Chromatographic Separation of the Milks of Native and Genetically Modified Mice

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PUBLICATIONS

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ABSTRACT

Selective breeding of dairy cattle has enabled milk composition to be modified by manipulation of the relatively small naturally occurring gene pool. Advances in molecular biology have widened our understanding of the control of gene structure and expression and may be utilised to construct genes encoding protein variants, whose physical properties have been altered. The mouse has proved to be an ideal choice of animal for the study of genetic manipulation of milk proteins because of its short generation time (21 days), large litter sizes and low cost in comparison to larger ruminants. The main drawback however is the relatively small volumes of milk (about 1 ml) produced by the animals. The principal aim of this project was to develop methods for the quantitative separation of the main protein components of mouse milk and utilise these methods to analyse the expression of proteins in the milk of genetically modified animals.

Separation of the major caseins and whey proteins was achieved by cation exchange FPLC. The conditions necessary for the complete dissociation of the murine casein micelles were more extreme than those used in the analysis of milk from other species, particularly the inclusion of the non-ionic detergent n-octyl β-D-glucopyranoside together with 8M-urea. κ-, β- and α-caseins were identified by comparing the amino acid compositions of the purified proteins with their translated cDNA sequences. Another, unidentified acid precipitable casein was also detected. The concentrations of the major acid precipitable proteins in mouse milk were calculated to be WAP, 15.3; κ-casein 4.7; β-casein 25.2 and α-casein 51.7 mg ml^{-1}. The

separation was then scaled up using the Hi-Load system which gave sufficient amounts of the β- and α-casein components to be used in the raising of antibodies to these individual proteins.

Milk from two lines of transgenic mice which expressed a foreign protein in their milk was also analysed. The first line secreted normal ovine β-lactoglobulin in their milk and the effect on the expression of individual mouse milk proteins was investigated. It had previously been shown that in this line, the amount of total protein secreted in the milk was unaltered even although it contained a relatively high proportion of ovine β-lactoglobulin. The actual amounts of the individual murine milk proteins secreted were reduced, the most obvious of which was the α-casein. The concentrations of milk proteins were calculated as WAP, 9.6 mg ml^{-1}; κ-casein, 2.8 mg ml^{-1}, β-casein, 14.3 mg ml^{-1}, α-casein, 20.4 mg ml^{-1} and ovine β-lactoglobulin, 50.1 mg ml^{-1}.

The other line of transgenic mice expressed ovine β-lactoglobulin which had been genetically modified to contain an oligomer containing a casein kinase recognition sequence on a readily accessible part of the protein molecule. It was hoped that the inclusion of this oligomer would result in the normally unphosphorylated protein being phosphorylated. As was demonstrated by its staining with the cationic dye Stains-all, the protein did not appear to be phosphorylated. To rule out the possibility of dephosphorylation due to the presence of endogenous phosphatases in the milk, the milk was collected and stored in a phosphatase inhibitor. Since this had no effect on protein staining with Stains-all, it can be concluded that protein phosphorylation requires more than just the appropriate kinase

recognition sequence.

The last line of mice to be studied were ones in which the murine β-casein gene had been deleted. In this line the overall level of casein decreased by 11.5% and that of whey protein increased by about 16%. The increased level of whey protein could however be accounted for by changes in the partitioning of these proteins between the acid precipitated and supernatant fractions and suggests that β-casein may be important in influencing the isoelectric precipitation of whey proteins in native mouse milks. Again, changes in the levels of expression of the remaining caseins were not uniform and may indicate that the proteins are secreted by different pathways.

CHAPTER 1

1. GENERAL INTRODUCTION

1. 1 COMPOSITION OF MILK

Milk is a complex fluid the principal of which is to supply nutrients to the young. Since mammals are born at different stages of development, variations in the composition of milk will exist between species in order to suit the requirements of the offspring. For some animals, milk provides all of the nutritional requirements for many months and must therefore be a source of vitamins, minerals, energy and amino acids in order to sustain growth and development.

Although the composition of milk has been investigated in a number of species, much remains unknown about many others. Interspecies differences occur in the quantitative composition of milk. Table 1 shows the gross composition of milks from thirty species. In addition to these interspecies differences, variations exist in the composition of milk within a given species depending upon the age of animal, its stage of lactation and also genetic variation within that species. Gross compositional changes in milk arise from differences in the relative rates of synthesis and secretion of milk components. Studies of lactational changes in milk have been carried out for a few species. Changes in the composition of bovine milk at successive milkings after parturition are shown in Figure 1A (Jenness, 1985), and Figure 1B

TABLE 1: Composition of milks of various species

Species	Percentage by Weight						Energy (kcal/ 100g)
	Water	Fat	Casein	Whey protein	Lactose	Ash	
Echidna (*Tachyglossus aculeatus*)		19.6	8.4	2.9	2.8	0.8	233
Opussum (*Didelphis virginiana*)	76.8	11.3	8.4[a]		1.6	1.7	142
Red kangaroo (*Macropus rufus*)	80.0	3.4	2.3	2.3	6.7	1.4	76
Hedgehog (*Erina Europaeus*)	79.4	10.1	7.2[a]		2.0	2.3	100
Fringed bat (*Myotis thysanodes*)	59.5	17.9	12.1[a]		3.4	1.6	223
Tree shrew (*Tupaia belangeri*)	59.6	25.6	10.4[a]		1.5		278
Human (*Homo sapiens*)	87.1	4.5	0.4	0.5	7.1	0.2	72
Sloth (*Bradypus variegatus*)	83.1	2.7	6.5[a]		2.8	0.9	62
Rabbit (*Oryctolagus cuniculus*)	67.2	15.3	9.3	4.6	2.1	1.8	202
Gray squirrel (*Sciurus carolinensis*)	60.4	24.7	5.0	2.4	3.7	1.0	267
Rat (*Rattus norvegicus*)	79.0	10.3	6.4	2.0	2.6	1.3	137
Guinea pig (*Cavia porcellus*)	83.6	3.9	6.6	1.5	3.0	0.8	80

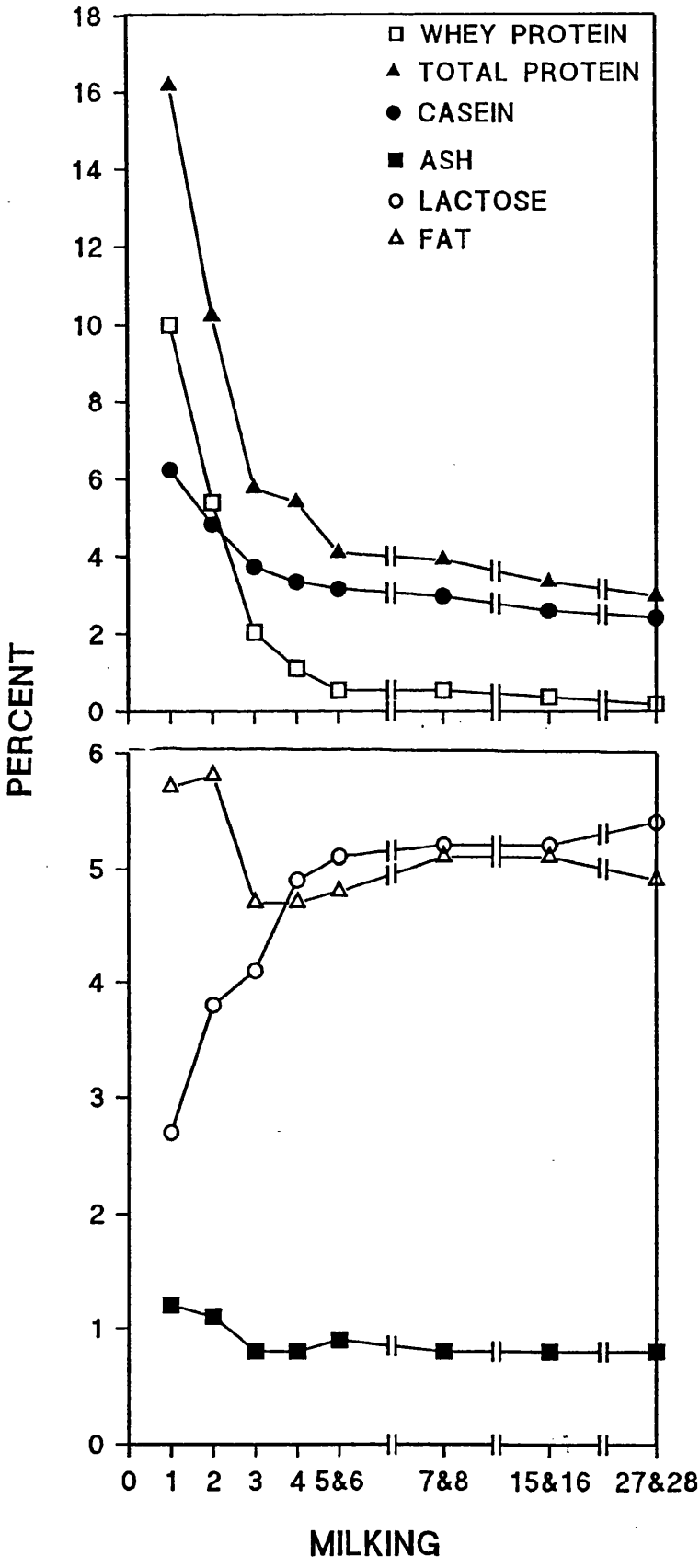
Species	Percentage by Weight						Energy (kcal/ 100g)
	Water	Fat	Casein	Whey protein	Lactose	Ash	
Dolphin (*Tursiops truncatus*)	58.3	33.0	3.9	2.9	1.1	0.7	329
Dog (*Canis familiaris*)	76.4	10.7	5.1	2.3	3.3	1.2	139
Black bear (*Ursus americanus*)	55.5	24.5	8.8	5.7	0.4	1.8	280
Fur seal (*Callorhinus ursinus*)	34.6	53.3	4.6	4.3	0.1		
Aardvark (*Orycteropus afer*)	68.5	12.1	9.5	4.8	4.6	1.4	184
Indian elephant (*Elephas maximus*)	78.1	11.6	1.9	3.0	4.7	0.7	143
Manatee (*Tricheehus manatus*)	87.0	6.9	6.3[a]		0.3	1.0	88
Horse (*Equus caballus*)	88.8	1.9	1.3	1.2	6.2	0.5	52
Donkey (*Equus asinus*)	88.3	1.4	1.0	1.0	7.4	0.5	44
Pig (*Sus scrofa*)	81.2	6.8	2.8	2.0	5.5	1.0	102
Camel (*Camelus dromedarius*)	86.5	4.0	2.7	0.9	5.0	0.8	70
Reindeer (*Rangifer tarandus*)	66.7	18.0	8.6	1.5	2.8	1.5	214
Cow (*Bos taurus*)	87.3	3.9	2.6	0.6	4.6	0.7	66
Zebu (*Bos indicus*)	86.5	4.7	2.6	0.6	4.7	0.7	74

Species	Percentage by Weight						Energy (kcal/ 100g)
	Water	Fat	Casein	Whey protein	Lactose	Ash	
Yak (*Bos grunniens*)	82.7	6.5	5.8[a]		4.6	0.9	100
Water buffalo (*Bubalus bubalis*)	82.8	7.4	3.2	0.6	4.8	0.8	101
Goat (*Capra hircus*)	86.7	4.5	2.6	0.6	4.3	0.8	70
Sheep (*Ovis aries*)	82.0	7.2	3.9	0.7	4.8	0.9	102

[a] Percentage of combined protein

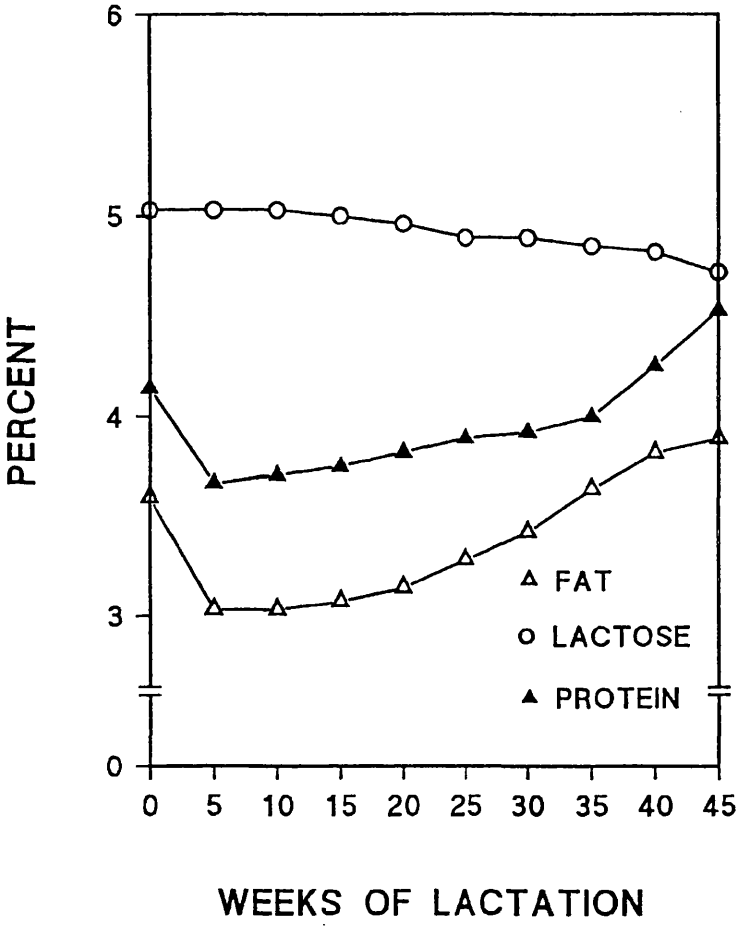
Data from Jenness, 1985

FIGURE 1(A)



Change in composition of milk at successive milkings after parturition.

FIGURE 1(B)



Changes in protein, fat and lactose contents of milk from dairy cows over the lactation period.

demonstrates the changes which occur in milk over the whole lactation period (Jenness, 1985). In addition to its nutritional role, milk is also important in some species as a means of conveying immunological protection from mother to her offspring. Mammals can be divided into three groups on the basis of their mode of transmitting immunoglobulins. Group I(humans, rabbits and monkeys), acquire all immunoglobulins *in utero*; group II(mice, rats, cats and dogs) acquire maternal immunoglobulins *in utero* and also from colostrum while group III(cows, horses, pigs and goats) acquire maternal immunoglobulins from colostrum in the first few hours after birth. The immunoglobulin composition of colostrum from groups I, II and III is also different, with group I colostrum being predominantly composed of IgA, group III composed of IgG and group II being intermediate.

Milk proteins of all species examined to date can be conveniently divided into two main classes, the caseins and the serum or whey proteins. The caseins have been defined as those proteins which are precipitated when skimmed milk is acidified to pH 4.6 at 20°C(Jenness *et al.*, 1956; Eigel *et al.*, 1984) and the whey proteins are those which remain in solution. Although this definition may be accurate for bovine milk which, due to its economic importance, is the species most extensively studied, the optimum pH for precipitating the caseins of other species may vary.

1.1.1 WHEY PROTEINS

The whey proteins identified to date include β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin, transferrin, immunoglobulins,

proteose peptones and whey acidic protein. Some whey proteins, ie. β-lactoglobulin, α-lactalbumin and whey acidic protein, are synthesized by the mammary gland whilst others are derived directly from the blood. Interspecies variations occur in the variety of whey proteins present in milk, with β-lactoglobulin being present in caprine, ovine and bovine milks and whey acidic protein being restricted to the milk of rodents. Unlike the caseins, the whey proteins of most species are not phosphorylated, contain more cysteine residues and possess a folded globular structure. In the case of β-lactoglobulin, five half cysteines are present in the polypeptide chain, four of which are paired in disulphide bonds. At physiological pH, bovine β-lactoglobulin exists mainly as a dimer whereas that of the horse and pig is monomeric. Despite a great deal of research into all aspects of its structure and possible function, the actual role(s) of β-lactoglobulin is still unclear. α-Lactalbumin contains four cysteine residues present as two disulphide bridges, and exists as a monomer in milk (Davies *et al.* 1983). The whey acidic protein present in rodent milk is a cysteine-rich protein. Its structure will be discussed later. As with β-lactoglobulin, the role of this protein has yet to be determined.

1.1.2 CASEINS

The caseins are a group of phosphoproteins which represent the major protein fraction in the milk of most species (Jenness, 1973, 1979), accounting for approximately 80% of the total milk protein. Their high proline content, with the proline residues being widely distributed along the length of

the polypeptide chain, inhibits folding and results in a relatively open tertiary structure. This facilitates their digestion by proteolytic enzymes. These proteins contain few or no cysteine residues and therefore lack the capacity to stabilise their internal structure by disulphide bridge formation. The chemistry of the caseins has recently been reviewed by Swaisgood (1992).

Bovine, caprine and ovine caseins have been purified, quantified and named α_{s1}-, α_{s2}, β-(β_1 and β_2 for caprine and ovine caseins), κ- and γ-caseins. Similarly, murine caseins have been identified and named α_{s1}-, α_{s2}- ,β_1-, β_2-, γ-, δ-, κ- and more recently ϵ-casein (Hennighausen & Sippel, 1982a). The caseins were one of the first group of proteins discovered to contain covalently bound phosphorus. Studies of phosphoproteins have shown that only six amino acid residues, namely serine, threonine, histidine, lysine, aspartic acid and glutamic acid are involved in phosphorylation. In the caseins, phosphorylation is limited to serine and some threonine residues. Phosphoserine residues frequently occur in clusters which in the caseins consist of up to four residues.

The degree of phosphorylation is very variable. Bovine α_{s1}-, α_{s2}-, β- and κ-casein contain 8/9, 10-13, 5 and 1 phosphorylated serine residues respectively. Phosphorylation of seryl and threonyl residues occurs when the sequence Ser/Thr-X-Glu/Ser-P (where X is any amino acid residue), is recognised by an enzyme, casein kinase (Mercier *et al.*, 1972) There are exceptions to this rule, as demonstrated by the case of bovine α_{s2}- and κ-caseins. In bovine κ-casein, the amino acid residues (Ser, Thr, Thr) at positions 127, 135 and 145 are not phosphorylated although they are all one residue away from the N-terminal end of a glutamic acid residue. There are

two possible explanations for this. Either these residues are not accessible to the kinase because of steric hindrance arising from the tertiary structure of the molecule (Mercier *et al.*, 1972), or the recognition sequences are masked by the carbohydrate chains which are bound to this portion of the molecule. The lack of phosphorylated residues in bovine β-lactoglobulin and α-lactalbumin reinforces the recognition sequence theory of phosphorylation. Both of these proteins contain serine and threonine residues. However, only one of these occurs in the sequence Ser/Thr-X-Glu/Ser-P (Brew *et al.*, 1970; Lyster, 1972). It is of course possible that in these highly folded, globular proteins, the residues which could be phosphorylated are simply not located in regions accessible to the enzyme(s) which catalyses the reaction. This possibility was investigated as part of the current project and will be reported later in this work.

As with phosphorylation, glycosylation is a post-translational modification. Glycosylation occurs by the sequential transfer of monosaccharide units from nucleotide sugars to the polypeptide chains. The process may start in the smooth encloplasmic reticulum and end in the Golgi apparatus (Lyster, 1972; Soulier & Gaye, 1981). In the bovine caseins, only three threonyl residues in κ-casein are glycosylated (Kanamori *et al.* 1980). All of the carbohydrate moieties are located on the macropeptide portion of κ-casein, i.e. that part of the molecule at the C-terminal end which is cleaved by the enzyme, chymosin, at the Phe-Met bond at residues 105-106. It is currently believed that glycosylation occurs on a β-turn (Loucheux -Lefebvre *et al.*, 1978).

Caseins possess hydrophobic surfaces (Bigelow, 1967) and are secreted into milk as stable aggregates known as micelles. The structure of the casein micelles is still a matter of some debate. The various models which have been proposed will be considered briefly.

1.2 CASEIN MICELLES

The caseins are present in milk in the form of complex aggregates known as micelles and it is these micelles and their light-scattering properties which give milk its opalescent appearance. The only species whose micellar structure has been examined in any detail is the cow, once again because of its economic importance. The structure of casein micelles has recently been reviewed by Holt (1992).

1.2.1 CHEMICAL PROPERTIES

Micelles are highly hydrated, colloidal particles which contain about 3.7 g H_20/g protein. However, only 0.5 g of water is actually protein bound, the remainder being occluded within the micelle (Bloomfield & Mead, 1975; McMahon & Brown, 1984). Casein forms about 93% of the dry weight of bovine casein micelles. α_{s1}-, α_{s2}-, β- and κ-Caseins are present in a ratio of approximately 3:1:3:1, respectively. The remaining 7% of the dry weight of casein micelles consists mainly of calcium and phosphate (known as colloidal calcium phosphate) and small amounts of citrate and magnesium. Table 2 shows the typical composition of bovine casein micelles (Davies & Law, 1977a, b, 1980,; Barry & Donnelly, 1980; Schmidt 1980; McMahon &

TABLE 2: Composition of Casein Micelles (g/100g) in Bovine Milk

α_{s1}-Casein	35.6	Davies & Law 1977a, b Barry & Donnelly, 1980
α_{s2}-Casein	9	"
β-Casein	33.6	"
κ-Casein	11.9	"
Minor Caseins	2.3	"
Calcium	2.9	McMeekin & Groves, 1965
Magnesium	0.1	"
Inorganic Phosphate	2.9	"
Citrate	0.4	"
Sodium	0.1	"
Potassium	0.3	"
Sialic Acid	0.3	Schmidt & Van Markwijk, 1968
Galactose	0.2	"
Galactose Amide	0.2	"

Data from McMahon & Brown, 1984

Brown, 1984). Micelles, therefore, in addition to being a source of amino acids for young animals, also enable calcium and phosphorus to be transported in milk in a stable form.

1.2.2 PHYSICAL PROPERTIES

Micelles are roughly spherical, and electron microscopy places micellar size in the region of 20-300 nm (Schmidt *et al.*, 1973). The existence of larger particles has also been demonstrated (Brooker & Holt, 1978). Inelastic light scattering techniques have shown that 80% of micelles by weight have diameters in the region of 100-200 nm, with 95% of micelles having a diameter between 80 and 440 nm(Lin *et al.*, 1971). Micellar size was found to be dependent on the proportion of κ-casein, with smaller micelles containing relatively more κ-casein (Garnier, 1973). Characteristics of casein micelles are listed in Table 3.

1.2.3 MICELLAR STABILITY

Casein micelles are generally very stable. However, under certain conditions they may disintegrate or aggregate. The integrity of micellar structure is dependent on calcium ion activity and on factors such as pH, temperature and citrate concentration. Removal of calcium from micelles using EDTA has been shown to cause micellar dissociation. Removal of subcritical amounts of calcium results in a progressive decrease in the sedimentation velocities of micelles but without altering their hydrodynamic radii. This suggests that calcium removal initially results in dissociation of weakly bound

TABLE 3: Average characteristics of bovine casein micelles

Characteristics	Value
Diameter	130-160 nm
Surface	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (Hydrated)	1.0632 g/cm^3
Mass	2.2×10^{-15} g
Water Content	63%
Hydration	3.7 g H_2O/g Protein
Voluminosity	4.4 cm^2/g
Molecular Weight (Hydrated)	1.3×10^9 Daltons
Molecular Weight (Dehydrated)	5×10^8 Daltons
Number of Peptide Chains (MW:30000)	10^4
Number of Particle/ml Milk	10^{14}-10^{16}
Whole Surface of Particle	5×10^4 cm^{2}ml^{-1} Milk

(McMahon and Brown, 1984)

casein from the micelle (Lin *et al.* 1972). Solubilisation of colloidal calcium phosphate was also achieved by dialysing micelles against calcium-free buffer resulting in micellar dissociation (Schmidt & Buchheim, 1970).

A decrease in pH to 6.0 also causes great changes within the micelle, mainly due to dissolution of colloidal calcium phosphate. At even lower pH values(pH 5.5), there is an increase in the formation of salt bridges. Colloidal calcium phosphate affects many properties of casein in milk and its removal results in both an increased sensitivity to calcium and increased coagulability when treated with rennet or heat (McGann & Pyne, 1960).

A decrease in temperature results in an increased micellar volume, presumably due to protruding hairs of mainly β-casein(Walstra, 1990). At temperatures above 70°C, parts of the casein molecules become more flexible. Micelles are even stable at the very high temperatures used during sterilization and pasteurization demonstrated by the fact that bulk bovine milk coagulates in 20 minutes when heated to 140°C. Coagulation is thought to be due to a variety of causes such as hydrolysis of κ-casein, dephosphorylation of casein, a decrease in pH due to thermal decomposition of lactose, precipitation of calcium phosphate and the neutralization of carboxylic acid groups by binding of calcium (Rose, 1965; Fox & Morrissey, 1977; Fox, 1982). Micelles possess surface potential charge of approximately 17 mV (Green & Pearce) and this potential provides a barrier against micellar coagulation. However, the exact contribution which surface charge makes to this stability has not fully been determined, since hydrophobic colloids with surface potentials of less than 20-30 mv are generally unstable (McMahon &

Brown, 1984; Darling & Dickson, 1979). Treatment of micelles with rennet reduces the surface potential (zeta-potential) by 5-7 mv, due to the loss of glycomacropeptide which creates a more hydrophobic micellar surface carrying para-κ-casein and leads to micellar coagulation (Green & Crutchfield, 1971; Pearce, 1976; Dalgleish, 1982a,b; 1992). This is the basis of cheese manufacture.

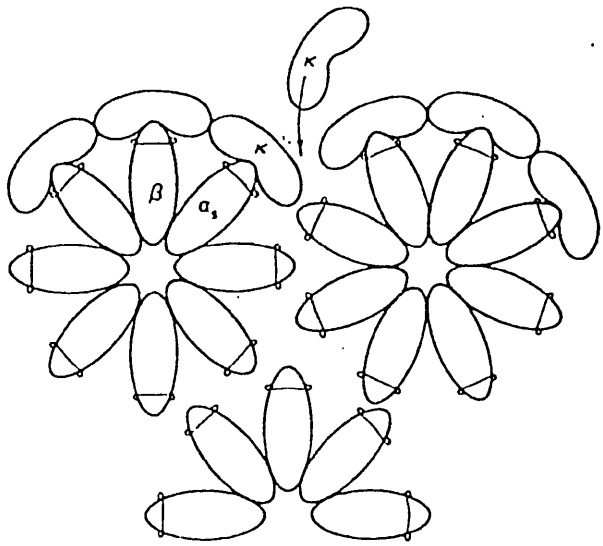
1.3 MICELLAR MODELS

A number of theories on the structure of casein micelles exist and these have been classified into three categories; coat-core models, internal structure models and submicellar models (Schmidt, 1982).

1.3.1 COAT- CORE MODELS

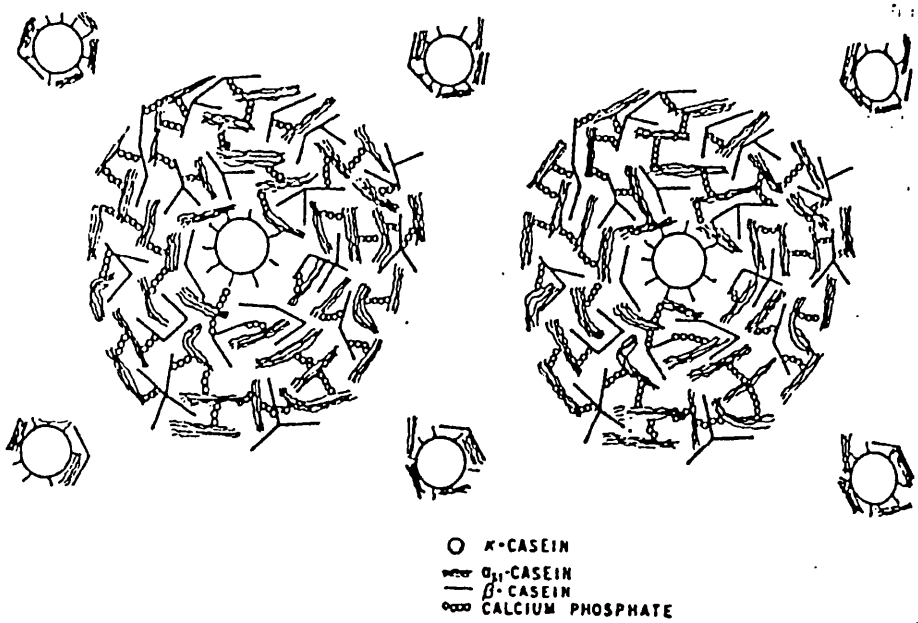
This model is based on the solubilities of caseins in calcium solutions. The monomers of α_{s1}- and β-caseins have charged phosphate groups and in the presence of calcium, these monomers aggregate to a limiting size to form the core. This caseinate core is then stabilized by a monolayer of κ-casein over its entire surface (Figure 2A). The micelle size is therefore dictated by the amount of κ-casein available (Waugh & Noble 1965; Waugh *et al.* 1970; Waugh, 1971; Garnier, 1973). This model explains the rennet-induced aggregation of micelles, where the rennet hydrolyses κ-casein at or near the surface of the micelle. However, Ashoor *et al.* (1971) found that α_s-, β-, and κ-caseins were all cleaved at similar rates by high molecular weight polymers of the proteolytic enzyme, papain. This suggests that all three components

Figure 2A



Waugh's coat-core model of the casein micelle. H.A. McKenzie (ed.)., *Milk Protein*, **2**, 58, Academic Press, London, 1971.

Figure 2B



Coat-core model of the casein micelle proposed by Parry and Carroll (1966).

occupy surface positions on the micelle in relatively the same proportions that they occur in milk (Ashoor *et al.* 1971). In another model, Parry & Carroll, (1969), concluded that κ-casein serves as a nucleation point where calcium-insoluble caseins cluster and are then stablized by colloidal calcium phosphate (Figure 2B).

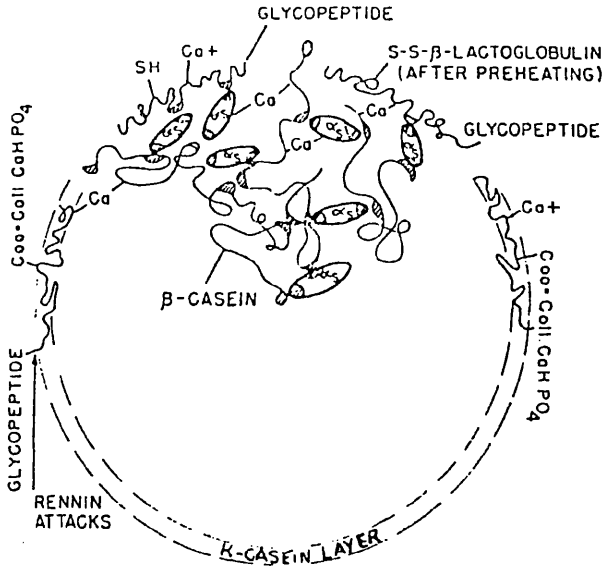
1.3.2 INTERNAL STRUCTURE MODEL

In one of the first models, Payens (1966), postulated that micelles consisted of a mesh of β-casein threads to which α_s-casein molecules were attached by hydrophobic forces. These were then surrounded by a layer of κ-casein and calcium phosphate(Figures 2C). This model was revised by Rose (1969), who proposed that micelles are built up from polymers of β-casein to which α_{s1}- and κ-caseins are attached by hydrophobic bonds and interconnected by colloidal calcium phosphate bridges (Figure 2D). Another model proposed that trimers of κ-casein are linked to three chains of α_{s1}-casein and β-casein nodes, forming a loosely packed network (Figure 2E) (Garnier & Ribadeau-Dumas, 1970; Garnier, 1973).

1.3.3 SUBMICELLAR MODELS

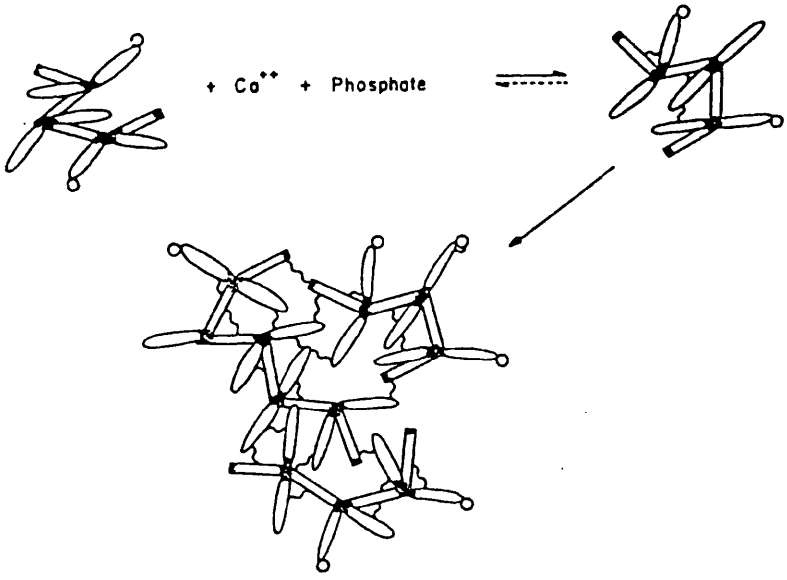
Morr (1967), proposed a submicellar model for casein micelles, consisting of a core of β- and α_s-casein complexes with an outer layer of κ- and α_s-casein complexes. These submicelles are stabilized by hydrophobic bonding and calcium caseinate bridges, being finally aggregated into a micellar structure by colloidal calcium phosphate (Figure 3). Another

Figure 2C



Internal structure model of the casein micelle proposed by Payens (1966).

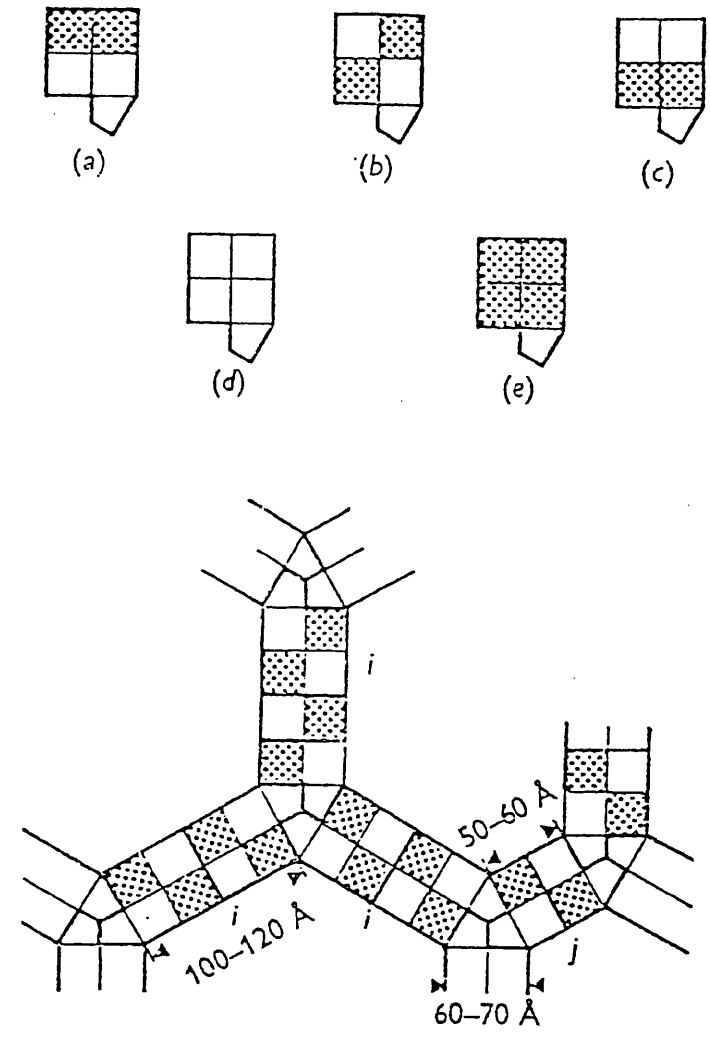
Figure 2D



Internal structure model of the casein micelle proposed by Rose (1969).

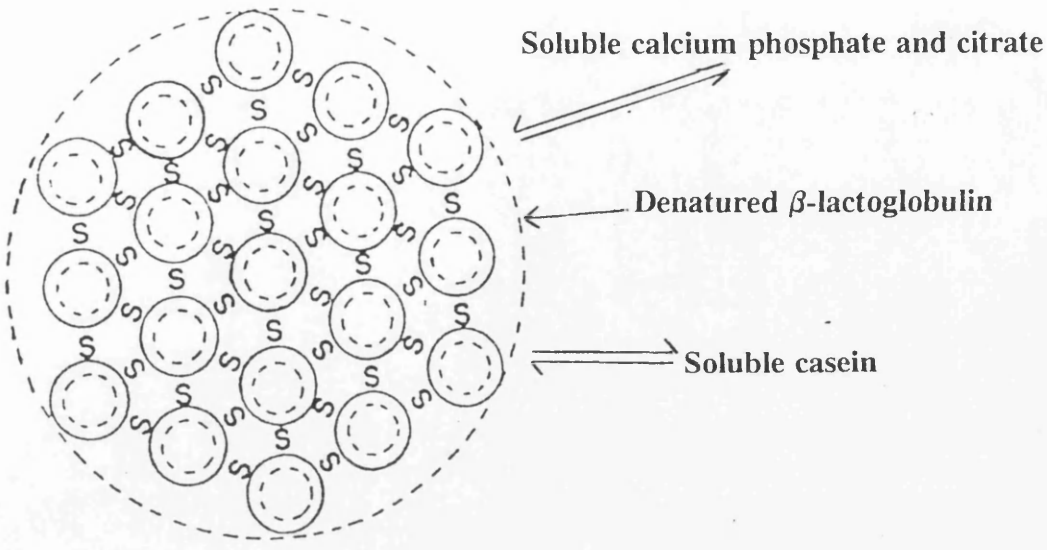
Rods represent β-casein, circles represent κ-casein, more eliptical rods represent α_{s1}- casein and s-shaped lines represent apatite chain formation.

Figure 2E



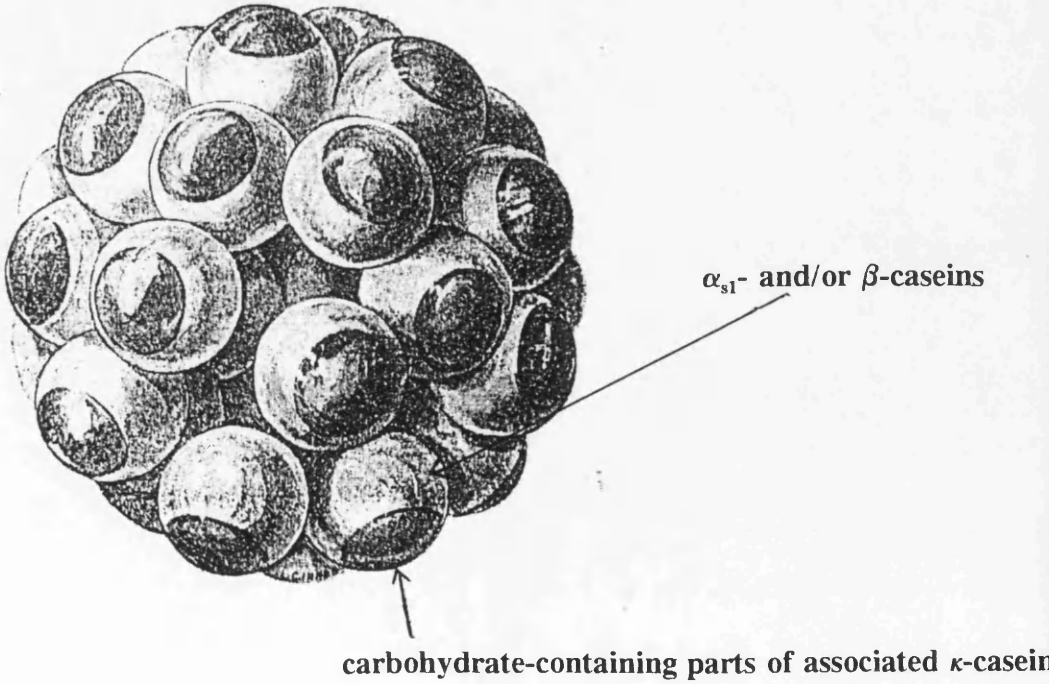
Repeating unit of the internal structure model of the casein micelle proposed by Garnier and Ribadeau-Dumas (1970).

Figure 3



Submicellar model of the casein proposed by Morr (1967). The s-shaped lines represent calcium phosphate linkages between small spherical complexes of the α_{s1}-, β-, and κ-caseins.

Figure 4

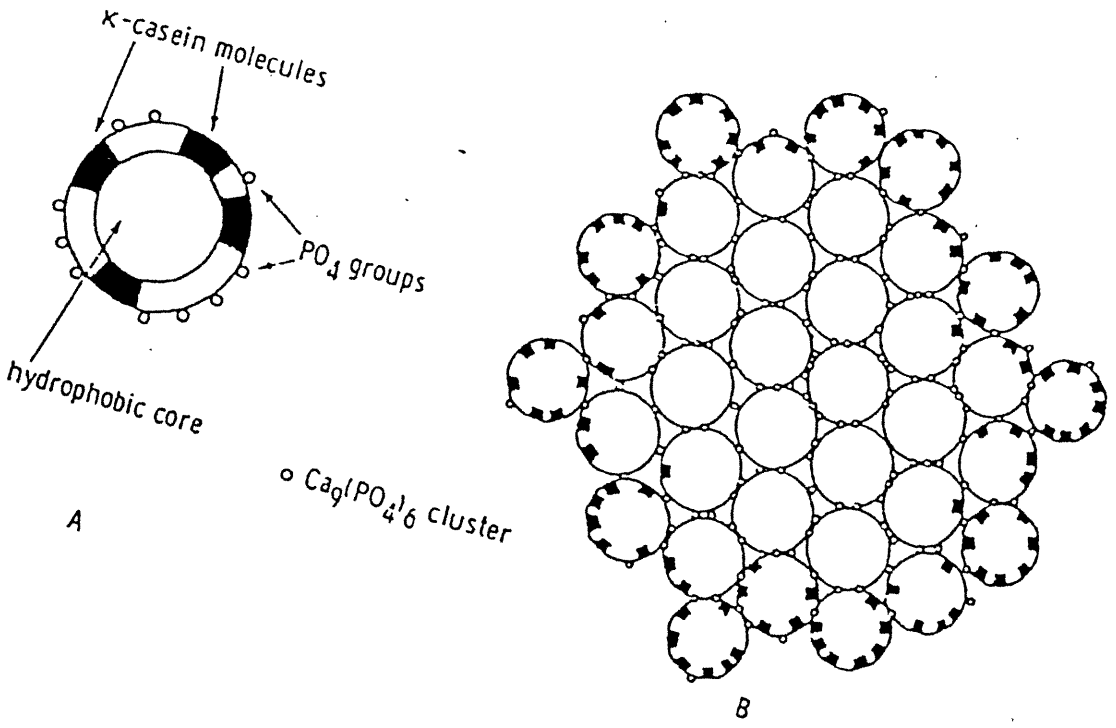


Submicellar model of the casein micelle proposed by Slattery and Evard (1973). The micelle was proposed to consist of approximately forty subunits.

submicellar structure was proposed based on the above model in which submicelles consisted of a hydrophobic core and a hydrophilic coat of carboxylic and phosphate groups linked by calcium, magnesium and colloidal calcium phosphate.

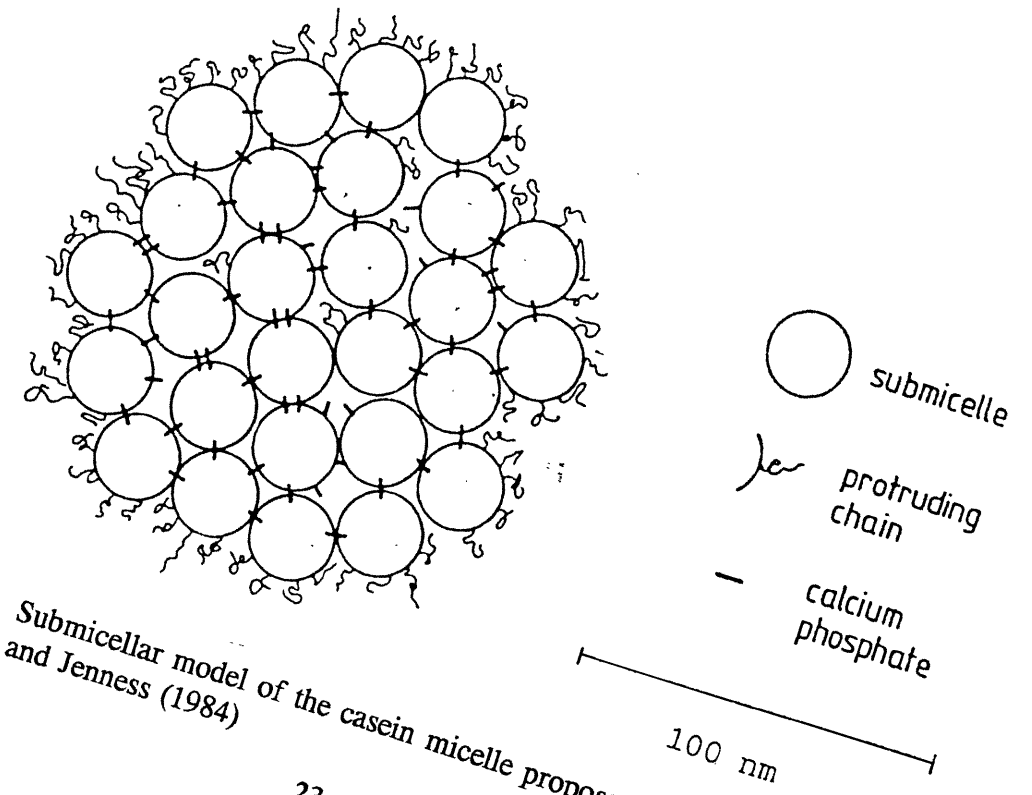
Slattery & Evard (1973) and Schmidt & Payens (1976) proposed the existence of submicelles having a variable casein composition, in which κ-casein monomers associate to form a hydrophilic area on the surface of the submicelle. These submicelles then aggregate to form micelles via hydrophobic bonds. Such micelles would then have a surface consisting of κ-casein (Figure 4). This idea was adapted again by Schmidt (1982), when it was postulated that submicelles had a hydrophobic core and a hydrophilic coat in which polar moieties of κ-casein were accumulated in one area. The remainder of the coat consisted of polar areas of other caseins - mainly the phosphoserine side chains of α_{s1} -, α_{s2}- and β-caseins (Figure 5A). Another submicellar model of the casein micelle has also been proposed, (Walstra and Jennes 1984, Walstra 1990) in which a micelle is composed of 15-25 submicelles with diameters of between 10 and 15 nm. The submicelles are then linked together by colloidal calcium phosphate and stabilised by hydrophobic and electrostatic bonds. Two major types of submicelles have also been proposed, those with and without κ-casein, and submicelles containing κ-casein predominantly located at the surface of the micelle (Figure 5B). Although the exact distribution of the various caseins in the micelle is still a matter for debate it is now generally accepted that the surface isstabilised by a "hairy" layer of κ-casein. Addition of chymosin or ethanol to

Figure 5A



Submicellar model of the casein micelle proposed by Schmidt and Payens (1976).

Figure 5B



Submicellar model of the casein micelle proposed by Walstra and Jenness (1984)

micelles leads to a shaving or flattening of this layer resulting in a reduction in the size of the micelles followed by an increase as the individual micelles aggregate. The structure and stability of bovine casein micelles has recently been reviewed by Holt (1992).

1.4 MODIFICATION OF MILK COMPOSITION

Ever since the domestication of dairy animals, selective breeding has enabled milk composition to be modified by using the relatively small, naturally occurring variations in the genetic pool. In recent years, increased understanding of the control of gene structure and expression has afforded the potential to modify the structure of milk in a much more flexible fashion. Molecular biology permits the construction of a whole new series of genes encoding for protein variants whose physical properties have been altered in a beneficial way, at least from the point of view of food manufacturers. By the use of transgenic animals, these changes in gene structure can be transmitted from one generation to the next. Transgenic animals can be defined as those in which foreign DNA has been integrated into their germline as a consequence of the experimental introduction of DNA. The most common way of producing a transgenic animal is by the micro-injection of recombinant DNA directly into the pronuclei of fertilized eggs (Gordon *et al.*, 1980). This foreign DNA is therefore carried in both the somatic and germ cells. Genes can be isolated from one animal, modified as required *in vitro* and then reintroduced into embryos of the same or a different species. These

animals are now transgenic and can pass on the modified gene to their offspring in a Mendelian fashion.

Since milk is such an easily processed and relatively simple source of protein, totally foreign, pharmacologically active and industrially useful proteins may also be expressed in milk. A number of such modifications are listed in Table 4 (Bremel *et al.*, 1989). Some potentially useful changes to bovine milk proteins are also listed in Table 5 (Jimenez-Flores & Richardson, 1988). Unlike bacteria which have also been used to produce foreign proteins, animals are capable of carrying out post-translational modifications of proteins, ensuring that the final protein product is fully functional. Tissue cultures can also be employed in the production of recombinant proteins but they are expensive and difficult to work with. Transgenic animals, although initially expensive, ultimately provide a cheaper source of recombinant proteins.

Transgenic mice have been bred that carry the ovine β-lactoglobulin gene (Simons *et al.*, 1987). This protein was found to be abundantly expressed in the mouse mammary gland during lactation. The rat and caprine β-casein genes, and guinea pig and bovine α-lactalbumin genes, have also been expressed in mouse milk in a similar fashion (Lee *et al.*, 1988, 1989; Maschio *et al.*, 1991; Vilotte *et al.*, 1989; Persuy, 1992). Pigs have also been used in transgenic experiments, with the gene encoding for mouse whey acidic protein being integrated into the pig genome (Shamay *et al.*, 1991; Wall *et al.*, 1991).

TABLE 4: Mammary expression in transgenic animals (Bremel *et al.*, 1989)

Protein	Gene Source	Host	Promoter	Reference
α-Lactalbumin	Bovine	Mouse	α-Lactalbumin	Villote *et al.*, 1989
β-Lactoglobulin	Sheep	Mouse	Sheep-β-lactoglobulin	Simons *et al.*, 1987
Thymidine kinase	Herpes simplex virus	Mouse	Mouse metallothionein-I	Brinster *et al.*, 1982
Clotting factor IX*	Human	Sheep	Sheep β-lactoglobulin	Simons *et al.*, 1988
α-1 antitrypsin*	Human	Sheep	Sheep β-lactoglobulin	Simons *et al.*, 1988
T-PA*	Human	Mouse	Rat WAP	Gordon *et al.*, 1987
CAT	Bacteria	Mouse	Rat β-casein	Lee *et al.* 1988a
Ha-Ras	Human	Mouse	Rat WAP	Andres *et al.*, 1987
Casein*	Rat	Mouse	Rat β-casein	Lee *et al.*, 1988b

* Secreted into the milk

Data from Bremel *et al.* 1989

TABLE 5: Some proposed mutations on milk proteins to study their functionality

Protein	Potential Change	Potential Effect
α_{s1}-Casein	-$SerP_{115}$ to Ile_{115}(AGT to ATT) -Addition of a glycosylation sites in either $Asn_{17,19,74}$ or $_{114}$ (all possible sites need a change of one or two bases)	-Reduce charge and increase the hydrophobicity -Enhance amphiphilicity and solubility
β-Casein	-Addition of a glycosylation site in Asn_7 or Asn_{27} -Addition of the phosphorylation domain (a.a. 10 to 25) at the C terminus of the protein -Change Glu_{36} and $_{37}$ to Val(GAG to GTG and GAA to GTA)	-Enhance solubility, increase amphiphilicity -Enhance binding for calcium and other divalent cations -Enhance the hydrophobic patch on the amphipathic helix (predicted by Holt and Sawyer, 1988) -Turn the C terminus of β-casein into a better substrate for transglutaminase
κ-Casein	-Change Arg_{97} to Cys_{97} (CGT to TGT) -Change Phe_{105}-Met_{106} to Phe_{105}-Phe_{106} -Addition of a poly-Met residues at the N- terminus -Change Ser_{149} to Glu_{149} (TCT to GAA)	-Increase the heat stability of the protein and improve the thermalization characteristics of milk. -Increase the hydrolysis rate by chymosin -Improve the nutritional characteristics and possible enhancement of cheese flavour development -Increase calcium stability of the protein and micelle
α-Lactalbumin	-Modification of the binding site to galactosyl transferase -Anti-sense mRNA -Modification of calcium binding site	-Alter the efficiency of lactose synthesis
β-Lactoglobulin	-Anti-sense mRNA	-Reduction of β-lactoglobulin in milk

Data from Jiminez-Flores & Richardson, 1988

Although ultimately, the larger ruminants such as the sheep, goat and cow would be the animals of choice for the expression of both modified milk proteins and pharmacologically active proteins/peptides, the production of transgenic animals is still extremely difficult. Successful production of transgenic animals is very low and expression of the modified proteins is variable. Because of their large size and long generation times, together with the small numbers of offspring, the use of large ruminants in preliminary experiments would be prohibitively expensive. Therefore, various smaller animals have been used in laboratory experiments. The most common of these is the mouse. Its small size, short gestation period (21 days), rapid maturity and large litter size make it an ideal model. However, its low yield is a drawback since each dam typically yields less than 1 ml of milk per milking. Furthermore, although the mouse has been used in many studies related not only to the expression of modified and foreign proteins in its milk but also to the development and control of mammary function, its milk protein composition is poorly characterized.

The original intention of this work was to develop chromatographic methods to separate the proteins, particularly the caseins, of mouse milk, and then, by using transgenic animals expressing bovine β-casein in their milk to investigate the effects on protein composition. The bovine β-casein gene would then have been modified so as to alter the functional properties of the protein, and the modified protein(s) could then be isolated and their physical properties determined. Unfortunately, difficulties were encountered with the cloning of the bovine β-casein gene. Therefore, this thesis deals with: (1) the

development of techniques to separate, identify and quantify the major proteins of mouse milk; (2) the effect of expression of a foreign protein (ovine β-lactolgobulin) on the levels of expression of endogenous proteins; (3) the expression of a modified ovine β-lactoglobulin containing the potential phosphorylation site, and (4) the effect of deletion of a mouse casein gene on the levels of expression of other proteins in the milk.

CHAPTER 2

SEPARATION AND PROPERTIES OF MOUSE MILK PROTEINS

2.1 INTRODUCTION

2.1.1 GROSS CHEMICAL COMPOSITION

Analysis of the overall composition of murine milk did not begin until 1964 when investigations on the composition of the milk were made by Meier *et al.* (1964). This was apparently due to the difficulties encountered in obtaining a large enough sample for analysis. Analysis was carried out on two unrelated inbred strains of mice, namely DBA 2/J and C51BL/6J, which were fed diets identical in protein content and source.

The gross compositional analysis of mouse milk (Table 6) shows that it is very concentrated, containing approximately 70% water compared with bovine milk which contains about 88% water and rat milk which contains about 74% water. The amount of lipid present in murine milk is also much greater than in that of the cow, approximately 13% compared to approximately 3.9% (Nagasawa *et al.*, 1989). A high lipid content, as demonstrated in the kangaroo rat (Klooyman, 1963) and seals and whales, is thought to be advantageous for water conservation (Jenness & Sloan, 1970; Baverstock *et al.*, 1976).

TABLE 6: Gross composition of mouse milk of a number of strains

	Mouse Strains				JENNESS & SLOAN[a]	BAVERSTOCK *et al.*[b]
	SHN	SLN	C_3H/HE	GRIA		
	% of Total					
FAT I[c]	14.8	15.2	14.2	13.2	13.1	21.4
II	15.5	16.0	17.8	11.7		
III	17.5	16.3	23.0	11.9		
Protein I	10.2	10.2	10.5	10.5	9.0	6.4
II	9.3	10.0	11.0	9.9		
III	10.3	11.0	12.1	10.5		
Lactose I	2.6	2.2	1.8	2.2	3.0	2.3
II	2.3	2.2	1.4	2.1		
III	2.4	2.0	1.9	2.0		
Ash I	1.8	1.8	1.9	1.8	1.3	
II	1.7	1.7	1.8	1.7		
III	1.8	1.8	1.8	1.8		
Moisture I	69.8	68.3	66.4	68.4	70.7	63.5
II	67.7	67.5	64.1	70.7		
III	66.4	66.5	59.8	69.6		

Data from Nagasawa, Naito & Kataoka, 1989

(a) In white Swiss strain
(b) Mean values obtained from three periods during the first lactation in BALB/C mice
(c) Lactation number

The protein concentration of murine milk is generally in the region of 90 mg ml^{-1} but it can be as high as 213 mg ml^{-1} depending on the species of mouse and stage of lactation (Piletz & Ganshow, 1981). Compared with bovine milk which contains 35 mg ml^{-1}, the protein content of murine milk is 2 to 6 times greater.

2.1.2 IDENTIFICATION OF MURINE CASEINS

Early studies of the composition of the protein fraction of mouse milk were carried out using starch gel electrophoresis. Rennin-precipitated mouse casein ran as four bands on the starch gel (Turkington *et al.*, 1965). Later, urea-polyacrylamide gel electrophoresis revealed five casein bands (Ceriani, 1969).

Immunologic and electrophoretic analyses have been performed on mouse and rat caseins (Feldman & Ceriani, 1970). Immunodiffusion techniques showed that of the five casein bands visible on urea-gel electrophorosis, γ-globulins recognized only two components. The same analysis of rat casein was even less successful since only one casein component was immunologically recognizable. In the case of the single rat casein component and one of the mouse caseins, cross-reactivity with the antiserum to casein was incomplete as demonstrated by the presence of a spur formed on the immunodiffusion plates.

Immunoelectrophoretic analysis demonstrated only one band when mouse milk and rat milk reacted with mouse antiserum (Feldman & Ceriani, 1970). A similar analysis was also carried out using antibodies raised to rat

milk. Four components were found to react in the rat milk and one in the mouse milk. From these results it was concluded that the single mouse casein component which reacted to both mouse antiserum and rat antiserum shares determinants with one rat casein component. Since mouse casein was originally shown to contain five components on gel electrophoresis, two components using immunodiffusion techniques and only one using immunoelectrophoresis, it is likely that the other caseins are only weakly antigenic. The weak antibody response elicited apparently goes undetected using immunodiffusion and immunolectrophoretic techniques, with immunoelectrophoresis being less sensitive than immunodiffusion.

Later analyses used antibodies specific to mouse casein as well as antibodies to mouse serum proteins. The results showed that the two fastest moving components of the five bands separated on gel electrophoresis are contaminated with serum components and that the casein in three remaining bands is uncontaminated (Feldman & Hohmann, 1971). These techniques demonstrated that caseins precipitated using rennin and calcium ions yielded quite impure products and so made quantification difficult.

To try to overcome the problems encountered previously, a radioimmunoassay was developed for the detection of mouse caseins (Feldman, 1974). The assay involved raising rabbit anti-mouse casein antibodies and then using these antibodies to precipitate radiolabelled mouse casein (154 counts min^{-1}). Of the total precipitable mouse casein present, only 61.7% was precipitated by the antibodies, i.e. was immunologically recognizable. It was thought that the reason for this could be due to:

(a) An insufficient distribution of antibodies to precipitate all of the five casein components as detected by urea-polyacrylamide gel electrophoresis, and/or,

(b) The presence of non-casein serum-like proteins in the rennin-calcium precipitated material which do not react to serum-absorbed antisera (Feldman & Hohmann, 1971).

Although the assay did not precipitate all of the available radiolabelled mouse casein, it could detect very small quantities of mouse casein i.e. 0.6 μg of immunologically recognizable mouse casein. Further work was carried out by Enami & Nandi (1977) which was also based on radioimmunoassay. This assay was more specific than that of Feldman (1974), and was developed in order to purify a single casein component the molecular weight of which was estimated to be 22,000Da. The assay is capable of detecting as little as 0.1 mg of casein and was successfully used to show casein production in cultured mouse mammary explants.

Further analyses of mouse milk proteins was carried out using various electrophoretic methods. Acid-urea gels of mouse casein showed that all the caseins migrated with a similar mobility and clustered near to the origin. Separations performed using alkaline-urea gels revealed six casein components. Phosphorylation of the caseins was also shown to play a role in their migration in the gel under acidic and alkaline conditions.

Dephosphorylated casein was prepared using highly purified alkaline phosphatase. Under acidic conditions, the dephosphorylated caseins migrated more quickly towards the cathode than native caseins, and under alkaline conditions dephosphorylated caseins moved more slowly towards the anode

than native caseins (Green & Pastewka, 1976a). Mouse caseins were further characterized using staining and enzymatic procedures. Whole mouse casein separated by SDS-PAGE using various concentrations of acrylamide gave rise to four blue bands when stained with Stains-all. These were named C1-C4, where C1 was the slowest migrating band. Staining with Coomassie blue also revealed four bands. However, C2 was poorly stained (Green & Pastewka, 1976b). Molecular weights of three of the proteins were determined by SDS-PAGE. This method is accurate only for proteins that bind SDS in a manner similar to that of marker proteins of known molecular weight. SDS-binding was investigated by looking at plots of relative mobilities of marker proteins on a logarithmic scale versus percentage gel concentration. The lines formed tend to intersect at the same spot when extrapolated to zero percent gel concentration, thus indicating a nearly constant free mobility. Plots of three of the mouse caseins were found to intersect at the same point as the standards and their molecular weights were calculated as C-1, 43,000Da; C-3, 27,700Da; C-4, 25,900Da. Bovine caseins were similarly analysed and were found to have a higher molecular weight than the value calculated from amino acid data. This phenomemon may be due to characteristics of charge and hydrophobicity of the proteins.

Dephosphorylation of mouse caseins by alkaline phosphatase treatment was investigated using SDS-PAGE and staining with Stains-all. Scanning of SDS gels of mouse caseins before and after dephosphorylation indicated that dephosphorylation resulted in a decrease in the intensity of staining of all casein components. Fraction C-1 failed to stain when treated with alkaline

phosphatase and staining of C-2 was reduced by 42%, C-3 by 90% and C-4 by 95%. The C-2 component did not stain when treated with neuraminidase. This component was found to behave in a manner similar to bovine κ-casein on SDS-PAGE. It is a glycoprotein and glycoproteins bind less SDS than marker proteins. C-2 was also found to be rennin-sensitive and, was therefore, considered to be mouse-κ-casein (Green & Pastewka, 1976b).

2.1.3 CHARACTERIZATION OF LACTATION SPECIFIC mRNA

The characterization and cloning of lactation specific mRNA coding for mouse caseins has been made possible by advances in genetic engineering techniques. Seven casein mRNAs have been identified on the basis of the size and antigenic properties of their translation products *in vitro* and also by characterization of their respective cDNA clones (Hennighausen & Sippel, 1982a). In order to identify individual mouse caseins, the total mouse milk protein pattern was compared with the protein pattern from mouse breast homogenate.

Milk proteins are secretory proteins and as such possess amino-terminal signal peptides. Subsequent analysis was carried out to determine whether those proteins synthesized *in vitro* were further processed in the presence of microsomes. The apparent *in vitro* mRNA length (number of base pairs) and the apparent protein size are listed in Table 7.

All the mouse milk proteins were found to be co-translationally processed to an extent of about 80%. Most of the *in vitro* synthesized proteins could be correlated with specific milk proteins as separated by SDS-

TABLE 7: Molecular weights of major mouse milk proteins

Protein Specificity	Occurrence in Milk	Apparent Protein Size			Apparent Length	
		In vivo Da	*In vitro* Da	*In vitro* + microsomes Da	mRNA Bases	mRNA.cDNA Base Pairs
α_1-casein	+	43,000	43,000	43,000	1,600	1,460
α_2-casein	+	39,000	39,000	39,000	1,600	1,460
β_1-casein	+	26,500	27,500	26,000	1,450	1,140
β_2-casein	+	26,500	27,000	26,000	1,450	1,140
γ-casein	+	23,700	24,500	22,500	860	840
δ-casein	+	21,000	22,500	21,500	1,150	950
ϵ-casein	[a]	[a]	16,100	14,500	860	790
Whey acidic protein	+	13,100	15,200	13,700	680	620

Data from Hennighausen & Sippel, 1982[a]

[a] ϵ-casein has so far not been isolated from mouse milk, and the apparent molecular weight of the *in vivo* protein unknown

PAGE. Seven caseins were identified by *in vitro* synthesis and named α_1, α_2, β_1, β_2, γ, δ and ϵ- caseins with molecular weights of 43, 39, 27.5, 27, 24.5, 22.5 and 16.1 kDa, respectively. The nomenclature of the murine caseins however, does not correspond to that of the bovine caseins. SDS-PAGE of mouse milk revealed only five caseins, α_1-, α_2-, β-, γ- and δ-caseins with molecular weights of 43, 39, 26, 23.7 and 21 kDa respectively. No protein corresponding to the ϵ-casein clone was identified and only one β-casein was apparent. Immunoprecipitation experiments were carried out on β- and α-caseins. Antisera prepared against the 43 kDa α-casein gave rise to a double precipitin line with total milk proteins in Ouchterlony gel diffusion assays, indicating the presence of two antigens which had cross-reacting determinants. The same analysis using antisera to precipitate α_1- and α_2-casein mRNAs from total mRNA also precipitated two mRNAs. Antisera raised against the 26 kDa β-casein from SDS-PAGE was similarly found to precipitate the 27.5 and 27 kDa mRNAs from total mRNA (Hennighausen & Sippel, 1982a).

It was not until 1985 that a cDNA coding for mouse κ-casein was isolated. The nucleotide sequence encoding κ-casein was also deduced and the molecular weight of the protein was calculated to be 18,400 Da (Thompson, Dave & Nakhasi, 1985).

2.1.4 MOUSE α_1-CASEIN

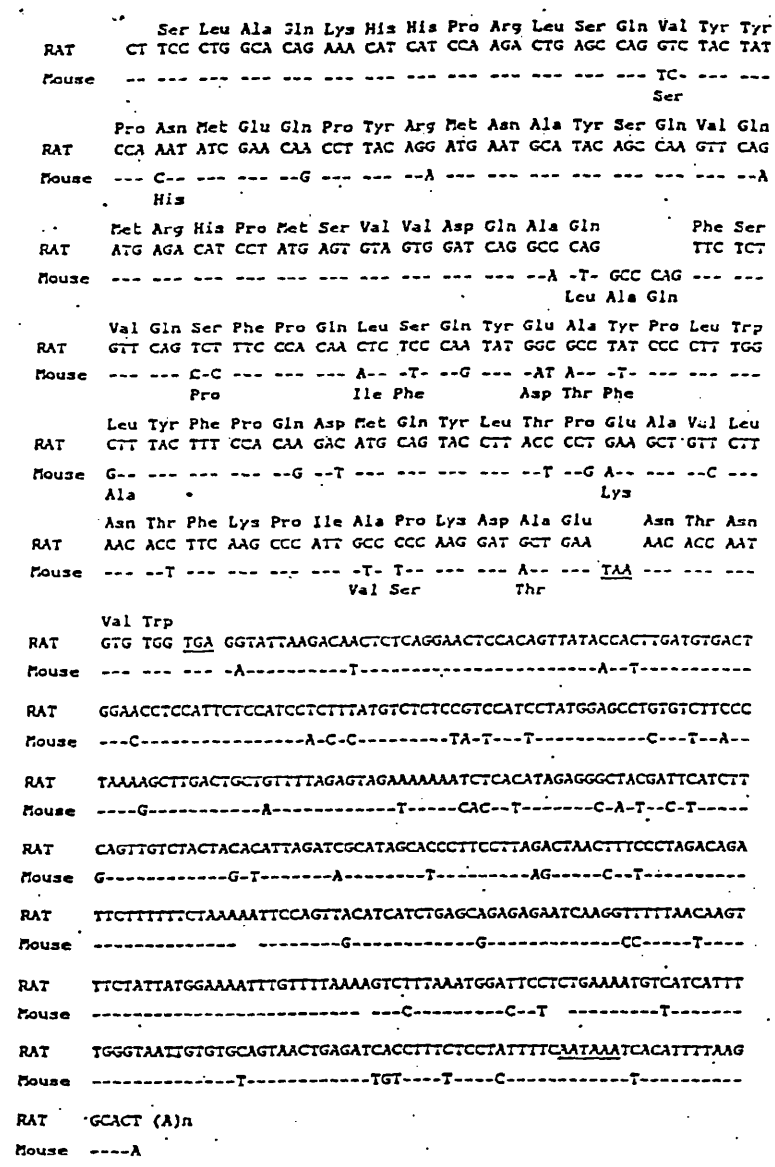
The mouse α_1-casein gene was cloned in 1982 but the nucleotide sequence encoding protein remained unknown. However, under stringent hybridization criteria the mouse α-casein mRNA cross-hybridized with the respective rat mRNA, demonstrating reasonable conservation between the two

species (Hennighausen & Sippel, 1982a). Mouse α_1-casein has an estimated molecular weight by SDS-PAGE of 43 kDa, approximately the same as the 42 kDa calculated for rat α-casein. The calculated molecular weight from cDNA analysis was 31,683 Da (Rosen, Woo & Comstock, 1975). The cDNA sequence encoding the rat α-casein has also been isolated (Hobbs & Rosen, 1982). Comparison with the mouse α_1-casein cDNA sequence has revealed that there is a 90% homology in the coding regions and an 80% homology in the 3' untranslated region. Initially, seven hundred bases of the mouse α-casein cDNA sequence were identified and found to correspond to a region of about 719 bases of rat α-casein mRNA (Faruque *et al.*, 1987; Faruque & Skidmore, 1988). A comparison of the partial cDNA sequence of mouse α_1-casein to that of rat α-casein cDNA is shown in Figure 6 (Faruque, 1990).

A full length cDNA for mouse α_1-casein was not elucidated until 1990. The full length clone of 313 amino acid residues is shown in Figure 7 (Grusby *et al.*, 1990). A comparison of the rat α-casein clones (Figure 7) also demonstrates their homology. The amino acid sequence for mouse α-casein was found to be 81% homologous to that of rat α-casein. Both species have signal peptides consisting of fifteen amino acid residues which are identical with the exception of one residue. The mouse cDNA was also shown to contain fifteen repeated elements of six residues, shown boxed in Figure 8. These elements are also present in the rat sequence (Hobbs & Rosen, 1982).

Mouse α_1-casein was initially thought to contain no cysteine residues since translation of mRNA *in vitro* in the presence of [^{35}S] cysteine resulted in no incorporation of the radioactive label (Hennighausen & Sippel, 1982a).

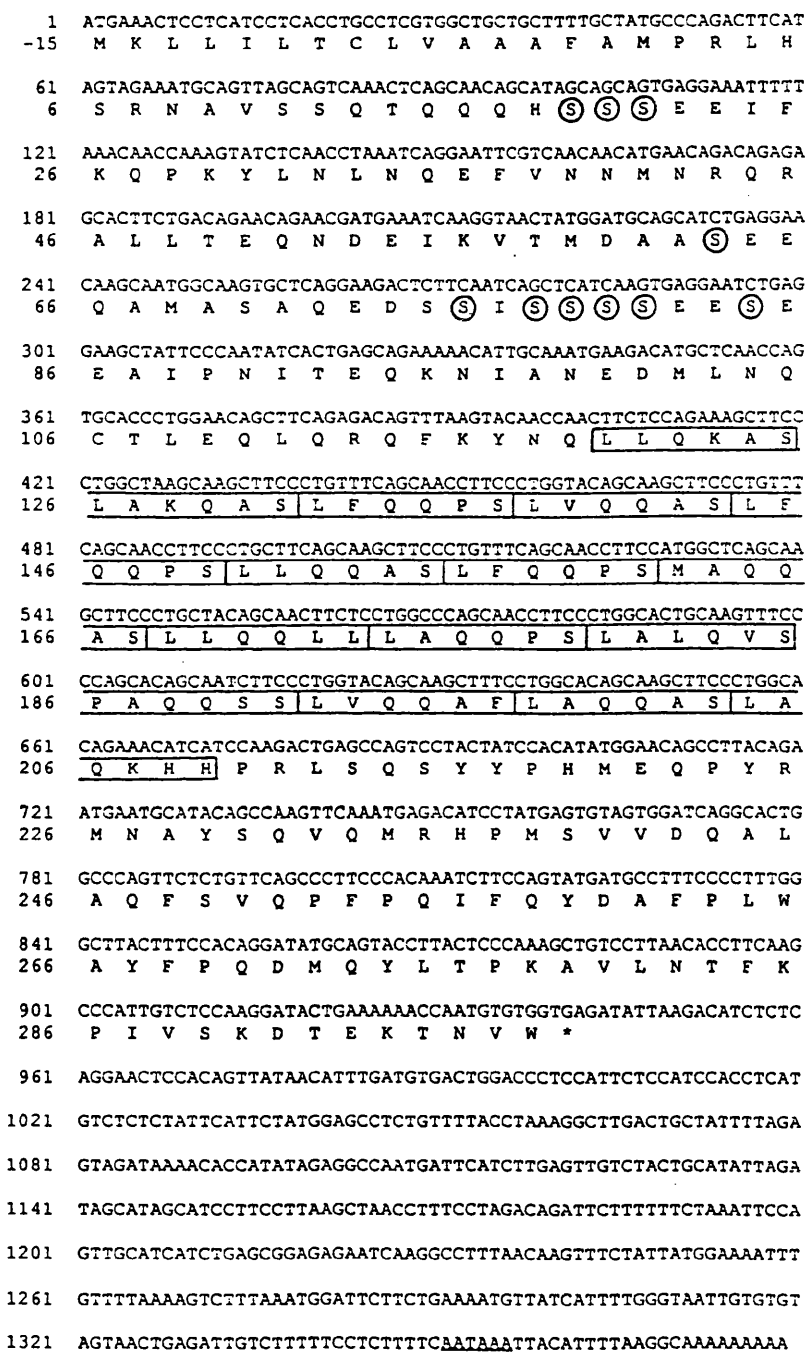
Figure 6



Comparison of the partial cDNA sequence for the mouse α_1-casein with the corresponding sequence from the rat.

The sequence representing the coding regions are arranged in triplet codes. Dashed lines represent homologous sequences and spaces introduced in the rat or mouse sequence represent deletions or insertions. The termination codons and the polyadenylation signals are underlined. Data from Faruque, 1990.

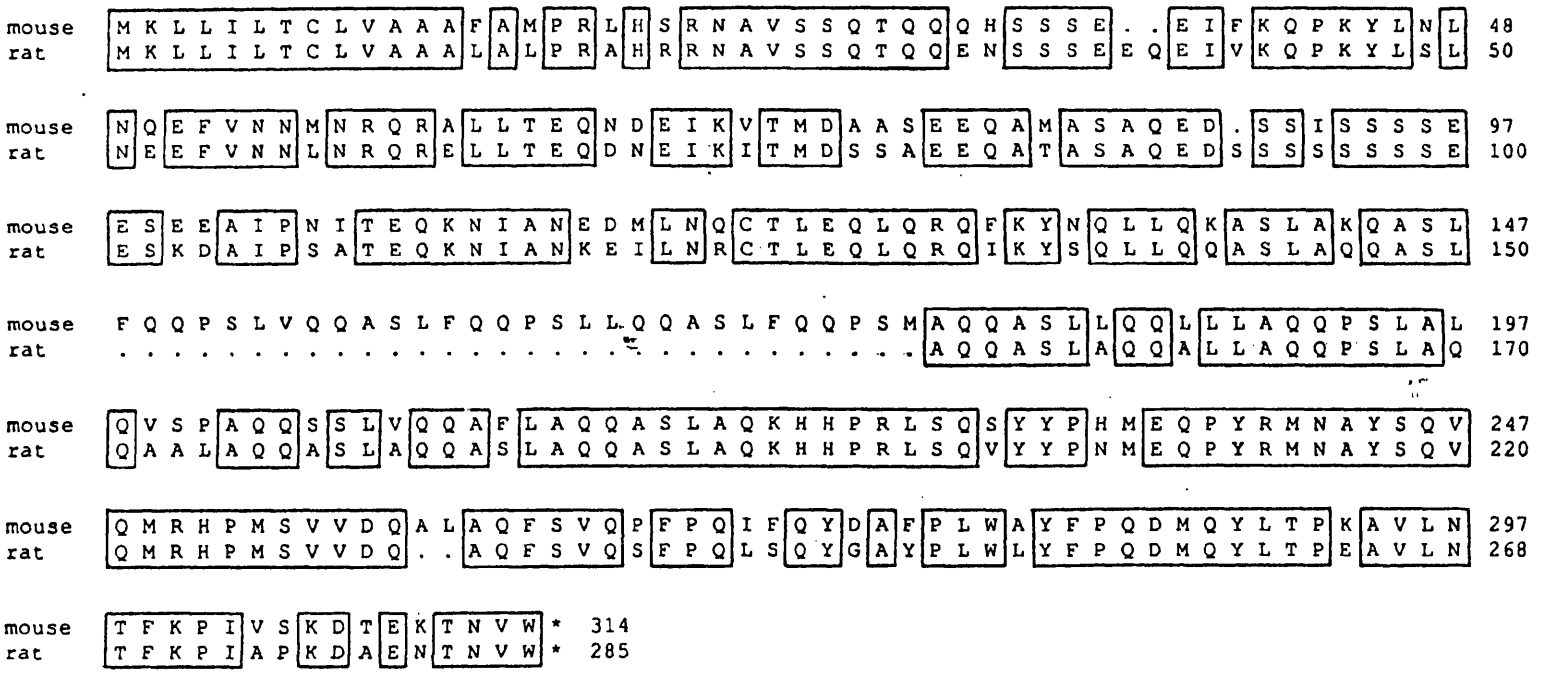
Figure 7



Nucleotide and deduced amino acid sequences of the full length clone of mouse α-casein. Potential casein kinase phosphorylation sites are circled and repeated units are boxed. A concensus polyadenylation signal is underlines and the star indicates translation termination.

Data from Grusby *et al.* 1990.

Figure 8



Comparison of the deduced amino acid sequence of mouse α-casein clone with corresponding rat α-casein sequence.

Residues identical in both sequences are shown boxed in figure.
Data from Grusby *et al.* 1990.

However, on elucidation of the amino acid sequence it was evident that the precursor form of mouse α_1-casein contained 2 cysteine residues and the mature protein one cysteine residue (Grusby *et al.*, 1990).

Mouse α_1-casein may also be phosphorylated. There are two areas on the protein that have clusters of casein-kinase recognition sites, i.e. serine-x-acidic amino acid/phosphoserine, where x is any amino acid. In total there are ten serine residues which could be phosphorylated. These residues are circled in Figure 7.

2.1.5 MOUSE α_2-CASEIN

A mRNA coding for a 39 kDa protein was isolated and cloned (Table 7). The protein was named α_2-casein on the basis of results from immunological experiments, in which antibodies raised to α_1-casein precipitated both α_1- and another mRNA from total mammary gland mRNA (Hennighausen & Sippel, 1982a). The amino acid sequence of α_2-casein has not yet been elucidated.

2.1.6 MOUSE β-CASEIN

The presence of two β-caseins was postulated on the basis of the results of cDNA analysis and immunoprecipitation. The two caseins have calculated molecular weights of 27.5 and 27 kDa (Hennighausen & Sippel, 1982a; Table 7). However, only one band however is visible on SDS-PAGE. The cDNA sequence of mouse β-casein has been determined (Yoshimura *et al.* 1986; Figure 9).

Figure 9

1	M K V F I L A C L V A L A L A R E T T F T V [S] S E T D [S] I [S]
31	[S] E E [S] V E H I N E Q K L Q K V N L M G Q L Q A E D V L Q A
61	K V H S S I Q S Q P Q A F P Y A Q A Q T I S C N P V P Q N I
91	Q P I A Q P P V V P S L G P V I [S] P E L E S F L K A K A T I
121	L P K H K Q M P L L N S E T V L R L I N S Q I P S L A S L A
151	N L H L P Q S L V Q L L A Q V V Q A F P Q T H L V S S Q T Q
181	L S L P Q S K V L Y F L Q Q V A P F L P Q D M S V Q D L L Q
211	Y L E L L N P T V Q F P A T P Q H S V S V

Amino Acid sequence of mouse β-casein derived from the β-casein cDNA sequence.
The first fifteen residues sequenced form the signal peptide of the pre-protein.

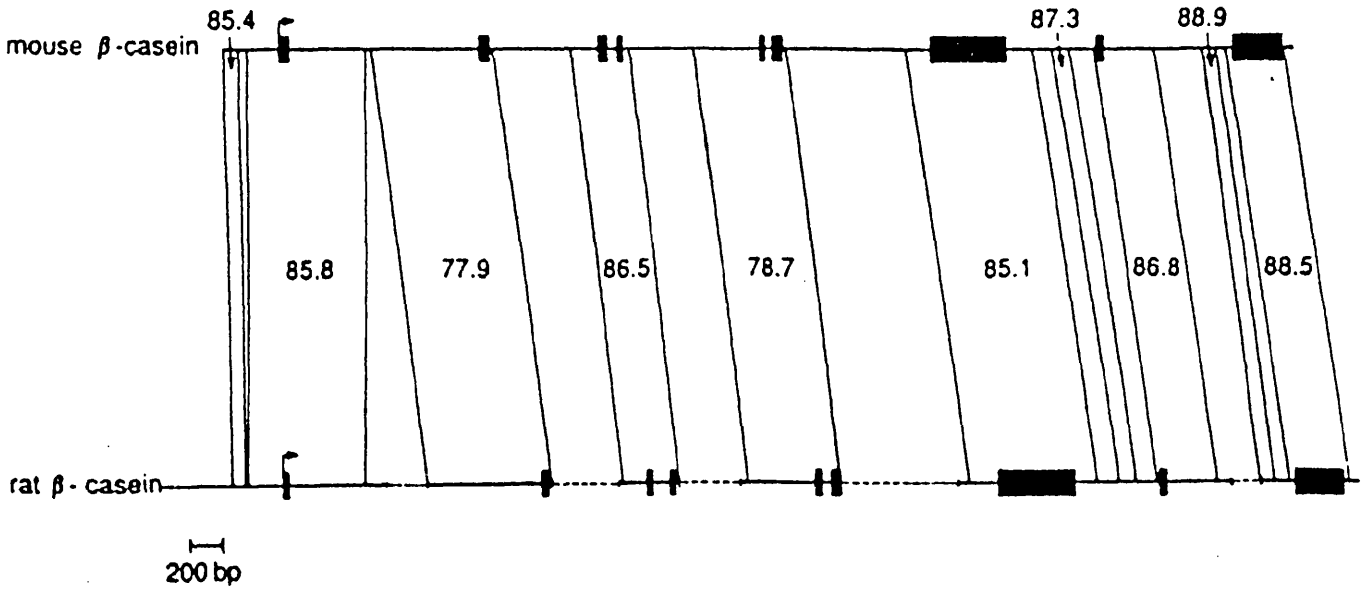
Data adapted from Yoshimura *et al.* 1986

Mouse β-casein cDNA was found to cross-hybridize with rat β-casein under strict hybridization criteria, indicating some homology with the rat casein (Hennighausen & Sippel, 1982a). A comparison of the two cDNA sequences revealed that the intron/exon organization of the two genes was highly conserved. Both sequences contained nine exons and eight introns all with similar lengths except for the first intron of rat β-casein which had an extra sequence of approximately 400 base pairs (Figure 10). The nine exons were found to be 88.4, 95.2, 74.1, 95.2, 95.8, 84.4, 86.3, 85.4 and 87.0% homologous, respectively (Yoshimura & Oka 1989). At the amino acid level, the two caseins are still quite homologous (approximately 70%), with 100% homology for the fifteen amino acid residues in the signal peptides. Unlike bovine β-casein, the mouse β-casein contains two cysteine residues, one of which occurs in the signal peptide. The full-length mouse β-casein has been calculated to be 231 amino acid residues long with a molecular weight of 25,308Da. Mouse β-casein may also be phosphorylated since it contains six casein-kinase recognition sites, i.e. ser-x-glutamic acid/phosphoserine (shown boxed in Figure 9).

2.1.7 MOUSE κ-CASEIN

A cDNA clone for mouse κ-casein was not isolated until 1985. The nucleotide sequence was determined and from this, the amino acid sequence. The deduced amino acid analysis revealed that the mature protein is 160 amino acid residues long, has a signal peptide of 21 amino acid residues and a calculated molecular weight of 18,400Da (Thompson *et al.*, 1985). The deduced amino acid sequence is shown in Figure 11. The sequence of mouse

Figure 10



A comparison between the mouse and rat β-casein genes.

Sequence alligned 5' - to 3' . Blackened boxes represent exons, thick lines sequenced regions, dashed lines non-sequenced regions. Lines conecting the two sequences indicate the range of homologous regions with the percentage homology numbered. Transcription start points are indicated by bent arrows.
Data from Yoshimura and Oka, 1989.

Figure 11

1	M M R N F I V V M N I L A L T L P F L A A E I Q N P D S N C
31	R G E K N D I V Y D E Q R V L Y T P V R S V L N F N Q Y E P
61	N Y Y H Y R P S L P A T A S P Y M Y Y P L V V R L L L L R S
91	P A P I S K W Q S M P N F P Q S A G V P Y A I P [N] P S F L A
121	M P [T] N E N Q D N T A I P T I D P I T P I V S T P V P [T] M E
151	S I V N T V A N P E A S T V S I N [T] P E T T T V P V S S T A
181	A

Amino Acid sequence of mouse κ-casein

The first twenty-one residues sequenced form the signal peptide of the pre-protein. The probable site of glycosylation at residue 94 is shown by a hatched box and the three putative phosphorylation sites shown boxed.

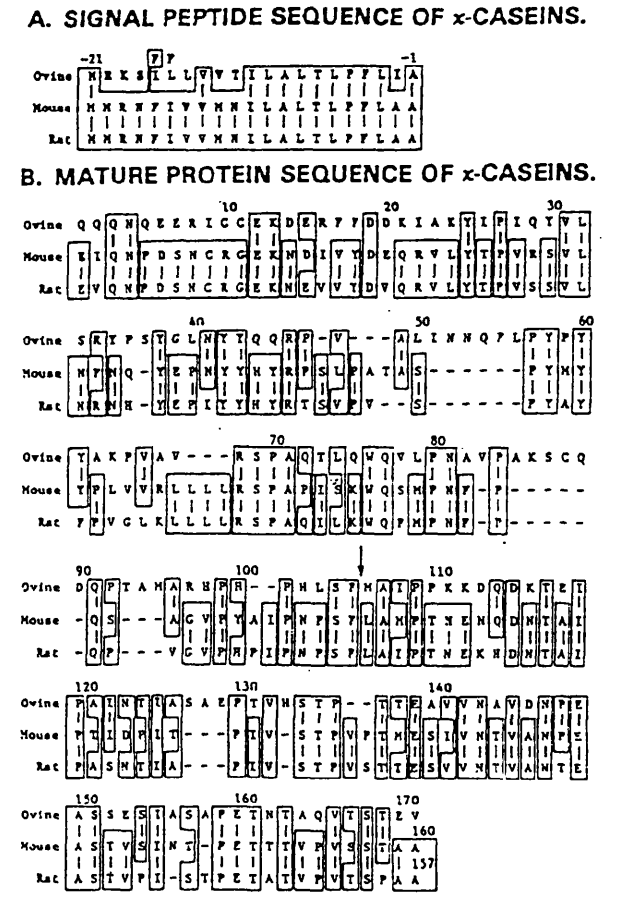
Data from Thompson *et al.* 1985

κ-casein has also been compared with the corresponding rat and ovine caseins(Figure 12). The signal peptides of mouse and rat κ-casein are completely homologous and both are 62% homologous with the ovine κ-casein signal peptide. The mature caseins were found to be less homologous, and both were only 43-46% homologous with ovine κ-casein.

Sequence homologies are shown in Figures 12A-C. Homologous regions are found in the C-terminal end from residues 105-106. This bond occurs between phenylalanine and leucine residues in both mouse and rat but between phenylalanine and methionine in both bovine and ovine caseins. (Mercier & Chobert, 1976). This could indicate that the enzyme responsible for cleaving this bond may differ among species. A comparison of peptides 1-105 and 106-160 revealed that the peptides of different species have diverged during evolution. In peptide 1-105 of mouse κ-casein three amino acids have been deleted as well as an insertion of four leucines at positions 61-64 in both mouse and rat κ-caseins.

Amino acid sequencing data have also revealed a single putative glycosylation site at residue 94 of mouse κ-casein. The protein may also be phosphorylated and the three putative sites are shown in Figure 11. In this instance, phosphate groups may be present on threonine residues as well as serine residues. Phosphorylation of threonine residues obeys a similar rule to that of serine residues i.e. threonine-x-acidic amino acid/phosphothreonine, where x is any amino acid.

Figure 12



C. COMPARISON OF AMINO ACID COMPOSITION OF OVINE, MOUSE AND RAT x-CASEINS.

	# of A.A. CONSERVED	# of A.A. SUBSTITUTED	# of A.A. INSERTED	# of A.A. DELETED	% HOMOLOGY
Signal Peptide					
Ovine vs Rat and Mouse	13/21	8/21	—	—	62
Mouse vs Rat	21/21	—	—	—	100
Mature Protein					
Ovine vs Mouse	68/157	78/157	22/157	11/157	43
Ovine vs Rat	74/157	72/157	21/157	11/157	46
Rat vs Mouse	115/157	40/157	1/157	3/157	73

Comparison of amino acid sequences of mouse, rat and ovine κ-caseins.
Conserved residues are boxed and maximum alignment achieved by introducing gaps represented by dashes. The proteolytic cleavage site is marked by an arrow.
Data from Thompson *et al*, 1985.

2.1.8 MOUSE ε-CASEIN

A clone for mouse ε-casein was isolated in 1982 which coded for a 16.1kDa protein (Hennighausen & Sippel, 1982a; Table 7). Sequence analysis has shown that mouse ε-casein contains regions homologous to bovine α_{s2}-casein but not to bovine β-casein (Hennighausen *et al.* unpublished results). ε-Casein cDNA contains a Pst1 restriction site in the 3′ non-coding region similar to that found in mouse β-casein cDNA. A rat clone similar to the mouse ε-casein clone has been detected. It was however named β'-casein because its Pst1 restriction site was found in a similar position to that of rat β-casein (Hennighausen & Sippel, 1982a). The amino acid sequence of mouse ε-casein has been deduced (Hennighausen *et al.*, 1982; Figure 13). Six potential phosphorylation sites are also indicated in Figure 13. As in bovine caseins which have phosphorylated residues clustered in regions rich in acidic amino acids, the potential phosphorylation sites in mouse ε-casein occur in the region of amino acids 5-48 which contains ten acidic and only one basic amino acid.

2.1.9 MOUSE γ-CASEIN

A clone for mouse γ-casein has been isolated (Hennighausen & Sippel, 1982a). However, the amino acid sequence of the protein has not been elucidated to-date. The mature protein is estimated to have a molecular weight of 23.7 kDa (Table 7). Under strict hybridization criteria mouse γ-casein cDNA cross-hybridizes with rat γ-casein cDNA, demonstrating reasonable conservation between the two species.

Figure 13

1	M K F I I L T C L L A V A L A K Q R M E Q Y I [S] [S] E E [S] M D
31	N [S] Q E N F K Q N M D V A F F P [S] Q E T V E N I Y I P Q M E
61	[S] V E A P M K V S D I I S Q Q Q Y N Q K M M D M S V S A R E
91	K T V M T E E S K N I Q D Y M N K M K R Y S K I T W P Q F V
121	K L L H Q Y Q T M T P W S Y Y P S T P S Q V

Amino Acid sequence of Mouse ϵ-casein

The first fifteen residues sequenced correspond to the signal peptide of the pre-protein. Putative phosphorylation sites are shown boxed.

The amino acid sequence of mouse ϵ-casein was derived from the cDNA sequence published by Hennighausen *et al.* 1982.

2.1.10 MOUSE δ-CASEIN

δ-Casein has a molecular weight of 21kDa (Table 7). The amino acid sequence for this protein has not yet been elucidated although a cDNA clone has been isolated (Hennighausen & Sippel, 1982a). Unlike the α-, β- and γ-caseins, mouse δ-casein cDNA does not cross-hybridize to a rat mammary gland mRNA.

2.1.11 IDENTIFICATION OF MURINE WHEY PROTEINS

As with the caseins, mouse whey proteins were initially investigated using various electrophoretic techniques. Acid-urea gels of mouse whey proteins revealed two bands as did alkaline-urea gels (Green & Pastewka, 1976a). SDS-PAGE of whole whey and of ammonium sulphate fractions also showed two proteins. The slower migrating of the two whey proteins was found to have the same mobility as mouse and bovine serum albumin. The faster of the two migrating bands was found to have the same mobility as bovine α-lactalbumin. These proteins were eluted from the gel and analysed by immunodiffusion.

Goat-anti-mouse serum albumin confirmed that the slower migrating whey protein was mouse serum albumin. The faster migrating band was named α-lactalbumin because of its activity in a lactose synthetase assay (Green & Pastewka, 1976a). It was not until 1981 that another mouse whey protein was discovered (Piletz, Heinlen & Ganschow, 1981). The protein was named whey acidic protein (WAP) due to its acidic isoelectric point and it was found to be the major protein in mouse whey. This protein was previously reported as being α-lactalbumin but was later confirmed as WAP since it

lacked lactose synthetase B-protein activity (Piletz *et al.* 1981).

2.1.12 WHEY ACIDIC PROTEIN (WAP)

This protein is the major protein in mouse whey and it constitutes a minimum of 2.4% of total mouse protein. WAP has an acidic isoelectric point: WAP-A, 4.7; WAP-B, 4.8(Piletz *et al.* 1981) has a molecular weight of 14,000 Da and is synthesized in the mammary gland of the mouse. To date, WAP has only been identified in the milk of rodents and its function remains unknown. WAP has been purified using a 33% ammonium sulphate fractionation of whey, and was subsequently purified by gel permeation and anion exchange chromatography. Estimates of the molecular weight of WAP using gel electrophoresis, SDS-PAGE and gel permeation chromatography are quite similar being 12,500, 13,500 and 15000 Da, respectively.

A cDNA for mouse WAP was isolated in 1982 (Hennighausen & Sippel, 1982a; Table 7). The amino acid sequence of the protein was deduced and is shown in Figure 14. Mouse WAP was found to be 134 amino acid residues long and to contain a signal peptide of 19 residues (Hennighausen & Sipple, 1982b, c). Two allelic forms of mouse WAP have been identified, WAP-A and WAP-B. WAP-B has one less cysteine residue and one more arginine residue than WAP-A. The amino acid compositions of both WAP forms are tabulated in Table 8 (Hennighausen & Sippel, 1982b; Piletz *et al.* 1981) with WAP-A designated the wild type and WAP-B the mutant allele.

Mouse WAP is a cysteine rich protein with cysteine residues grouped in two domains. This pattern of cysteine residues is similar to that seen in other small proteins such as snake venom neurotoxins and wheat germ

Figure 14

1	M R C L I S L V L G L L A L E V A L A H N L E E Q V F N S V
31	Q S M F P K A S P I E G T E C I I C Q T N E E C A Q N A M C
61	C P G S C G R T R K T P V N I G V P K A G F C P W N L L Q T
91	I S S T G P C P M Q I E C S S D R E C S G N M K C C N V D C
121	V M T C T P P V P V I T L Q

Amino Acid sequence of mouse whey acidic protein

The first nineteen residues sequenced form the signal peptide of the pre-protein.

Data from Hennighausen and Sippel, 1982

TABLE 8: Amino acid composition of mouse whey acidic protein

	Mouse WAP[1]	Mouse WAP-A[2]	Mouse WAP-B[2]
	mol amino acids/mol WAP		
Gly	7	7.8	7.8
Ala	4	4.5	4.5
Leu	4	4.4	4.5
Ile	7	6.5	6.5
Val	8	8.3	7.8
Met	5	7.8	7.9
Ser	9	8.7	9.1
Thr	9	8.2	8.1
Tyr	0	0.0	0.0
Trp	1	not done	not done
Phe	3	3.2	3.2
Glu	8	16.2	16.1
GlN	8	-	-
Asp	2	10.9	10.8
AsN	3	-	-
Cys	14	7.5	6.8
Pro	11	11.8	11.7
His	0	0.0	0.0
Lys	4	4.7	4.5
Arg	3	3.0	3.9

Data from Hennighausen & Sippel, 1982b

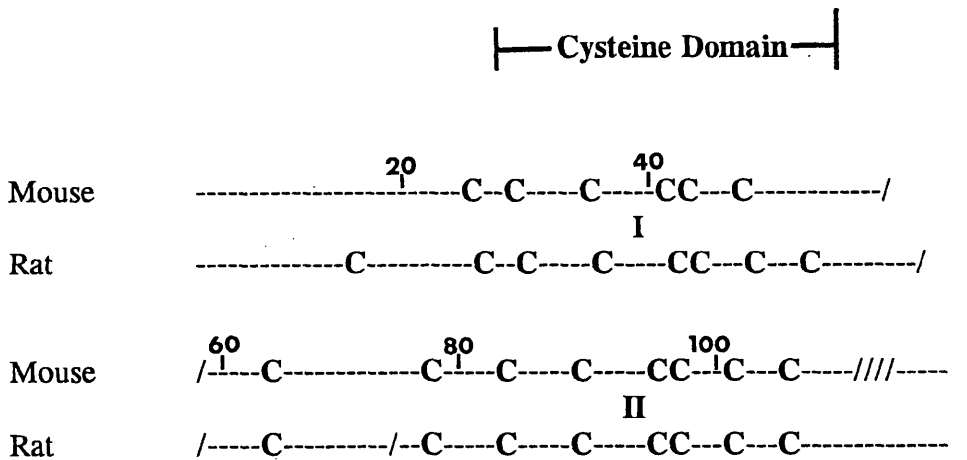
[1] As calculated from the amino acid sequence of the whey acidic protein (WAP) which was deduced from the nucleotide sequence of cloned cDNA.
[2] As determined from authentic WAP by Piletz *et al.* 1981. The figures are corrected per 115 amino acid residues per molecule of WAP.

agglutin (Drenth *et al.*, 1980). The distribution of cysteine residues in mouse WAP is identical to that found in rat WAP, i.e. five cysteines have been identified in the N-terminal domain of mouse WAP and six in that of rat WAP. The C-terminal domain of both mouse and rat WAP contains six cysteines residues.

In both parts of the polypeptide chain, the cysteine residues match exactly in their distance from each other (Hennighausen & Sippel, 1982b,c). The two domain structures of the mouse and rat WAP polypeptides are shown diagramatically in Figure 15. A comparison of mouse and rat WAP (Figure 16) demonstrates reasonable homology between the two proteins. The signal peptides show 95% conservation, the cysteine domain I show 81% conservation and all other areas including cysteine domain II only show 60-65% homology. Compared with the levels of conservation of other proteins, whey acidic proteins are among the most rapidly diverging proteins known (Hennighausen & Sippel, 1982c).

WAP-A and WAP-B have also been tested for the presence of phosphate groups and carbohydrate moieties, by specific staining of polyacrylamide gels with periodic acid-Schiff, Stains-all and methyl-green dyes. Since no positive result was obtained, enzymatic procedures were tested i.e. treatment with neuraminidase, acid phosphatase and alkaline phosphatase. These enzymes also had no effect on the electrophoretic mobilities of phosphate nor sugar groups. Using Sudan Black B, the lipid content of WAP was investigated. WAP A and B stained well for lipid. The nature of the lipid association with WAP was determined by delipidating WAP using a

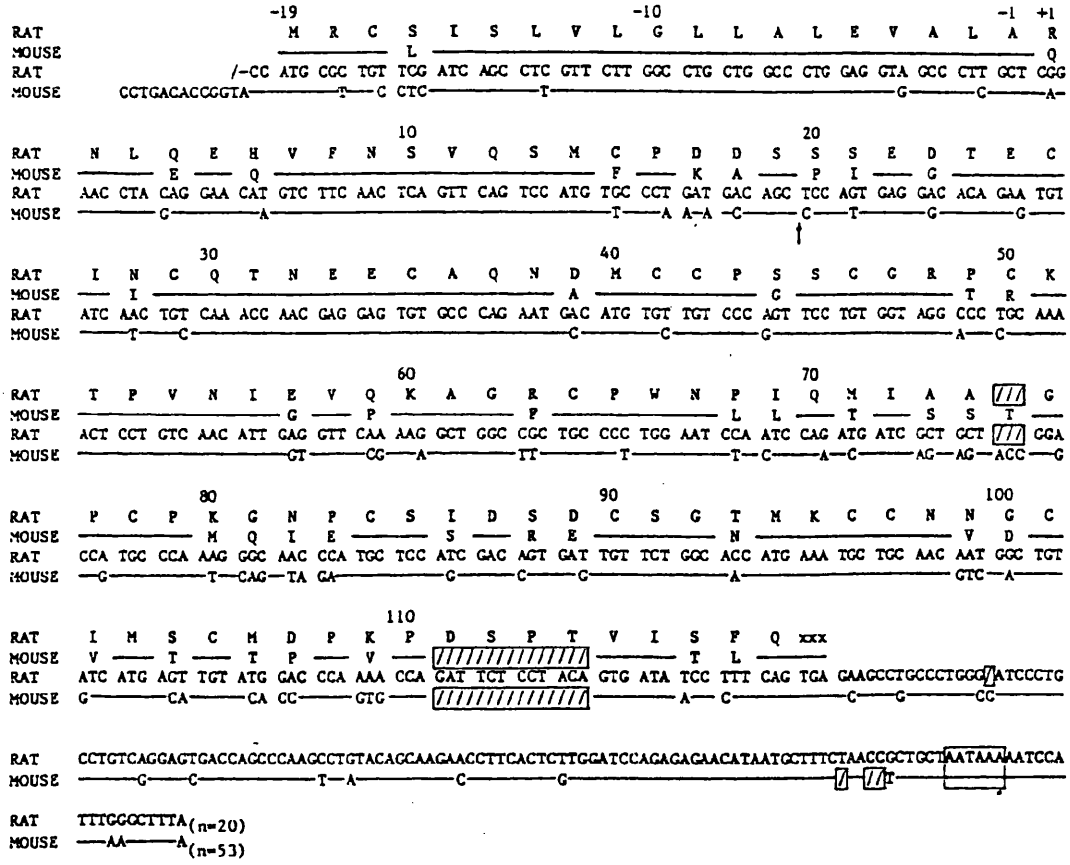
Figure 15



Alignment of the Cysteine Domains of Rat and Mouse Whey Acidic Protein.
Only the cysteine residues are shown. Each - represents an amino acid.
Hatched areas represent introduced gaps in sequence.

Data from Hennighausen & Sippel, 1982c

Figure 16



Comparison of the nucleotide sequences of the cDNAs and deduced amino acid sequence of rat and mouse whey acidic proteins. Hatched areas represent introduced gaps.
Data from Hennighausen and Sippel, 1982(c).

chloroform/methanol extraction. Triglyceride and cholesterol were found to account for 20-25% of the total mass of WAP. The density of WAP was also found to be greater than that of other lipoproteins (Piletz *et al.* 1981).

2.1.13 α-LACTALBUMIN

Like α-lactalbumin of other species, this protein is synthesized in the mammary gland. α-Lactalbumin plays a major role in the biosynthesis of lactose in milk. In the presence of α-lactalbumin, galactosyltransferase catalyses the formation of lactose by the transfer of the galactosyl residue of UDP-galactose to glucose rather than to the usual galactose acceptor, N-acetylglucosamine. α-Lactalbumin has been purified from mouse milk, (Nagamatsu & Oka, 1980) but the purification was complicated. The purification procedure included precipitating the caseins at pH 4.6, ammonium sulphate fractionation of the whey, and two gel permeation chromatography steps followed by ion-exchange chromatography. The results indicated that α-lactalbumin existed in two forms with molecular weights of 14,000 and 18,500 Da, as estimated by SDS-PAGE. The 14,000 Da form also includes at least four differently charged forms and represents >90% of total mouse α-lactalbumin. The 18,500 Da form of α-lactalbumin which constitues only 5-10% of the total α-lactalbumin also appears to be a glycoprotein.

The amino acid composition of the two major α-lactalbumins were found to be generally similar to rat α-lactalbumin (Brown *et al.* 1977) with the exception of the proline content. The amino acid composition of mouse α-lactalbumin is shown in Table 9 (Nagamatsu & Oka, 1980). The multiple

charged forms of α-lactalbumin are thought to exist due to deamination of residues such as asparagine or glutamine by agents such as urea or ammonium sulphate. Such phenomena have been shown to occur in the case of other small proteins (Lewis et al 1970). Studies of mammary gland explants have also shown that α-lactalbumin accounts for only 0.2% of total protein and is therefore a minor whey component. A mRNA for α-lactalbumin has been isolated, in keeping with previous studies (Nagamatsu & Oka, 1980). α-Lactalbumin mRNA was shown to account for only 2.8% of total mRNA, compared with casein mRNA which comprised about 90% of total mRNA (Takemoto *et al.* 1980). The deduced amino acid sequence is shown in Figure 17 (Vilotte *et al.* 1992; Vilotte & Soulier, 1992). A radioimmunoassay developed to detect α-lactalbumin in whole mouse milk indicated that α-lactalbumin accounted for no more than 0.83% of total milk protein i.e. a concentration of less than 1 mg ml^{-1} milk (Zamierowski & Ebner, 1980).

2.1.14 SERUM ALBUMIN AND LACTOFERRIN

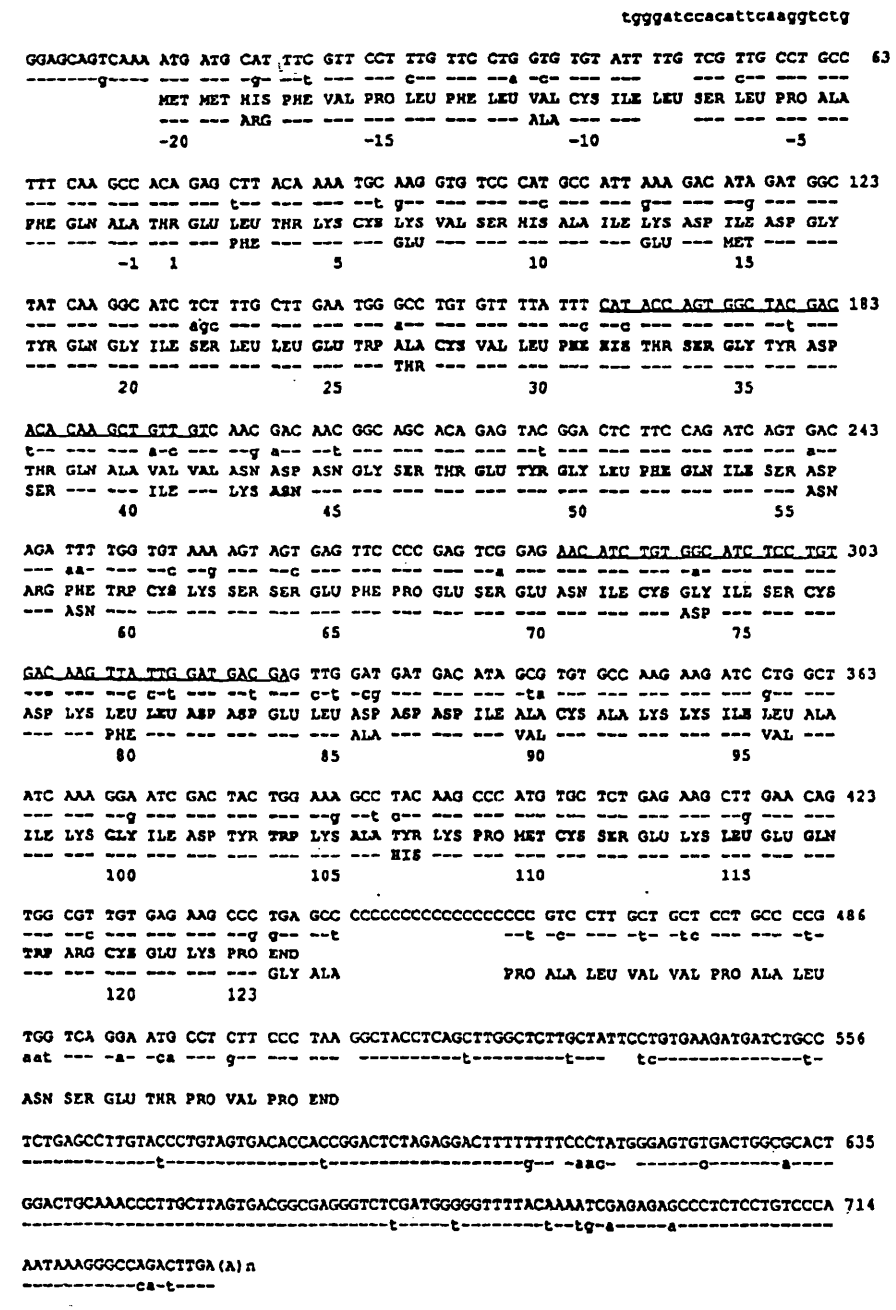
SDS-PAGE of mouse milk and mouse breast homogenate has revealed two proteins with molecular weights of 78 and 67 kDa (Hennighausen & Sippel, 1982a). By comparison with other species, the 67kDa protein could be milk serum albumin and the 78 kDa protein, lactoferrin.

TABLE 9: Amino acid composition of mouse α-lactalbumin

	Preparation I			Preparation II		
	(mol/100 mol)	(estimated no. of residues/molecule)		(mol/100 mol)	(estimated no. of residues/molecule)	
Asx	12.40	15.3	(15)	12.60	15.5	(15)
Thr	3.99	4.9	(5)	4.22	5.2	(5)
Ser	8.70	10.7	(11)	8.71	10.7	(11)
Glx	12.60	15.5	(16)	12.42	15.3	(16)
Pro	2.68	3.3	(3)	2.68	3.3	(3)
Gly	7.09	8.7	(9)	6.98	8.6	(9)
Ala	6.67	8.2	(8)	6.50	8.0	(8)
Cys(½)	4.90	6.0[5.1]	(6)	5.17	6.4	(6)
Val	2.70	3.3	(3)	2.49	3.1	(3)
Met	0.71	0.9[0.8]	(1)	0.88	1.1	(1)
Ile	7.31	9.0	(9)	7.18	8.9	(9)
Leu	7.92	9.8	(10)	7.93	9.8	(10)
Tyr	4.02	5.0	(5)	3.61	4.5	(5)
Phe	3.38	4.2	(4)	3.41	4.2	(4)
Trp	2.70	3.3	(3)	2.82	3.5	(3)
Lys	8.89	11.0	(11)	9.14	11.3	(11)
His	1.69	2.1	(2)	1.84	2.3	(2)
Arg	1.65	2.0	(2)	1.48	1.8	(2)
Total Residues		**123**	**(123)**		**123**	**(123)**

Data from Nagamatsu & Oka, 1980

Figure 17



Nucleotide sequence of mouse α-lactalbumin cDNA and comparison with its rat counterpart. Each upper line (upper case letters): nucleotide sequence of mouse α-lactalbumin cDNA. Each lower line (lower case letters): nucleotide sequence of rat and lactalbumin cDNA.

Dashes represent identical nucleotides, and blanks represent gaps. The amino acid sequences of mouse and rat α-lactalbumin are the bottom two lines respectively. The first twenty residues represent the signal peptide and stop codon marked by END.

2.1.15 AIM OF THE WORK

Despite the enormous amount of research which has been conducted on mouse milk, its protein composition remains relatively poorly characterised and the individual caseins have so far not been isolated. Currently, the method most widely used to determine the amounts of the individual proteins in mouse milk is densitometric scanning of stained polyacrylamide gels. This method is, however, very difficult to quantify accurately and does not allow sufficient of the individual proteins to be separated to enable antibodies to be raised to them, as is required in some projects involving protein expression in the mouse mammary gland.

The aim of this part of the project was, therefore, to develop a quantitative single-step chromatographic method to separate the proteins of mouse milk so as to analyse very small amounts of milk from individual mice, and if possible to scale-up the separation to obtain relatively large amounts of individual proteins to make antibody production feasible.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Chromatography columns, FPLC/HPLC systems, the Phast System, PhastGels/buffer strips and agarose (IEF grade) were purchased from Pharmacia Biotech, Milton Keynes, UK. The Spherisorb ODS column was purchased from Capital HPLC Specialists, Waverley Industrial Estate, Waverley Street, Bathgate, West Lothian.

Sodium acetate, Tris, EDTA, glacial acetic acid, methanol, propan-2-ol, glycerol, formaldehyde, hydrochloric acid, acetonitrile (HPLC-grade), di-potassium hydrogen orthophosphate, potassium dihydrogen orthophosphate and trifluroacetic acid (TFA) were purchased from Fisons Scientific Equipment, Bishop Meadow Rd., Loughborough, Leicestershire, UK. All were analytical reagent grade, except where specified.

Rennin (chymosin;EC 3.4.23.4), silver nitrate, 2-mercaptoethanol, iodoacetamide, triethylamine, n-octyl-β-D-glucopyranoside, acrylamide, bisacrylamide, phenylisothiocyanate, dimethylaminopropionitrile, molecular weight markers, guanidine hydrochloride and 1-ethyl-2-[3-(ethylnaphtho-[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naptho-[1,2-d]thiazoliumbromide (Stains-all) were purchased from Sigma Chemical Company Ltd., Poole, Dorset, UK.

Barbitone, ammonium persulphate, urea(Analar), sodium carbonate urea and sodium thiosulphate were purchased from BDH Chemicals Ltd, Poole, Dorset, UK.

E-C470 gel cell, E-C Apparatus Corporation, St. Petersbourg, Florida.

Hypnorm, Hypnovel, oxytocin and saline were purchased from The Veterinary Drug Co, 10 Castinop Road, Middlefield Industrial Est., Falkirk.

Potato acid phosphatase (EC 3.1.3.2) was purchased from Boehringer Mannheim U.K., (Diagnostics and Biochemicals) Ltd, Bell Lane, Lewes, East Sussex.

CRMX-pellets - Special Diets Services, P.O. Box 705, Witham, Essex, England.

Mice: (Tux, No 1) from A. Tuck & Son, Beaches Road, Battlesbridge, Essex.

OLAC: (CBAx C57 BL6) from Harlan OLAC Ltd., Shaws Farm, Blackthorn, Bicester.

Animals: (Tucks No. 1) and OLAC CBA x C57BL6 mice were mated between the ages of 7 to 9 weeks when they weighed about 25 g for Tucks N°1 and about 19g for CBAX C57BL6 mice. Mice were fed a diet of CRMX pellets (~6 g daily); milk was obtained 10-12 days *post partum* from suckling dams.

2.2.2 MILK COLLECTION

Dams were separated from their young by means of a cage partition for four hours prior to milking. Oxytocin (0.1 units) and 0.1 ml of a solution of Hypnorm, Hypnovel and sterile water (1:1:2 v/v/v) per 10 g of body weight were injected intraperitoneally fifteen minutes before milking. Milk was then expressed by gentle manipulation of the mammary gland and collected via small bore capillary tubes.

2.2.3 MILK FRACTIONATION

Milk was centrifuged for fifteen minutes at 3000 rpm, cooled at 4°C for ten minutes and the fat layer removed.

Caseins were precipitated by reducing the pH of skimmed milk to 4.27 by the addition of acetate buffer (0.2 M-sodium acetate and acetic acid). Precipitated casein was pelleted by centrifuging at 3000 rpm for five minutes. The whey was decanted and the casein pellet washed a further four times by resuspending in acetate buffer and recentrifuging. The washed casein pellet was resuspended in distilled water and the pH adjusted to pH 7.0 with sodium hydroxide. The pH of the soluble whey fraction was similarly adjusted to 7.0 with sodium hydroxide. The casein and whey proteins were dialysed at 4°C for 60 hours against five changes of distilled water. After dialysis, the pH of the casein and whey fractions was adjusted to 7.0 and the samples filtered through a cellulose nitrate filter (1.2 μm pore) to remove any residual fat and then freeze dried.

2.2.4 ALKYLATION

Acid-precipitated, freeze-dried casein was dissolved at a protein concentration of 1.5 mg ml^{-1} by stirring overnight at 4°C in 5 mM-bis-tris propane buffer, pH 7.0, containing 6M-guanidine-HCl and 0.5% n-octyl β-D-glucopyranoside. Disulphide bridges in the proteins were then reduced by adding 2-mercaptoethanol at a concentration of 10 μl ml^{-1} and stirring at room temperature for 1 hour. Iodoacetamide was then added to a final concentration of 8mM and the solution stirred for 15 min. An additional 20 μl of 2-mercaptoethanol were added and the solution stirred for a further 15 min.

The casein was then dialysed against 6x3l of distilled water over 60 hours. The pH of the solution was adjusted to 7.0 and the samples were then freeze-dried.

2.2.5 ION EXCHANGE CHROMATOGRAPHY

2.2.5.1 ANION EXCHANGE CHROMATOGRAPHY: MONO Q

Sample Preparations

***Casein/whey proteins*:** Freeze dried casein and whey proteins were dissolved by stirring overnight (16 h) at 4°C in 5 mM-bis-tris propane buffer pH 7.0, containing a range of urea concentrations (3, 6, 8M). The proteins were then reduced at room temperature for 1 hour by addition of excess 2-mercaptoethanol (final concentration of 20 μl ml^{-1}) and the solutions filtered through a 0.22 μm filter.

***Skimmed milk*:** Skimmed milk was dissolved overnight (25 μl ml^{-1}) at 4°C in 5 mM-bis-tris propane buffer, pH 7.0, containing 20 mM-EDTA, 8M-urea and 0.5% n-octyl-β-D-glucopyranoside. The samples were then dialysed for 4 hours at room temperature against 500 ml of a 5 mM-bis-tris propane buffer, pH 7.0, containing 8M-urea and the proteins were then reduced by the addition of 2-mercaptoethanol (final concentration 20 μl ml^{-1}) and filtered through a 0.22 μm filter.

Protein separations

Proteins were separated at room temperature on a Pharmacia Mono Q column (HR 5/5) on an FPLC system which was used in accordance with the manufacturer's instructions. Proteins were eluted using a gradient of sodium chloride in a 5 mM-bis-tris propane buffer, pH 7.0, containing urea at various

concentrations (3, 6 and 8 M) at room temperature with detection at 280 nm. Individual peak fractions were collected, dialysed against 6x3l of distilled water for 60 hours to remove buffer salts and freeze-dried.

2.2.5.2 CATION EXCHANGE CHROMATOGRAPHY: MONO S

Sample Preparation

Casein:

(a) Alkylated mouse casein was dissolved overnight at 4°C in 20 mM-sodium acetate buffer, pH 7.0, containing either 6 or 8 M-urea. After stirring for 1 hour with 2-mercaptoethanol (10 μl ml^{-1}), the pH of the solution was reduced to either 2, 3 or 5 with hydrochloric acid and the solution filtered through a 0.22 μm filter (Table 10).

(b) Freeze-dried mouse casein was dissolved overnight at 4°C in either 20 mM-formate buffer, pH 7.0, or in 20 mM-sodium acetate buffer, pH 7.0, containing either 6 or 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. The solutions were reduced for 1 hour with 2-mercaptoethanol (10 μl ml^{-1}). The pH of the samples was reduced to pH 3.0 using 1M-HCl acid and the solutions filtered through a 0.22 μm filter (Table 11).

***Whey protein*:** The freeze-dried whey protein fraction was dissolved overnight at 4°C in 20mM-sodium acetate buffer pH 7.0 containing either 6 or 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. The protein was reduced for 1 hour with 2-mercaptoethanol (20 μl ml^{-1}), the pH of the solution being reduced to either 3.0 or 5.0 and the solution filtered through a 0.22 μm filter.

TABLE 10: Cation exchange chromatography of mouse casein

Alkylated -6 M Guanidine	Alkylated -6 M Urea	Sample Prep. -6 M Urea	Sample Prep. -8 M Urea	Running buffer -6 M Urea	Final pH of protein 2	3	5	NaCl gradient (M)	Length of Run (min)	[Protein] (mg ml^{-1})	Figure	Vol. Load. (μl)
	*	*		*			*	0-0.4	27.5	2		500
	*	*		*			*	0-0.31	36.5	2		500
*		*		*			*	0-0.21	26.5	3	29A	500
*		*		*		*		0-0.4	27.5	3	29B	500
*		*		*		*		0-0.55	34.5	2		500
	*	*		*		*		0-0.55	34.5	2		500
	*		*	*			*	0-0.55	34.5	2		500
	*		*	*			*	0-0.31	36.5	2		500
	*	*		*	*			0-0.31	36.5	2	29C	500
*		*		*	*			0-0.55	34.5	2		500
*		*		*	*			0-0.31	36.5	2		500

TABLE 11: Cation exchange chromatography of mouse casein

Casein dissolved overnight in buffer containing 0.5% n-octyl β-D-glucopyranoside				pH	Running buffer, pH 3				[protein] (mg ml^{-1})	NaCl gradient (M)	Run Time (min)	Fig.	Volume loaded (μl)
A	B	C	D		A	B	C	D					
		*		3			*		2	0-0.6	40		500
		*		7			*		2	0-0.6	40	31A	500
*				7	*				2	0-0.6	40		500
	*			7		*			2	0-0.50	40		500
			*	3				*	2	0-0.25	~35		500
			*	7				*	2	0-0.35	35		500
			*	7				*	2	0-0.45	35.3		500
			*	7				*	2	0-0.28	38.3		500
	*		*	7				*	2	0-0.28	42		500
	*			7		*			2	0-1.0	46	31B	1000
	*			7		*			2	0-1.0	35		1000
	*			7		*				0-1.0	46		1000
				7		*				0-1.0	58		1000

A = 20 mM - Sodium Acetate, 6 M-urea B = 20 mM - Sodium Acetate, 8 M-urea
C = 20 mM - Formate, 6 M-urea D = 20 mM - Formate, 8 M-urea

2.2.5.3 CATION EXCHANGE CHROMATOGRAPHY: HI-LOAD S

Sample Preparation

Casein: Mouse casein (400 mg) was dissolved overnight at 4°C in 150 ml of 20 mM-sodium acetate buffer, pH 7.0, containing 8M urea and 0.5% n-octyl β-D-glucopyranoside. The casein solution was reduced for 1 hour with 2-mercaptoethanol (10 μl ml^{-1}). The pH of the sample was then reduced to 3.0 using 1M-HCl and the sample filtered through a 0.22 μm filter.

Whey protein: The freeze-dried whey protein fraction was dissolved overnight at 4°C in 20 mM-sodium acetate buffer, pH 7.0, containing either 6- or 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. The protein was reduced for 1 hour with 2-mercaptoethanol (20 μl ml^{-1}), the pH of the solution being reduced to either 3.0 or 5.0 and the solution filtered through a 0.22 μm filter.

Skimmed milk: Skimmed mouse milk was stirred overnight at 4°C in 20 mM-sodium acetate buffer, pH 7.0, containing 20 mM-EDTA, 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. The samples were dialysed for 4 hours against 500 ml of 20 mM-sodium acetate buffer pH 7.0 containing 8 M-urea and then reduced with 2-mercaptoethanol (10 μl ml^{-1}) for 1 hour before reducing the pH to 3.0 and filtering through a 0.22 μm filter.

Protein Separations

a) Analytical

Protein samples were separated at room temperature on a Pharmacia Mono S cation exchange column (HR 5/5). The flow rate was 0.5 ml min^{-1} and detection was at 280 nm.

Casein: Casein fractionation was carried out using a sodium chloride gradient in either 20 mM-sodium acetate or 20 mM-formate buffer containing 6 or 8M-urea at a variety of pH values (Tables 10 and 11).

Whey protein: Whey proteins were fractionated using a sodium chloride gradient in 20 mM-sodium acetate buffer containing 8 or 6 M-urea at pH 3.0 and pH 5.0, respectively.

Skimmed milk: Milk was fractionated using a gradient of sodium chloride in 20mM-sodium acetate buffer, pH 3.0, containing 8 M-urea.

b) Preparative: Hi-Load S

Casein: Protein was loaded at a rate of 4 ml min^{-1} and eluted using a gradient of sodium chloride in 20 mM-sodium acetate buffer, pH 3.0 containing 8 M-urea.

2.2.6 REVERSE PHASE CHROMATOGRAPHY

Sample Preparation

Casein: Alkylated freeze-dried casein was dissolved by stirring overnight at 4°C in 5 mM-bis-tris propane buffer, pH 7.0, containing either 6 M-urea or 6M-guanidine-HCl and 0.5% n-octyl β-D-glucopyranoside and then filtered through a 0.22 μm filter.

Protein Separation

Protein samples were separated at room temperature on a Pharmacia ProRPC column (HR 10/5). The separations were performed using a linear gradient of acetonitrile in 0.1% trifluoracetic acid. The gradient was 25-55% for casein separations and 10-33% for whey protein separations (Table 12).

TABLE 12: Reverse phase liquid chromatography of mouse casein

Protein Type	Dissociation buffer	pH	Eluting buffer	Protein conc. (mg ml^{-1})	Sample Volume (ml)	Elution Rate (ml min^{-1})	Run Time (min)	Wave length (nm)	Fig.
Mouse Casein	5 mM-BTP 6 M-urea	7.0	10-55% Acetonitrile 0.1% TFA	3	1	0.8	100	280	
"	"	7.0	"	"	"	"	"	214	23A
"	5 mM-BTP 6 M-urea 0.5%n-octyl β-D-glucopyranoside	7.0	"	"	"	"	"	214	
"	5 mM-BTP 6 M-guanidine-HCl	7.0	"	"	"	"	"	214	
"	5 mM-BTP, 8 M guanidine-HCl	7.0	"	"	"	"	"	280	
"	5 mM-BTP, 6 M guanidine-HCl	7.0	"	2	"	"	"	214	23B

2.2.7 GEL PERMEATION CHROMATOGRAPHY

Two gel permeation columns were assessed for separation of mouse milk proteins; Superose 12 (analytical and prep grade) and Superdex 75.

Sample Preparation

a) Superose 12

Whey: In order to optimise the separation, mouse whey protein was dissolved overnight at 4°C in a variety of buffers (Table 13A,B). The protein was then reduced in some samples using 2-mercaptoethanol (15 μl ml^{-1}) and on occasions, the pH was reduced to pH 5.0. All samples were then filtered through a 0.22 μm filter.

Casein: Casein was dissolved overnight at 4°C in either 5 mM-bis-tris propane buffer, pH 7.0, or 20 mM-sodium acetate buffer, pH 7.0, containing either 6 or 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. The protein was reduced with 2-mercaptoethanol (25 μl ml^{-1}) for 1 hour and filtered through a 0.22 μm filter (Table 13C,D).

Milk: Skimmed mouse milk was stirred overnight in 20 mM-sodium acetate buffer, pH 7.0, containing 8 M-urea, 20 mM-EDTA and 0.5% n-octyl β-D-glucopyranoside. After dialysing for four hours at room temperature against 500 ml of 20 mM-sodium acetate buffer, pH 7.0, containing 8M-urea in order to remove EDTA, the sample was reduced with 2-mercaptoethanol (15 μl ml^{-1}) for 1 hour and then filtered through a 0.22 μm filter.

Protein separation: All separations on gel permeation columns were performed at room temperature using an FPLC system. Buffers and running conditions are summarised in Tables 13A, B, C and D for the different sample

TABLE 13A: Gel permeation chromatography of mouse whey protein

Protein Type	Column Type	Run Time (min)	Dissociation Buffer	pH	Elution Buffer	pH	Sample (ml)	Elution Rate (ml mn^{-1})	[Protein] (mg ml^{-1})	Fig.
Mouse whey protein	Superose 12 analytical	50	0.1 M-Tris	7	0.1 M-Tris 5 mM-NaCl	7	0.1	0.5	3	20A
		"	6 M-guanidine-HCl, 5 mM-BTP, 0.5% n-octyl β-D-glucopyranoside	"	6 M-urea, 5 mM-BTP, 5 mM-NaCl	"	0.5	"	2	20C
"	"	"	8 M-guanidine-HCl, 5 mM-BTP, 0.5% n-octyl β-D-glucopyranoside	"	6 M-urea, 5 mM-BTP 5 mM-NaCl	"	"	"	2	
"	"	"	6 M-urea, 20 mM-Na acetate	"	6 M-urea, 20 mM-Na acetate 5 mM-NaCl	"	"	"	4	
"	"	"	6 M-urea, 0.1 M-Tris 0.5% n-octyl β-D-glycopyranoside	"	6 M-urea, 0.1 M Tris 5 mM-NaCl	"			4	20B

TABLE 13B: Gel permeation chromatography of mouse whey protein

Protein Type	Column Type	Run Time (min)	Dissociation Buffer	pH	Elution Buffer	pH	Sample (ml)	Elution Rate (ml min^{-1})	[Protein] (mg ml^{-1})	Fig.
Mouse Whey Protein	Superose 12 analytical	50	6 M-urea, 0.1 M-Tris	7	6 M-urea, 0.1 M-Tris 5 mM-NaCl	7	1	0.5	3	
"	Superdex 75	50	8 M-urea, 0.1 M-Tris 0.5% n-octyl β-D-glucopyranoside	"	8 M-urea 0.1 M-Tris 5 mM-NaCl	5	"	"	3	20D
"	"	"	0.1 M-Tris 0.5% n-octyl β-D-glucopyranoside	"	0.1 M-Tris 5 mM-NaCl	7	"	"	2	20E

TABLE 13C: Gel permeation chromatography of mouse casein

Protein Type	Column Type	Run Time (min)	Dissociation Buffer	pH	Elution Buffer	pH	Sample vol. (ml)	Elution Rate (ml min^{-1})	[Protein] (mg ml^{-1})
Mouse Casein	Superose 12 Prep-Grade	912	8 M-urea, 5 mM-BTP	7	8 M-urea, 5 mM-BTP 5 mM-NaCl	7	0.2	0.12	50
"	"	302	8 M-urea, 5 mM-BTP	"	"	"	0.2	0.5	"
"	"	200	8 M-urea, 5 mM-BTP	"	"	"	0.1	0.5	"
	"	200	8 M-urea, 5 mM-BTP	"	"	"	1	1.0	"

TABLE 13D: Gel permeation chromatography of mouse casein

Protein Type	Column Type	Run Time (min)	Dissociation Buffer	pH	Elution Buffer	pH	Sample vol. (ml)	Elution Rate (ml min^{-1})	[Protein] mg ml^{-1}	Fig.
Mouse Casein	Superose 12 Analytical	50	6 M-urea, 5 mM-BTP	7	6 M-urea, 5 mM-BTP, 5 mM-NaCl	7	0.1	0.5	2.4	19A
"	Superdex 75	"	8 M-urea, 20 mM-Na acetate 0.5% n-octyl β-D-glucopyranoside	"	8 M-urea, 20 mM Na acetate 5 mM-NaCl	"	1	"	3	19B
Native Mouse Casein	"	"	8 M-urea, 20 mM-Na acetate 20 mM-EDTA 0.5% n-octyl β-D-glucopyranoside	"	8 M-urea 20 mM Na acetate 5 mM-NaCl	5	"	"	2.5	

and column types.

2.2.8 PROTEIN DESALTING

Proteins which had previously been separated by means of ion-exchange chromatography in aqueous buffers were desalted by reverse-phase liquid chromatography. Desalting was carried out on a ProRPC column and was achieved using a 0-70% gradient of acetonitrile containing 0.1% TFA over 18.5 minutes at a flow rate of 1.5 ml min^{-1}.

Volumes of up to 2.0 ml were desalted in a single run giving rise in most cases to single protein peaks which were collected and freeze dried.

2.2.9 ELECTROPHORESIS

2.2.9.1 SDS-PAGE

Sample Preparation: Approximately 100 μg of protein were dissolved in 11 μl of stock SDS buffer (60 μl of 20% SDS , 24 μl 2-mercaptoethanol, 366 μl of a solution of 10mM-Tris-HCl and 1mM-EDTA, pH 8.0, and 30 μl 0.05% bromophenol blue). Samples were then denatured by placing them in a boiling water bath for 5 minutes.

Run conditions: A maximum of 12 x 0.3 μl samples was analysed on a Pharmacia 20% homogeneous PhastGel using SDS buffer strips. The apparatus was used in accordance with the manufacturer's instructions and run at 250 Volts at 15°C for 95 volt hours(vh).

Development:

(a) *Coomassie stain*: Gels were stained in a 2% solution of Coomassie blue for 8 minutes at 50°C. Gels were then destained for a total of 23 minutes at 50°C

using a solution of methanol, water, glacial acetic acid (3:6:1, v/v/v), with three changes of buffer. After destaining, gels were preserved by incubating them with a solution of 10% glycerol and 10% glacial acetic acid at 50°C for 7.5 minutes.

(b) *Silver stain*: Gels were stained initially with Coomassie blue and destained as described above. The gel was then washed with distilled water for 4 minutes at 40°C and overstained with a 0.4% solution of silver nitrate. The gel was developed in a 2.5% solution of sodium carbonate containing 50 μl formaldehyde/150 ml for 5 minutes at 30°C. The intensity of background staining was reduced by incubating the gel in a solution of sodium thiosulphate(0.1 M) and tris(0.3 M) for 1.5 minutes at 30°C and the gel was preserved as previously described.

(c) *Stains-all*: Proteins were fixed in the gel by incubating with a 25% propan-2-ol solution for 10 minutes at 50°C. The gel was then stained for 16 hours at room temperature with Stains-all solution (10 mg Stains-all in 3M-Tris-HCl pH 8.8, formamide, propan-2-ol and distilled water, 1:20:50:129, v/v/v/v). The gel was then destained in distilled water at room temperature until the background was reduced.

2.2.9.2 ALKALINE GEL ELECTROPHORESIS

i) Phast System

Sample preparation: Whole mouse casein and individual casein fractions were dissolved in 25 mM-Tris, 3.2 mM-EDTA, 27 mM-Barbitone buffer, pH 7.9, containing 8M-urea. Whole casein was used at a concentration of 2 mg ml^{-1} and casein fractions at 1 mg ml^{-1}. Samples were reduced with 2-

mercaptoethanol (10 μl ml^{-1}) for 1 hour at room temperature.

Gel preparation: A 12.5% homogeneous PhastGel was soaked in a 25 mM-Tris, 3.2 mM-EDTA, 27 mM-Barbitone buffer, pH 7.9, containing 4.5 M-urea for 1 hour with constant agitation. The surface of the gel was then allowed to dry (30 minutes) before loading samples in order to prevent the protein diffusing into any surface liquid when it was applied to the gel.

Buffer strip preparation: 1 g of agarose (IEF grade) was dissolved, with heating, in 50 ml of 25 mM-Tris, 3.2 mM-EDTA and 27 mM-Barbitone buffer, pH 7.9. After cooling to 50°C, 2.5 ml aliquots were dispensed into buffer strip moulds.

Running conditions: Electrophoresis was performed on a Pharmacia Phast System, at 400 V at 15°C for 98 vh.

Development: Gels were stained and destained as previously described for SDS-PAGE.

ii) Slab gel electrophoresis

Sample preparation: Casein was dissolved in 25 mM-Tris, 3.2 mM-EDTA, 27 mM-barbitone buffer, pH 7.9, containing 8 M-urea, 0.5% n-octyl β-D-glucopyranoside and 0.05% bromophenol blue at a protein concentration of 20 mg ml^{-1}. Alternatively, skimmed milk was mixed with the above buffer at a ratio of 3 parts buffer to 1 part milk. Sufficient solid urea was added to the mixture to bring the concentration to 8M. The samples were then reduced with 2-mercaptoethanol (10 μl ml^{-1}) for 1 hour at room temperature.

Gel preparation: Acrylamide/bisacrylamide (96:4, w/w) was dissolved in Tris-EDTA-Barbitone buffer, pH 7.9, containing 4.5 M-urea, the final pH of the

solution being 8.0 +/- 0.1.

Ammonium persulphate (0.2%) was dissolved in the solution followed by 300 μl of dimethylaminopropionitrile per 100 ml of gel solution. The gel solution was poured immediately into an E-C470 gel cell and allowed to polymerise for 30 minutes.

Running conditions: A pre-electrophoresis run was performed for 30 minutes at 300 volts, to remove excess catalysts and impurities. Samples were then loaded and separated in accordance with the manufacturer's instructions for 6 hours at 200 volts.

Development: Proteins were fixed in the gel by incubating it in a solution of methanol, water and glacial acetic acid (5:10:1, v/v/v) for 10 minutes at room temperature. The gel was stained with a 1% solution of napthalene black 12B in methanol, water, glacial acetic acid (5:10:1, v/v/v) for 10 minutes at room temperature. The gel background was destained with several changes of methanol, water, glacial acetic acid(5:10:1, v/v/v) over a period of 2-3 days.

2.2.10 DEPHOSPHORYLATION

(a) Aqueous solutions of bovine and murine caseins (4%, w/v), with or without 20 mM-EDTA, were incubated at 38°C for 2 hours in the presence and absence of 0.04 units ml^{-1} potato acid phosphatase.

(b) Skimmed mouse milk (25 μl) was diluted with 50 μl of water with or without 20 mM-EDTA and incubated at 38°C for 2 hours in the presence or absence of 0.02 units ml^{-1} potato acid phosphatase.

Aliquots (50 μl) of the incubated casein solutions were diluted with 300 μl of 25 mM-Tris, 3.2 mM EDTA and 27 mM-barbitone buffer, pH 7.9, containing 8M-urea. Aliquots (25 μl) of incubated milk solutions were diluted with 150 μl of the Tris-EDTA-barbitone buffer containing 8M-urea. All samples were then reduced with 28 μl of 2-mercaptoethanol for 1 hour and separated on alkaline gels.

2.2.11 RENNETING

Murine and bovine caseins were dissolved in phosphate buffer (20 mM-di-sodium hydrogen orthophosphate, 46 mM-potassium dihydrogen orthophosphate, pH 6.5) at a concentration of 10 mg ml^{-1}. Protein solutions were incubated with or without various amounts of chymosin solution (specific activity 91 units mg^{-1}, 100 μg chymosin in 100 μl phosphate buffer = 9.1 units of activity) for 30, 60, 90 and 120 minutes at room temperature. The reaction was stopped by the addition of 200 μl of stock SDS-buffer to the samples which were then heated in a boiling water bath for 5 minutes. The volume of the samples was then reduced in a freeze dryer to approximately 150 μl and then analysed by SDS-PAGE. The experimental layout is shown in Table 14.

2.2.12 AMINO ACID ANALYSIS

Approximately 20 μg of protein were subjected to gas phase hydrolysis at 120°C for 20 hours under vacuum in 6 M-HCl containing 1% phenol. The hydrolysate was treated with a mixture of ethanol, water and triethylamine (2:2:1, v/v/v) to ensure complete removal of HCl. The samples was dried

TABLE 14: Rennet/Chymosin treatment of murine and bovine casein

Casein	Amount (mg)	Volume Phosphate Buffer (μl)	Volume of Rennet/Chymosin	Incubation Time (min)	Incubation Time	Vol.of SDS-Buffer
Bovine	4 x 1	100	3 μl/5,10,15 μl	30	Room Temp.	200
Murine	4 x 1	100	3 μl/5,10,15 μl	30	Room Temp.	200
Bovine	4 x 1	100	3 μl/5,10,15 μl	60	Room Temp.	200
Murine	4 x 1	100	3 μl/5,10,15 μl	60	Room Temp.	200
Bovine	4 x 1	100	3 μl/5,10,15 μl	90	Room Temp.	200
Murine	4 x 1	100	3 μl/5,10,15 μl	90	Room Temp.	200
Bovine	4 x 1	100	3 μl/5,10,15 μl	120	Room Temp.	200
Murine	4 x 1	100	3 μl/5,10,15 μl	120	Room Temp.	200

in a Gyrovap for 30-60 minutes at 45°C.

Phenylthiocarbamyl (PTC) derivatives of the amino acids were prepared by the addition of 60μl of a derivatisation buffer containing ethanol, water, triethylamine and phenylisothiocyanate (7:1:1:1, v/v/v/v). Tubes were incubated for 15 minutes at room temperature to allow the derivitisation to go to completion before drying at 30°C for 20 minutes. The PTC-amino acids were dissolved in 70 μl of acetonitrile and water (1:1, v/v) and transferred to HPLC sample tubes. Redistilled water(630 μl) was then added before separating the PTC-amino acids on a Spherisorb ODS reverse phase column (200 X 4.6 mM i.d). The separation was carried out at 38°C using a gradient of 5-25% acetonitrile in 12.5 mM-sodium phosphate buffer, pH 6.4, at a flow rate of 1.5 ml min^{-1} and detection at 254 nm.

2.3 RESULTS

2.3.1 MILK PROTEIN FRACTIONATION

2.3.1.1 OPTIMISATION OF THE pH OF CASEIN PRECIPITATION

The optimum pH for the precipitation of mouse casein in skimmed milk was determined by SDS-PAGE analysis of the precipitated and soluble protein fractions at a variety of pH values (Figure 18). Very little casein could be detected in the whey fraction at pH 4.27 and this pH was subsequently routinely used in all isoelectric precipitations of mouse caseins.

2.3.1.2 QUANTITY OF CASEIN AND WHEY PROTEINS IN MOUSE MILK

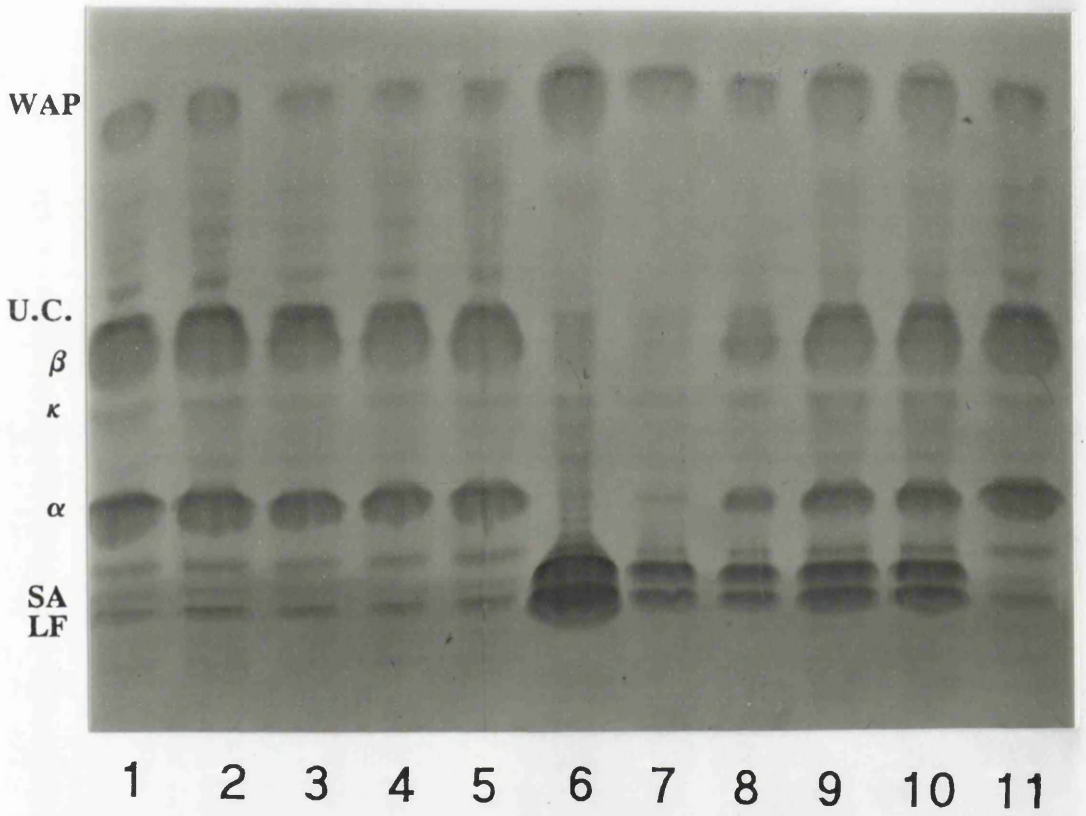
The casein and whey protein content of the milks from 19 individual mice milked in mid-lactation (10-12 days *post partum*) was calculated (Table 15). Casein was present in milk at a concentration of between 50 and 100 mg ml^{-1}, corresponding to 65-89% of the total protein. Whey protein was found at concentrations of between 11 and 28 mg ml^{-1}, which corresponds to 10-34% of the total protein. The total protein content of the milk ranged from 62 to 112 mg ml^{-1}.

2.3.2 CHROMATOGRAPHIC SEPARATION OF PROTEINS

2.3.2.1 GEL PERMEATION CHROMATOGRAPHY

i) Casein: Casein was analysed on two types of Superose 12 and Superdex 75 (Table 13C, D). Typical elution profiles are shown in Figure 19A and B, respectively. Peak fractions were collected and analysed by SDS-PAGE. No fractionation of the individual caseins was achieved with peaks numbered 1 found to consist of whole casein and peaks numbered 2 consisting of the reducing agent 2-mercaptoethanol. Gel permeation chromotography was

FIGURE 18



SDS-PAGE analysis of skimmed mouse milk precipitated over a range of pH values.

Tracks 1-5 and 11 contained casein and 6-10 whey protein fractions from the following precipitations at pH: (1) 4.02; (2) 4.27; (3) 4.45; (4) 4.63; (5) 4.80; (6) 4.02; (7) 4.27; (8) 4.45; (9) 4.63; (10) 4.80; (11) 4.02.

WAP: Whey acidic protein
U.C: Unidentified casein
β: β-casein
κ: k-casein
α: α-casein
LF: Lactoferrin
SA: Serum albumin

TABLE 15: Concentration of casein and whey protein in mouse milk as calculated from 19 animals

Sample	[Casein] mg ml^{-1}	[Whey protein] mg ml^{-1}	[Total Protein] mg ml^{-1}	Casein %	Whey %
1	53.2	21.9	75.1	70.8	29.2
2	49.5	19.7	69.2	71.5	28.5
3	63.0	19.5	82.5	76.4	23.6
4	65.0	11.8	76.9	84.6	15.4
5	60.8	25.0	85.8	70.8	29.2
6	56.3	20.6	76.9	73.1	26.9
7	50.4	18.7	69.1	72.8	27.2
8	53.5	13.8	67.3	79.4	22.6
9	100.0	12.0	112	89.2	10.8
10	66.6	11.8	78.4	85.0	15.0
11	51.5	10.9	62.4	82.6	17.4
12	54.8	28.3	83.0	66.0	34.0
13	75.9	13.8	89.6	84.7	15.3
14	71.0	11.4	82.4	86.2	13.8
15	50.9	14.2	65.0	78.3	21.7
16	67.0	14.6	81.7	82.0	18.0
17	60.0	11.4	71.4	84.0	16.0
18	74.3	14.3	88.6	83.9	16.1
19	83.8	16.3	100.0	83.8	16.2

FIGURE 19: Gel permeation chromatography of mouse casein

(A) Protein (2.4 mg) dissolved in Bis-tris-propane buffer (5 mM, pH 7.0) containing 6 M-urea (final concentraion 2.5 mg ml^{-1}) and protein reduced as described in the text. Separation developed using the same buffer containing sodium chloride (5 mM) on a Superose 12 column, at 0.5 ml min^{-1} with detection at 280 nm.

(B) Protein (3 mg) dissolved in sodium acetate buffer (20 mM, pH 7.0) containing 8 M-urea and 0.5% n-octyl β-D-glucopyranoside (final concentration 3 mg ml^{-1}) and reduced as described in the text. Separation developed on a Superdex-75 column. The separation was then carried out as described in (A) above.

SUPEROSE 12 SEPARATION OF MOUSE CASEIN

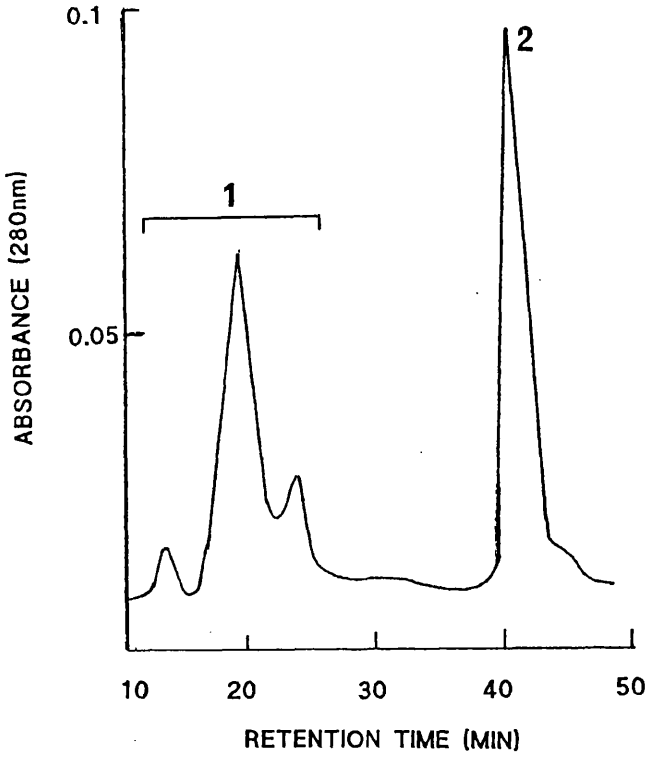


FIGURE 19(A)

SUPERDEX 75 SEPARATION OF MOUSE CASEIN

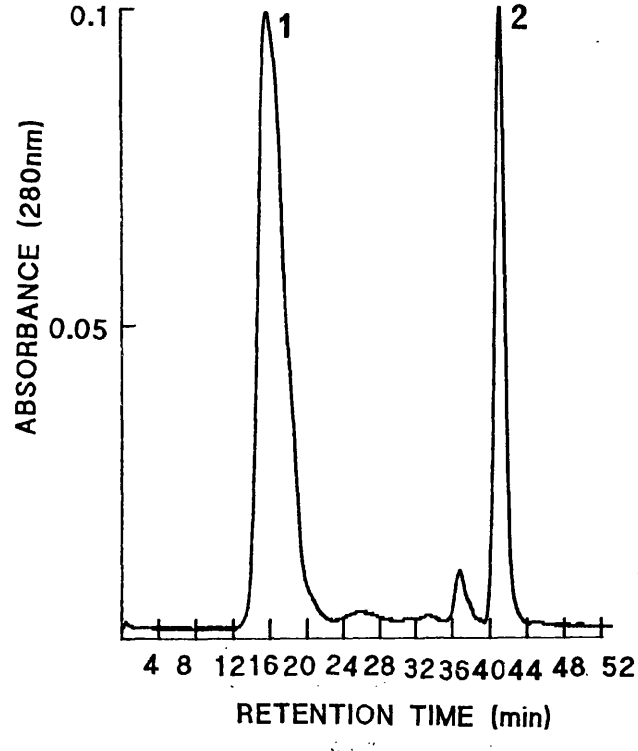


FIGURE 19(B)

therefore deemed to be unsuitable as a means of purifying mouse caseins.

ii) ***Whey***: Native whey proteins were separated on Superose 12 and Superdex 75 columns using a variety of dissociation conditions and elution buffers(Table 13A, B). Results of Superose 12 analyses are shown in Figures 20A, B and C; Superdex 75 results are shown in Figures 20D and E. Peak fractions were analysed by SDS-PAGE. The results indicated that WAP could be separated as a pure fraction. Milk serum albumin and lactoferrin, due to the similarity of their molecular weights, were usually eluted together. The last peak in all separations contained only the reducing agent 2-mercaptoethanol. (Figure 21).

iii) ***Milk***: Native milk was analysed on a Superdex 75 gel permeation column. Results of the analysis are shown in Figure 22. Individual peak fractions were collected, desalted by reverse phase chromatography and examined by SDS-PAGE. It is evident from the figure that the proteins in the milk could not be sufficiently separated by this method of chromatography. The desalting step gave rise to a slightly improved separation but peak fractions were still found to contain mixtures of proteins when examined by SDS-PAGE.

2.3.2.2 REVERSE PHASE CHROMATOGRAPHY

Casein: Various dissociation conditions and buffers were tested in an attempt to separate mouse caseins by reverse phase chromatography. The elution profiles shown in Figure 23A and B were obtained from separations of whole casein dissolved in 5mM-Bis-tris propane buffer containing 0.5% n-octyl β-D-glucopyranoside and either 6 M-urea or 6 M-guanidine(Table 12). SDS-PAGE analysis of individual peak fractions showed some to consist of pure fractions of κ-, β- and α-casein. However, the remaining peak fractions were

FIGURE 20: Gel permeation chromatography of mouse whey protein

(A) Protein (3 mg) dissolved in Tris buffer (0.1 M, pH 7.0) at a concentration of 3 mg ml^{-1}. Separation developed using the same buffer at 0.5 ml min^{-1} on a Superose-12 column with detection at 280 nm. Numbered fractions were collected and analysed by SDS-PAGE.

(B) Protein (3 mg) dissolved in the Tris buffer (0.1 M pH 7.0) containing 6 M-urea and 0.5% n-octyl β-D-glycopyranoside (final concentration 4 mg ml^{-1}). Separation developed in the same buffer on a Superose-12 column with detection at 280 nm.

(C) Protein (2 mg) dissolved in Bis-tris propane buffer (pH 7) containg 6 M-guanidine hydrochloride and 0.5% n-octyl β-D-glycopyranoside (final concentration 2 mg ml^{-1}). Separation developed in same buffer on a Superose-12 column with detection at 280 nm.

SUPEROSE 12 SEPARATION OF MOUSE WHEY PROTEIN

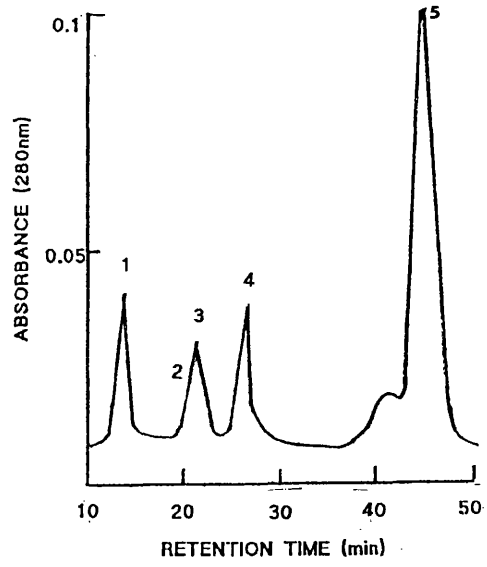


FIGURE 20(A)

SUPEROSE 12 SEPARATION OF MOUSE WHEY PROTEIN

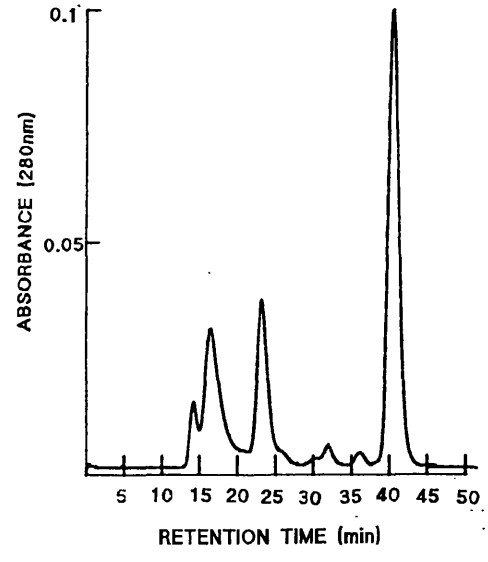


FIGURE 20(B)

SUPEROSE 12 SEPARATION OF MOUSE WHEY PROTEIN

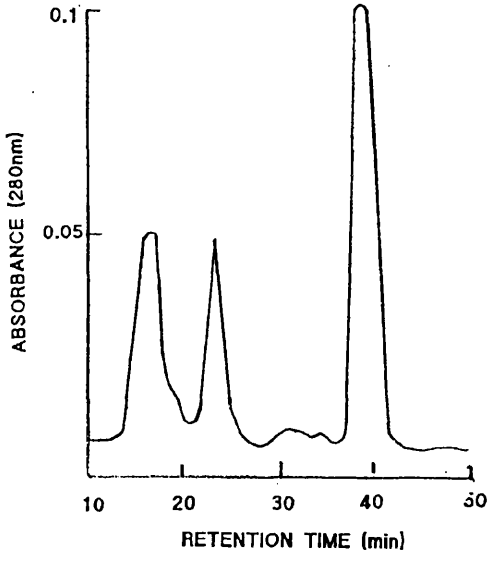


FIGURE 20(C)

FIGURE 20: Gel permeation chromatography of mouse whey protein

(D) Protein (3 mg) dissolved in Tris buffer (0.1 M, pH 7.0 final concentration 3 mg ml^{-1}) containing 0.5% n-octyl β-D-glucopyranoside, for 16 hours at 4°C and reduced as described in the text. Separation developed on a Superdex 75 column.

(E) Protein (2 mg) dissolved in Tris buffer (0.1 M, pH 7.0, final concentration 2 mg ml^{-1}) containing 8 M-urea and 0.5% n-octyl β-D-glucopyranoside for 16 hours at 4°C and reduced as described in the text. Separation developed as described in (D) above.

SUPERDEX 75 SEPARATION OF MOUSE WHEY PROTEIN

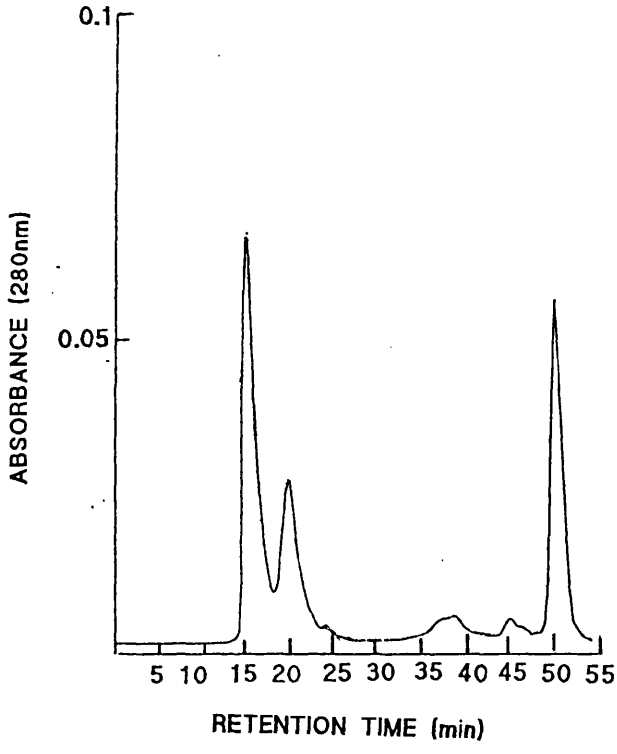


FIGURE 20(D)

SUPERDEX 75 SEPARATION OF MOUSE WHEY PROTEIN

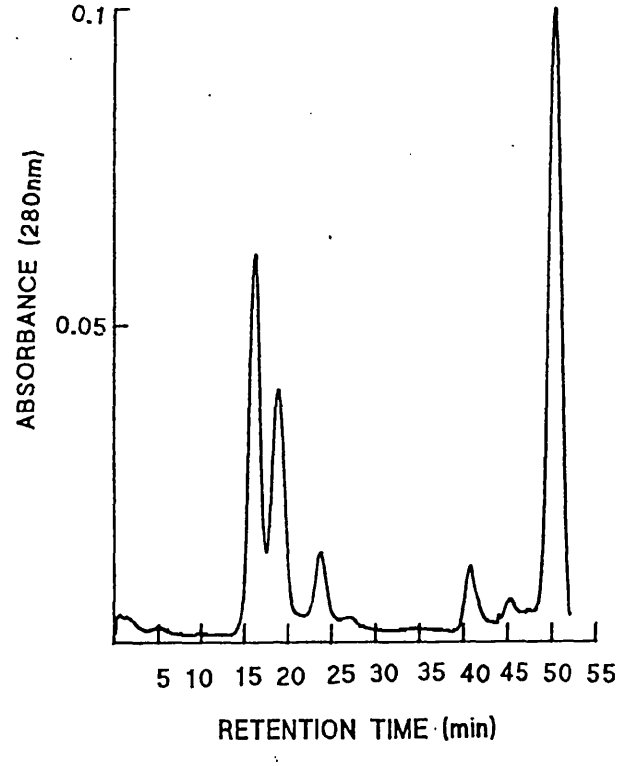
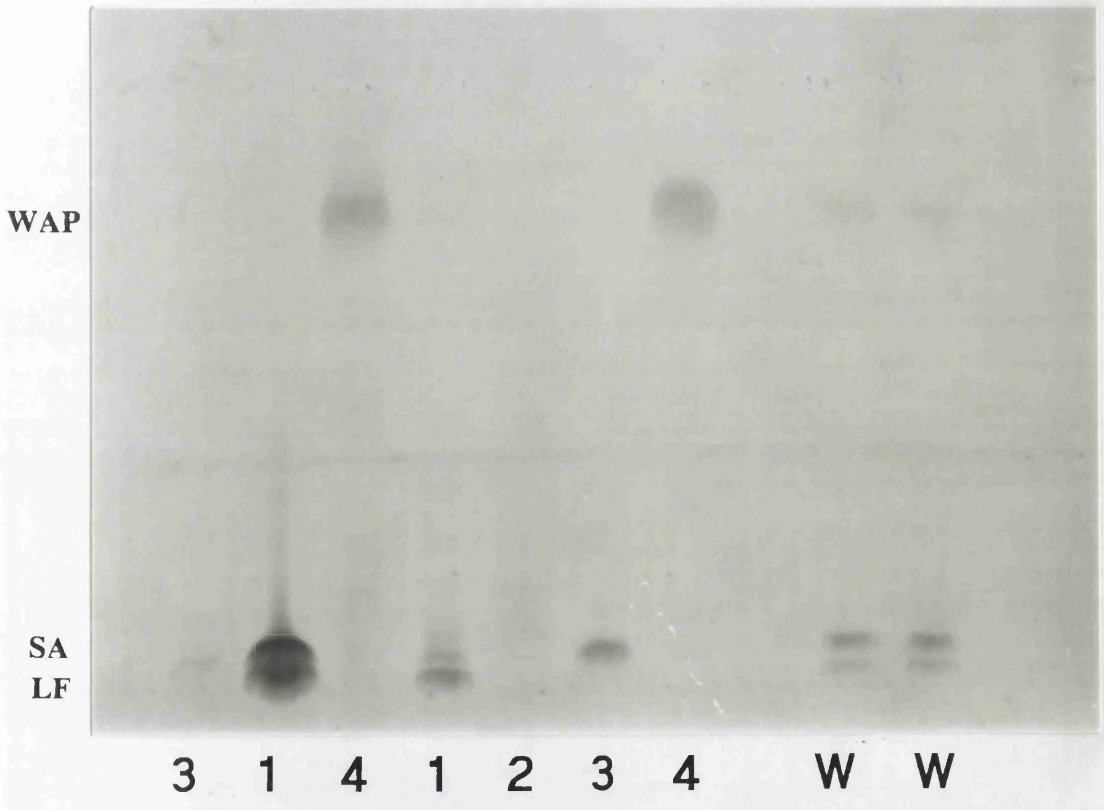


FIGURE 20(E)

FIGURE 21



SDS-PAGE analysis of gel permeation chromatography of mouse whey protein. Fractions collected from a Superose 12 column.

Tracks numbered 1-4 correspond to peak fractions collected from Figure 20A.
W: denotes freeze-dried whey protein.

WAP: Whey acidic protein
LF: Lactoferrin
SA: Serum albumin

SUPERDEX-75 SEPARATION OF NATIVE MOUSE MILK

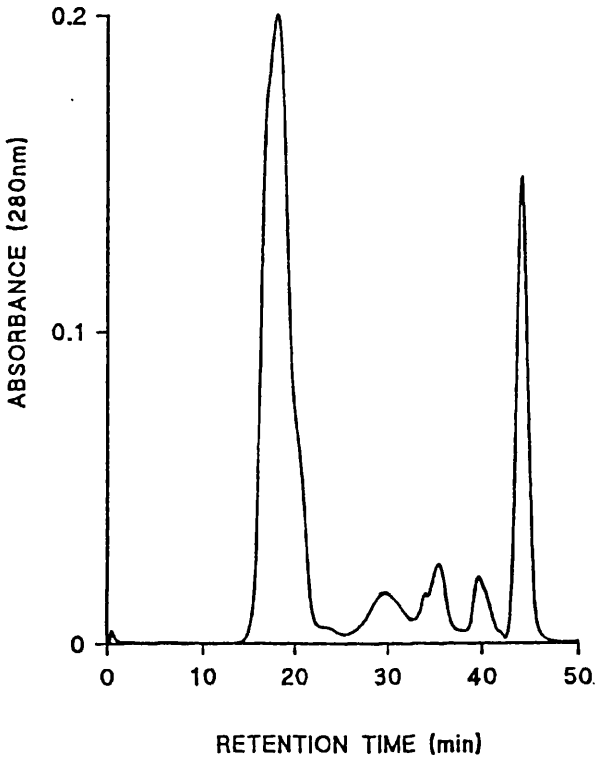


FIGURE 22

Native milk: 20μl skimmed milk (~2mg protein) dissolved in 1ml sodium acetate buffer (20mM, pH7.0 containing 8M-urea, 20mM-EDTA and 0.5% n-octyl β-D-glucopyranoside for 16 hours at 4°C. Protein solution dialysed and reduced as described in the text. Separation developed in sodium acetate buffer above containing 8M-urea on a Superdex-75 column at 0.6ml mn^{-1} flow rate and detection at 280nm.

FIGURE 23: Reverse phase chromatography of mouse casein

(A) Protein (3mg) dissolved in Bis-tris propane buffer (5mM, pH7.0 final concentration 3mg ml^{-1}) containing 6M-urea and reduced as described in text. The separation was developed on a ProRPC column and eluted with a gradient of acetonitrile with detection at 214nm.

(B) Protein (2mg) dissolved in Bis-tris-propane buffer (5mM, pH7 final concentration 2mg ml^{-1}) containing 6M-guanidine hydrochloride and 0.5% n-octyl β-D-glucopyranoside and reduced as described in the text. Numbered fractions were analysed by SDS-PAGE.

ProRPC SEPARATION OF MOUSE CASEIN

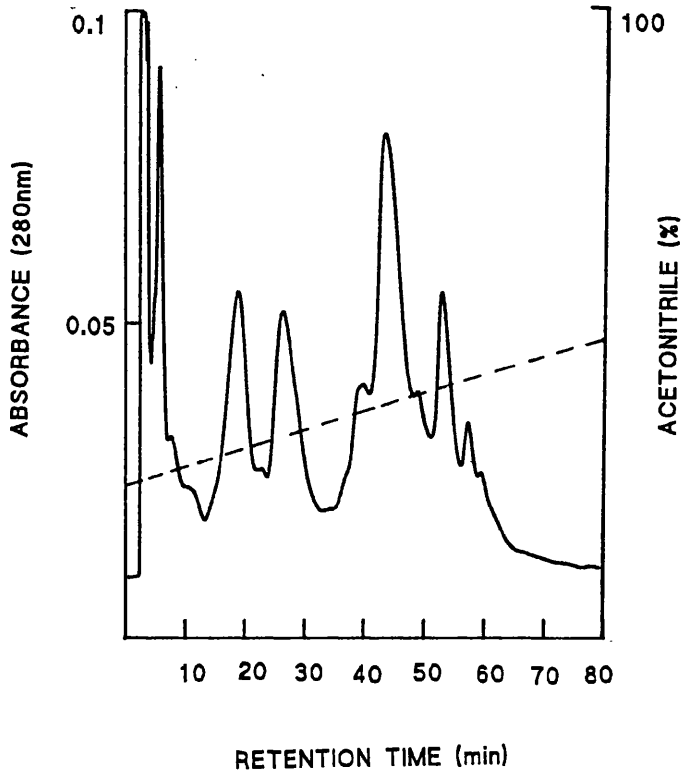


FIGURE 23(A)

ProRPC SEPARATION OF MOUSE CASEIN

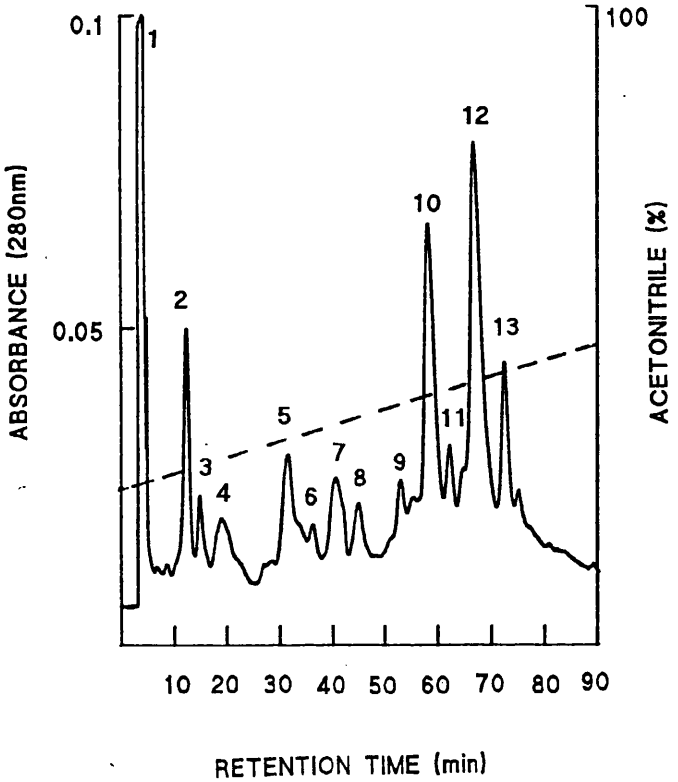


FIGURE 23(B)

found to contain either mixtures of proteins or low molecular weight components that did not stain on the gel (Figure 24).

2.3.2.3 ANION EXCHANGE CHROMOTOGRAPHY

Casein: Various dissociation conditions and elution buffers were tested in an attempt to purify individual caseins from whole mouse casein on a Pharmacia Mono Q column. Bis-tris-propane buffers(5 mM, pH 7) containing either 6 or 8M-urea, gave the best casein separations. The proteins were eluted as a number of peaks (Figure 25). However, analysis of individual peaks by SDS-PAGE showed them to contain mixtures of proteins (Figure 26).

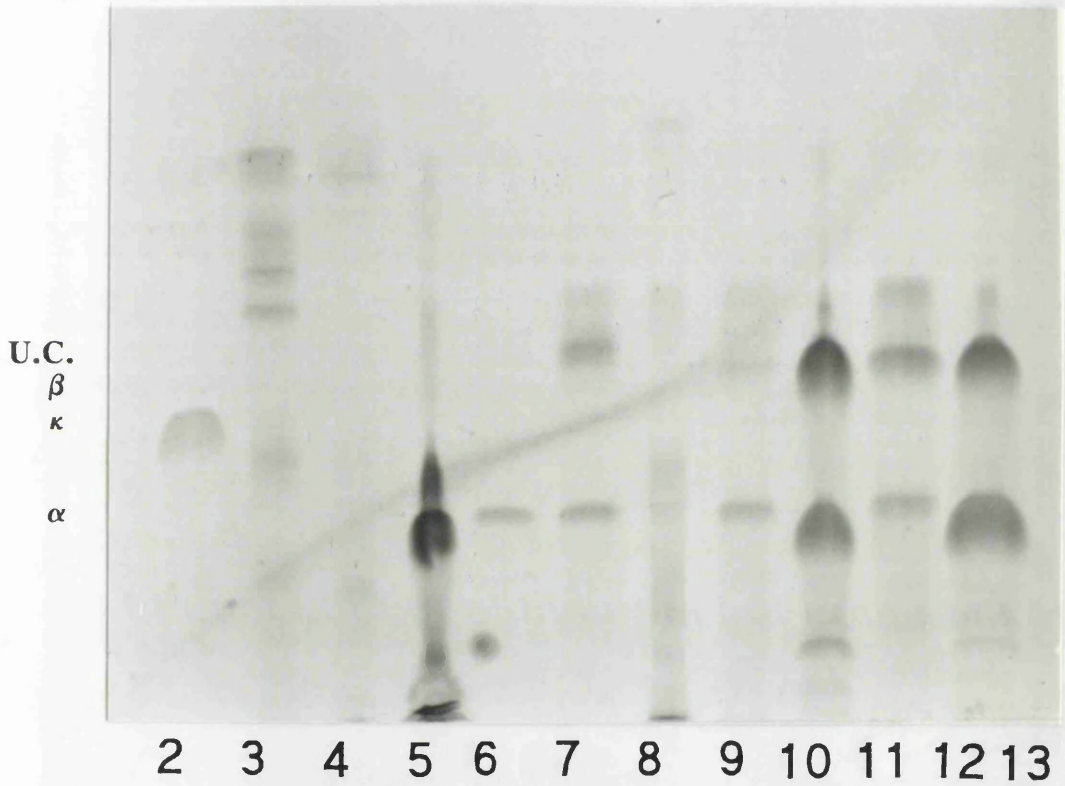
Whey: Anion-exchange chromatography of mouse whey protein was not a successful means of separating individual proteins. Peak 1 contained only the reducing agent and peak 2 a mixture of whey proteins (Figure 27). As a result, anion-exchange chromatography was not pursued as a means of separating mouse whey protein.

Milk: Native and mouse milk when separated on the Mono Q column gave a number of protein peaks similar to those observed in the casein separations. A large peak was also eluted when the column was washed with buffer containing 1M-sodium chloride (Figure 28). SDS-PAGE showed that individual peaks contained mixtures of caseins and that the large end peak consisted of undissociated casein.

2.3.2.4 CATION EXCHANGE CHROMATOGRAPHY

Casein: A variety of dissociation conditions, elution buffers, alkylation conditions and pH values were tested in order to optimise the separation of murine casein by cation exchange FPLC on a Pharmacia Mono S column.

FIGURE 24



SDS-PAGE analysis of reverse-phase chromatography of mouse casein on a Pro RPC column.

Tracks numbered 2-13 contained fractions 2-13 collected from Figure 23B.

U.C: Unidentified casein
β: β-casein
κ: κ-casein
α: α-casein

MONO Q SEPARATION OF NATIVE MOUSE CASEIN

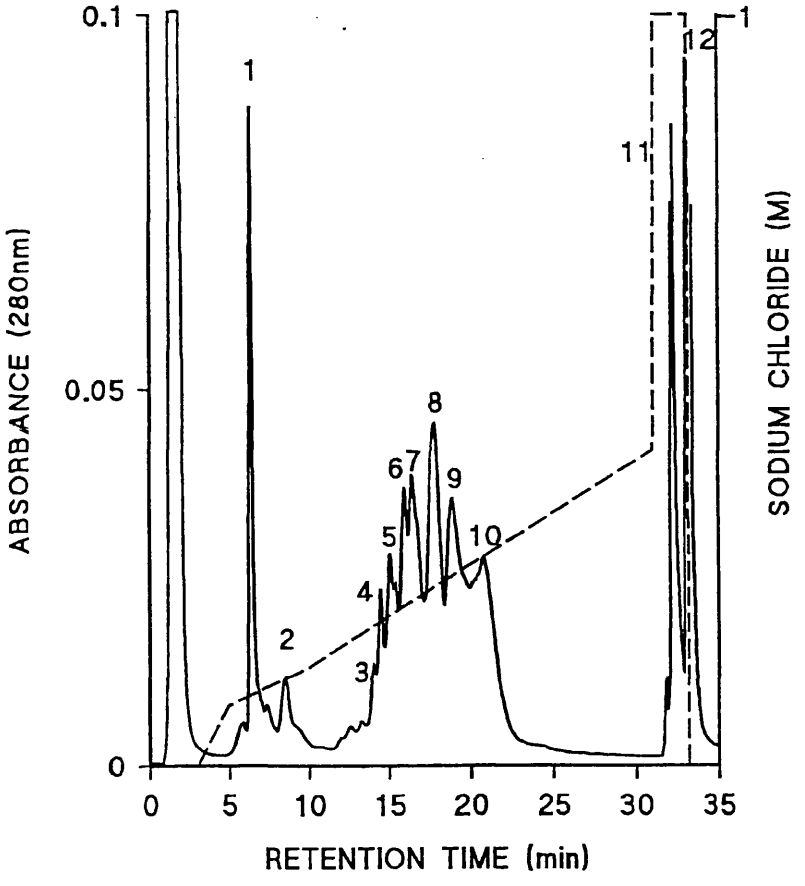
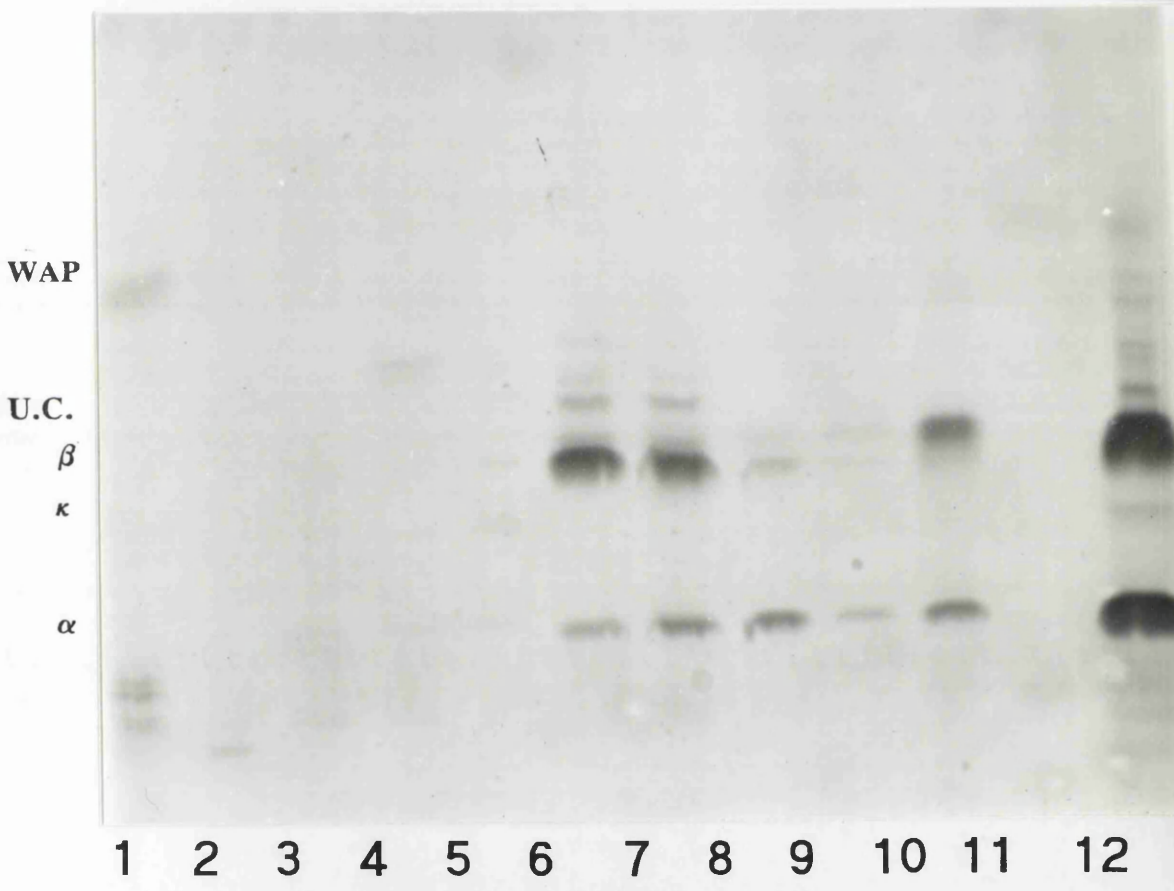


FIGURE 25

Protein (2 mg) was dissolved in Bis-tris-propane buffer (5 mM, pH 7.0, final protein concentration 2 mg ml^{-1}) containing 6M-urea for 16 hours at 4°C and reduced as described in the text. Proteins were eluted from the Mono Q column using a gradient of sodium chloride, with detection at 280 nm. Numbered fractions were analysed by SDS-PAGE (Figure 26).

FIGURE 26



SDS-PAGE analysis of mouse casein peak fractions obtained from anion-exchange chromatography of freeze-dried mouse casein on a Mono Q column.

Tracks 1-12 contained peak fractions numbered 1-12 on Figure 25.

WAP: Whey acidic protein
U.C: Unidentified casein
β: β-casein
κ: κ-casein
α: α-casein

MONO Q SEPARATION OF NATIVE MOUSE WHEY PROTEIN

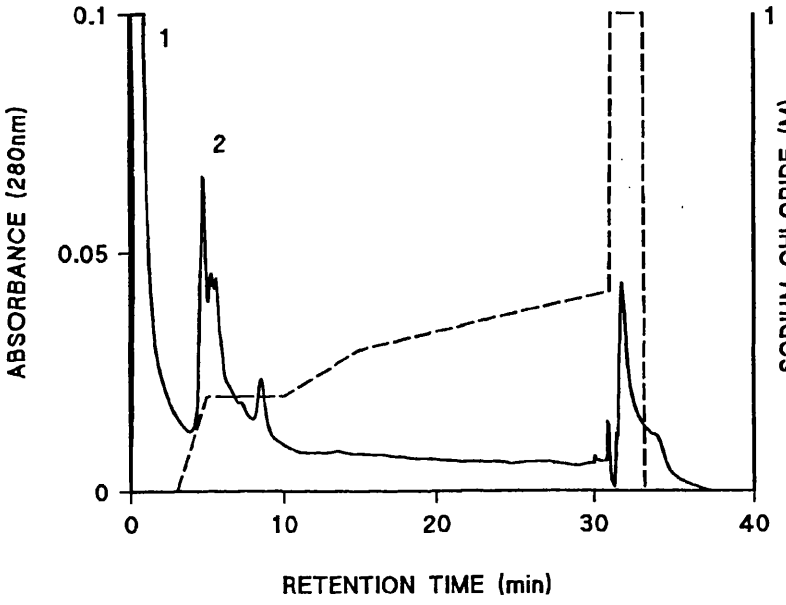


FIGURE 27

Protein (2 mg) was dissolved in 5 ml Bis-tris-propane buffer (5 mM, pH 7.0) containing 8M-urea for 16 hours at 4°C and reduced as described in the text. Proteins were eluted from a Mono Q column using a gradient of sodium chloride with detection at 280 nm.

MONO Q SEPARATION OF NATIVE MOUSE MILK

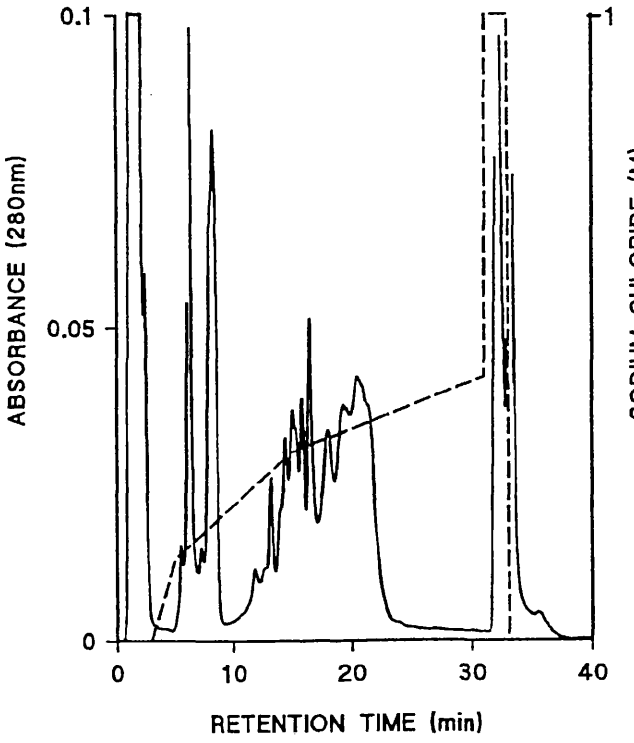


FIGURE 28

Native milk: Skimmed milk (20 μl, ~2 mg protein) was dissolved in 1 ml Bis-tris-propane buffer (5 mM, pH 7) containing 6M-urea for 16 hours at 4°C and reduced as described in the text. Proteins were eluted from the Mono Q column using a gradient of sodium chloride, with detection at 280 nm.

The pH of the elution buffer was found to be critically important with respect to the efficiency of the separation of the caseins, as is evident from the dramatic changes observed between elution profiles of separations performed at pH 5.0, pH 3.0 or pH 2.0 (Figures 29A,B and C). Numbered fractions were collected and analysed by SDS-PAGE(Figures 30A, B and C). Pure fractions were obtained only for the unidentified casein and α-casein in fractions numbered 3 and 5, 6 and 5 and 3 and 4 of Figures 29A, B and C respectively.

Differences were also apparent in elution profiles in which the dissociation conditions of the caseins were varied. The most notable difference was observed when the non-ionic detergent, n-octyl β-D-glucopyranoside, was included in the dissociation buffer (Figure 31B). The presence of the detergent resulted in enhanced dissociation of the caseins. This is evident by the increase in the area of individual peak fractions, together with a reduction in the size of undissociated casein peak which was eluted by 1 M-sodium chloride during the re-equilibration phase of the chromatographic run. SDS-PAGE analysis of those peak fractions collected in the absence of detergent (Figure 31A) revealed two almost pure caseins; α-casein and the unidentified casein(Fractions numbered 5 and 6, respectively, gel not shown). In the presence of detergent, fractions 2, 3, 4 and 5 consisted of pure WAP, κ-casein, β-casein and α-casein, respectively, with fraction 6 containing mainly the unidentified casein (Figure 32). In some samples analysed, fraction 6 was also found to contain a number of low molecular weight peptides.

Whey: Mouse whey proteins were separated by cation-exchange

FIGURE 29: Effect of pH on the separation of the alkylated mouse casein on cation exchange chromatography

(A) Protein (2 mg), alkylated in the presence of 6M-guanidine hydrochloride as described in the text, was dissolved in sodium acetate buffer (20 mM, pH 7.0, final protein concentration 2 mg ml^{-1}) containing 6M-urea for 16 hours at 4°C. Separation performed at pH 5.0 on a Mono S column using a gradient of sodium chloride. Numbered fractions were analysed by SDS-PAGE (Figure 30A).

(B) Protein was prepared as described above and separation performed at pH 3.0. Numbered fractions were analysed by SDS-PAGE (Figure 30B).

(C) Protein was prepared as described above and separation performed at pH 2.0. Numbered fractions were analysed by SDS-PAGE (Figure 30C).

MONO S SEPARATION OF MOUSE CASEIN

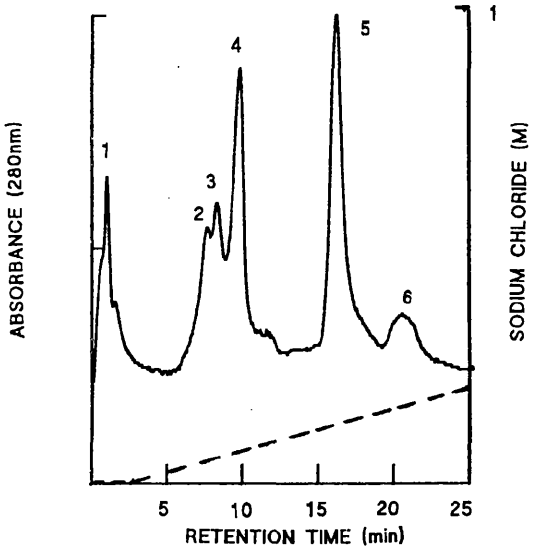


FIGURE 29(A)

MONO S SEPARATION OF MOUSE CASEIN

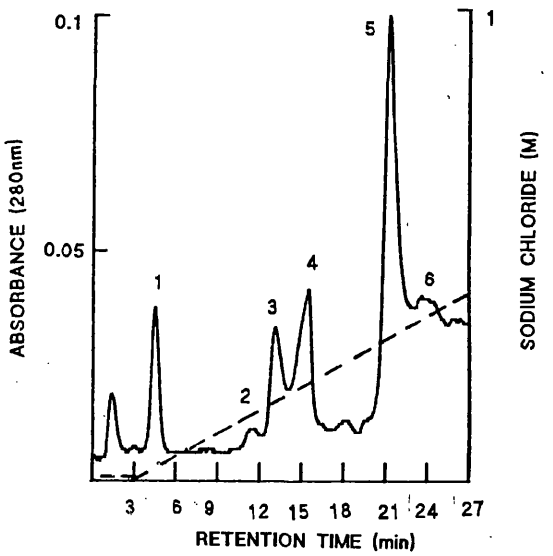


FIGURE 29(B)

MONO S SEPARATION OF MOUSE CASEIN

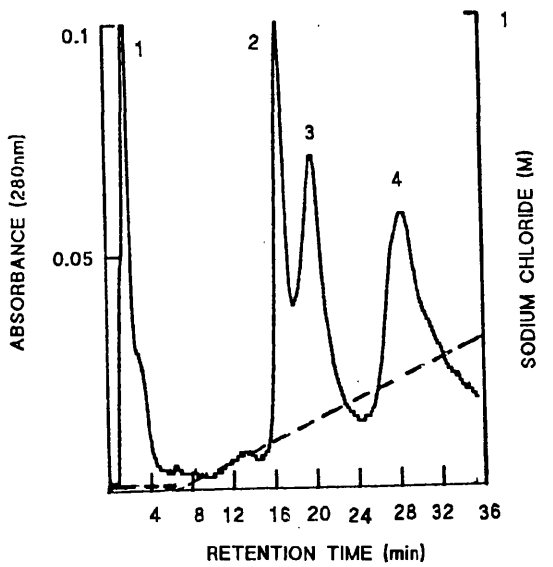


FIGURE 29(C)

FIGURES 30A, B, C:

SDS-PAGE analysis of peak fractions obtained from cation exchange chromatography of alkylated freeze-dried mouse casein on a Mono S column at pH5, 3 and 2 respectively.

(A) Tracks (W.C.) contain whole mouse casein and tracks numbered 2-6 contain peak fractions numbered 2-6 on Figure 29A.

(B) Tracks (W.C.) contain whole mouse casein and tracks numbered 1-6, contain peak fractions numbered 1-6 on Figure 29B.

(C) Tracks (W.C.) contain whole mouse casein and tracks numbered 1-4, contain peak fractions numbered 1-4 on Figure 29C.

U.C. Unidentified casein

β β-casein

κ κ-casein

α α-casein

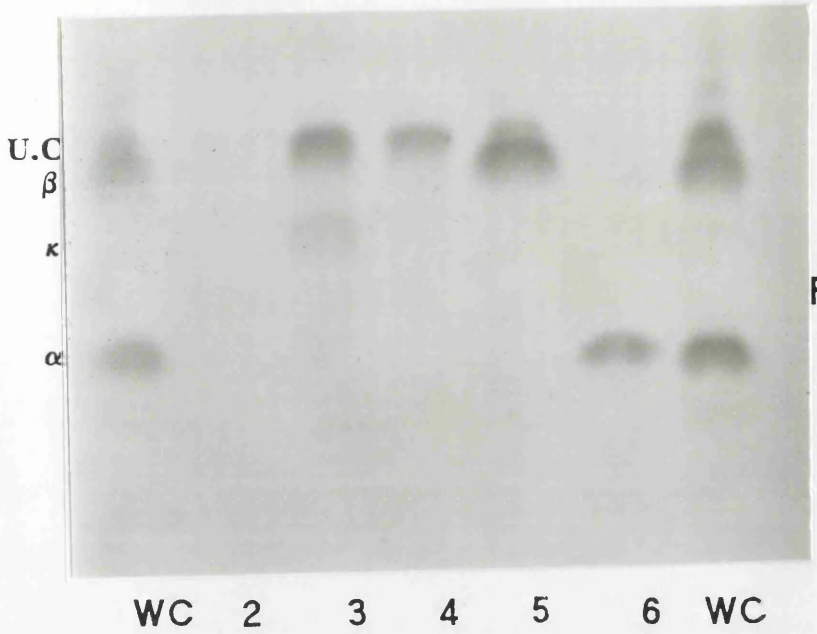


FIGURE 30(A)

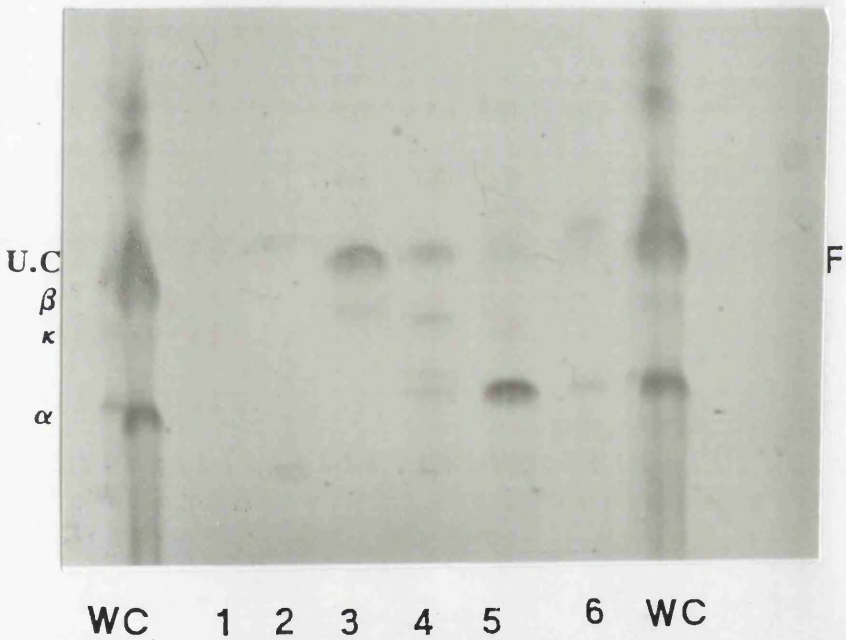


FIGURE 30(B)

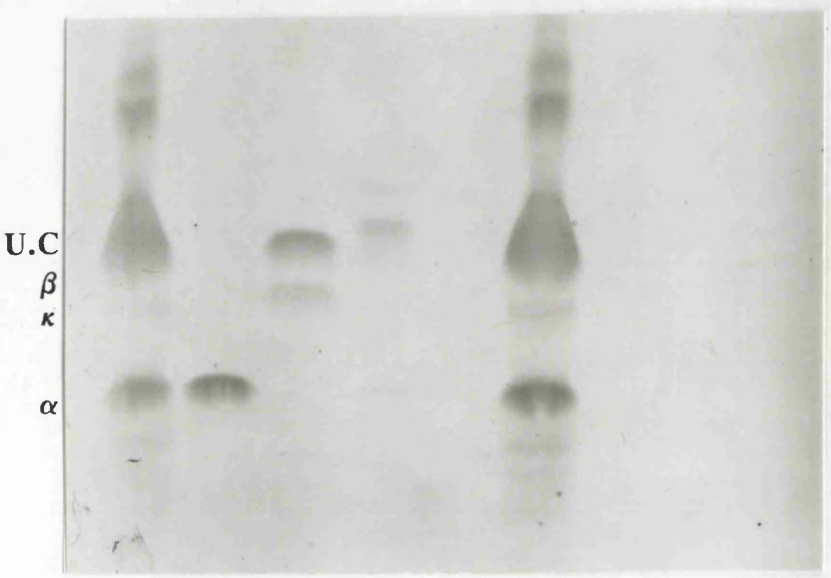


FIGURE 30(C)

FIGURE 31: Cation exchange chromatography of mouse casein

(A) Protein (2 mg) dissolved in Formate buffer (20 mM, pH 7.0, final protein concentration 2 mg ml^{-1}) containing 6M-urea. Protein was reduced as described in the text and the pH adjusted to 3.0. Proteins were eluted from the Mono S column at pH 3.0 using a gradient of sodium chloride with detection at 280 nm.

(B) Protein (2 mg) dissolved in sodium acetate buffer (20 mM, pH 7.0 final protein concentration 2 mg ml^{-1}) containing 8M-urea and 0.5% n-octyl β-D-glucopyranoside. Protein preparation and separation on the Mono S column are described above. Numbered fractions were analysed by SDS-PAGE (Figure 32).

MONO S SEPARATION OF MOUSE CASEIN

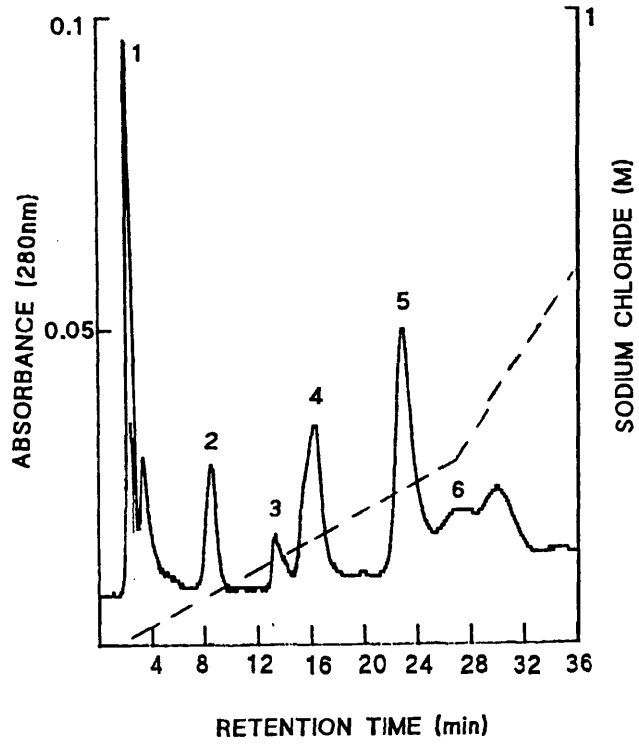


FIGURE 31(A)

MONO S SEPARATION OF MOUSE CASEIN

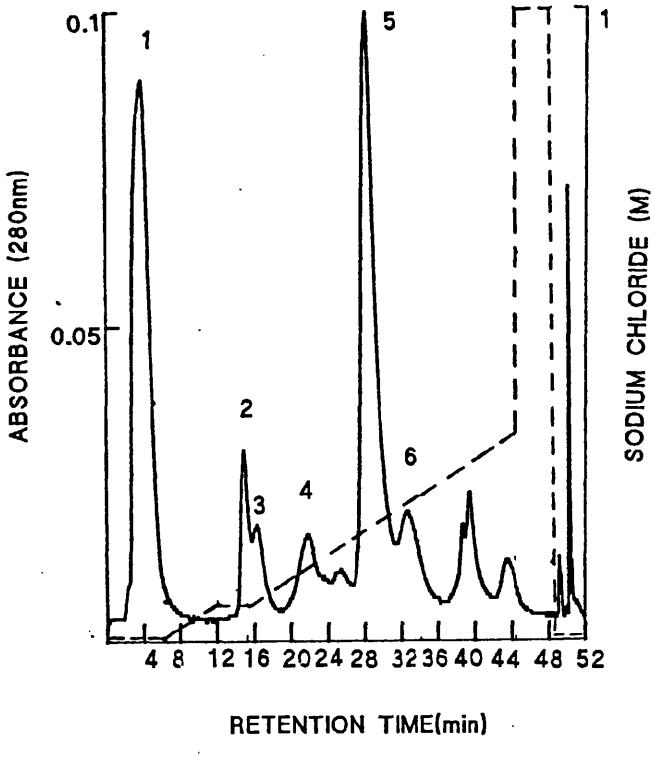
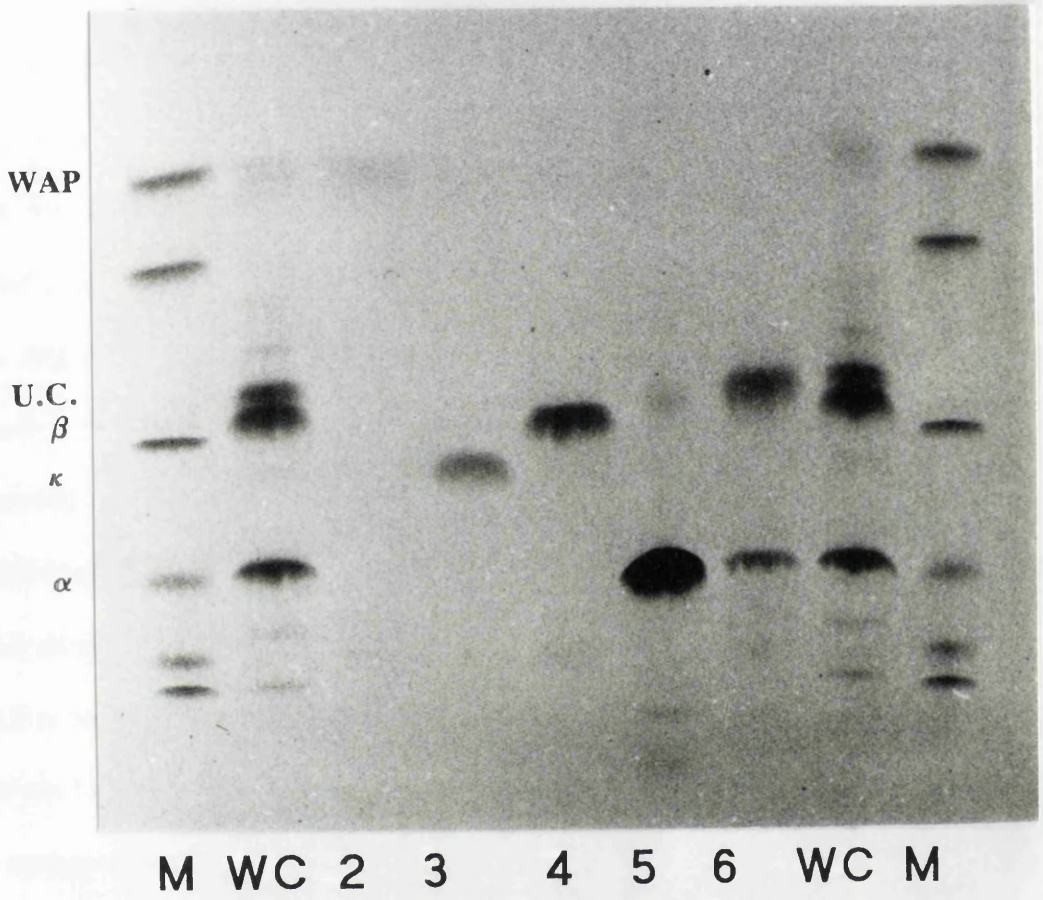


FIGURE 31(B)

FIGURE 32



SDS-PAGE analysis of peak fractions from cation exchange chromatography of freeze-dried mouse casein on a Mono S column at pH 3.0 in the presence of the non-ionic detergent n-octyl β-D-glucopyranoside.

Tracks labelled (M) and (W.C.) contain molecular weight markers and freeze-dried whole mouse casein respectively. Tracks numbered 2-6, contain peak fractions numbered 2-6 in Figure 31B.

WAP: Whey acidic protein
U.C: Unidentified casein
β: β-casein
κ: κ-casein
α: α-casein

chromotography using 20mM -sodium acetate buffers, pH 7.0, containing either 8 M-urea and 0.5% n-octyl β-D-glucopyranoside or 6 M-urea only. Samples were reduced for 1 hour with 10 μl ml^{-1} of 2-mercaptoethanol and the pH adjusted to pH 3.0 or 5.0. Results of the analyses are shown in Figure 33A and B, respectively. The pH at which the chromatographic separation was performed again significantly affected the separation. SDS-PAGE analysis of peak fractions (Figure 34A) showed that at pH 3.0, WAP (fraction 2), was eluted as a pure protein with milk serum albumin and lactoferrin being eluted as a single peak (fraction 3). At pH 5.0, the separation of the albumin and lactoferrin components (fractions 3 and 4 in Figure 34B) was significantly improved over that obtained at pH 3.0 (fraction 3). WAP appeared to be contaminated with κ-casein (Figure 34B). However, the relative amount of WAP detected was greater at pH 5.0 perhaps due to the fact that at pH 5.0 WAP is not highly charged as it nears its isolectric point of 4.7-4.8, therefore does not bind strongly to the cation exchange resin and elutes very soon after the mercaptoethanol peak in the void volume resulting in what appears to be a large WAP peak.

Milk: On the basis of the successful separation of the component caseins and whey proteins, separations of native milks (Tux No.1; CBAx C57 BL6 mice) were also attempted using cation-exchange chromotography on the Mono S resin. A profile of the separation achieved when milk was dissociated in 20 mM-sodium acetate buffer obtaining 8 M-urea 20 mM-EDTA and 0.5% n-octyl β-D-glucopyranoside at pH 7.0 is shown in Figure 35. SDS-PAGE analysis of the peak fractions collected from both control milks showed them

FIGURE 33: Effect of pH on the separation of native mouse whey protein by cation exchange chromatography

(A) Protein (2 mg) dissolved in 1ml of sodium acetate buffer (20 mM, pH 7.0) containing 8M-urea and 0.5% n-octyl β-D-glucopyranoside. Protein was reduced as described in the text and the pH of the protein solutions adjusted to that of the elution buffer. Proteins were eluted from a Mono S column at pH 3.0 using a gradient of sodium chloride with detection at 280 nm. Numbered fractions were analysed by SDS-PAGE (Figure 34A).

(B) Protein (2 mg) was prepared as described above. Elution from the mono S column was achieved using a gradient of sodium chloride at pH 5.0, with detection at 280 nm. Numbered fractions were analysed by SDS-PAGE (Figure 34B).

MONO S SEPARATION OF MOUSE WHEY PROTEIN

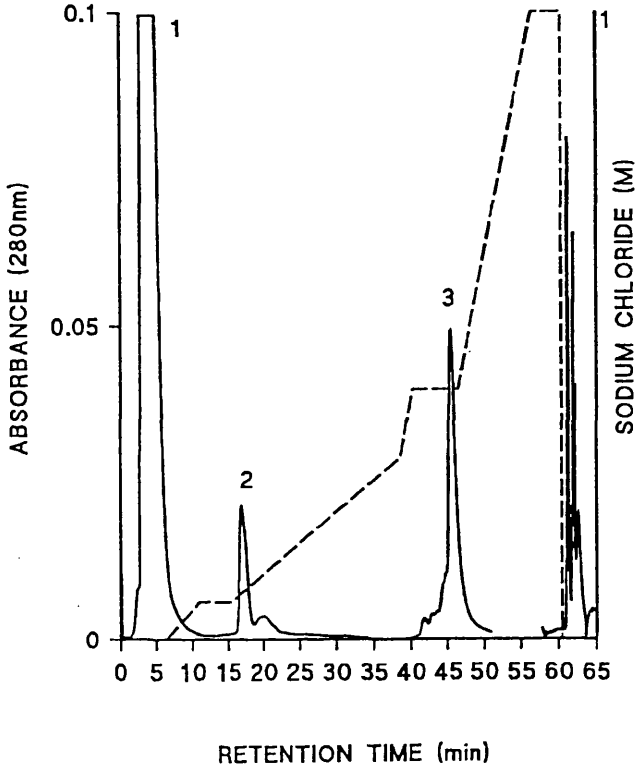


FIGURE 33(A)

MONO S SEPARATION OF MOUSE WHEY PROTEIN

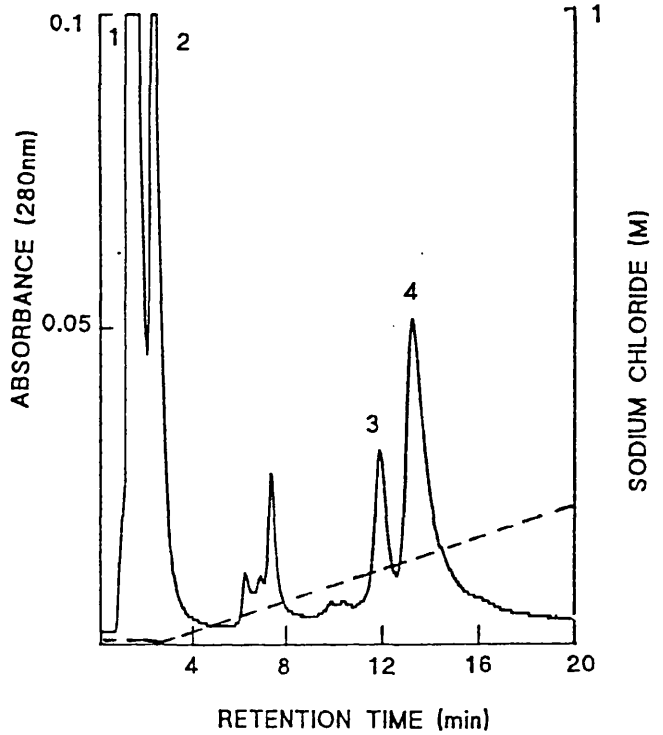


FIGURE 33(B)

FIGURE 34A,34B SDS-PAGE analysis of peak fractions collected from cation exchange chromatography of mouse whey protein at pH3 and pH5 respectively.

(A) Tracks labelled (M) and (W) contain molecular weight markers and freeze-dried whey protein respectively. Remaining tracks contain peak-fractions numbered 2 and 3 on Figure 33A, with fraction 3 being split further by desalting by reverse phase liquid chromatography.

(B) Tracks labelled (M) and (W) contain molecular weight markers and freeze-dried whey protein respectively. Remaining tracks contain peak fractions numbered 2, 3 and 4 on Figure 33B.

WAP: Whey acidic protein
SA: Serum albumin
LF: Lactoferrin

FIGURE 34(A)

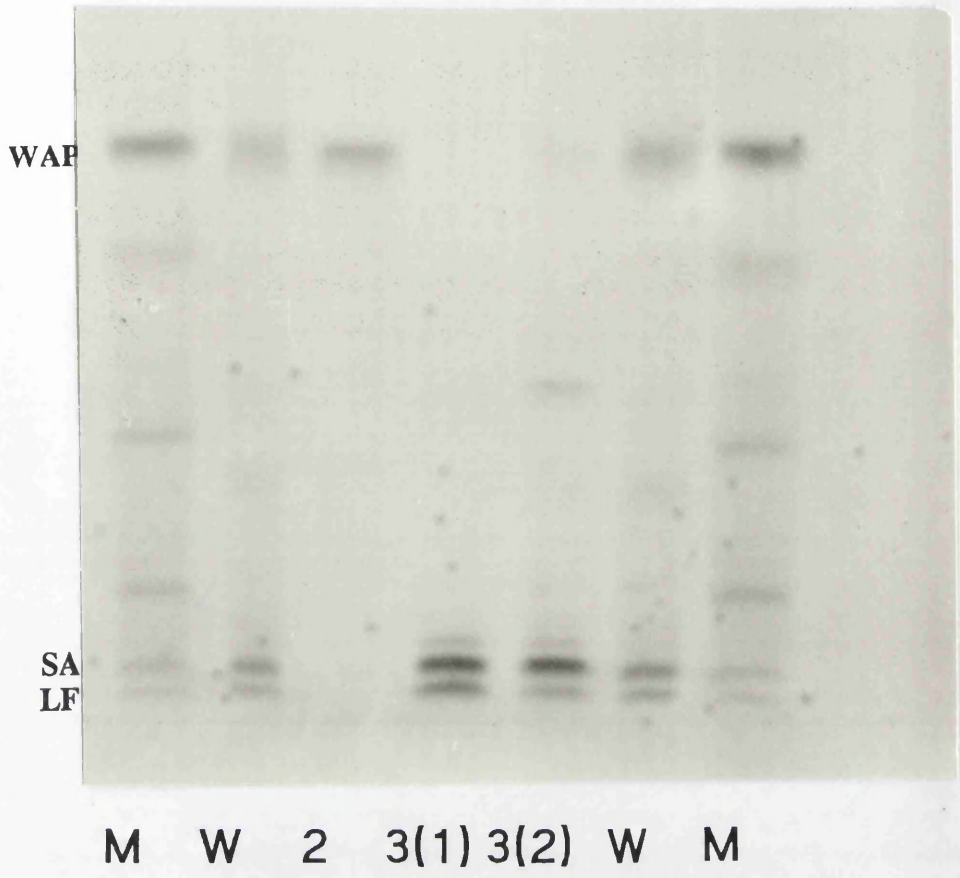
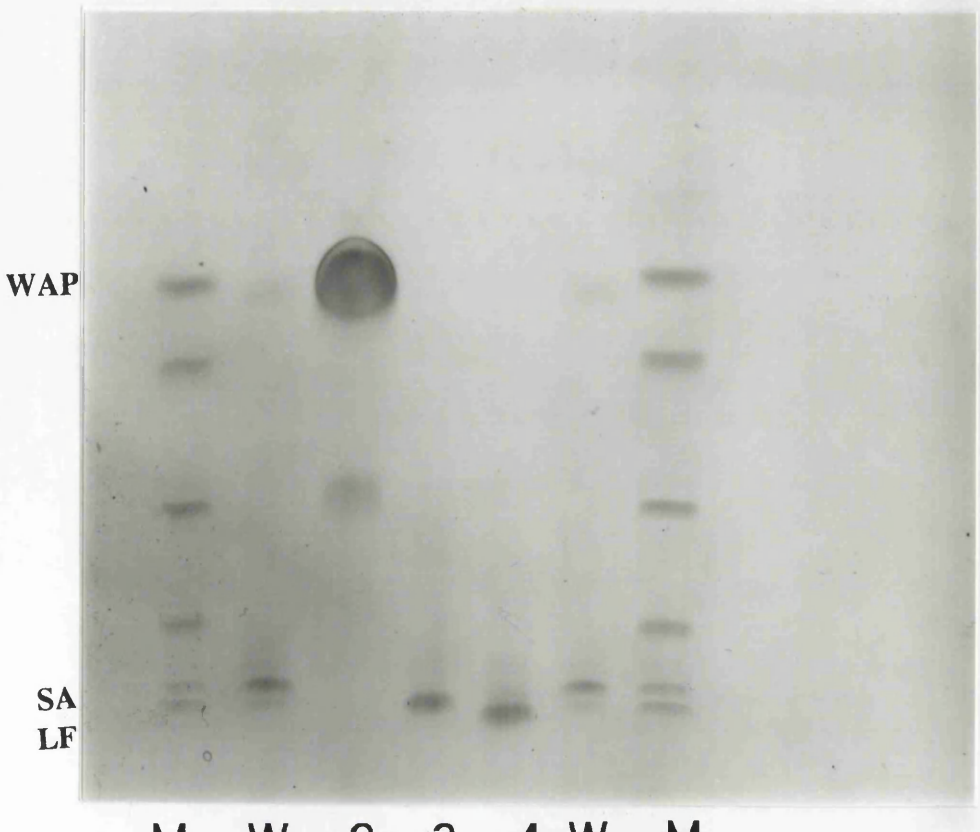


FIGURE 34(B)



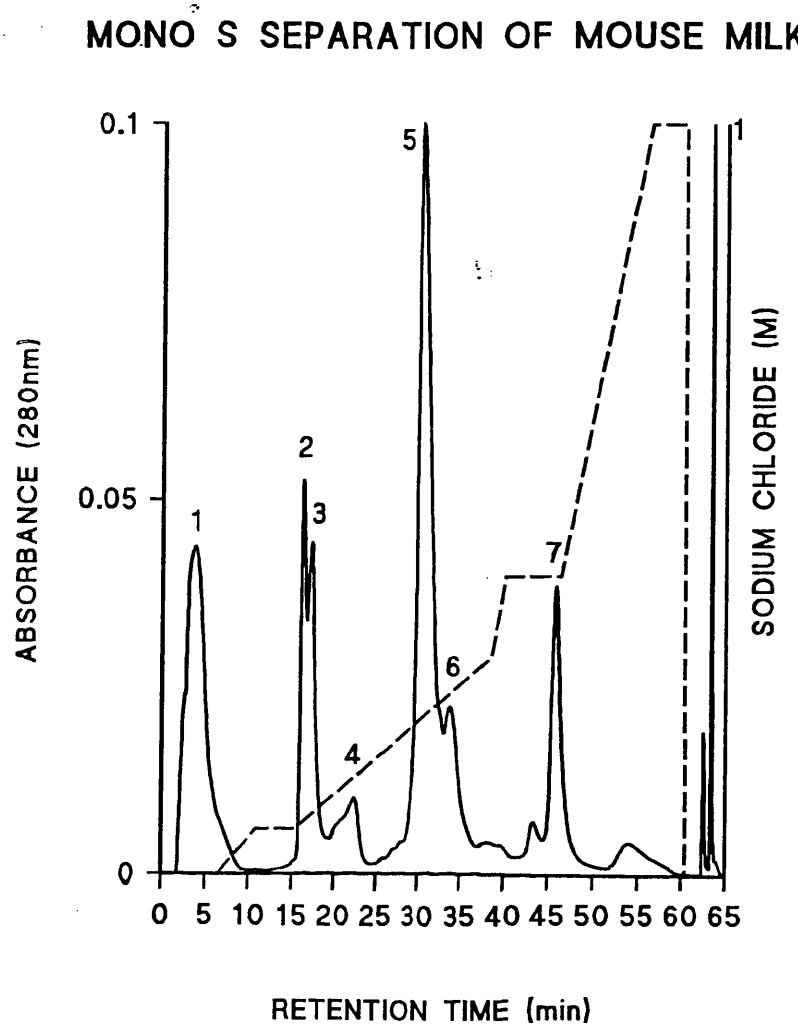


FIGURE 35

Skimmed milk (20 μl, ~2 mg protein) was dissolved in 1 ml sodium acetate buffer (20 mM, pH 7.0,) containing 8M-urea, 20 mM EDTA and 0.5% n-octyl β-D-glucopyranoside for 16 hours at 4°C. The solution was then dialysed and reduced as described in the text. The pH of the sample was reduced to pH 3.0 and proteins eluted from a Mono S column at pH 3.0 using a gradient of sodium chloride with detection at 280 nm. Numbered fractions were analysed by SDS-PAGE (Figure 36).

to consist of pure or almost pure caseins and whey proteins. The protein fractions were identified as WAP, κ-casein, β-casein, α-casein, unidentified casein and milk serum albumin /lactoferrin (Figure 36).

2.3.2.5 HI-LOAD S

The separation of whole mouse casein on the Hi-Load S column is shown in Figure 37, SDS-PAGE analyses of the fractions are shown in Figure 38. Fractions of pure β-casein and of almost pure α-casein were obtained from a single run. The other fractions were found to be impure and the order of elution of the WAP and κ-casein was reversed from that from the Mono S column.

2.3.3 PROTEIN DESALTING

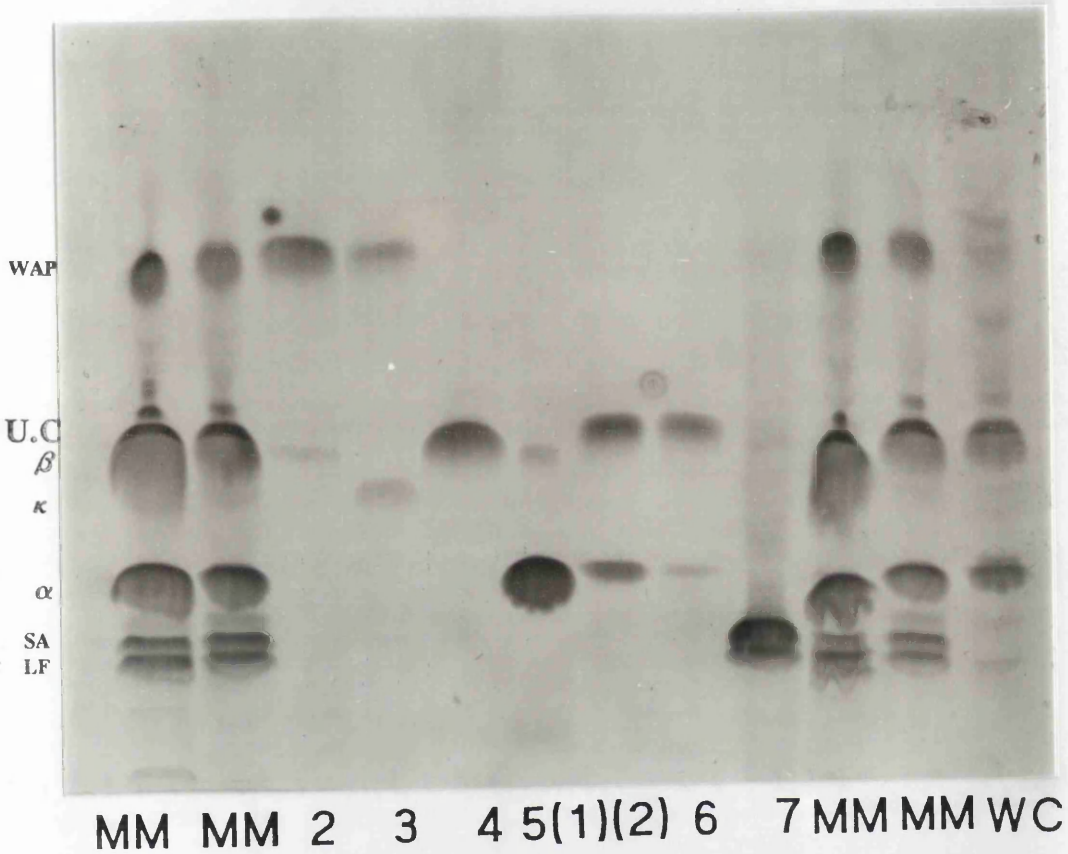
Initially, fractions collected from column chromatography separations were dialysed in order to remove buffer salts. However, yields, especially of the whey proteins, were poor due to the proteins adhering to the dialysis membrane. As an alternative, reverse-phase liquid chromatography, using a gradient of acetonitrile in 0.1% TFA, was evaluated. This proved to be a much better way of desalting small volumes of protein solutions. A dramatic increase in protein recovery was achieved. Furthermore, the volatile buffer was removed more quickly on freeze drying, facilitating the analysis of samples.

2.3.4 PROTEIN CHARACTERIZATION

2.3.4.1 AMINO ACID ANALYSIS

Amino acid analysis was performed on the purest protein fractions

FIGURE 36

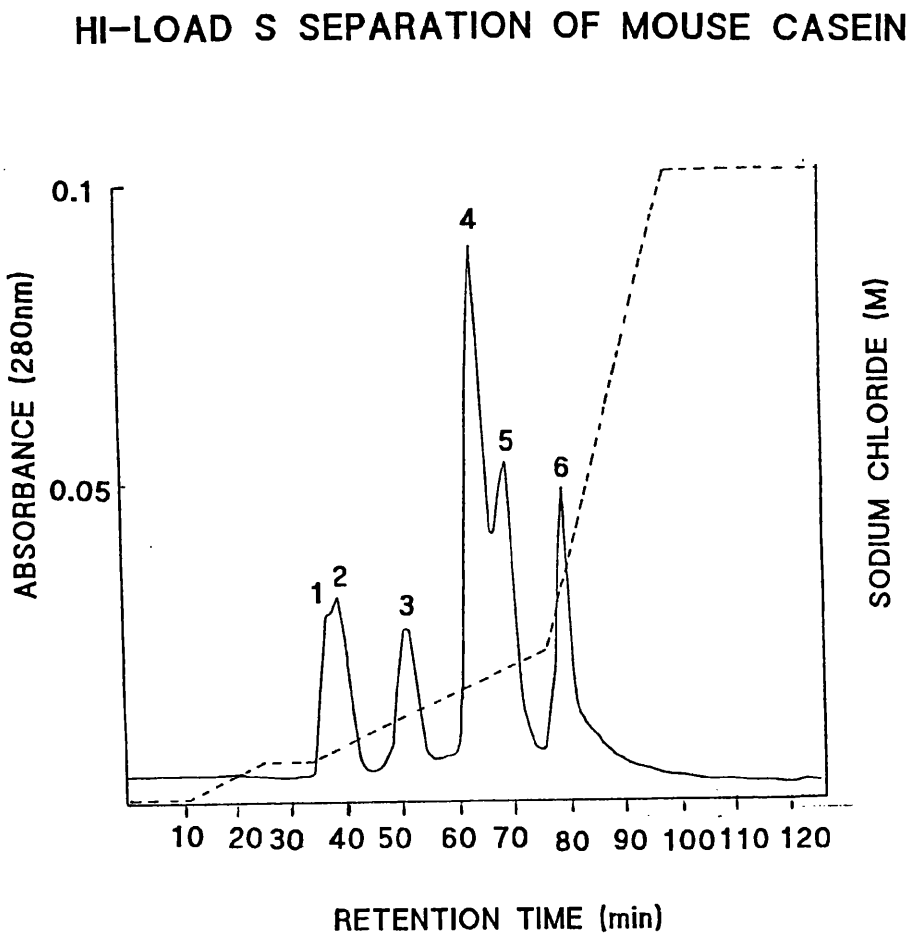


SDS-PAGE analysis of skimmed milk separated by cation-exchange chromatography on a Mono S column.

Tracks labelled (MM) and (WC) contain skimmed mouse milk and freeze-dried whole casein respectively. Remaining tracks contain peak fractions numbered 2-7 on Figure 35, with fraction 5 being split on desalting by reverse phase chromatography.

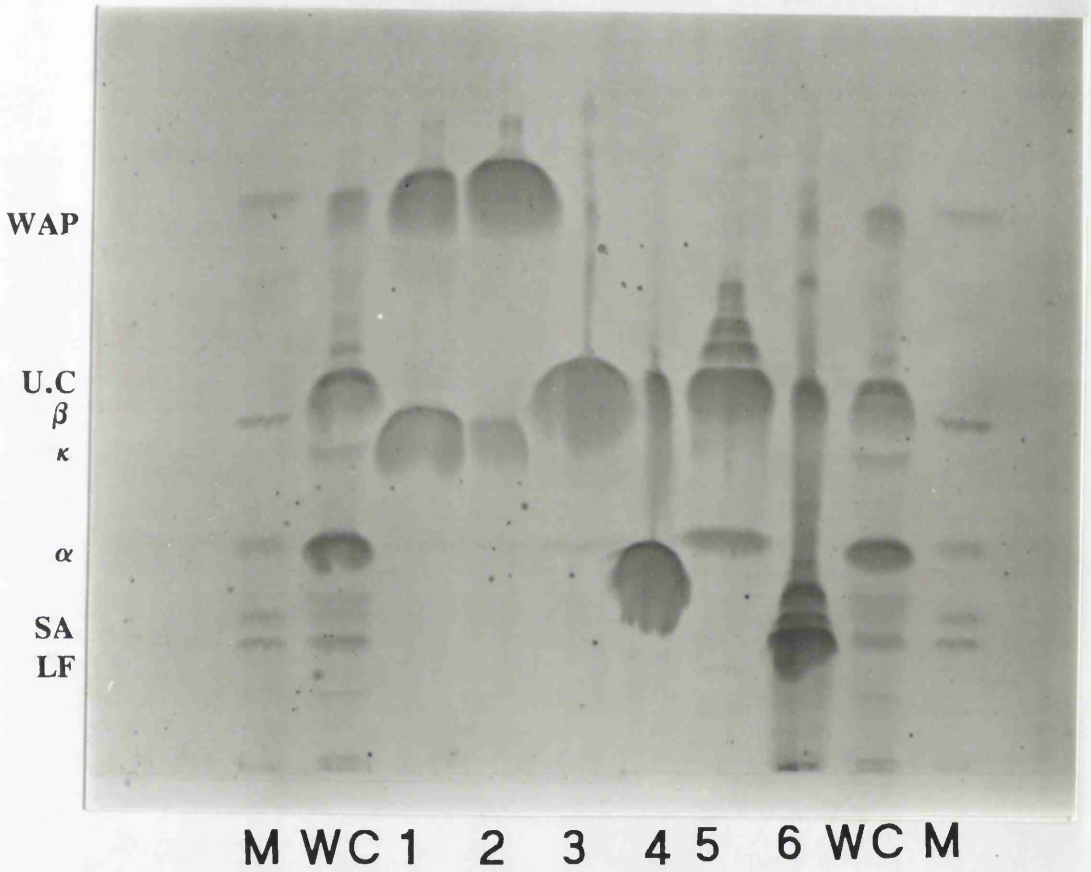
WAP: Whey acidic protein
U.C: Unidentified casein
β: β-casein
κ: κ-casein
α: α-casein

FIGURE 37



Protein (400 mg) dissolved in 150 ml of sodium acetate buffer (20 mM, pH 7.0) containing 8M-urea and 0.5% n-octyl β-D-glucopyranoside for 16 hours at 4°C. Protein was reduced as described in text and the pH adjusted to 3.0 and eluted from a Hi-Load S column using a gradient of sodium chloride with detection at 280 nm. Numbered fractions were analysed by SDS-PAGE (Figure 38).

FIGURE 38



SDS-PAGE analysis of mouse casein separated by cation exchange chromatography on a Hi-Load S column.

Tracks labelled (M) and (WC) contain molecular weight markers and freeze-dried mouse casein respectively. Remaining tracks contain peak fractions numbered 1-6 on Figure 37.

WAP: Whey acidic protein
U.C: Unidentified casein
β: β-casein
κ: κ-casein
α: α-casein
LF: Lactoferrin
SA: Serum Albumin

obtained from cation-exchange chromatography of whole casein. The results are shown in Table 16A and B. By comparing the measured amino acid composition with that derived from the published cDNA sequences, all except one of these proteins could be identified. A comparison of the murine casein amino acid compositions with that of the bovine casein amino acid compositions is shown in Table 16C. The first protein eluted by cation-exchange chromatography was found to be WAP. Although this protein is usually found in the whey fraction, variable amounts co-precipitated with the caseins. The remaining caseins were then eluted in the order κ-casein, β-casein and α-casein. The amino acid composition of the remaining component, which was contaminated with some α-casein did not appear to correspond to any of the published cDNA compositions.

2.3.4.2 MOLECULAR WEIGHTS

The molecular weights of individual mouse caseins as determined by cDNA sequence analysis and SDS-PAGE against protein standards are shown in Table 17. Mouse β-casein had an apparent molecular weight similar to that predicted from the cDNA sequence whereas the apparent molecular weight of the α- and κ-caseins were considerably higher than their predicted values. This was more evident in the case of κ-casein which appeared to have a higher molecular weight than β-casein on SDS-PAGE.

2.3.4.3 DEPHOSPHORYLATION OF MOUSE CASEIN AND MOUSE MILK

Dephosphorylation of both murine and bovine caseins with potato acid phosphatase gave rise to a number of protein bands on alkaline-urea gel electrophoresis. The presence of EDTA also appeared to enhance the reaction

TABLE 16A: Amino acid composition of identified murine caseins and WAP

Amino Acid	α-Casein		κ-Casein		β-Casein		WAP	
	AAA	cDNA	AAA	cDNA	AAA	cDNA	AAA	cDNA
D/N	25.2	23	20.2	19	12.9	12	12.1	10
E/Q	69.8	70	12.2	14	40.3	41	15.7	16
S	38.1	34	13.0	15	27.6	25	9.8	10
G	1.2	0	2.3	2	2.2	2	6.7	6
H	5.1	6	1.6	1	5.3	6	0	0
T	11.4	9	10.6	15	10.3	11	6.0	9
A	22.4	29	11.7	11	12.1	15	4.4	4
R	7.0	8	6.4	6	1.0	1	2.3	3
P	18.0	18	16.8	23	21.0	21	10.5	10
Y	9.4	9	8.5	10	4.4	3	0	0
V	14.4	13	14.8	14	18.3	20	8.0	9
M	11.0	11	5.5	4	3.3	3	5.4	5
I	12.0	8	9.4	10	10.3	10	7.7	7
L	27.8	32	9.9	9	26.2	30	3.6	3
F	11.7	13	3.2	3	7.1	7	2.5	3
K	12.1	12	3.7	2	9.3	8	4.5	5

Amino acid composition as determined by amino acid analysis (AAA) and from the published cDNA sequences expressed as residues per molecule.

TABLE 16B: Amino acid composition of mouse milk serum albumin (Fraction 3) and lactoferrin (Fraction 4)

Amino Acid	Fraction 3(moles%)	Fraction 4 (moles %)
D/N	11	11.4
E/Q	15	13.1
S	9	11.9
G	7.5	10.5
H	2	2
T	4.5	4.2
A	3.5	3.4
P	6.5	4.5
R	ND	ND
Y	2	2.2
V	2	2.9
M	1	0.7
C	ND	ND
I	3.5	4.1
L	7.5	17.5
F	3	3.0
W	2	-
K	13.5	6.8

ND = not determined

TABLE 16C: Comparison of the amino acid composition of the major bovine and murine caseins

Amino Acid	Bovine α_{s1}-casein	Murine α-casein	Bovine α_{s2}-casein	Bovine κ-casein	Murine κ-casein	Bovine β-casein	Murine β-casein
D/N	15	23	18	11	19	9	12
E/Q	39	70	40	26	14	39	41
S	16	34	17	13	15	16	25
G	9	0	2	2	2	5	2
H	5	6	3	3	1	5	6
T	5	9	15	14	15	9	11
A	9	29	8	15	11	5	15
R	6	8	6	5	6	4	1
P	17	18	10	20	23	35	21
Y	10	9	12	9	10	4	3
V	11	13	14	11	14	19	20
M	5	11	4	2	4	6	3
C	0	1	2	2	1	0	1
I	11	8	11	13	10	10	10
L	17	32	13	8	9	22	30
F	8	13	6	4	3	9	7
K	14	12	24	9	2	11	8
W	2	2	2	1	1	1	0

TABLE 17: Molecular weights of murine caseins

Casein	Molecular weight (kDa)	
	From cDNA sequence	From SDS-PAGE
α	33.6	44.1
β	23.4	26.3
κ	17.6	30.9
unidentified	-	25.1

The molecular weights of murine caseins as calculated from their mobility on SDS-PAGE (see text) compared to that calculated from their amino acid composition as determined from the cDNA sequence

Figure 39 **Alkaline urea gel of bovine and murine freeze-dried casein and potato acid phosphatase treated bovine and murine caseins in the presence and absence of EDTA.**

(A) Tracks numbered (1) (5) and (9) contain bovine caseim, (2) (6) and (10) dephosphorylated bovine casein (de-P), (3) (7) and (11) murine casein, (4) (8) and (12) de-P murine casein.

(B) Tracks numbered (1) and (3) contain bovine casein, (2) de-P bovine casein, (4) de-P bovine casein + EDTA, (5) skimmed mouse milk, (6) and (7) de-P mouse milk, (8) and (10) modified β-lactoglobulin transgenic mouse milk, (9) and (11) de-P modified β-lactoglobulin transgenic mouse milk + EDTA.

FIGURE 39(A)

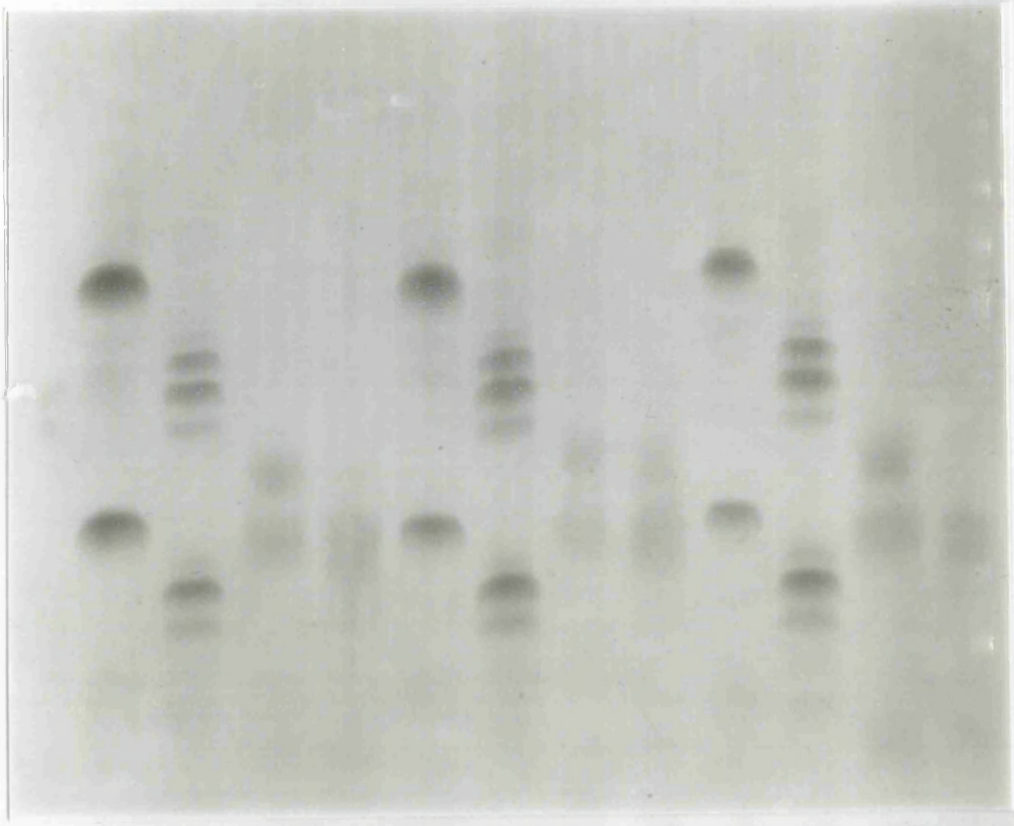
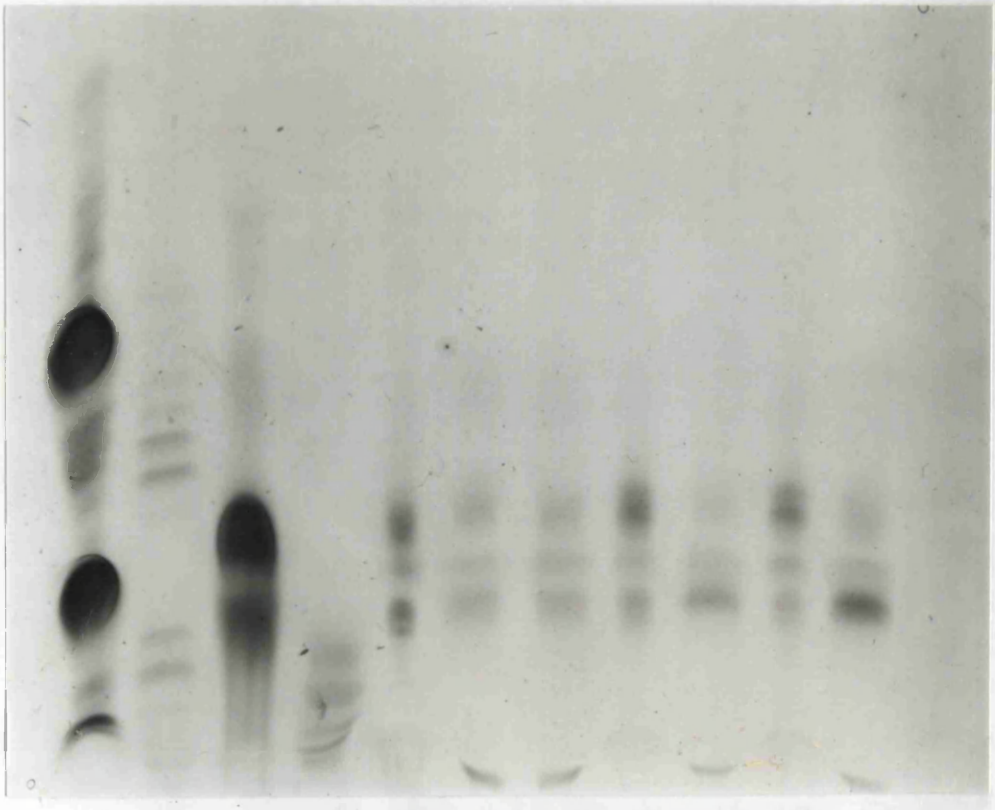


FIGURE 39(B)



as shown in the case of bovine casein in Figure 39B. Dephosphorylation of mouse casein was less obvious in the absence of EDTA (Figure 39A). Mouse milk samples were also very difficult to dephosphorylate. However, in the case of modified β-lactoglobulin milk the acid phosphatase was more effective on the isolated proteins as seen in tracks 8-11 of Figure 39B.

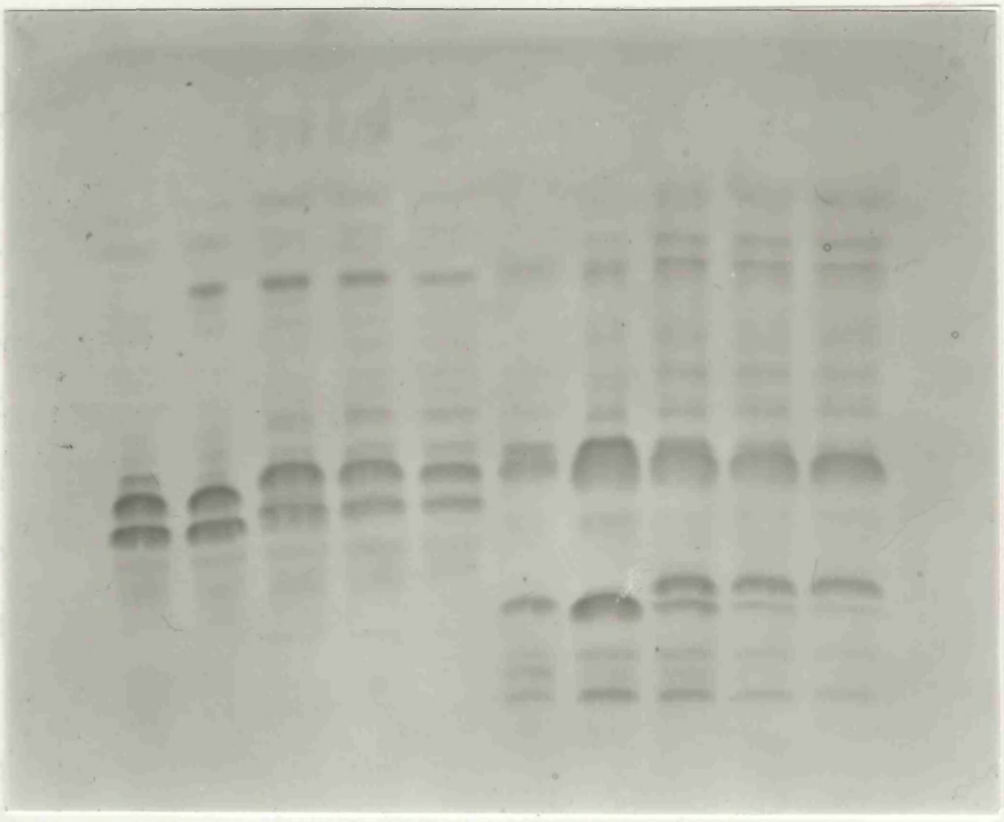
2.3.4.4 RENNETING

Bovine and murine caseins were treated with rennet and various volumes of rennin solution for 30, 60, 90 and 120 minutes(Table 14). Proteins were then analysed by SDS-PAGE (Figures 40A and B). With the caseins from both species numerous smaller protein bands are observed upon renneting. Mouse α-casein appears to be sensitive to calf-stomach chymosin since the intensity of the α-casein band decreased and that of another smaller peptide band which migrate immediately in front of it on SDS-PAGE (Figure 40B) increased with duration of reaction. The unidentified casein also appears to be sensitive to chymosin treatment as determined by its gradual disappearance on SDS-PAGE with both increasing amount of chymosin and incubation time. Bovine κ-casein is obviously highly sensitive to chymosin and even at the lowest concentration of enzyme and shortest incubation time, the band corresponding to para κ-casein can be readily observed. Mouse κ-casein was also hydrolysed, but more slowly.

2.3.5 CASEIN COMPOSITION

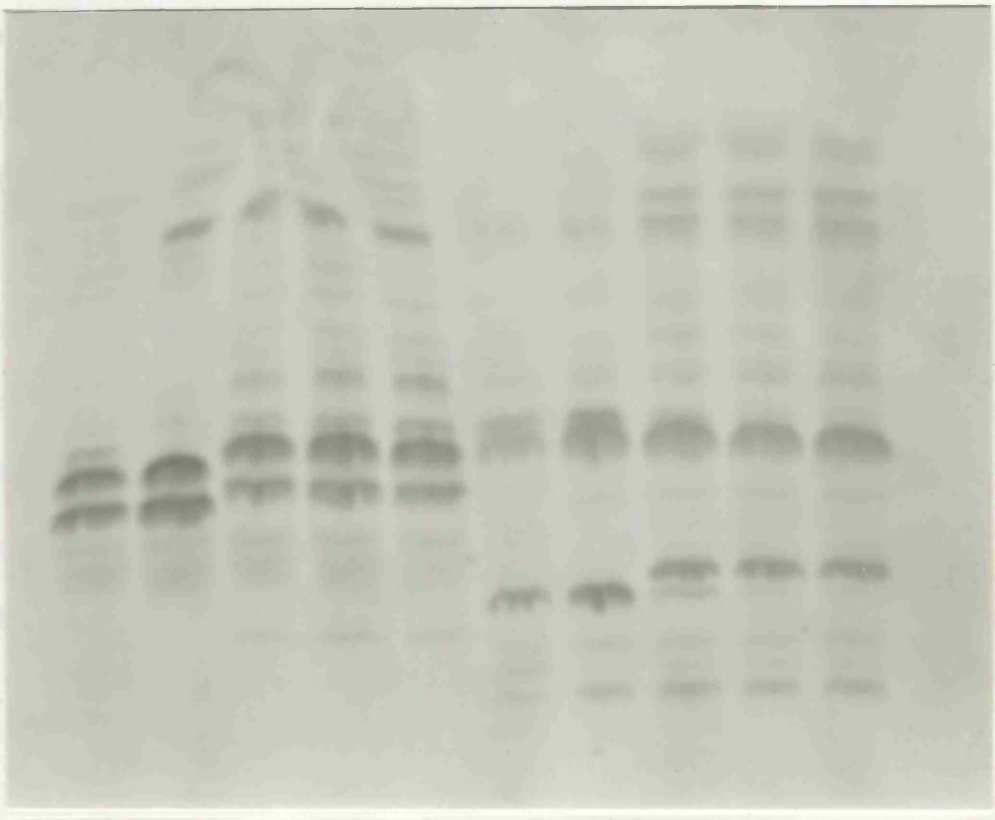
The extinction coefficients of the three major mouse caseins and ovine β-lactoglobulin were estimated using the known extinction coefficients for tyrosine and tryptophan (the only amino acids which absorb significantly at

FIGURE 40(A)



1 2 3 4 5 6 7 8 9 10

FIGURE 40(B)



1 2 3 4 5 6 7 8 9 10

278 nm) and the amino acid composition of the mouse casein and ovine β-lactoglobulin determined from cDNA sequence analysis. The results are shown in Table 18. The calculated values for the various bovine caseins were very similar to the measured values thus showing the applicability of the method. The calculated absorption values for the individual mouse caseins were then applied to the corresponding integrated peak areas from Mono S separations. From this, the relative amounts of the major caseins were calculated. The results shown in Tables 19A and 19B are for the milks obtained from individual mice. Although the relative levels were rather variable between animals. the mean values for the α-and β-caseins were similar and both were generally greatly in excess of the κ-casein level with the mean ratio of κ:β:α being 1:11.7:17.4. The differences in the ratios between individual mice is not due to proteolysis since milk left at room temperature for 2-3 days did not show an increase in low molecular weight bands.

When large amounts of the murine β-casein became available as a result of the large-scale separation on the Hi-Load S, the extinction coefficient of this protein was measured by determining the protein nitrogen content of a freeze-dried sample by micro-Kjeldahl and then measuring the absorbance of a solution of the protein at 278 nm. The determined value was found to be 1.43 which is almost identical to the calculated value. The absolute amounts of the 3 identified caseins in mouse milk and WAP were determined on the basis of their peak areas in the Mono S separations. These values are shown for a number of individual animals in Table 20, the mean values being α-casein, 51.7, β-casein, 25.2, κ 4.7 and WAP 15.3 mg ml^{-1}.

TABLE 18: Absorbance values for bovine and murine caseins

	Bovine			Murine				Ovine
	α_{s1}	β	κ	α	β	κ	WAP	β-lg
Calculated	10.4	4.5	9.4	4.9	1.4	9.3	3.5	8.33
Measured	10.7	4.6	10.5	-	-	-	-	-

. Values are the absorbance of a 1% (w/v) solution in a 1 cm path length at 278 nm. Calculated values are based on the amino acid composition either from protein hydrolysis or cDNA sequencing and molar absorptivity values at 278 nm and pH 6.0 of 5100 and 1200 for tryptophan and tyrosine respectively

TABLE 19A: Relative amounts of major milk protein components in acid-precipitated freeze-dried mouse casein

κ-casein	β-casein	α-casein
1	10.1	17.7
1	12.2	19.8
1	12.7	11.1
1	12.1	29.9
1	11.1	17.8
1	9.0	16.8
1	10.6	17.8
1	9.7	16.7
1	13.1	20.1
1	10.7	13.7
1	9.4	14.8
1	12.4	19.3
1	16.0	18.2
1	9.7	18.1
1	13.4	20.8
1	13.2	20.0
1	10.3	16.4
1	9.7	16.4
1	12.1	20.4
1	8.5	15.5
1	10.9	11.6
1	12.0	12.0
1	14.5	18.5
1	13.8	13.8
1	15.2	18.2
Mean 1	**11.7**	**17.4**

TABLE 19B: Relative amount of major milk proteins in mouse skimmed milk

WAP	κ-casein	β-casein	α-casein
2.7	1	5.3	9.4
2.3	1	5.6	9.4
2.3	1	4.4	8.3
2.1	1	1.6	8.8
2.9	1	7.0	15.9
3.2	1	7.0	14.0
3.2	1	6.4	15.7
3.6	1	6.2	16.5
4.1	1	5.4	9.5
3.3	1	6.1	15.8
3.1	1	6.4	15.9
4.0	1	7.2	10.9
3.8	1	6.0	10.2
3.3	1	6.5	9.5
4.8	1	5.9	9.9
4.0	1	4.1	8.5
3.7	1	5.0	8.6
Mean 3.3	**1**	**5.7**	**11.6**

TABLE 20: Absolute amounts of the major milk proteins in mouse milk expressed as concentration of protein (mg ml^{-1})

WAP	κ-Casein	β-Casein	α-Casein
14.2	5.3	28.0	49.6
12.2	5.3	26.7	49.8
13.9	6.1	26.7	50.3
15.1	7.2	11.5	63.2
10.5	3.6	25.3	57.5
12.3	3.8	26.9	53.9
11.8	3.7	23.6	58.0
12.8	3.6	22.0	58.6
19.9	4.9	26.2	46.1
12.2	3.7	22.6	58.5
11.4	3.7	23.5	58.4
16.8	4.2	30.2	45.8
17.6	4.6	27.7	47.1
15.8	4.8	31.1	45.4
21.6	4.5	26.5	44.5
22.0	5.5	22.6	46.8
19.6	5.3	26.5	45.6
Mean 15.3	**4.7**	**25.2**	**51.7**

2.4 DISCUSSION

2.4.1 PROTEIN COMPOSITION OF NATIVE MOUSE MILK

The most striking feature of mouse milk is its appearance. Compared to bovine milk, mouse milk appears to be more creamy and viscous, reflecting differences in composition. Mouse milk is generally more concentrated than bovine milk, containing only 70% water with 13% lipid and a reported protein concentration in the region of 90 mg ml^{-1} (Nagasawa, Naito and Kataoka 1989) compared to the 87% water, 3.9% lipid and 35 mg ml^{-1} of protein in bovine milk (Jenness 1985). Total mouse milk protein was measured for a number of individual animals and was found to be 97 mg ml^{-1}, of which the whey component accounted for 18 mg ml^{-1}.

The fractionation of mouse milk into casein and whey proteins by isoelectric precipitation was examined in detail between pH 4.0 and 5.0. At pH 4.27, casein was precipitated and whey protein remained in solution with very little cross contamination. This pH value is lower than that required to precipitate bovine casein the optimum pH for which is 4.6.

In view of the widespread use of the mouse as a model system in a number of areas associated with milk secretion ranging from the hormonal control of milk production, to the evaluation of DNA constructs designed to express foreign proteins in the milk of transgenic animals (Gordon *et al.* 1987; Simons *et al.* 1987, 1988; Lee *et al.* 1988, 1989; Pittius *et al.* 1988; Bayna and Rosen, 1990; Meade *et al.* 1990; Vilotte *et al.* 1989 and Wilde *et al.* 1992), it is somewhat surprising that no column chromatographic methods have been developed to separate and quantify the milk proteins. Ideally,

quantitative fractionation of the component caseins and whey proteins should be via a single chromatographic step in order to minimise losses between stages. A number of chromatographic techniques were evaluated in an attempt to purify individual caseins and whey proteins from freeze-dried acid precipitated whole casein and whey, and skimmed mouse milk from both native and transgenic animals.

By evaluating a variety of dissociation and chromatographic techniques, this project has resulted in methods to separate, identify and quantify a number of the individual murine proteins, particularly the caseins.

In gel permeation chromatography, separation is based on molecular size, with higher molecular weight proteins being eluted first. This technique has been successfully used to separate whey proteins from intact micelles, polymerised bovine κ-casein from the other caseins and also to separate a number of individual whey proteins. In view of the differences in apparent molecular weight of some of the mouse caseins and whey proteins, it was felt worthwhile to attempt the use of this technique to achieve at least a partial separation of these proteins. Superose 12 separates proteins with a molecular weight in the range of 1-300 kDa and Superdex 75 separates proteins with molecular weights in the range of 30-70 kDa. However, gel permeation chromatography proved to be unsuccessful as a means of purifying individual caseins. Caseins were eluted together as a single peak on both resin types. Similarly, separations of skimmed milk proteins were unsuccessful. Separations of freeze-dried mouse whey proteins were slightly more successful resulting in the purification of the 14 kDa whey acidic protein (WAP). Milk

serum albumin and lactoferrin, however, were always eluted together as a single peak, irrespective of dissociation and running conditions.

The failure of gel permeation chromatography to separate individual caseins and whey proteins is a reflection of the limitations imposed by the column resins together with the similarity of the molecular weights of the individual caseins and of the high molecular weight whey proteins.

Reverse phase chromatography separates proteins on the basis of their relative hydrophobicity. Bovine caseins have been successfully fractionated into their individual components using this technique (Carles 1986; Visser *et al.* 1991). Pure fractions of mouse κ-, β- and α-caseins were obtained by chromatography on the Pro RPC, column as well as a pure fraction of WAP, some of which co-precipitated with the caseins at pH 4.27 during the initial fractionation of casein and whey protein from skimmed milk. The efficiency of the separations of proteins by reverse phase was found to be dependent upon the dissociation conditions used in sample preparation. Buffers containing 6 M-guanidine-HCl dissociated the caseins better than buffers containing 6 M-urea. However, the best separationd were achieved when the detergent, n-octyl β-D-glucopyranoside, was added. Nevertheless, even the best chromatographic separation of mouse caseins by reverse phase chromatography resulted in only a small proportion of the total casein being eluted as pure individual components. Several of the major peaks consisted of mixtures of proteins. Although useful as a means of obtaining pure samples of most of the caseins, reverse phase chromatography was not suitable as a means of quantification. However, this did prove to be a very useful

method of desalting protein fractions.

Ion exchange chromatography separates proteins according to their charge. Caseins from bovine (Andrews *et al.* 1985; Davies & Law 1987), caprine (Brignon *et al.* 1989) and ovine (Hassnoot *et al.* 1986) species have been quantitatively fractionated by anion exchange chromatography on Mono Q resin using a sodium chloride gradient in 3.3 M-urea. However, attempts to fractionate mouse caseins under these conditions were unsuccessful. Even though a number of peaks were obtained, all were found to consist of mixtures of caseins. Buffers containing high concentrations of the dissociating agent urea (6 or 8 M) gave the best separations of mouse casein on anion-exchange chromatography with a series of sharp peaks being observed. However, these peak fractions were found to consist of mixtures of caseins when examined by SDS-PAGE. In addition, a large proportion of the protein remained undissociated, as demonstrated by the large peak which was eluted with 1M-sodium chloride and which was shown by SDS-PAGE to consist of a mixture of caseins. Again, WAP was found to be present in the casein preparations and was eluted as a pure fraction by this chromatographic procedure.

Mono Q analysis of skimmed mouse milk from both native and ovine β-lactoglobulin transgenic mice gave a similar separation to that of the casein. Even with the detergent n-octyl β-D-glucopyranoside and 8 M-urea present in the dissociating buffer no pure, individual casein fractions were separated at pH 7.0.

The separation obtained for whey protein was also disappointing at pH 7.0; one protein peak was detected. However, once again this was shown to be a mixture of whey proteins (Figure 27). Therefore, attempts to separate and quantify murine proteins by anion-exchange chromatography were discontinued.

Cation exchange chromatography on Mono S columns at pH 5 in 6 M-urea has been used successfully in the separation of bovine (Andrews *et al.* 1985), ovine (Law *et al.* 1992) and caprine caseins (Law & Tziboula, 1992). Cation exchange chromatography of mouse milk proteins was evaluated on a Mono S resin at pH 5, 3 and 2. Separations at all three pH's produced pure fractions of the κ-casein, unidentified casein and an almost pure fraction of β-casein. From the profiles obtained and analysis of peak fractions by SDS-PAGE, the caseins appeared to be more fully dissociated when separated at pH 3.0. The pH of the dissociation buffer did not appear to have a pronounced effect on the overall separation obtained. Two buffer salts, acetate and formate were also tested but did not appear to have any significant effect on the overall separation obtained at pH 3.0. The factor which had the greatest effect on the separation of the murine caseins was found to be the inclusion of n-octyl β-D-glucopyranoside into the dissociation buffer. This non-ionic detergent has been found to be very effective in the solubilization of intrinsic membrane-bound proteins (Baron & Thompson, 1975; Stubbs & Litman, 1978a, b; Petri & Wagner, 1979; Schneider *et al.*, 1980, Gould *et al.*, 1979; Gould *et al.* 1981) and proved ideal for the disaggregation of mouse caseins. In addition, it was also easily removed by dialysis. The role

that this detergent plays in the dissociation of the precipitated casein has not been determined. However, it may function by opening up the hydrophobic core to the chaotropic agent, allowing complete dissolution of the ca seins. Furthermore, it may also prevent reaggregation of the dissociated proteins. Inclusion of the detergent resulted in near baseline separations of the individual caseins, and improved the dissociation of the aggregated casein peak, which in its absence eluted at high salt concentrations, it increased the size of the individual casein peaks, particularly the α-casein.

Pure fractions corresponding to κ-casein, β-casein and α-casein were obtained under these conditions with an almost pure fraction of the unidentified casein. Once again, a pure fraction of mouse WAP was obtained.

A similar baseline separation was achieved with mouse skimmed milk. The milk was dissolved in the same buffer as was used for the casein separation, with the addition of 20 mM-EDTA to sequester the calcium. All the major caseins and whey proteins were separated. However, serum albumin and lactoferrin were eluted together as a single peak. Only very small quantities of milk (20 μl) were required for quantitative analysis of the proteins, enabling the milk of individual animals to be analysed without sacrificing the animal.

Mouse whey proteins were separated at pH 3.0 and pH 5.0 under the same conditions, as described for the caseins. At pH 5.0, however, the relative amount of WAP to milk serum albumin and lactoferrin appeared to be much greater than at pH 3.0. This is due to the fact that at pH 5 WAP is

very close to its isolectric point of 4.7-4.8 and therefore is hardly charged and elutes very quickly from the column. As a result it elutes along with or soon after the mercaptoethanol peak resulting in what appears to be a large protein peak but which is not.

Having separated the proteins by cation-exchange chromatography it was necessary to remove the urea and salts before further analysis of the protein. Initially, mouse caseins and whey proteins were desalted by dialysing against distilled water over a period of 3 days. Protein recovery by this method proved to be very inefficient, especially in the case of the whey proteins which, since they account for only approximately 20% of the total protein, are present in relatively small quantitites. Protein losses were due to the proteins adhering to the the dialysis tubing as the detergent and urea were removed. That was perhaps not totally unexpected considering the pronounced tendency for the mouse milk proteins to aggregate.

Desalting using reverse-phase liquid chromatography resulted in a much improved recovery of protein. This desalting method was also less time consuming, being complete in twenty minutes. Using this method, even individual protein samples from the ion-exchange chromatography were visible after freeze-drying, unlike the situation with samples that had been desalted by dialysis.

As a result of the successful separations of mouse caseins on the Mono-S column, attempts were made to scale-up the separation of the mouse casein fraction using the larger Hi-Load S column. Up to 400 mg of mouse casein were separated which represented a two hundred-fold increase in the

amount of casein that could be loaded onto the Mono S column. The separation was largely similar to that achieved on the Mono S column. However, the order of elution of WAP and κ-casein was reversed. These proteins were eluted as a single broad protein peak with a shoulder. SDS-PAGE showed that the leading edge of the peak contained more WAP than κ-casein, and the trailing edge contained more of κ-casein. A similar pattern emerged with Hi-Load S separations of the caseins of other species, and is perhaps due to slight differences in the column resin.

Using the Hi-Load System sufficient of the β and α-casein component was obtained to make the raising of antibodies to these individual proteins, feasible.

2.4.2 IDENTIFICATION OF THE CASEINS

Four major mouse milk proteins have been purified using ion-exchange chromatography. Amino acid compositions were obtained for all four proteins and by comparisons with published cDNA sequences (Table 16A), three mouse caseins, κ-, β- and α-casein, and one mouse whey protein, WAP, were positively identified. Two other mouse milk proteins were purified from the whey and an additional protein which was believed to be a casein but which was always contaminated by α-casein were also analysed. Amino acid compositions were obtained for the two whey proteins which are presumed to be milk serum albumin and lactoferrin on the basis of their molecular weights derived from SDS-PAGE. However, an absolute identification was not possible since cDNA sequences for these proteins have not yet been

published. The third protein to be analysed as yet remains unidentified, and, as for the whey proteins none of the published cDNA sequences including the ϵ-casein sequence show any similarity to this protein(Table 21). This may however be due the fact that a pure fraction of this protein has not yet been obtained. However, visualisation using Stains-all of the various proteins separated by SDS-PAGE showed that like the three major casein bands and unlike the whey proteins, this protein was phosphorylated and was therefore probably a casein.

2.4.3 MOLECULAR WEIGHTS

The mouse caseins exhibited a lower mobility on SDS-PAGE and hence a higher molecular weight than expected from their amino acid composition as determined by cDNA analysis (Table 17). This feature has also been observed with caseins from other species and is due to the unusually extended nature of the molecules and anomalous binding of SDS (Cheeseman & Jeffcoat, 1970).

In the case of mouse κ-casein this might be wrongly identified as β-casein if no other methods of protein characterization were used.

2.4.4 PROTEIN COMPOSITION

Freeze-dried casein was shown to consist of κ-, β-, α-caseins in the proportions 1:11.7:17.4. In skimmed milks however, the relative proportions of WAP and these proteins were 3.3:1:5.7:11.6 (Table 19B)respectively, i.e., lower amounts of α and β casein components relative to the κ-casein. All the milks were dissociated in buffer containing the detergent n-octyl β-D-

TABLE 21: Amino acid analysis of the unidentified mouse milk protein compared with the published cDNA sequence for mouse ϵ-casein.

Amino Acid	Amino acid residues (mole) per mole of protein	
	Unidentified	ϵ-casein
Asx	20.0	12
Glx	21.9	26
Ser	25.2	15
Gly	1.6	-
His	3.8	1
Thr	9.1	8
Ala	7.2	6
Arg/Pro	12.4	3+7
Tyr	6.7	8
Val	8.1	9
Met	1.5	13
Ile	10.4	9
Leu	8.4	6
Phe	6.1	4
Lys	6.4	9
Trp	-	2
Cys	-	1

glucopyranoside and no large protein peak was evident at the end of the milk separation indicating that dissociation was complete. The reduction in the α- and β-casein levels may once again be due to protein adhering to the dialysis tubing during sample preparation. Alternatively, the change in the casein ratio could also have been due to tailing of the enlarged WAP peak into the κ-casein peak in the skimmed milk samples leading to an apparent increase in the level of κ-casein on which the ratios were normalized.

When the protein levels were expressed in absolute units, the concentrations of these proteins were calculated to be WAP 15.3, 4.7 κ-casein, β-casein 25.2 and α-casein 51.7 mg ml^{-1}. α-Lactalbumin was not detected in either freeze-dried whey or in skimmed milk. This confirms the findings of Zamierowski and Ebner (1980) who used a radioimmunoassay and found that α-lactalbumin was a very minor component of mouse milk, accounting for no more than 0.83% of the total protein in mouse milk.

2.4.5 PHOSPHORYLATION

On the basis of their staining behaviour on SDS-PAGE after treatment with Stains-all, murine κ-, β- and α-caseins were all shown to be phosphorylated. This had previously been noted by Green & Pastewka (1976a, b). Stains-all was found to be a better dye than Coomassie blue for detecting the mouse κ-casein component which normally did not stain as well as the other caseins. This is probably due to the low content of κ-casein in mouse milk and also to glycosylation of the molecule, interfering with dye binding. It is interesting to note that in some papers dealing with mouse milk

proteins, the κ-casein band was not visible in gels stained with Coomassie blue and was not therefore identified as a casein by these authors The unidentified mouse milk protein was also shown to be a phosphoprotein as indicated by its blue colour in the presence of Stains-all. It is therefore probably a casein.

CHAPTER 3

EXPRESSION OF OVINE AND MODIFIED OVINE β-LACTOGLOBULIN IN MOUSE MILK

3.1 INTRODUCTION

β-Lactoglobulin is present in the whey of a number of species, including horse, dog, pig and dolphin (Jenness, 1985; Pervaiz and Brew, 1986) and is the major component of ruminant whey protein. Ruminant β-lactoglobulin is encoded by a gene, thought to be single copy and to be 800 nucleotides in length (Kolde and Braunitzer, 1983; Gaye *et al.* 1986; Godovac-Zimmermann and Braunitzer, 1987). In ruminant milks, β-lactoglobulin exists predominantly as a dimer with a protein chain length of 162 amino acid residues. Rodent milk does not contain β-lactoglobulin, but DNA containing the ovine β-lactoglobulin gene has successfully been incorporated into the mouse genome. Several lines of transgenic animals expressing this foreign protein in their milk have been produced with levels of β-lactoglobulin as high as 23 mg ml^{-1} being expressed. This is more than five times the concentration of β-lactoglobulin found in ovine milk (Simons *et al.*, 1987).

Wilde *et al.*(1992) used column chromatography to partially separate the casein fraction from the ovine β-lactoglobulin transgenic mouse milk. Their results indicated that although the overall level of protein in the transgenic milk was the same as that in native milks, the level of the caseins

apparently decreased to compensate for the expression of the foreign protein. However, they did not separate the mouse caseins into their individual components and could not therefore comment on the levels of the individual caseins. It was therefore proposed in consultation with workers at AFRC Roslin Institute to use the chromatographic system reported here to determine the levels of individual caseins in the transgenic milks and so determine whether expression of all of the endogenous caseins was controlled by the same mechanism. As an extension of the work with the β-lactoglobulin transgenic animals, it was proposed that a modified β-lactoglobulin gene could be constructed to investigate protein phosphorylation. Relatively little is known about the control of the mechanism by which milk proteins are phosphorylated, and none of the major whey proteins are apparently phosphorylated. Therefore in the modified gene construct, an oligonucleotide coding for the sequence of amino acids necessary to form a phosphate kinase recognition site on an external loop of the β-lactoglobulin molecule was inserted. By examining the phosphorylation state of this modified protein when expressed in mouse milk, it was hoped to determine whether phosphorylation merely required the presence of an accessible kinase reconition site or if the control was much more complex. This chapter will deal with the analysis of the β-lactoglobulin milks and the determination of the phosphorylation state of the modified whey protein.

3.2 MATERIALS AND METHODS

Milk was obtained from both native ovine β-lactoglobulin and modified ovine β-lactoglobulin mice. The milk was skimmed and fractionated into the acid precipitated and whey protein fractions as previously described. Skimmed milk and freeze-dried whey proteins were then separated by cation exchange FPLC on the Mono S resin.

Phosphatase inhibitor - a solution of sodium fluoride (50-100 mM); Pyrophosphate (5-10 mM) and sodium vanadate (0.1 mM).

3.2.1 SAMPLE PREPARATION

Whey proteins: Mouse whey protein was dissolved overnight (2 mg ml^{-1}) at 4°C in 20 mM-sodium acetate buffer, pH 7.0, containing either 8M-urea and 0.5% n-octyl β-D-glucopyranoside or 6 M-urea only. Samples were reduced for 1 hour with 10 μl ml^{-1} of 2-mercaptoethanol and the pH adjusted to pH 3.0 for samples containing 8 M-urea and pH 5.0 for samples containing 6 M-urea and filtered through an 0.22 μm filter.

Skimmed milks: Skimmed milk was dissociated overnight (25 μl ml^{-1}) at 4°C in 20 mM-sodium acetate buffer, pH 7.0, containing, 8 M-urea 20 mM-EDTA and 0.5% n-octyl β-D-glucopyranoside. The milks were then dialysed against the starting buffer for 4 hours. The protein was then reduced with 2-mercaptoethanol (10 μl ml^{-1}) for 1 hour before reducing the pH to 3.0 and filtering through an 0.22 μm filter.

Protein separation: All samples were separated at room temperature on a Pharmacia Mono S cation exchange column (HR 5/5), with a flow rate of 0.5 ml min^{-1} with the exception of the whey protein separation carried out at pH 5.0 which was run at 1 ml min^{-1}.

Whey protein: Whey proteins were fractionated using a gradient of sodium chloride in 20 mM-sodium acetate buffer at both pH 5.0 and pH 3.0 containing 6M-urea and 8M-urea, respectively.

Skimmed milk: Milk was fractionated using a gradient of sodium chloride in 20 mM-sodium acetate buffer pH 3.0 containing 8 M-urea.

3.3 RESULTS AND DISCUSSION

The sequences of ovine β-lactoglobulin and the serine-rich oligomer inserted in the protein, in an attempt to phosphorylate it by casein kinase recognition of the oligomer sequence, are shown in Figures 41A and 41B respectively, Figure 41A also shows the point in the protein sequence at which the oligomer was inserted.

3.3.1 CHROMATOGRAPHIC SEPARATION OF TRANSGENIC MOUSE MILK

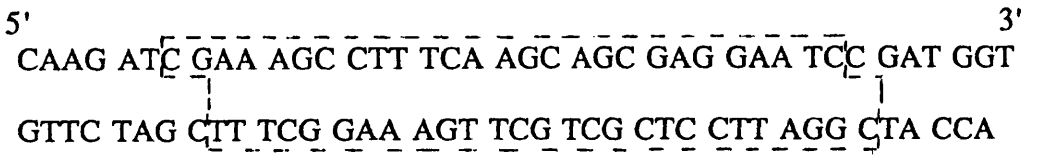
Whey protein: Separations were carried out at pH 3.0 and pH 5.0 and peak fractions were analysed by SDS-PAGE. The order of elution of the proteins was, WAP (fraction 2), then the transgenic proteins (fraction 3; Figure 42A, fraction 3; Figure 42B, 42C) and finally milk serum albumin and lactoferrin fraction 5 and 6 of Figure 42A, and 5 & 4 of Figures 42B and C,

FIGURE 41(A)

DNA Sequence of β-lactoglobuin gene SS1. Exon sequences are shown in upper case and flanking sequences in lower case. The predicted protein sequence is shown immediately above the DNA sequence. CAT, TATAA and AATAAA signals are underlined. The putative mRNA cap site is shown (*).

FIGURE 41(B)

INSERTED OLIGOMER



The boxed sequence represents the inserted oligomer encoding the amino acid sequence

Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser

FIGURE 42A, B and C: Cation exchange chromatography of transgenic mouse whey proteins

(A) Protein (2 mg) dissolved in sodium acetate buffer (20 mM), pH 7.0, containing 6M-urea (Figure 42A) or 8M-urea and 0.5% n-octyl β-D-glucopyranoside (Figures 42B and C) Samples were reduced as described in the text and the pH reduced to pH 5 (Figure 42A) and/or pH 3 (Figure 42B and C). The separation was developed using the same buffers at 1 ml min^{-1} on a Mono S column with detection at 280 nm. Numbered fractions were collected and analysed by SDS-PAGE (Figures 43A, B and C).

MONO S SEPARATION OF β-Lg TRANSGENIC MOUSE WHEY PROTEIN

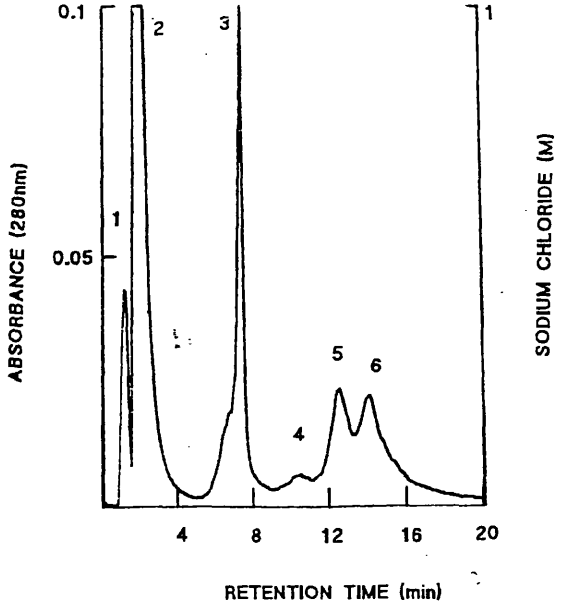


FIGURE 42(A)

MONO S SEPARATION OF β-Lg TRANSGENIC MOUSE WHEY PROTEIN

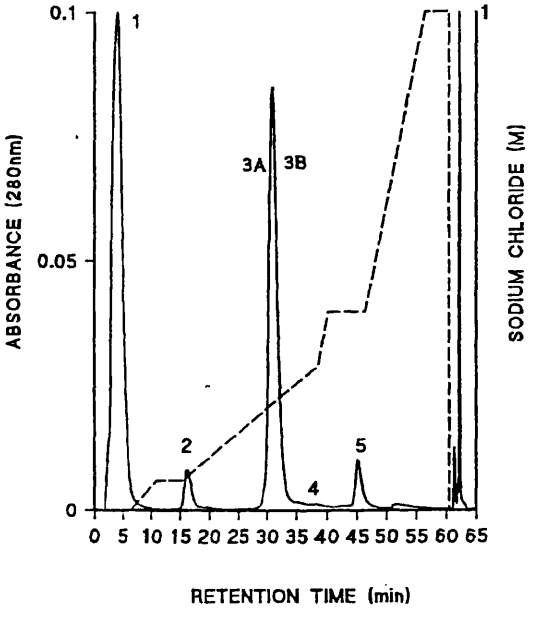


FIGURE 42(B)

MONO S SEPARATION OF MODIFIED β-Lg TRANSGENIC MOUSE WHEY PROTEIN

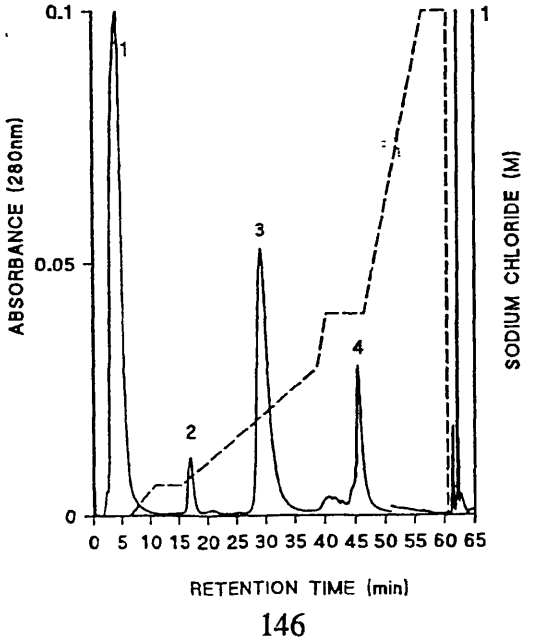
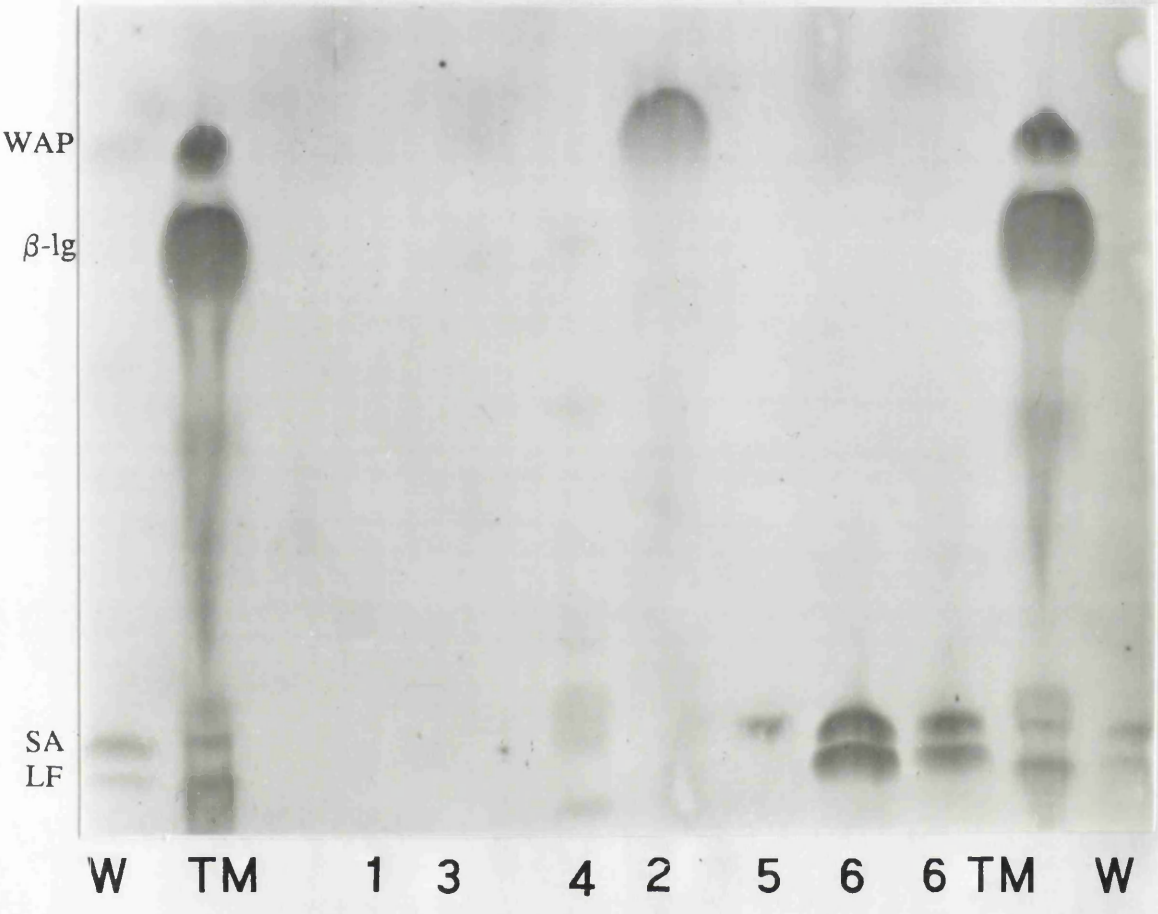


FIGURE 42(C)

FIGURE 43(A)



SDS-PAGE analysis of peak fractions collected from cation exchange chromatography of transgenic mouse whey proteins

Tracks (W) and (TM) contain whole whey protein and transgenic mouse milk respectively. Remaining numbered tracks 1-6 contain peak fractions numbered 1-6 on Figure 42(A).

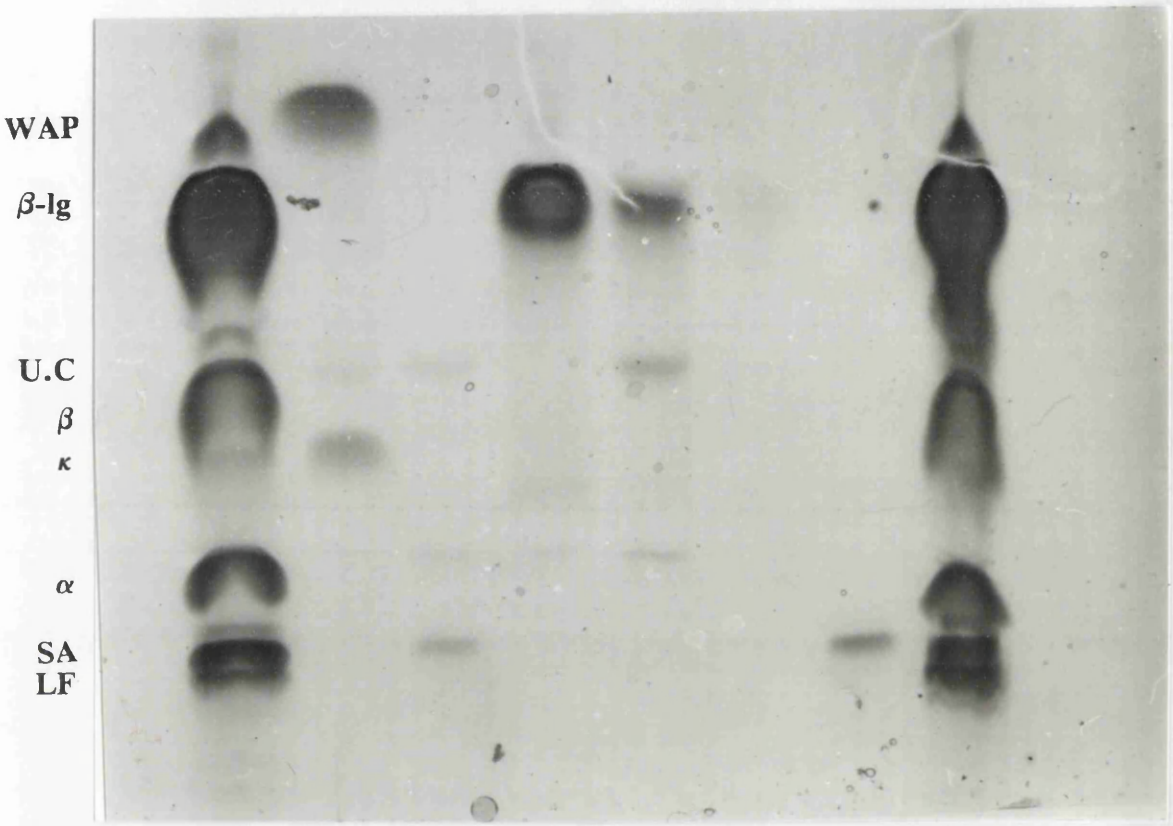
WAP: Whey acidic protein

β-lg: Ovine β-lactoglobulin

SA: Serum albumin

LF: Lactoferrin

FIGURE 43(B)

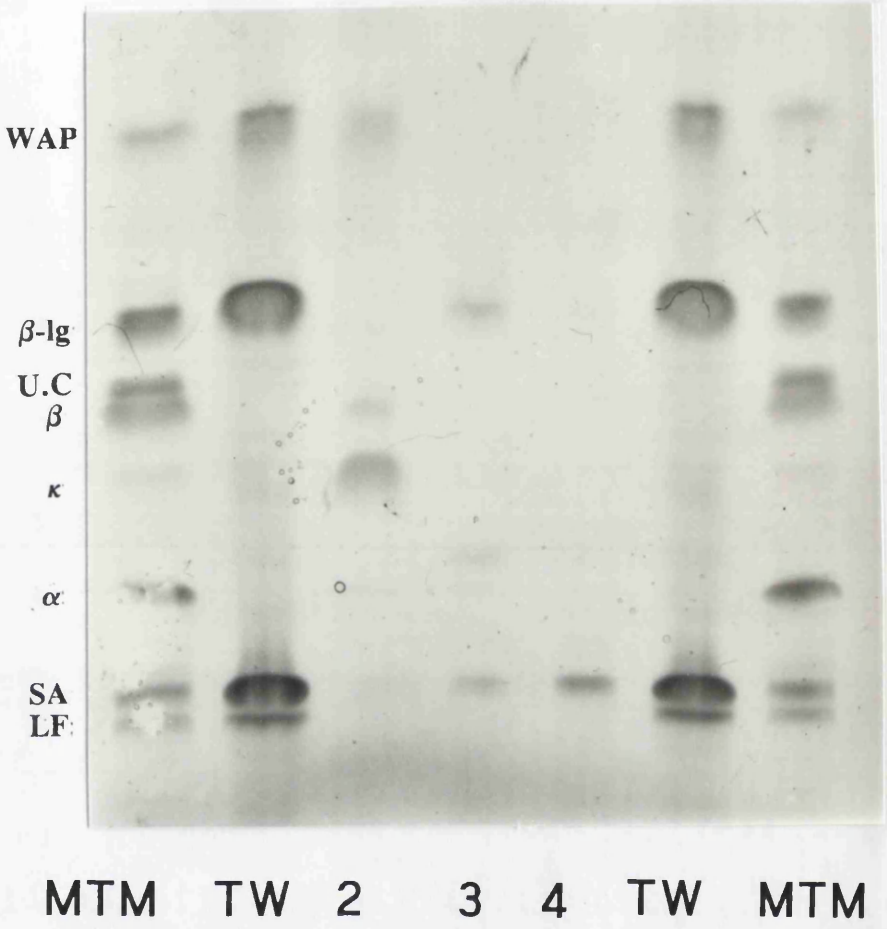


SDS-PAGE analysis of peak fractions collected from cation exchange chromatography of transgenic mouse whey proteins

Tracks labelled (TM) contain transgenic mouse milk. Remaining numbered tracks 2-5 contain peak fractions numbered on Figure 42(B), with fraction 3 being split further by desalting on reverse phase liquid chromatography.

WAP: Whey acidic protein
β-lg: Ovine β-lactoglobulin
U.C: Unidentified casein
κ: κ-casein
β: β-casein
α: α-casein
SA: Serum albumin
LF: Lactoferrin

FIGURE 43(C)



SDS-PAGE analysis of peak fractions collected from cation exchange chromatography of transgenic mouse whey proteins

Tracks labelled (MTM) and (TW) contain modified ovine β-lactoglobulin transgenic mouse milk and modified ovine β-lactoglobulin transgenic whey protein. Remaining numbered tracks contain peak fractions as numbered on Figure 42(C)

WAP: Whey acidic protein

β-lg: Modified ovine β-lactoglobulin

U.C: Unidentified casein

κ: κ-casein

β: β-casein

α: α-casein

SA: Serum albumin

LF: Lactoferrin

respectively: SDS-PAGE analyses of protein fractions are shown in Figures 43A, B, and C.

Skimmed milk: Separations of both the ovine β-lactoglobulin transgenic and modified ovine β-lactoglobulin transgenic milks are shown in Figures 44A and B. SDS-PAGE analysis of peak fractions revealed that as with the native mouse milk, fractions 2, 3, 4 and 7 consisted of WAP, κ-casein, β-casein and a mixture of serum albumin and lactoferrin respectively. In the separation of ovine β-lactoglobulin transgenic milk, fraction 5 was further separated during the desalting on reverse-phase chromatography and was found to contain α-casein (almost pure), the unidentified casein (almost pure) and some α-casein contaminated with β-casein. Fraction 6 was found to be an almost pure fraction of ovine β-lactoglobulin (Figure 45A). Fraction 5 of the modified ovine β-lactoglobulin milk was similarly separated further on desalting, giving rise to a pure fraction of the transgenic protein and the unidentified casein, as well as mixtures containing the α-casein. Fraction 6 also contained the transgenic protein as well as some low molecular weight peptides (Figure 45B).

3.4 THE EXPRESSION OF FOREIGN PROTEINS IN MILK

Expression of foreign proteins in mouse milk has been reported to cause no overall increase in the total level of protein in the milk (Wilde *et al.*, 1992). Instead, the foreign protein is apparently expressed at the expense of the native proteins, the levels of which decreased as that of the foreign protein increased. The cation exchange chromatographic separation which was

FIGURE 44A and B: Cation exchange chromatography of transgenic mouse milks

Milk from β-lactoglobulin transgenic and modified β-lactoglobulin transgenic mice was dissociated (25 μl ml^{-1}) in sodium acetate buffer. (20 mM) pH 7 containing 8M-urea, 20 mM-EDTA and 0.5% n-octyl β-D-glucopyranoside. The proteins were then reduced as described in the text and dialysed. The protein solution was reduced to pH 3 and the separations developed in sodium acetate buffer (20 mM) containing 8M-urea at 0.5 ml min^{-1} and detection at 280 nm. Numbered fractions were collected and analysed by SDS-PAGE (Figures 45A and B).

MONO S SEPARATION OF β-Lg TRANSGENIC MOUSE MILK

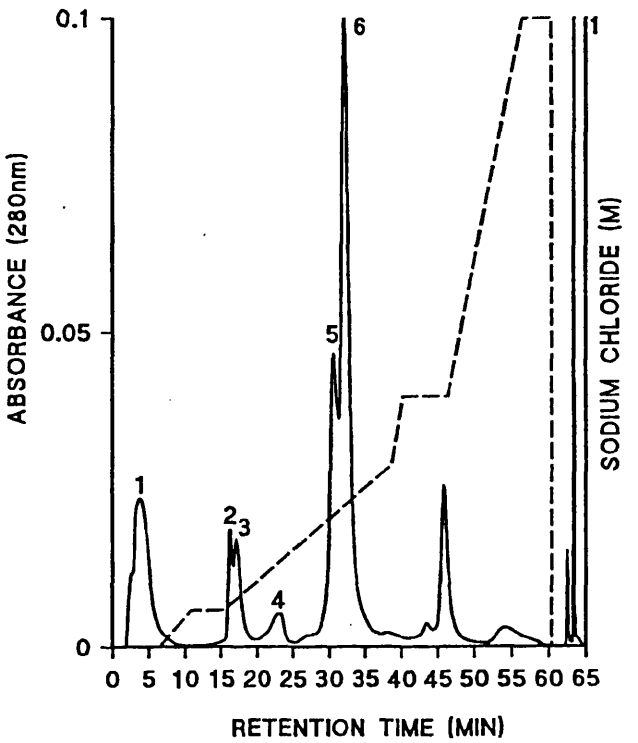


FIGURE 44(A)

MONO S SEPARATION OF MODIFIED β-Lg TRANSGENIC MOUSE MILK

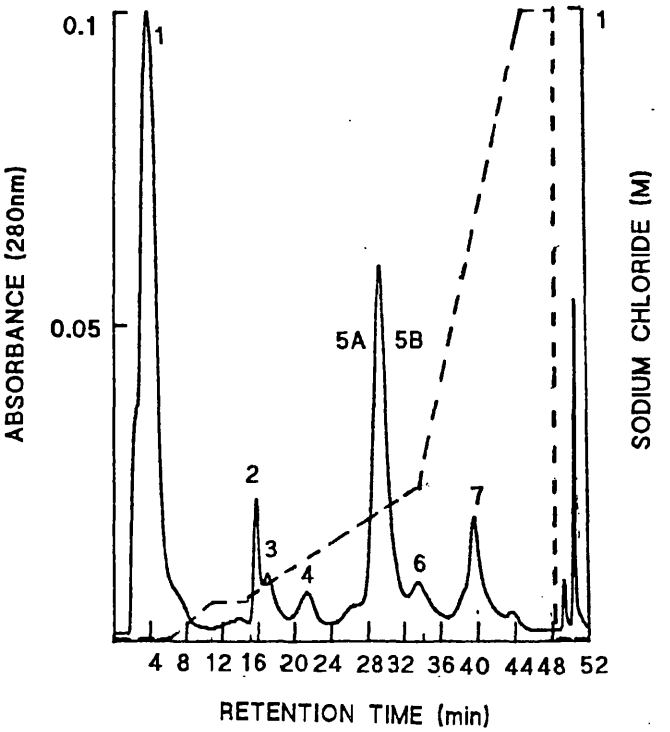
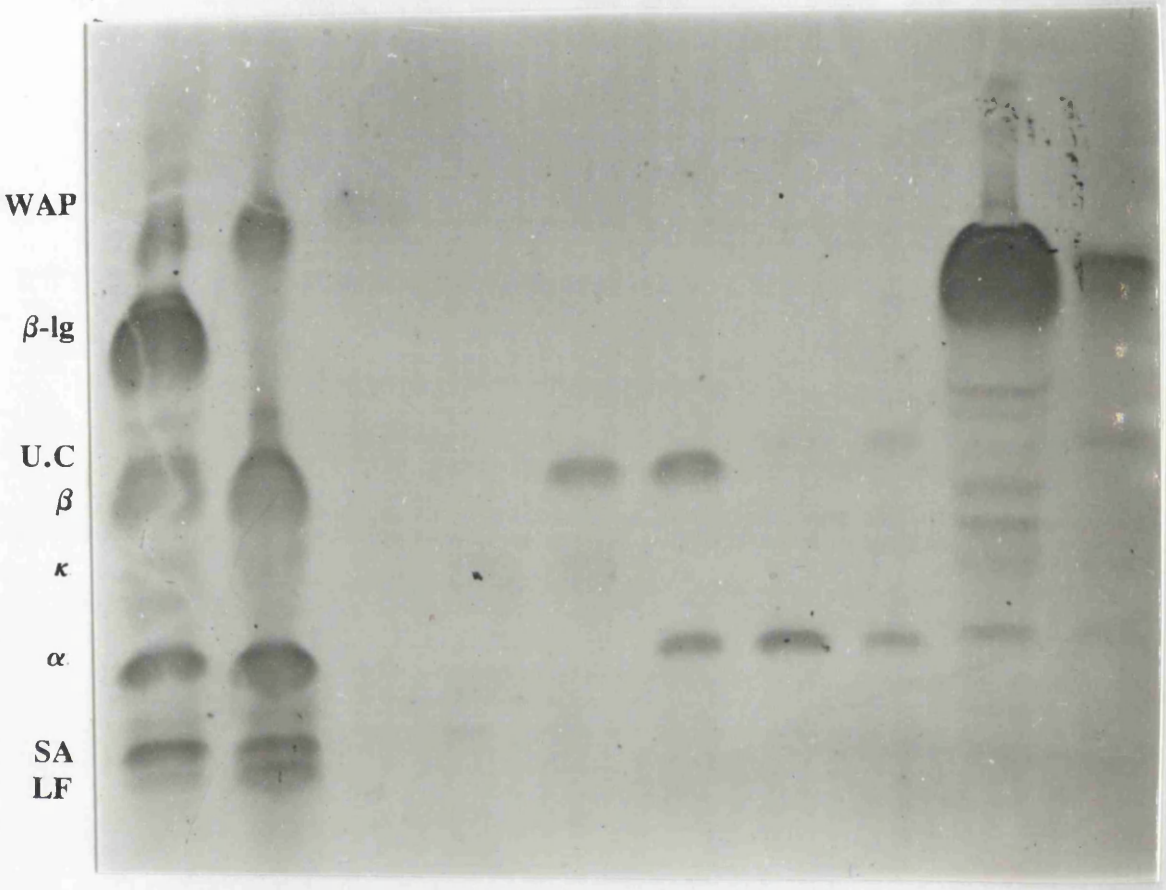


FIGURE 44(B)

FIGURE 45(A)

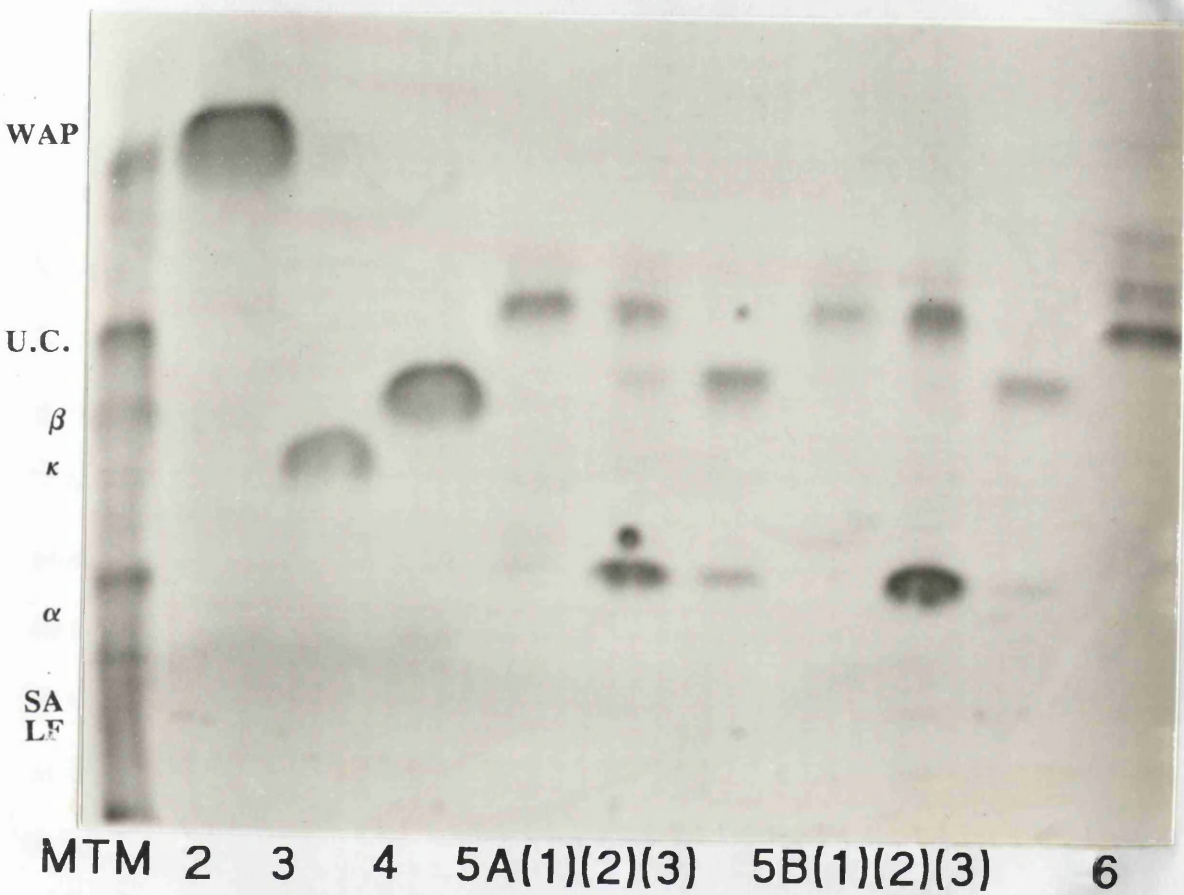


SDS-PAGE analysis of peak fractions obtained from cation exchange chromatography of β-lactoglobulin transgenic mouse milk.

Tracks labelled (TM) and (MM) contain ovine β-lactoglobulin transgenic mouse milk and native skimmed mouse milk respectively. Remaining numbered tracks 2-6, contain peak fractions as numbered in Figure 44(A), with fractions 5 and 6 being split further on desalting by reverse phase chromatography.

WAP: Whey acidic protein
β-lg: Ovine β-lactoglobulin
U.C: Unidentified casein
κ: κ-casein
β: β-casein
α: α-casein
SA: Serum albumin
LF: Lactoferrin

FIGURE 45(B)



SDS-PAGE analysis of peak fractions obtained from cation exchange chromatography of modified β-lactoglobulin transgenic mouse milk.

Track (1) modified β-lactoglobulin transgenic mouse milk (M.T.M.) and remaining numbered tracks, peak fractions as numbered in Figure 44B.

WAP: Whey acidic protein

U.C: Unidentified casein

β: β-casein

κ: κ-casein

α: α-casein

developed in this project enabled the levels of individual casein and native whey proteins to be determined as well as that of the ovine β-lactoglobulin in transgenic mouse milk. Overall, the casein level decreased, but the decrease did not appear to be uniform. Expression of the α-casein component was apparently more suppressed than that of the other caseins.

Ovine β-lactoglobulin transgenic mouse milk (Table 22) was found to contain a reduced level of α-casein and a high proportion of ovine β-lactoglobulin. WAP:κ-casein:β-casein:α-casein; ovine β-lactoglobulin = 3.5:1:5.2:7.4:19.0. Native mouse milk was found to contain WAP, κ-casein, β-casein and α-casein in the proportions of 3.3:1:5.7:11.6. The results in Table 23 demonstrate the effect of the transgenic protein on endogeneous milk protein secretion. WAP, κ-casein, β-casein and α-casein were found to be decreased by 29.7, 46.0, 41.0 and 62.3%, respectively. The proportion of WAP in transgenic and non-transgenic milk samples was calculated as ~10% and 113-15% of total protein, respectively, i.e., a decrease of ~4% of the total protein. The absolute amount of ovine β-lactoglobulin found in the transgenic mouse milk was calculated as 50.1 mg ml^{-1}, which was more than that calculated by Simmons *et al.*(1987). This is a result of the fact that the mice used by Simmons *et al.*(1987) were heterozygotes while those used for the calculations in Table 23 were homozygotes. It was found using anion-exchange chromatography that a protein peak containing mouse α-lactalbumin and WAP was greatly reduced in the transgenic milk sample i.e. from 30% to 17% of the total protein Wilde *et al.* (1992). α-Lactalbumin however, was not detected in any transgenic or native milks analysed by chromatographic or

TABLE 22: Relative amounts of the major proteins in the milks of two lines of transgenic mice

Milk Sample	Relative amounts				
	WAP	κ-Casein	β-Casein	α-Casein	Ovine β-lg
β-lg					
1	3.4	1	7.6	9.3	15.7
2	4.0	1	5.7	6.6	24.3
3	4.1	1	4.6	7.3	18.8
4	3.4	1	4.3	7.1	27.6
5	2.9	1	4.7	6.2	22.9
6	3.3	1	5.1	7.7	14.2
7	3.3	1	4.5	7.3	9.6
Mean	**3.5**	**1**	**5.2**	**7.4**	**19.0**
Modified β-lg					
1	2.6	1	2.9	10.3	
2	4.9	1	6.4	16.4	
3	3.9	1	6.9	16.4	
4	3.4	1	3.2	17.8	
5	2.9	1	6.5	13.4	
6	3.3	1	7.5	19.5	
Mean	**3.5**	**1**	**6.4**	**15.6**	

TABLE 23: Absolute amounts of the major proteins in native and β-lactoglobulin transgenic mouse milks

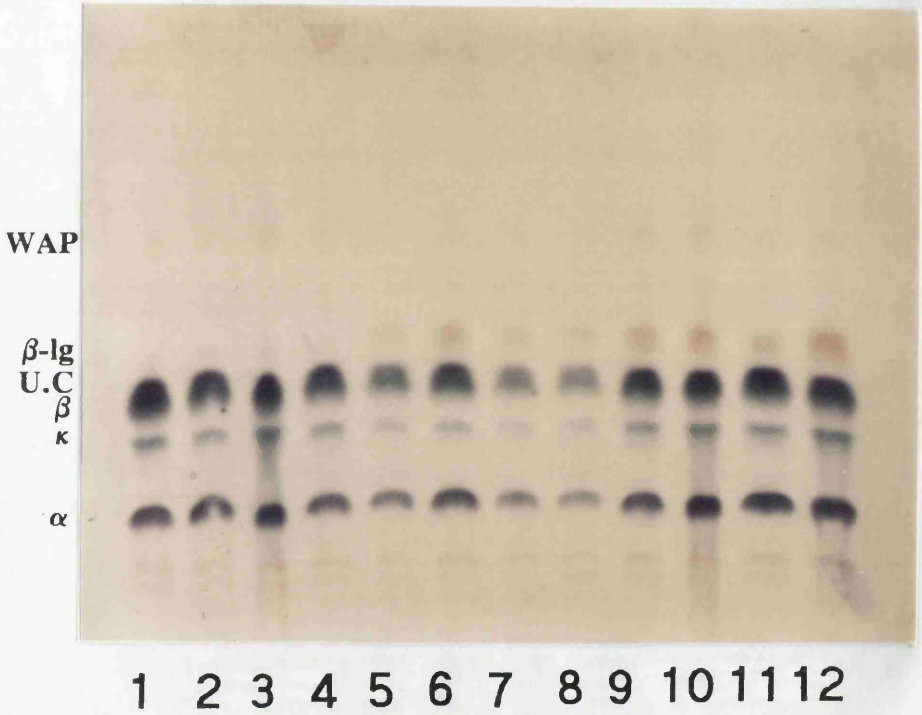
β-Lg milks (1 ml)	WAP mg ml^{-1}	κ-Casein mg ml^{-1}	β-Casein mg ml^{-1}	α-Casein mg ml^{-1}	Ovine β-lg mg ml^{-1}
1	8.9	2.6	19.9	24.4	41.2
2	9.3	2.3	13.3	15.4	56.7
3	11.1	2.7	12.5	19.8	50.9
4	7.6	2.2	9.6	15.8	61.7
5	7.5	2.6	12.1	16.0	60.0
6	10.2	3.1	15.8	23.9	44.0
7	12.5	3.8	17.0	27.6	36.2
Mean	**9.6**	**2.8**	**14.3**	**20.4**	**50.1**
Native Milk (1 ml)	**WAP mg ml^{-1}**	**κ-Casein mg ml^{-1}**	**β-Casein mg ml^{-1}**	**α-Casein mg ml^{-1}**	
1	14.2	5.3	28.0	49.6	
2	12.2	5.3	26.7	49.8	
3	13.9	6.1	26.7	50.3	
4	15.1	7.2	11.5	63.2	
5	10.5	3.6	25.3	57.5	
6	12.3	3.8	26.9	53.9	
Mean	**13.0**	**5.2**	**24.2**	**54.1**	
% Decrease	**29.7%**	**46.0%**	**41.0%**	**62.3%**	

electrophoretic method throughout the duration of this project, as previously discussed in the section on protein composition.

An estimate of the amount of modified β-lactoglobulin in the other transgenic line was not possible since this protein co-eluted with the mouse α-casein component as shown by SDS-PAGE. However, if this protein peak was assumed to contain the same relative amount of α-casein as the native ovine β-lactoglobulin line, an estimate of the amount of modified β-lactoglobulin can be made. The results (Table 22) show that the relative amount of α-casein increased in comparison to β-lactoglobulin transgenic milks. The relative amounts of the major milk proteins in the modified β-lactoglobulin line would therefore be WAP:κ-casein:β-casein:α-casein:modified β-lactoglobulin, 3.5:1:6.4:7.4:8.2. Expressed in absolute amounts they would be, WAP 12.8, κ-casein 3.7, β-casein 23.4, α-casein 27.1 and modified β-lactoglobulin 30.0 mg ml^{-1}.

A simple method to determine whether the modified β-lactoglobulin was phosphorylated was to separate the proteins by SDS-PAGE and stain with Stains-all. This requires only minute amounts of protein. If the novel β-lactoglobulin in the modified β-lactoglobulin transgenic milk had been phosphorylated, it should have stained blue in the presence of Stains-all. However, the foreign protein, which due to the incorporation of the kinase recognition peptide sequence appeared to have a higher molecular weight than native ovine β-lactoglobulin, stained pink (Figure 45C). This indicated that it was not phosphorylated. There were two possible explanations for this. ne was that the protein was not phosphorylated despite the possession of the

FIGURE 45(C)



SDS-PAGE of native and modified β-lg transgenic mouse milk stained with Stains all.

Tracks numbered (1) to (4) native mouse milk and tracks numbered (5) to (12) modified β-lg mouse milk.

WAP: Whey acidic protein

β-lg: Modified ovine β-lactoglobulin

U.C: Unidentified casein

β: β-casein

κ: κ-casein

α: α-casein

requisite kinase recognition site and serine residues which could undergo phosphorylation. The second was that the serine residues were phosphorylated but being on the exposed loop of the β-lactoglobulin, they were also rapidly dephosphorylated by phosphatases in the milk. Milk samples were therefore collected and stored in the presence of a phosphatase inhibitor to rule out any dephosphorylation of the inserted oligomer by endogenous phophatases. This had no effect on the staining of the protein in transgenic milks (Figure 45C), strongly suggesting that the modified β-lactoglobulin was not phosphorylated. It can therefore be concluded that phosphorylation of milk proteins is not simply dependent upon the possession of the correct sequence of amino acids in an exposed position on the protein.

CHAPTER 4

EFFECT OF DELETING MURINE β-CASEIN GENE ON PROTEIN EXPRESSION

4.1 INTRODUCTION

In Chapter 3, the effect of expressing a foreign whey protein on the levels of the native mouse milk proteins was investigated. Mr S. Kumar and Dr P. Simons at AFRC Roslin Institute have succeeded in producing several lines of mice in which the native mouse β-casein gene has been successfully deleted. This is to our knowledge the first occasion in which an individual casein gene has been deleted. Although the absence of expression of β-casein from the milk could be determined by SDS-PAGE, quantification of the levels of expression of the other mouse milk proteins, particularly the caseins, was much more difficult. Milk from these β-casein "knock-out" lines was therefore analysed using the column chromatographic techniques developed and described earlier in this project, and the levels of expression of the various proteins compared with that in native mice. Analysis of these milks effectively complements that of the β-lactoglobulin mouse milks where expression of a foreign protein was shown to cause a general reduction in the levels of endogenous proteins but this, as shown in Chapter 3 was, not a uniform change.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Milk was obtained from a number of native and homozygous β-casein "knock-out" mice. Individual samples were pooled and the milk was skimmed and fractionated into the acid precipitated and whey protein fractions as described previously. Every effort was made to ensure that analysis of the two types of milk was as quantitative as possible.

4.2.2 SAMPLE PREPARATION

Acid precipitated casein/whey protein: Proteins were dissociated by stirring overnight at a protein concentration of approximately 2 mg ml^{-1} in 20 mM sodium acetate buffer, pH 7.0, containing 8M-urea and 0.5% n-octyl β-D-glucopyranoside. The proteins were then reduced for 1 hour with 2-mercaptoethanol (10 μl ml^{-1}) after which the pH of the protein solution was reduced to 3.0 and filtered through an 0.22 μm filter.

4.2.3 PROTEIN SEPARATION

Casein and whey proteins were separated on a Mono S cation exchange column (HR 5/5) with a flow rate of 0.5 ml min^{-1} using a gradient of sodium chloride in 20 mM-sodium acetate buffer pH 3.0 containing 8M-urea as described previously.

4.3 RESULTS & DISCUSSION

The Mono S elution profiles of the pooled acid precipitated and whey protein fractions (Figures 46 and 47) confirmed the SDS-PAGE results obtained by Kumar and Simons(submitted for publication) showing that the synthesis of β-casein in the "knock-out" lines had been completely blocked. The effect of the deletion of the β-casein gene on the levels of expression of the other endogenous proteins was investigated and these results are summarized in Tables 24 to 26. The level of total protein in these animals was decreased from 97 to 87 mg ml^{-1} of which the whey accounted for 21 mg ml^{-1} compared to 18 mg ml^{-1} in native mice. The changes in the levels of the individual proteins were variable with the levels of the κ-casein in the "knock-out" milk increasing by 25% compared to the native milk whilst that of the α-casein increased by only 4% and that of unidentified "casein" actually decreased by 10%. Changes in the levels of the whey proteins were also detected with the levels of WAP and lactoferrin/serum albumin increasing in the whey protein fraction by 36 and 25%, respectively, and an even greater increase (68%) in the level of a minor whey component (B) which was originally thought to be serum κ-casein but was clearly shown in the "knock-out" whey to have a slightly different retention time.

The levels of most of the proteins increased in the whey fraction. However, the increase could be accounted for by changes in the partitioning of these components between the acid precipitated and supernatant fractions. Overall, the levels of these components in the β-casein "knock-out" milk decreased slightly ($\sim$10%). The overall level of the whey protein increased

MONO S SEPARATION OF NATIVE (....)

AND

KNOCKOUT (——) MOUSE CASEINS

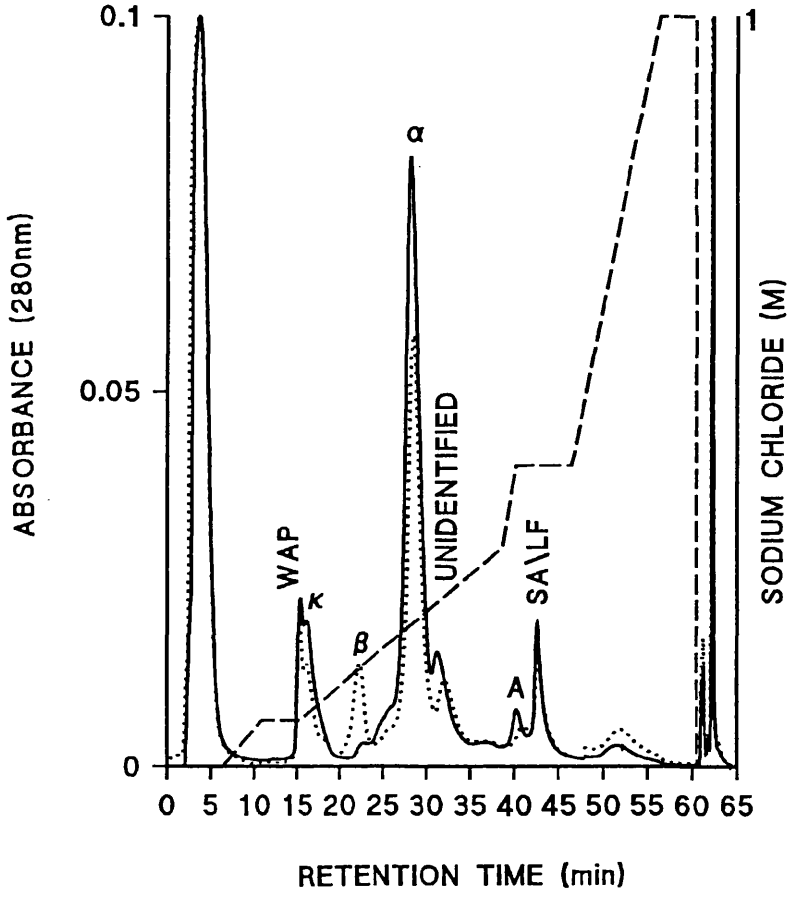


FIGURE 46

Protein (2 mg) was dissociated in sodium acetate buffer (20 mM), pH 7, containing 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. Proteins were reduced as described in the text and adjusted to pH 3.0. The separation was developed on a Mono S column at a rate of 0.5 ml min^{-1} with detection at 280 nm.

MONO S SEPARATION OF NATIVE (...)

AND

KNOCKOUT (——) MOUSE WHEY PROTEINS

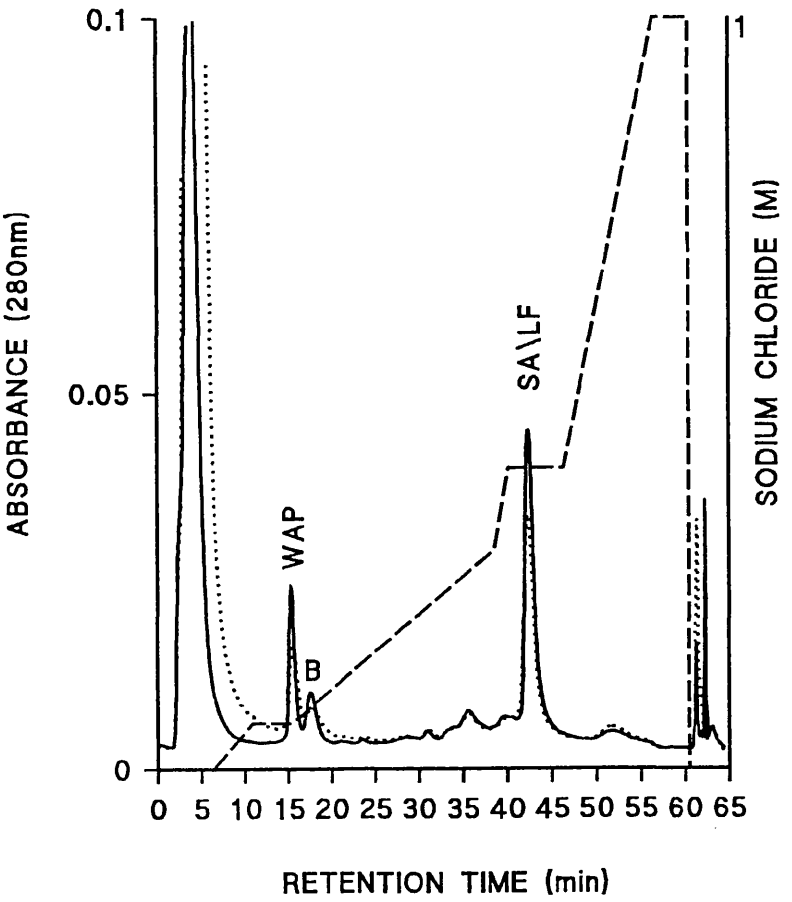


FIGURE 47

Protein (2 mg) was dissociated in a sodium acetate buffer (20 mM), pH 7, containing 8 M-urea and 0.5% n-octyl β-D-glucopyranoside. Proteins were reduced as described in the text and adjusted to pH 3. The separation was developed on a Mono S column at a rate of 0.5 ml min^{-1} with detection at 280 nm.

TABLE 24: Changes in the levels of various proteins in the acid precipitated fraction of β-casein deleted milk compared with native mouse milk.

Protein	Change (%)
WAP	-22
κ-casein	+25
β-casein	-100
α-casein	+4
unidentified casein	-10
A	+22
Lactoferrin/serum albumin	-24

TABLE 25: Changes in the levels of various proteins in the acid soluble whey fraction of β-casein deleted milk compared with native mouse milk

Protein	Change (%)
WAP	+36
B	+68
Lactoferrin/serum albumin	+25

TABLE 26: Overall changes in the levels of the main "whey" proteins in β-casein deleted milk compared with native mouse milk

Protein	Change (%)
WAP	-11
Lactoferrin/serum albumin	-9

by ~16% and casein decreased by ~16%. This change in partitioning suggests that β-casein may be important in influencing the isoelectric precipitation of these components in the native milk.

The implication of the effect of deleting the β-casein gene on the levels of expression of the other caseins will be discussed in Chapter 5.

CHAPTER 5

GENERAL DISCUSSION

The principal aim of this project was to develop methods for the quantitative separation of the main protein components of mouse milk and to utilise these methods to analyse the expression of proteins in the milk of transgenic animals. This was achieved using cation exchange chromatography although the separation proved to be more difficult than had been anticipated. More extreme conditions were required for the initial dissociation of the mouse casein micelles than for the milks of other species. The main difference was the necessity of including a non-ionic detergent, n-octyl β-D-glucopyranoside, as well as 8M-urea, in the dissociation buffer. In the absence of the detergent, a large peak of undissociated protein was eluted from the column during the re-equilibration step. When the detergent was included, the areas of all of the casein peaks increased, but not uniformly for all of the caseins. The increase in the amount of the α-casein component was much greater. A possible explanation for this observation is postulated later in this section.

The α-, β- and κ-caseins were isolated and identified on the basis of their amino acid compositions. An additional acid precipitable protein was obtained in an almost pure form. Although this has not yet been identified, on the basis of its staining behaviour, it has been shown to be phosphorylated and is therefore presumably a casein. The various whey proteins were also

separated by cation exchange FPLC, although in the absence of sequence data, the high molecular weight components could not be quantified. The separation of the caseins was scaled-up to permit sufficient of some of them to be obtained in a pure form to make the raising of antibodies feasible. Together with the development of a method for the chromatographic separation of the proteins from as little as 25 μl of skimmed milk, antisera to individual caseins should be useful in a number of areas of research involving lactation in the mouse as a model system.

Applying the analytical chromatographic techniques to the composition of modified milks i.e. those from transgenic mice expressing ovine β-lactoglobulin and those from animals in which the endogenous β-casein gene had been deleted, yielded data which may be important with regard to the mechanism of milk protein secretion.

Studies of protein secretion from different cell types have identified two pathways by which protein is secreted via the mechanism of exocytosis; the constitutive pathway and the regulated pathway. Constitutive exocytosis results in proteins being secreted immediately after they are packaged into secretory vesicles. Regulated secretion of protein results in proteins being stored in secretory vesicles or granules for variable periods of time until their release in response to cell activation which in turn results in a rise in either intracellular Ca^{2+} or another second messenger (Burgess & Kelly, 1987). Some cell types possess both secretory pathways. In such cases, proteins usually show a preference for one pathway over the other. The constitutive pathway has also been considered to be a default pathway, by which proteins

that have been transported through the trans-golgi network but which lack the information to enable them to be targeted to the correct organelle are secreted.

Milk protein secretion is usually considered a constitutive process (Franke *et al.* 1976; Linzell and Peaker, 1971; Saacke and Heald, 1974). A recent study of milk protein secretion examined secretory acini isolated from the glands of lactating mice and concluded that lactating mammary cells possess both a Ca^{2+}- independent constitutive pathway and a Ca^{2+}-activated regulated pathway for protein secretion (Turner *et al.*, 1992). [^{35}S]-Methionine was found to be incorporated into protein linearly for at least 5 hours with no lag period. However, protein secretion was detected only after a lag of about 1 hour. This was consistent with constitutive exocytosis. Secretion was unaffected by the addition of 8-bromo-cAMP and 8-bromo-cGMP, but was doubled by addition of the Ca^{2+} ionophore, ionomycin. A pulse-chase experiment designed to examine the importance of Ca^{2+} concentration in the regulation of exocytosis revealed that constitutive secretion was unaffected by the depletion of cytosolic Ca^{2+} but ionomycin gave a two-fold increase in the stimulation of presynthesized protein in a Ca^{2+}-dependent manner. Ionomycin was also found to stimulate protein secretion after constitutive secretion had ceased. These results suggested the involvement of both pathways in milk protein secretion.

Analysis of milks from native and ovine β-lactoglobulin transgenic mice as reported in Chapter 3 revealed that although the overall level of protein secretion was unchanged between the two types, the levels of the endogenous milk proteins, WAP, κ-casein, β-casein and α-casein were

reduced by 29.7, 46.0, 41.0 and 62.3%, respectively in the transgenic milk. These results reinforce the idea of a regulated pathway, as well as a constitutive pathway of milk protein secretion, since if proteins were only secreted constitutively, it would be expected that the levels of all the milk proteins would be reduced to the same extent.

Results from the β-casein 'knockout' transgenic lines also proved interesting. The total milk protein secreted by the mammary gland was reduced in these animals by around 10% even though β-casein accounts for 21% of the milk protein. Therefore, it could be concluded that the gland is compensating to a limited extent for the loss of the β-casein gene by secreting more of the other milk proteins. The results showed that total whey protein increased by 16% and although the overall level of casein was reduced by ~16%, the amount of κ-casein being synthesized by the gland actually increased by 25%. Again, this fact could reinforce the idea of both regulated and constitutive secretory pathways being involved since the deletion of the β-casein gene did not have the same effect on all the milk proteins.

Recent studies of protein secretion in the mouse mammary gland have investigated the process of protein phosphorylation using the fungal metabolite, brefeldin A (BFA)(Turner *et al.* 1993). Addition of BFA results in a functional dissection of Golgi cisternae from the trans-Golgi network and examining the effect of BFA on phosphorylation should indicate whether phosphorylation of newly synthesized protein occurs either in the Golgi cisternae or the trans-Golgi network. The results suggest that α-casein phosphorylation is primarily mediated via a kinase located within the Golgi

cisternae since phosphorylation of newly synthesized α-casein can occur even in the presence of BFA which causes the collapse of cisternal enzymes back into the endosplasmic reticulum. Phosphorylation of β-casein however was inhibited by the presence of BFA, indicating that the kinase required for phosphorylation of mouse β-casein must be located in the trans-Golgi network. In addition, protein produced by ionomycin-activated regulated secretion was found to contain a higher ratio of α- to β-casein, than that produced by the constitutive pathway of secretion (Turner *et al.* submitted for publication). The significance of these results is still unclear. However, they could indicate that the mouse casein micelle consists of a core of α-casein since it is phosphorylated before β-casein. This in turn could explain why addition of n-octyl β-D-glucopyranoside resulted in an increased α-casein peak as the core region was disrupted. In addition, if α-casein is secreted primarily via a regulated pathway, this would perhaps begin to explain the differential effects on casein secretion observed in both the β-lactoglobulin transgenic mouse line and the β-casein deleted lines.

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