FREQUENCY OF OCCURRENCE OF NOVEL MILK PROTEIN VARIANTS IN A NEW ZEALAND DAIRY CATTLE STUDY POPULATION

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ABSTRACT

Since the discovery of genetic polymorphism within milk protein genes, a considerable volume of research has been published relating milk protein genetic variants and milk production properties. Polymorphism of milk proteins can result in two effects:

(a) changes in the biological and physico-chemical properties of systems containing the variant protein,

(b) changes in the synthesis level of variant proteins.

As a result several studies of milk protein variants have identified phenotypes which may be commercially advantageous for specific products.

Currently employed methods to determine milk protein phenotypes are generally limited to electrophoretic techniques. The gel electrophoretic techniques commonly used are able to detect most milk protein variants that differ by their net electrical charge. However single amino acid substitutions that result in a change in net charge account for only 25% of the possible substitutions that could occur. The remaining 75% of potential variants are the result of a neutral residue substituted by another neutral residue - a 'silent' variant. Thus it is likely that some substitutions, and hence genetic variants have gone undetected in the past.

The purpose of this study was to develop new methods for determining the phenotype of milk proteins, and to determine the frequency of occurrence of silent or other novel variants in a New Zealand dairy cattle study population.

Polyacrylamide gel electrophoresis (PAGE), free zone capillary electrophoresis (CE), peptide mapping by reverse-phase HPLC and electrospray mass spectrometry (ESI-MS) were used in the characterisation of milk proteins purified from 109 individual dairy cows.

Three different PAGE systems were used. Alkaline-urea PAGE enabled the detection of α_{S1}-casein variants B and C, β-casein variants group A (variants A^1, A^2 and A^3) and B, and κ-casein variants A and B in the study population. Beta-casein variants A^1, A^2 and A^3 were subsequently resolved in an acid-urea PAGE system. The whey proteins were very poorly resolved in PAGE systems containing urea. Alpha-lactalbumin A, and β-lactoglobulin

(β-LG) variants A and B were resolved in a non-denaturing 'native' PAGE system. The frequencies of the various milk protein variants corresponded closely to figures previously published.

A free zone CE method that is able to resolve β-LG variants A, B and C was used to check the phenotype of purified β-LG samples. Three samples previously typed as β-LG BB were subsequently determined to be β-LG CC; one sample typed as β-LG BB was re-assigned as β-LG BC. This highlighted the limitations of PAGE systems for the detection of known variants.

Tryptic hydrolysis of purified casein proteins and β-LG, followed by reverse-phase HPLC separation of the resultant peptides was used to create peptide 'maps' of the hydrolysis products. Differences in peptide maps were noted between protein variants. The differences corresponded to peptides containing a substitution site. All samples analysed in this way contained more peptide peaks than expected. Analysis revealed that some were the result of incomplete digestion; others the result of chymotryptic-like cleavages. No aberrant peptide maps, indicative of a silent mutation, were detected.

Purified casein proteins and β-LG were subjected to ESI-MS for mass analysis. The mass of each protein species was determined as follows:

Protein	Average mass	Std. dev.
α_{S1}-CN B-8P	23614.9 Da	1.2 Da
α_{S2}-CN A-11P	25228.9 Da	1.5 Da
β-CN A^1-5P	24023.9 Da	3.1 Da
β-CN A^2-5P	23983.5 Da	1.8 Da
β-CN B-5P	24092.6 Da	n.d.
κ-CN A-1P	19038.8 Da	1.5 Da
κ-CN B-1P	19003.8 Da	n.d.
β-LG A	18362.6 Da	1.0 Da
β-LG B	18277.0 Da	0.9 Da
β-LG C	18287.2 Da	0.6 Da

In all cases the experimentally determined mass corresponded to the mass calculated from published primary sequences of milk protein variants.

In addition to the expected β-LG variant in each mass spectrum, additional species were detected differing from the mass of the β-LG species by increments of approximately 324 Da. Although less pronounced, the +324 Da molecular weight species were also detected in a sample of β-LG purchased from the Sigma Chemical Company. The additional species were also detected in whey prepared by ultra-centrifugation, although at a much lower level.

The 324 Da molecular weight adducts observed in ESI-MS spectra of purified β-LG are consistent with an addition of a lactosyl residue to the protein. The observation that these species remain after heat denaturation, reduction and RP-HPLC treatment suggest that the linkage is covalent. Lactulosyl-lysine is known to form in milk products during some processing conditions, particularly during heating. The observation of these glycated species in gently treated, unheated milk suggests that glycation may occur to some extent in the udder of the cow.

The association of the 324 Da molecule with β-LG does not alter the charge, molecular weight or hydrophobicity sufficiently to be detected by PAGE, CE or RP-HPLC.

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ABBREVIATIONS AND TERMINOLOGY

α-La	Alpha lactalbumin
α_{s1}-CN	Alpha S1 casein
α_{s2}-CN	Alpha S2 casein
β-CN	Beta casein
β-LG	Beta-lactoglobulin
κ-CN	Kappa casein
2-D	Two dimensional
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CHO	Carbohydrate
Da	Dalton
DAD	Diode array detector
DNA	Deoxyribose nucleic acid
ESI-MS	Electrospray ionisation - mass spectrometry
FAB-MS	Fast-atom bombardment mass spectrometry
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IEX	Ion exchange
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RP	Reversed phase
UHT	Ultra-high temperature

In the context of this work the following terms are defined as:

Genotype - the genetic constitution of an individual organism. The genetic make up of an animal which codes for the synthesis of an individual milk protein.

Phenotype - the characteristics of an expressed milk protein as a result of the interaction of its genotype with its environment. Post-translational modifications such as phosphorylation and glycosylation frequently alter the gene product (protein) prior to expression in milk.

Electrophoretic variant - a protein variant that is able to be resolved from other genetic variants by electrophoretic techniques.

Silent variant - a protein variant with (an) amino acid substitution(s) that has no net effect on the overall electrical charge of the protein - generally undetected by standard electrophoretic techniques. Silent variants may occur when a neutral amino acid residue is substituted by another neutral residue, or when a residue carrying a charge is substituted by a similarly charged residue.

Introduction

Since the domestication of cattle and the adoption of pastoral agriculture, milk has been an important food source for man. Internationally, dairy production has developed into a 518 million tonnes of milk per year industry (International Dairy Federation, 1994). New Zealand production accounts for about 1.7% of this or 8.3 million litres (Evans, 1995). Successive advances in technology have seen the traditional core products of cheese, fresh milks, cream and butter, greatly expanded to include dietary formulations, concentrated and dried milk products, ultra-heat-treated milk products, food ingredients, new functional products and non-food technical applications.

Traditional protein sources are increasingly being utilised as ingredients in a growing number of formulated foods. The benefits of milk proteins as ingredients in other foods stems from their excellent nutritional characteristics and their ability to contribute unique and essential functional properties to the final product. As we begin to more fully understand the elements which determine the behaviour of a protein, we have seen a rapid expansion in the technological opportunities to utilise these characteristics in novel ways.

However, proteins that are adapted to human technological needs are not necessarily as nature would have designed them. In the past ten years we have witnessed a rapid expansion in the number of genetically altered or engineered organisms that express proteins with superior characteristics for specific applications (Jiminéz-Flores and Richardson, 1988, 1991; Krimpenfort *et al.*, 1991; Bian, 1991; Martin and Grosclaude, 1993). This technology has begun to be used in applications involving higher organisms. A modified genomic alpha-1-antitrypsin gene construct has been successfully introduced into the DNA of inseminated eggs of sheep and goats (Bian, 1991). Alpha-1-antitrypsin, a serum glycoprotein used in the treatment of emphysema, is expressed in the milk of these transgenic animals. Tissue plasminogen activator (t-PA), used to dissolve fibrin clots in coronary occlusions, has similarly been expressed in the milk of transgenic goats. Intact lactoferrin DNA has been incorporated into the DNA of bovine embryos by microinjection of pronuclei of the

oocytes after *in vitro* fertilisation. The anti-bacterial human iron-binding/transport protein lactoferrin is a highly desirable ingredient that could be included in infant formula milk powders.

Yet in the present global environment there is an overwhelming rejection of genetically engineered milk or milk products for human consumption (Dionysius, 1991; Coghlan, 1991; Joyce, 1991; Bruhn, 1992; Driesel, 1992; Nathan, 1992; Watson, 1994; Rifkin and Howard, 1993; Anon., 1995). The options available to meet specific processing, functional and nutritive needs are thus presently limited to exploration and utilisation of the full range of naturally occurring genetic variations within the dairy cow population.

Since the original finding of genetic polymorphism (variance in the protein primary sequence) within the bovine β-lactoglobulin (β-LG) gene by Aschaffenburg and Drewry (1955), a considerable volume of research has been recently published relating milk protein genetic variants and milk production properties (McLean *et al.*, 1984; Ng-Kwai-Hang *et al.*, 1984; Ng-Kwai-Hang *et al.*, 1986; Ng-Kwai-Hang *et al.*, 1987; Aleandri *et al.*, 1990; Bech and Kristiansen, 1990; Ng-Kwai-Hang and Monardes, 1990a; Van Eenennaam and Medrano, 1991; Lawrence, 1993; Rahali and Ménard, 1991; van den Berg *et al.*, 1990, 1992; Bovenhuis *et al.*, 1992; Jakob and Puhan, 1992; Mariani *et al.*, 1992; Oloffs *et al.*, 1992; Delacroix-Buchet *et al.*, 1993; Hill, 1993; Hill and Paterson, 1994; Hill *et al.*, 1995a, 1995b, 1995c; Macheboeuf *et al.*, 1993; Sacchi *et al.*, 1993; Jakob, 1994a, 1994b; Puhan and Jakob, 1993). Reported results indicate genetic polymorphism of milk proteins can give rise to two effects:

(a) changes in the biological and physico-chemical properties of systems containing the variant protein such as gelation, viscosity, syneresis, heat stability, aggregation and emulsification

(b) changes in the synthesis levels of the variant proteins - despite having the mutation occurring in the coding sequence, rather than the regulation region of the milk protein gene (Bouniol *et al.*, 1993; Schild *et al.*, 1994; Levéziel *et al.* 1994; Wagner *et al.*, 1994). Other mutations are also seen in promoter and regulatory sequences (Schild *et al.*, 1994; Wagner *et al.*, 1994).

Subsequent studies of the combinations of milk protein variants expressed in milk (phenotypes) have identified milk types that are commercially advantageous for specific products such as cheese (Aleandri *et al.*, 1990; Marziali and Ng-Kwai-Hang, 1986a, 1986b; Davoli *et al.*, 1990), UHT milk (McLean *et al.*, 1987; Kristiansen, 1990; van den Berg *et al.*, 1990) or yogurt (Vegarud *et al.*, 1990; McLean and Schaar, 1989).

Consequently the concept of segregating milk for particular milk products, based on desirable milk protein phenotype, has been the topic of considerable debate (Jakob and Puhan, 1992; Puhan and Jakob, 1993; Jakob, 1994a). Effective implementation of milk segregation strategies rely on

(a) accurate identification of the milk protein phenotype of individual animals, and

(b) an appropriate breeding programme to align the number of animals with the desired volume of product.

Our current knowledge of milk protein phenotypes extends mainly to those that are detectable by electrophoresis (electrophoretic variants). These are due to amino acid substitutions and deletions which generate a net difference in the overall charge on the protein and hence facilitates their separation by electrophoretic techniques.

It is important to note that polymorphism detected by electrophoresis provides incomplete information as to the real polymorphism at a genetic level. Only three out of four mutations of the codon generate a substitution of one amino acid for another due to redundancy of the genetic code (multiple codons, or nucleotide triplets, generate the same amino acid). Additionally only one out of three amino acid substitutions (on average) modify the net charge of the protein which is then able to be resolved by electrophoresis. Substitutions involving neutral amino acid groups that do not alter the net charge of the protein ('silent' variants) subsequently would not be detected by electrophoresis. Theoretically electrophoretic techniques allow the detection of only 23% of the mutations occurring at the coding level of the corresponding gene (3 out of 4 mutations at the DNA level resulting in an amino acid

substitution; 1 out of 3 amino acid substitutions resulting in a modification of the net charge of the protein) .

Silent substitutions are likely to be associated with changes in milk physico-chemical properties, composition, yield of milk components, and functionality of milk as already observed with electrophoretic variants.

Some studies of milk protein phenotypes and their effect on milk composition, milk yield and milk functional behaviour, have generated results that are confusing and contradict previous studies. The experimental designs in some studies have taken into account many of the variables encountered such as differences in breed, age, stage of lactation and geographic location (Ng-Kwai-Hang *et al.*, 1984; Ng-Kwai-Hang *et al.*, 1986; Ng-Kwai-Hang *et al.*, 1987; Ng-Kwai-Hang and Monardes, 1990b; Bovenhuis and van Arendonk, 1991; Van Eenennaam and Medrano, 1991, Bovenhuis *et al.*, 1992; Hill, 1993; Hill and Paterson, 1994). Additionally linkage effects between genes physically clustered on a chromosome have been incorporated. Despite this some effects attributed to phenotype are still inconclusive.

Given the limitations of electrophoretic techniques currently used for determination of milk protein phenotype, it is likely that some phenotypes have been incorrectly assigned in the past. It is possible that other modifications of milk proteins, such as phosphorylation and glycosylation could have an influence on milk production, milk composition and milk functional behaviour.

Thus using traditional electrophoretic techniques we are seeing only part of the phenotypic picture.

The objectives of this current work was to:

(1) develop new methods for determining the phenotype (both silent and electrophoretic) of the casein proteins and β-LG

(2) determine the frequency of occurrence of silent or other novel variants in a New Zealand dairy cattle study population

Once the true phenotype of an individual cow is known, then we may be able to isolate more precisely the effects of milk protein polymorphism on composition, yield and functionality from environmental and other effects. Genotyping of sires and dams additionally enables milk protein genes to be incorporated as genetic markers in accelerated breeding programmes for desirable milk types. Utilisation of naturally occurring genetic variations in this way provides a consumer acceptable means to control aspects of milk production and composition, and improve processing.

Literature Review

1. MILK COMPOSITION

Milk is a complex mixture of lipids, proteins, carbohydrates, vitamins and minerals, structured to provide a complete diet for infant mammals. The average composition of bovine milk from Western cattle breeds (Guernsey, Jersey, Ayrshire, Brown Swiss, Shorthorn and Holstein/Friesian) is 86.6% water, 4.1% fat, 3.6% protein, 5.0% lactose and 0.7% minerals (Swaisgood, 1985). The lipid fraction shows a bewildering number of components, however triglycerides account for 97-98% of total lipids (Jenness, 1974; Johnston, 1974). Environmental and genetic influences also contribute to variations in milk composition and the proportion of lipid to other milk constituents (Hill and Paterson, 1994). Lactose is the predominant carbohydrate, accounting for 50% of the solids in skim milk (Morrisey, 1985). Its synthesis is associated with α-lactalbumin, which is part of the UDP-galactosyltransferase enzyme complex. Milk salts consist principally of chlorides, phosphates, citrates and bicarbonates of sodium, potassium, calcium and magnesium (Pyne, 1958). The distribution of calcium, magnesium, phosphate and citrate between soluble and colloidal phases of milk and their interaction with milk proteins are important factors in the stability of dairy products.

The major milk proteins may be subdivided into casein or whey proteins on the basis of their solubility at pH 4.6 at 20°C. The isoelectrically precipitated proteins are classified as caseins and account for about 80% of the total protein. The remaining soluble proteins are classified as whey proteins. The phenotype and relative proportions of casein and whey proteins have major influences on the manufacturing properties of milk (see discussion Section 6, page 29-38).

2. HETEROGENEITY OF BOVINE MILK PROTEINS

Bovine milk contains 30-35 g l^{-1} of total protein (Figure 1) of which about 80% is present as large spherical complexes with inorganic species, particularly calcium phosphate, known as micelles. The mammary gland produces six major secretory protein products : α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-casein (β-CN), κ-casein (κ-CN), α-lactalbumin (α-La) and β-lactoglobulin (β-Lg). They are all products of codominant, allelic autosomal genes. Genetic polymorphisms, or genetic variants, of each are also observed. In addition to the primary gene product several milk proteins are subject to post-translational modifications such as glycosylation and phosphorylation.

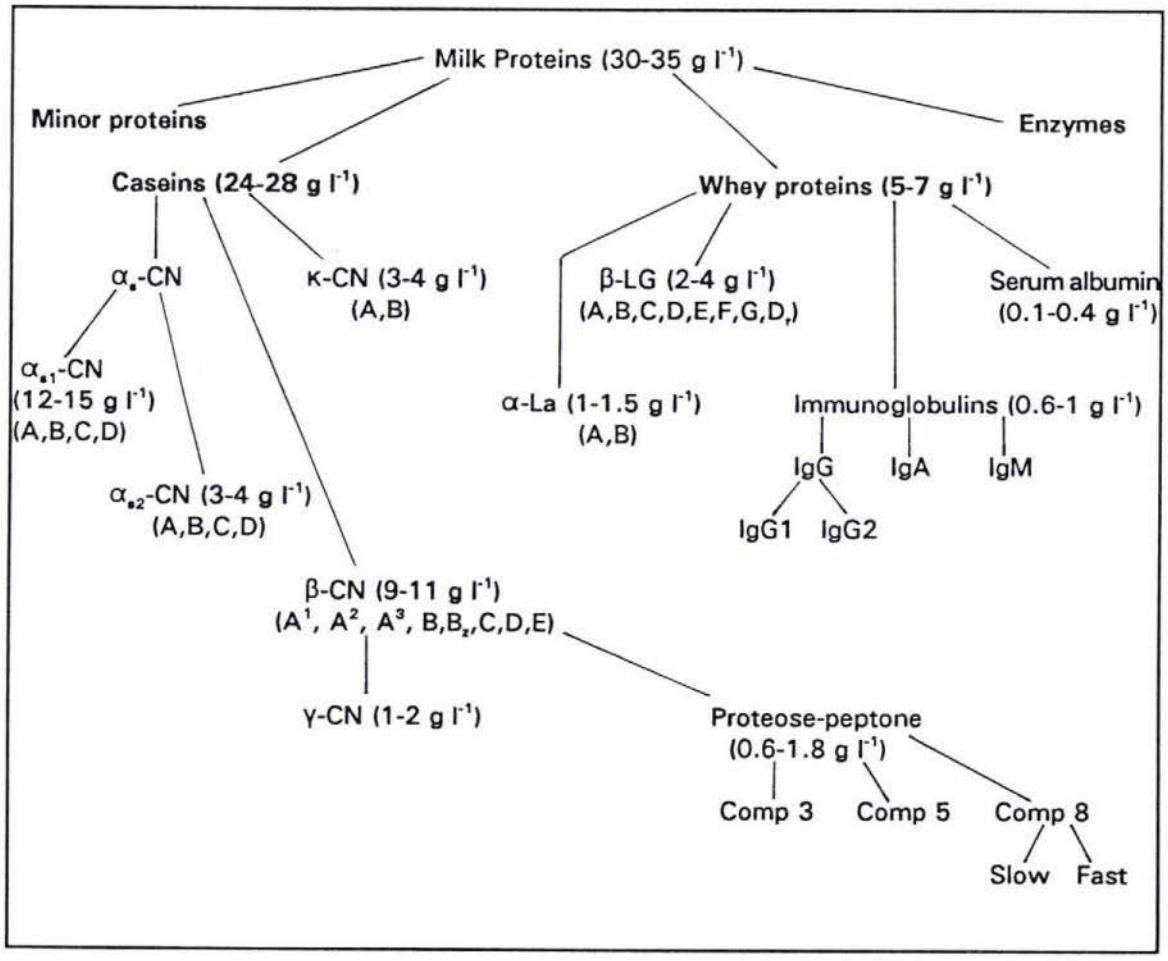


FIGURE 1 Distribution of major milk proteins and peptides in bovine milk

2.1 CASEINS

Caseins were originally subdivided on the basis of their solubility in 0.25 M Ca^{2+} at pH 7 and 37°C into 'calcium sensitive' or 'calcium insensitive' fractions (Waugh and von Hippel, 1956). The advent of starch gel, and later alkaline urea polyacrylamide gel electrophoresis (PAGE) allowed the identification of individual families. With the determination of their primary sequence, it was then possible to define them on the basis of their chemical structure, rather than solubility under specific conditions.

2.1.1 α_{s1}-CN

The primary sequence of α_{s1}-CN B was established by Mercier *et al.* in 1971. It is a polypeptide of 199 residues (see Figure 2). Three hydrophobic regions are discernable - residues 1 to 44, 90 to 113 and 132 to 199. Additionally the region 41 to 80 contains a cluster of phosphoseryl residues. These observations suggest a dipolar structure with a globular hydrophobic domain and a highly solvated and charged domain. Amongst the caseins there is a strong sequence homology in these hydrophobic and phosphoseryl regions. This conservation of structural domains suggests that these proteins may have evolved from a common ancestral gene.

Five genetic variants of α_{s1}-CN are currently recognised - A, D, B, C and E (in decreasing order of mobility in alkaline urea gel electrophoresis) (Eigel *et al.*, 1984). The polymorphs are breed specific with the B variant predominant in *Bos taurus* and C predominant in *B. indicus* (zebu) and *B. grunniens* (yak). The E variant has only been observed in some *B. grunniens* milk. The A variant differs from the B variant by the deletion of residues 14 to 26, and is rarely seen in *B. taurus* milk. Erhardt (1993a) reported a new α_{s1}-CN variant with a low frequency occurring in German Black and White cattle, differing from other known variants by its electrophoretic mobility in alkaline polyacrylamide gels. The exact nature of the mutation has yet to be determined.

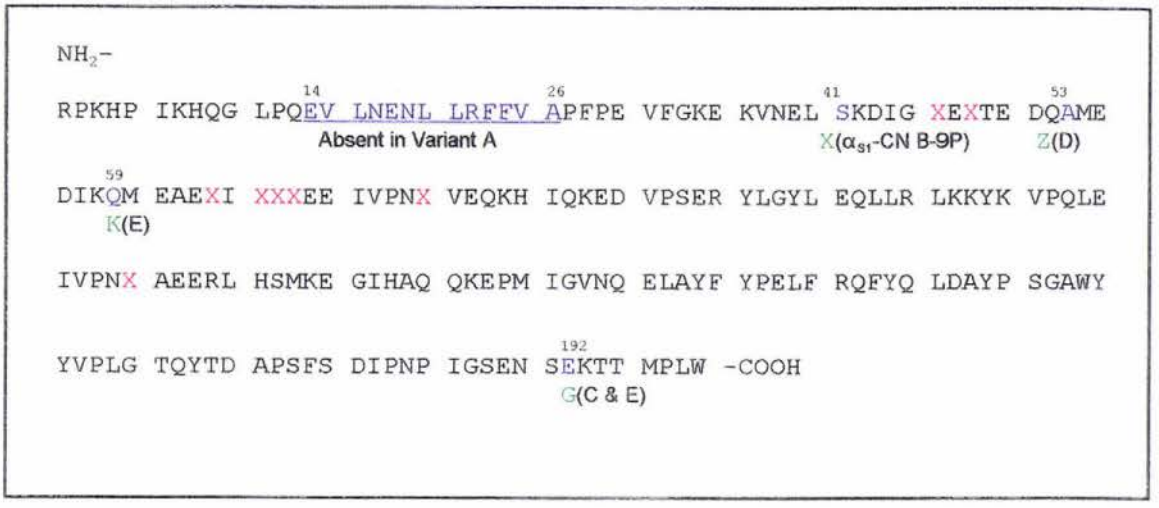


FIGURE 2 Primary structure of α_{S1}-CN B-8P. Sites of mutational differences between the B variant and other variants are indicated in blue and green respectively. The alternative phosphoserine (X) at position 41 represents an additional phosphorylation site in the α_{S1}-CN B-9P variant. The D variant differs from the B variant by a phosphothreonine (Z) at position 53. Phosphoserine residues (X) are highlighted in red.

2.1.2 α_{S2}-CN

Brignon *et al.* (1976, 1977) determined the primary structure of α_{S2}-CN (see Figure 3). It consists of 207 residues, possibly existing as dimers linked via a disulphide bond (Hoagland *et al.*, 1971). A cluster of negative charges at the N-terminus and positive charges at the C-terminus create a strong dipolar arrangement, suggesting that electrostatic interactions may be an important factor in influencing structural characteristics.

The four genetic variants of this protein have been designated as A, B, C, and D. Whilst variants A and D have been observed in *B. taurus*, B is the predominant variant. C is observed specifically in *B. grunniens*. α_{S2}-CN D differs by a deletion of 9 residues around the phosphoserine residue cluster SerP56-SerP58 (Grosclaude *et al.*, 1978, 1979) although the exact location of the deletion has not yet been established.

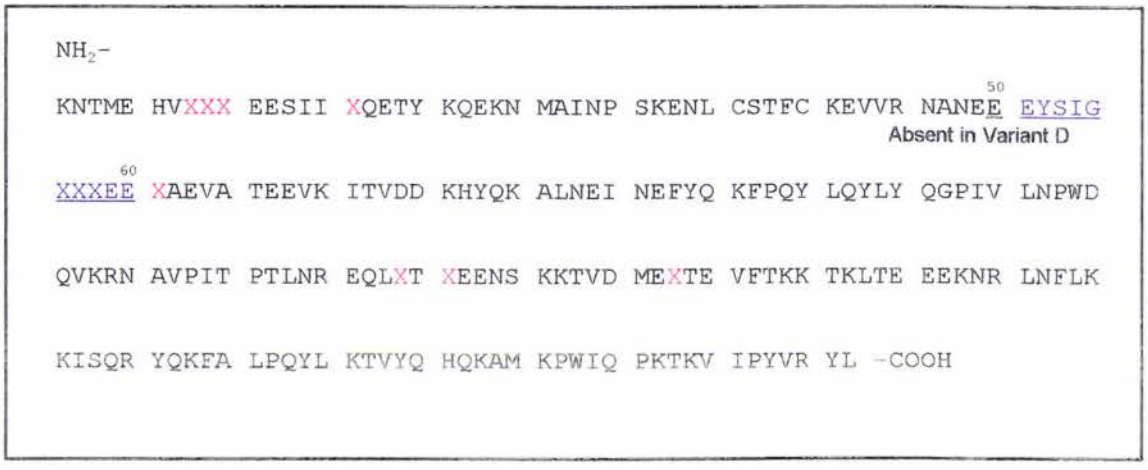


FIGURE 3 Primary structure of α_{S2}-CN A-11P. The D variant differs from the A variant by a deletion of 9 residues between positions 50 and 60 (indicated in blue). The exact location has not been determined. The notation X (in red) indicates phosphoserine.

2.1.3 β-CN

The β-CN family consists of at least seven genetic variants (A^1, A^2, A^3, B, C, D, E), of which A^1, A^2, A^3 and B are the predominant types seen in Western cattle breeds. A^1, A^2 and A^3 variants are only distinguishable by acid urea gel electrophoresis or isoelectric focusing. Another possible variant, B_z, has been observed in Indian and African zebu (*B. indicus*) cattle (Aschaffenburg, 1968). Although it has the same electrophoretic mobility as the B variant, chymotryptic peptide mapping has revealed some differences in peptide elution profile. Visser *et al.* (1995) recently identified a further β-CN variant, differing from the A^1 variant by a 'silent' substitution of a leucine residue at position 152 for a proline residue. The new genetic variant has been named β-CN F-5P in accordance with guidelines for the nomenclature of milk proteins (Eigel *et al.*, 1984).

β-CN A^2 consists of 209 amino acids (see Figure 4). The primary structure was determined by Ribadeau-Dumas *et al.* (1972). β-CN is the most hydrophobic of the caseins, with a highly charged domain clearly separated from a large hydrophobic domain. The unusually high frequency (0.17) of prolyl residues most likely influences the number of β-turns present (Andrews *et al.*, 1979; Graham *et al.*, 1984).

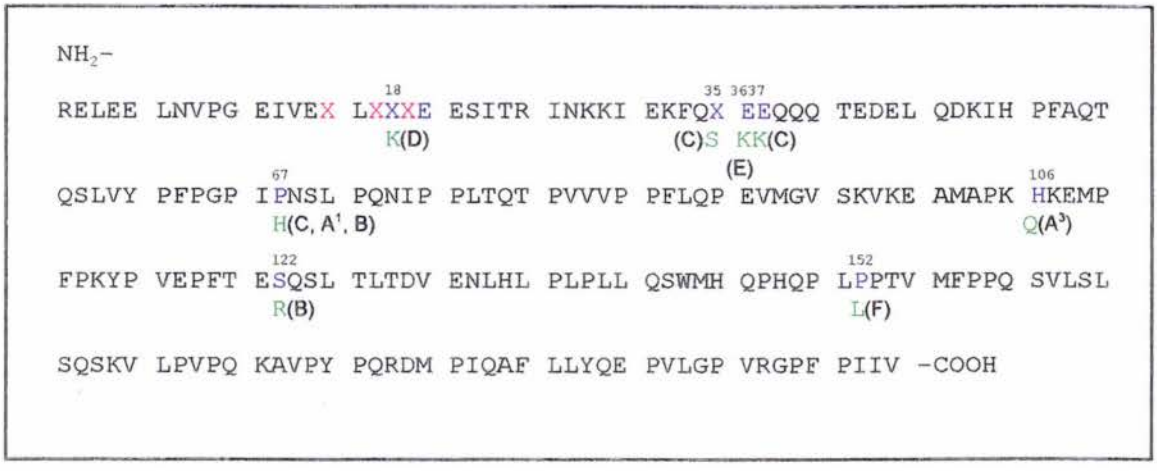


FIGURE 4 Primary structure of β-CN A^2-5P. Mutational differences between the A^2 variant and the A^1, A^3, B, C, D, E and F variants are indicated in green. X denotes phosphoserine (shown in red).

2.1.4 κ-CN

The primary structure of κ-CN, determined by Mercier *et al.* (1973), is shown in Figure 5. In comparison with other caseins it is notable for its carbohydrate moieties attached via threonyl residues, and the absence of phosphoseryl clusters. κ-CN contains a single phosphoserine residue. Consequently κ-CN does not bind Ca^{2+} to the same degree as the other caseins; thus its solubility is independent of this ion. The amphipathic nature of this milk protein has been appreciated for many years due to the specific chymosin-catalysed hydrolysis of the Phe105-Met106 bond. This releases the polar macropeptide from κ-CN, destabilising the casein micelle and results in clotting of milk. This is the central process in the production of many cheeses.

Approximately two thirds of κ-CN molecules are glycosylated; all κ-CN molecules carry at least one phosphate group. Considerable heterogeneity is seen in this protein, with at least seventeen minor components varying in carbohydrate type and content, and/or phosphate content (Vreeman *et al.*, 1977; Doi *et al.*, 1979; Fournet *et al.*, 1975; Mollé and Léonil, 1995). κ-CN, as isolated from milk, occurs in the form of a mixture of polymers linked by intermolecular disulphide bonding, (Swaisgood and Brunner, 1963) although Beeby (1964) suggests that the native form may be the reduced monomer.

Two variants, A and B, are common in *B. taurus*. The A variant is seen in the Friesian breed with a frequency of approximately 0.68. The opposite is seen in the Jersey breed, where the B variant is seen at a high frequency (0.77) (McLean *et al.*, 1984).

NH_2-

EEQNQ EQPIR CEKDE RFFSD KIAKY IPIQY VLSRY PSYGL NYYQQ KPVAL INNQF

LPYPY YAKPA AVRSP AQILQ WQVLS DTVPA KSCQA QPTTM ARHPH PHLSF MAIPP

KKNQD KTEIP TINTI ASGEP TSTPT ^{136}IEAVE STVAT LEA^{148}XP EVIES PPEIN TVQVT
T(A) D(A)

STAV -COOH

FIGURE 5 Primary structure of κ-CN B-1P. The A variant differs from the B by substitutions at positions 136 and 148(indicated in green). Phosphoserine is represented as X (in red); pyroglutamate as E.

2.2 WHEY PROTEINS

The major families of proteins included in this class are β-LG, α-La, serum albumin and immunoglobulins. Previously the proteose-peptones were included. When these fragments were sequenced it was found that these were proteolysis products of β-CN and are subsequently now included in the casein family.

2.2.1 α-La

Of the three genetic variants of α-La that are known, only the B variant is observed in milk from Western cattle. Brew *et al.* (1970) determined that α-La is a polypeptide consisting of 123 amino acids (Figure 6). Four intramolecular disulphide bridges occur in the molecule as shown. Minor forms of α-La have been reported, most containing some form of carbohydrate moiety - hexosamine (Gordon, 1971), mannose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid (Barman, 1970). Barman (1973) also reported an additional variant form of α-La differing by one less disulphide bridge than that described by Brew *et al.* (1970).

α-La forms part of an enzyme complex in the synthesis of lactose. It interacts with galactosyltransferase, an enzyme that catalyses the transfer of galactose from uridine diphosphate galactose to N-acetylglucose. Without α-La, glucose is an extremely poor substrate for galactosyltransferase.

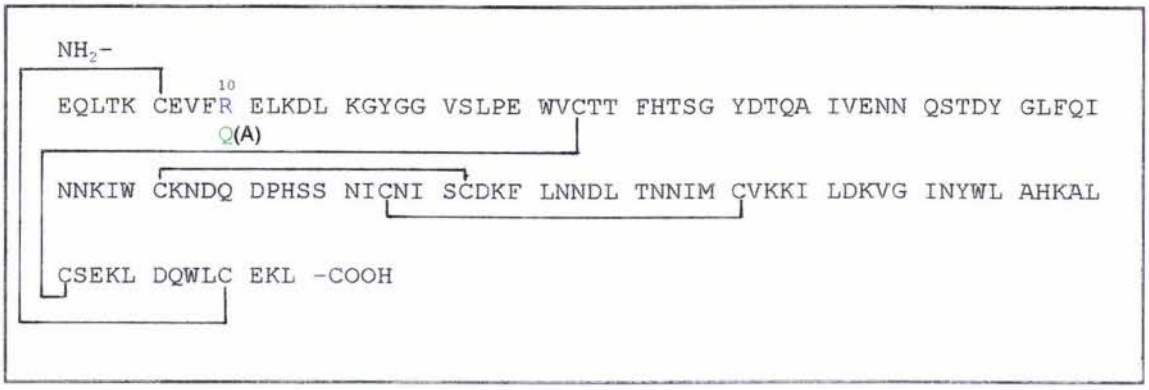


FIGURE 6 Primary sequence of α-Lac B. The A variant differs from the B by a substitution at position 10 (shown in green). Intramolecular disulphide bridges are indicated between residues 6 and 120, 28 and 111, 61 and 77, and 73 and 91.

2.2.2 β-LG

β-LG is the principal whey protein in bovine milk. Two genetic variants, A and B are found in all breeds of *B. taurus* and *B. indicus*. Some breeds of *B. taurus* also express variants C and D. The Droughtmaster breed of *B. taurus* includes a further variant, β-LG_{Dr}, containing both an amino acid substitution and a single glycosylation (Bell *et al.*, 1970). Several more variants are seen in the Yak[1] (*B. grunniens*) and in Bali (Bentang) cattle (*B. javanicus*) (Bell *et al.*, 1981). Baranyi *et al.* (1993) identified a possible further variant in Hungarian Grey cattle. The nature of this mutation is yet to be determined.

Bovine β-LG contains 162 residues. Five of these are cysteine. Four occur as disulphides and one as a free thiol. One of the disulphides occurs invariably between residues 66 and 160 (Figure 7). The second is estimated to be distributed approximately 50% between residues 106 and 119, or between residues 106 and 121. In the pH range from 5.2 to 5.7, all the genetic variants investigated have been shown to exist primarily as dimers. Initially it was thought that hybrid dimers could not exist. Subsequent experiments have clearly shown hybrid β-LG AB dimers. Below the isoionic point, and particularly below pH 3.5, the dimer dissociates into monomers. Dissociation also occurs above pH 7.5, although there

[1] Referred to as β-LG D_{yak} by Grosclaude et al. (1976) or as β-LG E as suggested by the Report of the Committee on the Nomenclature and Methodology of Milk Proteins of the Dairy Foods Research Section, Dairy Foods Division, American Dairy Science Association (Eigel et al., 1984).

is some debate as to the extent (McKenzie, 1967; Zimmerman *et al.*, 1970). In the isoelectric region octomerisation is also known to occur. The pH dependence with maximum octomerisation at pH 4.6 suggests that carboxyl groups are involved. Three carboxyl groups occur in the vicinity of Asp64 in β-LG A. This residue is replaced by a Gly residue in the B variant. Swaisgood (1982) observed the B variant as having a greater negative change in entropy during octomerisation than the A variant. This tends to support the concept of Asp64 as being at the contact site as fewer water molecules would be released from the B variant monomers than the A during octomerisation.

The first determination of the primary sequence of β-LG was made by Braunitzer *et al.* (1972).

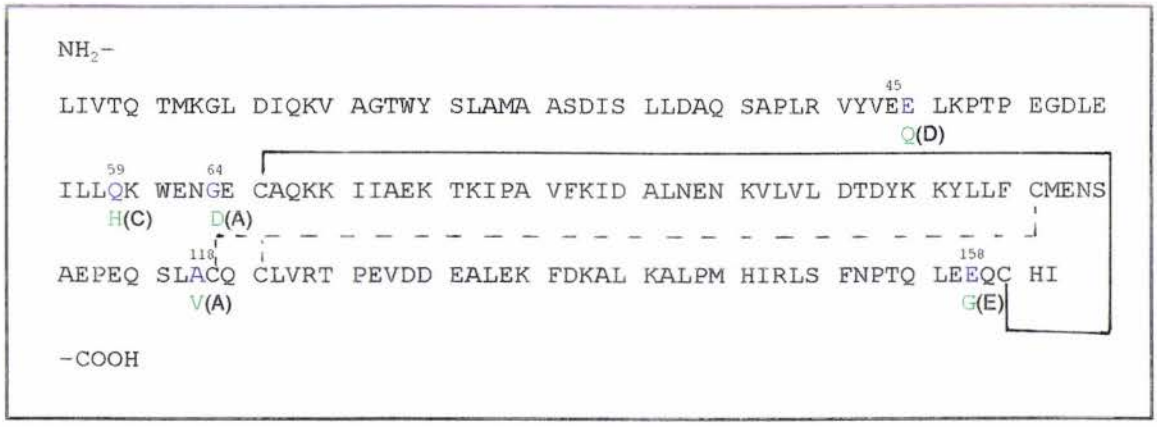


FIGURE 7 Primary sequence of β-LG B. Mutational differences between the B variant and A, C and D variants are as indicated. Intramolecular disulphide bridges are as indicated between residues 66 and 160; the second disulphide bridge occurs between residues 106 and 119, or 106 and 121 as the free thiol group exists in a 50:50 distribution between residue 119 and 121.

2.2.3 Serum albumins and immunoglobulins

Serum albumin accounts for approximately 1.2% of the total milk proteins. Serum albumin (SA) prepared from milk is physically and immunologically identical to blood SA. Analysis by isoelectric focusing (IEF) shows considerable micro-heterogeneity in SA (Spenser and King, 1971) although no variant species are known to exist. *Bos* SA consists of 582 amino acid residues with 17 intramolecular disulphide bonds and a single free thiol (Figure 8). The complete primary sequence was determined by Brown (1975).

The immunoglobulins form an extremely heterogeneous family of proteins and are classified primarily by immunochemical criteria. Four classes have been identified in bovine milk (IgG, IgA, IgM and IgE), all existing as glycoprotein monomers or polymers of a basic unit composed of four polypeptide chains linked covalently by disulphide bonds.

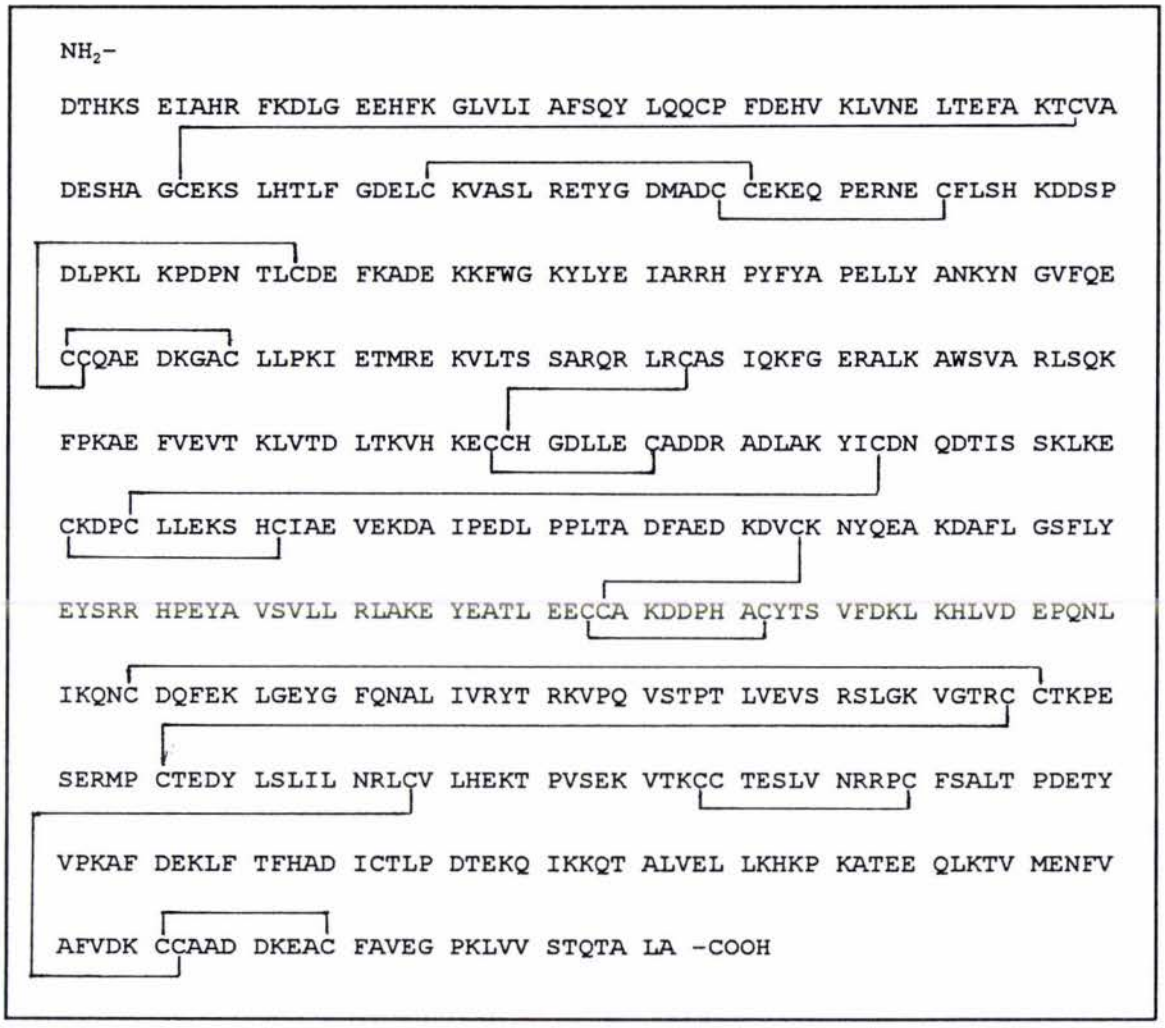


FIGURE 8 Primary sequence of bovine serum albumin. Intramolecular disulphide bridges are as indicated.

3. THE MOLECULAR BASIS OF POLYMORPHISM IN MILK PROTEINS

The central dogma of molecular genetics proposed by F.H.C. Crick in 1953 states that genetic information flows from DNA to RNA to protein. Three major processes are involved in the preservation and transmission of genetic information. The first is replication, the copying of DNA to form identical daughter molecules. The second is transcription, the process by which the genetic message in DNA is transcribed into the form of messenger RNA, to be carried to the ribosomes. The third is translation -the process by which the genetic message is decoded on the ribosomes. RNA is used as a template in directing the specific amino acid sequence from the nucleotide triplets during protein biosynthesis. This central dogma is supported by clear demonstration that the sequence of nucleotides in a gene bears a linear correspondence to the sequence of amino acids in the protein coded by the gene.

The somatic cells of eucaryotic organisms are usually diploid and contain twice the number of chromosomes found in the germ cells, which are haploid. Each gene in a eucaryote thus occurs in two forms, or alleles. Eucaryotic organisms usually reproduce by sexual conjugation, during which genes from both parents are exchanged and incorporated into the genome of the progeny by the process of recombination.

Various types of chemical changes in DNA can lead to mutant gene products. Single point mutations can be divided into four major classes on the basis of the change produced in the DNA. In translational mutants one purine-pyrimidine base pair is replaced by another, *i.e.* A-T for G-C or *visa versa*. In transversional mutations a purine-pyrimidine base pair is replaced by a pyrimidine-purine pair (A-T to T-A, G-C to C-G). The third type involves insertion of an extra base pair. This results in a frame-shift mutation where the normal reading-frame of the nucleotide triplets is pushed out of register by the insertion. Similarly a deletion of one or more bases from the DNA results in a frame-shift. Transitions and transversions are relatively benign as they result in the substitution of only one amino acid in the peptide chain. It is possible for the defective protein to still be functional. Insertions or deletions, unless in a multiple of three base pairs, cause all the DNA beyond the point of the mutation to be misread. Such mutations are often fatal.

Eucaryotic organisms contain far more DNA than actually codes for protein. It has been estimated that in mammalian DNA, perhaps only 2% codes for proteins. The function of the remaining DNA largely remains a mystery. Coding sequences (exons) in these split genes are separated by intervening sequences (introns), which are removed in the conversion of the primary transcript into mRNA. Many exons encode discrete structural and functional units of proteins. We now know that introns control aspects of the timing and level of gene expression. These include promoters and enhancers of transcription (*cis*-acting elements) and factors specific for the transcriptional machinery or modulators of DNA binding (*trans*-acting elements).

A further source of polymorphism is derived from 'exon skipping'. Aberrant splicing of primary transcripts, where intron sequences are removed and the remaining exon sequences are spliced together, is suspected to be responsible for polymorphism of α_{s1}- and α_{s2}-CN in ruminant species (Grosclaude *et al.,* 1970; Grosclaude *et al.,* 1979; Brignon *et al.*, 1990; Bouniol *et al.,* 1993).

Recently it has been shown that the point mutation in the fourth intron of the α_{s1}-CN A gene from a cow in New Zealand responsible for causing exon skipping is different to that published in the literature by Mohr *et al.* (1994) (R. Wilkins, personal communication).

Many mammary-gland nuclear factors (mammary gland factor, milk protein-binding factor, pregnancy-specific mammary nuclear factor, mammary cell-activating factor) bind specifically in the 5'-flanking regions of milk-protein genes. Point mutations in these 5'-flanking regions may affect the binding of these factors and hence the transcription activity of the gene.

4. POST TRANSLATIONAL MODIFICATIONS

As a result of translocation many proteins are modified in the lumen of the endoplasmic reticulum. Amino-terminal signal sequences are cleaved by signal peptidase, disulphide bonds are formed and some proteins are glycosylated. Further processing may occur in the Golgi apparatus - O-linked sugars are fashioned and N-linked ones may be modified. Serine and threonine residues may be phosphorylated. These post-translational modifications are functionally distinct from genetic mutations as they occur after translation of RNA.

Phosphorylation of casein occurs post-translationally in the Golgi apparatus where casein kinases have been identified (Bingham, 1979). Sequence homology at the site of phosphorylation has led to a postulated recognition sequence of Ser/Thr-X-Glu/SerP (Mercier *et al.*, 1972). It also appears that Asp can replace Glu in the recognition sequence as Ser41 is sometimes phosphorylated in the α_{S1}-CN sequence yielding α_{s0}-CN (Manson *et al.*, 1977). However Thr-X-Asp does not appear to be a recognition site as this site is not phosphorylated in α_{S1}- (Thr39), α_{S2}- (Thr72, Thr138) or β-CN (Thr41). Only one case of phosphorylation of a threonyl residue is known (α_{S1}-CN D) although the Thr-X-Glu sequence occurs four times in α_{S2}-CN. Thus it appears that the recognition sequence is not sufficient *per se* for recognition by the phosphorylating enzyme. In α-La and β-LG these sequences are buried within secondary structure, rendering them inaccessible. For interaction with casein kinases these residues must be exposed - typical in the unstructured polar domains of caseins. Phosphorylation of κ-CN is likely to be influenced by previous glycosylation at or near the susceptible residues.

Glycosylation of κ-CN probably also occurs in the Golgi apparatus. It is thought that glycosylation always occurs on a β-turn (Loucheux-Lefebvre *et al.*, 1978). Comparison with predicted structures of human κ-CN macropeptide, containing roughly three times more carbohydrate than bovine, shows twice as many Thr residues in β-turn regions (Fiat *et al.*, 1980).

4.1 PHOSPHORYLATION

The major proportion of α_{s1}-CN contains eight phosphate groups. A minor component previously designated α_{s0}-CN was found to have a primary amino acid sequence identical to α_{s1}-CN apart from a substitution of a serine for a phosphoserine. The α_{s1}-CN D variant differs from the α_{s1}-CN B variant by an alanine to phosphothreonine substitution. Thus the major component of α_{s1}-CN D contains nine phosphate groups and the minor component ten phosphate groups.

At least one major and four minor components are seen in the α_{s2}-CN family corresponding to differences in phosphate content. The major component contains eleven phosphorylated serine residues although the exact location of them has yet to be determined.

β-CN A^1, A^2, A^3, B and E variants contain five phosphoserines. In the C variant one phosphoserine is substituted by a serine. In the D variant a phosphoserine is substituted by a lysine residue.

Phosphorylation increases the viscosity, water adsorption and solubility of casein in acidic medium, but decreases the emulsifying capacity (Matheis *et al.*, 1983; Girerd *et al.*, 1984). Courthaudon *et al.* (1989) reported similar results. They concluded that the binding of acidic phosphate groups and consequent increase in electrostatic repulsion was responsible for the observed differences between phosphorylated and de-phosphorylated casein. Chemically induced phosphorylation of β-LG increased viscosity and emulsion stability compared to native β-LG (Woo and Richardson, 1983).

The high proportion of ester-bound phosphate observed in the casein proteins is closely associated with the calcium binding capacity. Modulation of the calcium and inorganic phosphate content of milk strongly influences intermolecular interactions and hence the stability of the casein micelle. In nutritional terms the casein proteins not only serve as a source of amino acids but also serve to increase the content of dispersed calcium and phosphate above that which is possible on the basis of solubility alone. Phosphorylation in combination with micellar structure provide a mechanism for

transportation of calcium and phosphate to appropriate sites in the gut for subsequent absorption.

4.2 GLYCOSYLATION

The κ-CN family contains a major carbohydrate free component plus at least seventeen other components (Mollé and Léonil, 1995). Vreeman *et al.* (1977) concluded that seven of the various groups were :

- a major carbohydrate (CHO) free component containing one phosphate group
- a component containing one phosphate and one CHO moiety with a N-acetylneuraminic acid (NeuNAc) residue
- a component containing one phosphate and two NeuNAc in a branched configuration
- a component containing two phosphates and no NeuNAc
- a component containing one phosphate and two CHO groups of two NeuNAc
- a component containing two phosphates and one CHO group of two NeuNAc
- a component containing either two phosphates, two CHO groups of two NeuNAc, or one phosphate and three CHO groups.

In contrast Doi *et al.* (1979) concluded that there are five major and two minor components, all containing one phosphate group. The major components were :

- a CHO free component
- a component containing one galactose (Gal) and one galactosamine (GalNAc)
- a component containing one Gal, one GalNAc and one NeuNAc
- a component containing three Gal, two GalNAc and two NeuNAc
- a component containing four Gal, three GalNAc and three NeuNAc

D-glucose and D-mannose (Man) were also detected in κ-CN preparations from colostrum milk (Wheelock and Sinkinson, 1973).

Jollés *et al.* (1973), Fournet *et al.* (1975, 1979) and Kanamori *et al.* (1980) determined that the points of attachment of oligosaccharides occur at threonine residues 131, 133, 135 and 136 via O-glycosidic linkages. Mollé and Léonil (1995) reported a maximum of three of the potential glycosylation sites as being modified by carbohydrate chains, containing up to six NeuNAc residues per molecule of κ-CN.

Covalently attached carbohydrates are also seen in the β-LG, but only in the Droughtmaster breed. NeuNAc, N-acetylglucosamine (GlcNAc), GalNAc, Man and galactose (Gal) were determined to be present in the proportions 1.0:3.4:0.9:1.9:0.8 (Bell *et al.*, 1970).

Minor forms of α-La that are glycosylated are known to exist. Barman (1970) reported a glycosylated form of α-La containing Man, Gal, fucose, GlcNAc, GalNAc and NeuNAc in the proportions 4.1:1.4:1.0:3.1:1.1:0.64.

Glycosylation of milk proteins has been shown to enhance solubility, heat stability and viscosity of protein solutions, and alter surface properties. Kitabatake *et al.* (1985) covalently attached gluconic or melibionic acids to the amino groups of β-LG. The synthetic glycoproteins exhibited enhanced solubility, particularly at low ionic strength or at the isoelectric point of native β-LG. Heat stability was also improved compared to native β-LG. Glycosylation of β-LG with maltose or glucosamine increased the viscosity over native β-LG in proportion to the degree of substitution (Waniska and Kinsella, 1984).

4.3 DISULPHIDE CROSS-LINKING

Disulphide cross-linking is observed in several of the milk proteins. Intramolecular disulphide bridges are observed in α-Lac (Figure 6), β-LG (Figure 7) and BSA (Figure 8). Two disulphide bridges occur in β-LG. One occurs between residues 66 and 160. One free thiol in β-LG is distributed equally between residues 119 and 121. The second

disulphide bridge is distributed between residues 106 and 119, and 106 and 121 (McKenzie, 1971; Braunitzer *et al.*, 1972; McKenzie *et al.*, 1972).

Intermolecular cross-linking is observed in α_{s2}- and κ-CN. A minor component previously designated as α_{s5}-CN is now known to be a dimer of α_{s2}-CN A-12P and α_{s2}-CN A-11P linked by a disulphide bond. κ-CN occurs as a mixture of polymers linked by intermolecular disulphide bonds (Swaisgood and Brunner, 1963). In raw milk κ-CN does not appear to form disulphide-linked polymers with α_{s2}-CN or with whey proteins.

Following heat denaturation β-LG, containing a buried unreactive sulphydryl in the native state, forms disulphide linked complexes with κ-CN with major effects on many technologically important properties of milk. κ-CN may also undergo sulphydryl-disulphide interchange reactions with α-La and α_{s2}-CN during heating. Little is known about the exact location and processes involved in disulphide bridge formation during the synthesis of milk proteins.

5. GENETIC ORGANISATION OF THE MILK PROTEINS

All of the major milk proteins reflect the action of autosomal genes transmitted from parent to offspring by Mendelian inheritance. The protein products are the result of expression of co-dominant alleles, found as identical pairs in homozygotes, or non-identical pairs in heterozygotes. Thus an individual cow that carries both alleles coding for the same variant of the protein (AA, BB, CC etc.) is described as homozygous for that particular genotype. Conversely an individual cow carrying dissimilar alleles for a protein (AB, AC, BC etc.) is described as heterozygous.

Phenotype describes the protein product(s) expressed by an individual. This is of primary importance in determining the physical characteristics of milk from an individual. Usually the phenotype of an individual for a particular milk protein corresponds to the individual's genotype. However Bouniol *et al.* (1993) reported an example of three homozygous cows, two carrying the α_{s2}-CN D (CasD) allele and one carrying the α_{s2}-CN A (CasA) allele, the two variants differing by a deletion of 9 amino acids (see Figure 2, page 10). Sequencing of genomic DNA revealed no differences in the coding region for α_{s2}-CN. The two cows carrying the CasD allele differed from the cow carrying the CasA allele by two identical nucleotide substitutions in the non-coding region of the α_{s2}-CN A cDNA. This suggests that during processing of the α_{s2}-CN D-encoding-pre-mRNA, the nucleotide substitution results in skipping of exon VIII, coding for the 9 amino acids deleted in the α_{s2}-CN D variant. Similar observations of exon skipping in milk protein genes have been reported in ovine α_{s2}-CN (Boisnard *et al.*, 1991), caprine α_{s1}-CN D and F (Leroux *et al.*, 1992), human β-CN (Martin and Leroux, 1992; Menon *et al.*, 1992) and bovine α_{s1}-CN A (Mohr *et al.*, 1994).

Using classical linkage analysis Hines *et al.* (1981) demonstrated the close linkage of the casein genes. Threadgill and Womack (1990) concluded that the four casein genes reside on less than 200 kb of DNA in the order α_{s1}-CN, β-CN, α_{s2} -CN, κ-CN on chromosome 6. In contrast the β-LG locus is genetically independent of the casein cluster. Population data further suggest that the loci of β-LG and α-La are not closely linked (Grosclaude *et al.*, 1974). Threadgill and Womack (1990) assigned α-La to the bovine syntenic group U3 on chromosome 5, whilst the β-LG gene was located on the

syntenic group U16 of a yet to be identified chromosome.

The non-independent inheritance of casein alleles may be observed in the genotypic structure of populations. King *et al.* (1965) first reported the apparent absence of particular combinations of α_{s1}-CN and β-CN genotypes. The absence was interpreted as a consequence of close linkage between the two loci. These early interpretations have led to postulations of the phylogeny of the casein alleles (Figure 9) and their distribution, particularly throughout Europe. The reader is referred to Ng-Kwai-Hang and Grosclaude (1992) for an excellent review of this topic.

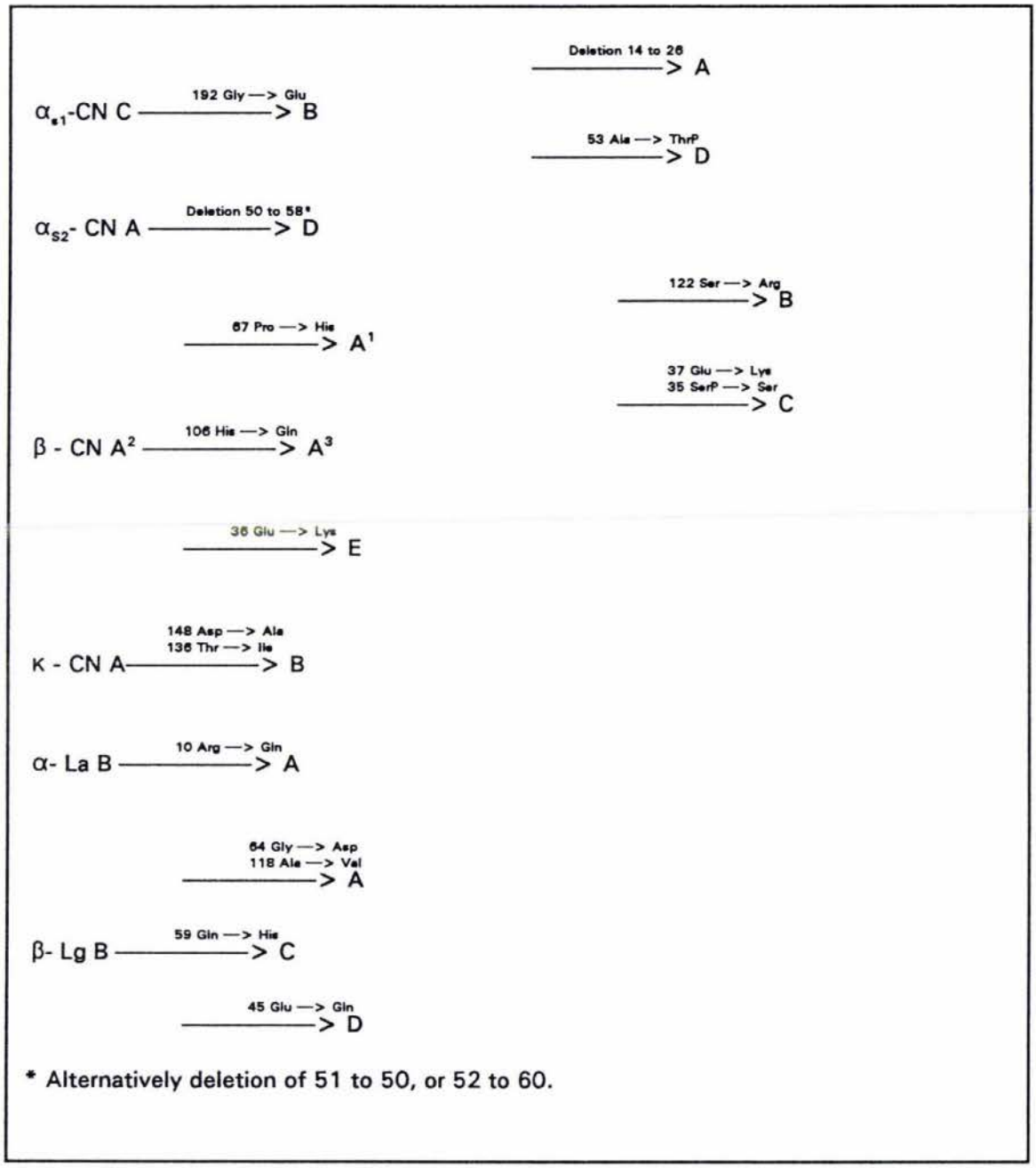


FIGURE 9 Phylogenetic relationships between genetic variants of the major bovine milk proteins.

6. SIGNIFICANCE OF POLYMORPHISM

Since the first report of genetic polymorphism in β-LG in bovine milk by Aschaffenburg and Drewry in 1955, a considerable volume of information has been amassed which examines the relationship between milk protein polymorphisms and milk production traits, milk composition and technological properties of milk. Several comprehensive reviews have been published in this area (Grosclaude, 1988; Jacob and Puhan, 1992; Ng-Kwai-Hang and Grosclaude, 1992; Puhan and Jacob, 1993; Jacob, 1994a). The ability to phenotype cows for milk proteins and examine the physical characteristics of each type has offered plausible explanations for the variation in properties of milk from individual animals. Consequently considerable interest has been generated in using milk protein genes as genetic markers for selective breeding to increase milk production, control milk composition and improve processing related traits.

6.1 MILK PRODUCTION

There are conflicting reports on the relationship between genetic variants and milk production. McLean *et al.* (1984), Ng-Kwai-Hang *et al.* (1984), Ng-Kwai-Hang and Monardes (1990a, 1990b) and Lin *et al.* (1986) all reported no relationship between β-LG variant and milk production. Jairam and Nair (1983) reported higher production for the BB phenotype, whereas Ng-Kwai-Hang *et al.* (1986) determined that cows homozygous for the β-LG A gene were higher milk producers. Similarly the effects of casein phenotype on milk production are unclear. Whilst McLean *et al.* (1984) found no significant correlation between milk yield and genetic variants of α_{s1}-, β- and κ-CN, others have reported superiority of α_{s1}-CN BB phenotype over AB or BC (Ng-Kwai-Hang *et al.*, 1984, 1986; Lin *et al.*, 1986), the A variant (particularly the A^2 and A^3 variants) of β-CN (Ng-Kwai-Hang *et al.*, 1984, 1986; Lin *et al.*, 1986) and the AB phenotype of κ-CN (Ng-Kwai-Hang *et al.*, 1986). Ng-Kwai-Hang and Grosclaude (1992) in their review draw our attention to the variable nature of the studies undertaken and the difficulty in making a valid comparison between them.

6.2 MILK COMPOSITION

The results of studies relating milk protein phenotype and different milk components is somewhat clearer, although not without conflicting results. Milk collected from β-LG BB phenotype cows is associated with higher fat content (McLean *et al.,* 1984; Ng-Kwai-Hang *et al.,* 1984, 1986; Aleandri *et al.,* 1990; van den Berg *et al.,* 1992; Bovenhuis *et al.,* 1992; Hill, 1993; Hill and Paterson, 1994). Similar associations have been made between α_{s1}-CN BC phenotypes, β-CN BB phenotypes and κ-CN BB phenotypes (Ng-Kwai-Hang *et al.,* 1986). The BB phenotype of α_{s1}-CN was found to produce more protein in the study by Ng-Kwai-Hang *et al.* (1984), as did β-CN phenotypes A^2A^3 and BC (Ng-Kwai-Hang *et al.,* 1984 and Graml *et al.,* 1985 respectively). In all studies β-LG AA phenotype was associated with more protein than the milk from AB or BB phenotype cows. McLean *et al.* (1984) examined the effect of three casein polymorphisms on the level of synthesis of these proteins and on their proportion relative to total casein synthesis. They concluded that the level of β-CN synthesis is only affected by the polymorphism of the β-CN gene. The levels of α_{s1}-CN and κ-CN synthesis were found to be always affected by the polymorphism of other casein genes. Whatever changes occurred in individual casein levels, the total casein level remained constant.

The difference between the protein content of milks from β-LG phenotype AA, AB and BB cows is largely due to the altered level of β-LG synthesis (Aschaffenburg and Drewry, 1957; McLean *et al.,* 1984, Ng-Kwai-Hang *et al.,* 1987). The higher β-LG content of AA phenotype milk results in a higher proportion of whey protein fraction in milk. If the level of synthesis of casein remains constant, the ratio of casein to total protein (or casein number) declines. This casein number is frequently used as a measure of cheese yielding capacity of milk. Most studies confirm that β-LG AA phenotype milk is associated with a lowered casein content, compared to BB phenotype (Mariani *et al.,* 1979; Buchberger *et al.,* 1982; McLean *et al.,* 1984; Ng-Kwai-Hang *et al.,* 1986; Hill, 1993; Hill and Paterson, 1994; Hill *et al.,* 1995a, 1995b, 1995c). Ng-Kwai-Hang *et al.* (1987) determined the effects of β-LG polymorphism on the contents of individual caseins and whey proteins. They found β-LG AA phenotype to be associated with lower contents of α_s-CN, α-La, serum albumin and

immunoglobulins, but higher content of β-LG. Ng-Kwai-Hang and Grosclaude (1992) suggest that the β-LG gene is a major gene determining the amount of this protein synthesised. Hill (1993) further proposes that the β-LG gene may be more active than the other milk protein genes, diverting the available supply of amino acids away from the synthesis of these proteins. In studies of the effect of bovine somatotropin (bST) on milk protein distribution, Ozimek *et al.* (1989) observed that bST increases in α-La and β-LG B were greater than the increase in β-LG A, indicating that control of the two β-LG variant genes may be different.

6.3 MILK PROPERTIES

Milk, as a food, is subjected to various technological processes prior to its consumption either as liquid milk or as a component of a food system. The factors which influence the manufacturing processes of milk have been studied for many years. In particular, the inheritable traits of cows that influence cheese manufacture have been scrutinised since 1923 (Köstler, 1923). It has long been recognised that the technological behaviour of milk is determined by its composition. However this does not fully account for the variation in the behaviour of milk from individual cows or breeds.

The discovery of genetic polymorphism of milk proteins offered other avenues of research to explain these variations. It has become clear that genetic variants of all the major milk proteins influence the behaviour of milk (Grosclaude, 1988).

6.3.1 Heat stability

The heat stability of milk was the first property to be studied in relation to genetic polymorphism of β-LG. Gough and Jenness (1962), Dupont (1965) and Lyster (1970) reported that β-LG in milk containing the B variant of β-LG denatured faster than β-LG in milk containing the A variant when heated at temperatures up to 90-95°C. Many other studies have confirmed these findings (Sawyer *et al.*, 1971; McKenzie, 1971; Puhan and Flueler, 1974; Hillier and Lyster, 1979; Hillier *et al.*, 1979; Marshall, 1986, Dannenberg and Kessler, 1988; Luff, 1988). Above 100°C the situation appears to

reverse, with the A variant of isolated β-LG (Hillier and Lyster, 1979; Hillier *et al.*, 1979) and reconstituted whole milk containing the A variant of β-LG (Anema *et al.*, 1995) to be less stable than the B variant. In contrast to these findings Manji and Kakuda (1986) found that the A variant was slightly more stable than the B variant upon heating skimmed milk. Parnell-Clunies *et al.* (1988) reported similar results in ultra-high temperature treated milk. Sawyer (1968) found that the susceptibility to denaturation of the variants of β-LG was in the order C>B>A. Other studies have found the order of susceptibility to be B>C>A (G. Manderson and L. Creamer, personal communication).

The pH of the protein solution also influences the heat stability. Laligant *et al.* (1991) found that at pH 6.5 and heating between 75-90°C, and pH 7.5 and heating at 90°C, β-LG B was more heat sensitive than β-LG A. The opposite trend was observed at pH 7.5 at 75°C, with β-LG B being more heat stable.

The only report of thermal properties of genetic variants of casein was that of κ-CN A, AB and B variants by Ma *et al.* (1990). In calcium chloride solutions they found that the order of heat stability was A>AB>B.

In milk systems McLean *et al.* (1987) and Schulte-Coerne *et al.* (1992) associated κ-CN B with higher heat stability. For κ-CN BB milk the heat coagulation time at 140°C was reported to be between 40 and 90% longer than κ-CN AA milk (Schulte-Coerne *et al.*, 1992). The same authors also reported higher amounts of deposits fouling heat exchangers in κ-CN BB milks relative to κ-CN AB and κ-CN AA milks.

The effects of genetic variation of β-LG on the heat stability of milk is less clear. Some studies have found that the apparatus used to measure heat coagulation time had a marked effect on the results obtained (G. Paterson, personal communication). The general trend found in a number of studies (Rose, 1962; Feagan *et al.*, 1971; McLean *et al.*, 1987) is for the maximum in the heat coagulation time-pH curve to follow the order of β-LG phenotype AA>AB>BB.

6.3.2 Association, dissociation and aggregation behaviour of β-LG

In its native form in milk β-LG exists as a dimer. Under certain conditions the dimeric form of β-LG dissociates into monomers (Hambling *et al.*, 1992). McKenzie and Sawyer (1972) reported the tendency of β-LG dimers to dissociate follows the order A>B>C. The dissociation is a key step in the mechanism leading to an unfolding of β-LG and possible exposure of the free thiol group (Hambling *et al.*, 1992). Using unreduced, discontinuous sodium dodecylsulphate-PAGE, Hill *et al.* (1995, unpublished) found that purified β-LG A, B and C variants heated to 110°C, produced two bands. A single band was observed from the same samples prior to heating. It is likely that the two bands are a result of differences in the distribution of the free thiol group (see Figure 7 and discussion section 4.3). The B and C variants exhibited similar distributions between the two bands. The A variant was markedly different, possibly due to the valine substitution at position 118 influencing the accessibility of cystine 119 for disulphide bonding.

Thresher *et al.* (1994) examined the subunit interactions of β-LG A and B variants in simulated milk ultrafiltrate by affinity chromatography. They found the interaction of soluble β-LG B with immobilised β-LG B to be stronger than the interaction of soluble β-LG A with immobilised β-LG A. Mixed variant interactions were weaker than either the A or B variant interactions.

The influence of genetic variation of β-LG on heat induced aggregation in sweet whey has been examined by Parris *et al.* (1993). Sweet whey containing β-LG B tended to form a greater proportion of soluble aggregates than insoluble aggregates upon heating. The opposite was found for the A variant. They suggested that this may be related to an Asp64 to glycine substitution in the B variant, lowering the net charge, consequently binding less calcium and therefore lowering the proportion of insoluble aggregates. An alternative explanation is that the proportion of soluble or insoluble aggregates is related to differences in the reactivity of the thiol group between the variants.

6.3.3. β-LG interaction with κ-CN

During heat treatment of milk, β-LG and κ-CN interact to form a complex (Sawyer, 1969; Fox, 1982). The reaction between denatured β-LG and κ-CN via disulphide bonding also has a significant effect on the heat stability-pH profile of milk (Singh and Creamer, 1991). Studies by McKenzie (1971) and Parnell-Clunies *et al.* (1988) showed the reaction between β-LG B and κ-CN is faster than β-LG A and κ-CN.

6.3.4. Heat induced gelation of β-LG

Huang *et al.* (1994a) examined the gelation of purified β-LG A and B variants at pH 7.0. Although both variants formed viscoelastic gels, the gelation point and initial gelation rate was higher in the A variant. They concluded that the gel matrix structures formed in the gels of the two variants must involve different molecular interactions between partially unfolded chains of the protein. McSwiney *et al.* (1994) found that the strength of gels made from β-LG A was higher than those made from β-LG B, particularly at concentrations above 5%. They also found the strength of gels made with β-LG B was markedly decreased at lower pH values, whereas the strength of gels made from β-LG A was independent of pH.

6.3.5. Cheese making

The most significant economic consequence of genetic polymorphism of milk proteins may be seen during cheese manufacture. For some milk products special demands are made of the processing parameters of the milk. For example a very fast renneting time is required in the manufacture of Parmesan cheese. Speed of renneting (or coagulation), curd strength, cheese yield and quality are all important economic parameters which are influenced by polymorphisms of not only the caseins, but β-LG as well.

6.3.5.1. Renneting

A clear agreement exists on the effects of milk protein polymorphism on the renneting

properties of milk. The favourable effects of κ-CN B over the A variant for cheese making (shorter coagulation time and higher curd firmness) has been confirmed by several groups (Sherbon *et al.*, 1967; Jakob and Puhan, 1986; Aaltonen and Antila, 1987; Menard *et al.*, 1986; Mariani and Leoni, 1985; van den Berg *et al.*, 1990; Kristiansen, 1990; Veguard *et al.*, 1990; van den Berg *et al.*, 1992; Delacroix-Buchet *et al.*, 1993; Jakob, 1993). The enzymatic rate of cleavage at the chymosin sensitive region is similar between the A and B variants (Jakob, 1993), however the clotting time differs appreciably. Several theories have been postulated to explain the differences in clotting activity including:

- differences in citric acid content and hence calcium chelating capacity (Mariani *et al.*, 1979, 1983; Schaar, 1985). Jakob and Puhan (1986), and van den Berg *et al.* (1990) found the differences between the κ-CN AA and BB type milks could be minimised by the addition of calcium chloride and/or slight acidification of the milk.
- differences in the number and nature of the carbohydrate groups involved in glycosylation of κ-CN between the two variants.
- differences in micelle size distribution (Morini *et al.*,1975).
- differences in the relative proportions of the caseins (Mariani *et al.*,1983; Menard *et al.*,1986).
- differences in micellar size (Ekstrand *et al.*, 1980).
- differences in electrostatic repulsion of the micelles due to net differences in the charge of the two variants.

Horne *et al.* (1994) proposed a simple mechanical model to explain the differences in rennet gel strength between the A and B variants. They assumed that gel strength is a function of the number and strength of bonds formed. If each micelle is assigned an average coordination number (the maximum number of bonds each is able to form), then the number of micelles in the system determines the gel strength. To accommodate all of the κ-CN at the micellar surface, a milk with a higher κ-CN content must have a larger micellar surface area, and consequently smaller micelles. In support of this they found κ-CN BB type milks to have a higher κ-CN content, smaller micelle size and higher gel strength than κ-CN AA type milks.

The influence of α_{s1}-CN variants on renneting time is less conclusive. In general α_{s1}-CN C variant milk is associated with a higher curd firmness than the B variant (Sherbon *et al.*, 1967; El-Negoumy, 1972; Mariani *et al.*, 1988), whereas the A variant produces very soft curd (Sadler *et al.*, 1968).

Various reports have established the order of β-CN variants in both renneting time and curd strength is C≥B>A (El-Negoumy, 1972; Mariani *et al.*, 1982; Mariani and Leoni, 1985; Jakob and Puhan, 1986). The various differences reported between different β-CN A variants (A^1, A^2, A^3) are very small and inconsistent.

Even though β-LG is not directly involved in the process of enzymatic coagulation, a number of studies have associated superior curd firmness with β-LG BB milk over β-LG AA milk (Sherbon *et al.*, 1967; Feagan *et al.*, 1971; Mariani *et al.*, 1979; Rahli and Menard, 1991; Hill *et al.*, 1994a). Marziali and Ng-Kwai-Hang (1986b) found β-LG AA phenotype milk to give firmer curds.

6.3.5.2. Syneresis

Mariani *et al.* (1976) observed that cheese made from κ-CN BB type milk lost approximately 15% more weight within 24 hours than cheese derived from κ-CN AA milk. McLean and Schaar's (1989) results confirmed these findings. They further concluded that if syneresis in acid coagulated curds followed the same trend, it could impact on yogurt manufacture. Vegarud *et al.* (1990) reported that mixed variant κ-CN AB milk resulted in yogurt with better water binding than κ-CN AA variant milk.

6.3.5.3. Yield and quality

Compared to κ-CN AA type milk, κ-CN BB type milk has proven to be significantly better for manufacture of Parmigiana-Reggiano cheese (Mariani *et al.*, 1976). Firmer curd and more uniform curd size resulted in nearly 10% higher yield of cheese in κ-CN BB type milk. Fat losses in cheese whey were approximately 50% lower, moisture content was 4% lower and a higher proportion of cheese made from κ-CN BB type milk were graded as first class. Graham *et al.* (1986), and Marziali and Ng-Kwai-Hang

(1986a) found a similar trend in Cheddar cheese manufacture on a laboratory scale. Similar trends have also been observed by Mariani *et al.* (1976) in Parmesan cheese, Rahali and Menard (1990, 1991) with Camembert cheese, and van den Berg *et al.* (1990) with Gouda cheese. In contrast Schaar *et al.* (1984), using pasteurised milk, were unable to associate κ-CN polymorphism with cheese yield or composition, except for fat content.

Cows homozygous for the β-LG B variant also appear to produce milk with better cheese making properties than the A variant (Morini *et al.*, 1982; Schaar *et al.*, 1985; Marziali and Ng-Kwai-Hang, 1986a, 1986b; Aleandri *et al.*, 1990; Rahali and Menard, 1990,1991; Hill *et al.*, 1995a). The increase in yields varied from 1% in Parmesan cheese (Morini *et al.*, 1982) to 10% in Cheddar cheese (Hill *et al.*, 1995a). The finding of significantly higher protein recovery from β-LG BB type milk than AB or AA type milks (Marziali and Ng-Kwai-Hang, 1986b; Rahali and Menard, 1991) can be attributed to higher relative casein content in the milk of β-LG BB phenotype cows (Grosclaude, 1988).

Combined effects of polymorphisms of κ-CN and β-LG support independent evidence of the superiority of the B variants of these two milk proteins (Feagan *et al.*, 1971; El-Negoumy, 1972; Mariani and Leoni, 1985). The study by Graham *et al.* (1984) compared cheese making properties of milk from type B phenotypes (α_{S1}-CN BB, κ-CN BB, β-CN AB or BB, β-LG AB or BB) with that of type A phenotype milk (α_{S1}-CN BB, κ-CN AA, β-CN AA, β-LG AA). Type B milks had a higher casein content (25.2 g kg^{-1} milk compared to 22.9 g kg^{-1}), reached a given curd firmness 15 minutes earlier and resulted in an 8% (actual) and 9% (dry matter) greater yield of cheese than the type A milk.

6.3.6 Other effects

Moisture adjusted yield of rennet casein was 6% higher when manufactured from β-LG BB phenotype milk relative to β-LG AA phenotype milk (Hill *et al.*, 1995c). Yield losses during processing were also lower. Other functional properties such as solubility, viscosity, colour, hydration and extrusion properties of rennet casein manufactured

from β-LG AA and BB phenotype milks were found to be similar.

Polymorphs of all caseins have been found to affect calcium sensitivity of the micelle (Thompson and Kiddy, 1964; Thompson *et al.*, 1969; El-Negoumy, 1968, 1971, 1974; Ng-Kwai-Hang and Imafidon, 1990). The polymorphs of α_{s1}-CN may be ranked A<B<C in calcium sensitivity and the κ-CN variants A<B for micellar stabilising effects (El-Negoumy, 1971, 1974).

Water sorption of casein was found to be correlated with polymorphisms of β-CN (AA/AB>AC) by Kirchmeier *et al.* (1983).

The effects of casein variants in fermented milks was reported by Vegarud *et al.* (1990). A better consistency and viscosity was associated with α_{s1}-CN BB, κ-CN BB and β-LG BB phenotype milks.

Plasmin activity in β-LG AA type milks was reported to be lower than β-LG BB type milks by Schaar (1985), resulting in lower levels of proteose peptones.

Differences in dye binding characteristics of β-LG A and B variant proteins have also been noted. Reimerdes and Mehrens (1978) found β-LG B bound nearly twice as much Coomassie Blue G 250 dye than β-LG A. Graml *et al.* (1989) also found β-LG B to have a higher dye binding capacity for Amido Black dye relative to β-LG A.

7. DETECTION OF POLYMORPHISMS IN MILK PROTEINS

Aschaffenburg and Drewry (1955) first recognised the occurrence of polymorphism in β-LG using paper electrophoresis. Prior to this proteins in milk were considered to be homogeneous. The two electrophoretically distinct bands were denoted originally as β_1- and β_2-LG, revised to A and B when they discovered that the synthesis of both types of β-LG were under control of paired autosomal genes (Aschaffenburg and Drewry, 1957). Since then a range of techniques have been developed capable of detecting polymorphisms within milk proteins.

7.1 ELECTROPHORESIS

Electrophoretic techniques have been extensively used for phenotyping due to their resolving power and relative simplicity of use in rapid analysis of large numbers of samples. Under the influence of an electric field charged molecules migrate in the direction of the electrode of the opposite charge. Due to the varying charges and masses, different molecules migrate at different speeds and are thus separated into discrete fractions. The electrophoretic mobility is a characteristic parameter of a charged molecule and is dependent on the pK value of the charged group and the size of the molecule. It is influenced by the type, concentration and pH of the buffer, temperature and field strength as well as the nature of the support material. Electrophoretic separations may be carried out in free solution, as in capillary electrophoresis, or in stabilising media such as thin-layer plates, films and gels.

7.1.1 Paper electrophoresis

As mentioned above Aschaffenburg and Drewry (1955) first used paper electrophoresis to detect variants of β-LG. The inclusion of 6.0 M urea as a casein dissociating agent enabled Aschaffenburg (1961) to overcome the problem of association of casein proteins in the micelle. This allowed him to detect A, B and C variants of β-CN.

7.1.2 Starch gel electrophoresis

Starch gel electrophoresis expanded the range of detectable genetic variants to α_{s1}-CN A, B and C (Thompson *et al.,* 1962). The inclusion of 2-mercaptoethanol as a reducing agent led to the detection of further β-LG variants (Grosclaude *et al.,* 1966) and κ-CN variants A and B (Swaisgood and Brunner, 1963; Neelin, 1964; Schmidt, 1964; Woychik, 1964). Further resolution of β-CN variants A^1, A^2 and A^3 was achieved by electrophoresis under acidic conditions (Kiddy *et al.,* 1966; Grosclaude *et al.,* 1966; Arave, 1967) as opposed to alkaline conditions previously used.

7.1.3 PAGE

Polyacrylamide gel electrophoresis (PAGE) has largely superseded starch and paper electrophoresis for the analysis of proteins due to its ease of preparation, staining and robustness. Most of the known variants of caseins, except β-CN A variants, have been resolved by alkaline PAGE (Creamer and Richardson, 1975; Grosclaude *et al.,* 1978, 1979; Medrano and Sharrow, 1989; Creamer, 1991). The New Zealand Dairy Research Institute has found that slight modifications to the alkaline urea mini PAGE gel method reported by Creamer (1991) have been suitable for the resolution of α_{s1}-CN A, B and C, α_{s2}-CN B, β-CN A, B and C, and κ-CN A and B variants after reduction with 2-mercaptoethanol (Coker - personal communication, 1995). Similarly modifications to the acid urea mini PAGE method (Creamer, 1991) are routinely used for resolving β-CN variants A^1, A^2 and A^3. Whey proteins are better resolved without the addition of urea or reducing agents.

7.2 Isoelectric focusing (IEF)

Isoelectric focusing has recently been developed to the point where the majority of milk protein polymorphisms are able to be resolved in a single analysis. Prior to the development of ultra thin (< 0.5 mm) gels and narrow pH range ampholytes, resolution was somewhat limited and run times long. Using 100 μm thick gels Siebert *et al.* (1985) were able to resolve all the milk protein variants in a single run within 45

minutes. Using the Phast System (Pharmacia LKB, Uppsala, Sweden), Bovenhuis and Verstege (1989) further improved the method to enable complete processing through to destained gel within 45 minutes. They were able to resolve proteins differing in isoelectric point by as little as 0.01 pH units. This level of resolution has led to the discovery of other variants of κ-CN (Siebert *et al.,* 1987; Erhardt, 1989) and β-LG (Krause *et al.,* 1988). The β-LG W variant in the Murnau-Werdenfelder breed of cattle differs from the B variant by a proposed 'silent' substitution of neutral amino acids. Observations of other amino acid substitutions in β-LG and β-CN variants shows a shift in pI of 0.100-0.125 pH units per unit of molecular charge. The pI determined for the W variant of β-LG differed by only 0.005 pH units from the B variant. Upwards of 20 bands may be observed in the analysis of whole casein. Given the extremely small change in pI observed in silent variants and the large number of bands observed in partially purified proteins, it is possible that some variants may be overlooked or misassigned.

Similar silent substitutions in human haemoglobins have been detected by IEF (Altland and Rossmann, 1985).

7.3 2-D ELECTROPHORESIS

Two dimensional electrophoresis has also been used in the study of protein polymorphisms. Isoelectric focusing, followed by SDS-PAGE was used in the study of caprine α_{s1}- and α_{s2}-CN genetic variants by Tutta *et al.* (1991). Lopez *et al.* (1995) utilised a broad pH range (2.5 - 7.0) for IEF prior to SDS-PAGE in the analysis of ovine whey proteins. In the absence of urea, proteins separated by this method were able to be blotted and detected by immunological means. The incorporation of urea as a dissociating agent altered the isoelectric focusing pattern of whey proteins considerably.

The combination of IEF and PAGE is capable of resolving many of the protein components in milk. However, substitutions involving non charged residues are unlikely to be easily detected.

7.4 Liquid Chromatography

Many liquid chromatographic techniques have been applied to the separation of milk proteins. Yaguchi and Rose (1971) provided an excellent review of column chromatographic techniques for general milk protein separations. More recently Ng-Kwai-Hang and Grosclaude (1992) reviewed chromatographic techniques as applied to separation of milk protein variants.

7.4.1 Ion-exchange

Prior to the development of high performance chromatographic media, few examples of the separation of milk protein genetic variants existed. Thompson and Pepper (1964) and Ng-Kwai-Hang and Pélissier (1989) achieved separation of the A and C variants of β-CN on a DEAE-cellulose and QAE-cellulose media respectively. Using a DEAE-cellulose column Thompson (1966) could resolve κ-CN variants A and B. The advent of Fast Protein Liquid Chromatography (FPLC®, Pharmacia AB, Uppsala, Sweden) and high performance liquid chromatography (HPLC) columns began an era of routine separation of some milk protein variants. Humphrey and Newsome (1984) and Andrews (1986) reported resolution of β-LG variants A and B by anion-exchange chromatography. Guillou *et al.* (1987) separated κ-CN variants A and B, and β-CN variants A^1 and C also using anion-exchange chromatography. Hollar *et al.* (1991b) were further able to resolve the A^2 variant of β-CN as well using cation-exchange chromatography. Ng-Kwai-Hang and Dong Chin (1994) reported a series of four peaks corresponding to κ-CN using a DEAE anion exchange column. The first appeared to be the B variant and the remaining three representing both A and B variants with differing degrees of glycosylation.

In all of these cases the protein variants that were resolved differed in the net charge of the molecule. Variants with amino acid substitutions that do not alter the net charge of the protein are not expected to be resolved from other similarly charged variants, thus limiting the use of ion-exchange chromatography to phenotyping only those milk proteins variants which differ in charge.

7.4.2 Reverse phase

Reverse phase chromatography exploits the property of hydrophobicity to separate molecules. Each amino acid in a protein or peptide contributes to the overall hydrophobicity of the molecule. Thus any alteration in the primary sequence of a protein would be expected to alter the hydrophobicity of that molecule. Several groups have used this approach to separate some of the polymorphs of milk proteins (Barrefors *et al.,* 1985; Carles, 1986; Bican and Spahni, 1991; de Frutos *et al.,* 1991; Visser *et al.,* 1991, 1995). Using a C_{18} column Visser *et al.* (1991) were able to resolve glycomacropeptide (GMP) A and B, κ-CN A and B, α_{S2}-CN A, α_{S1}-CN A, B/C and D, α-La/β-CN B, A^1, A^2, A^3 and a silent variant termed X (see footnote[2]), β-LG B and A respectively in an increasing gradient of acetonitrile in water-trifluoroacetic acid.

The contribution of a single amino acid substitution to a change in the net hydrophobicity of a protein is likely to be small. Despite the resolving power of reverse phase chromatography silent variants of intact milk proteins are unlikely to be detected easily by this technique.

7.5 Peptide Mapping

The contribution of a single substitution to the net hydrophobicity of a whole protein may be too small to detect in all cases. Complete hydrolysis of the protein followed by reverse phase separation of the peptide fragments has been successfully used to detect protein variants, including silent substitutions (Carles, 1986; Dalgalarrondo *et al.,* 1990; Visser *et al.,* 1991, 1995; Dong Chin, 1992). In the cases cited trypsin was used to cleave a purified milk protein. The resulting peptide fragments were separated on a reverse phase column in an increasing acetonitrile concentration gradient in water-trifluoroacetic acid. The resultant peptide maps were compared with digests of standard proteins. Amino acid analysis, amino acid sequencing or mass spectroscopic analysis of aberrant peaks were used to determine the substitutions involved. Although the technique is able to detect silent and electrophoretic protein variants, it

[2]The β-CN variant X reported by Visser *et al.* (1991) was subsequently shown to differ from the A^1 variant by a Pro to Leu substitution at position 152 by Visser *et al.* (1995).

requires the initial purification of the suspect protein. The procedure of protein purification, hydrolysis and peptide mapping is unlikely to be used on a large scale for phenotyping due to its complexity and time required.

7.6 DNA Sequencing

The limitations of traditional electrophoretic and chromatographic techniques to phenotyping only lactating females has begun to be overcome by the development of DNA techniques in conjunction with gene amplification using the polymerase chain reaction (PCR). DNA sequences derived from nucleated cell samples have made possible the identification of milk protein genotypes independent of lactation, age and sex.

The κ-CN A and B alleles were first differentiated using labelled DNA probes by Levéziel *et al.* (1988). Restriction fragment length polymorphism (RFLP) involves separation of fragments of amplified DNA by agarose-gel electrophoresis after digestion by different restriction enzymes. This method has been used by several groups for typing variants at the κ-CN (Denicourt *et al.*, 1990; Schlieben *et al.*, 1991; Schlee and Rottmann, 1992a), β-LG (Medrano and Aguilar-Cordova, 1990), α_{s1}-CN (David and Deutch, 1992) and α-La locus (Schlee and Rottmann, 1992).

The success of RFLP relies on the occurrence of a recognition site for the enzyme at the mutation point. Other methods such as allele-specific PCR have overcome this limitation and have been used for typing β-CN (Schlee and Rottmann, 1992), κ-CN (Medrano and Aguilar-Cordova, 1990) and α_{s1}-CN (David and Deutch, 1992). However genotyping at the DNA level, using these techniques, is reliant on the appropriate DNA primers or restriction enzymes to detect known variants. Alleles other than the already known protein polymorphisms have been demonstrated at the DNA level (Threadgill and Womack, 1990; Lien and Rogne, 1993; Levéziel *et al.*, 1994) but have involved detailed and labourious DNA sequencing to determine the exact nature of the mutation.

The advent of recombinant DNA and PCR technologies, and development of variant

specific probes has allowed genotyping of both parents, offspring and even unborn foetuses. The commercial implications of these developments are significant. It means that herds with desirable genotypes can be assembled or increased very quickly and at relatively low cost compared to traditional methods requiring progeny to achieve lactation before typing is possible.

7.7 Mass Spectroscopy

Recent advances in ionisation technology has seen mass spectrometry emerge as a means of measuring the molecular mass of large molecules. Electrospray ionisation mass spectroscopy (ESI-MS) in particular has proven to be extremely useful in the analysis of large, labile biomolecules up to 200,000 Da with a precision of better than 0.01% (Fenn *et al.,* 1989). This far exceeds the accuracy of conventional techniques such as SDS-PAGE, chromatography or ultracentrifugation, typically accurate to only 5% at best. For proteins and peptides within the range of 100 to 100,000 Da, it is possible to identify post-translational modifications such as phosphorylation, glycosylation, acetylation, methylation and hydroxylation solely by the increase in mass. Subunits or prosthetic groups not covalently bound will be dissociated and measured as separate species. Conversely, covalently bound groups will show a characteristic increase in mass. Also, the mass change produced by a single amino acid substitution or deletion may often be used to establish or confirm the presence of variants.

Recently Léonil *et al.* (1995) published a comprehensive analysis of the major bovine milk proteins by reverse phase HPLC coupled to ESI-MS. The method permitted the simultaneous identification of caseins and whey protein variants by comparison of molecular masses derived by ESI-MS to molecular masses calculated from primary structures derived from chemical sequencing or DNA sequences. Protein masses were resolved to within of 3.2 Da or less. This degree of accuracy in mass determination allows cross checking of genotype and phenotype. If the measured mass of a protein agrees with that calculated from the gene sequence, it is likely that the deduced sequence is correct, the amino and carboxyl terminals of the mature protein have been correctly assigned, and the protein contains no post-translationally modified amino acid

residues. However a difference between the measured and predicted molecular weights implies either an error in the cDNA deduced sequence or a post-translational modification or processing of the protein. Léonil *et al.* (1995) suggested two corrections to the primary sequences, as determined from cDNA sequences, of α_{S2}-CN A (Leu 193 to Trp - Stewart *et al.*, 1987) and β-CN A^1 and A^2 (Leu 93 to Met - Jimenéz-Florez *et al.*, 1987). In addition to the rapid identification of genetic variants it is possible to observe co- and post-translational modifications, protein-protein interactions and metal binding phenomena (Léonil *et al.*, 1995, Mollé and Léonil, 1995).

The electrospray ionisation process produces intact protein molecules in an ionised form from a dilute protein solution by nebulisation in the presence of a strong electric field (Figure 10). As a consequence of the strong electric field between the end of the capillary (~4 kV) and the counter electrode (~1 kV), the sample solution emerging from the capillary is dispersed into an aerosol of highly charged droplets. The fine droplets formed carry excess positive or negative charge (depending on the polarity of the electric field). The droplets diminish in size by evaporation, assisted by dry gas and or heat. A point is reached where multiply charged ions are released free of solvent resulting in multiply ionised proteins in the gas phase. The exact process by which this occurs is still unknown. These are directed into the mass analyser (usually a quadrupole filter) held under high vacuum for mass to charge ratio (*m/z*) analysis (Figure 11).

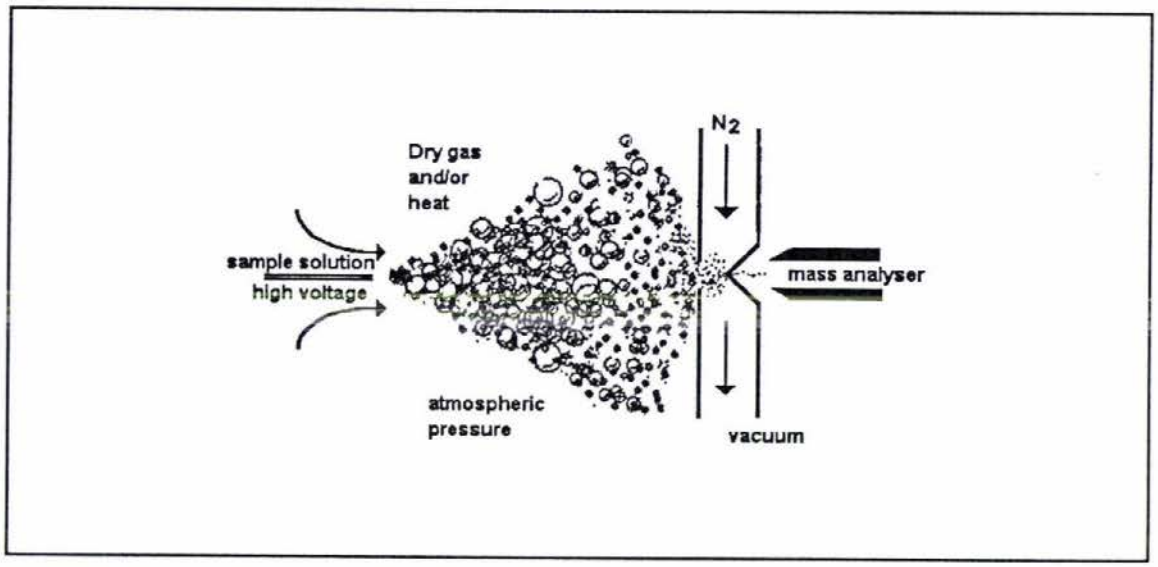


FIGURE 10 Production of vapour phase ionised molecules from a liquid source. The electrospray ionisation source uses a combination of a stream of nitrogen or air, vacuum and heat to facilitate desolvation of the nebulised liquid. (Adapted from Siuzdak, 1996).

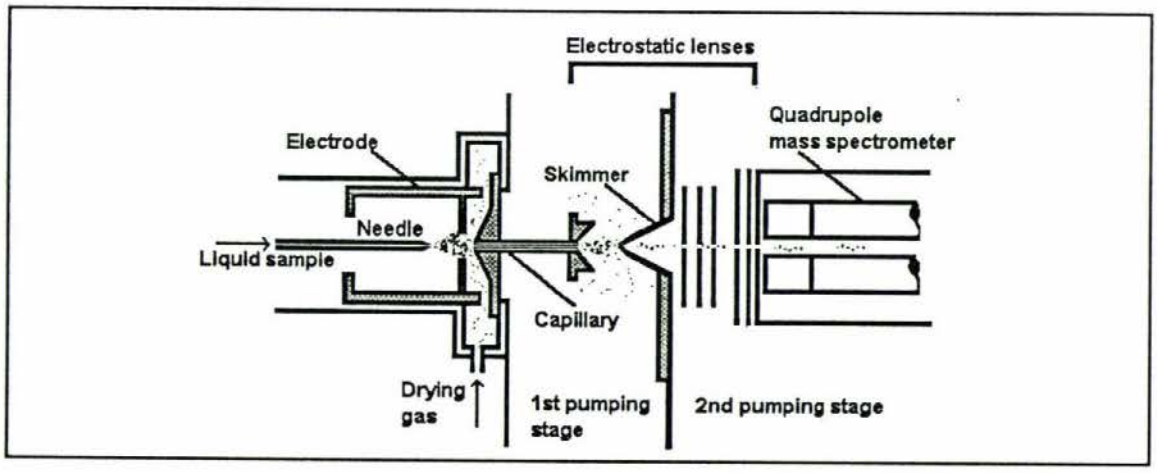


FIGURE 11 Schematic diagram of an electrospray mass spectrometer. (Adapted from Mann and Fenn, 1992).

A characteristic of an ESI mass spectrum is the production of intact ions with extensive multiple charging, resulting in a mass spectra containing a family of charge states arising from a single protein. On average one charge is added to the protein per 1,000 Daltons. Each member of the series of charge states for a particular protein is related to its nearest neighbour by having one more or one less proton.

Proteins are normally analysed as positive ions where the charges are produced by added protons. The ions have the general form:

$$(M+nH)^{n+}$$

where ***M*** is the molecular mass of the protein
n is an integer number of protons (charges)
and ***H*** is the mass of the proton (1.00794).

The mass to charge ratio (***m***) of each peak is given by:

$$m=\frac{M+nH}{n}$$

The mass spectrometer measures the mass to charge ratio (*m/z*) of each peak. Thus a protein of molecular mass of 10,000 with 10 added protons would appear at a mass/charge ratio of 1001.0.

It follows then that the molecular mass may be calculated from the measured mass to charge ratio if ***n*** can be found. To determine ***n***, any two consecutive peaks differing by one proton in the series may be used:

$$m_2=\frac{M+nH}{n}$$

$$m_1=\frac{M+(n+1)H}{n+1}$$

where m2 and m1 are the measured mass/charge ratios of two peaks with n and n + 1 protons respectively. Solving the two simultaneous equations, the charge (n) on m2 is determined as:

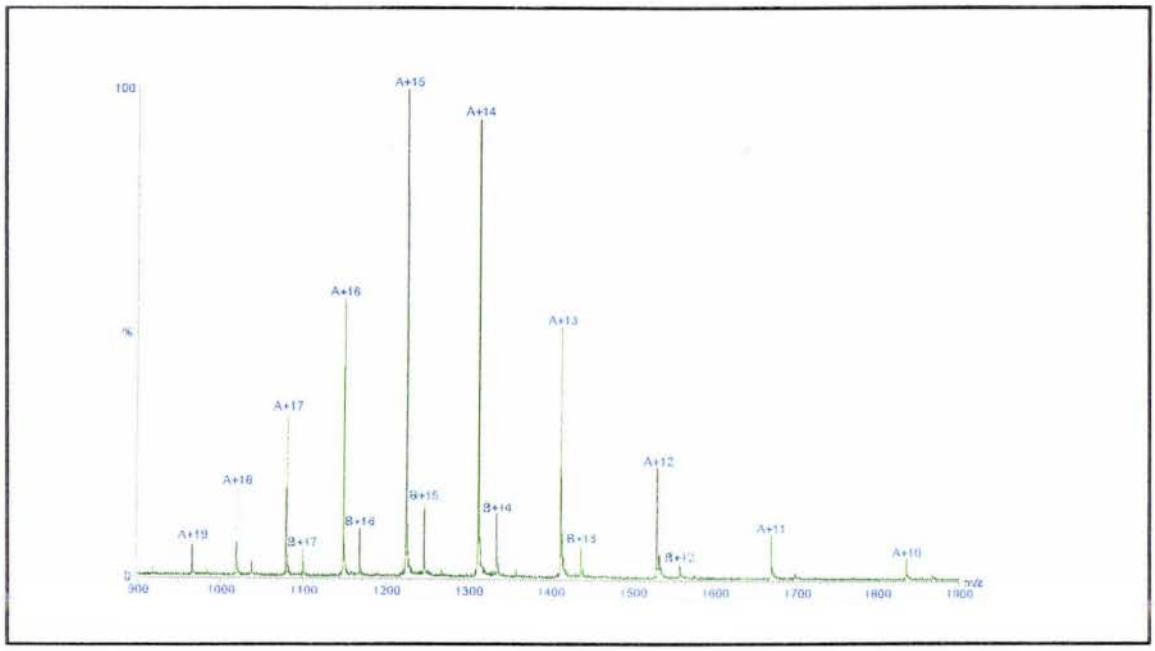
$$n=\frac{m_1-H}{m_2-m_1}$$

The molecular mass is calculated for every peak using:

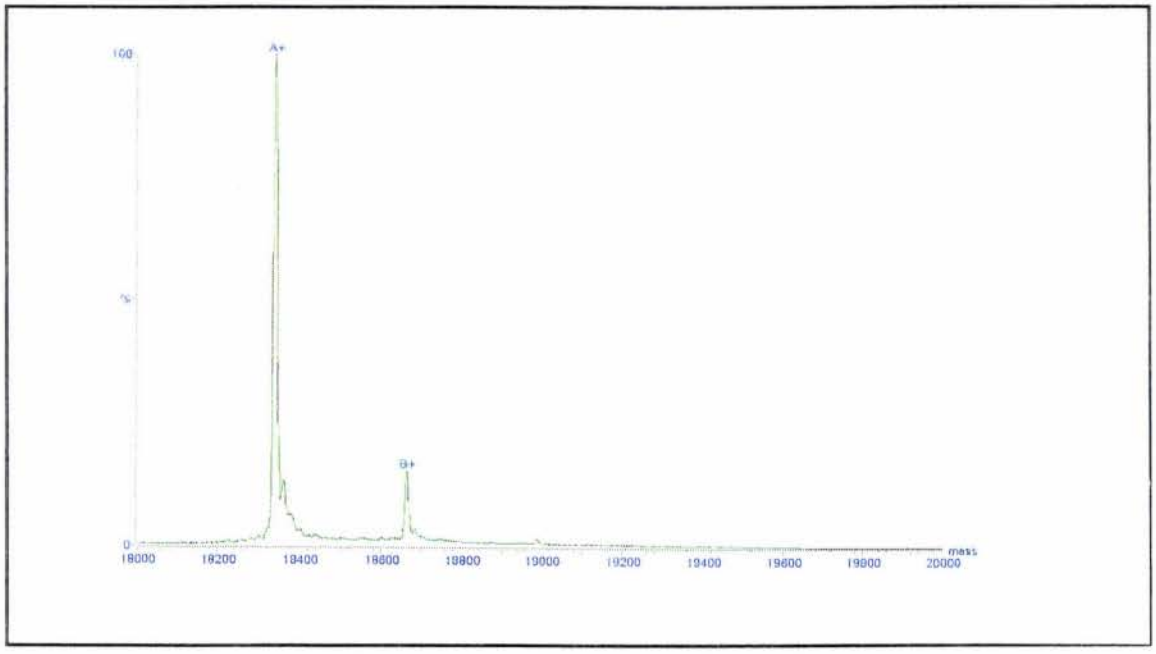
$$M=n(m-H)$$

- the masses are then summed and averaged. Molecular masses are normally given as average (chemical) values based on the atomic weights of the elements C = 12.011, H = 1.00794, N = 14.0067, O = 15.9994 and S = 32.06.

To facilitate interpretation electrospray spectra are normally transformed by data analysis procedures so that all the peaks in the original spectrum from a given protein are combined and presented as a single peak on a true molecular mass scale (Figure 12).



(a) Raw spectrum of a mixture of two proteins. Numbers above the peaks designate the charge states of each ion species.



(b) Reconstructed mass spectrum of (a) after transformation to a true molecular mass spectrum.

FIGURE 12 Raw and transformed electrospray mass spectra

In tandem MS (or MS-MS) two consecutive stages of mass analysis are utilised. The first stage of mass analysis is used to select the $(M + nH)^{n+}$ ion of a peptide from the other parent ions produced during ionisation. Following this mass filtering the relatively stable ion is directed to a collision cell. It is bombarded with a neutral gas such as argon or helium to cause fragmentation in a process called collision-induced dissociation (CID). The family of fragment ions, or daughter ions, is directed to a second mass analyser for similar mass to charge analysis. Partial, if not complete, amino acid sequence information may be deduced from the mass differences between successive fragments. Interpretation of sequence information becomes more difficult as molecular weight increases, realistically limiting tandem MS sequence analysis to peptides of less than 40 residues.

An important attribute of ESI-MS is the capability of interfacing with high-resolution liquid chromatography and capillary electrophoresis separation methods. With the automation capabilities of autosamplers and data analysis software, routine analysis of multiple milk samples for simultaneous phenotyping of the major milk proteins, and detection of silent or new protein variants is likely to become the analysis method of choice.

For comprehensive reviews of the principles of ESI-MS the reader is referred to Mann and Fenn (1992) , Smith *et al.* (1992) and Mann and Wilm (1995).

Experimental

The aim of the research undertaken was to examine peptide mapping and mass spectroscopy as techniques for determining the phenotype of milk proteins, and to determine the frequency of silent or novel milk protein variants in a study dairy cow population. These methods are contrasted with standard PAGE techniques commonly used. This entailed several phases of work

- collection of milk samples and preparation of casein and whey
- determination of electrophoretic phenotype of milk proteins by standard PAGE techniques
- purification of milk proteins
- hydrolysis, peptide mapping and analysis of peptides
- mass spectroscopy of intact milk proteins

1 PRELIMINARY SAMPLE PREPARATION

Milk samples were processed by several means to provide purified milk proteins for further analysis. Preliminary sample preparation consisted of centrifugation of milk samples to remove the fat prior to PAGE, and isolation of casein and whey as a primary step in individual protein purification.

1.1 PREPARATION OF SKIM MILK

A total of 109 fresh milk samples were collected from individual Jersey or Jersey/Friesian cross cows in a local seasonal supply dairy herd. Approximately 200 ml of raw milk from each cow was heated to 40°C in a water bath for a period ranging from 5 minutes to 30 minutes to facilitate cream separation. After heating samples were centrifuged at 5,000 x g for 10 minutes (unrefrigerated) in a Sorvall GSA rotor. Skimmed milk was separated from fat by carefully aspirating the cream layer. At this stage a 1 ml aliquot from each milk sample of skimmed milk was taken for analysis by PAGE.

1.2 CASEIN AND WHEY PREPARATION

The skimmed milk was then adjusted to pH 4.6 by slow addition of 1M HCl with continuous stirring. After standing for a period of 20 minutes the isoelectrically precipitated casein was separated from the whey by centrifugation at 5,000 x g for 15 minutes. The casein curd was crumbled into small particles and washed extensively with reverse osmosis water and finally with MilliQ deionised water (Millipore Corp., Bedford, MA.). After removing excess water the casein was frozen at -20°C until required.

2 DETERMINATION OF ELECTROPHORETIC PHENOTYPE

2.1 MATERIALS AND METHODS

The electrophoretic phenotype of casein and whey proteins from each cow were determined by various polyacrylamide gel electrophoresis (PAGE) techniques. To date no single electrophoretic technique has been shown to unequivocally resolve all of the known major milk protein variants. Alkaline urea PAGE was used to phenotype α_{s1}-, β- and κ-casein variants. β-casein variants A^1, A^2 and A^3 could not be resolved from each other by this technique. Acid urea PAGE was used to resolve β-casein A^1, A^2 and A^3 variants, and 'native' PAGE for phenotyping β-LG variants A and B or C. β-LG B and C variants were unable to be resolved using any of these gel methods. All methods utilised 60 x 100 x 0.75 mm gels run on Mini-Protean II equipment (BioRad Corp., Richmond, CA). After electrophoresis each gel was stained in a 0.05% (w/v) solution of Coomassie Brilliant Blue R (BioRad Corp., Richmond, CA) in isopropanol:acetic acid:water (2.5:1:6.5 v/v) for 1 hour, before destaining in two changes of isopropanol:acetic acid:water (1:1:8 v/v). Unless stated otherwise all reagents were obtained from the Sigma Chemical Company (St. Louis, MO, USA).

2.1.1 Alkaline Urea PAGE

Alkaline urea PAGE was carried out using a modification of the nondissociating discontinuous buffer system used by Creamer (1991). The resolving gel (12%T, 2.6%C) consisted of 8 ml of a stock 30% w/v solution of acrylamide:N,N'-Methylene-bis-acrylamide (BIS) (37.5:1) (BioRad) in 11.9 ml of resolving gel buffer (0.38 M Tris-HCl, 4.5 M urea), polymerised with 100 µl of a 10% solution ammonium persulphate (APS) and 10 µl of tetramethylenediamine (TEMED). This was overlaid by a stacking gel (3.9%T, 2.7%C) consisting of 1.3 ml of 30% acrylamide:BIS stock in 8.65 ml of stacking gel buffer (90 mM Tris-HCl, 0.6 M urea, 90 mM boric acid, 2.5 mM ethylenediaminetetraacetate (EDTA), pH 8.4) and polymerised with 50 µl 10% APS and 5 µl TEMED. The electrode buffer was 17.6 mM Tris-HCl, 17.8 mM boric acid, 0.55 mM EDTA, pH 8.4. Gels were left overnight to ensure completion of polymerisation.

Samples were prepared by adding 50 µl of skimmed milk to 0.95 ml of stacking gel

buffer, to which 0.1% w/v bromophenol blue, 10% glycerol and 10 µl of 2-mercaptoethanol had been added. After vortexing and standing for at least 1 hour, 10 µl of reduced sample was loaded per lane. Gels were run in pairs at 210 V, 70 mA, 6.5 W (limit values) for 1.7 hours and stained immediately after electrophoresis.

2.1.2 Acid Urea PAGE

Similarly, acid urea PAGE was performed using a modified nondissociating discontinuous buffer system based on that used by Creamer (1991). The resolving gel (6.5%T, 5%C) consisted of 9.75 g acrylamide:BIS (5%C) (Serva Research Grade) dissolved in 150 ml resolving gel solution (4.5 M urea, 1 M acetic acid, 62 mM ammonium acetate, 23 mM thiourea, pH 3.86) and polymerised by 3 µl of hydrogen peroxide (30% w/v) per ml of solution. Stacking gel (5%T, 5%C) consisted of 5.0 g acrylamide:BIS (5%C) (Serva Research Grade) dissolved in 100 ml stacking gel buffer (6 M urea, 0.25 M acetic acid, 15 mM ammonium acetate, 23 mM thiourea, pH 4.1), and polymerised by 3 µl of hydrogen peroxide (30% w/v) per ml of solution. Electrode buffer was 1 M acetic acid. Gels were left overnight to ensure completion of polymerisation.

Samples were prepared by adding 25 µl of skimmed milk to 750 µl of sample buffer (6.8 M urea, 0.25 M acetic acid, 15 mM ammonium acetate, pH 4.16, and 1 µl of 10% basic fuschin dye) and 10 µl of 2-mercaptoethanol. Samples were vortexed and stood for at least 1 hour before loading 10 µl per lane. Gels were run in pairs with the electrodes reversed to the configuration used with alkaline urea and native gels. The values used for electrophoresis were 210 V, 70 mA, 6.5 W (limit values) for 1 hour. Gels were stained immediately following electrophoresis.

2.1.3 Native PAGE

Native PAGE was performed using a modification of the discontinuous buffer system reported by Creamer (1991). Resolving gel (15%T, 2.6%C) was prepared by mixing 5 ml of a stock acrylamide:BIS solution (30% w/v) with 1.25 ml resolving gel buffer (3 M Tris-HCl, pH 8.8), 3.75 ml of water, 50 µl APS (10% w/v) and 5 µl TEMED. Stacking gel (3.75%T, 2.6%C) was prepared by mixing 1 ml of stock acrylamide:BIS

with 2 ml stacking gel buffer (0.5 M Tris-HCl, pH 6.8), 5 ml water, 40 μl of APS and 8 μl TEMED. Electrode buffer consisted of 25 mM Tris-HCl, 0.2 M glycine, pH 8.3. Gels were left overnight to polymerise.

Samples were prepared by adding 50 μl of skimmed milk to 950 μl of sample buffer (0.1 M Tris-HCl, 0.01% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8). Ten microlitres of prepared sample was loaded per lane. Gels were run in pairs at 210 V, 70 mA, 6.5 W (limit values) for 1.5 hours and stained immediately after.

2.2 RESULTS

Typical results of alkaline-urea PAGE, acid-urea PAGE and native PAGE are seen in Figure 13 (a), (b) and © respectively. The major milk protein variants were identified by their relative mobilities in each of the PAGE systems used.

Under alkaline urea conditions α_{s1}-CN had the highest mobility, followed by α_{s2}-CN, α-La, β-LG, β-CN, κ-CN and BSA in order. Lactoferrin and immunoglobulins essentially did not penetrate the stacking gel at all. In milk α-La is present in such low quantities relative to the casein proteins, that at the loadings used it was not visualised clearly. β-LG appeared as a diffuse band in approximately the same position as β-CN. The variants of β-LG could not be clearly resolved.

Under acid urea conditions α_{s1}-CN migrated the furthest, followed by κ- and β-CN. The variants of α_{s1}- and κ-CN were not resolved. The whey proteins appeared as faint, diffuse bands.

Non-reducing conditions in the absence of urea resulted in clearly resolved bands corresponding to α-La, β-LG B or C, and β-LG A. Under these conditions the casein proteins do not dissociate from casein micelles to any appreciable degree. The caseins appear as a broad, heavily stained smeared band extending from the loading well at the top of the gel, through to approximately half the length of the resolving gel.

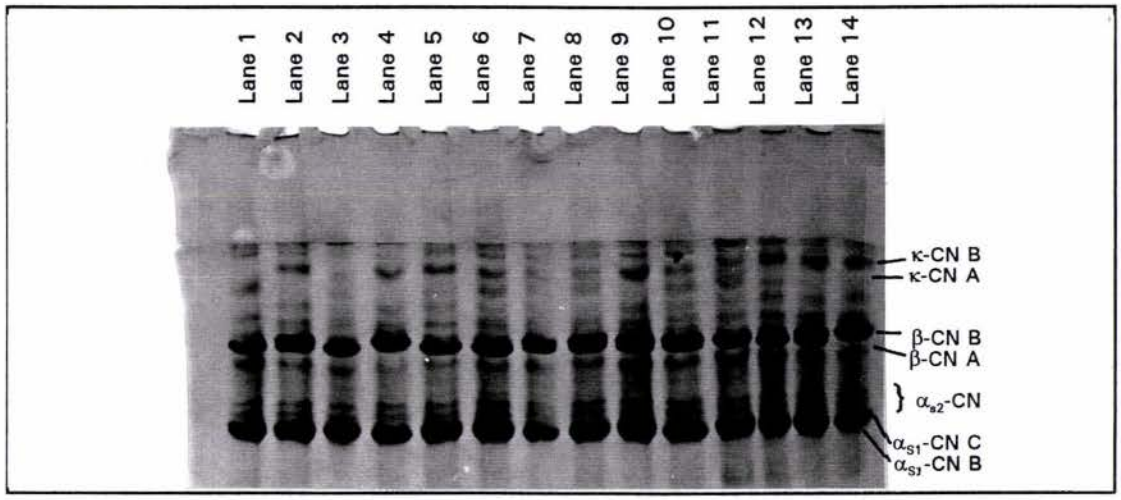


FIGURE 13 (A) Alkaline-urea PAGE of whole milk samples from individual cows. The phenotypes determined are as follows:

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
κ-CN	AA	BB	AB	BB	BB	AB	??	AB	BB	AB	AB	BB	BB	BB
β-CN	AA	AB	AA	A?	AA	AA	AA	AB	AB	AB	AA	AA	AA	AA
α_{S1}-CN	BB	BB	BB	BB	BB	BC	BB	BB	BC	BB	BB	BB	BB	BB

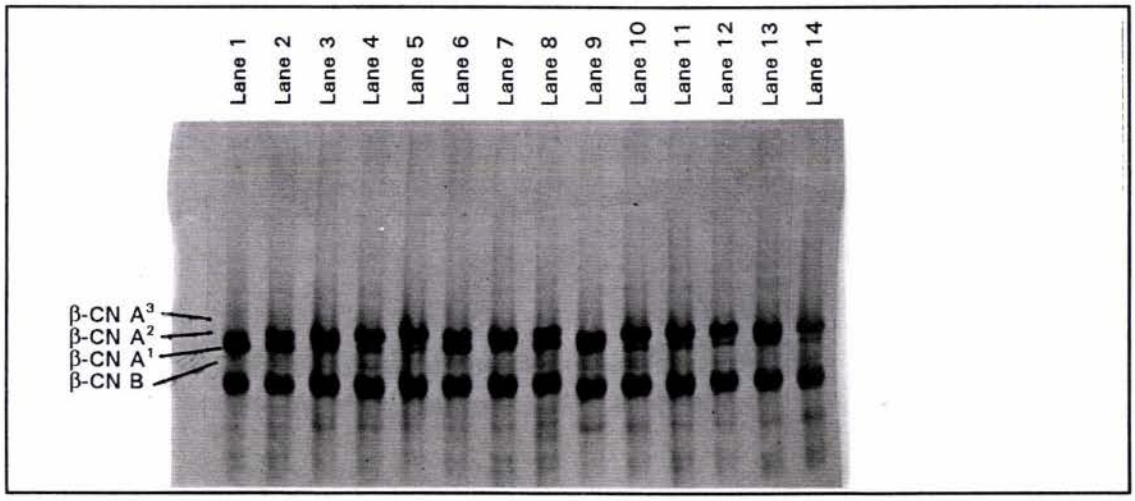


FIGURE 13 (B) Acid-urea PAGE of whole milk samples from individual cows. The phenotypes determined are as follows:

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
β-CN	A1	A1	A2	A2	A2	A1	A2	A1	A1	A2	A2	A2	A2	A2
	A1	A2	A2	A2	A2	A2	A2	A2	A1	A2	A2	A2	A2	A2

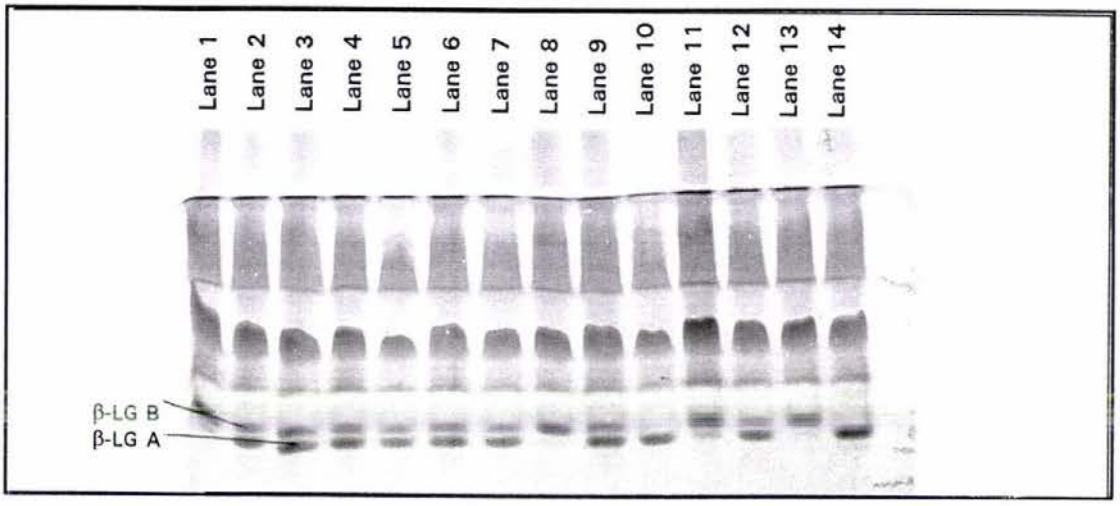


FIGURE 13 (c) Non-denaturing 'native' PAGE of whole milk samples from individual cows. The β-LG phenotypes determined are as follows:

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
β-LG	BB	AB	AB	AB	AB	AB	AB	BB	AB	AA	BB	AB	BB	AA

2.2.1 Alkaline-urea PAGE

Alkaline-urea PAGE enabled the determination of casein phenotypes with the exception of β-CN A^1, A^2 and A^3 variants.

α_{s1}-CN variants B and C were identified by alkaline-urea PAGE. The B variant had a slightly higher relative mobility than the C variant. Often mixed BC phenotypes appeared as a broader band than monovariant B or C types.

α_{s2}-CN appeared as a two distinct and several faint bands, corresponding to variously phosphorylated species. All samples analysed appeared identical in the area corresponding to the bands ascribed to α_{s2}-CN. As only the A variant has been identified in *Bos taurus*, all samples were assumed to be α_{s2}-CN AA phenotype.

Alpha-lactalbumin appeared as a very faint, smeared band with a relative mobility higher than β-LG, but less than α_{s2}-CN.

Beta-lactoglobulin showed a similar mobility to β-CN, but the quantity loaded onto the gel from a skimmed milk sample was negligible. This did not unduly interfere in assessment of β-CN phenotype.

The incorporation of urea in alkaline-urea PAGE to dissociate the casein species results in the whey proteins appearing as indistinct broad bands. Combined with the broad bands seen in comparison to the caseins, determination of β-LG phenotype in this gel system could not be accomplished.

Beta-casein appeared as either a single band, corresponding to either A group (A^1, A^2 or A^3) or B variants, or as two bands indicating a mixed A(*x*)B phenotype. The A group of β-CN variants had a slightly higher relative mobility than the B variant. Some samples were difficult to assign. The combination of the high protein loading level (required to simultaneously visualise κ-CN) and the small difference in relative mobility between the A group and B variants often resulted in broad bands that tended to overlap. In all cases further analysis by acid-urea PAGE allowed complete determination of β-CN phenotype.

Mono variant κ-CN appeared as a single faint band. Mixed κ-CN AB variant appeared as two faint bands, corresponding to the unglycosylated forms of each variant. The A variant has a higher relative mobility than the B variant. Additionally a series of fainter bands, assumed to be the variously glycosylated forms of κ-CN, were seen.

Kappa casein represents only 10% of the total casein in skimmed milk. The intensity of the bands attributed to κ-CN, relative to the intensity of the other caseins, was consistent with the ratio of κ-CN to the other caseins.

2.2.2 Acid-urea PAGE

Acid-urea PAGE allowed the further characterisation of β-CN phenotypes. Variants A^1, A^2, A^3 and B were resolved from each other. The order of relative mobility was $B > A^1 > A^2 > A^3$. Under acidic conditions the other milk proteins appeared as smeared, diffuse bands.

Immediately after the completion of electrophoresis gels were immersed in Coomassie stain. For acid-urea gels it was found that the β-CN phenotype was best determined after 5 to 10 minutes staining time. The β-CN bands were stained more intensely than the background and were relatively well defined. After phenotyping the gels were returned to stain, processed and photographed as normal. After the standard staining and destaining procedure the bands were less defined and tended to overlap.

2.2.3 Native PAGE

Alpha-lactalbumin appears as a single band with a relative mobility higher than that of the caseins, but lower than any of the β-LG variants.

Beta-lactoglobulin variants A and B were clearly resolved with the A variant having a higher relative mobility than the B. The B and C variants were not able to be resolved in this gel system. The B variant differs from the C variant by a Gln59 to His substitution. At the pH of the buffer systems used, His is unprotonated. Thus in this system the B and C variants are expected to have the same net charge. The C variant was assumed to be rare in the New Zealand dairy population (J. Hill and G. Paterson, personal communication). All samples with a single band corresponding to the same mobility as β-LG B were assigned as the β-LG BB phenotype. Those samples with two bands corresponding to the same mobility as β-LG A or B were assigned as mixed β-LG AB phenotype.

Protein	Phenotype	Number of cows	% of study population
α_{s1}-CN	BB	82	75.2
	BC	27	24.8
α_{s2}-CN	AA	109	100.0
β-CN	A^1A^1	15	13.8
	A^1A^2	20	18.3
	A^2A^2	56	51.4
	A^1B	11	10.1
	A^2B	7	6.4
κ-CN	AA	36	33.0
	AB	52	47.7
	BB	21	19.3
α-Lac	AA	109	109
β-LG	AA	31	28.4
	AB	62	14.7
	BB	16	56.9

TABLE 1 Tabulated results of phenotyping by PAGE methods

2.3 DISCUSSION

The milk protein phenotypes found in the samples analysed by the three PAGE systems used included α_{s1}-CN AB, BB and BC; α_{s2}-CN AA; β-CN A^1A^1, A^1A^2, A^2A^2, A^1B, A^2B and BB; κ-CN AA, AB and BB; α-La BB and β-LG AA, AB and BB. These phenotypes are consistent with the phenotypes reported in Western cattle breeds by Aschaffenburg (1968), Swaisgood (1982), Eigel *et al.* (1984) and Ng-Kwai-Hang and Grosclaude (1992). The rarer milk protein phenotypes (α_{s1}-CN AA and β-CN CC) were not observed in the samples analysed by alkaline-urea PAGE, acid-urea PAGE or native-PAGE. The β-LG C variant was unable to be resolved from the B variant in any of the systems used.

No single PAGE system has been able to resolve all of the milk protein variants. Hence a systematic study of milk protein polymorphism requires three runs under alkaline, acid and non denaturing conditions.

The majority of the casein genetic variants are resolved from each other by alkaline-urea PAGE. A high concentration of urea is incorporated into both the gel system and sample buffer. The chaotropic nature of urea solution effectively disrupts the association between casein species in the micelle. Once dissociated the individual species are able to be separated by a combination of molecular sieving and charge under the influence of an electric field in the gel. The relative mobility of each species under these conditions is a function of the apparent size and net charge of the molecule.

The major influence on relative mobility of protein species in the three gel systems used is the net charge of the molecule. Asp and Glu residues have a negative charge only above the pK of their lateral carboxyl groups (pH 3 to 4), whilst His is positively charged at a pH below the pK of the imidazole ring NH (below pH 6). SerP and ThrP are negatively charged and Arg and Lys are positively charged throughout the pH range which can be used in electrophoresis. Thus substitutions of SerP, ThrP, Arg and Lys by a neutral amino acid may be detected at both alkaline and acid pH values. This type of substitution (or deletion) is seen in α_{s1}-CN A, D and E relative to α_{s1}-CN B (see Figure 2, page 10), α_{s2}-CN D relative to α_{s2}-CN A (Figure 3, page 11), β-CN B, C, E relative to β-CN A^2 (Figure 4, page 12) and α-La A relative to α-La B (Figure 6, page 15). Substitution of Asp and Gly residues by neutral amino acids may be detected at a pH above 3 to 4. Examples of this type of substitution or deletion may be found in α_{s1}-CN A, C, E relative to α_{s1}-CN B (Figure 2, page 10), α_{s2}-CN D relative to α_{s2}-CN A (Figure 3, page 11), κ-CN A relative to κ-CN B (Figure 5, page 13) and β-LG A, D, E relative to β-LG B (Figure 7, page 16). Acidic buffers must be used to detect substitutions involving His and neutral residues. These are found in β-CN A^1, A^3, B and C relative to β-CN A^2 (Figure 4, page 12) and β-LG C relative to β-LG B (Figure 7, page 16).

IEF has been shown to be useful in the analysis of the polymorphism of casein (Rigehetti *et al.*, 1980; Trieu-Cuot and Grippon, 1981; Addeo *et al.*, 1983; Siebert *et al.*, 1985) and whey proteins (Krause *et al.*, 1988). The technique has advanced from resolving some of the known variants of individual proteins (Trieu-Cuot and Gripon, 1981) to phenotyping in a single run all milk proteins in ultrathin layer polyacrylamide gels (Siebert *et al.*, 1985; Bovenhuis and Verstege, 1989). With the development of ultra-narrow pH range, immobilised pH gradient gels further variants of κ-CN (Siebert *et al.*, 1987; Erhardt, 1989) and β-LG (Krause *et al.*, 1988) have been discovered. The β-LG W variant reported by Krause *et al.* (1988) differed by only 0.007 pH units from that of the β-LG B variant. They suggest that this may be a result of substitution of amino acids with uncharged side chains.

The substitutions mentioned cover most of the known polymorphisms found in *Bos taurus*. The inability of the native PAGE method used to resolve the B and C variants of β-LG highlights the limitation of PAGE methods alone to accurately determine milk protein phenotype. Silent substitutions involving non-charged amino acids are unlikely to be detected by standard electrophoretic techniques. It is possible that the extremely small shifts in pI seen with silent variants may be overlooked when using IEF methods.

3 MILK PROTEIN PURIFICATION

3.1 β-LACTOGLOBULIN PURIFICATION

Detailed analysis of milk proteins for unknown polymorphisms required purification of the major proteins species from individual cow's milk. Beta-lactoglobulin was prepared from whey after isoelectric precipitation of the casein.

3.1.1 Materials and Methods

All the whey proteins with the exception of β-LG were precipitated from the whey supernatant by adaptations to the method of Mailliart and Ribadeau-Dumas (1988). This involved slow addition of NaCl to 7% (w/v) to acid whey (pH 4.6). Once the salt had completely dissolved the pH was adjusted to 2.0 by slow addition of 6 M HCl drop wise with continuous stirring. After standing for 20 minutes the precipitated proteins were separated by centrifugation at 8 000 x g for 15 minutes.

The β-LG containing supernatant was decanted into 10,000 molecular weight cut off dialysis tubing (Union Carbide), and dialysed against 300 mM Tris buffer, pH 7.0, overnight. Two subsequent dialysis steps were performed against distilled water for 24 hours each before freezing samples on a Just-a-Tilt Model SF-4 shell freezer (FTS Systems Inc., Stone Ridge, NY), followed by freeze drying on a Virtis freeze drier Model 20L (The Virtis Co. Ltd., Gardiner, NY). Confirmation of phenotype and assessment of purity were made by native PAGE as described above, using a loading of 2.5 μg per lane. Gels were analysed by scanning densitometry using a Personal Densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Protein purity was determined by comparing the band volume of purified protein (calculated from the area and density of staining of each band) to the band volume of any contaminating protein.

3.1.2 Results.

Native PAGE analysis of β-LG purified by the method above indicated a purity of greater than 95% when analysed by native PAGE (Figure 14). Alpha-lactalbumin was

identified as the contaminant in β-LG preparations. The β-LG preparations were deemed to be sufficiently pure for use in further experiments.

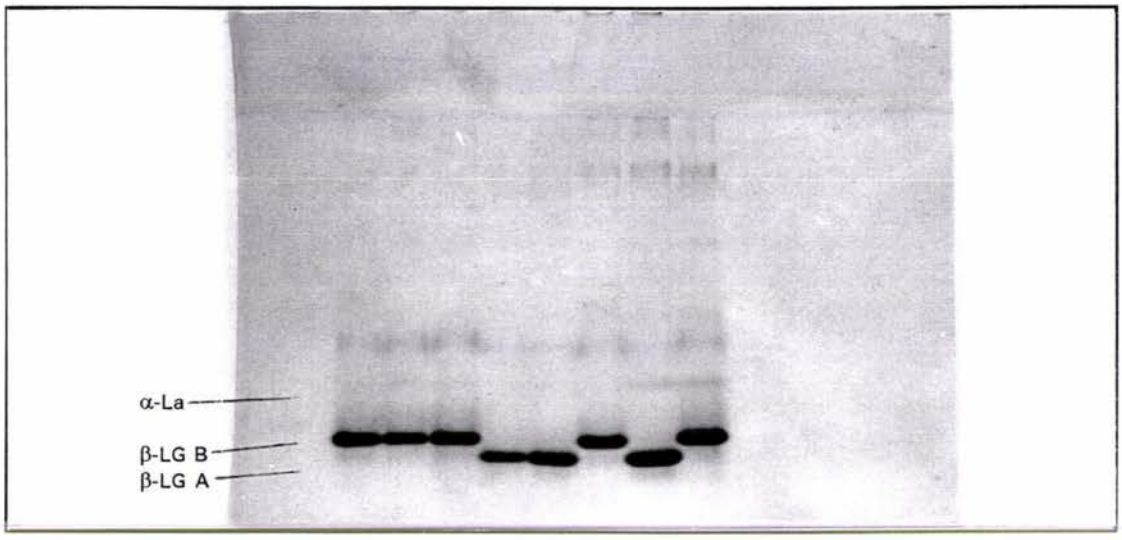


FIGURE 14 Non-denaturing 'native' PAGE of salt precipitate prepared β-LG. Each lane represents a sample of β-LG prepared from an individual cow.

3.2 CASEIN PURIFICATION

Similarly purified caseins were required for detailed analysis of the range of polymorphisms occurring in the study population. Caseins were purified by ion exchange chromatography.

3.2.1 Materials

Urea was obtained from PetroChem NZ Ltd., (Hawera, N.Z.). This was de-ionised by passing a solution of 8 M urea through a 2.6 x 50 cm column of AG501-X8 mixed bed resin (BioRad, Richmond, CA.). After de-ionisation the solution was filtered through Whatman GF/A filter paper, and finally through a 0.45 μm pore size filter (both from Millipore, Bedford, MA.)

Chromatography was performed on a BioPilot chromatography station fitted with a Frac 300 R fraction collector. Data was collected and analysed using FPLC Manager software (all Pharmacia, Uppsala, Sweden).

Equipment and methods used for PAGE analysis of purified casein fractions were the same as for electrophoretic phenotype determination.

Casein samples were selected for further purification based on the phenotypes of each protein. Twenty two samples that were homozygous for α_{s1}-CN B, and homozygous for either β-CN A^1, β-CN A^2 or β-CN B proteins were selected.

3.2.2 Methods

Thawed wet casein, prepared as above, was dissolved in buffer A (6 M urea, 20 mM sodium acetate, pH 5.0) to 2% (w/v). This was titrated to pH 7.0 with 2 M NaOH and treated with 0.1% dithiothreitol for 1 hour before careful titration back to pH 5.0 with 2 M HCl. The sample was then stood overnight at room temperature before centrifugation at 5,000 x g for 10 minutes. 100 ml of solubilised, reduced casein was then chromatographed on a 50 x 150 mm column of S-Sepharose Fast Flow ion-exchange media (Pharmacia, Uppsala, Sweden) at a flow rate of 5 ml min^{-1}, using a 0-0.4 M NaCl gradient over 3 column volumes (60 minutes). Eluate was collected in 5 ml fractions. The eluate was monitored by UV absorbence at 280 nm throughout the run. The elution profile was recorded during each run and used to assess which fractions to analyse by PAGE, based on the UV absorbence. Following analytical chromatography the column was regenerated by washing for 10 minutes at a flow rate of 5 ml min^{-1} first with buffer A + 1 M NaCl, followed by buffer A for 30 minutes.

Collected fractions were analysed by alkaline-urea PAGE as outlined above. The fractions that corresponded to pure α_{s1}-, α_{s2}-, β- and κ-CN, as indicated by the appearance of band(s) corresponding to the purified protein, were individually pooled. They were then dialysed against 5 l of 300 mM Tris, pH 7.0 overnight, followed by two further 24 hour, 5 l dialysis steps against purified water. Dialysed, purified caseins were then lyophilised by shell freezing liquid samples prior to freeze drying and storage at -20°C.

The purity of each freeze dried casein sample was checked by alkaline PAGE analysis as described above. 2.5 μg of α_{s1}- and β-CN, and 5.0 μg of α_{s2}- and κ-CN were loaded per lane. Samples were run in conjunction with a whole casein standard. The relative

order of migration of caseins in alkaline PAGE was α_{s1}-CN>α_{s2}-CN>β-CN>κ-CN.

3.2.3 Results

Figure 15 shows a typical elution profile of acid precipitated casein on S-Sepharose Fast Flow. The collected fractions A to F were examined by alkaline PAGE. These corresponded to γ-, β-, κ-, α_{s2}-, α_{s2}- D and α_{s1}-CN respectively. The fraction identified as α_{s2}- D appeared to be a dimeric form of α_{s2}-CN. Given the sample preparation conditions, all proteins were expected to be dissociated to monomeric forms. It appears that reduction of α_{s2}-CN proceeds at a much slower rate than κ-CN.

By alkaline-urea PAGE purified caseins appeared homogeneous (Figure 16).

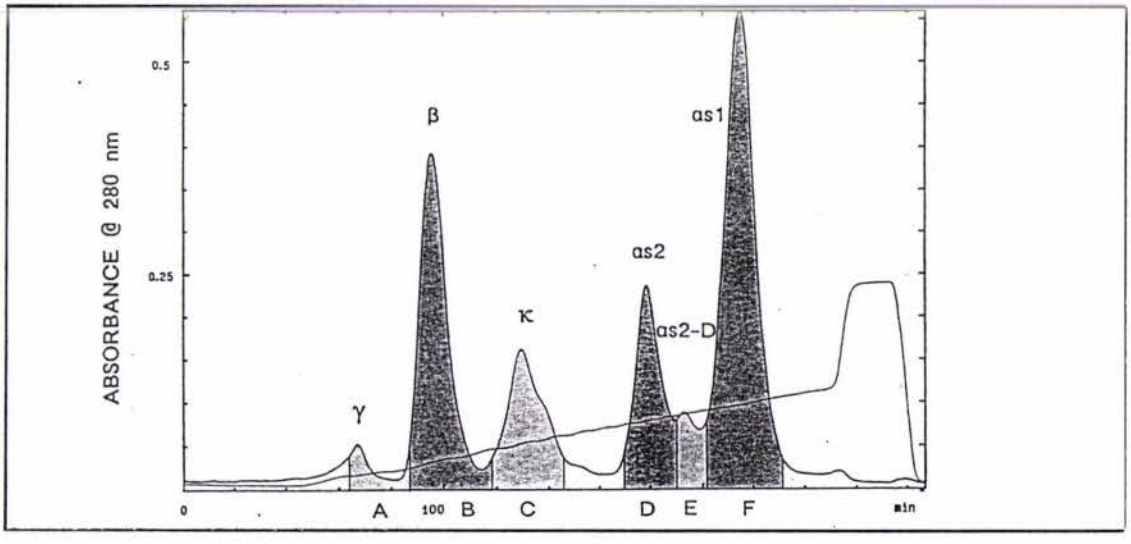


FIGURE 15 Elution profile of casein proteins from S-Sepharose Fast Flow ion-exchange column. Fractions are - γ: γ-CN; β: β-CN; κ: κ-CN; αs2: α_{s2}-CN; αs2-D: α_{s2}-CN dimer; αs1: α_{s1}-CN. Shaded areas indicate fraction cuts. The ionic gradient overlays the spectral absorbence.

Using the method outlined above, purified α_{S1}-, α_{S2}-, β- and κ-CN were prepared from 22 milk samples selected. On the basis of this selection 22 samples of α_{S1}-CN B, 22 samples of α_{S2}-CN A, 14 samples of β-CN A^2, 7 samples of β-CN A^1, 1 sample of β-CN B, 8 samples of κ-CN A, 1 sample of κ-CN B and 13 samples of mixed κ-CN A and B were purified.

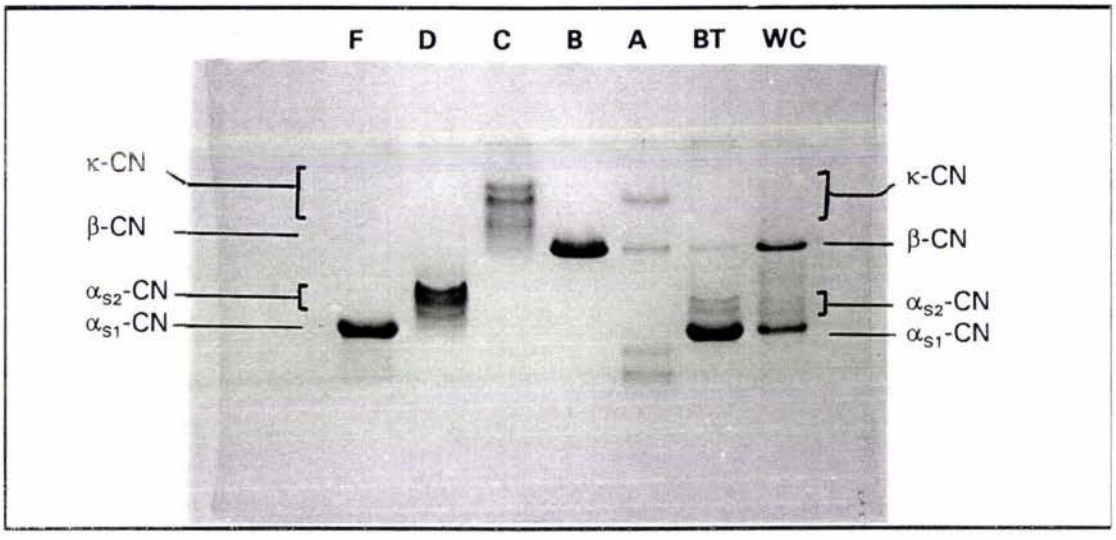


FIGURE 16 Alkaline-urea PAGE of peak fractions collected from ion-exchange chromatography of whole casein. Lanes A, B, C, D and F correspond to fractions collected from the chromatogram shown in Figure 15. BT represents the breakthrough peak (not shown in chromatogram; WC represents the sample of whole casein loaded onto the column.

3.2.4 Discussion

The success of this chromatographic method relies on the careful preparation of the sample. Dalgleish (personal communication) recommended dissolving acid casein in 6 M urea at pH 5.0, titration to pH 7.0 and reduction with 2-mercaptoethanol for 1 hour prior to careful titration back to pH 5.0. In this separation the less noxious dithiothreitol was substituted as reductant.

Both reducing agents are far more effective at neutral pH. Thus samples were titrated to pH 7.0 prior to reduction. The minimum quantity of acid or base was added to meet the required pH for reduction and chromatography. It was found that an excess of acid or base increased the ionic concentration of the sample enough to effect dissociation with the media. This resulted in a higher proportion of the sample

appearing in the break though peak (data not shown).

Hollar *et al.* (1991a) recommended a sample concentration of between 0.3 to 0.75%. It was found that this could be increased to 2% without precipitation.

Temperature also appears to be an important factor. Sample storage of the reduced samples overnight at 4°C resulted in selective precipitation of approximately half of the α_{s1}-CN. This was avoided by storage at room temperature.

4 B-LG PHENOTYPING BY CAPILLARY ELECTROPHORESIS

The native PAGE method, used for determining β-LG phenotype proved to be unable to resolve variants B and C. Previously it was assumed that the frequency of the C variant in New Zealand dairy cattle was extremely low as no C variant had been detected (Hill, 1993). Using purified β-LG A, B and C standards, Paterson *et al.* (1995) developed a method of separating the three β-LG variants by free zone capillary electrophoresis. This method was used to check the β-LG phenotype previously assigned using native PAGE.

4.1 METHODS AND MATERIALS

Samples of purified β-LG, prepared as outlined above, were dissolved in 50 mM 2-(N-morpholino)ethane sulphonic acid (MES) (BDH Chemical Ltd., Poole, England) buffer, pH 8.0, 0.1% Tween 20, to approximately 0.5 mg ml^{-1}. Each sample was then spiked with a solution of α-La, bringing the final concentration of α-La to approximately 0.15 mg ml^{-1}. Samples were then filtered through a 0.45 μm syringe filter to remove any residual particulate material.

Free zone capillary electrophoresis was carried out using an Applied Biosystems 270A-HT capillary electrophoresis system (Applied Biosystems, San Jose, CA). The capillary used was an Applied Biosystems 72 cm uncoated silica capillary with 50 μm internal diameter. Injections were carried out using vacuum (17 kPa) for 4 seconds, and the separation was carried out at 20 kV. Eluent was monitored at 215 nm. In all cases, a separation buffer consisting of 50 mM MES, pH 8.0 with 0.1% Tween 20 was used. To retain separation reproducibility, the capillary was flushed between injections with 0.1 M NaOH, Milli-Q water and buffer successively for 2 minutes each (at 68 kPa).

The data were collected via a PE Nelson 900 series interface and analysed using a PE Nelson Turbochrom 3.3 software package (PE Nelson, Cupertino, CA).

4.2 RESULTS

Typical electropherograms of purified β-LG from individual cows, spiked with and aligned to α-La are seen in Figure 17. Some drift in injection to elution time of α-La was observed between samples. Paterson *et al.* (1995) suggest that this is due to ion depletion of the cathodal buffer. Aligning electropherograms to the α-La peak resulted in consistent relative elution times for β-LG variants A, B and C.

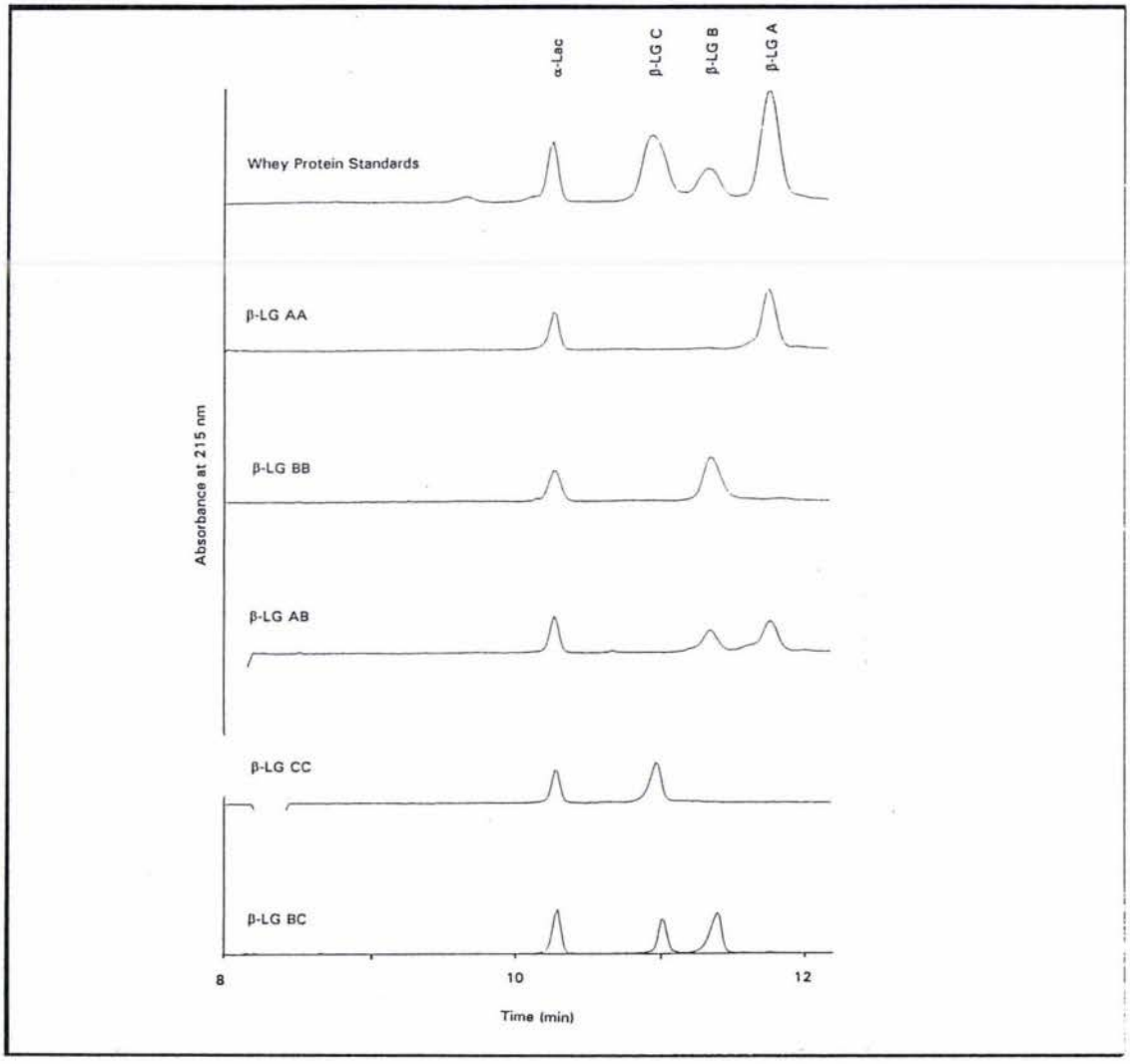


FIGURE 17 Capillary electrophoresis of (a) whey protein standards, and β-LG purified from acid whey of a β-LG AA phenotype (b), a β-LG BB phenotype (c), a β-LG AB phenotype, a β-LG CC phenotype, and a β-LG BC phenotype using the method of Paterson *et al.* (1995).

All samples of purified β-LG were also analysed by CE to check the accuracy of the phenotype assessed by native PAGE. Seven of the samples previously phenotyped as β-LG BB were found to be either BC (2) or CC (5) phenotypes. No β-LG AC phenotype samples were identified. All samples examined resulted in peak retention times that corresponded to standard β-LG A, B or C variants. No peaks were observed that fell outside of the expected ranges for the A, B or C variant.

4.3 Discussion

Although β-LG A and B are the predominant variants, the C variant has been observed in some populations of Jersey and Ayrshire breed cows at frequencies between 0.01 and 0.11 (Aschaffenburg and Drewry, 1955; McLean *et al.*, 1984; Bech and Kristiansen, 1990; Paterson *et al.*, 1995). Given the inability to distinguish the B and C variants in the gel electrophoretic systems described, the accuracy of phenotypic determination by PAGE alone appears questionable.

Previous studies have indicated the presence of the β-LG C variant may influence the composition and functional properties of milk and milk products (Whitney, 1988; Motion and Hill, 1994). Obviously the determination of the physical characteristics and effect that milk protein variants have on the behaviour of milk and milk products are dependent on accurate phenotyping.

Phenotyping of β-LG by native PAGE and CE highlights the limitations of traditional PAGE methods for the detection of variants, even those involving substitutions of charged residues.

5 TRYPSIN PURIFICATION

Trypsin is frequently used in hydrolysis studies of proteins. Its specificity for cleavage at the carboxyl side of arginine and lysine residues results in a limited number of peptides compared to less specific enzymes. Theoretically each tryptic peptide, except for the carboxyl-terminal peptide of the protein, will end with an arginine or lysine residue. However, trypsin preparations are often contaminated with chymotrypsin (Jany *et al.*, 1976). Chymotrypsin cleaves preferentially on the carboxyl-side of aromatic and other bulky non polar residues. Chymotrypsin action may be specifically blocked by α-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK). Trypsin preparations are often treated with TPCK to block contaminating chymotrypsin activity.

5.1 MATERIALS AND METHODS

TPCK treated trypsin type XIII (from bovine pancreas) and chymotrypsin were obtained from Sigma Chemical Company (St. Louis, MO.) An FPLC low pressure chromatography station and MonoQ HR5/5 column (Pharmacia, Uppsala, Sweden) were used for the analysis of trypsin and chymotrypsin.

Trypsin and chymotrypsin standards were dissolved in 50 mM phosphate buffer, pH 7.0 to 0.5 mg ml^{-1}. A trypsin plus chymotrypsin solution was prepared by mixing the two standards in a 1:1 ratio. Fifty microlitres of each protein solution was injected onto the column, previously equilibrated with 50 mM phosphate buffer, pH 7.0. Protein was eluted from the column in a 10 column volume gradient of 0 to 1 M NaCl in phosphate buffer at 1 ml min^{-1}. Eluent was monitored at 280 nm.

5.2 RESULTS

The elution profiles of TPCK trypsin, chymotrypsin and a mixture of both are seen in Figure 18. Chymotrypsin elutes earlier in the gradient than trypsin. The TPCK trypsin standard used showed no traces of chymotrypsin contamination.

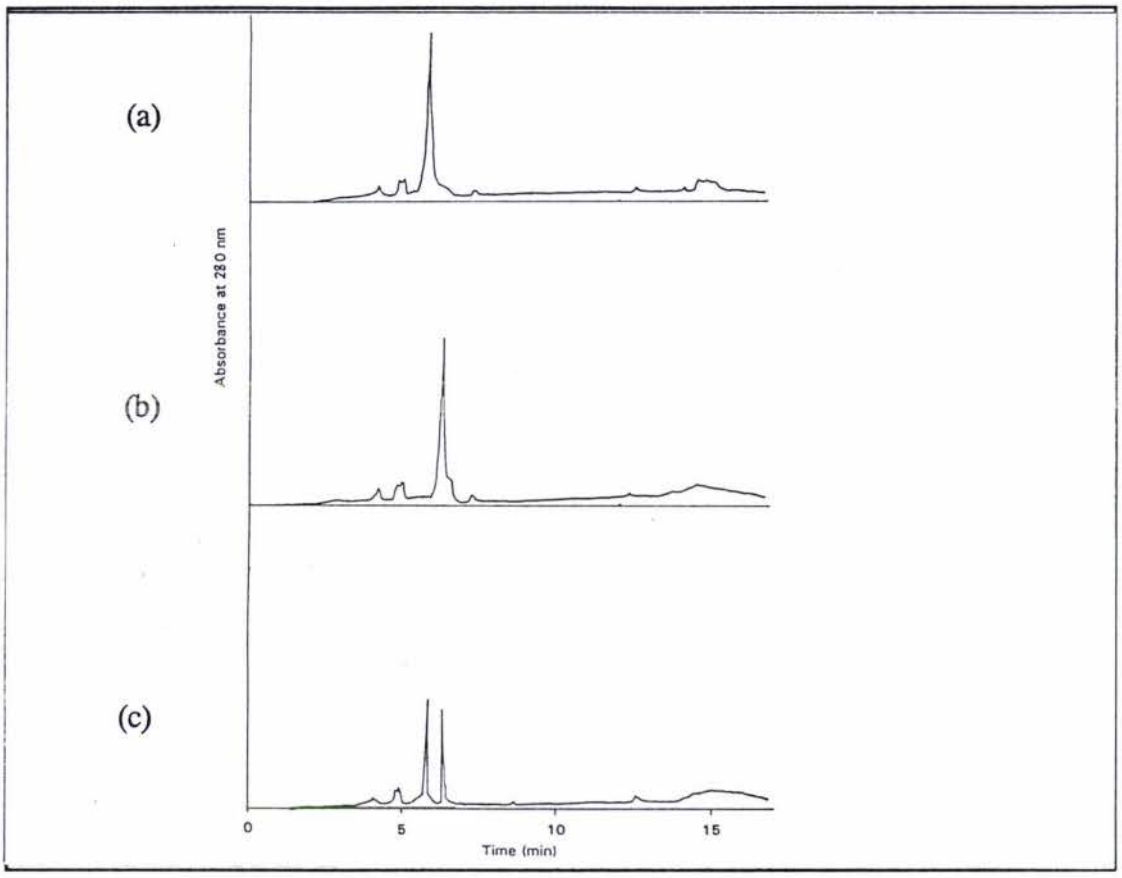


FIGURE 18 Analysis of trypsin purity. Chymotrypsin (a), trypsin (b), and a mixture of both standards were analysed by ion-exchange chromatography. Neither trypsin or chymotrypsin showed cross contamination with the other enzyme.

6 TRYPTIC HYDROLYSIS

Enzymatic hydrolysis of a protein followed by separation of the resultant peptides is frequently used as the first step in determining the primary sequence of the protein. Comparison of the elution profiles of the peptides, or peptide mapping is a useful tool for detecting alterations in the amino acid sequence from that of the standard protein.

Reversed phase (RP) chromatography separates compounds on the basis of their hydrophobicity. The hydrophobicity of a peptide is determined primarily by the amino acid composition. To a lesser degree a peptide's net hydrophobicity is influenced by the environment around each residue. The slight change in hydrophobicity resulting from a single amino acid residue substitution is likely to have little net effect on the overall hydrophobicity of the intact protein. By prior cleavage of the protein to subsequent smaller peptides the hydrophobic effect of amino acid substitution on a peptide is much easier to detect. Trypsin is frequently used for hydrolysis due to its high degree of specificity and subsequently limited number of resultant peptides to analyse.

Peptide mapping using RP-HPLC has been used for the detection of silent mutations in haemoglobin (Schroeder *et al.*, 1982). Eluent fractions corresponding to peaks with different retention times than the standard elution profile may be further analysed by amino acid analysis, amino acid sequencing, mass spectroscopy or a combination of these techniques to determine the substitution involved. Mass spectroscopy enables the detection of many post-translational modifications such as phosphorylation that may not be detected by amino acid analysis or sequencing.

6.1 METHODS AND MATERIALS

6.1.1 Hydrolysis

TPCK treated trypsin (analysed above) was dissolved in phosphate buffer (0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, pH 7.45) to 1.0 mg ml^{-1}. Purified milk proteins, prepared above, were dissolved in phosphate buffer to 5 ml ml^{-1}. 50 μl of trypsin was added

to each ml of protein solution (1:100 enzyme:substrate w/w) and digested at 37°C with shaking for the required time. The hydrolysis was terminated by acidification with 5 μl of trifluoroacetic acid (TFA) (SpectroSol, BDH Laboratory Supplies, Poole, England). After mixing and standing at room temperature for 30 minutes any precipitated material was separated by centrifugation at 10,000 x g for 15 minutes. The supernatant was either analysed immediately or stored at -20°C until required.

6.1.2 Peptide Mapping

Hydrolysed milk protein peptides were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using linear gradients of several steps on a octadecyl silane porous silica column (3.9 x 300 mm Bondclone 10μ C18 with 3.9 x 30 mm Bondclone C18 guard column, Phenomenex, Torrance, CA) at 60°C. Two HPLC systems were utilised. One system comprised a Waters 600 E multi solvent delivery system, Waters 700 Satellite WISP autosampler (Waters Division, Millipore Corp., Milford, MA), Hewlett-Packard 1040A diode array detector (HP DAD) and Hewlett-Packard 300/7946 data station running HPLC ChemStation software (Hewlett-Packard, Palo Alto, CA). The alternative system comprised two LC-6A pumps, SIL-6A autoinjector, SCL-6A system controller, CTO-6A oven, SPD-6AV UV-Vis spectrophotometric detector, C-R3A Chromatopak integrator, FDD-1A floppy disc drive data storage (all Shimadzu Corp., Kyoto, Japan) and Gilson FC 203 fraction collector (Gilson Medical Electronics Inc., Middleton, WI).

The solvents used during reversed phase analysis were 5% HiPerSolv 'Far UV' grade acetonitrile, 0.05% TFA for solvent A (both BDH Laboratory Supplies, Poole, England), and 60% acetonitrile, 0.05% TFA as solvent B. Solvents were sparged with helium prior to use.

Prior to use TFA was re-distilled from $KMnO_4$ over 4Å sieves. Coloured fractions from the start and end of distillation were discarded and only the clear mid-distillate was used.

A start-up programme linearly increased the flow rate from 0 to 1 ml min^{-1} over 10 minutes prior to equilibrating the column in solvent A. Similarly a shut-down

programme decreased the flow rate to 0 ml min^{-1} at the end of a series of chromatographic runs.

A series of gradients were run to determine optimum separation parameters. The gradient that was found to resolve most peptides across the range of proteins used consisted of a linear gradient from 0 to 15% B over 15 minutes after sample injection, followed by a linear gradient from 15 to 50% B over 10 minutes. The gradient was increased from 50 to 60% B over 15 minutes prior to an increase to 100% B over the next 10 minutes. The column was held at 100% B for 3 minutes prior to re-equilibration by a decreasing gradient to 0% B over 0.5 minutes, then holding at 0% B for 6.5 minutes. The separation programme used a consistent flow rate of 1 ml/min throughout. The programme from sample injection to completion of re-equilibration took 60 minutes (see appendix A).

A sample injection volume of 50 ul was used, applied via the autoinjector with thorough rinsing between samples.

Detection was either at 214 or 280 nm (as indicated) on the Shimadzu HPLC, or between 205 and 300 nm in 2 nm steps with the HP DAD system. Multiple wavelength monitoring of column eluate provides supplementary information as to the possible amino acid composition of each peptide. The peptide bond absorbs strongly at 205 to 214 nm. Thus the peak height, in the absence of strongly absorbing aromatic amino acid residues, is proportional to the number of peptide bonds. At 280 nm the aromatic amino acids tyrosine and tryptophan absorb strongly. These two amino acids may be differentiated at 295 nm where tryptophan has a much stronger absorbence.

Peptide maps, obtained from reversed phase separation of the tryptic peptides, were compared. Typical retention times and peak patterns were determined for each variant of the casein proteins. Samples exhibiting aberrant peptide maps were re-analysed. Peptides with altered retention times or peak shapes were collected automatically using changes in peak slope as an indicator of a new peak. Peptides were either sequenced directly or dried using a SpeedVac and stored at -20°C until required.

6.1.3 Peptide sequencing

Peptides were sequenced by the Biochemistry Department of Massey University, Palmerston North, New Zealand. The instrument used was a Applied Biosystems Model 476A protein sequencer (Applied Biosystems Inc., Foster City, CA), utilising Edman N-terminal sequencing chemistry.

6.1.4 Fast atom bombardment - mass spectrometry (FAB-MS)

Selected peptides isolated from tryptic hydrolyses were subjected to mass analysis by FAB-MS. The analyses were completed by the Mass Spectroscopy Unit of AgResearch Grasslands, Palmerston North, New Zealand. Mass spectra were obtained on a VG70-250S double focusing, magnetic sector mass spectrometer (VG Analytical, Manchester, U.K.). This was equipped with a Liquid Secondary Ion Mass Spectrometry ion source (LSIMS) and associated caesium ion gun. The primary beam was 15 KeV caesium ions at a total current of 1 mA measured at the gun cathode. The secondary ion beam used was 6 kV at the source.

Samples were dissolved in water/70% formic acid to approximately 10 μg ml^{-1}. One microlitre of sample was spotted on 1 μl of glycerol matrix on the stainless steel LSIMS probe tip. A mass range of 10 to 1600 AMU was scanned at 5 sec./decade scan rate, using a resolving power of 1000.

6.2 Results

Typical tryptic peptide profiles after RP-HPLC of α_{s1}-CN B, α_{s2}-CN A, β-CN A^1, and κ-CN B may be seen in Figure 19.

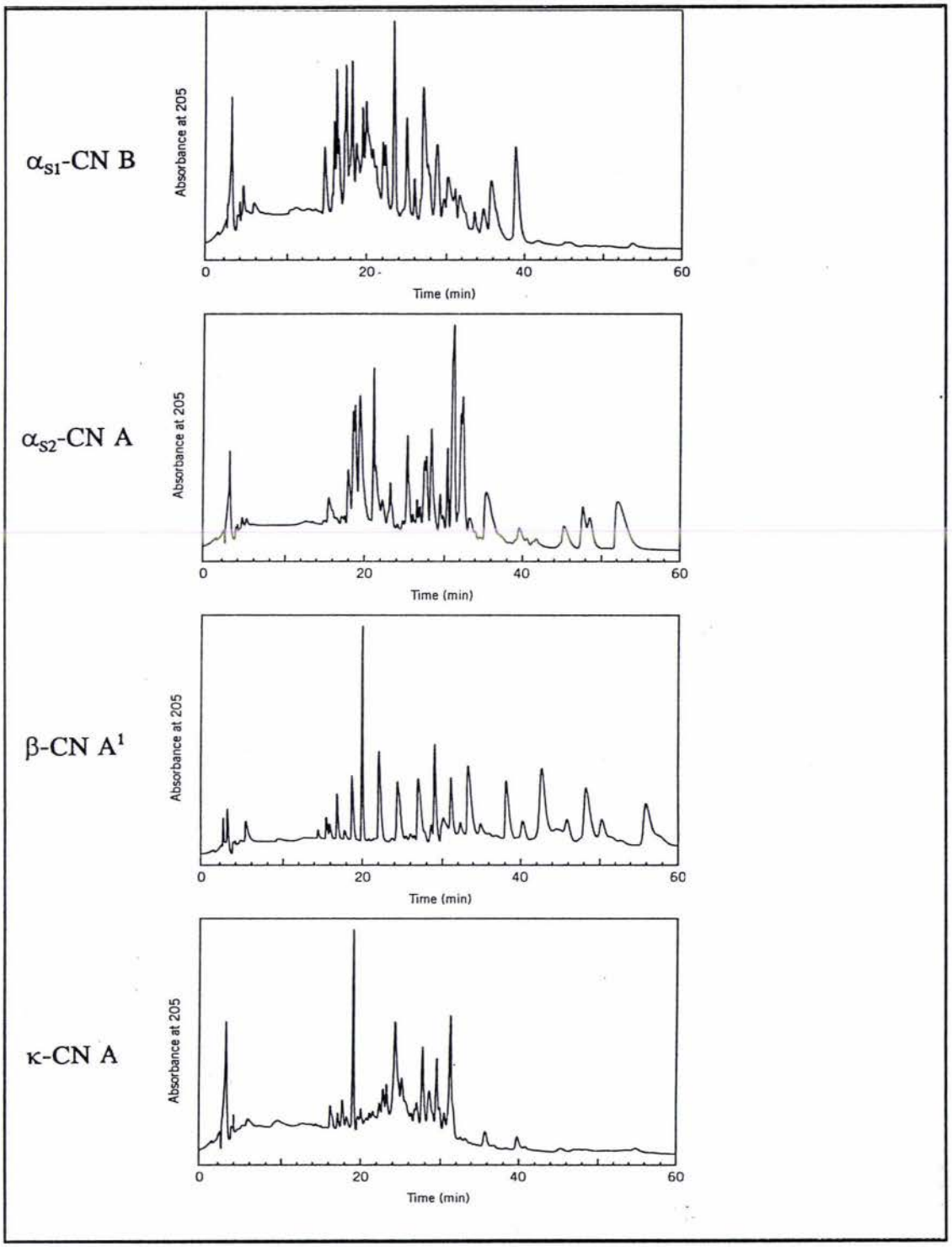


FIGURE 19 Typical RP-HPLC tryptic peptide profiles for the casein proteins α_{s1}-CN B, α_{s2}-CN A, β-CN A^1 and κ-CN A.

6.2.1 α_{s1}-CN

The primary sequence of α_{s1}-CN B-8P indicates that there are 18 peptide bonds that are theoretically cleaved by trypsin (see Table 2). About 30 peaks may be seen resolved in 60 minutes on the typical peptide map of α_{s1}-CN B. The profile obtained bore little resemblance to that obtained by other researchers (Lemieux and Amiot, 1990; Dong Chin, 1992). This is not surprising due to differences in RP columns and gradient conditions used. The additional peaks observed are likely due to incomplete hydrolysis of the protein, resulting in additional intermediate peptides containing trypsin susceptible bonds which had not been cleaved. Alternatively the protein may have been cleaved at sites not normally associated with trypsin specificity (see discussion 6.2.2).

Pept #	Residue	Mass (Da)	Sequence
1	A(1-3)	399.3	RPK
2	A(4-7)	493.3	HPIK
3	A(8-22)	1758.	HQGLP QEVLN ENLLR
4	A(23-34)	1383.7	FFVAP FPEVF GK
5	A(35-36)	275.1	EK
6	A(37-42)	688.4	VNELS K
7	A(43-58)	1926.7	DIGXE XTEDQ AMEDI K
8	A(59-79)	2720.9	QMEAE XIXXX EEIVP NXVEQ K
9	A(80-83)	524.3	HIQK
10	A(84-90)	830.4	EDVPS ER
11	A(91-100)	1266.7	YLGYL EQLLR
12	A(101-102)	259.2	LK
13	A(103-103)	146.1	K
14	A(104-105)	309.2	YK
15	A(106-119)	1659.8	VPQLE IVPNX AEER
16	A(120-124)	614.3	LHSMK
17	A(125-132)	909.5	EGIHA QQK
18	A(133-151)	2316.1	EPMIG VNQEL AYFYP ELFR
19	A(152-193)	4717.2	QFYQL DAYPS GAWYY VPLGT QYTDA PSFSD IPNPI GSENS EK
20	A(194-199)	747.4	TTMPL W

TABLE 2 Expected tryptic peptides from α_{s1}-CN B and their calculated mass.

All of the peptide profiles of the samples of α_{s1}-CN B examined were very similar. By superimposing profiles (Figure 20) it may be seen that peak retention times are nearly identical with minor differences in peak heights.

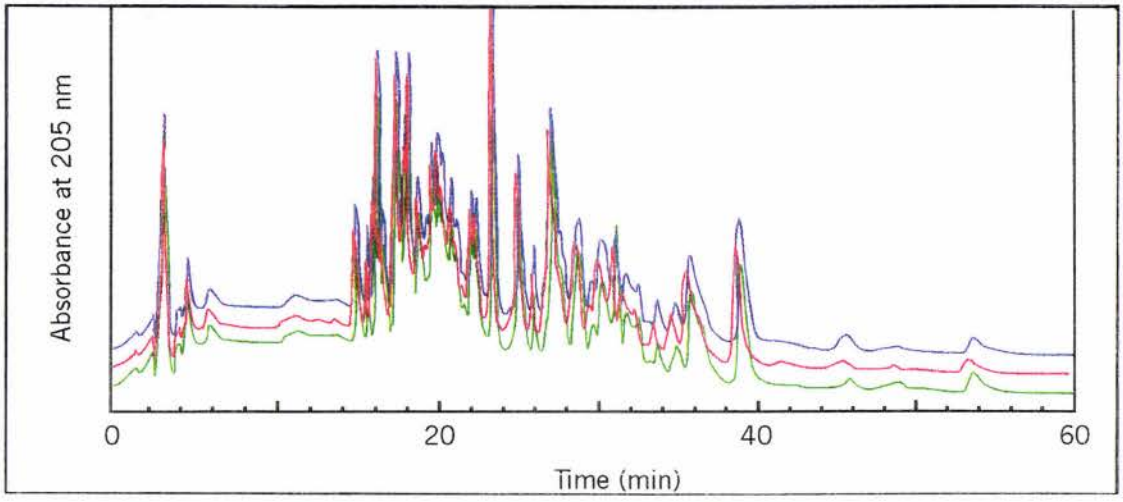


FIGURE 20 Overlay of three α_{s1}-CN tryptic peptide maps.

6.2.2 α_{s2}-CN

A theoretical trypsin hydrolysis of α_{s2}-CN should result in 28 peptides (Table 3). About 35 peaks were resolved in 60 minutes on a typical tryptic peptide profile of α_{s2}-CN B. The majority of peaks observed were collected and the peptides contained within each peak identified by amino-acid sequencing. FAB-MS was used to corroborate the primary sequence of the peptides by comparison of measured mass and calculated mass. In addition to the expected tryptic peptides, several peptides were identified by sequencing that were the result of chymotryptic like activity (Table 4). Chymotrypsin cleaves carboxyl-terminal to aromatic and bulky hydrophobic residues (tryptophan, tyrosine and phenylalanine) Peptide bonds preceded by other hydrophobic residues may also be cleaved but generally at a slower rate.

Previous analysis of the trypsin used showed no traces of chymotrypsin present. Furthermore TPCK treatment of the trypsin is expected to block the activity of any chymotrypsin present. The chymotrypsin-like activity seen is thus likely to be endogenous to the trypsin used. It is likely that at least some of the additional peaks seen in the α_{s1}-CN B and κ-CN A tryptic hydrolyses were also peptides resulting from endogenous chymotryptic-like activity.

Pept #	Residue	Mass (Da)	Sequence
1	A(1-1)	146.1	K
2	A(2-21)	2618.9	NTMEH VXXXE ESIIX QETYK
3	A(22-24)	403.2	QEK
4	A(25-32)	873.4	NMAIN PSK
5	A(33-41)	1043.4	ENLCS TFCK
6	A(42-45)	501.3	EVVR
7	A(46-70)	3008.0	NANEE EYSIG XXXEE XAEVA TEEVK
8	A(71-76)	689.4	ITVDD K
9	A(77-80)	574.3	HYQK
10	A(81-91)	1367.7	ALNEI NEFYQ K
11	A(92-113)	2709.4	FPQYL QYLYQ GPIVL NPWDQ VK
12	A(114-114)	174.1	R
13	A(115-125)	1194.7	NAVPI TPTLN R
14	A(126-136)	1410.5	EQLXT XEENS K
15	A(137-137)	146.1	K
16	A(138-149)	1465.6	TVDME XTEVF TK
17	A(150-150)	146.1	K
18	A(151-152)	247.2	TK
19	A(153-158)	747.4	LTEEE K
20	A(159-160)	288.2	NR
21	A(161-165)	633.4	LNFLK
22	A(166-166)	146.1	K
23	A(167-170)	502.3	ISQR
24	A(171-173)	437.2	YQK
25	A(174-181)	978.6	FALPQ YLK
26	A(182-188)	902.5	TVYQH QK
27	A(189-197)	1097.6	AMKPW IQPK
28	A(198-199)	247.2	TK
29	A(200-205)	745.4	VIPYV R
30	A(206-207)	294.2	YL

TABLE 3 Theoretical tryptic α_{s2}-CN A peptides and their calculated mass.

There were several α_{s2}-CN peptide profiles that differed slightly from the majority of the α_{s2}-CN profiles. These samples had a relatively small peak eluting at approximately 15.8 minutes. This was absent in the standard samples (Figure 21). Collection and sequencing of this peptide identified it as peptide 138-149 (TVDMESTEVFTK). The same peptide was also identified as eluting at approximately 16.4 minutes in both the standard and aberrant samples. FAB-MS analysis of the peptide eluting at approximately 15.8 minutes revealed that this peptide had a measured mass of 1467 mass units (Figure 22), 80 mass units higher than the calculated and measured mass of the peptide 138-149. This is consistent with a phosphorylation of a single residue.

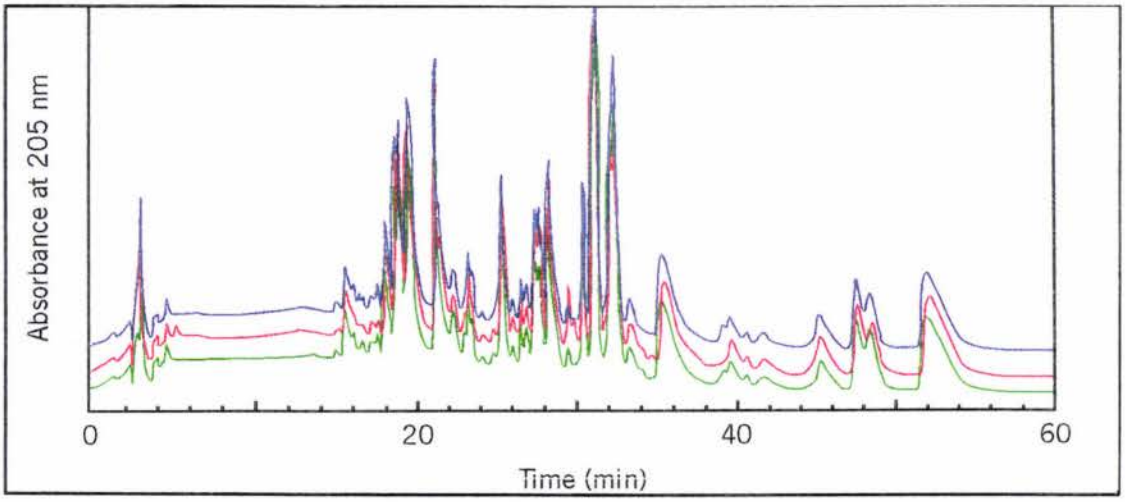


FIGURE 21 Tryptic hydrolysis peptide map of standard α_{s2}-CN A.

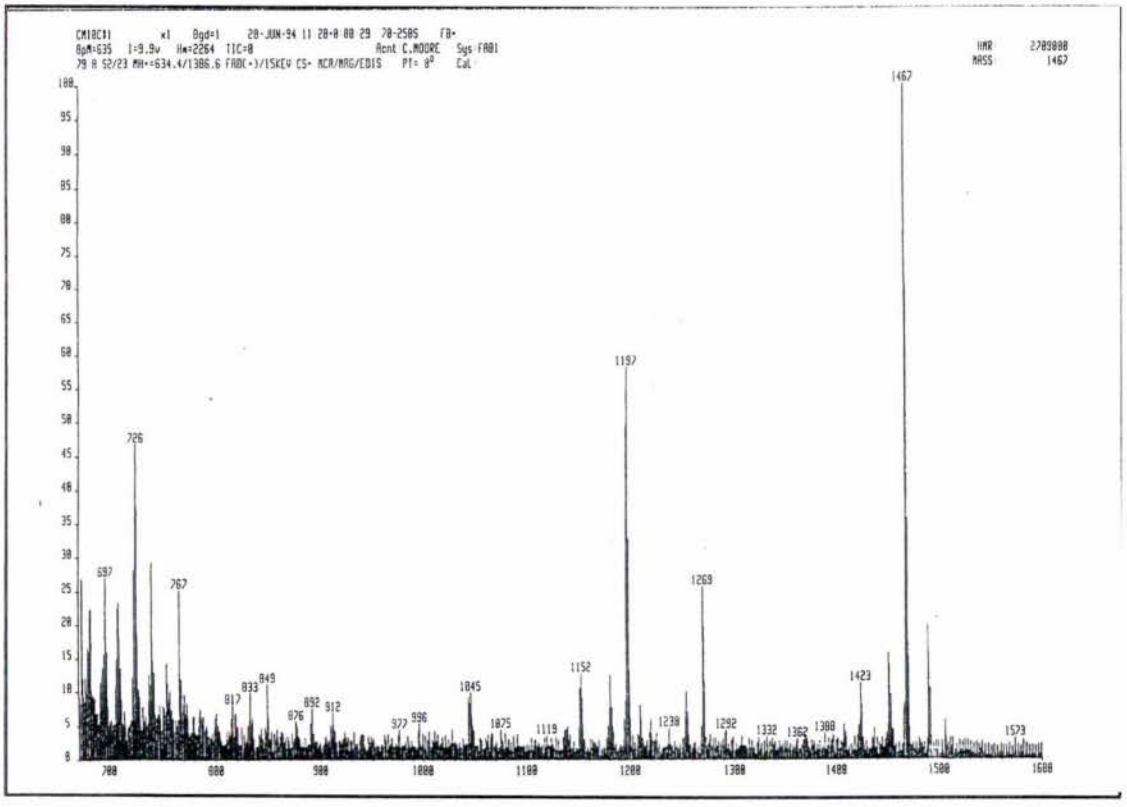


FIGURE 22 FAB-MS of peak eluting at 16.4 minutes in RP-HPLC chromatogram of α_{s2}-CN A tryptic digest.

Ser143 has been identified as phosphorylated in at least one α_{S2}-CN fraction (Brignon *et al.*, 1977). Evidence to date suggest that all of the fractions of α_{S2}-CN A separated by gel electrophoresis (formerly classified as α_{S3}-, α_{S4}-, α_{S5}- and α_{S6}-CN) have the same amino acid sequence but differ in phosphate content (Brignon *et al.*, 1976, 1977; Eigel *et al.*, 1984). From this experimental evidence it appears that in at least some individual cows that Ser143 is a site of variable phosphorylation.

A similar phenomenon is seen in ovine α_{S1}-CN. Ferranti *et al.* (1995) found nine serine residues fully phosphorylated in ovine α_{S1}-CN A, whereas Ser41 and Ser115 were phosphorylated by approximately 20 and 50% respectively.

Expected tryptic peptide	Fragment	Chymotryptic-like peptide identified	Fragment observed
VIPYVR	200-205	VIPY	200-203
NMAINPSK	25-32	NMAINP	25-30
AMKPWIQPK	189-197	AMKPW	189-193
FPQYLQYLYQGPIVLNPWDQVK	92-113	FPQYLQY	92-98
		LYQGPIVLNPWDQV	99-112
		FPQYLQYLYQGPI	92-104

TABLE 4 Additional chymotryptic-like peptides generated from the tryptic hydrolysis of α_{S2}-CN A.

6.2.3 β-CN

Three variants of β-CN were examined by this peptide mapping technique. Eleven samples of β-CN A^1, 10 of β-CN A^2 and one of β-CN B were hydrolysed under identical conditions, the resultant peptides separated by RP-HPLC and the elution profiles compared.

Theoretically 16 peptides are expected from a tryptic hydrolysis (Table 5). Approximately 27 peptides were resolved in each variant within 60 minutes. An overlay of three samples of β-CN A^1 is presented in Figure 23(a). Similarly an overlay of three samples of β-CN A^2 is presented in Figure 23(b). These two figures indicate the reproducibility of hydrolysate elution profiles between samples of the same protein.

The elution profile of β-CN B may be seen in Figure 23(c).

Pept #	Residue	Mass (Da)	Sequence
1	A(1-1)	174.1	R
2	A(2-25)	2966.2	ELEEL NVPGE IVEXL XXXEE SITR
3	A(26-28)	373.2	INK
4	A(29-29)	146.1	K
5	A(30-32)	388.2	IEK
6	A(33-48)	2061.8	FQXEE QQQTE DELQD K
7	A(49-97)	5318.8	IHPFA QTQSL VYPFP GPIPN SLPQN IPPLT QTPVV VPPFL QPEVM GVSK
8	A(98-99)	245.2	VK
9	A(100-105)	645.3	EAMAP K
10	A(106-107)	283.2	HK
11	A(108-113)	747.4	EMPFP K
12	A(114-169)	6362.2	YPVEP FTESQ SLTLT DVENL HLPLP LLQSW MHQPH QPLPP TVMFP PQSVL SLSQS K
13	A(170-176)	779.5	VLPVP QK
14	A(177-183)	829.4	AVPYP QR
15	A(184-202)	2186.2	DMPIQ AFLLY QEPVL GPVR
16	A(203-209)	741.4	GPFPI IV

TABLE 5 Expected peptides from a tryptic hydrolysate of β-CN A^2.

The differences in the elution profiles of β-CN A^1 and β-CN A^2 are highlighted in Figure 24 by arrows. Theoretical tryptic hydrolysis of the A^1 and A^2 variants indicates that the substitution of His67 to Pro occurs in the peptide comprising residues 49-97. Accordingly it is expected that the only difference in the peptide elution profiles of β-CN A^1 and β-CN A^2 is the elution time of peptide 49-97. Figure 24 clearly shows much larger peaks eluting at 30.3 and 42.6 minutes, peaks of reduced size eluting at 31.4 and 48.5 minutes, peaks at 35.1 and 45.9 minutes missing, and a new peak at 37.6 minutes for β-CN A^2 relative to β-CN A^1. This suggests that there are several peptide bonds within β-CN that are susceptible to cleavage with trypsin that do not conform to the expected specificity of trypsin (see discussion section 6.2.2)

In the β-CN samples analysed there were no elution profiles that did not conform to standard β-CN A^1, A^2 or B variant profiles.

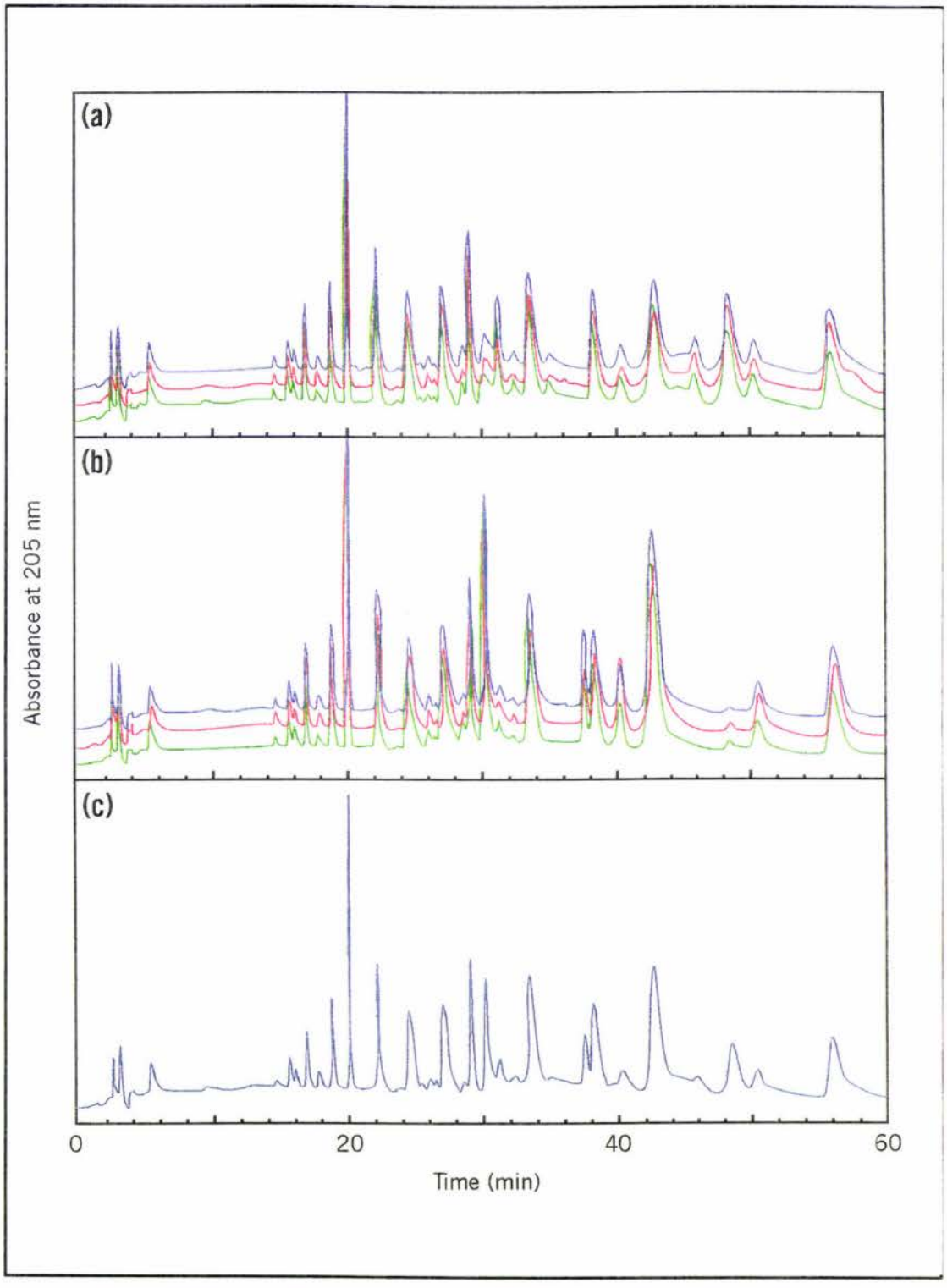


FIGURE 23 Overlays of three tryptic hydrolyses of (a) β-CN A^1 and (b) β-CN A^2 ; and a single trace (c) of β-CN B.

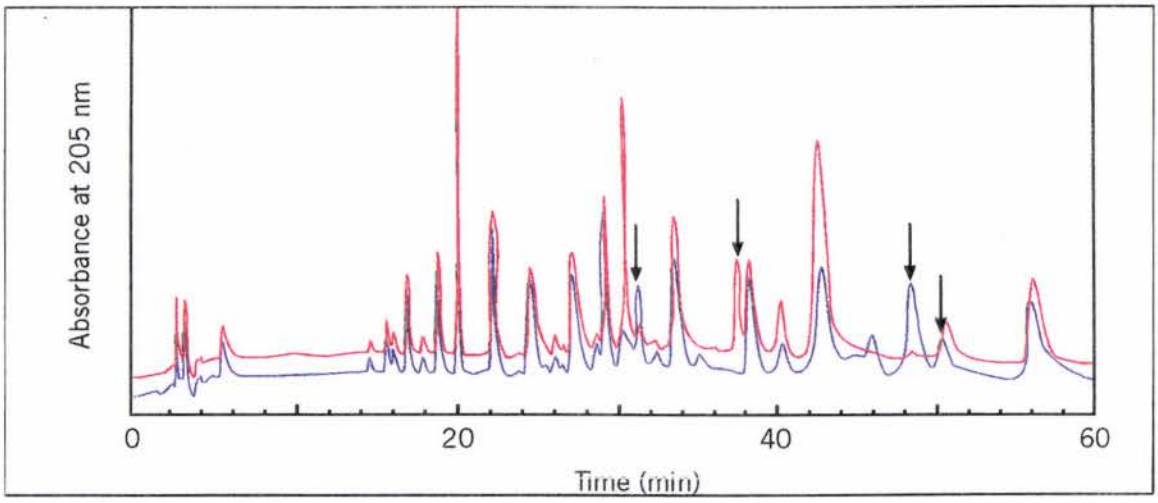


FIGURE 24 Overlays of tryptic hydrolyses of β-CN variants A^1 and A^2. Differences in the elution profiles are highlighted by arrows.

6.2.4 κ-CN

Figure 25 shows an overlay of three elution profiles of hydrolysed κ-CN A. The majority of peaks eluted in a concentrated time period from 16 to 32 minutes. A theoretical trypsin hydrolysis is expected to yield 13 peptides (Table 6).

Pept #	Residue	Mass (Da)	Sequence
1	A(1-10)	1249.4	EEQNQ EQPIR
2	A(11-13)	378.2	CEK
3	A(14-16)	418.2	DER
4	A(17-21)	642.3	FFSDK
5	A(22-24)	330.2	IAK
6	A(25-34)	1250.7	YIPIQ YVLSR
7	A(35-68)	4011.1	YPSYG LNYYQ QKPVA LINNQ FLPYP YYAKP AAVR
8	A(69-86)	1981.1	SPAQI LQWQV LSDTV PAK
9	A(87-97)	1192.5	SCQAQ PTTMA R
10	A(98-111)	1607.8	HPHPH LSFMA IPPK
11	A(112-112)	146.1	K
12	A(113-116)	503.2	NQDK
13	A(117-169)	5535.7	TEIPT INTIA SGEPT STPTT EAVES TVATL EDXPE VIESP PEINT VQVTS TAV

TABLE 6 Theoretical tryptic peptides from hydrolysis of κ-CN A.

In the samples analysed no aberrant profiles were detected.

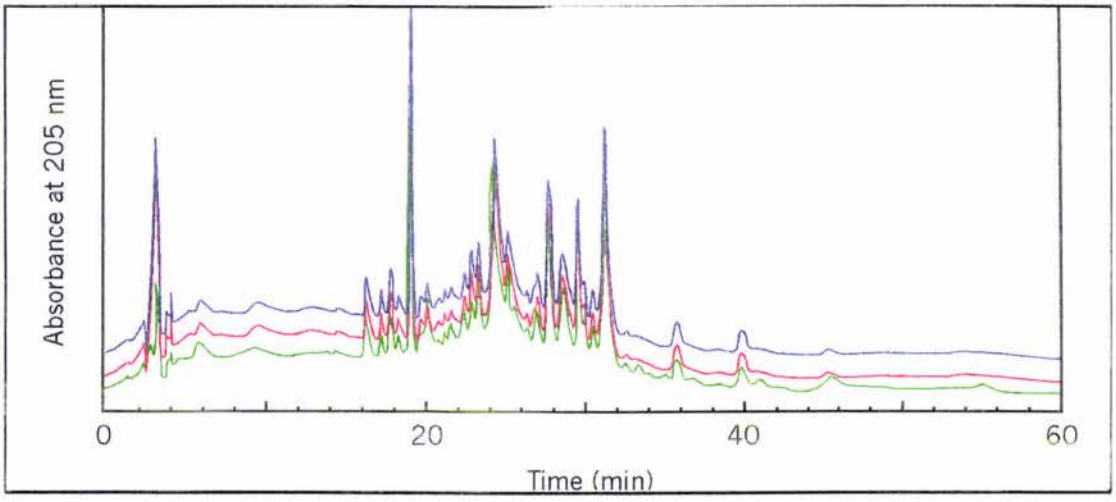


FIGURE 25 Overlay of three κ-CN A tryptic peptide maps

6.3 DISCUSSION

Peptide sequence information derived from Edman degradation chemistry is often not unequivocal. Terminal residues may be modified such that they do not cleave. Other modifications may affect the retention time of a residue during chromatography resulting in mis-identification. The value of cross checking peptide sequencing information by peptide mass measurement was highlighted in the analysis of two peptides.

During amino acid sequencing of the α_{s2}-CN tryptic peptide 189-193 (AMKPW), the elution time of the methionine residue was slightly different than the standard. This suggested that the methionine residue may have been modified. A frequent modification of methionine is oxidation. If the methionine residue was oxidised the mass of the peptide would be either 16 (sulphoxide) or 32 (sulphone) mass units higher, consistent with an additional one or two oxygen atoms respectively. Mass spectroscopic analysis of the peptide revealed a mass of 632. This is consistent with the calculated mass of the unoxidised peptide. This confirmed the methionine residue was unmodified. The reason for the slight difference in elution time of the methionine residue remains unknown.

The second peptide was the α_{s2}-CN tryptic peptide 138-149 (TVDMEX[3]TEVFTK). Sequencing was unable to differentiate between a serine or phosphoserine residue at position 143. The measured mass of this peptide was 80 units higher than the calculated mass of the peptide TVDMESTEVFTK indicating a post-translational phosphorylation of Ser143.

Of the 74 samples of purified casein proteins hydrolysed and analysed by peptide mapping none exhibited an abnormal profile that would indicate a potential variant, other than the recognised electrophoretic variants. The frequencies of potential silent variants detected by Dong Chin (1992) were 9.1, 15.8, 9.1 and 4.3% for α_{s1}-CN B, β-CN A^1, β-CN A^2 and κ-CN A respectively. Based on these frequencies it is expected that few potential variants would be detected in this study population.

The results of this study and those of Dong Chin (1992) indicate that random mutation involving neutral residues in milk proteins is not as frequent as mutations that alter the net charge of the protein.

[3]X denotes a phosphorylated serine residue

7 MASS SPECTROSCOPY

Recent developments in ionisation techniques has made mass spectroscopic analysis of proteins and large peptides possible. Electrospray ionisation (nebulisation in the presence of an electric field) and matrix assisted desorption, has seen mass spectroscopic analysis of proteins and peptides become a routine tool in protein analysis. The ability to measure the mass of intact proteins up to approximately 100,000 Daltons to within 0.01% has simplified the process of identifying protein variants. In some cases mixtures of several proteins may be simultaneously analysed making complex purifications redundant.

Electrospray mass spectroscopy was investigated as a means of identifying milk protein variants from purified samples and acid casein.

7.1 MATERIALS AND METHODS

Samples of purified casein proteins, acid casein and purified β-LG were prepared as outlined earlier. Samples were dissolved in 50% acetonitrile, 0.05% formic acid to approximately 0.1 mg ml^{-1} prior to filtering through a 0.22 μm syringe filter. The instrument used was a VG Platform single-quadrupole mass spectrometer (VG BioTech, Altrincham, UK) equipped with a pneumatically assisted electrospray ion source. Positive multi-charged protein ions were generated by introducing filtered sample diluted in 50% acetonitrile, 0.05 % formic acid to approximately 1 μg ml^{-1}, into a stream of 50% acetonitrile pumped at 10 μl min^{-1} by a Spectra-Physics pump through a 75 μm ID fused-silica capillary. This was housed in a stainless steel capillary held at a potential of 5.0 kV.

The interface between the electrospray source and mass analyser consisted of a small conical orifice of 100 μm diameter held at +60 V. A gas curtain of 0.8 l min^{-1} of dry nitrogen in the interface region prevented entry of neutral molecules into the mass analyser.

10-15 μl aliquots were introduced into the solvent stream via a Rheodyne sample injection loop. This allowed at least 8 acquisitions to be accumulated and averaged

over a period of approximately 1 minute. Each acquisition was acquired over the mass-to-charge range of 600 to 2500, with a step size of m/z = 0.25 and a dwell time of 0.5 sec. The charge number of the multi-charged ions, the m/z ratio of each peak, the deconvoluted mass spectra and molecular weight determinations were derived using MassLynx software.

7.2 Mass Spectrum Data Processing

The raw spectra acquired were subjected to a series of processes to reduce background noise, differentiate signal from spurious spikes, identify series of multiply charged ions and to deconvolute the data to a true mass scale. The process parameters used were, in order :

Background subtraction - a polynomial of order 1 was fitted to data such that 40% of the data points lay below the polynomial. The operation was performed to an arithmetic tolerance of 0.01. This reduced a large proportion of non-specific background noise.

Smoothing - high frequency noise was reduced by smoothing the data twice using a moving mean technique with an average peak width of 3.75 Da.

Peak Centre - used all the points across a peak in a continuum trace to calculate the mass of the peak centre. A minimum of 4 data points across a peak at half height defined a significant peak. This process was used to produce stick spectra for each sample. From this the series of multiply charged ions were then identified manually.

Transform - once the multi-charged ion components were identified in the spectrum, the data system assigns charge states to each peak. The Transform algorithm uses this information to display the m/z spectrum on a true molecular mass axis. The data was transformed over an appropriate mass range for each protein (18000 to 20000 Da for β-LG, 18000 to 28000 Da for caseins) with a resolution of 0.125 Da.

7.3 Results

7.3.1 α_{s1}-CN

Twenty two samples of purified α_{s1}-CN were analysed by ESI-MS. A raw spectrum with inset transformed spectra may be seen in Figure 26. The group mean mass determined was 23614.9 ± 1.2 Da. This compares favourably with the calculated mass of α_{s1}-CN B-8P of 23614, 23614.8 and 23614.87 from Eigel *et al.*, (1984), Léonil *et al.* (1995) and PeptideTools[4]. The average individual variation from the mean was ± 3.54 Da (Table 7, page 99; Figure 26). All of the samples analysed corresponded to the calculated mass of α_{s1}-CN B-8P within error. The 7 or 9 phosphoseryl forms of this protein detected by Léonil *et al.* (1995) were not seen.

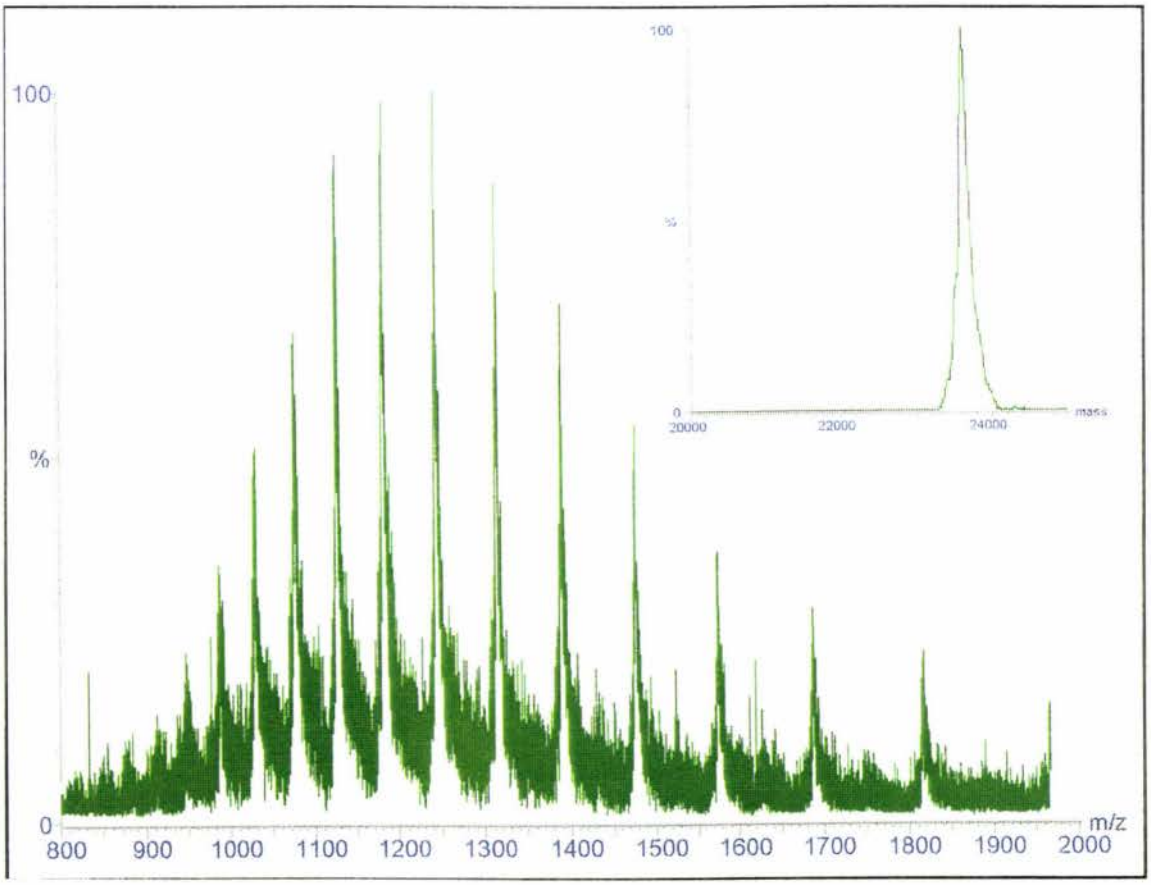


Figure 26 Raw ESI-MS spectrum and transformed spectra (inset) of a sample of purified α_{s1}-CN.

[4]Average mass calculated from the primary sequence as published by Eigel *et al.* (1984) by PeptideTools software (Hewlett-Packard, Palo Alto, CA.).

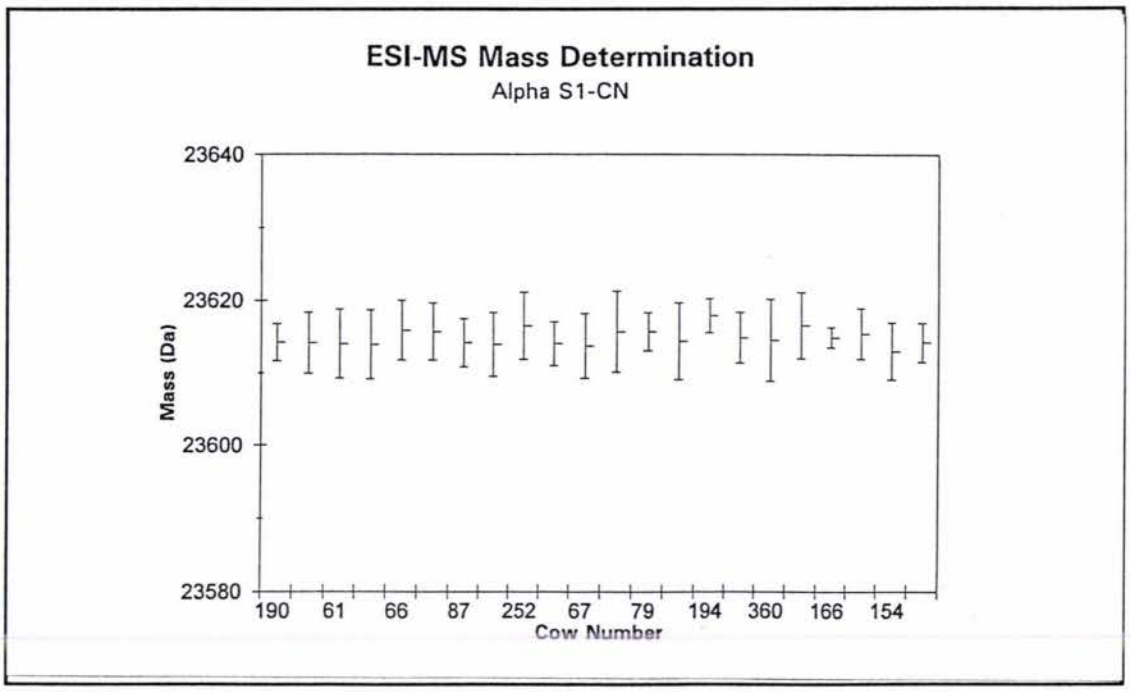


FIGURE 27 Average individual mass determinations for α_{s1}-CN B samples. The individual error of each mass determined is indicated by an error bar.

7.3.2 α_{S2}-CN

The group mean mass of the 22 samples of α_{S2}-CN analysed was 25228.9 ± 1.5 Da with an average individual variation of ± 1.5 Da (Figure 28). This corresponds to the mass of α_{S2}-CN A-11P, calculated to be 25230, 25228.4 and 25229.48 by Eigel *et al.* (1984), Léonil *et al.* (1995) and PeptideTools respectively. The micro heterogeneity, indicative of variations in the level of phosphorylation seen by Léonil *et al.* (1995) was not observed. Within error all samples corresponded to the calculated mass of α_{S2}-CN A-11P.

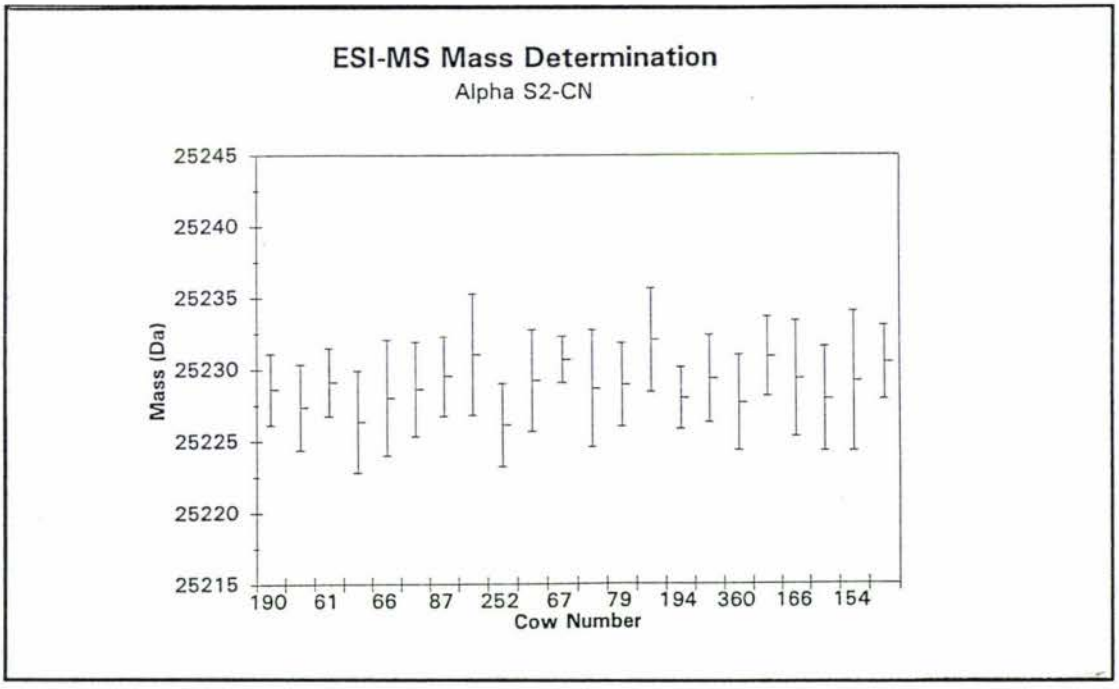


FIGURE 28 Average individual mass determinations for each α_{S2}-CN A sample. The individual error of each mass determination is indicated by an error bar.

7.3.3 β-CN

Three groups of β-CN, differentiated by mass may be seen in Figure 29. The three mass groupings calculated were 23983.5 ± 1.8 Da, 24023.9 ± 3.1 Da and 24092.6 ± 4.1 Da. These correspond to the calculated masses of the 5 phosphoseryl form of β-CN variants A^2, A^1 and B respectively (Table 7). All of the masses determined corresponded to one of the variants described.

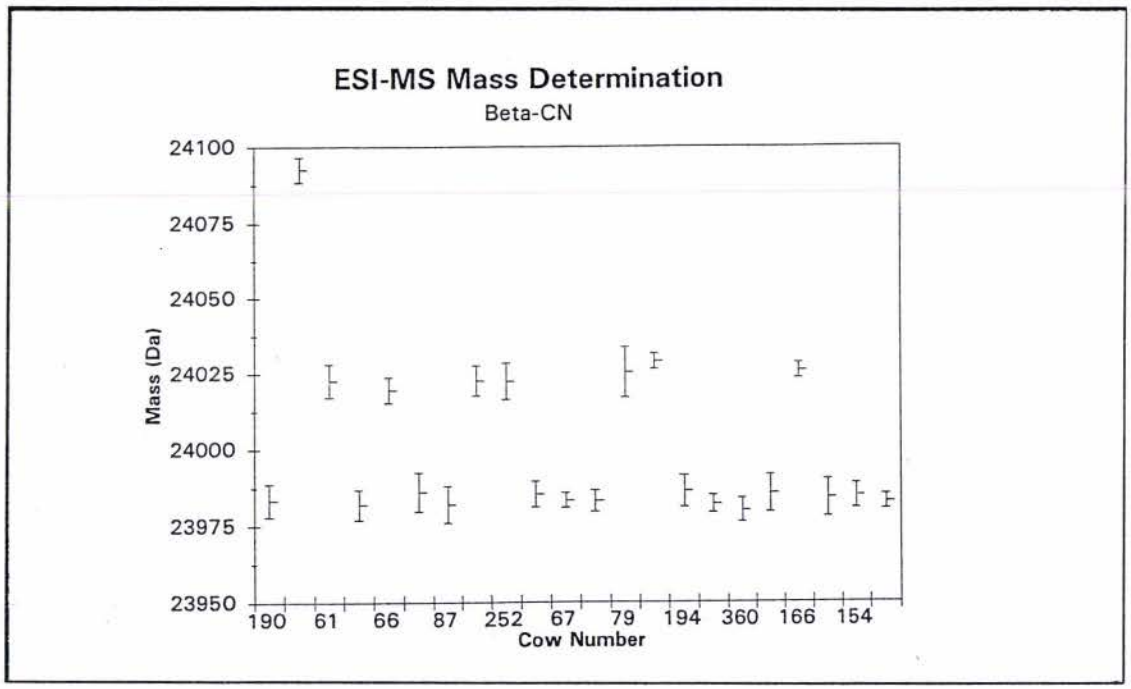


FIGURE 29 Average mass determinations for each β-CN sample analysed. The individual error of each mass determination is represented by an error bar.

7.3.4 κ-CN

Nine samples of κ-CN were examined. The raw spectra for all of the samples consisted of a large proportion of background noise relative to the ion signal. This made it difficult to differentiate spurious peaks from the true signal. Despite this spectra were able to be processed and a mass for each sample was determined. Eight samples had a group average mass of 19038.5 ± 1.5 Da, with an average individual variation of ± 3.3 Da. This corresponds within error to the calculated mass of κ-CN A-1P. The remaining sample of κ-CN analysed had a measured mass of 19003.8 ± 3.6 Da, corresponding to κ-CN B-1P (Figure 30).

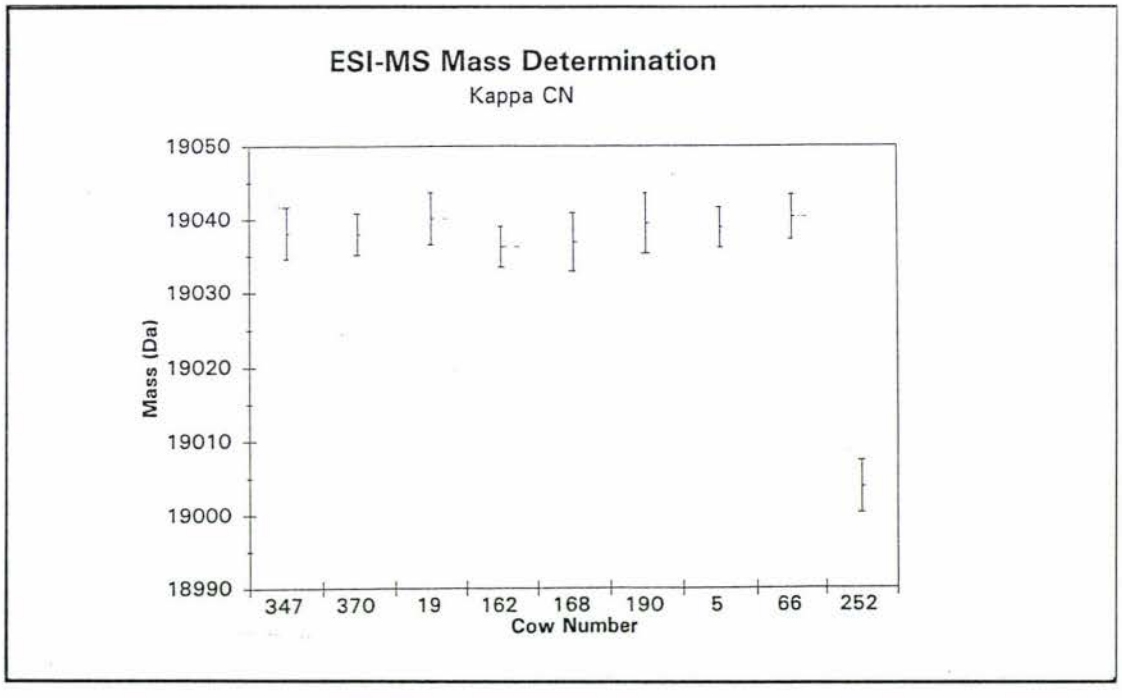


FIGURE 30 Average individual mass determinations for each κ-CN sample analysed. The individual error of each mass determination is indicated by an error bar.

Previous experience in purification of κ-CN by the ion-exchange method used indicates that despite the extensive dialysis, a significant proportion of the dried material collected from the κ-CN fraction is salt or urea. The presence of salts during ESI-MS is known to induce the protonation of the analyte with particularly sodium and potassium ions, and to a lesser extent calcium ions (MNa^+, MK^+ and MCa^{2+} respectively) as well as the hydrogen ions (MH^+). As a result the MS signal for an

analyte may consist of a series of multiply charged species for each of the protonating species - protonated, sodiated, potassiated and calciated spectra all overlaying on the one spectrum.

It is known that a proportion of κ-CN exhibits micro heterogeneity in the form of various degrees of glycosylation (Vreeman *et al.*, 1977). None of these forms were detected. It is probable that minor quantities of these glycosylated forms were present during analysis, but were unable to be resolved from the background.

Protein	Average observed mass	Std. dev.	n	Individual error	Theoretical mass calculated by Eigel *et al.* (1984)	Léonil *et al.* (1995)	Peptide Tools
α_{s1}-CN B-8P	23614.9	1.2	22	3.5	23614	23614.8	23614.9
α_{s2}-CN A-11P	25228.9	1.5	22	3.2	2230	25228.4	25229.5
β-CN A^1-5P	24023.9	3.1	7	4.6	24023	24023.3	24023.4
β-CN A^2-5P	23983.5	1.8	14	4.5	23983	23983.3	23983.4
β-CN B-5P	24092.6		1	4.1	24092	24092.4	
κ-CN A-1P	19038.5	1.5	8	3.3	19039	19037.3	19036.3
κ-CN B-1P	19003.8		1	3.6	19007	19005.5	19004.4
β-LG A	18362.6	1.0	40	1.6	18363	18363.4	18363.4
β-LG B	18277.0	0.9	56	1.8	18277	18278.3	18277.3
β-LG C	18287.2	0.6	4	1.1	18286		18286.3

TABLE 7 Comparison of milk protein masses determined experimentally and calculated from the primary sequence.

7.3.5 Casein

A number of acid precipitated caseins were also analysed by ESI-MS. Samples were dissolved in 50% acetonitrile, 0.1% formic acid, filtered and diluted in 50% acetonitrile prior to injection. In general the spectra obtained had a low signal to noise ratio, possibly caused by the inclusion of an unacceptably high salt content. The masses of two species could be determined in each sample. In all cases these corresponded to α_{s1}-CN B-8P, and either the β-CN A^1-5P or β-CN A^2-5P variant. However the average individual variation from the calculated mean was of the order ± 10-20 Da for both species. This degree of error meant that several potential substitutions with a net mass change of less than 40 Da may not be detected. The use of ESI-MS for phenotyping casein proteins by this particular method is thus limited to detection of gross changes in the mass of α_{s1}-CN and β-CN only. With suitable modifications to the methodology it is possible that the resolution may be improved such that all of the casein proteins may be characterised.

7.3.6 β-LG

One hundred and nine samples of β-LG, prepared from acid whey were analysed by ESI-MS. Several groups of β-LG variants could be distinguished by mass determination. A group of 38 samples had a calculated group average mass of 18362.6 ± 1.0 Da (Figure 31a). Within error this coincides with a mass of 18363 Da calculated from the primary sequence of β-LG A (Eigel *et al.*, 1984). A second group of 54 samples had a group average mass of 18277.0 ± 0.9 Da (Figure 31b), consistent with a mass of 18277 Da calculated from the primary sequence data of β-LG B (Eigel *et al.*, 1984). A third group of 3 samples had a group average mass of 18287.2 ± 0.6 Da (Figure 31c), consistent with a mass of 18286 Da calculated from the primary sequence of β-LG C (Eigel *et al.*, 1984). Fourteen samples showed two species present (Figure 31d). Thirteen matched the calculated molecular weights of β-LG variants A and B. The same samples had been previously phenotyped as β-LG AB by both PAGE and CE techniques. A single sample matched the calculated molecular weights of β-LG B and C variants. This sample had previously been phenotyped as β-LG BB by PAGE and corrected to β-LG BC by CE. All of the samples analysed corresponded within error with one or more of the β-LG variants A, B or C.

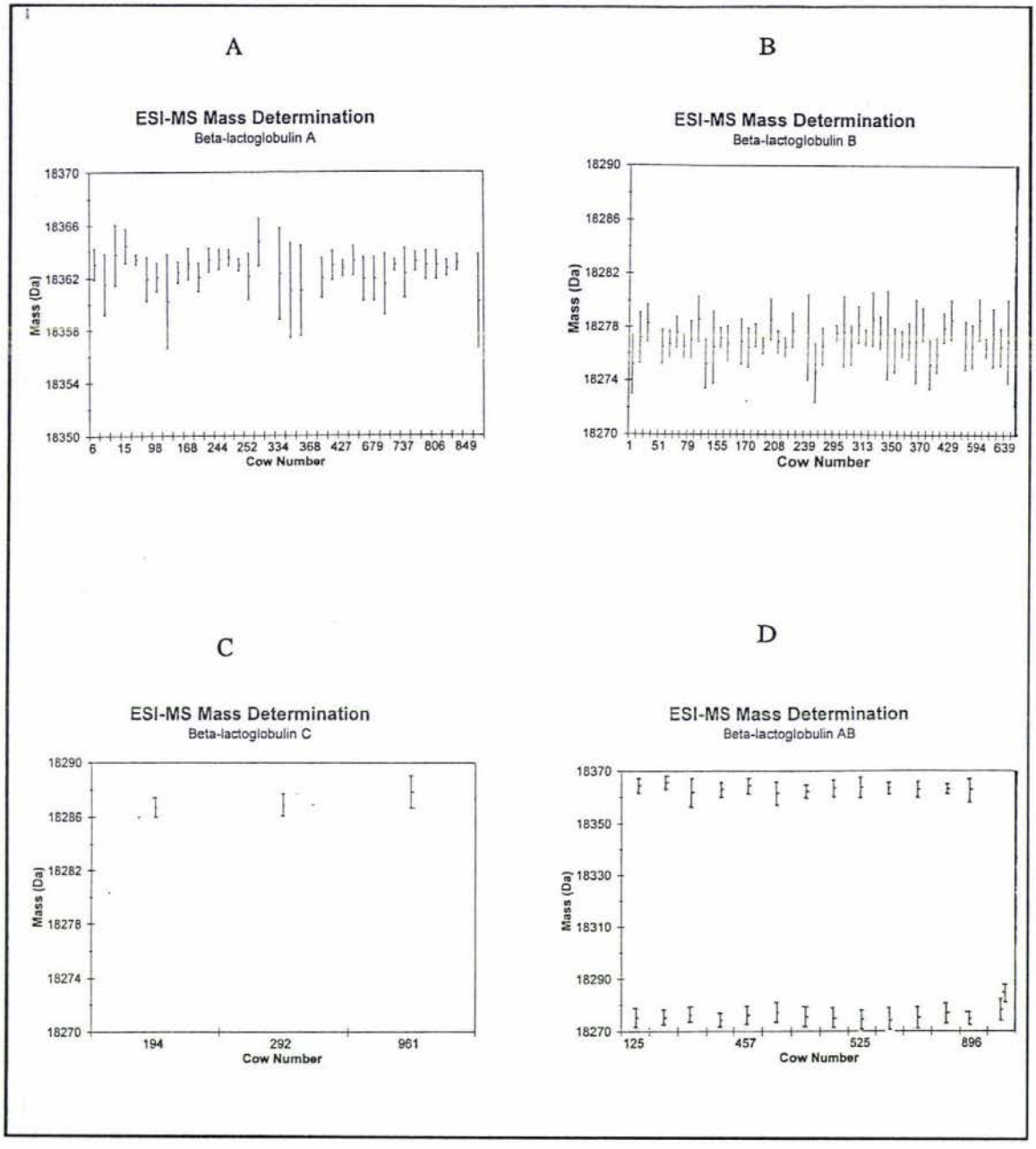


FIGURE 31 Individual mass determinations for each β-LG sample analysed. The individual error of each mass determination is indicated by an error bar. The figures A to D are β-LG A, β-LG B, β-LG C and mixed phenotype β-LGs respectively.

In all the samples analysed up to 4 other species were detected (Figure 32). In each case the lowest molecular weight species detected corresponded to the calculated mass of one of the β-LG variants A, B or C. The mass of the subsequent species were always of the form

$$\textit{Mass of species detected} = \textit{Mass of } \beta\textit{-LG variant} + n\ (324.4\ Da)$$

where ***n*** is an integer between 1 and 4.

The average increment to the β-LG variant was 324.4 ± 1.1 Da. This phenomenon was examined more extensively (see section 8).

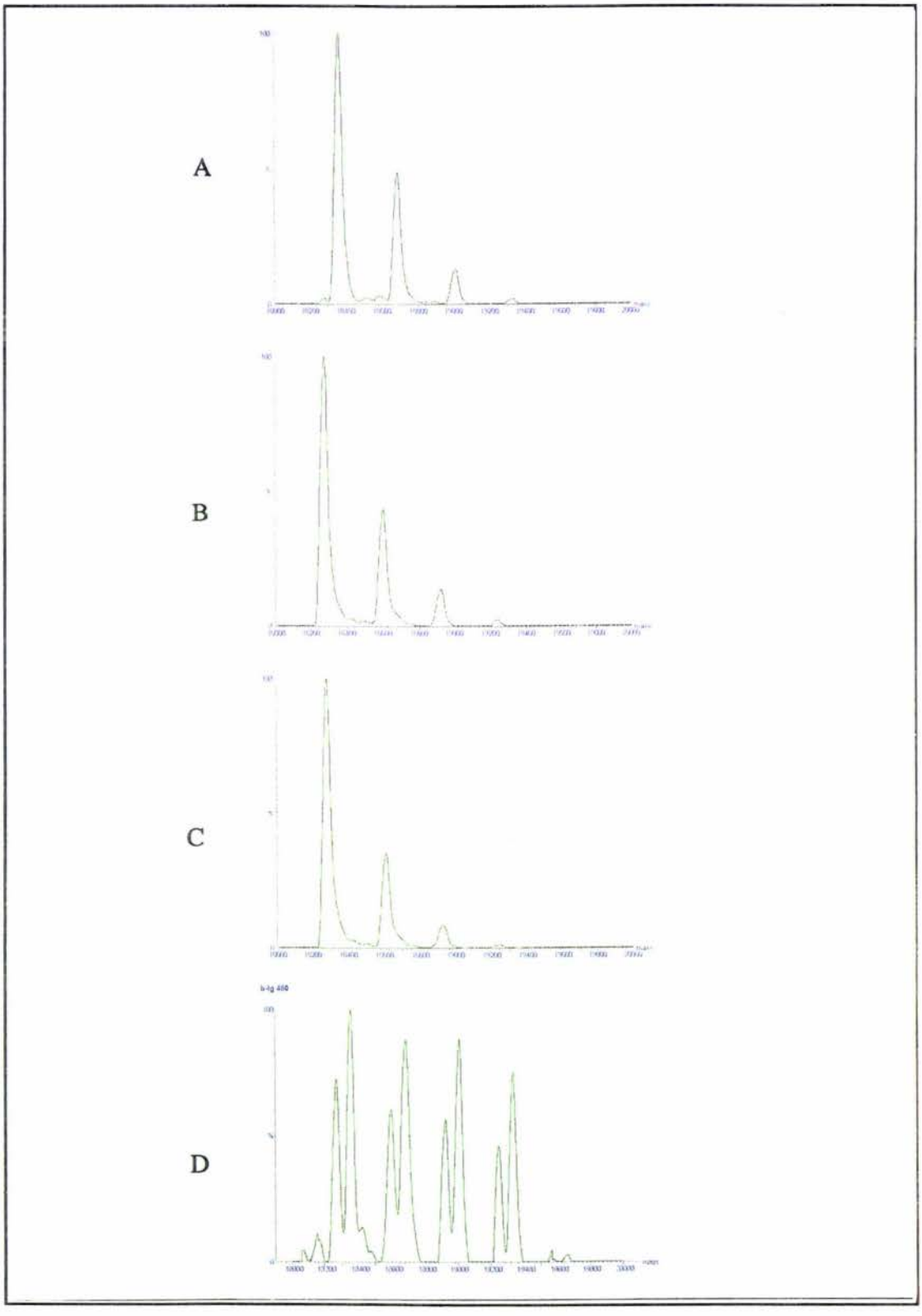


FIGURE 32 Multiple molecular weight species detected in β-LG samples. Figures A to D are samples of β-LG A, β-LG B, β-LG C and β-LG AB respectively.

7.4 Discussion

The determination of the mass of intact proteins to within ± 2-3 Da has been successfully applied to the identification of some common genetic variants of bovine milk proteins. This information has been used to confirm the primary sequence and post-translational modifications of these proteins by comparison of observed and calculated mass.

The group average masses for each protein variant agreed very closely with the expected mass. Individual average masses were calculated from up to 12 determinations of mass, based on the *m/z* ratios of adjacent pairs of ionised species. Although the average mass agreed closely with the expected mass, there was a much larger variation in individual determinations. This is graphically demonstrated in Figures 26 to 30. In these the average mass is plotted for each sample with an error bar indicating the variation in individual determinations. The various parameters used in deconvoluting the raw data which the user has control over, had significant effects on the average mass determined and the variation observed. Using the values suggested by the software manufacturer generally resulted in the lowest variation, although in some cases this could be marginally reduced by altering some of the processing variables.

The degree of accuracy in mass determination obtained during these experiments is consistent with that quoted by the manufacturer (0.01%) and other groups (Chianese *et al.*, 1995; Léonil *et al.*, 1995; Visser *et al.*, 1995). The major milk proteins vary in mass from approximately 19000 to 25000 Da. Given an error of ± 2 Da most single amino acid residue substitutions should be detectable. The exceptions may involve:

Pro (97.1 Da) and Val (99.1 Da);
Cys (103.1 Da) and Thr (101.1 Da);
Ile (113.2 Da), Leu (113.2 Da), Asn (114.1 Da) and Asp (115.1 Da);
Gln (128.1 Da), Lys (128.2 Da), Glu (129.1) and Met (131.2 Da).

The number of potentially undetectable substitutions (14) accounts for 7.3% of the 190 possible single substitutions amongst the 20 common amino acids. This does not include other modifications such as phosphorylation, amidation, oxidation and glycosylation. If multiple substitutions, additions and deletions are considered the number of potentially undetected alterations rises considerably. However the ratio of undetected to potential substitutions remains relatively constant.

Although ESI-MS may not detect all potential substitutions it is a major improvement on electrophoretic techniques. Assuming that only the substitutions that alter the net charge of a protein are detected, electrophoresis has the potential to detect 87 (45.8%) of the potential 190 single residue substitutions[5]. The substitution may be detected but little indication is given as to the nature of the substitution.

Not all substitutions involving a change in the net charge of a milk protein are easily detected by electrophoresis as is evidenced by the inability to differentiate β-LG B and C variants in the native PAGE system used earlier. In some cases this may be a result of the substitution site being buried within the tertiary structure of a protein. Conversely some 'silent' substitutions have been detected by ultra-narrow range IEF (Altland and Rossmann, 1985; Krause *et al.*, 1988). The mechanism whereby these substitutions are differentiated is largely unknown. It is possible that these silent substitutions are located in positions that influence the tertiary structure of the protein sufficiently to cause a significant change in the overall charge or isoelectric point.

Purification of the milk proteins was required prior to ESI-MS analysis due to the limitations of the equipment available. Sample solutions could only be introduced into the electrospray solvent line via a sample loop or included in the solvent solution. The complexity of the raw spectrum obtained from sample mixtures is proportional to the number of species present. This makes spectral peak assignment to an ionised species in an *m/z* series increasingly difficult, especially as series from different species often overlap. Consequently sample solutions were restricted to containing as few protein species as possible. To avoid having more than one protein species present in a

[5]The substitutions potentially detectable by electrophoresis are:
His, Lys and Arg may be substituted by 15 neutral residues plus Asp and Glu (17 x 3);
Asp and Glu may be substituted by 15 neutral residues plus His, Lys and Arg (18 x 2).

sample of purified protein, only casein samples homozygous for α_{s1}-CN, α_{s2}-CN and β-CN were selected for ion exchange purification. Thus only 22 casein samples were selected for purification of casein proteins.

Simultaneous analysis of more than one species in a sample solution is possible and was demonstrated in several β-LG samples that contained both the A and B protein variants. Given this it is likely that caseins from cows heterozygous for casein proteins could have been successfully analysed.

Léonil *et al.* (1995) took a different approach in protein purification prior to ESI-MS analysis. They were able to separate the major milk proteins in a skim milk sample on a RP-HPLC column, directing the eluent into an electrospray interface to a mass spectrometer. Simultaneous separation and mass analysis of all the major milk proteins was accomplished in less than 30 minutes. This compares favourably with traditional PAGE phenotyping. Although PAGE may be used for simultaneous analysis of a number of samples (up to 15 samples per mini-gel), three gel systems and their associated sample preparations are required to determine all known 'electrophoretic' phenotypes. The system used by Léonil *et al.* (1995) lends itself to routine automated determination of protein variants in a milk sample - including 'silent' variants and other post-translational modifications.

The species differing from the mass of β-LG variants A, B and C in multiples of approximately 324 Da detected by ESI-MS were not detected by either PAGE or CE. The nature of this adduct or modification was not immediately apparent. Elucidation of the nature of these species was the subject of further investigation (see section 8).

8 ANALYSIS OF B-LG ADDUCT SPECIES

While its biological role is unclear, β-LG is known to bind many non-polar ligands such as retinol and fatty acids (Hamblin *et al.*, 1992; Papiz *et al.*, 1986). It is thought that a possible function of β-LG could be to bind and protect small hydrophobic molecules during their passage through the stomach in order to deliver these ligands to specific receptors located in the intestine (Papiz *et al.*, 1986).

It was postulated that the additional species seen in the analysis of β-LG samples may have been due to non-covalent association of multiple small non-polar molecules in the retinol binding cleft of β-LG. However the observed mass additions of 324 Da does not match the calculated mass of any small molecule likely to be found in bovine milk. The categories of molecules searched included vitamins, fatty acids, hormones, sugars, mineral salts and small non-polar compounds.

A number of small molecules were re-examined with a view to potential covalent interactions that they could undergo with the protein. Of the small molecules likely to be found in milk only a covalent linkage of a lactosyl residue to the protein matched the observed change in mass of 324 Da within error.

The hypothesis that the observed adducts to β-LG were multiple lactosyl residues covalently bound to one or more amino acid residues was the subject of the following section of experiments.

A number of experiments were conducted to establish

- whether lactose was the adduct species
- the nature of the association between adduct species and β-LG
- identification and location of any modified amino acid residues in the primary sequence of β-LG
- whether adduct formation was the result of purification or analysis conditions, or whether the adduct was formed *in vivo*

8.1 Reducing sugar test

A simple colorimetric analysis to determine the presence of a reducing sugar in the prepared β-LG samples was performed.

8.1.1 Methods and materials

Beta-lactoglobulin A and B was prepared by salt precipitation from acid whey, as described earlier. A sample of β-LG A was further purified by ion-exchange chromatography. This involved dissolving the lyophilised protein in 50 mM sodium acetate buffer, pH 6.0 to 1 mg/ml^{-1}. Aliquots of 100 μl of protein were injected onto a MonoQ HR5/5 column (Pharmacia BioTech, Uppsala, Sweden) previously equilibrated in 50 mM sodium acetate buffer, pH 6.0. Protein was eluted with a linear gradient of 0 to 0.5 M NaCl over 15 minutes at a flow rate of 1 ml min^{-1}. Eluent with an absorbence at 280 nm of greater than 0.2 units was pooled from 20 consecutive chromatographic runs. This was dialysed in 10,000 MW cut off dialysis tubing (Union Carbide) against three changes of RO water prior to lyophilisation. A further sample of commercially prepared mixed β-LG A and B was purchased (Sigma Chemical Co., St. Louis, MO.).

The presence of reducing sugars was determined by a modification of the phenol-sulphuric acid method of McKelvy and Lee (1969). Protein solutions were prepared from samples of salt precipitate purified β-LG, β-LG purified by ion-exchange chromatography and commercial β-LG by dissolving 1 mg of protein in 1 ml of 1% sodium dodecyl sulphate (SDS) (BDH Chemicals, Poole, UK). 0.5 ml of protein solution was added to 0.3 ml of 10% aqueous phenol solution. 1.8 ml of concentrated sulphuric acid was added to the solution while vortexing. After cooling to room temperature the absorbence was read at 480 nm and compared to a standard curve prepared with lactose in 1% SDS.

8.1.2 Results

Phenol-sulphuric acid analysis of salt precipitation prepared β-LG samples demonstrated the presence of reducing sugar in the ratio of 2 to 3 moles of sugar to 1 mole of β-LG. Analysis of Sigma and ion-exchange purified β-LG revealed only trace quantities of sugar.

8.1.3 Discussion

The method for detection of reducing sugars by McKelvy and Lee (1969) is not particularly sensitive nor specific. Some difficulty was experienced in obtaining an acceptable standard curve as the absorbence reading was influenced by the reaction time and temperature at which the reading was taken. Consequently the ratio of sugar to β-LG is at best a crude estimate. On the basis of the molar quantities of sugar involved, lactose is indicated as the species involved. Although other sugars are found in milk, they are present in very low quantities.

8.2 Nature of adduct association

Samples of salt precipitate prepared β-LG, Sigma and ion-exchange prepared β-LG were subjected to heat denaturation under reducing conditions. Samples were analysed by RP-HPLC and ESI-MS after denaturation.

8.2.1 Materials and methods

Sample β-LG solutions (1 mg ml^{-1}) from salt precipitate prepared β-LG, Sigma and ion-exchange prepared β-LG were prepared in 4 M urea containing 50 mM dithiothreitol. The samples were heated at 100°C in a water bath for 10 minutes, followed by cooling to room temperature prior to 0.22 μm filtration.

Undenatured samples (1 mg ml^{-1}) were prepared in water.

Samples were analysed by RP-HPLC on a Shimadzu HPLC (Shimadzu Corp, Osaka, Japan). Sample injections of 50 μl were injected onto a Vydac C4 4.6 x 150 mm column (Separation Group, Vesperia, CA.) at 60°C. Protein was eluted in a gradient of 0 to 60% solvent B (60% acetonitrile, 0.05% trifluoroacetic acid) over 60 minutes at a flow rate of 1 ml min^{-1}. Eluent was monitored at 214 nm. Eluted peaks were collected and dried prior to ESI-MS analysis.

Collected samples were dissolved in 50% acetonitrile:water to approximately 1 mg ml^{-1} with the addition of 0.5% formic acid. Samples were subsequently diluted 1000 fold in 50% acetonitrile prior to injection onto the mass spectrometer.

The mass spectrometer and conditions used were identical to those used for earlier analyses. Reference solutions of horse heart myoglobin and cytochrome C were used to calibrate the equipment prior to sample analysis.

8.2.2 Results

RP-HPLC (Figure 33) was able to confirm the purity of monovariant samples with only a single peak being observed in all chromatograms. Sigma β-LG exhibited two peaks consistent with β-LG variants A and B. No differences were observed between chromatograms of native β-LG and β-LG samples that had undergone heat denaturation.

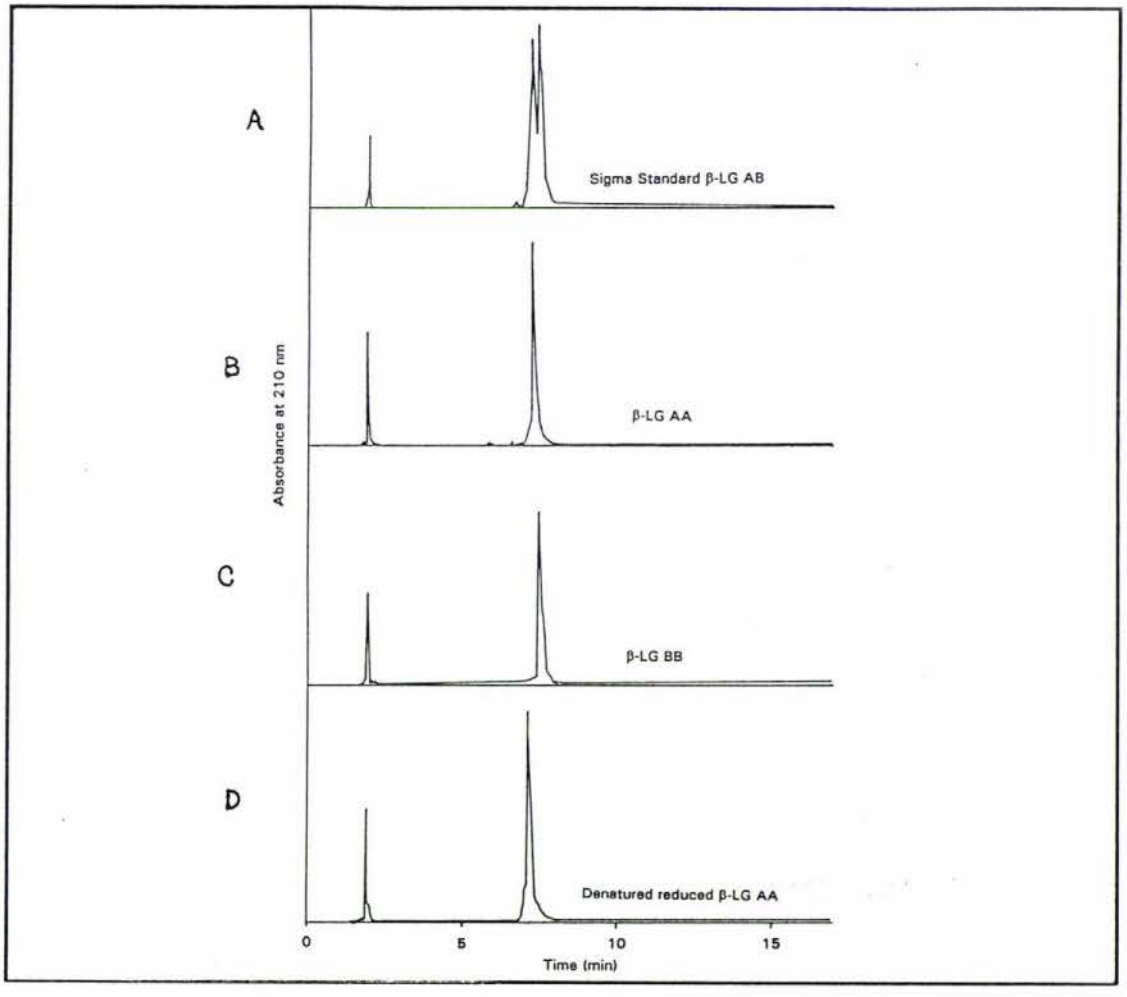


FIGURE 33 RP-HPLC chromatograms of (a) Sigma β-LG, β-LG purified from acid whey from a β-LG AA phenotype cow (b), a β-LG BB phenotype cow (c), and heat denatured and DTT reduced β-LG from an AA phenotype cow.

Figure 34 shows the transformed ESI-MS spectra of a number of native β-LG samples. The multiple molecular weight β-LG species are clearly visible. As in previous analyses the lowest molecular weight species corresponded to the calculated molecular weight of one of the β-LG variants A, B or C within error. Up to four additional species of higher molecular weight, differing by multiples of 324 Da, were present. The samples of Sigma and ion-exchange purified β-LG showed a single additional species of each of the β-LG variants present. Integration of the peak areas in transformed spectra revealed that in the β-LG samples prepared by salt precipitation, adduct species accounted for between 31.9 and 88.7% of the total peak area. In Sigma and ion-exchange prepared β-LG samples adduct peaks accounted for 7.5 and 9.0% of total peak areas respectively.

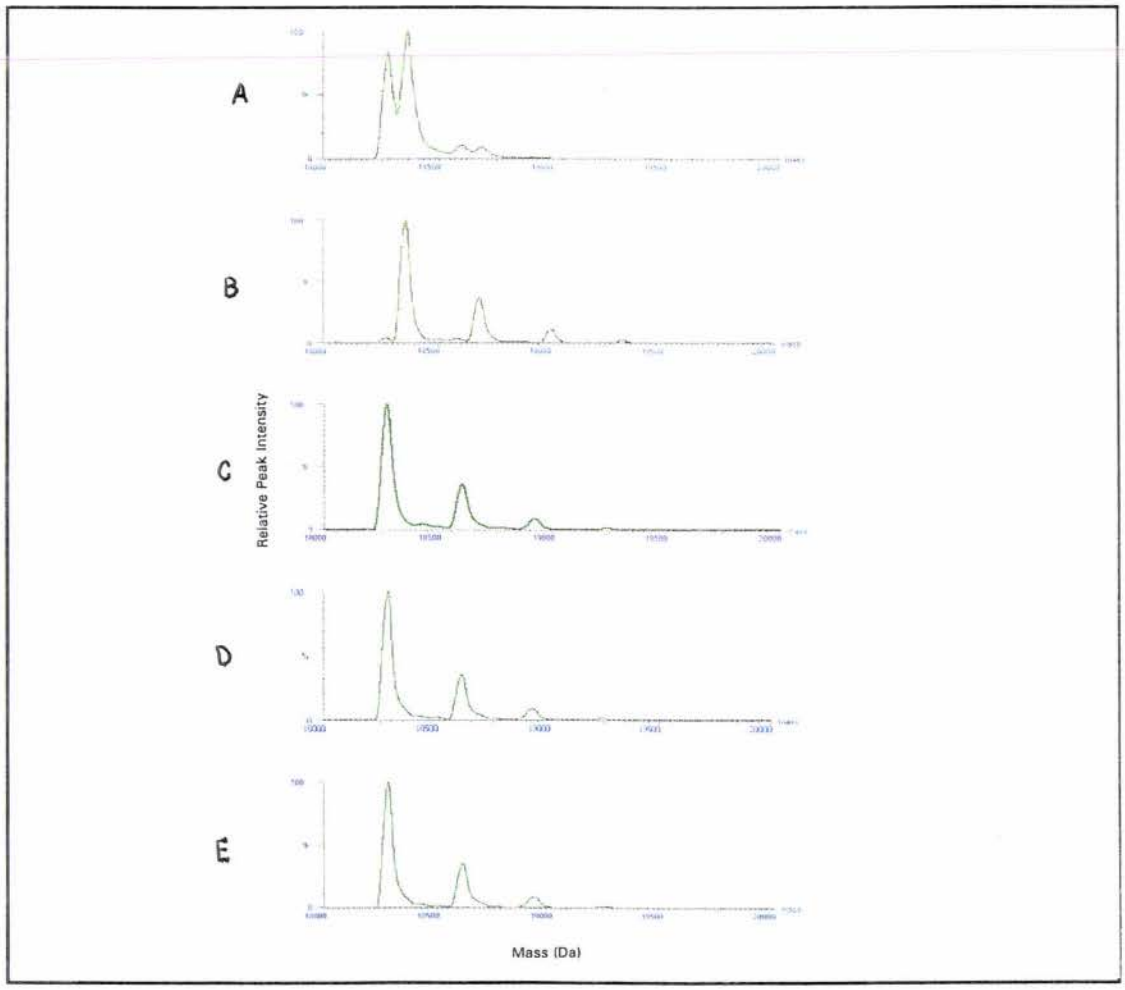


FIGURE 34 Transformed ESI-MS spectra of (a) Sigma purified β-LG, β-LG purified from acid whey from a β-LG AA phenotype cow (b), a β-LG BB phenotype cow (c), a β-LG CC phenotype cow (d), and heat-denatured, reduced β-LG from an AA phenotype cow.

Heat denaturation, reduction and RP-HPLC, followed by ESI-MS of β-LG samples was found to increase the observed mass of β-LG by an average of 4 Da. This is consistent with a reduction of two disulphide bonds in the protein. There was no effect on the number or proportion of +324 Da molecular weight derivatives.

8.2.3 Discussion

Milk is often heat treated to effect pasteurisation or sterilisation. Besides these desirable effects heating also results in undesirable effects such as browning, off flavour development and loss of nutritional value. In heated milks and milk products the Maillard reaction is frequently responsible for the browning seen.

The Maillard reaction is characterised by the condensation of the carbonyl group of lactose with particularly the ϵ-amino group of lysine residues of milk proteins. A Schiff's base is formed first and subsequently the Amadori rearrangement leads to the formation of the protein-bound Amadori product lactulosyllysine (Figure 35).

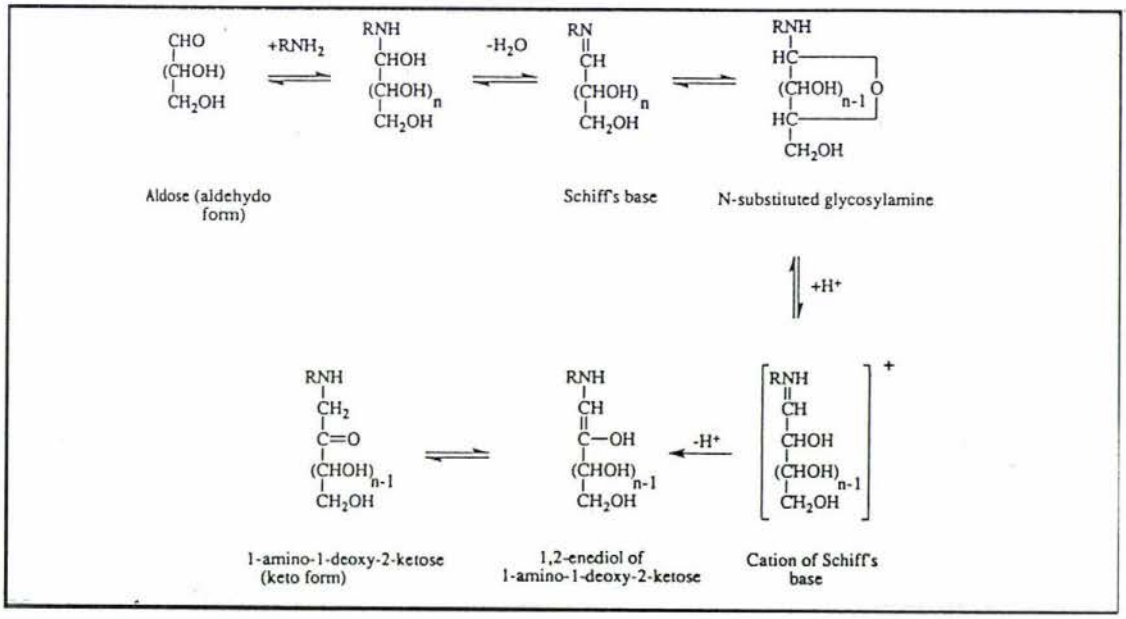


FIGURE 35 Initial steps of the Maillard reaction. The first step involves the nucleophilic attack by the nitrogen atom of an amino compound (lysine) on the electrophilic carbonyl group of an aldehyde or ketone (such as lactose). A water molecule is eliminated in the formation of a Schiff's base, which re-arranges to form the lactosylamine intermediate. The unstable lactosylamine reacts, via the spontaneous Amadori rearrangement, to form the keto derivative (1-amino-1-deoxy-2-ketose). (Adapted from Berg and van Boekel, 1994).

The Maillard reaction is known to occur predominantly in the casein proteins, particularly kappa casein (Berg and van Boekel, 1994), but is also known in the whey proteins (Pelligrino *et al.*, 1994).

The observed adducts may be intermediate products of the Maillard reaction. The mass shift of 324 Da is consistent with a lactosyl residue covalently bound to β-LG. The existence of the adducts after heat denaturation and reduction suggests that the association is covalent rather than electrostatic or hydrophobic. Given the concentration of organic solvent used in ES-MS and the ionisation voltages, it seems very unlikely that associations other than covalent would survive the process of sample ionisation.

As β-LG contains 15 Lys residues, and only two to four adducts are seen in the samples this suggests that the process of multiple glycation[6] targets specific Lys residues. Henle and Klostermeyer (1993) reported that lysine residues which were highly reactive to reducing sugars in β-casein A^1 variant were located directly adjacent to the charged side chains of glutamic acid and lysine, respectively. In β-LG, four such sites occur - Lys69-Lys70, Glu74-Lys75, Lys100-Lys101 and Glu134-Lys135. This suggests that the microenvironment may influence the reactivity of lysine residues. Henle and Klostermeyer further suggested that the charged side chains of lysine and glutamic acid residues may be involved in the intramolecular catalysis of Amadori rearrangement products. It is possible that adjacent lysine or glutamic acid residues participate in modulating the supply of a proton between the N-substituted glycosylamine and the cation of Schiff's base.

Confirmation of the particular lysine residues involved will be the subject of further experimentation. It is expected that tryptic hydrolysis of β-LG, followed by ESI-MS characterisation of the resultant peptides will enable the location of residues that have been modified in the process of glycation.

It is of interest to note that the samples of β-CN previously analysed (Section 7.3.3,

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[6]In this discussion glycation denotes the process of non enzymatic covalent bonding of sugar groups to reactive residues. This is contrasted with the enzyme catalysed process of glycosylation.

page 97) exhibited no signs of multiple glycation. Nor did any other purified milk protein analysed by ESI-MS. This suggests that β-LG is much more susceptable to Maillard reactions under the conditions used during purification and mass determination.

Maillard reactions are normally associated with alkaline conditions and elevated temperatures (Hurrell and Carpenter, 1974; O'Brian, 1995; Berg and van Boekel, 1994; Mauron, 1981). The relatively low temperatures and acidic conditions used during purification and ESI-MS analysis in this current study are not thought to be conducive to Maillard product formation.

During the development of this series of experiments the characterisation of a lactosyl-β-LG conjugate occurring during the heating of whey by ESI-MS was reported. Maubois *et al.* (1995) reported that even very mild heat treatment (63°C, 20 sec) applied to milk prior to whey separation resulted in modification of the molecular weight of native β-LG by 324 Da. At more severe heat treatments (70°C, 60 minutes) up to 35% of β-LG appeared to be of the modified form. Although not discussed in their report, similar adducts were seen in the ESI-MS spectrum of a bovine β-LG sample analysed by Hutton *et al.* (1995).

The much lower levels of glycated species observed in Sigma and ion-exchange purified β-LG suggest that the degree of modification is influenced by the purification process. Alternatively the glycated species are removed at some stage of preparing the Sigma and ion-exchange purified samples. The observation of a lactosyl-β-LG conjugate in whey prepared from very mildly heated milk (Maubois *et al.*, 1995) suggests the possibility that glycation may occur *in vivo* at physiological temperatures.

8.3 Preparation of native whey proteins

To examine the effects of acidification, salt precipitation and lyophilisation on the degree of glycation, a 'gentle' method of protein preparation was required to provide unmodified 'native' β-LG. Ultra-centrifugation of whole milk was used to isolate native whey from sedimented casein micelles and supernatant fats. Native whey proteins were variously dried in the presence of lactose, dialysed and dried, and salt

precipitated and dried. The resultant products were analysed for adduct species by ESI-MS.

8.3.1 Methods and materials

Fresh milks were obtained from 6 cows homozygous for the β-LG B variant protein. Samples were kept at 30 to 35°C throughout. Approximately 5 ml of whole milk was centrifuged in 13 x 51 mm Beckman Quick-Seal centrifuge tubes at 60,000 x g for 60 minutes in a Beckman L8-80M ultra-centrifuge (Beckman Instruments Inc., Palo Alto, CA.). A portion of the translucent whey fraction was carefully aspirated from each sample. An aliquot of each sample was held at -20°C until ESI-MS analysis. A further aliquot was lyophilised. A sample of each whey was extensively dialysed in 10,000 MW cut off dialysis tubing (Union Carbide) and then freeze dried. Beta-lactoglobulin was prepared from the remaining portion of native whey by addition of NaCl to 7% and acidification to pH 2.0 with 1M HCl. After standing for 1 hour at room temperature samples were centrifuged at 5,000 x g in a benchtop centrifuge for 15 minutes. The β-LG containing supernatant was decanted into 10,000 MW cut off dialysis tubing, dialysed against purified water and lyophilised.

The variously prepared samples of whey proteins were dissolved in, or diluted with 50% acetonitrile to approximately 1 μg ml^{-1} and 0.22 μm filtered prior to ESI-MS. Conditions used for ESI-MS were identical to those used in previous analyses.

8.3.2 Results

ESI-MS analysis of liquid native whey prepared by ultracentrifugation resulted in a poor signal to noise ratio in the raw spectrum. Despite this a signal was able to be extracted. Deconvolution of the signal revealed that β-LG, and a small quantity of glycated β-LG with one and two lactulosyllysine residues was present. In the un-dialysed, lyophilised sample no signal could be reasonably extracted from the background noise. The dialysed and lyophilised sample provided a clearly distinguishable signal with very low background noise. Deconvolution and transformation show that the major component is unmodified β-LG B (Figure 36). Two minor components corresponding to β-LG B plus 1 and 2 lactulosyl adducts are

also present. The proportions of glycated β-LG appear to be similar between the liquid sample and the dialysed, lyophilised sample.

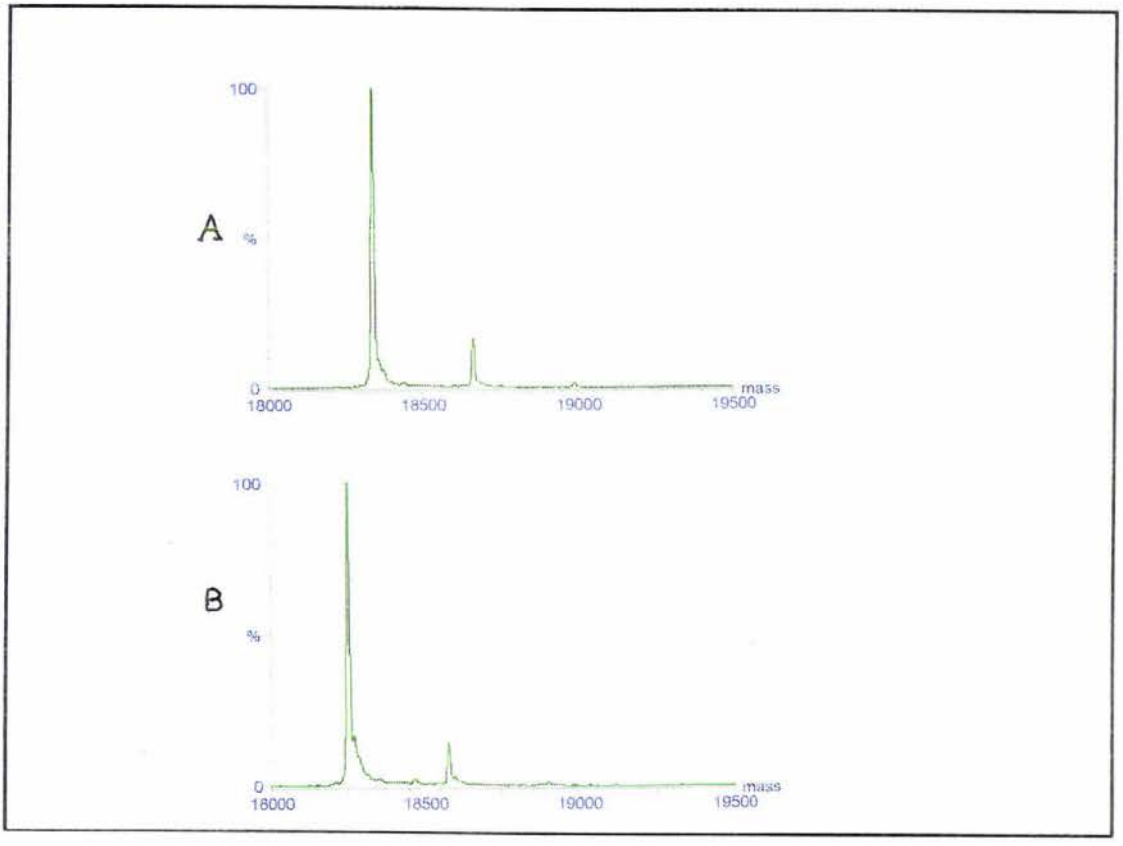

FIGURE 36 Transformed ESI-MS spectra of β-LG in whey prepared by ultracentrifugation of the milk from a β-LG AA phenotype (a) and a β-LG BB phenotype cow (b).

8.3.3 Discussion

The quantity of salts present in whey is likely to severely suppress ionisation of the protein species present. A high proportion of sodiated, potassiated and calciated ionised species (MNa^+, MK^+ and MCa^{2+}) is also expected. This was the case with the aliquot of whey that had been freeze dried. Dialysis of the whey sample prior to lyophilisation reduced the salt concentration to a level sufficiently low, such that only the MH^+ ionised species series is seen.

Although the signal in samples containing salts was difficult or impossible to extract, the results indicate that the ratio of glycated β-LG to unmodified β-LG remains

relatively constant. This observation suggests that the process of dialysis or lyophilisation does not impact on the process of glycation. Why samples that had been prepared by salt precipitation from acid whey appeared to have a higher proportion of glycated species is still unknown. These samples were held at 40°C for up to 30 minutes prior to cream separation. Evidence from Maubois *et al.* (1995) indicates that heat is a factor in the formation of glycated species.

All samples, with the exception of the unresolved freeze dried sample, clearly showed glycated species present in the whey of ultracentrifuged milk. This process did not expose the samples to any extreme changes in heat, pH or shear stress. Thus it is likely that glycation occurs in the udder under physiological conditions.

The elucidation of the process of formation of glycated β-LG species will be the focus of ongoing research.

9 DISCUSSION AND CONCLUSION

Ng-Kwai-Hang and Grosclaude (1992) theorised that mutations in proteins due to amino acid substitutions not leading to a change in the net charge of the protein should occur three times more frequently than those resulting in an alteration of net charge. A study of milk proteins purified from 281 Holstein and Ayrshire cows in Canada identified a number of potential silent variants by RP-HPLC tryptic peptide mapping (Dong Chin, 1992). She found that 24 of 264 samples of α_{s1}-CN (9.1%), 9 of 57 samples of β-CN A^1A^1 (15.8%), 5 of 55 samples of β-CN A^2A^2 (9.1%) and 8 of 188 samples of κ-CN AA (4.3%) were potential candidates for silent genetic variants. In this study population no proposed silent mutations were observed by RP-HPLC peptide mapping and ESI-MS of 109 samples of β-LG, 22 samples of α_{s1}-CN, 22 samples of α_{s2}-CN, 22 samples of β-CN, and 9 samples of κ-CN.

Of the 190 possible single amino acid substitutions, only eight result in a mass shift of less than 5 Da and do not involve a change in net charge. Given the mass resolution obtained in these experiments, these are the substitutions which may not be detected by either ESI-MS or electrophoretic methods. It is expected that all other substitutions would be detected by one or more of the techniques used in this work. The lack of diversity in the mass of milk proteins outside of the known variants indicates only the known electrophoretic variants are present in the study population. If this is so, it raises the question as to why the majority of milk protein variants involve substitutions resulting in a change of net charge. Possible explanations proposed include -

the number of mutations involving neutral residues is lower than those involving charged residues,

a higher proportions of mutations involving neutral residues may be lethal,

the substitutions involved in the known 'electrophoretic' protein variants are associated with desirable traits. The process of intensive selection pressure and the extensive use of artificial insemination from selected sires in the study population has excluded many variants that may exist in the gene pool of the

larger national and international dairy population.

It is assumed that the proposal that silent mutations should occur three times more frequently than electrophoretic mutations is based on the ratio of neutral to charged residues (15 neutral : 5 charged = 3:1). At the DNA level not all point mutations in the codon result in an alteration of amino acid (Table 8). For example there are six times as many codons specifying an arginine, serine or leucine residue as there are for a methionine residue.

Residue	No. of codons	Residue	No. of codons
Gly	4	Glu	2
Asp	2	Val	4
Ala	4	Arg	6
Ser	6	Lys	2
Asn	2	Met	1
Ile	3	Thr	4
Trp	1	Cys	2
Tyr	2	Leu	6
Phe	2	Pro	4
Gln	2	His	2

TABLE 8 Number of codons specifying a particular amino acid

Recalculating the ratio of neutral to charged residues based on random point mutations of nucleotides results in a slightly higher proportion of charged residues (3:1.17 - 46 codons specifying neutral residues, 18 codons specifying charged residues, 3 codons specify an end signal).

Ignoring deletions, analysis of the substitutions occurring in the known variants of *Bos taurus* milk proteins reveals that 16 mutations involve substitution of neutral to charged residues, or positively charged and negatively charged residues. Only four mutations involve substitutions of neutral residues. An interspecies comparison of mutations occurring in casein proteins between bovine, ovine and caprine species shows charged to neutral residue substitutions account for 35.1% of changes in

primary sequences. Silent substitutions (neutral to neutral residue substitutions) account for 60.6%, and charged to similar charged residue substitutions account for the remaining 4.3%. The larger proportion of silent substitutions evident in a cross-species comparison suggests that it is possible that a larger number of silent substitutions may exist within a species but to date have gone undetected.

Alternatively silent substitutions may be associated with less desirable traits and have been selectively bred out of the study population. In an attempt to maximise the genetic potential of dairy herds, the New Zealand dairy industry has made extensive use of artificial insemination to a restricted number of superior sires for a number of years. It could be argued that the gene pool within the New Zealand dairy population is weighted towards the genes of a small number of high performing dams and sires.

Mutations involving hydrophobic residues may have adverse effects on casein micelle stability. A large number of apolar residues occur in the primary sequences of α_{s1}-, β- and κ-CN. In general these hydrophobic residues are somewhat clustered. Hydrophobic bonding contributes significantly to the stability of the casein micelle. Substitution of key hydrophobic residues with more polar residues may reduce hydrophobic bonding to such an extent as to disrupt micellar structure.

Intra- and intermolecular ionic bonds between charged residues also contribute to casein micelle stability. The total number of charged groups of the casein monomer indicate that in the formation of a casein micelle, not all of the ionic groups can occupy a surface position (Farrell, 1988). Either a large amount of energy is used to bury these groups or the structure is porous and accessible to water. Current evidence suggest the latter (Ribadeau-Dumas and Garnier, 1970). Sufficient alteration of the solvation of the micelle is known to destabilise milk.

Similar comments may be made for residues involved in disulphide bonds, and binding of inorganic phosphate and calcium. Substitutions of residues that are involved in key stabilising interactions may be more likely to result in lethal conditions where the milk becomes either unpalatable or unable to be expressed from the udder. Thus it is likely that the range of substitutions in milk proteins is limited by their effect on the functionality of the protein.

The proposed glycation of β-LG may have several functional implications. MacRichie (1978) observed that glycosylation of native β-LG reduced surface properties in the isoelectric pH range. Thus glycosylated proteins might be less strongly attracted to the hydrophobic air-water interface and less easily engaged in the hydrophobic interactions critical in the formation of cohesive films. Covalent bonding of gluconic or melibionic acids to amino groups of β-LG by Kitabake *et al.* (1985) increased the heat stability and solubility of the synthetic glycoprotein compared to native β-LG. Glycosylation of β-LG with maltose or glucosamine increased the viscosity over native β-LG in proportion to the degree of substitution (Waniska and Kinsella, 1984).

Further investigation into the process of glycation of β-LG may provide a measure of control over the degree of modification, or a means of isolating modified from unmodified species. In this way we may be able to alter the functional behaviour of this milk protein to meet technological requirements in a consumer acceptable way.

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11 APPENDIX

11.1 Appendix A - Liquid chromatography time and parameter programmes for RP-HPLC separation of milk protein tryptic hydrolyses on Shimadzu system.

LC Time Program
File 2 (B.Ge)

Time	Func	Value
0.01	T.Flow	1
0.01	Wave	214
0.01	B.Conc	0
15	B.Conc	15
25	B.Conc	50
40	B.Conc	60
50	B.Conc	100
53	B.Conc	100
53.5	B.Conc	0
60	B.Conc	0
60	Stop	

LC Parameter
File 2 (B.Ge)

T.Flow	1
B.Conc	0
B.Curve	0
C.Flow	0
P.Max	240
P.Min	0
Oven.T	60
T.Max	65
Wave	214
SV	
RV.A	0
RV.B	0
RV.C	1
RV.D	1
Event	

11.2 Appendix B - FAB-MS spectra of α_{S2}-CN A tryptic peptides.

Peptide	Calculated mass	
VIPY	492	
AMKPW	633	
NAVPI TPTLN RK	1197	
TVDME XTEVF TK	1467	(X denotes a phosphoserine residue)
Glycerol (matrix)	369	

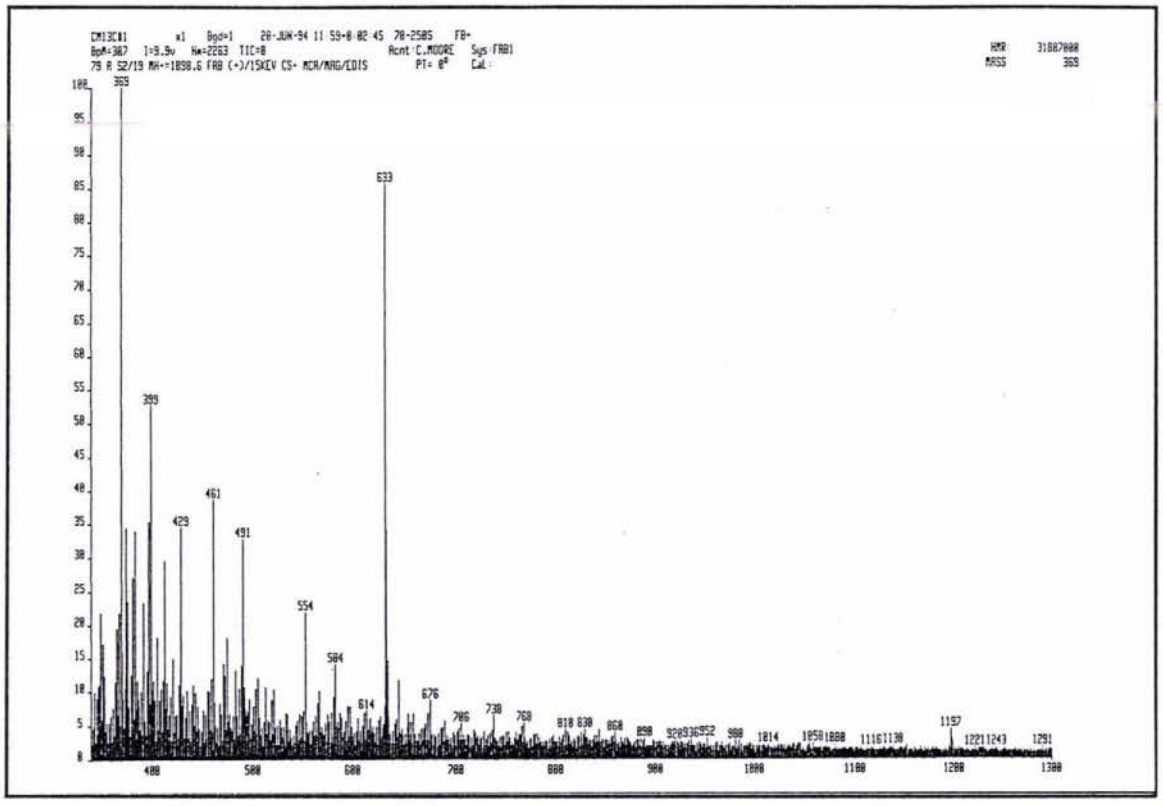


FIGURE A FAB-MS analysis of α_{S2}-CN A tryptic hydrolysis RP-HPLC peak eluting at 18.1 min.

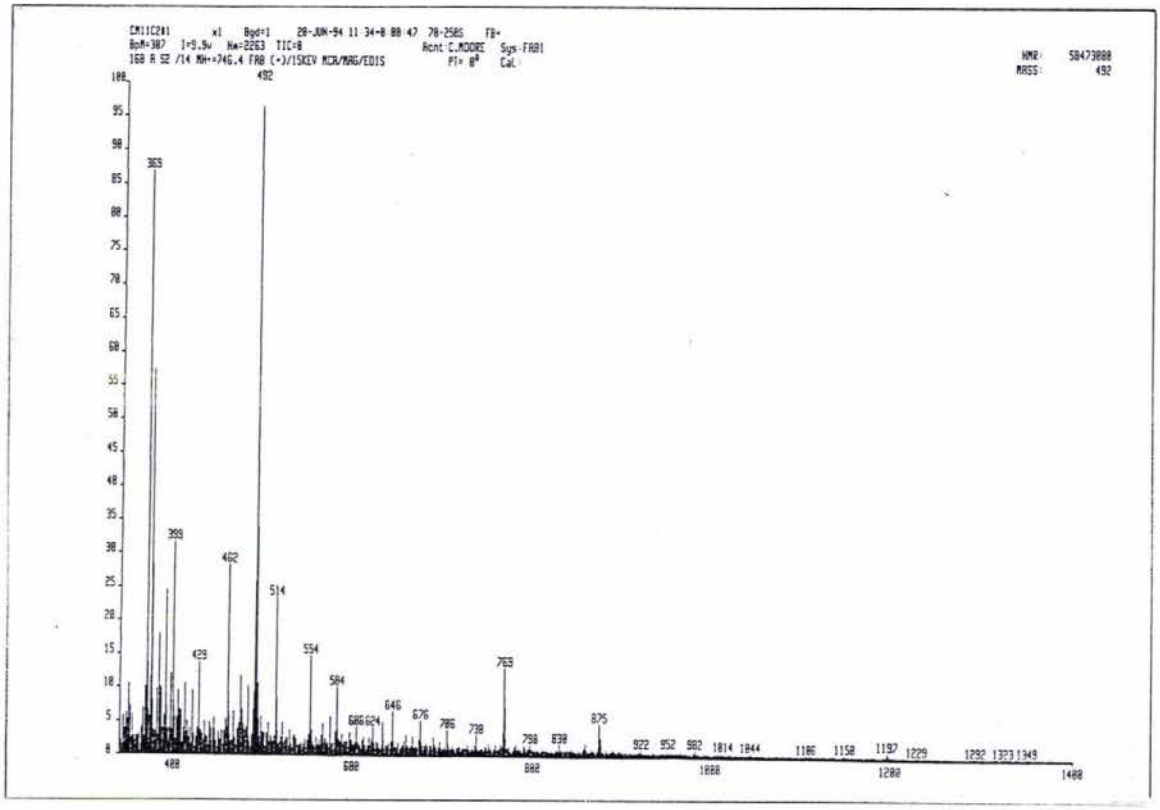


FIGURE B FAB-MS analysis of α_{S2}-CN A tryptic hydrolysis RP-HPLC peak eluting at 18.4 min.

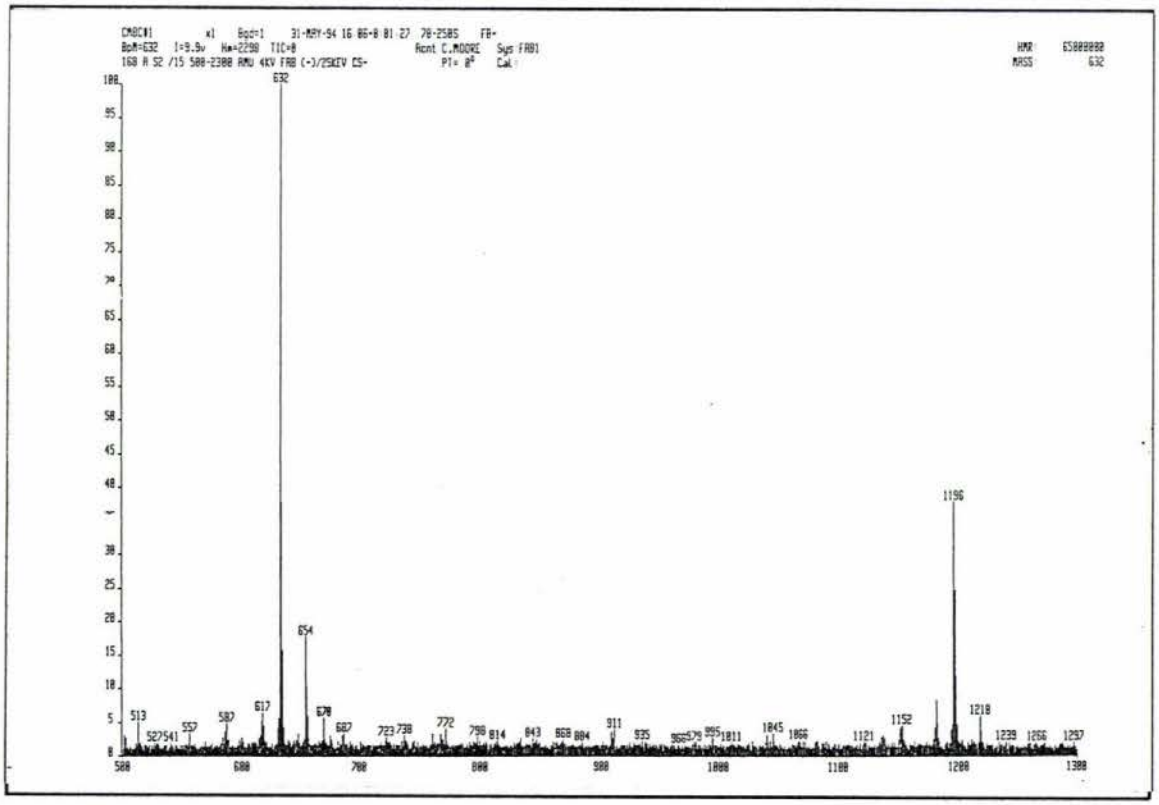


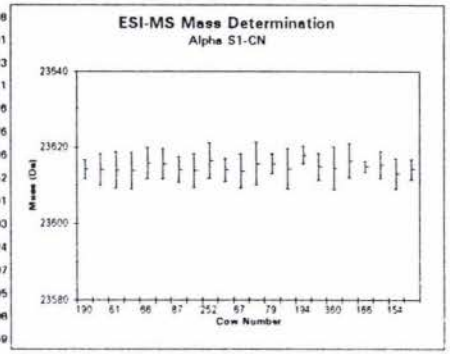
FIGURE C FAB-MS analysis of α_{S2}-CN A tryptic hydrolysis RP-HPLC peak eluting at 18.7 min.

11.3 Appendix C - ESI-MS data spreadsheets and plots of observed masses for milk proteins analysed

Casein ESI-MS Data Sheet

alpha S1

Cow	Mass	Error	High	Low
190	23614.24	2.56	23616.8	23611.68
292	23614.12	4.21	23618.33	23609.91
61	23614	4.77	23618.77	23609.23
19	23613.87	4.77	23618.64	23609.1
66	23615.88	4.12	23620	23611.76
5	23615.7	3.94	23619.64	23611.76
87	23614.17	3.31	23617.48	23610.86
51	23613.93	4.41	23618.34	23609.52
252	23616.52	4.61	23621.13	23611.91
370	23614.05	3.02	23617.07	23611.03
67	23613.71	4.47	23618.18	23609.24
168	23615.68	5.61	23621.29	23610.07
79	23615.67	2.62	23618.29	23613.05
162	23614.38	5.3	23619.68	23609.08
194	23617.93	2.34	23620.27	23615.59
347	23614.89	3.47	23618.36	23611.42
360	23614.51	5.66	23620.17	23608.85
160	23616.55	4.57	23621.12	23611.98
166	23614.88	1.42	23616.3	23613.46
209	23615.43	3.47	23618.9	23611.96
154	23613.01	3.93	23616.94	23609.08
125	23614.23	2.67	23616.9	23611.56

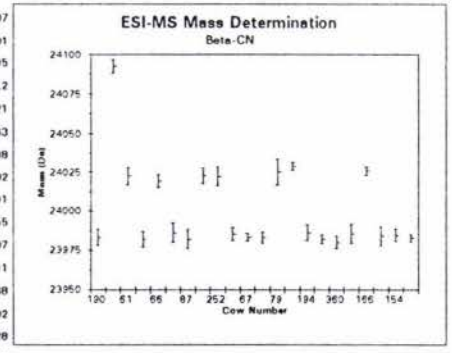


Mass stat aS1-CN B

Mean	23614.87955
Standard Error	0.248687009
Median	23614.445
Mode	NA
Standard Deviation	1.166445465
Variance	1.360595022
Kurtosis	0.696784857
Skewness	0.904724436
Range	4.92
Minimum	23613.01
Maximum	23617.93
Sum	519527.35
Count	22
Confidence Level(0.950000)	0.487417582

b-CN

Cow	Mass	Error	High	Low
360	23979.96	3.89	23983.85	23976.07
19	23981.89	4.88	23986.77	23977.01
87	23981.98	6.03	23988.01	23975.95
347	23981.98	2.78	23984.76	23979.2
125	23982.62	2.41	23985.03	23980.21
168	23983.06	3.43	23986.49	23979.63
67	23983.25	2.37	23985.62	23980.88
190	23983.35	5.33	23988.68	23978.02
209	23983.94	6.03	23989.97	23977.91
154	23984.58	4.03	23988.61	23980.55
370	23985.24	4.17	23989.41	23981.07
160	23985.45	6.04	23991.49	23979.41
5	23986	6.32	23992.32	23979.68
194	23986.16	5.14	23991.3	23981.02
66	24019.5	4.22	24023.72	24015.28
252	24022.34	6.09	24028.43	24016.25
51	24022.55	4.95	24027.5	24017.6
61	24022.59	5.5	24028.09	24017.09
79	24025.3	8.4	24033.7	24016.9
166	24025.89	2.54	24028.43	24023.35
162	24028.96	2.62	24031.58	24026.34
292	24092.57	4.08	24096.65	24088.49

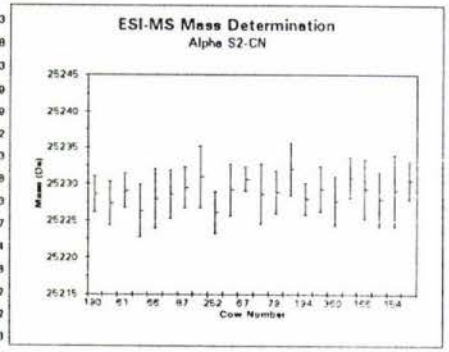


Mass stat b-CN A2

Mean	23983.53286
Standard Error	0.482066159
Median	23983.3
Mode	23981.98
Standard Deviation	1.803801237
Variance	3.253698901
Kurtosis	-0.482395994
Skewness	-0.184110477
Range	6.2
Minimum	23979.96
Maximum	23986.16
Sum	335769.46
Count	14
Confidence Level(0.950000)	0.944871512

Alpha S2-CN

Cow	Mass	Error	High	Low
190	25228.61	2.48	25231.09	25226.13
292	25227.36	2.98	25230.34	25224.38
61	25229.1	2.37	25231.47	25226.73
19	25226.35	3.56	25229.91	25222.79
66	25228.01	4.02	25232.03	25223.99
5	25228.61	3.29	25231.9	25225.32
87	25229.51	2.78	25232.29	25226.73
51	25231.03	4.23	25235.26	25226.8
252	25226.12	2.89	25229.01	25223.23
370	25229.21	3.54	25232.75	25225.67
67	25230.66	1.62	25232.28	25229.04
168	25228.65	4.07	25232.72	25224.58
79	25228.91	2.89	25231.8	25226.02
162	25232.04	3.62	25235.66	25228.42
194	25227.98	2.15	25230.13	25225.83
347	25229.32	3.05	25232.37	25226.27
360	25227.65	3.33	25230.98	25224.32
160	25230.87	2.77	25233.64	25228.1
166	25229.32	4.05	25233.37	25225.27
209	25227.89	3.64	25231.53	25224.25
154	25229.13	4.87	25234	25224.26
125	25230.45	2.57	25233.02	25227.88

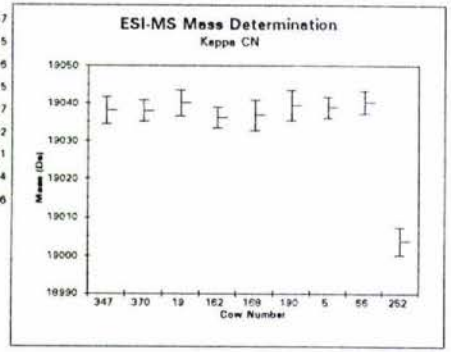


Mass Stat aS2-CN A

Mean	25228.94455
Standard Error	0.314519304
Median	25229.005
Mode	25228.61
Standard Deviation	1.475226302
Variance	2.175292641
Kurtosis	-0.008871226
Skewness	0.089887859
Range	5.92
Minimum	25226.12
Maximum	25232.04
Sum	555036.78
Count	22
Confidence Level(0.950000)	0.616446511

Kappa CN

Cow	Mass	Error	High	Low
347	19038.12	3.55	19041.67	19034.57
125	19037.99	2.84	19040.83	19035.15
19	19040.11	3.51	19043.62	19036.6
162	19036.23	2.78	19039.01	19033.45
168	19036.88	4.01	19040.89	19032.87
190	19039.44	4.12	19043.56	19035.32
5	19038.88	2.77	19041.65	19036.11
66	19040.29	3.05	19043.34	19037.24
292	19003.82	3.56	19007.38	19000.26



Mass stat k-CN A

Mean	19038.4925
Standard Error	0.517644907
Median	19038.5
Mode	NA
Standard Deviation	1.464120897
Variance	2.14365
Kurtosis	-1.082878571
Skewness	0.303713766
Range	4.06
Minimum	19036.23
Maximum	19040.29
Sum	152307.94
Count	8
Confidence Level(0.950000)	1.014565379

b-LG ESI-MS Data Sheet

b-LG BB

	Mass				Mass +1				Mass +2				Mass +3				Mass +4			
Cow no.	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo
1	18275.2	2.19	18277.39	18273.01	18599.57	3.1	18602.67	18596.47	18925.52	1.96	18927.48	18923.56	19250.45	0.86	19251.32	19249.6	19574.65	1.22	19575.87	19573.43
5	18277.2	1.9	18279.1	18275.3	18601.53	0.8	18602.43	18600.83	18927.4	2.8	18930.2	18924.6	19250.73	2	19252.73	19248.73	19572.71	2.2	19574.91	19570.51
17	18278.29	1.4	18279.69	18276.89	18603.34	0.5	18603.84	18602.84	18927.47	3.3	18930.77	18924.17								
19					18599	3.8	18602.8	18595.2	18926.14	0.6	18926.74	18925.54	19249.79	1.1	19250.89	19248.69	19575.84	3.4	19579.24	19572.44
51	18276.53	1.3	18277.83	18275.23	18602.22	2.56	18604.78	18599.66	18926.95	0.82	18927.77	18926.13	19251.79	0.39	19252.18	19251.4	19576.4	1.23	19577.63	19575.17
61	18276.73	1.01	18277.74	18275.72	18601.45	3.23	18604.68	18598.22	18926.14	1.39	18927.53	18924.75	19252.57	1.29	19253.86	19251.28				
65	18277.59	1.2	18278.79	18276.39	18601.69	1.9	18603.59	18599.79	18926.98	1.2	18928.18	18925.78	19249.42	0.9	19270.32	19268.52	19577.34	1.5	19578.84	19575.84
67	18276.55	0.89	18277.44	18275.66	18600.88	1.67	18602.55	18599.21	18926.66	0.85	18926.51	18924.81	19250.46	0.4	19250.86	19250.06	19576.23	1.12	19577.35	19575.11
79	18277.05	1.41	18278.46	18275.64	18601.25	1.12	18602.37	18600.13	18927.42	4.57	18931.99	18922.85	19251.01	1.86	19252.87	19249.15	19577.41	0.99	19578.4	19576.42
87	18278.59	1.7	18280.29	18276.89	18600.88	2.9	18603.78	18597.98	18927.2	1.5	18928.7	18925.7	19251.82	1.8	19253.62	19250.02	19576.21	1	19577.21	19575.21
118	18275.23	1.84	18277.07	18273.39	18600.48	0.92	18601.4	18599.56	18926.08	2.86	18927.94	18923.22	19249.47	0.96	19250.43	19248.51	19577.08	1.96	19579.04	19575.12
154	18276.46	2.7	18279.16	18273.76	18602.63	1.7	18604.33	18600.93	18926.5	1.7	18928.2	18924.8	19251.73	0.9	19252.63	19250.83	19578.89	1.8	19580.69	19577.09
155	18277.18	0.79	18277.97	18276.39	18603.23	0.83	18604.06	18602.4	18926.21	1.42	18929.63	18926.79	19253.76	0.58	19254.34	19253.18	19578.66	0.97	19579.63	19577.69
159	18276.76	1.33	18278.09	18275.43	18602.33	1.53	18604.46	18601.4	18927.76	1.7	18929.46	18926.06	19252.62	0.66	19253.28	19251.95	19577.39	0.78	19578.17	19576.61
160					18600.38	5.88	18606.26	18594.5	18927.11	1.91	18929.02	18925.2	19252.36	0.7	19253.06	19251.66	19577.33	1.19	19578.52	19576.14
162	18276.21	1.71	18278.62	18275.2	18602.09	1.14	18603.23	18600.95	18927.52	1.01	18928.53	18926.51	19253.02	1.67	19254.69	19251.35	19577.08	0.87	19577.95	19576.21
170	18276.43	1.51	18277.94	18274.92	18602.06	1.21	18603.27	18600.85	18927.13	1.57	18928.7	18925.56	19254.06	1.11	19255.17	19252.95				
182	18277.36	0.83	18278.25	18276.47	18601.66	1.54	18603.2	18600.12	18923.77	1.88	18925.65	18921.89								
194	18275.57	0.62	18277.19	18275.95	18600.58	2.67	18603.35	18598.01	18924.65	2.54	18927.19	18922.11	19249.6	0.94	19250.54	19248.66	19574.16	2.41	19576.57	19571.75
206	19278.55	1.55	18280.1	18277	18603.25	0.98	18604.23	18602.27	18927.63	1.66	18929.29	18925.97	19252.19	0.81	19253	19251.38	19576.7	2.18	19578.88	19574.52
208	18276.98	0.83	18277.71	18276.05	18602	0.58	18602.58	18601.42	18926.9	0.61	18927.51	18926.29	19251.34	0.74	19252.08	19250.6	19576.01	1.46	19577.47	19574.55
209	18276.45	0.7	18277.15	18275.76	18600.85	0.5	18601.35	18600.35	18923.42	1	18924.42	18922.42								
234	18277.75	1.3	18279.05	18276.45	18603.37	1	18604.37	18602.37	18927.64	0.7	18928.34	18926.94	19250.95	5.2	19256.15	19245.75	19578.15	0.7	19578.85	19577.45
236					18602.3	2.12	18604.42	18600.18	18926.24	3.35	18929.59	18922.89	19250.71	1.69	19252.4	19249.02	19577.52	5.4	19582.92	19572.12
238	18277.21	3.2	18280.41	18274.01	18602.61	0.7	18603.31	18601.91												
250	18274.56	2.2	18276.76	18272.36	18599.82	3.8	18603.62	18596.02	18924.98	1.9	18926.88	18923.08	19246.6	3.3	19249.9	19243.3				
279	18276.54	1.4	18277.94	18275.14	18600.64	3.8	18604.44	18596.84	18926.49	2.2	18928.69	18924.29	19250.98	3.3	19254.28	19247.68				
282					18603.45	2.94	18606.39	18600.51	18924.58	2.58	18927.25	18922.1	19250.23	1.89	19252.12	19248.34				
295	18277.56	0.58	18278.14	18276.98	18601.76	2	18603.76	18599.76	18927.28	0.65	18927.93	18926.63	19250.7	0.42	19251.12	19250.28	19576.22	0.73	19576.95	19575.49
296	18277.64	2.65	18280.29	18274.99	18603.14	2.86	18606	18600.28	18928.19	1.56	18929.75	18926.63	19254.54	3.76	19258.3	19250.78	19575.29	1.19	19576.48	19574.1
301	18276.53	1.47	18278.1	18275.16	18602.47	1.05	18603.52	18601.42	18926.86	0.86	18927.72	18926	19251.22	1.11	19252.33	19250.11	19576.85	1.16	19578.01	19575.69
302	18278.18	1.4	18279.58	18276.78	18602.53	1.03	18603.56	18601.5	18926.25	1.03	18927.28	18925.22								
313	18277.23	0.6	18277.83	18276.63	18603.74	1.9	18605.64	18601.84	18928.58	1.7	18930.38	18926.98	19255.22	2.7	19257.92	19252.52	19580.38	1	19581.38	19579.38
323	18278.59	2	18280.59	18276.59	18602.07	1.7	18603.77	18600.37	18926.34	0.8	18927.14	18925.54	19250.35	0.8	19251.15	19249.55	19575.09	1.7	19576.79	19573.39
326	18277.58	1.24	18278.82	18276.34	18602.5	1.93	18604.43	18600.57	18926.33	1.27	18927.6	18925.06	19252.51	0.54	19253.05	19251.97	19577.21	1.88	19579.09	19575.33
344	18277.42	3.3	18280.72	18274.12	18599.04	8.1	18607.14	18590.94	18926.59	1.6	18928.29	18925.09	19250.55	1.7	19252.25	19248.85	19573.43	1.4	19574.83	19572.03
350	18276.27	1.67	18277.94	18274.6	18600.85	2.05	18602.9	18598.8	18926.18	0.72	18926.9	18925.46	19250.76	0.85	19251.61	19249.91	19575.76	0.55	19576.31	19575.21
360	18276.73	1.01	18277.74	18275.72	18601.45	3.23	18604.68	18598.22	18926.14	1.39	18927.53	18924.75	19252.57	1.29	19253.86	19251.28				
363	18276.94	1.4	18278.34	18275.54	18602.19	0.8	18602.99	18601.39	18927.71	1.2	18928.91	18926.51	19252.55	0.9	19253.45	19251.65	19577.55	3.4	19580.95	19574.15
366	18276.95	3.11	18280.06	18273.84	18602.68	2.96	18605.64	18599.72	18922.83	4.24	18927.07	18918.59								
370	18278.24	1.24	18279.48	18277	18602.39	1.29	18603.68	18601.1	18927.76	0.7	18928.46	18927.06	19253.11	0.63	19253.74	19252.48	19578.31	1.24	19579.55	19577.07
375	18275.23	1.84	18277.07	18273.39	18600.48	0.92	18601.4	18599.56	18926.08	2.86	18927.94	18922.22	19249.47	0.96	19250.43	19248.51	19577.08	1.96	19579.04	19575.12
403	18275.92	1.31	18277.23	18274.61	18602.54	0.48	18603.02	18602.06	18927.09	0.54	18927.63	18926.55	19251.51	0.96	19252.46	19250.56				
413	18277.95	1.1	18279.05	18276.85	18601.43	2.5	18603.93	18598.93	18925.41	1.4	18926.81	18924.01	19251.41	0.8	19252.21	19250.61	19576.51	0.8	19577.31	19575.71
429	18278.54	1.5	18280.04	18277.04	18603.57	1.9	18605.47	18601.67	18927.37	3.5	18930.87	18923.87	19254.11	3.5	19257.61	19250.61	19579.35	0.9	19580.25	19578.45
434					18601.86	2.5	18604.36	18599.36	18926.73	3.9	18930.63	18922.83	19253.6	0.7	19254.3	19252.9	19578.55	2.4	19580.95	19576.15
439	18276.63	1.8	18278.43	18274.83	18601.71	1.9	18603.61	18599.81	18927.35	1.1	18928.45	18926.25	19252.12	1.7	19253.82	19250.42	19577.57	0.9	19578.47	19576.67
568	18276.57	1.62	18278.19	18274.95	18600.68	2.67	18603.35	18598.01	18924.65	2.54	18927.19	18922.11	19249.6	0.94	19250.54	19248.66	19574.16	2.41	19576.57	19571.75
594	18278.55	1.55	18280.1	18277	18603.25	0.98	18604.23	18602.27	18927.63	1.66	18929.29	18925.97	19252.19	0.81	19253	19251.38	19576.7	2.18	19578.88	19574.52
596	18276.46	0.7	18277.16	18276.76	18600.85	0.5	18601.35	18600.35	18923.42	1	18924.42	18922.42								
606	18277.21	2.2	18279.41	18275.01	18602.61	0.7	18603.31	18601.91												
609	18276.54	1.4	18277.94	18275.14	18600.64	3.8	18604.44	18596.84	18926.49	2.2	18928.69	18924.29	19250.98	3.3	19254.28	19247.68				
639	18276.95	3.11	18280.06	18273.84	18602.68	2.96	18605.64	18599.72	18922.83	4.24	18927.07	18918.59								
7	18276.06	3.9	18279.96	18272.16	18599.38	4	18603.38	18595.38	18927.3	2.9	18930.2	18924.4	19251.01	1.4	19252.41	19249.61				

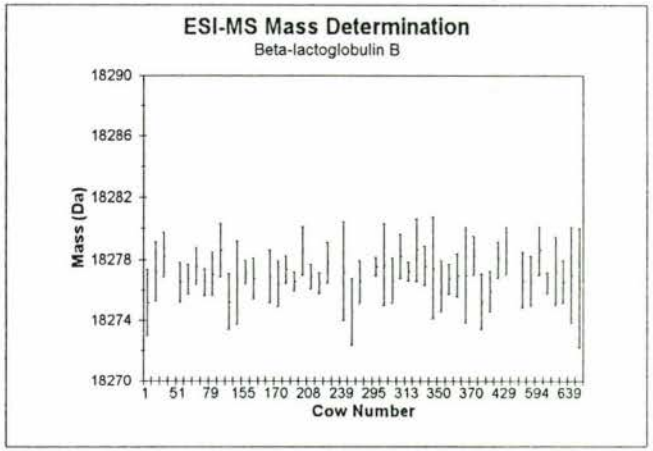
b-LG AA	Mass				Mass +1				Mass +2				Mass +3				Mass +4			
Cow nu	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo	Mass	"+/-	Hi	Lo
6	18363.07	1.18	18364.25	18361.89	18687.25	2.27	18689.52	18684.98	19012.98	1.03	19014.01	19011.95	19337.33	0.74	19338.07	19336.59	19663.03	1.58	19664.61	19661.45
8	18361.52	2.33	18363.85	18359.19	18687.77	1	18688.77	18686.77	19012.43	1.03	19013.46	19011.4	19337.17	1.51	19338.68	19335.66	19664.75	4.19	19668.94	19660.56
10	18363.77	2.3	18366.07	18361.47	18685.61	2.7	18688.31	18683.91												
15	18364.43	1.3	18365.73	18363.13	18688.44	0.8	18689.24	18687.64	19011.91	1.3	19013.21	19010.61								
18	18363.42	0.37	18363.79	18363.05	18688.45	0.7	18689.15	18687.75	19011.69	0.95	19012.64	19010.74								
76	18361.93	1.67	18363.6	18360.26	18687.67	0.4	18688.07	18687.27	19011.89	0.78	19012.67	19011.11	19334.93	3.86	19338.79	19331.07				
98	18362.07	1.1	18363.17	18360.97	18685.42	5.3	18690.72	18680.12	19011.87	1	19012.87	19010.87	19335.16	0.9	19337.06	19335.26	19660.44	2.4	19662.84	19658.04
114	18360.2	3.6	18363.8	18356.6	18686.36	5.1	18691.46	18681.26	19012.04	0.8	19012.84	19011.24	19335.69	1.4	19337.09	19334.29	19658.77	1.4	19660.17	19657.37
166	18362.43	0.8	18363.23	18361.63	18686.7	1.1	18687.8	18685.6	19010.14	2.1	19012.24	19008.04								
168	18363.07	1.18	18364.25	18361.89	18687.25	2.27	18689.52	18684.98	19012.98	1.03	19014.01	19011.95	19337.33	0.74	19338.07	19336.59	19663.03	1.58	19664.61	19661.45
175	18362.07	1.1	18363.17	18360.97	18685.42	5.3	18690.72	18680.12	19011.87	1	19012.87	19010.87	19335.16	0.9	19337.06	19335.26	19660.44	2.4	19662.84	19658.04
190	18363.38	0.9	18364.28	18362.48	18688.29	1	18689.29	18687.29	19013	0.7	19013.7	19012.3	19337.41	0.4	19337.81	19337.01	19662.25	2	19664.25	19660.25
244	18363.42	0.79	18364.21	18362.63	18688.48	0.86	18689.34	18687.62	19013.17	1.41	19014.58	19011.76	19337.17	4.21	19341.38	19332.96				
246	18363.58	0.59	18364.17	18362.99	18688.16	1.34	18689.5	18686.82	19011.38	1.2	19012.58	19010.18								
248	18362.99	0.45	18363.44	18362.54	18687.84	0.85	18688.69	18686.99	19012.46	0.94	19013.4	19011.52	19337.81	0.79	19338.6	19337.02	19662.98	1.46	19664.44	19661.52
252	18362.11	1.75	18363.86	18360.36	18683.83	3.46	18693.29	18686.37	19012.82	0.44	19013.26	19012.38	19335.76	1.22	19336.98	19334.54	19662.55	2.18	19664.73	19660.37
312	18364.75	1.8	18366.55	18362.95	18690.18	0.5	18690.68	18689.68	19015.04	1.3	19016.34	19013.74	19339.37	1	19340.37	19338.37	19658.85	2.1	19660.95	19656.75
329					18689.27	1.3	18690.57	18687.97	19014.08	1.2	19015.28	19012.88	19338.34	1.4	19339.74	19336.94	19663.89	2	19665.89	19661.89
334	18362.32	3.5	18365.82	18358.82	18688.14	1	18689.14	18687.14	19012.43	1.4	19013.83	19011.03	19337.57	1.1	19338.67	19336.47	19661.93	2	19663.93	19659.93
347	18361.05	3.63	18364.68	18357.42	18687.81	0.59	18688.4	18687.22	19011.32	2.94	19014.26	19008.38	19335.89	1.27	19336.16	19335.62	19661.67	0.48	19662.15	19661.19
357	18361.03	3.46	18364.49	18357.57	18686.31	1.33	18687.64	18684.98	19011.32	1.34	19012.66	19009.98	19335.09	1.61	19336.7	19333.48	19658.05	1.04	19659.1	19657.02
358					18689.83	1.46	18691.35	18688.43	19013.42	1.79	19015.21	19011.63	19337.79	0.7	19338.49	19337.09	19663.09	1.02	19664.11	19662.07
399	18362	1.52	18363.52	18360.48	18687.71	1.2	18688.91	18686.51	19013.31	1.16	19014.49	19012.13	19336.74	1.27	19338.01	19335.47	19659.96	1.83	19661.79	19658.13
420	18362.95	1.09	18364.05	18361.87	18687.54	1.37	18688.91	18686.17	19012.11	0.88	19012.99	19011.23	19335.92	1.39	19337.31	19334.53				
427	18362.73	0.61	18363.34	18362.12	18686.88	1.86	18688.74	18685.02	19011.07	4.32	19015.39	19006.75	19334.66	1.64	19336.3	19333.02	19655.86	3.59	19659.45	19652.27
428	18363.32	1.1	18364.42	18362.22	18687.69	3.3	18690.99	18684.39	19012.7	0.8	19013.5	19011.9	19336.71	1	19337.71	19335.71	19661.85	1.9	19663.75	19659.95
629	18361.93	1.67	18363.6	18360.26	18687.67	0.4	18688.07	18687.27	19011.89	0.78	19012.67	19011.11	19334.93	3.86	19338.79	19331.07				
679	18361.93	1.67	18363.6	18360.26	18687.67	0.4	18688.07	18687.27	19011.89	0.78	19012.67	19011.11	19334.93	3.86	19338.79	19331.07				
692	18361.52	2.33	18363.85	18359.19	18687.77	1	18688.77	18686.77	19012.43	1.03	19013.46	19011.4	19337.17	1.51	19338.68	19335.66	19664.75	4.19	19668.94	19660.56
733	18362.39	0.45	18363.44	18362.54	18687.84	0.85	18688.69	18686.99	19012.46	0.94	19013.4	19011.52	19337.81	0.79	19338.6	19337.02	19662.98	1.46	19664.44	19661.52
737	18362.35	1.69	18364.25	18360.47	18688.46	1.38	18689.84	18687.08	19013.43	3.06	19016.49	19010.37	19338.43	0.96	19339.39	19337.47	19660.74	2.58	19663.32	19658.16
766	18363.25	0.73	18363.98	18362.52	18687.93	0.56	18688.49	18687.37	19012.35	0.53	19012.88	19011.82	19336.34	0.5	19336.84	19335.84				
768	18362.95	1.09	18364.05	18361.87	18687.54	1.37	18688.91	18686.17	19012.11	0.88	19012.99	19011.23	19335.92	1.39	19337.31	19334.53				
806	18362.95	1.09	18364.05	18361.87	18687.54	1.37	18688.91	18686.17	19012.11	0.88	19012.99	19011.23	19335.92	1.39	19337.31	19334.53				
820	18362.73	0.61	18363.34	18362.12	18686.88	1.86	18688.74	18685.02	19011.07	4.32	19015.39	19006.75	19334.66	1.64	19336.3	19333.02	19655.86	3.59	19659.45	19652.27
827	18363.15	0.61	18363.76	18362.54	18686.88	1.86	18688.74	18685.02	19011.07	4.32	19015.39	19006.75	19334.66	1.64	19336.3	19333.02	19655.86	3.59	19659.45	19652.27
849					18689.83	1.46	18691.35	18688.43	19013.42	1.79	19015.21	19011.63	19337.79	0.7	19338.49	19337.09	19663.09	1.02	19664.11	19662.07
976	18360.2	3.6	18363.8	18356.6	18686.36	5.1	18691.46	18681.26	19012.04	0.8	19012.84	19011.24	19335.69	1.4	19337.09	19334.29	19658.77	1.4	19660.17	19657.37
b-LG AA	18365.47	2.9	18368.37	18362.57	18688.09	6.4	18694.49	18681.69												
b-LG CC																				
194	18286.71	0.7	18287.41	18286.01	18610.74	2.9	18613.64	18607.84	18934.52	1.1	18935.62	18933.42	19257.03	1.5	19258.53	19255.53	19582.47	0.9	19583.37	19581.57
292	18286.89	0.8	18287.69	18286.09	18610.51	1.3	18613.81	18609.21	18935.14	1.8	18936.94	18933.34								
951	18287.86	1.2	18289.06	18286.66	18610.19	1.3	18611.49	18610.19	18936.14	1.8	18937.94	18934.34								
b-LG BC																				
129	18275.78	1.8	18277.58	18273.98	18286.22	1	18287.22	18285.22	18601.51	1	18602.5	18600.5	18609.95	0.4	18610.35	18609.55	18925.26	1.2	18926.46	18924.06
b-LG AB																				
125	18274.32	3.4	18277.72	18270.92					18597.13	7	18604.13	18590.13	18666.43	3.6	18690.03	18662.83	18929.87	3.5	18933.37	18926.37
406									18599.53	2.46	18601.99	18597.07	18685.66	3.84	18689.5	18681.82	18925.56	1.75	18927.31	18923.81
411									18597.19	5.2	18602.39	18591.99	18684.53	5.9	18690.43	18678.63	18924.88	2.8	18927.68	18922.08
449																	18928.73	2.04	18930.77	18926.69
457									18598.47	2.67	18601.14	18595.8	18687.61	2.82	18690.43	18684.79	18926.51	1.11	18927.62	18925.4
460	18276.93	2.6	18279.53	18274.33	18361.29	2.9	18364.19	18358.39	18600.88	2.8	18603.68	18598.08	18687.52	3.8	18691.32	18683.72	18928.11	1.9	18930.01	18926.21
462	18276.58	2.8	18279.38	18272.78	18362.38	1.5	18363.88	18360.88	18598.31	5.5	18603.81	18592.81	18686.87	2.9	18689.77	18683.97	18926.99	2.3	18929.29	18924.69
469	18275.1	3.2	18278.3	18271.9	18362.72	1.8	18364.52	18360.92	18601.19	2.5	18603.69	18598.69	18687.56	0.9	18688.46	18686.66	18927.26	2.5	18929.76	18924.76
525	18274.32	3.7	18277.02	18271.62					18597.13	7	18604.13	18590.13	18666.43	3.6	18690.03	18662.83	18929.87	3.5	18933.37	18926.37
529	18274.32	3.7	18278.02	18270.62					18597.13	7	18604.13	18590.13	18666.43	3.6	18690.03	18662.83	18929.87	3.5	18933.37	18926.37
648	18275.1	3.2	18278.3	18271.9	18362.72	1.8	18364.52	18360.92	18601.19	2.5	18603.69	18598.69	18687.56	0.9	18688.46	18686.66	18927.26	2.5	18929.76	18924.76
860																	18928.73	2.04	18930.77	18926.69
896									18599.53	2.46	18601.99	18597.07	18685.66	3.84	18689.5	18681.82	18925.56	1.75	18927.31	18923.81
Sigma b-	18278.07	3.09	18281.16	18274.98	18362.29	2.9	18365.19	18359.39	18598.4	1.1	18599.5	18597.3	18685.81	1.2	18687.11	18684.71				

Statistical analysis

b-LG B mass		mass error		mass +1 lac		mass error +1 la		mass +2 lac		mass error +2 lac		mass + 3 lac		mass error + 3 lac		mass + 4 lac		mass error +4 lac	
Mean	18277.00327	Mean	1.61776	Mean	18601.7561	Mean	2.076	Mean	18926.369	Mean	1.8296	Mean	19251.9502	Mean	1.4402	Mean	19548.9381	Mean	1.8161111
Standard Error	0.125982064	Standard Error	0.10385	Standard Error	0.16349336	Standard Error	0.195	Standard Error	0.1908483	Standard Error	0.1427	Standard Error	0.42197203	Standard Error	0.1439	Standard Error	22.6200158	Standard Error	0.1311481
Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR
Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR
Standard Deviatio	0.925775323	Standard Deviatio	0.76314	Standard Deviatio	1.20142591	Standard Deviatio	1.431	Standard Deviatio	1.4024428	Standard Deviatio	1.0484	Standard Deviatio	3.10084848	Standard Deviatio	1.0572	Standard Deviatio	166.22249	Standard Deviatio	0.9637376
Variance	0.857059949	Variance	0.58238	Variance	1.44342421	Variance	2.048	Variance	1.9668457	Variance	1.0992	Variance	9.61526131	Variance	1.1177	Variance	27629.9163	Variance	0.9287902
Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR
Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR
Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR
Minimum	18274.56	Minimum	0.58	Minimum	18599	Minimum	0.48	Minimum	18922.83	Minimum	0.54	Minimum	19246.6	Minimum	0.39	Minimum	18579.35	Minimum	0.55
Maximum	18278.59	Maximum	3.9	Maximum	18603.74	Maximum	9.1	Maximum	18928.68	Maximum	4.57	Maximum	19269.42	Maximum	5.2	Maximum	19580.38	Maximum	5.4
Sum	895573.16	Sum	79.27	Sum	1004494.83	Sum	112.1	Sum	984171.18	Sum	95.14	Sum	866337.76	Sum	64.81	Sum	703761.77	Sum	58.18
Count	49	Count	49	Count	54	Count	54	Count	52	Count	52	Count	45	Count	45	Count	36	Count	36
Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR

b-LG A mass		mass error		mass +1 lac		mass error + 1 la		mass + 2 lac		mass error + 2 la		mass + 3 lac		mass error + 3 lac		mass + 4 lac		mass error + 4 lac	
Mean	18362.56	Mean	1.53886	Mean	18687.7313	Mean	1.736	Mean	19012.316	Mean	1.4316	Mean	19336.5642	Mean	1.4755	Mean	19661.022	Mean	2.1192
Standard Error	0.186932811	Standard Error	0.16083	Standard Error	0.18182072	Standard Error	0.228	Standard Error	0.1524456	Standard Error	0.1674	Standard Error	0.20196609	Standard Error	0.1614	Standard Error	0.43442869	Standard Error	0.1634597
Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR	Median	ERR
Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR	Mode	ERR
Standard Deviatio	1.029942959	Standard Deviatio	0.9914	Standard Deviatio	1.12081816	Standard Deviatio	1.404	Standard Deviatio	0.939738	Standard Deviatio	1.0317	Standard Deviatio	1.24500259	Standard Deviatio	0.995	Standard Deviatio	2.67799832	Standard Deviatio	1.007633
Variance	1.059929412	Variance	0.98287	Variance	1.25623336	Variance	1.971	Variance	0.8831075	Variance	1.0645	Variance	1.55003144	Variance	0.99	Variance	7.171675	Variance	1.0153243
Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR
Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR	Skewness	ERR
Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR	Range	ERR
Minimum	18360.2	Minimum	0.37	Minimum	18685.42	Minimum	0.4	Minimum	19010.14	Minimum	0.44	Minimum	19334.56	Minimum	0.4	Minimum	19655.86	Minimum	0.48
Maximum	18364.75	Maximum	3.63	Maximum	18690.18	Maximum	5.3	Maximum	19015.04	Maximum	4.32	Maximum	19339.37	Maximum	4.21	Maximum	19664.75	Maximum	4.19
Sum	642689.6	Sum	53.86	Sum	710133.79	Sum	65.97	Sum	703455.7	Sum	52.97	Sum	638106.23	Sum	48.69	Sum	491525.55	Sum	52.98
Count	35	Count	35	Count	38	Count	38	Count	37	Count	37	Count	33	Count	33	Count	25	Count	25
Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR

b-LG C mass		mass error		mass +1 lac		mass error + 1 la		mass + 2 lac		mass error + 2 la		mass + 3 lac		mass error + 3 lac		mass + 4 lac		mass error + 4 lac	
Mean	18287.15333	Mean	0.9	Mean	18610.48	Mean	1.833	Mean	18935.267	Mean	1.5667	Mean	19257.03	Mean	1.5	Mean	19582.47	Mean	0.9
Standard Error	0.357133651	Standard Error	0.15275	Standard Error	0.15947832	Standard Error	0.533	Standard Error	0.4719228	Standard Error	0.2333	Standard Error	ERR	Standard Error	ERR	Standard Error	ERR	Standard Error	ERR
Median	18286.89	Median	0.8	Median	18610.51	Median	1.3	Median	18935.14	Median	1.8	Median	0	Median	0	Median	0	Median	0
Mode	NA	Mode	NA	Mode	NA	Mode	1.3	Mode	NA	Mode	1.8	Mode	0	Mode	0	Mode	0	Mode	0
Standard Deviatio	0.618573528	Standard Deviatio	0.26458	Standard Deviatio	0.27622455	Standard Deviatio	0.924	Standard Deviatio	0.8173942	Standard Deviatio	0.4041	Standard Deviatio	ERR	Standard Deviatio	ERR	Standard Deviatio	ERR	Standard Deviatio	ERR
Variance	0.382633333	Variance	0.07	Variance	0.0763	Variance	0.853	Variance	0.6681333	Variance	0.1633	Variance	ERR	Variance	ERR	Variance	ERR	Variance	ERR
Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR	Kurtosis	ERR
Skewness	1.568516679	Skewness	1.45786	Skewness	-0.4829679	Skewness	1.732	Skewness	0.6805922	Skewness	-1.7321	Skewness	1.73205081	Skewness	1.7321	Skewness	1.73205081	Skewness	1.7320508
Range	1.15	Range	0.5	Range	0.55	Range	1.6	Range	1.62	Range	0.7	Range	19257.03	Range	1.5	Range	19582.47	Range	0.9
Minimum	18286.71	Minimum	0.7	Minimum	18610.19	Minimum	1.3	Minimum	18934.52	Minimum	1.1	Minimum	19257.03	Minimum	1.5	Minimum	19582.47	Minimum	0.9
Maximum	18287.86	Maximum	1.2	Maximum	18610.74	Maximum	2.9	Maximum	18936.14	Maximum	1.8	Maximum	19257.03	Maximum	1.5	Maximum	19582.47	Maximum	0.9
Sum	54861.46	Sum	2.7	Sum	55831.44	Sum	5.5	Sum	56805.8	Sum	4.7	Sum	19257.03	Sum	1.5	Sum	19582.47	Sum	0.9
Count	3	Count	3	Count	3	Count	3	Count	3	Count	3	Count	1	Count	1	Count	1	Count	1
Confidence Level(	0.699969096	Confidence Level(	0.29939	Confidence Level(	0.31257176	Confidence Level(	1.045	Confidence Level(	0.9249517	Confidence Level(	0.4573	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR	Confidence Level(	ERR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18278
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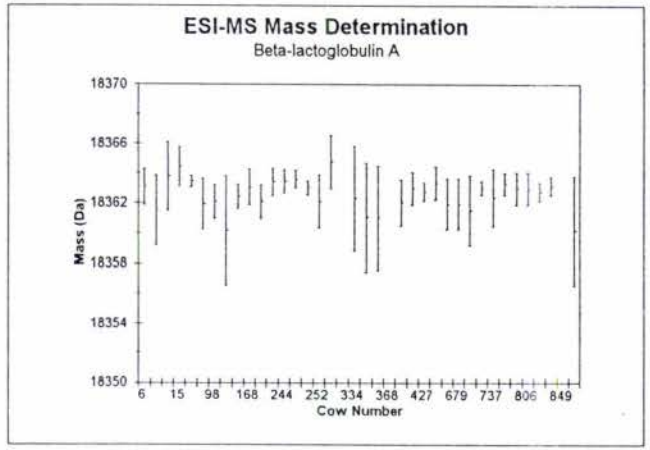
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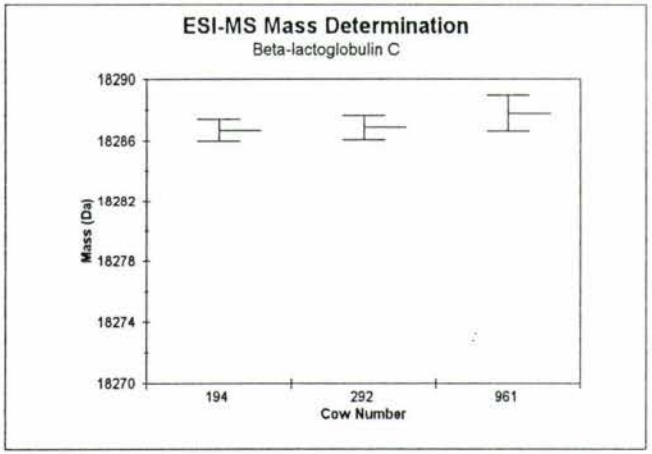
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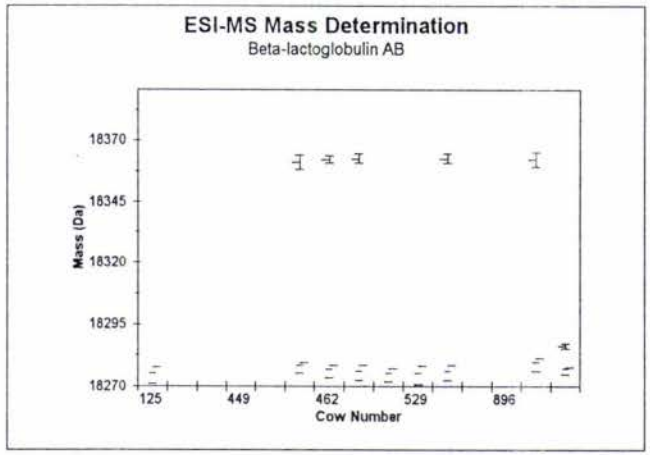
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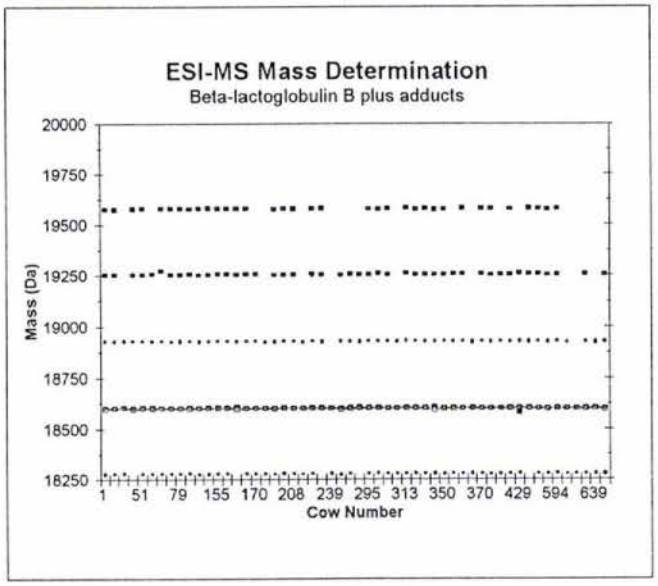
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19000
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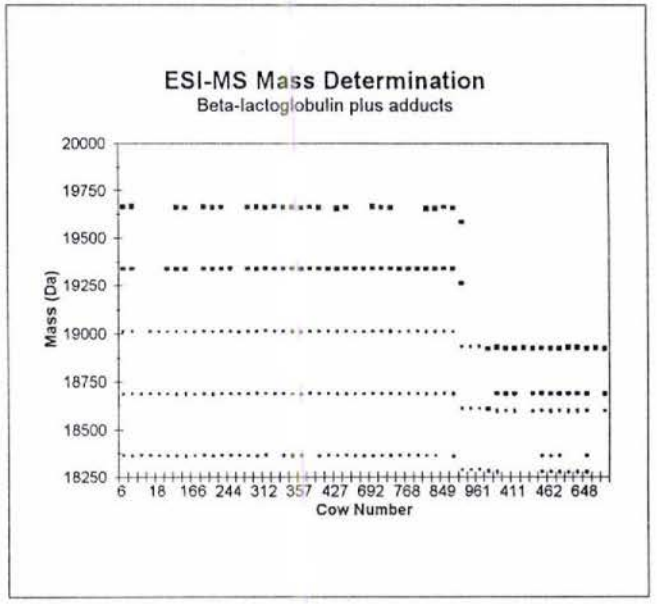
1 51 79 155 170 208 239 295 313 350 370 429 594 639
Cow Number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Beta-lactoglobulin plus adducts
Mass (Da)
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19500
19250
19000
18750
18500
18250
6 18 166 244 312 357 427 692 768 849 961 411 462 648
Cow Number

12 PUBLICATIONS

12.1 Burr, R., Moore, C.H., Hill, J.P. (1996)
Evidence of multiple glycosylation of bovine β-lactoglobulin by electrospray ionisation mass spectrometry. *Milchwissenschaft*, (in print).

Evidence of Multiple Glycosylation of Bovine β-Lactoglobulin by Electrospray Ionisation Mass Spectrometry

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1. Introduction

The major milk proteins (α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin) are found in a number of variant forms in cattle [1]. The milk proteins and their variants have traditionally been detected through differences in charge that result from amino acid substitutions or deletions in the primary structure [1, 2]. A number of methods have been used in the analysis of milk proteins in milk and other systems, including electrophoretic techniques such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), various column chromatography techniques, including gel filtration, ion exchange and reverse phase HPLC [3]. Electrophoretic and other techniques are also able to separate the various post-translationally phosphorylated and glycosylated forms of the milk proteins. Although these techniques are likely to separate proteins on the basis of size and/or charge, they are less likely to separate or detect variants which arise from substitutions involving neutral residues (silent variants). Similarly, post-translation changes to proteins which do not result in a major change in protein size or charge could also remain undetected by such techniques.

The advent of modern mass spectrometric techniques, such as electrospray ionisation mass spectrometry (ESI-MS) has provided a powerful new tool to investigate the primary structure of proteins and peptides. Accurate molecular weight determination (within 0.01% for compounds up to 100,000 Da) enables identification of primary sequence and post-translational modifications such as phosphorylation and glycosylation of proteins to be detected [4, 5]. This technique has been used in the analysis of the milk proteins by Léonil et al. (6), where in combination with reverse phase HPLC (RP-HPLC-ESI-MS) the observed masses of the A and B variants of β-LG were found to correspond closely to the calculated mass of these proteins derived from their primary sequences. In both cases a single peak was observed in the reconstructed spectra.

Recently ESI-MS has been used in the characterisation of α_{s1}-casein (α_{s1}-CN) from sheep [7]. The ovine α_{s1}-CN A variant was found to be present as two species differing in molecular weight by approximately 1000 Da. The extent of the phosphorylation of these species of α_{s1}-CN A was also examined by the ESI-MS technique.

In another recent report [8] ESI-MS was used in the identification of a new genetic variant of bovine β-casein (β-CN). The new variant differs from the β-CN A1 variant at position 152 in the primary protein sequence, where a leucine residue was found instead of a proline and thus represents a silent substitution which would not have been detected by classical electrophoretic techniques.

In this report ESI-MS was used in the characterisation of purified β-LG samples prepared from individual cows and the identification of multiple glycosylations of this protein.

2. Materials and methods

All chemicals used were analytical grade or better. Solvents used were HPLC 'UV grade'. Water was purified by reverse osmosis, followed by deionisation through MilliQ (Millipore, Bedford, MA.) equipment. Bovine β-LG AB, horse heart myoglobin and horse heart cytochrome C were all obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1 Purification and phenotyping of β-LG

Fresh milk samples were collected from 109 individual Jersey or Jersey-Friesian cross cows. Approximately 200 ml of raw milks were heated to 40°C in a water bath for a period from 5 minutes to 30 minutes to facilitate cream separation. After heating, samples were centrifuged at 5 000 x g for 10 minutes (unrefrigerated). The fat layer was aspirated prior to acidification to pH 4.6 by addition of 1M HCl with continuous stirring. After standing for a period of 20 minutes the isoelectrically precipitated casein was separated from the whey by centrifugation at 5 000 x g for 15 minutes. All whey proteins with the exception of β-LG were precipitated from the whey supernatant by adaptations to the method of Maillard and Ribadeau-Dumas [9]. This involved slow addition of NaCl to 7% (w/v). Once the salt had completely dissolved the pH was adjusted to 2.0 by addition of 6M HCl drop-wise with continuous stirring. After standing for 20 minutes the precipitated proteins were separated by centrifugation at 8 000 x g for 15 minutes. The β-LG supernatant was decanted into 10 000 molecular weight cut off dialysis tubing (Union Carbide), and dialysed against 300 mM Tris buffer, pH 7.0, overnight. Two subsequent dialysis steps were performed against deionised water for 24 hours each before freeze drying.

The β-LG phenotype of the samples and assessment of purity was made by capillary electrophoresis according to the method of Paterson et al. [10]. For this purified β-LG was dissolved in water to 1 mg ml^{-1} and 0.22 μm filtered (Millex PVDF 0.22 μm, Millipore, Bedford, MA). An internal standard of α-lactalbumin (α-Lac) was added to all samples and electropherograms were aligned using this standard.

2.2 Preparation of β-casein (β-CN)

β-CN was purified from acid precipitated casein on a BioPilot chromatography station (Pharmacia, Uppsala, Sweden). Whole casein was dissolved in buffer A (6 M urea, 20 mM sodium acetate, pH 5.0) to 2% (w/v). Prior to chromatography the sample solution was titrated to pH 7.0 with 2 M NaOH, treated with 0.1% dithiothreitol for 1 hour before titration back to pH 5.0. Three hundred mls of sample casein (6 g) was loaded onto a 113 x 150 mm column of S-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) and eluted at 25 ml min^{-1} in a 3 column volume (4.5 l) gradient from 0-0.4 M NaCl, with eluent monitored at 280 nm. β-CN was eluted immediately following a small γ-CN peak, and prior to κ-, α_{s2}- and α_{s1}-CN respectively. The collected eluent was dialysed exhaustively against water before lyophilisation. Identification and purity was assessed by alkaline urea PAGE according to the method of Creamer [11].

2.3 Preparation of Whey by Ultracentrifugation of Whole Milk.

Fresh milk samples (5 ml) collected from individual cows were centrifuged at 60 000g Richard for 30 mins in a Beckman L8-M ultracentrifuge. Following centrifugation the soluble fraction in the samples was separated from the casein (which had formed a pellet) and fat (which had formed a layer above the aqueous phase). The whey samples were

then subjected to mass spectroscopy as described below.

2.4 Reverse phase-HPLC analysis of β-LG

β-LG samples were analyzed by reverse phase HPLC (RP-HPLC) on a Shimadzu HPLC (Shimadzu Corp, Osaka, Japan). Samples were dissolved to 1 mg ml^{-1} in solvent A (5% acetonitrile, 0.05% trifluoroacetic acid) and 0.22 μm filtered prior to injection onto a Vydac C4 4.6 x 150 mm column (Separation Group, Vesperia, CA, USA) at 60°C. Protein was eluted in a gradient of 0 to 60% solvent B (60% acetonitrile, 0.05% trifluoroacetic acid) over 60 minutes at a flow rate of 1 ml min^{-1} and detected at 214 nm.

2.5 Mass Spectroscopy

The mass spectrometer used was a VG Platform single-quadrupole mass spectrometer (VG BioTech, Altrincham, UK) equipped with a pneumatically assisted electrospray ion source. Multi-charged protein ions were generated in the positive ionisation mode by introducing sample into a stream of 50% acetonitrile pumped at 10 μl/min by a Spectra-Physics pump through a 75 μm ID fused-silica capillary. This was housed in a stainless steel capillary held at a potential of 3.2 kV. The electrospray source-mass analyzer interface consisted of a 100 μm diameter conical orifice held at +30 V, at a temperature of 60°C. A gas curtain of 0.8 l min^{-1} of dry nitrogen in the interface region prevented entry of neutral molecules into the mass analyzer.

Samples were dissolved in 50% acetonitrile:MilliQ water to 1 mg ml^{-1} with the addition of 0.5% formic acid prior to filtration through a 0.22 μm filter. Samples were subsequently diluted 1000 fold in 50% acetonitrile prior to injection onto the mass

spectrometer. Reference solutions of horse heart myoglobin and cytochrome C were run prior to sample solutions for instrument calibration.

Aliquots of protein sample (10-15 μl) were introduced into the solvent stream via a Rheodyne (Cotati, CA, USA.) sample injection loop. This allowed at least 8 acquisitions to be accumulated and averaged over a period of approximately 1 minute. Each acquisition was obtained over the mass-to-charge range of 300 to 2100. The raw spectra acquired were subjected to a series of processes to reduce background noise (background subtraction), differentiate signal from spurious spikes (smoothing), identify series of multiply charged ions (peak centering) and to deconvolute the data to a true mass scale (transformation) using MassLynx software (Fissons Instruments, Altrincham, UK). The data were transformed over an appropriate mass range for each protein (18000 to 20000 Da for β-LG) with a resolution of 0.125 Da.

2.6 Theoretical Calculation of Protein Mass

Theoretical calculations of the mass of each β-LG variant were obtained with the use of Peptide Tools software (Hewlet-Packard Co., San Diego, CA), based on the milk protein variant primary sequences published by Eigel *et al.* (1).

2.7 Heat Denaturation and Reduction

Sample β-LG B solutions (1 mg ml^{-1}) were prepared in 4 M urea containing 50 mM dithiothreitol. The samples were heated at 100°C for 10 minutes, followed by cooling to room temperature and 0.22μm filtered.

3. Results

Figure 1 shows a series of CE electropherograms of the purified β-LG samples. As was observed by PAGE, the only species present in the samples were found to correspond to either the A, B or C variant forms of β-LG [10].

RP-HPLC (Figure 2) was able to confirm the purity of the β-LG samples, with only a single peak being observed in all chromatograms. No differences were observed in the chromatograms of native β-LG and β-LG which had been heat-denatured under reducing conditions.

Figure 3 shows the transposed ESI-MS spectrums of a number of purified β-LG samples. It is clear that multiple molecular weight β-LG species are observed by this technique. The species with the lowest molecular weight was found in the case of each purified β-LG sample analysed to correspond to the known molecular weight (within error) of one of the variants (A = 18363.4 Da, B = 18277.3 Da or C = 18286.4Da) of this protein (Table 1). Additional species of higher molecular weight were also observed to varying degrees. These adducts differed from the known molecular weights of the β-LG variants by multiples of 324.5 ± 0.9 Da (up to four additional species). Although less marked, analysis of a sample of purified mixed β-LG A and B variants purchased from the Sigma Chemical Company was also found to contain one adduct of the +324 molecular weight species attached to both the A and B variants (Figure 3). As with the other samples of purified β-LG, the sample from Sigma gave only single peaks corresponding to either β-LG A or B upon RP-HPLC.

In the transposed ESI-MS spectra of the whey samples prepared by ultrcentrifugation peaks corresponding to the +324 molecular weight adduct species of β-LG were also observed (Figure 4).

The heat denaturation, reduction and RP-HPLC, followed by ESI-MS of β-LG samples was found to increase the observed mass of β-LG by an average of 4 Da, as would be expected due to the reduction of the two disulphide bonds in the protein. However, this treatment had no effect on the number or proportion of the +324 molecular weight derivatives.

Analysis of five monovariant samples of purified β-CN A1 by ESI-MS determined that a single component existed with a molecular weight of 24,024 Da, consistent with a calculated weight derived from the primary sequence. Unlike β-LG no adducts were observed with β-CN.

4. Conclusions

The 324 Da molecular weight adducts observed in ESI-MS of the purified samples of β-LG are consistent with the covalent linkage of a lactosyl residues to the protein. The observation that these species remain after heat denaturation, reduction and RP-HPLC treatment is supporting evidence that the linkage is covalent. It is probable that such a series of treatments would remove any molecules which were associated with β-LG by noncovalent bonding. Similarly the samples of β-LG were subjected to extensive dialysis during preparation, thus making it unlikely that any molecules would remained associated with the protein unless the dissociation constant for the binding of that molecule to β-LG

was extremely low. It is unlikely that any noncovalent associations would survive the ionisation conditions used in the ESI-MS experiments.

Henle and Klostermeyer [12] chemically induced the glycosylation of purified β-CN A1 by heating this protein in the presence of lactose. They found the highly reactive lysine residues to reducing sugars in β-CN A1 were located directly adjacent to the charged side chains of glutamic acid and lysine, respectively. Henle and Klostermeyer [12] suggested that the charged side chains of lysine and glutamic acid residues may be involved in the intramolecular catalysis of the Maillard reaction Amadori rearrangement products. It is possible that the lysine or glutamic acid residues adjacent to the lysine residue which reacts with the sugar participate in modulating the supply of a proton between the N-substituted glycosylamine and the cation of Schiff's base. This suggests that the microenvironment of the protein may influence the reactivity of lysine residues in the Maillard reaction. In this investigation there was no evidence for the formation of 324 Da adduct species in the β-CN samples purified as descibed in the methods section. It is interesting to note that in β-LG there are four sites where a lysine residue is adjacent to either another lysine residue or to a glutamic acid residue (Lys69-Lys70, Glu74-Lys75, Lys100-Lys101 and Glu134-Lys135) and that the adducts species of β-LG were observed in multiples of up to four 324 Da units.

The fact that the multiple forms of β-LG were not observed by CE or RP-HPLC would suggests that the association of the 324 Da molecule with β-LG does not alter the charge, molecular weight or hydrophobicity of this protein enough to be detected under the conditions used with these techniques.

Future experiments will look to confirm that the modified species of β-LG involve the covalent attachment of lactose to lysine residues on the protein and the location in the primary sequence of these modified residues. The nature of the reaction mechanism is also to be studied. The stage in the isolation β-LG at which the adduct species of this protein are formed is also of interest. The individual milk samples were held at 40°C for up to 30 mins during the purification process. It is possible that this step induced a reaction between milk lactose and β-LG. If this were the case then it is also possible that the reaction would also occur at physiological temperature in the udder of the cow. The finding that β-LG in whey samples prepared from fresh milk by ultracentrifugation contain +324 Da molecular weight species would suggest that this is in fact the case.

Acknowledgments

We wish to thank all our colleagues for their helpful discussions during preparation of this manuscript. Thanks also go to the University of Waikato for access to their mass spectrometer facilities and Wendy Jackson for her invaluable help with ESI-MS. This work was supported by funding from the Foundation for Research, Science and Technology (Contract No. DRI 401) and the New Zealand Dairy Board.

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5. Summary

Electrospray ionisation mass spectrometry (ESI-MS) was used in the characterisation of β-lactoglobulin (β-LG) A, B and C variants purified from the milks of 109 individual cows. Capillary electrophoresis and reverse phase-HPLC were used to confirm the purity of the samples, which contained either one species in the case of samples prepared from cows homozygous for a particular β-LG variant or two species in the case of samples prepared from cows heterozygous for this protein. However, all samples were found to contain multiple molecular weight species of β-LG when analysed by ESI-MS. In addtion to spectral peaks corresponding to the known molecular masses of the A, B and C variants, additonal peaks were observed which differed in molecular weight from β-LG by increments of approximatetly 324 mass units. Although less marked, the +324 Da molecular weight species were also present in a sample of β-LG purchased from the Sigma Chemical Company.

Figure 1: Capillary electropherograms of (a) whey protein standards, and β-LG purified from acid whey of a (b) β-LG AA phenotype, (c) β-LG BB phenotype, (d) β-LG AB phenotype (e) β-LG CC phenotype (f) β-LG BC phenotype cow using the method of Paterson *et al.* (10).

Figure 2: RP-HPLC chromatograms of (a) Sigma purified β-LG, β-LG prepared from acid whey from a (b) β-LG AA phenotype cow, (c) β-LG BB phenotype cow, and (d) heat denatured and DTT reduced β-LG from an AA phenotype cow.

Figure 3: Transformed ESI-MS spectrums of (a) Sigma purified β-LG, β-LG prepared from acid whey from a (b) β-LG AA phenotype cow, (c) β-LG BB phenotype cow, (d) β-LG CC phenotype cow and (e) heat denatured and DTT reduced β-LG from an AA phenotype cow.

Figure 4: Transformed ESI-MS spectrums of β-LG in whey prepared by ultracentrifugation of the milk from a (a) β-LG AA phenotype cow and (b) β-LG BB cow.

Table 1: Average molecular mass of β-LG variants and adducts as determined by ESI-MS

Mass (Da)	Variant A	Varinat B	Variant C
'Native' protein	18362.3 ± 1.1	18277.0 ± 1.6	18286.2 ± 1.0
+ 1 adduct	18686.3 ± 1.2	18601.5 ± 1.5	18609.3 ± 0.5
+ 2 adduct	19011.0 ± 1.4	18924.9 ± 1.4	18934.4 ± 1.3
+ 3 adduct	19334.7 ± 1.3	19249.6 ± 2.2	19258.6 ± 1.7
+ 4 adduct	19661.0 ± 1.6	19575.0 ± 2.6	19583.5 ± 2.5

Figure 1

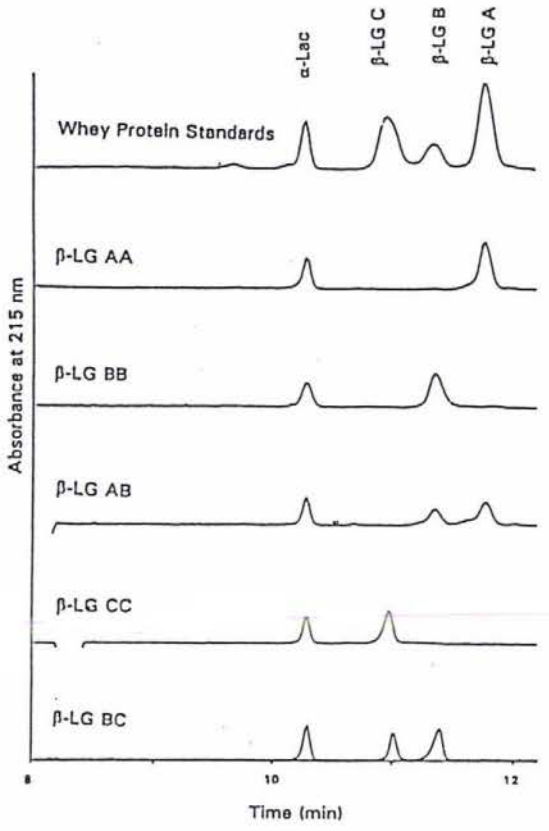


Figure 2

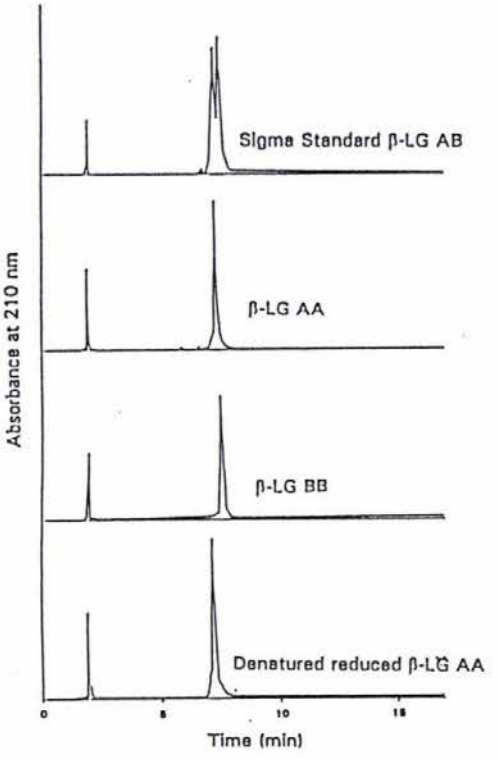
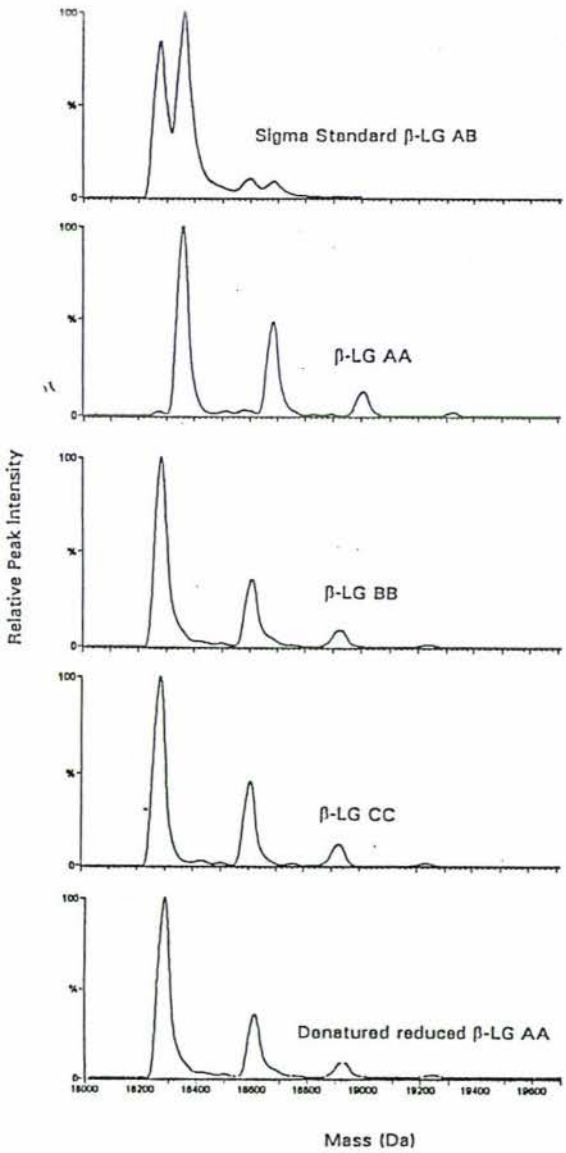


Figure 3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β-LG AA
β-LG BB
β-LG CC
Denatured reduced β-LG AA
Relative Peak Intensity
Mass (Da)

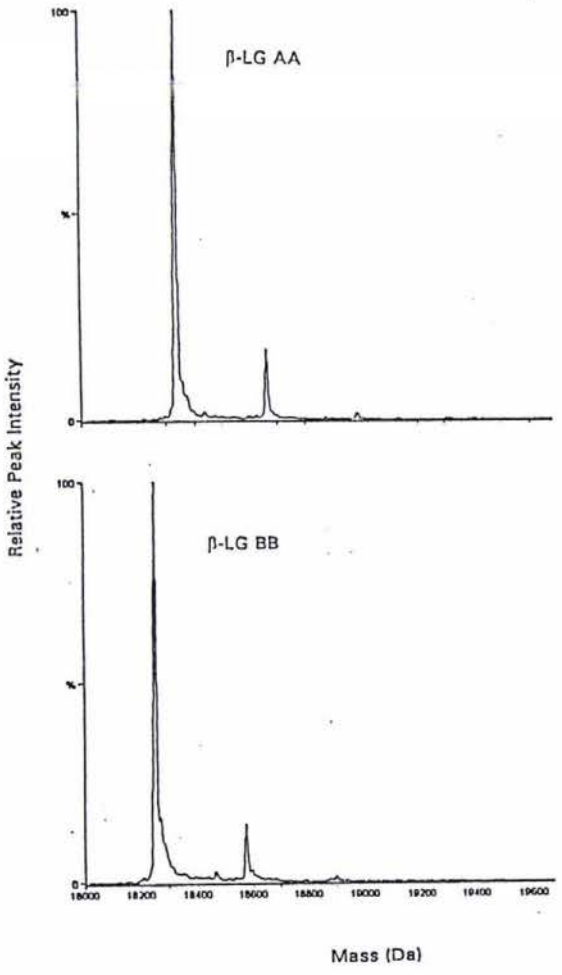
β-LG AA
β-LG BB
Relative Peak Intensity
100
%
0
18000
18200
18400
18600
18800
19000
19200
19400
19600
Mass (Da)

Figure 4

12.2 Burr, R. (1996)

Detection of "silent" milk protein variants. *Dairy Technology*, **10** (2), 5

Detection of "silent" milk protein variants

by Richard Burr, Food Science Section, NZDRI

It has been known for some time that genetic variants of milk proteins are closely associated with significant changes in milk production traits (Ng-Kwai-Hang *et al.*, 1984, 1986), milk composition (McLean *et al.*, 1984; Ng-Kwai-Hang *et al.*, 1984, 1986; Hill, 1993) and processing characteristics such as heat stability and cheese making properties (Marziali and Ng-Kwai-Hang, 1986a, 1986b; McLean *et al.*, 1987, 1989; Aleandri *et al.*, 1990; Ng-Kwai-Hang, 1990).

Genetic polymorphism of milk proteins are the result of amino acid substitutions or deletions in the protein primary sequence. To date these genetic variants have been identified by mainly electrophoretic methods. These methods are able to differentiate proteins of different net charge at a given pH, due to the substitution or deletion of an amino acid carrying a ionisable side chain group.

However, of the 20 common amino acids, only 5 carry positive or negative charge. Changes involving the other 15 neutral residues theoretically will not affect the net charge of the protein appreciably, and will therefore go undetected by normal electrophoresis. Assuming that mutation leading to amino acid substitution is random, we could expect neutral or "silent" variants to occur 3 times as frequently as known electrophoretic variants.

A procedure for detection of silent variants using a combination of enzymatic hydrolysis, reverse phase chromatography, mass spectroscopy and amino acid sequencing is being developed in the Food Science Section.

Enzymatic hydrolysis of purified proteins results in a group of peptides as a result of the specific cleavage of certain peptide bonds by an enzyme. Individual peptides are then isolated by reverse phase chromatography, resulting in a characteristic elution pattern, or peptide "fingerprint". Amino acid substitutions influence the hydrophobicity of peptides involved and are detected as a change in the peptide fingerprint. Peaks that have altered retention times may be isolated for further study by mass spectroscopy and amino acid sequencing to determine the nature of any substitution that has occurred.

Given the observed association between known variants and milk production, composition and physiochemical properties, it is possible that similar associations also exist for silent variants. If this is so, it is to the advantage of the New Zealand dairy industry to develop quick and widely applicable methods for detection of desirable (or undesirable!) silent variants to supplement our existing methods for detection of protein variants due to change differences.

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New Zealand Dairy Research Institute

Use of Electrospray Ionisation Mass Spectrometry to Elucidate the Multiple Glycation of Bovine β-Lactoglobulin

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Introduction

In cattle the major milk proteins (the caseins, α-lactalbumin and β-lactoglobulin (β-LG) exist in a number of variant forms which can be post-translationally phosphorylated and glycosylated to various extents. These forms have traditionally been detected through differences in size or charge using electrophoretic (including CE) and HPLC techniques. Variants arising from substitutions involving neutral residues (silent variants) and post-translation changes not resulting in major changes in protein size or charge may remain undetected by such techniques.

Mass spectrometric techniques, such as electrospray ionisation (ESI-MS), are powerful new tools to investigate the primary structure of proteins. Accurate molecular weight determination (within 0.01% for compounds up to 100 kDa) enables detection of primary sequence and post-translational modifications such as phosphorylation and glycosylation. ESI-MS has been used used to characterise purified β-LG samples and to identify multiple glycations of this protein.

Methods

Fresh milk was collected from individual Jersey or Jersey-Friesian cross cows.
Whey was produced either by acidification of milk to pH 4.6 with HCl and centrifugation at 5000 xg for 15 min or by ultracentrifugation of fresh milk at 60,000 xg for 30 min.
β-LG was prepared from whey by NaCl precipitation of the other whey proteins at pH 2.0.
β-LG (1mg/ml in 4M urea/50mM dithiothreitol) was heat denatured under reducing conditions at 100°C for 10 min.

The β-LG phenotype of the samples and assessment of purity was made by CE (Paterson et al.) and reverse phase-HPLC on a Shimadzu HPLC (Shimadzu Corp, Osaka, Japan) using a Vydac C4 4.6 x 150 mm column (Separation Group, Vesperia, CA, USA) with a gradient of 0 to 36 % acetonitrile, 0.05 % trifluoroacetic acid.

Mass spectrometry was performed on a VG Platform single-quadrupole MS (VG BioTech, Altrincham, UK) equipped with a pneumatically assisted electrospray ion source. Each sample was the average of at least 8 acquisitions over a period of 1 min. Raw spectra were analysed using MassLynx software (Fisons Instruments, Altrincham, UK) and the theoretical mass of each β-LG variant calculated using Peptide Tools software (Hewlett-Packard Co., San Diego, CA).

Conclusions

The 324 Da molecular weight adducts observed in ESI-MS of the purified samples of β-LG are consistent with the covalent linkage of a lactosyl residues to the protein.
The observation that these species remain after heat denaturation, reduction and RP-HPLC treatment suggested that the linkage was covalent.
The association of the 324 Da molecule with β-LG does not alter the charge, molecular weight or hydrophobicity of β-LG enough to be detected by CE or RP-HPLC.
The presence of the 324 Da molecular weight species in β-LG prepared by ultracentrifugation suggests that the reaction between milk lactose and β-LG may occur in the udder of the cow.

Reference

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Acknowledgments

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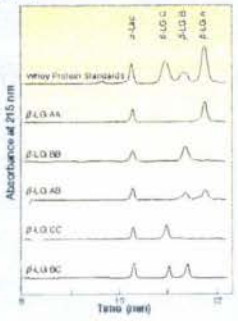
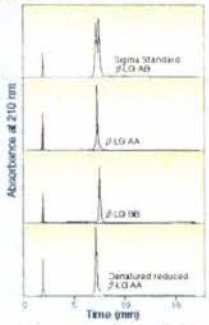
Results

Figure 1



The CE electropherograms in Figure 1 were used to determine the phenotype of the purified β-LG samples.

Figure 2



The purity of the β-LG samples was also confirmed by RP-HPLC (Figure 2). There were no differences in the chromatograms of native β-LG and β-LG which had been heat-denatured under reducing conditions

Figure 3

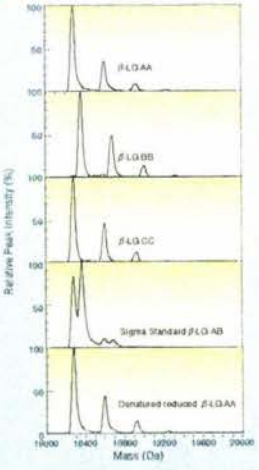


Figure 3 shows the multiple molecular weight β-LG species observed by ESI-MS of a number of purified β-LG samples. The lowest molecular weight species of each sample corresponded to the molecular weight (within error) of the corresponding β-LG variant (Table 1).

Additional adducts differing from the known molecular weights of the β-LG variants by multiples of 324.5 ± 0.9 Da (up to four additional species) were also observed.

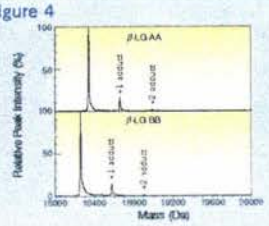
Although less marked, a mixed β-LG A and B sample (Sigma Chem. Co.) also contained one adduct of the +324 molecular weight species attached to both the A and B variants (Figure 3 -arrowed). This sample gave only single peaks (β-LG A and B) upon RP-HPLC.

Heat denaturation, reduction and RP-HPLC, followed by ESI-MS resulted in an increased observed mass of β-LG of 4 Da (due to the reduction of two disulphide bonds). This treatment did not effect the number of +324 molecular weight derivatives.

Table 1: Average molecular mass of β-LG variants and adducts as determined by ESI-MS

Mass (Da)	Variant A	Variant B	Variant C
'Native' protein	18362.3±1.6	18277.0±1.6	18286.2±1.0
+1 adduct	18686.3±1.2	18601.5±1.5	18609.3±0.5
+2 adduct	19011.0±1.4	18924.9±1.4	18934.4±1.3
+3 adduct	19334.7±1.3	19249.6±2.2	19258.6±1.7
+4 adduct	19661.0±1.6	19575.0±2.6	19583.5±2.5

Figure 4



The +324 molecular weight adduct species of β-LG were also observed in the whey samples prepared very 'gently' by ultracentrifugation (Figure 4).