide-linked α_{s2}- and κ-casein from bovine milk was ascertained on a Phenyl Superose HR5/5 column (Syväoja, 1992).

The elution consisted of a linear gradient from 0.48 M sodium phosphate (pH 6.3) to 0.037 M same buffer at pH 6.5 with the presence of 2.5 M urea and 0.02% sodium azide.

2.7.2.1.6. Size exclusion

The chromatographic separation of protein mixture by size-exclusion is dependent upon molecular sizes of the components. Theorically, proteins larger than the pore size of the chromatographic matrix will be excluded and eluted in the void volume. Those with sizes smaller than the pore size of the matrix will penetrate into the pore and follow longer flow paths in the order of large to small. It was also of interest to study the application of size exclusion method milk protein separation in a rapid and reliable way (Barrefors *et al.*, 1985). During the earlier study, disulphide-linked α_{s2}- and κ-casein had been fractionated by gel chromatography from bovine skim milk (Toma and Nakai, 1973). Later naturally occurring disulphide-linked α_{s2}- and κ-casein in bovine milk was purified by gel chromatography using a Sepharose CL-6B column (Rasmussen and Petersen, 1991). In most of the cases, the caseins were too similar in their size to be separated, whereas, high speed gel filtration can provide satisfactory separation of all the whey proteins on Sephacryl-S-1000 (Ekstrand and Larsson-Raznikiewicz, 1978) and Superose 12 column (Andrews *et al.*, 1985). The analysis of glycomacropeptide in rennet milk was studied by Sharma *et al.* (1993) with HPLC on a protein pak-125 column. Size

exclusion was performed on TSK gel G3000 PW_{xl} column (Kawakamiet *et al.*, 1992) and TSK 2000 SW column (Lopez-Fandino *et al.*, 1993) for determination of κ-casein glycomacropeptide.

FPLC and HPLC have been used for gel filtration method where the resolution is based on size of molecules. Andrews *et al.* (1985) reported for whey protein separation on a Superose 12 column with 0.1 M Tris-HCl buffer containing 0.5 M NaCl and 10 mM NaN_3. More recently, purification and characterization of proteose peptone fractions of bovine milk were achieved on Sephadex G-75 column with 0.1 M NH_4HCO_3 buffer at pH 8.0 (Sorensen and Petersen, 1993).

Characterization of bovine κ-casein was achieved by HP gel-permeation chromatography based on their difference in N-acetylneuraminic acid (NeuAc) and/or phosphorus content (van Hooydonk and Olieman , 1982; Vreeman *et al.*, 1986; Davies and Law, 1987).

2.7.2.1.7. Reversed-phase HPLC

Utilization of reversed-phase column has greatly expanded the analytical capacity of chromatography. Reversed-phase HPLC became the choice for resolving mixtures containing small proteins and peptides since it enables the delivery of good separation efficiency with low sample requirement (Covey *et al.*, 1991). Reversed-phase separation of casein components were carried out on C-18 column (Carles, 1986; Visser *et al.*, 1986; Strange *et al.*, 1991), C-4 column (Parris *et al.*, 1990) as well

as reversed-phase phenyl column (Visser *et al.*, 1986). Traditionally, the fractionation was achieved by increasing the gradient of acetonitrile with 0.1% TFA inside the mobile phase (Gonzalez-Llano *et al.*, 1990; Swaisgood, 1993). Mikkelsen *et al.*(1987) purified α_{s1}-casein by reversed-phase HPLC.

2.7.2.2. Detection of genetic variants by chromatography

Application of column chromatography for milk proteins are mainly focused on protein separation and purification by using either ion-exchange or size exclusion techniques. Regarding phenotyping of milk proteins, the differences between genetic variants, in most of cases, are not sufficient to be discriminated by size exclusion method. During the past decades, a few papers have been published involving in identification of genetic variant by ion-exchange chromatography and reversed-phase HPLC. Generally, chromatographic methods are relatively laborious and time-consuming compared to electrophoresis.

2.7.2.2.1. Ion-exchange chromatography

In 1960s, the use of DEAE-cellulose ion-exchange column chromatography containing urea helped to separate β-casein A variant from C (Thompson and Pepper, 1964) and κ-casein A and B variants (Thompson, 1966). In these cases, the separation of genetic variants is also ascertained according to the difference of net charges of the molecules. Later, resolution of β-casein A and C variants was achieved by QAE-cellulose ion-exchange stationary phase (Ng-Kwai-Hang and Pélissier, 1989).

The application of Fast Protein Liquid Chromatography and High Performance Liquid Chromatography significantly decreased possible running time as well as the increased resolution. Separation of β-lactoglobulin A and B variants was reported by others (Pearce, 1983; Humphrey and Newsome, 1984; Andrews *et al.*, 1985). Guillou *et al.* (1987) differentiated κ-casein A and B variants as well as β-casein A^1 and C variants by anion-exchange chromatography. The separation for κ-casein A and B variants as well as their glycosylation components had been ascertained by DEAE-cellulose anion-exchange chromatography (Dong, 1992). Quantitative examination of β-casein polymorphism by cation-exchange chromatography using acetate-urea buffer was described by Hollar *et al.* (1991a, 1991b) and Law (1993). Caprine κ-casein fractionation and its polymorphism has been studied on the same kind of column (Law and Tziboula, 1993). But somehow the result was less attractive than that from anionic columns.

2.7.2.2.2. Reversed-phase HPLC

Based on their difference on hydrophobicity, Jaubert and Martin (1992) reported the distinguishable α_{s1}- and α_{s2}- genetic variants on a Vydac C-4 column. Visser *et al.* (1991) reported the resolution of glycomacropeptide (GMP) A and B, α_{s1}-casein B/C and D, β-casein A^1, A^2 and A^3, κ-casein A and B as well as β-lactoglobulin A and B variants on a C18 column using an increasing gradient of acetonitrile. Moreover, the separation of

two variants of porcine β-Lg was elucidated by Dalglarrondo *et al.* (1992) after a subsequent salting-out at low pH.

2.7.3. Casein hydrolysis

A great advantage of reversed-phase HPLC is its application for peptide mapping after casein hydrolysis. Reversed-phase HPLC was initially utilized by Bican (1983) to resolve the products of tryptic digests of total casein and concluded that this method had attractive potential for use in food industry. Later on, the separation of casein hydrolysates after treatment with proteases such as chymotrypsin, bovine plasmin and trypsin have been elucidated (Leadbeater and Ward, 1987; Lemieux and Amiot, 1990; Le Bars and Gripon, 1993). The examination of the extent of whey protein denaturation by reversed-phase HPLC was also described (Parris and Baginski, 1991).

2.7.3.1. Plasmin

As a native proteinases of milk, hydrolysis of casein by bovine plasmin was first studied by Eigel (1977). In bovine milk, β- and α_{s2}-casein are easier to cleave than α_{s1}-casein while, κ-casein and whey proteins are more slowly hydrolysed and more resistant. According to Le Bars and Gripon (1993), twenty peptide bonds consisting of Lys-X or Arg-X in α_{s1}-casein can be potentially cleaved by plasmin. However, they only identified fourteen of them. This is possibly due to the fact that α_{s1}-

casein is less susceptible to plasmin hydrolysis when compared to other protein such as β-casein (Visser *et al.*, 1989).

2.7.3.2. Chymosin

Generally, peptide bonds which had an aromatic or hydrophobic residue at the N-terminal side of the scission bond were found to be susceptible to hydrolysis by chymosin. The primary action of chymosin during cheesemaking occurs at the Phe_{105}-Met_{106} of κ-casein and triggers the coagulation process of milk. A specific study for the action of chymosin by HPLC was reported by van Hooydonk and Olieman (1982). The recent study for proteolytic specificity of chymosin on bovine α_{s1}-casein was performed by McSweeney *et al.* (1993) under variable pH conditions. The comparative study of CMP from cows, ewes and goats milk by RP-HPLC was investigated for the fraction soluble in 4% trichoroacetic acid after treatment with chymosin (Lopez-Fandino *et al.*, 1993).

2.7.3.3. Trypsin

As the most documented proteolyetic enzyme, trypsin specifically cleaves after Lys and Arg residues. However, it is also suggested that tryptic hydrolysis takes place more slowly when a basic residue is adjacent in sequence to an acidic residue or to cysteine (Léonil and Mollé, 1990). Carles and Ribadeau-Dumas (1986) analysed tryptic hydrolystaes of β-casein A^1. Later, an HPLC study for bitter peptides resulting from

tryptic hydrolysis of β-casein A^2 was reported by Leadbeater and Ward (1987) where sixteen theoretical fragments could be expected. Later, the liberation of tryptic caseinomacropeptide of κ-casein was determined by HPLC after trichloroacetic acid precipitation and anion-exchange chromatography (Léonil and Mollé, 1990). At the same time, the comprehensive study on trypsin hydrolysis of whole caseins were reported by Lemieux and Amiot (1990) using peptide mapping.

2.7.4. New technology and silent variants

Within the past four decades, the major caseins and whey proteins of bovine milk have been revealed to contain numerous genetic variants. Among the 20 amino acids, three of them carry positive (His, Arg, Lys) and two carry negative (Asp, Glu) charges, whereas, the remaining 15 belong to the neutral amino acid group. With regard to this condition, it could be predicted that the presence of some genetic variants of milk proteins due to mutations not invloving changes in net charges cannot be established by electrophoresis. These are so-called non-electrophoretic variants or "silent variants". Silent variants resulting from neutral amino acid substitution was first demonstrated in human hemoglobin by tryptic peptide mapping using reversed-phase HPLC (Schroeder *et al.*, 1982). Shortly after, the same group found the substitution of Met → Thr in Hb Aztec peptide by Vydac C4 reversed-phase column with a shallow gradient of acetonitrile from 44-55.6% in 60 min (Schroeder *et*

al., 1985). Later on, another silent variant involving Val to Leu substitution was distinguished from tryptic hydrolysate of HbA (Jones *et al.*, 1990). In milk proteins, the neutral amino acid substitution of Pro to Leu was found in β-casein fragment 114-169 after trypsin hydrolysis of A^1 variant (Carles, 1986). Another discovery of aberrant variant termed as "W" was revealed from β-lactoglobulin B (Ile → Leu) (Presnell *et al.*, 1990).

Most recently, the nature of heterogeneity of multiple phosphorylation in milk protein peptides was studied by Mass Spectrometry combined with other techniques. The determination of peptide mapping of human serum albumin by chemical and enzymatic cleavage using HPLC and fast-atom bombardment mass spectrometry was described by Compagnini *et al.* (1994). The study on proteolytic specificity of chymosin on bovine α_{s1}-casein was investigated by HPLC and plasma desorption time-of-flight mass spectrometer (McSweeney *et al.*, 1993). In caprine milk, electrospray mass spectrometry (ESMS) analysis of the chromatographic fractions of β-casein was performed on a Bio-Q triplequadrupole mass spectrometer to investigate the difference in molecular weight of individual components and to identify the heterogeneity of attached phosphate residues (Chianese *et al.*, 1993). Similar approach to locate phosphorylation on synthesized β-casein phosphopeptide was reported by Liao *et al.* (1994). Most recently, localization of phosphorylation sites of ovine α_{s1}-casein and its new simple silent substitution (Ser → Pro) at

the position 13 of C variant was ascertained by HPLC and mass spectrometry (Ferranti *et al.*, 1995).

A highly informational approach, mass spectrometry has recently emerged as an indispensable and fast tool for peptide and protein structure analysis (Covey *et al.*, 1988; Fenn *et al.*, 1989; Carr *et al.*, 1991; Yates *III et al.*, 1993) for providing precision information on minor structural difference or modifications of protein and peptide. In Electrospray Mass Spectrometry, the ionisation process produces ionised form from intact protein or peptide molecules by nebulisation in the presence of strong electric field. Figure 1 is a schematic diagram of a triple-quadrupole mass spectrometer and the electrospray interface. When the sample-containing liquid is being pumped through an ionsprayer which has been maintained at a high voltage, a mist of highly charged droplets is produced by the formation of adducts with several protons. As the droplets evaporate from the probe, ions are ejected into the gas phase by a very low energy process which does not induce fragmentation. Compounds containing one or more charged sites are observed as singly or multiply charged ions as they are being introduced into a quadropole mass analyzer in the form of quasimolecular ions. In the mass determinations, peak centroid of the experimental multicharged ion series were considered in order to get experimental average molecular mass for comparison purposes (Rouimi *et al.*, 1995). As ionspray can produce multiply charged ions predominantly by the formation of adducts with several protons, any pair of ions can be used to determine the charge

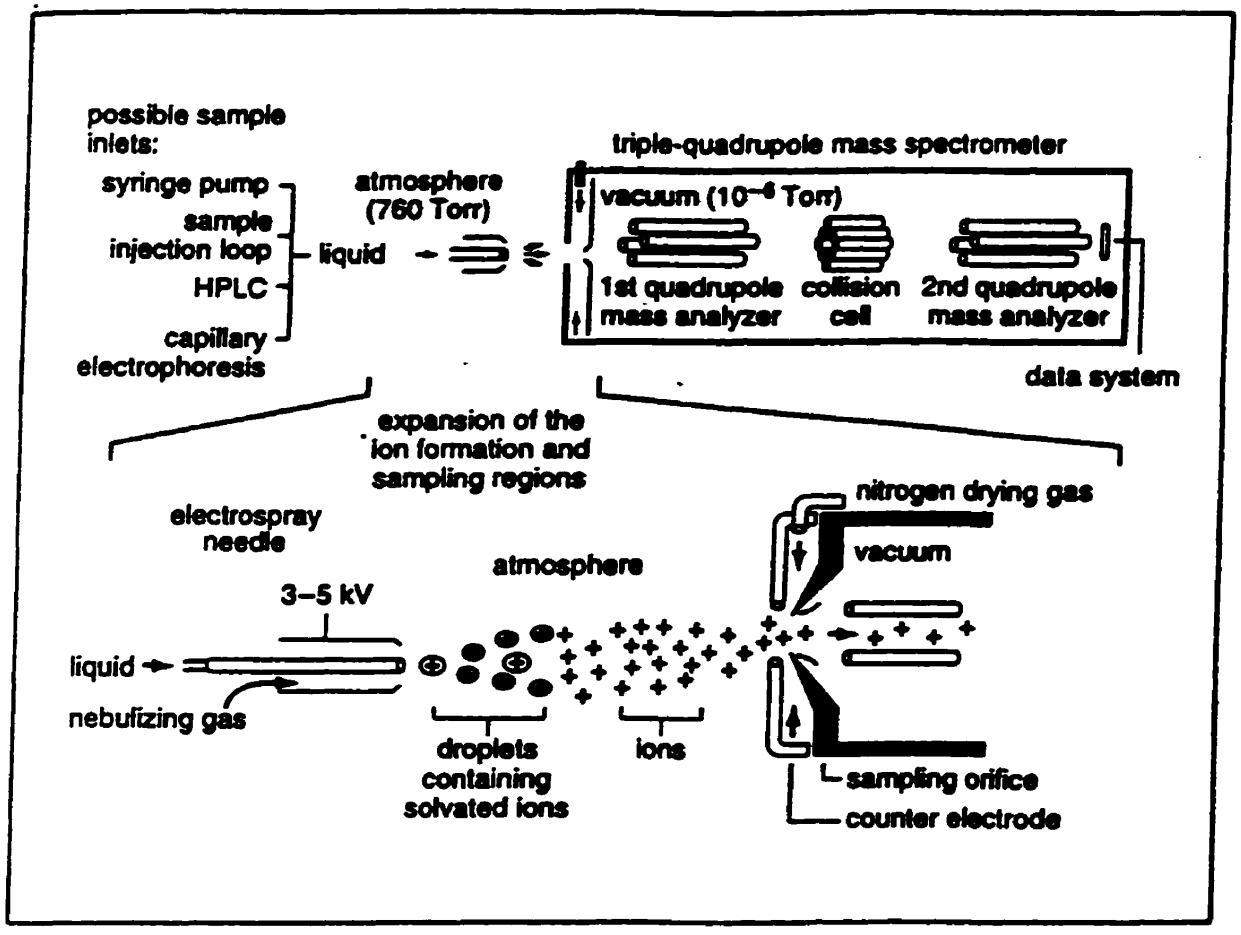


Figure 1. Schematic diagram of a triple-quadrupole mass spectrometer (top) and the electrospray interface (bottom) (Adopted from Carr and Annan, 1996)

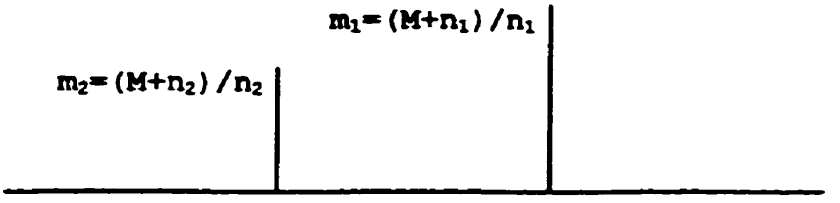
state and molecular weight. However, additional ions provide confirmation of the charge state as well as independent measurement of molecular weight. The basic theory for "Hyper Mass" function in API *III* for molecular weight determination using multiply charged ions is described in the following relationship:

If: $n_2 = n_1 + 1$

and assuming that the ions are adducts of the neutral molecule and protons

Then: $n_2 = (m_1 - 1)/(m_1 - m_2)$

$M = n_2 \times (m_2 - 1)$



While: n_1, n_2: the number of charges

m_1, m_2: mass to charge ratio

M: the calculated molecular weight

Sensitivity and speed of the MS-based approach such as tandem MS and on-line liquid chromatography/mass spectrometry using fast-atom bombardment or electrospray ionization have been integrated with more conventional techniques in order to increase the accuracy and speed of peptide and protein structure characterization (Carr *et al.*, 1991; Mollé and Léonil, 1995). Conversely, current techniques for structural analysis of peptide and proteins such as nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, are relatively time-

consuming and typically require milligram amounts of pure materials (Müir *et al.*, 1995).

3. Materials and Method

3.1. Origin of milk samples

From a previous study (Ng-Kwai-Hang *et al.*, 1990), Holstein cows enrolled in the Programme d'Analyse des Troupeaux Laitiers du Quebec were phenotyped by electrophoresis for the genetic variants of α_{s1}-casein, β-casein, κ-casein and β-lactoglobulin. In the present project, milk samples from identified cows were selected to provide representives of different genetic variants of milk proteins. It was possible to obtain α_{s1}-casein A, B, C; β-casein A^1, A^2, A^3 and B; κ-casein A and B in either the homozygous or heterozygous form.

3.2. Preparation of whole casein

Approximately 25 mL of fresh milk samples were collected from the selected cows and skimmed twice by centrifugation at 3000 X g for 15 min at 4°C and the top layer of fat was removed. The skim milk was adjusted to pH 4.6 by the addition of 1 M HCl at room temperature. The precipitated whole casein was separated out by centrifugation at 3000 X g and the supernatant which contained whey proteins was discarded. The coagulated whole casein was subsequently washed with deionized water and stored at -20°C pending future analysis. Prior to chromatographic fractionation of the whole casein, electrophoresis were performed on a subsample to ascertain the phenotypes for α_{s1}-casein, β-casein and κ-casein.

3.3. Determination of genetic variants of caseins by electrophoresis

Genetic variants of α_{s1}-casein, β-casein and κ-casein were determined by polyacrylamide gel electrophoresis in a vertical Bio-Rad minigel system (Bio-Rad Laboratories, Richmond, CA). The system contained two gel slabs with the dimension of 10 X 8 X 0.1 cm and having 10 sample loading wells each. The identification of genetic variants 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 separation gel contained 4.5 M urea; 8% acrylamide; 0.3% N,N'-methylene-bis-acrylamide; 0.025 M Trizma base; 0.065 M glycine; 0.003 M EDTA. The gel formation was promoted by the addition of 0.6% of freshly prepared 10% ammonium persulfate and 0.06% of N,N,N'-tetramethylethylenediamine. The whole casein samples were dissolved in sample buffer at the concentration of 50 mg/mL. The sample buffer had the following components: 4.5 M urea; 0.025 M Trizma base; 0.065 M glycine; 0.003 M EDTA and 2% β-mercaptoethanol. Two drops of 0.1% bromophenol blue which was used as tracking dye were added and mixed with every 10 mL of sampling buffer. For the electrophoresis, 3 μL of the solubilised whole casein sample were loaded into each well of the gel slab. Electrophoresis was performed with an electrode buffer (pH 8.3) having the same concentration of Trizma base, glycine and EDTA as in the polyacrylamide gel. Electrophoresis was carried out at a constant voltage of 100 V for 100 min.

After electrophoresis, the gel was removed between the glass plates and transferred into staining solution containing 40% methanol, 7% acetic acid and 0.1% Coomassie brilliant blue R-250 in distilled water. After staining for 10 min, the gel was carefully removed and transferred into 7% acetic acid solution for destaining by diffusion. The phenotyping of each casein component was assessed by visualization of the corresponding bands through transmitted light. Under the above conditions, α_{s1}-casein had the highest mobility followed by β-casein and κ-casein. Within α_{s1}-casein, the following order of migration was observed for the different variants: A > B > C. For β-casein, variant A moved faster than B and C. κ-Casein A had faster electrophoretic mobility than κ-casein B.

With polyacrylamide gel electrophoresis under alkaline conditions, it was not possible to differentiate A^1, A^2 and A^3 variants of β-casein. Therefore, electrophoresis under acidic conditions (pH 3.0) as described by Ng-Kwai-Hang *et al.* (1984) was performed to phenotype β-casein. The separation gel contained 4 M urea, 6% acrylamide; 0.22% N,N′-methylene-bis-acrylamide; 8.6% acetic acid; 2.5% formic acid. Polymerization was initiated with 0.23% ammonium persulphate and 0.65% TEMED. Approximately 50 mg of wet whole casein was completely dispersed in 1 mL of sample buffer containing 6.6 M urea, 0.2% reducing reagent β-mercaptoethanol and 0.5% methyl red as tracking dye. Under acidic conditions, β-casein carries net positive charges

and hence migrates toward the negative electrode. Electrophoresis was run with a constant voltage of 150 V until the tracking dye reached the bottom edge of gel slab. After staining and destaining, β-casein variants were visualized and had the following order of migration: $B>A^1>A^2>A^3$.

3.4. Protocol for determination of variants not detectable by electrophoresis

3.4.1. Chromatographic fractionation of whole casein

In the native state, casein exists as large micelle, and therefore, it is necessary to denature its tertiary structure before fractionation. Urea, a common protein denaturant was utilized throughout this study for this purpose. In order to avoid introduction of contamination from commercially purchased urea (J.T.Baker), 4.5 M urea solution was passed through a glass column containing 500g of mixing bed AG 501-X8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA). The solution was then filtered through a 0.45 micron pore size filter membrane (Whatman, Maidstone, England). Additional deionization of the urea solution was ascertained by pumping it through a QAE Zeta Prep 250 cartridge (LKB Produkter AB, Bromma, Sweden).

The isolation of individual casein from whole casein was performed on three different liquid chromatographic systems:

3.4.1.1. High performance liquid chromatography

The high performance ion-exchange chromatography was carried out on a Waters HPLC system which consisted of a 600E

multi-channel solvent delivery pump, a multi-wavelength detector and a Waters 745/745B data acquisation module.

Approximately 250 mg of thawed whole casein were dissloved in 5 mL of Tris-urea buffer which contained 5 mM Tris in 4.5 M urea solution. The casein solution was adjusted to pH 6.0 by dropwise addition of 1 M NaOH. Either 50 μL of β-mercapoethanol or 1 mg of DTT was added to the reconstituted solution one hour prior to chromatographic fractionation.

The separation of whole casein was ascertained on an AP-2 Protein PAK DEAE-15HR anion-exchanger packed inside a thick-wall glass column (20 X 2 cm, Waters Associates, Milford, MA). The HPLC separation was carried out as previously described (Ng-Kwai-Hang and Dong, 1994) with a gradient of sodium chloride from 0 - 0.4 M in Tris-urea buffer. For optimum separation, the pumping flow rate was maintained at 3.0 mL/min with a back pressure ranging from 300 to 350 psi. The eluant was monitored through a UV detector at 280 nm. Each chromatographic run lasted for 120 min including 10 min column equilibration with solvent A, 20 min increasing gradient up to 30% solvent B, 15 min linear gradient up to 40%, 65 min increasing gradient to 80% solvent B, 10 min decreasing gradient to 0% solvent B and a 10 min column reequilibration by 100% solvent A.

3.4.1.2 Fast protein liquid chromatography

Chromatographic separations of whole caseins were also carried out by a Fast Protein Liquid Chromatography (FPLC) system (LKB Lab, Uppsala, Sweden) coupled with the previously

described ion-exchange column. The system included a single wavelength detector at 280 nm, data acquisition device as well as a Frac-100 fraction collector (LKB Lab, Uppsala, Sweden). Elution was performed at a constant flow rate of 3.0 mL/min with a linear gradient of sodium chloride from 0 M to 0.4 M in the buffer containing 5 mM Tris and 4.5 M urea over a period of 70 min as previously described (Dong, 1992).

3.4.1.3. Bio-Rad Econo liquid chromatography

A method for large scale isolation of electrophoretically pure κ-casein, β-casein, α_{s2}-casein as well as α_{s1}-casein from 2 g of thawed whole casein was also developed using Bio-Rad Econo Liquid chromatography (Bio-Rad Laboratories, Richmond, CA). The system consisted of a programmable solvent delivery system, a single wavelength UV detector at 280 nm, a salt conductivity monitor, a fraction collector as well as a chart recorder which responded to UV absorbancy and salt conductivity. The whole casein fractionation was achieved by increasing NaCl from 0 M to 0.5 M in a stepwise manner in 5 mM Tris, 4.5 M urea solution at pH of 7.0. Approximately 2 g of wet casein which originated from 25 mL of fresh milk were reconstituted in 20 mL of Tris-urea buffer and adjusted to pH 7.0 by dropwise addition of 1 M NaOH. Then 5 mg of DTT was added to the casein solution one hour prior to loading. The casein solution was loaded onto a glass column (25 X 2.5 cm) packed with Macro 50Q anion exchanger (Bio-Rad Laboratories, Richmond, CA). The whole casein separation was also achieved on High Q anion exchanger from the same supplier.

A sequential stepwise elution procedure for different casein components was carried out with the following NaCl concentration: 0.10 M, 0.15 M, 0.20 M and 0.25 M in Tris-urea buffer. The flow rate was set at 3 mL/min and each of the elution with each salt concentration lasted for 30 min. Prior to the whole elution procedure, there was a 30 min equilibration step with buffer containing 0 M NaCl as well as a 15 min clean up step with 0.5 M NaCl Tris-urea buffer after each run. Absorbance of the eluant was monitored at 280 nm and fractions corresponding to each absorption peak were collected individually by the fraction collector. κ-Casein eluted faster than β-casein which eluted faster than α_{s2}-casein and α_{s1}-casein.

3.4.2. Concentration and characterization of casein fractions

Fractions corresponding to each type of casein were collected according to their chromatographic peaks and elution times. The concentration and desalting of each casein fractions were achieved by a model 8400 ultrafiltration cell (Amicon Division, Danvers, MA) fitted with YM10 membrane which has a molecular weight cut-off of 10000 dalton (Whatman, Maidstone, England). The liquid flowed through the cell by a stream of nitrogen at 40 psi with constant stirring. Each fraction from HPLC and FPLC separation was concentrated to a final volume of approximately 1 mL while the final sample volume from large scale separation in Bio-Rad system was 5 mL.

The identity and purity of each casein sample was ascertained by polyacrylamide gel electrophoresis under alkaline

conditions (pH 8.3) in the presence of urea and β-mercaptoenthanol as previously described. A volume of 10 μL of the concentrated casein fraction was diluted with the same amount of sample buffer as described before. For band identification, a standard whole casein sample was loaded together with individual casein fractions on the same gel slab. The electrophoresis was performed at a constant voltage of 100 v until the tracking dye reached the bottom end of the gel. After staining and destaining, casein fractions exhibited the following relative order of migration: α_{s0}->α_{s1}->α_{s2}->β->κ-casein.

3.4.3. Enzymatic hydrolysis of caseins

Exactly 10 mg of trypsin crystallized powder from bovine pancreas (ICN Biochemicals, Cleveland, OH) were weighed and dissolved in 100 mL of pH 7.44 phosphate buffer which contains 0.04 M Na_2HPO_4 and 0.01 M KH_2PO_4. The enzyme working solution was diluted five folds with the same phosphate buffer. The isolated, desalted and concentrated casein fractions were mixed with the enzyme solution in the ratio of 1:1 (v:v) for κ-casein, 3:1 (v:v) for β-casein as well as α_{s1}-casein. The final volume for the mixture was 400 μL.

Each casein/enzyme preparation was thoroughly mixed using a vortex mixer and then incubated for 30 h in a water-bath set at 35°C. It was observed that the extent of hydrolysis of the individual caseins as indicated by maximum number of peptides was reproducible under those conditions. The hydrolysis reaction

was stopped by decreasing the pH of the reaction mixture to 2.2 with the addition of 10 µL 10% trifluoroacetic acid. After allowing the hydrolysate to stand at room temperature for one hour, the mixture was centrifuged at 10000 rpm for 15 min in order to separate out the residual protein which was not hydrolysed. Then the supernatant containing a mixture of peptides was transferred to a 1.8 mL glass autosampler vial pending further analysis by HPLC.

3.4.4. Analysis of casein hydrolysates

Casein hydrolysates obtained from the above procedure were analysed by reversed-phase high performance liquid chromatography. The separation of the different peptides was achieved by using a set-up comprised of a Waters 600E multisolvent delivery system, a multiwavelength 490E UV detector, a Waters 745/745A data integrator, an in-line C-18 guard column, a reversed-phase µBondapak phenyl SS analytical column (3.9 X 300 mm, 10 micron, Waters Division, Millipore Corp., Milford, MA). The system was automatically operated via a programmable Varian 9090 autosampler (Walnut Creek Division, CA).

The solvents used during reversed-phase separation were prepared from deionized water which were obtained from NANO ultrapure water system (Barnstead Thermolyne Corp., Dubuque, IW). The eluant A contained 0.1% trifluoroacetic acid (TFA) in deionized water and solvent B contained 60% acetonitrile in 0.1% TFA. Solvent A and B were degassed with helium at a flow rate of

100 mL/min for at least 15 min run and filtered through 0.45 μm pore size filters prior to the chromatographic run.

Exactly 150 μL of casein hydrolysate was applied via the automatic injector into reversed-phase column. Profile of casein hydrolysate was monitored at wavelength of 280 nm. Operating flow rate was maintained constantly at 1.0 mL/min while the temperature of column was maintained at 40°C with the performing back pressure ranging from 800 to 900 psi. The optimum separation conditions were determined according to the maximum resolution of the mixture of peptides from hydrolysates. The column was equilibrated by passing through solvent A for 5 min. After sample injection, the peptides were eluted by a linear gradient of 0% to 100% solvent B within 75 min. Elution with 100% solvent B was maintained for 5 min as a clean-up procedure. This was followed with a shift from 100% B to 0% B in 2 min and a completion of column re-equilibration with 100% A for 5 min before the next sample injection.

Peptide maps were classified according to pre-established electrophoretic phenotypes. In order to avoid confusion of tryptic peptides from heterozygous phenotype, only the samples with homozygous variants were carefully compared. Typical peak retention time corresponding to each peptide from hydrolysate of α_{s1}-casein BB; β-casein A^1A^1, A^2A^2, BB; κ-casein AA and BB were recorded and used as reference. The standard profiles for each casein category were selected by comparison of peptide maps with the same electrophoretic phenotype. The sample corresponding to the most representative elution pattern was chosen for each

casein type. To establish the identity of individual peaks generated by peptide mapping, each peaks collected from standard tryptic mapping was dried immediately under vacuum and stored at -20°C. The identification of each standard peaks was achieved by amino acid composition as well as mass analysis. The comparison with previously described casein elution pattern was also employed in this study.

The aberrant profile was also identified during the comparison with standard profile with the same electrophortic pattern. The tryptic hydrolysates from aberrant samples were rechromatographed and peaks with aberrant retention time were collected and vacuum dried prior to further analysis. In this case, the identity of aberrant peptide fragment was established by a combination of the results from analysis of amino acid composition, molecular weight determination by mass spectrometry and amino acid sequencing.

Peptide mapping of tryptic casein fragments were also ascertained by a Inertsil 150A/ODS2 (0.46 x 10 cm, 5 micron, CSC, Montreal) reversed-phase column at the flow rate of 0.7 mL/min. After the injection of 60 µL hydrolysate, the program was initialized by holding at 100% solvent A (5% acetonitrile in water with 0.1% TFA) for 5 min. Separation gradient consisted of increasing to 30% solvent B in 40 min. Then, solvent B was linearly increased to 80% in 5 min and then 100% in 2 min. After return to 100% A in 2 min, the column was then equilibrated with solvent A for 5 min prior to next injection. The total analysis time was 60 min.

3.4.5. Mass spectrometry

Mass spectrometry analysis of whole casein and selected peptides from casein hydrolysates were performed on a Sciex API *III* triple-quadrupole instrument (Sciex, Toronto). Only the first quadrupole was used for determination of the mass to charge ratio. Mass scale calibration was carried out using the multiple charged ions of a standard lysozyme which has a molecular weight of 14305. Quantitative analysis of lysozyme was performed by integration of the multiple charged ions of the single specie, the acceptance criteria was 14305 ± 2. The instrument Q1 scan was operated over a mass range of m/z from 200 to 2500. The dry peptide fractions in the eppendrof tubes were reconstituted in 20 µL of 10% acetic acid prior to injection and loaded into the ion source at a flow rate of 2 µL/min by using a syringe pump (Harvest Apparatus, MA) via a pneumatically assisted electrospray interface. All spectra were recorded using an interface sprayer at a potential of 5000 V. Positive ions were generated by the ion-spray process and entered the mass spectrometer through a 100 µm orifice at an interface potential of 650 V. All data acquisition was achieved on an Macintosh II computer equipped with the software package Mac Spec 3.22.

A computer search algorithm "Mac Pro Mass" (Terri Lee, City of Hope, Duarte, CA) was used to identify protein sequences in the previous established database with peptide mass information obtained from tryptic digests of caseins. The

average mass of each tryptic peptide of casein was used to locate peptide fragment on the whole sequence.

3.4.6. Determination of amino acid composition

The Pico-Tag method using precolumn phenylisothio-cyanate (PITC) derivatization and liquid chromatography was employed for the determination of amino acid composition in tryptic peptides (Sarwar *et al.*, 1988).

Peptide from Fibronectin type III connecting segment (peptide 1 - 25, Sigma, St-Louis, MO) contains amino acids comparable to the casein peptide of interests, especially a high proportion of Pro, was used as a reference peptide to determine recovery for all amino acid residues. In order to have an adequate sample size for analysis, it was necessary to pool the fraction corresponding to a peptide of interests from six different chromatographic separation runs as previously described (Section 3.4.4.). The pooled fractions were collected in a 6 x 50 mm culture tube and dried under vacuum. Exactly 200 µl of 6 N HCl were added and solution was well mixed by a vortex mixer. Then, one drop each of phenol, β-mercaptoethanol and octanoic acid were added and the mixture was flash frozen in liquid nitrogen and followed by thawing under vacuum in order to release the dissolved oxygen. The tube was sealed and maintained at 110°C for 24 h. After cooling down to room temperature, the hydrolysate was passed through a 0.45 µm pore size filter by centrifuging at 3000 rpm for approximately 5 min.

A mixture consisting of 10 µL of hydrolysate and 10 µL of norleucine as internal standard were transferred and evaporated to dryness under vacuum in a 5 x 50 mm culture tube. Then, 50 µL of reducing solution containing methanol, water, and triethylamine (TEA) in the proportion of 2:2:1 were added. The contents of the tube were well mixed and followed by evaporation under vacuum at room temperature. The dry substrate at the bottom of the tube was dissolved in 50µL of PITC derivatization reagent containing methanol, water, TEA, and PITC in the ratio 7:1:1:1 and the content were evaporated to dryness under vacuum. Finally, the sample was reconstituted in 200 µL of sample diluent for HPLC analysis (Waters Cooporation) and centrifuged at 3000 rpm for 5 min to settle the precipitates. The supernatant was transferred into an insert located in a sample injection vial pending analysis by HPLC.

The chromatographic system was coupled with a Waters Pico-Tag amino acid analysis column (0.39 x 15 cm) being maintained at 38°C and a UV detector monitored at 254 nm. The mobile phase A consisted of 60 mL of acetonitrile and 940 mL of mixture which contained 19.0 g sodium acetate, 500 µL of TEA with pH adjusted to 6.4 by the addition of acetic acid. The mobile phase B was made up of 60% acetonitrile and 40% water. The gradient are listed in the Table 1 which consisted of a 13.5 min gradient separation at the flow rate of 1.0 mL/min and a 7 min equilibration with mobile phase A for the next analysis. The total running time was 20.5 min.

Table 1. Separation Gradient for Pico-Tag Amino Acid Analysis

Time (min)	Flow rate (mL/min)	A%	B%	Curve*
0.0	1.0	100	0	5
1.0	1.0	88	12	6
3.0	1.0	80	20	6
11.0	1.0	60	40	6
11.5	1.0	25	75	6
13.0	1.0	0	100	6
13.5	1.5	0	100	6
14.0	1.5	100	0	6
20.0	1.5	100	0	6
20.5	1.0	100	0	0

* 5=concave; 6=linear

The application of the PITC derivatization, however, can provide satisfactory resolution for most of the amino acid residues except for Trp, Met as Met sulfate and Cys as cysteic acid. Herein, Trp needs basic hydrolysis as well as a decrease in column temperature (Sarwar *et al.*, 1988; Marty and Chavez, 1995).

3.4.7. Amino acid sequencing

The N-terminal sequencing of the reference and aberrant peptide of β-casein A^1 fragment 114-169 was carried out by automated Edman degradation method on an Applied Biosystem protein sequencer. In order to achieve enough quantity, twenty collections of the aberrant peptide were pooled and dried under vacuum. Briefly, each degradation cycle consists of three steps: coupling, cleavage and conversion as described by Allen (1981). In the coupling step, the N-terminus of the peptide is modified by coupling with phenyl isothiocyanate under basic conditions and formed a phenylthiocarbamoyl (PTC) peptide. In the following step, the PTC-peptide is cleaved from polypeptide by trifluoroacetic acid (TFA) to generate an anilinothiazoline (ATZ)-amino acid derivative of the original N-terminal residues. During the conversion step, the unstable ATZ-amino acid is converted to stable phenylthiohydantoin (PTH)-amino acid by acid. At the same time, the new N-terminus for the (n-1) peptide is available for the next cycle.

4. Results and Discussion

4.1. Identification of genetic variants of casein by electrophoresis

A total of 635 individual milk samples from Holstein-Friesian were used for this study. Electrophoretic variants of caseins were determined by polyacrylamide gel electrophoresis under alkaline and acidic conditions. Based on the alkaline separation, four casein components have the relative migration order of α_{s1}->α_{s2}->β->κ-casein. Electrophoresis under acidic condition was performed to differentiate A^1, A^2 and A^3 variants of β-casein. Most of the major phenotypes for caseins had been observed in this study and they are included the following: α_{s1}-casein AB, BB, BC; β-casein A^1A^1, A^1A^2, A^2A^2, A^1B, A^2B, A^1A^3; and for κ-casein AA, AB, BB.

4.1.1. α_{s1}-Casein

The typical result of electrophoretic separation of whole casein under alkaline condition is shown in Figure 2. In alkaline pH, α_{s1}-casein carries more negative charges than α_{s2}-casein and β-casein which are followed by κ-casein while genetic variants of α_{s1}-casein exhibit the relative mobility of A>B>C. As indicated in Figure 2, 9 out of 10 individual whole caseins have homozygous BB type while whole casein sample in the lane 7

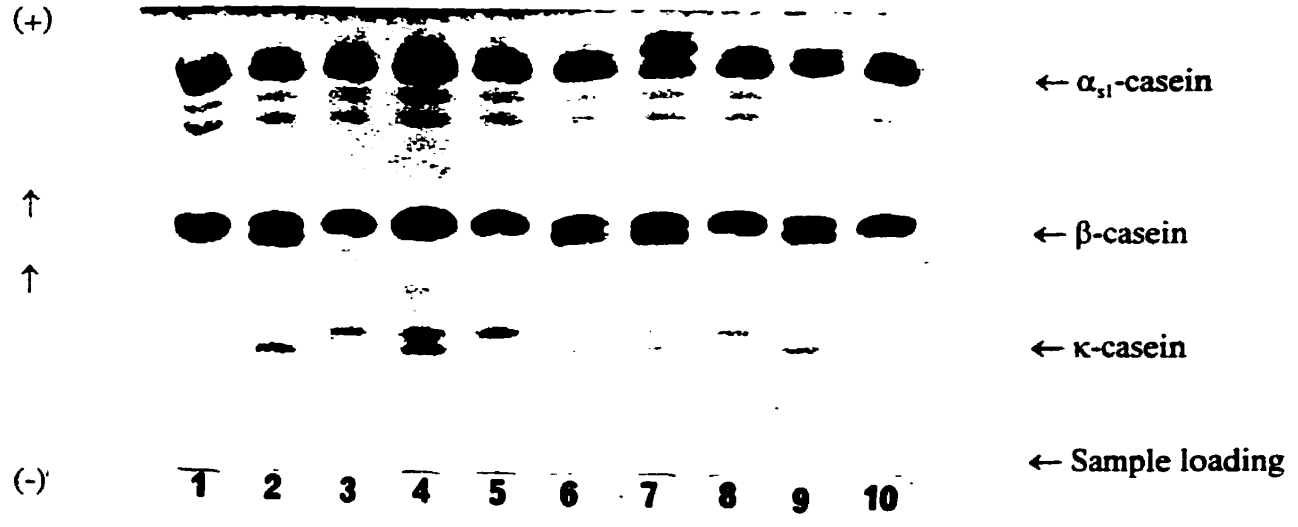


Figure 2. Phenotyping whole casein by polyacrylamide gel electrophoresis under alkaline condition

	1	2	3	4	5	6	7	8	9	10
α_{s1}-casein:	BB	BB	BB	BB	BB	BB	AB	BB	BB	BB
β-casein:	AA	AB	AA	AA	AA	AB	AB	AA	AB	AA
κ-casein:	AB	BB	AA	AB	AA	AB	AB	AA	BB	AB

contains heterozygous AB for α_{s1}-casein. The reason of this resolution is based on the difference of net charges of these variants since there is a 13 amino acid residues deletion from position 14 to 26 in A variant. This deletion gives rise to a distinctive negative charge when compared with B variant which consists of a positive residue Arg at position 22. As rare variant, α_{s1}-casein C was also observed as heterozygous BC type in this study which has a slower mobility than B variant, however, it is not shown in Figure 2.

4.1.2. β-Casein

The genetic variants of ß-casein are resolved by both alkaline and acidic gel electrophoresis. Under alkaline conditions, β-casein A variant could be distinguished from B variant by having a faster mobility. In Figure 2, the samples at lane 2, 6, 7 and 9 show two identical bands which represent β-casein AB type while samples at lane 1, 3, 4, 5, 8 and 10 have single band which belong to β-casein AA type. No differentiation of β-casein A^1, A^2 and A^3 is possible due to their similar mobility in the electric field. In order to unambiguously identify the nature of β-casein A variants, it is necessary to perform electrophoresis under acidic condition. Figure 3 shows the separation of β-casein variants on acidic electrophoretic gel. It is observed that the samples at lane 2, 3, 4, 8, 9 and 10 have two bands and thus would be classified as heterozygous

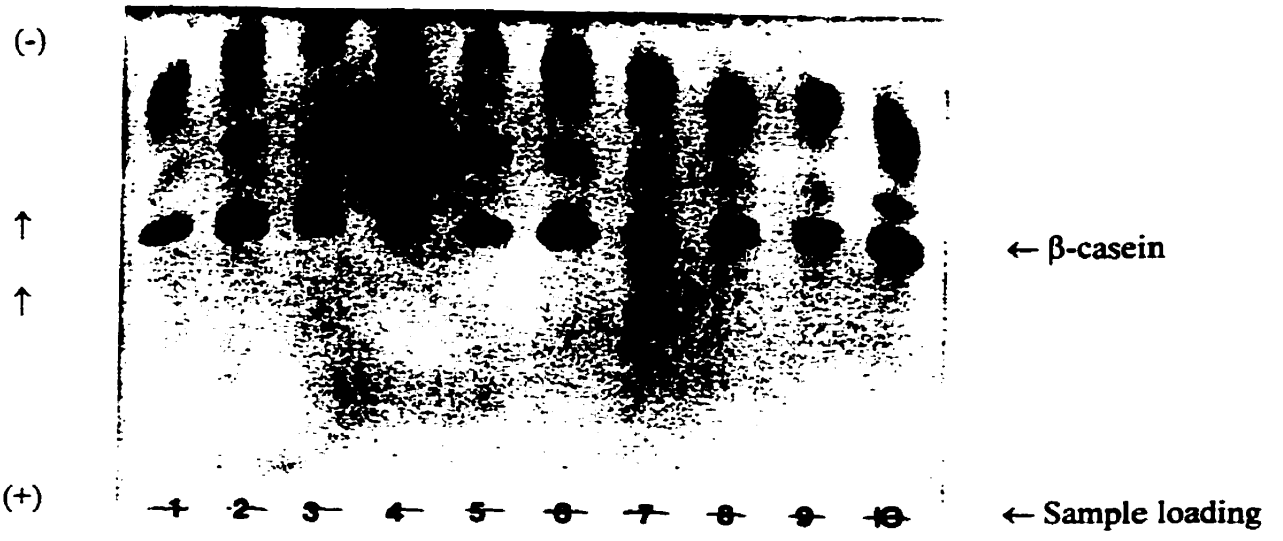


Figure 3. Phenotyping β-casein by polyacrylamide gel electrophoresis under acidic condition

	1	2	3	4	5	6	7	8	9	10
β-casein:	A^1A^1	A^1A^2	A^1A^2	A^1A^2	A^2A^2	A^2A^2	A^2A^2	A^1A^2	A^1A^2	A^1A^2

β-casein A^1A^2. The sample at lane 1 has single band which has faster mobility when compared with samples at lane 5, 6 and 7. Therefore, it could be phenotyped as homozygous A^1A^1 while samples at lane 5, 6 and 7 could be termed as β-casein A^2A^2. The difference of mobility of β-casein A^1 and A^2 is due to the amino acid substitution at the position 67 where A^1 has a positive residue His and A^2 has a Pro residue which belongs to neutral amino acid. Therefore, variant A^1 moves faster toward anode than A^2 under acidic condition. Other variants such as β-casein A^3 and B were observed in this study with relative mobility of $B>A^1>A^2>A^3$. The separation of β-casein A^3 and B was not included in Figure 3. Similar to previous result from the same population, no β-casein C, D and E variant was identified during the study (Ng-Kwai-Hang *et al.*, 1986).

4.1.3. κ-Casein

κ-Casein has the lowest mobility when compared with α_{s1}-casein and β-casein in alkaline pH. As shown in Figure 2, samples in lane 1, 4, 6, 7 and 10 consist of double bands and could be classified as heterozygous AB type. Samples in lane 3, 5 and 8 contain single faster moving band as termed as homozygous AA. At the same time, samples in lane 2 and 9 have single band which has slower mobility and therefore could be recognized as BB type. The difference of mobility of κ-casein A and B variants is due to the amino acid substitution at position

148 where A has a negative residue Asp and B consists of neutral residue Ala. Under alkaline condition, A variant carries one more negative charge than B variant, therefore, A moves faster toward the positive side of electric field than B variant.

Successful separation of κ-casein A and B variants are based on the presence of reducing agent which catalyzes the disulfide bond between κ-casein polypeptide. As clearly observed in lane 1, 6 and 7, B variant appears to be more intense than A variant on the basis of band density. The difference has been attributed to uncertain gene regulation which results in the difference in gene expression of κ-casein two alleles. Also, κ-casein appears to be composed of several bands above two major electrophoretic bands as indicated in most of the samples. It is suggested that the origin of this heterogeneity is depended on multiple glycosylation and probably phosphorylation groups.

4.2. Frequency distribution of electrophoretic variants.

As noted by previous investigation, the distribution of difference electrophoretic variants of caseins are closely related to variation of breed, geographic location as well as production trait adopted (Aschaffenburg, 1968; Ng-Kwai-Hang and Grosclaude, 1992; Kim, 1994; Kim *et al.*, 1996, 1998). The summary of the occurrence of electrophoretic variants among a total of 635 Holstein cows involved in this study are shown in Table 2.

Table 2. Frequency Distribution of Genetic Variants of Major Caseins

Proteins	Phenotype	Number	Percent
α_{s1}-Casein	AB	1	0.16
(632)	BB	627	99.2
	BC	4	0.63
ß-Casein	A^1A^1	158	24.9
(634)	A^1A^2	283	44.6
	A^2A^2	128	20.2
	A^1B	33	5.21
	A^2B	27	4.26
	A^1A^3	1	0.16
	A^3A^3	1	0.16
	BB	3	0.47
κ-Casein	AA	415	65.4
(635)	AB	198	31.2
	BB	22	3.7

4.2.1. α_{s1}-Casein

With the accordance of previous literature, α_{s1}-casein BB is predominant in Holstein and accounts for 99.2% of 632 samples analyzed as indicated in Table 2. As rare variants, α_{s1}-casein A and C were only observed in heterozygous type with low frequency of 0.63% for BC and 0.16% for AB phenotypes respectively. All these results are closely in agreement with previous finding by Ng-Kwai-Hang *et al.* (1986) that α_{s1}-casein BB, BC and AB consisted of 94.03%, 5.38%, and 0.59% respectively in a total of 2045 Holstein cows.

4.2.2. β-Casein

Among 634 individual cows being tested in this study, four variants of β-casein A^1, A^2, A^3 and B with 8 different phenotypes were observed. β-Casein A^1A^1 and A^2A^2 account for 24.9% and 20.2% of the population respectively. β-Casein A^1A^2 occurs with the highest frequency and represents 44.6%. β-Casein A^1B, A^2B and BB had frequencies of 5.21%, 4.26% and 0.47% respectively. There was no observation for β-casein A^2A^3 in this group while there was a single observation for A^3A^3 and A^1A^3 was identified. β-Casein B was also observed in homozygous and heterozygous types such as A^1B, A^2B and BB. These findings are in accordance with previous reports (Aschaffenburg, 1968, Ng-Kwai-Hang *et al.*, 1986).

4.2.3. κ-Casein

It is known that variant A of κ-casein is predominant in most of the western breeds except for Jersey in which B variant is found to be predominant (Ng-Kwai-Hang and Grosclaude, 1992, Kim, 1994). As indicated in Table 2, among 635 samples being investigated in this project, κ-casein AA, AB and BB phenotypes consist of 65.4%, 31.2% and 3.46% respectively. In addition, the overall frequency of A and B variants account for 80.94% and 19.05% respectively which are similar to those from previous investigation (Ng-Kwai-Hang *et al.*, 1986).

4.3. Chromatographic separation of caseins

All the whole casein samples isolated from milk of 635 individual cows were subjected to ion-exchange chromatography using either gradient or stepwise elution procedure.

4.3.1. Fractionation of whole casein by gradient elution

The fractionation of whole casein was performed on a DEAE cellulose anion-exchange column by HPLC and FPLC. The typical elution pattern is presented in Figure 4. The optimal condition for reproducible result was achieved by using Tris-urea buffer at pH 6.0 and a linear increasing of NaCl molarity in the mobile phase as previously described (Dong, 1992; Ng-Kwai-Hang and Dong, 1994). Due to the strong tendency of aggregation for casein micelle, a sampling buffer containing 4.5M urea as dissociation agent was utilized in order to promote dissociation

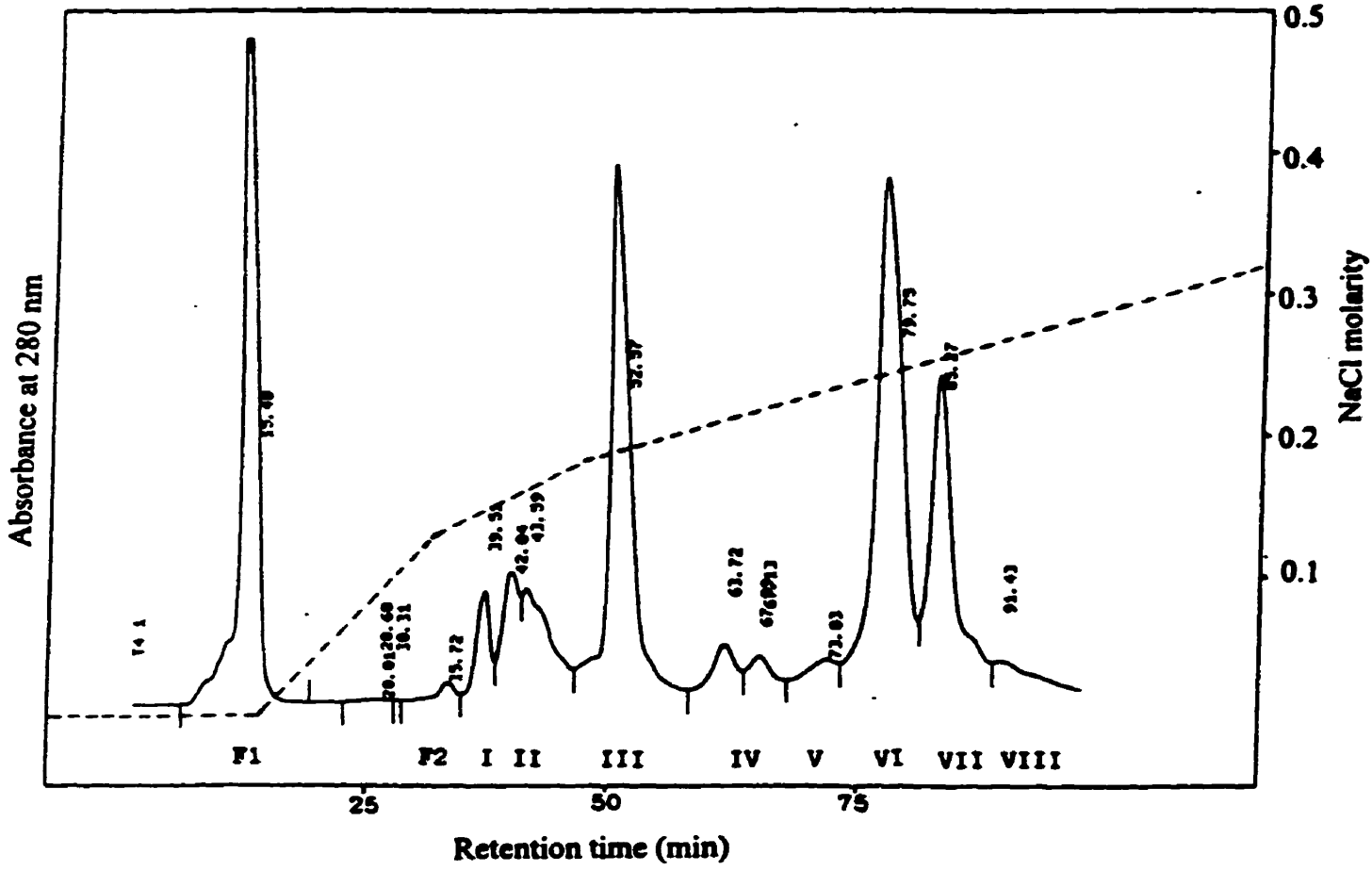


Figure 4. Fractionation of whole casein by gradient elution ion-exchange chromatography

of micellar components. At the same time, due to the presence of disulfide bond between α_{s2}-casein and κ-casein, it is also necessary to add reducing agent to the alkalized sample prior to fractionation. The addition of reducing agent prevents the formation of casein complex as well as κ-casein dimer.

Elution characteristic of proteins is quite dependent on their net charge under the performed pH. Elution of protein from anion exchanger is ascertained by increasing the concentration of chloride ion. Figure 4 shows the elution profile of whole casein fractionation by NaCl gradient. In the present study, the gradual increase of NaCl molarity from 0 to 0.5M gives rise to 8 well-defined casein fractions denoted as I to VIII according to their relative elution order. Their identity and purity were determined by polyacrylamide gel electrophoresis with the standard whole casein on the gel. The first peak, denoted as F1, eluted before NaCl gradient corresponds to the reducing agent and unbound component from loaded sample. The first peak eluted by salt gradient, denoted as F2 consists of proteolysis component in casein such as γ-casein which was considered as breakdown product of β-casein polypeptide. The representative electrophoresis gel with regard to identification of casein fractions is shown in Figure 5. According to their relative migration when compared with whole casein sample on the gel, fraction I and II contained κ-casein, fraction III corresponds to β-casein, fractions IV and V represent minor α_s-casein (α_{s2}-, α_{s1}- and α_{s6}-casein) while fraction VI represents for α_{s1}-casein.

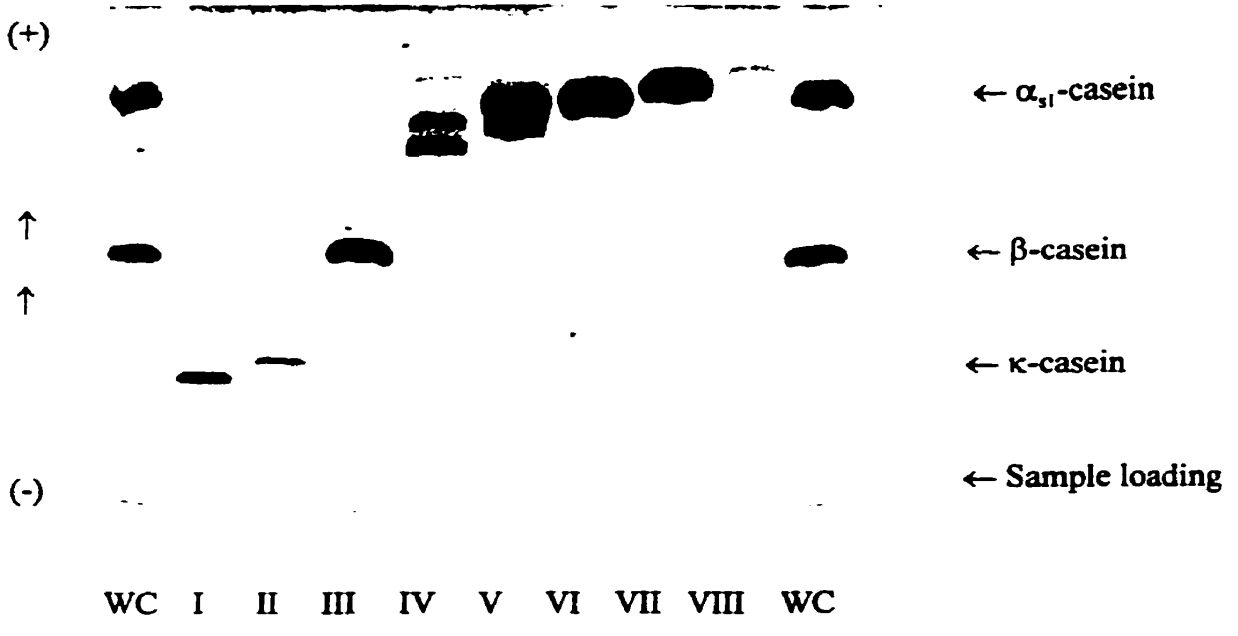


Figure 5. Characterisation of casein fractions by polyacrylamide gel electrophoresis

Fraction VII shows the presence of α_{s0}-casein. The last fraction VIII is probably attributed to small amount of non-reduced κ-casein or to a complex between κ-casein and α_{s2}-casein due to intermolecular -s-s- linkage. It was implied that all the major casein components (fractions I, II, III and VI corresponding to κ-casein, β-casein and α_{s1}-casein respectively) are in electrophoretically pure forms.

For the κ-casein fraction, electrophoresis indicates the resolution of multiple bands which are due to different degree of glycosylation. This also implies that 5 or 6 κ-casein subcomponents can be distinguished under the described condition.

4.3.2. Fractionation of whole casein by stepwise elution

The fractionation of approximately 2.0 g precipitated casein was achieved on an Bio-Rad Econo LC system equipped with a Macro 50Q anion-exchanger packed inside a glass column. The casein separation was performed by five elution procedures with increasing molarity of NaCl in Tris buffer containing 4.5M urea. Isolation of large quantities of casein components was achieved by stepwise elution with 0.1 M, 0.15 M, 0.2 M, 0.25 M and 0.5 M NaCl eluant with initial equilibrium and reequilibrium by salt-free mobile phase. The typical elution profile is presented in Figure 6. The identity and purity of each casein fraction was ascertained by electrophoresis which exhibits similar pattern as that shown in Figure 5. The result showed that κ-casein was

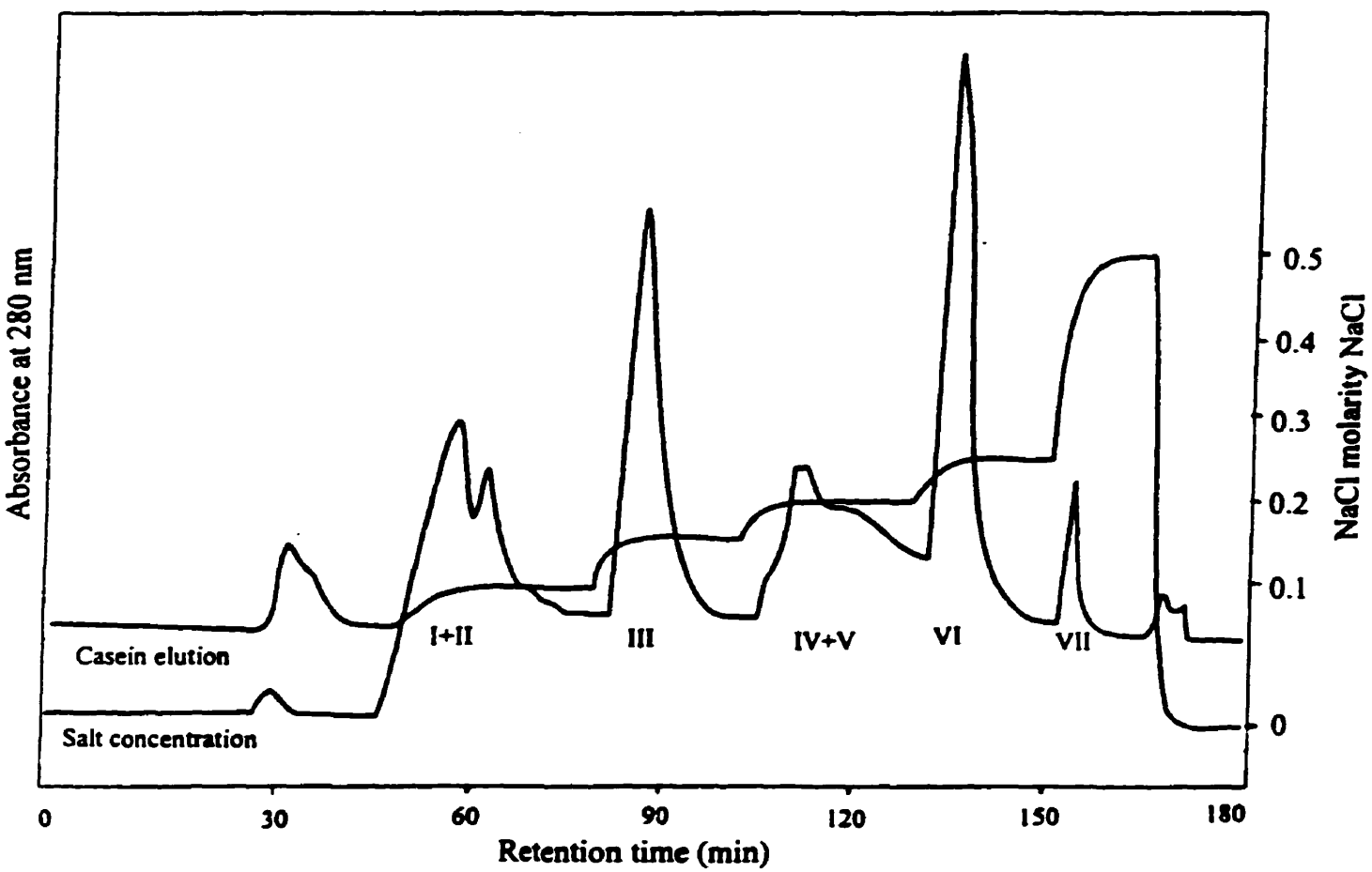


Figure 6. Fractionation of whole casein by step-wise elution ion-exchange chromatography

eluted by the Tris-urea buffer containing 0.1M NaCl. β-Casein, α_{s2}-casein and α_{s1}-casein were eluted by the buffer containing 0.15M NaCl, 0.20M NaCl and 0.25M NaCl respectively. An appropriate ratio of 1:3:1:3 was observed for the relative proportion of peak area of κ-casein, β-casein, α_{s2}-casein and α_{s1}-casein. This described technique was found to provide enough resolution for all major caseins components of κ-casein, β-casein, α_{s2}-casein and α_{s1}-casein in electrophoretically pure forms. Thus, it could be performed in the range of gram quantity of precipitated whole casein.

4.3.3. Separation genetic variants of casein by anion-exchange chromatography

Chromatography of proteins on ion-exchanger involves the establishment of multiple electrostatic bonds between ionized groups on the surface of the exchanger and opposite charges on the protein, followed by a selective release of these bonds by changing in the concentration of Cl^- or pH of the eluant (Yaguchi and Rose, 1972). According to Swaisgood (1982), charges for different casein variants at pH 6.6 range from -20.0 to -22.6 for α_{s1}-casein; -18.0 to -13.2 for α_{s2}-casein; -12.8 to -8.2 for β-casein and -3.9 to -3.0 for κ-casein respectively. These phenomenon would be used to explain the relative elution order for different casein components (κ-casein > β-casein > α_{s2}-casein > α_{s1}-casein).

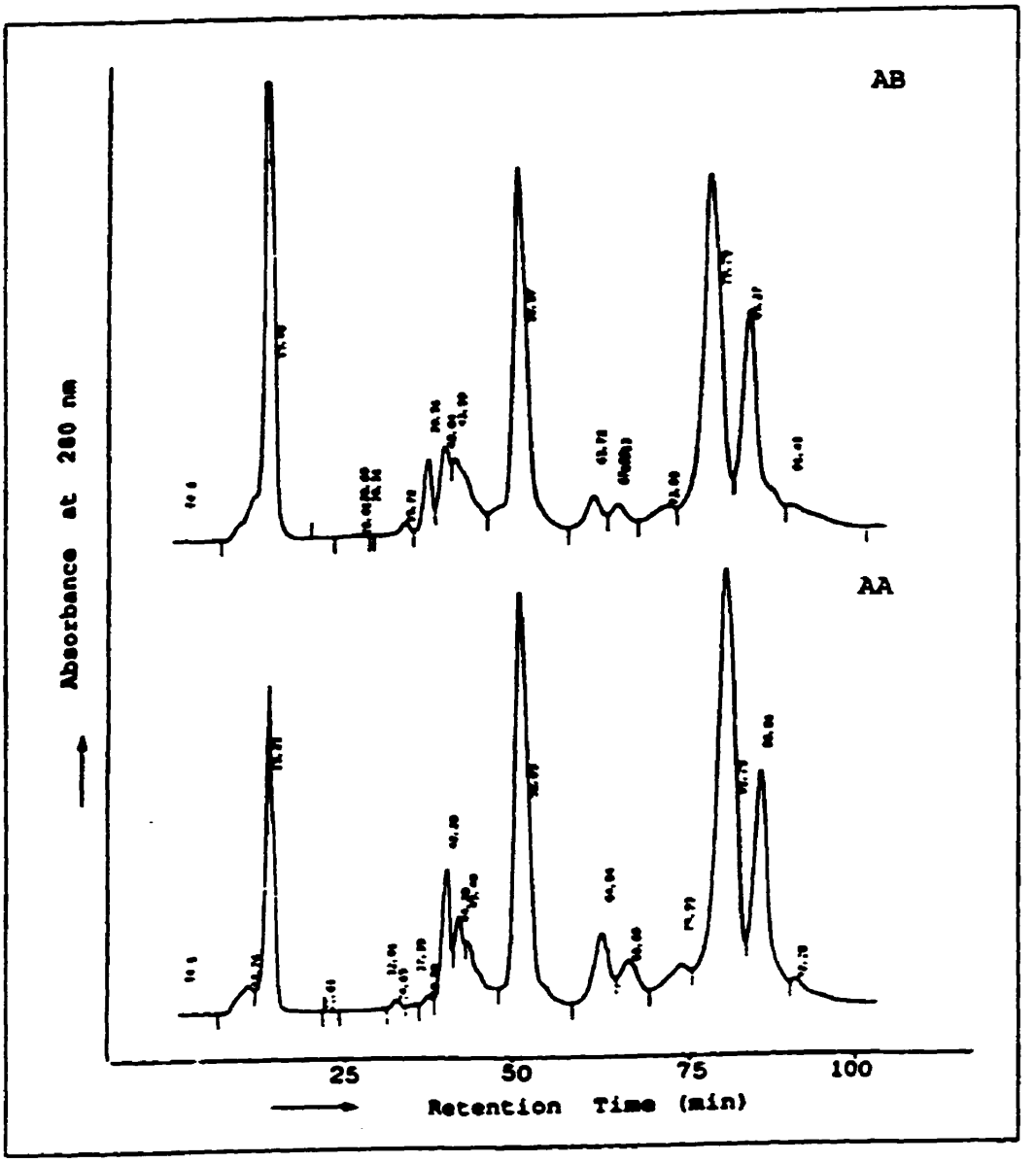


Figure 7. Identification of genetic variants of κ-casein A and B by ion-exchange chromatography

Upon the comparison of elution profile of κ-casein AA with κ-casein AB samples in Figure 7, it is observed that B variant exhibits an extra peak in the front of major κ-casein fraction. In order to unambiguously prove its identity, electrophoresis at alkaline pH was carried out for this collected fraction. The difference in migration behavior confirmed that B variant κ-casein eluted faster than A variant. The influence of genetic variants of κ-casein A and B on the elution pattern could be ascribed to the fact that A variant carries one more negative charge than B variant due to amino acid substitution at position 148 from Ala (in B variant) to Asp (in A variant). Therefore, the latter variant was retained stronger and had longer retention time during elution. The origin of heterogeneity of κ-casein in fraction II (Figure 6) were also dependent on the existence of glycosylation such as N-acetylneuraminic acid groups which mainly attached to Thr residues at positions 131 and 133 in the κ-casein polypeptide.

The separation of genetic variants of β-casein and α_{s1}-casein by chromatographic methods was somewhat less satisfactory. No consistent separation was achieved for the differentiation of β-casein genetic variants since the differences of net charges between the variants are too slight to be separated by column chromatographic method. Thus, the differences between A^1, A^2 and A^3 polymorphs in β-casein are not significant enough for separation by chromatographic separation.

Partial resolution were observed in some samples for β-casein A and B variants during gradient elution. For the same reason, no significant resolutions of α_{s1}-casein A, B and C variants were observed.

4.3.4. Recovery and purity of caseins

Based on the elution profiles of chromatographic separation of whole casein and the results of electrophoresis of the collected fractions, all the major caseins were found to be pure. Also, they were quantitatively recovered as indicated by the values of integrated areas corresponding to each peak. Correction are made for differences in absorbencies of the individual caseins. According to Swaisgood (1982), there are differences in the absorptivities for casein components at 280 nm. The absorptivities are 0.46 cm^2/mg for β-casein, 0.95 cm^2/mg for κ-casein, 1.05 cm^2/mg for α_{s1}-casein B and 1.11 cm^2/mg for α_{s2}-casein. The relative proportion of κ-casein:β-casein:α_{s2}-casein:α_{s1}-casein were 1:3:1:3 which agree with values quoted in the literature.

With regard to casein fractionation, the described methods for separation by HPLC and FPLC with a gradient of NaCl appear to be promising methods based on the high degree of purity of the four casein fractions. These procedures could also be used for the quantitative estimation of the four caseins in whole casein samples due to the good resolutions obtained.

Separation of casein components by elution with different concentrations of NaCl in a stepwise method is also of interests for obtaining individual casein in a pure form as well as in relatively large quantity. With this method, α_{s2}-casein could be distinctly separated from α_{s1}-casein. Most of the chromatographic techniques described in the literature vary in their ability to separate those two casein components.

The application of column chromatography for the identification of genetic variants in caseins is limited since it is more tedious and time-consuming when compared with electrophoretic methods. However, the resolution of κ-casein A and B variants have been observed during whole casein fractionation.

4.4. Hydrolysis of caseins by trypsin

Silent variant could not be detected by electrophoretic methods since it does not involve differences of net charges. The approach for identification of silent variant of milk proteins is based on differences of hydrophobicities of the peptides resulted from mutation. The separation of milk proteins and some of their genetic variants have been ascertained on reversed-phase HPLC (Visser *et al.*, 1991). Major casein components consist of approximately 200 amino acids. Amino acid substitutions giving rise to slight changes in hydrophobicities might not be sufficient enough to be detected in the whole polypeptide. Therefore, enzyme hydrolysis of the protein was

introduced to produce smaller peptide fragments. The analysis of the hydrolysate by reversed-phase HPLC could then be used to detect any changes in hydrophobicities within the smaller peptides which are resulted from mutation.

Trypsin is specific for the cleavage of peptide bond at the carboxyl adjacent to Lys or Arg. In theory, one could predict the number of peptides from hydrolyzed protein by the number of internal Arg and Lys residues. Based on their primary structures, tryptic hydrolysis of α_{s1}-casein BB will give a mixture of 21 peptides compared to 16 for β-casein A^1A^1 or A^2A^2, and 15 for κ-casein AA. It is also known that the cleavage by trypsin takes place more slowly when the basic amino acid residues is adjacent to an acidic residue or to Cys. In practice, a smaller number of peptides are obtained after tryptic hydrolysis because of incomplete hydrolysis. The described enzyme/substrate ratio, incubation buffer pH, temperature, as well as time course of the tryptic hydrolysis were chosen in this study so as to give maximum proteolysis products (Dong, 1992).

The peptide mapping of casein tryptic hydrolysates was performed by reversed-phase HPLC on the C18 stationary phase. Although better separation could have been achieved by using shallow gradient and varying the flow rate, the described gradient represents a compromise between the maximum resolution and running time as well as eluting peak shape of interest. Generally, the peptides behaved predictably according to their

average hydrophobicity and also as suggested by Lemieux and Amiot (1990) by its molecular weight and composition.

4.4.1. Peptides in α_{s1}-casein hydrolysate

The result of electrophoretic analysis in Table 2 shows that more than 99% of α_{s1}-casein were BB phenotype. A total of 632 α_{s1}-casein samples were subjected to tryptic hydrolysis followed by peptide mapping and 627 of them contain α_{s1}-casein BB.

The primary structure of bovine α_{s1}-casein B-8P is shown in Figure 8. It consists of 14 Lys and 6 Arg residues which are trypsin-sensitive bonds as shown by arrows in Figure 8. The separation of tryptic hydrolysate from intact α_{s1}-casein was achieved by reversed-phase HPLC. The peptides were separated by eluting with a linear gradient of acetonitrile from 0 to 60% in 0.1% TFA for 75 min. A series of experiments were previously carried out in order to obtain optimum condition for the separation. Potential silent variants were identified based on the comparison of peptide maps with the same electrophoretic phenotype. The profile representing most of the samples was identified and considered as standard profile. In order to avoid confusion from heterozygous phenotype, only homozygous samples of α_{s1}-casein BB, β-casein A^1A^1, β-casein A^2A^2, β-casein BB, κ-casein AA and κ-casein BB were analyzed in this project.

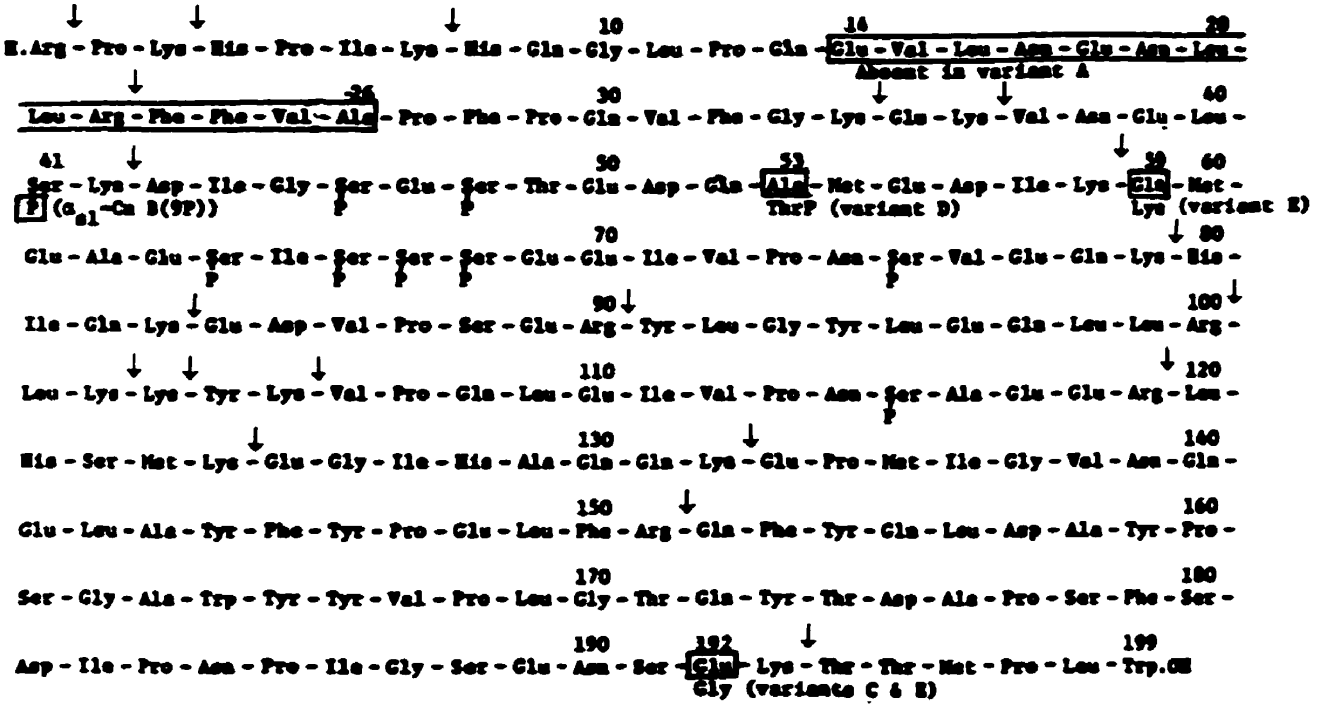


Figure 8. Primary structure of bovine α_{s1}-casein B-8P and its tryptic cleavage sites as indicated by arrows

As an example, the reversed-phase HPLC elution profile for tryptic hydrolysate of α_{s1}-casein BB phenotype obtained from a C18 column is shown in Figure 9. Approximately 20 tryptic peptides were resolved over a period of 61 min.

Individual peaks were evaluated for purity on the basis of symmetry and narrowness of the peak as suggested by Lemieux and Amiot (1990). All the peptide maps obtained from the 627 hydrolysates of α_{s1}-casein BB were compared with respect to the elution time of each peptide. The profile which represents the most common elution pattern was identified. For peak assignments, approximately 18 eluted peptide fractions from tryptic hydrolysate originating from standard α_{s1}-casein BB were collected into individual Eppendorf tubes during the rechromatographic fractionation. The fractions were evaporated to dryness under vacuum and then subjected to amino acid analysis. Table 3 summarizes the result of amino acid analysis which were conducted to determine the identity of tryptic hydrolysates of α_{s1}-casein BB. As a example, peak 6 has the relative retention time of 36.54 min and it contains 15 amino acids from sequence 8-22. Although the fragment 120-124 has not been identified, there are 18 peaks being well located on the remaining part of primary structure of α_{s1}-casein B containing various size of tryptic fragments which range from 3 to 61 residues. Generally, smaller and more polar peptides eluted faster than bigger and more hydrophobic peptides, however, amino

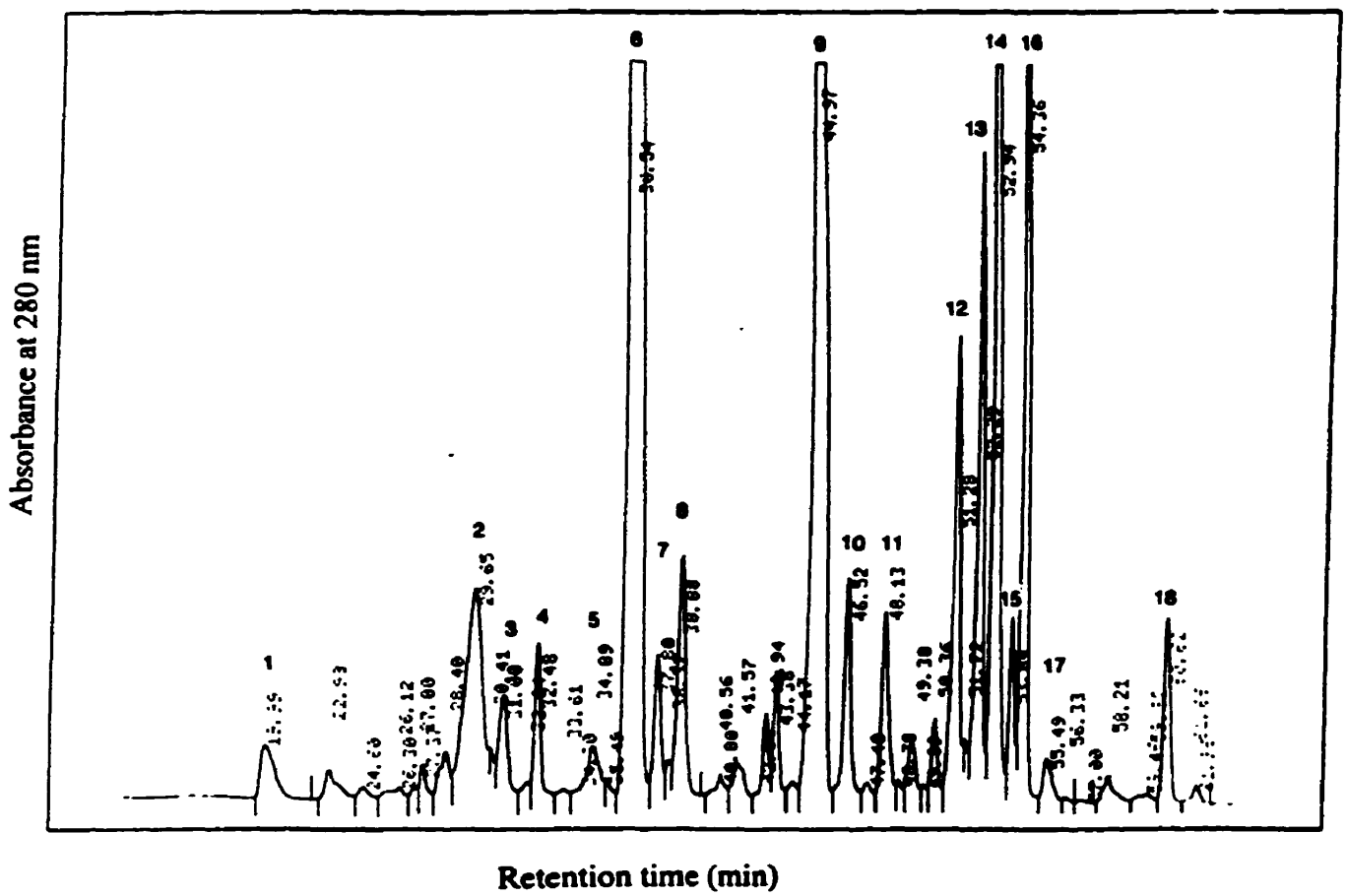


Figure 9. HPLC profile of α_{s1}-casein B tryptic hydrolysates

Table 3. Identity of peptides from tryptic hydrolysates of α_{s1}-casein BB

Peak Number	Retention Time (min)	Position in Sequence	Number of Residues
1	19.99	1-3, 103-105	3,3
2	29.65	80-83	4
3	31.00	4-7	4
4	32.48	91-100	10
5	34.89	194-199	6
6	36.54	8-22	15
7	37.80	84-100	17
8	38.88	35-42	8
9	44.97	23-34	12
10	46.52	125-151	27
11	48.13	43-58	16
12	51.28	133-151	19
13	52.29	59-79	21
14	52.94	106-119	14
15	53.84	152-193	42
16	54.36	43-79	37
17	55.49	152-199	48
18	60.62	133-193	61

acid composition of peptide also plays an important role during elution.

Aberrant peptide maps were identified by comparison of peptide profiles with standard peptide mapping. There were 25 out of the 627 α_{s1}-casein BB samples which could be identified as having abnormal elution pattern due to differences in the retention times of certain peaks. An example of comparison between standard and aberrant peptide maps of α_{s1}-casein B is shown in Figure 10. Although most of the elution peaks are corresponding to each other in standard and aberrant profiles, there are two extra peaks which are indicated by arrows as being aberrant profile. This is possible due to amino acid substitution which results in heterozygous α_{s1}-casein phenotype and this substitution could not be detected by electrophoresis. All the aberrant samples were reanalyzed and aberrant peaks, if still exist, were collected in order to characterize the amino acid substitution which resulted in the change in hydrophobicities of the peptide.

4.4.2. Peptides in κ-casein hydrolysate

A total of 415 κ-casein AA, 198 κ-casein AB and 22 κ-casein BB was subjected to trypsin hydrolysis followed by reversed-phase HPLC in order to identify possible silent variants of κ-casein.

Figure 11 shows the primary structure of κ-casein which consists of 169 amino acid residues with 9 Lys and 5 Arg

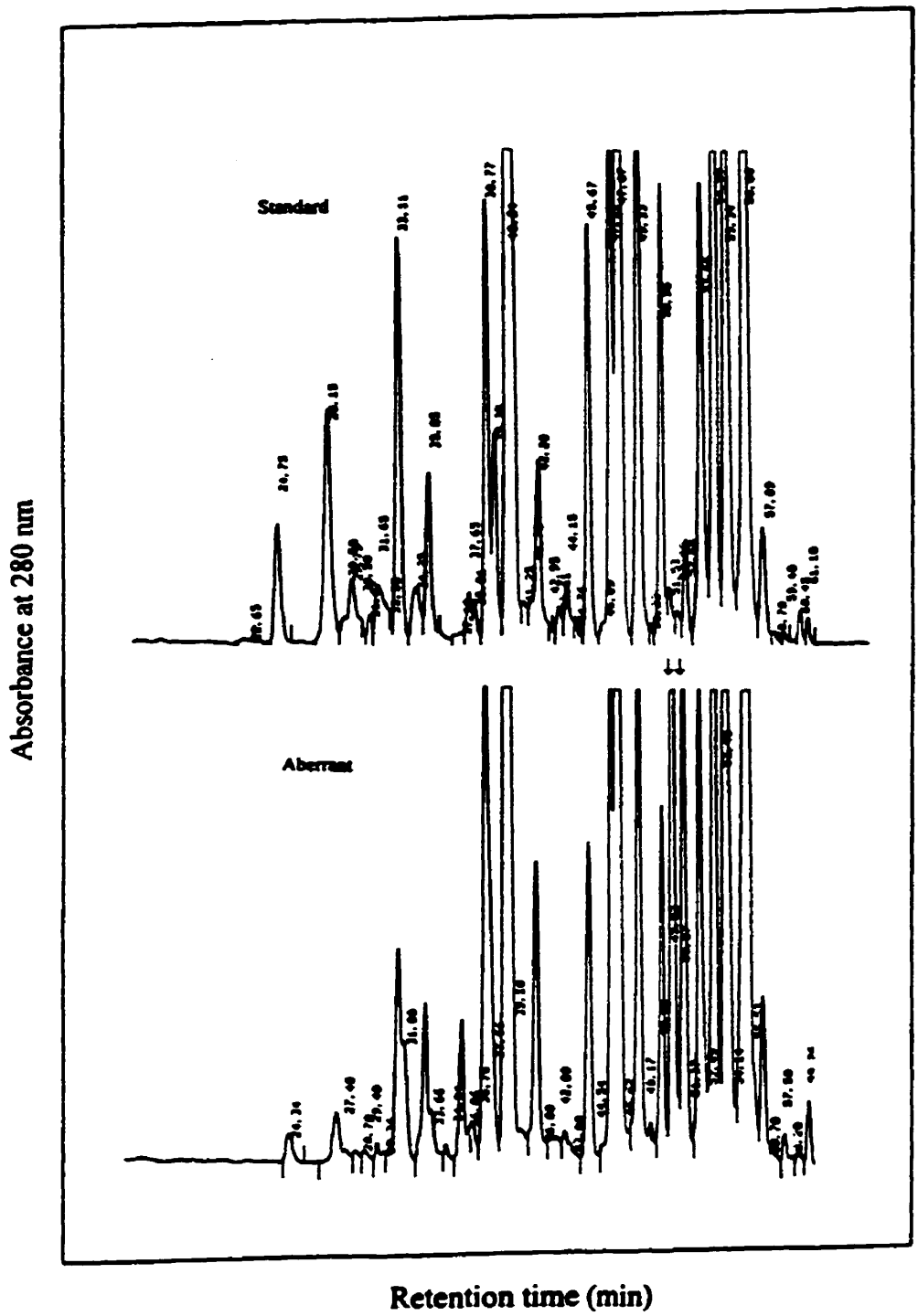


Figure 10. HPLC profiles of standard and aberrant α_{s1}-casein B tryptic hydrolysates.

residues. There are 15 tryptic cleavage sites as shown by arrows along the primary structure of κ-casein. The same conditions used for peptide mapping of hydrolysates of α_{s1}-casein were applied to hydrolysates of κ-casein. More than 20 fragments were observed on the HPLC profile of κ-casein as indicated in Figure 12. This could probably be ascribed to the fact that in addition to fifteen peptides indicated above, there could be some residual containing uncleavaged peptides with Lys or Arg within. As consistent with Léonil and Mollé (1990), certain intermediate products could be expected to appear during proteolysis.

Table 4 summarizes the relationship between elution peak and their identity on primary structure of κ-casein A. Nine peaks on the peptide map of κ-casein A have been located and covered most sequence of the primary structure of κ-casein excluding sequence 35-68. For example, peak number 8 has 57 amino acid residues from position 113 to 169. This peptide includes an uncleavaged site at Lys_{116}-Thr_{117}. According to literature (Kesharavarz and Nakai, 1979; Chaplin, 1986), the difference in elution behavior of each peptide relates with the effective hydrophobicity which is based on amino acid composition. Peak number 9 with larger retention time contains tryptic fragment 117-169 which is smaller than that in peak number 8. This could be explained by the fact that three polar amino acid residues (Asn_{113}, Gln_{114}, Asp_{115}) clustering in the beginning of peptide 113-169 and hence decreasing its hydrophobicity.

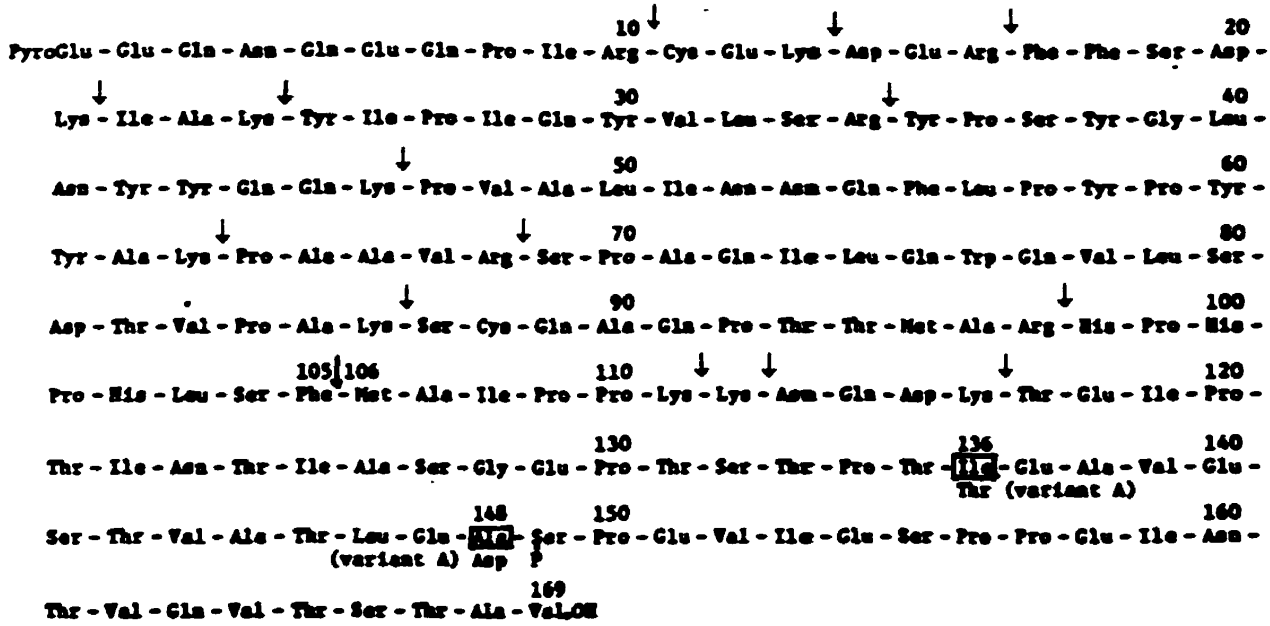


Figure 11. Primary structure of bovine κ-casein B-1P and its tryptic cleavage sites as indicated by arrows

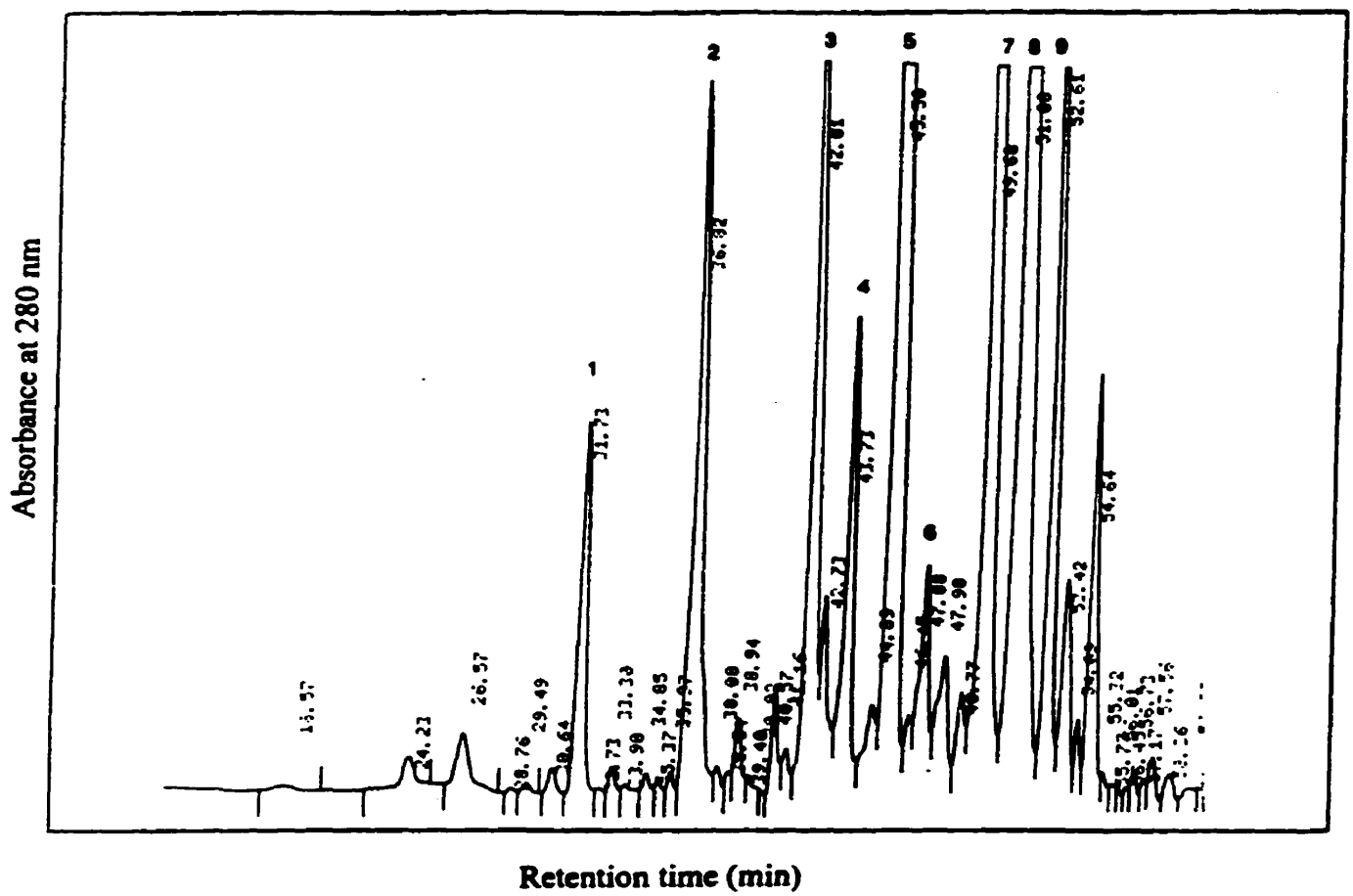


Figure 12. HPLC profile of κ-casein A tryptic hydrolysates

Table 4. Identity of peptides from tryptic hydrolysates of κ-casein AA

Peak Number	Retention Time (min)	Position in Sequence	Number of Residues
1	31.73	1-10	10
2	36.82	11-16	6
3	42.01	98-111	14
4	43.73	25-34	10
5	45.50	1-21	21
6	47.08	87-111	27
7	49.68	69-86	18
8	51.08	113-169	57
9	52.61	117-169	53

Potential silent variants were identified by comparing all peptide profiles with the standard profile. There were 11 out of 415 κ-casein AA samples which have been found to contain possible silent variants due to a shifting in the retention time for one or more elution peaks. Figure 13 shows the comparison of peptide maps from standard and aberrant κ-casein A hydrolysates. In spite of high consistency between standard and aberrant maps, an extra big peak with the retention time at 55.03 min, as being indicated by arrow, is observed in the aberrant profile following the peak with the retention time at 54.12 min. Thus, both peaks have similar peak height. According to the results in Table 4, the peak with the retention time of 54.12 min represents tryptic peptide 117-169. It could be deduced that potential silent variant might take place in this fragment and this variant can not be differentiated from κ-casein A variant by convential electrophoretic techniques.

Although 22 homozygous κ-casein BB samples have been analyzed in this study, no significant difference on peptide maps of κ-casein A and B has been observed. It could be explained by the fact that the difference in hydrophobicities between κ-casein A and B variants is not sufficient for changing the retention time of tryptic fragments 117-169 or 113-169. These two tryptic fragments contain both amino acid substitution sites at position 136 and 148. Thus, there is no potential silent variant being found in κ-casein BB.

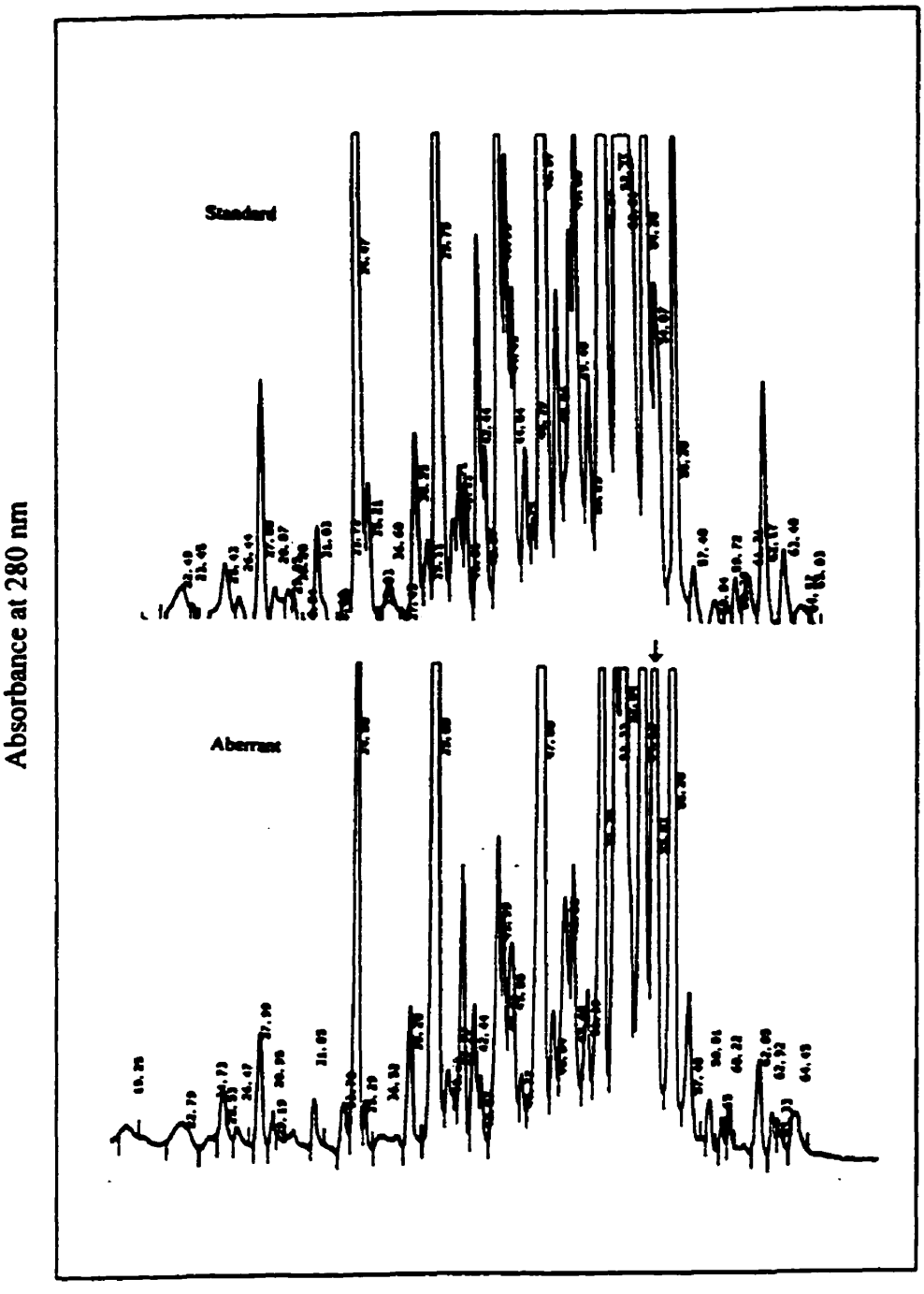


Figure 13. HPLC profiles of standard and aberrant κ-casein A tryptic hydrolysates.

4.4.3. Peptides in β-casein hydrolysate

β-Casein is the most hydrophobic casein and its hydrolysis products have been relatively well documented due to their utilization in food ingredients as well as their potential to release biologically active peptides such as oligo-element (β-casein 1 - 25), opioids β-casomorphin 7 (β-casein 60 - 66) and pharmaceutical compound for antihypertension (β-casein 177 - 183)(Bican, 1983; Carles, 1986; Leadbeater and Ward, 1987; Lemieux and Amiot, 1990). The primary structure of β-casein A^2-5P presented in Figure 14, consists of 11 Lys and 4 Arg residues and could provide 15 trypsin susceptible bonds. Theoretically, sixteen fragments could be expected to result from trypsin hydrolysis.

A total of 158 β-casein A^1A^1, 128 β-casein A^2A^2, 3 β-casein BB and 1 β-casein A^3A^3 were hydrolyzed by trypsin and their hydrolysates were analyzed by reversed-phase HPLC. The peptide maps for homozygous β-casein A^1, β-casein A^2 and β-casein B variants are shown in Figures 15, 16 and 17 respectively. The specific peaks for each variants are marked on each profiles that β-casein A^1 consists of specific peaks 10 and 11; β-casein A^2 has special peaks 12 and 13 while the profile of β-casein B variant has specific peak 4 which is absent in the profiles of β-casein A^1 and β-casein A^2. The results of identity of tryptic peptides from β-casein hydrolysates are summarized in Table 5.

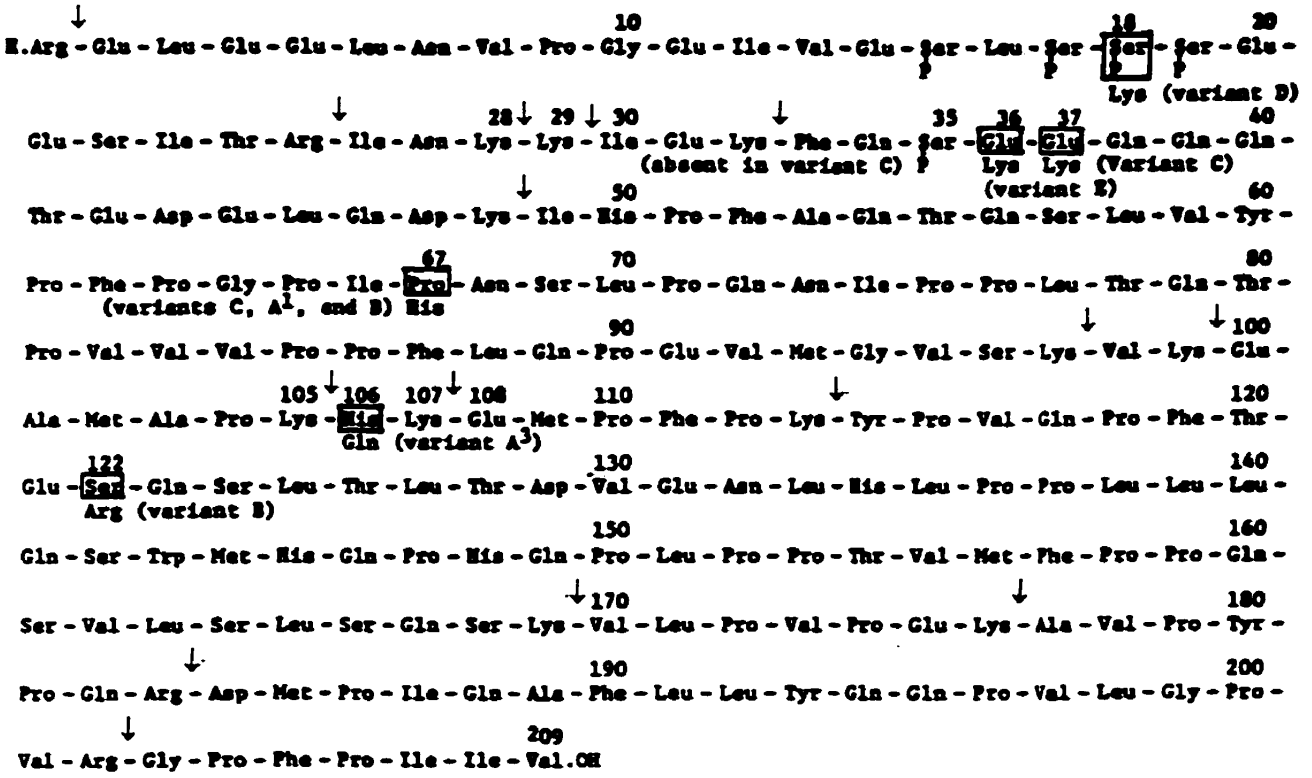


Figure 14. Primary structure of bovine ß-casein A^2-5P and its tryptic cleavage sites as indicated by arrows

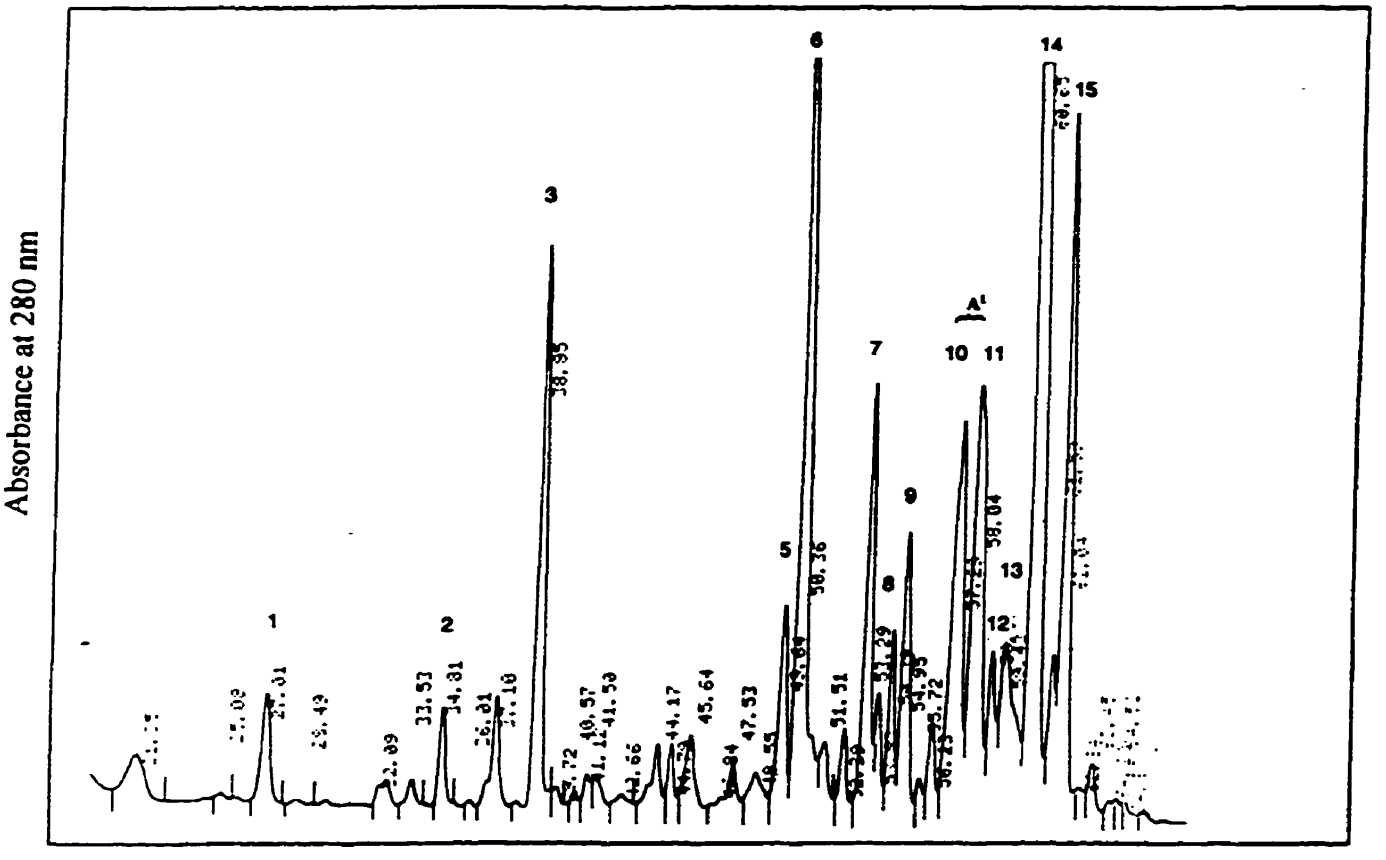


Figure 15. HPLC profile of ß-casein A^1 tryptic hydrolysates

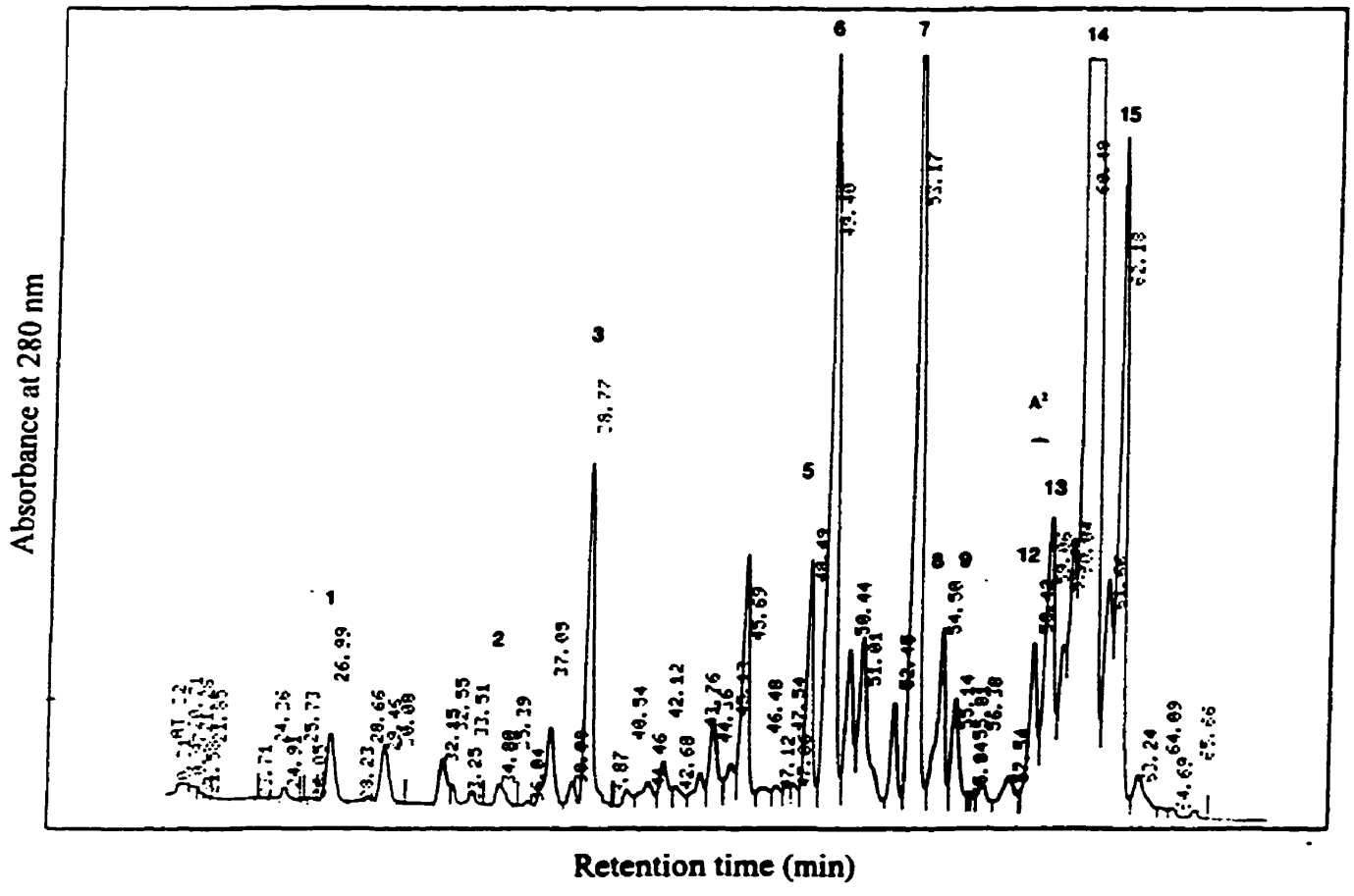


Figure 16. HPLC profile of ß-casein A^2 tryptic hydrolysates

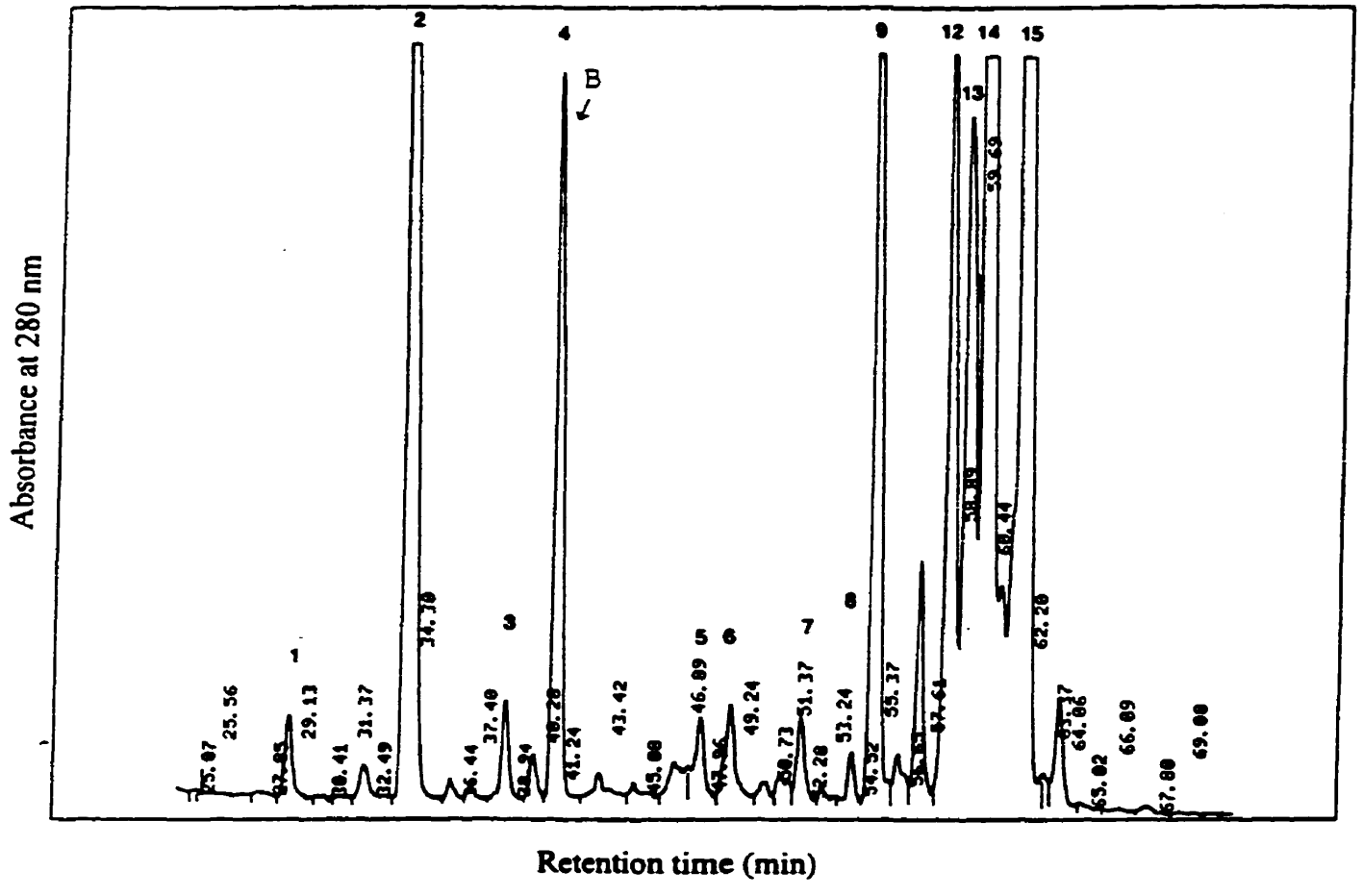


Figure 17. HPLC profile of ß-casein B tryptic hydrolysates

Table 5. Identity of peptides from tyrptic hydrolysates of β-caseins

Peak Number	Retention Time (min)	Position in Sequence			Number of Residues
		A^1	A^2	B	
1	26.99-29.13	108-113,98-105	108-113,98-105	108-113,98-105	6,8
2	34.30-34.81	106-107,29-32	106-107,29-32	106-107,29-32	2,4
3	38.77-38.94	177-183	177-183	177-183	7
4	41.24	—	—	114-122	9
5	47.96-48.64	184-202	184-202	184-202	19
6	49.24-50.36	2-25	2-25	2-25	24
7	52.28-53.29	33-48	33-48	33-48	16
8	54.39-54.52	30-48	30-48	30-48	19
9	54.95-55.37	1-25	1-25	1-25	25
10	57.29	49-97	—	—	49
11	58.04	33-97	—	—	65
12	58.42-58.89	—	49-97	49-97	49
13	59.06-59.69	—	33-97	33-97	65
14	60.44-60.65	114-169	114-169	114-169	56
15	62.04-62.20	114-176	114-176	114-176	63

By amino acid analysis, it indicates that peak 10 on β-casein A^1 map contains β-casein sequence 49 - 97 while peak 11 has incompletely hydrolyzed β-casein 33-97. Whereas, for β-casein A^2, peak 12 consists of fragment 49-97 and peak 13 represents 33-97. This difference is due to the amino acid substitution at position 67 from His (β-casein A^1) to Pro (β-casein A^2) and thus results in significant increase of hydrophobicity of fragments 49-97 and 33-97 since Pro is more hydrophobic than His. In β-casein B variant, the amino acid substitution at position 122 from Ser (A^1 and A^2 variants) to Arg (B variant) gives rise to one more tryptic sensitive bond and therefore results in extra fragment of 114-122 which elutes in peak 4 as shown in Figure 17. Generally, a tryptic hydrolysis of β-casein under the condition described in materials and method results in more fragments than theoretically predicted. The additional peaks may be due to incomplete hydrolysis of β-casein polypeptide which results in intermediate peptide containing one or more trypsin specific bonds. Moreover, these results indicate that reversed-phase HPLC could be applied for identification of genetic variants of β-casein since it has great potential in differentiation of peptides based on the difference of hydrophobicity.

The aberrant profile was identified by comparison with standard profile containing the same electrophoretical type. The typical examples of standard and aberrant β-casein A^1A^1 peptide

maps are shown in Figure 18. By superimposing profiles, two profiles are close resemblance. However, in the standard profile of β-casein A^1A^1, all the well identified peaks can be concordant to the profile of aberrant β-casein A^1A^1. Except two additional peaks were observed in aberrant profile with the retention times of 61.79 min and 63.35 min respectively. Moreover, these two peaks matched the standard peaks with the retention time of 60.98 min and 62.57 min but with longer retention times and slightly lower peak heights. Therefore, the addition of two extra peaks in the aberrant β-casein A^1A^1 tryptic profile would be explained by the fact that this electrophoretically homozygous β-casein A^1A^1 sample possibly consist of heterozygous type in which most likely contains amino acid substitution which does not involve in the difference on net charge. The increasing of retention time being observed for the aberrant peaks suggests that very slightly chance of the occurrence of amino acid deletion in this case. Based on the results depicted in Table 5, it could be expected that the increasing of hydrophobicity of the mutated amino acid residue in the sequence 114 - 169 of β-casein A^1A^1. Since this amino acid substitution could not be differentiated from standard β-casein A^1 variant by electrophoresis, it confirmed the existence of silent variants in milk proteins and it could be identified due to their difference in hydrophobicity by reversed-phase HPLC. In a total of 158 β-casein A^1A^1, 16 of them have been found containing possible silent variants based on their changes on

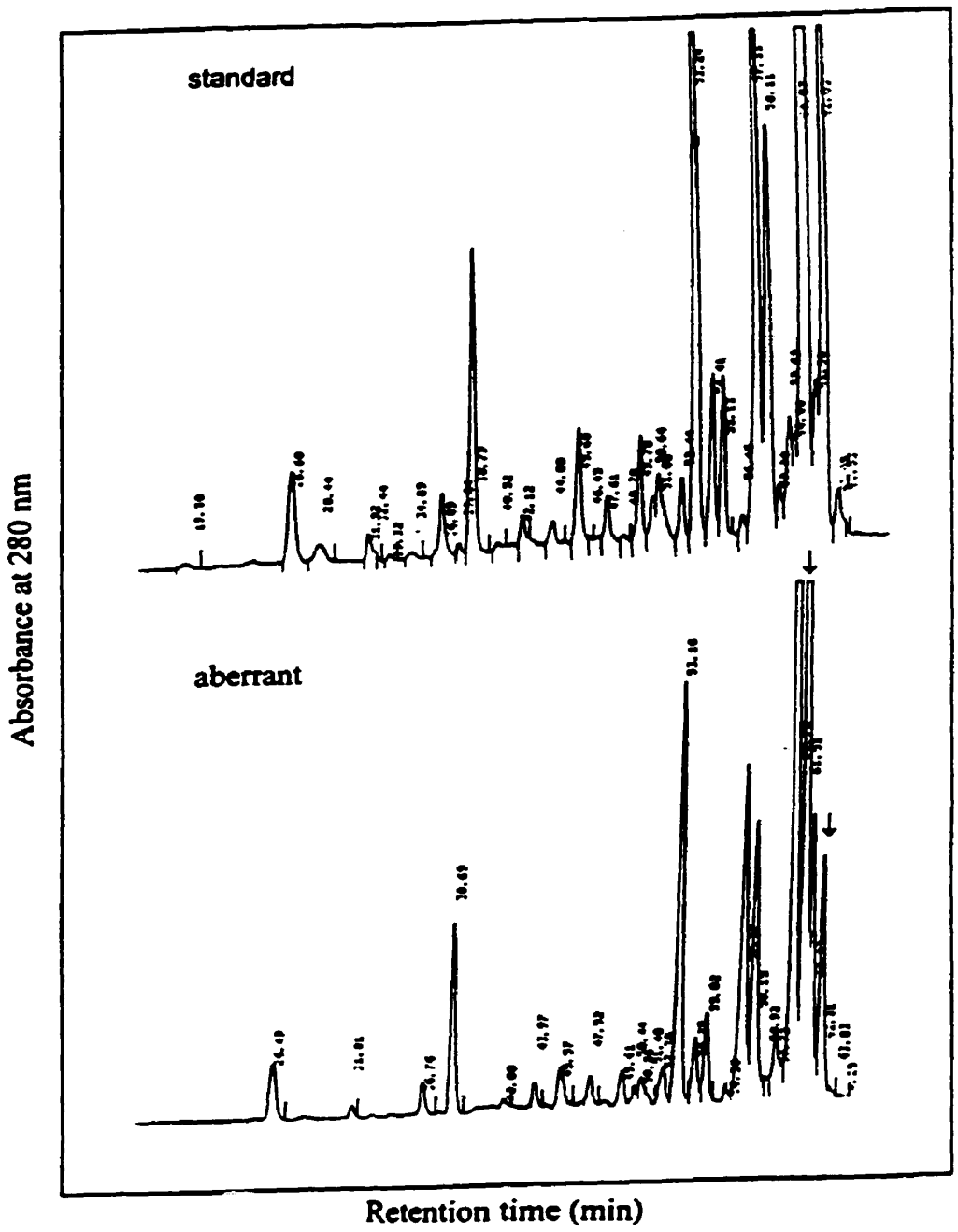


Figure 18. HPLC profiles of standard and aberrant ß-casein A^1A^1 tryptic hydrolysates

hydrophobicities of tryptic peptides while 7 out of them contain the same mutation involved in peptide 114-169.

Figure 19 shows a comparison of standard and aberrant peptide maps for β-casein A^2A^2. It is observed that the peak with retention time of 48.49 min in standard profile is absent in aberrant profile as shown by arrow. According to the result in Table 5, the peak with the retention time of 48.49 min consists of tryptic peptide 184-202. Therefore, it could be predicted that there is a substitution or deletion occurring in the sequence 184-202 and this potential silent variant can not be differentiated from β-casein A^2 variant by electrophoresis. In a total of 128 β-casein A^2A^2 samples, 7 potential silent variants have been observed according to their changes in retention time of β-casein tryptic hydrolysates.

Due to relatively small sample size, no potential silent variants have been identified in homozygous β-casein BB and A^3A^3 samples.

Enzyme hydrolysis was repeated for all the samples containing standard and aberrant samples. Most the aberrant samples of α_{s1}-casein BB, β-casein A^2A^2 and κ-casein AA show no significant difference during reanalysis. However, 7 out of 16 previously identified aberrant β-casein A^1A^1 still show the same type of silent variant. In order to further characterize this silent variant as well as other potential silent variants in α_{s1}-

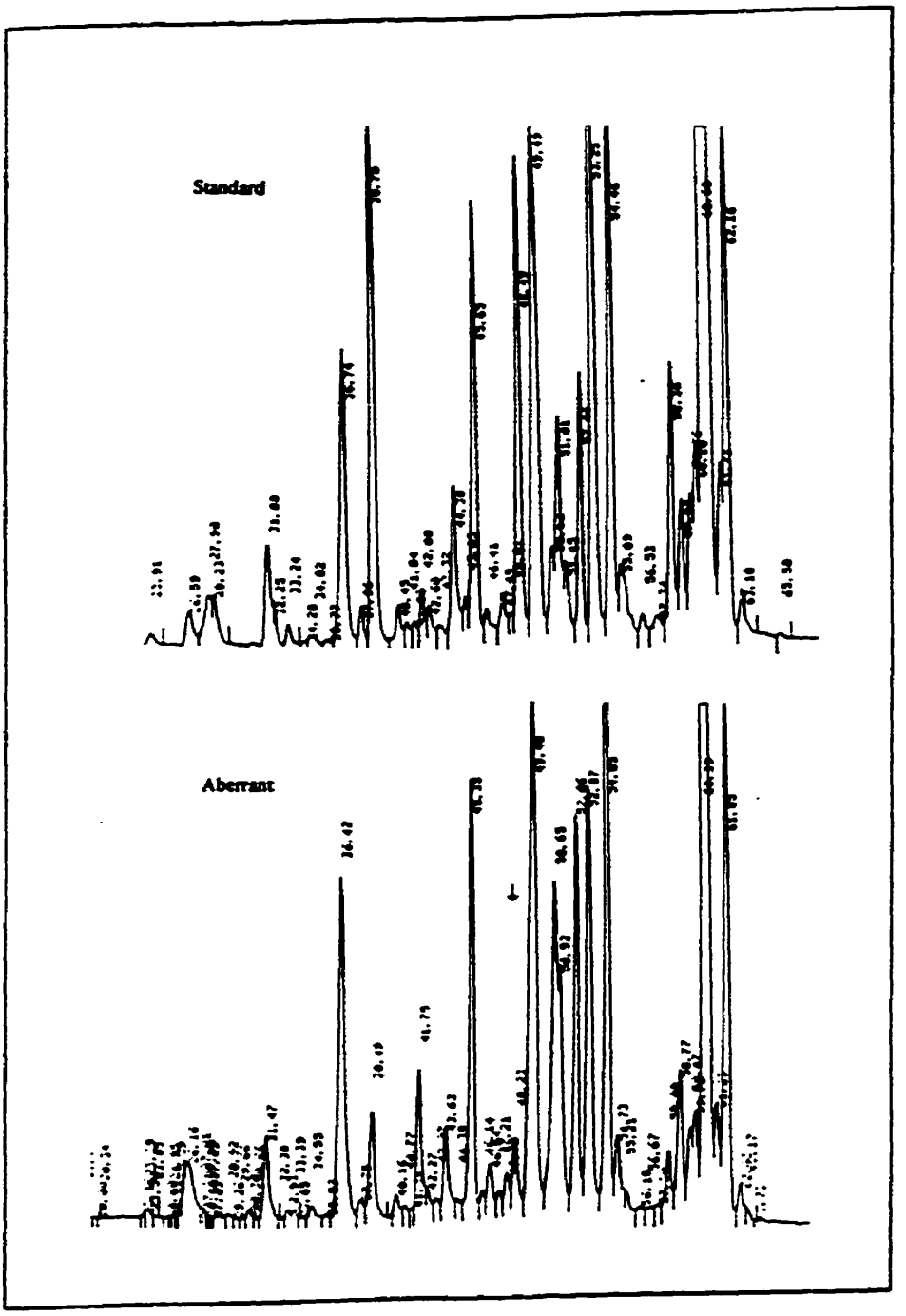


Figure 19. HPLC profiles of standard and aberrant ß-casein A^2 tryptic hydrolysates.

casein BB, β-casein A^2A^2 and κ-casein AA, all aberrant peaks were collected and subjected to mass analysis.

4.5. Mass Spectrometry

As an indispensable tool for peptide and protein analysis, mass spectrometry is now mostly demanded for its high accuracy, low sample requirement and especially its speed for determination of molecular weight of peptides. In the original design of this project, mass spectrometry was chosen for identification of silent variants by the means of molecular weight determination of the fractionated α_{s1}-casein, β-casein and κ-casein. However, the assay for these intact milk protein components was interfered by the impurity of small molecules such as urea and sodium chloride used for gradient elution since the detection of electrospray ionization was greatly restricted due to its low tolerance for non volatile buffers and alkali metal salts.

For each intact casein component, the average deviation from mean calculated mass ranged from 10 to 20 Da. This meant that several potential substitutions with a net mass change around 40 dalton might be uncertain. Therefore, the ability of rapid and highly efficient of mass spectrometry was adapted to determine the identity of tryptic fragments collected from aberrant peptide hydrolysates and confirm the possible change in molecular weight between the standard and abnormal tryptic peptides.

4.5.1. Standard

Prior to analysis each batch of sample, lysozyme was used as standard for calibration of mass detector. Normally, three signals at the mass to charge ratio of 1590, 1789 and 2045 were observed for lysozyme which were generated from the fragments containing 9, 8 and 7 protons. Therefore, the mass for lysozyme would be calculated as 14305±2 which is in good accordance with its theoretical value, and thus, indicates good accuracy for the mass detection.

4.5.2. Mass analysis of peptides from α_{s1}-casein hydrolysate

Approximately 18 tryptic fractions corresponding to Figure 9 were individually collected and subjected to mass analysis. Mass spectrum of the ascertained tryptic fragments could be calculated based on Mac Spec procedure as described in the previous section 3.4.5.. All the signals were assigned to the corresponding peptides from the reference sequence on the basis of their mass value and enzyme specificity. The cleaved carboxyl terminal of Lys and Arg in addition to His and the amino terminus are the most likely protonated sites under electrospray conditions. The typical mass spectrum for peak number 12 in α_{s1}-casein B hydrolysate is shown in Figure 20. The signals with m/z ratio of 1159.6 and 2317.6 originated from peptide with the observed mass of 2316.6 which matches with α_{s1}-casein peptide 133-151 with the expected mass of 2316.1. Table 6 summarizes the results of expected and observed molecular weight for identified

peptides as well as their primary sequence. Eleven peaks from α_{s1}-casein B tryptic hydrolysate gave satisfactory results in that their observed results match with expected mass. These identified peptides ranging from 748 to 4716 Da account for a total coverage of about 75% of the entire sequence of α_{s1}-casein. Peaks number 1, 2, 3, 7, 11, 17 and 18 have not been confirmed due to certain possible interference from protein phosphorylation or impurity during collection. The remaining portion of unidentified peptides might be coeluted or do not have enough absorbancy at the operating wavelength (λ = 280 nm) which results in the loss during fraction collection. None of the 25 α_{s1}-casein BB which were previously found to contain potential silent variant was confirmed during the reanalysis.

4.5.3. Mass analysis of peptides from κ-casein hydrolysate

Approximately 9 tryptic fractions from κ-casein hydrolysate were resolved by peptide mapping using Reversed-phase HPLC and collected individually. Figure 21 represents the typical mass spectrum for peptide 69-86 from κ-casein tryptic hydrolysate with the retention time of 49.68 min. Two major signals with m/z ratio of 992.0 and 1981.5 result from the peptide with mass of 1980.5 which matches with the peptide 69-86 with predicted mass of 1981.1. The results for fast peak assignment were achieved by mass analysis and shown in Table 7. Table 7 summarizes amino acid sequence for each identified tryptic fragment with their expected and observed molecular weight. Five out of nine tryptic

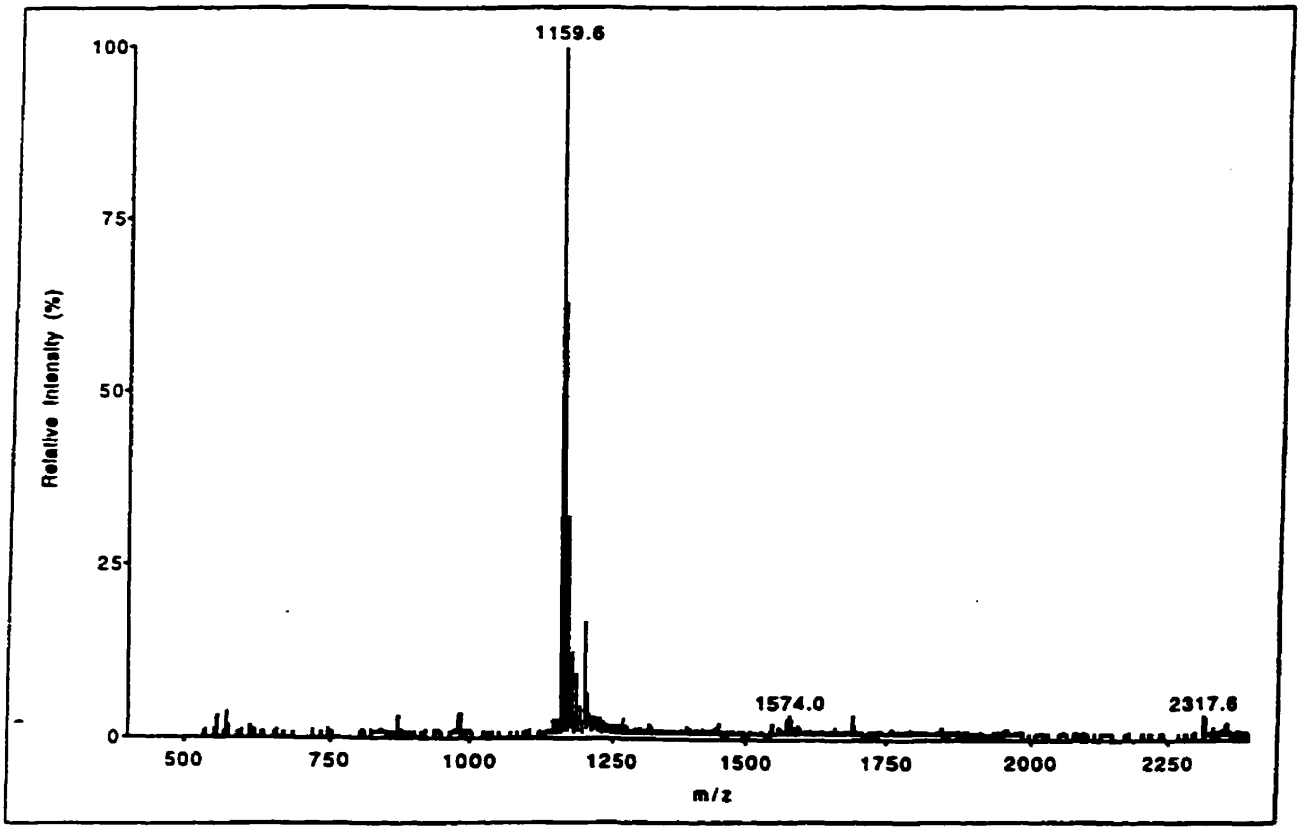


Figure 20. Mass spectrum for peptide 133-151 from α_{s1}-casein B tryptic hydrolysates.

Table 6. Mass analysis of peptides from α_{s1}-casein BB tryptic hydrolysates

Peak Number*	Retention Time (min)	Amino Acid Sequence	Mass	
			Expected	Observed
4	37.80	YLGYLEQLLR	1267.7	1266.9
5	34.89	TTMPLW	748.4	748.5
6	36.54	HQGLPQEVLNENLLR	1760.0	1759.2
8	38.88	EKVNELSK	1026.5	1028.5
9	44.97	FFVAPFPQVFGK	1383.8	1383.7
10	46.52	EGIHAQQKEPMIGVNQELAYFYPELFR	3207.6	3209.2
12	51.28	EDVPSERYLGYLEQLLR	2316.1	2316.6
13	52.29	QMEAESISSSEEIVPNSVEQK	2321.1	2320.8
14	52.94	VPQLEIVPNSAEER	1580.8	1580.8
15	53.84	QFYQLDAYPSGAWYYVPLGTQYTDAPSFS DIPNPIGSENSER	4716.2	4719.2
16	54.36	DIGSESTEDQAMEDIKQMEAESISSSEEIVP NSVEQK	4069.8	4066.8

* Peak number and retention time corresponding to Figure 9

fragments of κ-casein gave the matching results when compared with predicted mass.

In κ-casein, approximately 62% of tryptic fragments have been identified by mass spectrometry. The relatively less number of fragments being located by mass spectrometry might be due to the following reasons: Firstly, impurity being introduced during casein isolation and enzyme hydrolysis. Secondly, possible cross-contamination during tryptic fraction collection. Thirdly, the existence of carbohydrate compounds as well as disulfide bond in the κ-casein polypeptide. As we are aware, κ-casein is heterogeneous with respect to its carbohydrate moiety which is exclusively linked to its caseinomacropeptide part. Caseinomacropeptide comprises of fragment 106 - 169 which includes different extent of glycosylation (MacKinlay *et al.*, 1966). According to Swaisgood (1982), it is also known that κ-casein contains two Cys residues at positions 11 and 88 which would lead to linkage between two remote peptides. This might result in the co-elution of two fragments during peptide mapping and therefore, influence the retention time for some of the tryptic fragments during elution.

4.5.4. Mass analysis of peptides from β-casein hydrolysate

As the most successful example during this project, β-casein peptide map shows outstanding pattern for each β-casein variant as represented in Figures 15, 16 and 17 for A^1, A^2 and B respectively. For fast identification of elution peaks, 15

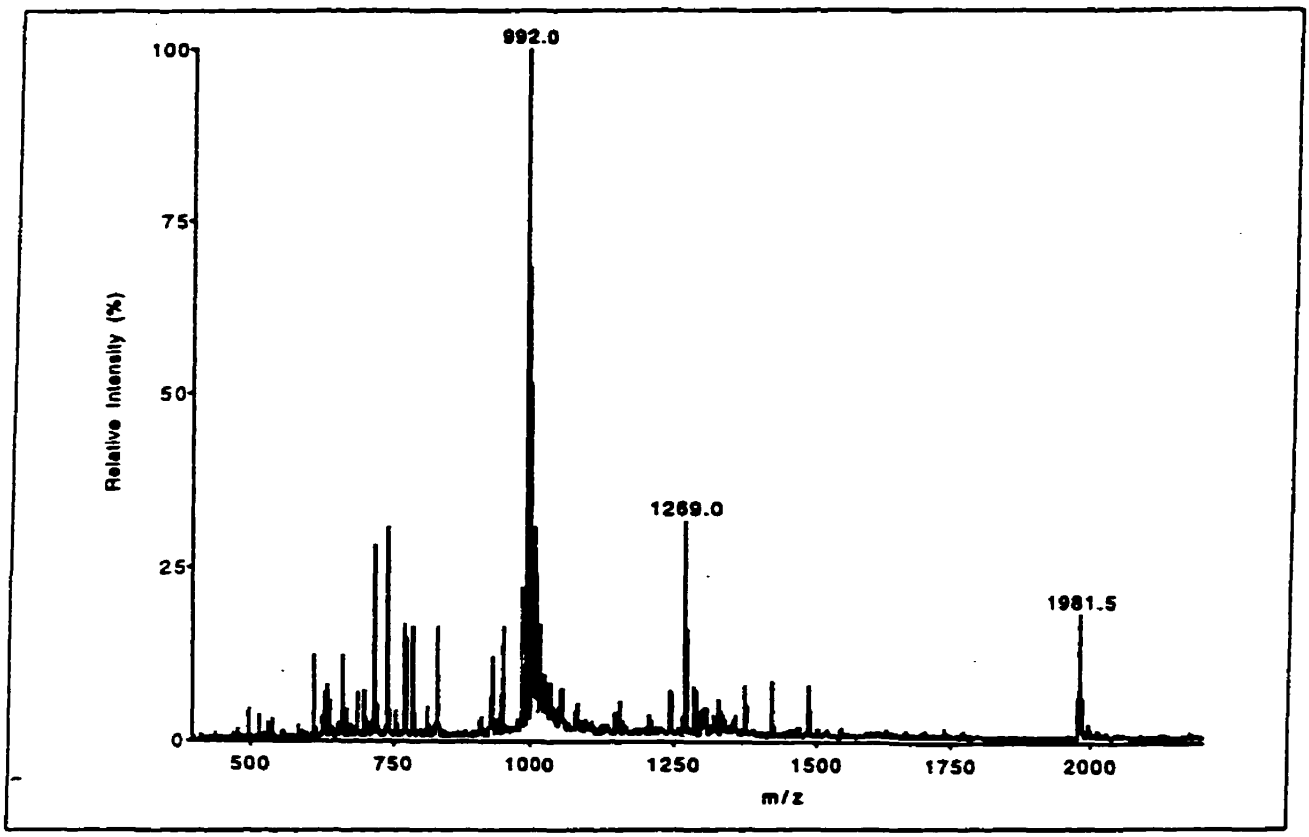


Figure 21. Mass spectrum for peptide 69-86 from κ-casein A tryptic hydrolysates.

Table 7. Mass analysis of peptides from κ-casein tryptic hydrolysates

Peak Number	Retention Time (min)	Amino Acid Sequence	Mass	
			Expected	Observed
2	36.82	CEKDER	796.4	796.5
3	42.01	HPHPHLSFMAIPPK	1607.8	1608.0
4	43.73	YIPIQYVLSR	1250.7	1250.0
7	49.68	SPAQILQWQVLSDTVPAK	1981.1	1980.5
8	51.08	NQDKTEIPTINTIASGEPTSTPTTEAVES	6038.9	6032.2
		TVATLEDXPEVIESPPEINTVQVTSTAV		

* Peak number and retention time corresponding to Figure12.

fractions originating from A^1, A^2 and B variants of β-casein were collected and subjected to mass analysis. Table 8 tabulates the results of expected and observed mass for identified fractions as well as their relative retention times and primary sequence.

Both β-casein A^1A^1 and A^2A^2 have been found to contain potential silent variants during the first analysis using peptide mapping. However, no aberrant samples has been confirmed for β-casein A^2A^2 during reanalysis. However, there are 7 out of 158 β-casein A^1A^1 samples containing silent variant which results in two extra peaks on the aberrant peptide profiles (Figure 18). According to the previously described result in Table 5, within standard β-casein A^1A^1 profile, the peak with the retention time of 60.98 min was correlated to the peptide fragment of 114-169 of β-casein A^1 primary structure while the peak at the retention time of 62.57 min contained incomplete digest fragment of 114-176 which included a tryptic susceptible bond at the Lys 169. For identification of possible silent variants involved in them, mass analysis were carried out to determine the difference of their molecular weight. Figure 22 shows the mass spectrum for the standard β-casein A^1 fragment 114-169 and mass spectrum for aberrant β-casein A^1 as denoted to A^{1a} is represented in Figure 23. In Figure 22, all the major signal shown in the spectrum can be interpreted with the presence of series mass to charge ratios of 1061.1, 1273.6, 1591.4 and 2121.6 with the corresponding series of charges: 6, 5, 4, and 3 protons respectively. According to the calculation formula of $m=(M+n)/n$, the estimated

Table 8. Mass analysis of peptides from β-casein tryptic hydrolysates

Peak Number*	Retention Time (min)	Amino Acid Sequence	Mass	
			Expected	Observed
1	26.99-29.13	EMPFPK	748.4	748
		VKEAMAPK	873.5	873.5
2	34.30-34.81	HK	284.2	284.4
3	38.77-38.94	KIEK	517.3	517.3
4	41.24	YPVQPFTER	1280.3	1280
5	47.96-48.64	DMPIQAFLLYQQPVLGPVR	2185.2	2186.5
6	49.24-50.36	ELEELNVPGEIVESLSSSEESITR	2966.3	2968.2
7	52.28-53.29	FQSEEQQQTEDELQDK	1981.9	1981.2
8	54.39-54.52	IEKFQSEEQQQTEDELQDK	2352.1	2351.6
10	57.29	IHPFAQTQSLVYPFPGPIHNSLPQNIPPLTQT	5356.9	5356.5
		PVVVPPFLQPEVMGVSK		
11	58.04	FQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGP	7399.7	7399.5
		IHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK		
14	60.44-60.65	YPVEPFTESQSLTLTDVENLHIPLPLLQSWMHQP	6362.2	6362.8
		HQPLPPTVMFPPQSVLSLSQSK		

* Peak number and retention time corresponding to Figures 15, 16 and 17.

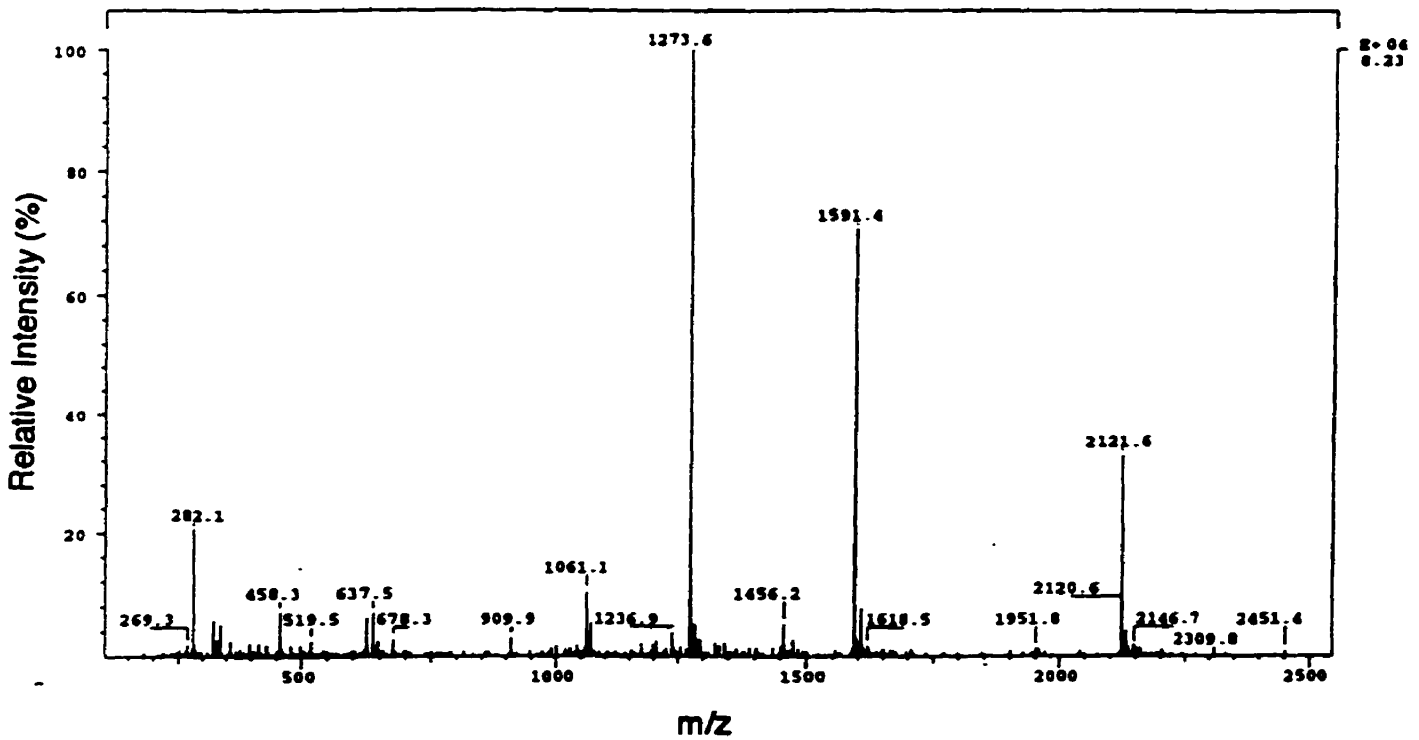


Figure 22. Mass spectrum for peptide 114-169 from standard ß-casein A[1] tryptic hydrolysates

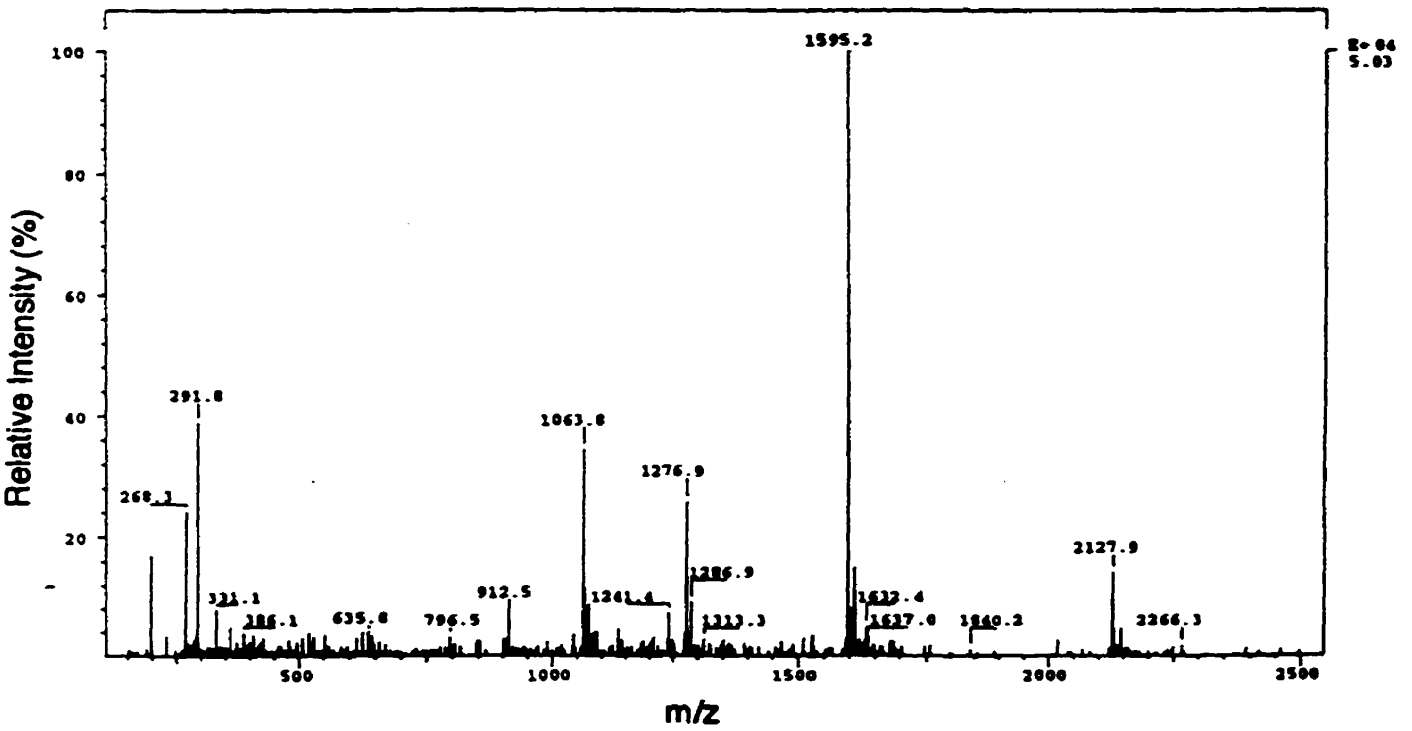


Figure 23.Mass spectrum for peptide 114-169 from aberrant ß-casein A[1] tryptic hydrolysates

molecular weight of standard β-casein A^1 114-169 fragment is 6362. With a series of signal at 1063.8, 1276.9, 1595.2 and 2127.9 corresponding to 6, 5, 4 and 3 protons as shown in Figure 23, the molecular weight for abnormal β-casein A^{1a} fragment 114-169 is calculated to be 6378. This indicated that there is an amino acid substitution occurring within the fragment 114-169 of β-casein A^1 and this substitution lead to a increasing of hydrophobicity and also an 16 dalton difference in molecular weight for the aberrant peptide fragment when compared with the matching part in standard profile.

There are 15 different types of amino acids in β-casein tryptic peptide 114-169. Table 9 lists the molecular weight of those 15 residues, their respective molecular weight and the possible molecular weight after 16 Da increase. The following possibility could be expected for the substitution involving the 16 Da increase in molecular weight: Pro→Leu or Ile; Val→Asp; Phe→Tyr; Ser→Cys; Leu→Glu; Asp→Met; Met→Phe. Based on the hydrophobicity of each residues (Walstra and Jenness, 1984), the substitutions consisting of Val→Asp and Leu→Glu will result in the decrease in hydrophobicity because the hydrophobicities of these amino acids are respectively 7.1, 0, 10.0, 0. At nucleotide level, 61 triplet codons represent 20 amino acids , thus each amino acid apart from Met has several codons. The substitution in β-casein A^{1a} would be due to mutation of one base rather than mutation involving more than one base. Therefore, with regard to single-base change in codon, the following

Table 9. Amino acid composition of ß-casein peptide 114-169 and its possible mutations

Amino Acid Composition	Molecular Weight (MW)	MW + 16	Possible Amino Acid
Tyrosine	181	197	—
Proline	115	131	Leu, Ile
Valine	117	133	Asp
Phenylalanine	165	181	Tyr
Threonine	119	135	—
Glutamic acid	147	163	—
Glutamine	146	162	—
Serine	105	121	Cys
Leucine	131	147	Glu
Aspartic acid	133	149	Met
Asparigine	132	148	—
Histidine	155	171	—
Tryptophan	204	220	—
Methionine	149	165	Phe
Lysine	146	162	—

substitutions could be excluded: Pro→Ile and Asp→Met which should involve at least two base mutations. Furthermore, the substitutions of Asp→Met, Leu→Glu and Val→Glu would be excluded because they involved the difference in net charges and hence would be identified by electrophoretic methods. The rest of the possible amino acid substitutions in β-casein A^{1a} will be limited to: Pro→Leu, Phe→Tyr, Ser→Cys or Met→Phe because such a change will increase the hydrophobicity and involve only one base mutation. Amino acid analysis was carried out to further identify the exact amino acids involved in this substitution.

4.6. Amino acid composition of β-casein A^1 peptide 114-169.

As suggested by peptide mapping results from reversed-phase HPLC, the silent amino acid substitution occurring within β-casein A^1 polypeptide fragment 114 - 169 involved an increase in hydrophobicity of the fragment. In addition, the results from electrophoresis indicated that there is no difference on net charge between standard β-casein A^1 and aberrant β-casein A^{1a} variants. As complementary results from mass spectrometry, this substitution gives rise to an increase of 16 dalton in molecular weight.

Therefore, it is of great interest to identify the exact amino acid substitution involved in this case. Amino acid composition analysis by standard Pico-Tag method after acidic hydrolysis of both standard β-casein A^1 and aberrant β-casein A^{1a} peptide 114-169 were performed. The determination of amino acid

composition of tryptic peptide is based on the releasing the amino acid residues quantitatively without concomitant degradation. Under the acidic hydrolysis by 6 N HCl for 24 hr at 110°C under vacuum, several amino acid will undergo certain modification. Asn and Gln will be converted quantitatively to Asp and Glu. A loss of 5 to 10% will occur to Thr and Ser respectively. Tyr can be halogenated during HCl hydrolysis which would be prevented by the addition of one drop of phenol (approximately 0.1%, w/v). Oxidation of Met would be avoided by adding β-mercaptoethanol. Trp is not generally recovered in acid hydrolysates and therefore, alkaline hydrolysis is required to quantitatively determine this residue. Previously, it was argued that the possible substitutions could be restricted to: Pro→Leu, Phe→Tyr, Ser→Cys, or Met→Phe. Therefore, the non-discriminations of Asn from Asp and Gln from Glu are of no concern here. Likewise, Trp is most probably not involved in the substitution and one would expect to find one residue of Trp in either the reference or aberrant peptide.

It was observed that a large proportion of Pro was lost during acid hydrolysis in the standard Pico-Tag method. This loss could be prevented by avoiding the addition of β-mercaptoethanol and ocatanoic acid. The comparable peptide, fibronectin type III connecting segment which contains a high percentage of Pro and Leu residues is used as reference to ensure the accuracy of this assay. This 25 residue fragment contains 5 Pro and 5 Leu residues with the molecular weight of 2732.1. The result for theoretical and obtained amino acid

composition of fibronectin type III connecting segment is depicted in Table 10. The matching data for all the residues indicate that this modification provided promissing recovery for Pro residue and has no influence on the accuracy of assay for other amino acid residues.

Table 11 summarizes the results from amino acid composition of reference β-casein A^1 peptide 114-169 and aberrant β-casein A^{1a} peptide 114-169. These 56-residue fragments consist of 15 different types of amino acid residues with one of each Tyr, Asp, Asn, Trp and Lys. The reference fragment contains 10 Pro and 10 Leu residues whereas there are 9.2 Pro and 11.1 Leu residues found in the aberrant fragment 114-169. This clearly indicated that there is a Pro to Leu replacement which occurred within this region and gives rise to a molecular weight difference of +16 dalton as well as an increase in hydrophobicity for the tryptic fragment. This is in good agreement with previous observation by peptide mapping and mass analysis.

From a genetic point of view, and a comparison of four of the codons for Leu (CUU, CUC, CUA, CUG) and Pro (CCU, CCC, CCA, CCG), it could be deduced that both amino acids have the same first and third bases. Therefore, the aberrant β-casein A^{1a} and standard β-casein A^1 is due to a point mutation resulting from the second base replacement of U by C. However, since there are 10 Pro residues in the β-casein A^1 peptide 114-169, the exact location of this mutation awaits for amino acid sequencing.

Table 10. Amino acid composition of fibronectin type III connecting segment

Amino acid residue	Theoretical number	Observed number
Aspartic acid/Asparigine	2+1	3.1
Glutamic acid/Glutamine	2+1	3.1
Leucine	5	5.3
Proline	5	5.2
Valine	2	2.1
Threonine	2	1.8
Histidine	2	2.0
Glycine	1	1.1
Isoleucine	1	0.9
Serine	1	1.0

Table 11. Amino acid composition of peptide 114-169 from reference and aberrant ß-casein A^1A^1

Amino acid residues	Number of residues	
	Reference ß-casein	Aberrant ß-casein
Tyrosine	1	0.8
Proline	10	9.2
Valine	4	4.0
Phenylalanine	2	1.7
Threonine	4	3.7
Aspartic acid/Asparigine	1+1	1.8
Glutamic acid/Glutamine	2+7	8.8
Serine	7	6.9
Leucine	10	11.1
Histidine	3	2.7
Tryptophan	1	0.8
Methionine	2	1.8
Lysine	1	0.9

4.7. Amino acid sequencing of β-casein A^{1a} peptide 114-169

The identification of N-terminal sequence of aberrant β-casein A^{1a} were ascertained by automated Edman degradation procedure. In order to obtain sufficient sample quantity as well as purity, twenty collections of aberrant β-casein A^{1a} fragment 114-169 were prepared, pooled and further purified. The amino acid sequencing started from the position 114. The results indicated good concordance with standard β-casein A^{1} sequence except there is a mutation being observed at position 137 from Pro to Leu residue which is in agreement with the previous observation. Therefore, it could be concluded that, in the sample which is termed as β-casein A^{1a}, there is neutral amino acid substitution from Pro to Leu at position 137 of the polypeptide and this substitution gives rise to an increase in hydrophobicity of β-casein A^{1} 114-169 fragment and an increase in molecular weight of 16 dalton. More importantly, this substitution can be designated as silent variant since it involves a neutral amino acid replacement which could not be detected by electrophoretic methods.

4.8. Distribution frequency of silent variants

It is well known that among the 20 standard amino acid residues, only five of them carry either positive or negative charges, the remaining 15 residues belong to neutral amino acids. As theorized by Ng-Kwai-Hang and Grosclaude (1992), the occurrence of amino acid substitution or deletion without any

charge difference should be much higher than that resulted from the alteration of net charge. In the other words, the occurrence of silent variants should be at least three times more than those known electrophoretic variants based on the ratio of neutral to charged residues (15 neutral residues : 5 charged residues, 3:1). Similar results could be assumed according to the calculation of genetic codons related to neutral or charged amino acid residues. It is also known that the possibility of silent variants being detected during the amino acid sequencing or other alternative molecular techniques are rather small and it is too expensive and time consuming.

The combination of peptide mapping and mass spectrometry involved a relatively fast approach for identification of silent variants according to their difference of hydrophobicity and mass shift.

In this study, a population consisting of 635 samples containing homozygous α_{s1}-casein BB, β-casein A^1A^1, β-casein A^2A^2, κ-casein AA and κ-casein BB were analyzed using peptide mapping and mass spectrometry. During peptide mapping, it was found that 25 out of 627 samples of α_{s1}-casein BB, 16 out of 158 β-casein A^1A^1, 7 out of 128 β-casein A^2A^2 and 11 of 415 κ-casein AA samples contained potential silent variants due to their changes on peptide profiles. However, most of them show no indication of the existence of silent variant during further reanalysis for α_{s1}-casein, β-casein A^2A^2 and κ-casein. As the confirmed silent variant, 7 of β-casein A^{1a} variant have been identified within

the 158 pre-screened β-casein A^1A^1 samples respectively a distribution frequency of approximately 4.4% in β-casein A^1 and 0.55% in β-casein loci. Moreover, since only heterozygous β-casein A^1A^{1a} was observed in this study with relatively low frequency, it suggests that the silent variant β-casein A^{1a} originated from mutation of β-casein A^1 during evolution. This newly found silent variant, termed as β-casein A^{1a}, involves the neutral amino acid substitution from Pro to Leu at the position 137 on β-casein A^1 polypeptide.

Among 190 possible amino acid substitutions which could result in genetic variants, only nine of them consist of mass shift of less than 5 dalton. Moreover, each amino acid residues contain specific hydrophobicity. Therefore, the combined approach by peptide mapping and mass analysis show great potential in identification of silent variants. The lack of specificity of the combination of peptide mapping and mass analysis for location of silent mutant make it necessary to employ the complimentary characteristic by amino acid composition analysis, or sometimes, amino acid sequencing. Although, it could be expected that all the possible silent variant would be detected by one or more of the previous described techniques, a study of scanning silent variants for β-lactoglobulin in New Zealand cow population indicated no silent mutation identified among 109 β-lactoglobulin, 9 κ-casein, 22 each of α_{s1}-casein, α_{s2}-casein and β-casein samples (Burr, 1996).

Together with previous reports (Carles, 1986; Addeo *et al.*, 1995; Visser *et al.*, 1995), it indicates the fact that silent variants occur in much lower frequency than expected. This phenomenon would be due to the following reasons:

First of all, the determination method developed in this study might not be sufficient to reveal all the possible silent variants of milk proteins. Silent variants occurring within small tryptic peptides might be missed due to their shorter retention during chromatography. Therefore, less resolution would be expected between those standard and aberrant peptides during peptide mapping.

Secondly, silent variants, which involve neutral amino acid substitutions or deletion might be lethal and therefore be lost during long term evolution.

Also, silent variants might be associated with low milk production and decreased during animal selection.

Silent variants as genetic variants are of great interest for their possible effect on production trait. The methodology approach for identification of silent variants in this study explored the new field for study of genetic variants of milk proteins.

5. Summary and Conclusion

The identification of genetic variants for α_{s1}-casein, β-casein and κ-casein were achieved by polyacrylamide gel electrophoresis under alkaline condition. Further separation of ß-casein A^1, A^2 and A^3 variants were carried out by electrophoresis under acidic condition. A total of 635 individual milk samples was analyzed in this project. The distribution frequency of different electrophoretic variants in these major casein components are in good accordance with previously described results.

The isolation and purification of four caseins were ascertained by anion-exchange chromatography by either gradient or step-wise separation. Gradient elution provided high purity of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein forms with minimum cross-contamination. While, step-wise elution was performed for large quantity of whole casein samples. Both fractionations were employed by using Tris-urea buffer with increasing molarity of NaCl in the mobile phase. In this study, the identity and purity of each casein forms were achieved by polyacrylamide gel electrophoresis and the results indicate that the obtained casein components are in electrophoretically pure forms.

Silent variants arise from amino acid substitutions or deletions which do not involve in the difference on net charges and hence would not be detected by conventional electrophoretic

methods. Identification of silent variant, however, would be approached either by changes on hydrophobicity or molecular weight. In order to rapidly target the possible mutations which result from neutral amino acid substitution or deletion, trypsin hydrolysis was performed on each casein forms. Peptide mapping of the tryptic hydrolysates were ascertained by reversed-phase HPLC with increasing gradient of acetonitrile. Tryptic peptide profiles with the same homozygous electrophoretic phenotype were compared and standard profiles were chosen. Potential silent variants were determined by comparison of peptide maps with standard refernce profiles.

Identity for each peak on the peptide profile was obtained by amino acid analysis and confirmed by mass analysis. As part of the result, this study demonstrated that tryptic peptide mapping by reversed-phase HPLC provides great potential in phenotyping of genetic variants of milk proteins.

Although there is no indication of the existence of silent variants being found in α_{s1}-casein, β-casein A^2A^2 and κ-casein, there are 7 out of 158 samples, which were originally classified as β-casein A^1A^1, being found to contain silent variants according to their presence of two extra peaks on peptide profiles. The increased retention time for β-casein A^1 tryptic peptide 114-169 indicates the occurrence of mutation in this region which results in the increasing of hydrophobicity. Mass analysis for both standard and aberrant fragments suggested that it involves an amino acid substitution and this substitution gives rise to a increase of 16 Da in molecular

mass. Further analysis by amino acid composition shows a substitution from Pro to Leu in the aberrant peptide. In order to characterize the exact mutation involved, amino acid sequencing for the aberrant peptide was performed. The result suggests that this substitution is located in position 137 along the β-casein A^1 polypeptide and it arises from the amino acid replacement from two neutral amino acid residues from Pro to Leu. Only heterozygous β-casein A^1A^{1a} was observed in this study which suggested that it originated from mutation of β-casein A^1 polypeptide. The newly identified silent variant, termed as β-casein A^{1a}, shows distribution frequency of 0.55% out of 634 β-casein samples.

6. Claims of Originality and Contribution to Knowledge

Based on the author's knowledge, the present study is the first attempt to identify on a large scale, genetic variants of milk proteins which are not identifiable by electrophoresis. The following aspects of this study constitute original contributions to knowledge:

1. Whole casein fractionation on AP-2 Protein PAK DEAE-15HR anion-exchanger column provides complete separation of electrophoretically pure forms of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. Thus, this fractionation could be performed to provide gram quantities of individual caseins.

2. Differentiation between genetic variants A and B of κ-casein was achieved by anion-exchange chromatography using AP-2 Protein PAK DEAE-15HR column.

3. Differentiation of genetic variants A^1, A^2 and B of β-casein based on changes in hydrophobicities of peptides due to amino acid substitution.

4. First application of mass spectrometry to complement results of reversed-phase HPLC and amino acid analysis for characterization of non-electrophoretic variants of caseins.

5. In the process of screening for non-electrophoretic variants of caseins, this project is the first to report the existence of a non-electrophoretic variant denoted as β-casein A^{1a}. This new variant has a Leu at position 137 instead of a Pro as in β-casein A^1.

6. The first report of a frequency of 4.4% within β-casein A^1 of the newly discovered variant.

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