HEAT-INDUCED INTERACTIONS OF β-LACTOGLOBULIN, α-LACTALBUMIN AND CASEIN MICELLES.

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ABSTRACT

The denaturation and aggregation of β-lactoglobulin and α-lactalbumin were studied in the following mixtures, designed to simulate the protein concentrations and ionic environment in normal skim milk.

1. β-lactoglobulin (0.4% w/v),
2. α-lactalbumin (0.15% w/v),
3. β-lactoglobulin (0.4% w/v) and casein micelles (~ 2.6% w/v),
4. α-lactalbumin (0.15% w/v) and casein micelles (~ 2.6% w/v),
5. β-lactoglobulin (0.4% w/v) and α-lactalbumin (0.15% w/v) and
6. β-lactoglobulin (0.4% w/v), α-lactalbumin (0.15% w/v) and casein micelles (~ 2.6% w/v)

Proteins were dissolved in SMUF, pH 6.7, and heated at 80 and 95°C for various times and centrifuged at 100,000 **g** for 60 min. The supernatants and pellets obtained were analysed using gel electrophoresis under non-dissociating (Native-PAGE in the absence of dissociating and reducing agents), dissociating but non-reducing (SDSNR-PAGE) and dissociating and reducing conditions (SDSR-PAGE).

When β-lactoglobulin was heated alone and examined by native-PAGE, the quantity of native protein decreased with increasing heating time at 80°C. Addition of α-lactalbumin to the β-lactoglobulin solution increased the loss of β-lactoglobulin during the initial stages of heating. Addition of casein micelles to the β-lactoglobulin solution markedly increased the loss of native β-lactoglobulin throughout the heating period. The loss of β-lactoglobulin from the mixture containing β-lactoglobulin, α-lactalbumin and casein micelles was similar to that from the mixture of β-lactoglobulin and casein micelles. The loss of β-lactoglobulin from these protein mixtures could be described by second-order reaction kinetics. Heating these mixtures at 95°C caused very rapid loss of native β-lactoglobulin, but the effects of the addition of casein micelles and α-lactalbumin were generally similar to those observed at 80°C.

When α-lactalbumin was heated at 80°C either alone or in the presence of casein micelles, there was only a slight loss of the native α-lactalbumin. However the

corresponding losses of native α-lactalbumin were considerable greater on heating at 95°C. At both temperatures, the addition of β-lactoglobulin increased the rate of loss of α-lactalbumin substantially. The addition of casein micelles to the mixture of α-lactalbumin and β-lactoglobulin had little further effect on the loss of native α-lactalbumin. The rates of loss of α-lactalbumin at 95°C in all mixtures could be adequately described by first-order kinetics.

When β-lactoglobulin was heated either alone or in the presence of casein micelles and examined by SDSNR-PAGE, the loss of SDS-monomeric β-lactoglobulin was less than the loss of native β-lactoglobulin. In contrast, when α-lactalbumin was added to β-lactoglobulin or β-lactoglobulin and casein micelles mixture, the loss of SDS-monomeric β-lactoglobulin was comparable to that of native β-lactoglobulin. The difference between native and SDS-monomeric β-lactoglobulin represents aggregates that are linked by non-covalent (hydrophobic) interactions. Thus the protein mixtures containing α-lactalbumin, contain no or little non-covalently linked β-lactoglobulin aggregates, and consequently, all the β-lactoglobulin aggregates would be disulphide linked.

The results for the loss of SDS-monomeric and native α-lactalbumin at 95°C showed that both non-covalent and disulphide-linked aggregates of α-lactalbumin were present in all the protein mixtures studied.

When β-lactoglobulin solution was heated at 95°C, large aggregates were formed which could be sedimented at 100,000 **g** for 60 min. Addition of casein micelles to β-lactoglobulin solution caused greater sedimentation of β-lactoglobulin. Similar results were obtained when the mixture containing β-lactoglobulin, α-lactalbumin and casein micelles was heated at 95°C. In contrast, the mixture containing β-lactoglobulin and α-lactalbumin behaved in a similar manner to β-lactoglobulin alone.

When α-lactalbumin was heated at 95°C alone or in the presence of casein micelles, it did not interact to form large sedimentable aggregates. However when β-lactoglobulin was added to the above protein solutions, there was a considerable increase in sedimentation of α-lactalbumin.

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CHAPTER 1
INTRODUCTION

In the dairy industry, most of the milk is subjected to heat during processing. Heating is invariably used in order to preserve milk by killing pathogenic or spoilage organisms, inactivating enzymes as well as altering the physico-chemical state of milk. High temperature heat processing, such as forewarming and sterilisation causes changes in the physico-chemical and functional properties of milk proteins. The principal changes in milk proteins caused by heating include: whey protein denaturation, interaction of denatured whey proteins with casein micelles and Maillard browning reaction of proteins with lactose or other added sugars.

Although denaturation of whey proteins has been studied extensively in model systems and in milk (Baer *et al.*, 1976; Elfagm and Wheelock, 1978a,b; Harwalker, 1980a,b; Park and Lund, 1984; Relkin and Launay, 1990; Calvo *et al.*, 1993; McSwiney *et al.*, 1994), the effects of constituent milk proteins, e.g. α-lactalbumin, β-lactoglobulin and casein micelles on denaturation of β-lactoglobulin or α-lactalbumin have not been fully explored. The details of interaction pathways between different whey proteins during heating are virtually unknown. The thermal denaturation processes that take place in milk and whey on exposure to heat are very complex because of the many protein reactions occurring simultaneously. One possible way to have a better understanding of these processes is to use a simple model system. This will help predict the denaturation behaviour of the whey proteins in milk and whey.

In the present study, an investigation into the denaturation and aggregation behaviour of β-lactoglobulin and α-lactalbumin at protein concentrations and ionic environment similar to that which exists in milk has been carried out. The interactions between β-lactoglobulin, α-lactalbumin and casein micelles have also been investigated.

CHAPTER 2
LITERATURE REVIEW

2.1 Milk proteins

Normal bovine milk contains about 3.5% protein. The role of milk proteins is to supply the calf with nitrogenous compounds, especially the essential amino acids which the calf requires for development of muscular and other protein-containing tissues. Milk protein can be fractionated into two well defined groups, namely the casein and whey proteins. Both the casein and whey protein groups are very heterogenous.

2.1.1 Casein proteins

Casein may be defined as a group of proteins precipitated from milk by acidification to pH 4.6 at 20°C. Casein can be fractionated into four distinct groups or subunits, namely α_{s1}-, α_{s2}-, β- and *k*-. There are also several derived caseins which result from the action of indigenous milk proteinases, the prominent reaction being the action of plasmin on the casein micelles. These are usually referred to as γ-caseins. The composition and some properties of casein proteins are shown in Table 2.1.

Each of the caseins contain variable numbers of serine phosphate, proline and cysteine groups that strongly affect their chemical and functional properties. The high concentration and uniform distribution of proline groups impart a random coil secondary structure to casein that renders them highly reactive and subject to interaction via hydrophobic and ionic bonding. Thus, although their monomer molecular weights range from about 19,000 to 25,000, they occur in milk as large complex structures, termed casein micelles, that range in molecular weight up to 2-18 x 10^8 daltons (Da).

Casein micelle diameter ranges from 15-600 nm with an average diameter of 150 nm (Rollema, 1992). k-Casein is predominantly found at the surface of the micelle. At this position, its hydrophillic portion protrudes into the surrounding solution and provide steric stability to the micelle (Rollema, 1992). Hydrophobic and hydrogen bonds also contribute to the micelle structure and stability.

Table 2.1: Properties of casein proteins

Casein	Concentration in milk (g/kg)	Molecular weight (Da)	Cysteine (mol/mol)
α_{s1}-	10.0	23614	0
α_{s2}-	2.6	25230	2
β-	9.3	23983	0
k-	3.3	19023	2

From Walstra and Jenness (1984)

2.1.2 Whey proteins

Whey proteins, also known as serum or non-casein proteins, are the milk proteins that remain soluble after destabilisation of casein by acidification to pH 4.6, or through the action of the enzyme chymosin. Whey proteins, which constitute about 20% of the total bovine milk proteins, include β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, minor proteins such as proteose-peptone, transferrin, lactoferrin, β2-microglobulin, and enzymes such as lysozyme, lipase, xanthine oxidase, plasmin, acid and alkaline phosphotases. The structure and properties of various whey proteins have been reviewed by Swaisgood (1982), Eigel *et al.* (1984) and Mulvihill and Donovan (1987). Table 2.2. summaries the general characteristics of whey proteins.

2.1.2.1 β-Lactoglobulin

β-Lactoglobulin (β-lg) is the principal protein in whey, and comprises about 50% of the total serum protein in bovine milk. The molecule binds retinol strongly, and its proposed biological function is to transfer vitamin A from maternal milk to the neonate via specific receptors in the intestine (Papinez *et al.*, 1986). The molecular weight of β-lactoglobulin is 18,300 Da, and it exists as a stable dimer between pH 5.5 and 7.5. β-lactoglobulin exhibits well developed secondary, tertiary and quaternary structures. Of the secondary structure, approximately 15% is α-helix, 51% β-sheet and 47% is

unordered structure (Jelen and Rattray, 1995). Much of the tertiary structure, which may be roughly spherical, (Green *et al.*, 1979) is stabilized by the presence of thiol groups. Depending on pH and temperature, β-lactoglobulin may exist as a monomer, dimer or octamer (Mackenzie and Sawyer, 1967). The quartenary structure is maintained largely by electrostatic forces.

Table 2.2: General characteristics of whey proteins.

Whey protein	Concentration in milk (g/kg)	Molecular weight (Da)	Isoelectric point	Disulphide bonds
β-Lactoglobulin	3.3	18,363	5.13	2
α-Lactalbumin	1.2	14,147	4.2-4.5	4
Bovine serum albumin	0.4	66,267	4.7-4.9	17
Immunoglobulins	0.7	$(1,5\text{-}10)\times10^3$	5.5-8.3	21
Proteose peptone and minor whey proteins	0.8	$(4,1\text{-}40.8)\times10^3$ of proteose peptones	-	0

Adapted from Mulhill and Donovan (1987) and Walstra and Jenness (1984)

Known genetic variants of bovine β-lactoglobulin are A, B, C, E, G, Dr, D (D and D_{yak}) and F (Bell *et al.*, 1981). Of these variants, only variants A and B occur commonly in the cow's milk. The difference between all these variants is the substitution of amino acids at different positions of the molecule (Bell *et al.*, 1981).

2.1.2.2 α-Lactalbumin (α-la)

This is the second most important whey protein, consisting of about 20% of serum protein. In milk, α-lactalbumin participates in lactose synthesis where it acts as a co-

factor for the enzyme, lactose synthetase. α-Lactalbumin is a small, compact, globular protein with a molecular weight of 14,200 Da. It consists of 123 amino acid residues and has a stable conformation between pH 5.4 and pH 9.0, which is stabilized by four intramolecular disulphide bonds (Eigel *et al.*, 1984; McKenzie and White, 1991; Brew and Grobler, 1992). Three genetic variants (A, B and C) are known to exist.

At physiological pH, the secondary structure of α-lactalbumin consists of 26% α-helix, 14% β-sheet and 60% unordered structure (Creamer *et al.*, 1983). Being a calcium metalloprotein, binding one mole calcium per mole of protein (Hiraoka *et al.*, 1980), it requires calcium for maintenance of the native conformation. The importance of this is emphasized by the fact that α-lactalbumin denatures at acidic pH (3.5) due to dissociation of calcium (Desmet *et al.*, 1987).

2.1.2.3 Bovine serum albumin (BSA)

Bovine serum albumin represents about 10% of the serum protein in bovine milk. BSA has a molecular weight of 66,300 Da and contains 17 intramolecular disulphide bridges and one free thiol group (Reed *et al.*, 1980). The molecule contains no long-distance disulphide bonds and is, thus, relatively flexible. The secondary structure of this compact globular protein consist of 55% α-helix, 16% β-sheet and 25% unordered structure (Reed *et al.*, 1975).

2.1.2.4 Immunoglobulins

This is a complex mixture of large glycoproteins with molecular weights of 150,000 to 900,000 Da. The immunoglobulins exhibit antibody activity, and their function is to confer passive immunity on the neonate during the first few days of life. These immunoglobulins are not unique to whey, but are part of the immune system of bovine serum. There are five known classes of immunoglobilins, IgA, IgD, IgE, IgM and IgG (IgG1 and IgG2). IgG is the principal type found in bovine milk and comprises about 80% of the total content of immunoglobulins (Eigel *et al.*, 1984). All classes of immunoglobulin exist as either monomers or polymers of a basic subunit, the subunit being composed of four polypeptide chains linked covalently by disulphide bonds. These proteins are easily denatured by heat (Lyster, 1972). Although immunoglobulins

make up about 10% of the total whey protein in milk, their contribution to the functional properties of milk and dairy products has been largely ignored.

2.1.2.5 Proteose peptones

Milk and whey contain a number of polypeptides known as proteose peptones. These are a heterogenous group of phosphopeptides, many of which are the result of proteolysis by plasmin of the casein, especially β-casein. Like the casein, the proteose peptones do not exhibit well developed secondary, and hence, tertiary structure. The amount of, and composition of the proteose-protein fraction present may have some significant effect on the functionability of whey protein concentrate and isolates, as they are not denatured by heat and can bind calcium (Kinsella and Whitehead, 1989).

2.2 Thermal denaturation and aggregation of whey proteins

The native three dimensional structure of proteins is maintained by a variety of non-covalent interactions between amino acid residues within the polypeptide chain and between residues and solvent molecules. These interactions include hydrogen bonding, electrostatic, van der Waals' and hydrophobic interactions. Any change in the environment of the protein molecule, which can influence these non-covalent interactions, results in an alteration of the secondary and tertiary structures. It is this change in secondary and tertiary structure that is termed denaturation. Denaturation of the protein structure can be induced by heating, freezing, pressure, extremes of pH, chaotrophic agents, urea, guanidium chloride, sodium dodecyl sulphate and organic solvents such as ethanol and mercaptoethanol (Paulsson, 1990). Of the environmental conditions that can lead to changes in protein structure, temperature represents the most important factor. The strength of hydrogen bonds decreases progressively with increasing temperature, while the strength of the hydrophobic bonds increases with increasing temperature up to 60°C but then decreases at higher temperatures, and are probably non-existent above 100°C. Disulphide bonds can undergo sulphydryl-disulphide interchange to become oxidized on heating. Not surprisingly, then, protein structure undergoes temperature dependent changes over a very broad temperature range. The changes that occur at low temperatures (0 to 60°C) are usually reversible, but exposure to high temperatures, (above 60°C) frequently causes irreversible denaturation,

although some proteins renature readily when the temperature is reduced. If the heat treatment is excessive, covalent bonds may rupture, leading to thermal degradation of the protein molecule.

Following denaturation, proteins often interact either with themselves or other molecules to form aggregates, precipitates or gels which are virtually irreversible. Sulphydryl groups are essential to initiate aggregation.

Whey protein denaturation can produce a number of undesired effects, such as deposits. However, the same denaturation can be used to obtain desired modifications and improvements in products (for example, texture of yoghurt) or processes (for example increase in yield during production of fresh cheese or increased running time of heating plants by reduction of deposit formation). Furthermore heat-induced protein interactions are of great importance in the processing of dairy products; for example, milk must be forewarmed to form the β-lactoglobulin-*k*-casein complex in order to provide adequate heat stability to evaporated milk. Heat induced milk protein complexes adversely affect the dispersibility and solubility of dried milk products. Whey protein-casein micelle complexes in highly heated milk exhibit poor cheese curd formation properties. Heat induced whey protein-casein micelle complexes provide improved viscosity and gel properties of yoghurt. It is also important to use processing conditions that will minimise protein denaturation and interaction in the manufacture of soluble and functional whey protein concentrate and isolate (Morr, 1979). On the other hand, processing conditions must be carefully selected to optimise whey protein-whey protein interactions, if the objective is to promote gelation of whey protein concentrates (WPC) or whey protein isolate (WPI). From these reasons it is, therefore, necessary to study the denaturation of whey proteins.

2.2.1 Methods used to evaluate the thermal denaturation and aggregation of whey protein

Although a range of methods are available to measure protein denaturation and aggregation, each relies on measuring an aspect of the physico-chemical changes that the protein undergoes. The methods available are gel electrophoresis, high performance

liquid chromatography, differential scanning calorimetry, solubility, immunology and spectral properties.

2.2.1.1 Solubility

Thermal unfolding of globular proteins tends to enhance intermolecular interactions. This effect frequently leads to a loss of protein solubility which can be measured to assess the extent of whey protein denaturation (Larson and Rolleri, 1955; Harwalker, 1979, 1980a; Harwalker and Kalab, 1985a,b; Patocka *et al.*, 1986). Generally, maximum sensitivity is obtained when protein solubility is measured at pH values close to the isoelectric point, as electrostatic repulsive forces between proteins are minimal. At pH values removed from the isoelectric point, protein denaturation does not always lead to a loss in protein solubility. Harwalkar (1979) found that whey proteins remained highly soluble following heating at 90°C for 30 minutes at pH 2.5, but readily precipitated at pH 4.6. These same observations were confirmed by Patocka *et al.* (1986). Similarly, de Wit (1981) reported that solutions of β-lactoglobulin remained almost crystal clear after heat treatment (80°C for 10 min) at pH ≥ 6.8, but readily precipitated at pH 6.5. A number of studies have been carried out in which protein solutions were heated at a specific pH (e.g. pH 6.7) and then the denatured proteins were precipitated at pH 4.6. The precipitated coagulum was centrifuged, and the supernatant obtained was assumed to contain the native proteins. Therefore, the total native protein can be quantified by Kjeldahl or individual proteins by polyacrylamide gel electrophoresis or high performance liquid chromatography.

2.2.1.2 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is used for qualitative (e.g. phenotyping) and quantitative protein analysis. Separation is based on protein mobility across a voltage gradient and the molecular sieving action of the porous gel. Some of the methods that have been used to quantify the different bands of protein include fluorometric scanning and photometric scanning of stained gels, unstained gels, or photographic negatives (Hillier, 1976). Currently photometric scanning using a densitometer is the most common method used to quantify individual protein bands on a gel. Densitometers capable of integrating the absorbance of a band rather than measuring a single line

through the band are more accurate. Lateral spreading and distortion of the band produces errors in single line measurements. Comparison between gels should only be made with a standard run on both gels, as significant differences can result from staining/destaining methods, gel composition/manufacture and electrophoretic running conditions (Hillier, 1976; Andrews, 1988).

2.2.1.3 High performance liquid chromatography (HPLC)

The principle behind HPLC analysis is that the sample is injected into continuously flowing solvent and that, after separation, the analyses are determined in the eluent by either their physical or chemical properties. The chromatographic process depends on an interaction between the protein molecules and the packing particles, which result in retardation of the proteins compared to the eluent (Leppard, 1984). As the solvent flows through the column, the different proteins then separate with those held most strongly by the stationary phase moving slowest (Matissek, 1993). A mixture of denatured proteins will, therefore, separate according to size and reactive groups on them. The effluent from the column is passed into a detector, the electrical output of which is passed to a recorder for production of the chromatogram. The peaks on the chromatogram are caused by the separated proteins of the sample, and either the peak heights or peak areas are generally indicative of the amounts of each protein (Leppard, 1984). The time between injection of the sample and appearance of the maximum for the peak is referred to as the retention time for that peak.

2.2.1.4 Differential scanning calorimetry (DSC)

The thermal unfolding of globular proteins requires the uptake of thermal energy, needed primarily to break intramolecular hydrogen bonds (Privalov and Khechinashvili, 1974). This endothermic heat effect can be monitored by a technique called differential scanning calorimetry (DSC). In DSC, a sample of native protein and a reference sample are simultaneously subjected to the same heating regime. In this case, the reference may consist of solvent minus the protein, or an identical protein solution previously heat denatured under the same conditions. An example of a typical DSC thermogram showing an endothermic peak is presented in Figure 2.1.

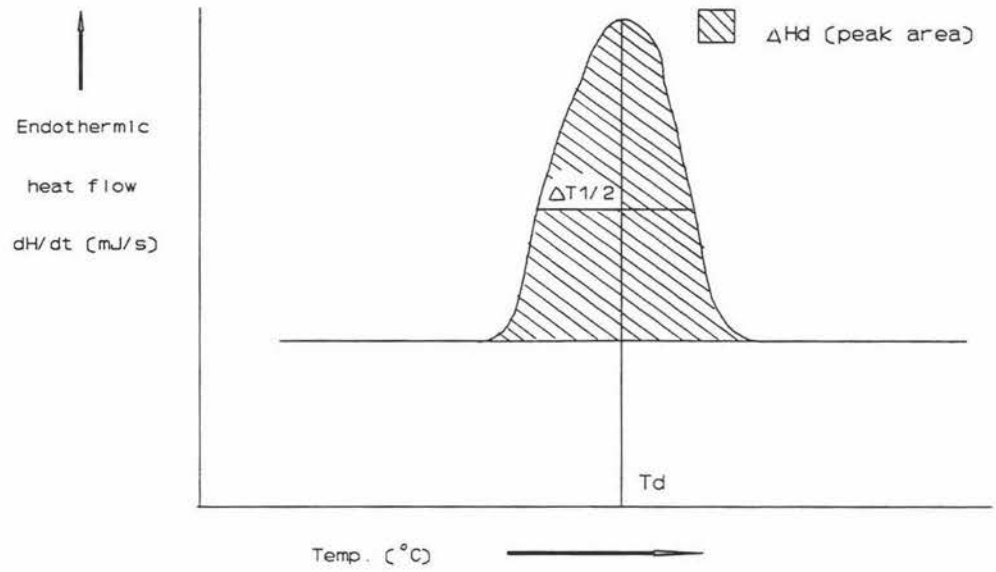


Figure 2.1 A typical DSC thermogram for the endothermic unfolding of a globular protein.

The temperature of protein denaturation (Td) is a measure of the heat stability while the enthalpy of denaturation (ΔHd) is related to the extent of secondary structure of the protein, particularly the α-helix content. The sharpness of the endothermic peaks can be related to ΔT1/2 (peak width at 1/2 peak height), which is an index of the cooperative nature of the transition from native to denatured state. It is important to note that, in DSC, characteristics may change as a function of the heating rate applied, for example Td; if a protein is subjected to very fast heating, then the Td value will be

higher.

The advantages of DSC in measuring protein denaturation are that the technique allows denaturation to be monitored directly, continuously and sensitively. Thermal characteristics can be obtained relatively quickly, and, as only small samples (several mg) are required, it is possible to assay large number of samples under a variety of conditions. A disadvantage of DSC is that it requires use of relatively high concentrations; thus, the method is not suitable for studies of denaturation behaviour in typical dairy systems such as skim milk or whey.

2.2.1.5 Immunology

Whey proteins are antigenic molecules and, therefore, small structural changes can affect their ability to bind to antibodies. Protein denaturation leads to a decrease in antigenic activity which is the underlying principle of several immunoassay techniques. One method is micro complement fixation which involves measuring the degree of complementarily between a partly denatured protein and its specific antibody (Levine and van Vunakis, 1967). The amount of complement (antibody) fixed at different concentrations of protein (antigen) is determined. By comparing the percent complement fixation between native and denatured proteins, the degree of protein denaturation can be inferred i.e. greater quantities of heated proteins are needed to achieve the same percent complement fixation compared to native protein. This technique is reported to be very sensitive and applicable to both simple model systems and complex media (Baer *et al.*, 1976, 1979). The technique can also be used to measure the ability of proteins to renature. This is done by performing the complement assay on solutions of heated whey protein allowed to cool for different periods of time.

2.2.1.6 Spectral properties

Whey proteins are optically active molecules, as they posses a large number of asymmetric carbon atoms. Structural changes alter their optical activity, and such changes can be monitored by optical rotary dispersion (ORD) and circular dichroism (CD). The thermal unfolding of β-lactoglobulin leads to increased exposure of asymmetric centres to solvent, which increases the laevorotation of plane polarized light

(Harwalker, 1980a,b,; Harwalker and Kalab, 1985a,b). The difference between the two techniques is that, generally, ORD measures changes in the angle of rotation of plane polarized light, while CD is concerned with the ability of the molecules to absorb circularly polarized light.

2.2.1.7 Other methods

There are other methods that can be used to assay for denaturation and aggregation of proteins. Biorecognition has been used as an indicator of the native structure of β-lactoglobulin (Jang and Swaisgood, 1990). This method works on the principle that denatured proteins have lower binding affinities for the given biological compound. Another method, gel filtration chromatography, separates the protein molecules according to their sizes.

2.2.2 Thermal denaturation of whey proteins in various media

The reaction rates and denaturation mechanism of whey proteins vary with environmental conditions; for example, the reaction orders and denaturation mechanism of whey protein dissolved in distilled water and phosphate buffer systems may be significantly different from that in milk. In this connection, whey proteins in heated acid whey did not denature to the same extent as whey proteins in skim milk (Parris *et al.* 1991). At 85°C, approximately 95% of the whey proteins in skim milk were denatured compared to about 70% in acid whey. It is because of these differences that the denaturation of the whey proteins will be discussed separately in three sections: denaturation in skim milk, in whey and in buffered solutions of isolated proteins.

2.2.2.1 Denaturation of whey proteins in skim milk

Denaturation behaviour of individual whey proteins

Larson and Rolleri (1955) assessed the denaturation of the individual whey proteins in heated skim milk using quantitative, free-boundary electrophoresis. The heat resistance of the individual proteins was in the order: immunoglobulins < BSA < β-lactoglobulin < α-lactalbumin. The 29% denaturation of total whey protein observed was due to the specific denaturation of 89% Ig, 52% BSA, 32% β-lactoglobulin and 6% α-lactalbumin. These researchers also noted that β-lactoglobulin, being the major whey protein, largely

determines the course of total whey protein i.e. the curve for total protein denaturation parallels that for β-lactoglobulin denaturation. This order of resistance, observed by Larson and Rolleri (1955), was later confirmed by Donovan and Mulvihill (1987), Singh and Creamer (1991b), Dannernberg and Kessler (1988) and Oldfield (1996).

Lyster (1970) measured the denaturation of α-lactalbumin and β-lactoglobulin in skim milk using the immuno-diffusion method. Denaturation of α-lactalbumin was found to follow first-order reaction kinetics, while β-lactoglobulin followed second-order kinetics. Hillier and Lyster (1979) also found similar results using quantitative polyacrylamide gel electrophoresis. They however pointed out that the denaturation of α-lactalbumin may possible be a second-order reaction displaying first-order kinetics. The denaturation of BSA was more complex and could equally well be described as first or second-order kinetics. It has been suggested that the complexity may result from the microheterogeneity of the protein as well as from the formation of heat stable intermediates (Ruegg *et al.*, 1977). In skim milk, the denaturation of immunoglobulins follow second order kinetics (Resmini *et al.*, 1989). Oldfield (1996) found the denaturation of immunoglobulins followed a reaction kinetics order of 2.2.

While Hillier and Lyster (1979) found the that both β-lactoglobulin A and B follow second-order kinetics with only their relative heat stability varying, some workers have found results that conflict with those discussed above. Oldfield (1996) found the reaction kinetics of the denaturation of β-lactoglobulin A to follow the order 1.1 and that of β-lactoglobulin B to be 1.6. His results, at least for β-lactoglobulin A, are in close agreement with those of Gough and Jenness (1962). Dannenberg and Kessler (1988) found the kinetics of the denaturation of β-lactoglobulin to follow the order 1.5 when assayed using gel electrophoresis. The B-variant of β-lactoglobulin was found to be less heat stable than the A-variant at all temperatures. They also found the kinetics of the denaturation of α-lactalbumin to be first order. These conflicting results may be a result of the different experimental conditions that were used e.g. protein concentrations and pH.

Dannenberg and Kessler (1988) found that the activation energies at lower temperatures,

(i.e. temperatures below 90°C, were typical for a denaturation reaction. In skim milk, they obtained activation energies of 265, 280 and 269 kJ mol^{-1} for β-lactoglobulin A, β-lactoglobulin B, and α-lactalbumin respectively. The corresponding entropies of activation ($\Delta S^{\#}$) range from 0.45 to 0.5 kJ $mol^{-1}K^{-1}$, indicating that denaturation caused an increase in the degree of freedom of translation and rotation. At temperatures above 90°C, the activation energies are 54, 48 and 69 kJ mol^{-1} for β-lactoglobulin A, β-lactoglobulin B, and α-lactalbumin respectively. All these are in the range of chemical reactions. Apparently, in this denaturation reaction the transitional states are more stable than the reactants, possibly through an aggregation mechanism (Dannenberg and Kessler, 1988). Activation energies for β-lactoglobulin obtained by other authors in the lower temperature ranges were 259 to 521 kJ mol^{-1} for temperatures below 90°C and 32 - 46 kJ mol^{-1} for temperatures above 80°C (Oldfield, 1996). α-Lactalbumin is generally more heat resistant than β-lactoglobulin, and this is shown in the higher activation energy of α-lactalbumin (69 to 72 kJ mol^{-1}) compared to β-lactoglobulin (32 to 46 kJ mol^{-1}) at temperatures above 85°C (Oldfield, 1996). In skim milk, an activation energy of 345 kJ mol^{-1} has been reported for immunoglobulins denaturation (Resmini *et al.*, 1989). The activation energy of BSA has not been reported.

Heat-induced interactions of whey proteins

Thermal denaturation causes the whey proteins to unfold, exposing the disulphide and thiol groups. The thiol groups are then free to interact, resulting in whey protein aggregation. Dagleish (1990) established that there was a direct relationship between denaturation and the formation of aggregates in skim milk. β-Lactoglobulin, a crucial protein in the aggregation process, contains four disulphide bonds and one thiol group. The disulphide linkages (S-S) formed by the thiol oxidation group (SH), or thiol-disulphide interchange, are important in the aggregation process.

The most significant effect of heating in fluid milk is related to the complexation of some of the heat denatured whey proteins with the casein micelles. This complexation is mainly through interactions between β-lactoglobulin and *k*-casein. Numerous studies have reported on these interactions in milk (Sawyer, 1969; Snoeren and van der Spek, 1977; Vissier *et al.*, 1986; Noh *et al.*, 1989a; Reddy and Kinsella, 1990). The complex

is formed via sulphydryl-disulphide interchange and hydrophobic interactions (Purkayastha *et al.*, 1967; Dagleish, 1990; Jang and Swaisgood, 1990). The extent of interaction is dependent upon several factors, such as heating conditions, protein concentration, and salt concentration, particularly calcium. Complex formation also occurs between β-lactoglobulin and α-lactalbumin and between β-lactoglobulin and BSA (Snoeren and van der Spek, 1977).

2.2.2.2 Denaturation of whey proteins in whey

Denaturation behaviour of individual whey proteins

Heating sweet whey resulted in the denaturation of all whey proteins (Guy *et al.*, 1967; Parris *et al.*, 1993); the loss of proteins increased with pH in the range 3.4 to 7.4, and also increased with temperature between of 60 and 90°C.

Hillier and Lyster (1979) analyzed protein denaturation in cheese whey. They found that β-lactoglobulin followed second-order reaction kinetics, and that α-lactalbumin followed first-order kinetics. They also found that the heat denaturation kinetics of bovine serum albumin were complex and could be described equally well by first and second order equations. Furthermore Hillier *et al.* (1979) found that α-lactalbumin was more susceptible to denaturation in cheese whey having a higher total solids content, especially at higher temperatures. This was in agreement with the results reported by Nielsen *et al.* (1973) for Colby cheese whey at pH 5.1. The reaction mechanism appears to change at about 90°C for all total solids concentrations studied, indicating that the point of change is apparently independent of concentration. In contrast to α-lactalbumin, β-lactoglobulin denaturation is retarded by increased total solids. In cheese whey at temperatures above 90°C, β-lactoglobulin A is more stable than β-lactoglobulin B (Hillier and Lyster, 1979). The B variant of β-lactoglobulin favoured formation of soluble protein aggregates, while the A variant formed more insoluble aggregates (Parris *et al.*, 1993).

The absence of casein in whey has some effect on the way in which the whey proteins are denatured. Elfagm and Wheelock (1977) reported that loss of α-lactalbumin was less in whey than in milk, while the loss of β-lactoglobulin was greater in whey below

70°C but greater in milk above 74°C.

Heat-induced interactions of whey proteins

In the absence of the casein fraction in whey, the thermodenaturation phenomena of the whey proteins is different to that found in milk. Because there are no caseins in whey systems, interaction can only occur between the whey proteins themselves. Aggregation of whey proteins in whey has been reported by Kenkare *et al.* (1964), Josephson and Morr (1968), Sabarwal and Ganguli (1972) and Parris *et al.* (1993). When sweet whey was heated at 75 and 85°C for 30 min, the size exclusion profiles showed a soluble aggregate peak, that was later revealed to contain all whey proteins (Parris *et al.*, 1993). Addition of micellar casein as well as several casein fractions, α-, α_s-, *k* and β-caseins to whey have been reported to stabilise the serum proteins against mass self aggregation (Kenkare *et al.*, 1964; Sabarwal and Ganguli, 1972).

2.2.2.3 Denaturation of whey proteins in buffered solutions

Denaturation behaviour of individual whey proteins

The thermal denaturation processes, that occur in skim milk and whey on exposure to heat are very complex because of the many proteins and substances present in milk, consequently researchers have used solutions of isolated pure proteins in distilled water, buffers and simulated milk ultrafiltrate (SMUF) in order to gain a better understanding of these reactions.

De Wit and Klarenbeek (1984), using DSC on individual proteins (8-10% concentration) in 0.7 M phosphate buffer (pH 6.0), showed that the order of heat resistance of whey proteins was: β-lactoglobulin > immunoglobulins > BSA > α-lactalbumin. This is different from the order: α-lactalbumin > β-lactoglobulin > BSA > immunoglobulins found in milk by some workers (Larson and Rolleri, 1955; Donovan and Mulvihill, 1987; Singh and Creamer, 1991b and Oldfield, 1996). Furthermore Paulsson *et al.* (1983) found that the order of denaturation varied with pH. Using DSC they found that, except at pH > 9.0, β-lactoglobulin was the most thermostable whey protein at all pH values: at acidic pH-values BSA was the least thermostable while at alkaline pH-values α-lactalbumin had lower thermal stability than BSA.

Denaturation of β-lactoglobulin when heated either separately or with other proteins has been extensively researched (Harwalkar, 1980a,b; de Wit and Swinkels, 1980; de Wit and Klarenbeek, 1981; Park and Lund, 1984; Paulsson and Dejmek, 1990; Relkin and Launay, 1990; Hines and Foegeding, 1993; McSwiney *et al.* 1994; Roefs and de Kruif, 1994; Gezimati, 1995). When heated alone, β-lactoglobulin self aggregates via both non-covalent and covalent bonding (Hines and Foegeding, 1993; McSwiney *et al.* 1994; Gezimati, 1995). The presence of casein micelles and casein fractions, increase the rate of β-lactoglobulin denaturation (Elfagm and Wheelock, 1978a; Park and Lund, 1984; Paulsson and Dejmek, 1990). However, α-lactalbumin does not have any effect on the loss of β-lactoglobulin (Hines and Foegeding, 1993; Elfagm and Wheelock, 1978a,b) while BSA increased denaturation of β-lactoglobulin (Hines and Foegeding, 1993; Gezimati, 1995).

There is no agreement on which of the two main genetic variants of β-lactoglobulin is the more heat stable. This because the protein concentration and media may be important in determining the relative stabilities of the variants. Sawyer (1968) and McKenzie *et al.* (1971) reported that purified β-lactoglobulin B was less stable that purified β-lactoglobulin A. However Imafidon *et al.* (1991) observed that 10% solutions of β-lactoglobulin A in SMUF were more heat sensitive that β-lactoglobulin B. This finding was supported by McSwiney *et al.* (1984) who used a 10% solution of β-lactoglobulin dispersed in 20mM imidazole buffer (0.1 M NaCl, pH 7.0) and reported that β-lactoglobulin B was more stable than β-lactoglobulin A when heated at 75-85°C. The rate of denaturation of β-lactoglobulin (combined A and B variants) increases as the concentration in solution increases (Relkin and Launay, 1990). Nielsen *et al.* (1996) showed that when β-lactoglobulin variants were heated at 75°C in an immidazole-HCl buffer at concentrations below 5% (w/v), β-lactoglobulin B aggregated faster than β-lactoglobulin A, but at concentrations above 5% (w/v) β-lactoglobulin A was more sensitive to aggregation. The pH and media composition also affect the denaturation of the variants (Imafidon *et al.*, 1991).

Sawyer (1968) reported that genetic variant C (β-lactoglobulin C) is the most heat labile of the three variants at 75 and 97.5°C. β-Lactoglobulin C forms an aggregated product

with less thiol/disulphide interactions than the A and B variants (Sawyer, 1968). Contrary to the results of Sawyer (1968), Phillips *et al.* (1967) found that the reactivity of β-lactoglobulin A and β-lactoglobulin B in a potassium phosphate buffer are the same, while β-lactoglobulin C is less reactive.

De Wit and Swinkles (1980) and Park and Lund (1984) used DSC to study the kinetics of β-lactoglobulin denaturation and reported conflicting results. De Wit and Swinkles (1980) found that the denaturation of β-lactoglobulin in phosphate buffer (pH 6.75) could be described as a first-order process between 65 and 70°C, but above 70°C denaturation deviated from first-order kinetics. However, Park and Lund (1984) found that the apparent reaction order of β-lactoglobulin denaturation was pH dependent. Between pH 6.0 and pH 9.0 denaturation could be described by a second-order process, and below pH 5.0 by a third-order process. This suggests that the denaturation mechanisms probably change near pH 6.0. These authors commented that although there appears to be wide discrepancies, a reaction order of 2.0 could be used to model adequately all data in the literature. The discrepancies in the above results may be explained by the fact that the researchers used different methods to obtain kinetic measurements of β-lactoglobulin denaturation from DSC curves. De Wit and Swinkels (1980) used similar buffers for the preparation of all β-lactoglobulin solutions and reference solutions for DSC measurements, while Park and Lund (1984) used different buffers to dissolve β-lactoglobulin at different pH values and used water as the reference solution in DSC. In addition to the above data, McSwiney *et al.* (1994) reported that the denaturation of 10% β-lactoglobulin dispersed in imidazole buffer followed second order reaction kinetics. Roefs and de Kruif (1994) showed that the loss of β-lactoglobulin followed the reaction rate order of 1.5.

The heat denaturation of α-lactalbumin in NaCl and KCl solutions and milk ultrafiltrate was studied using the method of micro complement fixation. It was established that this protein was very resistant to heat denaturation (Baer *et al.*, 1976; Calvo *et al.*, 1993). Baer *et al.* (1976) also reported that α-lactalbumin was more stable in milk ultrafiltrate than in the other media studied at temperatures up to 70°C. These findings are supported by Larson and Rolleri, (1955), Lyster, (1970) and de Wit, (1981), who showed

that α-lactalbumin is the most heat stable of the whey proteins. This is mainly because of its ability to revert to its native state following heat treatment (Baer *et al.*, 1976). Ruegg *et al.* (1977) observed renaturation levels of 90% in systems containing only α-lactalbumin. Research carried out using DSC showed that α-lactalbumin has the lowest denaturation temperature, but that it requires a large amount of heat for unfolding (de Wit and Klarenbeek, 1984).

Baer *et al.* (1976) studied the reaction kinetics of the denaturation of α-lactalbumin dispersed in milk ultrafiltrate. They described loss of α-lactalbumin when heated alone as pseudo first-order, and that of α-lactalbumin heated in the presence of β-lactoglobulin as first order. There is some loss of BSA due to denaturation when 0.79% (w/v) BSA in 50 mM TES buffer, pH 7.0, with 100 mM NaCl was heated at 80°C (Hines and Foegeding, 1993). However, the reaction kinetics of BSA do not seem to have been studied.

Heat-induced interactions of whey proteins

The labile whey proteins unfold on heating and are able to interact with each other, mainly through thiol-disulphide interchange or disulphide linkages, to produce aggregates. Heat-induced interactions among β-lactoglobulin, α-lactalbumin and BSA, studied in model systems, are discussed below.

Self aggregation of whey proteins

When β-lactoglobulin is heated above a critical temperature, it undergoes conformational changes followed by protein-protein interaction. Aggregation of β-lactoglobulin, when heated alone in a buffer, has been reported by several workers (Sawyer, 1968; Park and Lund, 1984; Hines and Foegeding, 1993; McSwiney *et al.*, 1994; Gezimati, 1995). Both hydrophobic interactions and intermolecular disulphide bonds are involved in this aggregation. BSA is also reported to form aggregates when heated on its own in a buffer (Oh and Richardson, 1991; Hines and Foegeding, 1993). However no aggregation of α-lactalbumin was detected by Calvo *et al.* (1993) after heating at 90°C for 24 min. In contrast Hines and Foegeding (1993) reported the self aggregation of α-lactalbumin, though its aggregation rate is very slow in comparison to the self aggregation of β-

lactoglobulin and BSA.

Interactions between β-lactoglobulin and α-lactalbumin

Besides self aggregation, β-lactoglobulin can form a heat-induced complex with α-lactalbumin (Melo and Hensen, 1978; Matsudomi, 1992; Calvo *et al.*, 1993). A soluble complex is formed mainly through thiol-disulphide interchange between β-lactoglobulin and α-lactalbumin (Matsudomi, 1992; Calvo *et al.*, 1993). Denaturation of α-lactalbumin is affected by the presence of β-lactoglobulin. The faster loss of α-lactalbumin when heated in the presence of β-lactoglobulin, compared to when it is heated alone, has been demonstrated using size exclusion chromatography (Elfagm and Wheelock, 1978a; Hines and Foegeding, 1993; Calvo *et al.*, 1993), DSC (Paulsson and Dejmek, 1990) and gel filtration and HPLC (Matsudomi *et al.*, 1992). In contrast loss of β-lactoglobulin was not affected by α-lactalbumin (Hines and Foegeding, 1993). Elfagm and Wheelock, (1978a) reported similar results at concentrations of 0.2% (w/v) β-lactoglobulin and 0.1% (w/v) α-lactalbumin. However, when there was a higher ratio of β-lactoglobulin to α-lactalbumin (3:1), α-lactalbumin had a marked effect in reducing the amount of aggregated β-lactoglobulin.

Interactions between β-lactoglobulin and BSA

Hines and Foegeding (1993), using a 1% (w/v) solution containing equimolar amounts of β-lactoglobulin and BSA heated at 80°C, showed that although the rate of β-lactoglobulin aggregation was enhanced by the presence of BSA, BSA aggregation appeared unaffected by β-lactoglobulin. The formation of soluble β-lactoglobulin/BSA aggregates occurs through disulphide crosslinks (Matsudomi *et al.*, 1994).

Interactions between α-lactalbumin and BSA

Baer *et al.* (1976) showed that BSA had no effect on α-lactalbumin (1 mg ml^{-1}) heated at 80°C for 30 min. However Calvo *et al.* (1993) reported a good correlation between BSA concentration and formation of α-lactalbumin/BSA aggregates in milk ultrafiltrate at 90°C (α-lactalbumin 1.5 mg ml^{-1}, BSA 0.3 to 10.0 mg ml^{-1}). Matsudomi *et al.* (1993) showed thiol-disulphide interchange to be involved in aggregate formation between 0.2 % α-lactalbumin and 0.2% BSA heated at 80°C for 30 min.

Interactions involving immunoglobulins

Oh and Richardson (1991) used radiolabelled immunoglobulins G (IgG) to study heat-induced interactions in skim milk at 95°C. Gel filtration chromatography of samples dispersed in 6 M guanidine HCl showed all the radioactivity eluted in the same volume as untreated IgG. Elution profiles of IgG heated alone indicated the formation of large complexes through both covalent interactions and thiol-disulphide interchange.

Interaction of the whey proteins and caseins

- Interaction between the whey proteins and k-casein

Interactions between β-lactoglobulin and *k*-casein in model micelle systems have been reported by Smits and Brouwershaven (1980), Noh *et al.* (1989a), Reddy and Kinsella (1990) and in β-lactoglobulin/*k*-casein mixtures by Zittle *et al.* (1962), Tessier *et al.* (1969), McKenzie *et al.* (1971), Itoh *et al.* (1976), Euber and Brunner (1982), Park and Lund, 1984; Haque *et al.* (1987), Haque and Kinsella, (1988) and Paulsson and Dejmek (1990).

The interaction between β-lactoglobulin and *k*-casein is affected by the proportion of the two proteins in the mixture. Increasing the proportion of β-lactoglobulin causes more of the β-lactoglobulin to interact with *k*-casein and the ratio of β-lactoglobulin to *k*-casein in the complex increases (Long *et al.*, 1963). Tessier *et al.* (1969) reported that when there is a large proportion of β-lactoglobulin in solution, the β-lactoglobulin:*k*-casein ratio in the complexes increase to a maximum of 3:1 on heating at 80°C. On heating at 85°C for 20 min, the maximum interaction ratio of β-lactoglobulin to *k*-casein is 2.2 β-lactoglobulin to 1 *k*-casein (Long *et al.*, 1963). Temperature has also been reported to have an effect on the ratio of β-lactoglobulin to *k*-casein in that complex. Long *et al.* (1963) found that the ratio of β-lactoglobulin to *k*-casein in the complex decreased from 2.2 to 1.4, when the heating temperature was raised from 85 to 99°C in a 1:1 mixture of β-lactoglobulin and *k*-casein, heated for 20 min at pH 6.5. The degree of β-lactoglobulin interaction with *k*-casein is at a maximum of 82% when heated at 85°C, decreasing to 76% at 99°C (Long *et al.*, 1963).

Disulphide bonds have been shown to be involved in the complex formation between

β-lactoglobulin and *k*-casein (Purkayastha *et al.*, 1967; Kresheck *et al.*, 1964; Euber and Brunner, 1982). Complex formation is inhibited by N-ethylmaimide, and disrupted by the reducing agent, 2-mercaptoethanol (Purkayastha *et al.*, 1967). Para-hydroxymercuribenzoate inhibits complex formation (Kresheck *et al.*, 1964), as does alkylated (S-carboxyamidomethyl) *k*-casein (Purkayastha *et al.*, 1967). Euber and Brunner (1982) used β-lactoglobulin immobilised on Sepharose gel, which they then heated at 80°C for 40 min, to demonstrate that a β-lactoglobulin/*k*-casein was linked by disulphide linkages. Aggregation of β-lactoglobulin is not essential for the interaction of *k*-casein (Euber and Brunner, 1982).

Other interactions have been suggested to be involved in the stabilisation of the complex. A 1:1 mixture of β-lactoglobulin and *k*-casein heated at 70°C in 20 mmol/L imidazole buffer (pH 6.8) showed that the initial stages of the complex formation are driven mainly by hydrophobic forces (Sedmerova *et al.*, 1972; Haque and Kinsella, 1988). At low temperatures (~70°C) where -SH group development is very slow, hydrophobic interactions are important in stabilising the complex, but as the temperature increases, hydrophobic interactions are weakened and the sulphydryl-disulphide interactions become more dominant.

No complex between *k*-casein and α-lactalbumin was observed after heating the two proteins at 85°C for 30 min (Hartman and Swanson, 1965). However, Doi *et al.* (1983) demonstrated that an α-lactalbumin/*k*-casein complex was formed in 35 mM phosphate buffer, pH 7.6, containing 0.4 M NaCl when heated at 90°C for 30 min. This complex was not observed under the same heating conditions in 10 mM imidazole-HCl buffer, pH 7.1, containing 0.07 KCl, suggesting that a high pH is necessary for complex formation (Doi *et al.*, 1983). α-Lactalbumin is thought to associate indirectly with *k*-casein, by complexing with β-Lactoglobulin (Baer *et al.*, 1976). Complex formation between BSA and *k*-casein has not been observed (Hartman and Swanson, 1965).

- Interaction between whey proteins and casein micelles

The heat-induced associations of β-lactoglobulin with the casein micelle is primarily

through *k*-casein located on the micelle surface. Both intermolecular disulphide bonds and hydrophobic interactions play a role in this association (Smits and Brouwershaven, 1980). Direct evidence for the interactions between β-lactoglobulin and *k*-casein was shown by Jang and Swaisgood (1990). Casein micelles, separated from skim milk by ultracentrifugation and then redispersed in SMUF, when heated, interacted with β-lactoglobulin immobilised on glass beads. At temperatures below 65°C, disulphide bond formation did not occur. At temperatures below 75°C, the complexes formed primarily through non-covalent interactions, however increasing the temperature from 75°C to 85°C resulted in a greater amount of *k*-casein forming disulphide bonds with β-lactoglobulin. Of the caseins, only *k*-casein was observed to react covalently with β-lactoglobulin (Jang and Swaisgood, 1990).

The association of β-lactoglobulin with casein micelles is dependent upon several factors, such as heating conditions, protein concentration and salt concentration, in particular calcium (Smits and Brouwershaven, 1980).

The presence of β-lactoglobulin is required for α-lactalbumin and casein micelles to interact. This is probably through the formation of a heat-induced β-lactoglobulin/α-lactalbumin complex which then interacts with the micelle (Elfagm and Wheelock, 1978b). α-Lactalbumin appears to have no influence on the complex formation between β-lactoglobulin and *k*-casein (Smits and Brouwershaven, 1980). Noh *et al.* (1989a) showed that although a mixture of α_{s2}-casein and β-lactoglobulin forms complexes readily, α_{s2}-casein is relatively unreactive in milk because of its position within the micelle.

Interaction between whey proteins and other casein fractions

Paulsson and Dejmek (1990) found no aggregation between β-casein and the three whey proteins β-lactoglobulin, α-lactalbumin and BSA. The α-casein fraction significantly lowered the transition temperature of β-lactoglobulin, α-lactalbumin and BSA and decreased the Van't Hoff enthalpy of β-lactoglobulin. Specific interaction between α-casein and β-lactoglobulin has been reported by Kresheck *et al.* (1964). *k*-Casein does not affect the denaturation behaviour of either BSA or α-lactalbumin (Paulsson and

Dejmek, 1990).

OBJECTIVES

* To determine the rates of denaturation and aggregation of β-lactoglobulin and α-lactalbumin during heating at 80 and 95°C.

* To determine the effect of casein micelles on the denaturation and aggregation of β-lactoglobulin and α-lactalbumin.

* To determine the distribution of non-covalently and covalently-linked aggregates in different proteins.

* To determine the sedimentation behaviour of β-lactoglobulin and α-lactalbumin during heating at 80 and 95°C.

* To determine the effect of casein micelles on the sedimentation of β-lactoglobulin and α-lactalbumin.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Fresh unpasteurised milk was obtained from the Massey University Dairy Farm No. 4, Palmerston North, New Zealand.

β-Lactoglobulin (β-Lg) (L-0130, Lots 98F8030 and 91H7005), and α-lactalbumin (α-La) (L-5385, Lot 92H7015) were obtained from Sigma Chemical Company, St Louis, USA.

All chemicals used were of analytical grade obtained from either BDH Chemicals (BDH Ltd, Poole, England) or Sigma Chemical Company, (St Louis, MO, USA) unless specified otherwise.

3.2 Preparation of Simulated Milk Ultrafiltrate (SMUF)

The simulated milk ultrafiltrate was prepared according to Jenness and Koops (1962). Two stock solutions were prepared.

Stock solution *I* was prepared by dissolving the salts listed below in deionised water and then making up to up to 200 ml.

Potassium dihydrogen orthophosphate (KH_2PO_4)	15.80g
(Ajax Chemicals Pty Ltd, Aurburn, NSW, Australia)	
Potassium citrate (K_3 citrate.H_2O)	12.00g
(Koch-Kight Laboratories Ltd England)	
Sodium citrate (Na_3 citrate.$5H_2O$)	21.20g
(Prolabo, Manchester)	
Potassium sulphate K_2SO_4	1.80g
Potassium chloride (KCl)	6.00g
(Scientific supplies Ltd, Auckland)	

Stock solution *II* was prepared by dissolving the salts listed below in a mixture of 150 ml of deionised water and 25 ml concentrated HCl in a beaker held at 100°C in a water

bath. The solution was evaporated to dryness at 100°C and then redissolved in deionised water and made up to 200 ml. This preparation method avoids the uncertainties associated with weighing hygroscopic calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$).

Calcium carbonate ($CaCO_3$)	8.98g
Magnesium carbonate ($MgCO_3$)	2.70g
(Lonover Scientific Supplies Ltd, London, England)	

The SMUF solution was prepared by dissolving 20 ml of each of the stock solutions *I* and *II* in 940 ml of deionised water together with 45g lactose (Ajax Chemicals Pty Ltd, Aurburn, NSW, Australia), 0.30g K_2CO_3, and 0.2g sodium azide (inhibits microbial growth). The pH was adjusted to 6.67 (0.03 below the desired pH of milk 6.7) with 1.5 N KOH (Scientific Supplies Ltd, Auckland) and made up to 1 litre with deionised water. This addition caused the pH to rise to 6.7. The composition of the milk salt solution described above is shown in the Table 3.1.

3.3 Experimental procedure

The overall experimental plan for the preparation of protein solutions, and their subsequent heat treatment and analysis is given in Figure 3.1.

3.3.1 Preparation of protein solutions

Fresh unpasteurised milk at 20°C was centrifuged at 3500 **g** for 20 min in a Sorvall SS-3 automatic centrifuge. This was filtered through glass wool and the skim milk (filtrate) was collected in a beaker. The skim milk at 20°C was ultracentrifuged at 90 000 **g** (Sorvall ultracentrifuge OTD combi) for 90 min in order to separate the casein micelles from the whey. The supernatant, which constituted the whey, was carefully decanted into a measuring cylinder and the volume noted. The pellet, which constituted the casein micelles, was rinsed twice with deionised water to remove traces of whey proteins. The pellet was redispersed (by thoroughly crushing in a pestle and motar) in the same volume of SMUF as the supernatant (whey). This redispersed solution was gently stirred overnight at 5^0C using a magnetic stirrer.

Table 3.1: Composition of SMUF buffer

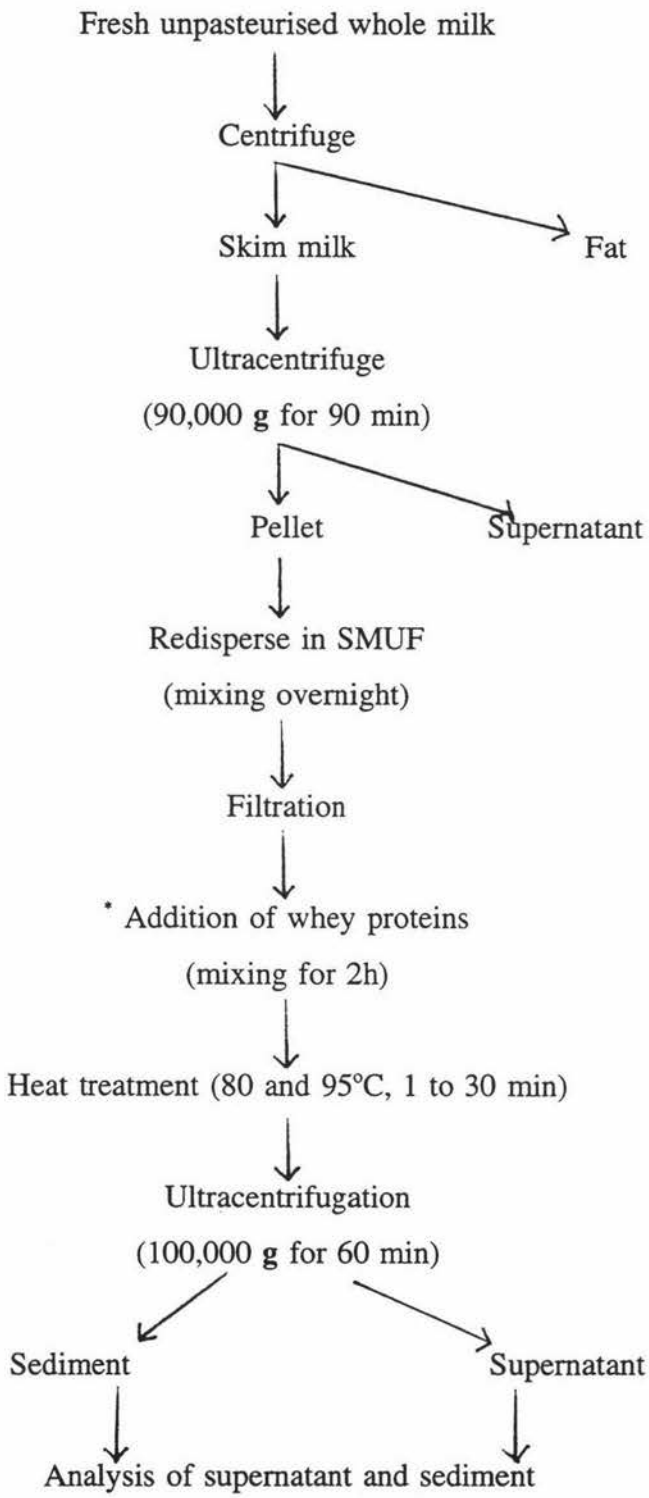
Ingredient	Weight (g/L)	Composition (mg/100 ml)								
		Na	K	Ca	Mg	P	Cl	Ci*	SO_4	CO_2
KH_2PO_4	1.58	-	45.4	-	-	36.0	-	-	-	-
K_3citrate.H_2O	1.20	-	43.4	-	-	-	-	70.0	-	-
Na_3citrate.$5H_2O$	2.12	42.0	-	-	-	-	-	115.0	-	-
K_2SO_4	0.18	-	8.0	-	-	-	-	-	9.9	-
$CaCl_2.2H_2O$	1.32	-	-	35.9	-	-	63.8	-	-	-
$MgCl_2.6H_2O$	0.65	-	-	-	7.8	-	22.7	-	-	-
K_2CO_3	0.30	-	17.0	-	-	-	-	-	-	9.5
KCl	0.60	-	31.4	-	-	-	28.5	-	-	-
KOH**	2.25***	-	8.8	-	-	-	-	-	-	-
Lactose	45.00									
Total	mg/ 100 ml	42.0	154.0	35.9	7.8	36.0	115.0	185.1	9.9	9.5

** = KOH added to adjust pH to 6.7 Ci* = Citrate as citric acid

*** = ml equivalent

The pH of the redispersed solution was adjusted to 6.7 if necessary. The solution was then filtered though glass wool to remove any large undissolved pieces of the casein pellet. The solution was then mixed with the relevant amount and type of whey proteins and stirred gently for 2 h at room temperature using a magnetic stirrer. The amount of casein in the solution should be similar to that in the original milk, i.e about 2.6%.

A series of solutions of whey proteins, without casein micelles, were prepared by dissolving the appropriate amount of whey protein in SMUF buffer and stirring for 2 h. Using these methods, the following protein solutions were prepared: 0.4% (w/v) β-lactoglobulin in SMUF buffer, 0.4% (w/v) β-lactoglobulin and casein micelles in SMUF buffer, 0.15% (w/v) α-lactalbumin in SMUF buffer, 0.15% (w/v) α-lactalbumin and



* Procedure starts at this point for the preparation at whey protein solutions without casein micelles.

Figure 3.1 Experimental protocol.

casein micelles in SMUF buffer, 0.4% (w/v) β-lactoglobulin and 0.15% (w/v) α-lactalbumin in SMUF buffer, 0.4% (w/v) β-lactoglobulin and 0.15% (w/v) α-lactalbumin and casein micelles in SMUF buffer.

3.3.2 Heat treatment

2 ml of each protein solution was placed in a thin glass tube (6 mm internal diameter, 1.4 mm thickness and 100 mm length). A thermocouple connected to a digital thermometer was placed in one of the tubes in order to monitor the temperature of the samples during heating. Each tube was covered with parafilm in order to prevent evaporation of fluid. The tubes were placed in a water bath, which had previously been heated to the required temperature, making sure the level of protein solution was below the water level in the bath. The water bath was covered with polystyrene balls in order to maintain a constant temperature. The protein solutions were allowed to reach the temperature of the water bath before starting to record heating time. This "come up" time was 80 s at both 80 and 95°C. After heating for a series of different time intervals, the tubes were taken out of the water bath and placed in a beaker of water at about 20°C. At 80 and 95°C, it took 6 s and 8 s respectively for the temperature to drop to 60°C.

3.3.3 Centrifugation and analysis

1 ml of the heated protein solution was ultracentrifuged at 100 000 **g** (Beckman TL100 Ultracentrifuge) for 1 h. Immediately after ultracentrifugation the supernatant was decanted to prevent the redissolution of the pellet into the supernatant. The supernatant was analysed by native-, SDS non-reducing-, and SDS reducing-PAGE. The pellet was crushed and added to 2 ml of the SDS non reducing sample buffer with no dye. The pellet dissolved slowly over a 7 day period and was then analysed by SDS non reducing- and SDS reducing-PAGE.

3.4 Analysis of protein

Total protein was measured by determining total nitrogen by the macro Kjedahl method (AOAC, 1974) and multiplying it by a factor of 6.38. The samples were digested using a Kjeltec 1007 Digester and distilled using a Kjeltec 1026 Distilling Unit (Tecator,

Sweden). The protein contents of various protein mixtures used in this study are shown in Table A1.1 (*Appendix*).

3.5 Electrophoresis

Native and sodium dodecyl sulphate polyacrylamide gel electrophoresis (native and SDS-PAGE respectively) were carried out using the method of Laemmli (1970), as described by Singh and Creamer (1991a).

3.5.1 Preparation of stock solutions for native-PAGE

Bis/Acrylamide (30% T, 2.6% C)

Acrylamide (30g) and N, N-Bis acrylamide (0.8g) were dissolved in deionised water (Milli Q reagent water system, Millipore corporation, Bedford, MA, USA), to give a final volume of 100 ml. This was stored in the dark (dark bottle) at 5°C.

3 M TRIS-HCL buffer, pH 8.8

TRIS (tris hydroxymethyl aminoethane, 36.3g), obtained from United States Biochemicals Corp. (Cleveland, OH, USA), was dissolved in approximately 60 ml of deionised water. The pH was adjusted to 8.8 with 6 M HCL, the volume made to 100 ml with deionised water and the buffer was stored at 5°C.

0.5 M TRIS-HCL buffer, pH 6.8

TRIS (6g) was dissolved in approximately 60 ml of deionised water. The pH was adjusted to 6.8 with 1 M HCL, the volume made to 100 ml with deionised water, and the buffer stored at 5°C.

Native sample buffer

0.5 M TRIS-HCL buffer (20 ml), glycerol (8 ml), and 0.1% (w/v) bromophenol blue (2 ml) were added to 60ml of deionised water. The sample buffer was stored at 5^0C.

Native electrode buffer, pH 6.8

The Native Electrode buffer was made by dissolving TRIS (15g) and glycine (72g) in deionised water. The pH was adjusted to 8.3 and the volume made up to 1l. The buffer

was stored at 5°C. For each electrophoresis run, 60 ml of Native electrode buffer was diluted to 300 ml with deionised water.

3.5.2 Preparation of gels for native-PAGE

Preparation of resolving gel (15%, w/v, 0.375 M TRIS, pH 8.8)

For preparation of 10 ml of resolving gel buffer, the following solutions were mixed: deionised water (3.75 ml), 3 M TRIS-HCL buffer (1.25 ml), and acrylamide/bis mixture (5 ml). The mixture was degassed for 15 min. Immediately after that, 50 µl of 10% (w/v) ammonium persulphate (prepared earlier the same day and 5 µl of TEMED (tetramethylethylenediamine) were added. After gentle mixing, the contents were poured between electrophoresis casting plates (Bio-Rad Mini Protean, Bio-Rad, Richmond, CA, USA). A small quantity of deionised water was added to give a level gel surface and the acrylamide solutions were allowed to polymerise at 20°C for 35 min. The water layer was then poured out, and pieces of filter paper used to dry the gel (to absorb the last drops of water) before pouring the stacking gel.

Preparation of stacking gel (3.75%, w/v, 0.125 M TRIS, pH 6.8)

For preparation of 5 ml of stacking gel buffer, the following solutions were mixed: deionised water (3.125 ml), 0.5 M TRIS-HCL buffer (1.25 ml), and acrylamide/bis mixture (0.625 ml). The mixture was degassed for 15 min. Immediately after that, 25 µl of 10% (w/v) ammonium persulphate and 5 µl of TEMED were added and the mixture poured on top of the resolving gel. The slot former (plastic comb) was immediately placed on top of the stacking gel (between the plates) to form appropriate slots for samples. Polymerisation was carried out at 20°C. After about 35 min the combs were pulled out and the formed slots were rinsed with deionised water. Water was removed using pieces of filter paper. Gel plates were then placed in the electrode chamber and samples (see sample preparation below) were applied to the gel slots.

3.5.3 Preparation of stock solutions for SDS-PAGE

The Bis/Acrylamide (30% T, 2.6% C) solution and 0.5 M TRIS-HCL buffer, pH 6.8 were made in the same way as for the native-PAGE.

1.5 M TRIS-HCL buffer, pH 8.8

TRIS (tris hydroxymethyl aminoethane, 18.15g), obtained from United States Biochemicals Corp. (Cleveland, OH, USA), was dissolved in approximately 60 ml of deionised water. The pH was adjusted to 8.8 with 6 M HCL, the volume made to 100 ml with deionised water, and the buffer was stored at 5°C.

10% SDS

SDS (10 g) was dissolved with gentle stirring in deionised water and the volume made to 100 ml. This was stored at room temperature.

SDS-non-reducing buffer (sample buffer)

The following solutions were added to 55 ml of deionised water: 0.5 M TRIS-HCL buffer (12.5), glycerol (10 ml), 10% (w/v) SDS (20 ml), and 0.05% (w/v) bromophenol blue (2.5 ml). The sample buffer was stored at room temperature.

SDS-reducing buffer (sample buffer)

The following solutions were added to 54.5 ml of deionised water: 0.5 M TRIS-HCL buffer (12.5 ml), glycerol (10 ml), 10% (w/v) SDS (20 ml), β-mercaptoethanol (0.5 ml) and 0.05% (w/v) bromophenol blue (2.5 ml). Fresh sample buffer was made each day since the β-mercaptoethanol oxidises.

5X Electrode buffer pH 8.3

Electrode buffer (5X) was made by dissolving TRIS (9g), glycine (43.2g) and SDS (3g) in deionised water. The pH was adjusted to 8.3 and the volume to 600 ml. The buffer was stored at 5°C. For each electrophoresis run, 60 ml of 5X electrode buffer was diluted to 300 ml with deionised water.

Preparation of resolving gel (16%, w/v, 0.375 M TRIS, pH 8.8)

The preparation of the native-PAGE resolving gel (15%, w/v, 0.375 M TRIS, pH 8.8) was followed. The volumes of the following solutions were different, deionised water (2.02 ml), 1.5 M TRIS-HCL buffer (2.50 ml), 10% w/v SDS solution (100 µl) and acrylamide/bis mixture (5.3 ml).

Preparation of stacking gel (4%, w/v, 0.125 M TRIS, pH 6.8)

Again the preparation of the native-PAGE (3.75%, w/v, 0.125 M TRIS, pH 6.8) was followed. The volumes of the following solutions were different, deionised water (3.05 ml), 0.5 M TRIS-HCL buffer (1.25 ml), 10% w/v SDS solution (50 µl) and acrylamide/bis mixture (0.65 ml).

3.5.5 Sample preparation and electrophoretic running of gels

The supernatant samples were dispersed in measured aliquots of the relevant sample buffer (100 µl of sample : 300 µl of sample buffer). 5 µl of 0.1% bromophenol blue was added to 0.8 ml of the dissolved pellet solution before application to the non reducing SDS gel. For reducing SDS gels, 0.5% 2-mecaptoethanol and 5 µl 0.1% bromophenol blue were added to 0.8 ml of dissolved pellet solution, heated in a water bath at 95°C for 4 min and cooled to room temperature before being applied to the gel.

10 µl of each solution was applied to the slots of the SDS gel. The gels were run on a Mini-Protean system (Bio-Rad, Richmond, CA, USA) at 200 Volts using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA, USA), until the tracking dye moved out of the gel. The approximate running time was 60 min for SDS-PAGE and 80 min for native-PAGE. After that the gels were removed from the plates and transferred gently to staining solution.

Staining and destaining of gels

The gels were put into plastic containers and 50 ml of Coomassie Blue R solution (1 g brilliant blue R was dissolved in 500 ml of isopropyl alcohol and 200 ml acetic acid and made up to 2 l with deionised water) was added. The gels were rocked gently so that they were uniformly stained.

The staining solution was drained off carefully after 1 h and replaced with destaining solution (10% isopropyl alcohol, 10% acetic acid and 80% deionised water). After 1 h the destaining solution was replaced with a fresh destaining solution and destaining continued for 19 h. This destaining solution was replaced with deionised water to stop destaining.

3.5.7 Densitometry

Quantitative determination of the protein components of the mixture, separated by the different electrophoresis runs, was performed by densitometry using a laser densitometer (LKB Ultrascan XL, LKB Produkter, AB, Sweden. The scanning procedure involved cleaning the densitometer gel plate, placing the stained gel on the densitometer gel plate and defining the tracks for the laser beam to follow. The protein bands were scanned by the beam and absorbance at 522 nm plotted as a function of track distance. The printout gave a graph of individual peak areas. The output from the densitometer was quantified by measuring the areas under individual peaks. Standard proteins were run in conjunction with samples to aid identification of unknown protein bands in the sample.

3.5.8 Interpretation of PAGE results

The PAGE methods used enabled the following to be determined (Fig. 3.2):

(a) the amount of native whey protein in the supernatant;
native PAGE

(b) the amount of non-covalently linked aggregated (e.g. hydrophobic) whey protein in the supernatant;
SDS non reducing-PAGE (SDSNR-PAGE) less native-PAGE.

(c) the amount of disulphide-linked aggregated whey protein in the supernatant;
SDS reducing-PAGE (SDSR-PAGE) less SDSNR-PAGE.

(d) the amount of whey protein associated either among themselves (when casein micelles were not added) or with the casein micelles (when the casein micelles are added);
Total whey protein less SDSR-PAGE.

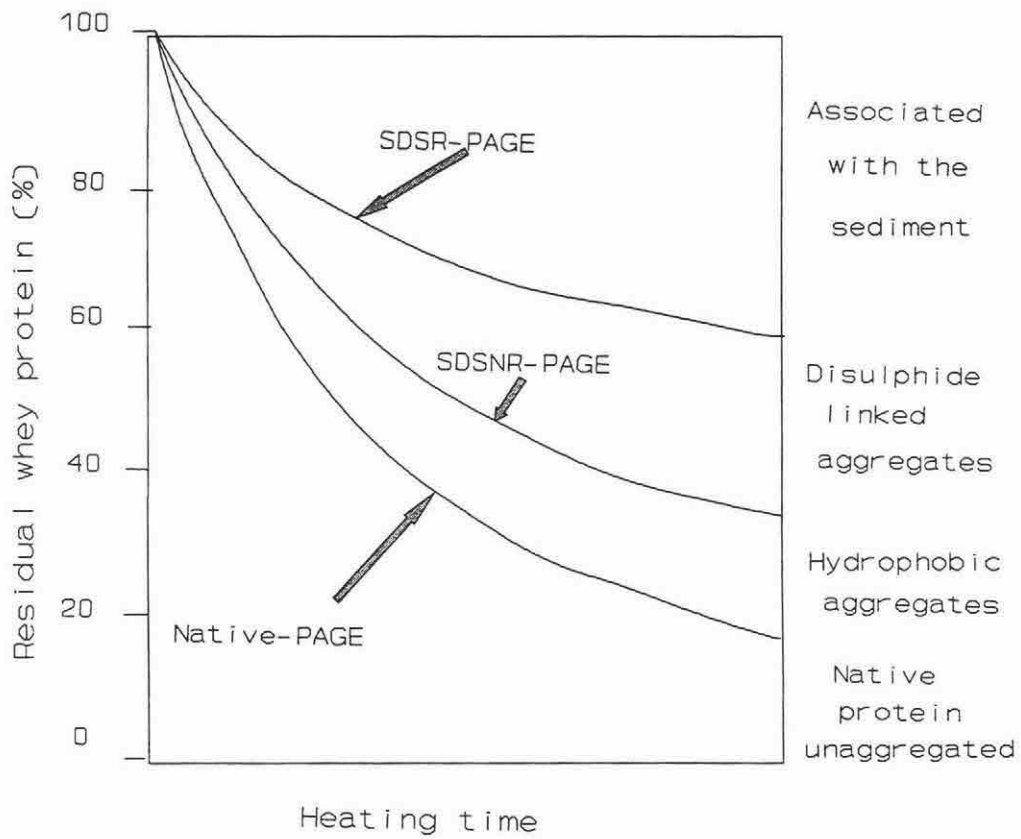


Figure 3.2 Diagrammatic representation of the loss of electrophoretic patterns from the different gels types and their interpreted results.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Denaturation and aggregation of β-lactoglobulin and α-lactalbumin

The denaturation and aggregation of β-lactoglobulin and α-lactalbumin were determined using native-PAGE in the following systems, designed to simulate the protein concentrations and ionic environment in normal skim milk.

1. β-lactoglobulin (0.4% w/v)
2. α-lactalbumin (0.15% w/v)
3. β-lactoglobulin (0.4% w/v) and casein micelles (2.6% w/v)
4. α-lactalbumin (0.15% w/v) and casein micelles (2.6% w/v)
5. β-lactoglobulin (0.4% w/v) and α-lactalbumin (0.15% w/v)
6. β-lactoglobulin (0.4% w/v), α-lactalbumin (0.15% w/v) and casein micelles (2.6% w/v)

Proteins were dissolved in SMUF, pH 6.7, heated at 80°C and 95°C for various times and centrifuged at 100,000 **g** for 60 min. The supernatant was then analysed for the concentrations of individual proteins using polyacrylamide gel electrophoresis (PAGE). The experimental protocol is shown in Figure 3.1 and the results of the experiments are discussed in the following sections.

4.1.1 Changes in native β-lactoglobulin concentrations at 80°C (Native-PAGE)

Ultracentrifugal supernatants obtained from solutions of pure proteins and mixtures containing β-lactoglobulin, α-lactalbumin and casein micelles heated at 80°C for various times, were dispersed in non-dissociating buffers (0.5 mol/l^3 Tris-HCl buffer pH 6.8), and analysed by native-PAGE. The typical gel electrophoretic patterns obtained are shown in Figure 4.1. When 0.4% β-lactoglobulin was heated alone, the intensity of β-lactoglobulin A and β-lactoglobulin B decreased with increase in heating time from 1 to 30 min (Fig. 4.1A). All heated samples (lanes 3 to 9) showed some protein material which failed to migrate into the stacking gel. This material probably contained high molecular weight protein aggregates cross-linked by non-covalent interactions and disulphide bonds. It appears that two different types of aggregates were formed. Some

protein aggregates presumably of larger molecular weight could not migrate into the stacking gel while smaller aggregates migrated through the stacking gel, but stopped at the top the resolving gel (Fig. 4.1A). It must be pointed out that very large "insoluble" aggregates would be sedimented during ultracentrifugation, therefore the aggregates seen in the gels are referred to as "soluble" aggregates.

The intensity of the β-lactoglobulin band also decreased with heating time when mixtures of β-lactoglobulin (0.4%) and casein micelles (~ 2.6%) were heated at 80°C (Fig. 4.1B). It is interesting to note that in this system, only one type of aggregate that could not migrate through the stacking gel was apparent (Fig. 4.1B).

When mixtures of 0.4% β-lactoglobulin and 0.15% α-lactalbumin were heated at 80°C, a decrease in the intensity of both the β-lactoglobulin and α-lactalbumin bands was observed as the heating time increased from 1 to 30 min (lanes 3 to 9) (Fig. 4.1C). Similar trends were observed for mixtures of β-lactoglobulin, α-lactalbumin and casein micelles (4.1D). Aggregates were formed in all these systems.

The gels from all four systems (Fig. 4.1 A-D) showed that β-lactoglobulin bands became slightly "blurred" as heating time was increased, especially above 20 min. Consequently it became difficult to obtain accurate quantitative data of the native β-lactoglobulin for the samples heated for more than 20 min. Thus only heating times of up to 20 min were used for quantitative analysis. Since the separation of proteins on native-PAGE is mainly on the basis of their net charge and molecular weight, the "blurring" of protein bands may suggest random modification of charged groups on the polypeptide chain. This in turn may result in a population of molecules with a range of net charges and charge distributions resulting in "blurred" bands (Singh and Creamer, 1991c). Maillard type reactions between lactose and amino groups of proteins during heating may be partly responsible for this phenomenon.

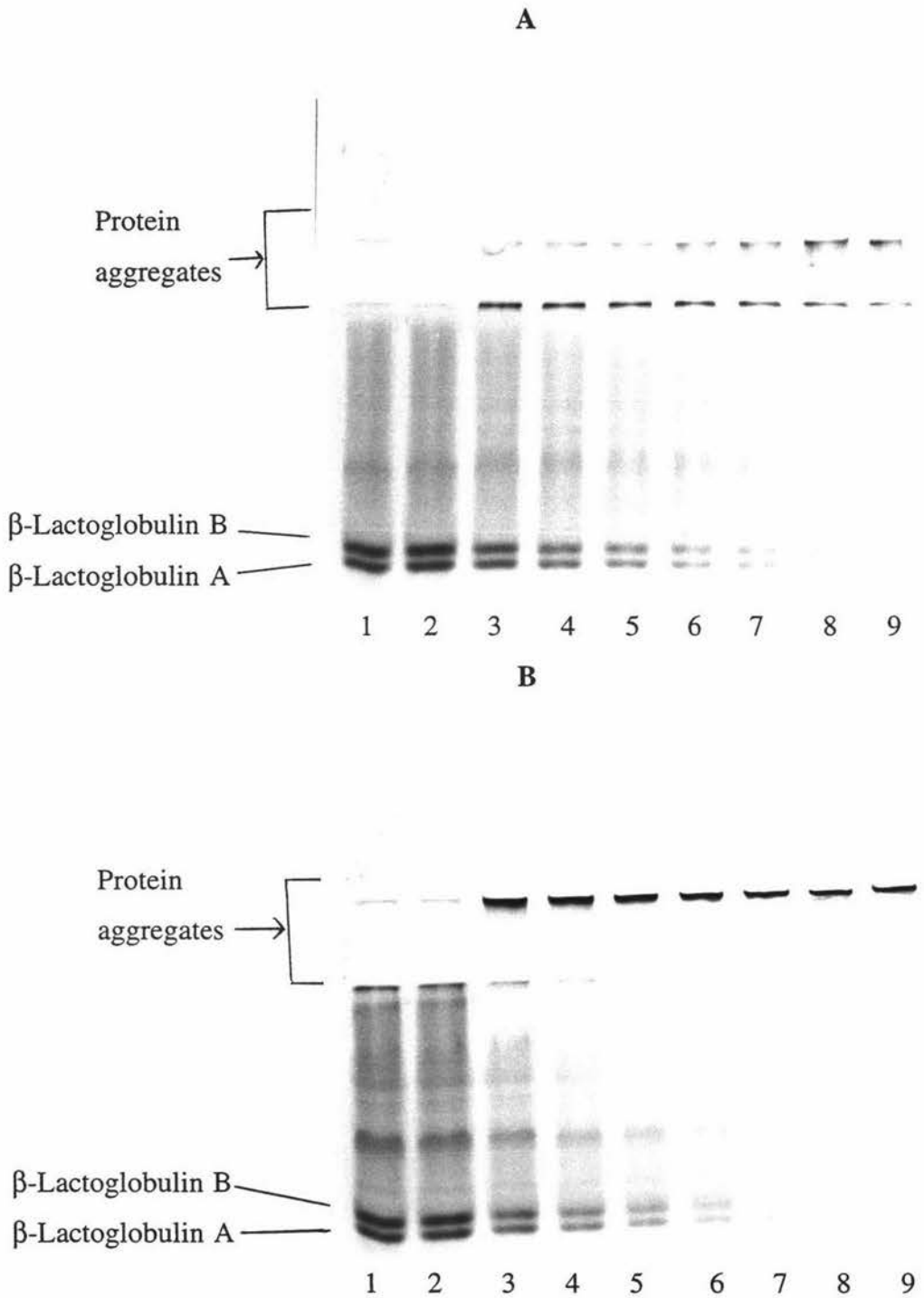


Figure 4.1 Native-PAGE on the supernatants (100, 000 **g** for 60 min) obtained from solutions containing (A) 0.4% (w/v) β-lactoglobulin and (B) 0.4% (w/v) β-lactoglobulin and ~ 2.6% (w/v) casein micelles, heated at 80°C for 0(1,2), 1(3), 2(4), 3(5), 5(6), 8(7), 15(8) and 30(9) min.

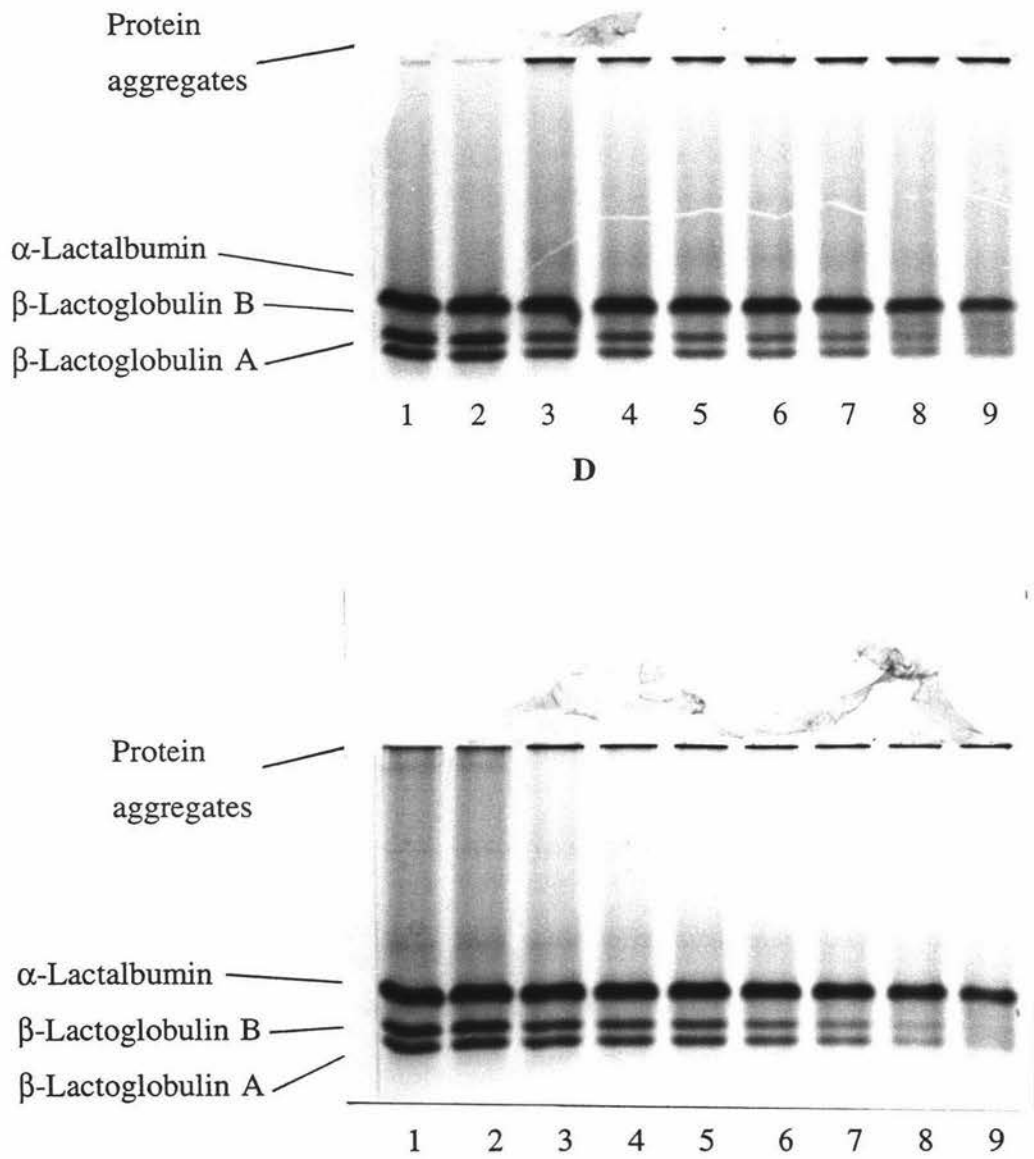


Figure 4.1 Native-PAGE on the supernatants (100, 000 **g** for 60 min) obtained from solutions containing (C) 0.4% (w/v) β-lactoglobulin and 0.15% (w/v) α-lactalbumin and (D) 0.4% (w/v) β-lactoglobulin, 0.15% (w/v) α-lactalbumin and ~ 2.6% (w/v) casein micelles, heated at 80°C for 0(1,2), 1(3), 2(4), 3(5), 5(6), 8(7), 15(8) and 30(9) min.

Figure 4.2 shows the changes in the intensity of stained bands obtained from densitometric scanning of the gels. When 0.4% β-lactoglobulin was heated separately the quantity of native β-lactoglobulin (total β-lactoglobulin A and B), i.e. the protein which showed as monomeric under native-PAGE conditions, decreased with heating time at 80°C (Fig. 4.2).

When β-lactoglobulin was heated in the presence of casein micelles, the loss of β-lactoglobulin from the mixtures was faster than that from β-lactoglobulin solution heated alone (Fig. 4.2).

During the initial stages of heating at 80°C, the loss of β-lactoglobulin was faster from the mixture of β-lactoglobulin and α-lactalbumin than from β-lactoglobulin alone. However after 8 min heating, the loss of β-lactoglobulin was somewhat slower from the mixture. The loss of β-lactoglobulin from β-lactoglobulin and α-lactalbumin mixture was slower than the loss from a mixture of casein micelles and β-lactoglobulin, especially during the later stages of heating (> 8 min).

When β-lactoglobulin was heated together with both α-lactalbumin and casein micelles the loss of native β-lactoglobulin was faster initially (first 8 min), but became slower for the remainder of the heating time. The loss of β-lactoglobulin from this mixture was faster than the loss of β-lactoglobulin when heated alone or in the presence of α-lactalbumin. However, the loss of β-lactoglobulin was slightly slower than that from β-lactoglobulin heated in the presence of casein micelles.

Analysis of the variants of β-lactoglobulin, i.e. β-lactoglobulin A and β-lactoglobulin B, showed that each variant followed the same trend as described for total native β-lactoglobulin. In all cases, the loss of β-lactoglobulin B was faster than β-lactoglobulin A (Tables A2.2-5; Figs. A1-4 *Appendix*).

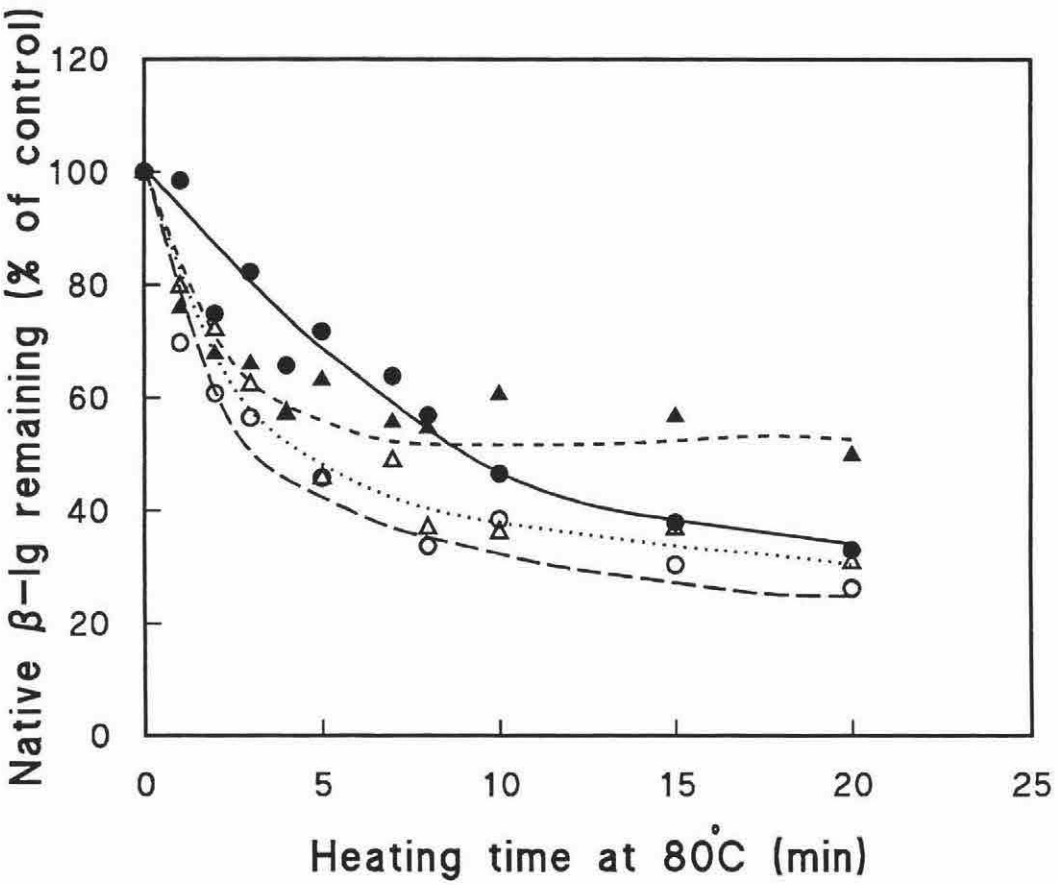


Figure 4.2 The effect of heating time at 80°C on the loss of native β-lactoglobulin from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin, 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.1.1.1 Kinetics of loss of native β-lactoglobulin

The general rate equation (Eqn 4.1) enables the order of a reaction and hence the rate constant to be obtained. This is done by using the linear forms of the general rate equation (Eqn 4.1).

$$-\frac{dC}{dt}=k_n C^n \qquad \textbf{4.1}$$

C = concentration of native protein

t = time

k_n = reaction rate constant

n = order of the reaction with respect to C

when $n \neq 1$

$$(\frac{C_t}{C_0})^{1-n}=1+(n-1)\,K_n C_o^{n-1} t \qquad \textbf{4.2}$$

where

C_t = concentration of native protein at time t=t

C_0 = concentration of native protein at time t=0

when n = 1 (first order reaction)

$$\ln\frac{C_t}{C_0}=-k_n t \qquad \textbf{4.3}$$

The left hand side of the linear forms of the general rate equation (Eqns 4.2 and 4.3) was plotted against time and K_n was calculated from the slope. A close fit to the regression line indicated that the results followed the order that had been predicted.

The initial rate of decrease (up to 8 min) of native β-lactoglobulin from all four systems

heated at 80°C could be fitted to a second-order rate equation (Fig. 4.3) and the derived empirical rate constants are shown in Table 4.1. The reaction rate constant for the loss of β-lactoglobulin when heated alone was found to be 0.0919 $gl^{-1}min^{-1}$. The value of the rate constant increased by 2.7 times when the mixture of casein micelles and β-lactoglobulin was heated. Addition of α-lactalbumin to the β-lactoglobulin solution also increased the initial reaction rate constant to 0.1246 $gl^{-1}min^{-1}$, which was about 1.4 times the value obtained for β-lactoglobulin alone. The rate constant value for the loss of β-lactoglobulin in the presence of both α-lactalbumin and casein micelles was intermediate between the rate constant value for the loss of β-lactoglobulin in the presence of casein micelles and the value for β-lactoglobulin in the presence of α-lactalbumin. Overall the initial reaction rate seems to follow the order: β-lactoglobulin and casein micelles mixture > β-lactoglobulin, α-lactalbumin and casein micelles mixture > β-lactoglobulin and α-lactalbumin mixture > β-lactoglobulin (Table 4.1).

Comparison of the reaction rate constants for the variants of β-lactoglobulin (Tables A2.3, A2.5; Figs. A2,4 *Appendix*) showed that the value for the reaction rate constant for β-lactoglobulin B was always higher than that of β-lactoglobulin A.

Table 4.1 Kinetic data for the loss of native β-lactoglobulin (β-lg) from different mixtures at 80°C (second-order reaction equation).

Sample	Degrees of Freedom	r^2	Rate constant (K_n) $gl^{-1}min^{-1}$	Standard Error for K_n
β-lg alone	7	0.8673	0.0919	0.0076
β-lg + casein micelles	5	0.9855	0.2492	0.0110
β-lg + α-la	7	0.8148	0.1246	0.0132
β-lg + α-la + casein micelles	7	0.9220	0.1962	0.0121

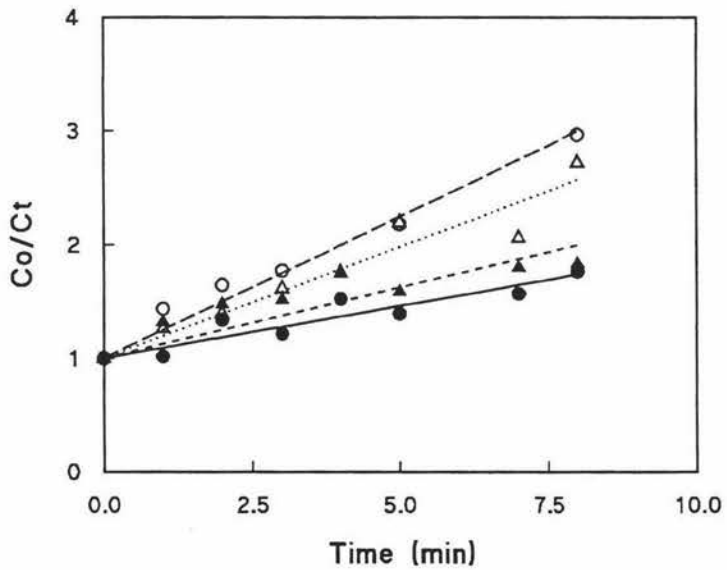


Figure 4.3 Kinetic plots (second order reaction equation) for the loss of native β-lactoglobulin at 80°C from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin, 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.1.2 Changes in native β-lactoglobulin concentrations at 95°C (Native-PAGE)

When various protein mixtures were heated at 95°C the loss of β-lactoglobulin occurred much more rapidly than at 80°C; for example, when β-lactoglobulin was heated alone at 95°C, ~ 80% of native β-lactoglobulin was lost within 1 min heating compared to ~ 5% within the same heating time at 80°C (Fig. 4.2 vs Fig. 4.4). All quantitative data is shown in the Appendix (Tables A2.9 to A2.15). The loss of native β-lactoglobulin from the β-lactoglobulin solution heated alone was comparable to that from the solution containing β-lactoglobulin and casein micelles (Fig. 4.4). However, the presence of α-lactalbumin in both these systems slightly decreased the rate at which native β-lactoglobulin was lost. In all the four systems, most of the β-lactoglobulin was lost within the first 1 min, suggesting that a significant amount of β-lactoglobulin was lost during the heating-up time to this temperature, which was 1 min 20 sec.

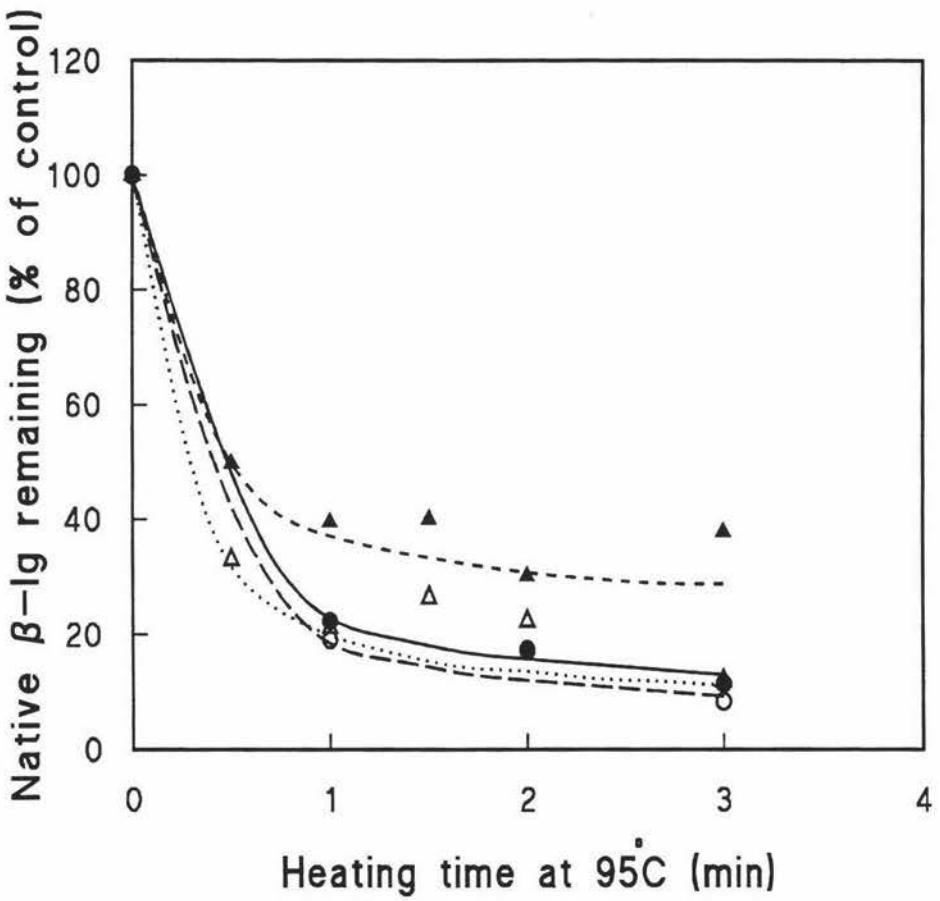


Figure 4.4 The effect of heating time at 95°C on the loss of native β-lactoglobulin from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.1.3 Discussion

When β-lactoglobulin was heated, cooled, ultracentrifuged and the supernatants examined by native-PAGE, the quantity of native protein was found to decrease with increase in severity of heating (Fig. 4.2). There was also an increase in the extent of the formation of high molecular weight aggregates. The loss of β-lactoglobulin was found to be more rapid from the mixture containing casein micelles (Fig. 4.2). These results confirm the earlier findings of Elfagm and Wheelock (1977, 1978b) that β-lactoglobulin denatures more rapidly in the presence of casein.

Elfagm and Wheelock (1977) examined the effects of casein micelles on the denaturation of β-lactoglobulin, by heating whole bulk milk and whey prepared from the same milk. Denaturation was determined as the loss of monomeric protein by gel filtration, with phosphate buffer, pH 7.0 as an eluent. It was found that at 80 and 90°C the extent of denaturation of β-lactoglobulin was greater in milk than in whey, for example, about 98% of the β-lactoglobulin was lost from milk when heated at 80°C for 20 min, compared to about 82% from whey. Elfagm and Wheelock (1978b) later found that when the concentration of casein in milk was reduced, there was an increase in the amount of native β-lactoglobulin recovered after heating, confirming that the presence of casein micelles increases the extent of heat-induced whey protein denaturation.

The effects of *k*-casein on the loss of β-lactoglobulin were studied by Park and Lund (1984), who dispersed β-lactoglobulin in SMUF at pH 7.0. They used DSC to analyse protein denaturation and reported that the denaturation rate constant of β-lactoglobulin increased with increasing concentrations of *k*-casein. They postulated that the hydrophobic amino acid residues of β-lactoglobulin were exposed by adding *k*-casein, thus making β-lactoglobulin more reactive. DSC work by Paulsson and Dejmek (1990) also showed that the thermal behaviour of β-lactoglobulin dissolved in SMUF at pH 6.6 and 6.8 was affected by *k*-casein. The presence of *k*-casein lowered the denaturation temperature of β-lactoglobulin by 3°C i.e at pH 6.6, T_{max} was reduced from 77.5 to 74.2°C, while at pH 6.8, T_{max} shifted from 76.5 to 73.3°C.

Increased loss of native β-lactoglobulin in the presence of casein micelles, observed in

this study, may be because in the absence of casein micelles, β-lactoglobulin can only interact with itself, whereas in the presence of casein micelles β-lactoglobulin can interact with itself as well as with the *k*-casein exposed on the micelle surface. The presence of casein micelles also increases the "effective" concentration of β-lactoglobulin in the system, possibly promoting the extent of denaturation. Some workers (Elfagm and Wheelock, 1977; Hillier and Lyster, 1979) have reported that the effect of casein micelles on the loss of β-lactoglobulin was dependent upon temperature of heat treatment. Elfagm and Wheelock (1977) found that at temperatures below 70°C, casein micelles inhibited the loss of β-lactoglobulin from solution. Hillier and Lyster (1979) reported that β-lactoglobulin was more stable in the absence of casein micelles after heating between 70 and 95°C, but at higher temperatures β-lactoglobulin became less stable.

In contrast to the findings of Elfagm and Wheelock (1977, 1978b), Park and Lund (1984) and Paulsson and Dejmek (1990), and the results of this study, Kenkare *et al.* (1964), Morr and Josephson (1968) and Sarbawal and Ganguli (1972) have reported that the presence of casein micelles decreased the rate of denaturation of β-lactoglobulin. Sarbawal and Ganguli (1972) compared denaturation of whey proteins in casein free milk sera, and the sera containing increasing concentrations of micellar casein at boiling water temperature. They found that the extent of denaturation decreased with increase in micellar casein in milk sera. Denaturation also decreased when micellar casein was added to skim milk. They reasoned that this trend was due the to removal of free-SH groups from β-lactoglobulin through complex formation with *k*-casein. However, they also pointed out that this presumption was not supported by data which indicated that small *k*-casein-rich casein micelles have the least stabilising capacity.

Kenkare *et al.* (1964) used gel filtration to determine the effect of casein on thermal denaturation of serum protein components in the temperature range 68.3 to 90.5°C. They also studied aggregation destabilisation by centrifuging the heated solutions at 1,000 **g** for 30 min and expressed protein destabilisation as the percent reduction of nitrogen content in the serum, before and after centrifugation. They found that addition of whole casein and various casein fractions (α-, α_s-, *k*-, and β-) to skim milk sera

increased the stability of the proteins to heat, and all casein additives were equally effective. Morr and Josephson (1968) used low- and high-speed centrifugation, gel filtration and PAGE to investigate the nature and extent of protein aggregation produced by heating whey and skimmilk systems. These workers also reported that casein stabilised whey proteins against heat-induced gross aggregation in skimmilk when heated at 90°C for 10 min. The results and conclusions of Kenkare *et al.* (1964) and Morr and Josephson (1968) indicate that added individual caseins, whole caseinate or casein micelles may not stabilise the protein against denaturation *per se* but prevent precipitation of denatured whey proteins in heated whey systems. They further concluded that this stabilising effect is not due to the sulphydryl-disulphide interchange mechanism but to the formation of non specific calcium-linked complexes between caseins and whey proteins.

The results of this study show that the presence of α-lactalbumin increased the loss of β-lactoglobulin during the initial stages of heating at 80°C but had an opposite effect at the later stages of heating (Fig. 4.2). Elfagm and Wheelock (1978a) dissolved β-lactoglobulin and α-lactalbumin in SMUF (pH 6.4 to 7.2) and heated the mixture at 80°C for 20 min. They found that the amount of native β-lactoglobulin recovered from the mixture of β-lactoglobulin and α-lactalbumin was similar to that when the protein was heated by itself. Hines and Foegeding (1993) using size exclusion chromatography found that when α-lactalbumin was heated at 80°C in the presence of an equimolar quantity of β-lactoglobulin, the aggregation rate was similar to that of β-lactoglobulin heated alone. The differences between results of this study and those in the literature may possibly be attributed to different buffers, heating methods and methods used in analysing protein denaturation.

This study shows that the loss of β-lactoglobulin from heated systems could be adequately fitted to a second-order rate equation (Fig. 4.3, Table 4.1). This is in agreement with earlier studies which showed that the denaturation of β-lactoglobulin in milk and buffer systems can be described by second-order reaction kinetics (Lyster, 1970; Hillier and Lyster, 1979; de Wit and Swinkels, 1980; Park and Lund, 1984; Relkin and Launay, 1990; McSwiney *et al.*, 1994). Lyster (1970), and Hillier and Lyster (1979)

used skim milk while Relkin and Launay (1990) dispersed β-lactoglobulin in distilled water at concentrations from 3.5% to 24% at pH 3.2. Park and Lund (1984) dissolved β-lactoglobulin in SMUF similar to that used in this study, and used DSC to determine denaturation. DSC data from the work of de Wit and Swinkels (1980) on β-lactoglobulin dispersed in 0.07M phosphate buffer at pH 6.75 also described the denaturation of β-lactoglobulin as a second-order reaction at temperatures between 75 to 100°C. McSwiney *et al.* (1994) used gel electrophoresis to study heat-induced aggregation of β-lactoglobulin at 75 and 80°C. They demonstrated that 10% (w/v) β-lactoglobulin dispersed in imidazole-HCl buffer, pH 7.0 followed second-order kinetics.

On the other hand, Dannenburg and Kessler (1988) studied the kinetics of the heat-induced irreversible denaturation of β-lactoglobulin A and B in skim milk over a wide range of temperatures and times (70 to 150°C, 2 to 5400 sec) and found that the reaction can be best described with an apparent reaction order of 1.5. Later Roefs and de Kruif (1994) determined reaction rates at 65°C using light scattering and described the reaction as following a 1.5 order. de Wit and Swinkels (1980) concluded that the denaturation of β-lactoglobulin followed first-order kinetics at temperatures 65 to 75°C. Dalgleish (1990) used gel filtration to study the denaturation and aggregation of β-lactoglobulin in skim milk and demonstrated that at temperatures between 75 to 90°C β-lactoglobulin denatured by a pseudo first-order.

In all cases, the loss of β-lactoglobulin B was faster than β-lactoglobulin A, which is in agreement with the earlier studies (Dannenberg and Kessler, 1988; Gough and Jenness, 1962; Oldfield, 1996).

4.1.4 Changes in native α-lactalbumin concentrations at 80°C (Native-PAGE)

Typical native gel patterns obtained after heating solutions of α-lactalbumin and α-lactalbumin and other proteins for various times at 80°C are shown in Figures 4.1 and 4.5. Heating a solution of 0.15% α-lactalbumin or a solution containing α-lactalbumin and casein micelles at 80°C for up to 30 min caused no apparent polymerisation of the protein as indicated by the lack of formation of aggregates (Fig. 4.5A). A slight reduction in the intensity of the α-lactalbumin band, however, did occur with increase in heating time (Fig. 4.5A,B).

In the mixture of α-lactalbumin and β-lactoglobulin solution, aggregates that could not migrate through the stacking gel were formed (lanes 3 to 9) (Fig.4.1C). In this system, there was a considerable reduction in the intensity of the α-lactalbumin band with increase in heating time. Similar observations were made for the mixture containing α-lactalbumin, β-lactoglobulin and casein micelles (Fig. 4.1D).

In order to quantify the amount of α-lactalbumin in different samples, the gels were scanned and the results are shown in Fig. 4.6. A small loss of α-lactalbumin was apparent when α-lactalbumin was heated alone or in the presence of casein micelles. On the other hand, heating a mixture of α-lactalbumin and β-lactoglobulin for up to 20 min resulted in a considerable decrease in the concentrations of native α-lactalbumin. Approximately 55% of α-lactalbumin was lost after 20 min heating. The decrease in the concentration of α-lactalbumin from a mixture containing α-lactalbumin, β-lactoglobulin and casein micelles was comparable to that from the α-lactalbumin and β-lactoglobulin mixture (Fig. 4.6).

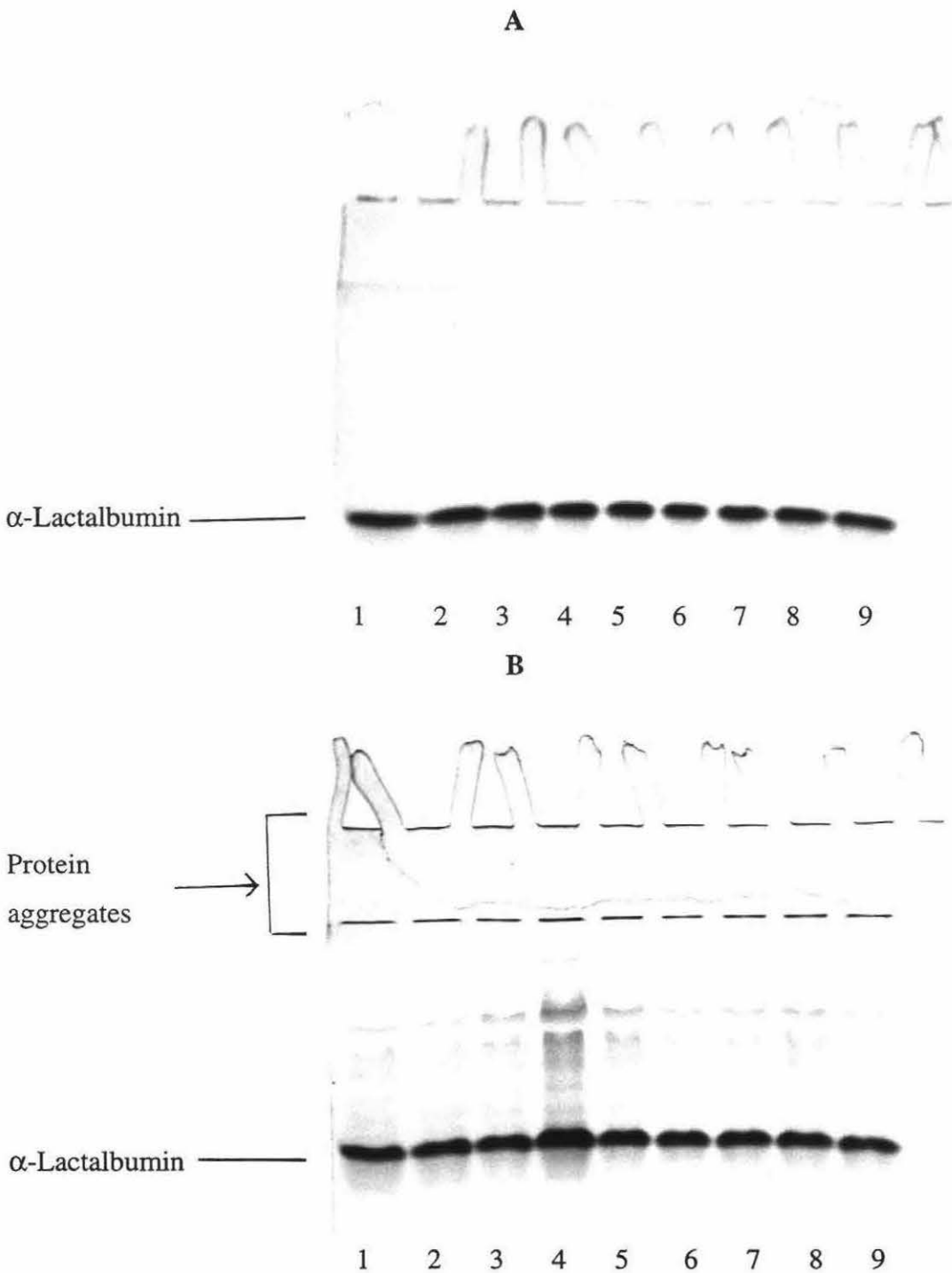


Figure 4.5 Native-PAGE on the supernatants (100 000 **g** for 60 min) obtained from solutions containing (A) 0.15% α-lactalbumin and (B) 0.15% α-lactalbumin and ~ 2.6% casein micelles solution, heated at 80°C for 0(1,2), 2(3), 4(4), 7(5), 10(6), 15(7), 20(8) and 25(9) min.

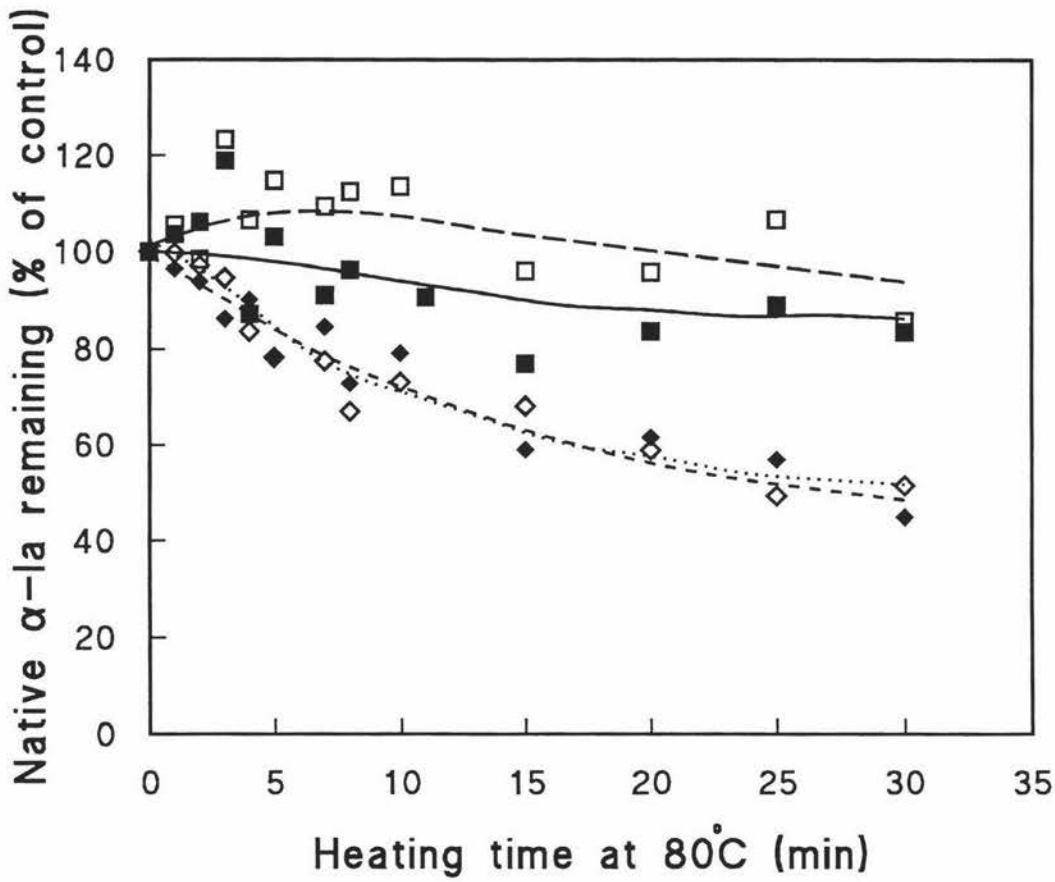


Figure 4.6 The effect of heating time at 80°C on the loss of native α-lactalbumin from solutions containing 0.15% α-lactalbumin (■) 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), and 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.1.4.1 Kinetics of loss of native α-lactalbumin

Data obtained for the mixtures containing α-lactalbumin and β-lactoglobulin, and α-lactalbumin, β-lactoglobulin and casein micelles were analysed using equations 4.2 and 4.3. In both cases, the loss of α-lactalbumin could be described by a first-order reaction (Equation 4.3). The kinetic parameters and plots are shown in Table 4.2 and Figure 4.7, respectively. The value of the reaction rate constant for the loss of α-lactalbumin from the two systems was similar i.e. 0.0265 and 0.0266 min^{-1}, respectively.

Table 4.2 Kinetic data for the loss of native α-lactalbumin (α-la) from different mixtures at 80°C (first-order reaction equation).

Sample	Degrees of Freedom	r^2	Rate constant (K_n) (min^{-1})	Standard Error for K_n
α-la + β-lg	12	0.9274	0.0265	0.0014
α-la + β-lg + casein micelles	12	0.9067	0.0266	0.0017

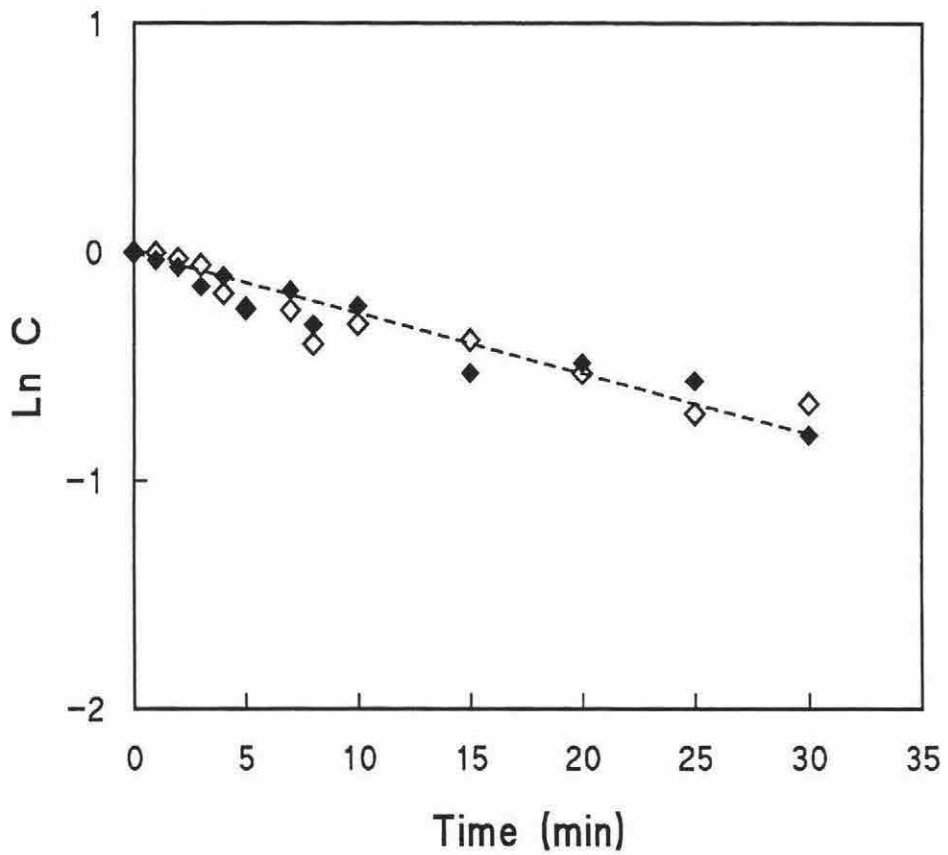


Figure 4.7 Kinetic plots (first-order reaction equation) for loss of native α-lactalbumin at 80°C from solutions containing 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), and 0.15% α-lactalbumin, 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.1.5. Changes in native α-lactalbumin concentrations at 95°C (Native-PAGE)

Figure 4.8A shows a typical native gel obtained when α-lactalbumin was heated at 95°C. When α-lactalbumin was heated either alone or with casein micelles at 95°C, the intensity of the α-lactalbumin bands decreased with time. Aggregates that could not migrate into the resolving gel were observed in heated α-lactalbumin- and casein micelles mixture (Fig. 4.8A,B). A hazy tract that became larger with time appeared just below the α-lactalbumin bands. When α-lactalbumin was heated in the presence of β-lactoglobulin and both β-lactoglobulin and casein micelles, the intensity of both α-lactalbumin and β-lactoglobulin decreased rapidly (gels not shown).

The changes in the concentrations of native α-lactalbumin in various heated protein mixtures are shown in Figure 4.9. When α-lactalbumin was heated separately at 95°C, the quantity of native α-lactalbumin decreased with heating time. Heating α-lactalbumin in the presence of casein micelles reduced the loss of α-lactalbumin slightly. In contrast, heating α-lactalbumin in the presence of β-lactoglobulin markedly increased the loss of α-lactalbumin. The loss of α-lactalbumin from a mixture of α-lactalbumin, β-lactoglobulin and casein micelles was faster than from α-lactalbumin heated either alone or in the presence of casein micelles, but it was slightly lower than that from the α-lactalbumin and β-lactoglobulin mixture.

4.1.5.1 Kinetics of loss of native α-lactalbumin

The rates of loss of α-lactalbumin at 95°C in all four α-lactalbumin solutions could be described by first-order reaction kinetics (Eqn 4.3, Table 4.3, Fig. 4.10). When α-lactalbumin was heated alone, the value of K_n was found to be 0.0268 min^{-1}. The K_n value decreased slightly when α-lactalbumin was heated together with casein micelles. Heating α-lactalbumin in the presence of either β-lactoglobulin only or both β-lactoglobulin and casein micelles, approximately doubled the value of K_n. The order of loss of α-lactalbumin in the four systems was: α-lactalbumin and β-lactoglobulin mixture ≥ α-lactalbumin, β-lactoglobulin and casein micelles mixture > α-lactalbumin alone ≥ α-lactalbumin and casein micelles mixture.

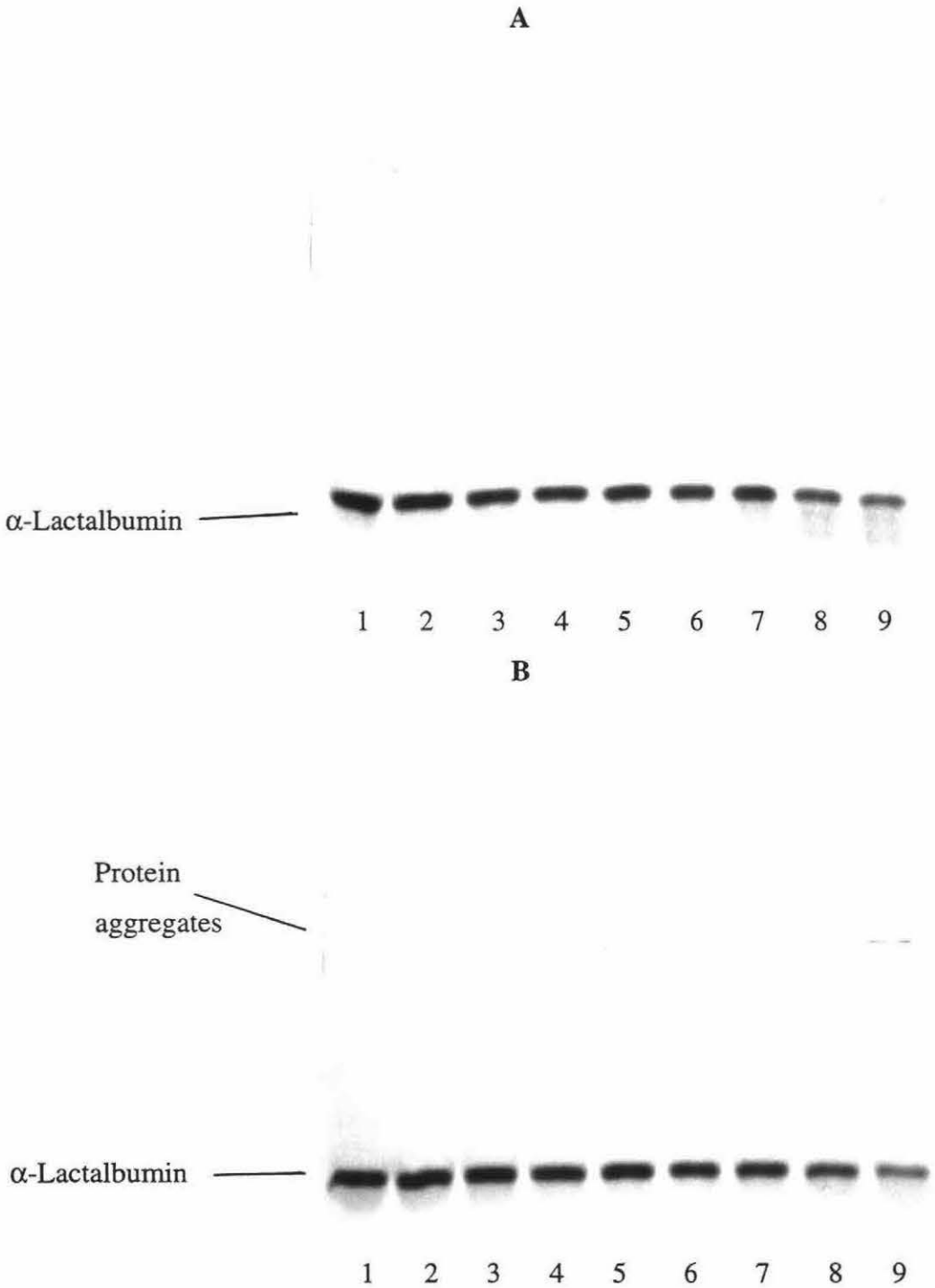


Figure 4.9 Native-PAGE on the supernatants (100 000 **g** for 60 min) obtained from solutions containing (A) 0.15% α-lactalbumin and (B) 0.05% α-lactalbumin and casein micelles, heated at 95°C for 0(1,2), 1(3), 2(4), 3(5), 5(6), 8(7), 15(8) and 30(9) min.

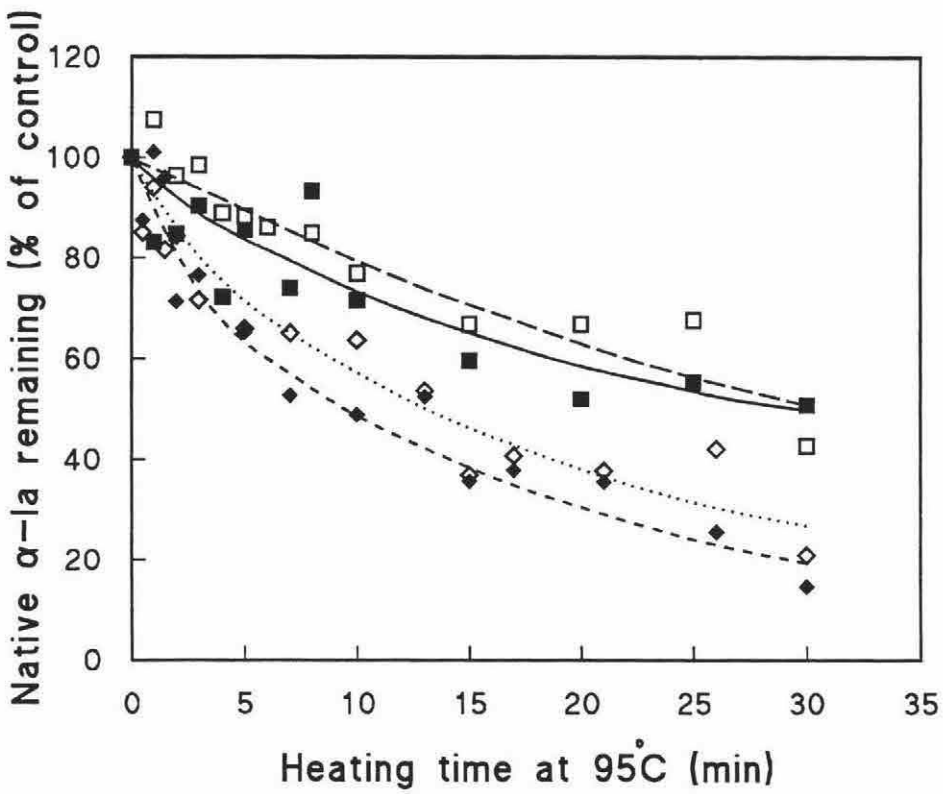


Figure 4.9 The effect of heating time at 95°C on the loss of native α-lactalbumin from solutions containing 0.15% α-lactalbumin solution (■), 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), and 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

Table 4.3 Kinetic data for the loss of native α-lactalbumin (α-la) from different mixtures at 95°C (first-order reaction equation).

Sample	Degrees of Freedom	r^2	Rate constant (K_n)(min^{-1})	Standard Error for K_n
α-la alone	12	0.8164	0.0268	0.0025
α-la + casein micelles	12	0.8731	0.0224	0.0018
α-la + β-lg	14	0.9471	0.0591	0.0027
α-la + β-lg + casein micelles	14	0.8984	0.0484	0.0030

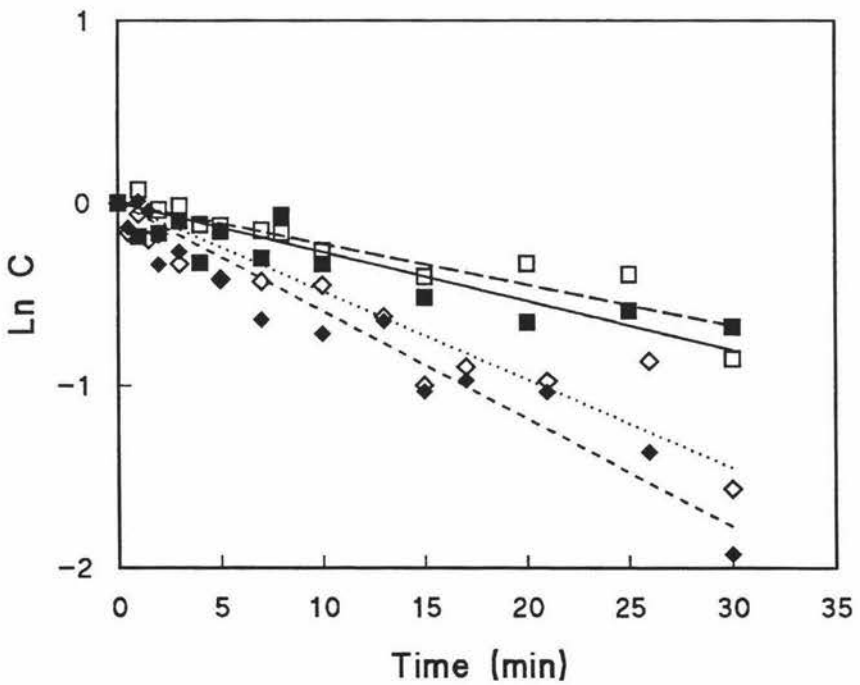


Figure 4.10 Kinetic plots (first-order reaction equation) for the loss of native α-lactalbumin at 95°C from solutions containing 0.15% α-lactalbumin (■), 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.1.6 Discussion

The present results show that when 0.15% α-lactalbumin was heated alone in SMUF at 80°C for up to 30 min, there was only a slight loss of the native protein (~ 15%)(Fig. 4.6). However, on heating at 95°C, considerable loss of α-lactalbumin was observed (Fig 4.9). Conflicting results are reported by previous workers on the heat effects on α-la. Elfagm and Wheelock (1978a) found that when 10% α-lactalbumin in SMUF was heated for 20 min at 80°C, about 32% of the protein was lost. Hines and Foegeding (1993) reported a slow loss of α-lactalbumin when heated alone; about 10% α-lactalbumin was lost after 30 min heating at 80°C. However, Matsudomi *et al.* (1992) reported that heating 0.2% α-lactalbumin at 80°C for 30 min did not cause any change in the elution pattern of gel filtration on an HPLC. Similarly, Calvo *et al.* (1993) while studying the influence of other whey proteins on the heat-induced aggregation of α-lactalbumin also found that no aggregation occurred after heating 0.15% α-lactalbumin alone in milk ultrafiltrate at 90°C for 24 min.

Serological studies on 0.1% α-lactalbumin in 0.15M NaCl solution, 0.15M KCl solution, milk ultrafiltrate and milk established that α-lactalbumin was very resistant to heat denaturation at 80°C (Baer *et al.*, 1976). α-Lactalbumin was more stable in milk ultrafiltrate than in NaCl and KCl solutions. Ruegg *et al.* (1977) used DSC to study the thermal transitions and degree of renaturation of whey proteins dissolved in SMUF, pH 6.7. They found that α-lactalbumin underwent some conformational changes when heated at 70°C. Rescanning the previously heated (denatured) samples after being cooled to 20°C, showed an 80-90% reversibility for α-lactalbumin at protein concentrations of 3-9%. This high degree of renaturation presumably accounts for the fact that α-lactalbumin has been considered as the most heat resistant of all whey proteins, when protein precipitation methods are used to assess denaturation (Larson and Rolleri, 1955; Donovan and Mulvihill, 1987; Singh and Creamer, 1991b and Oldfield, 1996).

When α-lactalbumin was heated in the presence of casein micelles little further change occurred in the quantities of native α-lactalbumin was observed (Fig. 4.6, 4.9). This suggests that there was no interaction between α-lactalbumin and casein micelles which

is in agreement with the results reported by Baer *et al.* (1976) who monitored the changes in immunological behaviour of α-lactalbumin after heat treatment.

Interaction between α-lactalbumin and casein micelles was, however, reported by Shalabi and Wheelock (1976) who used the action of chymosin to study the effect of casein micelles on whey proteins. They dispersed α-lactalbumin and casein micelles in either SMUF or distilled water and heated the solutions at 90°C for 20 min. Interaction may have been observed in their study because an indirect method of protein analysis was used.

When α-lactalbumin and β-lactoglobulin were heated together under the same conditions the quantity of native α-lactalbumin decreased markedly with time and aggregates were formed (Figs. 4.6, 4.9). This indicated that β-lactoglobulin must be present before α-lactalbumin can be incorporated into aggregates. Faster losses of native α-lactalbumin when heated in the presence of β-lactoglobulin, as compared to when it is heated alone, have also been demonstrated by other workers using size exclusion chromatography (Elfagm and Wheelock, 1978a; Matsudomi *et al.*, 1992; Hines and Foegeding, 1993; Calvo *et al.*, 1993) and DSC (Paulsson and Dejmek, 1990).

It appears that the addition of casein micelles to the mixture of α-lactalbumin and β-lactoglobulin did not affect the loss of native α-lactalbumin further (4.6, 4.9).

When α-lactalbumin is heated in the presence of β-lactoglobulin with or without casein micelles, aggregates are formed probably between β-lactoglobulin and β-lactoglobulin as well as between β-lactoglobulin and α-lactalbumin. In the system containing the three proteins i.e. α-lactalbumin, β-lactoglobulin and casein micelles, it is possible that in addition there might be aggregates formed between *k*-casein and β-lactoglobulin as well as β-lactoglobulin/α-lactalbumin aggregates may interact with *k*-casein. The role of β-lactoglobulin may be to act as a catalyst, presumably *via* its free-sulphydryl group, to open-up the disulphide bridges in either the polymerised *k*-casein or in the monomeric α-lactalbumin molecules allowing intermolecular sulphydryl-disulphide bridges to form *via* disulphide interchange (Calvo *et al.*, 1993).

The rate of loss of α-lactalbumin was found to be first-order. Reaction kinetics of the loss of α-lactalbumin in model systems was studied by Baer *et al.* (1976). They described loss of α-lactalbumin when heated alone in milk ultrafiltrate as pseudo first-order reaction, and that of α-lactalbumin heated in the presence of β-lactoglobulin as first-order. Similarly Lyster (1970), Hillier and Lyster (1979), Dannenberg and Kessler (1988) and Oldfield (1996) found the reaction kinetics of the α-lactalbumin to be first order in milk.

4.1.7 Changes in SDS-monomeric β-lactoglobulin concentrations at 80°C (SDSNR-PAGE)

Ultracentrifugal supernatants obtained from heated protein solutions were dispersed in a buffer (0.5 mol/dm^3 Tris-HCl buffer, pH 6.8 containing 2% SDS and no 2-mercaptoethanol), and analysed using SDS-non reducing PAGE (SDSNR-PAGE). This buffer presumably dispersed all non-covalently linked protein aggregates into monomers while aggregates linked through disulphide bonds remained intact.

Typical SDSNR-PAGE gels obtained after heating β-lactoglobulin solutions at 80°C for various times are shown in Figure 4.11. In unheated samples of β-lactoglobulin, some high molecular weight material was observed. When β-lactoglobulin was heated either alone or in the presence of other proteins, the intensity of the monomeric β-lactoglobulin bands decreased with heating time (Fig. 4.11B). All heated samples (lanes 3 to 9) showed some high molecular weight protein material that could not migrate into the stacking gel; this material was presumably the aggregates linked by intermolecular disulphide linkages. All samples also showed high molecular protein bands close to the top of the resolving gel, and these bands decreased in intensity with heating time. This was probably because some of these aggregates were being transformed into bigger ones.

The changes in the concentration of monomeric β-lactoglobulin resolved on SDSNR-PAGE when heated at 80°C in the four systems containing β-lactoglobulin are shown in Figure 4.12. For comparison with the data obtained from native-PAGE, the SDSNR data (Table A1.6) was plotted for up to 20 min heating (Fig. 4.12).

When heated either separately or with other proteins, the amount of SDS-monomeric β-lactoglobulin decreased with increase in heating time. About 40% total β-lactoglobulin was lost after 20 min heating when it was heated alone at 80°C. When β-lactoglobulin was heated in the presence of α-lactalbumin, the loss of β-lactoglobulin from the mixture was faster than when the protein was heated alone. Heating β-lactoglobulin in the presence of both α-lactalbumin and casein micelles further increased the rate of loss of β-lactoglobulin. After 20 min heating, the loss of β-lactoglobulin from the mixture

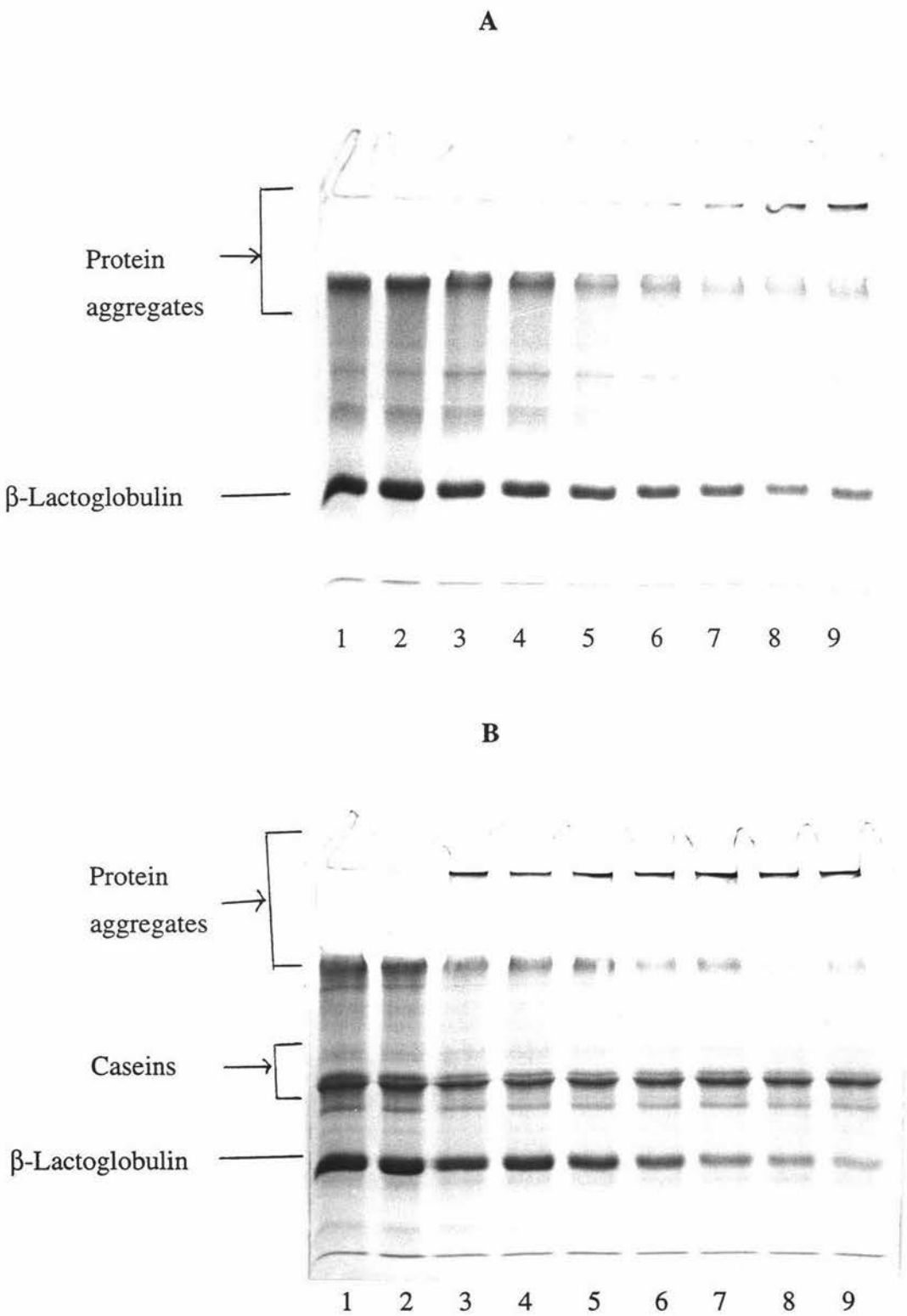


Figure 4.11 SDSNR-PAGE on the supernatants (100 000 **g** for 60 min) obtained from solutions containing (A) 0.4% β-lactoglobulin and (B) 0.4% β-lactoglobulin and ~ 2.6% casein micelles, heated at 80°C for 0(1,2), 2(3), 4(4), 7(5), 10(6), 15(7), 20(8) and 25(9) min.

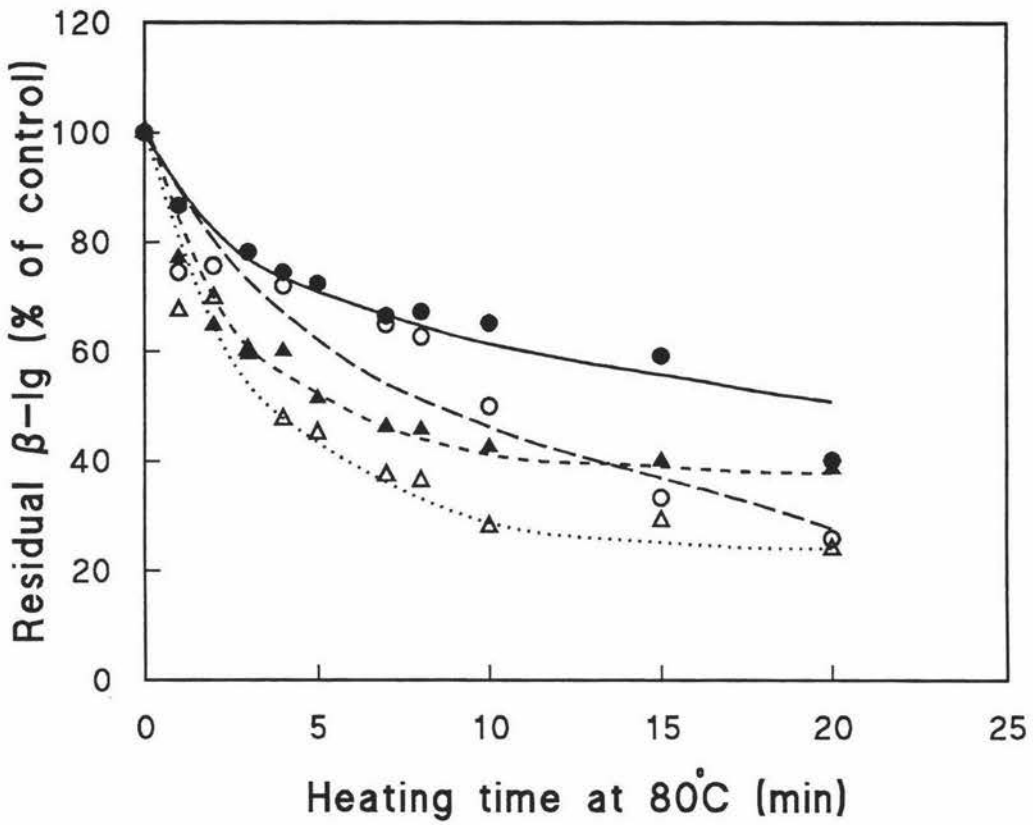


Figure 4.12 The effect of heating time at 80°C on the loss of residual monomeric β-lactoglobulin (quantitative SDSNR-PAGE) from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% -lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

of β-lactoglobulin and casein micelles was comparable to that from the mixture of β-lactoglobulin, α-lactalbumin and casein micelles. The loss of β-lactoglobulin from β-lactoglobulin and casein micelles mixture appeared to be gradual with heating time, while the loss of β-lactoglobulin from the other of β-lactoglobulin solutions was faster during the first 10 min of heating and slower thereafter.

4.1.8 Changes in SDS-monomeric β-lactoglobulin concentrations at 95°C (SDSNR-PAGE)

Heating a β-lactoglobulin solution at 95°C resulted in loss of monomeric β-lactoglobulin with increasing heating time (Fig. 4.13). Heating β-lactoglobulin in the presence of casein micelles markedly increased the rate of loss of β-lactoglobulin. However the presence of α-lactalbumin slightly decreased the loss of β-lactoglobulin. The rate of loss of β-lactoglobulin in the mixture containing β-lactoglobulin, α-lactalbumin and casein micelles was comparable to that containing β-lactoglobulin and casein micelles. Loss of β-lactoglobulin in all the β-lactoglobulin containing solution was much more rapid as compared to losses at 80°C.

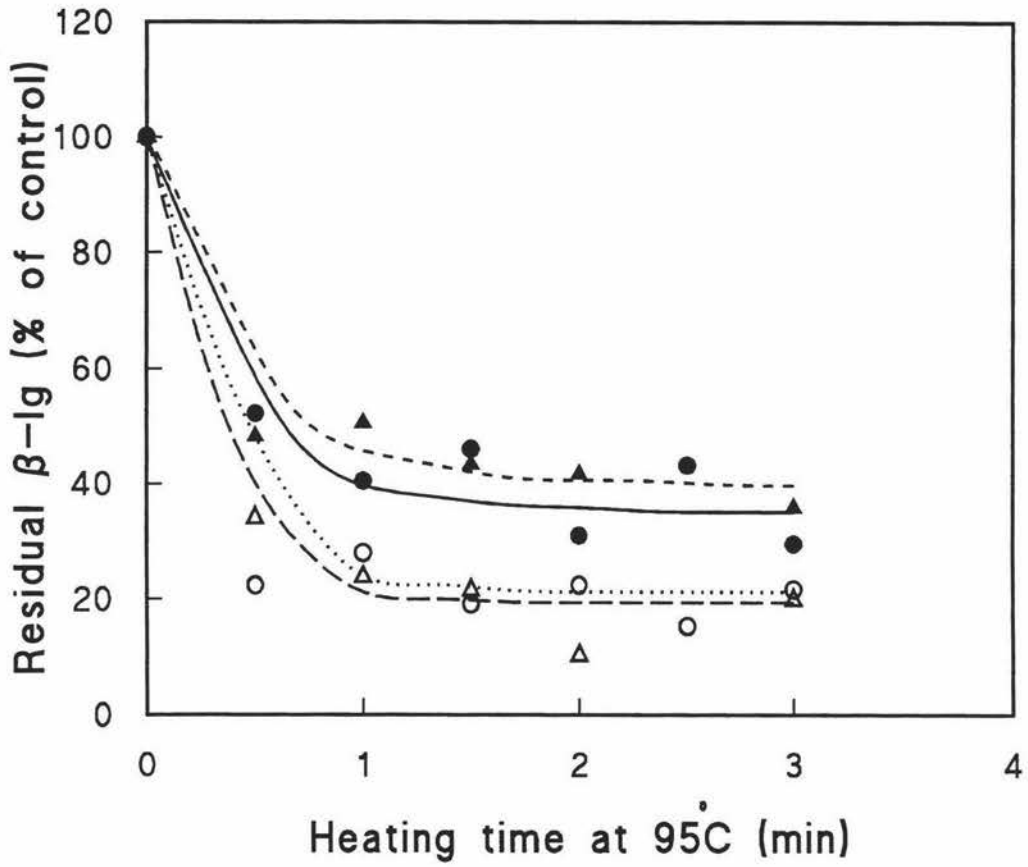


Figure 4.13 The effect of heating time at 95°C on the loss of residual monomeric β-lactoglobulin (quantitative SDSNR-PAGE) from solutions containning 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.1.9 Changes in SDS-monomeric α-lactalbumin concentrations at 80°C (SDSNR-PAGE)

When α-lactalbumin was heated either alone or in the presence of casein micelles and analysed by SDSNR-PAGE, it appeared that the intensity of the α-lactalbumin bands remained constant with time and no aggregates were formed (gels not shown). However, when mixtures of α-lactalbumin and β-lactoglobulin and that of α-lactalbumin, β-lactoglobulin and casein micelles were heated, high molecular weight material, located at the top of the stacking gel, was formed and the intensity of the monomeric α-lactalbumin band decreased with heating time.

No significant changes occurred in the quantity of SDS-monomeric α-lactalbumin, when it was heated alone or in the presence of casein micelles (Fig. 4.14). However, considerable losses of α-lactalbumin occurred when α-lactalbumin was heated in the presence of either β-lactoglobulin or both β-lactoglobulin and casein micelles. The losses of α-lactalbumin from these two latter mixtures were comparable (Fig. 4.14).

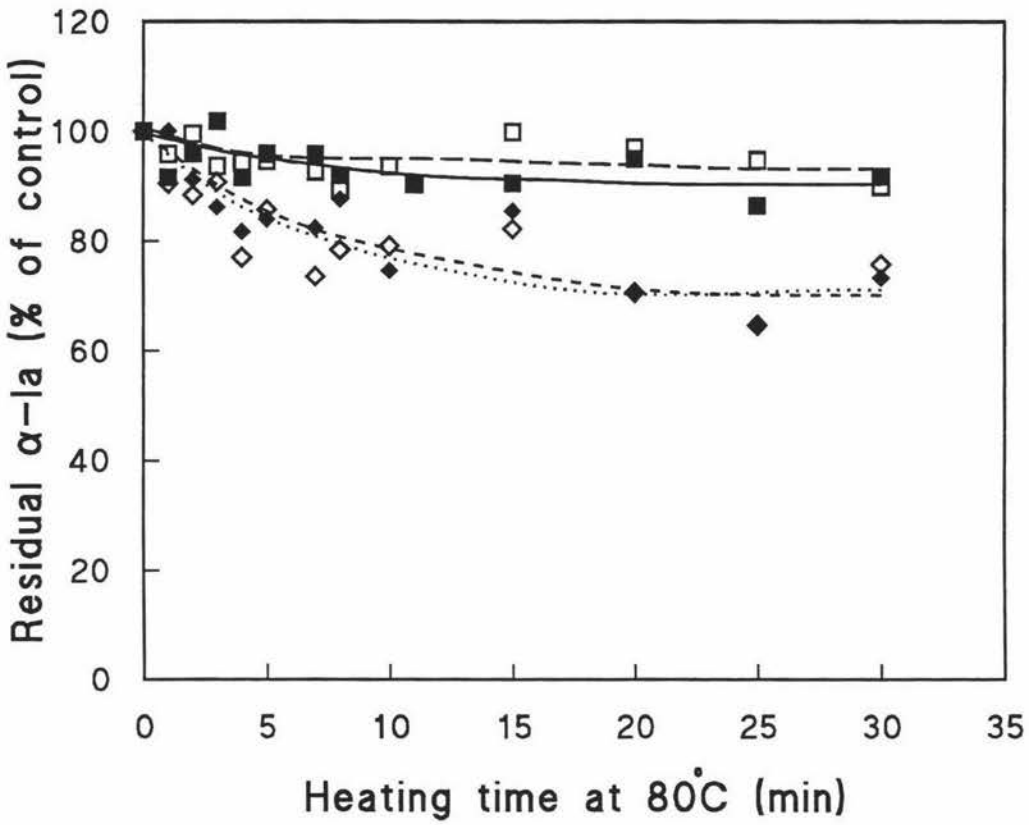


Figure 4.14 The effect of heating time at 80°C on the loss of residual monomeric α-lactalbumin (quantitative SDSNR-PAGE) from solutions containing 0.15% α-lactalbumin (■), 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), and 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.1.10 Changes in SDS-monomeric α-lactalbumin concentrations at 95°C (SDSNR-PAGE)

When α-lactalbumin was heated either separately or in the presence of casein micelles, no aggregates were observed, though the intensity of the α-lactalbumin bands decreased slightly with time (Fig. 4.15). Addition of β-lactoglobulin to these mixtures resulted in aggregate formation and rapid decreases in the intensity of the α-lactalbumin bands (gels not shown).

When heated separately, the amount of residual monomeric α-lactalbumin resolved on SDSNR-PAGE decreased with heating time (Fig. 4.16). Heating α-lactalbumin in the presence of either β-lactoglobulin or casein micelles, resulted in comparable losses of α-lactalbumin. Loss of α-lactalbumin in the mixture containing α-lactalbumin, β-lactoglobulin and casein micelles was about 20% faster than that in the other three mixtures.

Figure 4.15 SDSNR-PAGE on the supernatants (100 000 **g** for 60 min) obtained from a 0.15% α-lactalbumin solution, heated at 95°C for 0(1,2), 1(3), 2(4), 3(5), 5(6), 8(7), 15(8) and 30(9) min.

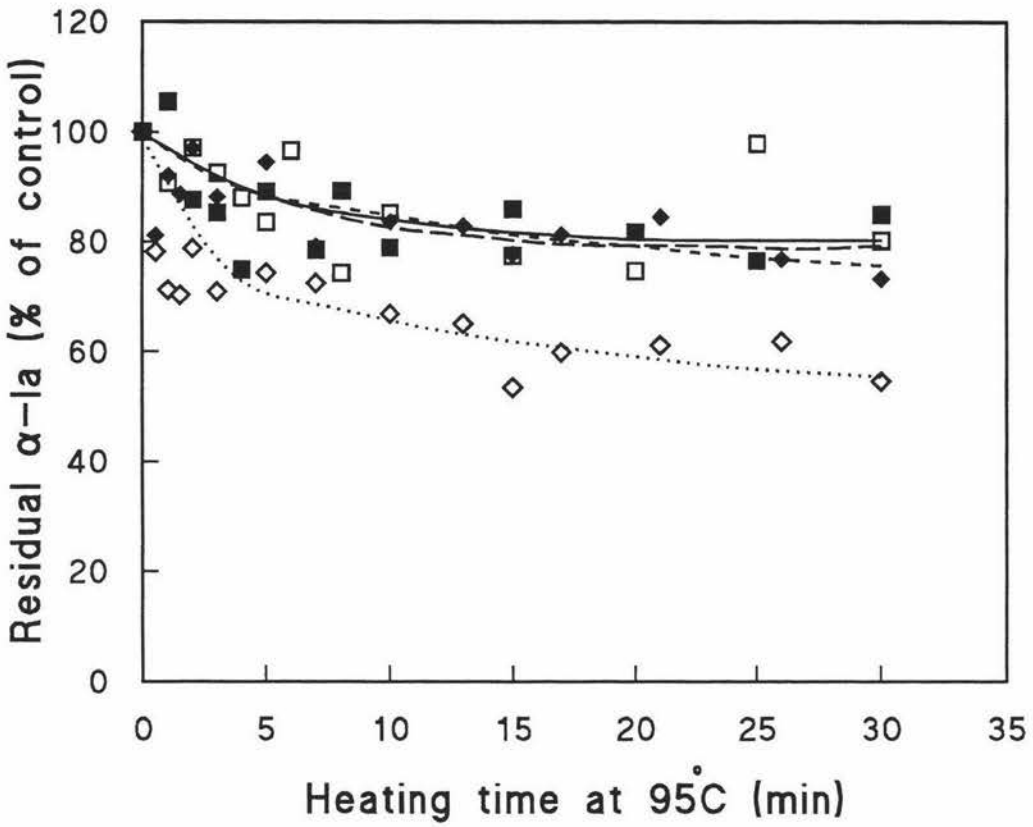


Figure 4.16 The effect of heating time at 95°C on the loss of residual monomeric α-lactalbumin (quantitative SDSNR-PAGE) from solutions containing 0.15% α-lactalbumin (■), 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), and 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.1.11 Analysis of the sediments

The sediments obtained from ultracentrifugation of heated β-lactoglobulin solution or solutions containing β-lactoglobulin, α-lactalbumin and casein micelles were also analysed by SDSNR-PAGE. The results showed aggregated material in the heated β-lactoglobulin containing systems which failed to migrate into the stacking gel. The intensity of aggregates increased with heating time, while that of β-lactoglobulin and α-lactalbumin bands remained constant (gels not shown).

All aggregates and high molecular weight protein bands located at the top of the separating gel disappeared when SDS-PAGE was carried out in the presence of 2-mercaptoethanol. This indicates that the aggregates that were present in the sediment were linked by disulphide bonds only and there was no or little non-covalently linked material in the sediment.

4.1.12 Comparison of native- and SDSNR-PAGE results for β-lactoglobulin

Figure 4.17 compares the loss of monomeric β-lactoglobulin from the native- and SDSNR-PAGE when it was heated at 80°C either separately or in the presence of casein micelles. In both systems the loss of monomeric protein from SDSNR-PAGE was lower than that from the native-PAGE. When β-lactoglobulin was heated alone the differences observed between the PAGE results were slight initially, but became greater in the latter stages of heating, indicating that less non-covalently linked aggregates were formed during the initial stages of heating (Fig. 4.17A).

However, in the mixture of β-lactoglobulin and casein micelles, the initial differences between the amounts of monomeric β-lactoglobulin in SDSNR-PAGE and native-PAGE were somewhat greater suggesting that during the heating-up period to this temperatures, β-lactoglobulin had aggregated through non-covalent interactions (Fig. 4.17B). This difference became less with heating time indicating the loss of the non-covalently bonded aggregates of β-lactoglobulin.

The mixture containing β-lactoglobulin and α-lactalbumin, showed that the amount of β-lactoglobulin resolved on native-PAGE was slightly greater than that resolved on SDSNR-PAGE (Fig. A5 *Appendix*). In the mixture containing β-lactoglobulin, α-lactalbumin and casein micelles, the amounts of β-lactoglobulin resolved on native-PAGE were comparable with that on resolved on SDSNR-PAGE.

At 95°C, with the exception of the solution where β-lactoglobulin was heated alone, the differences observed between native- and SDSNR-PAGE results for β-lactoglobulin were slight implying that these mixtures did not contain much β-lactoglobulin that was non-covalently aggregated.

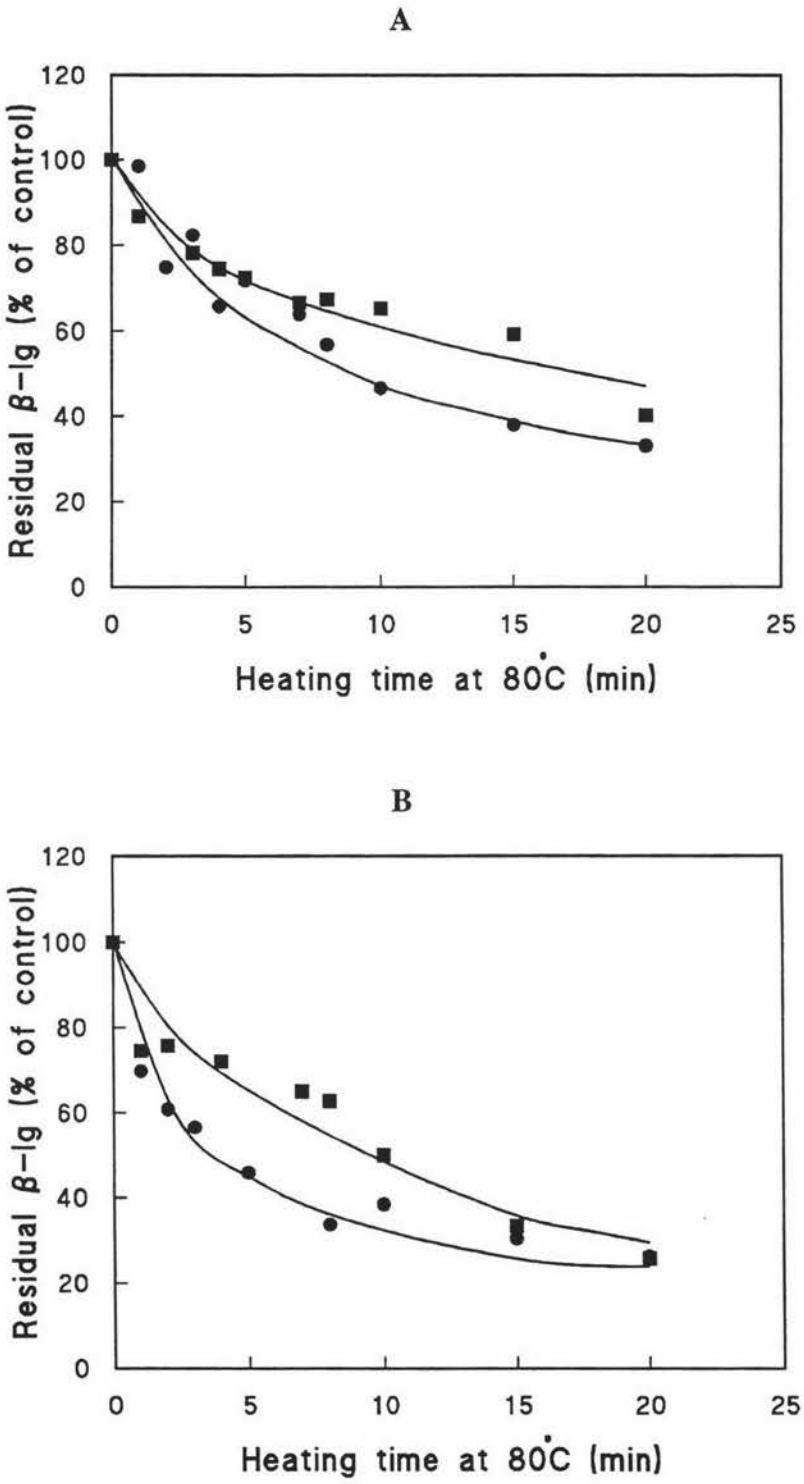


Figure 4.17 The effect of heating time at 80°C on the quantity of β-lactoglobulin remaining from solutions containing (A) 0.4% β-lactoglobulin and (B) 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native-(●)and SDSNR-PAGE (■).

4.1.13 Comparison of native- and SDSNR-PAGE results for α-lactalbumin

In the mixtures containing α-lactalbumin and β-lactoglobulin or α-lactalbumin, β-lactoglobulin and casein micelles heated at 80°C, the amount of α-lactalbumin resolved on SDSNR-PAGE was slightly greater than that resolved on native-PAGE (Fig. 4.18). In both cases, the differences increased with time, suggesting that more non-covalent aggregates were formed with time.

Figures 4.19 and 4.20 compares the loss of monomeric α-lactalbumin when it was heated at 95°C in various mixtures as analysed by native-and SDSNR-PAGE. In all α-lactalbumin mixtures studied, the amounts of monomeric α-lactalbumin in SDSNR-PAGE were generally greater (Fig. 4.19 and 4.20). The differences between the two PAGE results increased with heating time, in all systems, suggesting that more non-covalent linked aggregates were formed as heating time increased.

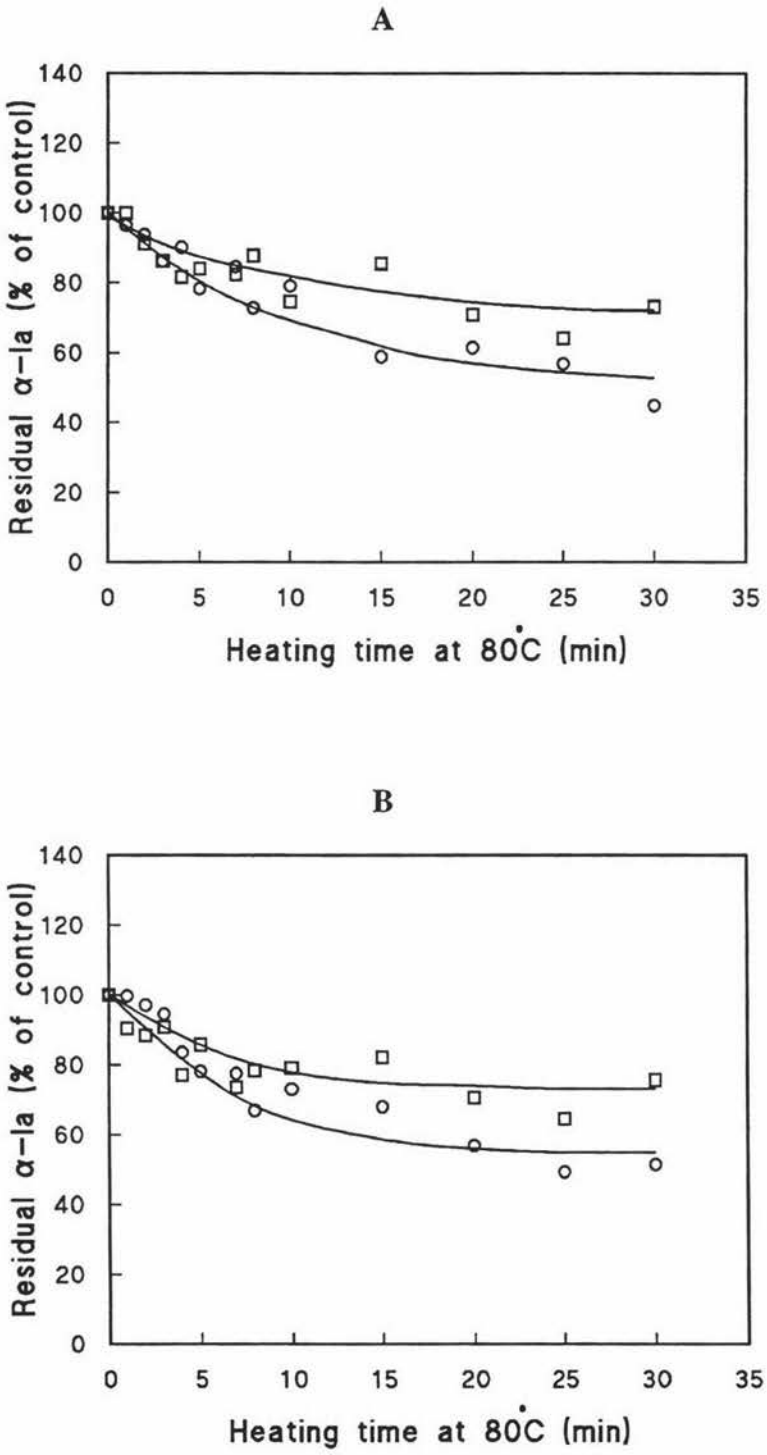


Figure 4.18 The effect of heating time at 80°C on the quantity of α-lactalbumin remaining from solutions containing (A) 0.15% α-lactalbumin and 0.4% β-lactoglobulin and (B) 0.15% α-lactalbumin, 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native- (O) and SDSNR-PAGE (□).

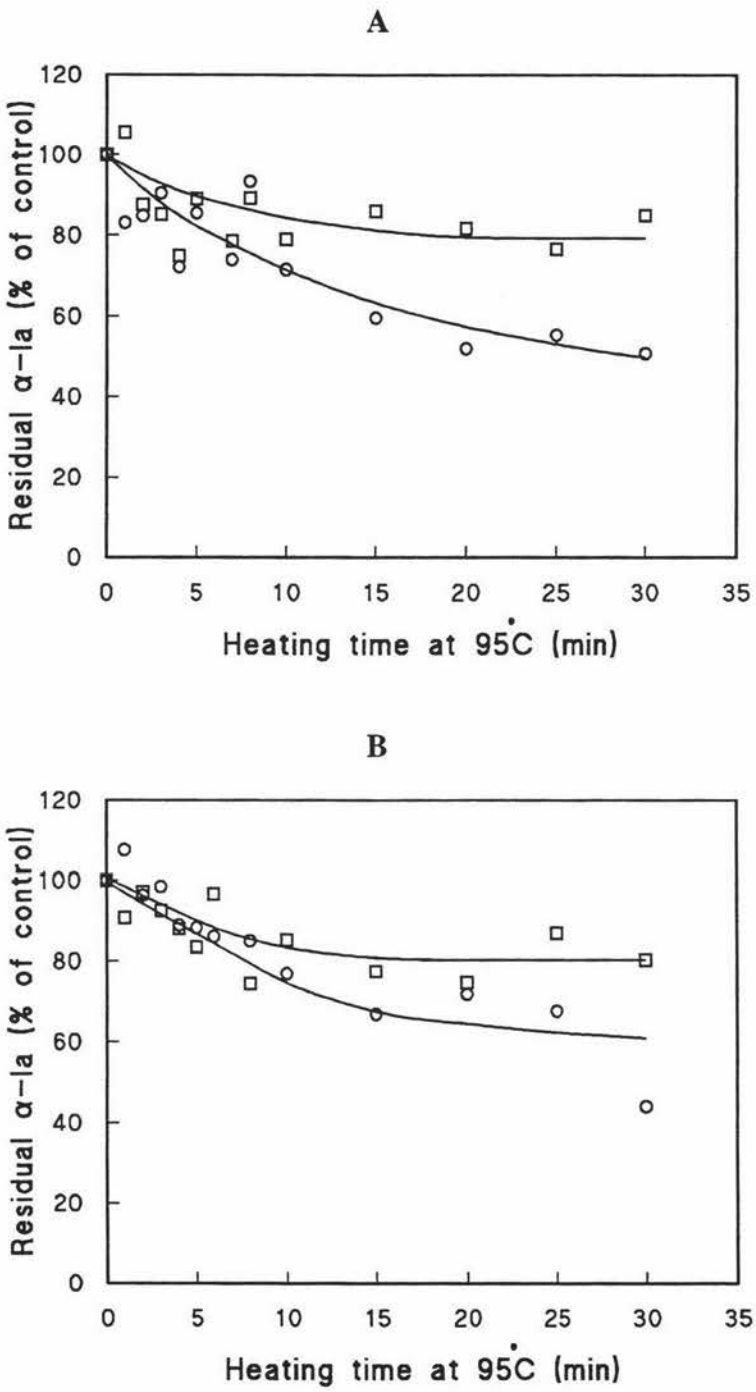


Figure 4.19 The effect of heating time at 95°C on the quantity of α-lactalbumin remaining from solutions containing (A) 0.15% α-lactalbumin and (B) 0.15% α-lactalbumin and ~ 2.6% casein micelles as resolved on native- (O) and SDSNR-PAGE (□).

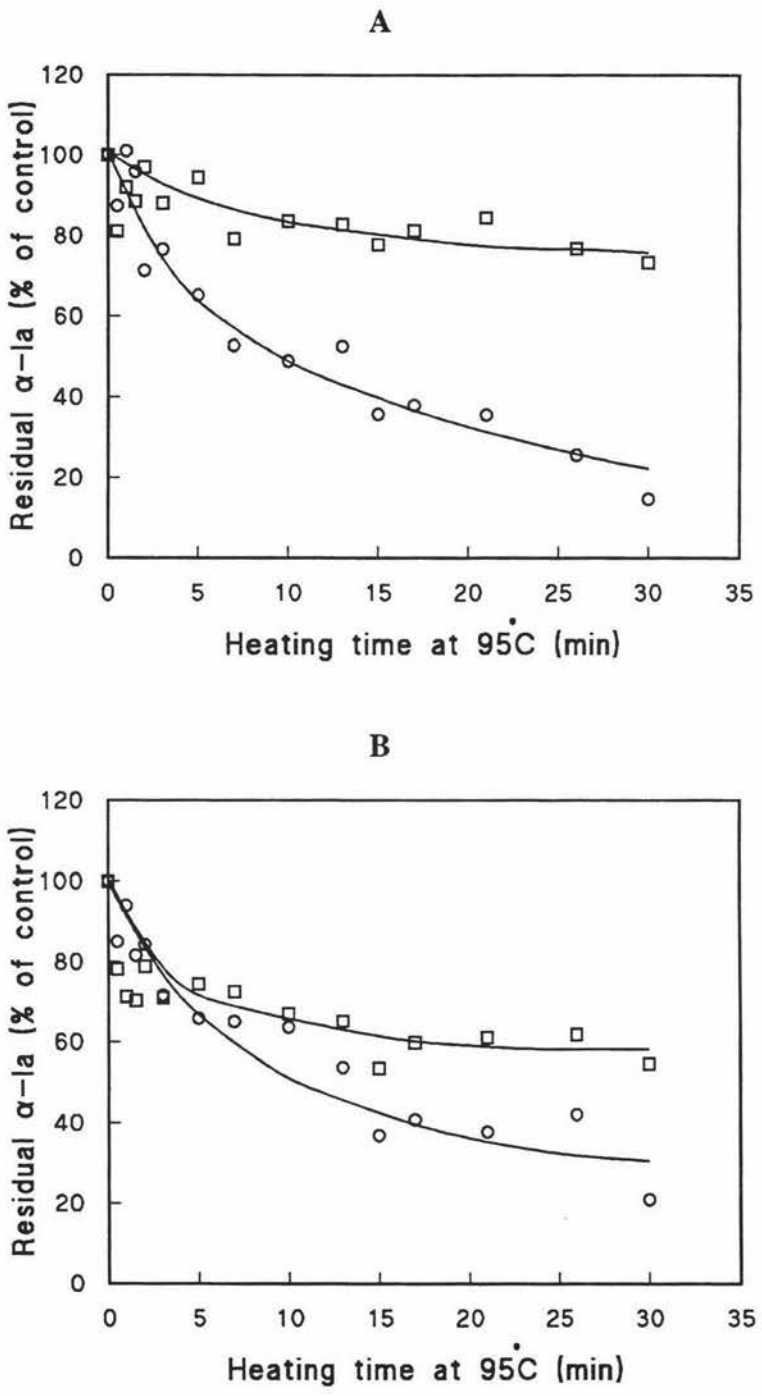


Figure 4.20 The effect of heating time at 95°C on the quantity of α-lactalbumin remaining from solutions containing (A) 0.15% α-lactalbumin and 0.4% β-lactoglobulin and (B) 0.15% α-lactalbumin, 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native- (○) and SDSNR-PAGE (□).

4.1.14 Discussion

The results of this study show that when β-lactoglobulin was heated and examined by electrophoresis, the quantity of SDS-monomeric protein decreased as a consequence of heating (Figs. 4.12). However, the extent of decrease was somewhat smaller than that found for the decrease in native β-lactoglobulin (Fig. 4.2). β-Lactoglobulin material that does not to give native-like monomers in native-PAGE sample buffer (0.05M TRIS, pH 8.4) but does disperse in 0.1% SDS to give monomeric β-lactoglobulin has been postulated to be linked by hydrophobic interactions (McSwiney *et al.*, 1994; Gezimati *et al.*, 1996). The extent of hydrophobic aggregate formation can be seen as the difference between β-lactoglobulin concentrations on native-PAGE and that on SDSNR-PAGE (Fig 4.17). It was found that no hydrophobic aggregates of β-lactoglobulin were present in the sediment. Thus the hydrophobic aggregates determined in the supernatant represent "total" hydrophobic aggregates formed in the system as a consequence of heating.

It appears that from this study that on heating β-lactoglobulin at 0.4% (w/v) concentrations, less hydrophobic aggregates were formed compared to the results reported by McSwiney *et al.* (1994), who heated β-lactoglobulin at 10% concentration. Data presented in Figure 4.17 showed that when β-lactoglobulin was heated in the presence of casein micelles, some hydrophobic aggregates were formed, especially during the initial stages of heating. This is consistent with the results reported by Jang and Swaisgood (1990) who found predominance of hydrophobic interactions in the initial stages of complex formation between β-lactoglobulin and casein micelles. Haque and Kinsella (1988), using model systems of *k*-casein and β-lactoglobulin (20mM imidazole buffer, pH 6.8), found that the two proteins interacted primarily *via* hydrophobic interactions on heating at 70°C for up to 12 min, but covalent interactions took place with further heating.

In the mixtures containing α-lactalbumin, i.e. β-lactoglobulin and α-lactalbumin or β-lactoglobulin, α-lactalbumin and casein micelles mixture, no or very little hydrophobic aggregates of β-lactoglobulin were formed. Since α-lactalbumin possesses two disulphide bonds per molecules, it is likely that in presence of α-lactalbumin during

heating, thiol-disulphide interchange reactions are favoured over the hydrophobic interactions between different protein molecules. Thus α-lactalbumin appears to promote thiol-disulphide interactions in heated protein mixtures.

Heating α-lactalbumin either separately or in the presence of casein micelles at 80°C caused no decrease in the concentrations of SDS-monomeric α-lactalbumin (Fig 4.14). However decreases occurred during heating at 95°C, suggesting that a more severe (higher) temperature is essential for loss of the SDS-monomeric α-lactalbumin (Fig. 4.16) Figures 4.20A,B suggest that at this higher temperature, α-lactalbumin aggregates held together by hydrophobic interactions were formed.

In the mixtures containing α-lactalbumin and β-lactoglobulin and that containing α-lactalbumin, β-lactoglobulin and casein micelles, considerable decreases of α-lactalbumin occurred, possibly due to hydrophobic interactions between β-lactoglobulin and α-lactalbumin, and between α-lactalbumin and α-lactalbumin. In addition there was also an evidence for the formation of disulphide linked aggregates. More interactions took place at 95°C than at 80°C.

Overall it can be concluded that the "soluble" β-lactoglobulin aggregates (i.e. non-sedimentable at 100.000 **g** for 60 min) formed during heating of various protein mixtures were primarily linked by disulphide bonds, but hydrophobic interactions were more important in the formation of α-lactalbumin aggregates.

4.2 Sedimentation of β-lactoglobulin and α-lactalbumin

The unheated and heated protein solutions were centrifuged at 100,000 **g** for 60 min, and the supernatants dispersed in a buffer containing 2% SDS and 0.05% and 2-mercaptoethanol, i.e. SDS reducing-PAGE (SDSR-PAGE). This system solubilised and dispersed all of the aggregates that had been excluded from the native- and SDSNR gels and converted them into their individual components. Therefore, this PAGE-PAGE system measured the total amounts of non-sedimentable β-lactoglobulin and α-lactalbumin. The loss of β-lactoglobulin and α-lactalbumin from the supernatant is due to sedimentation of large aggregated material.

4.2.1 Changes in non-sedimentable β-lactoglobulin concentrations at 80°C (SDSR-PAGE)

Typical electrophoretic patterns of the supernatants samples that had been dispersed in SDS reducing-buffer are shown in Figure 4.21. In unheated β-lactoglobulin containing systems, a number of bands which moved faster than the β-lactoglobulin band were observed. When comparing this with SDSNR-PAGE results, it appears the presence of mercaptoethanol affected the mobility of these bands. In the heated samples, the high molecular weight protein aggregates located at the top of the stacking and resolving gels that were present in SDSNR-PAGE disappeared completely. This suggested that the polymerised protein material was cross-linked via disulphide bonds. The intensities of β-lactoglobulin bands in all four systems studied decreased with increasing heating time at 80°C.

The changes in the concentrations of non-sedimentable β-lactoglobulin are shown in Figure 4.22. The quantity of non-sedimentable β-lactoglobulin decreased slightly with heating time when β-lactoglobulin solution was heated separately. Heating β-lactoglobulin in the presence of casein micelles had no further effect on the loss of β-lactoglobulin. The loss of β-lactoglobulin from the β-lactoglobulin and α-lactalbumin mixture was slightly lower than that of β-lactoglobulin alone. However, heating β-lactoglobulin, α-lactalbumin and casein micelles mixture resulted in a marked increase in the loss of β-lactoglobulin.

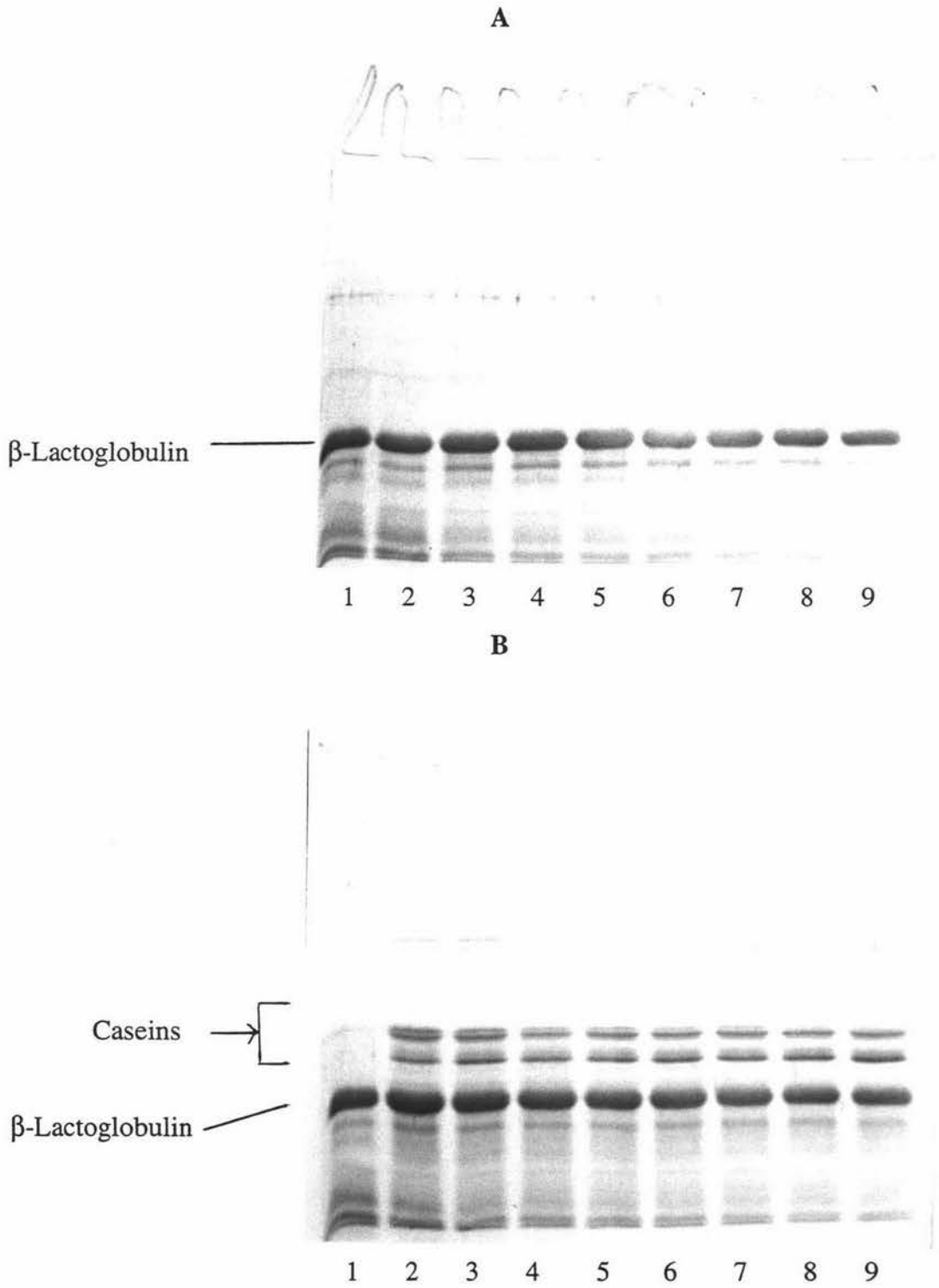


Figure 4.21 SDSR-PAGE on the supernatants (100 000 **g** for 60 min) obtained from solutions containing (A) a 0.4% β-lactoglobulin and (B) 0.4% β-lactoglobulin and ~ 2.6% casein micelles, heated at 80°C for 0(1,2), 1(3), 2(4), 3(5), 5(6), 8(7), 15(8) and 30(9) min.

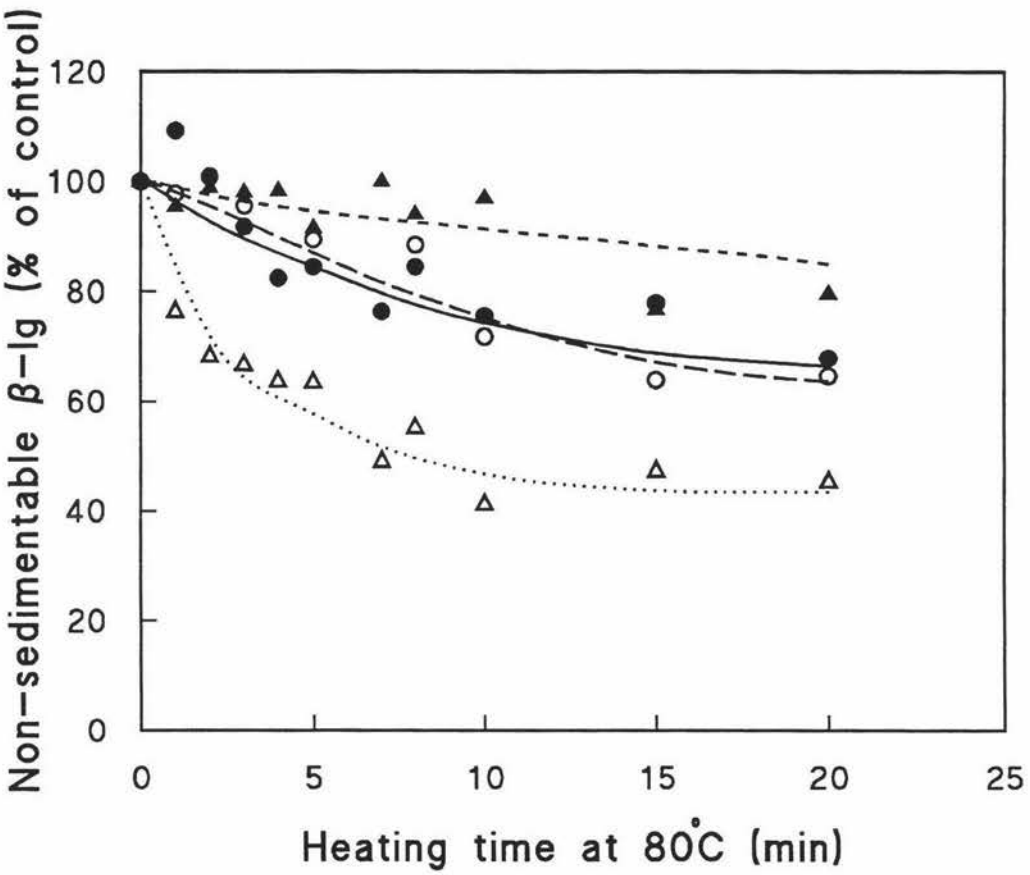


Figure 4.22 The effect of heating time at 80°C on the loss of non-sedimentable β-lactoglobulin (quantitative SDSR-PAGE) from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.2.1.1 Kinetics of loss of non-sedimentable β-lactoglobulin

The initial rate of loss of non-sedimentable β-lactoglobulin (up to 8 min heating) in the four mixtures studied could approximately be described by second-order reaction kinetics (Fig. 4.23) although r^2 values were lower than those obtained for the loss of native proteins (Fig. 4.3). The kinetic data is shown in Table 4.3. K_n value for loss of β-lactoglobulin when heated separately was 0.0332 $g^{-1}min^{-1}l$. It appeared that the addition of α-lactalbumin or casein micelles to the β-lactoglobulin solution, reduced the value of K_n. In contrast, when they were added together, K_n for β-lactoglobulin increased approximately 4 fold compared to that of β-lactoglobulin heated alone. The K_n values were in the order : β-lactoglobulin + α-lactalbumin + casein mixture > β-lactoglobulin alone > β-lactoglobulin + casein micelle mixture > β-lactoglobulin + α-lactalbumin mixture.

Table 4.3 Kinetic data for the loss of β-lactoglobulin from different mixtures at 80°C (second-order reaction equation).

Sample	Degrees of Freedom	r^2	Rate constant (K_n) $g^{-1}min^{-1}l$	Standard Error for K_n
β-lg alone	7	0.7260	0.0332	0.0057
β-lg + casein micelles	5	0.8401	0.0173	0.0024
β-lg + α-la	7	0.8160	0.0069	0.0025
β-lg + α-la + casein micelles	7	0.8494	0.1300	0.0119

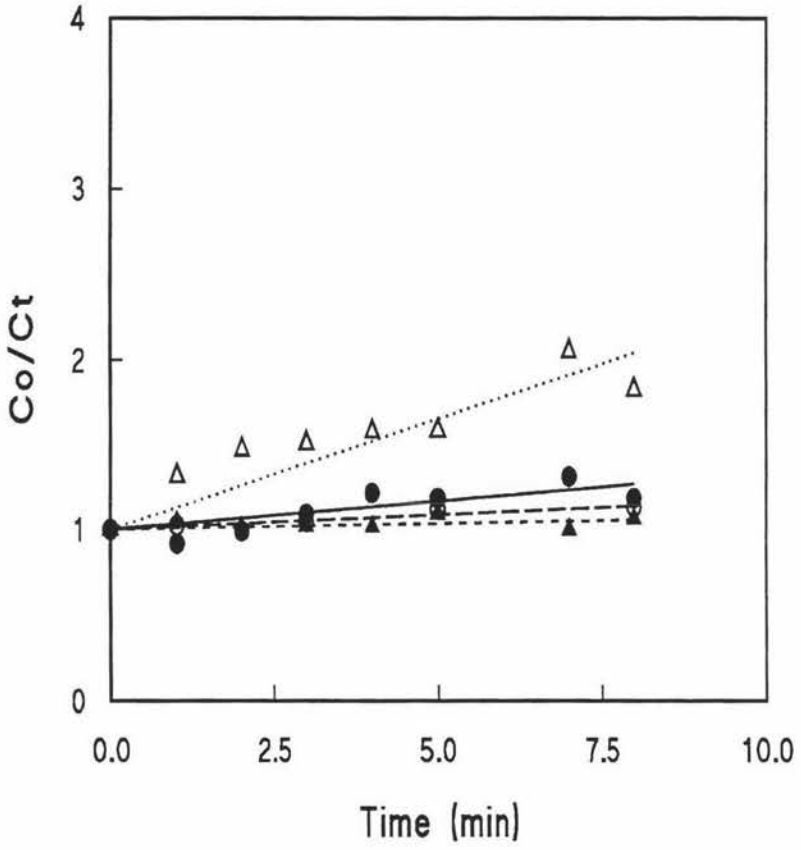


Figure 4.23 Kinetic plots (second order reaction equation) for the loss of non-sedimentable β-lactoglobulin (quantitative SDSR-PAGE) at 80°C from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (o), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.2.2 Changes in non-sedimentable β-lactoglobulin concentrations at 95°C (SDSR-PAGE)

Figure 4.24 shows the initial changes (0 to 5 min) in the concentration of non-sedimentable β-lactoglobulin when heated at 95°C in the four mixtures. Heating β-lactoglobulin either alone or in the presence of α-lactalbumin resulted in similar rates of depletion of β-lactoglobulin. However, the loss of β-lactoglobulin was substantially faster when β-lactoglobulin was heated in the presence of casein micelles or both casein micelles and α-lactalbumin.

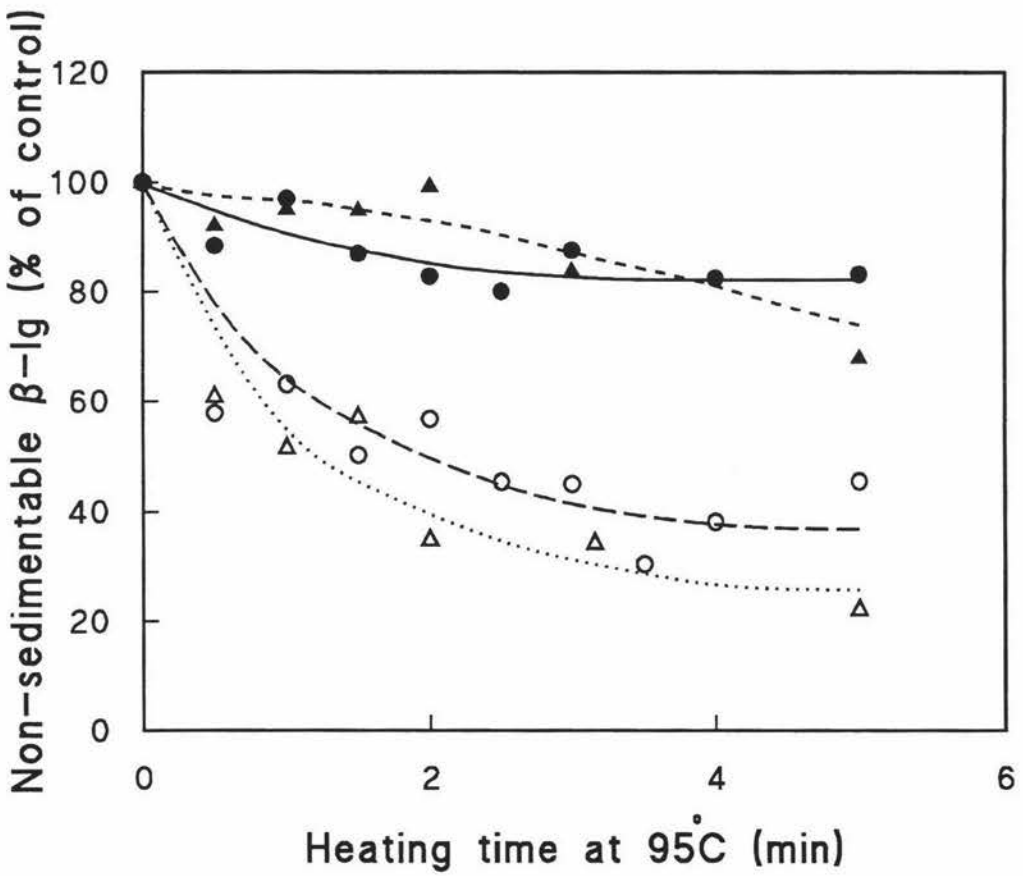


Figure 4.24 The effect of heating time at 95°C on the loss of non-sedimentable β-lactoglobulin (quantitative SDSR-PAGE) from solutions containing 0.4% β-lactoglobulin solution (•), 0.4% β-lactoglobulin and ~ 2.6% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.6% casein micelles (△).

4.2.3 Changes in non-sedimentable α-lactalbumin concentrations at 80°C (SDSR-PAGE)

The intensity of α-lactalbumin bands from the solutions of either α-lactalbumin heated alone or with casein micelles remained constant with time. However, the α-lactalbumin bands from the mixtures containing β-lactoglobulin decreased in intensity with increase in heating time (gels not shown).

There were only minor changes in the quantities of non-sedimentable α-lactalbumin when α-lactalbumin was heated either alone or in the presence of casein micelles. Loss of α-lactalbumin was ~ 10% faster when it was heated in the presence of β-lactoglobulin. Heating the mixture containing α-lactalbumin, β-lactoglobulin and casein micelles further increased the rate of loss of α-lactalbumin (Fig 4.25).

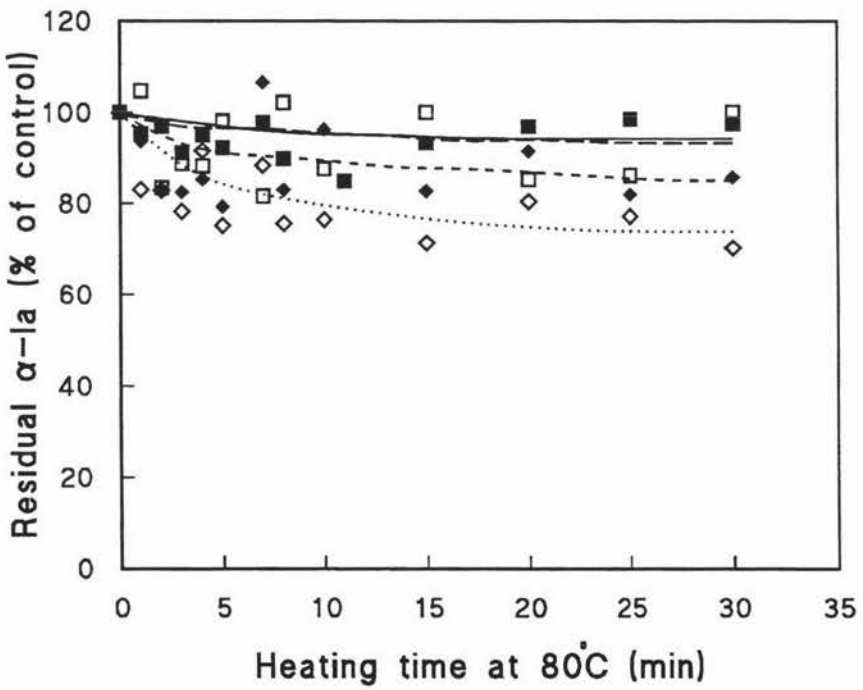


Figure 4.25 The effect of heating time at 80°C on the loss of non-sedimentable α-lactalbumin (quantitative SDSR-PAGE) from a 0.15% α-lactalbumin solution (■) and solutions containing 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.2.4 Changes in non-sedimentable α-lactalbumin concentrations at 95°C (SDSR-PAGE)

The changes in the quantities of non-sedimentable α-lactalbumin are shown in Figure 4.26). Minimal losses of α-lactalbumin occurred when an α-lactalbumin solution was heated at 95°C. Heating α-lactalbumin in the presence of β-lactoglobulin or casein micelles slightly increased the loss of α-lactalbumin. However, the presence of both proteins in the α-lactalbumin solution markedly increased the rate of loss of non-sedimentable α-lactalbumin. About 40% of total α-lactalbumin was lost after 30 min heating.

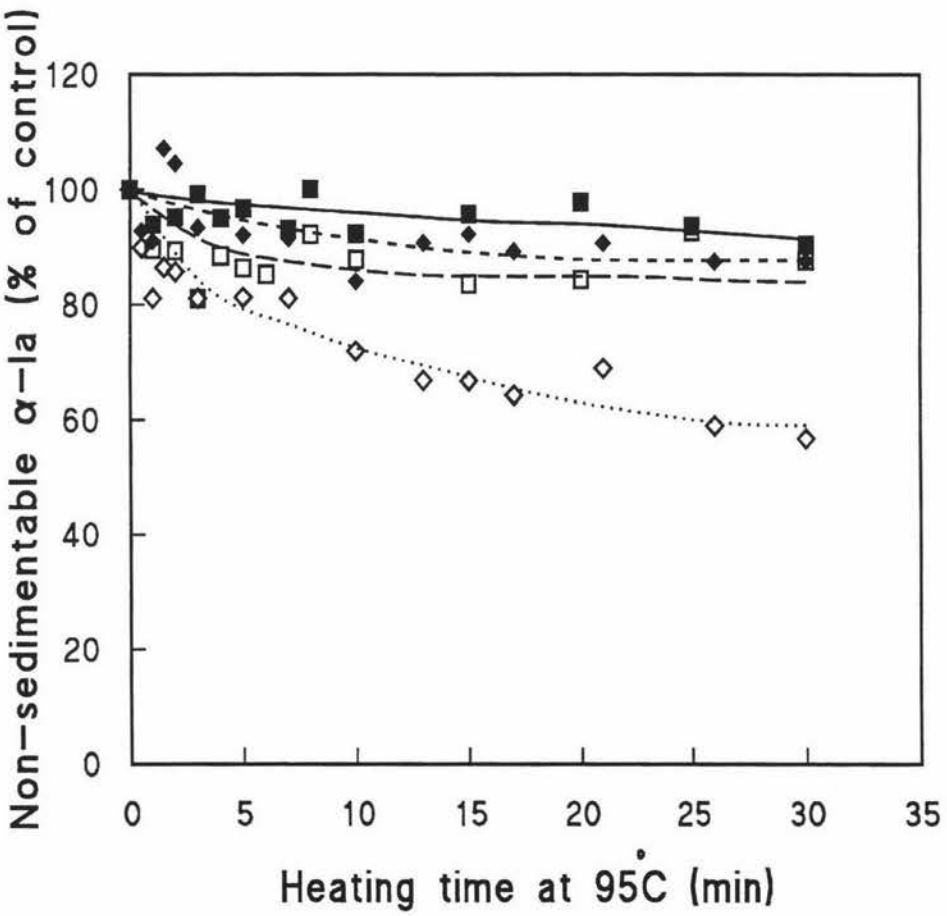


Figure 4.26 The effect of heating time at 95°C on the loss of non-sedimentable α-lactalbumin (quantitative SDSR-PAGE) from solutions containing 0.15% α-lactalbumin (■), 0.15% α-lactalbumin and ~ 2.6% casein micelles (□), 0.15% α-lactalbumin and 0.4% β-lactoglobulin (♦), and 0.15% α-lactalbumin and 0.4% β-lactoglobulin and ~ 2.6% casein micelles (◊).

4.2.5 Analysis of sediments

Qualitatively, β-lactoglobulin and α-lactalbumin in the sediments appear to increase with heating time in all systems. Typical electrophoretic patterns of the sediment samples that had been dispersed in SDS reducing-buffer are shown in Figures A5, and A6 (*Appendix*). However, it was difficult to get reproducible quantitative results. In order to get enough β-lactoglobulin and α-lactalbumin to quantify, an overloading of casein micelles was required (Fig. A6). Considerable amounts of β-lactoglobulin and α-lactalbumin were present at zero heating times (Fig. A6), in the mixtures containing casein micelles, which may have been the proteins trapped within the casein micelles.

4.2.6 Comparison of native- and SDSR-PAGE results for β-lactoglobulin

Figures 4.27 and 4.28 compares the loss of non-sedimentable and native β-lactoglobulin in β-lactoglobulin containing protein solutions when they were heated at 80°C. In all four systems, the loss of non-sedimentable β-lactoglobulin was lower than that of native β-lactoglobulin. The difference between the two PAGE results represent the total "soluble" aggregates formed in the systems. When β-lactoglobulin was heated alone the differences observed between the PAGE results were slight initially and marked in the later stages of heating, indicating that few "soluble" aggregates were formed during the initial stages of heating (Fig 4.27A), but more were formed with increasing heating time. Large and consistent amounts of "soluble" β-lactoglobulin aggregates appeared to have been formed with heating time when β-lactoglobulin was heated in the presence of casein micelles or α-lactalbumin(Figs 4.27B and 4.28A). Relatively small amounts of "soluble" aggregates were observed in the mixture containing β-lactoglobulin, α-lactalbumin and casein micelles.

At 95°C, a very large amount of β-lactoglobulin"soluble" aggregates were formed when β-lactoglobulin was heated separately at 95°C (Fig. A.7A) Relatively fewer "soluble" aggregates were formed when β-lactoglobulin was heated in the presence of casein micelles (Fig. A7B). The presence of α-lactalbumin in the system resulted in aggregates that were less than those when β-lactoglobulin was heated alone, but more than those formed when β-lactoglobulin was heated in the presence of casein micelles (Fig. A8A). The least amount of β-lactoglobulin"soluble" aggregates were formed in the mixture containing β-lactoglobulin, α-lactalbumin and casein micelles (Fig A8B).

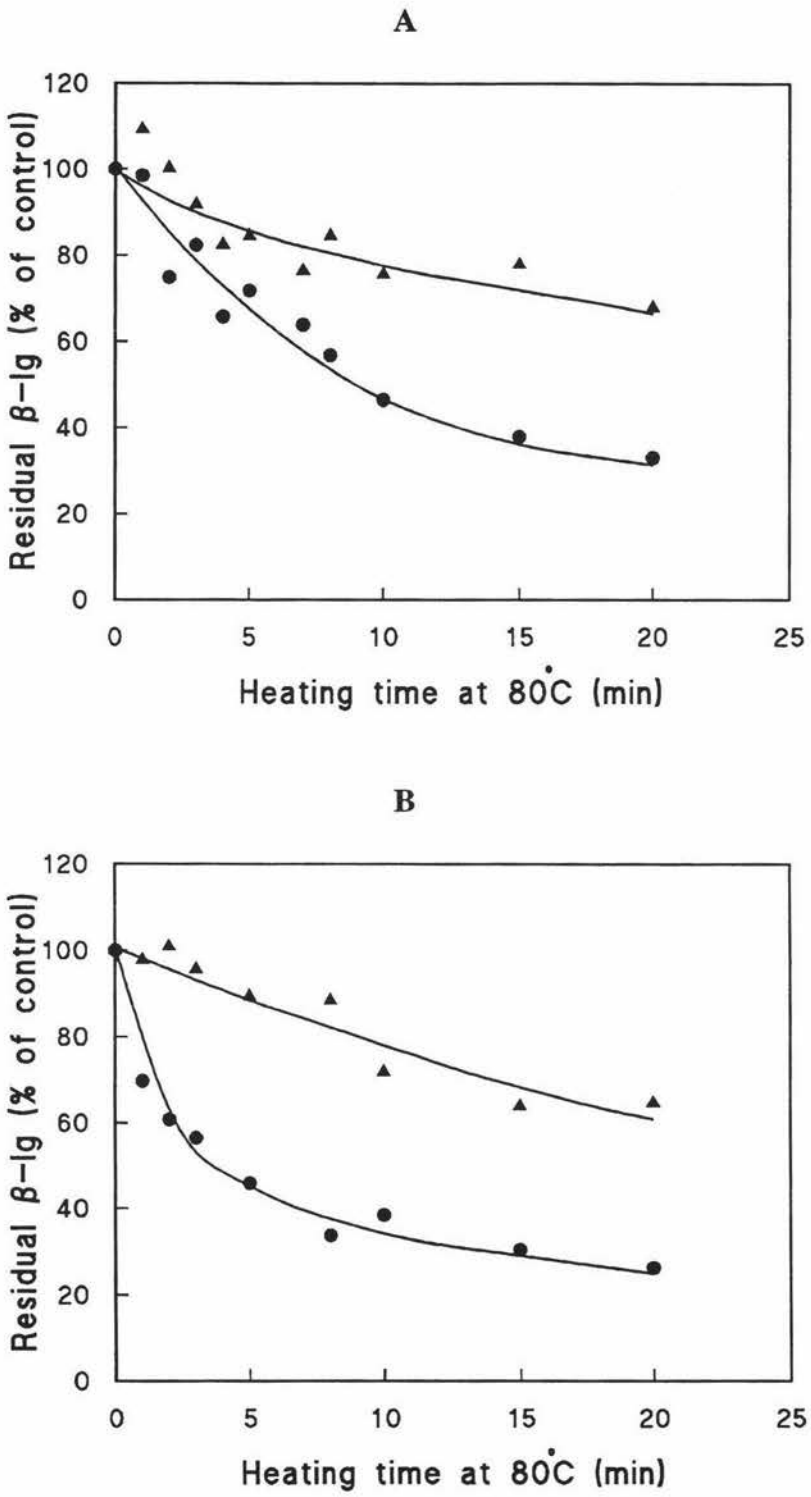


Figure 4.27 The effect of heating time at 80°C on the quantity of β-lactoglobulin remaining from solutions containing (A) 0.4% β-lactoglobulin and (B) 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native- (•) and SDSR-PAGE (▲).

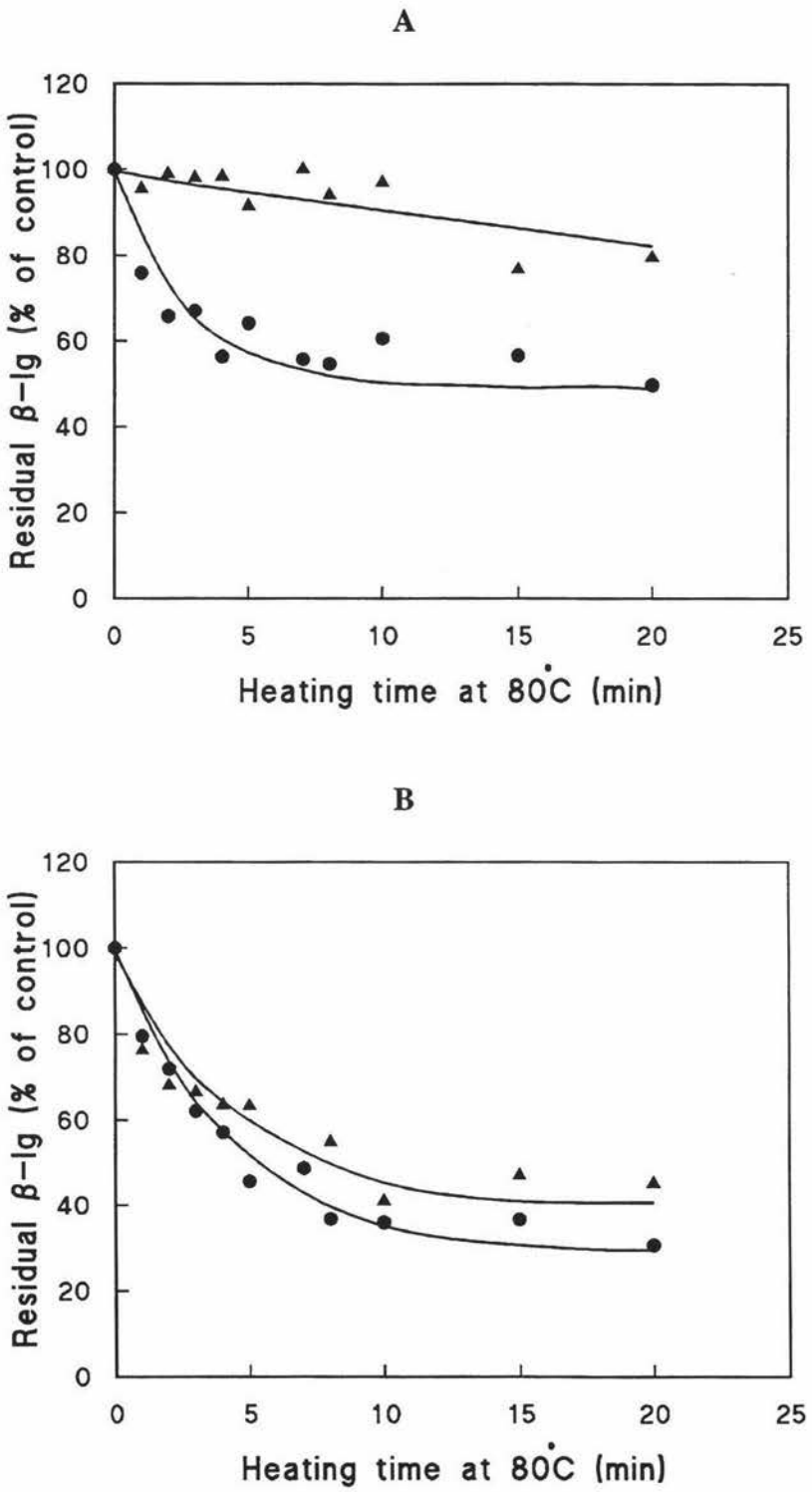


Figure 4.28 The effect of heating time at 80°C on the quantity of β-lactoglobulin remaining from solutions containing (A) 0.4% β-lactoglobulin and 0.15% α-lactalbumin (B) 0.4% β-lactoglobulin, 0.15% α-lactalbumin and ~ 2.6% casein micelles as resolved on native- (•) and SDSR-PAGE (▲).

4.2.7 Comparison of native- and SDSR-PAGE results for α-lactalbumin

Comparison of the rate of loss of non-sedimentable α-lactalbumin with native α-lactalbumin, in the mixture of α-lactalbumin and β-lactoglobulin or α-lactalbumin, β-lactoglobulin and casein micelles heated at 80°C, showed that the amounts of non-sedimentable α-lactalbumin were greater (Figs 4.29). It appeared that in both these systems, the differences between the amount of non-sedimentable α-lactalbumin and native α-lactalbumin increased with increasing heating time, suggesting that more "soluble" aggregates were formed as heating time increased (Fig 4.32). Slightly less "soluble" aggregates were formed in the mixture of α-lactalbumin, β-lactoglobulin and casein micelles, than in the mixtures containing α-lactalbumin and β-lactoglobulin when heated at 80°C.

Heating α-lactalbumin solutions at 95°C also produced increasing amount of "soluble" aggregates with time (Fig A10, A11 *Appendix*). The largest amount of "soluble" aggregates were formed in the solution containing α-lactalbumin and β-lactoglobulin followed by the α-lactalbumin solution. In the solutions containing α-lactalbumin and casein micelles or α-lactalbumin, β-lactoglobulin and casein micelles, comparable amounts of "soluble" aggregates were formed.

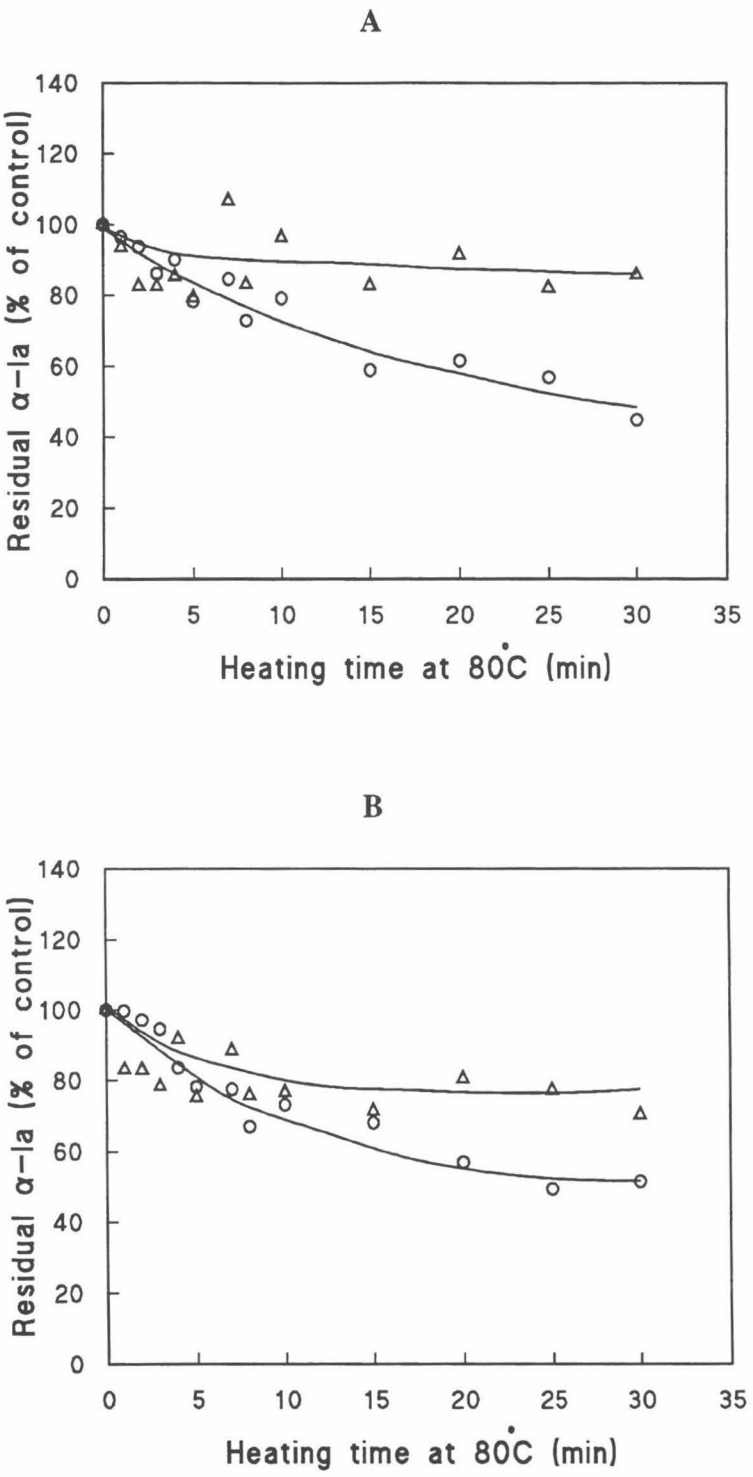


Figure 4.32 The effect of heating time at 80°C on the quantity of α-lactalbumin remaining from solutions containing (A) 0.15% α-lactalbumin and 0.4% β-lactoglobulin and (B) 0.15% α-lactalbumin, 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native- (○) and SDSR-PAGE (△).

4.2.8 Discussion

The present results show that when β-lactoglobulin solutions were heated at 80°C or 95°C, intermolecular aggregates were formed between β-lactoglobulin molecules, and some of these aggregates were large enough to become sedimented by ultracentrifugation at 100,000 **g** for 60 min (Figs. 4.22, 4.25). Analysis of the sediments by electrophoresis showed that these large aggregates were linked by disulphide bonds.

The addition of α-lactalbumin to β-lactoglobulin solutions caused slight decrease in the amounts of sedimentable aggregated β-lactoglobulin both 80 and 95°C, compared to β-lactoglobulin solutions heated alone (Fig. 22, 4.25). In this systems, the aggregates are probably formed between β-lactoglobulin and α-lactalbumin. It is possible that β-lactoglobulin/α-lactalbumin aggregates were somewhat smaller in size than β-lactoglobulin/β-lactoglobulin aggregates, and therefore did not sediment during ultracentrifugation.

The addition of casein micelles to β-lactoglobulin solution and subsequent heating at 80°C appeared to have little further influence on the formation of sedimentable aggregated β-lactoglobulin. However, heating β-lactoglobulin and casein micelles mixture at 95°C led to a faster sedimentation of β-lactoglobulin comparable to β-lactoglobulin heated alone. This is apparently due to interactions between β-lactoglobulin and *k*-casein on the micelles (Smits and Brouwershaven, 1980; Noh *et al.*, 1989; Reddy and Kinsella, 1990). Smits and Brouwershaven (1990) reported that ~ 60% of total β-lactoglobulin was sedimented with the casein micelles, when casein micelles and β-lactoglobulin mixtures were heated at 90°C for 20 min. In contrast no sedimentation of β-lactoglobulin was found to occur when heated alone under similar conditions. From the present results it appears that β-lactoglobulin interacted more efficiently with the casein micelles at 95°C than at 80°C.

When the solution containing β-lactoglobulin, α-lactalbumin and casein micelles was heated at 80 or 95°C, there was marked increase in the amount of sedimentable β-lactoglobulin. This suggests that the presence of α-lactalbumin in the mixture led to a faster interaction of β-lactoglobulin and casein micelles. At 95°C the loss of β-lactoglobulin was slightly faster than that in the mixture containing β-lactoglobulin and

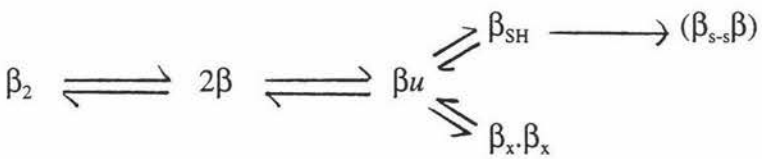
casein micelles. It is likely in this system that in addition to β-lactoglobulin/β-lactoglobulin aggregates, β-lactoglobulin/α-lactalbumin aggregates are also formed which interacted with casein micelles more efficiently. These results are contrary to those of Smits and Brouwershaven (1980) who reported that α-lactalbumin did not influence the heat-induced association of β-lactoglobulin and casein micelles during heating at 90°C for up to 20 min.

When α-lactalbumin was heated alone or in the presence of casein micelles, it did not interact to form large sedimentable aggregates. This is in agreement with previous studies (Baer *et al.*, 1976) which suggest that α-lactalbumin does not interact with casein micelles in the absence of β-lactoglobulin. It is clear that when α-lactalbumin was heated in the presence of β-lactoglobulin, some sedimentable aggregates, consisting of α-lactalbumin/β-lactoglobulin complexes, were formed which resulted in the loss of α-lactalbumin from the solution.

Heating α-lactalbumin in the presence of casein micelles and β-lactoglobulin caused considerable increase in sedimentable aggregated α-lactalbumin. This is due to interaction of β-lactoglobulin/α-lactalbumin complexes with the *k*-casein on the casein micelles. It can be concluded that in this system, α-lactalbumin interacted with β-lactoglobulin and these complexes then associate with the casein micelles. Similar conclusions have been reached by Baer *et al.* (1976) who found that the α-lactalbumin/β-lactoglobulin complex, which was dependent on the molar ratio of both proteins, changed the ability of α-lactalbumin to bind *k*-casein.

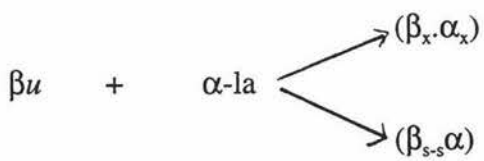
4.3 Possible overall denaturation and aggregation mechanisms

Based of the results of this study and work of other workers, a possible pathway of the reactions that take place when β-lactoglobulin solutions are heated can be postulated (**4.33**). At pH 6.7, β-lactoglobulin exists as a dimer (β_2) (Sawyer, 1968; Briggs and Hull, 1945) which dissociates into monomeric β-lactoglobulin at ~ 30°C (2β). Increase in temperature above 60°C may cause rearrangement of molecular structure, possibly the native structure changing into the molten globule conformation (β*u*) (Kuwajima, 1989). Characteristics of this molten globule state are the loosened tertiary structure, giving greater access to regions of the protein molecules that inaccessible in the native state. It is possible that the protein molecules in the molten globule state can interact through hydrophobic interactions to form non-covalently linked (hydrophobic) aggregates ($\beta_{x\text{-}x}\beta$). Electrophoretic data from this study provided some evidence of formation of such aggregates. With continued heating, β*u* species can further unfold to expose reactive thiol groups, which can undergo sulphydryl-disulphide interchange reactions to form disulphide-linked aggregates ($\beta_{s\text{-}s}\beta$).



4.33

When β-lactoglobulin is heated in the presence of α-lactalbumin, unfolded β-lactoglobulin molecules (β*u*) can also interact with α-lactalbumin molecules *via* thiol-disulphide interchange reactions to form $\beta_{s\text{-}s}\alpha$ aggregates or these two proteins can interact hydrophobicaly to form ($\beta_{x\text{-}x}\alpha$) (**4.33**).



4.34

The rates of formation of various species would be dependent on protein concentrations, pH, ionic strength and heating temperature. High heating

temperatures and low protein concentrations tend to favour the formation of disulphide-linked aggregates.

In the presence of casein micelles, the interactions of β-lactoglobulin molecules occur mainly via *k*-casein. The following interactions between β-lactoglobulin and casein micelles may occur:

(*i*) Unfolded β-lactoglobulin molecules with exposed thiol group (β_{SH}) can interact with *k*-casein on the micelle surface *via* thiol-disulphide interchange reaction.

(*ii*) Unfolded β-lactoglobulin molecules interact among themselves to form β-lactoglobulin aggregates which subsequently interact with *k*-casein.

$$\beta u \rightleftharpoons \beta_{SH} \longrightarrow \text{Interaction with } k\text{-casein in the casein micelles} \qquad \textbf{4.35}$$

$$\beta u \rightleftharpoons \beta_{SH} \longrightarrow (\beta_{s\text{-}s}\alpha)(\beta_x.\alpha_x) \quad \text{Interaction with } k\text{-casein in the casein micelles} \qquad \textbf{4.36}$$

The data presented in Figures 4.27 and 4.28 clearly show that the rate of interaction of β-lactoglobulin with casein micelles was much slower than the rate of aggregate formation. This may suggest that aggregates of β-lactoglobulin are formed first during heating which subsequently interact with casein micelles.

Finally, when β-lactoglobulin is heated in the presence of casein micelles and α-lactalbumin, α-lactalbumin is able to interact with β-lactoglobulin, and β-lactoglobulin/α-lactalbumin aggregates subsequently interact with the micelles.

$$\alpha\text{-La} + \beta u \begin{cases} \nearrow (\beta_{S\text{-}S}\alpha) \longrightarrow \text{Interaction} \\ \searrow (\beta_x.\alpha_x) \longrightarrow \text{with } k\text{-casein in the casein micelles} \end{cases} \qquad \textbf{4.37}$$

Under certain conditions, e.g. at 80°C, the presence of α-lactalbumin appears to promote the rate of β-lactoglobulin interaction with the casein micelles.

To clarify these mechanisms further, it would be necessary to isolate specific complexes between various proteins: β-lactoglobulin/*k*-casein, β-lactoglobulin/α-lactalbumin, β-lactoglobulin/α-lactalbumin/*k*-casein, and β-lactoglobulin/β-lactoglobulin, and determine their compositions and sizes.

4.4 Conclusions

* Heating β-lactoglobulin at 80 and 95°C resulted in loss of native structure of the proteins. Addition of casein micelles markedly increased the rate of loss of native β-lactoglobulin while addition of α-lactalbumin increased rate of loss of native β-lactoglobulin during the initial stages of heating, but the effect was much less during the latter stages of heating.

* Slight losses of native α-lactalbumin occurred during heating of α-lactalbumin either alone or in the presence of casein micelles at 80°C, although corresponding losses of native α-lactalbumin were greater at 95°C. The presence of β-lactoglobulin markedly increased the loss of native α-lactalbumin.

* The rate of loss of β-lactoglobulin at 80 and 95°C could be described by second-order reaction kinetics. The rate of loss of α-lactalbumin could be adequately be described by first-order reaction kinetics.

* The loss of SDS-monomeric β-lactoglobulin from the mixture containing β-lactoglobulin alone or β-lactoglobulin and casein micelles was less than the rate of loss of native β-lactoglobulin. In contrast, the losses of SDS-monomeric and native β-lactoglobulin from the β-lactoglobulin and α-lactalbumin mixture or mixtures containing β-lactoglobulin, α-lactalbumin and casein micelles were comparable.

* β-Lactoglobulin aggregated mainly *via* disulphide bonds when heated in the presence of α-lactalbumin, but non-covalent bonds were also formed when it was heated in the absence of α-lactalbumin. Both non-covalent and disulphide aggregates of α-lactalbumin were formed in all four α-lactalbumin solutions at 95°C.

* Large aggregates of β-lactoglobulin which sedimented at 100,000 **g** for 60 min, were formed when β-lactoglobulin solutions were heated at 95°C. Addition of casein micelles or casein micelles and α-lactalbumin to β-

lactoglobulin solutions and subsequent heating caused greater sedimentation of β-lactoglobulin.

* Heating α-lactalbumin at 95°C either alone or in the presence of casein micelles did not form sedimentable aggregates. However, the addition of β-lactoglobulin to both mixtures resulted in a marked increase in sedimentation.

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APPENDIX

The data for all the experiments using native-, SDSNR- and SDSR-PAGE are tabulated below. Also shown below are selected figures.

Table A1.1 Protein contents of solutions of various protein mixtures.

Sample	Nitrogen Percentage	Protein Percentage
β-lg	0.0523	0.3715
β-lg + casein micelles	0.4794	3.0584
α-la	0.0278	0.1774
α-la + casein micelles	0.4088	2.6079
β-lg + α-la	0.0739	0.4712
β-lg + α-la + casein micelles	0.5030	3.2095

β-lg = β-lactoglobulin α-la = α-lactalbumin

Loss of β-lactoglobulin at 80°C

Table A2.1 The concentrations of total native β-lactoglobulin (Native-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la + Casein micelles
0	100.00	100.00	100.00	100.00
1	98.45	69.74	75.81	79.50
2	74.84	60.81	67.73	71.94
3	82.30	56.48	65.94	62.14
4	65.72		57.34	57.15
5	71.74	45.82	63.11	45.60
7	63.84		55.69	48.64
8	56.83	33.72	54.65	36.81
10	46.54	38.42	60.57	36.00
15	37.89	30.42	56.65	36.69
20	33.02	26.27	49.82	30.67
25	33.02	14.97	50.09	35.27
30	23.60	17.87	36.90	29.86

Table A2.2 The concentrations of native β-lactoglobulin A (Native-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la + Casein micelles
0	100.00	100.00	100.00	100.00
1	108.21	73.03	75.00	80.65
2	85.82	65.79	68.86	84.00
3	90.30	63.16	68.64	76.61
4	64.71		58.59	59.38
5	82.71	51.97	63.29	61.29
7	60.29		55.46	51.50
8	68.66	40.79	57.93	47.58
10	47.79	41.67	61.63	39.80
15	46.27	33.33	56.36	41.94
20	36.76	25.00	46.71	27.88
25	32.35	18.06	41.18	34.59
30	29.10	21.05	38.50	29.03

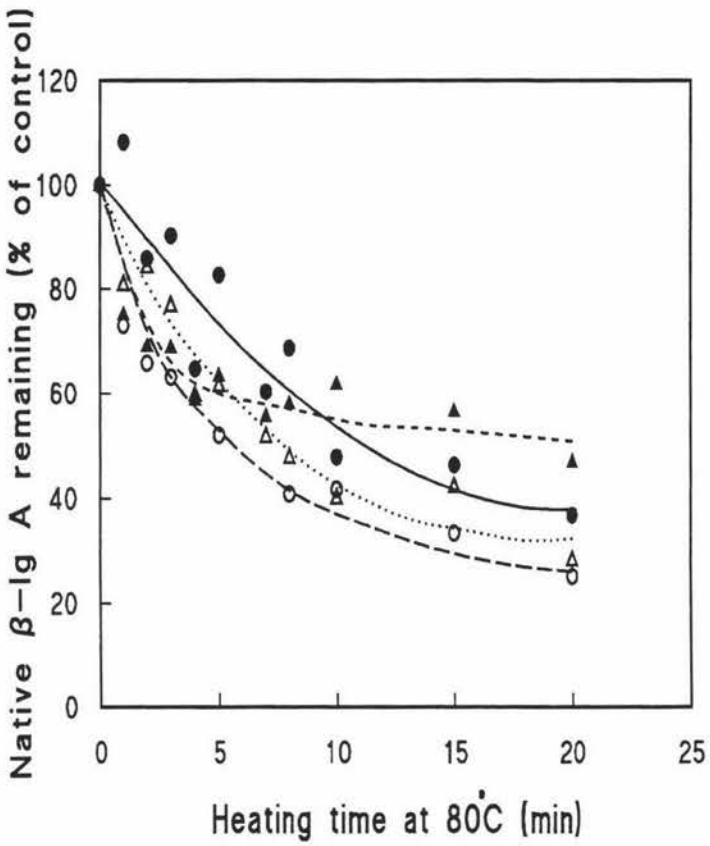


Figure A1 The effect of heating time at 80°C on the loss of native β-lactoglobulin A from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.5% casein micelles (o), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.5% casein micelles (△).

Table A2.3 Kinetic data for loss of native β-lactoglobulin A (Native-PAGE),from different mixtures (second-order reaction equation).

Sample	Degees of Freedom	r^2	Rate Constant K_n ($gl^{-1}min^{-1}$)	Standard Error for K_n
β-lg alone	7	0.6795	0.0718	0.0117
β-lg + casein micelles	5	0.9778	0.1882	0.0105
β-lg + α-la	7	0.7866	0.1177	0.0133
β-lg + α-la + casein micelles	7	0.9526	0.1359	0.01065

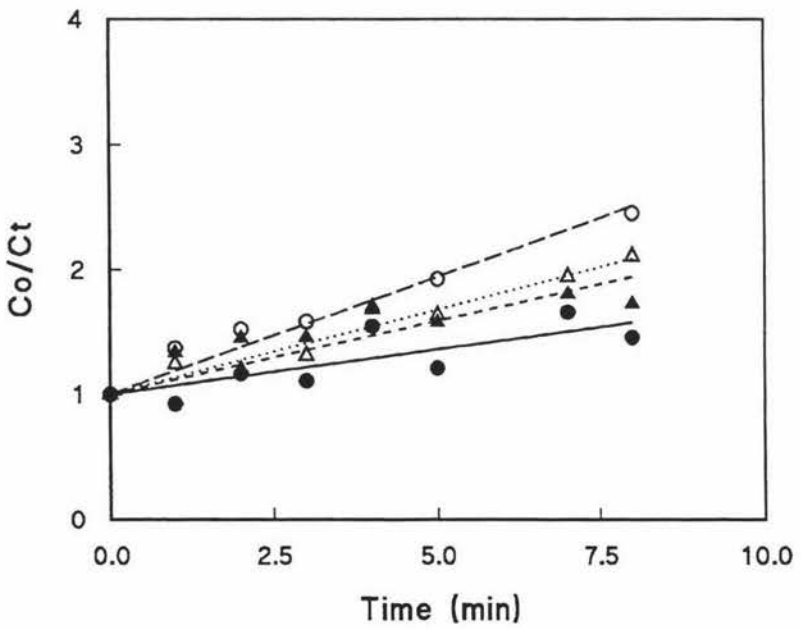


Figure A2 Kinetic plots (second order reaction equation) for loss of native β-lactoglobulin A from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.5% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.5% casein micelles (△).

Table A2.4 The concentrations of native β-lactoglobulin B (Native-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la + Casein micelles
0	100.00	100.00	100.00	100.00
1	91.49	67.18	74.36	77.59
2	67.02	56.92	64.04	77.59
3	76.60	51.28	63.77	64.10
4	66.48		56.35	54.39
5	63.83	41.03	62.04	46.79
7	66.48		55.86	46.19
8	48.40	28.21	51.17	38.46
10	45.60	36.19	59.73	32.75
15	31.91	28.10	56.07	32.05
20	30.22	27.14	52.27	33.04
25	33.51	12.86	57.13	32.15
30	19.68	15.38	36.68	32.15

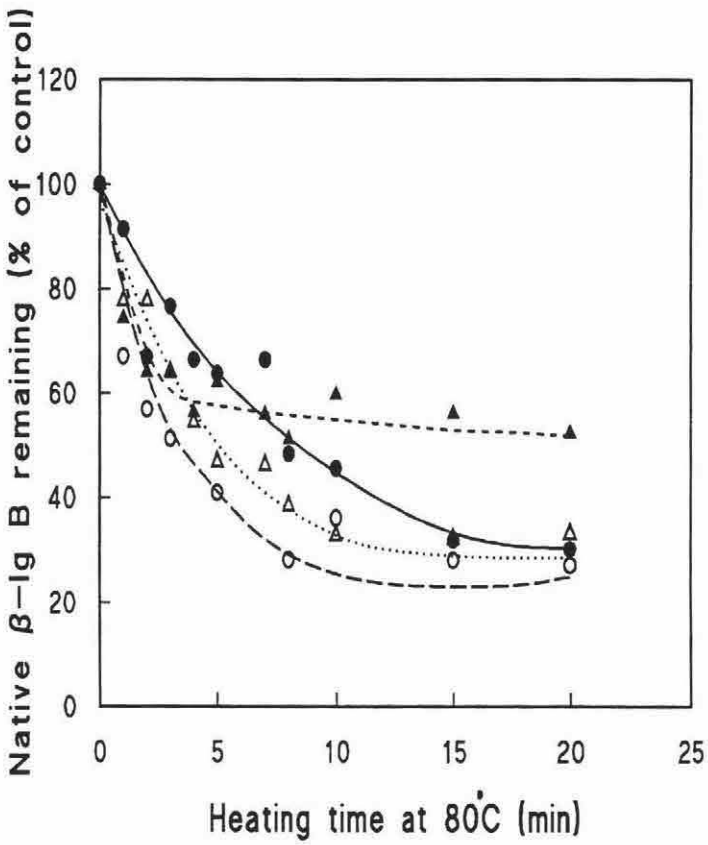


Figure A3 The effect of heating time at 80°C on the loss of native β-lactoglobulin B from solutions containing 0.4% β-lactoglobulin (•), 0.4% β-lactoglobulin and ~ 2.5% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.5% casein micelles (△).

Table A2.5 Kinetic data for loss of native β-lactoglobulin B (Native-PAGE) from different mixtures (second-order reaction equation).

Sample	Degees of Freedom	r^2	Rate Constant K_n ($gl^{-1}min^{-1}$)	Standard Error for K_n
β-lg alone	7	0.7679	0.1125	0.0012
β-lg + casein micelles	5	0.8801	0.2369	0.0274
β-lg + α-la	7	0.7997	0.1339	0.0147
β-lg + α-la + casein micelles	7	0.9571	0.1937	0.00881

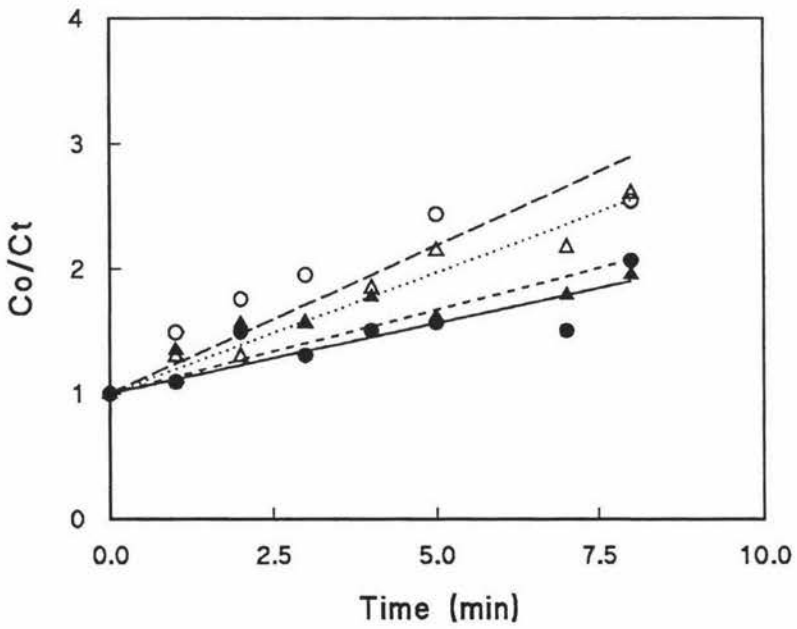


Figure A4 Kinetic plots (second-order reaction equation) for loss of native β-lactoglobulin B protein from solutions containing 0.4% β-lactoglobulin solution (•) 0.4% β-lactoglobulin and ~ 2.5% casein micelles (○), 0.4% β-lactoglobulin and 0.15% α-lactalbumin (▲), and 0.4% β-lactoglobulin and 0.15% α-lactalbumin and ~ 2.5% casein micelles (△).

Table A2.6 The concentrations of SDS-monomeric β-lactoglobulin (SDSNR-PAGE) when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la Casein micelles
0	100.00	100.00	100.00	100.00
1	86.78	74.53	77.14	67.39
2		75.66	64.76	69.58
3	78.16		59.52	60.13
4	74.45	72.00	60.05	47.46
5	72.41		51.43	44.91
7	66.52	65.00	46.18	37.22
8	67.24	62.73	45.71	36.22
10	65.20	50.00	42.55	27.93
15	59.20	33.33	40.00	28.97
20	40.10	25.83	38.59	23.83
25	42.73	15.42	28.12	24.54
30	42.53	16.93	38.10	20.64

Table A2.7 The concentrations of non-sedimentable β-lactoglobulin (SDSR-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la Casein micelles
0	100.00	100.00	100.00	100.00
1	109.22	97.81	95.45	76.24
2	100.72	100.88	98.85	68.10
3	91.75	95.61	98.01	66.52
4	82.42		98.29	63.57
5	84.47	89.42	91.48	63.35
7	76.37		100.00	48.81
8	84.47	88.46	94.03	54.98
10	75.55	71.84	96.93	41.01
15	77.91	63.94	76.70	47.06
20	67.86	64.66	79.47	45.09
25	64.01	62.36	64.70	45.67
30	56.20	66.83	66.48	44.12

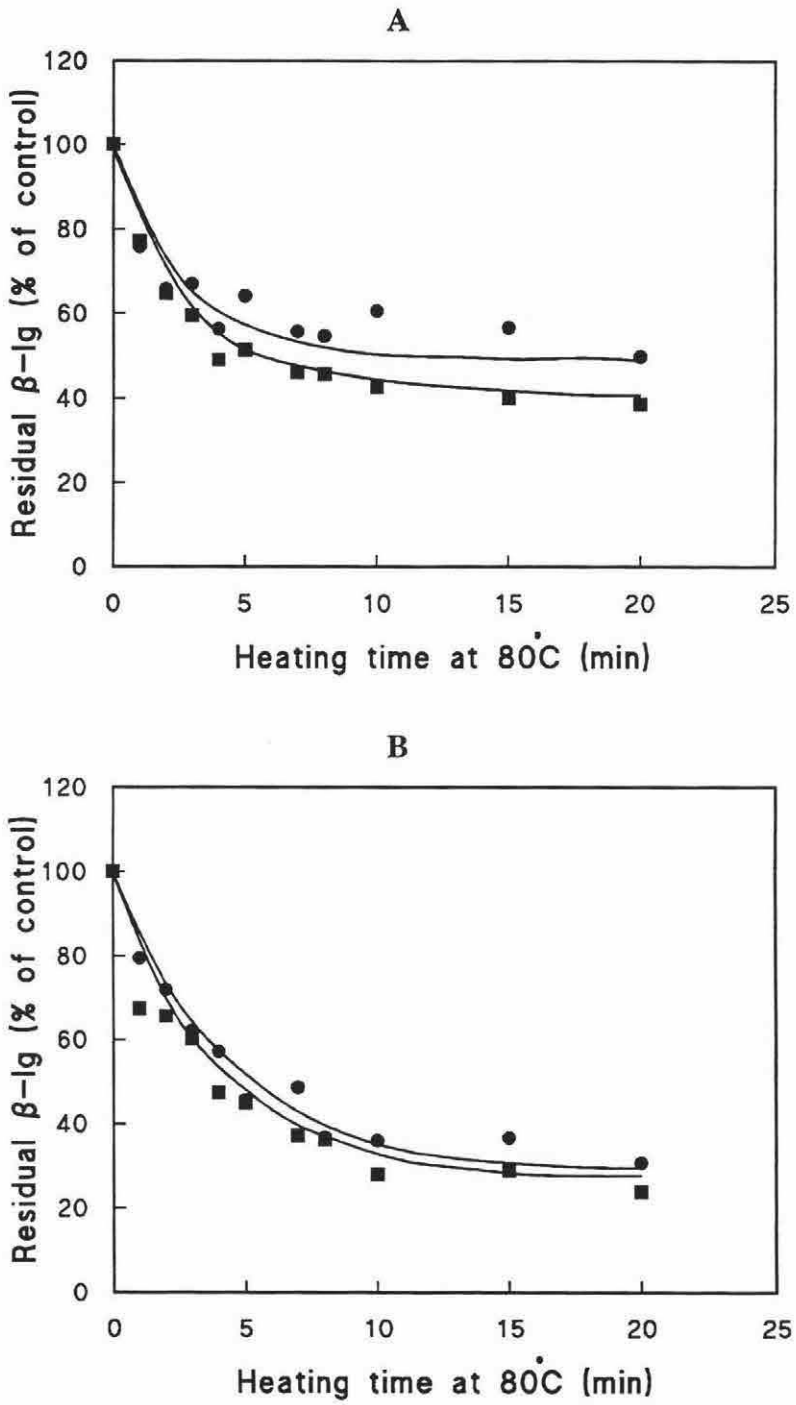


Figure A5 The effect of heating time at 80°C on the quantity of β-lactoglobulin remaining from solutions containing (A) 0.4% β-lactoglobulin and 0.15% α-lactalbumin and (B) 0.4% β-lactoglobulin, 0.15% α-lactalbumin and ~ 2.6% casein micelles as resolved on native- (●) and SDSNR-PAGE (■).

Table A2.8 The concentrations of SDS-monomeric β-lactoglobulin (SDSNR-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Pellet samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la Casein micelles
0	5.29	23.78	3.00	34.42
1	6.23	20.63	2.59	43.22
2	5.47	21.68	2.59	24.42
3	5.66	17.83	1.53	26.71
5	5.66	24.48	2.06	22.16
8	7.64	11.19	3.22	23.84
15	4.53	11.54	2.91	21.23
30	4.91	20.68	3.84	22.50

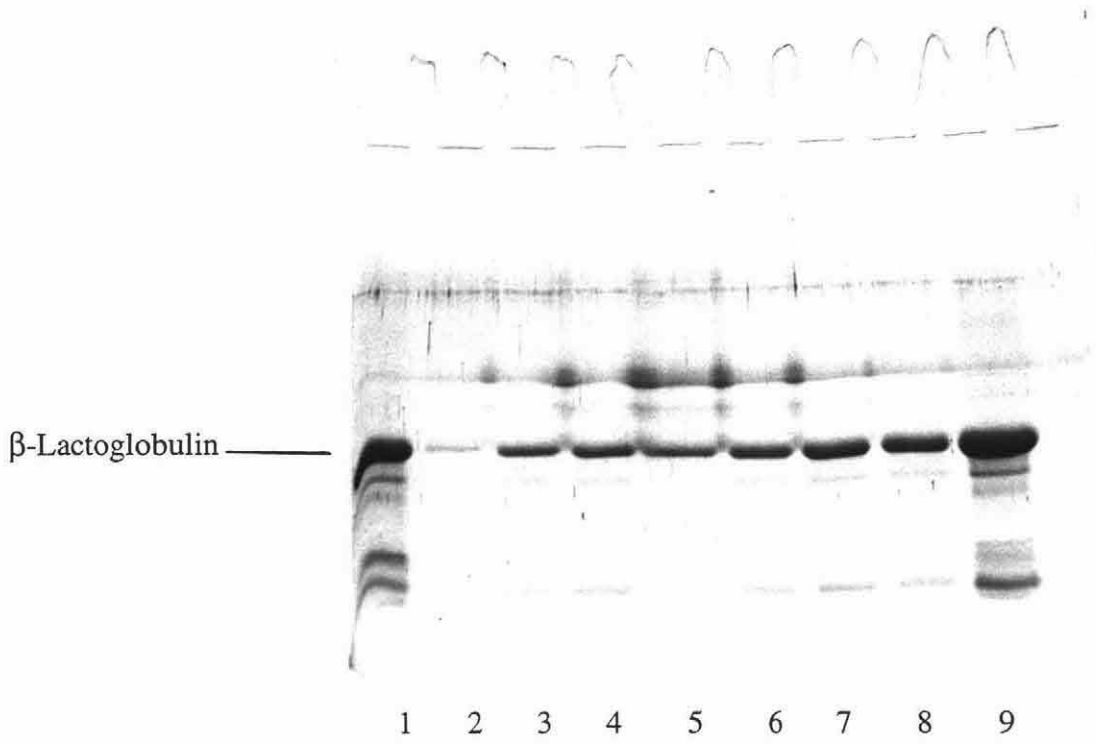


Figure A6 SDSR-PAGE on the sediments (100 000 **g** for 60 min) obtained from a 0.4% β-lactoglobulin solution, heated at 80°C, 0(2), 1(3), 2(4), 3(5), 5(6), 8(7), 15(8) and 30(9) min. Lane 1 contain the standard sample.

Loss of β-lactoglobulin at 95°C

Table A2.9 The concentrations of total native β-lactoglobulin (Native-PAGE), when heated at 95°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la + Casein micelles
0	100.00	100.00	100.00	100.00
0.5			49.72	32.85
1	22.30	19.06	39.49	21.12
1.5			40.00	26.26
2	17.42	17.46	30.00	22.16
2.5				
3	11.40	8.36	37.73	11.78
4	11.00			
5	9.72	8.46	31.99	16.67
7	8.50		45.80	
8	8.48			
10				15.69
15	8.03		45.80	
20				
25				
30			32.44	

Table A2.10 The concentrations of native β-lactoglobulin A (Native-PAGE), when heated at 95°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la + Casein micelles
0	100.00	100.00	100.00	100.00
0.5			46.05	34.56
1	18.75	19.06	43.29	28.44
1.5			46.32	27.22
2	17.13	17.46	39.87	10.05
2.5				
3	12.25	8.36	46.58	17.33
4	11.03			
5	7.00	8.46	41.18	16.67
7	9.31		45.00	
8	8.25			
10				15.69
15	8.00		47.89	
20				
25				
30			34.34	

Table A2.11 The concentrations of native β-lactoglobulin B (Native-PAGE), when heated at 95°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la + Casein micelles
0	100.00	100.00	100.00	100.00
0.5			52.50	32.13
1	25.20	17.88	36.60	19.54
1.5			35.20	29.46
2	17.65	16.10	23.90	27.83
2.5				
3	11.53	6.51	31.00	11.83
4	10.97			
5	11.94	6.99	25.00	16.67
7	7.74		46.40	
8	8.67			
10				15.69
15	8.67		44.20	
20				
25				
30			31.00	

Table A2.12 The concentrations of SDS-monomeric β-lactoglobulin (SDSNR-PAGE), when heated at 95°C in different mixtures.

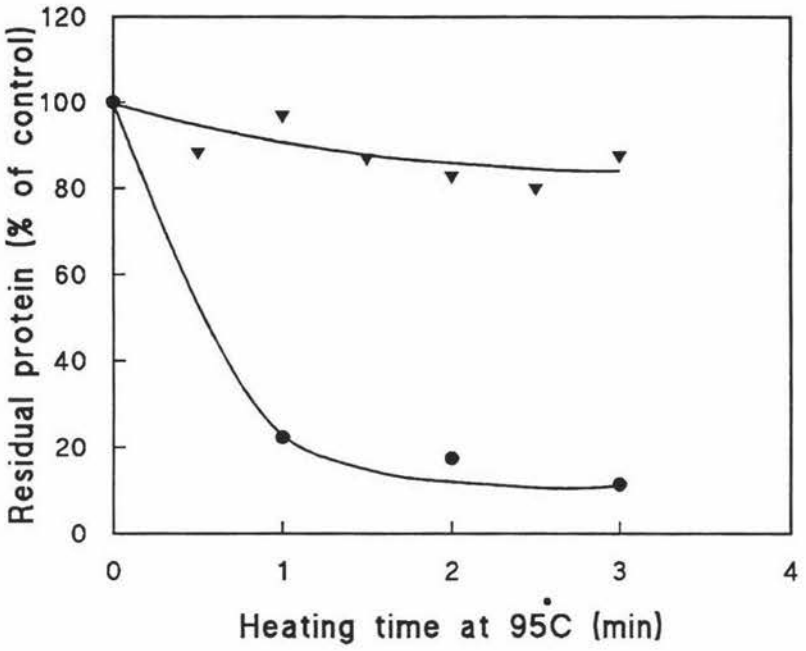
Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la Casein micelles
0	100.00	100.00	100.00	100.00
0.5	52.18	22.38	48.29	33.93
1	40.45	27.98	50.55	23.71
1.5	46.02	19.15	43.36	21.46
2	31.00	22.42	41.74	10.18
2.5	43.20	15.35		
3	29.50	21.67	35.89	19.78
3.5		10.73		
4	31.65	14.92		
5	31.20	21.27	30.23	9.96
7	21.80	12.54	24.66	11.97
8	15.10	17.78		
10	19.70	13.96	22.44	9.11
13			18.37	3.62
15	9.40	13.57	16.99	10.51
17			16.92	4.82
20	11.20	10.17		
21			15.29	2.90
25	8.00	10.00		
26			12.62	6.52
30	4.40	11.87	10.75	13.54

Table A2.13 The concentrations of non-sedimentable β-lactoglobulin (SDSR-PAGE), when heated at 95°C in different mixtures.

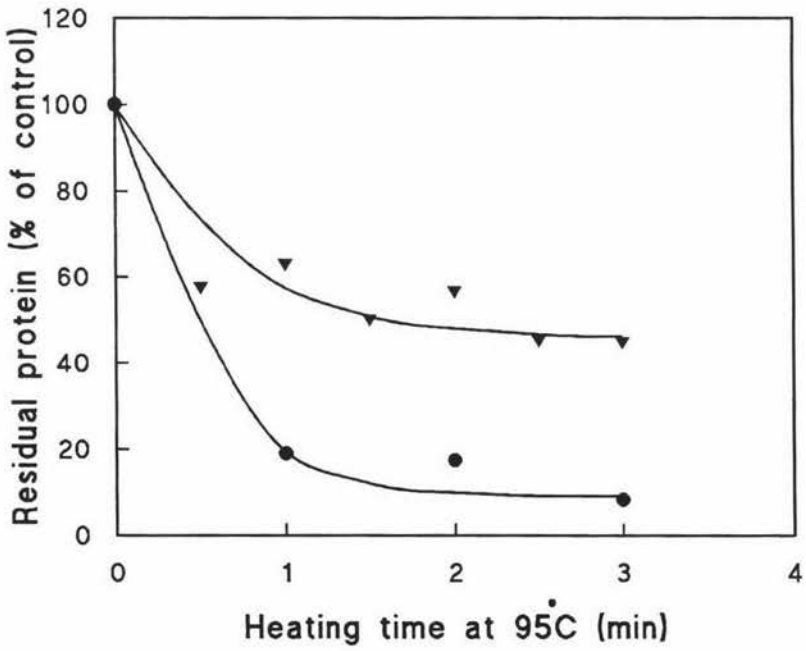
Heating time (min)	Supernatant samples (% of control)			
	β-lg alone	β-lg + Casein micelles	β-lg + α-la	β-lg + α-la Casein micelles
0	100.00	100.00	100.00	100.00
0.5	88.39	57.82	92.05	60.64
1	96.98	63.10	95.04	51.40
1.5	86.99	50.30	94.87	56.89
2	82.85	56.76	99.17	34.74
2.5	80.07	45.44		
3	87.57	45.07	83.71	34.09
3.5		30.50		
4	82.45	38.21		
5	83.19	45.56	67.79	22.70
7	69.89	31.72	77.50	20.80
8	75.42	38.10		
10	55.23	18.47	54.51	17.00
13			59.02	16.74
15	53.68	24.48	60.13	12.73
17			47.75	10.37
20	34.74	18.80		
21			43.38	10.15
25	33.99	10.04		
26			37.89	9.33
30	40.83	31.10	39.79	9.96

Table A2.14 The concentrations of SDS-monomeric β-lactoglobulin (SDSNR-PAGE) when heated at 95°C in different mixtures.

Heating time (min)	Pellets sample (% of control)	
	β-lg + α-la	β-lg + α-la Casein micelles
0	0.91	58.23
0.5	2.73	30.45
1	2.18	31.91
1.5	3.14	29.59
3	2.18	28.45
7	6.68	28.82
15	7.27	29.23
30	13.73	26.27

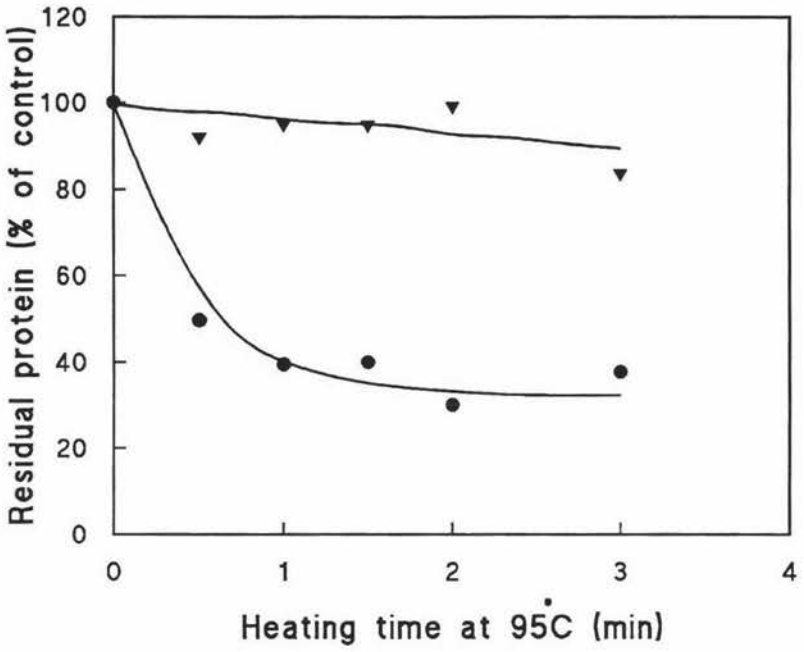


A



B

Figure A7 The effect of heating time at 95°C on the quantity of β-lactoglobulin remaining from solutions containing (A) 0.4% β-lactoglobulin and (B) 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native- (•) and SDSR-PAGE (▲).



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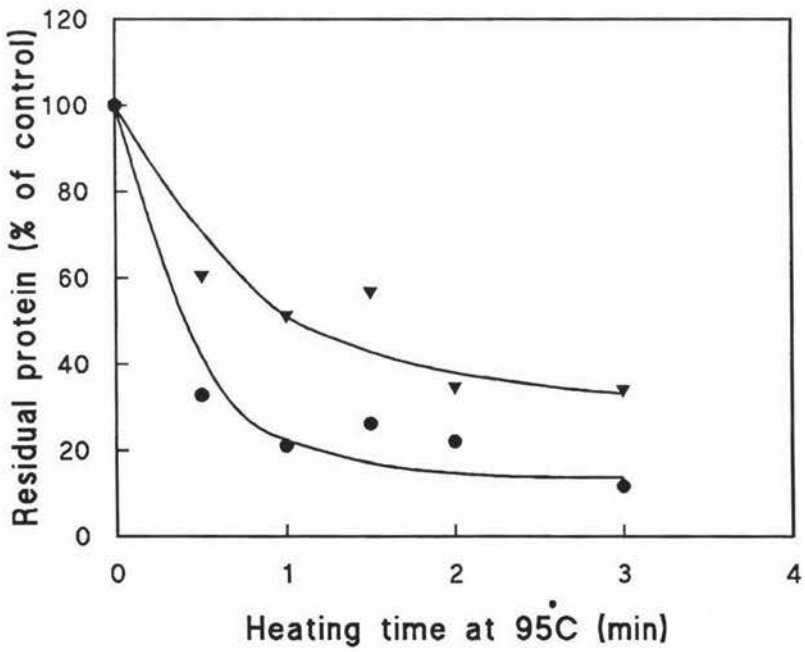

B

Figure A8 The effect of heating time at 95°C on the quantity of β-lactoglobulin remaining from solutions containing (A) 0.4% β-lactoglobulin and 0.15% α-lactalbumin (B) 0.4% β-lactoglobulin, 0.15% α-lactalbumin and ~ 2.6% casein micelles as resolved on native- (•) and SDSR-PAGE (▲).

Loss of α-lactalbumin at 80°C

Table A3.1 The concentrations of native α-lactalbumin (Native-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	100.00	100.00	100.00	100.00
1	103.62	105.54	96.47	99.71
2	106.16	98.39	93.75	97.11
3	118.88	123.28	86.14	94.55
4	87.21	106.62	90.10	83.57
5	103.11	114.79	78.26	78.17
7	91.03	109.39	84.55	77.52
8	96.24	112.48	72.83	66.97
10	90.56	113.55	79.10	73.13
15	76.93	96.03	58.97	68.10
20	83.62	95.76	61.53	58.90
25	88.89	106.62	56.88	49.34
30	83.46	85.60	44.84	51.45

Table A3.2 The concentrations of SDS-monomeric α-lactalbumin (SDSNR-PAGE) when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	100.00	100.00	100.00	100.00
1	91.69	95.94	100.00	90.53
2	95.98	99.51	91.19	88.46
3	101.83	93.68	86.19	90.83
4	91.66	94.41	81.68	77.03
5	95.93	94.62	83.96	85.80
7	95.81	92.74	82.36	73.56
8	92.00	89.29	87.74	78.40
10	90.60	93.70	74.63	79.12
15	90.60	99.86	85.53	82.25
20	95.03	97.10	70.99	70.62
25	86.53	94.80	64.25	64.65
30	91.91	90.04	73.27	75.74

Table A3.3 The concentrations of non-sedimentable α-lactalbumin (SDSR-PAGE), when heated at 80°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	100.00	100.00	100.00	100.00
1	95.40	104.71	93.56	82.96
2	96.94	83.38	82.43	82.87
3	91.03	88.65	82.43	78.25
4	94.96	88.20	85.27	91.56
5	92.12	98.07	79.21	75.11
7	97.76	81.55	106.54	88.34
8	89.78	102.14	82.93	75.56
10	84.82	87.59	96.17	76.43
15	93.21	100.00	82.67	71.30
20	96.85	85.08	91.31	80.35
25	98.45	86.08	81.88	77.10
30	97.44	100.00	85.64	70.18

Table A3.4 The concentrations of SDS-monomeric α-lactalbumin (SDSNR-PAGE) when heated at 80°C in different mixtures.

Heating time (min)	Pellet samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	2.41	24.36	1.45	24.64
1			0.78	24.64
2	2.84	25.46	1.74	19.81
3			2.21	22.24
4	2.84	22.98		
5			2.54	19.17
7	2.27	25.74		
8			2.64	23.29
11	2.56			
15	2.84	24.08	2.34	14.31
20	2.84	50.55		
25	3.27	26.38		
30			3.33	25.24

Loss of α-lactalbumin at 95°C

Table A3.5 The concentrations of native α-lactalbumin (Native-PAGE), when heated at 95°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	100.00	100.00	100.00	100.00
0.5			87.45	85.04
1	83.12	107.52	101.00	93.98
1.5			95.89	81.61
2	84.81	96.31	71.27	84.32
3	90.41	98.43	76.51	71.53
4	72.13	88.91		
5	85.49	88.25	65.13	65.82
7	73.92	86.09	52.71	65.00
8	93.31	84.96		
10	71.50	76.88	48.81	63.64
13			52.46	53.64
15	59.59	66.82	35.63	36.82
17			37.80	40.73
20	52.00	71.77		
21			35.47	37.68
25	55.38	67.63		
26			25.51	42.05
30	50.83	42.66	14.64	20.89

Table A3.6 The concentrations of SDS-monomeric α-lactalbumin (SDSNR-PAGE) when heated at 95°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	100.00	100.00	100.00	100.00
0.5			81.13	78.19
1	105.48	90.72	92.00	71.26
1.5			88.60	70.33
2	87.58	97.12	97.02	78.80
3	85.24	92.50	88.07	70.93
4	74.91	87.97		
5	89.05	83.52	94.44	74.34
7	78.53	96.59	79.20	72.47
8	89.21	74.36		
10	78.97	85.16	83.63	66.93
13			82.83	65.12
15	85.95	77.50	77.73	53.46
17			81.29	59.82
20	81.72	74.73		
21			84.47	61.14
25	76.64	97.91		
26			76.85	61.87
30	84.96	80.25	73.27	54.56

Table A3.7 The concentrations of non-sedimentable α-lactalbumin (SDSR-PAGE) when heated at 95°C in different mixtures.

Heating time (min)	Supernatant samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	100.00	100.00	100.00	100.00
0.5			92.05	60.64
1	93.87	89.62	95.04	51.40
1.5			94.87	56.89
2	95.18	89.37	99.17	34.74
3	99.25	81.11	83.71	34.09
4	95.06	88.46		
5	96.73	86.35	67.79	22.70
7	93.05	85.27	77.50	20.80
8	100.09	92.21		
10	92.27	87.84	54.51	17.00
13			59.02	16.74
15	95.68	83.64	60.13	12.73
17			47.75	10.37
20	97.80	84.36		
21			43.38	10.15
25	93.57	92.70		
26			37.89	9.33
30	90.39	87.63	39.79	9.96

Table A3.8 The concentrations of SDS-monomeric α-lactalbumin (SDSNR-PAGE) when heated at 95°C in different mixtures.

Heating time (min)	Pellet samples (% of control)			
	α-la alone	α-la + Casein micelles	α-la + β-lg	α-la + β-lg + Casein micelles
0	2.13	41.38	1.01	52.54
0.5			2.98	52.86
1			2.98	56.16
1.5	2.91		3.27	53.79
2		47.04		
3			2.93	53.62
4	1.83	48.59		
5				
6		42.66		
7	1.74		3.46	50.40
8				
10		45.43		
11				
15	1.95	39.01	2.93	50.40
20	1.86	49.59		
25	1.47	46.68		
30			2.69	59.46

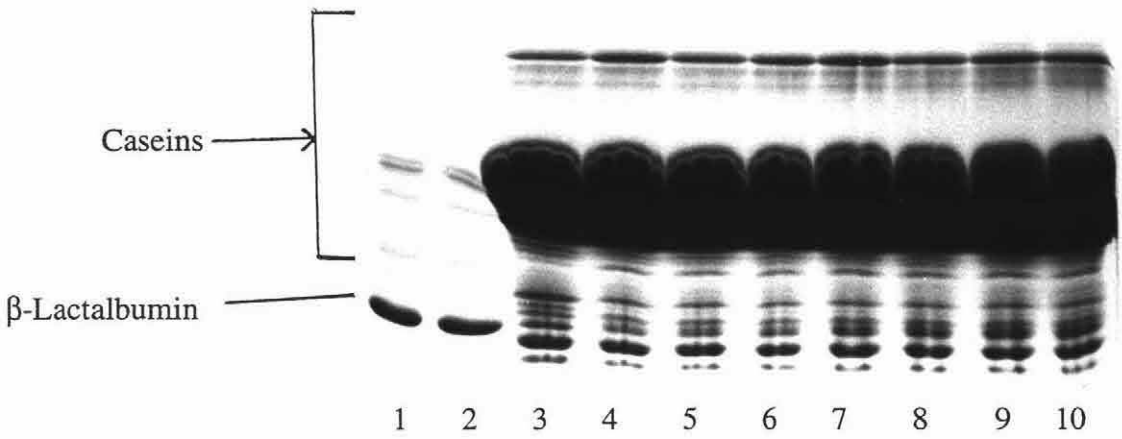

Figure A.9 SDSR-PAGE on the sediments (100 000 **g** for 60 min) obtained from a solution containing 0.15% α-lactalbumin and ~ 2.6% casein micelles, heated at 95°C for 1(3), 2(4), 4(5), 7(6), 10(7), 15(8) 20(9) and 25(10) min. Lanes 1 and 2 contain standard samples.

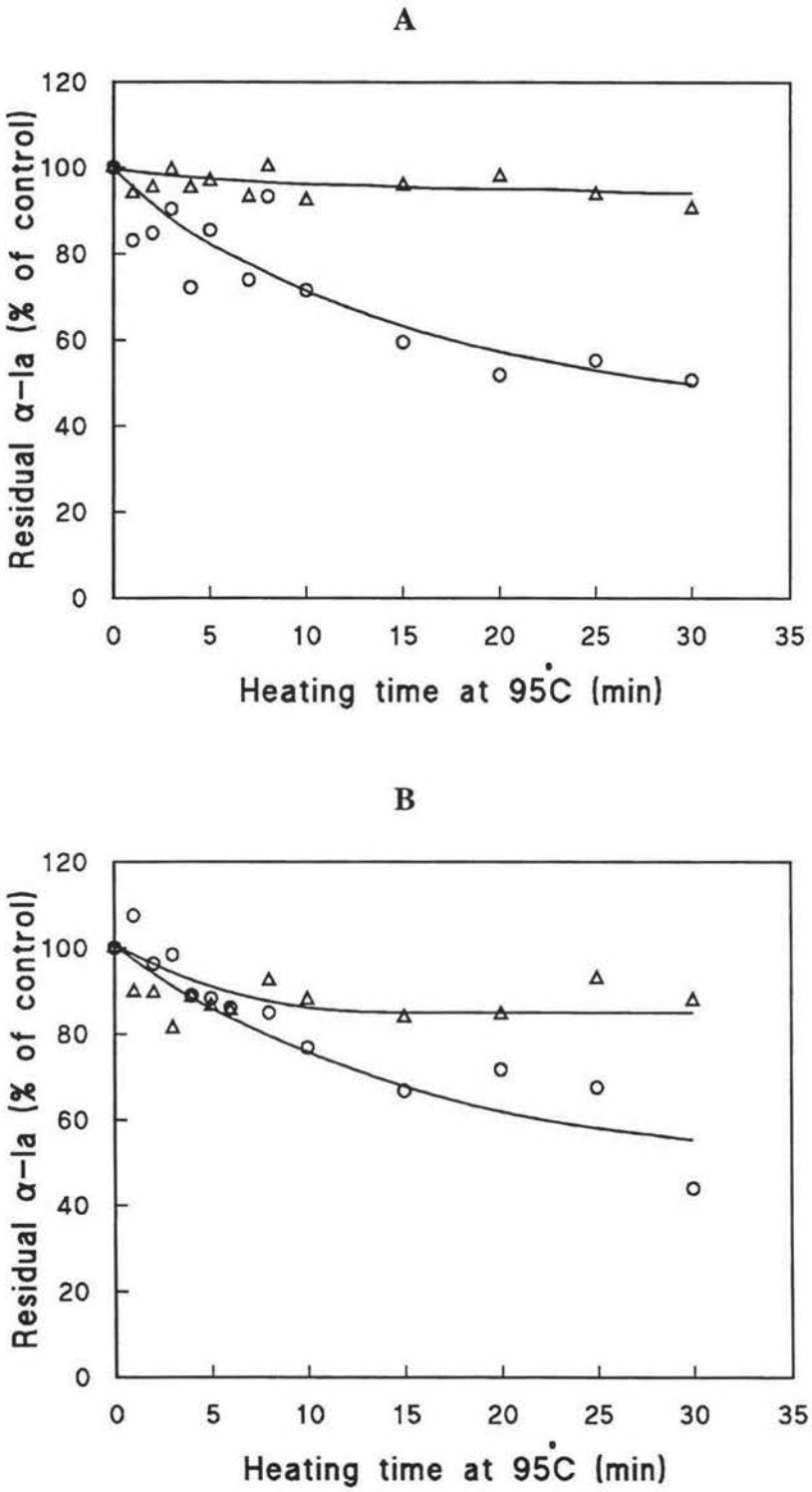

Figure A10 The effect of heating time at 95°C on the quantity of α-lactalbumin remaining from solutions containing (A) 0.15% α-lactalbumin and (B) 0.15% α-lactalbumin and ~ 2.6% casein micelles as resolved on native- (O) and SDSR-PAGE (Δ).

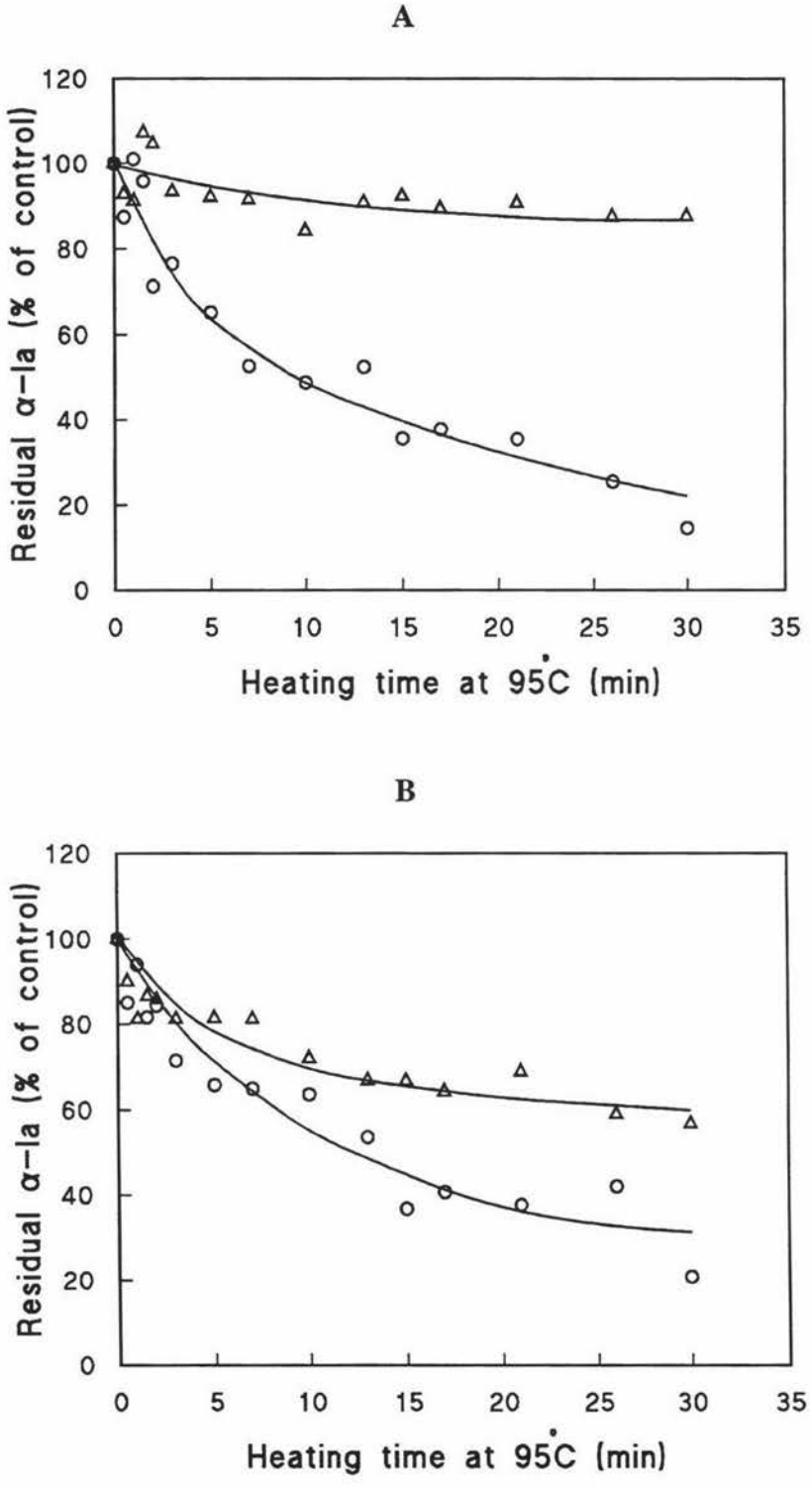

Figure A11 The effect of heating time at 95°C on the quantity of α-lactalbumin remaining from solutions containing (A) 0.15% α-lactalbumin and 0.4% β-lactoglobulin and (B) 0.15% α-lactalbumin, 0.4% β-lactoglobulin and ~ 2.6% casein micelles as resolved on native- (O) and SDSR-PAGE (△).