DEPARTMENT OF FOOD TECHNOLOGY

MASSEY UNIVERSITY

PALMERSTON NORTH

AGGREGATION AND GELATION OF

BOVINE β-LACTOGLOBULIN, α-LACTALBUMIN AND

SERUM ALBUMIN

A THESIS

PRESENTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF TECHNOLOGY IN FOOD TECHNOLOGY

AT MASSEY UNIVERSITY

JACQUELINE GEZIMATI

1995

DEDICATION

To my family, Luke, Rose, Joyce, Peter, Zvaitika, Luke (Jnr) and Chido.

ABSTRACT

Gelation is one of the most important functional properties of whey proteins in food systems. The properties of whey protein gels are affected by the chemical and physical properties of its protein components, β-lactoglobulin AB (β-Lg), α-lactalbumin (α-La) and bovine serum albumin (BSA).

Heat-induced aggregation and gelation of individual whey proteins, β-Lg, α-La and BSA and in mixture was studied by dynamic rheology and electrophoresis analysis. The proteins were dispersed in an ionic buffer containing 0.009 M $CaCl_2$, 0.012 M NaCl, 0.012 M K_2HPO_4 and 0.007 M Na_3citrate (pH 6.8) which was comparable to the ionic composition of 12% whey protein concentrate solution. Rheological properties of the protein solutions were measured using a Bohlin VOR rheometer after heating to 70, 75 and 80°C, holding at these temperatures for 60 min and after cooling to 25°C. Gel electrophoresis under non-dissociating (Native-PAGE in the absence of dissociating and reducing agents) and dissociating but non-reducing conditions (SDS-PAGE) was used to determine the extents of aggregation in some of the heated protein samples.

Gelation temperatures of 10%, w/v, protein solutions were found to be in the range 82.5 - 84°C for β-Lg and 68 - 70°C for BSA while α-La did not gel even at 90°C. Gelation temperatures of protein mixtures containing β-Lg and BSA were dependent on the relative proportion of the two proteins in the mixture. In contrast, the protein mixtures containing β-Lg and α-La gelled at temperatures (~ 83°C) comparable to that of β-Lg alone.

Rheological measurements on pure β-Lg and BSA showed that BSA solutions formed self-supporting gels at lower protein concentrations and lower temperatures. Increasing the heating temperature or protein concentration of either β-Lg or BSA resulted in higher values of the storage

modulus (G′).

It was apparent from the electrophoretic data that protein aggregates were formed as an intermediate prior to the formation of gel net-work. These aggregates appeared to be non-covalently linked initially and became increasingly disulphide-linked during heating.

Analysis of mixtures containing β-Lg and BSA during heat treatment showed that at both 70 and 75°C the gelation time decreased with the increasing proportion of BSA. Similarly, the values of G′ after 60 min of heating were greater for the gels containing more BSA. G′ values of these mixtures were dependent on the heating temperature and the relative proportion of the two proteins.

Gel electrophoresis data for a mixture of 5% β-Lg and 5% BSA heated at 70°C showed that prior to gelation most of the BSA had been transformed into aggregates while most of the β-Lg was essentially in the native form. Aggregates of both β-Lg and BSA were formed during heating at 75°C. At both temperatures, gelation commenced after most of the BSA had become covalently cross-linked but before all the β-Lg had become cross-linked. This effect was also apparent for other mixtures. Initially the aggregates appeared to be non-covalently linked and became increasingly disulphide linked with heating. From these results it is apparent that during heating at 70°C, BSA is the main protein forming the gel net-work and some β-Lg aggregates are probably attached to the net-work strand through either hydrophobic interactions or disulphide linkages. During heating at 75°C, two gel net-works are presumed to be formed independently, again with some interactions between the strands of the two net-works.

The rheological properties of protein mixtures containing β-Lg and α-La showed that β-Lg was the dominant gelling protein. G′ values decreased with increasing relative proportion of α-La in the mixture at both 75 and

80°C. Gelling times increased with increasing proportion of α-La in the mixture at both 75 and 80°C.

No aggregate formation was observed during heating of α-La at 75 or 80°C. However, in the presence of β-Lg, α-La aggregated rapidly during heating. This aggregation appears to involve sulphydryl disulphide interchange reactions particulary when the mixtures were heated at 80°C. Almost all the proteins had aggregated through disulphide linkages before any significant increase in G'. It is suggested that during heating and prior to gelation co-polymers of both β-Lg and α-La were formed and this resulted in heterogeneous net-work strands being formed.

The results presented in this study suggest that slight differences in the protein composition of WPC are unlikely to affect the gelation properties of WPC. Further studies into the effects of immunoglobulins (Igs) are needed in order to gain further understanding of the contributions of these proteins to rheological properties of WPC gels.

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ACKNOWLEDGEMENTS

I would like to sincerely acknowledge my supervisor, Dr. Harjinder Singh, for his guidance and assistance throughout the course of my study. Without his patience and helpful discussions this work could not have been completed.

I am also grateful to Dr. Lawrence Creamer for his expert advice and helpful long discussions in the preparation of the thesis. I wish to thank Dr. Osvaldo Campanella for his useful contributions in the initial stages of the project.

I would also like to thank June Latham and Steve Glassgow for their invaluable technical assistance.

I would like to acknowledge Prof. Ray Winger for providing me the opportunity to study in this Department. Mark Dorsey, Miss Leeann Wotjal and David Oldfield are also gratefully acknowledged for their computing, secretarial and training skills.

My thanks are also due to my fellow graduate students for their friendship and encouragement.

The following families are kindly acknowledged for their hospitality and for making my stay here in New Zealand a memorable one, the van Laar-Veths, Schaws, van Laars, Gartners and the Norrish's.

I am grateful to my family for their emotional support, their prayers and continued support through correspondence assuring me that all was well at home.

Last but not least, to my fiance for his unfailing support over the years.

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INTRODUCTION

The food industry continues to seek less expensive proteins for use in the manufacture of modern convenience foods. Proteins as isolates or concentrates are necessary ingredients in many food processes where they perform specific functions.

Interest in the use of whey protein isolates and their protein components as constituents in food products has grown steadily in recent years and are currently being used for a variety of applications in the food industry, some of which include use in processed meat, sugar confectionaries, desserts and baby foods. The functional properties of whey proteins include water-binding, emulsification, foaming and whipping and most important, gelation. Whey proteins improve the consistency of foods by forming thermally induced gels which provide a structural matrix for holding water, flavours, sugars and other food ingredients.

Whey protein isolates and concentrates are a heterogenous group of proteins each with different properties. The mechanism for gel formation of whey protein concentrates or isolates is not fully understood because of the complicated interactions among the different constituent proteins, i.e, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Igs).

Although the gelation of the individual whey proteins have been extensively studied (Paulsson *et al.* 1986; Yasuda *et al.* 1986; Mulvihill and Kinsella, 1988; Stading and Hermasson, 1990; Kuhn and Foegeding, 1991; Matsudomi *et al.* 1991; Foegeding *et al.* 1992; McSwiney *et al.* 1994a,b) the lack of close correlation between bench-scale functionality data for individual proteins and their predictive behaviour in complex food systems as well as the variability and complexity of whey proteins have hampered

the advancement of understanding of the roles that compositional and physicochemical factors play in gelation.

The roles of the constituent proteins in whey have not been studied extensively and it is not known if BSA and α-La interacts with β-Lg to produce a mixed protein gel matrix or whether they gel separately to form two interpenetrating gel networks. It is therefore appropriate to study the effects of possible interactions between the whey proteins on gel formation and to try and understand the possible protein-protein interactions in the aggregation process that leads to gel network formation in whey protein systems and therefore be able to predict the gelation behaviour of different whey protein powders.

In the present study, investigation into the aggregation behaviour of individual whey proteins and mixtures and their interactive contribution to gel formation in an ionic environment similar to that which exists in whey protein concentrates have been carried out.

REVIEW OF LITERATURE

2.1 Whey and whey products

Whey is the soluble fraction of milk rich in proteins, minerals and lactose that is removed from the casein curd during cheese or casein manufacture. Whey was traditionally regarded as a waste product and was usually disposed of or used as animal feed. Whey is now a significant source of functional proteins for the food industry worldwide. Whey protein concentrate (WPC) and whey protein isolate (WPI) are the powders manufactured from different whey types and they vary in the concentration of the protein, minerals, lipids and lactose. The manufacture of WPC and WPI involves several different processes such as electrodialysis, ultrafiltration, reverse osmosis, evaporation and spray-drying and these powders can be highly variable in their composition and functionality (Kinsella and Whitehead, 1989).

Whey protein products have applications in a wide range of foods depending on the amount of protein present which can vary from 15 to 90%, the type of product being made and the functional property required. The important functional properties of whey products include water-binding (baking\meat industry), emulsification (production of salad dressings), foaming and whipping (production of whipped creams and their substitutes) and gelation (meat industry) (Bech, 1981; Dybing and Smith, 1991).

2.1.1 *Composition of whey*

There are two basic types of whey; acid and sweet whey. Acid whey (pH 4.2 - 4.8) is obtained from cottage cheese and casein manufacture where acidification can be by direct addition of mineral acid or by "in situ" production of lactic acid by added starter bacteria. Sweet whey, also known as rennet whey, is obtained from the manufacture of cheese

products involving rennet coagulation, the resultant whey has pH values in the range pH 5.8 to 6.3. Components of whey ranked in decreasing order of relative quantity are water, lactose, nitrogenous compounds, minerals and lipids. The major mineral components of whey are calcium, phosphate, sodium, potassium and chloride. There are compositional differences between acid and sweet whey (Table 2.1). Sweet whey has a higher pH, more total solids, protein, lactose and lipid but less calcium and potassium than does acid whey. The primary causes of variations in the composition of whey are manufacturing processes and seasonal variations.

Table 2.1:   **Average composition and pH of sweet (rennet casein) and acid (lactic acid casein) wheys** (Mulvihill and Donovan, 1987b).

Component	Composition (g/l)	
	Rennet casein	Lactic acid casein
Total solids	66	64
Total protein (N x6.38)	6.57	6.20
Lactose	52.3	44.3
Minerals (ash)	5.0	7.5
Calcium	0.5	1.6
Phosphate	1.0	2.0
Sodium	0.53	0.51
pH	6.4	4.6

2.1.2 Whey proteins

Whey proteins are those proteins remaining soluble at pH 4.6 at 20°C after casein removal from whole or skim milk. Whey proteins are compact globular proteins ranging in molecular weight from 14,200 to 160,000 Da and are generally spherical in shape, soluble in water and in dilute salt solutions. Compared with caseins, whey proteins are more heat sensitive and can engage in thiol-disulphide interchange reactions at elevated temperatures or pHs to form oligomeric structures (Kinsella and Whitehead, 1989).

The principal whey proteins in all whey types are β-Lg and α-La (Table 2.2). The other proteins normally present in whey are bovine serum albumin (BSA) and immunoglobulins together with other heat stable proteins and peptides collectively termed proteose-peptones and a number of other enzymes and proteins in small amounts including lactoperoxidases, lysozyme, lactoferrin, lactollin and many others. These proteins have very different properties as a result of differences in amino acid composition and spatial arrangement.

Table 2.2: **Bovine whey protein composition**

Protein	Amount (g\L milk)	Approximate % of total whey protein
β-Lg	2.0 - 4.0	50
α-La	1.0 - 1.5	12
BSA	0.1 - 0.4	5
Proteose-peptone	0.6 - 0.8	8
Immunoglobulins	0.6 - 1.0	10

2.2 Whey protein structure

The properties of whey proteins have been well established (Lyster, 1972; Braunitzer *et al.* 1973; Fox and Mulvihill, 1982; Swaisgood, 1982; Creamer *et al.* 1983; Eigel *et al.* 1984; Pessen *et al.* 1985; McKenzie and White, 1991; Frapin *et al.* 1993). Table 2.3 shows some of the structural and chemical properties of β-Lg, α-La and BSA

Table 2.3: Structural and chemical properties of major whey proteins

	β-Lg	α-La	BSA
Isoelectric point	5.2	4.2-4.5	4.7-4.9
Molecular weight (g\mol)	18,400	14,200	66,000
Hydrophobicity (mol %)	35	36	28
Disulphide bonds	2	4	17
Sulphydryl	1	0	1
Secondary structure (mol %)			
α-helix (%)	10	26	54
β-sheets (%)	45	14	18
β-turns (%)	18	-	20
Random coil (%)	27	60	17

2.2.1 β-*Lactoglobulin*

β-Lg is the most abundant of the whey proteins in ruminant milks and comprises up to 50% of the total protein in whey. β-Lg is a globular protein of 162 amino acid residues in a single chain and exists as a stable dimer of two non-covalently linked monomeric subunits with a molecular weight of 36,800 Da between pH 5.5 and 7.5 and below pH 3.5 the dimer dissociates into spherical monomers. Dissociation increases with dilution as a consequence of a decrease in concentration and lower ionic strength

due to increased electrostatic repulsive forces (Lyster, 1972; McAlpine *et al*. 1992).

β-Lg exists in at least five different genetic variants A to E (Eigel *et al*. 1984; Ng-Kwai-Hang and Grosclaude, 1992). The two most common genetic variants, known as A and B, differ at position 63 and 118 where Asp and Val in the A variant are substituted by Gly and Ala in the B variant.

β-Lg has two disulphide bonds between residues 66 - 160 and 106 - 119 and one free thiol group (at position 121) which is normally unavailable and buried within the protein. The native conformation is sensitive to heat denaturation, pH and ionic changes at temperatures below 25°C and above pH 7.0 the protein forms octamers (Pessen *et al*. 1985). The secondary structure of β-Lg has been calculated from circular dichroism and infra-red spectroscopy data and it contains about 51% β-sheet, 17% reverse turns and 17% aperiodic structures (Creamer *et al*. 1983).

Although β-Lg is the major protein constituent of whey and occurs only in milk, its biological functions other than nutrition have not yet been established. There are speculations that it is a specific carrier of retinol from maternal milk to neonate *via* specific receptors in the intestine (Papiz *et al*. 1986) and possibly facilitates vitamin A esterification (Ong, 1985). At pH 2.0, β-Lg has a compact structure and is resistant to the enzyme pepsin and it has been shown to be a major contributor to milk allergenicity. β-Lg has also been shown to bind quite strongly long chains of less soluble fatty acids (Frapin *et al*. 1993).

2.2.2 α-*Lactalbumin*

α-La accounts for 25% of the whey proteins. It is a small compact globular protein consisting of 123 amino acid residues with a stable conformation between pH 5.4 and pH 9.0 which is stabilised by four disulphide bonds

(Eigel *et al*. 1984; McKenzie and White, 1991; Brew and Grobler, 1992). α-La possesses an essential amino acid profile, rich in lysine, leucine, threonine, tryptophan and cystine. Three genetic variants (A, B and C) are known to exist. Its secondary structure at physiological pH consists of 26% α-helix, 14% β-structure and 60% unordered structure (Robbins and Holmes, 1970). Its structure has also been demonstrated to be of close similarity to that of hen egg white (McKenzie and White, 1991).

At pH values below its isoelectric pH (pH 4.2), α-La forms dimers and trimers, the association being rapid and reversible and greater at 10°C than at 25°C. α-La has low solubility in water between pH 4.0 - 5.0. Above pH 9.0 and below pH 4.0, conformational changes occur at ambient temperature without causing irreversible aggregation (Lyster, 1972; Shukla, 1973).

On heating, conformational changes in α-La can be detected in buffer solutions at neutral pH in which no precipitation occurs (Baer *et al*. 1976). α-La has been shown to be a metalloprotein (Murakami *et al*. 1982) binding to different metals ions and one such cation is calcium which stabilises the protein against conformational changes which have been shown to occur when calcium is removed by a chelator (McKenzie and White, 1991).

The biological function of α-La is to modulate the substrate specificity of galactosyltransferase in the lactose synthetase complex which is responsible for the synthesis of lactose in lactating mammary tissue (Hill and Brew, 1975).

2.2.3 Bovine serum albumin (BSA)

BSA represents about 5% of the total whey proteins and the concentration varies with the stage of lactation and health of the animal. It is synthesised in the liver and gains entry into the milk through the

secretory cells. BSA has the longest single polypeptide chain of all the whey proteins consisting of 582 amino acid residues and has a molecular weight 66,000 Da (Eigel *et al.* 1984). It has 17 disulphide bonds which stabilise its tertiary structure and one free thiol group (at position 34). It is monomeric and the protein displays some microheterogeneity (Sogami *et al.* 1969; Spector, 1975). The secondary structure of BSA, estimated by comparison with proteins of known structure, has 55% α-helix, 16% β-pleated sheet and 29% unordered structure (Creamer *et al.* (1995). The precise three-dimensional crystal structure of BSA is not known and the current view of the structure is that the molecule exists in three major domains, each consisting of two large double loops and a small double loop with the overall shape as a 3:1 ellipsoid.

BSA is physically and immunologically identical to blood serum albumin. Little is known about its behaviour in milk and milk products and any possible influence on their properties but it can bind a wide range of small molecules, such as fatty acids (Puyol *et al.* 1991)

2.2.4 *Immunoglobulins*

These are antibodies and are a complex mixture of glycoproteins which represent about 10% of the whey proteins. There are two kinds of polypeptide chains, light (L) with molecular weight of 22,400 Da and heavy chain (H) with molecular weight of 50 - 60,000 Da and these chains are joined by disulphide bridges. Four types of immunoglobulins IgG, IgA, IgE and IgM occur in bovine milk. IgG is the principal type found in bovine milk and comprises of about 80% of the total content of immunoglobulins (Eigel *et al.* 1984). These immunoglobulins are not unique to whey, but are part of the immune system of bovine serum. These proteins are easily denatured by heat (Lyster, 1972). The functional properties of these large globular, heat-labile proteins has received little attention.

2.2.5 Proteose-peptone

Whey proteins contain variable amounts of proteose-peptone (2 - 20g/l). These are a heterogeneous mixture of heat-stable, acid-soluble (pH 4.6) polypeptides. Some of them are formed by proteolysis of β-casein by plasmin. These are amphiphilic because of the charged phosphate groups and sequences of hydrophobic amino acid residues. The amount and composition of the proteose-peptone fraction present may have some significant effect on the functionality of whey protein concentrates and isolates as they are not heat denaturable and can bind calcium (Kinsella and Whitehead, 1989).

2.3 Denaturation of proteins

Linked with structural stability of the proteins is the phenomenon of denaturation. Globular proteins maintain their stability and activity *via* a delicately balanced minimization of conformational free energy in the "native", compact structures. Small changes in the environment of the protein may result in a disturbance of the native structure, referred to as denaturation. The magnitude of such changes range from reversible ones in the spatial location of single amino acid residues to a total destruction of the secondary and tertiary structures (which maintain the protein molecular structure) which are largely irreversible (Pearce, 1989).

Denaturation is any major alteration in the original native or tertiary structure of the protein without hydrolysis of the primary covalent bonds, the changes are restricted mainly to those occurring in the secondary structures (de Wit, 1981; de Wit and Klarenbeek, 1984; Mulvihill and Donovan, 1987). Each protein has a unique structure and therefore displays different responses to changes in the environment. Depending on the protein and environmental conditions, denaturation may be confined to a segment of the protein (Lillford, 1978) or may involve the complete protein molecule (Brandts, 1967).

During denaturation the forces stabilising the native protein structure are disrupted, particularly the hydrogen bonds and van der Waals interactions, and the protein structure collapses and a new unidentified random coil structure is obtained. In some cases, it may involve the disruption of disulphide bonds leading to complete unfolding of the polypeptide chain. Denaturation exposes the hydrophobic amino acid residues buried deep within the protein structure causing an increase in the reactivity of such groups. There is increased reactivity of Cys residues since they can undergo oxidation to disulphide (S-S) or cysteic acid ($-S_3OH$) groups; disulphide interchange may occur leading to the formation of intermolecular disulphide bonds (de Wit, 1984; Mulvihill and Donovan, 1987; Kella and Kinsella, 1988).

Denaturation is often reversible but in most cases it is irreversible because of the interplay of many factors and subsequent reactions occurring after denaturation make it impossible for the proteins to return to their native state. The unfolded protein molecules may associate to form aggregates of irreversibly denatured molecules and such associations may finally lead to precipitation, coagulation and/or gelation. Oxidation of thiols and disulphides mainly results in irreversible aggregation (Donovan and Mulvihill, 1987b; de Wit, 1989).

Physical and chemical agents, such as heat, salts, pressure, organic solutes and solvents (ethanol, mercaptoethanol, extremes of pH (acids and alkali), chaotropic agents, urea, guanidinium chloride and sodium dodecyl sulphate (SDS) can induce denaturation (Donovan and Mulvihill, 1987b).

Manifestations of denaturation include loss of solubility and/or biological activity, changes in binding characteristics, alterations in spectral properties, a more negative optical rotation, a decrease in α-helix content, exposure of thiol groups and enhanced susceptibility to proteolytic digestion.

Denaturation of proteins may be assessed through measurements of loss of solubility, reactivity of the thiol groups, electrophoretic analysis, loss of antigenic activity and using differential scanning calorimetry (DSC) (de Wit and Klarenbeek, 1981; Bernal and Jelen, 1985). These methods are based on different physical or chemical properties of the protein and so it is difficult to compare the denaturation results obtained.

2.3.1 *Thermal denaturation of proteins*

An aqueous environment is essential for maintaining the native structure of protein. Heating globular proteins in an aqueous environment increases the thermal motion of the numerous structural elements in the polypeptide chain. These movements are all related to the energy supplied in the form of heat, leading to the rupture of intermolecular and intramolecular bonds, particularly the hydrogen bonds, van der Waals interactions and disulphide bonds.

Heat-induced unfolding of globular proteins is accompanied by an endothermal heat effect. Calorimetry can effectively be used to monitor the extent of heat denaturation as it is sensitive to the changes in enthalpy associated with denaturation. The main contributors to the heat effect are the rupture of intramolecular hydrogen and van der Waals's bonds, the formation of bonds between water and the exposure of certain amino acid residues on the protein. At high protein concentrations, aggregation of the unfolded protein molecules may proceed immediately. In contrast to unfolding, aggregation is generally an exothermic process.

Sulfhydryl and sulfhydryl-disulphide interchange reactions, molecular collision frequency, due to increased kinetic energy, are enhanced at higher temperatures. The rate of protein denaturation increases 20 - 30 times for every 10°C rise in temperature.

2.3.2 *Thermal denaturation and aggregation of whey proteins*

As heat treatments are commonly used in the production of whey protein products, this has a direct effect on the protein structure and can result in an alteration of their functions in food systems and thus have major influences on the quality of food products. Impaired functional properties include reduction in solubility, emulsifying, foaming and thermosetting properties.

Because of the heterogeneity of the whey protein system and with individual proteins exhibiting different responses towards heat, the thermal denaturation of total whey protein reflects the collective responses of the component proteins (de Wit and Klarenbeek, 1984). In addition there are synergistic effects of the proteins on one another.

The major whey proteins have different thermal transition temperatures between 60 - 100°C (Table 2.4).

Table 2.4: Thermal transition temperatures of whey proteins
(de Wit and Klarenbeek, 1984)

Protein	Td (°C)	Ttr (°C)
β-Lg	78	83
α-La	62	68
BSA	64	70
Igs	72	89

Td is the initial denaturation temperature and Ttr is the temperature at the DSC peak maximum (de Wit and Klarenbeek, 1984)

Heat-induced denaturation of whey proteins as analysed by reverse-phase HPLC indicates that denaturation proceeds in two stages (Parris *et al.* 1991). Between 60 and 70°C, denaturation of whey proteins increases slowly but progresses rapidly between 80 and 90°C. Reversible changes in whey protein structure occur mainly at temperatures up to 60°C as these reactions are governed mainly by hydrophobic bonding which is enhanced as the temperature increases up to 60°C (Scheraga *et al.* 1962; de Wit and Klarenbeek, 1984) and above this temperature the changes become irreversible. No denaturation effects are observed for β-Lg between 50 and 65°C. Secondary transition temperatures are observed above 100°C for β-Lg and BSA and De Wit and Klarenbeek, 1981 explained these to be unfolding of residual structures that are partially stabilised at 80°C.

α-La has been noted as the most heat stable of the whey proteins (Larson and Rolleri, 1955; de Wit, 1981; Lyster, 1970) mainly because of its ability to revert to its native state following heat treatment (Wong *et al.* 1988). Ruegg *et al.* (1977) observed renaturation levels of 90% in systems containing only α-La. Work using DSC by several workers (De Wit and Klarenbeek, 1984) showed that α-La has the lowest denaturation temperature but requires a large amount of heat for unfolding. DCS studies also show that the denaturation of α-La is irreversible in complex whey systems and when heated in the presence of calcium chelators, such as EDTA (Bernal and Jelen, 1984). The rate of denaturation of α-La was found by Dannenberg and Kessler (1988) to be of first order in the temperature range of 70-150°C.

BSA has an irreversible thermal transition with the free thiol group being responsible. The rate of denaturation of BSA has been observed follow be either first or second order kinetics because of the complexity of the reactions (Donovan and Mulvihill, 1987a). Fatty acids stabilise BSA against heat denaturation (de Wit, 1989).

The heat resistance of the individual whey proteins is in the order Ig < BSA < β-Lg < α-La (Larson and Rolleri, 1955) and since β-Lg is quantitatively the principal whey protein, it dominates the course of the total whey protein denaturation i.e, the denaturation curve for the total protein parallels that for β-Lg denaturation with differences in the transition temperatures. This difference can be explained by the combined stabilising effect exerted by the total proteins including the immunoglobulins and protease-peptone. Whey protein denaturation is a co-operative process (Mulvihill and Donovan, 1987).

The thermal denaturation of β-Lg has been studied by various techniques such as polarimetry, electrophoresis, light scattering or chromatography. Briggs and Hull (1945) and Sawyer (1968) observed that during denaturation of β-Lg, two distinct denaturation reactions occurred near pH 7. They noted that at pH 7.0, β-Lg exists as a dimer (N_2) and as the temperature is increased above 20°C, the dimer dissociates into its monomeric form (2N) (Figure 2.1). The first reaction is initiated at temperatures above 65°C, resulting in an increase in the β-Lg particle size followed by unfolding of the protein molecule. Molecular rearrangements increase the activity of the single sulfhydryl group and the unfolded species (2D) form aggregates Dn' as a result of disulphide interchange or sulfhydryl oxidation.

A further non-specific aggregation occurs to form heavy components (Dn") of about 29S. The unfolded molecules (2D) can form aggregates (Dx) by hydrophobic interactions when sulfhydryl blocking agents are used. The final denatured product following unfolding and aggregation reactions varies with the temperature of heating.

$$N_2$$

dimer-monomer dissociation

2N

molecular unfolding

Dx ⇌ 2D

SH/SS aggregation (primary reaction

Dn′

non-specific   aggregation (secondary reaction)

Dn‴ ⇌ Dn″
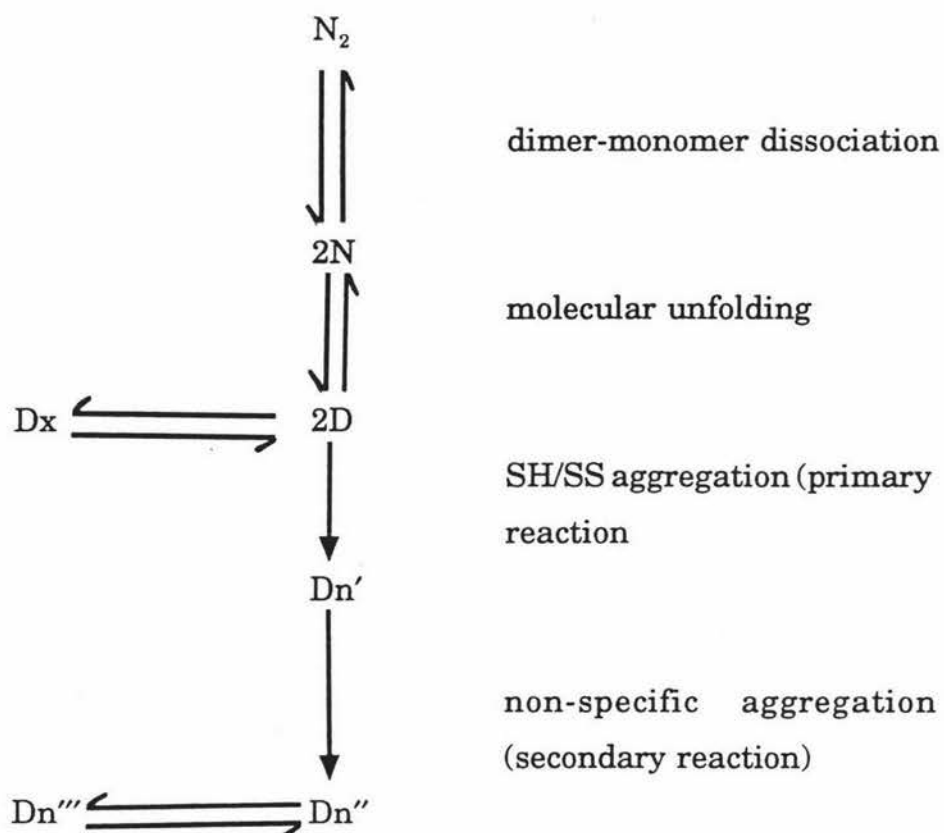
Figure 2.1 Denaturation pathway of β-Lg at pH 7 (Sawyer, 1968)

The susceptibility to heat denaturation of β-Lg genetic variants is different and in the order B-C > B-B > B-A (Sawyer, 1968).

The denaturation kinetics of β-Lg have been widely researched but the order of reaction remains unresolved due to the different methods used to assess denaturation.   Harwalkar (1980) studied the kinetics of denaturation of β-Lg at pH 2.5 in the temperature range 60-130°C by measuring optical rotation and its solubility at pH 4.5.   He detected denaturation above 75°C and observed it to occur in two stages, the first stage being faster than the second stage.   The extent of denaturation indicated a pseudo-first order or two consecutive first order reactions.   De Wit and Swinkel (1980), using DSC studies, observed denaturation of β-Lg as following first order kinetics at temperatures between 65-72°C.   A

reaction order of 1.5 was obtained by Dannenberg and Kessler (1988), whilst Lyster (1970) found a second-order reaction.

Reversible and irreversible changes occurring when whey proteins are heated are influenced further by environmental conditions such as pH, ionic strength and protein concentration.

2.3.2.1   The effect of pH

The thermodenaturation of total whey protein increases with increase in pH in the range 4.5-7.0 and the thermal responses of the individual whey proteins to pH differ. β-Lg is more sensitive to heat at high pH, BSA is most stable at the same pH while the thermal denaturation of α-La seems to be relatively independent of pH (Donovan and Mulvihill, 1987a). Whey proteins appear to be incompletely denatured on heating at pH 2.5 (Halwalkar, 1979; Li-Chan, 1983). The pH dependence of the thermal stability of whey proteins, especially β-Lg, is strongly related to electrostatic charge and thiol group activity.

Electrostatic charge is a major factor which influences protein denaturation. The different thermal responses of the individual whey proteins to pH changes may be a reflection of differences in content and distribution of polar residues in the different polypeptides. β-Lg at neutral pH has a high net negative charge resulting in strong intramolecular repulsive forces facilitating opening up of the protein and hence denaturation. Minimum thermodenaturation occurs at isoelectric pH values, where the absence of any substantial net charge means that intramolecular repulsions are very weak, the protein conformation is tight and more resistant to the unfolding action by heat.

Thermal aggregation of whey proteins is also markedly dependent on pH, aggregation being suppressed at high and low pH. This is due to intermolecular repulsive forces at the high net charge which tend to favour

protein-solvent interactions more than protein-protein interactions (Mulvihill and Donovan, 1987). Sulfhydryl reactions and sulfhydryl-disulphide reactions are promoted around pH 8 therefore the breaking of intramolecular bonds promotes denaturation whilst formation of these bonds would favour aggregation. The pH dependence of protein aggregation differs from that of denaturation thus suggesting that different molecular events are involved in the thermal process.

2.3.2.2 *The effect of salts*

The presence of salt seems to stabilise the protein quaternary structure against dissociation and denaturation. The effect of salt on denaturation temperature of whey proteins is pronounced outside the isoelectric point. The effects of salts on protein structure involves two mechanisms:

(i)   The electrostatic shielding effect, which is usually achieved at or below 0.2 ionic strength, is only dependent on the ionic strength of the medium.

(ii)   Salts exert an ion-specific effect on hydrophobic interactions thus affecting the stability of proteins at higher concentration through the modification of water structure which causes perturbations at the protein-water interface.

The addition of NaCl causes an upward shift in transition temperatures of whey proteins and these changes were greatest at low concentrations (Xiong *et al.* 1993). High concentrations of NaCl are capable of modifying the conformation and decreasing the thermal stability of whey proteins (Hermasson, 1979). Increasing ionic strength (NaCl concentration) may mask some exposed ionic groups and alter the electric double layer to facilitate interactions.

In the presence of Ca^{++}, whey proteins are less soluble and more sensitive to heating. In addition to charge depression, Ca^{++} is capable of forming bridges between adjacent peptides contributing to aggregation. Xiong *et al.* (1993) explained the different reactions observed during denaturation of β-Lg with NaCl and CaCl$_2$; they suggested that Ca^{++} binds to different structural domains of β-Lg whereas Na$^+$ interacts with all parts of the protein molecule so that a more simultaneous unfolding of the protein occurred resulting in associating in a narrower temperature range.

Denaturation is enhanced more by Ca^{++} ions than any other ions (Shimada and Matsushita, 1981a). Removal of calcium by a chelator (EDTA) decreased the denaturation temperature of α-La by 20°C, suggesting that binding of α-La (metalloprotein) with Ca^{++} is essential for stabilising its tertiary structure.

The effectiveness of various salts on the stability of proteins follows the Hofmeister series ie. $F^- > SO_4^= > Cl^- > ClO_4^- > SCN^-$ (Shimada and Matsushita, 1981a,b).

High concentrations of compounds that tend to break hydrogen bonds (for example urea, guanidine salts) also cause denaturation.

2.3.2.3   *The effect of concentration*

In general, the susceptibility of the whey proteins to heat denaturation appears to decrease as the total solid content of the solution increases (Mckenna and O'Sullivan, 1971). Mckenna and O'Sullivan (1971) reported that the amount of whey protein denaturing during heat treatment of concentrated skim milk was more dependent on the milk solids content than on temperature variations between 75 - 80°C. The effect of non-protein constituents on the thermal denaturation of whey proteins in cheese whey showed retardation of denaturation of β-Lg and α-La with increase in lactose concentration (Hillier *et al.* 1979). Bernal and Jelen

(1985) observed that the presence of milk sugars (lactose, glucose and galactose) appeared to increase the resistance of β-Lg to thermal denaturation. Hillier *et al.* (1979) noted that increasing total solids concentration up to 3-fold in cheese whey delayed the denaturation of β-Lg A and B but hastened denaturation of α-La during heating at 80°C.

2.4 Thermal gelation of globular proteins

One of the functional properties of proteins is their ability to form gels. Protein gels are responsible for the basic texture of a wide variety of foods and are the basis of certain formulated foods. Protein gels provide a structural matrix for holding water, flavours, sugars and food ingredients which are useful in food applications and product development.

Gelation results from aggregation of denatured protein molecules and involves the formation of a continuous network which exhibits a certain degree of order (Hermasson, 1979). The product of gelation, the gels, are composed of three-dimensional networks of intertwined partially associated polypeptides in which water is trapped (Clark and Lee-Tuffnell, 1986). The definition of gelation and that of its product, gels, depends on the observer's perspective and the technique used to observe it and this has resulted in a number of definitions of gels, as viewed by different scientists (Webster's Collegiate Dictionary, 1946 Ed.; 1987 Ed. (cited in Ziegler and Foegeding, 1990); Flory, 1974, 1983; Ferry, 1980; Tanaka, 1981; Russo, 1987; Ziegler and Foegeding, 1990).

Rheologically, gels have been defined as substantially dilute systems which exhibits no steady state flow thus classifying gels as viscoelastic solids rather than viscoelastic liquids (Ferry, 1980). Based on their optical properties, gels can be classified as transparent or opaque. Transparent gels consists of a homogenous network whereas opaque gels consist of colloidal particles or aggregates (Stading and Hermansson, 1991). Gels have infinite molecular weight and are concentrated systems that cannot

be substantially diluted.

2.4.1 Mechanism of gelation

Gelation occurs when there are specific protein-protein interactions and some protein denaturation is considered a prerequisite to the formation of a gel net-work. The primary importance of the denaturation process is to expose the functional groups (such as CO and NH of the peptide bond, side chain amide and -SH groups and hydrophobic groups) which, under appropriate conditions interact with each other to form a three-dimensional gel network.

Although the mechanism underlying the formation of gel networks are not fully understood the theoretical model proposed by Ferry (1948) has been widely accepted. The proposed theory of gelation is illustrated as follows :

$$xPn \underset{}{\overset{heating}{\rightleftarrows}} xPd \overset{heating\ and/or\ cooling}{\longrightarrow} (Pd)x$$
$$\qquad\qquad I \qquad\qquad\qquad\qquad II$$
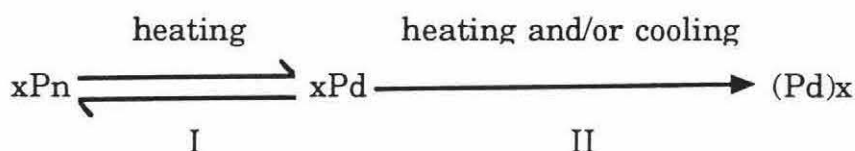
x= the number of native protein molecules, P

n= native state

d= denatured state

Thermally induced gelation is seen as a two-stage sequential process. During the first stage heat-induced changes in the protein molecule occur. This includes partial or complete unfolding of the polypeptide chain resulting in exposure of reactive side groups, such as hydrophobic residues and sulfyhydryl groups that are buried within the native protein. During the second stage of the gelation process, protein-protein interactions occur resulting in a progressive build-up of a three-dimensional network

structure under appropriate conditions (Figure 2.2). For the production of a highly ordered gel, it is essential that the aggregation step proceeds at a slower rate than the unfolding step.
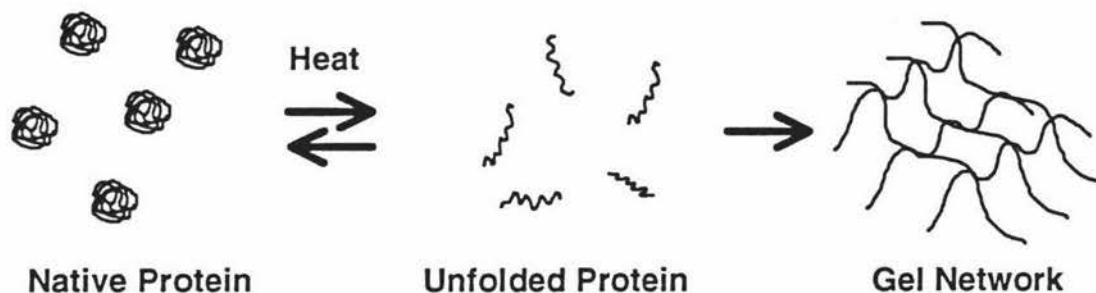
Figure 2.2 Schematic presentation of globular protein gelation.

The integrity of gels is maintained by balancing the attractive and repulsive forces between the polymer molecules and between the polymer network and the surrounding environment.

The kinetics of gel formation influence the gel microstructure, coarse networks being formed by rapid gelation and fine networks by a gradual increase in protein-protein interactions. Because denaturation is required to occur first before gel formation, complete denaturation prior to aggregation results in networks with greater homogeneity (lower opacity) and higher elasticity than if aggregation and denaturation occurred simultaneously or if aggregation preceded denaturation (Egelandsdal, 1984; Ziegler and Foegeding, 1990). Clear gels reflect the formation of uniform networks of filaments and there is greater linear aggregation with frequent cross-linking and/or branching. As the protein network density becomes less regular, the gels increase in turbidity (Kinsella and

Whitehead, 1989).

2.4.1.1   *Molecular forces governing protein-protein interactions during gelation*

Covalent disulphide bonds, non-covalent (electrostatic, van der Waals and hydrophobic interactions) and hydrogen bonds are the main molecular forces present within a protein and during interactions with other proteins (Figure 2.3).   Non-covalent interactions occur in the formation of both reversible and irreversible gels.   The extent and types of non-covalent interactions vary with the protein, pH, heat treatment and ions present. Cross-linking is essential for gel formation and the degree of cross-linking can be variable.   In the formation of irreversible set gels, covalent disulphide cross-linking may occur.   Electrostatic interactions are also critical in determining gel formation and gels properties.
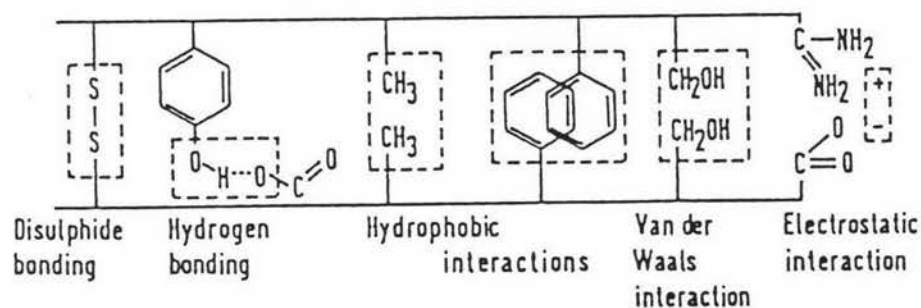
Figure 2.3   Molecular forces involved in protein interactions (Howell, 1992)

Table 2.5 shows the type of bonds and interactions that are presumed to contribute to the formation of the gel matrix.

Table 2.5:   Cross-link bonding of protein gel structures and their properties (Schmidt, 1981)

Type	Energy (kJ/mol)	Interaction distance (nm)	Groups involved	Role in gel matrix
Covalent bonding	335-375	0.1-0.2	-S-S-	Bridging Ordering
Hydrogen bonding	8-42	0.2-0.3	-NH..O=C- -OH..O=C-	Bridging Stabilising
Hydrophobic and related interactions	4-12	0.3-0.5	Non-specific	Strand thickening
Ionic bonding + interactions	42-84	0.2-0.3	-NH$_3^+$, -COO-	Solvent interactions Salt linking

2.4.2 *Whey protein gelation*

Whey proteins have excellent gelling characteristics, particularly above pH 7.0. The mechanism of gelation of whey proteins is presumed to be similar to that of other globular proteins where an initial denaturation occurs followed by an aggregation step of the denatured proteins resulting in the formation of a gel matrix. WPC gels can be formed under different conditions. There are no standard conditions for gelation and gelation will occur under favourable conditions of protein structure, ionic/pH environment and protein concentration and the resulting gels can possess different rheological properties.

Various methods have been used to characterise whey protein gels. These include visual comparison with a standard, gels strength and texture profile analysis, gel structure and their ability to take up water. Whey protein gels are described as irreversible gels and the gels formed range in properties from viscous fluid soft smooth pastes or curds to stiff, rubbery gels. These gels vary in hardness, cohesiveness, stickiness, colour and mouthfeel. WPC gels also vary in visual appearance from firm elastic transparent gels to opalescent curdlike coagula (Kinsella and Whitehead, 1989). In many cases, these evaluations are very subjective.

Studies on the gelation of whey proteins has been mainly concentrated on the factors that influence gelation, using reconstituted whey protein concentrates (WPC) (Hillier *et al.* 1980; Shimada and Cheftel, 1988; de Wit, 1989; O'Riordan *et al.* 1989; Kuhn and Foegeding, 1991).

2.4.3 *Factors affecting gelation of whey proteins*
The properties of whey protein gels are affected by a number of factors such as protein concentration, heating temperature and time and conditions of the medium, such as pH, salt concentration and the types of salts present.

2.4.3.1 *Protein concentration*
The hardness of heat-induced protein gels is affected by the concentration and purity of the protein (Hillier *et al.* 1980; Clark *et al.* 1981; Hegg, 1982). The minimum amount of protein required to form a gel is an important criterion for the gel forming abilities of specific proteins and is dependent on the environmental conditions such as, pH, ionic strength, heating temperature and time. Minimum protein concentrations required for gelation at pH 6.6 and in 1% NaCl were found to be 1% and 2% for BSA and β-Lg, respectively in the temperature range 60 - 90°C (Paulsson *et al.* 1986) but Schmidt (1981) found it to be > 7.5% at 100°C for 10 min at pH 7.0.

Opacity of the gels is increased as the protein concentration is increased (Mulvihill and Kinsella, 1987). As the protein concentration is increased the number of potential interactions between the protein molecules are enhanced resulting in increased gel strength, reduced gelling time and finer gel networks (Paulsson *et al.* 1986; Mulvihill and Kinsella, 1987; Ziegler and Foegeding, 1990; Mastudomi *et al.* 1991). Below a certain concentration, non-covalent interactions may be involved in protein-protein interactions while as the protein concentration is increased, disulphide cross-linking may become an additional mode of interaction, imparting a more elastic behaviour of gels (Kastuta, 1990; Matsudomi *et al.* 1991). The factors that affect gelation of proteins are all interrelated, so the effect of protein concentration is also dependent on the pH of the environment, the presence of salts and the heating temperature and time used.

The presence of lactose and ash may impair gelation by interfering with network formation, but generally increasing the protein concentration relative to non-protein components, improves gelation of the whey proteins (Kinsella and Whitehead, 1989).

2.4.3.2 *Temperature and heating time*

Gelation is temperature and time dependent. Heating protein solutions above the minimum denaturation temperature of the constituent proteins are required for gel formation (Matsudomi *et al.* 1991) although heating at temperatures below the denaturation temperature may also result in gelation but requires longer heating times before any significant structures begin to develop. The effect of the heating temperature is also dependent on the protein type and concentration. When heating time is increased, the firmness/strength of gel is affected (Schmidt *et al.* 1978a; Shimada and Cheftel, 1988), and when all the other factors are maintained, gel hardness is increased with increase in heating time.

Heating rates also affect the gelation process. Rapid heating does not

allow enough time for the proteins to unfold and aggregate in a sequential manner even if the temperature is above the denaturation temperature of the proteins while slow heating allows the proteins enough time for unfolding and aggregation resulting in much stronger gels (Stading and Hermansson, 1990).

2.4.3.3   pH

The net charge on proteins influences gel hardness. A certain amount of electrostatic repulsive and attractive forces seems to play a role in the gel network structures (Ferry, 1948; Hermansson, 1979; Schmidt, 1981) and where repulsive forces exceed a critical number, protein-protein interactions are reduced, resulting in weak gels (Hillier *et al.* 1980).

pH is one factor that regulates formation of WPC/WPI gels and the probability of gel formation is lowest at the isoelectric point of the protein. de Wit (1989) observed that weak WPC gels were formed at pH 4.0 and strong gels between pH 6.0 and pH 7.0. Shimada and Cheftel (1988) also noted a decreased firmness of WPC gels and an increase in their elasticity from pH 6.5 to pH 9.5. Gels formed at low pH (< 6.0) are more coagulated and less elastic than gels formed at pH 7 to 9 (Hillier *et al.* 1980; Schmidt, 1981).

The decrease in gel strength at elevated pH values is associated with an increase in protein solubility, suggesting that, at pH values which are favourable for disulphide formation, a decrease in protein-protein interactions (*via* electrostatic repulsion) lowers the effective protein concentration of the gel matrix (Shimada and Cheftel, 1988).

2.4.3.4   Salt concentration

The effect of salt on gelation is dependent on the salt concentration and species (Kinsella, 1982; Mulvihill and Kinsella, 1988). Salts cause charge neutralisation, suppressing repulsion by counter ions, thus enhancing

protein-protein interactions and resulting in more stable gel networks. The amount of salt added may influence the type of gel formed. Several researchers have carried out experiments on gelation and the effects of adding salts (Schmidt *et al.* 1979; Zirbel and Kinsella, 1988; Matsudomi *et al.* 1991; Kuhn and Foegeding, 1991).

Matsudomi *et al.* (1991) observed that the addition of 2 mM $CaCl_2$ resulted in the β-Lg gels which were more elastic and transparent whereas above 5 mM the gels appeared opaque and sponge-like. BSA formed elastic clear gels with $CaCl_2$ concentrations up to 5 mM. Kuhn and Foegeding (1991) noted translucent, gelatin-like gels with low levels of NaCl (25 - 30 mM) and opaque curd-like gels with 7.5 mM $CaCl_2$ using WPI. Experiments by Schmidt *et al.* (1979) on dialysed WPC showed that dialysed WPC produced more translucent, stronger and gummy, chewy gels than non-dialysed WPC gels.

$CaCl_2$ has been shown to be more effective than NaCl in affecting gel strength. Calcium addition may cause calcium bridging between negatively charged groups on adjacent unfolded protein molecules thereby increasing the strength of the matrix (Mulvihill and Kinsella, 1988). Excessive amounts of these ions have a detrimental effect on gel strength, resulting in the matrix collapsing (Mulvihill and Kinsella, 1988; Mangino *et al.* 1987), possibly through excessive cross-linking and aggregation (Schmidt *et al.* 1979). Chelation using EGTA and EDTA on WPC and WPI solutions showed a detrimental effect on gels formed, showing that calcium plays an important role in gelation of whey proteins (Foegeding *et al.* 1991)

The effects of different anions of sodium on the hardness of whey proteins (BSA, β-Lg) has been shown to follow the lyotropic series which is $SO_4^=$ > > Cl^- > Br^- > I^- > SCN^- (Matsudomi *et al.* 1991; O'Riordan *et al.* 1989)

The difference between the reaction of the salts has been shown to be monovalent or divalent cation effects (Kuhn and Foegeding, 1991). Salts of monovalent cations such as LiCl, NaCl, KCl had similar effects on WPC gels while salts of divalent cations ($MgCl_2$; $CaCl_2$; $BaCl_2$) also showed similar effects on WPC gels in their group. The divalent salt has a dominating effect when the two are in mixture even if the monovalent salt was in sufficient quantity to form strong gels (Kuhn and Foegeding, 1991).

2.4.3.5   Protein conformation

The strength of whey protein gels is related to the extent of denaturation and unfolding of the protein molecules under a given set of conditions. The higher the extent of denaturation, the higher is the exposure of functional groups and the greater the gel strength. The state of conformation of the protein at the onset of gelation seems to have a major influence on the ultimate physical characteristics of protein gels (Wang and Damodaran, 1991). Work on macroscopic network structure of gels (Clark and Tuffnell, 1980; Clark *et al.* 1981a) does not seem to provide information on the molecular structures of proteins in the gel network and their relationship to gel properties.

β-Sheet structure has been shown to be involved in aggregation and network formation in BSA gels (Wang and Damodaran, 1991) and the strength of the gels may be directly related to the extent of transformation of α-helical structures into β-sheets during the heating step.

2.4.3.6   The role of thiol groups in gelation

Thiol groups and disulphide bonds have been reported to play an important role in the heat induced gelation of whey proteins (Hillier *et al.* 1980; Utsumi and Kinsella, 1985; Shimada and Cheftel, 1989) by forming intermolecular cross-links. These covalent bonds can be induced by thiol oxidation or by thiol-induced disulphide interchange reactions (Hillier *et al.* 1980).

The addition of N-ethylmaleimide (NEM) has the effect of blocking free thiol groups, inhibiting their participation in thiol-disulphide interactions within or between protein molecules. Dithiothreitol (DTT) has the effect of reducing disulphide bonds in proteins. Addition of NEM or DTT to whey proteins resulted in decreased gel strength of β-Lg and BSA gels. Low levels of added DTT reduce the disulphide bonds and enhanced protein unfolding and protein-protein interactions resulting in an increase in gel strength but higher levels of DTT resulted in reduced gel strength due to excessive reductions resulting in coagula being formed and not structured networks. Electrostatic interactions and thiol-disulphide interactions are critical in the formation and stabilisation of whey protein gels (Matsudomi *et al.* 1991).

2.5 Gelation of individual whey proteins

2.5.1 *Gelation of* β-*Lactoglobulin*

β-Lg, the major whey protein, dominates the overall gelling behaviour of whey proteins and has been shown to have good gelling properties (Paulsson *et al.* 1986). When β-Lg is heated above a critical temperature it undergoes conformational changes followed by subsequent protein-protein interactions resulting in gelation depending on the protein concentration, heating methods and environmental conditions (such as pH and salt concentration). Disulphide and hydrophobic interactions are involved in the formation of the gel network but their relative contribution to the stability of the gel network is still unclear (Kinsella and Whitehead, 1989; Matsudomi *et al.* 1991).

McSwiney *et al.* (1994a,b) studied thermal aggregation and gelation of β-Lg and from their results a possible reaction mechanism occurring during gelation was suggested.

The sequence of events was depicted as follows:

$$Pn \rightleftharpoons Pu \rightleftharpoons (Pu)x \longrightarrow (Ps\text{-}s)x \longrightarrow \text{Gel network}$$

Where Pn represents the native monomeric protein, Pu represents partially unfolded or molten globule state of protein, and (Pu)x and (Ps-s)x represents the aggregated forms of the molten globule protein. The first step involved partial unfolding of the protein while retaining its secondary structure with increased exposure of hydrophobic groups. This state was referred to as the "molten globule state". As a result of unfolding, hydrophobic interactions between the exposed groups resulted in aggregation of the protein molecules while still in the molten globule state. The aggregates were further strengthened by the formation of inter- and intramolecular disulphide bonds via sulphydryl-disulphide interchange or sulphydryl oxidation reactions prior to polymerisation of the aggregates into a structured network. Both β-Lg variants (A and B) appear to contribute to the gelation of β-Lg although the gelation properties of the two variants are different (McSwiney *et al.* 1994a,b).

The gelation temperatures of β-Lg have been reported to be in the range 75 - 80°C and it increases with decreasing β-Lg concentration but is little affected by pH (Paulsson *et al.* 1986).

The minimum concentration of β-Lg required to form a self-supporting gel is dependent on the environment in which the protein is dispersed, the pH of the solution, the heating temperature and the rate of heating used. Matsudomi *et al.* (1991) found a minimum protein concentration of 5% in 100mM Tris-HCl buffer at pH 8.0 and heating temperature of 90°C for 15 min. A minimum β-Lg concentration of 2% (w/v) was required to form a gel at pH 6.6 and in 1% NaCl (Paulsson *et al.* 1986).

The hardness of β-Lg gels increases exponentially with increase in protein concentration (Hillier *et al.* 1980; Clark *et al.* 1981; Mulvihill and Kinsella, 1987; Matsudomi *et al.* 1991; McSwiney *et al.* 1994a,b). The gelation time decreased with increasing β-Lg concentration.

The gel properties of β-Lg gels are markedly influenced by pH. β-Lg gels formed at pH between 4 and 6 are opaque and are known as "particulate" or "aggregate" gels. At pHs below and above this range, fine-stranded transparent gels are formed (Stading and Hermansson, 1991; Langton and Hermansson, 1992). Depending on the pH β-Lg can form gels at different concentrations. Stading and Hermansson (1990) noted a 1% (wt/wt) β-Lg concentration at intermediate pH while concentrations >10% (wt/wt) were observed at high and low pH. Matsudomi *et al.* (1991) reported a maximum gel hardness of β-Lg gels at pH 6.5 while Stading and Hermansson (1990), Paulsson *et al.* (1986) and McSwiney *et al.* (1994) reported maximum gel hardness at pH 5.5, 5.0 and 7.0, respectively. Variations in the methods used to assess the gel strengths, the methods of heating and heating rates and the buffering systems used by the different workers allow for the wide variations in the results reported.

The addition of salts affects β-Lg gels (Mulvihill and Kinsella, 1988; Matsudomi *et al.* 1991; Foegeding *et al.* 1992). Maximum gel hardness was obtained after the addition of 20-40 mM NaCl or 2 mM $CaCl_2$ (Matsudomi *et al.* 1991) and Mulvihill and Kinsella (1988) obtained gels of maximum compressive strength with sodium and calcium chloride concentrations of 200 and 10 mM at pH 8.0, respectively. The effect on β-Lg gelation by various anions of sodium follow the lyotropic series.

Addition of NEM (< 5 mM) or DTT (2 mM) increased gel strength of β-Lg gels. Excessive addition increases of NEM and DDT result in negative effects on gel strength. Foegeding *et al.* (1992) using dynamic rheology observed that β-Lg gels containing 20 mM $CaCl_2$ had lower G' values than

gels containing 100 mM NaCl, the latter gels were less deformable requiring a greater shear strain to achieve fracture. Electrostatic interactions and disulphide bonds are involved in the formation of β-Lg gels (Matsudomi *et al*. 1991).

2.5.2 Gelation of BSA

BSA is a protein with strong gelling properties (Yasuda *et al*. 1986; Paulsson *et al*. 1986). Heating BSA solution above its denaturation temperature is required for gelation to occur (Paulsson *et al*. 1986; Matsudomi *et al*. 1991). Gelation temperature is dependent on the protein concentration and is lowered as the BSA concentration is increased. Paulsson *et al*. (1986) observed gelation temperatures of 90°C, 79°C and 70°C for 1, 2 and 5%, w/v, BSA in 1% NaCl at pH 6.6. Gelation temperatures have been shown to be lower on the acidic side than on the alkaline side suggesting that the same forces may not be involved in gels formed on both sides of the isoelectric point (Yasuda, 1986). BSA gels appear transparent, smooth textured and exhibiting good water holding properties (Matsudomi *et al*. 1991).

The minimum protein concentration required for gelation varies from 0.6-6.6% depending on the conditions for gelation (Clark *et al*. 1981). Matsudomi *et al*. (1991) found that a minimum BSA concentration of 4% was required in 100 mM Tris-HCl buffer at pH 8.0. At pH 6.6 in 1% NaCl a minimum concentration of 1% (w/v) was noted by Paulsson *et al*. (1986). Richardson and Ross-Murphy, (1981a) reported a minimum BSA concentration of ~ 6.8% (wt/wt) at pH 6.5.

The factors that influence gelation of β-Lg to some extent affect the gelation of BSA. Changes in the secondary structure associated with gelation showed that there was an increased β-sheet content (Clark *et al*. 1981).

Increasing the protein concentration increases the gel hardness of BSA gels (Hillier *et al.* 1980; Richardson and Ross-Murphy, 1981a; Clark *et al.* 1981; Matsudomi *et al.* 1991). Higher temperatures produce stronger gels, mainly through disulphide bonding and increased protein unfolding. Increasing the heating time results in a progressive increase in gel hardness (Matsudomi *et al.* 1991).

Variations in pH and ionic strength results in profound effects in BSA gels. Opaque gels are formed at low pH (3.5 - 4.5) although they are more rigid and less elastic. At high pH (5.8 - 8.0) weaker, clearer gels are obtained (Richardson and Ross-Murphy, 1981b; Matsudomi *et al.* 1991). Maximum gel hardness was observed at pH 6.5 (Matsudomi *et al.* 1991). BSA has a tendency of forming large compact aggregates when the net charge is low and well developed gels only occur at pH values where BSA molecules have a limited capacity for aggregation (Yasuda, 1986)

At high pH (pH 8.0), the addition of NaCl greatly affected the critical concentration of BSA required for gel formation and the gels formed at constant concentration are strengthened with increasing NaCl (Mastudomi *et al.* 1991). $CaCl_2$ has an effect on BSA gels. When $CaCl_2$ is added to BSA solutions clear elastic gels are formed; $CaCl_2$ appears to be more effective than NaCl in increasing the gel strength of the BSA gels (Matsudomi *et al.* 1991). Excessive amounts of the salts appear to have a detrimental effect on the gels formed; the hardness of BSA gels does not seem to be affected by NaCl concentrations of up to 400 mM. Concentrations of $CaCl_2$ above 10 mM formed gels which did not recover once deformed (Matsudomi *et al.* 1991).

BSA has one thiol group and 17 disulphide bonds per monomer. Addition of NEM (> 2 mM) decreased gel strength and the BSA gels formed were transparent, fragile and less elastic than the controls (Matsudomi *et al.* 1991). Up to 5 mM, DTT increased strength of BSA gels and excessive

amounts (> 20 mM) resulted in gelation of the protein without heating, due to extensive disulphide reductions thus resulting in excessive protein-protein interactions and formation of coagula (Matsudomi *et al*. 1991).

2.5.3 Gelation of α-Lactalbumin

The ability of α-La to form gels has been described as poor (Paulsson *et al*. 1986). Protein concentrations of up to 20% have been found not to produce any form of a gel network after heating (Paulsson *et al*. 1986; Calvo, 1993). After heating at 80°C for 3 hours, weak α-La gels have been obtained by Hines and Foegeding, (1993). In the presence of glutathione (which presumably reduces the disulphide bonds in α-La), α-La has been shown to form a gel (Legowo *et al*. 1993).

2.6 Mixed protein gels

Most food gels consist of several components, some of which can gel individually and others not. These mixtures may occur naturally as in WPC and WPI. Whey products have been shown to possess proteins with different gelling behaviour (β-Lg, BSA) when heated individually and some which do not show significant gelling ability (α-La). An understanding of the interactions of these proteins, how they interact and whether the effects are positive or negative for gel formation is important.

2.6.1 Types of gels

There are a variety of ways in which two or more proteins can interact which will affect the properties of such a multicomponent gel. Several of these possibilities are shown in Figure 2.4. Factors which influence the type of gel structure formed are the thermodynamic compatibility of the components, their mutual reactivity or their potential for interaction and their respective mechanism of gelation. Proteins can react in several different ways when mixed. They can be considered as compatible, incompatible or semi-compatible and can also be either immiscible or can partially mix at the molecular level (Ziegler and Foegeding, 1990).

Protein gels which result when one or both proteins gel or when the non-gelling protein is of low concentration or does not gel at any concentration, are called composite or multi-component gels. Filled gels are obtained when the protein is interspersed throughout the gel network. Two types of filled gels can be distinguished depending on the phase state of the system; single phase (Figure 2.4A) where the filler remains soluble and two-phase gels (Figure 2.4B) where thermodynamic incompatibility results in phase separation, with the gel filler existing as dispersed particles or as a secondary gel network.

Physical association can occur as a result of interactions among proteins in complex gels. Non-gelling proteins may interact randomly *via* non-specific interactions resulting in rigid and less flexible gels. In some cases the proteins can co-polymerise into a single network (Figure 2.4C). Two or more proteins may copolymerize to form a single, heterogeneous network (Figure 2.4D). A type of multi-component gel is the interpenetrating polymer network (Figure 2.4E) in which two protein networks are formed through out the sample and this is mainly observed in compatible proteins systems. In some cases, a degree of incompatibility is observed even though the proteins remain intimately mixed and the network resulting from such a mixture appears to exhibit a greater degree of continuity and this determines the mechanical properties of the gel. Addition of other proteins has the effect of reducing the critical concentration of gelation of the primary gelling protein (Ziegler and Foegeding, 1990).
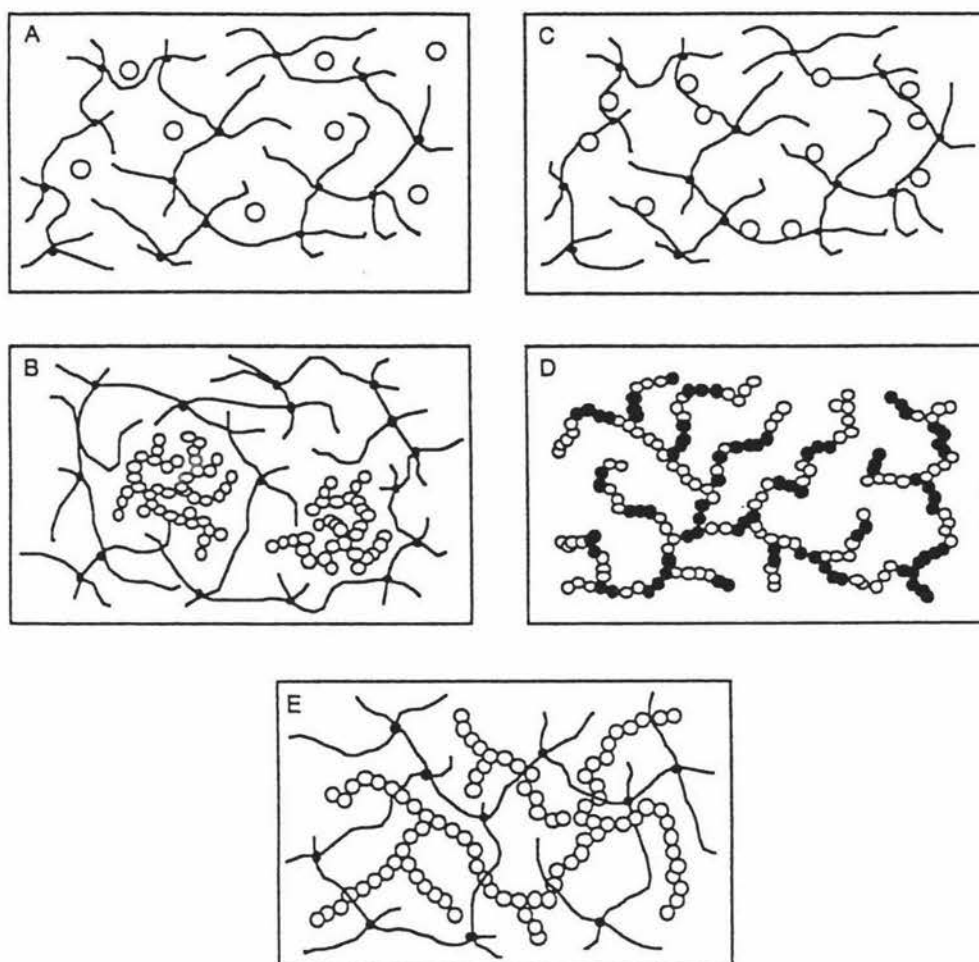
Figure 2.4 Types of mixed gels

A: Single phase gels

B: Two phase gels

C: Complex gels with a gelling and a non-gelling protein

D: Complex gel with co-polymerisation of the two networks

E: Complex gel also known as a interpenetrating gel

(from Ziegler and Foegeding, 1990)

2.6.2 *Gelation of mixed whey proteins*

The gelation process of any whey protein can be influenced by mixing with other whey proteins and it is difficult to predict the gelation pattern when the proteins are mixed. Little information is available on the gelation of mixed whey protein systems.

2.6.2.1 *Gelation of β-Lg and BSA*

When β-Lg and BSA are mixed and heated, the gelation temperatures have been shown to be affected by the amount of β-Lg or BSA in the mixture. Increasing the β-Lg concentration in the mixture shifted the gelation curves of the mixtures to higher temperatures. β-Lg and BSA have been shown not to be mutually interchangeable without changing the properties of the gel (Paulsson *et al*. 1986).

Matsudomi *et al*. (1994) studied heat-induced gelation in a mixed system of β-Lg and BSA. The hardness of the gels formed upon heating at 80°C for 30 minutes was significantly affected by the relative proportion of β-Lg and BSA. More intense interactions were developed between the two proteins at a mixing ratio of 1:1 (wt/wt) and these authors suggested that the gelation process of each of the protein was enhanced synergistically by the other causing the formation of the harder gels. Soluble protein aggregates were formed between β-Lg and BSA through thiol-oxidation reactions during gelation.

Hines and Foegeding (1993) studied mixed gel rheology using 7%, w/v, protein concentrations dispersed in 50 mM TES buffer at pH 7.0 and using protein mixtures containing β-Lg to BSA molar ratios of 5:1, 10:1, 20:1 and 50:1 and (corresponding to weight ratios of 1.39:1, 2.79:1, 5.6:1 and 13.9:1). The mixed protein solutions developed gel strength in a similar manner to that of β-Lg but the strength of the gels increased with the amount of BSA in the original protein solution. Protein-protein interaction studies using 1%, w/v, protein solutions showed that the rate of BSA aggregation was

not affected by the presence of equimolar quantities of β-Lg while the aggregation rate of β-Lg was faster in the presence of BSA.

2.6.2.2   *Gelation of β-Lg and α-La*

Interaction of β-Lg with α-La during heating has been reported by several workers (Baer *et al.* 1976; Elfagam and Wheelock, 1978a; Matsudomi *et al.* 1992; Calvo, 1993).   Renaturation of α-La after heating does not occur when heated in the presence of another protein, such as β-Lg.   The aggregation rates of α-La have been shown to increase when the protein was heated in the presence of β-Lg (Elfagam and Wheelock, 1978a; Matsudomi *et al.* 1992; Hines and Foegeding, 1993; Calvo, 1993).

Heat-induced gelation studies of β-Lg and α-La have shown that although α-La does not gel when heated alone, it seems to be involved in gelation when heated with proteins that possess SH groups.   Matsudomi *et al.* (1992) reported that α-La seemed to enhance the gelation of β-Lg by increasing the gel strength of the protein mixture even at concentrations where β-Lg can not gel.   Heating at temperatures (such as 70°C) where β-Lg can not form self-supporting gels the addition of α-La to the protein solution resulted in gelation of the protein solution.   Interaction of β-Lg and α-La during heating resulted in soluble aggregates being formed through thiol-disulphide reactions. Addition of increasing amounts of α-La to β-Lg resulted in increasing opaqueness of the gels (Matsudomi *et al.* 1992).

Hines and Foegeding (1993) observed that gels formed by β-Lg and α-La mixtures were similar to those obtained when β-Lg was heated alone. Molar ratios of 5:1, 10:1 and 20:1 (the corresponding weight ratios 1.39:1, 2.27:1, 5.6:1 and 13.9:1) with β-Lg predominating were used.

2.6.2.3   Gelation of α-La and BSA

Matsudomi *et al.* (1993) studied the gelation of α-La and BSA in mixture at 80°C. The proteins were dispersed in 100mM sodium phosphate buffer (pH 6.8). α-La appeared to enhance the gelation of BSA by increasing the gel hardness of the protein mixture. The two proteins interacted during heating resulting in formation of soluble aggregates through thiol-disulphide interchange reaction. These aggregates had lower molecular weights than those formed from BSA alone. Gels from the protein mixtures remained transparent, reflecting the interactions of these proteins during gelation.

OBJECTIVES

- To study the gelation properties of individual whey proteins and mixed protein systems in an ionic environment similar to that in whey protein concentrates.

- To study the denaturation and aggregation behaviour of individual whey proteins in mixed protein systems during gel formation.

- To identify the relationships between denaturation/aggregation of proteins and the rheological properties of the gels.

MATERIALS AND METHODS

3.1   Materials

3.1.1 Whey protein source

β-Lactoglobulin (β-Lg) (Lot 100H8185, L-2506), bovine serum albumin (BSA) (Lot 50H9300, L-4378), and α-lactalbumin (α-La) (Lot 92H7015, L-5385) were obtained from Sigma Chemical Company, St Louis, USA. Whey Protein Concentrate (WPC, Alacen 475) was obtained from the NZ Dairy Board, Wellington.

3.1.2 WPC buffer

A buffer was prepared using analytical grade chemicals to give a salt concentration similar to that of 12%, w/v, WPC solution (Table 1). The pH of the buffer was adjusted to pH 6.8 and it was stored at 5°C.

Table 3.1:   Composition of the buffer

Salt	Weight (mg/L)	Composition (mg/L)					
		Na	K	Ca	P	Cl	Citrate
K_2HPO_4	2030	-	911	-	361	-	-
NaCl	630	249	-	-	-	380	-
$Na_3Citrate.2H_2O$	2060	483	-	-	-	-	1324
$CaCl_2.2H_2O$	1310	-	-	356	-	632	-
Lactose	8000	-	-	-	-	-	-
Total weight		732	911	356	361	1012	1324

3.2   Experimental methods

3.2.1 Rheological Measurements

The proteins (β-Lg, BSA, α-La, WPC) were dissolved in WPC buffer (Table 3.1), pH 6.8. The solutions were stored overnight at 5°C, filtered through 0.45 µm filter (Millipore) and then degassed to remove air bubbles and the pH was readjusted to 6.8 using 0.1 M HCl or 0.1 M NaOH. A Bohlin Rheometer (Bohlin Rheologi, Lund, Sweden) was used to measure the rheological properties of the protein solutions and gels during heating and cooling.

The Bohlin VOR Rheometer is a computer controlled instrument working in three different modes performing oscillation, viscosity and relaxation measurements (Figure 3.1). The oscillatory mode was used in the present experiment. The instrument consists of a Couette type cup and a fixed bob system. The bob is suspended in a torsion bar and a torque shaft is suspended on an air bearing. The Bohlin instrument can measure forces between 10^{-5} and 10^{-2} Nm. The sensitivity of the instrument is determined by the choice of torque bar and the bob and cup size. The advantages of the Bohlin instrument include the possibility of unattended continuous or discontinuous measurement in a programmed course of temperatures and/or other measurement conditions; the amplitude for deformation can be kept small to prevent disruption of the gel. The properties of the gel, the elastic (storage) modulus G', the viscous (loss) modulus G'' and the combined resistance to deformation as the complex modulus G^* are measured at predetermined strains and frequencies as functions of time or temperature.

The protein solution (0.65 ml) was carefully placed in a measuring system consisting of a cup (outer cylinder of diameter 8.8 mm) and a bob (the inner cylinder , diameter of 8.0 mm). The bob was suspended on a torsion bar of 4.4×10^{-3} Nm.
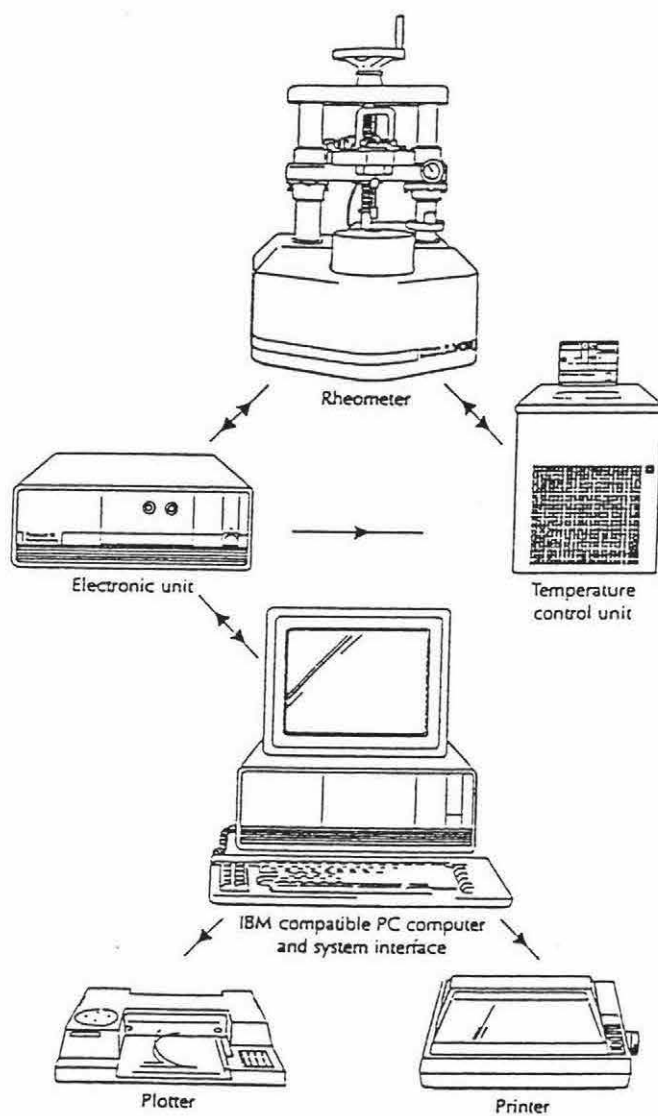
Figure 3.1 The Bohlin Rheometer set-up with associated equipment

The bob was then lowered very slowly into the cup until the protein solution just reached the top surface of the bob. The solution was covered with a thin layer of paraffin oil to prevent evaporation of water and subsequent surface drying. The oil was immiscible with the protein solution.

The protein solutions were heated "in situ" in the Bohlin cup at a rate of 1°C/min to the desired temperatures and the solutions held at the heating temperature for 60 min and gel characteristics continuously measured. On completion of measurements at the heating temperature, the temperature was lowered to 25°C at the rate of 1°C/min; the sample held at this temperature whilst measurements were made for 10 min (Figure 3.2)
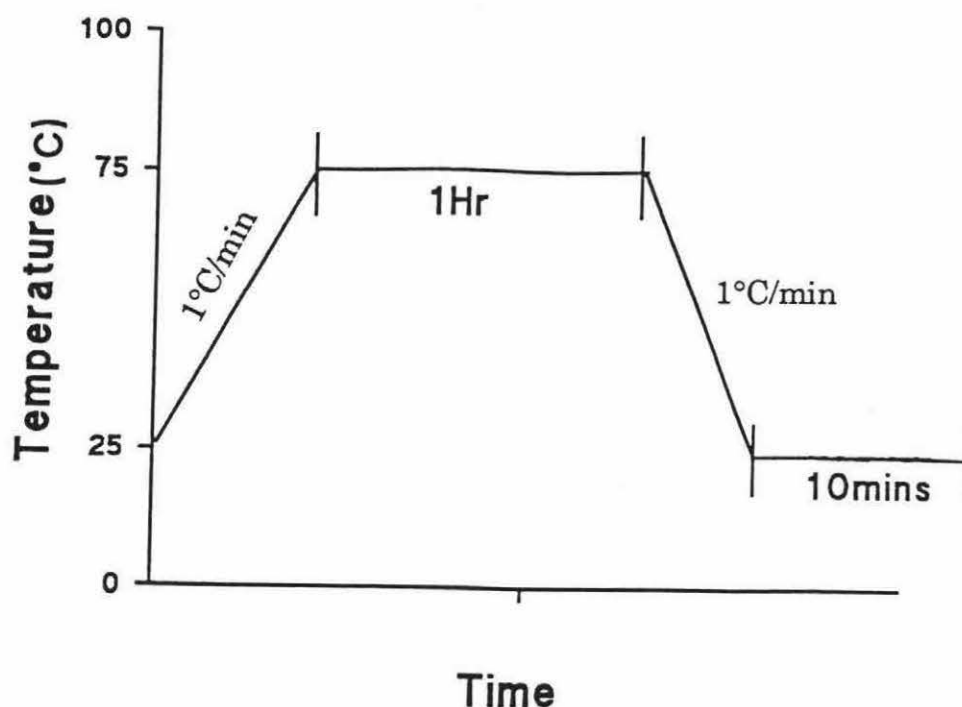
Figure 3.2 The heating profile of the protein solution during gel formation and measurement

During measurement when the cup is oscillating, the moment couple of the bob is sensed through the angular deflections and the signal is fed to a computer for calculations of G′, G″ and G* via fast Fourier transform. An attached temperature control unit is also attached and controlled by the computer.

4.2.1 *Viscoelastic properties of gels : theoretical considerations*
In dynamic rheological measurements, two fundamental parameters are measured, G′ and G″.

G′, (the storage modulus) is determined from the component of stress which is in phase with the strain and defined as:

$$G'(\omega) = (\frac{\tau_0}{\gamma_0}) \cos\delta \qquad (1)$$

where : γ_0 is the maximum amplitude ; ω is the frequency of oscillation, τ_0 is the maximum stress and δ is the loss angle.

G″ (the loss modulus), the viscous part of stress, which is the part of stress out-of-phase with the strain, is defined as :

$$G''(\omega) = (\frac{\tau_0}{\gamma_0}) \sin\delta \qquad (2)$$

The resultant stress wave is written as :

$$\sigma = \gamma_0 \ (G' \sin \omega t + G'' \cos \omega t) \tag{3}$$

where : σ is the stress at time (t).

The dynamic gel stiffness, G*, the complex modulus is expressed as :

$$|G*| = \frac{\tau_0}{\gamma_0} = \sqrt{(G')^2 + (G'')^2} \tag{4}$$

The loss angle (tan δ), which gives a ratio of the elastic and viscous elements of the gel is defined as :

$$\tan \delta = \frac{G''}{G'} \tag{5}$$

The relationship between G', G", G* and tan δ can be expressed vectorially as :
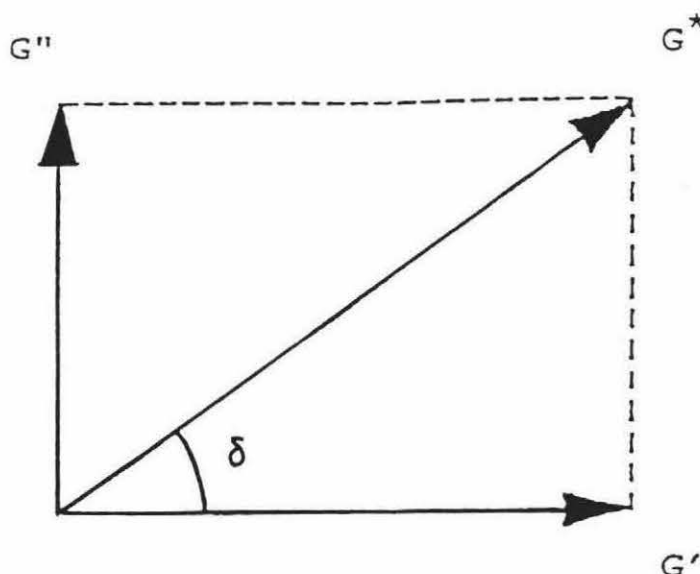
Figure 3.3 The relationship of G*, G', G", and δ

For a perfectly elastic material, all the energy is stored and the stress and strain will be in-phase and G" will be zero. For a liquid possessing no elastic character, all the energy is dissipated as heat and G' is zero and the stress and strain are out-of-phase. Subjecting protein gels to stress (shear stress), the energy for deformation is not completely conserved as in an ideal elastic solid nor is it dissipated as in liquid. Stress applied for a short time results in the elastic character of the gel predominating but if applied for a long time, the viscous character predominates. When viscoelastic protein gels are subjected to a sinusoidal oscillating strain, the stress is neither completely in-phase nor completely out-of-phase (Figure 3.4).
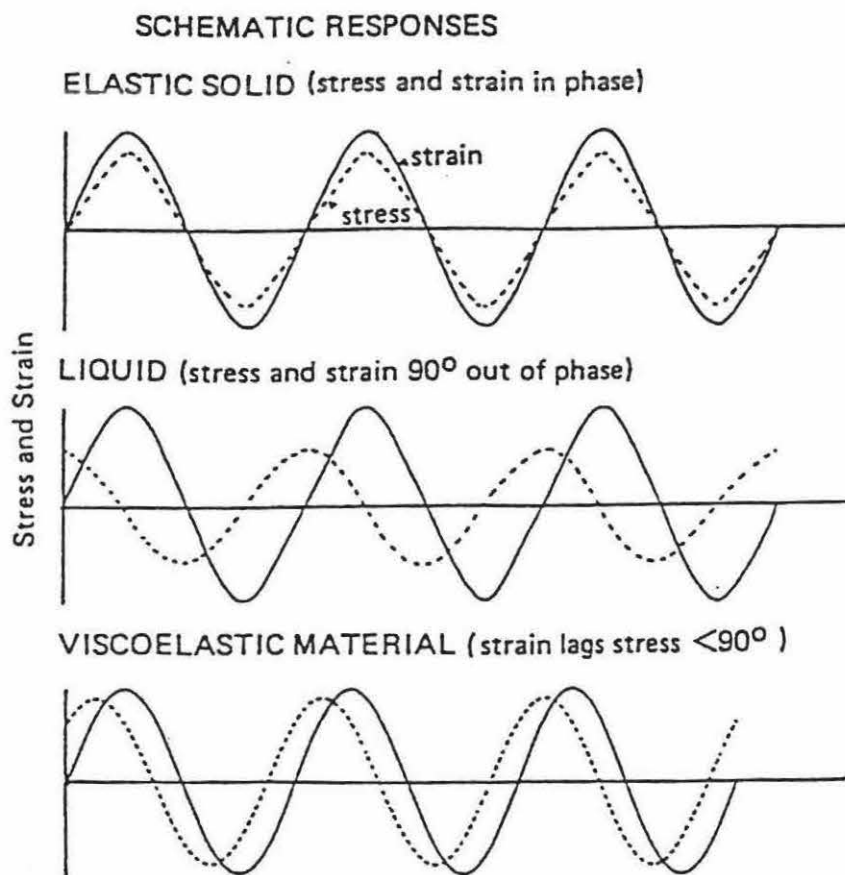
SCHEMATIC RESPONSES

ELASTIC SOLID (stress and strain in phase)

LIQUID (stress and strain 90° out of phase)

VISCOELASTIC MATERIAL (strain lags stress <90°)

Figure 3.4 Dynamic experiment showing response of elastic solid, liquid and viscoelastic material. (Ferry, 1980)

3.2.2 *Protein aggregate formation*

3.2.2.1   Heat treatment of protein solutions

The protein solutions (dissolved in WPC buffer) were heated at selected temperatures in the Bohlin Rheometer cup at 1°C/min under conditions similar to those used in the gelation studies. Aliquots were removed at 0 (when the heating temperature was just reached) 1, 2, 4, 6, 10 and 14

minutes (where possible) and mixed with chilled sample buffers for both native-PAGE and sodium dodecyl sulphate-PAGE gels (SDS-PAGE).

The native-PAGE sample buffer contained 20% 0.5M Tris-HCl buffer, 10% glycerol and 1.25 mg of bromophenol blue (tracking dye). In this buffer the native protein conformation presumably remains unaltered. The SDS-PAGE sample buffer contained 20% 0.5 M Tris-HCl buffer, 10% glycerol, 2% SDS and 1.25 mg of bromophenol blue. The presence of SDS in this buffer presumably dissociates all aggregated protein into individual polypeptide chains but the covalently-linked protein aggregates remain unaffected.

3.2.2.2   *Native-PAGE*
Undenatured whey proteins in unheated and heated samples were separated by native-PAGE using the Bio-Rad Mini Protean II system (Bio-Rad Laboratories, Richmond, CA, USA) and a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA, USA).

Gel preparation
The resolving gel was prepared by adding 10 ml of a 30% stock solution of *bis*-acrylamide mixture (37.5:1, 2.6% C) to 2.5 ml resolving gel buffer (36.3 g Tris base in 150 ml deionised water, adjusted the pH to 8.8 using 6 M HCl and brought to 100 ml with deionised water). The gel solution was degassed for 15 min at 20°C using a magnetic stirrer while evacuating with a water pump after which 10 µl of N,N,N',N' tetramethylenediamine (TEMED) was carefully mixed in. A 100 µl aliquot of freshly made ammonium persulphate solution (100 mg/1ml of deionised water) was then added and the solution mixed. The gel solution (3.3 ml) was poured between two glass plates and overlaid with deionised water. The water was removed after the gel was set and the stacking gel solution applied.

The stacking gel was prepared by adding 1.25 ml of acrylamide stock solution to 2.5 ml of stacking gel buffer solution (6.0 g Tris-base to 60 ml deionised water, adjusted the pH to 6.8 using 1 M HCl and brought to 100 ml with water). The gel solution was degassed for 15 min after which 10 µl of TEMED and 50 µl ammonium persulphate solution were added and gently mixed. A 10-slot comb was inserted once the stacking gel was poured on top of the resolving gel. The gel was allowed to set before the comb was removed. The gel slots were washed with water to remove the unpolymerised gel solution and excess water removed with filter paper.

Electrophoresis run

A pair of gels was fitted into the electrode assembly and placed into the electrode buffer chamber. A stock solution of electrode buffer (7.5 g Tris-base and 36.0 g of glycine in 400 ml deionised water, pH adjusted to 8.3 and the solution brought to 500 ml) diluted 1:5 with water was used to fill the inner buffer chamber. Protein solutions of 10 µl were applied to the gel and electrophoresed with the upper voltage limit set at 200 V. The electrophoresis run was terminated once the tracking dye moved out of the gel. The gels were then removed from the plates and transferred to plastic containers in which staining solution was present.

Staining / destaining

Each gel was stained with 50 ml of Coomassie brilliant Blue R solution (1 g brilliant blue R dissolved in 500 ml of isopropyl alcohol and 200 ml acetic acid and the solution made up to 2 l with water). The gels were rocked to ensure uniform staining. After staining for 1 hr, the staining solution was carefully drained and replaced with destaining solution which was made up of 100 ml isopropyl alcohol and 100 ml acetic acid diluted to 1 l. The destaining solution was replaced after 1 hr with fresh destaining solution and left to rock for 19 hr. The gels were then scanned and photographed.

Scanning

The gels were scanned on a UltraScan XL model laser densitometer (LKB Produkter AB, Bromma, Sweden) immediately after the destaining procedure ended. This instrument uses a helium-neon laser source to provide an intense, coherent beam of monochromatic light at 633 nm to automatically scan the gel. The amount of light which is not absorbed by the sample is determined electronically. From the results, the relative areas calculated were converted to percentages and plotted against time, from which the extent of denaturation and aggregation was determined.

4.2.2.3   SDS-PAGE

Gel preparation

The resolving gel was prepared by addition of 2.02 ml of deionised water to 2.50 ml 1.5 M Tris-HCl buffer solution (18.15 g Tris-base in 60 ml water, the pH adjusted to 8.8 using 1 M HCl and the buffer solution brought up to 100 ml), 100 µl of 10% SDS stock solution (10 g SDS dissolved in 100 ml water) and 5.3 ml acrylamide gel solution (as described in Section 2.2.2.). The gel solution was degassed for 15 min under vacuum using a magnetic stirrer after which 5 µl TEMED was added and mixed. 50 µl of freshly prepared ammonium persulphate solution (see Section 2.2.2) was gently added and carefully mixed. The gel solution (3.3 ml) was poured between two glass plates and overlaid with water. After the gel solution had polymerised the water was removed.

The stacking gel was prepared by mixing 6.1 ml deionised water, 2.5 ml 0.1 M Tris-HCl (which is the same as the stacking gel buffer described in Section 2.2.2), 100 µl of 10% SDS stock solution and 1.3 ml acrylamide gel solution. The gel solution was degassed using a magnetic stirrer under vacuum for 15 min. 10 µl TEMED and 50 µl ammonium persulphate solution were added, with careful mixing after each addition. The stacking gel was poured onto the resolving gel and a 10-slot comb inserted before

the gel began to polymerise. Once the gel had polymerised, the comb was removed and the unpolymerised gel solution washed out with water and excess water removed with filter paper.

Electrophoresis run

Sample application and the electrophoresis runs were carried out in the same way as that described for native-PAGE except that the electrode buffer was different. The stock electrode buffer (9.0 g Tris-base, 43.2 g of glycine and 3.0 g SDS in 1 l of water, pH 8.3) was diluted 1:4 with deionised water and put into the electrode buffer chamber.

Staining/Destaining/Scanning

The staining, destaining and scanning methods were the same as those described for native-PAGE.

PRELIMINARY EXPERIMENTS

The preliminary experiments were carried out using Whey Protein Concentrate (WPC) Alacen 475 in order to establish standard conditions for the gelation studies. A 12% (w/v) WPC solution was used to obtain approximately 10% (w/v) protein concentration. The WPC was reconstituted as described in Chapter 3.

4.1   Determination of the heating rate

A temperature gradient experiment was carried out from 25°C to 90°C using different heating rates (i.e 1°, 3° or 5°C/min). Transitions from a viscous solution to a viscoelastic structure during temperature gradients can be used to measure the temperature of gelation of protein solution. This transition is dependent on the time, heating rate and protein concentration.

Gelation of WPC solution was observed at 84.4°C using a heating rate of 1°C/min. Heating rates of 3 or 5°C/min did not show smooth transitions to a viscoelastic structure. Stading and Hermasson (1990) observed that the gelation temperature of β-Lg varied with the heating rate. At higher heating rates the protein solution is heated too rapidly and the reactions do not have time to develop and the gelation temperatures recorded are too high. Slower heating rates and increased protein concentration results in a shift to a lower gelation temperature. A heating rate of 1°C/min was chosen for use in the gelation studies.

4.2   Determination of frequency

The shear modulus G^*, which consists of the elastic modulus (G') and the loss modulus (G'') parts, is frequency dependent. The phase shift is usually independent of the frequency. The WPC solution was heated at 84.4°C for an hour and cooled at 25°C for 10 min and measurements taken

at the two temperatures. The gel was subjected to a frequency sweep over a frequency range of 0.1 - 20 Hz at 84.4°C and at 25°C. At 84.4°C, increasing the frequency in this range resulted in no significant change in G' but G' appeared to increase slightly at 25°C (Figure 4.1). A rapid increase observed at 20 Hz of G" and G' was disputed as this could have been due to the vibrations of the machine itself. Clark and Lee-Tuffnell (1986) also showed that log G' varied linearly with log frequency over a frequency range 10^{-2} to 10^{2} rad sec^{-1} of BSA gels at 15% (wt/wt) concentration. A frequency of 1Hz was chosen for further experiments.

4.3   Determination of the amplitude

Dynamic oscillatory measurements allow for the monitoring of the changes in the rheological properties during heating and gelation of the samples. Oscillatory strain (determines how often the cup oscillates) must be kept sufficiently low, in order to prevent disruption of the gel structure during measurements. All the measurements must be taken within the linear viscoelastic region. Strain sweeps were carried out in the 0.5 - 20% amplitude range after heating and measurements taken at 84.4°C and at 25°C . A viscoelastic gel shows a constant stress/strain ratio. A linear region was observed within the range used at both temperatures (Figure 4.2) and a amplitude of 10% which was within the linear range was chosen for further the gelation studies.

4.4   The effect on oscillation

The effect of either subjecting the sample to oscillations only during measurements at the required temperatures or both during the heating cycle and during measurements, was carried out. No conclusive differences were observed either when oscillation was carried out only during measurements or during heating and measurements.
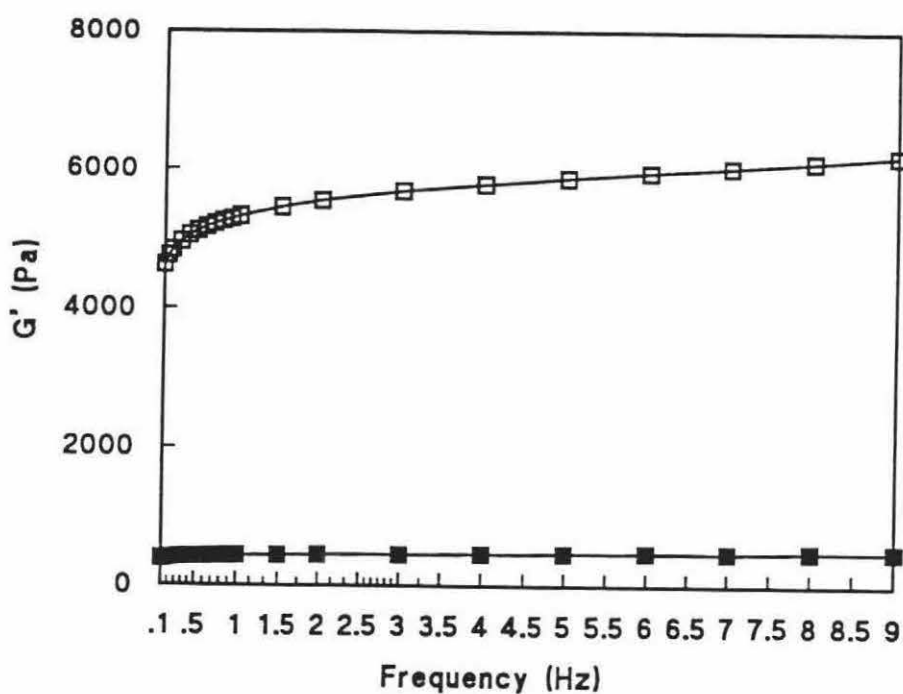
Figure 4.1 The effect of frequency (■, □) on G′ of WPC gels after heating
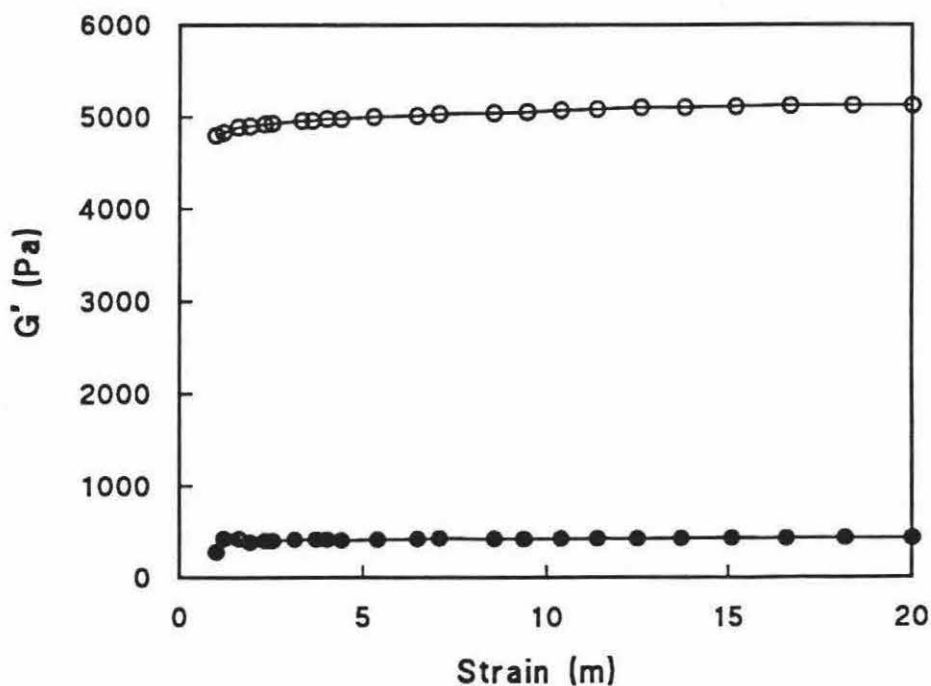at 84.4°C (■) and after the gels were cooled at 25°C (□).

Figure 4.2 The effect of strain (•, ○) on G′ of WPC gels after heating at
84.4°C (•) and after cooling the gels at 25°C (○).

Oscillating during measurements but not during the heating regime was chosen for the studies to follow.

Table 4.1 shows the selected parameters for use during the rheological characterisation of the whey protein gels.

Table 4.1: Selected parameters for gelation studies

PARAMETER	
Temperature	70 , 75 , 80°C
Frequency	1 Hz
Amplitude (strain)	10% (0.0206)
Torsion bar	4.62 g cm
Measuring system	C8
Heating/cooling rate	1°C/min

RESULTS AND DISCUSSION

5.1 Gelation temperatures of whey proteins

5.1.1 Pure proteins

When buffer solutions containing 10% β-Lg, BSA or α-La were heated from 25°C to 90°C at 1°C/min in the Bohlin VOR rheometer cup, G′ of the protein solutions increased (Figure 5.1). G′ of the β-Lg solution began to rise between 82°C and 84°C. G′ of BSA solution started to increase earlier than β-Lg, between 68°C and 70°C. G′ of α-La solution did not show any change in this temperature range (Figure 5.1).

These results are consistent with earlier findings by Paulsson *et al.* (1986). They observed that when 5% BSA or β-Lg, dissolved in 1% NaCl at pH 6.6, was heated from 30°C to 95°C at a rate of 1°C/min, the shear modulus (G*) of the protein solutions increased. G* of BSA began to increase at ~ 70°C and that of β-Lg at ~ 80°C. α-La did not show any change in G* when heated under the same conditions.

5.1.2 Mixtures of β-Lg and BSA

When 10% protein solutions containing varying concentrations of β-Lg and BSA were heated from 25°C to 90°C at 1°C/min, G′ of the protein mixtures started to increase (Figure 5.2). The G′ vs temperature curve gradually shifted from ~ 82.5°C to lower temperatures as the concentration of BSA in the mixture was increased.

These results are essentially in agreement with those of Paulsson *et al.* (1986) who heated mixtures of β-Lg and BSA from 30°C to 95°C at a heating rate of 1°C/min. They observed that the gelation curves (G* vs temperature) of the protein mixtures gradually shifted to a higher temperature with increasing amounts of β-Lg.
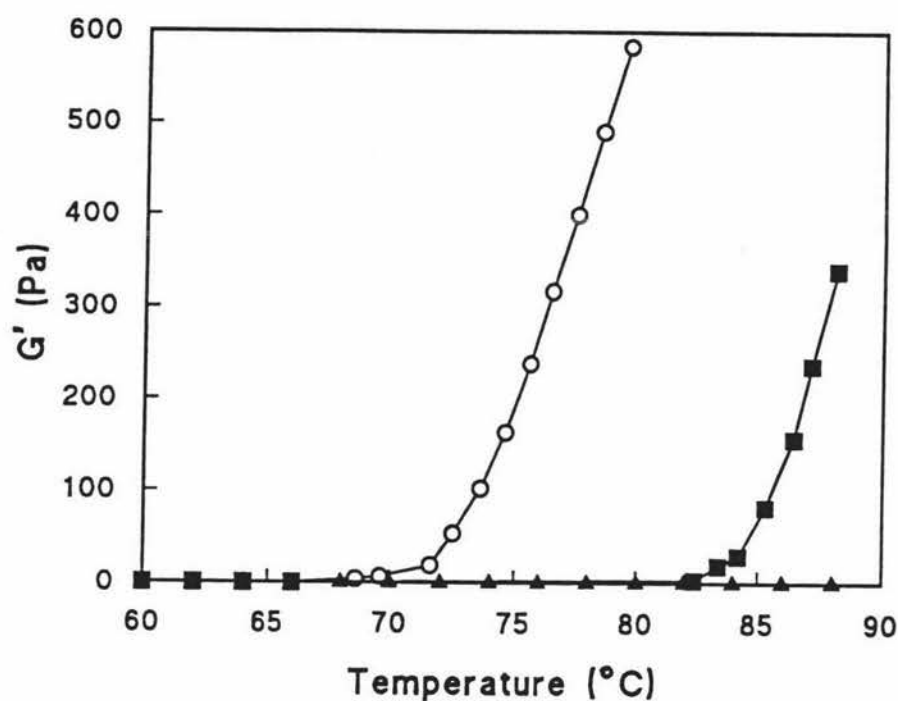
Figure 5.1 The gelation temperatures of individual whey proteins 10%, w/v, in simulated whey buffer, β-Lg (■), BSA (○) and α-La (▲).
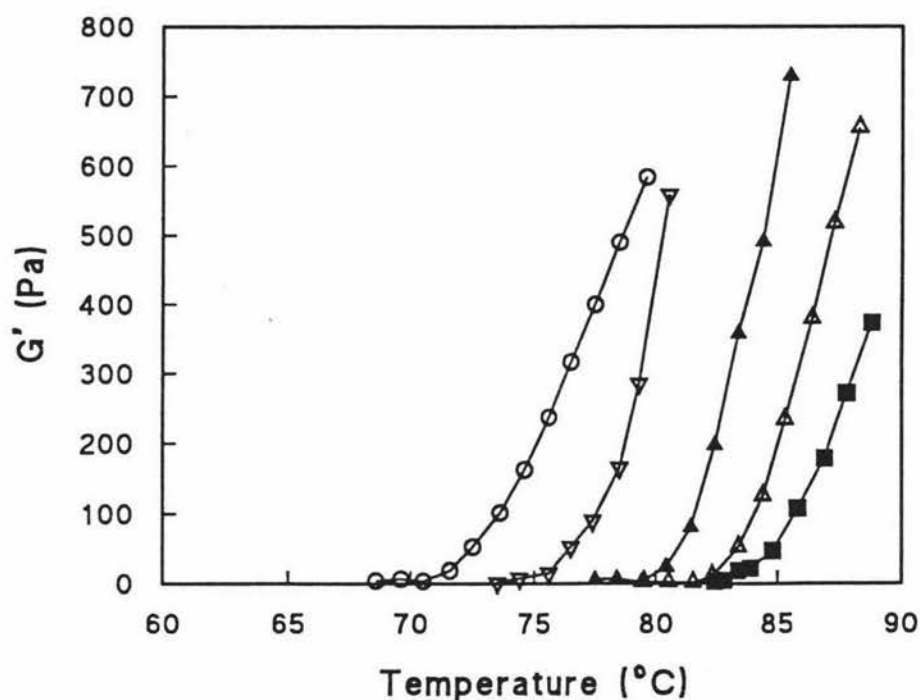

Figure 5.2 Gelation temperatures of 10% β-Lg (■); 10% BSA (○) and protein mixtures containing 8% β-Lg and 2% BSA (△), 5% β-Lg and 5% BSA (▲) and 2% β-Lg and 8% BSA (▽).

The value of the phase shift (δ) usually decreased markedly when G' increased steeply (results not shown). However, the results from the protein mixture containing 2% β-Lg and 8% BSA showed two sharp decreases in δ, one at 75.5°C (G' of 500 Pa) and the other at 85.3°C (G' of 2000 Pa). This observation may indicate that β-Lg and BSA were gelling independently of each other.

5.1.3   Mixtures of β-Lg and α-La

Protein solutions containing varying concentrations of β-Lg and α-La were heated from 25°C to 90°C at a heating rate of 1°C/min. The changes in G' of the protein mixtures as a function of temperature are shown in Figure 5.3.   G' of protein mixtures containing 8% β-Lg and 2% α-La or 5% β-Lg and 5% α-La began to increase between 81.5°C and 84°C; this range was similar to that observed for 10% β-Lg. A protein solution containing 2% β-Lg and 8% α-La did not show any change in G' when heated from 25°C to 90°C; the phase angle fluctuated between 90 and 20° during heating.

No earlier work has been reported on the gelation temperatures of β-Lg and α-La mixtures.

5.1.4   Comparison of gelation temperatures

The temperature at which G' began to increase during heating of whey proteins has been used in the present study as the temperature at which gelation commenced. The gelation temperatures observed for β-Lg (82 - 84°C) and BSA (68 - 70°C) were generally higher than their thermal transition temperatures, as determined using differential scanning calorimetry (DSC) which presumably detects changes in enthalpy during unfolding of globular proteins (Paulsson *et al.* 1985).
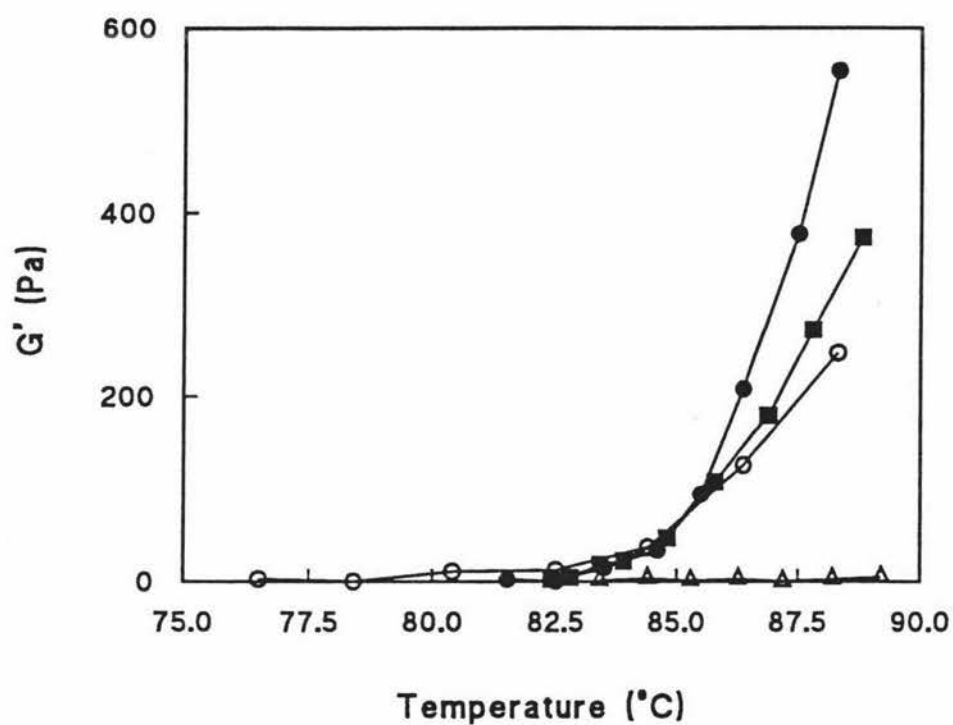
Figure 5.3 Gelation temperatures of 10% β-Lg (■) and protein mixtures containing 8% β-Lg and 2% α-La (○), 5% β-Lg and 5% α-La (•) and 2% β-Lg and 8% α-La (△).

The thermal transition temperatures were found to be 67°C for α-La, 65°C for BSA and 77°C for β-Lg at pH 6.6, when the proteins were dissolved in simulated milk ultrafiltrate (SMUF) (Paulsson and Dejemk, 1990). It is therefore likely that these thermal transitions precede aggregation and gelation.

When β-Lg and BSA were heated together, the gelation temperatures of the mixed protein solutions were dependent on the ratio of β-Lg to BSA (Figure 5.2). From the results obtained (Section 5.1.2) BSA and β-Lg appear to behave independently of each other, i.e the higher the proportion of BSA in the mixture the lower the gelation temperature.

When β-Lg and α-La were heated together, gelation temperatures were more or less similar to that of β-Lg (Figure 5.3). This may indicate that β-Lg dominated the gelation of the protein mixtures and that α-La seemed to be incorporated into the β-Lg gel network during heating.

5.2 The gelation of pure whey proteins

5.2.1 Rheological profile of β-Lg gels

5.2.1.1 The effect of heating at 75°C on G', G" and δ

When a 10% β-Lg solution was heated from 25°C to 75°C and held for 60 min, the rheological properties, G', G", and phase shift (δ) of the solution changed with time during heating. (Figure 5.4A). Initially the protein solution had a storage modulus, G', of zero, δ ~ 90° and a small value of G". The changes during heating were such that the G' and G" started to increase and δ started to decrease. The increase in G' was much greater than that in G" as the heating time increased. δ decreased from 90° to near zero. After 60 min, G' was still increasing. It is clear that gelation was not complete after heating for 60 min at 75°C, which is consistent with previous results reported by Paulsson et al. (1990) and McSwiney et al. (1994a,b).

Cooling the β-Lg gel to 25°C (at 1°C/min.) affected G', G" and δ (Figure 5.4B). G' increased markedly, from about 2000 Pa to about 8000 Pa while G" and δ increased only slightly. The gel was held at 25°C for 10 min during which measurements of G', G" and δ were made. G', G" and δ increased slightly during the first 2 min before remaining constant during the last 8 min of measurement at 25°C.

The increase in G' observed during cooling can be attributed to the increased formation of additional hydrogen bonds which are favoured at low temperatures (Beveridge et al. 1984). This increase during cooling has been shown to be reversible, i.e, when the cooled samples are reheated, the G' values decreased to almost the value that it had at the heating temperature (McSwiney et al. 1994a,b).
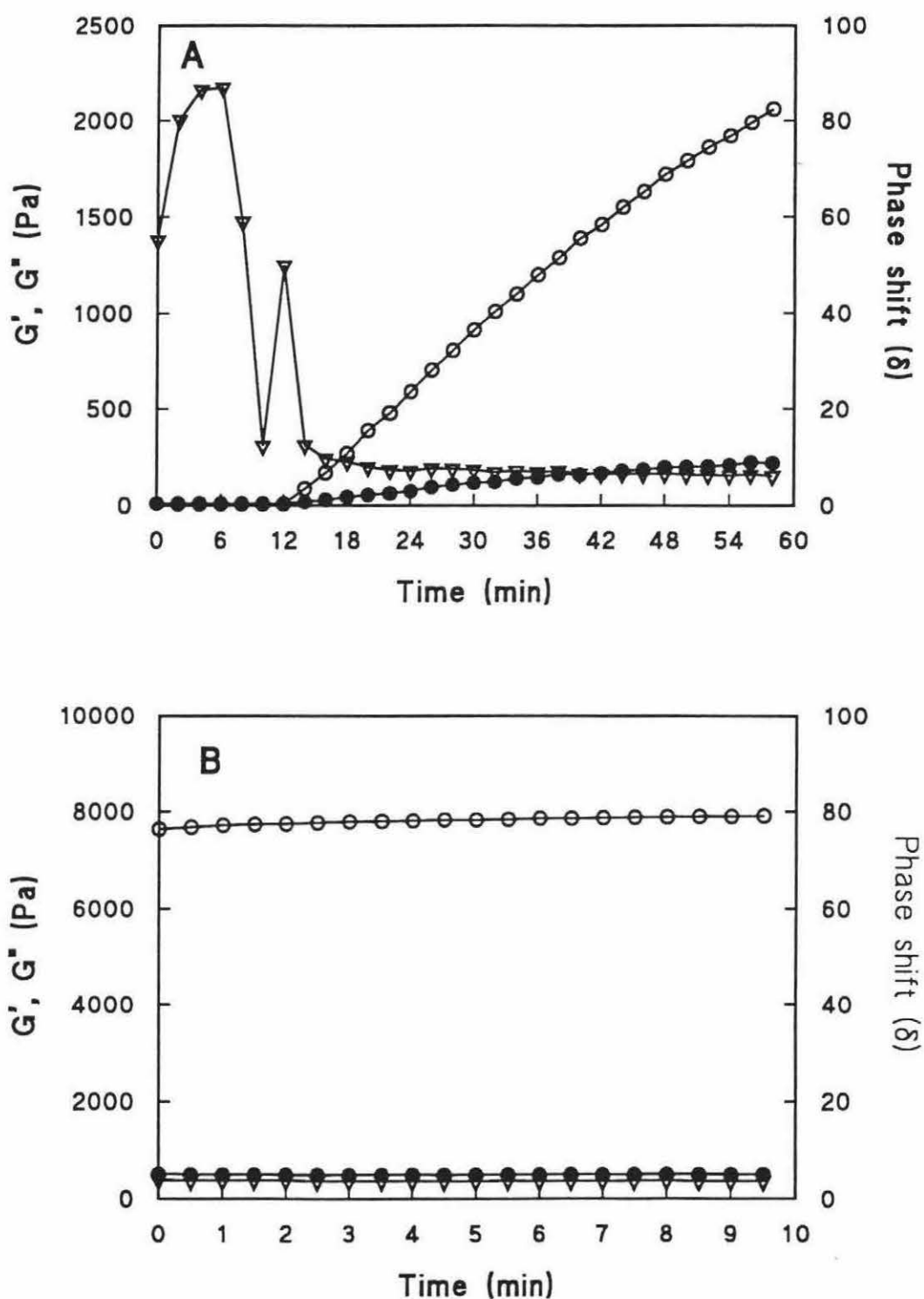
Figure 5.4 Changes in the storage modulus, G′ (○), loss modulus G″ (•)
and phase shift, δ (△) during heating of β-Lg solutions at 75°C
(A) and after cooling to 25°C (B).

5.2.1.2   The effect of heating temperature

Figure 5.5A,B shows the changes in G′ with time on heating a 10% β-Lg
solution at 70, 75 and 80°C and after cooling to 25°C. No measurable G′
was obtained after heating the protein solution at 70°C indicating that a
gel network had not formed. At 75 and 80°C, G′ increased as the heating
time was increased. G′ began to increase much earlier at 80°C than at
75°C. A crossover between the two G′ values was observed during heating
which finally resulted in G′ at 80°C being lower than G′ at 75°C (Figure
5.5A).

After cooling to 25°C, the values of G′ increased (Figure 5.5B). G′ value for
the sample that had been previously heated at 80°C was lower than that
heated at 75°C. However, the difference between G′ values of the gels
formed at 75°C and 80°C was less after cooling.

These results confirm the findings of McSwiney *et al.* (1994a,b) where G′
immediately after heating of 10% β-Lg A/B was higher for the solution
heated at 80°C than that at 85°C.

5.2.1.3   The effect of concentration on G′

β-Lg solutions at 10%, 8% and 5% were heated from 25°C to 80°C and held
for 60 min during which G′ was measured. The concentration-dependence
of β-Lg gel development was reflected in the changes in G′ with time
(Figure 5.6). No significant changes in G′ were observed during heating
of 5% β-Lg at 80°C. This may either indicate that 5% β-Lg did not form
a gel or a very weak gel was formed which could not be detected.
However, when the heated 5% β-Lg was cooled to 25°C, a G′ value of ~25
Pa was observed (Table 5.1).

At 8 and 10% β-Lg, G′ increased gradually with heating time at 80°C, the
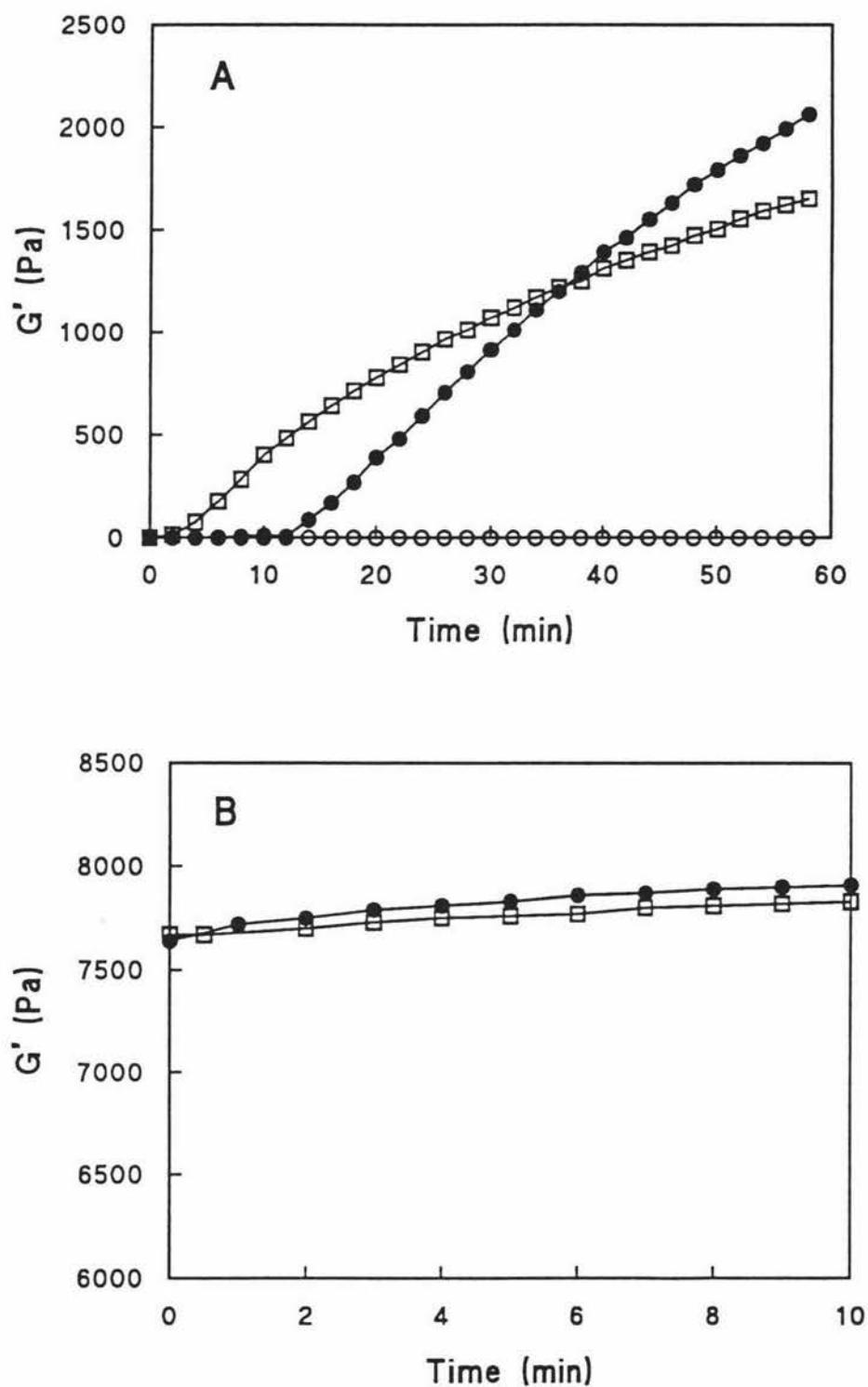rate of increase being greater at 10% protein concentration.

Figure 5.5 The effect of temperature on the storage modulus, G′ (•, o, □) during heating at 70°C (o), 75°C (•) and at 80°C (□) (A) and after cooling to 25°C (B) of a 10% β-Lg solution.
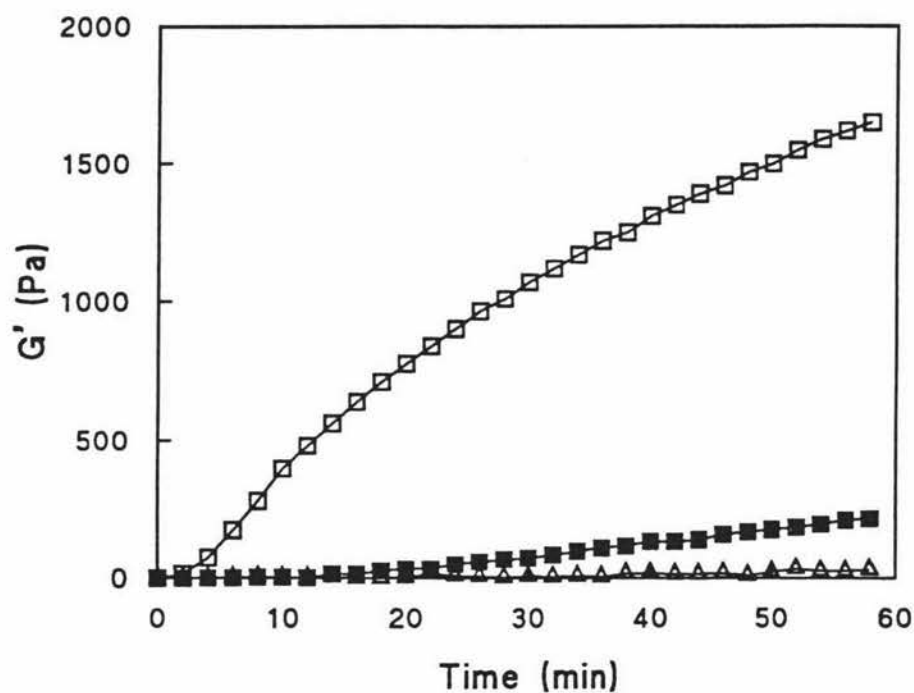
Figure 5.6 The effect of protein concentration on the storage modulus, G′ (□, ■, ▵) for β-Lg at 10%, w/v, (□), 8%, w/v, (■) and 5%, w/v, (▵) during heating at 80°C.

The final G′ values of 10% β-Lg measured at 80°C or 25°C (after cooling) were much greater than those at 8 and 5% β-Lg concentrations (Table 5.1).

Table 5.1   The effect of protein concentration on the final G′ of β-Lg gels

β-Lg concentration (g/100ml)	G′ at 80°C (Pa)	G′ at 25°C (Pa)
5	undetected	25.1
8	219	974
10	1650	7670

It was also observed that the gelation time, as indicated by an increase in G′, decreased as β-Lg concentration increased from 5 to 10% (Figure 5.6). This is in agreement with the results reported previously by Paulsson *et al.* (1986) on gelation of individual whey proteins.

5.2.2   Rheological profile of BSA gels

5.2.2.1   The effect of heating at 75°C on G′, G″ and tan δ

A 10% BSA solution was heated from 25°C to 75°C at 1°C/min and held at 75°C for 60 min while G′, G″ and δ were measured (Figure 5.7A). G′ of the BSA solution had already begun to rise at the beginning of the measurements, implying that a gel net-work had formed before the temperature reached 75°C. G′ increased further with increases in heating time and was still rising after 60 min. G″ did not show any change during heating at 75°C. There may have been a slight increase in δ at the beginning of the measurement period but subsequently decreased to near zero.
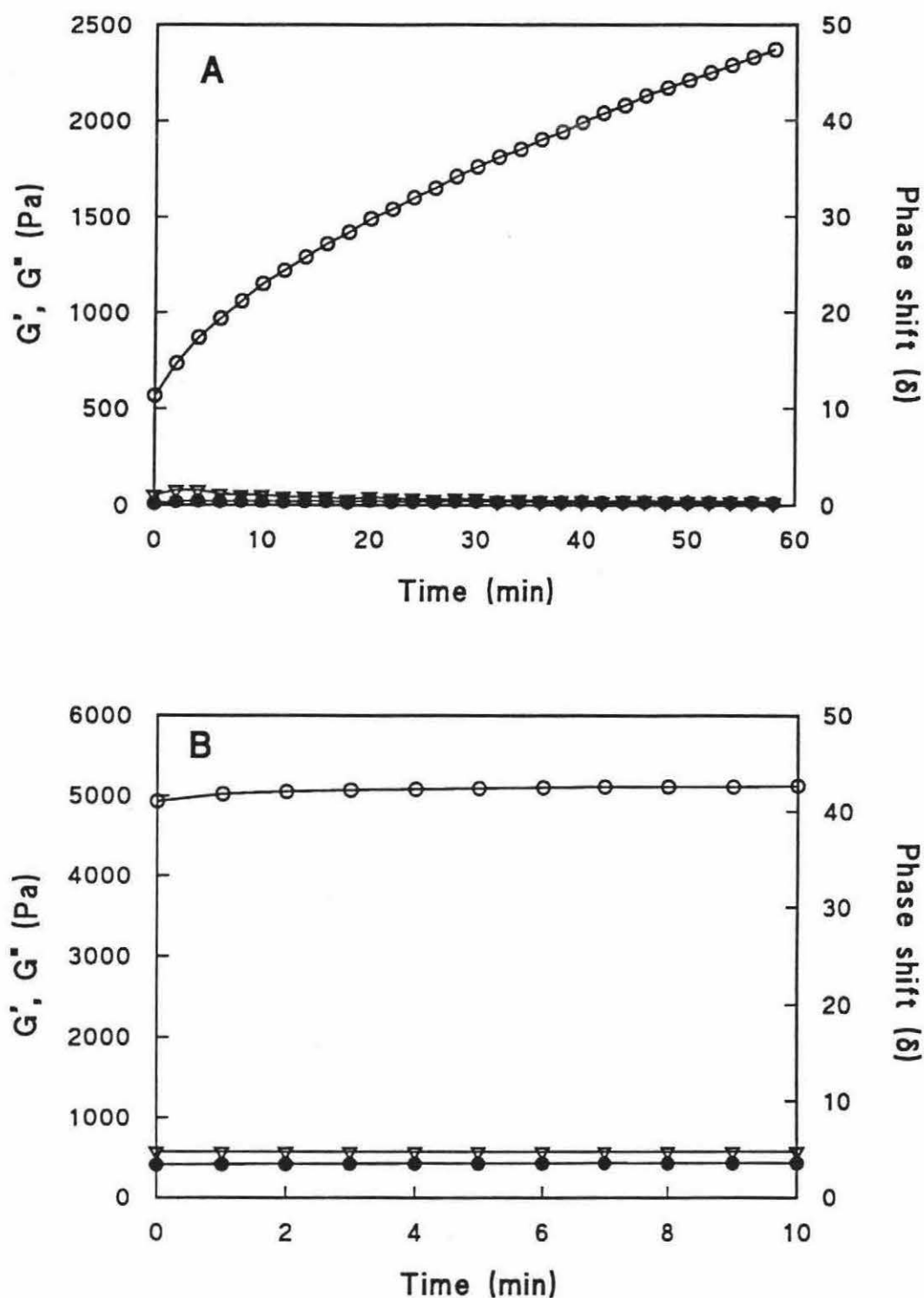
Figure 5.7 Changes in the storage modulus, G' (○), loss modulus, G" (•) and phase shift, δ (▽) during heating of 10%, w/v, BSA solutions at 75°C (A) and after cooling to 25°C (B).

When the BSA gel was cooled to 25°C, (Figure 5.7B) G′ increased two-fold while G″ and δ increased slightly. Initially G′, G″ and δ increased slightly with time at 25°C and then remained constant.

5.2.2.2 *The effect of heating temperature*

Figure 5.8A,B shows the changes in G′ on heating 10% BSA at 70 and 75°C and after cooling to 25°C. At 70°C, G′ began to increase at zero time, i.e, when the temperature reached 70°C. When the heating temperature was increased to 75°C, G′ began to rise before the temperature reached 75°C as shown by the high G′ value at zero time. The increase in G′ with time at 75°C was more rapid than at 70°C and the G′ values at 75°C remained higher than those at 70°C throughout the heating period. At both temperatures, G′ was still rising after 60 min.

When the gels were cooled to 25°C (Figure 5.8B), the G′ values for both gels increased. Higher G′ values were obtained for the BSA gels that had been heated at 75°C. G′ values of the BSA gels remained constant during the 10 min of measurement.

Richardson and Ross-Murphy (1981b) used the torsion pendulum method to show that G′ of BSA gels increased with increases in heating temperature in the range 65 - 90°C.

5.2.2.4 *The effect of concentration on G′*

The effects of protein concentration in the range 5 - 10% on changes in G′ of BSA were determined during heating at 70°C for 60 min (Figure 5.9) When a 5% BSA solution was heated G′ began to increase slowly after ~18 min. At 8% BSA, G′ increased when the temperature reached 70°C, and continued to do so throughout the heating period.
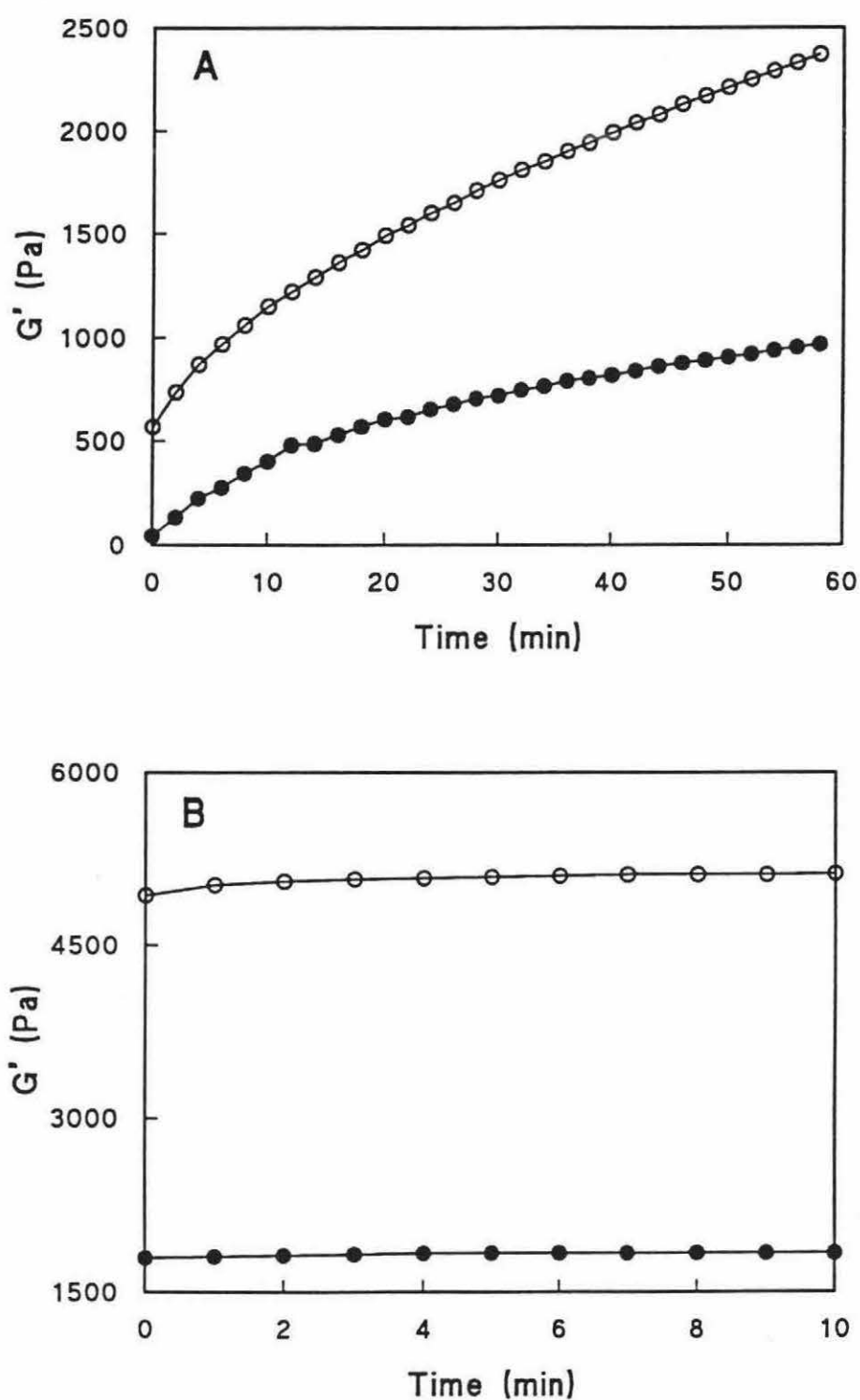
Figure 5.8 The effect of temperature on the storage modulus, G′ (•, o)
during heating of 10%, w/v, BSA solutions at 70°C (•) and at
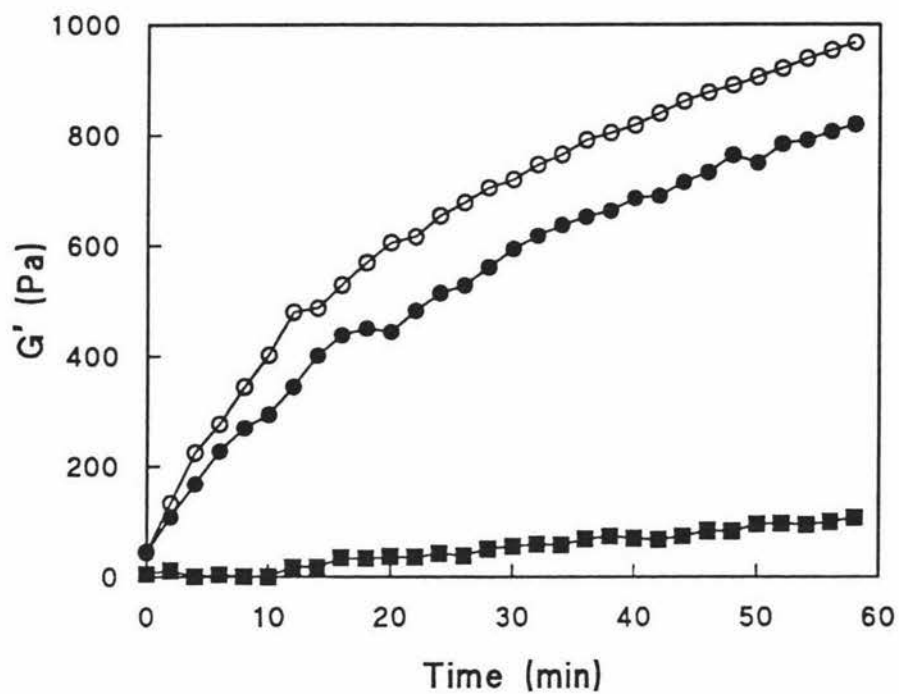75°C (o) (A) and after cooling to 25°C (B).

Figure 5.9 The effect of protein concentration on the storage modulus, G′ (○, •, ■) for BSA solutions at 10%, w/v, (○), 8%, w/v, (•) and 5%, w/v, (■) during heating at 70°C.

G′ of 10% BSA began to increase at about the same time as that of 8% BSA, although G′ values of the 10% BSA were much higher than G′ of 8% BSA (Table 5.2).

At all concentrations used, the G′ values showed marked increases after cooling to 25°C (Table 5.2).

Table 5.2   The effect of protein concentration on the final G′ of BSA solutions

BSA concentration (g/100ml)	G′ at 70°C (Pa)	G′ at 25°C (Pa)
5	107	219
8	819	1850
10	967	1800

These results confirm the work carried out earlier by Richardson and Ross-Murphy (1981a).

5.2.3 *Rheological profile of α-La gels*

When a solution containing 10% α-La was heated from 25°C to 75 or 80°C at 1°C/min and held for 60 min, no change in G′ was observed (Figure 5.10). This suggests that no gel network was formed during the heating period.

Similar results were obtained by Paulsson *et al.* (1986), where no change in G* for α-La was observed up to protein concentrations of 20% at pH 6.6. Under extreme heating conditions (180 minutes   at 80°C), Hines and Foegeding (1993) observed a rise in G′ for 7% α-La, dispersed in 50 mM TES buffer at pH 7.0.
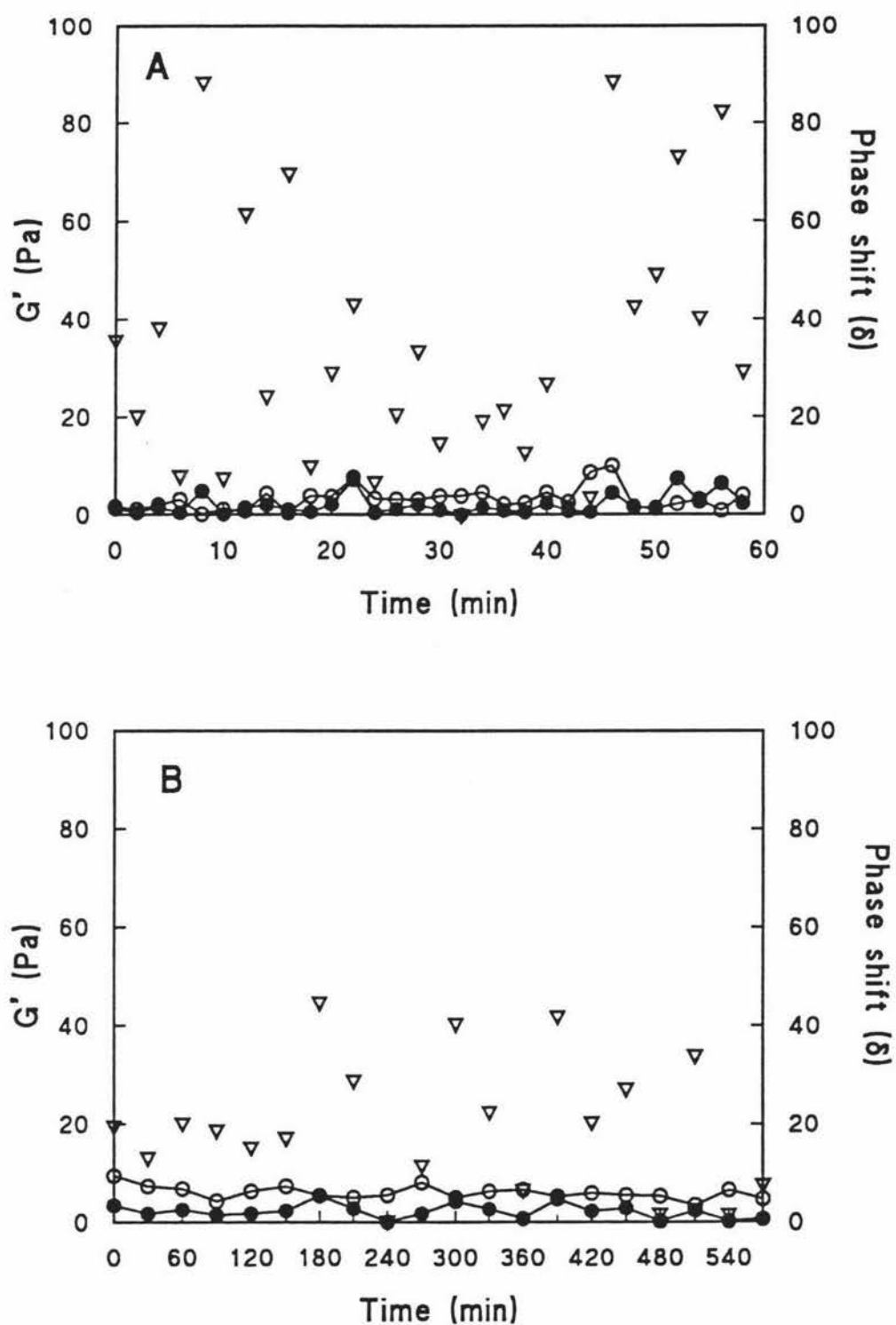
Figure 5.10 Changes in the storage modulus, G' (○), loss modulus, G" (•) and phase shift, δ (∇) during heating of 10%, w/v, α-La solutions at 75°C (A) and after cooling to 25°C.

During heating of 10% α-La, δ fluctuated between 90 and 0° reflecting the ability of the instrument to measure δ. It was noticed that the solution contained fine strands of material after heating. These may be contaminants and could have been the cause of the erratic responses. When the solution was cooled to 25°C, the values of δ were between 50 and 0° which indicated that the solution may have become more elastic.

5.2.4 Comparison of gelation characteristics of individual whey proteins

The present results clearly show that BSA solutions gel at lower temperatures and protein concentrations than β-Lg solutions and that α-La does not form a gel under the same conditions of heating (Figure 5.1). On heating at 75°C, BSA formed slightly stronger gels than β-Lg but after cooling to 25°C, β-Lg gels were stronger than BSA gels (Table 5.3).

Table 5.3   Comparison of G′ of β-Lg and BSA at 75 and 25°C.

Protein (10%, w/v)	G′ at 75°C (Pa)	G′ at 25°C (Pa)	Ratio
β-Lg	2060	7910	1 : 3.9
BSA	2370	5120	1 : 2.2
α-La	0	0	0 : 0

These observations indicate that different mechanisms are involved in the formation of β-Lg and BSA gels, although both proteins have a single thiol group which is probably important in catalysing gel formation.

Matsudomi *et al.* (1991) studied the gelation of β-Lg and BSA using the Instron Universal Testing Instrument to measure the gel hardness at 20°C. The proteins, dispersed in 0.1 M Tris-HCl buffer (pH 8.0), were heated at 90°C; the gels formed were left overnight at 4°C and were tempered for 30 min before gel hardness was measured. The hardness of the BSA gels formed after heating at 90°C were lower than β-Lg gels but as the heating time increased a crossover was observed resulting in BSA gels being harder than β-Lg gels. They reported that β-Lg (10%, w/v) formed self-supporting gels at 70°C while 10% BSA formed gels at 75°C.

In the present study, β-Lg and BSA were dispersed in a simulated whey protein concentrate buffer containing 356 mg/L $CaCl_2.2H_2O$ at pH 6.8. The proteins were heated gradually (heating rate of 1°C/min) to either 70, 75 or 80°C prior to G' measurements. Because of these major differences in the conditions used for gelation studies between the work done by Matsudomi *et al.* (1991) and the present study, it is therefore not suprising that the results obtained in the present study did not confirm the findings of these workers.

5.2.5 *Protein aggregate formation prior to gelation*

Heat-induced aggregation of β-Lg or BSA was studied using electrophoresis. The quantities of native protein and protein that became monomeric in SDS solution was measured and this indicated the degree to which each of the individual proteins in the mixture participated in the aggregation process.

A solution of 10% β-Lg or BSA was heated at 75°C in the Bohlin rheometer cup at 1°C/min until a gel formed. Aliqouts of the heated protein solution were removed at intervals and dispersed in non-dissociating buffer (0.5 M Tris-HCl buffer) or dissociating but non-reducing buffer (2% SDS in 0.5 M Tris-HCl buffer) at pH 6.8 and analysed using quantitative PAGE.

Addition of the sample to a solution of 2% SDS in 0.5 M Tris-HCl buffer, presumably dispersed all the non-covalently linked polymers including dimeric native β-Lg or BSA into monomers while leaving the cross-linked polymers bound by disulphide linkages intact.

The protein that appears monomeric in the non-dissociating system will be refered to as "native" protein while that appears monomeric in the dissociating system (SDS-PAGE) will be refered to as "residual monomeric" protein.

5.2.5.1   β-Lg

The amounts of both native and residual monomeric β-Lg decreased with heating time at 75°C (Figure 5.11).   The loss of native protein (native-Page) was faster than the loss of residual monomeric protein (SDS-PAGE) indicating that some non-covalently linked aggregates were formed during heating. The rate of formation of β-Lg aggregates was faster than the rate of formation of disulphide-linked aggregates.

The relationship between the loss of β-Lg from native-PAGE and SDS-PAGE and the changes in G' at 75°C are shown in Figure 5.12. It is clear that ~ 85% of the protein had aggregated (from native-PAGE), involving both non-covalent and covalent bond formation, before any appreciable rise in G'. A similar relationship was observed between the loss of protein from SDS-PAGE and the changes in G'; ~ 60% of the protein was cross-linked *via* disulphide bond formation before G' began to rise. This may suggest that soluble protein aggregates/homopolymers are formed prior to the development of a self-supporting gel. These results confirm the findings of McSwiney *et al.* (1994a,b).
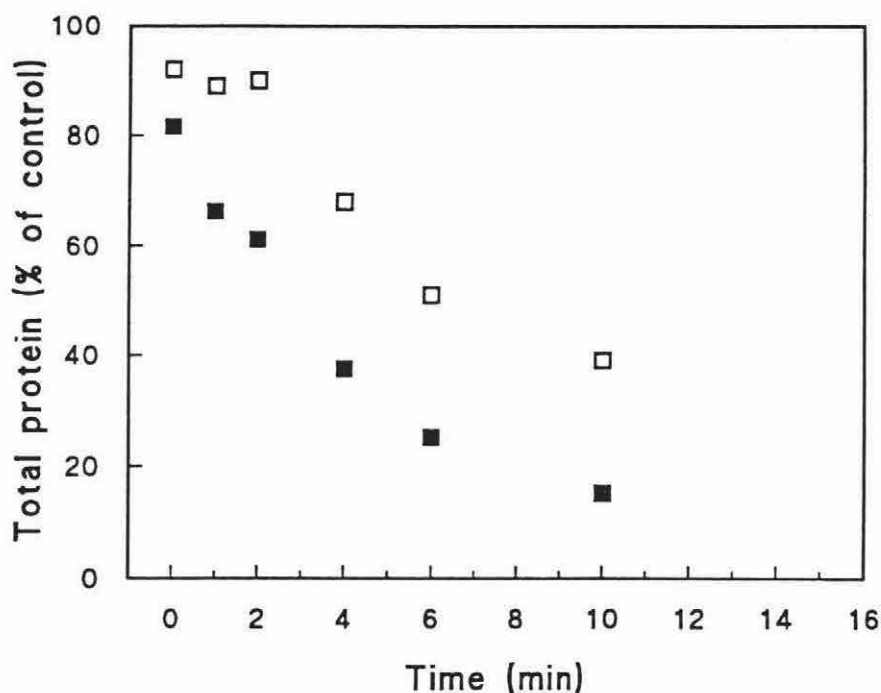
Figure 5.11   The effect of heating at 75°C on the quantity of native β-Lg (■) and residual monomeric β-Lg (□) remaining in a protein solution containing 10% β-Lg.
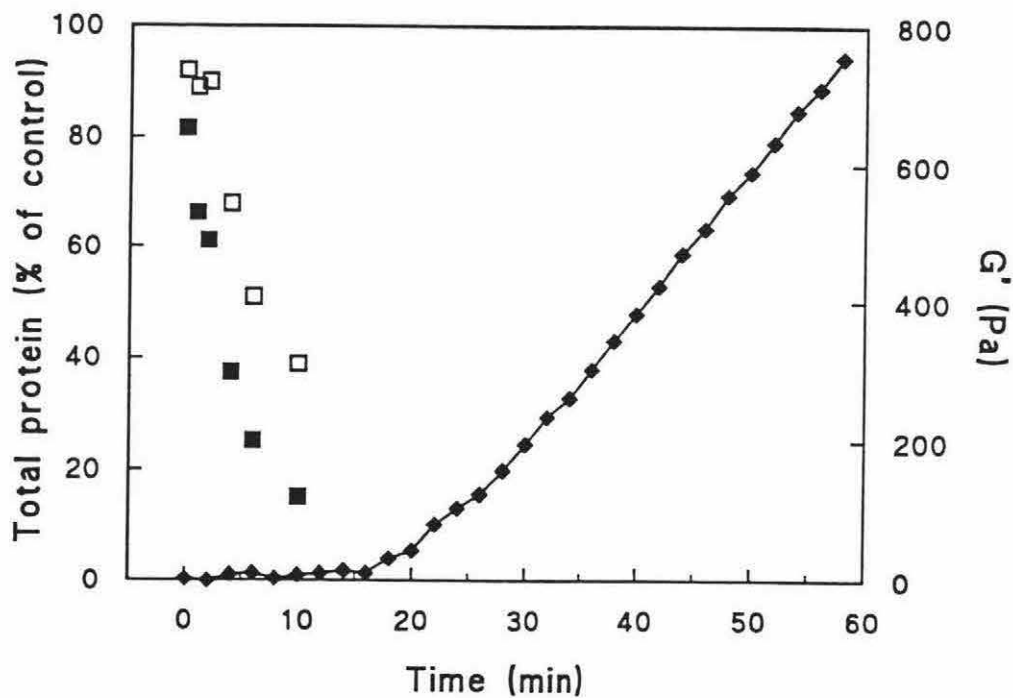

Figure 5.12   The effect of heating a protein solution containing 10% β-Lg at 75°C on G' (♦) and quantities of native β-Lg (■) and residual monomeric β-Lg (□).

5.2.5.2   BSA 5%

Figure 5.13 shows that the amounts of native and residual monomeric BSA decreased during heating of the protein solution at 75°C. There was a considerable loss of BSA during the heat-up time i.e, the time taken for the temperature to reach 75°C at 1°C/min. The loss of native protein was greater than the loss of residual monomeric protein.

The relationship between PAGE results and the development of G′ is shown in Figure 5.14. No detectable rise in G′ was observed until almost all the proteins had aggregated (Native-PAGE). About 85% of the proteins had aggregated *via* disulphide bonds. A 10% BSA solution gelled too quickly (Figure 5.7) under these conditions to determine the quantities of native or residual monomeric BSA.
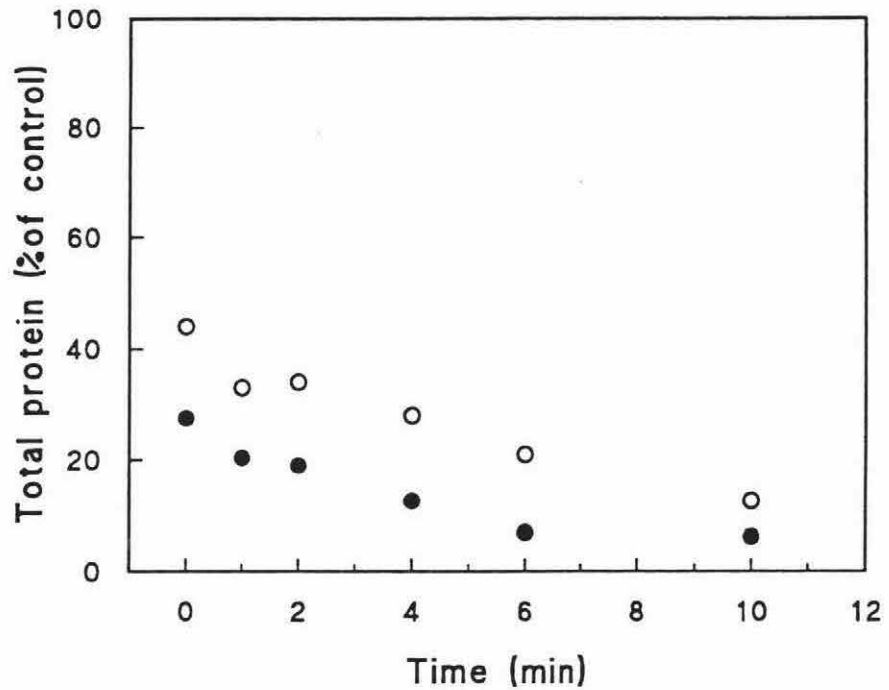
Figure 5.13   The effect of heating at 75°C on the quantity of native BSA (•) and residual monomeric BSA (o) remaining in a protein solution containing 5% BSA.
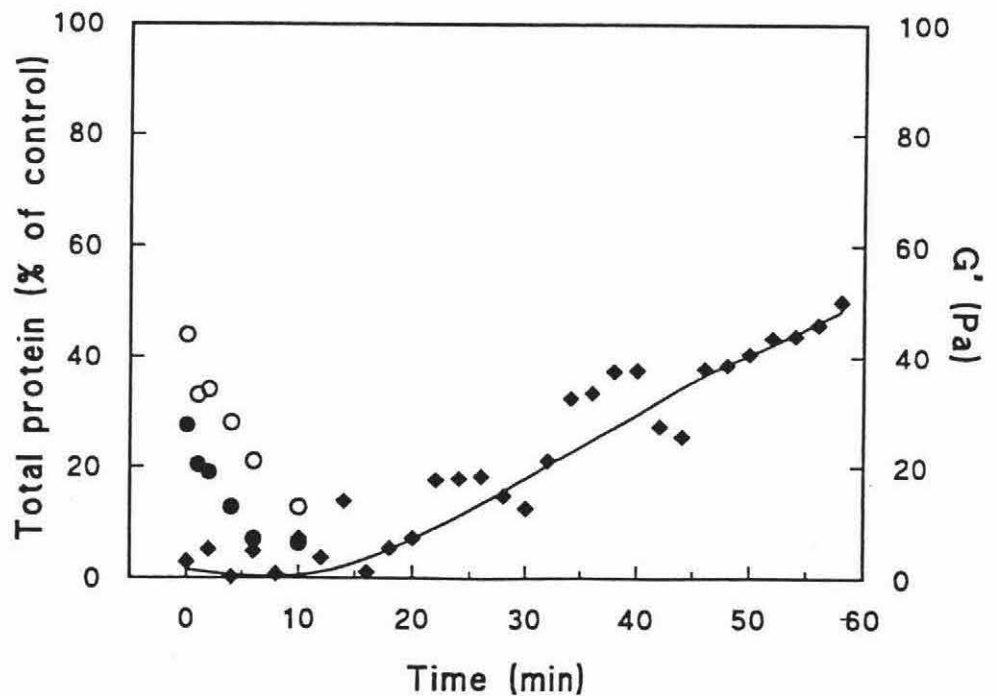

Figure 5.14   The effect of heating a protein solution containing 5% BSA at 75°C on G' (♦) and quantities of native BSA (•) and residual monomeric β-Lg (o).

5.2.6 Gelation mechanisms of whey proteins

Gelation mechanisms taking place during the formation of three dimensional gel networks during heating of proteins are not fully understood. Ferry (1948), on the basis of experiments with serum albumin, suggested that some protein denaturation or unfolding is a prerequisite to the formation of a protein gel network. The proposed mechanism can be formulated as follows:
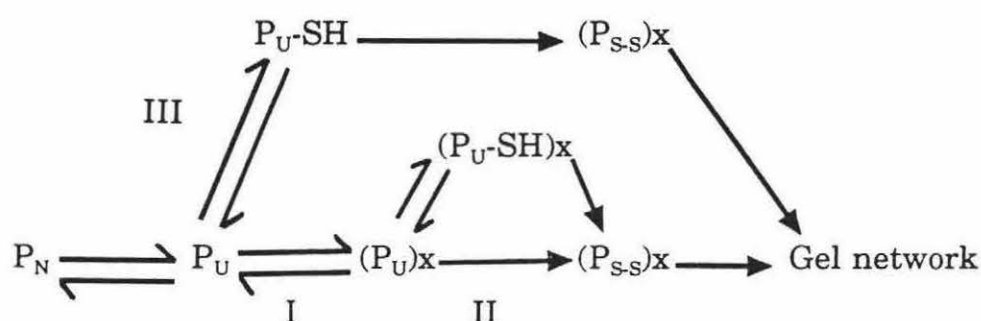
$$
xP_N \xrightleftharpoons[\quad I \quad]{\text{heating}} xP_U \xrightarrow{\text{heating and/or cooling}} (P_U)_x
$$
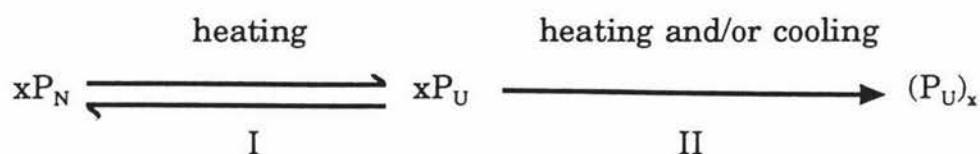
where x is the number of protein monomers, P_N is the native protein and P_U is the denatured or partially unfolded protein. During heating, the protein molecules undergo conformational changes due to changes in the environment. These thermal transitions can normally be detected by DSC. The proteins partially unfold exposing hydrophobic and thiol groups.

During the second aggregation step, the partially unfolded protein molecules aggregate, possibly through non-covalent and covalent interactions, to form a gel network. This step probably proceeds more slowly than the first allowing the unfolded protein molecules to orient themselves to a certain degree. Thus, the relative rates of unfolding and aggregation reactions are important in the formation of the gel network.

Since Ferry's proposal (1948) many aspects of the structure and folding of the protein have been elucidated. The idea that the protein attains a different conformation, however transitory, prior to the irreversible formation of aggregates, or gels, remains valid (Clark, 1992).

5.2.6.1   β-Lg

McSwiney *et al.* (1994a,b) studied the gelation and aggregation of β-Lg during gel formation. They noted that the development of rheologically significant structures did not begin until most of the protein had been denatured and aggregated to form soluble aggregates. The soluble aggregates were found to be linked through hydrophobic interactions and sulphydryl-disulphide interactions. A reaction pathway which is depicted as follows (Creamer *et al.* 1995):

$$P_U\text{-SH} \longrightarrow (P_{S\text{-}S})x$$
$$\text{III}$$
$$(P_U\text{-SH})x$$
$$P_N \rightleftharpoons P_U \rightleftharpoons (P_U)x \longrightarrow (P_{S\text{-}S})x \longrightarrow \text{Gel network}$$
$$\text{I} \qquad \text{II}$$

where P_N is the native protein, P_U is the partially unfolded protein molecules and $(P_U)x$ and $(P_{S\text{-}S})x$ represent the aggregated forms of the molten-globule/partially unfolded protein. The first step involves partial unfolding of the protein while retaining the native-like backbone secondary structure with increased exposure of hydrophobic groups. This state has been referred to as the molten-globule state i.e, partially unfolded protein molecules which can be differentiated from either the native or fully denatured protein molecules (Hirose, 1993). The protein can either unfold to present a reactive thiol group or form hydrophobically-associated aggregates. In the former case individual molecules can cross-link by thiol-disulphide interchange reactions to form aggregates (Reaction III). In the latter case the hydrophobic interactions occur between the exposed groups causing aggregation of the protein molecules while still in the molten-globule state.

These aggregates are further strengthened by the formation of inter- and intra-molecular disulphide bonds via sulphydryl-disulphide interchange or sulphydryl oxidation reactions (Reaction II) which leads to the formation of a gel network.

The rates of various reactions will be affected by protein concentration, temperature of heating and pH of the medium. For example, high pH's are likely to favour formation of disulphide-linked aggregates (Reaction III). Low pH's and/or high protein concentrations will increase the formation of non-covalently linked aggregates (Reaction I).
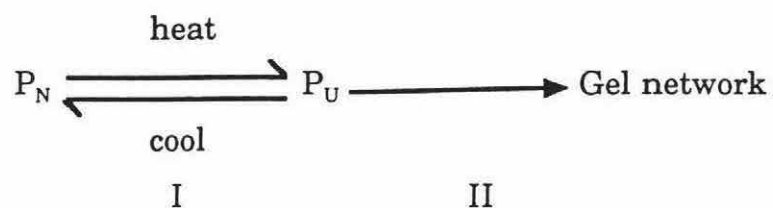
5.2.6.2   BSA

No studies have been carried out to elucidate the gelation mechanism of BSA. From HPLC studies, Mastudomi *et al.* (1993) observed that soluble aggregates were formed when BSA was heated. Using thiol reagents (DTT, NEM) they noted that these aggregates could be formed through hydrophobic interactions. Electrostatic interactions and thiol disulphide interactions have also been demonstrated to be critical in the formation of BSA gels (Matsudomi *et al.* 1991).

It seems likely that the gelation mechanism for BSA follows the same general principles outlined for β-Lg in the previous section (Section 5.2.6.1).

5.2.6.3   α-La

α-La has been shown to form a gel when heated under extreme conditions (Hines and Foegeding, 1993) or in the presence of free thiol groups (Calvo, 1993; Legowo, 1993). Thermal transitions have been detected by DSC during heating of α-La which suggests some conformational changes occur during heating (Ruegg *et al.* (1977).

The probable transition reactions occuring during heating are:

$$P_N \underset{\text{cool}}{\overset{\text{heat}}{\rightleftharpoons}} P_U \longrightarrow \text{Gel network}$$

$$\text{I} \qquad\qquad\qquad \text{II}$$

Reaction I, which is reversible is the main thermal transition giving the molten globule form of α-La. Reaction II is not well characterised but may involve molten globule aggregates as intermediates

5.3 The gelation of whey protein mixtures

5.3.1 *Gelation of β-Lg and BSA mixtures*

5.3.1.1 *Gelation at 70°C*

The gelation properties of mixtures of β-Lg and BSA were determined at 70°C and the evolution of G′ with time during heating and after cooling is shown in Figure 5.15A,B. The total protein concentration in each mixture was kept constant at 10%, w/v.

A solution of 10% β-Lg did not show any significant change in G′ after heating at 70°C for 60 min although on cooling (to 25°C) a G′ value of 79.5 Pa (Table 1.1. *Appendix*) was obtained. A solution containing 10% BSA began to gel before reaching 70°C as shown by the significant G′ value at zero time and G′ continued to increase during the heating period (Figure 5.15A).

Protein mixtures containing β-Lg and BSA showed a gradual increase in G′ with time during heating. The development of G′ for the mixed protein gels was slower than for BSA alone. The rate of G′ development increased as the relative amount of BSA in the original protein solution was increased. G′ of the protein gels was still rising after heating for 60 min (Figure 5.15A).

When the gels were cooled to 25°C, the values of G′ increased further. The G′ values for the protein mixtures containing 5% β-Lg and 5% BSA and 2% β-Lg and 8% BSA were greater than those for 10% BSA (Figure 5.15B) although their G′ values immediately after heating were much lower than those for BSA alone (Figure 5.15A).
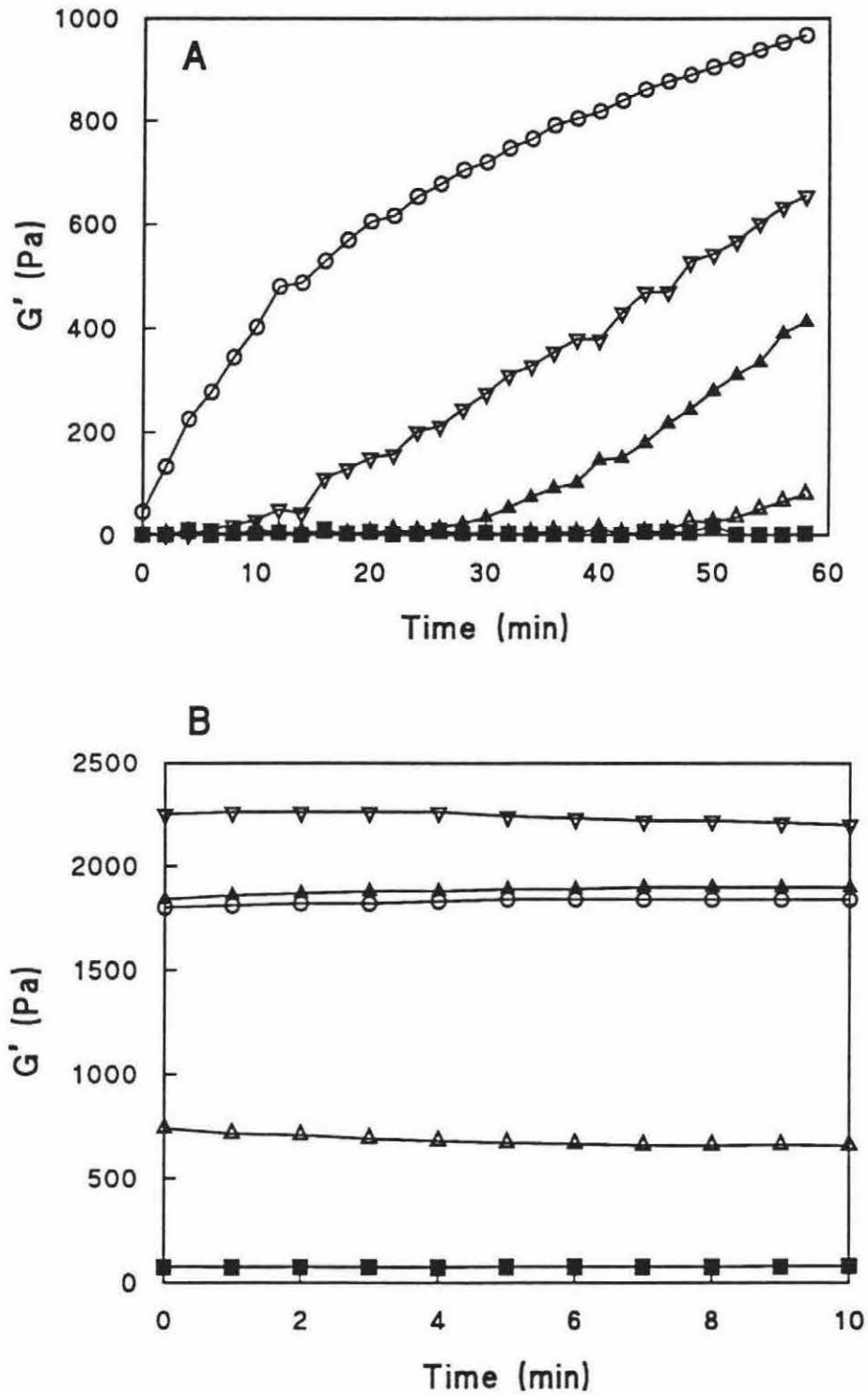
Figure 5.15   The effect of heating at 70°C (A) followed by cooling to 25°C (B) and holding on the storage modulus, (G') of 10%, w/v, β-Lg (■), 10%, w/v, BSA (○) and protein mixtures containing 8% β-Lg and 2% BSA (△), 5% β-Lg and 5% BSA (▲) and 2% β-Lg and 8% BSA (▽).

Gelation time, measured by the first clear signs of an increase in G′, decreased as the relative quantity of BSA in the protein mixtures increased (Table 1.1. *Appendix*).

Figure 5.16 shows the final G′ values for mixtures of β-Lg and BSA after heating at 70°C for 60 min and also at 25°C after cooling. G′ of the protein mixtures, measured at 70°C, increased almost linearly as the relative quantity of BSA in the mixtures increased. At 25°C, G′ of the protein gels increased markedly with increase in the proportion of BSA in the protein mixture up to 5%; further increase in the BSA concentration caused smaller further increases. The G′ values for the protein mixtures containing 1% β-Lg and 9% BSA or 2% β-Lg and 8% BSA were similar to each other but were greater than other mixtures or individual proteins.

5.3.1.2 Gelation at 75°C

Protein solutions containing β-Lg and BSA were heated at 75°C and Figure 5.17A,B shows the evolution of G′ as a function of time during heating and after cooling to 25°C. The total protein concentration was kept constant at 10% and the relative concentrations of β-Lg and BSA varied.

When a solution of 10% β-Lg was heated at 75°C, G′ began to increase after 20 min of heating. A solution of 10% BSA had already gelled before the heating temperature was attained, as shown by the high G′ value at zero time (Figure 5.17A).

Most of the protein mixtures showed a steady increase in G′ with heating time. G′ of most of the protein mixtures increased faster than when β-Lg was heated alone, except for the protein mixture containing 8% β-Lg and 2% BSA. In general, the rate of G′ development of the protein mixtures increased with increasing relative quantity of BSA. A crossover between G′ of a protein mixture containing 2% β-Lg and 8% BSA and that of 10% BSA was observed which resulted in G′ of the protein mixture being higher than G′ of 10% BSA immediately after heating. G′ of the protein mixtures was still rising after heating for 60 min.
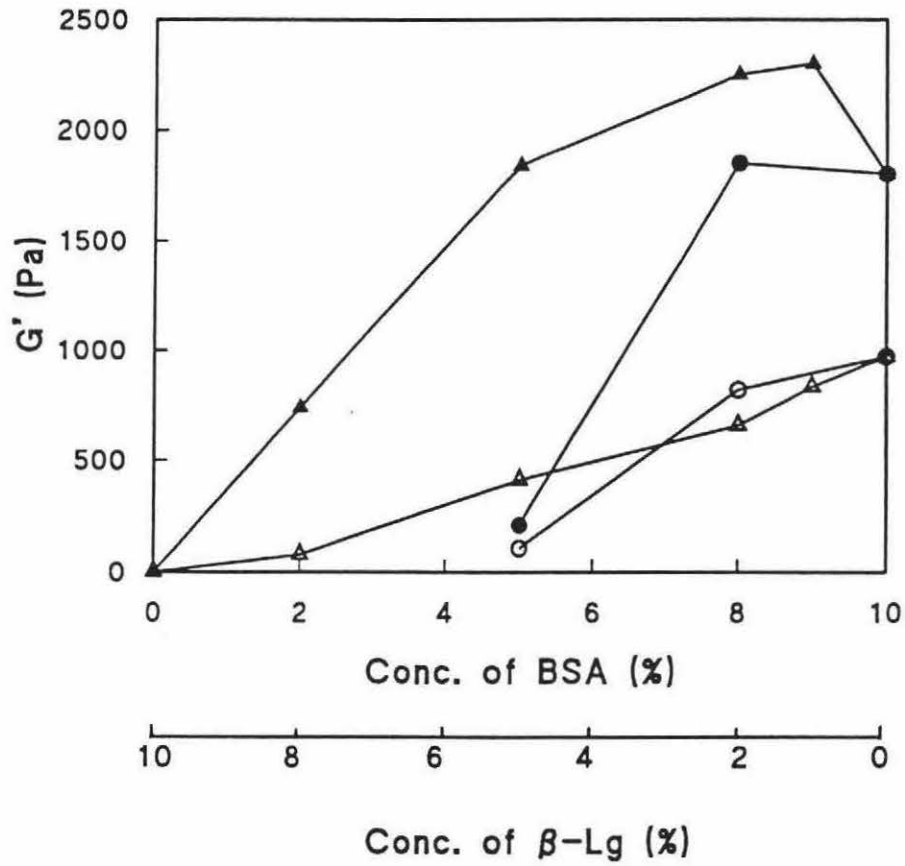
Figure 5.16   The effect of mixture composition on the final G' at 70°C (∇) and at 25°C (▲). For comparison the final G' values at 70°C (○) and at 25°C (•) for BSA at the concentrations indicated are included. (*Taken from Tables 1.1 & 1.2. Appendix*).
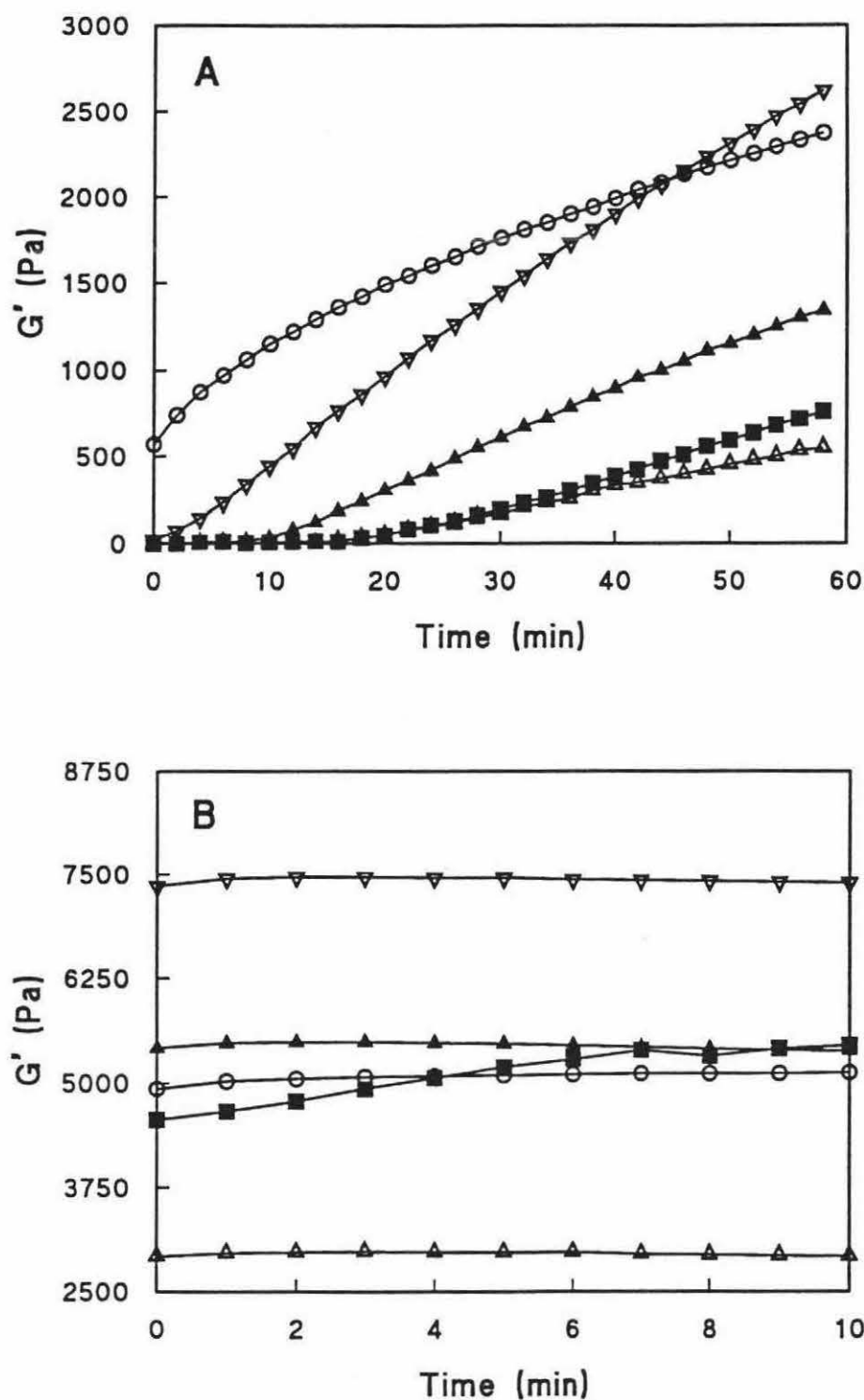
Figure 5.17   The effect of heating at 75°C (A) followed by cooling to 25°C (B) and holding on the storage modulus, G′ of 10% β-Lg (■) and BSA (○) solutions and protein mixtures containing 8% β-Lg and 2% BSA (△), 5% β-Lg and 5% BSA (▲) and 2% β-Lg and 8% BSA (▽).

When the protein gels were cooled to 25°C, G′ of all the protein gels increased further (Figure 5.17B). G′ of most protein gels increased slowly at the beginning of the measurements then remained constant during the 10 minute-hold. Initially, G′ of 10% β-Lg continued to increase slowly. The mixed protein gel containing 2% β-Lg and 8% BSA had the highest G′ value. These trends were examined in more detail and the results are reported in Tables 1.4 and 1.6 (*Appendix*).

Gelation time of the protein mixtures increased slightly as the relative quantity of BSA in the mixture increased from 1 to 4%. Further increases in the relative amount of BSA decreased the gelation time, except for the protein mixture containing 1% β-Lg and 9% BSA (Tables 1.4 and 1.6 *Appendix*).

Figure 5.18 shows the final G′ values for the protein mixtures containing β-Lg and BSA immediately after heating at 75°C for 60 min and after cooling to 25°C. As the relative quantity of BSA in the protein mixtures was increased from 1 to 4%, G′ values varied slightly with no consistent trend. However, there was a large increase in G′ when the concentration of BSA was increased to 5%. Above 5%, G′ increased gradually with increase in BSA concentration in the mixture. A slight decrease in G′ was observed for the protein mixture containing 1% β-Lg and 9% BSA.

After cooling to 25°C, G′ of all the protein gels increased further and with a marked increase in G′ for the β-Lg gel. Maximum G′ values were obtained for mixed protein gels containing 4% β-Lg and 6% BSA, 3% β-Lg and 7% BSA and 2% β-Lg and 8% BSA. G′ of the mixed protein gel containing 5% β-Lg and 5% BSA was more or less similar to that of both β-Lg and BSA.
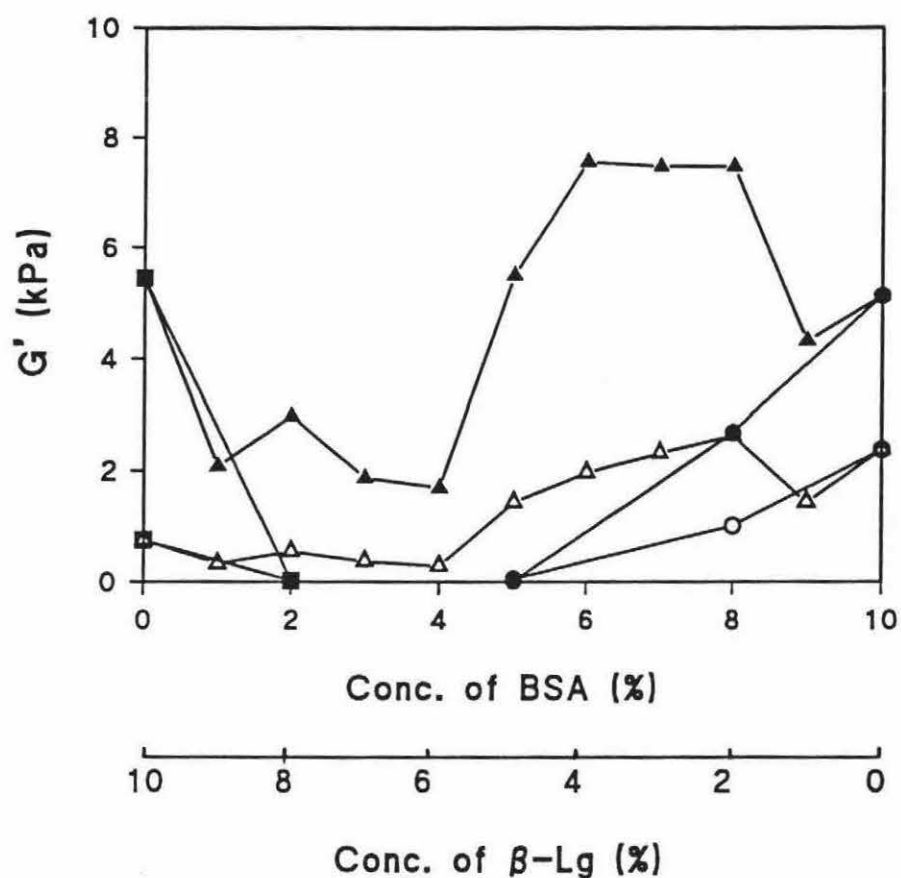
Figure 5.18   The effect of mixture composition on the final G' values at 75°C (∆) and 25°C (▲). For comparison the final G' values at 75°C (○, □) and 25°C (•, ■) for BSA (○, •) and β-Lg (■, □) at the concentrations indicated are included. (*Taken from Tables 1.4, 1.5 & 1.6. Appendix*).

5.3.1.3   The effect of added BSA on β-Lg gelation

Figure 5.19 shows the final G′ values for β-Lg/BSA mixtures containing 10% β-Lg and varying BSA concentrations measured at 70°C and after cooling to 25°C.

At 70°C, increasing the β-Lg concentration from 10 to 12% resulted in an increase in G′ but G′ decreased slightly at 13% β-Lg. The G′ values for the mixed protein gel consisting of 10% β-Lg and 1% BSA were similar to those of 11% β-Lg. In contrast, G′ values for the mixtures containing 2 or 3% BSA and 10% β-Lg were markedly greater than G′ values for β-Lg alone at 12 or 13% (Figure 5.19)

G′ values for the gels increased markedly after cooling to 25°C (Figure 5.19). The G′ values for the mixed protein gels were greater than values for β-Lg alone at the same total protein concentration.

The final G′ values for protein mixtures containing 10% β-Lg and varying BSA concentrations measured at 75°C after heating for 60 min and after cooling to 25°C are shown in Figure 5.20A,B.

No significant change in G′ was observed when the β-Lg concentration was increased between 10 and 12% but G′ increased markedly at 13%. G′ values of the protein mixtures increased substantially as the concentration of BSA in the protein mixture increased (Figure 5.20A).

When the protein gels were cooled to 25°C, G′ values of all the protein gels increased markedly. G′ values for the protein mixtures containing 1, 2 or 3% BSA and 10% β-Lg were greater than G′ values of β-Lg gels at 11, 12 and 13% concentration (Figure 5.20B).
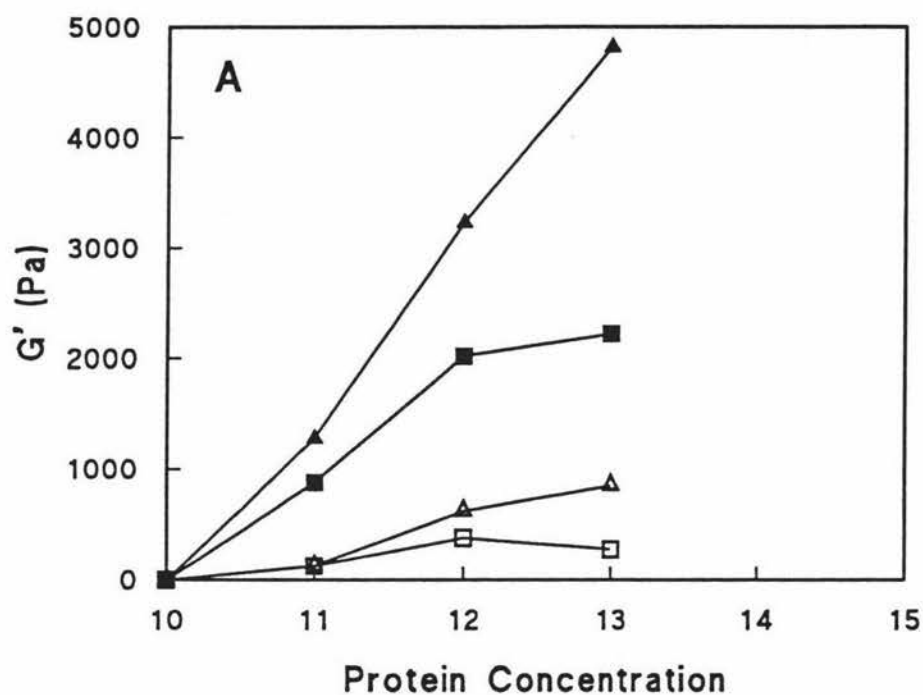
Figure 5.19   The effect of adding BSA to a 10% β-Lg on G′ at 70°C
(Δ) and 25°C (▲). Control samples at 70°C (□) and 25°C
(■) contained additional β-Lg so that the experimental
and control samples contained equal protein
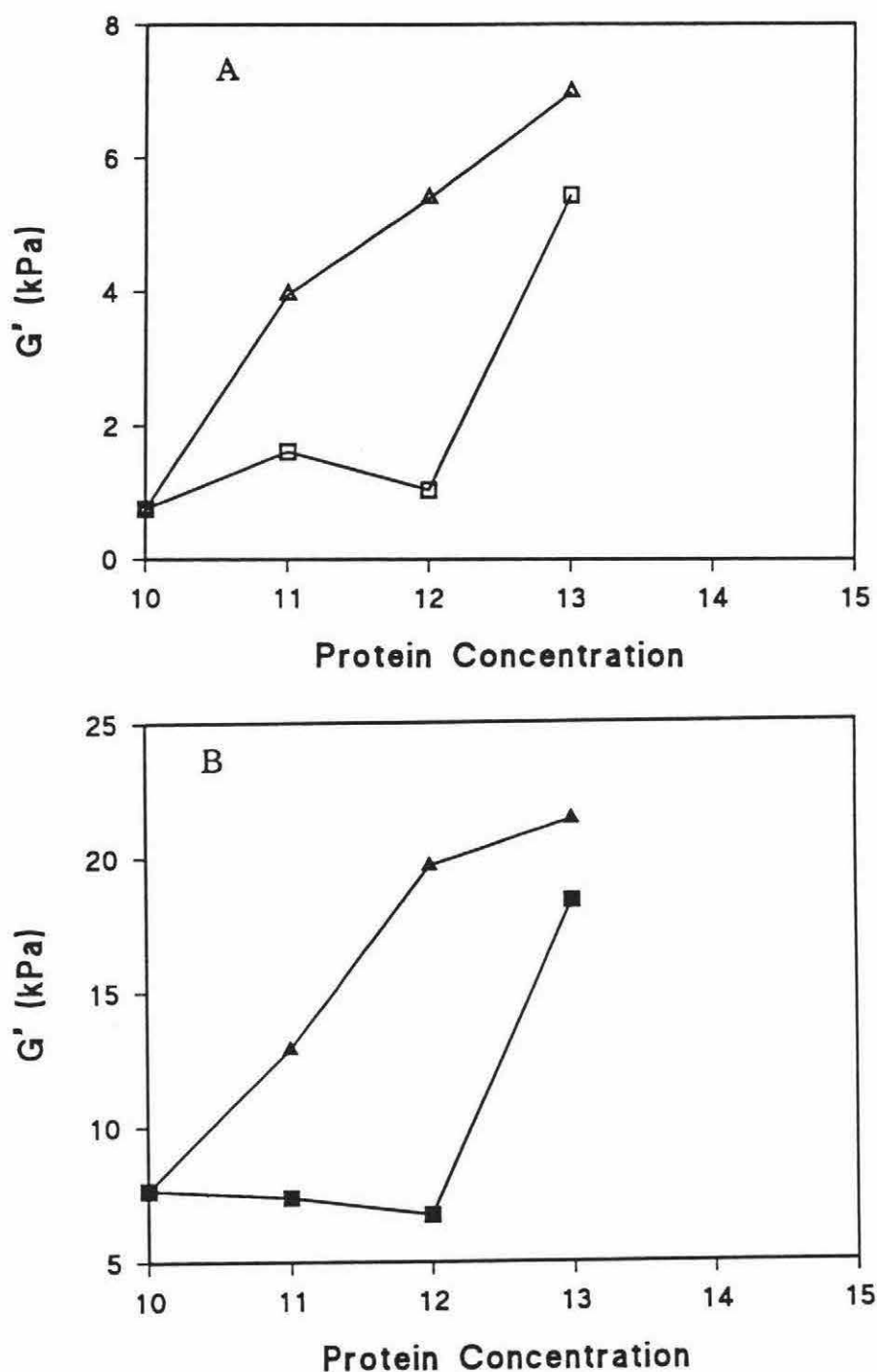concentrations. (*Taken from Tables 1.2 & 1.3,
Appendix*).

Figure 5.20   The effect of adding BSA to a 10% β-Lg on G' at 75°C
(A) (△) and 25°C (B) (▲). Control samples at 75°C (A)
(□) and 25°C (B) (■) contained additional β-Lg so that
the experimental and control samples contained equal
protein concentrations. (*Taken from Tables 1.7 & 1.8.
Appendix.*)

5.3.1.4 Gel characteristics of the protein mixtures at 70 and 75°C

β-Lg and BSA solutions heated alone produced transparent gels. Increasing the BSA concentration from 5 to 8 or 10% did not result in any change in the appearance of the gel; increasing the β-Lg concentration from 8 to 10, 11, 12 and 13% resulted in the gels becoming increasingly cloudy.

As the relative concentration of BSA in the protein mixtures increased from 1 to 5%, the mixed gels formed were clear but when the relative amount of BSA in the protein mixture increased beyond 5%, the gels became cloudy (Tables 1.4 and 1.6. *Appendix*). The cloudiest gel was obtained from the mixture containing 1% β-Lg and 9% BSA.

By comparing tan δ values (the ratio of the elastic and viscous properties of the gel) it is possible to determine the relative elastic or viscous character of the gels as the relative amount of BSA in the protein mixture increased. Tables 1.4 and 1.6 (*Appendix*) shows the values of tan δ for the protein mixtures after heating at 75°C and cooling to 25°C. At 75°C, tan δ decreased from 0.13 to 0.01 as the proportion of β-Lg in the mixture decreased. Tan δ (25°C) was constant at ~ 0.08 for the mixed protein gels as well as those of β-Lg and BSA, showing that the gels formed were more elastic than viscous. Paulsson *et al.* (1986) observed "ideally" elastic gels (tan δ near 0°) for BSA and viscoelastic gels with higher tan δ values for mixed gels.

5.3.1.5 Comparison of the protein mixtures at 70°C and 75°C

Although the initial gelation temperatures of the protein mixtures containing β-Lg and BSA (Figure 5.1) showed that the BSA appeared to dominate gelation, results obtained from the gelation studies seem to suggest that β-Lg does affect the gelation of the protein mixtures in some way.

Heating protein mixtures containing β-Lg and BSA at either 70 or 75°C showed that both proteins appeared to affect the character of the gels formed although β-Lg did not form any kind of gel when heated alone at 70°C.

When the heating temperature was increased to 75°C, G′ values for the mixed protein gels were higher than those formed at 70°C; both β-Lg and BSA were able to form self-supporting gels when heated alone at 75°C.

The final G′ values for the protein mixtures heated at 70°C were highest for the protein mixture with the highest relative amount of BSA while protein mixtures containing 4% β-Lg and 6% BSA, 3% β-Lg and 7% BSA and 2% β-Lg and 8% BSA had the highest G′ values after heating at 75°C (Figures 5.16 and 5.18).

Development of G′ was different for the mixed protein solutions during heating when compared to that of either β-Lg or BSA heated alone at the two temperatures studied. This is in conflict with the results obtained by Hines and Foegeding (1993). They observed that G′ of the protein mixtures developed in a similar pattern to that of β-Lg during heating at 80°C for 180 min although G′ increased proportionally with the amount of BSA in the original protein mixture. The differences observed by these workers with the present work can be attributed to variations in the buffering systems, pH, the heating temperatures used as well as the β-Lg and BSA ratios of the protein mixtures used, i.e, 5:1, 10:1, 20:1, 50:1 molar ratios with β-Lg predominating (the corresponding weight ratios are 1.39:1, 2.79:1, 5.6:1 and 13.9:1, respectively).

G′ of a protein mixture containing 6% β-Lg and 4% BSA was different from that containing 4% β-Lg and 6% BSA after heating at 70 or 75°C and after cooling to 25°C. This suggests that β-Lg and BSA are not interchangeable without altering the character of the gel. This is consistent with the work

carried out by Paulsson *et al.* (1986) and Matsudomi *et al.* (1994).

G' values for the mixed protein gels were greater than those formed from either proteins alone, both at constant total protein concentration and at constant β-Lg concentration. The highest G' was obtained for protein mixtures containing relatively higher BSA concentrations. Similar observations were noted by Matsudomi *et al.* (1994), but he reported that more intense intermolecular interactions were developed between β-Lg and BSA at a mixing ratio of 1:1 (wt/wt). The discrepancy between the results obtained in this study with those of Matsudomi *et al.* (1994) is possibly due to the different gelation conditions used and the method used to measure the gel properties.

Temperature may be considered as a major cause of the differences between the results obtained in this present study and some previous reports. Heating protein mixtures results in the degree of order of the protein molecules decreasing especially at high temperatures (e.g at 80°C; Hines and Foegeding, 1993 ; Matsudomi *et al.* 1994). The activation energy of the reaction is reached sooner at higher temperatures (e.g 75°C) and this may result in an alteration of the reaction mechanism and the ultimate rheological properties of the protein mixtures.

5.3.2   Gelation of β-lg/α-La mixtures

5.3.2.1   Gelation at 75°C

The rheological properties of protein solutions containing β-Lg and α-La were investigated at 75°C and Figure 5.21A,B shows the changes in G' with time during heating and after cooling to 25°C. The total protein concentration was kept constant at 10% while the concentrations of both β-Lg and α-La were varied.
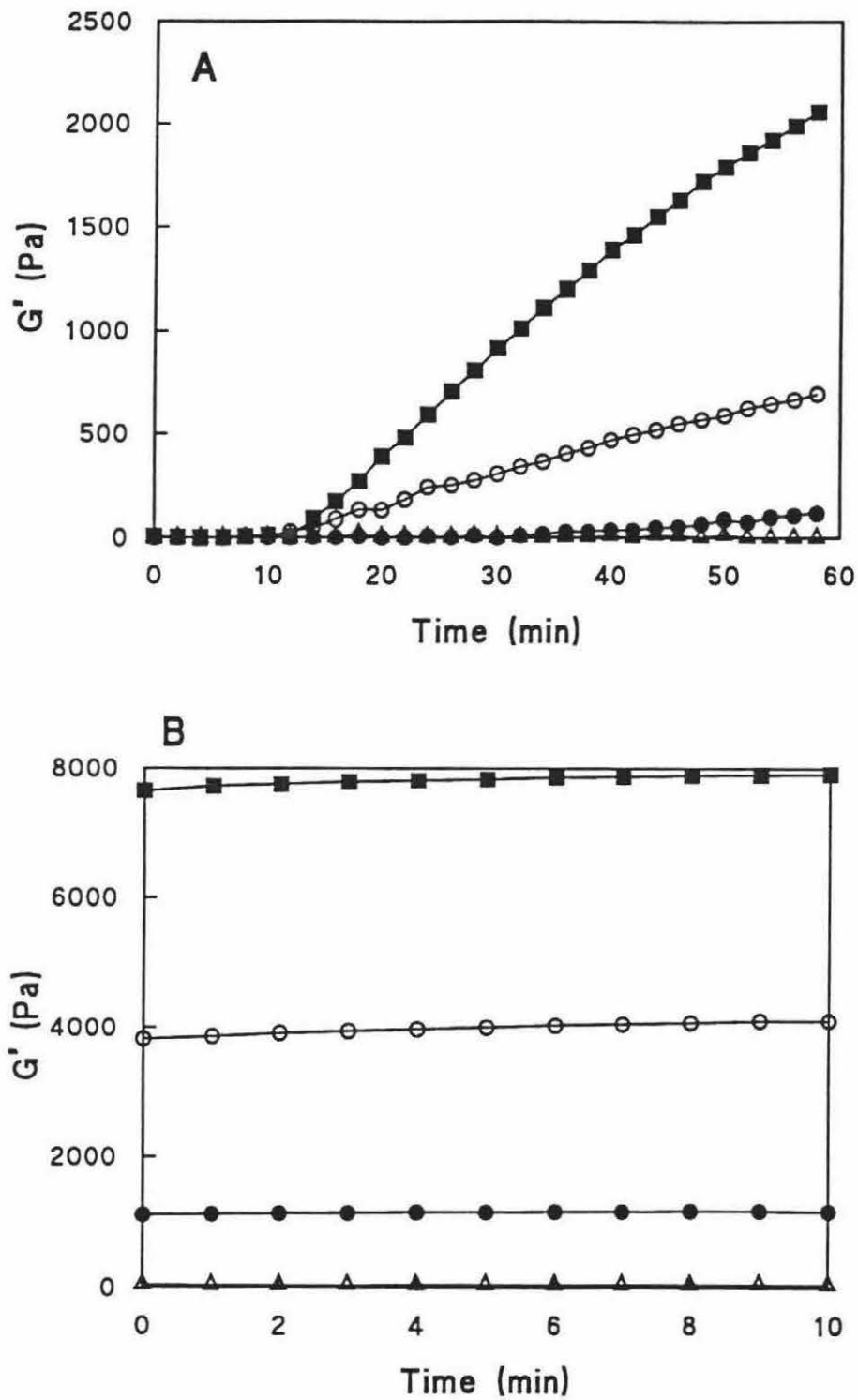
Figure 5.21   The effect of heating at 75°C (A) followed by cooling to 25°C (B) and holding on the storage modulus, G′, of 10%, w/v, β-Lg (■) and protein mixtures containing 8% β-Lg and 2% α-La (○), 5% β-Lg and 5% α-La (•) and 2% β-Lg and 8% α-La (△).

A protein solution containing 10% α-La did not show any change in G′ during heating at 75°C for 60 min (Figure 5.10). G′ of a protein solution containing 10% β-Lg began to increase after heating for 12 min and continued to do so during heating (Figure 5.21A).

Protein mixtures containing β-Lg and α-La showed a gradual increase in G′ with time. The rate of G′ development for the protein mixtures was slower than that of β-Lg alone and decreased as the relative concentration of α-La in the original protein mixture increased. No siginificant change in G′ was observed when a protein mixture containing 2% β-Lg and 8% α-La was heated for 60 min. G′ of the protein mixtures was still slowly rising after heating for 60 min.

When the gels were cooled to 25°C (Figure 5.21B), G′ of the protein gels increased by more than 5-fold. G′ of the protein gels increased slightly at the beginning then remained constant during the 10 minute-hold at 25°C. Lower G′ values were observed for the mixed protein gels containing increasing relative amounts of α-La. No change in G′ could be detected when a protein mixture containing 2% β-Lg and 8% α-La solution was cooled to 25°C.

Gelation time, determined as time to obtain an appreciable increase in G′, increased as the relative quantity of α-La in the mixture increased (Tables 1.9 and 1.10. *Appendix*).

Figure 5.22 shows the final G′ values after heating at 75°C and after cooling to 25°C for the protein mixtures containing different β-Lg and α-La concentrations. Both at 75°C and 25°C, the addition of 1% α-La to 9% β-Lg caused a sharp decrease in G′. G′ value increased slightly with further addition of α-La at 2%. Further additions, however, resulted in a gradual decrease in G′ of the protein mixtures.
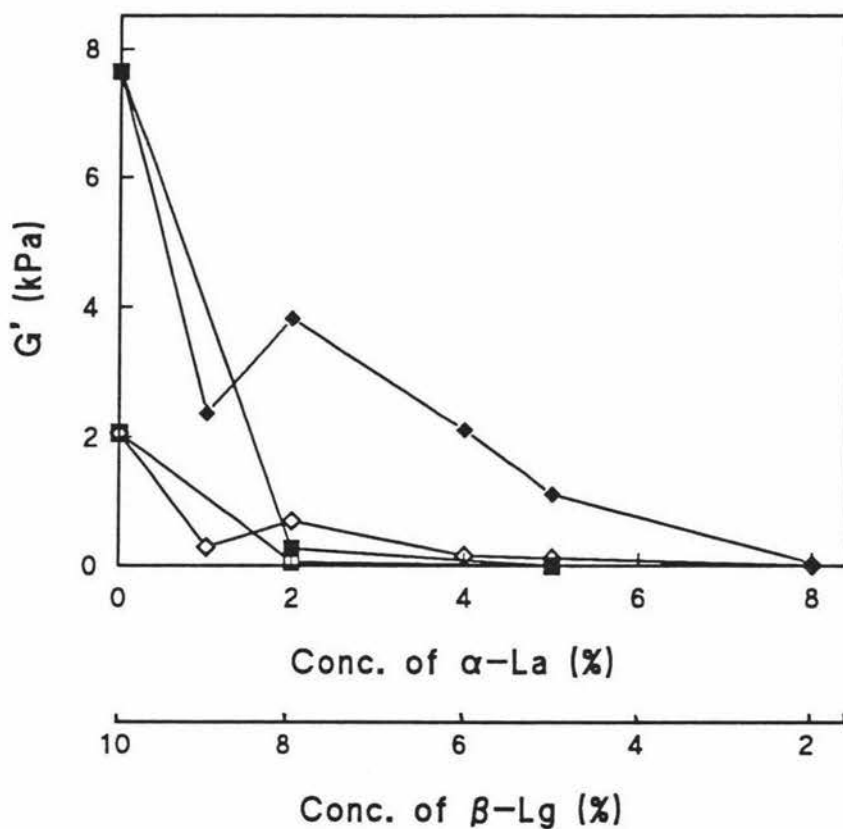
Figure 5.22 The effect of mixture composition on the final G' values at 75°C (◊) and 25°C (♦). For comparison the final G' values at 75°C (□) and 25°C (■) for β-Lg at concentrations indicated are included (*Taken from Tables 1.9 & 1.10. Appendix*).

5.3.2.2 *Gelation at 80°C*

Gelation behaviour of α-La and β-Lg protein mixtures was investigated at 80°C. Figure 5.23A,B shows the changes in G' with time for the protein mixtures during heating and after cooling to 25°C. The concentrations of β-Lg and α-La were varied while keeping the total protein concentration constant at 10%. G' of a solution containing 10% β-Lg started to increase after about 3 min of heating at 80°C. No change in G' was observed when a solution containing 10% α-La was heated.

G' of the protein mixtures increased gradually with increase in the heating time. The development of G' began much earlier for the protein mixture containing 8% β-Lg and 2% α-La than that for 10% β-Lg and as the relative quantity of α-La in the protein mixture increased the rate of increase of G' decreased with heating.

During cooling to 25°C, G' of all the protein mixtures increased drastically and G' changed slowly for some mixtures during the 10 minute-hold at 25°C (Figure 5.23B). G' values for protein mixtures containing 5% β-Lg and 5% α-La and 2% β-Lg and 8% α-La were higher than that of 10% β-Lg although their G' values immediately after heating were lower. Cooling appears to have a major effect on protein gels containing β-Lg and α-La.

Gelation time of most of the protein mixtures increased as the relative amount of α-La in the protein mixtures increased (Tables 1.11 and 1.12. *Appendix*).

Figure 5.24 shows the final G' values for the protein mixtures immediately after heating at 80°C and after cooling to 25°C. There were minor differences between the G' values immediately after heating for most of the protein mixtures except for the protein mixtures containing 8% β-Lg and 2% α-La and 2% β-Lg and 8% α-La, where the differences were more marked.
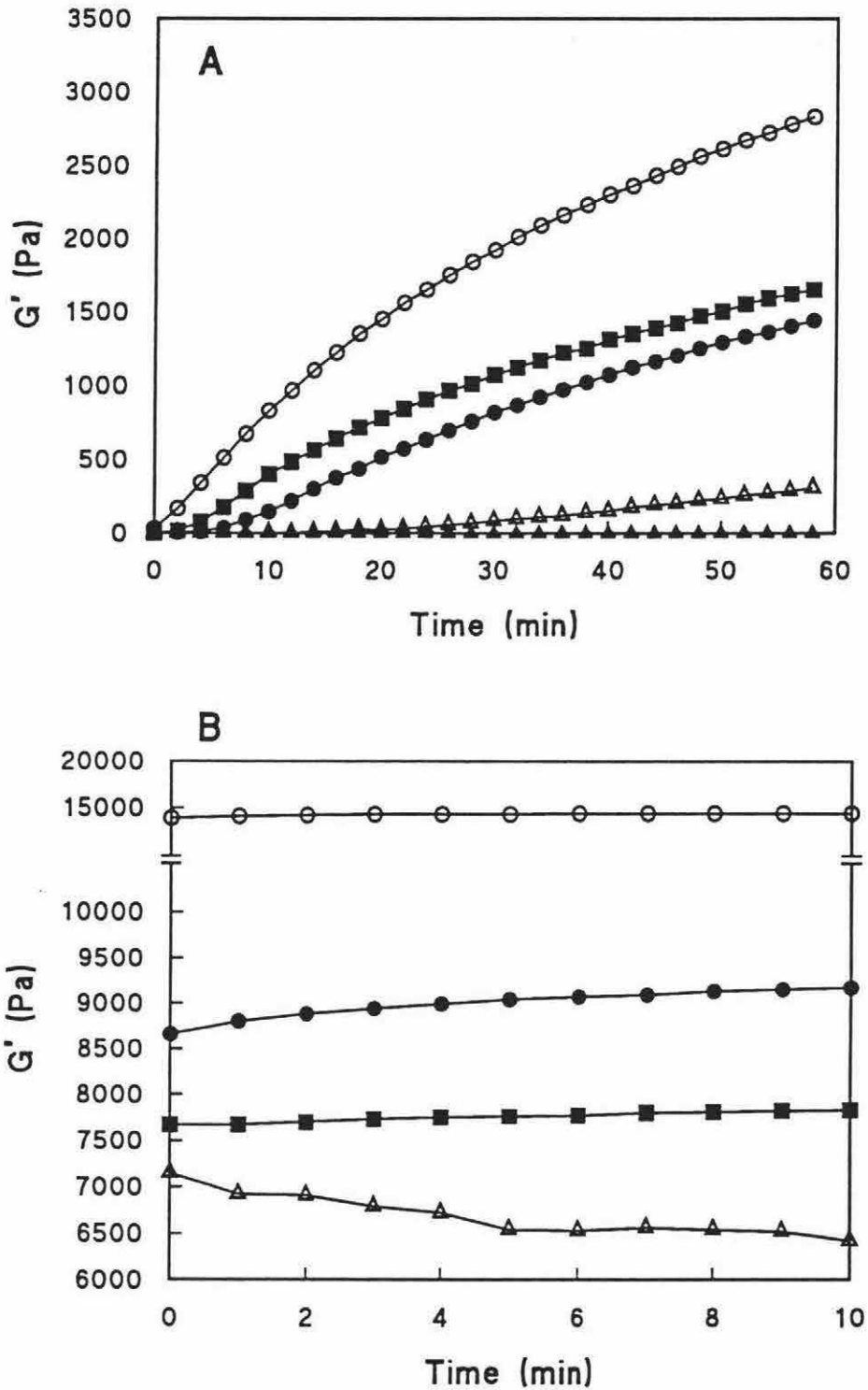
Figure 5.23   The effect of heating at 80°C (A) followed by cooling to 25°C (B) and holding on the storage modulus, G′ of 10%, w/v, β-Lg (■), 10%, w/v, α-La (▲) and protein mixtures containing 8% β-Lg and 2% α-La (○), 5% β-Lg and 5% α-La (•) and 2% β-Lg and 8% α-La (△).
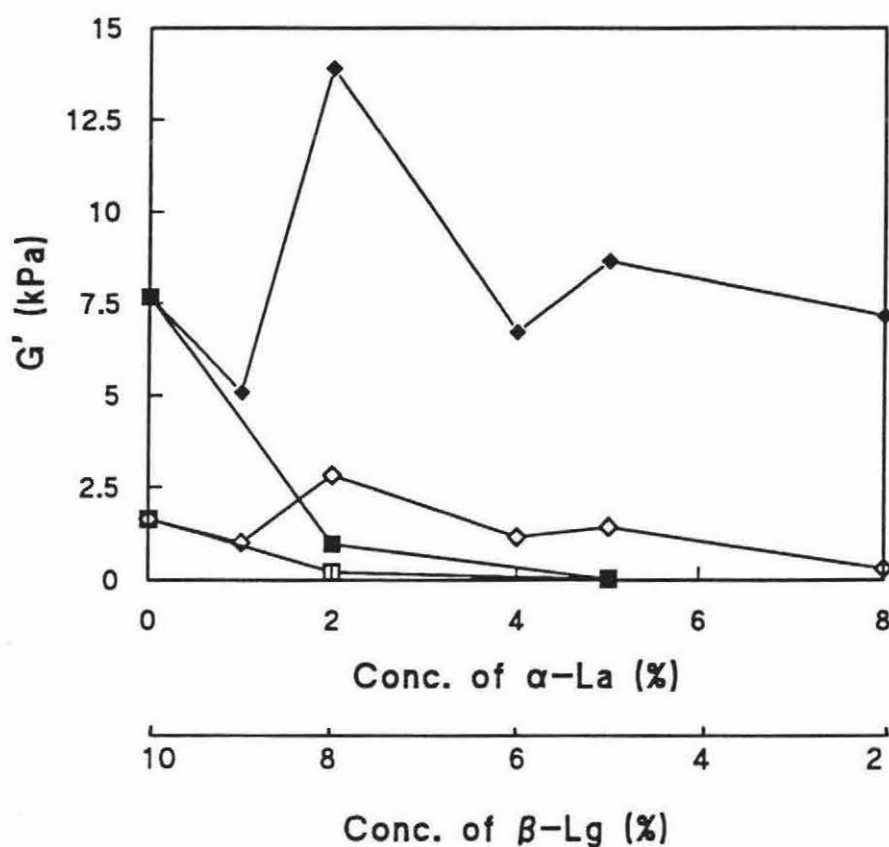
Figure 5.24   The effect of mixture composition on the final G′ values at 80°C (◊) and 25°C (♦). For comparison the final G′ values at 80°C (□) and 25°C (■) for β-Lg at the concentrations indicated are included (*Taken from Tables 1.11 & 1.12. Appendix*).

When the protein gels were cooled to 25°C, G′ of the protein gels increased markedly. G′ increased almost 5-fold for the protein mixture containing 8% β-Lg and 2% α-La. There were relatively minor differences between the G′ values for the protein mixtures containing 6% β-Lg and 4% α-La, 5% β-Lg and 5% α-La and 2% β-Lg and 8% α-La and 10% β-Lg.

5.3.2.3   Effect of added α-La on β-Lg gelation

The gelation properties of protein mixtures containing 10% β-Lg and varying concentrations of α-La were studied at 80°C, since a more interactive behaviour of α-La was observed at this temperature. Figure 5.25 shows G′ values for protein mixtures heated at 80°C and after cooling to 25°C. At 80°C and 25°C, there was no significant change in G′ when the β-Lg concentration was increased from 10 to 12% but G′ increased markedly at 13% during heating. G′ of protein mixtures increased markedly with increasing addition of α-La. G′ of the protein mixtures was higher than that of β-Lg heated alone.

Results reported by Matsudomi *et al*. (1992) after heating protein mixtures containing β-Lg and α-La at constant β-Lg (6%, w/v) are in agreement with the present work. They found that the hardness of the gels made from β-Lg and α-La increased with additions of α-La.

Contrary to observations in the present study, Hines and Foegeding (1993), using similar rheological methods, noted that gels made from protein mixtures of β-Lg and α-La at molar ratios of 5:1, 10:1 and 20:1 (corresponding weight ratios are 1.39:1, 2.79:1, 5.6:1 and 13.9:1, respectively) had similar transitions and ultimate values of G′ to the gels made from β-Lg alone during heating at 80°C for 180 min. This discrepancy can be attributed to the high β-Lg to α-La ratios used which possibly masked the effect of α-La.
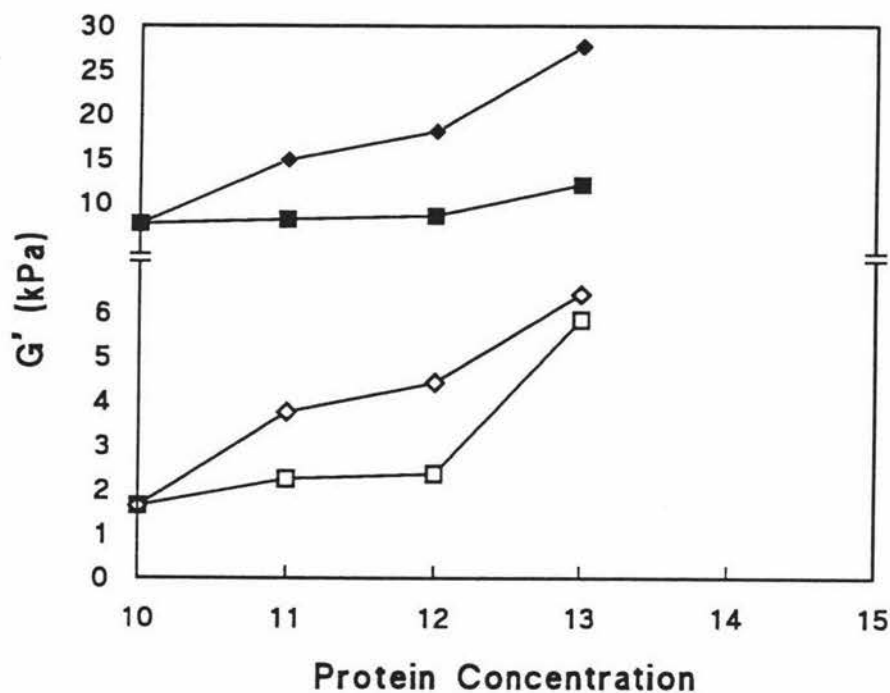
Figure 5.25   The effect of adding α-La to a 10% β-Lg on G′ at 80°C
(◊) and 25°C (♦). Controls samples at 80°C (□) and
25°C (■) contained additional β-Lg so that the
experimental and control samples contained equal
protein concentrations (*Taken from Table 1.13,
Appendix*).

5.3.2.4 Gel characteristics at 75°C and 80°C

Transparent gels were obtained when β-Lg was heated. As the concentration of β-Lg increased from 10 to 13% the gels became cloudy. As the relative quantity of α-La in the protein mixture increased the mixed protein gels became more turbid to a milky white. Similar observations were made by Matsudomi *et al.* (1992). When α-La was heated alone, small amounts of fine stranded material appeared, but the solutions were basically transparent.

Tables 1.9 - 1.13 (*Appendix*) show the values of tan δ for the different protein mixtures. Tan δ (25°C) for the protein mixtures at constant total protein concentration that were heated at either 75 or 80°C shifted from near 0° to values between 0 and 90° thus showing that the gels became increasingly viscoelastic as the relative proportion of α-La in the original protein mixture increased. Increasing the ratio of α-La to β-Lg resulted in increasing values of tan δ (25°C) indicating a diminishing elasticity.

5.3.2.5 Comparison of gelation of β-Lg and α-La at 75°C and 80°C

α-La did not show any changes in G′ during heating at 75 or 80°C and after subsequent cooling of the protein solution. Heating protein mixtures containing varying concentrations of β-Lg and α-La (while keeping the total concentration at 10%) at 75°C resulted in a decrease in the final G′ values for the protein mixtures when compared with G′ of a 10% β-Lg solution (Figure 5.22). When the same protein mixtures were heated at 80°C, there was little difference in the G′ values of most of the protein mixtures with that of 10% β-Lg (Figure 5.24). The protein mixture containing 2% β-Lg and 8% α-La had a final G′ value of 40 Pa after heating at 75°C; the same protein mixture had a G′ of 7150 Pa (similar to that of 10% β-Lg) after heating at 80°C. α-La appeared to be involved to some extent in the gelation of the protein mixtures at the two temperatures studied; the effect of α-La was more pronounced at 80°C than at 75°C (Figures 5.22 and 5.24).

Cooling the protein gels had a remarkable effect on the final G′ of the protein mixtures heated at 75 and 80°C and this may indicate that there is possibly increased crosslinking and molecular rearrangements occuring during cooling. It is also possible that there is increased hydrogen bonding during cooling since it has been shown to be favoured at low temperatures (Beveridge *et al.* 1984).

α-La has been shown to form gels when heated in the presence of some thiol groups (Calvo, 1993 ; Legowo, 1993). Native β-Lg has one thiol group and two disulphide groups and α-La has four disulphide groups (Fox, 1989). By heating above the thermal transition of both β-Lg (73°C) and α-La (77°C) for heat denaturation at pH 6.6 (Matsudomi *et al.* 1992) it is possible that the thiol group of β-Lg can reduce the disulphide bonds of α-La thus enhancing the participation of α-La in gel formation.

5.4 Protein aggregate formation prior to gelation

5.4.1 β-Lg/BSA mixtures

Native-PAGE

Solutions of protein mixtures, containing either β-Lg and BSA or β-Lg and α-La, and individual proteins were heated from 25°C to 70, 75 or 80°C in the Bohlin VOR rheometer cup at 1°C/min and held at these temperatures until a gel formed. Aliquots of heated protein solutions were removed at intervals and dispersed in non-dissociating buffer (0.5M Tris-HCl buffer) at pH 6.8, and analysed using PAGE followed by densitometry. Some protein solutions quickly became too viscous for sampling and were not analysed (see Table 3.1.*Appendix*).

Protein mixtures containing β-Lg and BSA were heated at 70 or 75°C from zero time (i.e, when the target heating temperature was reached) to 14 min (prior to gel formation). Figure 5.26 shows a typical gel electrophoretic pattern obtained under non-dissociating conditions when a protein solution containing 5% β-Lg and 5% BSA was heated at 75°C for various times. Lanes 1 and 2 show the unheated protein sample. A decrease in the intensity of the protein bands was observed as the heating time increased from 0 to 6 min (Lanes 3 to 7). After 6 min, the protein solution became too viscous for further sampling. Some protein material which failed to enter the stacking and separating gels was present in most of the heated samples. This protein material probably contained high molecular weight protein aggregates cross-linked *via* non-covalent interactions and disulphide bonds.

Protein aggregates ⟶

BSA ⟶

β-Lg ⟶

1　2　3　4　5　6　7
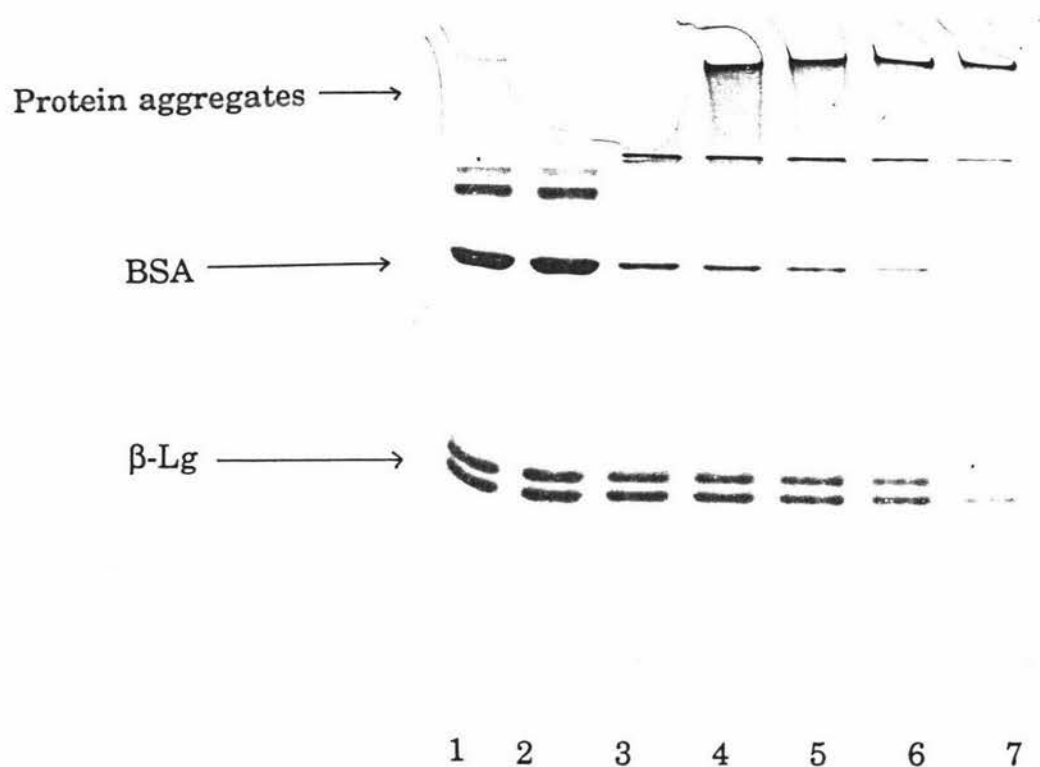
Figure 5.26　A typical native gel showing the effect of heating at 75°C on the amount of native β-Lg and BSA remaining. Lanes 1 - 7 are: unheated sample (duplicate), zero time sample, 1 min sample, 2 min sample, 4 min sample and 6 min sample.
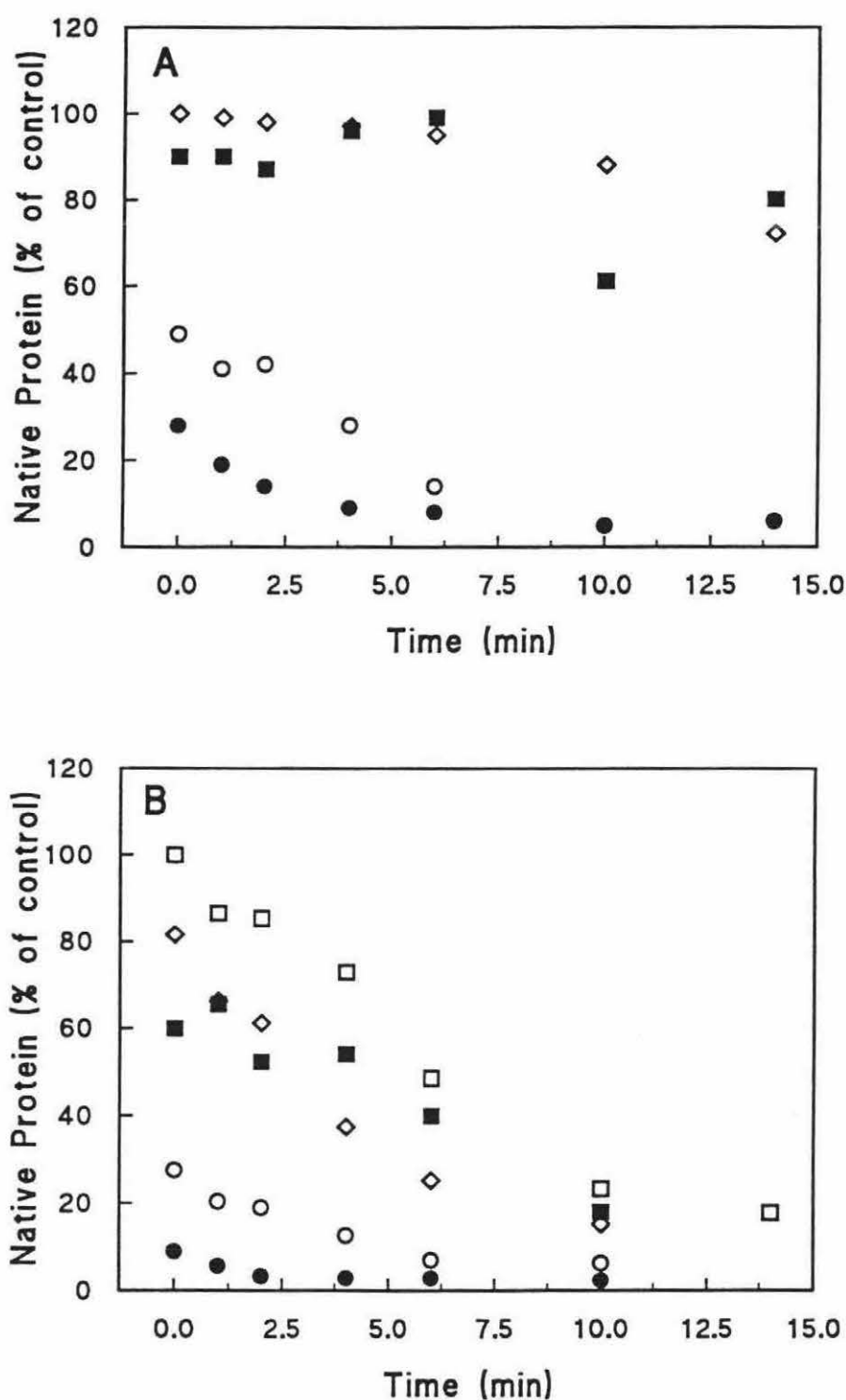
β-Lg/BSA ratio 5:5%

A protein mixture containing 5% β-Lg and 5% BSA was heated at 70°C and the amounts of native β-Lg and BSA in the heated sample are shown in Figure 5.27A.

When a 10% β-Lg solution was heated under similar conditions the amount of native β-Lg decreased slowly with heating time (Figure 5.27A). However, when a 10% BSA solution was heated, no samples could be taken for analysis as the protein solutions quickly became too viscous for sampling.

When the protein mixture containing 5% β-Lg and 5% BSA was heated, the amount of native BSA decreased rapidly with heating time while the amount of β-Lg decreased slowly. The loss of native BSA from the mixture was faster than when 5% BSA was heated alone (Figure 5.27A). There was a substantial loss of native BSA during the heat-up time.

When the same protein solutions were heated at 75°C, the quantities of native β-Lg and BSA decreased at a faster rate than at 70°C (Figure 5.27B). The loss of native β-Lg and BSA was faster from the mixed protein solutions than when either protein (at 5% concentration) was heated alone, although the loss of β-Lg (10%) was similar to that of β-Lg from the protein mixture. There was a considerable loss of the β-Lg from the mixture during the heat-up time and most of the native BSA (~ 80%) had disappeared from the gel under these conditions (Figure 5.27B).

Figure 5.27   The effect of heating time at 70°C (A) and 75°C (B) on
the loss of native β-Lg (◊, □, ■) and native BSA (○, •)
from a 10% β-Lg solution (◊), a 5% β-Lg solution (□), a
5% BSA solution (○) and a solution containing both 5%
β-Lg (■) and 5% BSA (•).

β-Lg/BSA ratio 8:2%

A protein solution containing 8% β-Lg and 2% BSA was heated at 70°C and the amounts of native proteins present determined (Figure 5.28A). Most of the native BSA in the mixture was lost during the heat-up time. Although ~ 25% of native β-Lg from the mixture was lost during the heat-up time, the rate of loss of the β-Lg from the mixture at 70°C was similar to that of 10% β-Lg heated alone.

When the same protein solution was heated at 75°C, the amount of native β-Lg decreased with heating time. The loss of native β-Lg from the mixture was somewhat similar to that from 10% and 5% β-Lg solutions.

β-Lg/BSA ratio 2:8%

When a protein solution containing 2% β-Lg and 8% BSA was heated at 70°C a decrease in the amount of native β-Lg and BSA was observed with heating time (Figure 5.29A). The loss of β-Lg from the 10% mixed protein solution was faster than 10% β-Lg heated alone (Figure 5.29A).

When the same protein mixture was heated to 75°C, there was a substantial loss of the native proteins. The loss of β-Lg from the mixture was similar to 10% β-Lg heated alone, whereas the loss of BSA appeared to be faster from the mixture (Figure 5.29B).
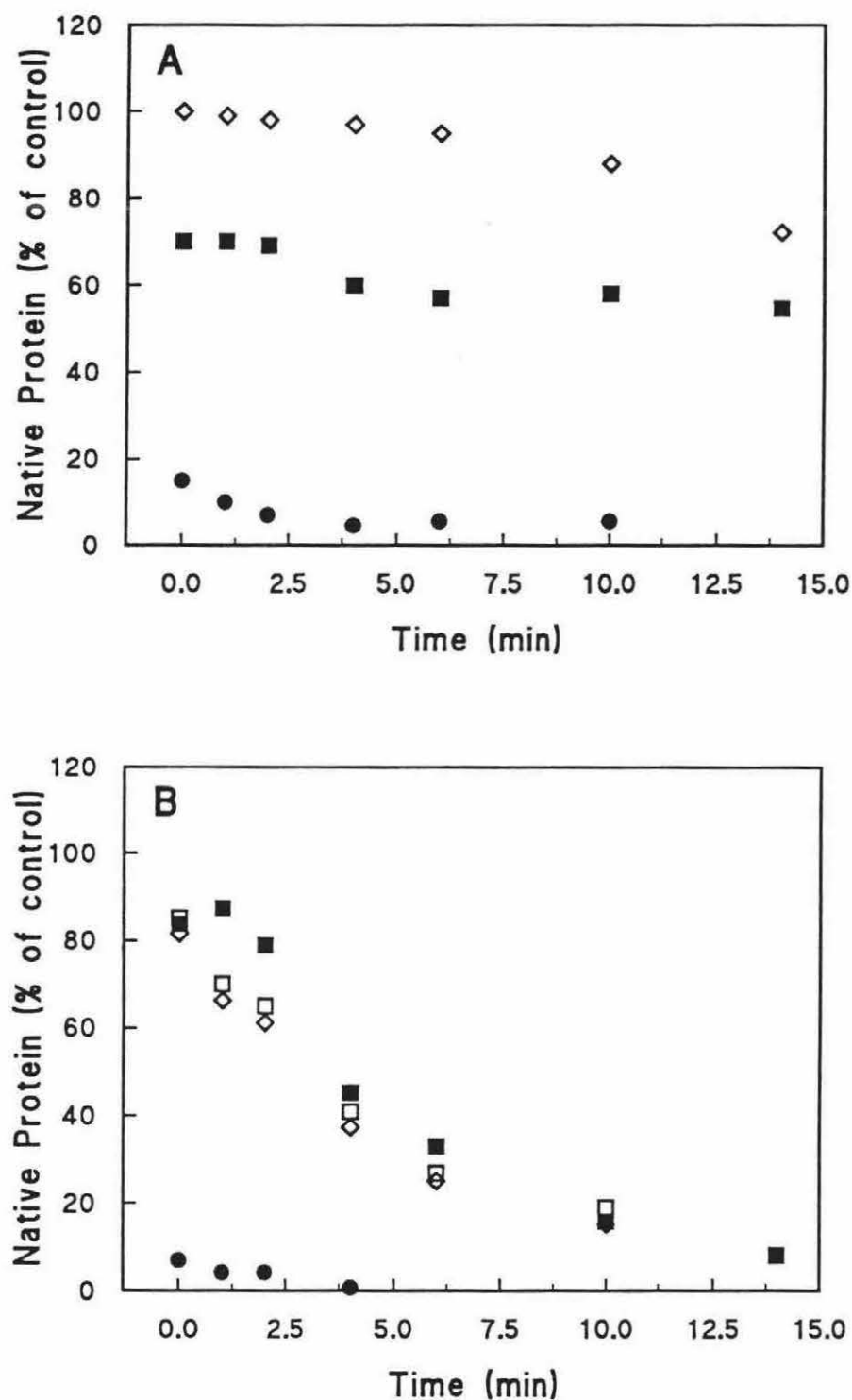
Figure 5.28   The effect of heating time at 70°C (A) and 75°C (B) on the loss of native β-Lg (◊, □, ■) and native BSA (•) from a 10% β-Lg (◊), a 8% β-Lg (□) and a solution containing 8% β-Lg (■) and 2% BSA (•).
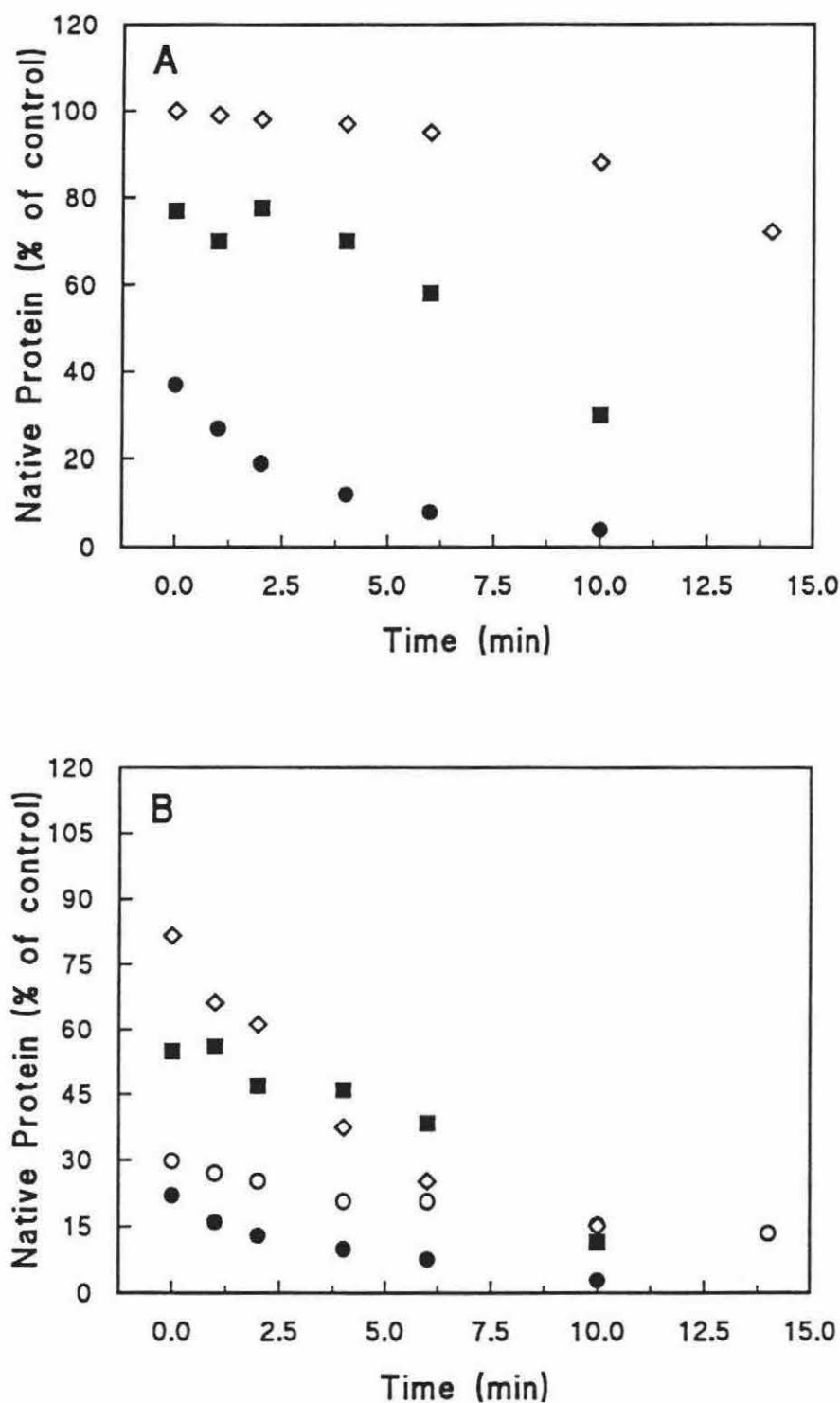
Figure 5.29   The effect of heating time at 70°C (A) and 75°C (B) on the loss of native β-Lg (◊, ■) and native BSA (○, •) from a 10% β-Lg solution (◊), a 5% BSA solution (○) and a solution containing 2% β-Lg (■) and 8% BSA (•).

SDS-PAGE

Figure 5.30 shows a typical SDS gel pattern obtained when a mixture of 7% β-Lg and 3% BSA was sampled and analysed during heating at 75°C. Lanes 1 and 2 show the unheated protein sample. The intensity of the protein bands gradually decreased with heating (Figure 5.30, lanes 3 to 8). All heated samples showed high molecular weight protein bands at the top of both the stacking and resolving gel; the intensity of these protein bands increased with heating time and later decreased with increased heating.

β-Lg/BSA ratio 5:5%

A protein mixture containing equal concentrations of β-Lg and BSA was heated at 70°C (Figure 5.31A). The amounts of residual BSA which showed as monomeric on SDS-PAGE, decreased much faster than β-Lg in the protein mixture. The amounts of residual monomeric β-Lg decreased only slightly with heating time and there was no significant difference between the mixture and 10% β-Lg heated alone. Approximately 55% residual monomeric BSA was lost during the heat-up time while there was no obvious change in the amounts of β-Lg.

When the same protein solution was heated at 75°C (Figure 5.31B), the amounts of residual monomeric β-Lg and BSA in the protein mixture decreased with heating. The decrease in residual monomeric β-Lg in the protein mixture was not significantly different from residual monomeric β-Lg levels found when 10 and 5% solutions of β-Lg were heated. Residual monomeric BSA in the mixture decreased much faster than BSA heated alone (Figure 5.31B).
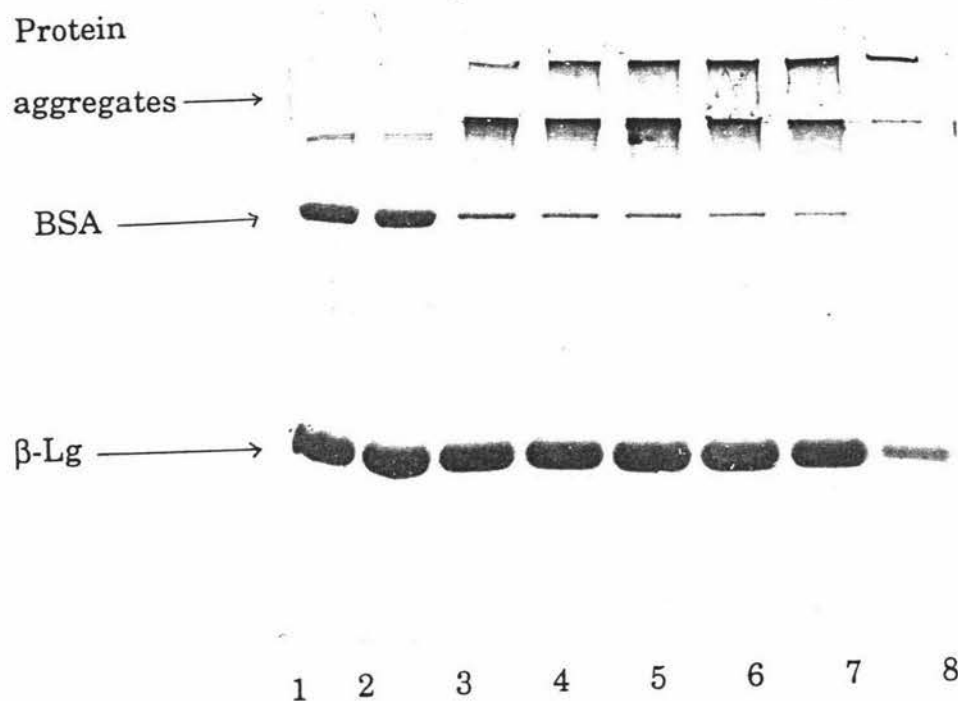
Figure 5.30   A typical SDS gel showing the effect of heating at 75°C on the amount of residual monomeric β-Lg and BSA remaining.   Lanes 1 - 8 are: unheated samples (duplicate), zero min sample, 1 min sample, 2 min sample, 4 min sample, 6 min sample and 10 min sample.

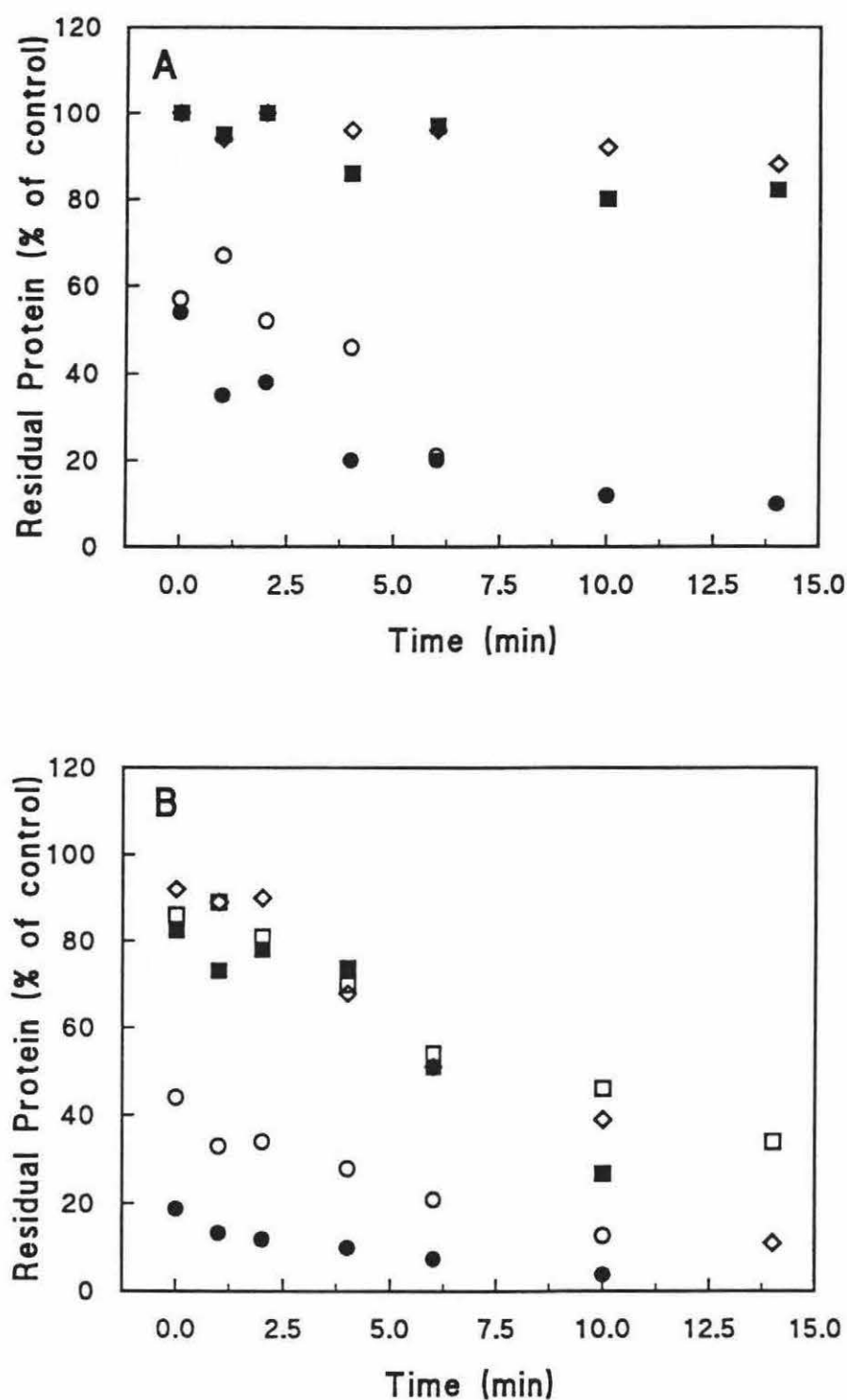**Figure 5.31**   The effect of heating time at 70°C (A) and 75°C (B) on the loss of residual monomeric β-Lg (■, □, ◊) and residual monomeric BSA (○, •) from a 10% β-Lg solution (◊), a 5% β-Lg solution (□) and a solution containing 5% β-Lg (■) and 5% BSA (•).

β-Lg/BSA ratio 8:2%

Figure 5.32A shows the intensity of the β-Lg and BSA bands after heating a protein solution containing 8% β-Lg and 2% BSA at 70°C. The loss of non-covalently linked β-Lg and BSA aggregates shown as monomeric on SDS-PAGE decreased more slowly for β-Lg than BSA with heating. During the heating period ~ 10% β-Lg and ~ 70% BSA was lost; it can be assumed that the proteins had already aggregated through disulphide bonds during heating. There was no significant change in the loss of residual β-Lg when β-Lg was heated alone.

When the heating temperature was increased to 75°C and the sample analysed (Figure 5.32B), the amounts of residual monomeric β-Lg and BSA in the mixture decreased at a faster rate than at 70°C. When heated alone, 10% β-Lg and 8% β-Lg decreased at the same rate as β-Lg in the mixture.

β-Lg/BSA ratio 2:8%

A mixture containing 2% β-Lg and 8% BSA was heated at 70°C (Figure 5.33A) and a gradual loss of the residual monomeric β-Lg and BSA was observed during heating.

At 75°C (Figure 5.33B), the loss of the residual monomeric proteins increased with heating time. The residual monomeric β-Lg in the protein solution decreased faster than when 10% β-Lg was heated alone.

Figure 5.32   The effect of heating time at 70°C (A) and 75°C (B) on the loss of residual monomeric β-Lg (◊, □, ■) and residual monomeric BSA (•) from a 10% β-Lg solution (◊), a 8% β-Lg (□) and a solution containing 8% β-Lg (■) and 2% BSA (•).
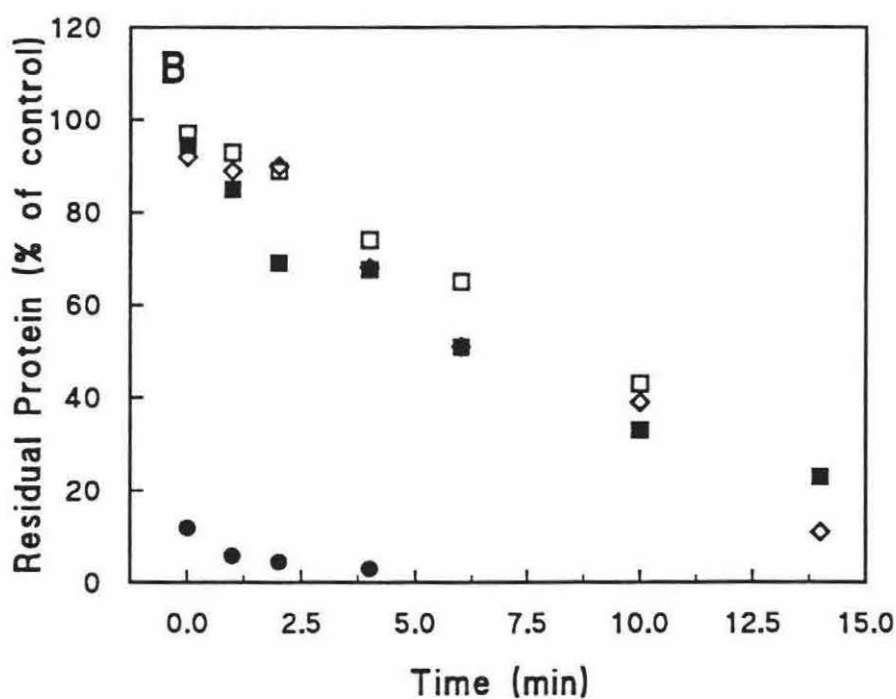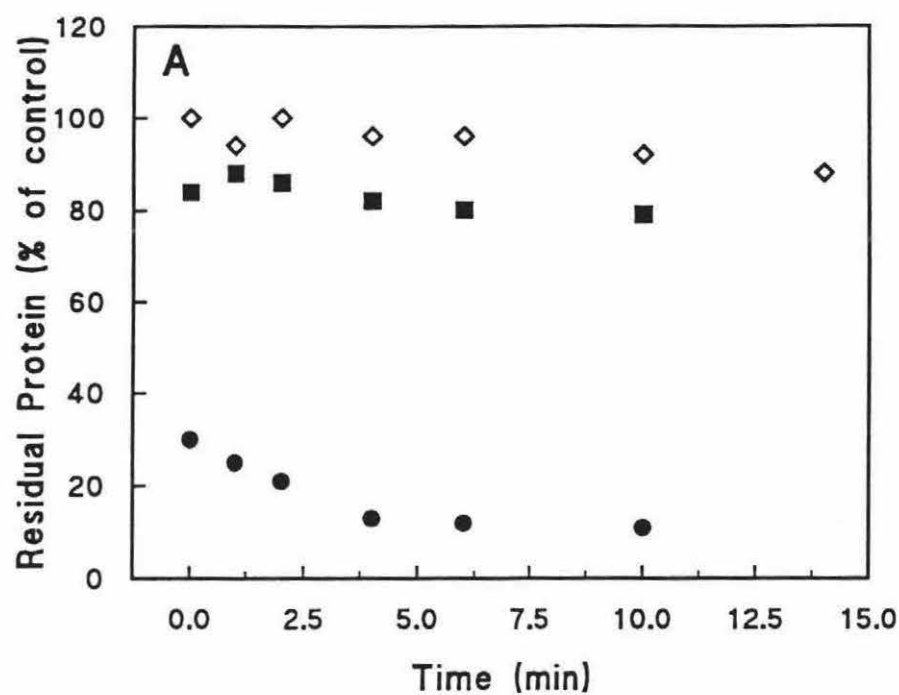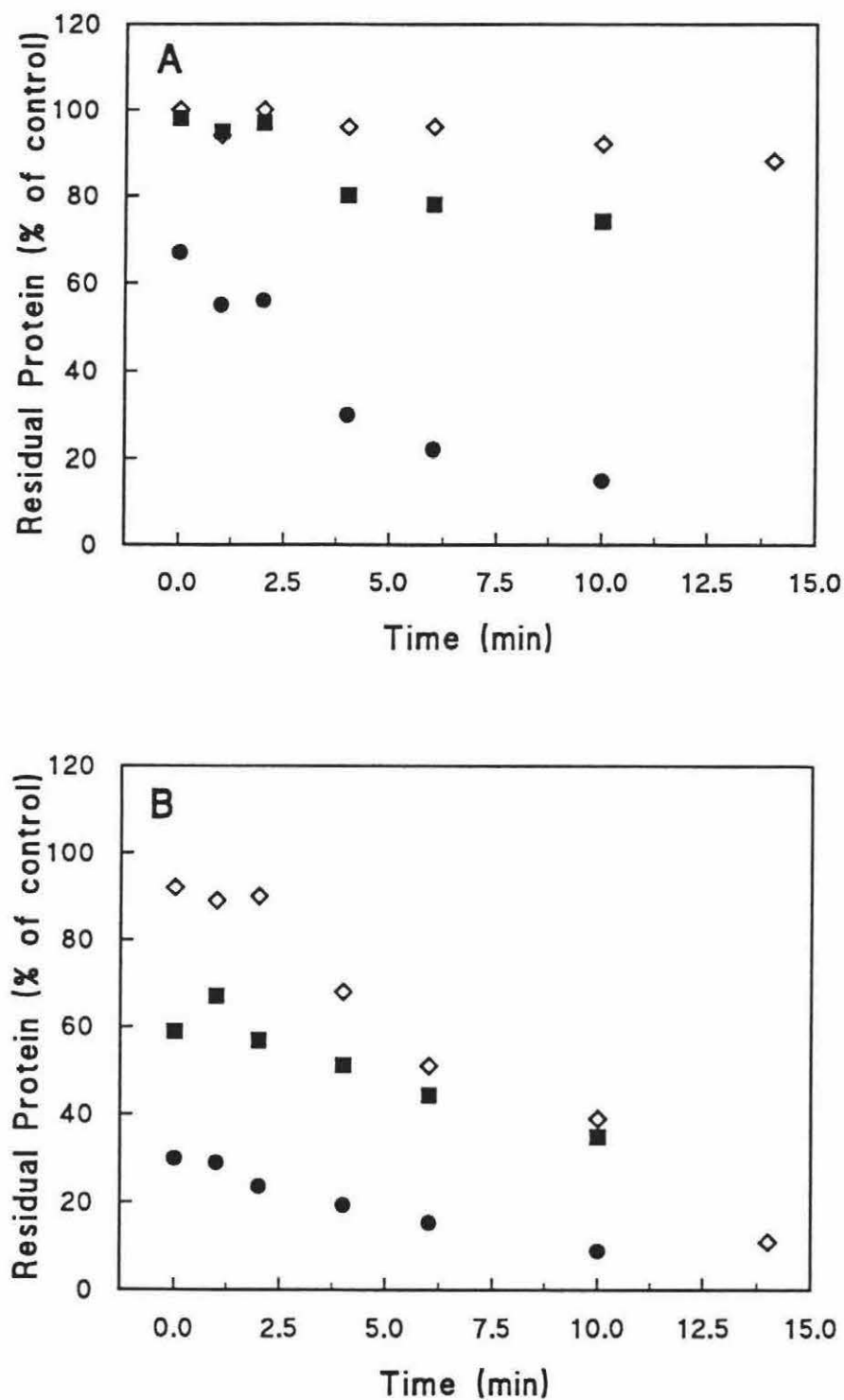
Figure 5.33   The effect of heating time at 70°C (A) and 75°C (B) on the loss of residual monomeric β-Lg (◊, ■) and residual monomeric BSA (•) from a 10% β-Lg solution (◊) and a solution containing 2% β-Lg (■) and 8% BSA (•).

The present results show that the amount of residual monomeric protein which includes native as well as non-covalently bonded aggregates but not disulphide cross-linked aggregates remaining during heating at 75°C and at 70°C (for BSA molecules) decreased with increased heating time.

5.4.1.1 Comparison of Native and SDS-PAGE results

β-Lg/BSA ratio 5:5%

Figure 5.34A compares the loss of monomeric protein from the protein mixtures heated at 70°C using native-PAGE and SDS-PAGE. The differences observed between the two PAGE results for β-Lg were slight implying that these mixtures did not contain very much β-Lg that was non-covalently aggregated. By contrast, the amount of monomeric BSA in SDS-PAGE were greater than the native-PAGE results indicating that some non-covalently linked BSA aggregates were formed during heating.

However, at 75°C, the initial (zero time) difference between the amounts of monomeric β-Lg in SDS-PAGE and native-PAGE were marked suggesting that during the heating-up period to this temperature β-Lg had aggregated through non-covalent interactions (Figure 5.34B). This difference became less with heating time indicating the loss of the non-covalently bonded aggregates of both β-Lg and BSA.

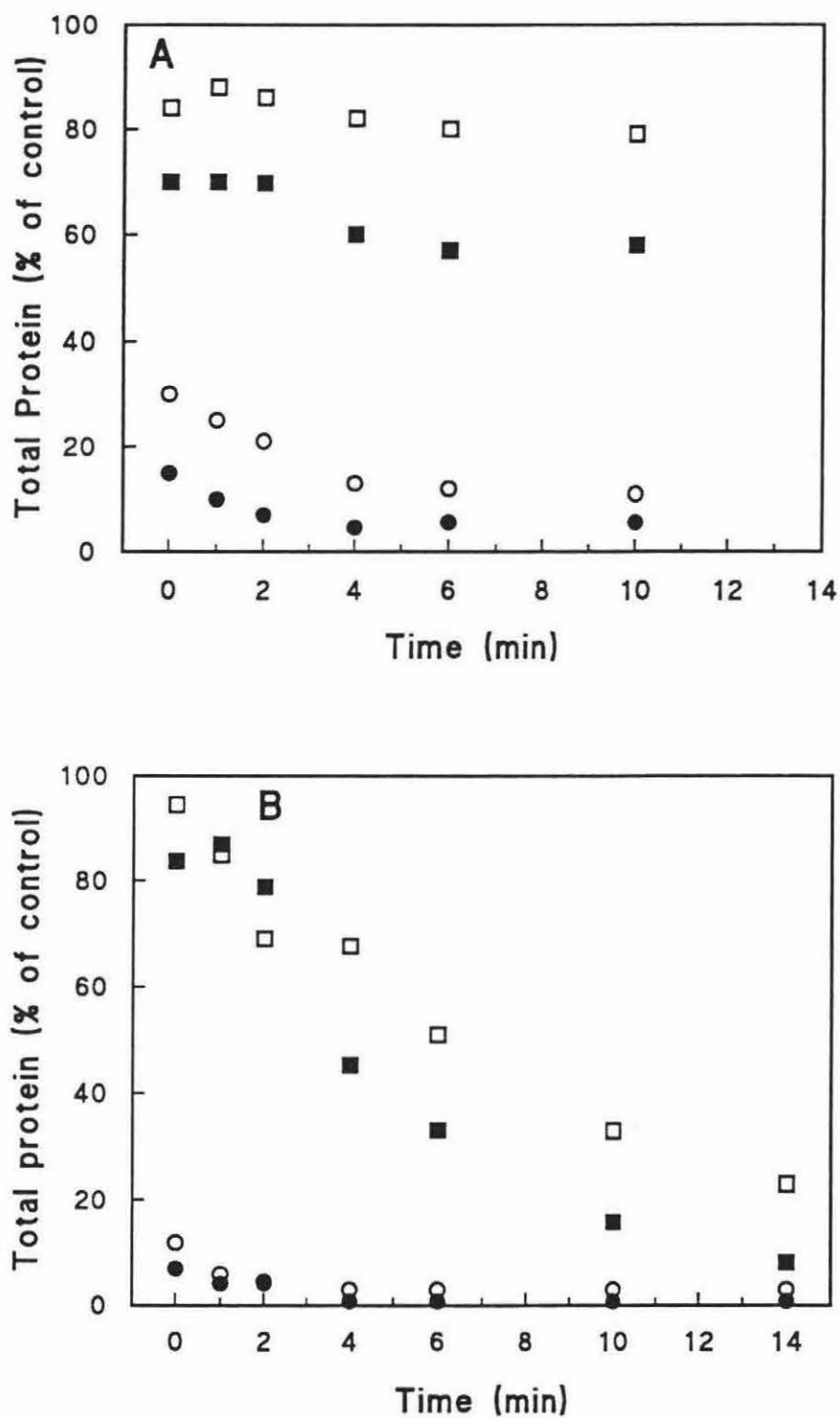**Figure 5.34**   The effect of heating at 70°C (A) and 75°C (B) on the quantity of native β-Lg (■) and native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○) remaining in a mixture containing 5% β-Lg and 5% BSA.

β-Lg/BSA ratio 8:2%

The rate of loss of monomeric protein from the native and SDS-PAGE is shown in Figure 5.35A for the protein mixture containing 8% β-Lg and 2% BSA during heating at 70°C. The loss of monomeric protein from native-PAGE was greater than that using SDS-PAGE. There was a constant difference in the two PAGE results for the β-Lg in the protein solution at 70°C.

At 75°C, there was hardly any significant difference in the loss of residual BSA from native-PAGE and SDS-PAGE, showing that most of the protein aggregates formed were linked *via* disulphide linkages (Figure 5.35B). Native β-Lg decreased at a faster rate than the residual monomeric β-Lg in the mixture.

β-Lg/BSA ratio 2:8%

Figure 5.36A compares the loss of monomeric protein from the native and SDS-PAGE for the protein mixture containing 2% β-Lg and 8% BSA during heating at 70°C. The amounts of monomeric proteins in SDS-PAGE were greater than in native-PAGE.

At 75°C (Figure 5.36B), the difference in the SDS-PAGE and native-PAGE results was less than that observed at 70°C, for both proteins.

From the present results, the difference in the native and SDS-PAGE results was greater at 70°C for BSA than at 75°C indicating there were more non-covalent linked BSA aggregates formed at 70°C. The difference in the PAGE results also appeared to be affected by the composition of the mixtures; the higher the proportion of BSA to β-Lg in the protein mixture the greater was the difference between the PAGE results at 70°C (for BSA) (Figures 5.34A, 5.35A and 5.36A).

Figure 5.35   The effect of heating at 70°C (A) and 75°C (B) on the quantity of native β-Lg (■) and native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (o) remaining in a mixture containing 8% β-Lg and 2% BSA.
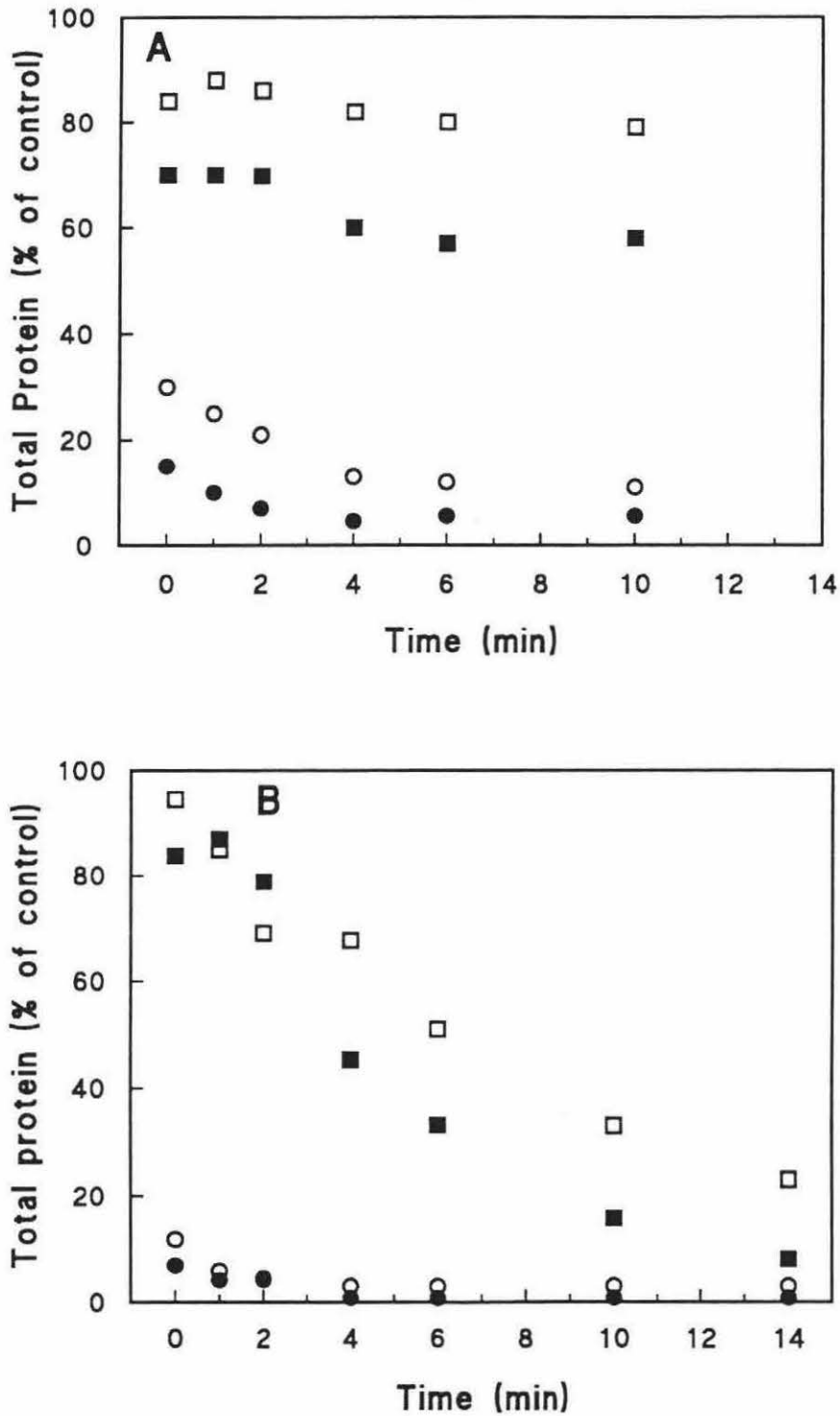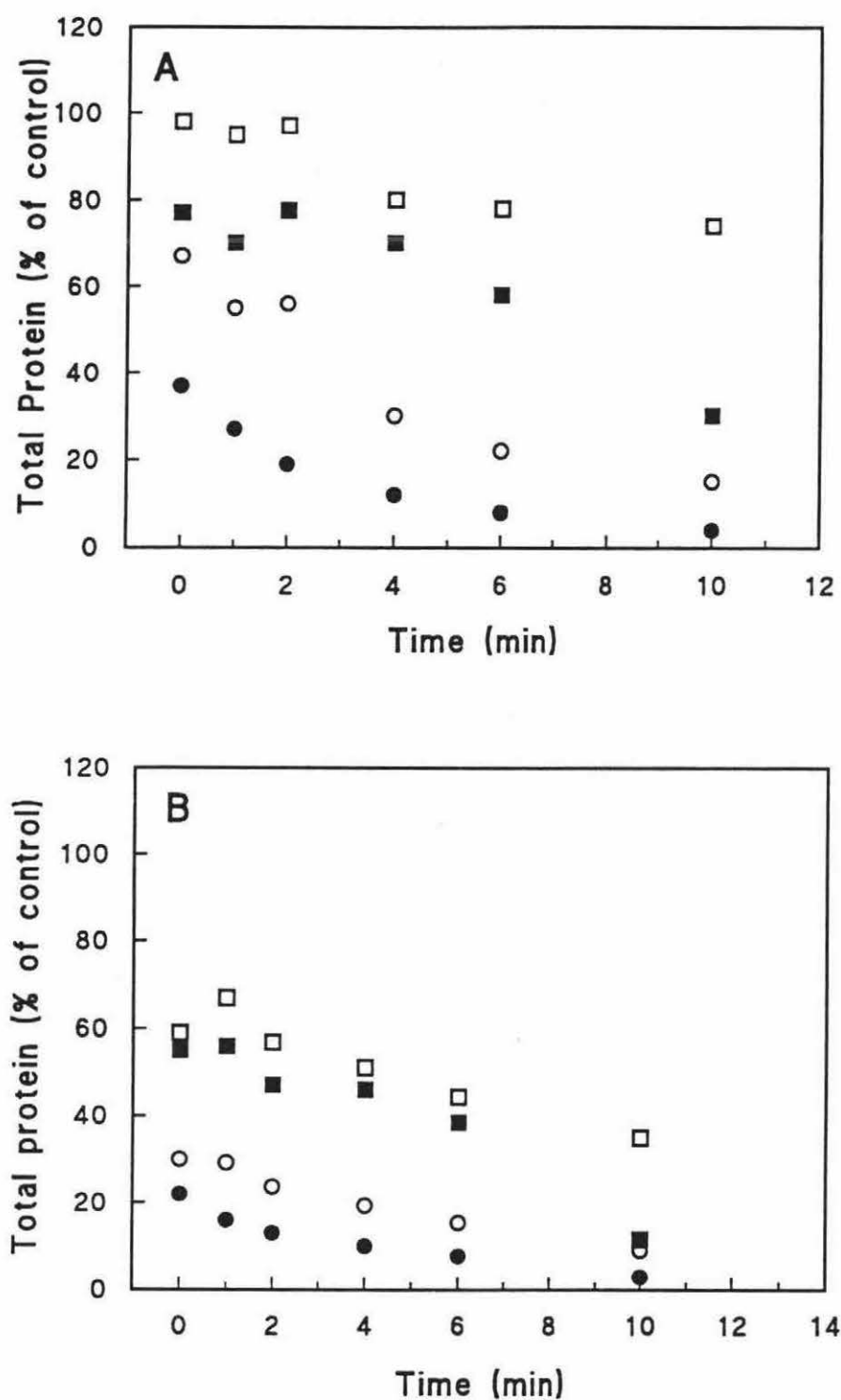
Figure 5.36   The effect of heating at 70°C (A) and 75°C (B) on the quantity of native β-Lg (■) and native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○) remaining in a mixture containing 2% β-Lg and 8% BSA.

From the results presented it appears that β-Lg and BSA are behaving independently of each other during heating. Heating at 70°C resulted only in BSA aggregates being formed for most of the protein mixtures except for the protein solution containing 2% β-Lg and 8% BSA. At this protein mixture BSA appears to have an effect on β-Lg. When the heating temperature was increased to 75°C, both proteins formed aggregates during heating of the protein mixtures.

Denaturation/aggregation rates of protein solutions containing β-Lg and BSA have been studied earlier (Hines and Foegeding, 1993; Matsudomi *et al.* 1994) but these studies have been concerned with denaturation *per se* in dilute solutions rather than with denaturation as a part of the gelation process. In the present studies, the denaturation/aggregation behaviour of proteins was determined under conditions identical to those used during gelation studies.

Hines and Foegeding, (1993) reported that the aggregtion rate of β-Lg was slightly influenced by the presence of BSA in the mixture although the aggregation of BSA was not affected by β-Lg during heating at 80°C. In contrast, in the present study, the aggregation rate of BSA was increased by the presence of β-Lg whereas the aggregation of β-Lg was not affected during heating at 70 and 75°C.

During heating at 75°C, aggregates of both β-Lg and BSA were formed; initially these aggregates were non-covalently linked and became disulphide linked with increased heating. However, it is not clear from the present results whether soluble aggregates were formed between β-Lg and BSA, although it appears that two structural gel matrices were formed during heating of protein solutions containing equal amounts of β-Lg and BSA during heating at 75°C (Figure 5.37). Matsudomi *et al.* (1994) using HPLC observed that β-Lg appeared to interacted with BSA to form soluble aggregates during heating of dilute protein solutions at 80°C, and these

aggregates later polymerised to form a structural gel matrix. Using NEM, they also observed that the soluble aggregates were formed through disulphide interactions during heating.

The present results could not confirm the reports by these workers due to variations in the methods used, the gelation temperatures employed and the protein concentrations used.

5.4.1.2 *Relationship between G' and PAGE results*

The relationships between the loss of β-Lg and BSA from native-PAGE and SDS-PAGE and the changes in G' during heating at 70 and at 75°C for three of the different protein mixtures are shown in Figures 5.37 - 5.39 and in Figures 2.1 - 2.3 (*Appendix*).

β-Lg / BSA ratio 5:5%

Figure 5.37A shows the relationship between the PAGE results and the development of G' during heating at 70°C for a protein mixture containing 5% β-Lg and 5% BSA. Most of the BSA molecules had already aggregated (native-PAGE) and become disulphide bonded (SDS-PAGE) before any detectable increase in G' was observed (25 min). About 20% of the β-Lg in the protein mixture had aggregated through disulphide bonding during the heating period.

When the protein mixture was heated at 75°C, all the BSA molecules and ~80% of the β-Lg had aggregated through non-covalent and/or disulphide bonding during the heat-up period (Figure 5.37B). G' did not begin to increase until all the BSA and ~75% of the β-Lg had aggregated *via* disulphide bonding.
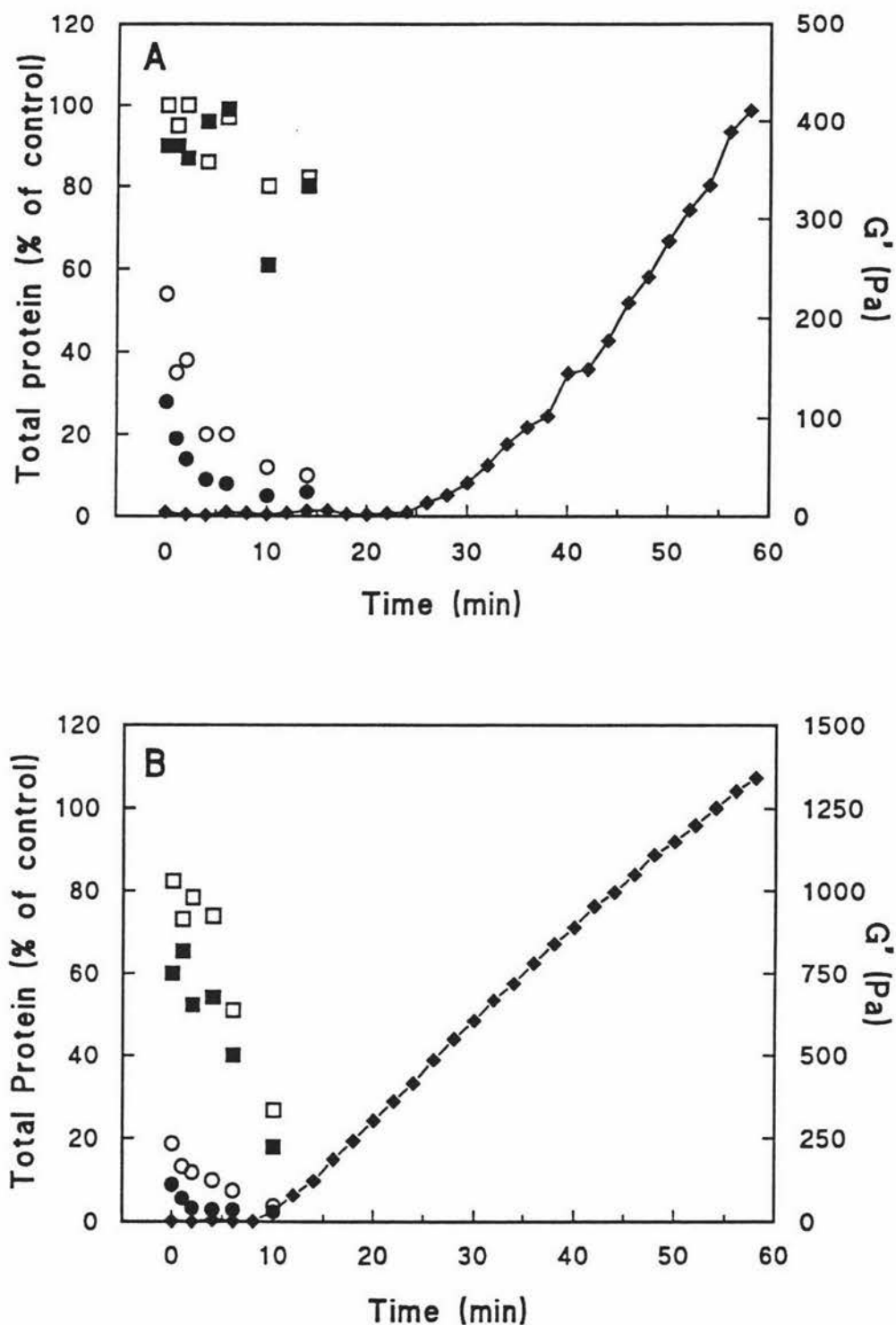
Figure 5.37　　The effect of heating a mixture of 5% β-Lg and 5% BSA at 70°C (A) and 75°C (B) on G' (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (o).

β-Lg / BSA ratio 8:2%

When a protein solution containing 8% β-Lg and 2% BSA was heated at 70°C, ~ 55% of native β-Lg and ~ 95% of the BSA had aggregated (native-PAGE) by either non-covalent interactions and/or disulphide bonding and ~ 20% β-Lg and ~ 90% BSA was cross-linked via disulphide bond formation during the heat-up period (Figure 5.38A). However, G′ of the mixture began to rise after about 45 min. This may have been due to the fact that a significant amount of β-Lg was required to unfold in order to participate in gel network formation.

At 75°C (Figure 5.38B), most of the β-Lg and BSA had aggregated by non-covalent interactions and disulphide bonding before there was any detectable increase in G′. About 80% of the β-Lg and all of the BSA had aggregated by disulphide linkages before G′ began to increase (21 min).

β-Lg / BSA ratio 2:8%

Figure 5.39A shows that ~95% BSA and ~70% β-Lg had already aggregated (native-PAGE) prior to G′ development during heating of a protein mixture containing 2% β-Lg and 8% BSA at 70°C. About 90% BSA and ~ 30% β-Lg was cross-linked *via* disulphide bonding. G′ began to rise when most of the BSA had aggregated through disulphide linkages.

When the same protein mixture was heated at 75°C (Figure 5.39B), G′ began to rise when almost all the BSA and ~60% β-Lg had aggregated.

Figure 5.38   The effect of heating a mixture of 8% β-Lg and 2% BSA
at 70°C (A) and 75°C (B) on G′ (♦) and quantities of
native β-Lg (■), native BSA (•), residual monomeric
β-Lg (□) and residual monomeric BSA (○).

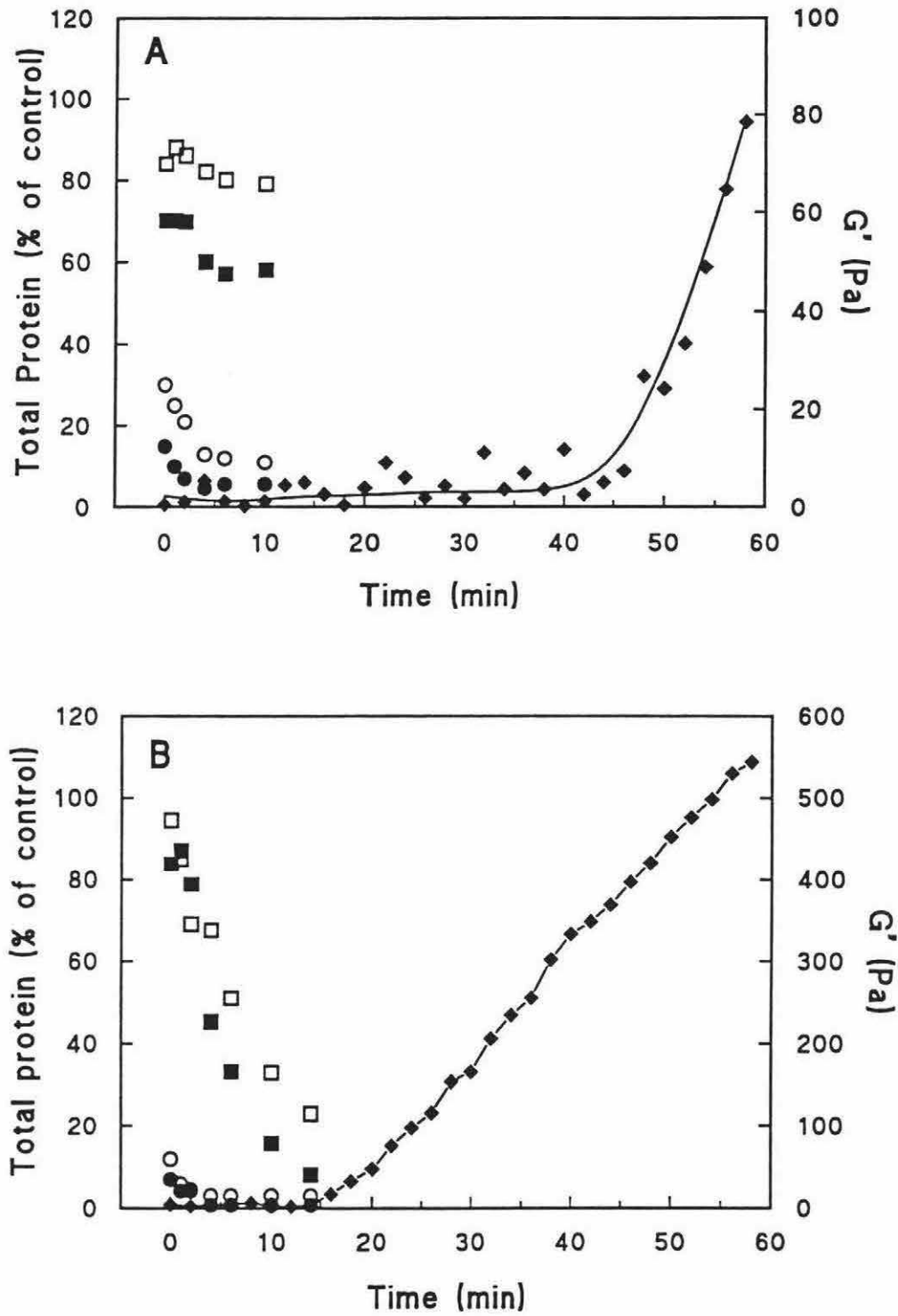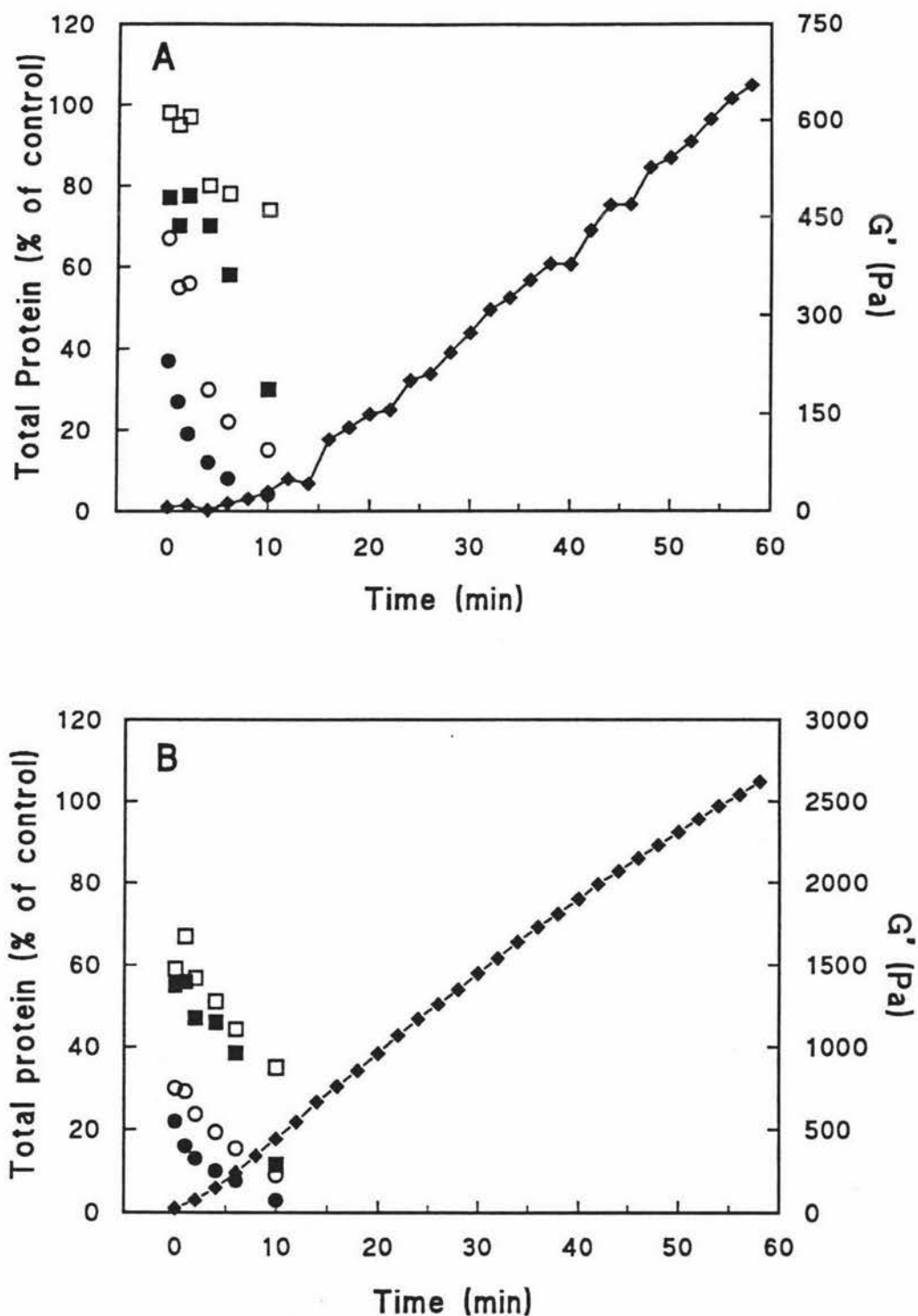**Figure 5.39**   The effect of heating a mixture of 2% β-Lg and 8% BSA at 70°C (A) and 75°C (B) on G' (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○).

These results suggest that the type of aggregation responsible for the increase in G' values was not the same as that associated with the loss of protein from PAGE. This supports the view that some protein aggregates are formed prior to the development of the self supporting gels. During heating of protein mixtures containing a relatively higher proportion or BSA to β-Lg in the mixture at 70°C, G' began to increase when almost all of the BSA had aggregated while in protein mixtures of predominantly β-Lg, there was a delay in the rise in G'.

5.4.1.3   *Possible gelation mechanism for β-Lg / BSA mixtures*

The molecular events which occur during gelation of pure β-Lg solutions have been described in section 5.2.5.1. On heating, β-Lg molecules undergo conformational changes which result in exposure of buried hydrophobic and sulphydryl groups. Once exposed, these groups interact to form soluble aggregates between the protein molecules initially through hydrophobic interactions then followed by disulphide bonding with increased severity of heating. Once these soluble aggregates or homo-polymers reach a certain critical concentration, form a gel matrix forms.

Limited rheological and electrophoretic data on BSA solutions suggest the same events may be occurring during thermal gelation of BSA solutions (Section 5.2.5.1). Soluble aggregates/homo-polymers have been observed during heating of BSA solutions (Figure 5.13; Matsudomi *et al.* 1993).

When a mixture of the two proteins (i.e β-Lg and BSA) is heated at 70°C, BSA aggregates are formed through hydrophobic and disulphide bonds. Under these conditions it is possible that some conformational changes in the β-Lg could result in the formation of aggregates linked through hydrophobic interactions. This is apparent in the electrophoretic data (Figures 5.34A, 5.35A, 5.36A) indicating small loss of native β-Lg.

However, this loss could also result from entrapment of native β-Lg molecules in the BSA aggregates. The rate of aggregation of BSA seemed to be enhanced by the presence of β-Lg in the mixture (Figure 5.27). This could either be attributed to the increase in effective concentration of BSA in the mixture due to the presence of β-Lg or some kind of interaction between the two proteins. Electrophoresis data from the present study however, does not provide any evidence of formation of β-Lg and BSA heteropolymers.

In the mixtures heated for 60 min at 70°C, G′ appeared to increase with the proportion of BSA in the mixture suggesting that gel network predominantly consists of BSA aggregates. Under these conditions, the role of β-Lg in this mixture is probably to act as a "filler" in the spaces between the BSA aggregates. These would result in greater G′ values for the mixtures than BSA heated alone at the same concentrations. The possibility of some unfolded β-Lg molecules or aggregates of β-Lg interacting with the gel matrix possibly *via* either hydrophobic or disulphide bonding can not be ruled out. A schematic presentation of the events occurring at the molecular level may be depicted as follows:

heat → heat →

* BSA
* β-Lg   Aggregated proteins   BSA gel network
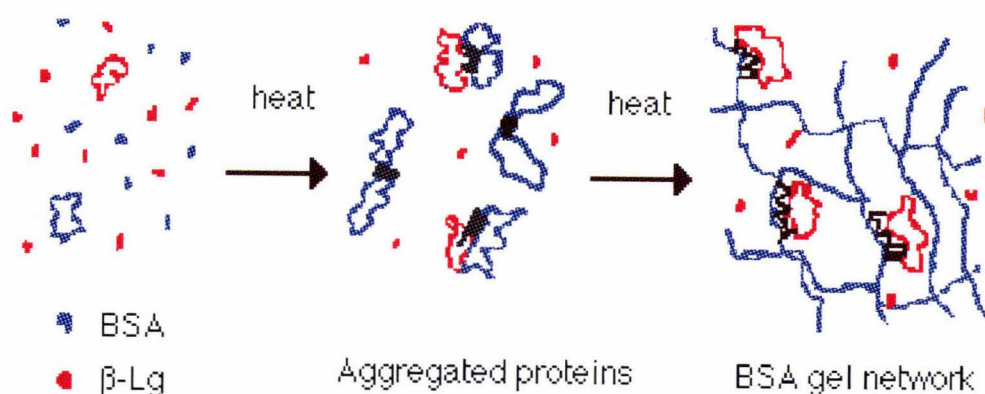
At 75°C, BSA molecules aggregated more rapidly with almost all the BSA molecules having aggregated before the temperature reached 75°C (under the conditions used in this study, i.e, heating rate of 1°C/min). β-Lg molecules began to aggregate after the temperature reached 75°C and its aggregation rate was not significant affected by the presence of BSA molecules/aggregates. Again there has been no evidence that β-Lg and BSA aggregates interact to form hetero-polymers.

Rheological characteristics (G′) of the gels were dependent on the concentration of β-Lg or BSA; there appeared to be two clear regions. For protein mixtures containing more than 5% β-Lg, gelling times were greater than 15 min and G′ after heating was less than 800 Pa (Figure 5.40) while protein mixtures containing more than 5% BSA the gelling times were less than 10 min and G′ after 60 min of heating was greater than 1200 Pa. If β-Lg and BSA each formed extensive homo-polymer networks then it would be expected that at high β-Lg ratios the overall gelling behaviour would be similar to that of pure β-Lg while at high BSA ratios the overall behaviour would be similar to that of pure BSA. Thus if the conditions favoured BSA gel formation but not β-Lg gel formation then the mixtures would be expected to gel in a fashion dictated by the level of BSA present, i.e, a 5:5 mixture would gel as though it were 5% BSA because the β-Lg would make no contribution. This appears to be apparent in the behaviour shown in Figure 5.40. Thus, it can be concluded that at high β-Lg concentrations the gels are predominantly β-Lg gels with possible entrapment/attachment of the BSA aggregates within the network when the BSA concentration is below the critical concentration for gel formation. As the relative concentration of the BSA in the mixture is increased there is a "net-work inversion" in which the BSA becomes the predominant gelling protein and the roles are reversed.
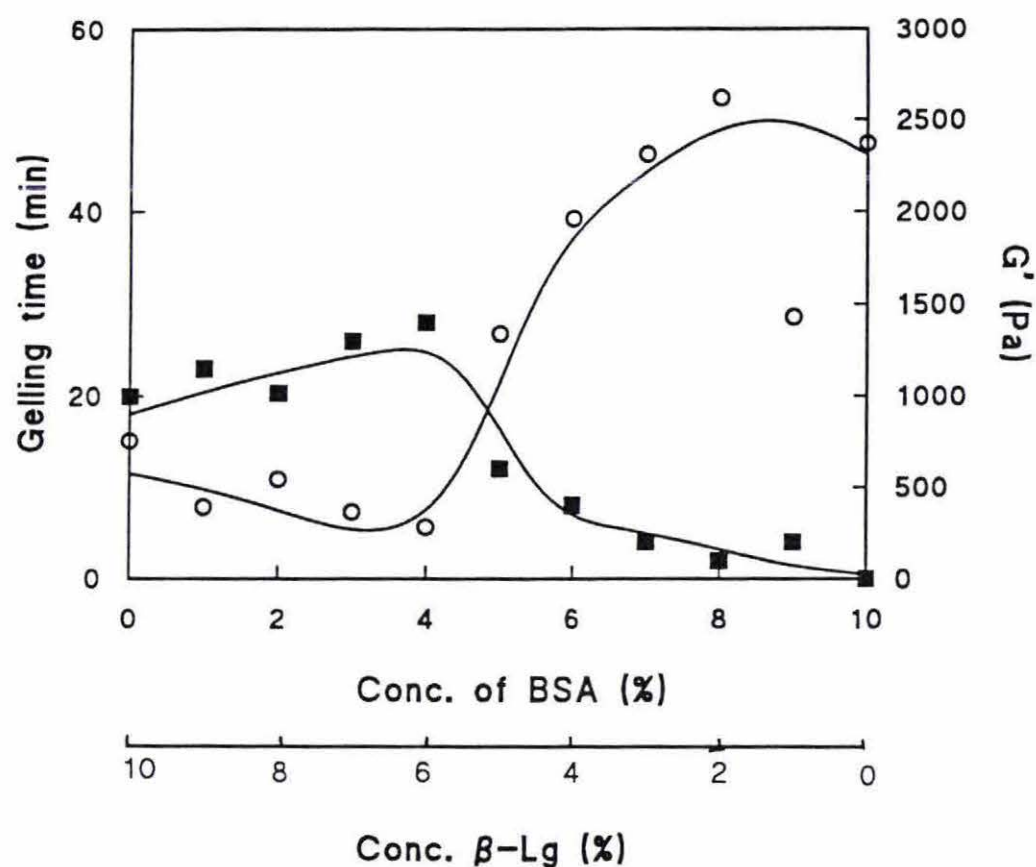
Figure 5.40 The effect of protein composition in mixtures of β-Lg and BSA on the gelation time (■) and G' (○) after heating at 75°C.

At equal protein concentration in the mixture, it is possible that two intertwined gel networks are formed with possible links between the network strands by either hydrophobic interactions or disulphide bonding. A schematic presentation of molecular events occuring during heating of a protein mixture containing equal quantities of β-Lg and BSA at 75°C is shown below:

Aggregated proteins

BSA and β-Lg gel networks
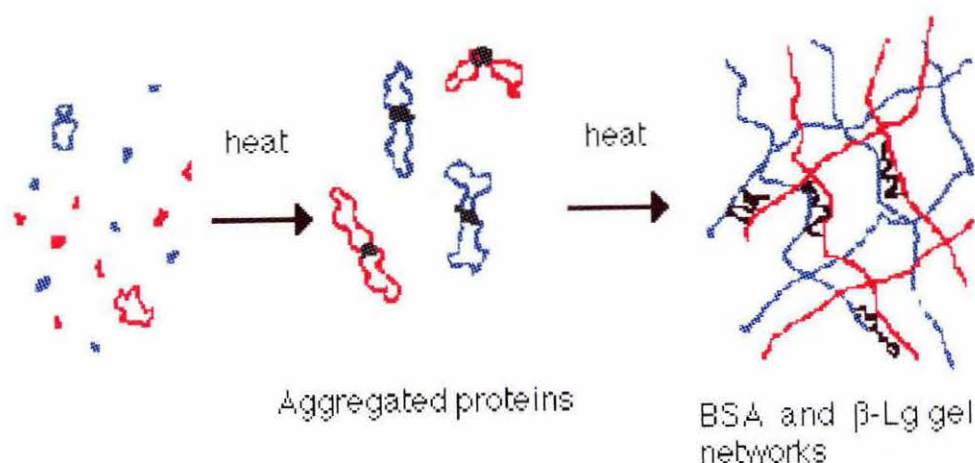
The gelation temperatures of the protein mixtures containing β-Lg and BSA was highly dependent upon the ratios of the two proteins (Figure 5.1). This conforms with the proposed gelation mechanism.

5.4.2 β-Lg/α-La mixtures

Native-PAGE

Figure 5.41 shows a typical gel electrophoretic pattern obtained under non-dissociating conditions during heating of a protein solution containing 5% β-Lg and 5% α-La. Lanes 1 and 2 show the unheated protein solution. It is apparent that the quantities of the native β-Lg and α-La decreased with heating times (Lanes 3 to 6) at 80°C. All heated samples showed some protein material which failed to migrate into the stacking and resolving gels. This material probably contained high molecular weight protein aggregates cross-linked via non-covalent interactions and disulphide bonds (McSwiney *et al*. 1994a,b).

The intensity of the stained protein bands, determined by densitometry, revealed the amount of protein present at each heating time. No samples for PAGE could be taken from some of the protein solutions containing β-Lg and α-La after heating at 80°C since the protein solution quickly became too viscous for sampling (Table 3.2. *Appendix*). The results obtained at 75°C will be used together with the few results obtained at 80°C.

β-Lg / α-La ratio 5:5%

A protein solution containing 5% β-Lg and 5% α-La was heated at 75°C and the amounts of native β-Lg and α-La present at different heating times determined by native-PAGE (Figure 5.42A). The amounts of native β-Lg and α-La decreased with increased heating time; the loss of native α-La was faster than that of β-Lg from the protein mixture. About 78% native β-Lg and 84% native α-La was lost during the heat-up time i.e, when the protein mixture was heated from 25 to 75°C at a heating rate of 1°C/min.
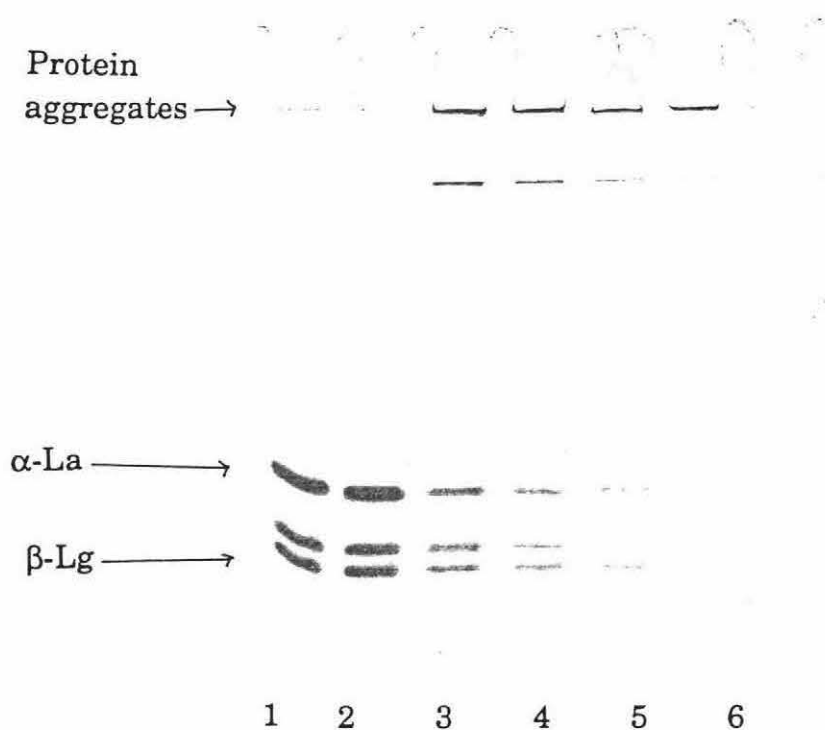
Figure 5.41   A typical native gel showing the effect of heating at 80°C on the amount of native β-Lg and α-La remaining.   Lanes 1 - 6 are: unheated sample (duplicate), zero time sample, 1 min sample, 2 min sample and 4 min sample.
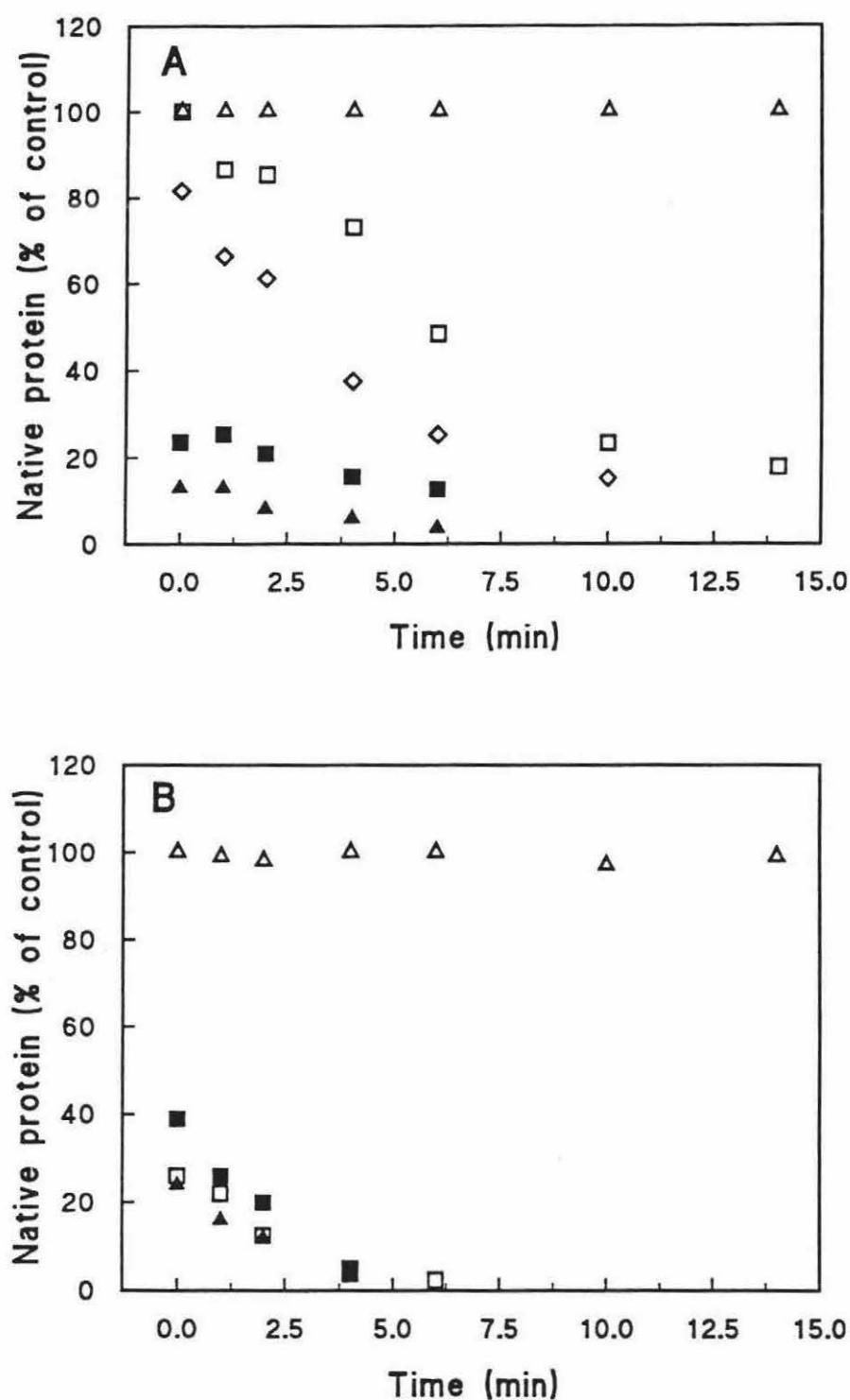
Figure 5.42   The effect of heating time at 75°C (A) and 80°C (B) on the loss of native β-Lg (◊, □, ■) and native α-La (△, ▲) from a 10% β-Lg solution (◊), a 5% β-Lg solution (□) an 8% α-La solution (△) and a protein solution containing 5% β-Lg (■) and 5% α-La (▲).

When 8% α-La solution was heated under the same conditions, there was no apparent loss of the native protein throughout the heating period (Figure 5.42A). The loss of native β-Lg from the mixture was much faster than when 5 or 10% β-Lg solutions were heated.

Similar observations were made when the same protein solution was heated at 80°C (Figure 5.42B). The quantities of native β-Lg and α-La from the mixture decreased with increased heating time. However, the rate of loss of both native proteins from the mixture was comparable to that of 5% β-Lg alone.

β-Lg / α-La ratio 8:2%

When a protein mixture containing 8% β-Lg and 2% α-La was heated at 75°C (Figure 5.43), the amounts of native β-Lg and α-La decreased to 20% of the original during the first 6 min of heating. The loss of native β-Lg from the protein mixture was similar to that of 10% and 8% β-Lg heated individually while the loss of α-La was much faster than when α-La was heated alone.

When the experiment was repeated at 80°C, the mixed protein solution gelled before the temperature reached 80°C (Figure 5.23A) and thus no samples were collected.

β-Lg / α-La ratio 2:8%

When a protein solution containing 2% β-Lg and 8% α-La was heated at 75°C, the amounts of both native β-Lg and α-La from the mixture decreased to 20% of the initial quantity in 14 minutes (Figure 5.44A). The rates of decrease for β-Lg and α-La were similar. The loss of β-Lg and α-La from the mixture was greater than that of 8% α-La and similar to 5% β-Lg heated alone, but less than 10% β-Lg heated alone.
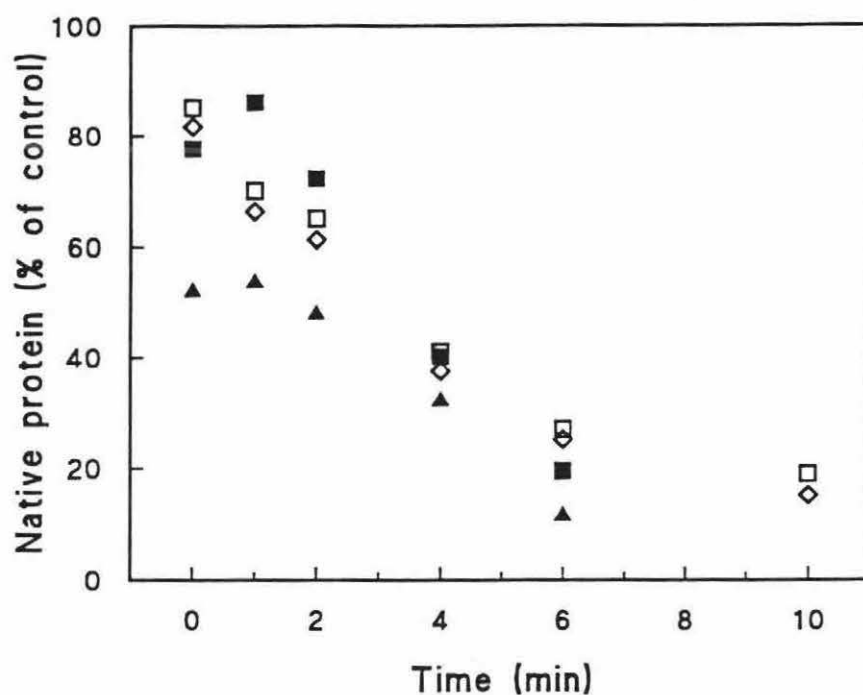
Figure 5.43   The effect of heating time at 75°C on the loss of native β-Lg (◊, □, ■) and native α-La (▲) from a 10% β-Lg solution (◊), a 8% β-Lg solution (□) and a protein solution containing 8% β-Lg (■) and 2% α-La (▲).

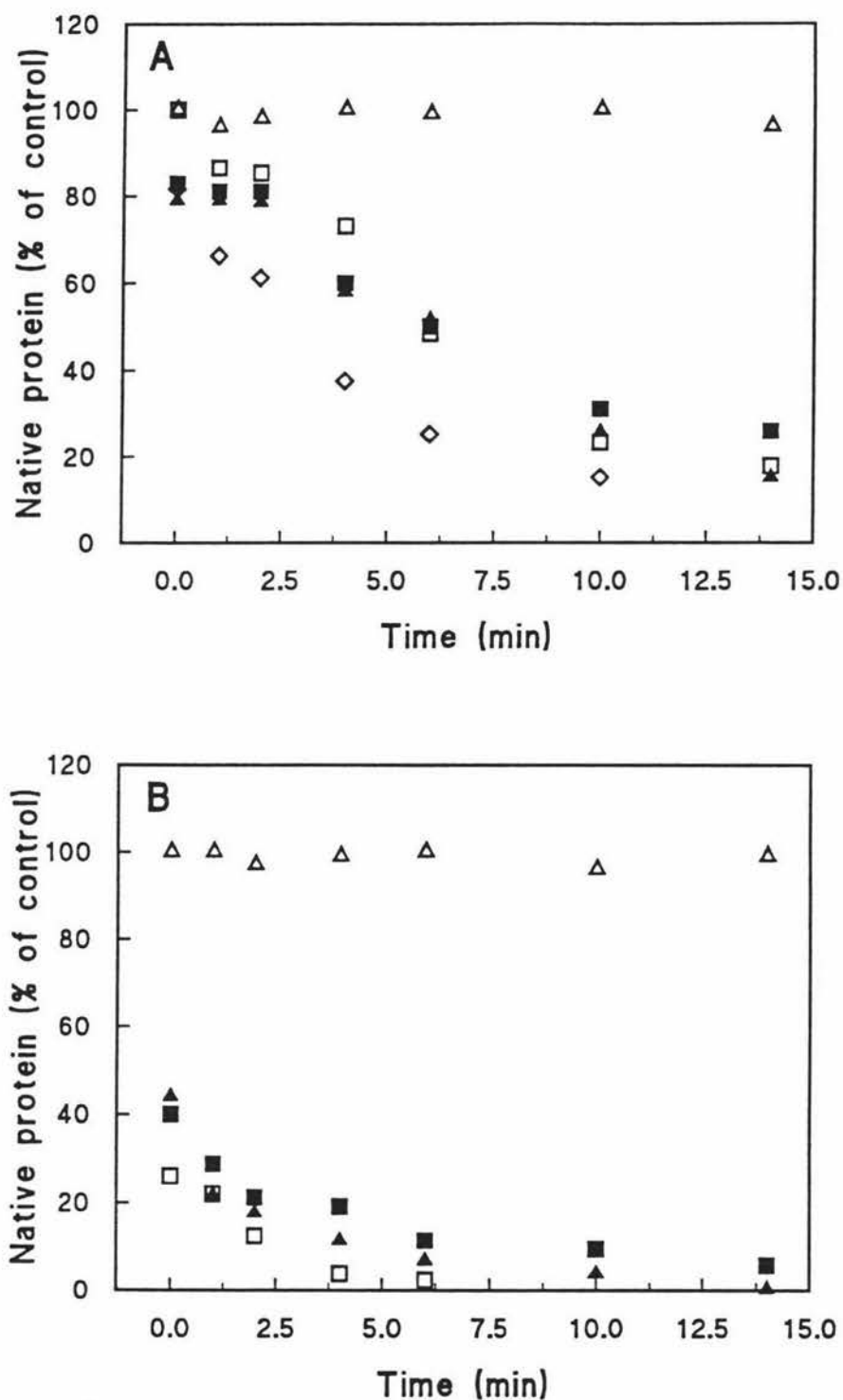**Figure 5.44**   The effect of heating time at 75°C (A) and 80°C (B) on the loss of native β-Lg (◊, □, ■) and native α-La (△, ▲) from a 10% β-Lg solution (◊), a 5% β-Lg solution (□), a 8% α-La solution (△) and a protein solution containing 2% β-Lg (■) and 8% α-La (▲).

Similar trends were obseved when the same protein solution was heated at 80°C, except that the loss of both β-Lg and α-La in the mixture was faster than at 75°C (Figure 5.44B). The loss of native β-Lg and α-La from the mixture was slower than the loss from 5% β-Lg solution heated alone.

SDS-PAGE

Figure 5.45 shows a typical gel electrophoretic pattern obtained when a protein mixture containing 2% β-Lg and 8% α-La was heated at 80°C and dispersed in an SDS buffer. Lanes 1 and 2 show the unheated β-Lg and α-La solution. The intensity of the protein bands gradually decreased with heating time (Lanes 3 to 9). Some high molecular weight protein aggregates which failed to enter the stacking and resolving gels were observed for some of the heated protein samples; some "intermediate" protein bands were also observed for most of the heated protein samples. The protein bands were scanned by densitometer to obtain the quantities of residual monomeric β-Lg and α-La.

β-Lg/α-La ratio 5:5%

When a protein solution containing 5% β-Lg and 5% α-La was heated from 25°C to 75°C and held for 60 min, the amounts of residual monomeric β-Lg and α-La gradually decreased with heating time and α-La decreased faster than β-Lg from the protein mixture (Figure 5.46A). About 55% of the total β-Lg and 70% of the total α-La was lost during the heat-up time which may indicate that some of the protein molecules had already aggregated *via* covalent bonding during the heating up period. Residual monomeric β-Lg from the protein mixture decreased faster than from the 5% β-Lg solution.

Protein aggregates →

β-Lg ⟶

α-La ⟶

1 2   3   4   5   6   7   8 9
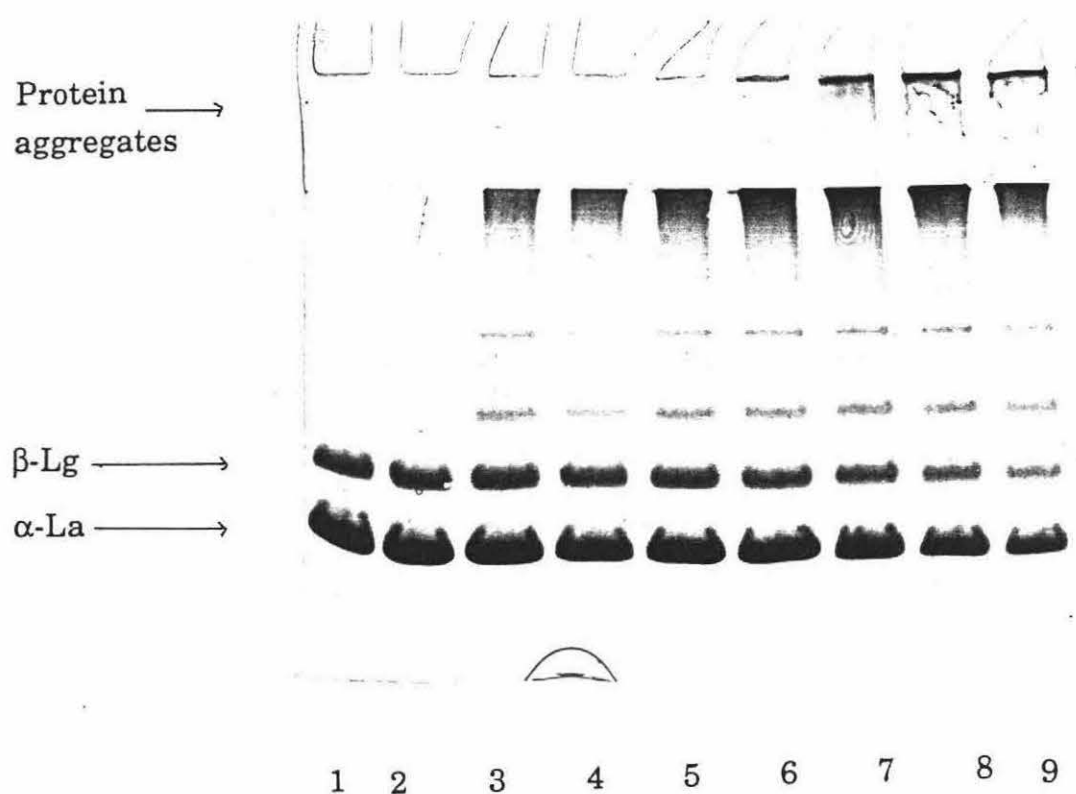
Figure 5.45   A typical SDS gel showing the effect of heating at 80°C on the residual monomeric β-Lg and α-La remaining. Lanes 1 - 9 are: unheated sample (duplicate), zero time sample, 1 min sample, 2 min sample, 4 min sample, 6 min sample, 10 min sample and 14 min sample.
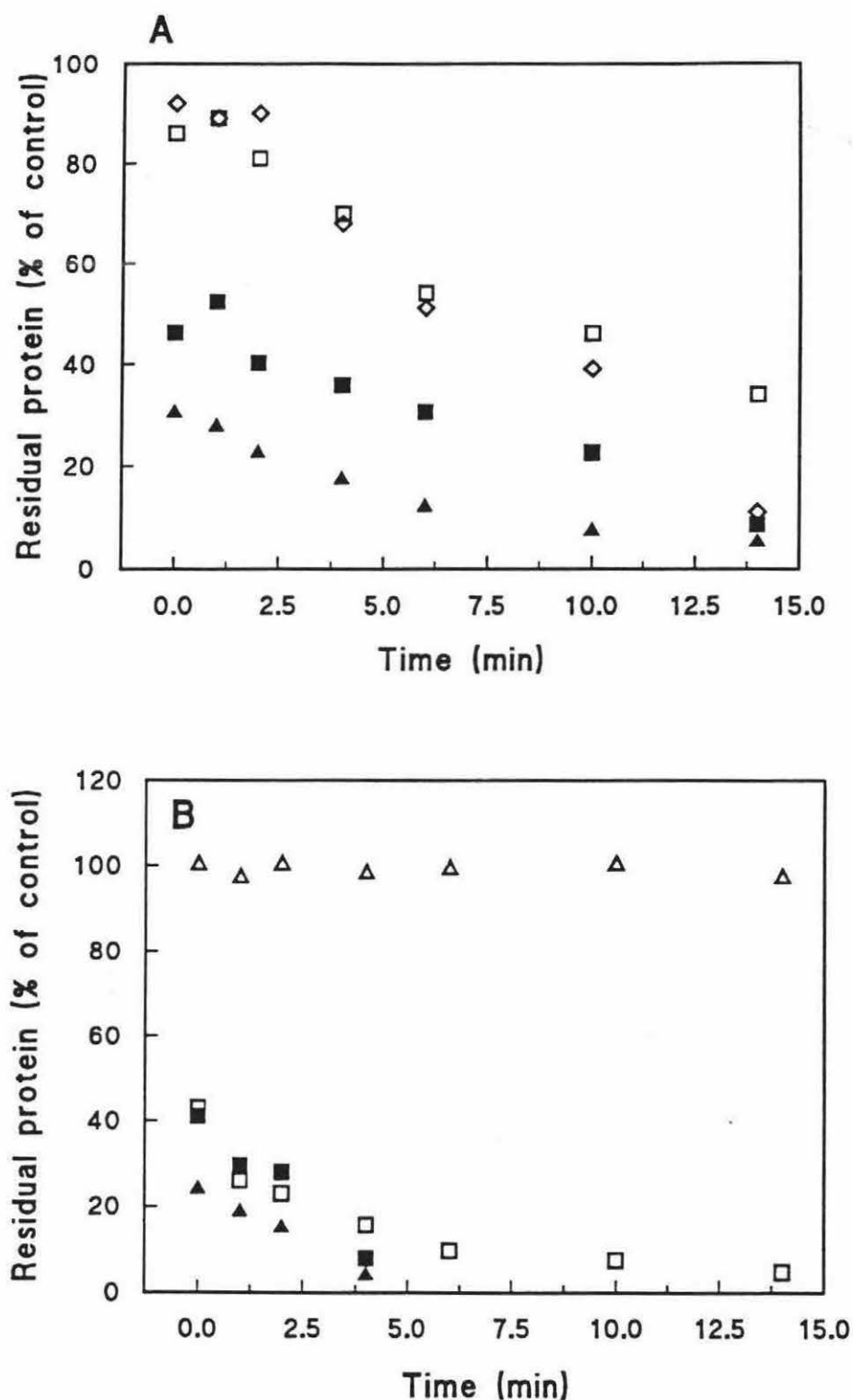
Figure 5.46   The effect of heating time at 75°C (A) and 80°C (B) on the loss of residual monomeric β-Lg (◊, □, ■) and residual monomeric α-La (△, ▲) from a 10% β-Lg solution (◊), a 5% β-Lg solution (□), an 8% α-La solution (△) and a solution containing 5% β-Lg (■) and 5% α-La (▲).

When the same protein mixture was heated at 80°C (Figure 5.46B), the amount of protein present at zero time was less than at 75°C. The loss of residual monomeric β-Lg and α-La seemed more similar to one another than at 75°C.

Protein aggregates which failed to enter the stacking and resolving gels on SDS-PAGE during heating of the protein solution at 80°C, were isolated and suspended in a sample buffer containing mercaptoethanol (ME) which cleaves the disulphide linked polymers. The aggregates dissociated into monomers of β-Lg and α-La on analysis with SDS-PAGE, indicating that these aggregates had polymerised by thiol-disulphide bonding during heating. Figure 5.47 shows the unheated samples (Lanes 1 and 2). Lane 3 shows the unreduced heated sample with the large aggregates which failed to enter the gel. Lanes 4 to 6 shows the reduced aggregates which were resolved into β-Lg and α-La monomers under reducing conditions with no evidence for the presence of any aggregates.

β-Lg / α-La ratio 8:2%

When a protein solution containing 8% β-Lg and 2% α-La was heated at 75°C (Figure 5.48), a similar trend was observed to that obtained when a protein solution containing 5% β-Lg and 5% α-La was heated at 75°C. There was no significant change in the amount of residual monomeric α-La after heating for 14 min, implying that no covalently bonded protein aggregates were formed during heating.

No samples were collected when this protein solution was heated at 80°C as the solution had gelled by the time 80°C was attained.
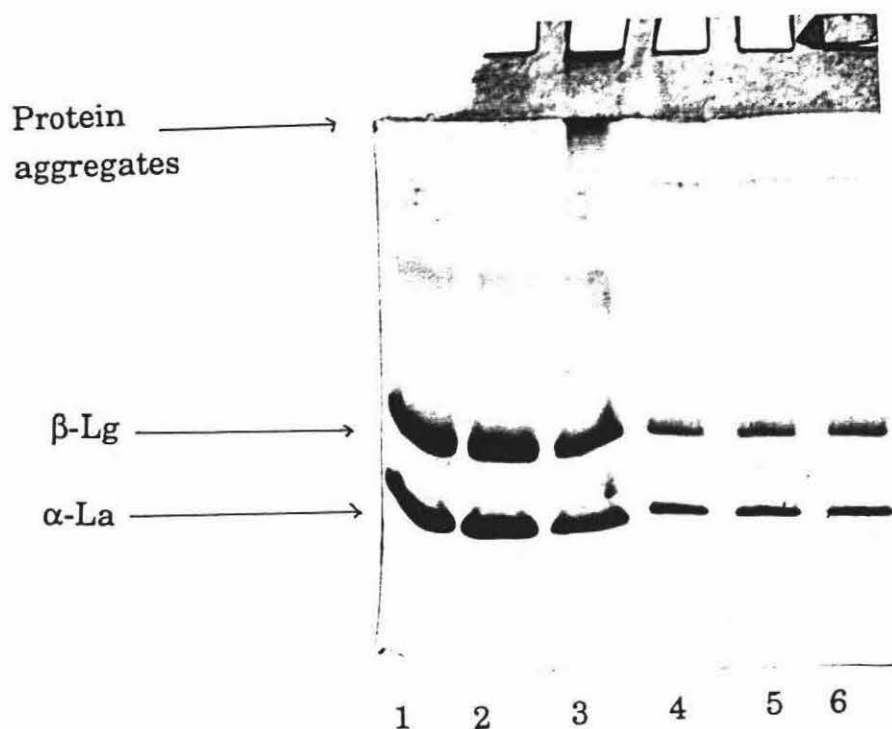
Figure 5.47   The effect of mercaptoethanol on the isolated large protein aggregates formed during heating a protein solution containing 5% β-Lg and 5% α-La. Lanes 1 and 2 show the unheated protein solution; Lane 3 shows the heated but unreduced protein solution with the large aggregates which failed to migrate the gel. Lane 4 to 6 shows the solubilised protein aggregates which had dissociated into β-Lg and α-La monomers.

Figure 5.48   The effect of heating time at 75°C on the loss of residual monomeric β-Lg (◊, □, ■) and residual monomeric α-La (△, ▲) from a 10% β-Lg solution (◊), an 8% β-Lg solution (□), an 8% α-La solution (△) and a solution containing 8% β-Lg (■) and 2% α-La (▲).

β-Lg / α-La ratio 2:8%

When a protein solution containing 2% β-Lg and 8% α-La was heated at 75°C, there was a gradual decrease in the amount of residual β-Lg and α-La from the mixture as the heating time was increased (Figure 5.49A). No change was observed when α-La was heated under the same conditions.

When the same protein mixture was heated at 80°C (Figure 5.49B), the extent of decrease of residual monomeric β-Lg and α-La with heating time was greater than at 75°C. SDS-PAGE patterns from which these results were obtained is shown in Figure 5.45.

5.4.2.1 Comparison of native and SDS-PAGE results

The depletion of native β-Lg and α-La from the gelling solutions was found to be faster than the loss of the residual monomeric proteins (Figures 5.50 to 5.52). The difference in depletion rates indicates an accumulation of hydrophobic aggregates that can be dispersed to monomers in SDS solution, as postulated by McSwiney *et al.* (1994a,b)

β-Lg / α-La ratio 5:5%

The depletion of β-Lg and α-La from solutions containing 5% β-Lg and 5% α-La is shown in Figure 5.50. The difference in depletion rates is greater at 75°C (Figure 5.50A) than at 80°C (Figure 5.50B) suggesting that the relative rate of the thiol interchange reaction compared with hydrophobic aggregation is faster at 80°C.

β-Lg / α-La ratio 8:2%

There were also differences between the native and SDS-PAGE results when a protein solution containing 8% β-Lg and 2% α-La was heated at 75°C (Figure 5.51). This result, and the higher β-Lg monomer concentrations at 2 and 4 min, indicate that much less of the protein is present as hydrophobic aggregates.
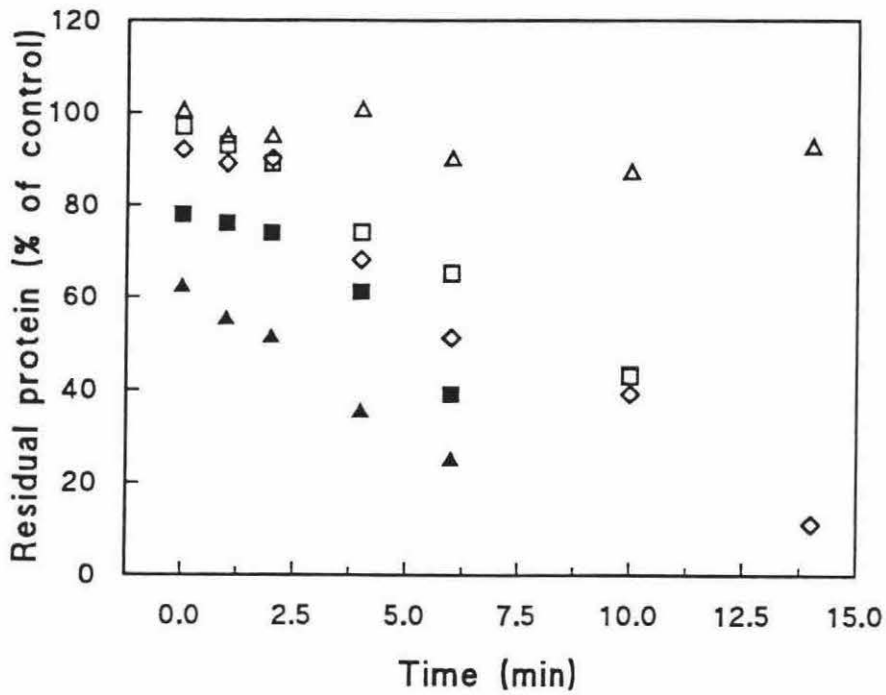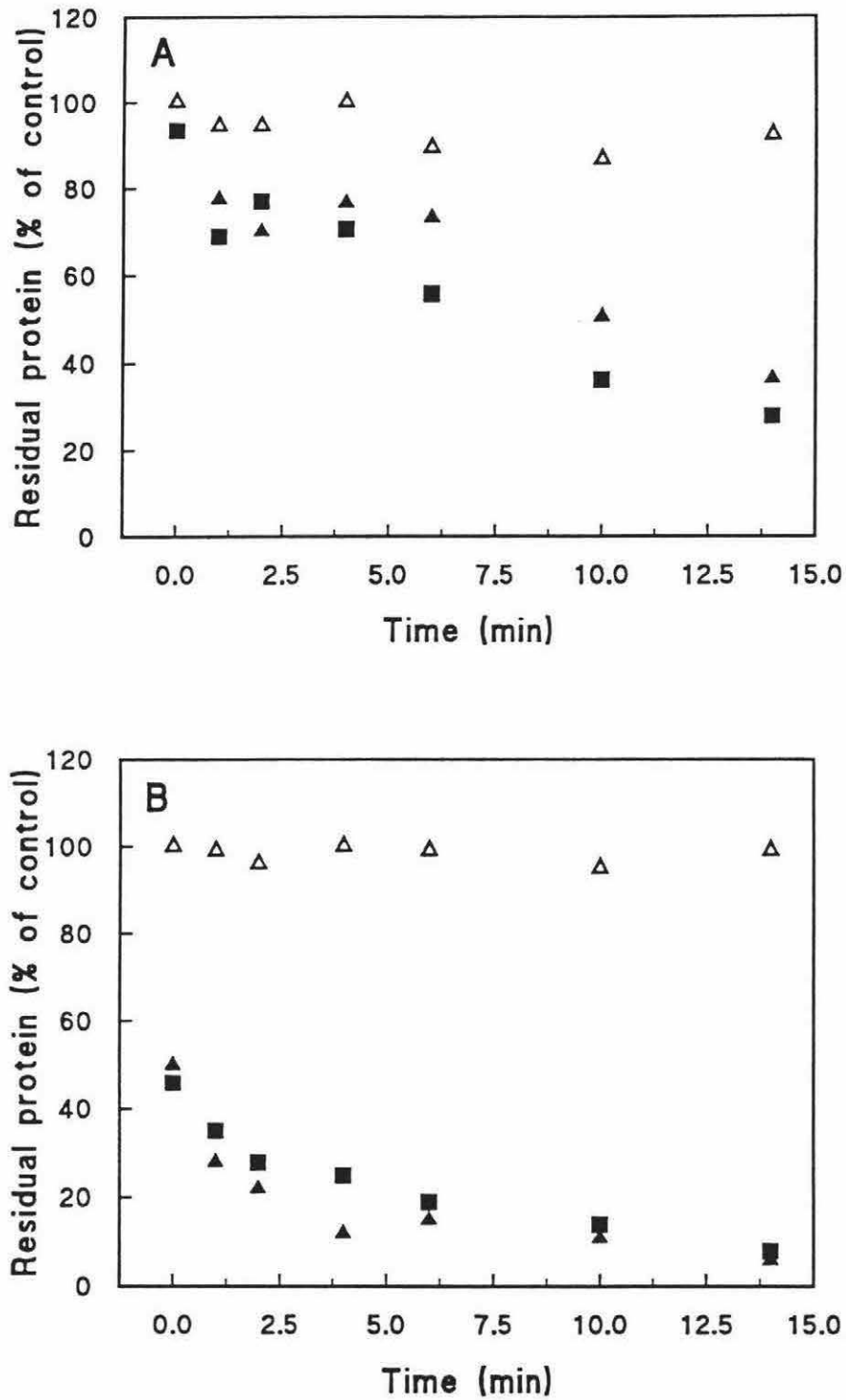
Figure 5.49   The effect of heating time at 75°C (A) and 80°C (B) on
the loss of residual monomeric β-Lg (■) and residual
monomeric α-La (△, ▲) from an 8% α-La solution (△)
and a solution containing 2% β-Lg (■) and 8% α-La (▲).

Figure 5.50   The effect of heating at 75°C (A) and 80°C (B) on the quantity of native β-Lg (■) and native α-La (▲), residual monomeric β-Lg (□) and residual monomeric α-La (△) remaining in a mixture containing 5% β-Lg and 5% α-La.

Figure 5.51   The effect of heating at 75°C on the quantity of native β-Lg (■) and native α-La (▲), residual monomeric β-Lg (□) and residual monomeric α-La (△) remaining in a mixture containing 8% β-Lg and 2% α-La.

The quantities of α-La on native-PAGE and SDS-PAGE were very close indicating the prevalence of disulphide bonding.

β-Lg / α-La ratio 2:8%

Figure 5.52A shows the comparative loss of monomeric proteins from native- and SDS-PAGE for the protein solution containing 2% β-Lg and 8% α-La during heating at 75°C. The loss of native α-La was greater than the loss of residual monomeric α-La indicating that α-La was forming hydrophobic aggregates. The similarity of the native and residual monomeric protein results for β-Lg indicates that very little β-Lg is incorporated into the hydrophobic aggregates.

At 80°C, the difference between the two PAGE results were smaller than that observed at 75°C (Figure 5.52B).

Heat-induced interactions of protein solutions containing β-Lg and α-La have been researched by several workers (Matsudomi *et al.* 1992; Calvo, 1993; Hines and Foegeding, 1993) although the studies have been concerned with interactions in dilute solutions rather than as part of the gelation process.

From the results presented in this study, it has been demonstrated that α-La does not aggregate when heated alone. However, it does aggregate and become part of the gel if it is heated in the presence of β-Lg. These results confirm those obtained by Mastudomi *et al.* (1992); Calvo, (1993) and Hines and Foegeding, (1993).

From the PAGE results more non-covalent interactions appeared to be involved in the formation of soluble protein aggregates during heating at 75°C than at 80°C and also in protein solutions containing a relatively higher proportion of β-Lg to α-La.
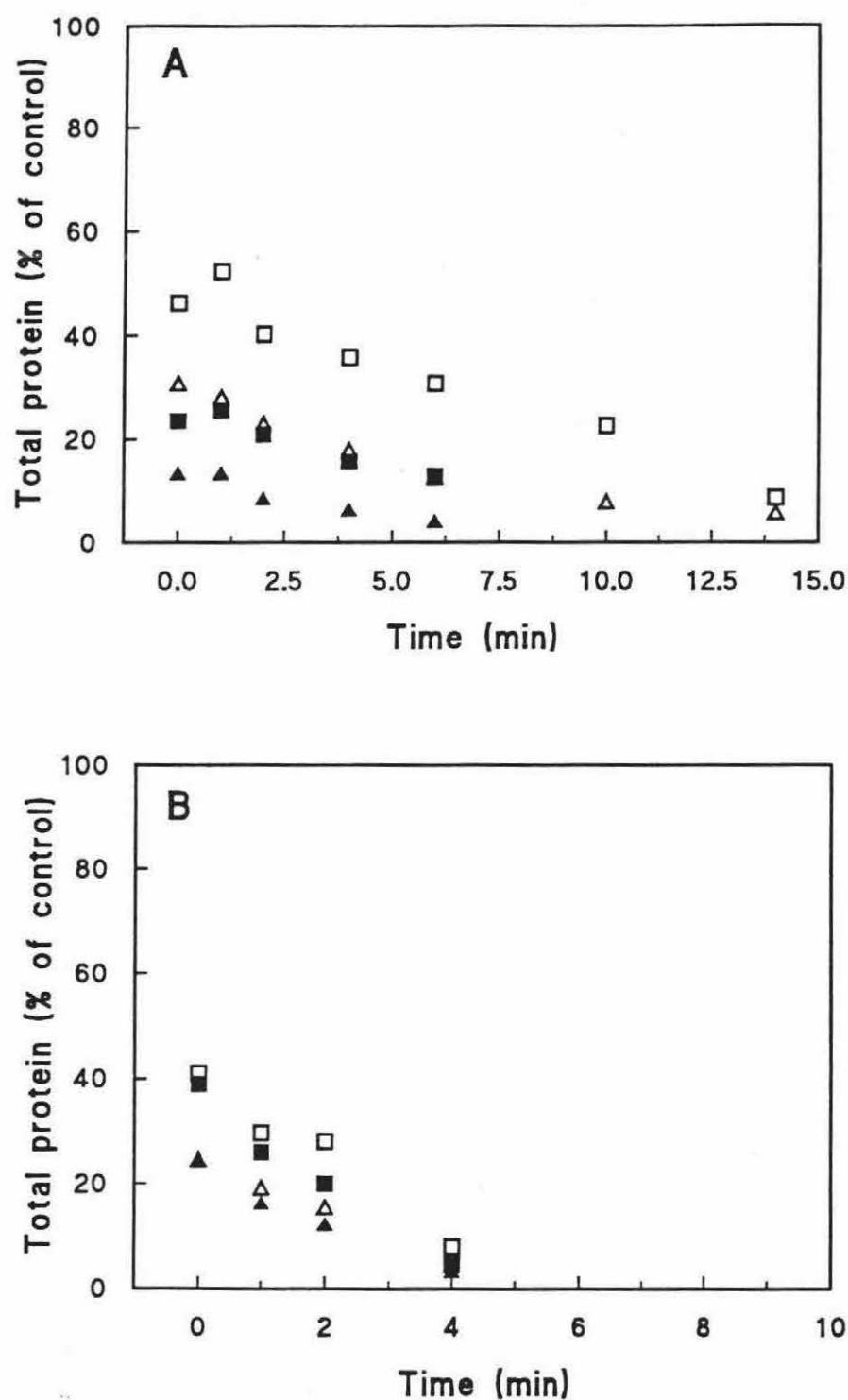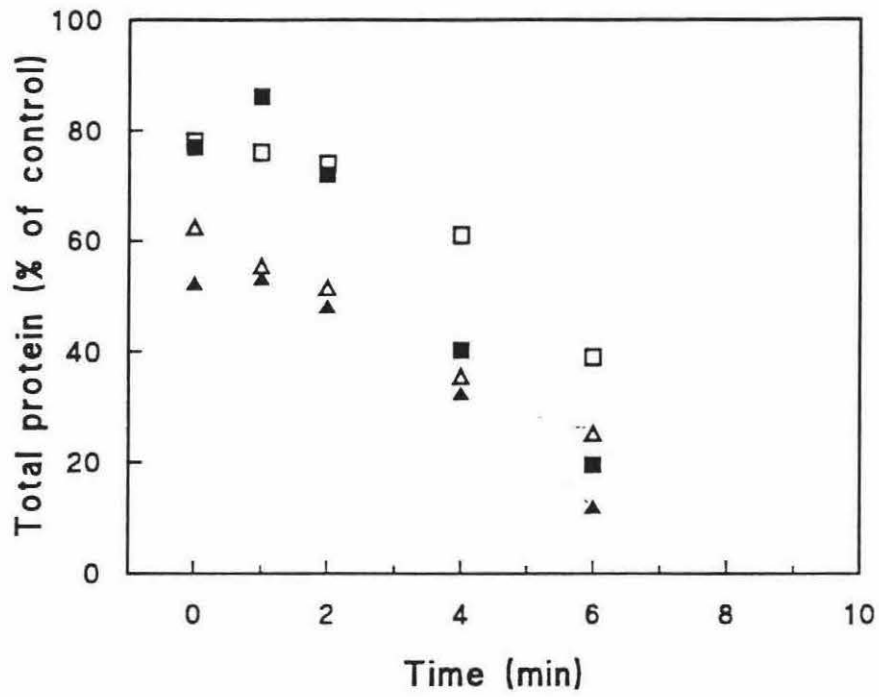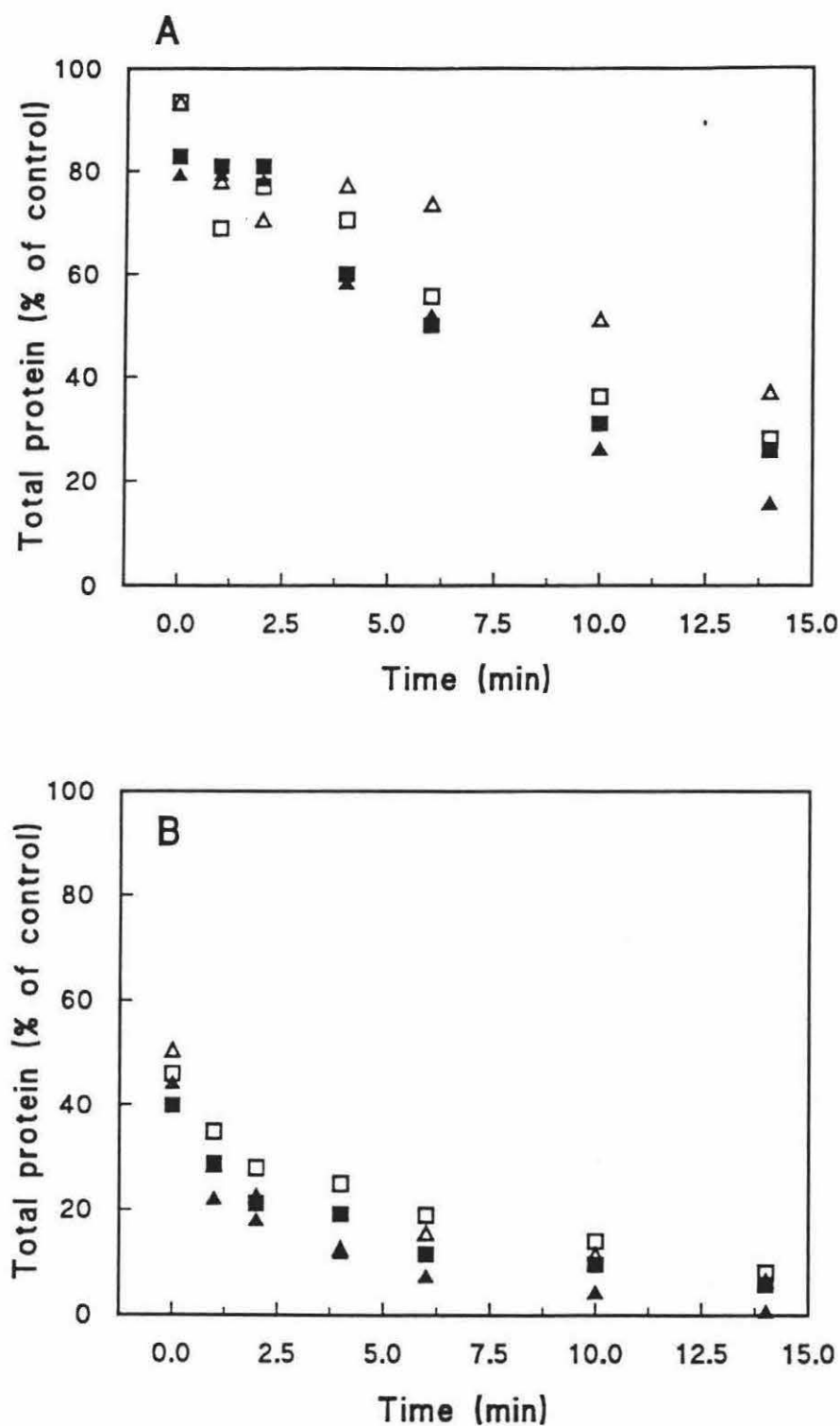
Figure 5.52   The effect of heating at 75°C (A) and 80°C (B) on the quantity of native β-Lg (■) and native α-La (▲), residual monomeric β-Lg (□) and residual monomeric α-La (△) remaining in a mixture containing 2% β-Lg and 8% α-La.

It is possible to assume that in such protein mixtures some protein aggregates probably, β-Lg, are formed through non-covalent interactions. Mastudomi *et al.* (1992) reported that β-Lg formed soluble aggregates with α-La mainly through a thiol-disulphide interchange during heating.

5.4.2.2 *Relationship between G' and PAGE results*

Figures 5.53A,B, 5.54 and 5.55 clearly show that both β-Lg and α-La had aggregated through disulphide bonding before any appreciable rise in G' was observed during heating at 75 and 80°C.

From these results it again appears that gel formation, which is responsible for the increase in G', does not occur until after all 10% of the protein present has been converted into disulphide-bonded aggregates.

Figure 5.53　　The effect of heating a mixture of 5% β-Lg and 5% α-La at 75°C (A) and 80°C (B) on G' (•) and the quantities of native β-Lg (■), native α-La (▲), residual monomeric β-Lg (□) and residual monomeric α-La (△).
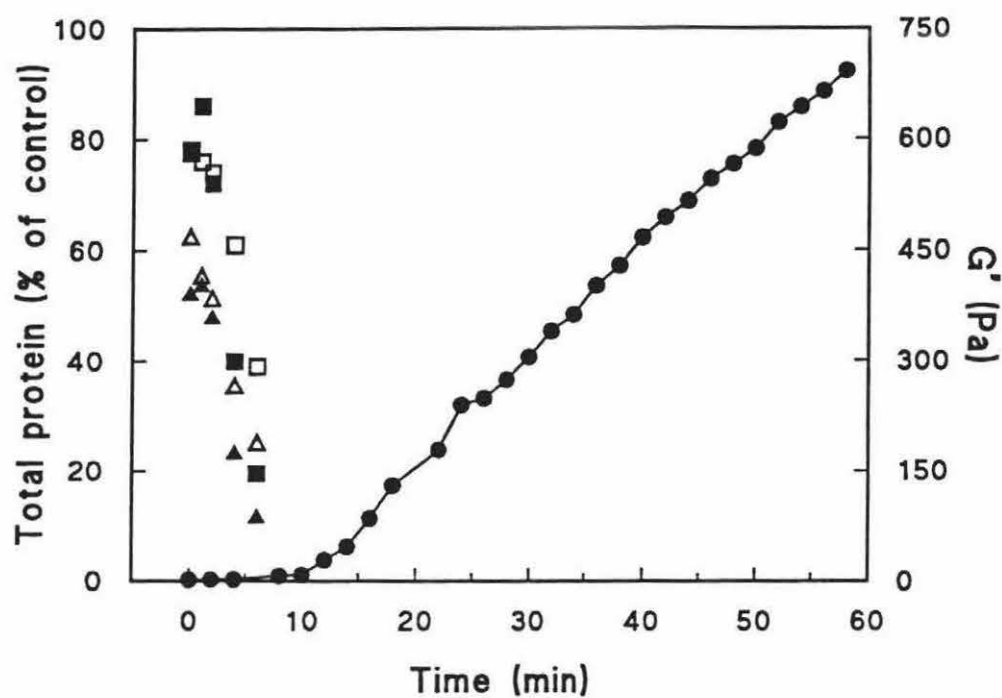
Figure 5.54   The effect of heating a mixture of 8% β-Lg and 2% α-La at 75°C on G' (•) and the quantities of native β-Lg (■), native α-La (▲), residual monomeric β-Lg (□) and residual monomeric α-La (△).
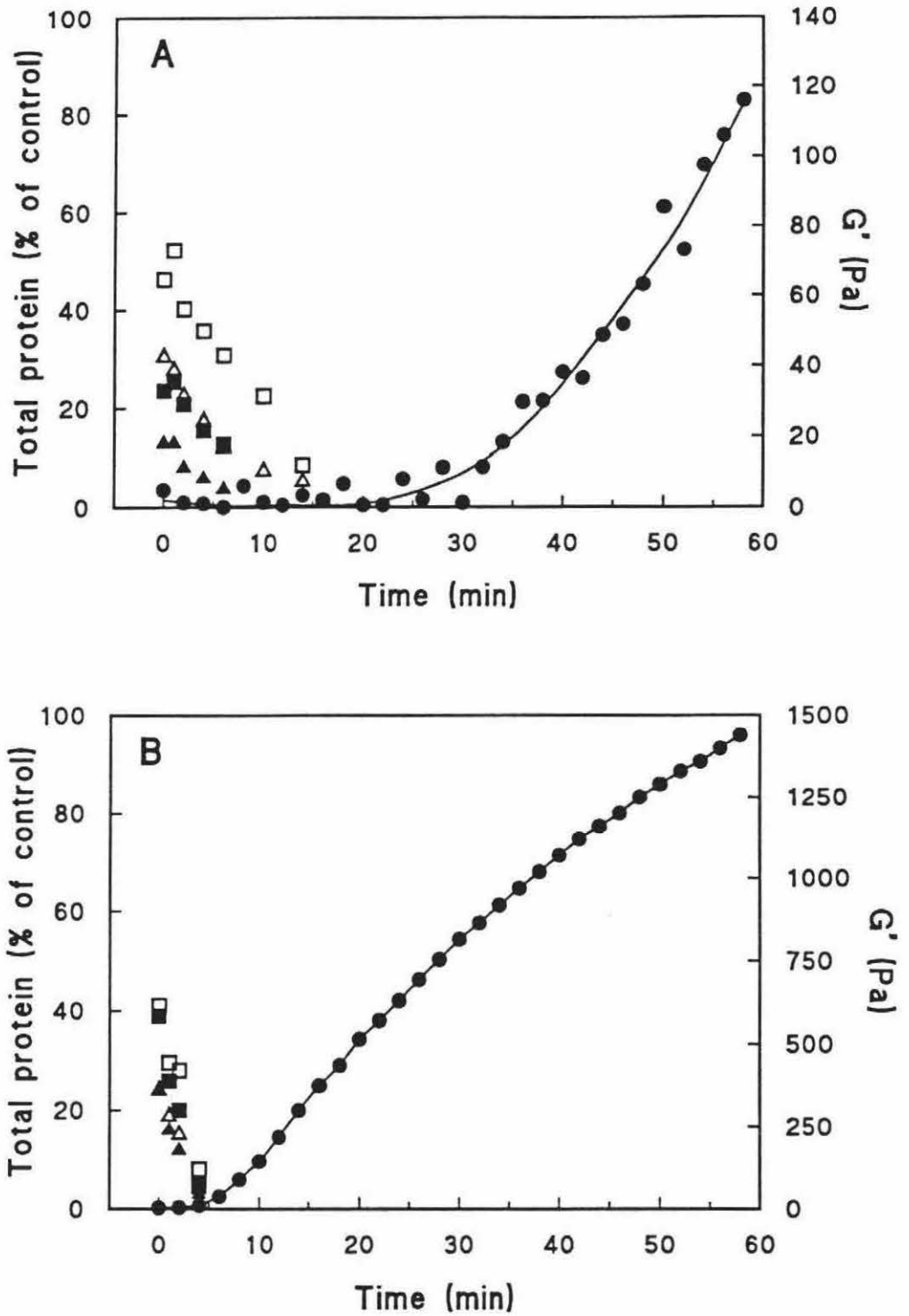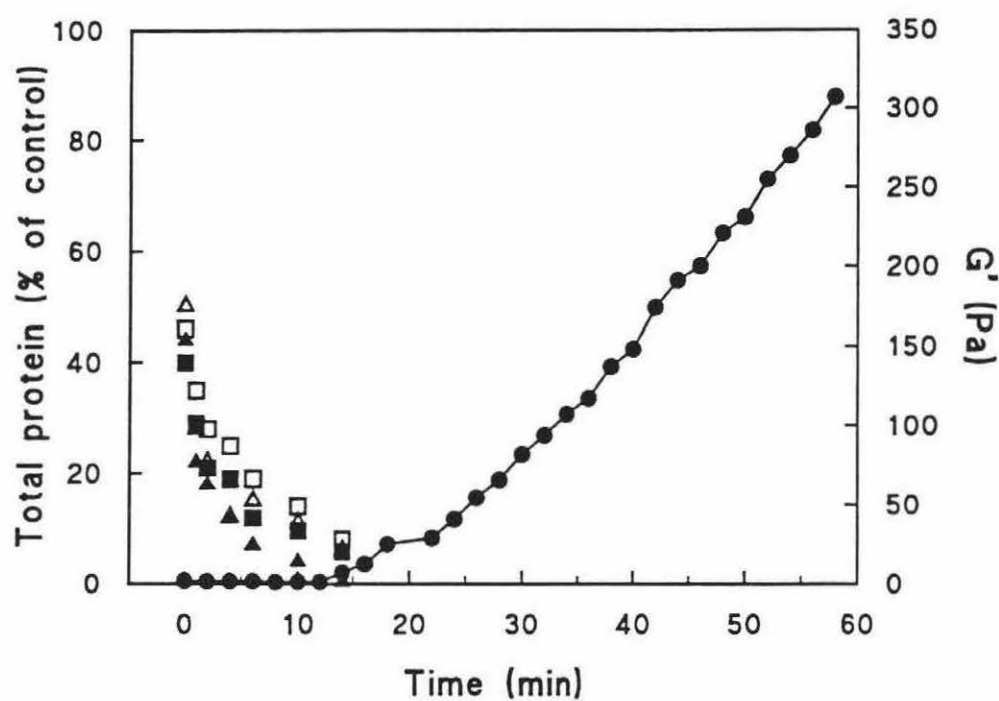
Figure 5.55   The effect of heating a mixture of 2% β-Lg and 8% α-La at 80°C on G' (•) and the quantities of native β-Lg (■), native α-La (▲), residual monomeric β-Lg (□) and residual monomeric α-La (△).

5.4.2.3 Possible gelation mechanism for β-Lg / α-La mixtures

Rheological and electrophoretic data clearly demonstrated that the aggregation and gelation behaviour of protein solutions containing β-Lg and α-La was different from that of protein solutions containing β-Lg and BSA.

α-La heated alone at 75 or 80°C did not form any aggregates or gel, although α-La has been reported to undergo thermal transitions at 63°C (Ruegg *et al*. 1977). It is possible that aggregates of α-La formed at these temperatures were rapidly reverted back to the native state. However, it is clear that α-La aggregated irreversibly when heated in the presence of β-Lg (Figures 5.42 - 5.44) or BSA (Matsudomi *et al*. 1994), possibly due to an interaction between the two proteins. At equal protein concentrations, α-La appeared to aggregate faster than β-Lg when heated at 75°C (Figure 5.42A) but at 80°C the aggregation of α-La was comparable to that of β-Lg (Figure 5.42B). Interactions between β-Lg and α-La during heating at 80°C appears to result mainly from sulphydryl-disulphide interchange reaction (Figures 5.50B - 5.52B.) but at 75°C, some non-covalently linked aggregates were also formed (Figures 5.50A - 5.52A).

At 75 or 80°C, the G' values for the mixed gels of β-Lg and α-La increased with increase in the relative proportion of β-Lg in the mixture (Figures 5.22 and 5.24), suggesting that β-Lg was the main protein supporting the gel net-work. However, G' values of the protein mixtures were greater than those of β-Lg alone (Figure 5. 25) indicating synergistic effect of α-La on β-Lg gelation. This enhancing effect of α-La on G' may be related to different kind of aggregates formed during heating. It can be implied that α-La co-polymerises with β-Lg to form aggregates, which may be of different shapes and sizes than those formed on heating β-Lg alone. The gel net-work can be visualised as consisting of co-polymers of β-Lg and α-La which form a single heterogenous network as shown diagrammatically below.

α-La

β-Lg

heat → Aggregated proteins

heat → β-Lg and α-La gel network
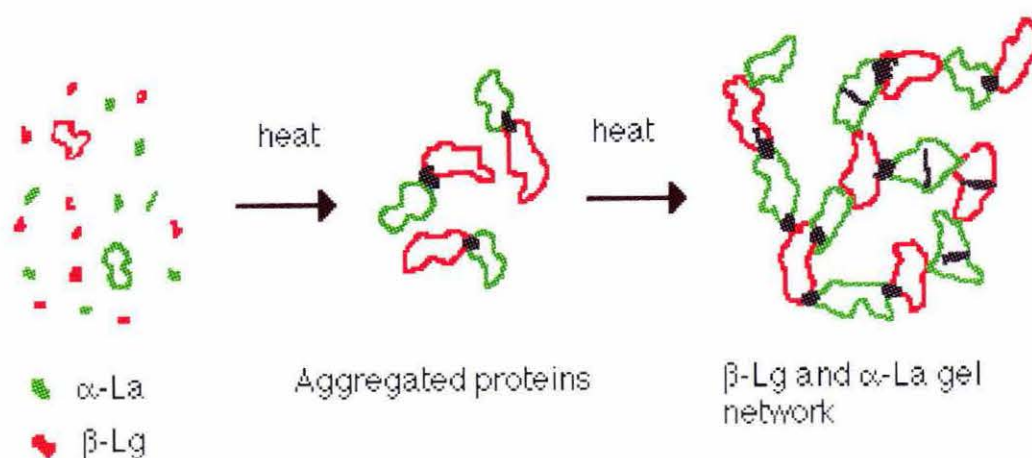
The constancy of gelation temperatures within the various protein mixtures (Figure 5.3) supports the above suggested mechanism.

5.5   WPC System

The concentration ratios of β-Lg, α-La and BSA in a typical WPC are 8: 3: 1, respectively.   From the results presented in this study and those reported by Matsudomi *et al.* (1993), it would be expected that on heating WPC solution, β-Lg would provide the three-dimensional structural gel matrix; α-La would be incorporated into β-Lg aggregates while BSA would aggregate independently or aggregate with α-La.   The presence of BSA aggregates or BSA/α-La aggregates would contribute to the strength and stability of the gel net-work.   Gel strength for the WPC solution would be expected to be higher than β-Lg heated alone at the same relative concentration.   However, the effects of Igs on the WPC gelation have not been investigated.   More investigations into the effects of Igs are needed in order to gain further understanding of the contributions of these proteins to rheological properties of WPC gels.

Overall the results of the present study suggest that slight differences in protein composition of WPC are unlikely to affect its gelation properties. Variations in the gelling properties of WPC during the season may be

related more to the non-protein components and/or processing effects rather than the changes in proportions of individual whey protein components.

The results of the present study could be useful in formulating new protein products containing different proportions of β-Lg, BSA and α-La with differentiated gelling properties.

CONCLUSIONS

*   The major pathway during gelation of β-Lg, BSA or protein mixtures initially involves formation of non-covalently linked aggregates followed by disulphide bonded aggregates leading to the formation of a three-dimensional gel net-work.

*   No aggregation of α-La occurs during heating of α-La solutions but in the presence of β-Lg aggregation occurs rapidly.

*   BSA does not seem to influence the loss of native β-Lg although the presence of β-Lg appears to slightly influence the loss of native BSA in the protein mixtures.

*   BSA solutions gel at lower temperatures and lower protein concentrations than β-Lg solutions.

*   Gelation temperatures of protein mixtures containing β-Lg and BSA are dependent on the relative proportions of the two proteins in the mixture while the protein mixtures containing β-g and α-La are comparable to that of β-Lg alone.

*   Rheological properties of β-Lg and BSA mixtures are dependent on the ratio of the two proteins and on the heating temperatures, however β-Lg and α-La mixtures are only dependent on the relative quantity of β-Lg in the mixture.

*   At 70°C, BSA forms the main gel net-work with possible β-Lg aggregates attached to the net-work strands while at 75°C, both proteins appear to form interpenetrating gel net-works with possible inter-strand linkages.

*   α-La appears to enhance the gelation of β-Lg by forming co-polymers with β-Lg resulting in a heterogenous gel net-work.

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1 Rheological Properties of Mixed Protein Gels

The results of most of the experiments using the Bohlin rheometer are tabulated below. Each table lists the experiments of a particular group and within the category (β-Lg/BSA or β-Lg/α-La). The tables are in chronological order. Within each table, the solutions were made up at the time using the same ingredients and measurements were made within a short period.

A *Gelation characteristics of β-Lg/BSA mixtures*

Table 1.1

β-LG Conc	BSA Conc	Gelation Time (s)	G′,70° (Pa)	G′,25° (Pa)	G′25/ G′70	Tan δ 70°	Tan δ 25°	Fig #	Fig # PAGE
8	2	3240	78.5	738	9.4	0.03	0.05	5.16	5.28A
5	5	1920	411	1840	4.5	0.1	0.06	"	5.27A
2	8	840	655	2250	3.4	0.03	0.08	"	5.29A
1	9	240	831	2300	2.8	0.02	0.09	"	-
0	10	0	967	1800	1.9	0.02	0.09	"	-
10	0	>3600	-	-	0	-	-	"	5.27A

Table 1.2

β-LG Conc	BSA Conc	Gelation Time (s)	G',70° (Pa)	G',25° (Pa)	G'25/ G'70	Tan δ 70°	Tan δ 25°	Fig #	Fig PAGE
0	5	1680	107	217	2.0	0.16	0.09	5.16	5.27A
0	8	2	819	1890	2.3	0.02	0.1	"	-
11	0	3120	123	879	0.12	0.09	7.1	5.19	-
12	0	2400	377	2020	5.4	0.13	0.07	"	-
13	0	2760	274	2220	8.1	0.09	0.07	"	-

Table 1.3

β-LG Conc	BSA Conc	Gelation Time (s)	G',70° (Pa)	G',25° (Pa)	G'25/ G'70	Tan δ 70°	Tan δ 25°	Fig #	Fig PAGE
10	1	3120	124	1280	1.0	0.15	0.07	5.19	-
10	2	2160	620	3230	5.2	0.11	0.06	"	-
10	3	1920	850	4820	5.7	0.11	0.06	"	-

Table 1.4

β-LG Conc	BSA Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
10	0	1200	755	5450	7.2	0.13	0.08	5.18	5.27B
8	2	1220	544	2970	5.5	0.07	0.06	"	5.28B
5	5	720	1340	5490	4.1	0.05	0.08	"	5.27B
2	8	120	2620	7470	2.9	0.02	0.08	"	5.29B
0	10	0	2370	5120	2.2	0.01	0.09	"	-

Table 1.5

β-LG Conc	BSA Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
5	0	>3600	-	-	0	-	-	5.18	5.27B
0	5	1920	50.4	129	2.6	0.05	0.14	"	5.27B
8	0	>3600	2.28	22.7	10	0.3	0.2	"	5.28B
0	8	0	1010	2480	2.5	0.01	0.09	"	-

Table 1.6

β-LG Conc	BSA Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
9	1	1400	392	2080	5.3	0.09	0.07	5.18	-
7	3	1560	365	1870	5.1	0.07	0.06	"	-
6	4	1680	282	1690	6.0	0.06	0.06	"	-
4	6	480	1960	7550	3.8	0.03	0.08	"	-
3	7	240	2310	7470	3.2	0.03	0.08	"	-
1	9	240	1430	4310	3.0	0.01	0.09	"	-

Table 1.7

β-LG Conc	BSA Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
10	0	480	2060	7640	3.7	0.12	0.06	5.20	-
11	0	600	2090	7390	3.5	0.09	0.05	"	-
12	0	600	2110	6750	3.1	0.09	0.05	"	-
13	0	600	5440	18600	3.4	0.10	0.05	"	-

Table 1.8

β-LG Conc	BSA Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
10	1	480	3950	12900	3.3	0.09	0.06	5.20	-
10	2	360	5370	19700	3.7	0.09	0.03	"	-
10	3	360	6960	21400	3.1	0.08	0.03	"	-

B   *Gelation characteristics of β-Lg/α-La mixtures.*

Table 1.9

β-LG Conc	α-La Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
10	0	720	2060	7640	3.7	0.12	0.06	5.22	5.42A
8	2	840	692	3830	5.5	0.08	0.09	"	5.43A
5	5	2760	116	1110	9.6	0.02	0.12	"	5.42A
2	8	>3600	-	-	-	-	-	"	5.44A
0	10	>3600	-	-	0	-	-	"	-

Table 1.10

β-LG Conc	α-La Conc	Gelation Time (s)	G',75° (Pa)	G',25° (Pa)	G'25/ G'75	Tan δ 75°	Tan δ 25°	Fig #	Fig PAGE
5	0	>3600	-	-	0	-	-	5.22	5.42
8	0	2160	53	237	4.8	0.17	0.09	"	5.43
0	8	-	-	-	-	-	-	"	5.44
6	4	2280	157	2110	13.4	0.13	0.11	"	-
9	1	1440	287	2370	8.3	0.12	0.06	"	-

Table 1.11

β-LG Conc	α-La Conc	Gelation Time (s)	G',80° (Pa)	G',25° (Pa)	G'25/ G'80	Tan δ 80°	Tan δ 25°	Fig #	Fig PAGE
8	2	0	2830	13900	4.9	0.07	0.08	5.24	-
5	5	480	1440	8660	6.0	0.04	0.12	"	5.42B
2	8	1560	307	7150	23.3	0.04	0.16	"	5.44B
0	10	>3600	-	-	0	-	-	-	-
10	0	240	1650	7670	4.6	0.08	0.07	"	-

Table 1.12

β-LG Conc	α-La Conc	Gelation Time (s)	G',80° (Pa)	G',25° (Pa)	G'25/ G'80	Tan δ 80°	Tan δ 25°	Fig #	Fig PAGE
5	0	2280	30.1	279	9.3	0.23	0.10	5.24	5.24B
0	8	-	-	-	-	-	-	-	5.42B
8	0	1440	219	984	4.5	0.07	0.06	"	5.43B
9	1	240	1010	5080	5.0	0.07	0.06	"	-
6	4	360	1170	6720	5.7	0.05	0.09	"	-

Table 1.13

β-LG Conc	α-La Conc	Gelation Time (s)	G',80° (Pa)	G',25° (Pa)	G'25/ G'80	Tan δ 80°	Tan δ 25°	Fig #	Fig PAGE
11	0	120	2260	8130	3.6	0.08	0.04	5.25	-
12	0	0	2370	8510	3.6	0.08	0.04	"	-
13	0	0	5830	12100	2.1	0.09	0.02	"	-
10	1	0	3760	14900	4.0	0.08	0.04	"	-
10	2	0	4420	18100	4.1	0.08	0.04	"	-
10	3	0	6410	27700	4.3	0.07	0.02	"	-

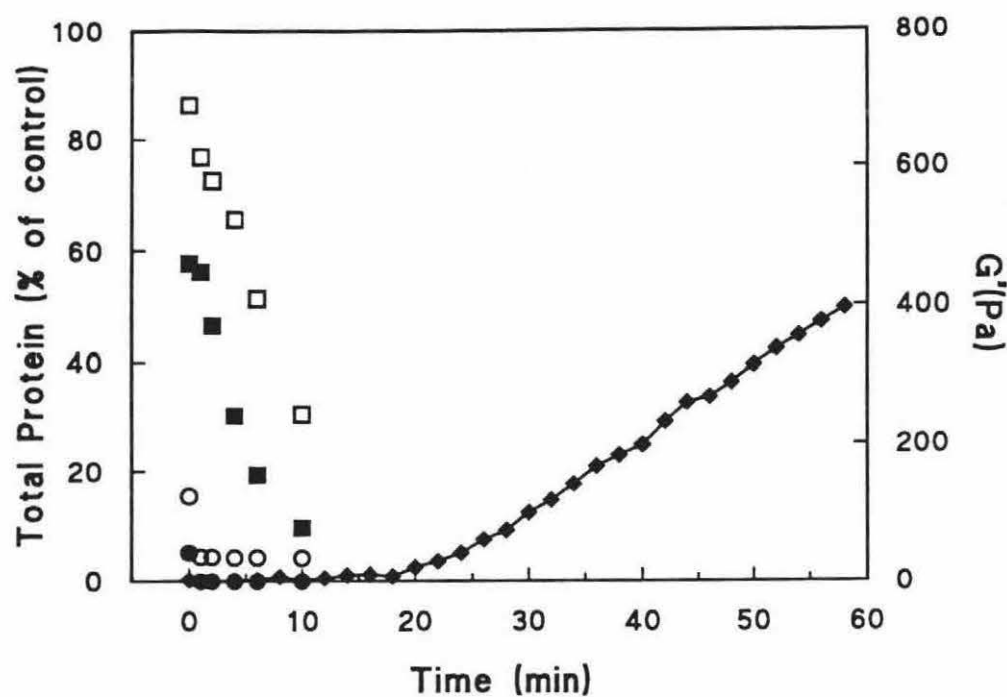## 2 Aggregation and Gelation of β-Lg/BSA mixtures

Figure 2.1 The effect of heating a mixture of 9% β-Lg and 1% BSA at 75°C on G′ (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○).
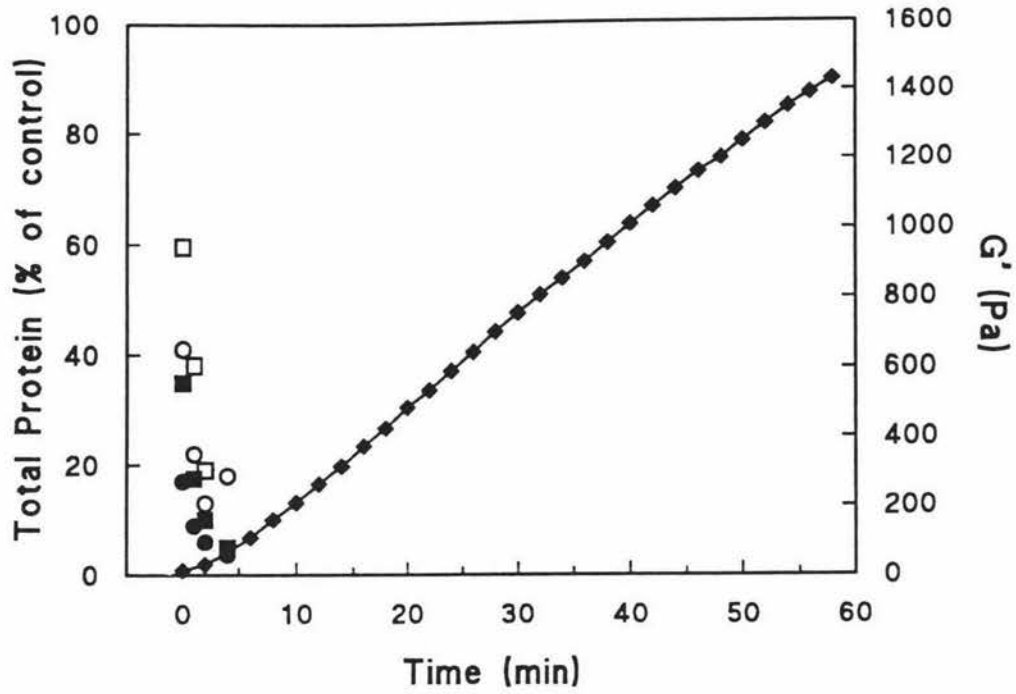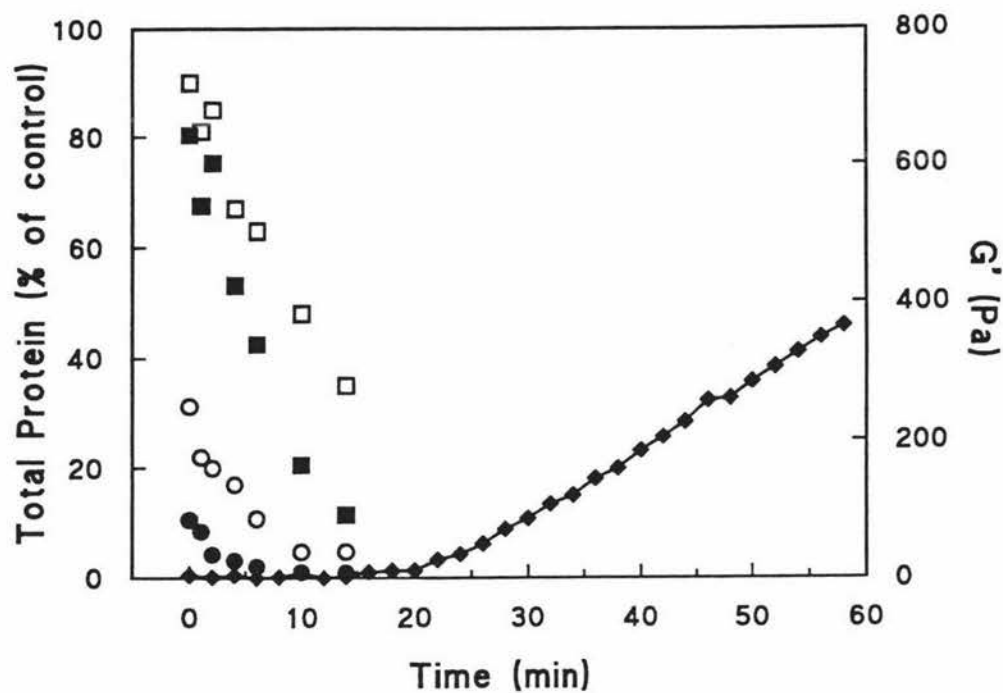
Figure 2.2 The effect of heating a mixture of 1% β-Lg and 9% BSA at 75°C on G′ (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○).

Figure 2.3 The effect of heating a mixture of 7% β-Lg and 3% BSA at 75°C on G' (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○).

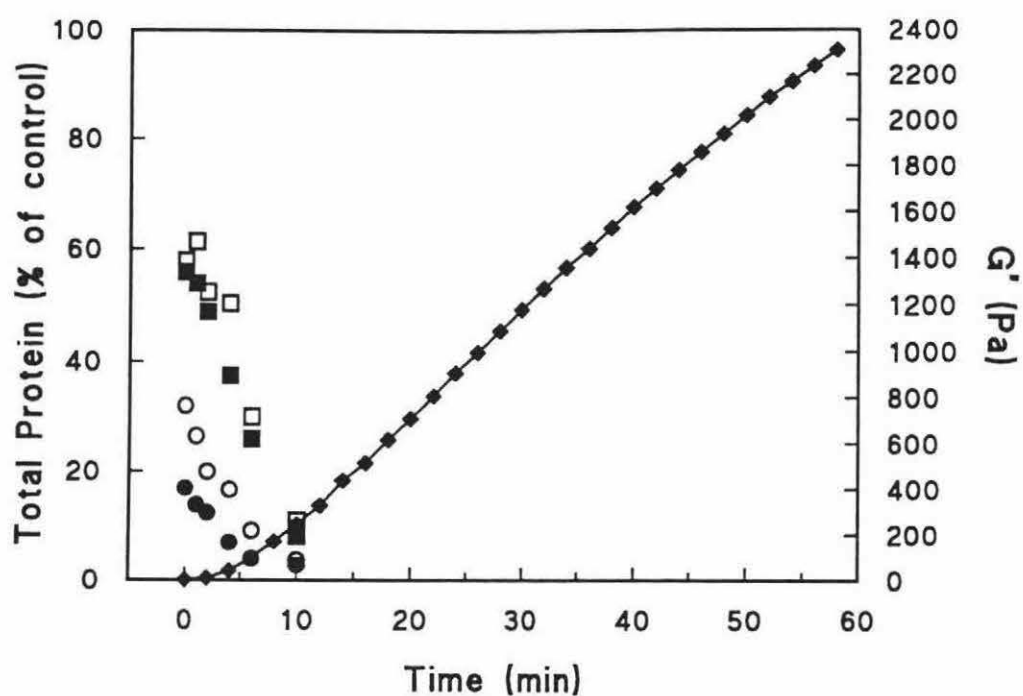**Figure 2.4** The effect of heating a mixture of 3% β-Lg and 7% BSA at
75°C on G′ (♦) and quantities of native β-Lg (■), native BSA
(•), residual monomeric β-Lg (□) and residual monomeric BSA
(○)

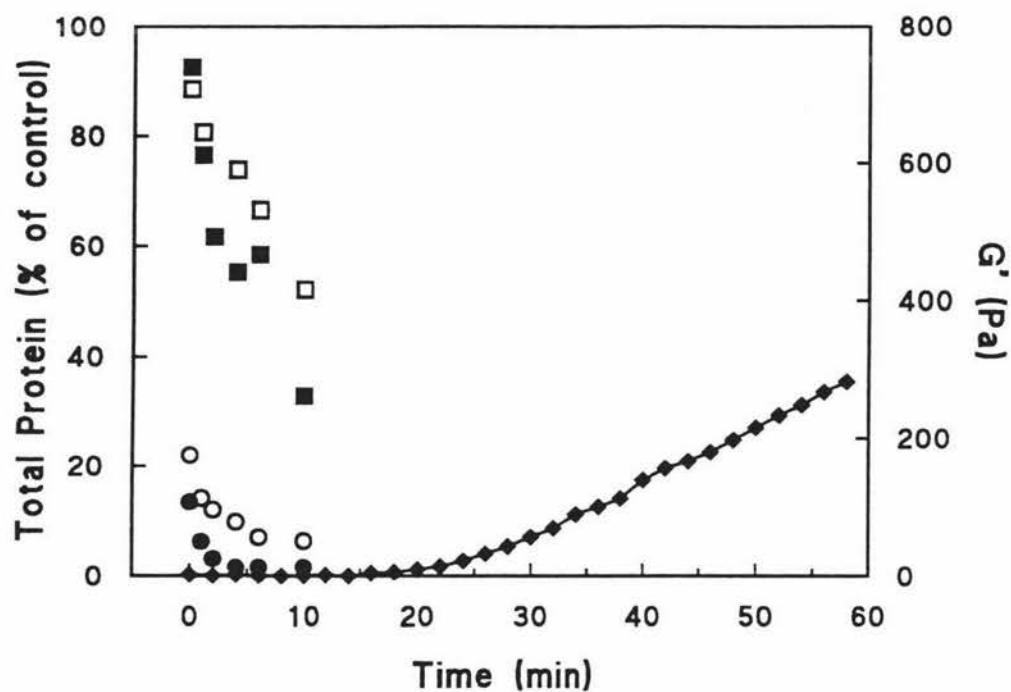**Figure 2.5** The effect of heating a mixture of 6% β-Lg and 4% BSA at 75°C on G' (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○).
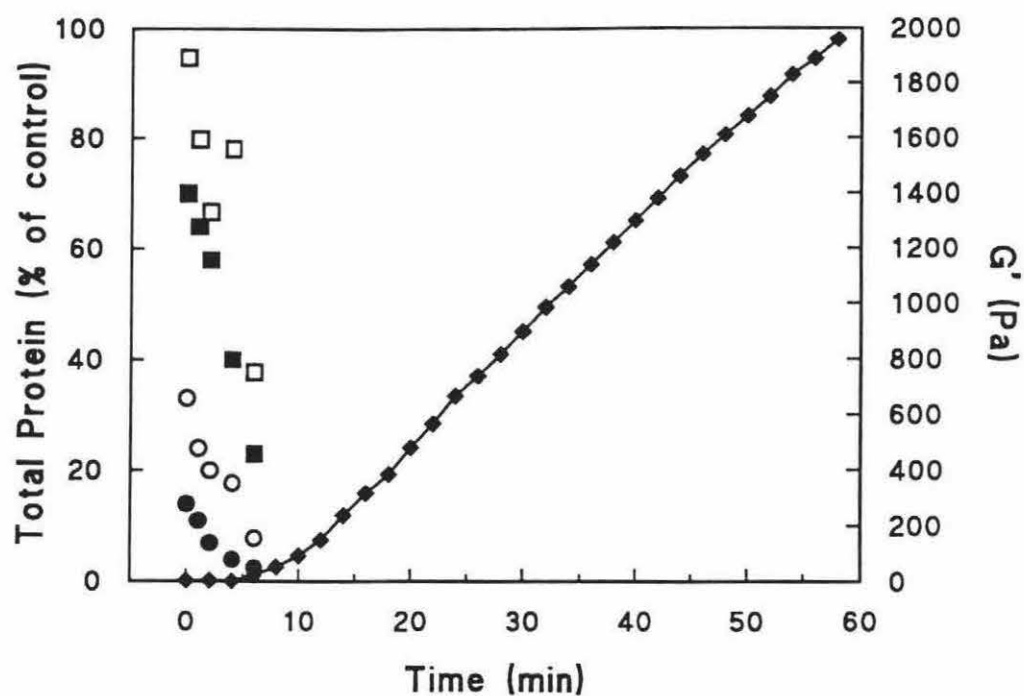
Figure 2.6 The effect of heating a mixture of 4% β-Lg and 6% BSA at 75°C on G′ (♦) and quantities of native β-Lg (■), native BSA (•), residual monomeric β-Lg (□) and residual monomeric BSA (○).

3 Aggregation and Gelation of Protein mixtures

Table 3.1   Results reported for β-Lg/α-La mixtures.

Protein		Heating Temperatures	
β-Lg Conc (%)	BSA Conc (%)	70°C	75°C
10	0	Aggregation (**)	Aggregation + Gel
8	0	(***)	Aggregation (**)
5	0	(***)	Aggregation (**)
0	8	Aggregation + Gel	Gel (*)
0	5	Aggregation + Gel	Aggregation + Gel
8	2	Aggregation + Gel	Aggregation + Gel
5	5	Aggregation + Gel	Aggregation + Gel
2	8	Aggregation + Gel	Aggregation + gel
0	10	Gel (*)	Gel (*)

** : No gel formed

* : Gelation was too fast to obtain samples for PAGE.

Table 3.2 Results reported for β-Lg/α-La mixtures

Protein		Heating Temperatures	
β-Lg Conc (%)	α-La Conc (%)	75°C	80°C
10	0	Aggregation + Gel	Gel (*)
8	0	Aggregation (**)	Aggregation + Gel
5	0	Aggregation (**)	Aggregation + Gel
0	8	Aggregation (**)	Aggregation (**)
8	2	Aggregation + Gel	Gel (*)
5	5	Aggregation + Gel	Aggregation + Gel
2	8	Aggregation (**)	Aggregation + Gel
0	10	(**)	(**)