Effect of bound ligands and κ-casein on the denaturation of β-lactoglobulin

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ABSTRACT

The objective of this study was to gain greater understanding of the behaviour of bovine β-lactoglobulin (β-lg) during thermal denaturation. The first part of the study was focused on gaining more knowledge of the structural changes in β-lg during heat-induced and urea-induced denaturation and the effect of ligands and the genetic variants of β-lg. The second part of the study explored the mechanism of the heat-induced interaction of β-lg with κ-casein (κ-CN) and the effects of genetic variants.

The reversible early steps during thermal denaturation of β-lg are not readily separated from the later irreversible steps which involve sulphydryl-disulphide interchange reactions. However, it should be possible to examine the behaviour of β-lg in the early steps without reactive thiols either by using a porcine β-lg, that does not have cysteine residue equivalent to Cys-121 of bovine β-lg, by blocking the free thiol or by using solvent denaturation.

Heating to 80 °C irreversibly altered near- and far-UV circular dichroism (CD) spectra, and 1,8-anilinonaphthalene sulphonate and retinol binding fluorescence spectra for bovine β-lg, but not for porcine β-lg which unfolded reversibly. Also the examination of the induced CD spectral changes of retinol and *cis*-parinaric acid (PnA) on binding to bovine β-lg B upon heating revealed that bovine β-lg lost the ability to bind retinol and PnA in a chiral environment. In contrast, porcine β-lg did not show significantly induced CD bands upon mixing with retinol, but PnA/β-lg mixtures showed induced CD bands of low intensity. In addition to the lack of a free thiol group in porcine β-lg, the sequence differences between bovine β-lg and porcine β-lg are also likely to affect the behaviour of these β-lgs during heat treatment and the binding of ligands. Although palmitic acid does not show any absorbance in CD spectra, it enhanced the stability of the bovine β-lg and porcine β-lg mixture.

The urea-induced unfolding of bovine β-lg at neutral pH (6.7) revealed that the stability of the genetic variants followed the order, β-lg B < β-lg A < β-lg C, as observed during thermal denaturation and tryptic hydrolysis. The stabilisation effect was also

observed by adding retinol, retinoic acid and palmitic acid during urea denaturation of β-lg, and by retinol, retinyl acetate and PnA during ammonium sulphate denatuation of β-lg. Blocking the sulphydryl group of β-lg destabilised the native protein against urea denaturation through the introduction of a bulky group to the compact structure of β-lg. This result, together with the results for porcine β-lg, confirms that the sulphydryl group plays an important role in the unfolding of bovine β-lg.

In the second part of the study, an attempt was made to investigate the effect of bovine κ-CN on the established heat-induced unfolding and aggregation pathway of bovine β-lg, by adding κ-CN A or κ-CN B to native or pre-heated β-lg A, B or C and heating the mixture. The CD band intensity of the mixture of β-lg and κ-CN at 270 nm, an index of significant alteration to the disulphide bond dihedral angle, indicated increasing structural changes involving disulphide bonds during heat treatment.

The rates of loss of β-lg and the distributions of intermediate products were determined using alkaline- and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). During reaction with β-lg, some monomeric κ-CN was found by SDS-PAGE, probably as a result of disulphide bond interchanges between κ-CN and β-lg, and two-dimensional PAGE also revealed disulphide-bonded β-lg/κ-CN aggregates. In the presence of κ-CN, the loss of monomeric β-lg increased and less non-native monomer and dimer were observed compared to β-lg alone and κ-CN reacted more rapidly with β-lg that had been unfolded by prior heat treatment than with native β-lg. This suggested that β-lg probably denatured (unfolded) independently and either simultaneously or consequently and contained higher sulphydryl reactivity than native β-lg, which lead to the reactions with κ-CN via disulphide bond interchange. It is possible that the equilibrium between native β-lg and denatured β-lg shifted rapidly, because κ-CN preferred to interact with denatured (unfolded) β-lg that has higher sulphydryl reactivity than native β-lg.

The kinetics of the interaction between β-lg and κ-CN were evaluated from the heat-induced loss of alkaline-monomeric β-lg at 80 °C. The interaction between β-lg and κ-CN could not be described by any reaction order between 1.0 and 2.0. The slopes of the plots changed at about 7.5-10 min heating time and this corresponded to intensity

changes in the alkaline-monomeric, non-native monomeric and dimeric β-lg bands, which increased during the first 10 min of heating and then slightly decreased or remained relatively constant for the rest of the heating.

The loss of native β-lg in β-lg/κ-CN mixtures during heating at 80 °C was shown to be significantly influenced by the genotypes of both β-lg and κ-CN. The κ-CN B variant showed considerably higher reactivity than κ-CN A, while the β-lg B variant was the most reactive. The greatest loss of native β-lg was observed from the β-lg B/κ-CN B mixture.

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CONTENTS

PART I. ROLE OF LIGANDS AND FREE CYSTEINE IN β-LACTOGLOBULIN DENATURTION

CHAPTER 1.
INTRODUCTION

Many of the functional properties of milk and dairy products are dependent on the behaviour of β-lactoglobulin (β-lg) as a consequence of the various heat treatments that are given to these products. Although its primary biological function is unknown, a wide range of compounds is known to bind to β-lg (Hambling et al., 1992). It is still unclear whether any of the ligands are important for the biological function of this protein, but it has been reported that β-lg is stabilised against denaturation by binding ligands, such as retinol or palmitic acid (Creamer, 1995; Hamada and Goto, 1997).

In milk systems, β-lg makes up more than 50 % of the whey protein mixture and its thermal behaviour is similar to that of whey protein concentrate. Bovine β-lg appears to denature through an initial dissociation of dimer to monomer, which is dependent on temperature and pH, followed by monomer unfolding to permit higher sulphydryl reactivity that can lead to disulphide interchange and aggregation, although aggregation also occurs without the involvement of the sulphydryl group (McSwiney et al., 1994a; Manderson, 1998; Schokker et al., 1999).

The objective of first part of the present study is to gain more understanding of how β-lg behaves during heat-induced denaturation. Bovine β-lg exists in a range of genetic variants; variants A and B of both proteins are dominant in dairy herds, but the β-lg C gene occurs at a frequency of 0.06 in the New Zealand Jersey cow population (Paterson et al., 1995). Earlier studies in our laboratory (Manderson, 1998) have shown the different thermostabilities and aggregation behaviours of β-lg A, B and C variants. Some questions raised about the reasons of the variant effects have been addressed by examining the equilibrium denaturation of β-lg using urea, the role of Cys-121 (the free thiol is critical in the thiol-catalysed disulphide bond interchange reactions that form the basis of heat-induced aggregation) and the effect of ligands on the denaturation behaviour of β-lg. It has been reported that urea denaturation of β-lg initially involves reversible dimer dissociation and unfolding of monomer and then the unfolded protein undergoes

irreversible sulphydryl-disulphide interchange reactions (McKenzie and Ralston, 1973; Creamer, 1995).

In the second part of this thesis, the effect of κ-casein (κ-CN) on the aggregation pathway of β-lg is investigated to extend the works of others in our group who examined the effect of α-lactalbumin (Gezemati et al., 1986; Schokker et al., 2000) or bovine serum albumin (Gezemati et al., 1987; Havea et al, 2000) on β-lg aggregation. Early studies (Long et al., 1963; Tessier and Rose, 1964; Sawyer, 1968, 1969) showed that β-lg interacts with κ-CN mainly through sulphydryl-disulphide interchange reactions, although the exact mechanism of the formation of this complex has not been clearly elucidated. As bovine β-lg and κ-CN exhibit genetic polymorphism, the genetic variant effects on the thermal denaturation of β-lg A, B and C in the absence and presence of the κ-CN A and B have been investigated using different protein concentrations and heating conditions.

Apart from the studies done in Palmerston North (Manderson et al., 1999a, b; Qin et al., 1999; Havea et al, 2000; Schokker et al., 1999, 2000) and Edinburgh (Wu et al., 1999; Sawyer et al., 1999; Uhrinova et al., 2000; Kontopidis and Sawyer, 2000; Sawyer, 2001), most studies published after 1999 are not considered in the Literature review because these studies were published after the work described in this thesis had been completed. However, all relevant references are considered in the appropriate Results and discussion or Conclusions sections of Chapters 4-10.

CHAPTER 2.
LITERATURE REVIEW

2.1. BOVINE β-LACTOGLOBULIN

β-Lg is the major whey protein in the milk of ruminants and many other mammals. Bovine β-lg is a small soluble protein, which is extremely acid stable and normally exists as a dimer of subunit molecular weight 18,300 Da. Each monomer comprises 162 amino acids, with one free cysteine and two disulphide bridges (Hambling et al., 1992).

The biological function of bovine β-lg is not fully understood. However, β-lg is stable at low pH (Kella and Kinsella, 1988), is resistant to proteolysis (Reddy et al., 1988) and remains mostly intact after it passes through the stomach at pH 2 (Yvon et al., 1984); its primary role may be to facilitate the transport of retinol or fatty acids to the intestinal tract of the neonate. Even though a retinol/β-lg complex receptor has been found in newborn calves (Papiz et al., 1986), the real physiological function of β-lg still remains a mystery. It has been suggested that β-lg is involved in the digestion of milk fat and/or in the transportation of retinol (Perez and Calvo, 1995).

The viscosity, gelation and water retention are important characteristics in heat-induced whey protein gelation in food products, which is a consequence of thermally induced disulphide interchange reactions of bovine β-lg involving the one free sulphydryl (cysteine) group and two cystine bridges. Heat results in the exposure of the thiol group of β-lg to the solvent. It can then participate in thiol-disulphide interchange reactions with the disulphide bonds of other β-lg molecules or other milk proteins, such as α-lactalbumin (α-la), bovine serum albumin (BSA), immunoglobulin (Ig) and κ-CN.

The main complex formed during the heat treatment of milk is believed to be the β-lg/κ-CN complex, the formation of which has profound implications in the rennet-coagulation process (Hill, 1989). At low heating temperatures, the reaction between β-lg and κ-CN seems to be driven mainly by hydrophobic interactions (Haque and Kinsella, 1988; Jang and Swaisgood, 1990), but at high temperature the main characteristic of the

complex is increased intermolecular disulphide bonding (Hill, 1989). The existence of this complex is also one of the major factors responsible for the heat stability-pH profile of milk (Singh and Creamer, 1992).

Note that bovine β-lg and bovine κ-CN are referred to as β-lg and κ-CN throughout this thesis, unless otherwise specified.

2.2. STRUCTURE OF β-LACTOGLOBULIN

2.2.1. Primary structure and genetic variants of β-lactoglobulin

To date, 13 genetic variants of β-lg have been identified: β-lgs A, B, C, D, E, F, G (Eigel et al., 1984), H (Lodes, 1995), I, J (Godovac-Zimmermann et al., 1996), W (Lodes, 1995), Dr, which is an N-glycosylated protein (Bell et al., 1970) and K^B (Sawyer, 2001).

In New Zealand herds, the A and B variants are predominant, with the C variant occurring at low levels in Jersey and related breeds at a frequency of 0.06 (Paterson et al., 1995). The differences in the primary sequences of these three variants are at residues 59, 64 and 118: β-lg A (Gln-59, Asp-64 and Val-118) and β-lg B (Gln-59, Gly-64 and Ala-118), which are found in the milk of both Friesian and Jersey cows, and β-lg C (His-59, Gly-64 and Ala-118), which is found only in the milk of Jersey cows. The amino acid sequences of β-lgs A, B and C (Eigel et al., 1984) are shown in Fig. 2.2.1.1.

Although many of the properties of β-lg are common to all genetic variants, there are several important differences in their chemical and physical behaviour, i.e. the electrophoretic mobilities (Aschaffenburg, 1965), the solubilities (Bell and McKenzie, 1967) and the isoelectric points (Tanford et al., 1959; Basch and Timasheff, 1967) of β-lgs A, B and C. In a native agar gel system at pH 8.6, β-lgs A, B and C migrate the greatest, intermediate and shortest distances, respectively (Aschaffenburg, 1965). At pH 5.2, β-lg A is the least soluble, whereas β-lg C is the most soluble (Bell and McKenzie, 1967). The isoelectric points of β-lgs A, B and C are 5.1, 5.2 and 5.3, respectively (Tanford et al., 1959; Basch and Timasheff, 1967; Ghose et al., 1968), and the masses of

monomeric β-lg variant A, B and C are 18,362.3 ± 1.1, 18,277 ± 1.6 and 18,286 ± 1.0 Da, respectively (Burr et al., 1997).

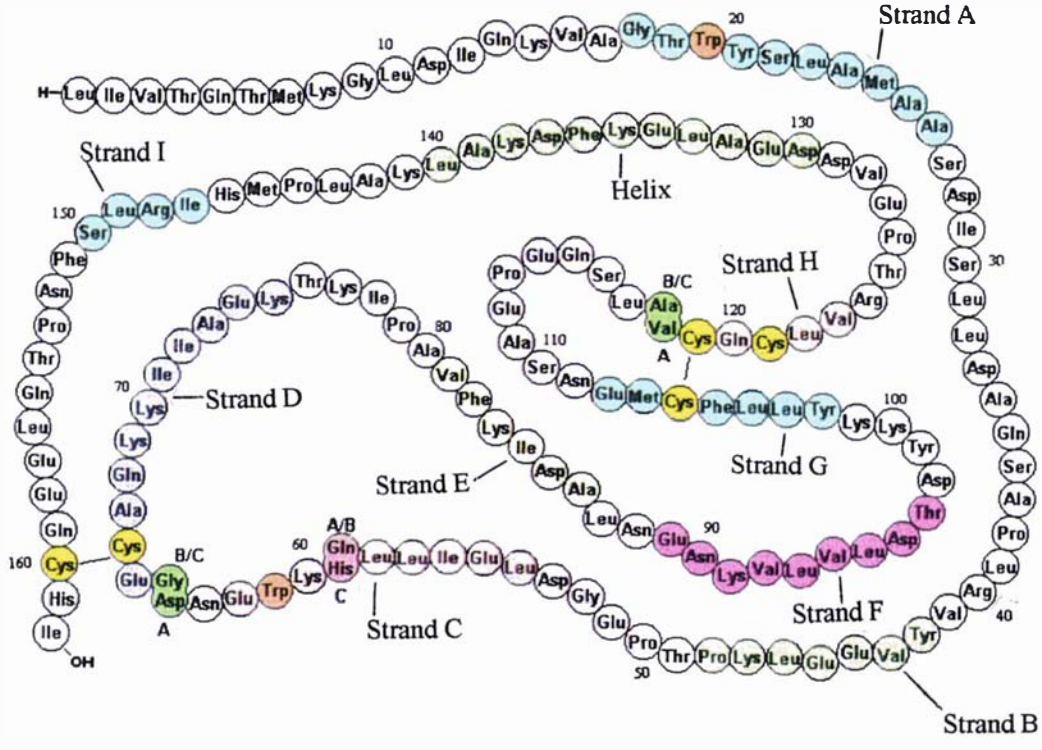

Figure 2.2.1.1. The amino acid sequences of bovine β-lg A, B and C (Eigel et al., 1984). The positions of the β-strands and the α-helix are marked, as well as two disulphide bonds between cysteine residues (Brownlow et al., 1997; Manderson, 1998).

2.2.2. Secondary and tertiary structures of β-lactoglobulin

The secondary structure of β-lg has been studied by various methods, i.e. optical rotary dispersion (ORD) (Timasheff et al., 1966), circular dichroism (CD) (Townend et al., 1967; Griffin et al., 1993; Matsuura and Manning, 1994; Qi et al., 1997), infrared (IR) spectroscopy (Timasheff and Susi, 1966; Casal et al., 1988; Qi et al., 1997) and preliminary nuclear magnetic resonance (NMR) structures (Molinari et al., 1996; Ragona et al., 1997; Belloque and Smith, 1998; Uhrinova et al., 1998). All methods show comparable results of around 10 % α-helix and 50 % β-sheet, the rest being random structure (Townend et al., 1967; Creamer et al., 1983; Griffin et al., 1993; Uhrinova et al., 1998).

The crystal structure of bovine β-lg was determined at medium resolution by Papiz et al. (1986) and Monaco et al. (1987), and at high resolution by Brownlow et al. (1997), Bewley et al. (1997) and Qin et al. (1998a, b, 1999). Also the high resolution structure of monomeric β-lg at pH 2.5 has been partially resolved by NMR spectroscopy (Kuwata et al., 1999; Uhrinova et al., 2000). In spite of the variation in primary structure between the A and B variants of β-lg, neither the secondary structure (Townend et al., 1967; Qi et al., 1997; Prabakaran and Damodaran, 1997) nor the medium resolution tertiary structure, determined by X-ray diffraction methods at 2.5 Å resolution (Monaco et al., 1987), show any significant differences.

The tertiary structure of β-lg, shown in Fig. 2.2.2.1, consists of nine anti-parallel β-strands, of which eight are wrapped into a β-barrel (Papiz et al., 1986; Brownlow et al., 1997). β-Strands A-D form one side of the calyx, and β-strands E-H and also part of strand A form the opposite side. The ninth strand, I, is on the outside, on the opposite side of strand A to strand H, and so is able to form part of dimer interface. The barrel is open to solvent at pH 8.2 (Qin et al., 1998b) at one end but the other end is closed by tight side chain packing. The three-turn α-helix is located on the outside of the barrel and aligned along strands A, G and H. The protein contains two disulphide bridges, between Cys residues 66-160 and 106-119, and one free sulphydryl group per monomer, at position 121 (Fig. 2.2.2.2) (Hambling et al., 1992). It should be noted that all three of the

amino acid substitutions are spatially located close to one or other of the disulphide bridges (Bewley et al., 1997).

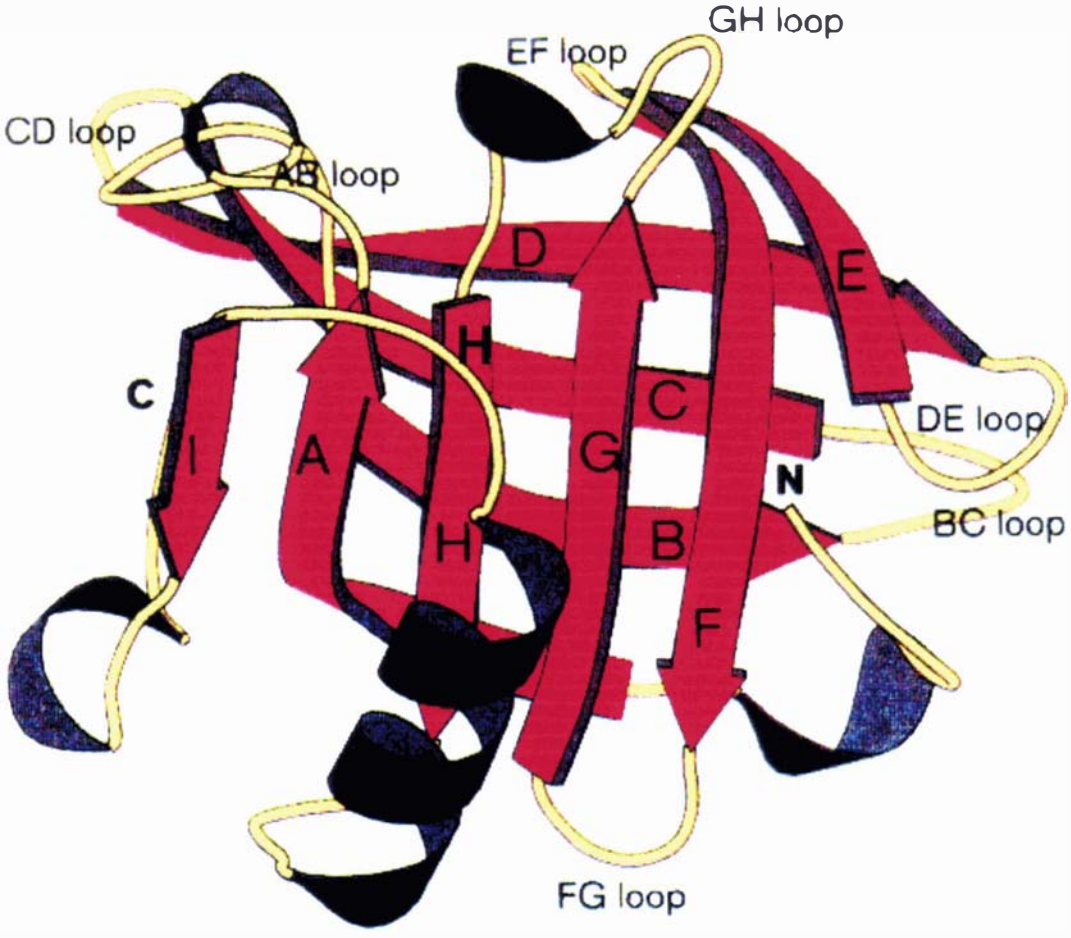

Figure 2.2.2.1. Ribbon diagram of a single subunit of β-lg lattice X, with the β-strands and joining loops labelled (Brownlow et al., 1997).

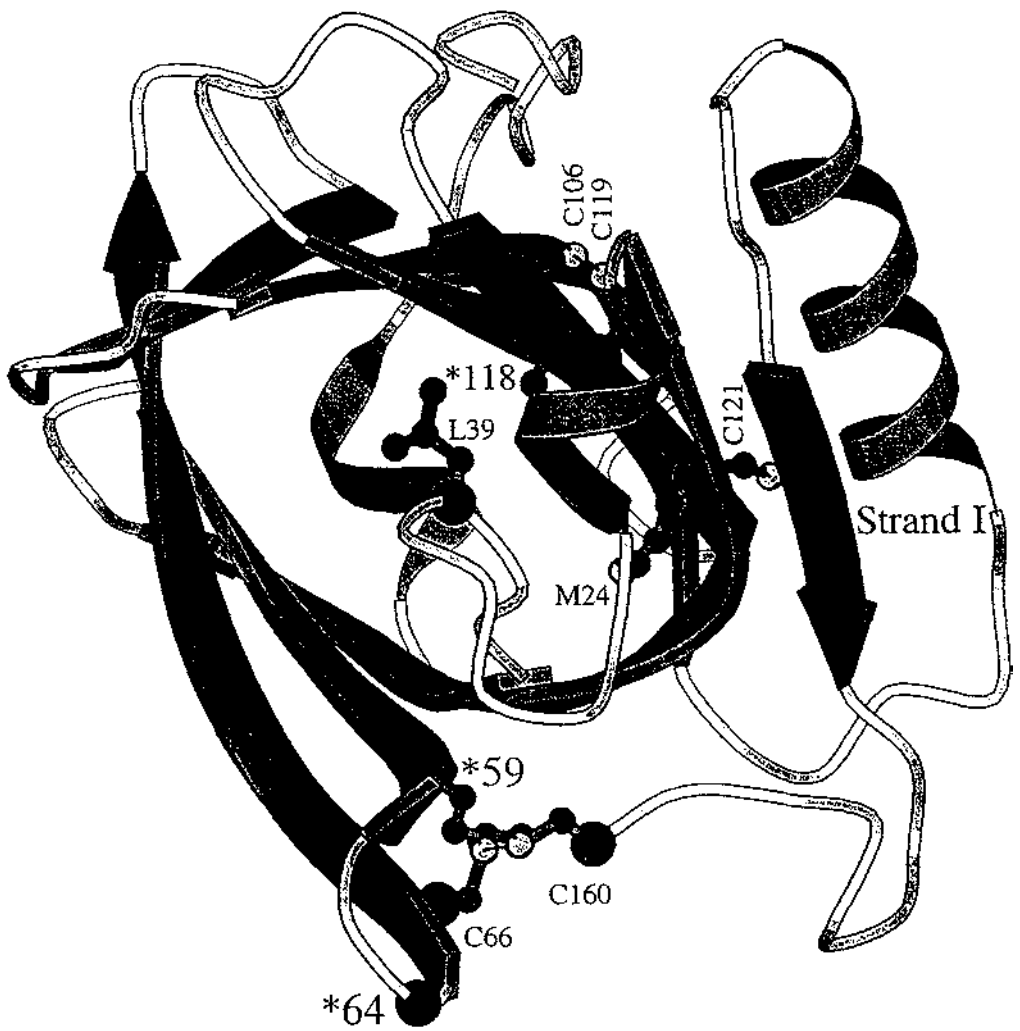

Figure 2.2.2.2. Molscript diagram of a single subunit of β-lg B. View looking down into the calyx. The variable residues among variants A, B and C are denoted *59, *64 and *118. Side chains Met-24 and Leu-39 move on the substitution Ala118Val in variant A. Cys-121 and cysteines in disulphide bonds are labelled (Bewley et al., 1997).

2.2.3. Binding properties of β-lactoglobulin

β-Lg is classified as a member of the lipocalin superfamily (Flower, 1996). Structural studies of typical lipocalins, e.g. retinol-binding protein (Newcomer et al., 1984), bilin binding protein (Huber et al., 1987), insecticyanin (Holden et al., 1987) and bovine β-lg (Papiz et al., 1986), show that these transporters of hydrophobic molecules share a common topology comprising an eight-stranded β-barrel with an α-helix; the barrel forms a calyx-shaped structure. Although the interior cavity is clearly specific for the ligand, the surface loops confer specificity for the receptor: the protein thus has dual molecular recognition properties, the external surface near the mouth of the calyx defining the address to which the ligand is to be delivered.

Most lipocalin molecules have a clear biological role, ligand-carrier, but the biological role of β-lg is not well understood. Some of the biologically important ligands in milk are the free fatty acids, which are prevalent, retinol, haemin and protoporphyrin IX, all have been shown to bind to bovine β-lg with relatively high affinity (Hambling et al., 1992; Sawyer, 2000), i.e. palmitic acid with a binding constant of 6.8×10^5 M^{-1} (Spector and Fletcher, 1970) and retinol with a binding constant of $5.0 \times 10^7 M^{-1}$ (Fugate and Song, 1980). Besides these physiologically important hydrophobic molecules, a variety of small hydrophobic molecules can be bound to β-lg with lower affinities (Sawyer, 2000).

As β-lg is able to bind fatty acids and to increase the activity of pregastric lipase, it has also been claimed that ruminant β-lgs can participate in fat digestion by the neonate (Perez et al., 1992). Bovine β-lg, purified by neutral-pH methods, has been found to even contain bound fatty acids (Perez et al., 1989). It has been also reported that binding palmitic acid to β-lg increased its conformational stability to trypsin degradation (Puyol et al., 1993) and thermal denaturation (Puyol et al., 1994), but no major differences found for retinol bound β-lg. Creamer (1995) also demonstrated enhanced stability of the protein to urea denaturation when complexed with either palmitic acid or a low concentration of sodium dodecyl sulphate (SDS). With its remarkable acid stability and similar topology to retinol-binding protein and even fatty-acid-binding protein (e.g. human tear lipocalins; Gasymov et al., 1998), β-lg has been postulated to serve as a

carrier for retinol or some other small hydrophobic molecules in neonates (Hambling et al., 1992).

Wu et al. (1999) and Sawyer et al. (1999) suggested that there are at least two independent ligand-binding sites per β-lg monomer; the central calyx, which binds in a similar manner to that of human retinol-binding protein, and an external channel, which is a solvent-accessible hydrophobic cleft located between the three-turn α-helix that is packed against the outer surface of the β-barrel and the β-barrel itself (Fig. 2.2.3.1).

A number of solution studies have been interpreted in terms of external binding sites, between the three-turn α-helix and the β-sheet (Futterman and Heller, 1972; Dufour et al., 1994). Frapin et al. (1993) and Narayan and Berliner (1997, 1998) suggested that retinol was possibly bound within the β-barrel of β-lg and that other ligands might be bound to the external site based on tryptophan fluorescence results, which suggested that fatty acids could bind to the β-lg/retinol complex. In contrast, Creamer (1995) concluded that the protein must bind fatty acids within the β-barrel or overlapping the retinol-binding site.

Earlier Monaco et al. (1987), based on a 2.5 Å structure, proposed that a retinol-binding site for β-lg was an external, solvent-accessible hydrophobic cleft located between the three-turn α-helix that is packed against the outer surface of the β-barrel and the β-barrel itself. They suggested that this model involved two key residues, Phe-136 and Lys-141 on the outside of the calyx; however, this model is incorrect, i.e. Phe-136 and Lys-141 are part of the helix (Qin et al., 1998a). Site mutation experiments, F136A and K141M, also did not support this idea (Cho et al., 1994). Because of the errors in the β-lg model of Monaco et al. (1987), there is no substantive evidence that retinol binds in the manner described.

The alternative model was based on the 2.8 Å tertiary model of β-lg (Papiz et al., 1986), which revealed the folding similarity between β-lg and retinol-binding protein. By direct analogy to the way in which retinol-binding protein binds retinol, retinol was fitted into the centre of the β-lg calyx. Site-directed mutations of β-lg (Cho et al., 1994; Katakura et al., 1994) have been made and studied to test this model. The site mutation W19A (Cho et al., 1994) indicated that Trp-19 is one of the invariant residues and so is

important in the structural stability of β-lg, whereas the site mutation W19Y (Katakura et al., 1994) showed only a slight change in the dissociation constant. Cho et al. (1994) also reported that Lys-69 could interact with the hydroxyl group of retinol.

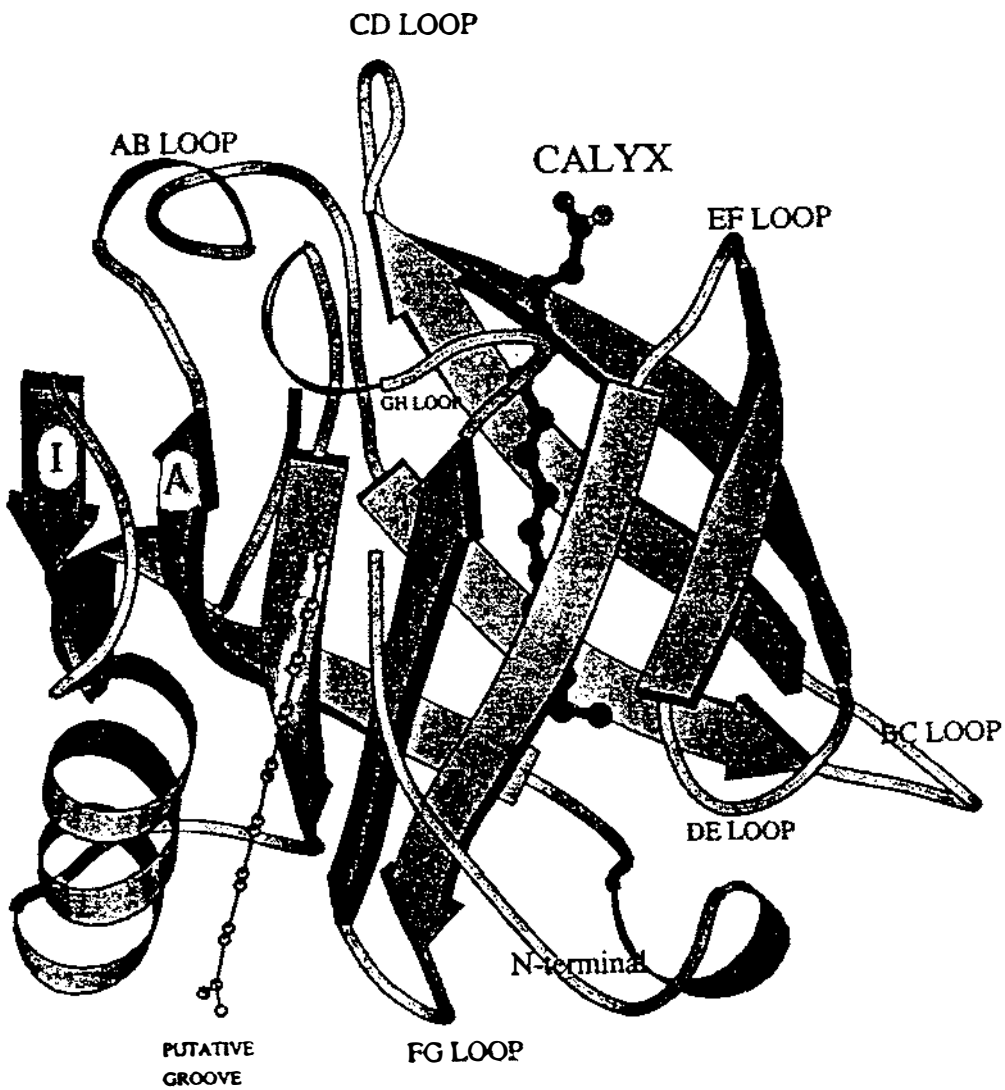

Figure 2.2.3.1. A general view of β-lg, a typical lipocalin. The binding site (*filled* atoms) is shown in the central calyx, and the putative binding site (*open* atoms) is indicated on the outer surface of the protein. The structurally conserved regions are at the rear of the molecule on strand A, the FG loop and the loop before the α-helix (Wu et al., 1999).

Until recently there has been no reliable crystallographic evidence for any binding site, either internal or on the β-lg surface. However, Qin et al. (1998a) showed 12-bromododecanoic acid bound inside the calyx of β-lg in an X-ray crystal structure at a resolution of 2.23 Å in the trigonal lattice Z form, and Wu et al. (1999) also found palmitic acid in the calyx and the carboxyl group bound to both Lys-60 and Lys-69 at the entrance to the cavity in a similar manner to the binding of retinol to the related lipocalin (retinol-binding protein). Ragona et al. (2000) also revealed that at neutral pH palmitic acid is bound within the central calyx of β-lg by NMR spectra. Furthermore, Kontopidis and Sawyer (2000) showed that retinol was within the central hydrophobic cavity of bovine β-lg; this clarified the speculation about the most probable binding site for small hydrophobic molecules.

The possibility of an external binding site for some ligands cannot be excluded. Ligand binding to β-lg is sensitive to extremes of pH: as the pH is increased, β-lg becomes very negatively charged, and electrostatically a less inviting host for negatively charged fatty acid species (Qin et al., 1998a).

Two subgroups have been identified in small-hydrophobic-molecule-binding proteins (Flower et al., 1993; Banaszak et al., 1994). One subgroup, known as *e*LBPs (*extra*-cellular lipid-binding proteins) (Banaszak et al., 1994) or lipocalins, forms the calyx or cup-shaped binding site (Flower et al., 1993), and includes β-lg, retinol-binding protein and bilin-binding protein. The structures of at least six proteins (Banaszak et al., 1994) of this subgroup have been determined. They all have an eight-stranded β-barrel with an associated three-turn helix and are structurally very similar to retinol-binding protein although the amino acid sequences are not especially similar (Flower et al., 1993). The proteins of the other subgroup, known as *i*LBPs (*intra*-cellular lipid-binding proteins) (Banaszak et al., 1994), include the retinoid and fatty-acid-binding proteins. In each case, the protein has the same basic three-dimensional structure, with a ten-stranded, slightly flattened β-barrel and two small helices, which may have a portal function, at one end of the barrel (Flower et al., 1993; Banaszak et al., 1994). Despite the differences between the proteins of the *e*LBP group and the *i*LBP group, with the high specificity of the binding site, some insight into the way in which ligands as diverse as retinol, SDS, phenyl

phosphate, hexane and palmitic acid bind to β-lg (Hambling et al., 1992) suggests that β-lg has a generalised binding function rather than a specific binding function.

It has been concluded that, for the *i*LBP group of proteins, the β-barrel is structurally stable and does not depend on the presence of a ligand. This is most probably because the barrel is held intact by a combination of hydrophobic and hydrogen bonding interactions between the side chains lining the inside of the β-calyx (or flattened β-barrel). Thus, replacement of a hydrophobic ligand within the β-calyx with water does not involve a large energy change. Specificity of binding seems to be related to the presence of a particular Arg residue in the case of cellular retinoic-acid-binding proteins (CRABPs, Banaszak et al., 1994) and fatty-acid-binding proteins (FABPs, Sacchettini and Gordon, 1993). For example, changing this Arg to Gln, which is present in some cellular retinol-binding proteins (CRBPs), in rat intestinal FABP diminished the binding of fatty acids but increased the binding of retinol and retinal (Jakoby et al., 1993). A converse modification of a Gln in CRABP-II to Arg diminished retinol binding and increased fatty acid binding but not retinoic acid binding.

2.3. CONFORMATIONAL CHANGES OF β-LACTOGLOBULIN IN SOLUTION

2.3.1. Self-association of β-lactoglobulin

The self-association behaviour of β-lg is complicated, and is dependent on several parameters such as pH, temperature, protein concentration and ionic conditions. In addition, the association properties differ between the genetic variants of the protein. In Fig. 2.3.1.1, the effects of pH and temperature are shown schematically.

The association-dissociation behaviour of β-lg has been studied over the pH range from pH 3.5 (Townend and Timasheff, 1960) to pH 8 (McKenzie and Sawyer, 1967) at room temperature. The dimer is dominant in solution at the concentration at which β-lg is present in bovine milk (approximately 3 g/L) (Walstra and Jenness, 1984), but the proportion of dimer increases with increasing protein concentration (Timasheff and

Townend, 1962). The monomer-dimer equilibrium shifts in the direction of monomers as the temperature of β-lg solutions at pH 7.0 is increased, and as the ionic strength of β-lg is decreased (Aymard et al., 1996).

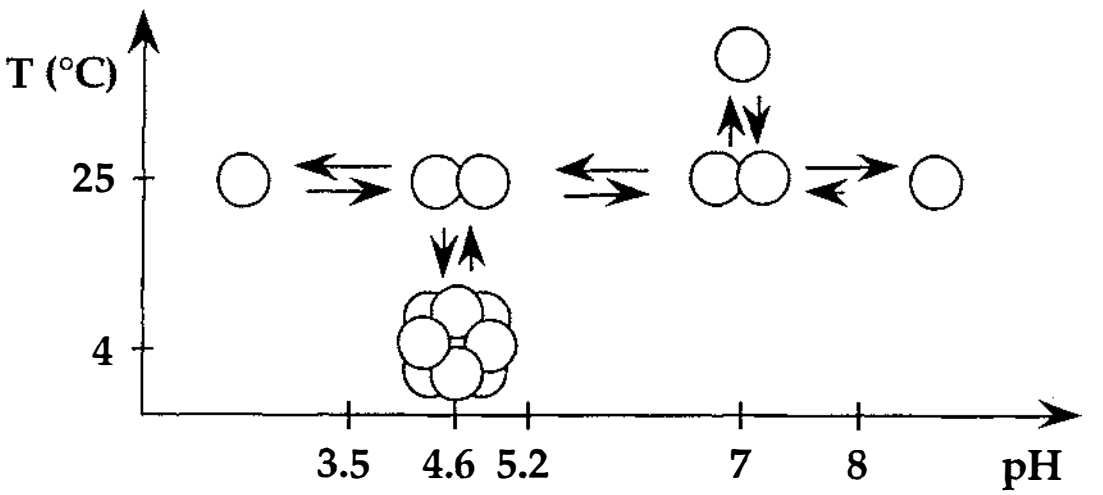

Figure 2.3.1.1. Illustration of the self-association behaviour of β-lg as a function of pH and temperature (Elofsson, 1996).

2.3.2. pH-induced reversible conformational changes of β-lactoglobulin

During an increase in pH from 4.0 to 7.8, β-lg undergoes two reversible conformational transitions: Q ↔ N ↔ R → S. The first reversible transition (Q ↔ N) occurs between pH 4.0 and pH 6.0; β-lg shrinks slightly in volume and the sedimentation coefficient increases a little. This transition is thought to arise as a consequence of the deprotonation of aqueous carboxyl groups (Timasheff et al., 1966). Between pH 6.5 and pH 7.8, β-lg undergoes a second reversible transition (N ↔ R), called the Tanford transition. Above pH 9, the protein molecule undergoes irreversible conformational changes.

Tanford et al. (1959) found that the unexpected titration behaviour of β-lg in the pH range 6-8 arose from the exposure of a buried COOH group at alkaline pH, which did

not titrate at the expected pH of about 4.5-5.5. Groves et al. (1951) reported an increase in optical laevorotation of β-lg with increasing pH, and Dunnill and Green (1965) found that the free sulphydryl group of β-lg could react with reagents containing Hg^{2+} much more readily above pH 6.7. In the crystal structures of β-lg A of Qin et al. (1998b), the number of hydrogen bonds formed by Glu-89, the conformation of loop EF (Fig. 2.3.2.1, Creamer et al., 2000) and the length of β-strand F were affected by the pH of the solutions in which the crystals were grown and clearly defined the structural basis of the Tanford transition.

2.3.3. Reversible unfolding of β-lactoglobulin

Pace and Tanford (1968) reported that, between pH 2.5 and pH 3.5, the unfolding of β-lg in urea was reversible. McKenzie and Ralston (1973) reported that the thiol-disulphide interchange reaction occurred when β-lg was unfolded in 7 M urea at pH 3.5 and pH 5.2. However, below pH 3.5, the protonated thiol groups are less likely to participate in thiol-disulphide interchange reactions, and consequently low pH structural changes are usually reversible. If thiol-disulphide interchange reactions do not occur, then equilibrium between native and non-native β-lg can be established because the irreversible aggregations do not occur. Similarly, Anantharamayanan et al. (1977) confirmed that the extent of thiol-disulphide interchange was minimal at pH values below 2.

2.3.4. Thermal unfolding and aggregation of β-lactoglobulin

2.3.4.1. Thermal unfolding of β-lactoglobulin

Most of the forces stabilising the native structure of globular proteins are ruptured during denaturation, resulting in new and unidentified, but predominantly random coil structures. As the three-dimensional conformation of the protein is only marginally balanced by the stabilising forces, the change in state of the structural elements does not occur independently (Privalov and Gill, 1988). This co-operativity can be confined to only a part of the molecule (partial unfolding) or can give rise to an "all or none" reaction, i.e. a two-state denaturation (Brandts, 1967).

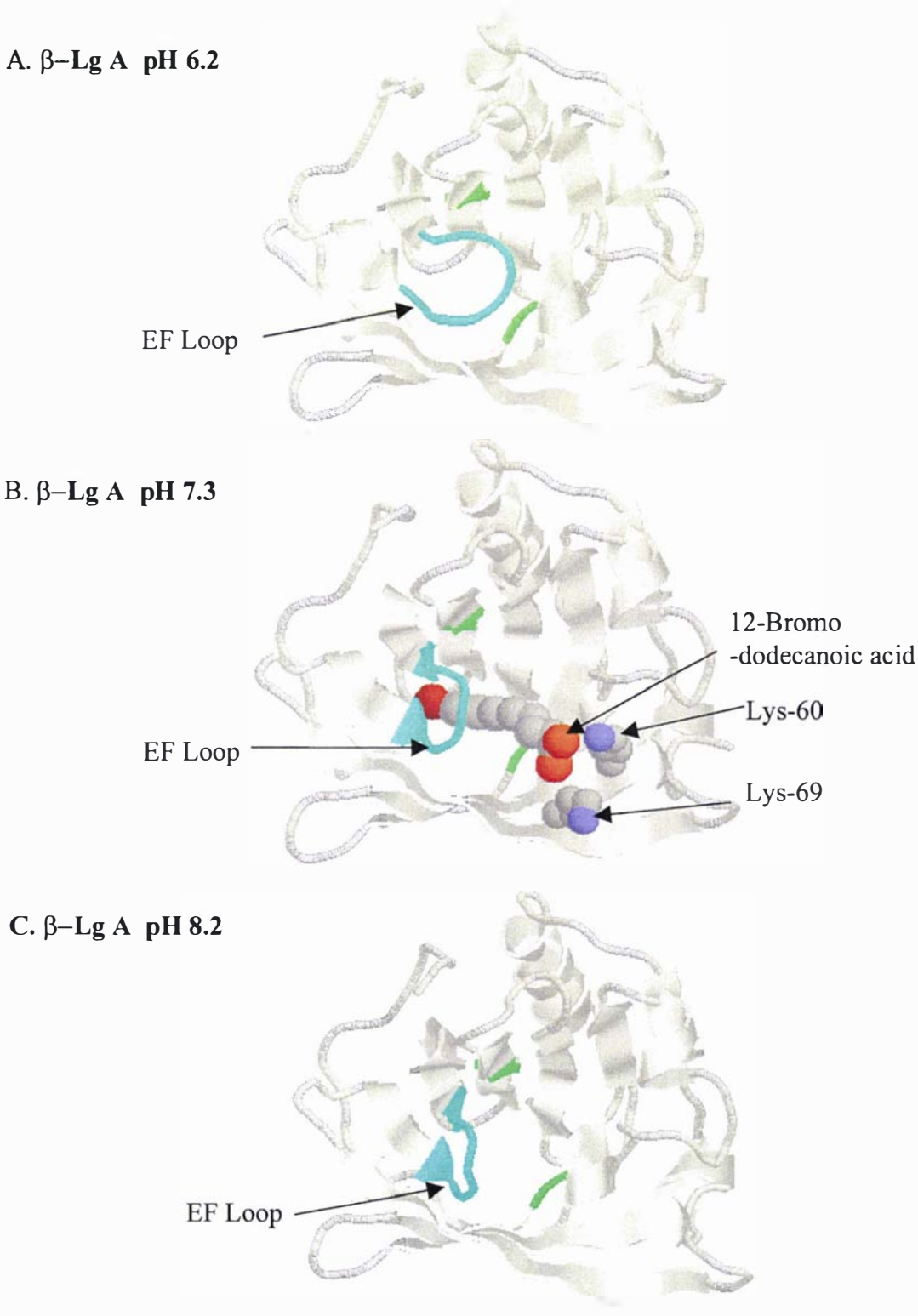

Figure 2.3.2.1. Secondary structural features of β-lg A in lattice Z at (A) pH 6.2; Loop EF (blue) covers the entrance to the calyx cavity. (B) pH 7.3; Loop EF flips to expose the interior of the calyx. 12-Bromododecanoic acid has been fitted into the calyx cavity. (C) pH 8.2; same orientation as for pH 6.2 and 7.3. Loop EF flips (Creamer et al., 2000).

A conformational state, with structural and dynamic properties intermediate between those of the native and completely unfolded states, a so-called molten globule state, has been found for some globular proteins under certain conditions by Kuwajima (1989). This state is suggested to have native-like secondary structure and a loosely ordered tertiary structure and may occur as a stable intermediate during the unfolding process (Qi et al., 1997).

β-Lg denaturation can be brought about by various treatments, such as alkali, heat, organic compounds or heavy metals (Hambling et al., 1992). The denaturation mechanisms resulting from these treatments are not clear, although the initial stages of denaturation by alkali and by heat at neutral pH seem to follow the same path (Casal et al., 1988). Kuwajima et al. (1987) and Cairoli et al. (1994) have suggested that a molten-globule-like state may represent an intermediate structure in the thermal denaturation of β-lg.

The effect of heat treatment on the structure of β-lg at neutral pH has been studied extensively using several spectroscopic techniques (Kella and Kinsella, 1988; Griffin et al., 1993; Qi et al., 1995, 1997; Bauer et al., 1998; Manderson et al., 1999a, b; Schokker et al., 1999). Heat-induced structural change in the vicinity of tryptophan side chains has been studied using near-UV CD spectroscopy (Griffin et al., 1993; Qi et al., 1997; Manderson et al., 1999b), intrinsic protein fluorescence (Mills, 1976; Iametti et al., 1996; Manderson et al., 1999a) and UV difference spectroscopy (Griffin et al., 1993).

It has been reported that the intensities of CD spectral bands, particularly at 287 and 293 nm, decrease with increasing temperature, which is an indication of a change in tertiary structure, particularly in the vicinity of the tryptophan side chains, i.e. Trp-19 and Trp-61 (Arakawa, 1989; Griffin et al., 1993; Manderson et al., 1999b). In the crystal structure of Brownlow et al. (1997), Trp-19 is surrounded by a greater number of side chains than Trp-61. Therefore, the contribution from Trp-19 to the near-UV CD spectrum is greater than that from Trp-61.

Mills (1976) reported that the fluorescence λ_{max} (fluorescence peak position) of the emission spectrum of β-lg was red shifted from 328 to 338 nm and the width of the emission peak at half height increased as the temperature increased. As the crystal structure (Brownlow et al., 1997; Qin et al., 1998b) indicates that Trp-61 is close to the

disulphide bond Cys66-Cys160, the extent of quenching of the fluorescence from Trp-61 is expected to be greater than that from Trp-19, which is located in a more hydrophobic environment. Mills (1976) suggested that the heat-induced increase in tryptophan emission intensity and the shift in λ_{max} are consistent with an increase in the solvent accessibility of tryptophan side chains and removal of the quenching group. Furthermore, reactions that involve the thiol group of β-lg occur at elevated temperatures (Gough and Jenness, 1962; Watanabe and Klostermeyer, 1976). Because the thiol group of Cys-121 is solvent inaccessible in native β-lg, these results suggest that structural changes in the vicinity of this side chain, which is a considerable distance from both Trp-19 and Trp-61, occur during heat treatment.

The far-UV CD results of Sawyer et al. (1971), Lapanje and Poklar (1989) and Griffin et al. (1993) indicated how the β-sheet structure of β-lg is affected by heat treatment. Sawyer et al. (1971) reported that the amount of β-sheet increased as a consequence of heat treatment, whereas Griffin et al. (1993) reported that the amount of β-sheet did not change appreciably during heat treatment. In contrast, Lapanje and Poklar (1989) suggested that β-lg possessed less β-sheet at elevated temperatures than at room temperature. Prabakaran and Damodaran (1997) reported from their far-UV CD results that the α-helix content was approximately 19 % for β-lg A and B and was not affected by temperatures between 26 and 81 °C and that the β-sheet content of β-lg A decreased from 60 % to 20 % at 81 °C, whereas the decrease for β-lg B was significantly less. However, Qi et al. (1997), using CD results extending to 170 nm together with Fourier transform IR results, concluded that the α-helix content decreased from about 11 % to about 2 % (with the midpoint transition at approximately 60 °C) and that the β-sheet content decreased from 50 % to 42 % (with the midpoint transition at approximately 50 °C) as a consequence of increased temperatures of β-lg A. Manderson et al. (1999b) found similar spectra patterns for β-lgs A, B and C both before and after heat treatment and suggested that the conformation protein backbone is most probably quite similar for all three variants.

2.3.4.2. Thermal aggregation of β-lactoglobulin

Several different mechanisms have been proposed and developed for the heat-induced aggregation of β-lg (McKenzie, 1971; Mulvihill and Donovan, 1987; Griffin et al., 1993; McSwiney et al., 1994b; Roefs and de Kruif, 1994; Qi et al., 1995, 1997; Elofsson et al., 1996; Elofsson et al., 1996; Iametti et al., 1996; Gezimati et al., 1997; Schokker et al., 1999). The unfolding of the protein, resulting in exposure and activation of the free sulphydryl group, is regarded as the rate-limiting step in the aggregation process by Roefs and de Kruif (1994).

A model with an overall reaction order of 1.5 for the aggregation of β-lg has been presented by Roefs and de Kruif (1994). The model proposes, in analogy with polymer radical chemistry, an initiation, a propagation and a termination step. The initiation reaction is suggested to be a first-order reaction in which the free sulphydryl group of β-lg becomes reactive. The propagation step in the model corresponds to the build-up of aggregates proceeding *via* disulphide interchange reactions. Termination occurs when two activated molecules meet and a disulphide bond is formed. This model accounts for the build-up of linear aggregates, suggesting that only one of the disulphide bridges in the monomeric form of β-lg can take part in the interchange reactions. It is also clear that intermolecular disulphide bridges are important in the aggregation of β-lg (Sawyer, 1968; Parris et al., 1993; Elofsson, 1996).

McSwiney et al. (1994a) showed that both non-covalently-linked and disulphide-linked aggregates of β-lg are formed during heat treatment. They concluded that the non-covalently-linked aggregates are probably intermediates during the β-lg aggregation pathway because only large disulphide-linked aggregates remained after extensive heating.

From the results obtained in their differential scanning calorimetry (DSC) study, Qi et al. (1995) suggested that the concentration of β-lg A influences whether unfolding occurs from the dimeric (above 25 mg/mL) or monomeric (less than 25 mg/mL) state during heat treatment. Qi et al. (1997) published a model for the aggregation of β-lg based on CD and IR spectroscopy results, which is similar to that of Griffin et al. (1993). They found that the proportion of β-sheet in β-lg decreases with increasing temperature

and suggested that β-lg forms a molten-globule-like species, which is more susceptible to aggregation than the native counterparts, at elevated temperature.

Heat-induced structural changes in β-lg A/B have been studied by Iametti and co-workers (Cairoli et al., 1994; Iametti et al., 1995, 1996). They found that, during a temperature increase from room temperature to 70 °C, dimers dissociate to monomers, the thiol group and the side chains of tryptophan residues become solvent accessible, the intensity of fluorescence from bound ANS increases and near-UV CD spectra suggest that the tertiary structure of β-lg changes. They suggested that the observed increase in fluorescence emission intensity from bound ANS is indicative of an increase in the solvent accessibility of the hydrophobic side chains of β-lg and that this may occur as a consequence of a loosening or "swelling" of the β-lg structure. Furthermore, all of the above changes, except possibly thiol group exposure, are reversible upon cooling from elevated temperatures as high as 70 °C down to room temperature.

From sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography results, Iametti et al. (1996) also reported that both disulphide-linked and non-covalently-linked aggregates of β-lg A/B are formed at temperatures greater than 70 °C. In addition, a correlation between rates of hydrophobic collapse and aggregate formation was suggested. They also suggested that, during heat treatment, β-lg monomers initially associate to form non-covalently-linked aggregates, and that thiol-disulphide interchange reactions lead to the stabilisation of these aggregates. They proposed that dimer dissociation is made evident by the exposure of a reactive –SH group, which is normally buried at the dimer interface and becomes accessible upon heating. However, the free sulphydryl group is located on the outside of the β-barrel and largely buried beneath the three-turn α-helix, and the exposure of sulphydryl group occurs after dimer dissociation on heating. Therefore, initial non-covalently-linked association of β-lg monomers cannot be prerequisite for thiol-disulphide interchange reactions.

Elofsson (1996) and Elofsson et al. (1996) used *in situ* dynamic and static light scattering to follow the increase in the size of β-lg aggregates during the heat treatment of solutions at pH values between 6.50 and 6.94. They found a lag phase, during which no

increase in hydrodynamic radius occurred, and the time lag was found to increase with heating temperature, pH and concentration. A comparison of the aggregation behaviour of β-lg A and β-lg B was also made and they suggested that the different aggregation rates between the two variants could be related to a different co-operativity in the thermal unfolding behaviour. Furthermore, they suggested that the smallest unit in the aggregates was monomeric for the A variant and dimeric for the B variant and that aggregation involved intermolecular disulphide interchange reactions.

Elofsson et al. (1996) used microcalorimetry to identify the T_{max} (the maximum in the thermogram) values of the different events that occur when solutions of β-lg are heat treated. They suggested that β-lg aggregation proceeds via two transitions and that the first and second transitions correspond to β-lg unfolding and thiol activation, respectively. They also suggested that, because the T_{max} for β-lg unfolding is lower than that for thiol activation, their results are consistent with those of McSwiney et al. (1994a), which indicate that the rates of loss of monomeric β-lg during heat treatment are greater than those for the formation of disulphide-linked aggregates.

After dimer dissociation and then thiol exposure in some β-lg monomers, McSwiney et al. (1994a) and Gezimati et al. (1997) postulated that there is a conversion of β-lg monomers to molten-globule-like species and the subsequent formation of non-covalently-linked aggregates. These non-covalently-linked aggregates may then be converted to disulphide-linked aggregates via either of two pathways. The thiol group of a monomeric β-lg molecule becomes solvent exposed and catalyses thiol-disulphide interchange reactions in non-covalently-linked aggregates or the thiol exposure may occur from within non-covalently-linked aggregates, initiating the conversion of these species to disulphide-linked aggregates (Manderson et al., 1998).

Recently, using size-exclusion chromatography in combination with multi-angle laser light scattering, Schokker et al. (1999) found that unfolding of β-lg native monomers resulted in the formation of reactive monomers. These monomers formed non-native dimers, via thiol-disulphide exchange, thiol-thiol oxidation and to a lesser extent non-covalent interactions, which reacted further to form a wide range of different oligomers.

Dimer dissociation at neutral pH occurs as a consequence of minimal structural changes, breaking the hydrogen bond network at the dimer interface (Brownlow et al., 1997). Iametti et al. (1996) stated that these apparently modest modifications result in increased exposure of the thiol group of Cys-121 and of adhesive hydrophobic surfaces, and these latter events represent one of the earliest steps in the thermal modification of β-lg. In proteins containing both cystine and cysteine, it has been observed that thiol-disulphide interchange reactions can occur in the molten globule state, resulting sometimes in almost randomised disulphide bonding (Creighton and Ewbank, 1994). It is proposed that β-lg forms the molten globule state at 65-70 °C and it is also known (Sawyer et al., 1963; Phelan and Malthouse, 1994) that thiol-catalysed disulphide bond interchange can occur readily in β-lg so that intermolecular aggregation can be expected during heat treatment.

In all of the models discussed above, dimer dissociation is assumed to be the first step in the aggregation pathway of β-lg. All of the aggregation models include structural changes in β-lg monomers after dimer dissociation. Studies in which heat-induced structural changes and aggregation were examined suggest that β-lg is converted to a molten-globule-like species at elevated temperatures (McSwiney et al., 1994a; Iametti et al., 1996; Qi et al., 1997; Gezimati et al., 1997). This species appears to possess no α-helix and slightly less β-sheet structure than native β-lg (Qi et al., 1997), and may be more hydrophobic than the corresponding native species (Iametti et al., 1996).

However, in the development of aggregates, Roefs and de Kruif (1994) assumed that the solvent exposure of the thiol group of partially unfolded β-lg monomers catalyses thiol-disulphide interchange reactions, leading to the formation of disulphide-linked aggregates. In other models, it is suggested that non-covalently-linked aggregates are initially formed from molten-globule-like species and that thiol-disulphide interchange reactions lead to the stabilisation of these aggregate species (McSwiney et al., 1994a; Iametti et al., 1996; Qi et al., 1997; Manderson et al., 1998). The formation of disulphide-linked aggregates of β-lg may also occur via both thiol-disulphide interchange reactions and association of molten-globule-like species, as suggested by McSwiney et al. (1994a) and Gezimati et al. (1997).

2.3.4.3. Comparison of thermal susceptibilities of β-lactoglobulin variants

As well as exhibiting differences in electrophoretic mobility and dissociation properties, the susceptibility of β-lgs A, B and C to thermal denaturation also differs. McKenzie (1971) reported that only β-lg A can form octamers under conditions of low temperature, higher concentrations and pHs close to the iso-ionic point; under these conditions, the other variants remain in the dimeric form. Hillier and Lyster (1979) studied thermal denaturation using PAGE to measure the soluble whey proteins remaining after heat treatment of skim milk and cheese whey. The results indicated that β-lg A was more resistant to thermal denaturation than β-lg B. When milk samples were heated to temperatures below 95 °C, the gel band intensities were larger for β-lg A than for β-lg B. This agrees with the results of Gough and Jenness (1962) and Sawyer et al. (1971). Gough and Jenness (1962) showed that β-lg B apparently unfolded more than β-lg A at 73 °C as measured by optical rotation, thiol availability and solubility at pH 5.0. Sawyer et al. (1971) used a turbidimetric method to show that, at 6 mg/mL in dilute tris-citrate buffer at pH 7.5, β-lg C solution was most turbid and β-lg A solution was least turbid after heating at 97.5 °C for 10 min. However, larger gel band intensities in SDS-PAGE were observed for β-lg B above 95 °C by McSwiney et al. (1994a), which agrees with the results of Hillier and Lyster (1979).

Manderson et al. (1998, 1999a, b) found that β-lg C denatured more slowly at 40 to 94 °C than β-lgs A and B using PAGE and various spectroscopic techniques and explained the differences in the behaviour of three variants caused by generalised electrostatic and hydrophobicity effects as well as specific amino acid effects. They also reported that the denaturation of β-Lg A was more like that of β-lg C at higher temperatures (approximately 90 °C), but more like that of β-lg B at lower temperatures. Phillips et al. (1967) reported that, at pH 7.5 and presumably at about 20 °C, the availability of the thiol was low and the order of reactivity was β-lg A ≈ β-lg B >> β-lg C and that, when SDS was bound to the proteins, the thiol reactivity was further decreased and the order was β-lg B > β-lg A > β-lg C. Thus β-lg C appears to be consistently less reactive.

2.4. EFFECT OF HEAT TREATMENT ON MIXTURES OF WHEY PROTEINS

The heat-induced interactions between β-lg, α-la and BSA have been studied in model systems (Hines and Foegeding, 1993; Matsudomi et al., 1994; Gezimati et al., 1996, 1997; Havea et al., 1998, 2000; Schokker et al., 2000). Hines and Foegeding (1993) found that the rate of loss of native β-lg from solutions heated at 80 °C increased in the presence of BSA, indicating some synergistic effect. Matsudomi et al. (1994) found that, when mixtures of BSA and β-lg were heated at 80 °C and examined at room temperature, the gels from the mixtures were stronger, again suggesting a synergistic effect.

The heat-induced aggregation and gelation of 10 % (w/w) solutions of a 1:1 mixture of β-lg and BSA (Gezimati et al., 1996) or β-lg and α-la (Gezimati et al., 1997) in a buffer that mimicked the whey protein concentrate (WPC) environment indicated that both covalent and non-covalent bonding were involved. Gezimati et al. (1996, 1997) suggested that BSA formed polymers prior to the unfolding of either α-la or β-lg at moderate temperature (75 °C). In a mixed BSA/β-lg system there was no obvious heat-induced interaction between the two proteins and it was suggested that β-lg probably unfolded and formed some sort of adduct with the BSA polymers. However, when a β-lg and α-la mixture was heated, it was concluded that these proteins interacted to form heterogeneous aggregates. Furthermore, Havea et al. (2000) examined the heat effects in 10 % (w/w) solutions of a 1:1 mixture of α-la and BSA in WPC permeate on the protein aggregation using PAGE and found that large disulphide-bonded aggregates and SDS-monomeric BSA and α-la were present. They suggested that BSA formed disulphide-bonded aggregates that contained available thiol groups that could catalyse the formation of differently structured α-la monomers, dimers, and higher polymers and adducts of α-la with BSA.

The heat-induced interactions between β-lg, α-la and BSA have also been studied extensively in whey and milk systems (Hillier and Lyster, 1979; Dannenberg and Kessler, 1988a, b; Havea et al., 1998). The mechanisms of aggregation in commercial WPC could

be quite different from those in these model systems which often do not contain glycomacropeptide, minor proteins, uncharacterised and diverse high molecular mass material, lactose or different levels of mineral components. However, Havea et al. (1998) showed that, when WPC solutions were heated at 75 °C, aggregates consisting of β-lg, α-la, BSA, caseins and minor proteins were formed and that both hydrophobic interactions and disulphide cross-linkages were involved in forming these aggregates, as suggested by model studies using binary mixtures of these proteins (Gezimati et al., 1996, 1997). Particularly at concentrations below 3 % (w/w), there was evidence when using two-dimensional (2D) PAGE of disulphide-bonded dimeric forms of α-la and of non-covalent aggregates involving α-la (Havea et al., 1998).

2.5. HEAT-INDUCED INTERACTIONS BETWEEN β-LACTOGLOBULIN AND κ-CASEIN

2.5.1. Effect of heat on individual caseins

Whole bovine casein comprises four major caseins, namely, α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN, and each has different properties. α_{s1}-CN consists of 199 amino acids, eight of which are phosphoserine. It shows strong self-association behaviour and is readily precipitated at low (4-5) pH or with calcium salts at neutral pH. α_{s2}-CN consists of 207 amino acids with 3-10 phosphoserines. It shows strong self-association behaviour and will precipitate at lower calcium chloride concentrations than α_{s1}-CN. β-CN consists of 209 amino acids, five of which are phosphoserine. It is readily precipitated at low pH or with calcium salts at neutral pH and at higher temperatures (> 8 °C). Under physiological conditions, α_{s1}-CN associates more strongly with β-CN than either protein self-associates (Creamer, 1991; Swaisgood, 1992). The characteristics of κ-CN are discussed in Section 2.5.2.

Heat treatment had little effect on the number- or weight-average molecular weight or on the gyration radius of whole casein; when individual caseins (α_s-CN, β-CN and κ-CN) were studied separately, only κ-CN showed any marked increase in molecular

weight and radius of gyration on heating at 90 °C (Kresheck et al., 1964). Guo et al. (1989) measured the turbidities of aqueous solutions of sodium caseinate, pH 7.0, after heating at temperatures of 120-150 °C for 60 min. The extent of aggregation, as indicated by an increase in turbidity, increased with increasing severity of heating to a maximum at 140 °C but decreased at higher temperatures, possibly indicating thermal degradation of the caseins. β-CN aggregated strongly on heating at 140 °C, but the aggregation appeared to be reversible on cooling to 0 °C. Changes in the gel electrophoresis patterns of sodium caseinate after heating (Fox, 1981; Guo et al., 1989) suggested that α_{s2}-CN and κ-CN (i.e. the –SH-containing caseins) are more susceptible to aggregation at high temperatures than α_{s1}-CN and β-CN.

2.5.2. Characterisation of κ-casein

2.5.2.1. Structure of κ-casein

κ-CN from bovine milk has a monomer molecular weight of 19,000 Da, contains two cysteine residues (Cys-11 and Cys-88) and occurs as a phosphoglycoprotein, displaying microheterogeneity with respect to phosphate and carbohydrate (Eigel et al., 1984). The naturally occurring κ-CN is cross-linked, apparently randomly, through -S-S- linkages to subunits containing three to eight monomers (Fig. 2.5.1.1, Creamer and MacGibbon, 1996). When purified from milk, κ-CN has a unique disulphide bonding pattern that can be detected by SDS-PAGE in the absence of reducing agents (Groves et al., 1991; Farrell et al., 1998). The molecular weight ranges from monomer to octamer and above and appears to be rather evenly distributed across this range. Rasmussen et al. (1992) reported an apparently random distribution of disulphides in these polymers involving Cys-11 and Cys-88 (88-88, 11-11 and 11-88).

κ-CN seems to be of crucial importance in stabilising the micelle (Walstra and Jenness, 1984; Creamer, 1991; Creamer et al., 1998). On the surface of the micelle, κ-CN functions as an interface between the hydrophobic caseins of the micelle interior and the aqueous environment. κ-CN is also involved in thiol-catalysed disulphide interchange reactions with whey proteins during heat treatment, and, after rennet cleavage, in the facilitation of micelle coagulation. One of the important factors cited is

that chymosin cleavage of κ-CN gives two large peptides of very different properties. Para-κ-CN (105 amino acids) is insoluble, carries a net positive charge at pH 7 and strongly associates with the calcium-sensitive caseins, whereas the macropeptide (64 amino acids) is soluble, carries a net negative charge and occupies a greater volume in buffer than in SDS solution (Creamer, 1991). Addition of chymosin to milk causes the micelles to coagulate as the κ-CN is converted to para-κ-CN, essentially because of the changes to the micelle surface.

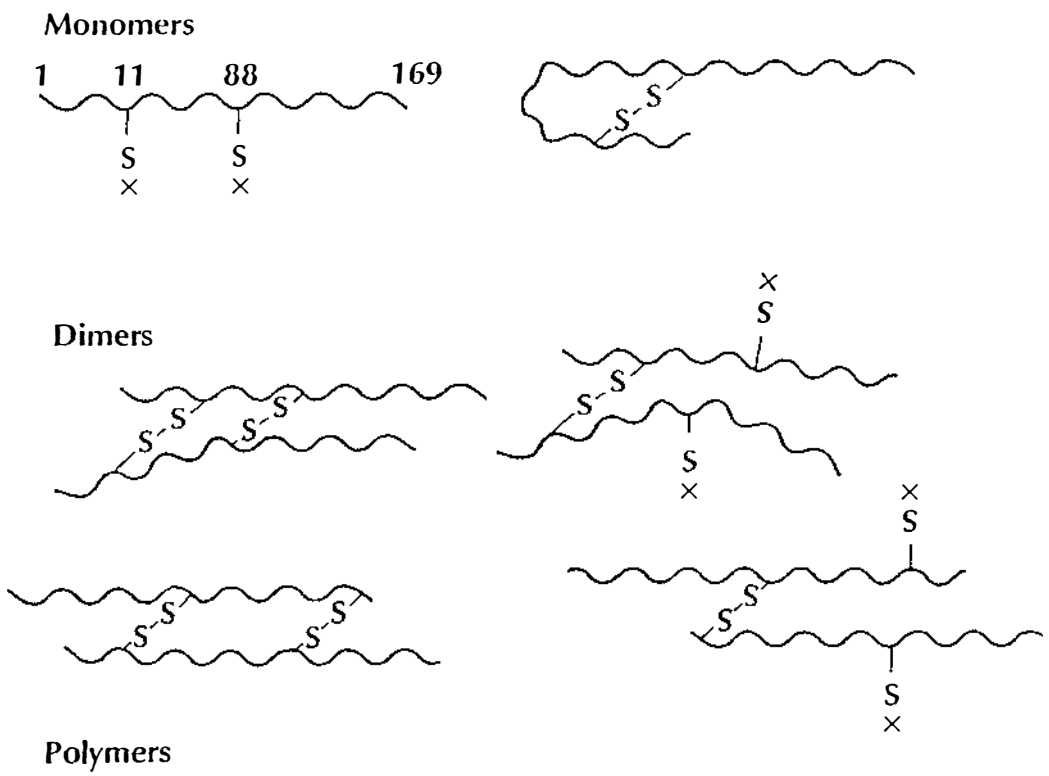

Figure 2.5.2.1. Disulphide bonding patterns of κ-CN. The cysteine residues are oxidised in the native protein and could bond to small molecules, shown as ×, or form disulphide cross-links to give polymers of varying size (Rasmussen et al., 1992; Creamer and MacGibbon, 1996).

The usual means of determining structure are not available for κ-CN because this protein is strongly self-associating and has never been crystallised. Instead, algorithms were used to predict selected secondary structures and CD spectroscopy was used for κ-CN and the macropeptide released by chymosin (Creamer et al., 1998). Loucheux-Lefebvre et al. (1978) predicted 22.5 % helix structures and 31 % sheet structures from the primary structure of κ-CN, although the spectral analysis using CD indicated 14 % helix structures and 31 % sheet structures. Kumosinski et al. (1993) used IR and CD and suggested 14 % helix structures and 30 % sheet structures. Other CD studies (Griffin and Roberts, 1985; Ono et al., 1987) have indicated that the helical structures are more likely to be in the macropeptide region of the protein and that the sheet structures are more likely to be in the para-κ-CN.

Groves et al. (1998) reported that heating purified κ-CN samples caused a significant increase in high molecular weight polymers as judged by electrophoresis and analytical ultracentrifugation, but an apparent decrease in polymeric distribution was caused by adding detergent (e.g. SDS). Polymerisation of κ-CN appeared to be driven by either one or both of the two cysteines in κ-CN. κ-CN polymers formed in the presence of reducing agents have a high specific volume. To accommodate the high hydration and aggregation number, a model consisting of a κ-CN shell and a hollow core has been proposed (Vreeman et al., 1981, 1986). The differences in methods of preparation and the degree of disulphide bonding could influence the κ-CN structure, and the properties of whole caseins as well. Several models of κ-CN structure have been proposed (Parry and Carroll, 1969; Schmidt and Buchheim, 1970; Thurn et al., 1987; de Kruif and May, 1991).

2.5.2.2. Genetic variants of κ-casein

κ-CN occurs in a number of genetic forms (A, B, C, E, F and G) but only the A (Thr-136 and Asp-148) and B (Ile-136 and Ala-148) variants are common (Fig. 2.5.2.2). The B variant is associated with higher expression levels of κ-CN (Ng-Kwai-Hang and Grosclaude, 1992; Jakob and Puhan, 1992; Creamer and Harris, 1997).

pyr-Glu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-LysAsp-Glu-Arg-Phe-Phe-Ser-Asp

Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu

Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr

Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser

81 98
Asn-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg-His-Pro-His

106 111
Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro

129 133
Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr-Ile-Glu-Ala-Val-Glu

146 163
Ser-Thr-Val-Ala-Thr-Leu-Glu-Ala-Ser-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn
P

169
Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val-OH

Figure 2.5.2.2. Amino acid sequence of the B variant of bovine κ-CN. The A variant has a Thr residue at position 136 and an Asp at position 148. The sole phosphoserine residue is at position 149 (Creamer and MacGibbon, 1996).

Macheboeuf et al. (1993) reported that κ-CN BB type milks had a shorter rennet clotting time, a shorter curd firming time and a greater curd firmness than κ-CN AA type milks, which agrees with Horne and Muir (1994) and FitzGerald and Hill (1997). κ-CN AB type milks had intermediate values for these properties (FitzGerald and Hill, 1997). These results are considered to be due to effects of variants on the compositional and physical properties of the micelles. It has been found that κ-CN variants significantly influence the concentration and proportion of κ-CN in milk in the order κ-CN BB > κ-

CN AB > κ-CN AA (McLean et al., 1984; Law et al., 1994). Milk containing higher κ-CN content has smaller average micellar diameters (Dalgleish et al., 1989). Smaller micelles have a greater surface-to-volume ratio and, because κ-CN is located on the surface of the micelle, this has a direct effect on renneting kinetics. Similarly, the increased level of the hydrophobic para-κ-CN in BB type milks after renneting, may explain the increased curd firmness observed in these milks (Creamer and Harris, 1997). In addition to the genetic variants, κ-CN shows different contents of carbohydrate (FitzGerald and Hill, 1997).

2.5.3. Interactions of casein with whey proteins

The heat stability of milk (the absence of coagulation, gelation and sedimentation of milk upon heating) is an important factor in the processing of milk and milk products, because almost all milk undergoes some form of heat treatment at one or more stages of processing. The heat-induced interactions between whey protein and casein have been studied in model systems (Ruegg and Moor, 1977; de Wit and Swinkel, 1980) and in milk systems (de Wit and Klarenbeek, 1984; Dalgleish, 1990; Oldfield et al., 1998).

At temperatures higher than 70 °C, denaturation of the major whey proteins occurs (de Wit and Klarenbeek, 1984; Singh, 1995). By studying the loss of solubility at pH 4.6 of whey proteins, and by DSC, it has been shown that Igs and BSA are the most easily denatured, β-lg is intermediate and α-la is the most heat stable (Ruegg and Moor, 1977). The free cysteine residue contained in the β-lg structure seems to be fundamental in the denaturation process. DSC studies show that α-la has a denaturation temperature of 64 °C and high reversibility in solution (Ruegg and Moor, 1977; de Wit and Swinkel, 1980). The cysteine residues present in α-la as four disulphide bridges seem to be responsible for the maintenance of the reversible conformational change upon denaturation, in the presence of calcium (Relkin et al., 1992). However, this thermal stability resulting from the high re-denaturation of α-la is not found when other proteins such as β-lg and BSA are present in the system (de Wit and Klarenbeek, 1984).

The β-lg/κ-CN complex is not the only complex that is formed during heat treatment, because the other whey proteins are involved (Creamer et al., 1978) and all of

the cysteine-containing proteins may play a role in the interactions (Dalgleish, 1990). Heat-induced intermolecular complexes may also form between α-la and β-lg (Shalabi and Wheelock, 1976), and the presence of both whey proteins in heated micelles has been described by Law et al. (1994) and Corredig and Dalgleish (1996). Corredig and Dalgleish (1996) also reported that faster reaction of the whey proteins with the micelles was found at lower pH among pH 6.8, 6.2 and 5.8 and higher temperatures in the range 75-90 °C. The rate and extent of interaction between whey proteins and casein micelle also changed when additional α-la and β-lg isolates were added to milk before heating (Dalgleish et al., 1997).

However, the mechanism of the reaction is not yet understood, and the results obtained are not in agreement. Elfagm and Wheelock (1978) reported that α-la can interact with κ-CN only in the presence of β-lg, because the initial formation of a heat-induced α-la/β-lg aggregate and then interaction with κ-CN occur. Smits and van Brouwershaven (1980) have also shown that, in the absence of β-lg, α-la hardly interacts with the casein micelles in milk. Conversely, the observation that α-la binds to κ-CN (Doi et al., 1983) at pH 7.6 in phosphate buffer suggests the possibility of an independent reaction of α-la with caseins, albeit under conditions not normally observed in milk. Oldfield et al. (1998) reported that the extent of β-lg and α-la denaturation and association with casein micelles in milk increased with an increase in both heating time and temperature; the rate of association was markedly less than that of denaturation. To determine the extent of β-lg and α-la denaturation and association with casein micelles, the heated samples were centrifuged and the supernatants analysed using quantitative PAGE. The association behaviour was affected by the heating temperature; during the initial stage of heating in the range 80-130 °C, mainly β-lg (not α-la) appeared to associate with the casein micelles, but, after prolonged heating, α-la began to associate with the micelles. In contrast, below 80 °C both β-lg and α-la appeared to associate simultaneously with the micelles.

Denatured whey proteins associated with the micelles on heating milk at pH < 6.8 in the temperature range 90-140 °C, but, at pH > 6.8, denatured whey proteins

precipitated in the intercellular fluid as fibrous strands (Creamer and Matheson, 1980; Singh and Fox, 1985, 1986).

Singh and Fox (1985, 1986) showed that whey proteins complexed and became co-sedimentable with casein micelles after heating milk in the temperature range 90-140 °C at pH values ≤ 6.7. Heating at pH values ≥ 6.9 caused dissociation of micellar κ-CN, probably as complexes with whey proteins. The physico-chemical properties of the whey-protein-coated micelles obtained on heating milk at pH ≤ 6.7 were different from those of the κ-CN-depleted micelles obtained at higher pH values (Singh and Fox, 1986). For example, κ-CN-depleted micelles were more sensitive to heat, Ca^{2+}, ethanol and rennet than whey-protein-coated micelles (Singh and Fox, 1986). Dissociation of micellar κ-CN on heating milk at pH values ≥ 6.9, which appeared to be governed by electrostatic forces, reduced the zeta potential, steric stabilisation and hydration sufficiently to induce coagulation of the κ-CN-depleted micelles in the presence of Ca^{2+} (Singh and Fox, 1987).

2.5.4. β-Lactoglobulin and κ-casein complex formation

The formation of a heat-induced complex between β-lg and κ-CN plays an important role in the heat stability of milk (Tessier and Rose, 1964). A number of studies have reported heat-induced interactions between β-lg and κ-CN in milk (Sawyer, 1969; Snoeren and van der Spek, 1977; Noh et al., 1989; Law et al., 1994; Corredig and Dalgleish, 1996) and in solutions containing pure β-lg and κ-CN (McKenzie et al., 1971; Euber and Brunner, 1982; Haque et al., 1987; Haque and Kinsella, 1988).

Despite extensive investigations, the exact nature of the interacting species and the forces stabilising the β-lg/κ-CN complex are not clear. Based on the electrophoretic patterns of skim milk heated in the presence and absence of reducing agents, Trautman and Swanson (1958) suggested that sulphydryl groups are involved in the reaction (Purkayastha et al., 1967). Sawyer (1969) suggested that the primary thermodenaturation of β-lg itself involves disulphide aggregation and it may be that only the aggregated form is capable of association with κ-CN in a non-specific manner. Sawyer (1969) and McKenzie et al. (1971) interpreted the restriction of β-lg aggregation during thermal

denaturation in the presence of κ-CN as evidence for κ-CN complexing with intermediate species of aggregated β-lg. Long et al. (1963) and Zittle et al. (1962) presented evidence indicating that the primary denaturation of β-lg precedes its interaction with κ-CN, although it was shown that only β-lg need be heated for an interaction to occur. Euber and Brunner (1982) and Jang and Swaisgood (1990) provided more direct evidence of β-lg and κ-CN intermolecular disulphide formation by using thermally denatured immobilised β-lg which, when exposed to κ-CN solution, yielded bound κ-CN that could be released only by disulphide reduction.

Alternative interactions (non-disulphide) are also possible (Parris et al., 1991), but there is strong evidence for the final interaction being via disulphide bond interchange (Jang and Swaisgood, 1990). However, Haque et al. (1987) and Haque and Kinsella (1988) reported the predominance of hydrophobic interactions especially in the initial stages of heated β-lg and κ-CN complex formation. They suggested that the primary force involved in initiating the interaction between these two proteins is mainly hydrophobic, i.e. entropic in origin.

Interaction between β-lg and κ-CN is influenced by factors such as pH, salt concentration, protein concentration and processing conditions (e.g. time, temperature and shear). When a 1:1 mixture of β-lg and κ-CN was heated at pH 6.5 for 20 min, the amount of β-lg reacting with κ-CN reached a maximum at 85 °C but was less at higher heating temperatures (99 °C), perhaps due to degradation of cysteine residues (Long et al., 1963). When the proportion of β-lg was increased, a higher percentage of the protein interacted with κ-CN after heating at 85 °C. Under conditions of excess β-lg, approximately two moles of β-lg associated with one mole of κ-CN after heating the protein mixture for 20 min at 85 °C. Haque et al. (1987) found that three molecules of β-lg interacted with one molecule of κ-CN, which is in agreement with the results of Tessier et al. (1969), when equimolar mixtures of the two proteins were heated at 70 °C.

2.5.5. Effect of genetic variants on the interaction between β-lactoglobulin and κ-casein

Although β-lg and κ-CN are known to form stable complexes during the processing of milk and milk products, little is known about the effect of genetic variation on complex formation. McKenzie et al. (1971) showed that κ-CN reacted faster with β-lg B than β-lg A in cacodylate buffer solutions at pH 6.6 and 74 °C. These findings were confirmed by Hillier and Lyster (1979) and Parnell-Clunies et al. (1988) during the heat treatment of milk.

Imafidon et al. (1991a, b), who studied the denaturation temperatures of different genetic variants of β-lg, individually and in solution with different genetic variants of κ-CN, concluded that genetic polymorphism significantly influenced the denaturation of these proteins, which also was dependent on the buffer characteristics. They reported that, in a 1:1 mixture of β-lg and κ-CN, κ-CN AA increased the heat stability of β-lg AB, but the reverse was observed for either κ-CN BB or κ-CN AB when using DSC.

For the HCT-pH curves of blended skim milk at 140 °C, Robitaille (1995) reported that the maximum heat stability was affected by the κ-CN genotype (κ-CN AB > κ-CN AA) but that the influence of the β-lg genotype was significant only when the κ-CN AA genotype was present (β-lg AA > β-lg BB). Minimum heat stability was significantly higher for milk containing β-lg AB and κ-CN BB. Hill et al. (1997a) observed the first signs of visual coagulation at 140 °C of pooled milk samples over the pH range 6.5-7.2. They reported that the β-lg and κ-CN phenotype combination AABB was the most stable at the maximum of the HCT-pH profile followed by the combinations AAAA, BBBB and BBAA.

Allmere et al. (1997) reported that, in skim milk containing κ-CN AA, the concentration of β-lg BB decreased more rapidly than that of β-lg AB during heating at 90 °C. Allmere et al. (1998) extended this combination to include κ-CN AB and BB variants and found that the β-lg BB and κ-CN BB combination had the highest reaction rate based on losses of native β-lg in the ultracentrifugal supernatant were determined by FPLC, i.e. approximately 1.4 times higher than that of β-lg AA and κ-CN BB.

2.6. RESEARCH OBJECTIVES

Extensive studies on the thermal denaturation of β-lg have established many of the features of heat-induced β-lg aggregation mechanism. Within native β-lg a free SH group is buried between the helix and sheet of the folded protein (Fig. 2.2.2.1: Brownlow et al., 1997) and conformation-steric constraints protect the SH group from the external solvent rendering it unreactive. The unfolding of the protein, resulting in exposure and activation of the free sulphydryl group after dimer dissociation during heat treatment, can lead to sulphydryl-disulphide interchange reactions along with hydrophobic interaction or ionic effect and result in the generation of dimers, trimers and other polyamers (McSwiney et al., 1994a; Roefs and de Kruif, 1994; Qi et al., 1995, 1997; Manderson et al., 1998, 1999a, b; Schokker et al., 1999).

The objective of the first part of the present study is to gain a greater understanding of how β-lg, the major whey protein, behaves during heat-induced denaturation. In 1997, when this study commenced, the effect of heat treatment on the structure and aggregation behaviour of bovine β-lg A, B and C was being studied by Manderson (1998) in our laboratory. He found that the variant C was the most stable of the three variants, whereas the heat stability of variants A and B was dependent on the measuring conditions and methods used (Manderson, 1998). But some questions on the effects of β-lg monomer-dimer and monomer-unfolded monomer equilibrium steps, which lead to sulphydryl-disulphide interchange, on different aggregation behaviours of β-lg A, B and C remain unanswered. Although the reversible early steps involved in heat denaturation are not readily separated from the later irreversible steps, it should be possible to examine the early steps using solvent denaturation and to examine the behaviour of β-lg without reactive thiols either by blocking the free thiol or by using a protein (e.g. porcine β-lg) that does not have cysteine residue equivalent to Cys-121 of bovine β-lg. Porcine β-lg is devoid of a free thiol group (Gallagher et al., 1996), so it is appropriate to study the role of the free sulphydryl group by comparing the structural changes and the ligand-binding properties of bovine β-lg and porcine β-lg

during heat treatment (Chapter 5). Urea denaturation of β-lg involves reversible dimer dissociation and unfolding of monomer without overall thermal factor. In order to throw more light on the unfolding and binding properties of three variants of β-lg, its structural changes during urea denaturation are studied and then extend this to the effect of ligands known to bind to β-lg (Chapter 6). Also in Chapter 6, the products of β-lg thiol blocked using 5,5'-dithio-bis-2-nitrobenzoic acid and *N*-ethylmaleimide are characterised and subjected to urea denaturation to elucidate the role of the free sulphydryl group in β-lg.

When milk is heated above 70 °C, β-lg reacts with κ-CN, and thus becomes associated with the casein micelles or partly with non-micellar casein. Despite the importance of the aggregates formed between β-lg and κ-CN, the mechanism of the formation of this complex has not been clearly elucidated (Tessier and Rose, 1964; Sawyer, 1969; Noh et al., 1989; Jang and Swaisgood, 1990). Therefore, in the second part of the present study, the interaction between β-lg A and κ-CN B during heat treatment is investigated using different protein concentrations and heating conditions (Chapter 8). As bovine β-lg and κ-CN exhibit genetic polymorphism, the study extends to examine the effect of genetic variants on the interaction between β-lg and κ-CN (Chapter 9). This work investigates how the stabilities and structural changes of the β-lg A, B and C variants differ from each other in the presence of the κ-CN A and B variants during heat treatment.

CHAPTER 3.

MATERIALS AND METHODS

3.1. MATERIALS

The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Glycine, glycerol, urea, tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulphate (SDS) (specially purified for biochemical work, Prod. No. 44215), $NaH_2PO_4.H_2O$, $Na_2B_4O_7.10H_2O$, NaCl, K_2HPO_4(anhydrous), $CaCl_2$, $CuSO_4$, ethylenediaminetetraacetic acid (EDTA), imidazole, NaN_3, boric acid, 2-mercaptoethanol, ethanol (99.8 % (w/v)), methanol (99.8 % (w/v)), acetonitrile, formic acid, ammonium sulphate, butylated hydroxytoluene (BHT), NaOH and HCl, all analytical reagent grade, were obtained from BDH Chemicals Ltd (Palmerston North, New Zealand). Glutaric acid was obtained from Koch-Light Laboratories Ltd (Colnbrook, Bucks, England). Trichloroacetic acid (TCA), analytical grade, was purchased from May and Baker Ltd (Palmerston North, New Zealand). Glacial acetic acid, industrial grade, was obtained from BP Chemicals (Palmerston North, New Zealand) and isopropanol was supplied by Shell Chemicals (Palmerston North, New Zealand). Oxygen-free dry nitrogen was from BOC Gases (NZ) Ltd (Palmerston North, New Zealand).

All-trans-retinol (retinol), retinyl acetate, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), ammonium d-camphor-10-sulphonic acid and N-acetyl-L-tryptophanamide (NATA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). "High purity" 1,8-anilinonaphthalene sulphonate (ANS) and *cis*-parinaric acid (PnA) were obtained from Molecular Probes Inc. (Eugene, OR, USA). Palmitic acid (puriss grade, standard for gas chromatography) was purchased from Fluka (Fluka Chemie AG, CH-9471 Buchs, Switzerland). Phast gel IEF 4-6.5 and Phast Gel Blue R (Coomassie R-250) were obtained from Pharmacia (Uppsala, Sweden).

Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and deionisation using a Milli-Q apparatus (Millipore Corp., Waltham, MA, USA) and referred to as Milli-Q water.

3.2. GENERAL METHODS

3.2.1. Electrophoresis

For all gel electrophoresis, the protein samples were analysed using the Mini-Protean II dual slab cell (Bio-Rad Laboratories, Hercules, CA, USA) discontinuous polyacrylamide gel electrophoresis (PAGE) system.

3.2.1.1. SDS-PAGE

SDS-PAGE was carried out using the method of Laemmli (1970), as described by Manderson et al. (1998) and Havea et al. (1998).

Preparation of stock solutions

Acrylamide/bis (30 % T, 2.67 % C). Acrylamide/bis mixture (30 g) was dissolved in approximately 60 mL of Milli-Q water and made up to 100 mL. The solution was stored in an amber bottle at 4 °C.

1.5 M Tris-HCl buffer (pH 8.8) (resolving gel buffer). Tris (18.15 g) was dissolved in approximately 60 mL of Milli-Q water and the pH was adjusted to 8.8 with 1 M HCl. The volume was made to 100 mL with Milli-Q water and this buffer was stored at 4 °C.

0.5 M Tris-HCl buffer (pH 6.8) (stacking gel buffer). Tris (6 g) was dissolved in approximately 60 mL of Milli-Q water and the pH was adjusted to 6.8 with 1 M HCl. The volume was made to 100 mL with Milli-Q water and the buffer was stored at 4 °C.

10 % SDS. SDS (10 g) was dissolved with gentle stirring in Milli-Q water and the volume was made to 100 mL. This was stored at room temperature.

SDS electrode buffer (pH 8.3) (5 × concentration). Tris (15 g), glycine (72 g) and SDS (5 g) were dissolved in Milli-Q water, the pH was adjusted to 8.3, the volume was made to 1

L and the buffer was stored at 4 °C. For each electrophoresis run, 60 mL of 5× electrode buffer was diluted to 300 mL with Milli-Q water.

SDS sample buffer (pH 6.8). The following solutions were added to 55 mL of Milli-Q water: 0.5 M Tris-HCl buffer (12.5 mL), glycerol (10 mL), 10 % (w/v) SDS (20 mL) and 0.1 % (w/v) bromophenol blue (2.5 mL). Then the pH of the mixture was adjusted to 6.8.

Preparation of resolving gel

For preparation of the resolving gel, the following solutions were mixed: Milli-Q water (2.0 mL), 1.5 M Tris-HCl (2.5 mL) and acrylamide/bis mixture (5.30 mL). The mixture was degassed for 15 min in a Buchner flask with rapid stirring. Then 10 % (w/v) SDS solution (100 μL), 50 μL of 10 % (w/v) ammonium persulphate (prepared earlier the same day) and 5 μL of TEMED (tetramethylethylenediamine) were added by gentle swirling. The contents were poured between electrophoresis casting plates (Bio-Rad Protean, Bio-Rad, Richmond, CA, USA). A small quantity of Milli-Q water was added to form an upper layer and the acrylamide solution was allowed to polymerise at room temperature for 1 h. The water was poured off carefully and removed with pieces of filter paper before pouring the stacking gel.

Preparation of stacking gel

For preparation of the stacking gel, the following solutions were mixed: Milli-Q water (3.05 mL), 0.5 M Tris-HCl (1.25 mL) and acrylamide/bis mixture (0.65 mL). The mixture was degassed for 15 min in a Buchner flask with rapid stirring. Then 10 % (w/v) SDS solution (50 μL), 25 μL of 10 % (w/v) ammonium persulphate (prepared earlier the same day) and 5 μL of TEMED were added by gentle swirling. The stacking gel was poured on the top of the set resolving gel and a slot former (10- or 15-slot plastic comb) was inserted between the plates to form appropriate slots for the samples. Polymerisation was carried out at room temperature for 35 min and then overnight at 4 °C.

Sample treatment

The protein samples were diluted with SDS sample buffer and run, or were mixed with 20 μL of 2-mercaptoethanol/mL of sample mixture, heated in a boiling water bath for 4 min, cooled and run. The molecular weight standard (Sigma Chemical Co., St. Louis, MO, USA) contained the following proteins: myosin (M_w 200,000), β-galactosidase (M_w 116,250), phosphorylase b (M_w 97,000), bovine serum albumin (M_w 66,200), ovalbumin (M_w 45,000), carbonic anhydrase (M_w 31,000), soybean trypsin inhibitor (M_w 21,500), lysozyme (M_w 14,400) and aprotinin (M_w 6,500).

SDS gel electrophoresis conditions

Two gels were placed in an electrode buffer chamber and SDS electrode buffer stock solution was used to fill the inner buffer chamber. The samples (10 μL) were injected into the slots of the gel. The gels were run on a Mini-Protean system (Bio-Rad, Hercules, CA, USA), with the voltage, current and power set at upper limits of 210 V, 70 mA and 3.25 W per gel using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Hercules, CA, USA), until the tracking dye moved out of the gel.

Staining and destaining

The gels were removed from the casting assembly and placed in plastic containers containing about 50 mL of staining solution (1.0 g of brilliant blue R dissolved in 500 mL of isopropanol and 200 mL of glacial acetic acid and made up to 2 L with distilled water). The gel containers were put on a rocking table for 1 h for uniform staining. The staining solution was then poured off and replaced with 100 mL of destaining solution (100 mL of isopropanol and 100 mL of glacial acetic acid made up to 1 L with distilled water). After rocking for 1 h, the destaining solution was replaced with 100 mL of fresh destaining solution and the container was rocked for a further 19 h.

Data analysis

The gels were then scanned using a Molecular Dynamics Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, USA) under the control of Windows NT. The stained proteins were scanned at 633 nm with a He/Ne laser with a spot size of 50 μm at a

resolution of 100 μm. The scanned images were processed using ImageQuant software, version 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) to obtain quantitative results. The total absorbance of each protein band was determined and compared with those of the standard samples on each gel as controls.

3.2.1.2. Alkaline-PAGE

Preparation of resolving gel

The alkaline resolving gel was made from a mixture of 3.0 mL of Milli-Q water, 2.5 mL of resolving gel buffer (3 M Tris-HCl, pH 8.8) and 4.0 mL of 30 % acrylamide/bis solution. The mixture was degassed for 15 min in a Buchner flask with rapid stirring. After degassing, 40 μL of freshly prepared 10 % (w/v) ammonium persulphate and 4 μL of TEMED were added by gentle swirling. The contents were poured and stored according to the procedures for SDS-PAGE.

Preparation of stacking gel

The stacking gel was prepared by mixing and then degassing 3.15 mL of Milli-Q water, 1.25 mL of stacking gel buffer (0.5 M Tris-HCl, pH 6.8) and 625 μL of 30 % acrylamide/bis solution. Then 25 μL of 10 % (w/v) ammonium persulphate (prepared earlier the same day) and 5 μL of TEMED were quickly mixed into the degassed solution. The stacking gel was poured and stored according to the procedures for SDS-PAGE.

Sample treatment

The protein samples were diluted with alkaline sample buffer as required. The buffer was prepared by mixing 600 mL of Milli-Q water, 200 mL of alkaline stacking gel buffer, 80 mL of glycerol and 20 mL of 0.4 % (w/v) bromophenol blue (2.5 mL) and then the pH was checked (which should be 6.8) and the buffer was stored at 4 °C.

Alkaline gel electrophoresis conditions

Gels were prepared for electrophoresis using a procedure similar to that described for SDS-PAGE. Alkaline electrode buffer (24 mM Tris, 192 mM glycine, pH 8.3) was

used to fill the inner buffer chamber. Alkaline-PAGE was run in a similar manner to that described for SDS-PAGE, including staining and destaining.

3.2.1.3. Two-dimensional PAGE

Two-dimensional (2D) PAGE procedures (non-reduced SDS-PAGE and then reduced SDS-PAGE) were essentially the same as those described by Havea et al. (1998). 2D-PAGE was used to identify the components in the protein aggregates in heated protein solutions.

Quadruplicate heated protein samples were run on one-dimensional (1D) gels; the samples were selected on the basis of the extent of the loss of monomeric β-lg. After electrophoresis, the gel was removed from between the glass plates and cut so that each strip contained all the protein bands from each sample, including the material that did not migrate into the stacking gel. For the SDS 2D gels, "first-dimension" SDS gels were treated with 2-mercaptoethanol to reduce the protein components. One of the unstained strips was placed in a test tube containing a solution of 5 mL/L of 2-mercaptoethanol in SDS sample buffer, and held in a water bath thermostatically controlled at 94 °C. After 2 min, the strip was removed from the test tube and washed with a little water to remove excess 2-mercaptoethanol solution and the surplus water was blotted from the strip surface with filter paper strips. The gel strip was then held at room temperature for 30 min, to allow evaporation of excess 2-mercaptoethanol from the surface of the strip (the 2-mercaptoethanol would have inhibited the acrylamide polymerisation). Two of the strips were stained to confirm that the separation in the "first dimension" was satisfactory.

The strip of reduced SDS gel was placed on one of the glass plates and perpendicular to the spacers (0.75 mm), the second plate was placed over it and the two plates were assembled into the gel setting equipment. SDS resolving gel solution (3.3 mL) was then carefully poured between the plates, leaving a space of about 12 mm between the top of the gel solution and the bottom of the gel strip. The gel solution was then overlaid with water in the usual fashion, taking care not to wet the gel strip. After the resolving gel had set, the water was removed, the equipment was flushed with a little stacking gel solution and the space was filled with stacking gel solution, with the equipment tilted at about 30°, so that no bubbles were trapped beneath the gel strip. A standard well-forming comb, which had most

of the teeth removed, was then inserted so that the wells formed were level with the centre of the gel strip and at least 4 mm distant from the end of the gel strip. After loading the appropriate chamber buffer and the control sample, the proteins were electrophoresed in the "second dimension". The resultant gel was then stained, destained and scanned as described above.

3.2.1.4. Alkaline-urea PAGE

Preparation of stock solutions

Alkaline-urea resolving gel buffer (pH 8.8). Tris (9.2 g), urea (54.0 g) and 0.8 mL of concentrated HCl were dissolved in Milli-Q water and the pH was adjusted to 8.8 with 1 M HCl. The volume was made to 200 mL with Milli-Q water and this buffer was stored at 4 °C.

Alkaline-urea stacking gel buffer (pH 8.4). Tris (1.08 g), urea (36.0 g), boric acid (0.55 g) and EDTA (92 mg) were dissolved in approximately 80 mL of Milli-Q water and the pH was adjusted to 8.4 with 1 M HCl. The volume was made to 100 mL with Milli-Q water and the buffer was stored at 4 °C.

Alkaline-urea electrode buffer (pH 8.4). Electrode buffer was made by dissolving Tris (10.7 g), boric acid (5.5 g) and EDTA (0.9 g) in approximately 800 mL of Milli-Q water. The pH was adjusted to 8.4 with 1 M HCl, the volume was made to 1 L and the buffer was stored at 4 °C. For each electrophoresis run, 80 mL of electrode buffer was diluted to 400 mL with Milli-Q water.

Alkaline-urea sample buffer (pH 8.4). Sample buffer was made by dissolving Tris (10.8 g), urea (360 g), boric acid (5.5 g), EDTA (0.92 g) and 0.4 % (w/v) bromophenol blue solution (25 mL) in approximately 500 mL of Milli-Q water. The pH was adjusted to 8.4, the volume was made to 1 L and the buffer was stored at 4 °C.

Preparation of resolving gel

The alkaline-urea resolving gel was made from a mixture of 5.95 mL of alkaline-urea resolving gel buffer and 4.0 mL of 30 % acrylamide/bis solution. The mixture was degassed for 15 min in a Buchner flask with rapid stirring. After degassing, 50 μL of freshly prepared 10 % (w/v) ammonium persulphate and 5 μL of TEMED were added by gentle swirling. The mixture was poured and stored according to the procedures for SDS-PAGE.

Preparation of stacking gel

The stacking gel was prepared by mixing and then degassing 4.33 mL of alkaline-urea stacking gel buffer and 0.65 mL of 30 % acrylamide/bis solution. Then 25 μL of 10 % (w/v) ammonium persulphate (prepared earlier the same day) and 5 μL of TEMED were quickly mixed into the degassed solution. The stacking gel was poured and set and the complete gels were stored overnight at 4 °C according to the procedures for SDS-PAGE.

Sample treatment

The protein samples were diluted to approximately 1 mg/mL with alkaline-urea sample buffer and then 10 μL of sample was loaded per well.

Alkaline-urea gel electrophoresis conditions

The set and stored gels were placed in the electrophoresis equipment, and samples and buffer were loaded as described for SDS-PAGE. Alkaline-urea electrode buffer was used to fill the inner buffer chamber. Alkaline-urea PAGE was run in a similar manner to that described for SDS-PAGE, including staining and destaining.

3.2.1.5. Borate PAGE

Preparation of stock solutions

Borate resolving gel buffer (pH 8.5). Boric acid (17.31 g) was dissolved in Milli-Q water to just under the final volume. The pH was adjusted to 8.5 with 1 M NaOH, the volume was made to 1 L with Milli-Q water and the buffer was stored at 4 °C.

Borate stacking gel buffer (pH 6.8). Boric acid (17.31 g) was dissolved in Milli-Q water to just under the final volume. The pH was adjusted to 6.8 with 1 M NaOH, the volume was made to 1 L with Milli-Q water and the buffer was stored at 4 °C.

Borate electrode buffer (pH 8.5). Boric acid (18.55 g) was dissolved in Milli-Q water to just under the final volume. The pH was adjusted to 8.5 with 1 M NaOH and the volume was made to 1 L with Milli-Q water. For each run, 400 mL of undiluted electrode buffer was used.

Borate sample buffer. The following solutions were added to 55 mL of Milli-Q water: resolving gel buffer (50 mL), glycerol (5 mL) and 0.4 % (w/v) bromophenol blue (10 mL).

Preparation of resolving gel

For preparation of the borate resolving gel, 30 % acrylamide/bis (2 mL) and resolving gel buffer (8 mL) were mixed. The mixture was degassed for 15 min in a Buchner flask with rapid stirring. Then 50 μL of 10 % (w/v) ammonium persulphate (prepared earlier the same day) and 5 μL of TEMED were added by gentle swirling. The contents were poured between electrophoresis casting plates (Bio-Rad Protean, Bio-Rad, Richmond, CA, USA). A small quantity of Milli-Q water was added to form an upper layer and the acrylamide solution was allowed to polymerise at room temperature for 35 min. The water was poured off carefully and removed with pieces of filter paper before pouring the stacking gel.

Preparation of stacking gel

For preparation of the borate stacking gel, 30 % acrylamide/bis (0.68 mL) and stacking gel buffer (4.35 mL) were mixed. The mixture was degassed with rapid stirring for 15 min. Then 37.5 μL of 10% (w/v) ammonium persulphate (prepared earlier the same day) and 5 μL of TEMED were quickly added and gently mixed. The stacking gel was poured on the top of the set resolving gel and a slot former (10- or 15-slot plastic comb) was inserted between the plates to form appropriate slots for the samples. Polymerisation was carried out at room temperature for 35 min and then overnight at 4 °C.

Sample treatment

The caseins of milk samples were precipitated by adjusting the pH to 4.6 and centrifuging for 10 min (greater than 500 rev/min) in a bench Microfuge to separate whey and casein. The supernatants were diluted with borate sample buffer.

Borate gel electrophoresis conditions

Two gels were placed in an electrode buffer chamber. The electrode buffer stock solution was used to fill the inner buffer chamber. The samples (5 μL) were injected into the slots of the gel. The gels were run on a Mini-Protean system (Bio-Rad, Hercules, CA, USA) as follows. S1 (constant voltage): the voltage, current and power were set at upper limits of 210 V, 500 mA and 250 W and the running time was 0.15 h. S2 (constant voltage): the voltage, current and power were set at upper limits of 40 V, 500 mA and 250 W and the running time was 3 h. Finally S3 read zero time, which programmed the pack to change the voltage through the run by using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Hercules, CA, USA), until the tracking dye moved out of the gel.

Staining and destaining

The gels were removed from the casting assembly and placed in plastic containers containing about 20 mL of staining solution (1.0 g of brilliant blue R dissolved in 500 mL of isopropanol and 200 mL of glacial acetic acid and made up to 2 L with distilled water)

for 5 min and then destained in 20 mL of destaining solution (100 mL of isopropanol and 100 mL of glacial acetic acid made up to 1 L with distilled water) for 10 min.

3.2.1.6. Isoelectric focusing

Overnight wash

Phast gels IEF 4-6.5 were soaked overnight in 25 mL (for two gels) of solution containing 8 M urea and 0.8 % Triton X-100 (BDH Chemicals Ltd, Palmerston North, New Zealand) prior to ampholyte (Pharmalytes pH ranges 4.2-4.9 and 4.5-5.4, Pharmacia, Uppsala, Sweden) coating. Components present in the commercial precast gel diffused out and urea, Triton and the correct mixture of ampholytes entered the gel.

Ampholyte solution

Gels were soaked for 15 min in a mixture of 8 M urea (1.84 mL), Triton (28 μL), carrier ampholyte pH 4.2-4.9 (54 μL) and carrier ampholyte pH 4.5-5.4 (82 μL). The excess liquid on the surface of the gel was removed by standing the Phast gel perpendicular for approximately 30-40 s.

Sample preparation

A 100 μL aliquot of whey solution was mixed with 300 μL of 8 M urea containing 3 % (v/v) 2-mercaptoethanol and left to stand at room temperature for 15 min.

Sample application and separation

Electrophoresis and gel development were performed using a PhastSystem (Pharmacia, Uppsala, Sweden). Sample (0.3 μL) was applied automatically at the anodic end of the gel. Application to the anodic end avoids any cathodic drift, which is a frequent problem with isoelectric focusing (IEF) methods (Bech and Munk, 1988). The gel development was programmed as follows:

Prefocusing of the ampholyte: 2000 V, 25 mA, 2.0 W, 20 °C, 40 Vh

Sample application: 200 V, 2.5 mA, 2.0 W, 20 °C, 15 Vh

Protein focusing: 2000 V, 25 mA, 4.0 W, 20 °C, 540 Vh

Sample staining and destaining

The gels were stained and destained automatically in the development unit of the PhastSystem. The programme (Bovenhuis and Vertege, 1989) was as follows:

Fix for 10 min in 20 % TCA at 20 °C

Wash for 2 min in 30 % methanol and 10 % glacial acetic acid at 20 °C

Stain for 10 min in 0.03 % Phast Gel Blue R (Coomassie R-350), 30 % methanol, 10 % glacial acetic acid and 0.1 % (w/v) $CuSO_4$ at 37 °C

Destain for 25 min in 30 % methanol and 10 % glacial acetic acid at 20 °C

3.2.2. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were obtained using a Jasco model J-720 spectropolarimeter (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan). The wavelength calibration was checked with benzene vapour (266.7 nm) and a neodymium filter (585.9 nm) using the polarimeter in the absorbance mode, and the sensitivity and rotation at 290.5 nm were checked using a solution of ammonium d-camphor-10-sulphonic acid. The spectra of the protein solutions were measured in 10 mm (for near-UV) and 1 mm (for far-UV) path length cylindrical quartz cells (Jasco, Ishikawa-cho, Hachioji city, Tokyo, Japan). For heat treatment experiments, a stoppered water-jacketed 10 mm cell was used for near-UV CD measurement. The solutions were scanned at 50 (near-UV: 250-400 nm) or 20 (far-UV: 185-250 nm) nm/min using a 2 s time constant, a 0.2 nm step resolution, a 1 nm bandwidth and a sensitivity of 10 m° (millidegrees). The average of five scans was recorded.

Near-UV CD measurements were recorded at 20 °C using a 1.0 mg/mL solution. Protein solutions were diluted to this concentration with the buffer against which the protein had originally been dialysed. Both protein and buffer were filtered prior to CD measurement. For far-UV CD, these solutions were diluted either 10-fold or 6-fold with filtered water and the sample compartment of the instrument was flushed with oxygen-free dry nitrogen prior to measurement.

The baseline spectrum was subtracted from each spectrum and the resultant rotation (m°) values were used. The results were normalised by taking the value of the parameter that did not affect any treatment (e.g. no urea in Chapter 6) as zero and the

value of the highest parameter as 1.00 and scaling the intermediate values between 0 and 1.00. In some instances, the maximum value of the parameter was below that of the maximum used and thus normalised values could be greater than 1.00.

3.2.3. Fluorescence spectroscopy

These measurements were made using 10 mm square quartz cells in a Perkin-Elmer MPF 2A spectrofluorimeter fitted with a water-jacketed cell holder held at 20 °C by a thermostatically controlled water bath (Neslab model RTE-100 thermostatted water bath). Each spectrum was scanned after the mixture had attained temperature equilibrium with the cell holder (20 min). Precise temperature control was needed primarily because the quantum yield of fluorophores is strongly dependent on temperature. During fluorescence data collection, this water bath was used to both heat and cool the circulating water. All data were acquired from cells placed in the same position in the fluorimeter cell holder to eliminate systematic errors resulting from systematic differences in temperature and cell alignment between the different cell compartments.

The excitation wavelength was 295 nm and the emission spectrum was scanned from 300 to 400 nm using excitation and emission spectral bandpasses of 8 nm and sensitivity settings of 4 or 5. After ANS addition, the mixture was excited at 370 nm and the emission spectrum was scanned from 375 to about 520 nm using excitation and emission bandpasses of 8 nm and sensitivity settings of 5 or 6. For both ANS and tryptophan (Trp) fluorescence measurements, the peak position, λ_{max}, and the peak heights at λ_{max} (I_{ANS} and I_{Trp}) were measured from the recorder chart. β-Lg and ANS concentrations were such that the absorbances of the solutions were less than 0.35 at the excitation wavelengths. Because the measurements were of a comparative nature, corrections for inner filter effects were not made. A filtered 0.2 μM NATA solution was used as a standard.

3.2.4. Mass spectrometry

All samples were dissolved in 50 % (v/v) acetonitrile and 0.05 % (v/v) formic acid in water to approximately 1 mg/mL. Samples (0.22 μL) were filtered through a syringe filter (Millex-GV_{13}, Millipore Corp., Bedford, MA, USA) prior to 100-fold dilution in 50 % (v/v) acetonitrile.

Samples were analysed on a VG Platform single-quadrupole mass spectrometer (VG Biotech, Altrincham, UK) equipped with a pneumatically assisted electrospray ion source. Positive multi-charged protein ions were generated by introducing sample, dissolved in 50 % (v/v) acetonitrile and 0.05 % (v/v) formic acid to approximately 1 μg/mL, into a stream of 50 % acetonitrile pumped at 10 μL/min into a 75 μm internal diameter fused silica capillary. This was housed in a stainless steel capillary held at a potential of 5.0 kV.

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

Aliquots (10-15 μL) were applied into the solvent stream via a Rheodyne sample injection loop. This allowed at least eight acquisitions to be accumulated and averaged over a period of approximately 60 s. Each acquisition was over the *m/z* range from 600 to 2500, with a step size of *m/z* = 0.25 and a dwell time of 0.5 s. The charge number of the multi-charged ions, the *m/z* = ratio of each peak, the deconvoluted mass spectra and molecular mass determinations were derived using MassLynx software (VG Biotech, Altrincham, UK).

CHAPTER 4.

DEVELOPMENT OF ISOLATION AND PURIFICATION METHODS FOR β-LACTOGLOBULIN AND κ-CASEIN

4.1. PREPARATION OF β-LACTOGLOBULIN

4.1.1. Introduction

The isolation of β-lg from milk usually involves four stages: the removal of milk fat, the removal of the caseins, the fractionation of the whey proteins and the purification of β-lg. As each stage can be carried out in a number of ways, there is a large range of possible protocols.

In early studies, Palmer (1934) used skim milk as the starting material and casein was removed by adding HCl to pH 4.6. After removing the precipitate, the resulting whey was frozen. The lumps of whey were spread evenly on a piece of muslin stretched tightly over the mouth of a large jar and some two-thirds to three-quarters of the water was frozen out. The first quarter of the total whey volume after thawing was retained and the remaining solid, mostly water, was then discarded. Na_2SO_4 was then used to fractionate the rest of the whey and β-lg was obtained after dialysis and re-precipitation.

The original method of Palmer (1934) was superseded by the method of Aschaffenburg and Drewry (1957), which involved adjusting the whey pH to 2 with 1 M HCl and salting out the β-lg by adding $(NH_4)_2SO_4$. Armstrong et al. (1967) used a similar procedure, but the whey proteins were fractionated by acid precipitation at pH 3.5. Mailliart and Ribadeau-Dumas (1988) devised a similar salting-out procedure to that of Aschaffenburg and Drewry (1957), which was suitable for large scale use. Mailliart and Ribadeau-Dumas (1988), using a concentrated whey (by ultrafiltration) as a starting material, separated β-lg from the other whey proteins using a salting-out procedure. At pH 2.0, 7 % NaCl (w/v) and 20 min holding time, nearly all the β-lg remained soluble and a precipitate containing all other whey proteins was formed. β-Lg was salted out of the supernatant by addition of NaCl to a total concentration of 25-30 % (w/v). The β-lg-

containing precipitate was recovered after centrifugation at 10,000 × *g* for 20 min. The β-lg solution was dialysed against distilled water and freeze dried. From 828.8 mg of β-lg in the original retentate, 696.2 mg of β-lg was recovered, representing 84 % of the initial β-lg. This β-lg preparation was contaminated with 0.5 % α-la and 0.5 % Igs. They claimed that the procedure was suitable for scaling up and was compatible with food industry requirements.

An alternative precipitation method used TCA (Fox et al., 1967) to remove the non-β-lg protein fraction from the whey and $(NH_4)_2SO_4$ (or additional TCA) to precipitate the β-lg. The yield of β-lg was 3.50-4.25 g/L of whey. Imafidon and Ng-Kwai-Hang (1992) described a procedure for the preparation of relatively pure β-lg in gram amounts from enriched fractions obtained after precipitation of acid whey with TCA to remove other whey proteins. They used mass ion-exchange chromatography (QAE-cellulosic anion-exchange cartridge) to extract the protein. β-Lg adsorbed on the QAE cartridge but was desorbed with 300 mM NaCl in 50 mM phosphate buffer at pH 6.0. The average yield of pure β-lg was 4.7 g from 1.8 L of whey. However, this method may not be useful for preparing food-grade β-lg because of the non-food-grade reagent, TCA, employed in the preparation (Imafidon et al., 1997).

Ion-exchange and size-exclusion chromatography have proven popular for preparing smaller quantities as the precipitation step is often thought to be unnecessary (Strange et al., 1993). McKenzie (1971) reported a procedure for the purification of β-lg and α-la and their resolution, in some cases, into the various genetic variants by chromatography on DEAE-Sephadex A-5 at pH 6.3 in imidazole-HCl buffer using a 0.0-150 mM NaCl gradient. Use of ion-exchange media for whey protein recovery has been criticised because of the deposition of milk salts in the system (Imafidon and Na-Kwai-Hang, 1992). Whey proteins were adsorbed on to DEAE-cellulose at pH 8-9 and subsequently eluted using NaCl and the formation of precipitates containing calcium phosphate was observed. However, this problem can be overcome by using diafiltration of the whey to remove the milk salts before pH adjustment (Imafidon et al., 1997). Ion exchange has the advantage that the β-lg A and B or β-lg A and C variants can be separated from one another as well as from other proteins. Monaco et al. (1987)

separated the whey proteins by DEAE-cellulose chromatography without the pH going below 6.6.

Affinity chromatography was used by Jang and Swaisgood (1990) to prepare pure β-lg; they used a column of immobilised retinal. Only the elution of β-lg from whey was retarded by bound retinal in 0.1 M phosphate buffer and native and denatured β-lg were separated.

In this study, β-lg A, B and C variants were prepared by three different methods. It was important that the starting material used in this study was pure and native. As ligand-free native protein was required, a gentle procedure that included a low pH separation step was needed. Processing that involves high shear can generate non-native proteins and freeze drying process can cause the attachment of lactosyl residues; therefore, the whey was not subjected to any high shear related process and stored frozen. Various spectroscopic and electrophoretic techniques were used to compare the products prepared by the three different methods.

4.1.2. Materials and methods

4.1.2.1. Materials

The raw material for all preparations was fresh unpasteurised whole milk from cows known to contain β-lg phenotype AA, BB or CC.

4.1.2.2. β-Lactoglobulin purification methods

(i) Method I (based on Mailliart and Ribadeau-Dumas;1988)

Milk from cows known to be homozygous for β-lg AA, BB or CC, by IEF or borate-PAGE, was pre-warmed to 40 °C and skimmed with a laboratory separator (Elecrem Buttermaschine, HÄKA GmbH, Stutensee, Germany) and the casein was precipitated with 1 M HCl at pH 4.6. Precipitated caseins were separated from the whey by filtration through a pad of loosely woven fabric.

The resultant whey was concentrated by a freeze-thaw technique based on that used by Palmer (1934) and essentially the same as that described by Manderson (1998) and the concentrated whey was warmed to 40 °C and adjusted to pH 2.0 with 1 M HCl.

The volume was measured and 7 % (w/v) NaCl was slowly added with constant stirring. After centrifugation (10,000 × *g*, 20 °C for 20 min, Sorvall SuperLite TM GSA), the supernatant was recovered and the NaCl content of the mixture was gradually increased to 30 % (w/v). Precipitated proteins were obtained by centrifugation (10,000 × *g*, 20 °C for 20 min, Sorvall SuperLite TM GSA) and the pellet was carefully re-dissolved in the minimum volume of water. Anhydrous K_2HPO_4 was slowly stirred into the mixture until the pH was approximately 4.0. The solution was then dialysed against five changes of Milli-Q water.

The protein was then purified by size-exclusion chromatography at 4 °C on a Superdex 75 column (50 mm × 600 mm) using the Pharmacia FPLC system (see Section 4.1.2.3). The column was equilibrated with pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) prior to chromatography. For each run, the absorbance of the eluate was monitored at 280 nm using a VWM 2141 detector and recorded using an REC 102 multi-channel chart recorder. The fractions were collected using a Frac-200 fraction collector and those likely to contain β-lg were analysed using alkaline-PAGE and selected fractions were pooled and stored frozen at -21 °C.

(ii) Method II (based on Monaco et al.; 1987)

Milk (3 L) from cows was de-fatted by centrifugation twice at 10,000 × *g* for 20 min at 4 °C (Sorvall SuperLite TM GSA). The skim milk was warmed to 40 °C and the pH was adjusted to 6.6 with 1 M HCl with constant stirring. $CaCl_2$ (2 M; 60 mL per 1 L of skim milk) solution was gradually stirred in, giving a final $CaCl_2$ concentration of 0.12 M, and the pH was re-adjusted to 6.6 with 1 M HCl. The skim milk was held for 10 min, warmed to 40 °C and centrifuged at 12,000 × *g* for 30 min (Sorvall SuperLite TM GSA), and the supernatant was retained.

The supernatant was cooled to 4 °C, adjusted to pH 5.9 and concentrated fourfold by stirred cell ultrafiltration (Amicon model 8400, Amicon Inc., Beverly, MA, USA) with a YM10 membrane (10,000 molecular weight cut-off) at 4 °C. The protein was then purified by size-exclusion chromatography as described in method I. The selected fractions were pooled and stored frozen at -21 °C.

(iii) Method III (based on Fox et al.;1967)

Acid whey was concentrated by the freeze-thaw technique described in method I. TCA (34.2 g per 1 L of whey) was dissolved in a minimal amount of water and slowly stirred into the acid whey at room temperature. The mixture was left for 30 min and then centrifuged for 20 min at 10,000 × *g* (Sorvall SuperLite TM GSA). The supernatant was retained and a saturated solution of ammonium sulphate was mixed into the supernatant, giving a final ammonium sulphate concentration of 0.021 M. Addition of ammonium sulphate ensured complete removal of any non-β-lg protein that did not precipitate using TCA. The mixture was centrifuged for 20 min at 10,000 × *g* (Sorvall SuperLite TM GSA) and the supernatant was carefully decanted. Crystalline ammonium sulphate was added to the supernatant until the solution was saturated (5.34 M). After standing for 12 h, the mixture was centrifuged for 20 min at 10,000 × *g*. The pellet was retained and dispersed into a saturated ammonium sulphate solution. The mixture was centrifuged again (10,000 × *g* for 20 min, Sorvall SuperLite TM GSA) and the resultant pellet was dissolved in water. The solution was dialysed against five changes of pH 6.0 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) and then stored frozen.

Table 4.1.2.1. Summary of protocols used to prepare β-lg.

	Method I	Method II	Method III
Fat removal	Separator	Centrifugation	Separator
Casein removal	HCl addition and isoelectric precipitation	$CaCl_2$ followed by centrifugation	HCl addition and isoelectric precipitation
Concentration	Freeze-thaw	Ultrafiltration	
Fractionation	NaCl fractionation followed by dialysis	Size-exclusion	TCA followed by centrifugation and $(NH_4)_2SO_4$ followed by centrifugation
Purification	Size-exclusion		Dialysis

4.1.2.3. General methods

Protein concentration

The protein concentrations were determined using 280 nm absorbance and an extinction coefficient of 9.6 for β-lg (Townend et al., 1967) and 12.2 for κ-CN (Zittle and Custer, 1963).

IEF and borate PAGE

IEF and borate PAGE used to identify various genetic variants of β-lg and experimental details are given in Sections 3.2.1.6 and 3.2.1.5, respectively.

Fast protein liquid chromatography (FPLC)

β-Lgs were purified further by size-exclusion chromatography using a Pharmacia FPLC system (Uppsala, Sweden) which consisted of two model P-500 pumps, a model P-1 pump, a model LCC-500 Plus controller, a model VWM 2141 detector, a model REC 102 multi-channel chart recorder, a model Frac-200 fraction collector and a column (50 mm × 600 mm, 1178 mL volume) of Superdex 75 (13 μm average bead size, preparation grade, Pharmacia). The absorbance of the eluate was monitored at 280 nm and recorded using the chart recorder for the duration of the run.

Size-exclusion chromatography multi-angle laser light scattering (SEC-MALLS)

Protein solutions were filtered through 0.22 μm membrane filter (Millipore Corporation, Bedford, MA, USA) and 50 μL aliquots were fractionated using a high performance gel chromatography system, consisting of a model LC 1150 HPLC pump, a 50 μL loop, a model LC 1200 UV/VIS detector, a model LC 1440 system organiser and WinChrom chromatography software (version 1.0, GBC Scientific Equipment, Dandenong, Victoria 3175, Australia). Separations were accomplished using a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden) or a Superose 6 HR 10/30 column (Pharmacia, Uppsala, Sweden) and a Superdex 75 HR 10/30 column in series, equilibrated with 0.02 M imidazole, 0.05 M NaCl, 0.003 M NaN_3, pH 7.0, and filtered through 0.25 μm filters (Millipore Corporation, Bedford, MA, USA), at flow rates of 1.0 and 0.5 mL/min, respectively. The Superose 6 column separates proteins from 5 to 5000

kDa, and the Superdex 75 column separates proteins from 3 to 70 kDa. The effluent stream was analysed by UV absorption at 280 nm and by changes in refractive index using a differential refractometer (Waters model R401, Millipore Waters, Milford, MA, USA). Particle size and molar mass were determined using a DAWN DSP laser photometer, consisting of a linearly polarised He-Ne laser (λ = 632.8 nm; 5 mW), a K5 flow cell, 18 detectors at various angles and Astra software (version 4.50, Wyatt Technology, Santa Barbara, CA, USA).

Heat treatment of samples

The three β-lg solutions were placed in stoppered glass test tubes, each containing a 4 mL β-lg aliquot of 1.0 mg/mL and were flushed with nitrogen to remove the oxygen. The solutions were heated at 90 °C for 10 min in a Neslab model RTE-100 thermostatically controlled water bath (Neslab Instruments Inc., Newington, NH, USA). Tubes and samples were removed after heating and immediately placed in ice water for 5 min and left at room temperature for 2 h. The above sets of experiments were carried out in duplicate.

The structural differences in the β-lg samples prepared using the three different methods were also compared using heat treated β-lg solutions determined by CD spectroscopy and ANS fluorescence.

CD measurement protocols

For near-UV CD measurement, β-lg samples were filtered through a 0.22 μm filtering unit (Millipore Corporation, Bedford, MA, USA) and diluted to about 1.0 mg/mL with filtered pH 6.7 phosphate buffer (26 mM sodium phosphate, 68 mM NaCl). The spectrum of each protein sample was recorded and manipulated as described in Section 3.2.2.

For far-UV spectra, β-lg solutions used for near-UV CD were diluted tenfold to 0.1 mg/mL with water. High values for the PMT voltage were noted below 210 nm, indicating low light intensity and hence diminished data quality (i.e. lower signal:noise ratio) in this portion of the far-UV CD spectra.

Fluorescence measurement

Aliquots (3.0 mL) of 1.0 mg/mL β-lg solution in pH 6.7 phosphate buffer were placed in fluorimeter cells and titrated with 1.41 mM ANS (4.2 mg of ANS dissolved in 1 mL of 99.8 % ethanol, and then diluted to 10 mL with water) in 20 or 40 μL increments up to 140 μL. After each addition of ANS, the cell contents were mixed by inversion and then placed in the fluorimeter cell holder. Solutions were left in the cell holder (20 °C) for 10 min before measurements were made.

Emission spectra were recorded using an excitation wavelength of 370 nm and scanned from 375 to about 520 nm using excitation and emission bandpasses of 8 nm, at a sensitivity 5 or 6, a scan speed of 25 nm/min and a chart recorder speed of 1 cm/min. At the conclusion of each titration, the molar ratio of ANS to monomeric β-lg was approximately 1:1.

4.1.3. Results and discussion

I. *Phenotyping and purification of crude β-lactoglobulin*

4.1.3.1. Phenotyping of β-lactoglobulin

Electrophoresis has been used to identify various genetic variants of both the caseins and β-lg. The β-lg A, B and C variants have slight differences in charge at pH 8.5 and can be separated based on this charge difference under suitable electrophoresis conditions. Alkaline-PAGE separates β-lg B and β-lg C efficiently. However, both IEF (Fig. 4.1.3.1.A) and borate-PAGE (Fig. 4.1.3.1.B) give good results and have demonstrated the existence of several genetic variants among two of the major whey proteins. IEF is a method (Section 3.2.1.6) specifically intended for the separation of molecular species differing only in net charge (Bovenhuis and Verstege, 1989). For the phenotyping of 350 cows' milks, IEF was used routinely because it was easy to use and all the milk proteins could be analysed simultaneously. Alternatively, the borate-PAGE method (Section 3.2.1.5) used to verify the A, B and C variants of β-lg and the results are shown in Fig. 4.1.3.1.B.

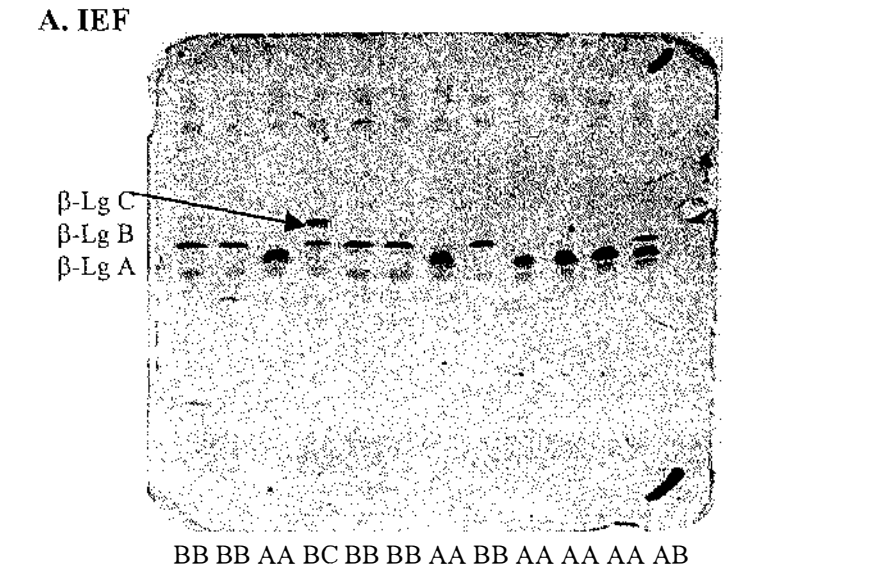

Figure 4.1.3.1. Representative phenotyping of β-lgs by (A) IEF and (B) borate-PAGE. Experimental details are given in Sections 3.2.1.6 and 3.2.1.5, respectively.

4.1.3.2. Size-exclusion chromatography

β-Lg prepared using methods I (based on Mailliart and Ribadeau-Dumas, 1988) and method II (based on Monaco et al., 1987) was purified further by size-exclusion chromatography. Alkaline-PAGE was used to check the purity of the protein. The FPLC (Fig. 4.1.3.2) and alkaline-PAGE (Fig. 4.1.3.3) measurements showed the co-existence of other milk proteins. The peaks 1, 2 and 3 represented aggregated whey proteins during former process. As shown in Fig. 4.1.3.2, samples prepared by method II showed more contamination with other proteins.

4.1.3.3. Yields of β-lactoglobulin

The final yields of β-lg prepared by method I, which were obtained from each size-exclusion FPLC run, usually ranged from 43 % to 55 % (i.e. assuming that the original concentration of β-lg in milk was 3 mg/mL). After chromatography, the average β-lg concentration in the five 4 mL fractions collected was 2.7 mg/mL. The yields of β-lg prepared by method II were similar to those of method I. The yields of β-lg prepared by method III, the TCA fractionation procedure of Fox et al. (1967), were approximately 17 %. These yields were low compared with those obtained using methods I and II.

Variant A showed the highest yields followed by variant B and variant C showed the lowest yields in all three methods.

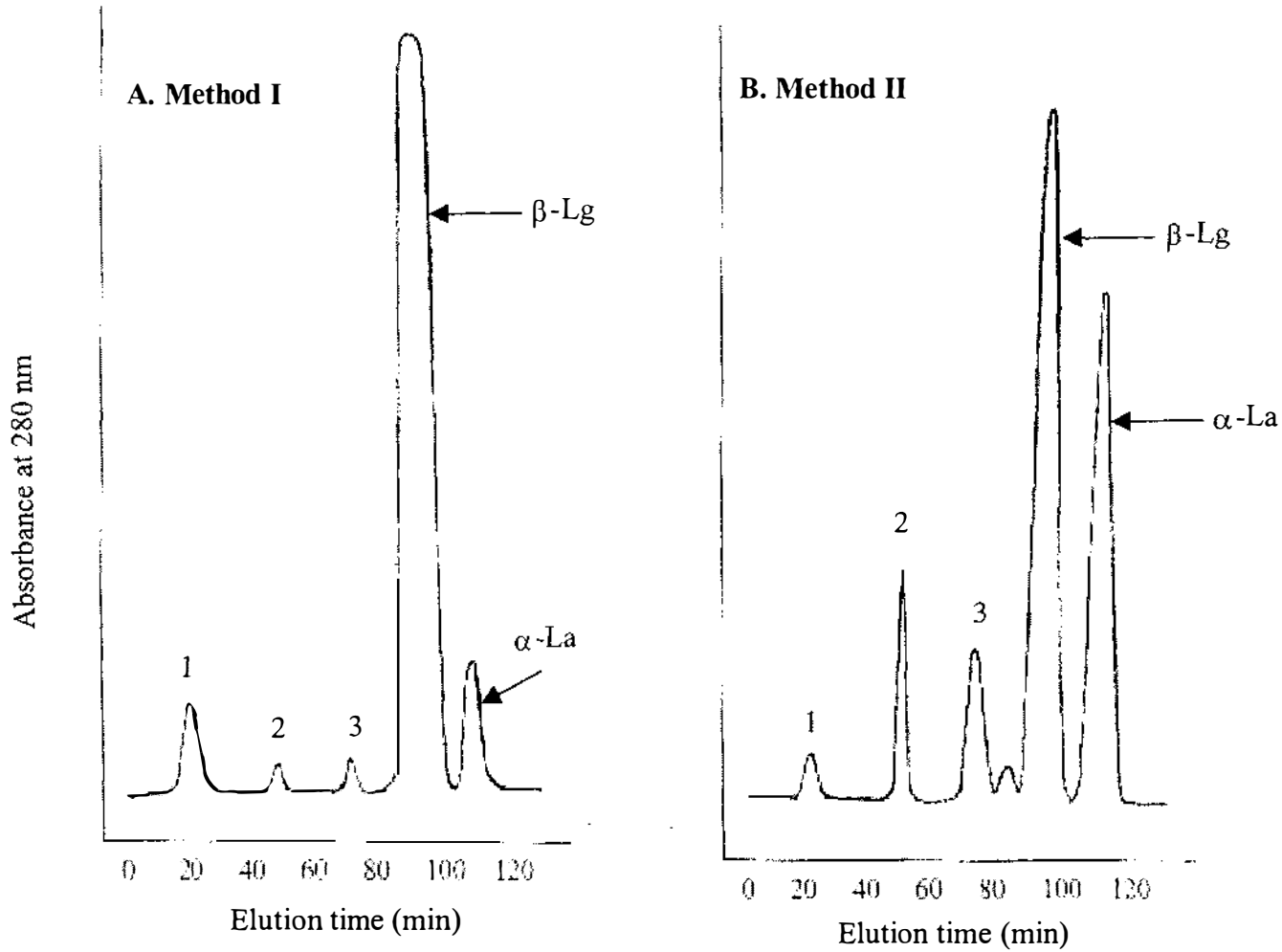

Figure 4.1.3.2. Size-exclusion FPLC chromatogram of β-lg B prepared using (A) method I or (B) method II. Proteins were separated on a Superdex 75 column (50 mm x 600 mm), equilibrated with pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl). The measurements were made using the FPLC system described in Section 4.1.2.3.

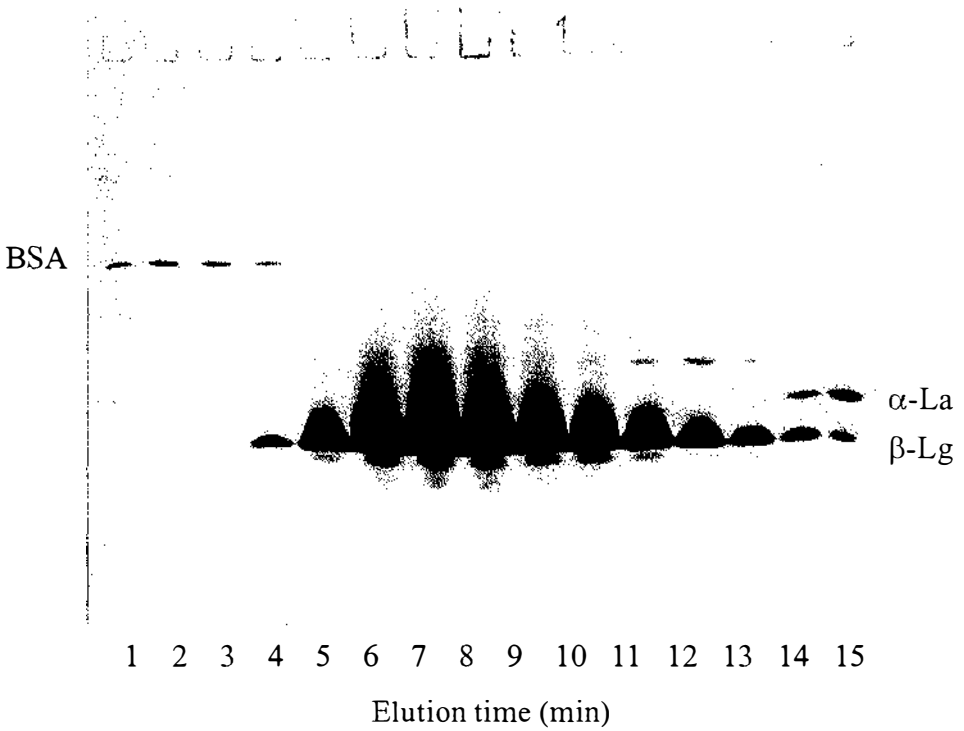

Figure 4.1.3.3. Alkaline-PAGE electropherogram of eluate fractions obtained when crude β-lg B was further purified by size-exclusion chromatography. Each fraction (1-15) was applied to one well and is shown in order of increasing elution time from left to right. The measurements were made using the alkaline-PAGE system described in Section 3.2.1.2.

II. Characterisation of purified β-lactoglobulin

The differences among β-lg A, B and C, which had been isolated by the three different methods, were determined by a number of methods, including SEC-MALLS, mass spectrometry (electrospray ionisation), CD and enhancement of ANS fluorescence.

The aim of the spectroscopic study was to identify which β-lg samples gave similar or different spectra, thus suggesting structural similarities or differences. The purified samples prepared by each method were pooled and stored frozen at -21 °C.

4.1.3.4. SEC-MALLS

Figure 4.1.3.4.A shows a typical example of the elution profile obtained with a Superose 6 column in combination with the MALLS photometer. The elution profiles obtained from a 3 mg/mL solution of β-lg A prepared by method I, which were detected by UV and by MALLS at the 90° angle, are shown in Fig. 4.1.3.4.A. The peak present in the UV trace and the MALLS trace with a retention time of approximately 40 min was β-lg. A peak with a retention time of 20 min can be seen in the MALLS trace of β-lg A prepared using method I (Fig. 4.1.3.4.A). This small initial peak was observed in all β-lg samples and was presumably caused by minute quantities of impurities, such as dust. As the light-scattering signal is proportional to the product of concentration and molecular mass, even a very small amount of a high molecular mass material will result in large scattering (Hoffmann et al., 1996; Schokker et al., 1999).

All three variants of β-lg prepared using the three different methods showed a similar range of weight-averaged molecular masses and similar patterns using SEC-MALLS. The MALLS software estimated that the weight-averaged molecular mass (M_w) for this peak was $27 \pm 2 \times 10^3$ Da, indicating that the β-lg existed mainly as a mixture of monomer and dimer with approximately half of the molecules self-associated (Fig. 4.1.3.4.B).

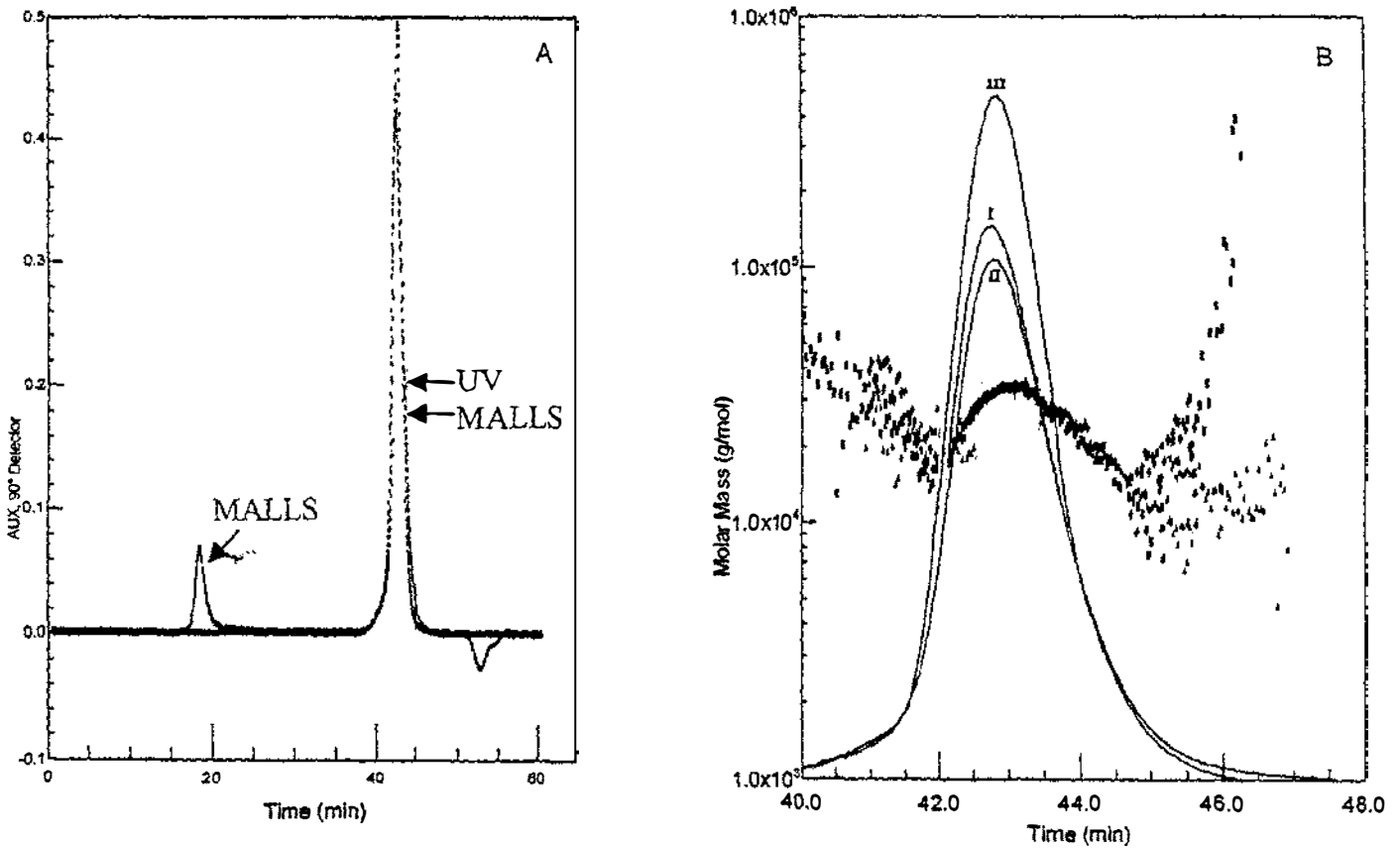

Figure 4.1.3.4. (A) Size-exclusion chromatography elution profile of 3 mg/mL of β-lg A prepared using method I by UV and MALLS at the 90° angle and (B) weight-averaged molecular masses of β-lg A prepared using methods I (■), II (◆) and III (▲), determined by MALLS detection and separated by a Superose 6 column in buffer containing 0.02 M imidazole, 0.05 M NaCl, 0.02 % Na_3N at pH 7.0. The measurements were made using the SEC-MALLS system described in Section 4.1.2.3.

4.1.3.5. Mass spectrometry

Morgan et al. (1997) reported that lactose reacts non-enzymatically with β-lg under mild heat treatment and monitored the formation of the complex by mass spectrometry. This study focused on detecting species differing from the mass of the β-lg species by increments of approximately 324 Da (M_w of lactose) and one species that corresponded to the expected mass of β-lg variant A, B or C.

Mass spectrometric analysis of the samples of β-lg prepared by the three different methods are shown in Figs 4.1.3.5, 4.1.3.6 and 4.1.3.7. One species corresponded to the expected mass of monomeric β-lg variant A, B or C (e.g. 18,362.3 ± 1.1, 18,277 ± 1.6 or 18,286 ± 1.0 Da, respectively). Each figure shows β-lg A, B and C prepared by the same method in parts (A), (B) and (C), respectively. In each mass spectrum of the three β-lg variants prepared by method III, in addition to the expected β-lg variant, up to two additional species differing from the mass of the β-lg species by increments of approximately 98.5 Da were detected (Fig. 4.1.3.7).

The β-lg + n(98.5 Da) adducts observed in the mass spectra of purified β-lg were consistent with the addition of sulphate residues to the protein. In method III, the β-lg preparation step included the addition of $(NH_4)_2SO_4$ for the fractionation of β-lg from other proteins. Although this solution was dialysed against five changes of buffer, it seems that the sulphate group remained associated with the β-lg, even after dialysis. Dialysis against neutral monovalent solutions may have prevented this.

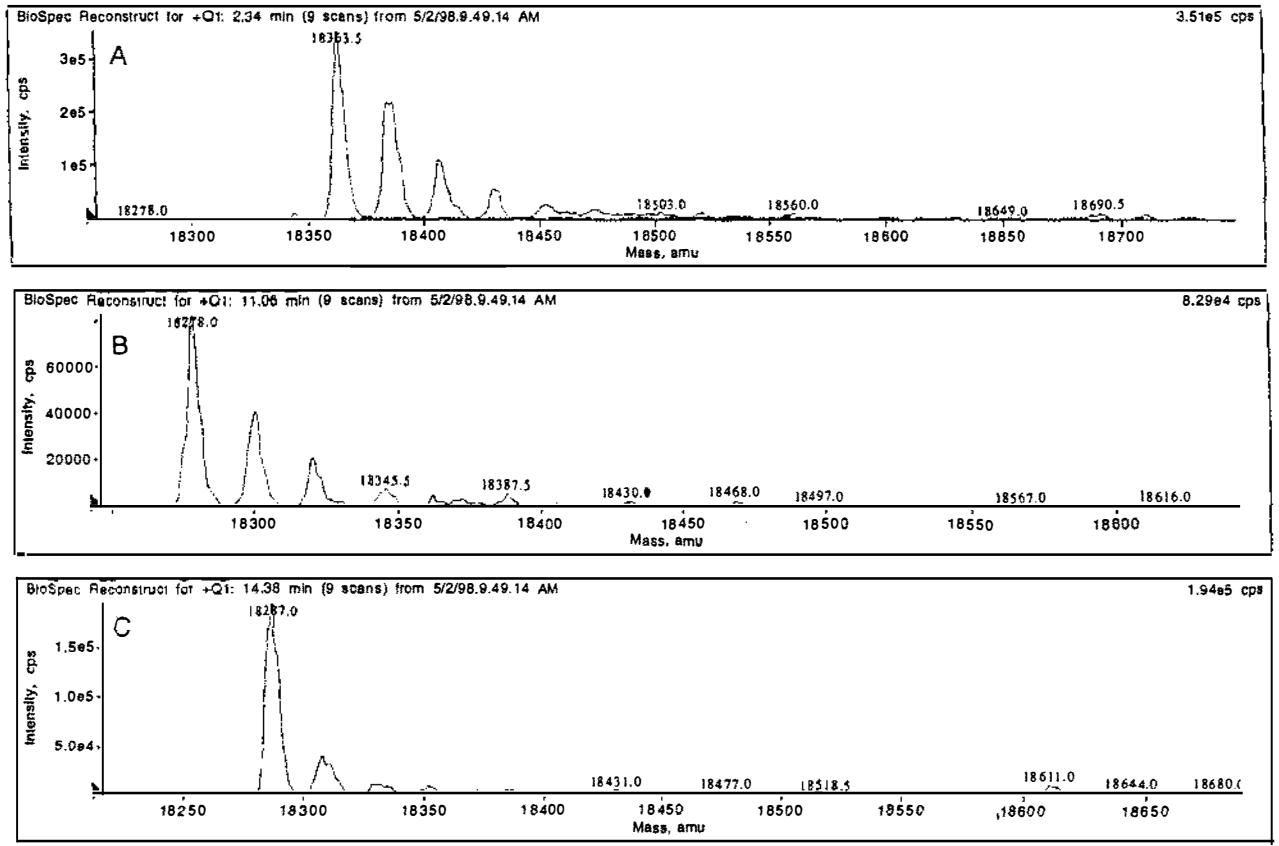

Figure 4.1.3.5. Mass spectrum of (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using method I. The measurements were made using the system described in Section 3.2.4.

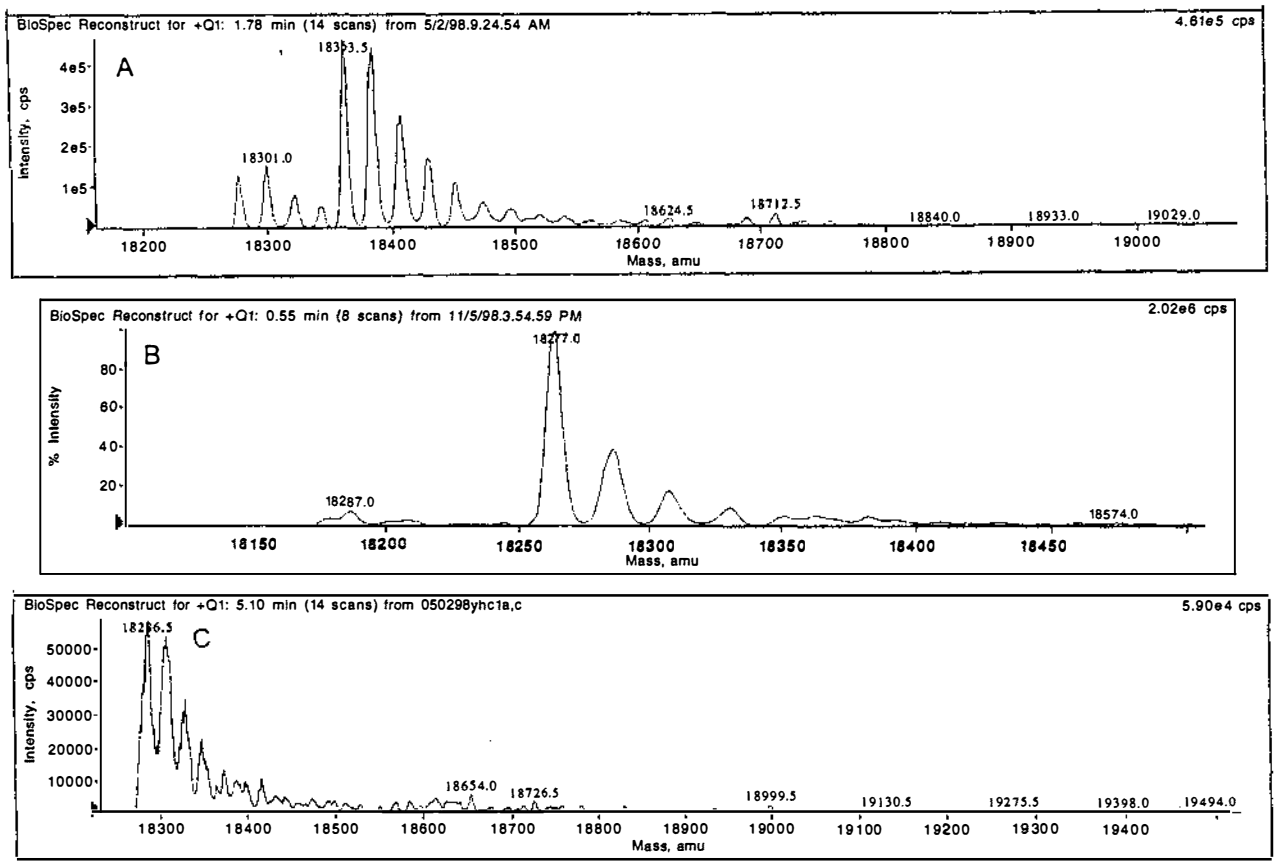

Figure 4.1.3.6. Mass spectrum of (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using method II. The measurements were made using the system described in Section 3.2.4.

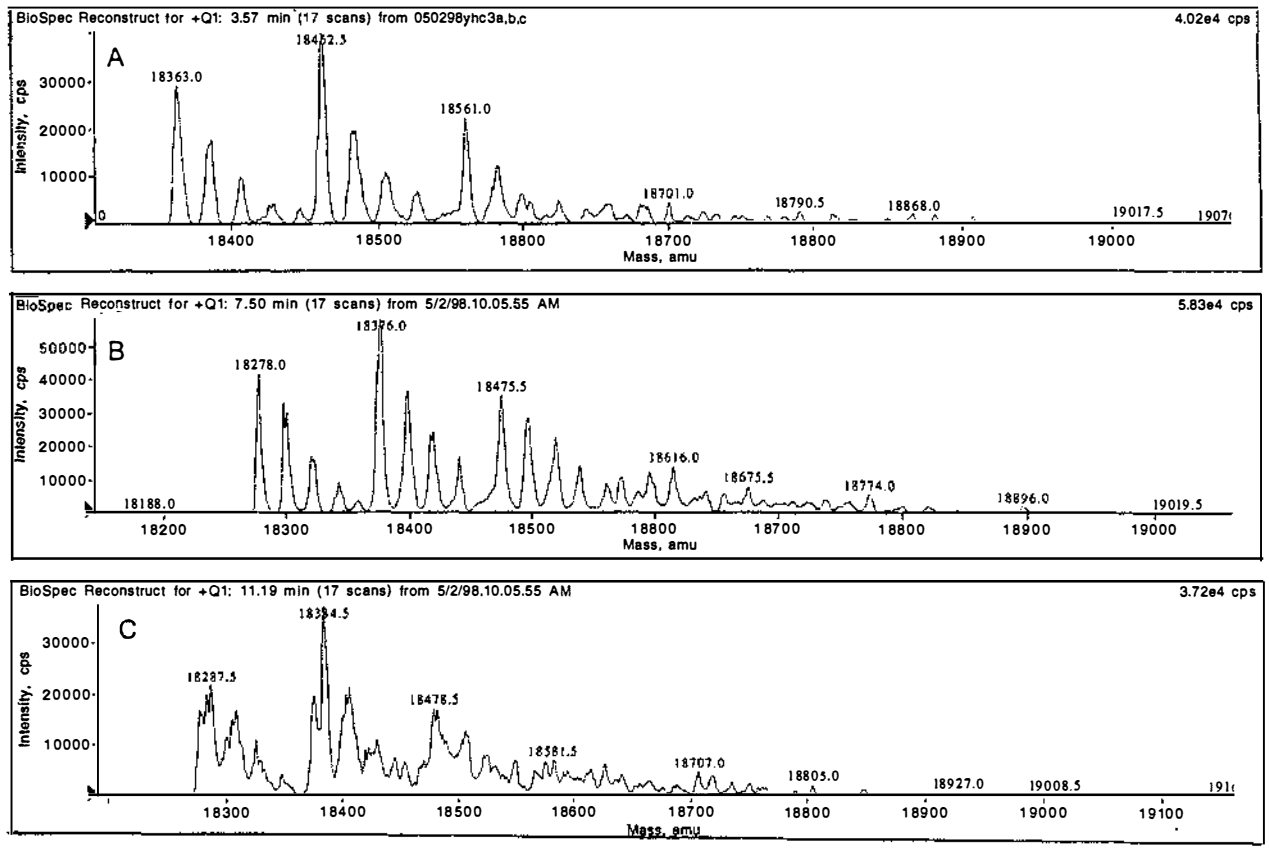

Figure 4.1.3.7. Mass spectrum of (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using method III. The measurements were made using the system described in Section 3.2.4.

4.1.3.6. Near-UV CD

Near-UV CD spectroscopy was used to identify changes in the vicinities of aromatic side chains and disulphide bond of the three variants of β-lg prepared by the three different methods. The CD spectra of the three preparations of each β-lg variant were overlaid and these overlays indicated that there were few differences among the different samples (Fig. 4.1.3.8) and that the spectra were similar to those previously reported (Timasheff et al., 1967; Manderson et al., 1999b). The β-lg from a commercial preparation also showed typical tryptophan bands at 285 and 293 nm (Fig. 4.1.3.9.A).

The 250-270 nm region of CD spectra has been reported to arise from the disulphide bonds of β-lg (Woody, 1973, 1995; Kuwajima et al., 1996). The band intensities of β-lg A, B and C at 257 nm were approximately 2, 1.5 and 0 m^{o}, respectively; the same trends were observed in all three preparation methods (Fig. 4.1.3.8). It is consistent with the results of Manderson. (1998).

The near-UV CD spectra of heat-treated β-lgs (90 °C for 10 min) were different from those of unheated β-lgs and the degree of reaction was different according to the source of the samples (i.e. preparation methods) and the variant type (Fig. 4.1.3.10). In the spectra of the heat-treated samples, the intensities of the bands at 293, 285 and 277 nm were less than those in the spectra of the unheated samples, which indicates a shift of the aromatic side chains of β-lg into less chiral environments. β-Lgs prepared by method III showed the most severe loss of band intensities among the three methods in the near-UV region (Fig. 4.1.3.10).

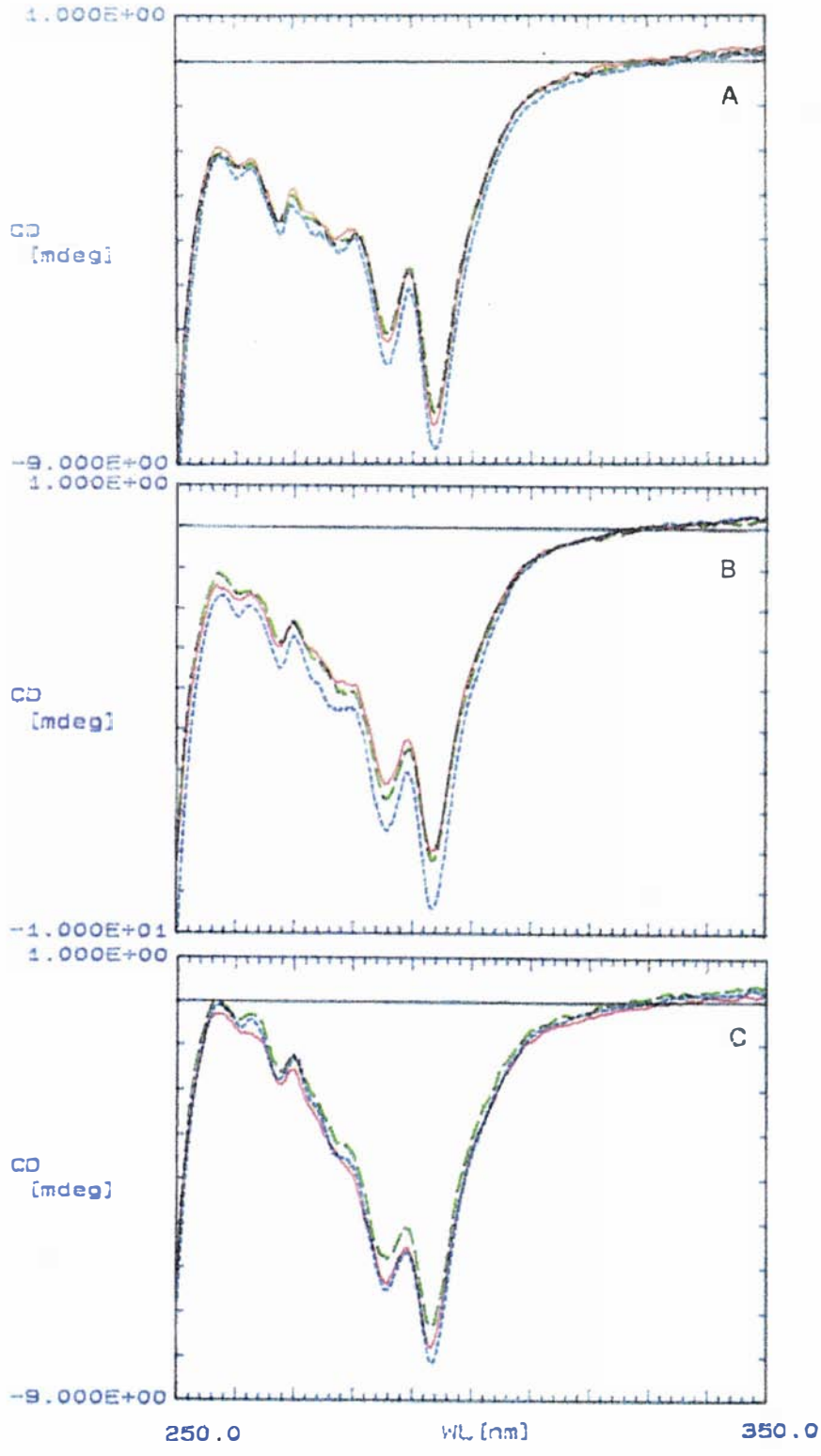

Figure 4.1.3.8. Near-UV CD spectra of (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using methods I (red solid), II (green dashed) and III (blue dotted) at concentrations of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. Protein CD spectra were acquired in 10 mm path length cells using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 50 nm/min, using a spectral band width of 1.0 nm, at a time constant of 2 s and a step resolution of 0.2 nm. In each case, the final spectrum is the average of five scans of the solution. See Section 3.2.2 for further experimental detail.

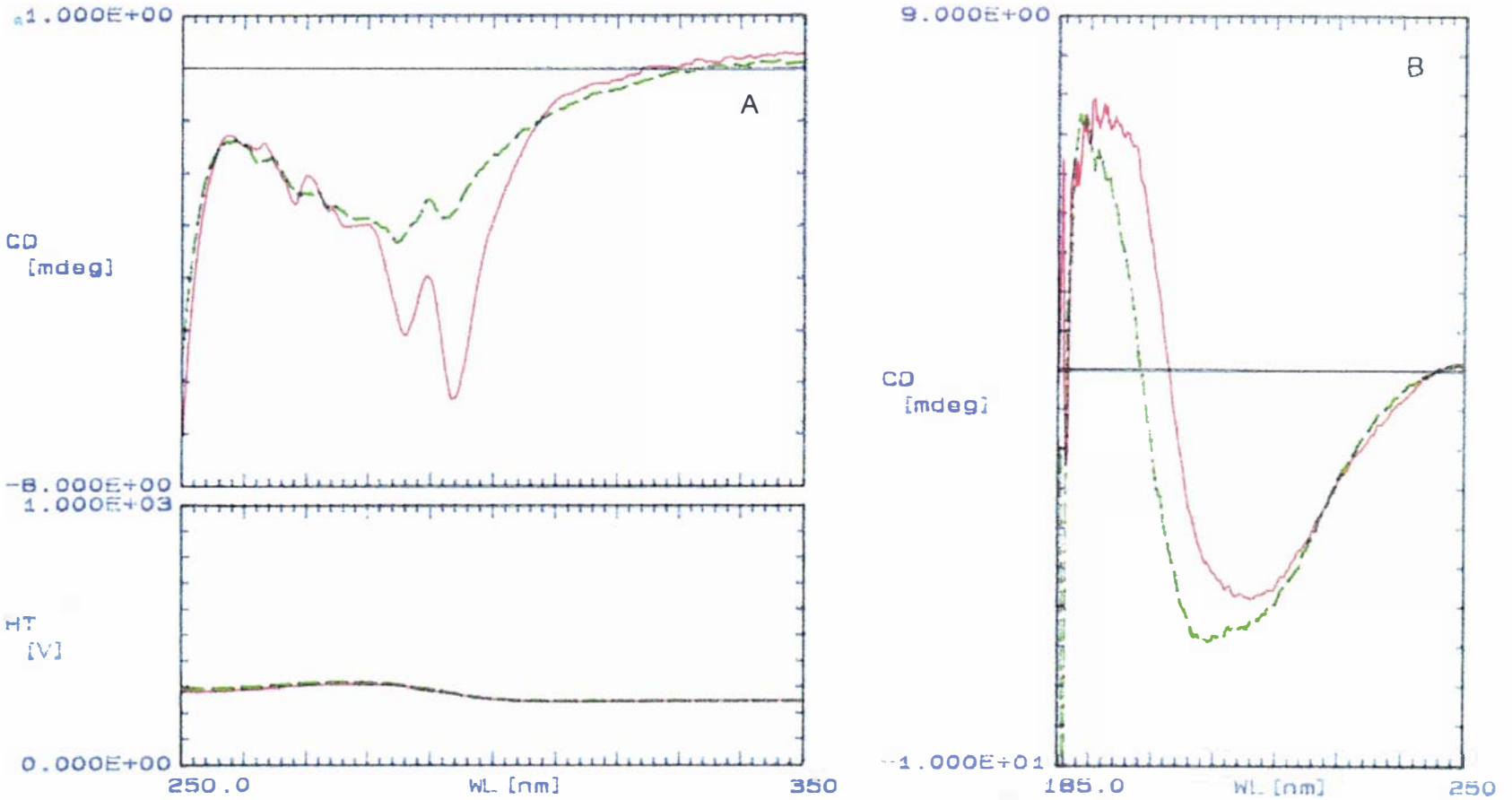

Figure 4.1.3.9. CD spectra of commercially prepared β-lg A/B in the (A) near-UV and (B) far-UV regions. Unheated β-lg (red solid) and heated (90 °C for 10 min) β-lg (green dashed) at concentrations of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. For far-UV measurement at concentrations of 0.1 mg/mL of β-lg and 1 mm path length cells were used. The spectra were acquired as described in Fig. 4.1.3.8. Further experimental details are given in Section 3.2.2.

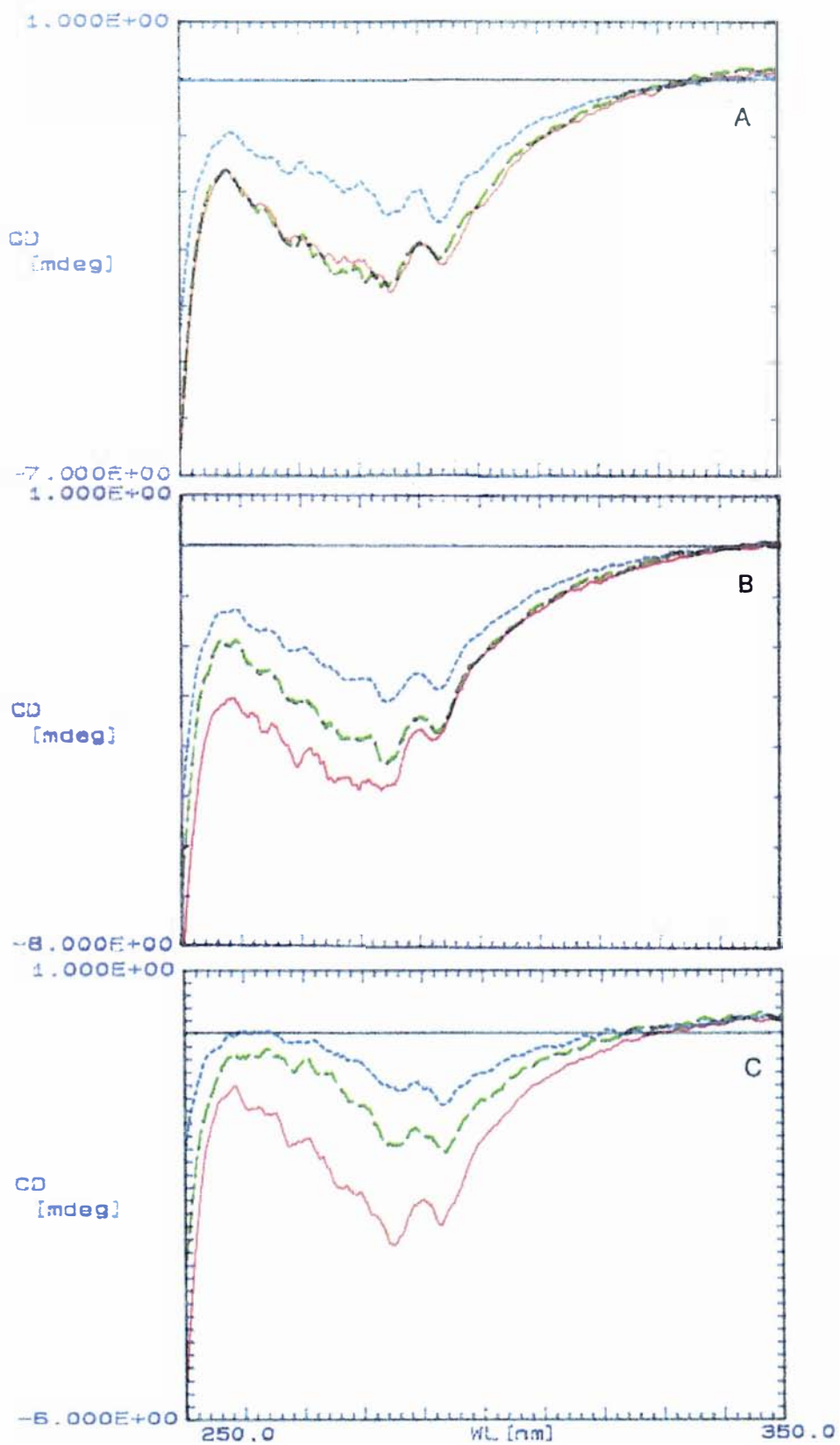

Figure 4.1.3.10. Near-UV CD spectra of heated (90 °C for 10 min) (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using methods I (red solid), II (green dashed) and III (blue dotted) at concentrations of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. The spectra were acquired as described in Fig. 4.1.3.8. Further experimental details are given in Section 3.2.2.

4.1.3.7. Far-UV CD

Far-UV CD spectroscopy was used to identify changes in the secondary structure of the three variants of β-lgs prepared by the three different methods. The CD spectra of three preparations of each β-lg variant were similar to each other (Fig. 4.1.3.11). In the spectra of the β-lg solutions, a peak at 193 nm and a trough at 216 nm were observed, but the noise level in these spectra was high compared with other spectroscopic techniques, reflecting the low signal from the chiral groups in the protein.

In the far-UV CD spectra of heat-treated solutions (90 °C for 10 min), the band at 216 nm became a shoulder on the long wavelength side of a new and more intense negative band centred at approximately 206 nm (Fig. 4.1.3.12). The heat-induced increase in the intensity at 206 nm in the spectra of the heat-treated solutions suggests that the proportion of random structure in the β-lg molecules increased as a consequence of the heat treatment. Commercially prepared β-lg samples showed similar spectra in the far-UV region (Fig. 4.1.3.9.B). The CD spectral results appear to be consistent with those of Manderson et al. (1999b). In the spectra of the β-lgs prepared using method III, the trough and peak intensities were least among the three methods in the far-UV region (Fig. 4.1.3.12).

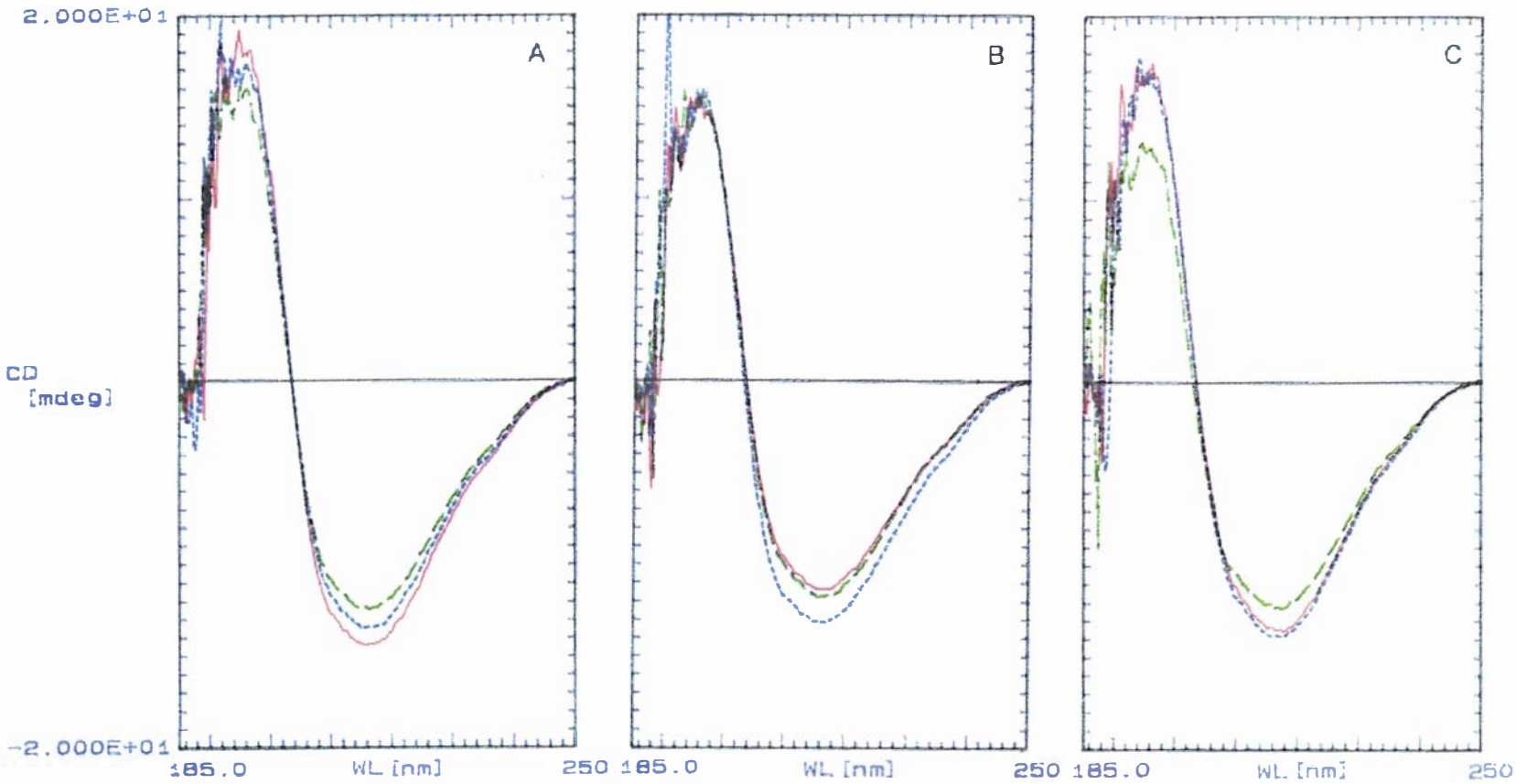

Figure 4.1.3.11. Far-UV CD spectra of (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using methods I (red solid), II (green dashed) and III (blue dotted) at concentrations of 0.1 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. The spectra were acquired as described in Fig. 4.1.3.8. Further experimental details are given in Section 3.2.2.

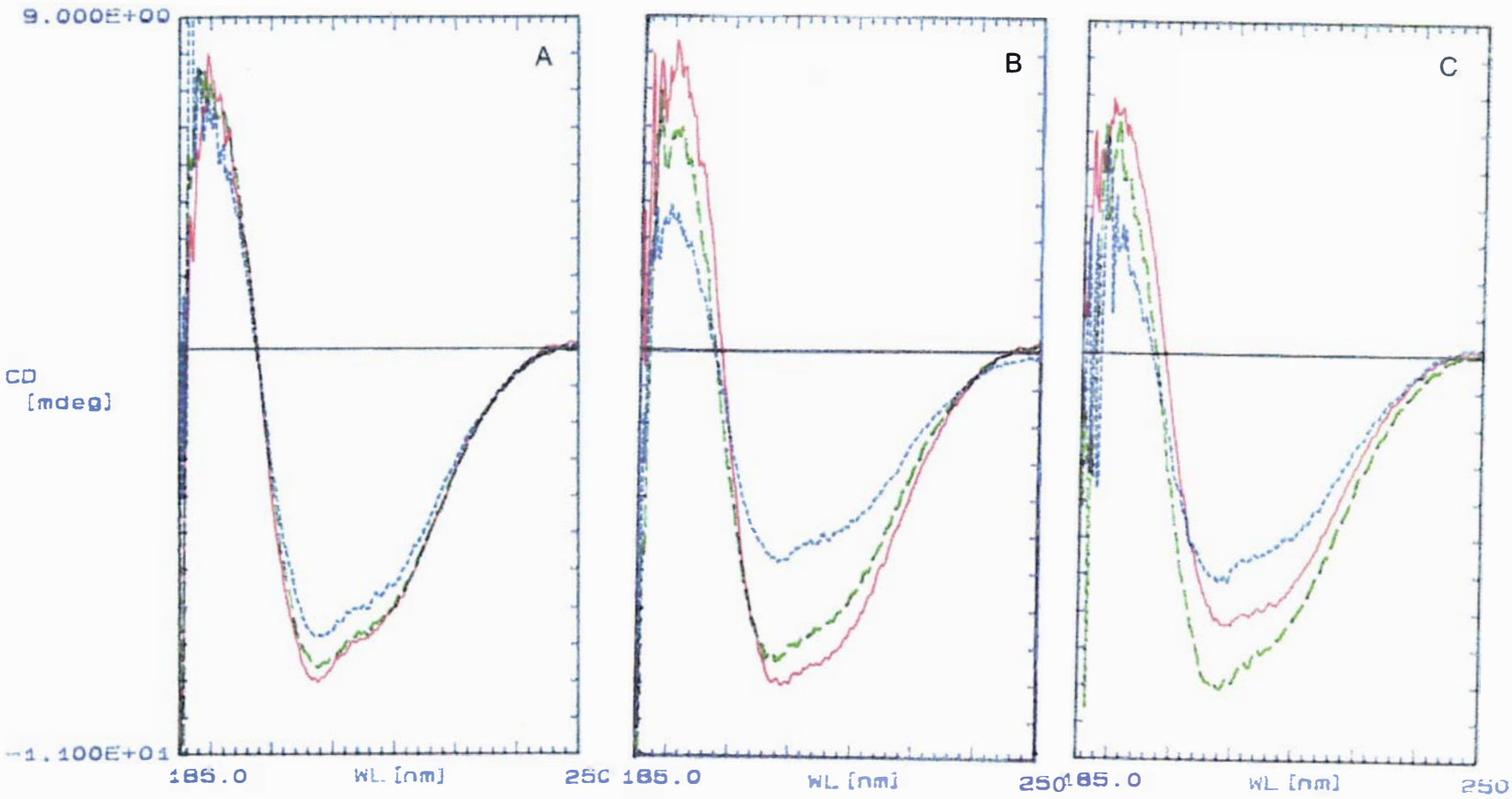

Figure 4.1.3.12. Far-UV CD spectra of heated (90 °C for 10 min) (A) β-lg A, (B) β-lg B and (C) β-lg C prepared using methods I (red solid), II (green dashed) and III (blue dotted) at concentrations of 0.1 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. The spectra were acquired as described in Fig. 4.1.3.8. Further experimental details are given in Section 3.2.2.

4.1.3.8. ANS fluorescence

Fluorescence from the molecules of the probe ANS bound to a β-lg was used to show changes in the structures of the solvent-accessible hydrophobic sites in β-lg and heat-denatured β-lg (Manderson et al., 1999a). Addition of ANS to β-lg solutions gave rise to a steady increase in the fluorescence emission at 470 nm as the molar ratio of ANS to monomeric β-lg increased (Fig. 4.1.3.13.A-C). β-Lg prepared using method II always showed the greatest increase in fluorescent intensity, and β-lg prepared using method III showed the lowest increase in values for all three variants.

All the heat-treated β-lg solutions showed an increase in ANS emission, but β-lgs prepared using method III all showed relatively smaller increases (Fig. 4.1.3.13.D-F). At a molar ratio ANS:monomeric β-lg of 1:1, the intensity of emission from ANS bound to heat-treated β-lg was approximately 10 × greater than that from ANS bound to unheated β-lg prepared using method I or II. This large difference suggests that heat treatment leads to irreversible structural change in molecules of β-lg.

β-Lg has some similarities to BSA, which also has a single thiol residue, such as the ability to bind hydrophobes and a propensity to form disulphide-linked polymers possibly catalysed by the free thiol residues under appropriate conditions. Therefore, ANS fluorescence of β-lg solutions mixed with 3% BSA was investigated; the results showed greater fluorescence enhancement than β-lg alone indicating that, if BSA was present in a sample as an impurity, it would enhance the ANS fluorescence disproportionately (Fig. 4.1.3.13.G-I).

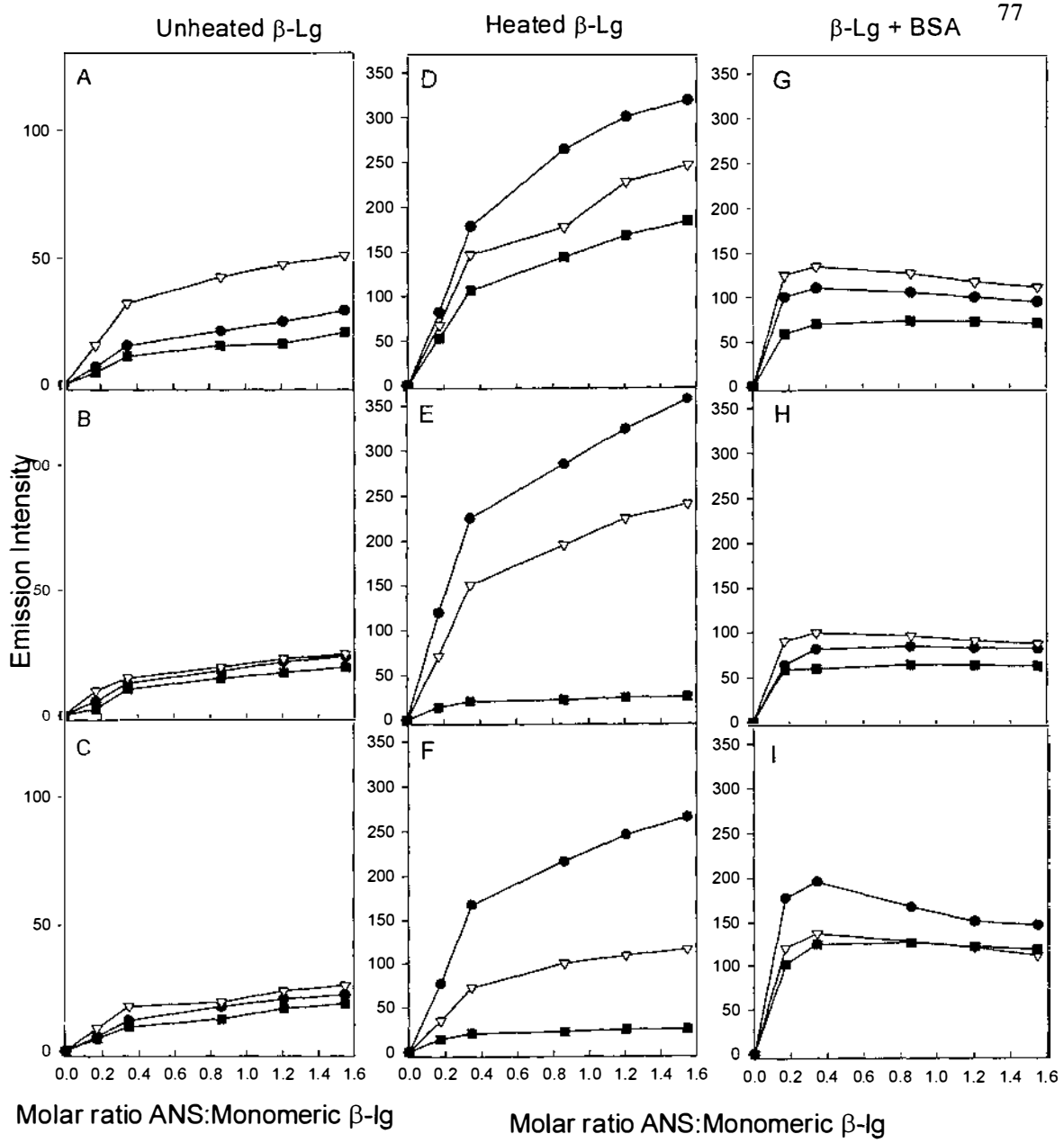

Figure 4.1.3.13. Plots of ANS emission intensity, I_{ANS}, versus molar ratio ANS:monomeric β-lg.

(A) Unheated β-lg A, (B) unheated β-lg B and (C) unheated β-lg C;

(D) Heated β-lg A (90 °C for 10 min), (E) heated β-lg B and (F) heated β-lg C;

(G) β-Lg A + 3 % (w/w) BSA, (H) β-lg B + 3 % BSA and (I) β-lg C + 3 % BSA prepared by methods I (●), II (▽) and III (■) in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. The spectra were acquired at β-lg concentrations of 1.0 mg/mL at 20 °C in a 10 × 10 mm path length cell using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The emission spectra were recorded between 310 and 510 nm, using an excitation wavelength of 370 nm and excitation and emission slit widths of 8 nm at a scan speed of 25 nm/min and a chart speed of 1 cm/min. Experimental details are given in Section 4.1.2.3.

4.1.4. Conclusions

SEC-MALLS gave similar weight-averaged molecular masses of β-lgs from the three different preparation methods. However, significant differences were identified in the mass spectra of β-lgs prepared using method III. The β-lg + n(98.5 Da) adducts observed in these purified β-lgs were consistent with the addition of one or more sulphate residues to the proteins. Also there was very little amount of lactosyl-β-lg A and lactosyl-β-lg C prepared using method I.

The near- and far-UV CD spectra obtained from β-lg samples prepared using methods I, II and III were very similar. Furthermore, plots of ANS emission intensity versus molar ratio of ANS to monomeric β-lg for these samples were similar. These results indicate that either the structures of the β-lg molecules in these samples were equivalent, or the selected spectroscopic techniques were not sensitive enough to detect minor structural differences or low concentrations of contaminating proteins.

However, near- and far-UV CD spectra obtained from heat-treated β-lg samples prepared using methods I and II versus method III showed some differences. At the conclusion of ANS titration, the intensity of the emission from ANS bound to heat-treated β-lg B prepared using methods I and II was approximately 14 × and 9 × greater, respectively, than that from ANS bound to heat-treated β-lg B prepared using method III. The similarities in the trends in these comparisons therefore suggest that an irreversible structural change in β-lg molecules occurred when β-lg was prepared using the TCA fractionation method of Fox et al. (1967). Also, they may indicate the effect of the sulphate group, as shown by mass spectrometry (Fig. 4.1.3.7).

In conclusion, method I, in which β-lg is purified using the salt fractionation procedure of Mailliart and Ribadeau-Dumas (1988) and then purified further by size-exclusion chromatography, appears to be appropriate for preparing gram quantities of pure native β-lg. The spectroscopic results obtained for samples prepared using methods I and II suggested that the structures of the β-lg molecules in these samples were similar. However, a number of time-consuming centrifugation steps are required in the initial stages of method II, when volumes of material are still high. Consequently method I was

used to prepare all the samples examined in the remainder of this study and reported in this thesis.

4.2. PREPARATION OF κ-CASEIN

4.2.1. Introduction

The simplest model of the casein micelle suggests that the casein particles can be considered to be spheres with a coating of κ-CN on their surfaces; this coating of κ-CN is considered to provide stability (Holt, 1998). Intermolecular disulphide bonding within κ-CN has not been considered to be of primary importance in casein micelle structure and stability (Swaisgood, 1992). This concept comes from the early work of Woychik (1965), who showed that reduced and alkylated κ-CN had the same propensity to reform model colloids as native κ-CN. When the disulphide bonding properties were discovered, it was suggested that discrepancies in the reported molecular weight for κ-CN aggregates could be related to alterations in the disulphide bonding patterns (Thurn et al., 1987; de Kruif and May, 1991; Groves et al., 1998). In this study, it was important to obtain "native κ-CN", i.e. to interfere with the strong association of κ-CN with the other caseins without breaking the κ-CN internal structure or the disulphide bonding patterns.

Various methods of κ-CN purification utilising precipitation, ion-exchange chromatography, gel filtration or various combinations of these have been described (Swaisgood, 1992). Some caution has been expressed over the years concerning the effect of purification conditions on the integrity and composition of κ-CN. Beeby and Nitschmann (1963) attributed κ-CN lability to the use of urea in the method of preparation, whereas Woychik (1965) attributed it to the increase in net negative charge found in κ-CN isolated by the urea-H_2SO_4 precipitation method of Zittle and Custer (1963) to either carbamylation of lysine by the cyanate ions found in urea solutions, or deamidation due to the strongly acidic conditions. Hill (1963) also suggested that there may be selective loss of sialic-acid-containing species of κ-CN during acid precipitation.

In milk micelles, and in preparations of whole casein such as acid-precipitated whole casein, κ-CN exists as a complex with α_{s} ($\alpha_{s1} + \alpha_{s2}$) and β-caseins. Urea dissociates this

complex (Payens, 1961); the α_s- and β-caseins are converted into monomeric forms whereas κ-CN remains as relatively large aggregates maintained by intermolecular disulphide bonding (McKenzie and Wake, 1961). Several reports (Nakai et al., 1966; Yamaguchi and Tarassuk, 1967; Yamaguchi et al., 1968; Rasmussen and Petersen, 1992) have suggested that gel filtration is a useful technique in the preparation of κ-CN from acid casein or κ-CN-rich materials; it is convenient and produces unmodified products with little or no contaminating protein. In this study, a gel filtration technique based on Yamaguchi et al. (1968) and Rasmussen and Petersen (1992) was used to obtain pure native κ-CN directly from casein micelles.

4.2.2. Materials and methods

4.2.2.1. Materials

Fresh unpasteurised whole milks, which were known to contain κ-CN AA or BB, were obtained from specific cows, which were known to produce the required phenotypes as determined using alkaline-urea PAGE (Section 3.2.1.4).

4.2.2.2. Preparation of κ-casein

An outline of the experimental procedure used for κ-CN A and κ-CN B purification is given in Figure 4.2.2.1. Each variant milk was treated separately.

Centrifugation of milk

The fresh unpasteurised whole milk was centrifuged at 8,000 *g* for 1 h at 20 ± 5 °C in a temperature-controlled centrifuge (Sorvall RC5B or RC5C, DuPont Company, Newtown, CT, USA) using a Sorvall SuperLite TM GSA type rotor. After centrifugation, three fractions were obtained, i.e. top (cream) layer, middle layer and pellet. The middle layer containing small casein micelles and most of the whey proteins was decanted off and retained. The top layer and the pellet, which contained fat and large casein micelles, respectively, were removed from the centrifuge tube.

The opaque middle layer was centrifuged at 90,000 *g* for 1 h at 20 ± 5 °C in a temperature-controlled centrifuge (Sorvall RC28S, DuPont Company, Newtown, CT, USA) using a Sorvall SS-34 type rotor. A heavy thick liquid layer was observed in

addition to the top (cream) layer, middle layer and pellet. This time both the thick liquid layer and the pellet were retained and stored frozen at -21 °C until required.

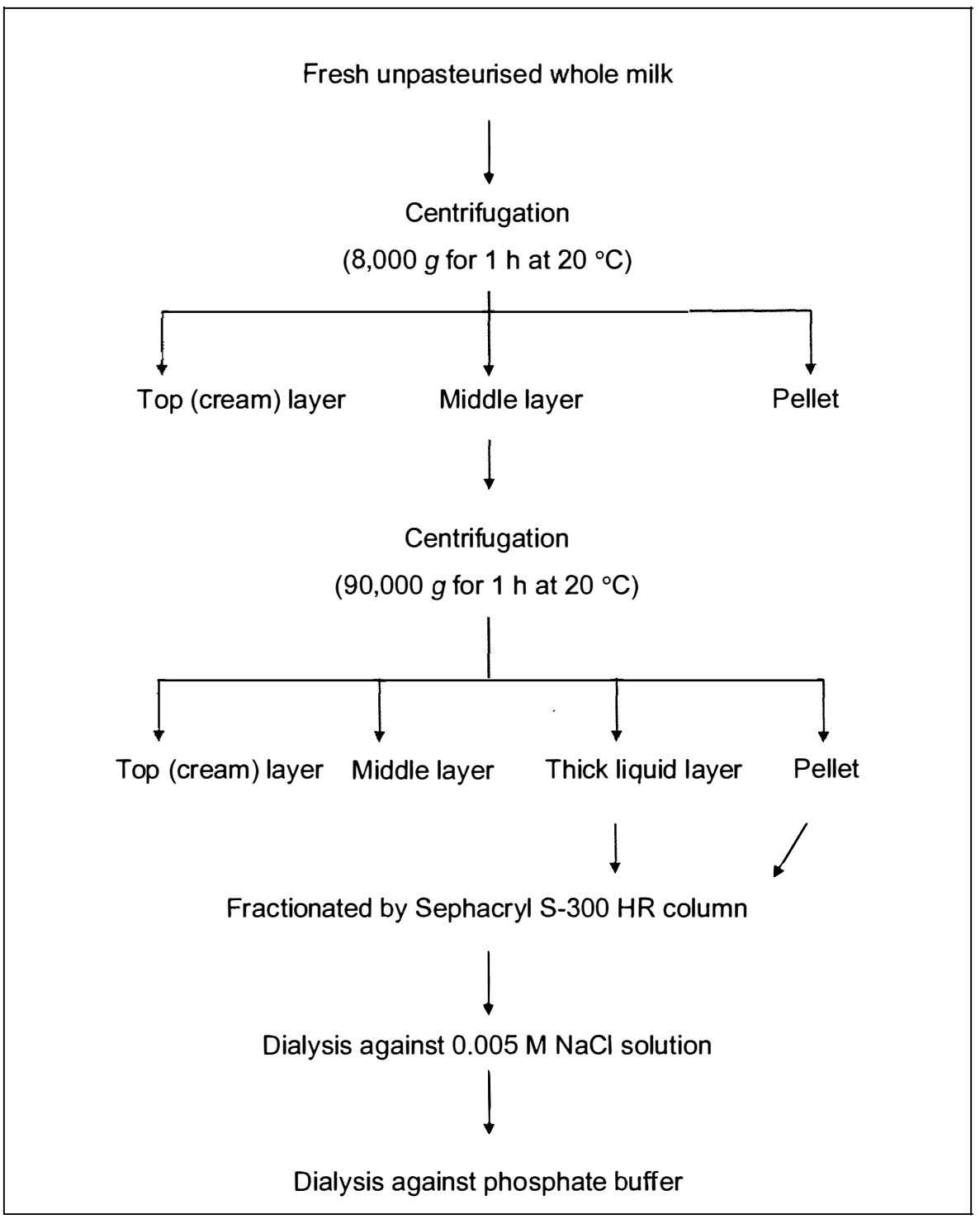

Figure 4.2.2.1. Experimental protocol of κ-CN purification.

Separation of κ-casein by size-exclusion chromatography

Size-exclusion chromatography was used to separate portions of κ-CN A or B samples. The frozen thick liquid layer and the pellet were thawed at 4 °C and the pellet was dissolved in pH 6.3 TCU buffer (0.005 M Tris-citrate buffer containing 6 M urea), whereas Yamaguchi et al. (1968) used pH 8.6 TCU buffer, and then both solutions were filtered using a 0.45 μM filtering unit (Millipore Corporation, Bedford, MA, USA). Proteins were separated on a Sephacryl S-300 HR column (50 mm × 400 mm) using the Pharmacia FPLC system (see Section 4.1.2.3). The column was equilibrated with TCU buffer prior to chromatography.

The absorbance of the eluate from each run was monitored at 280 nm by a VWM 2141 detector and recorded using an REC 102 multi-channel chart recorder. The fractions containing κ-CN were collected using a Frac-200 fraction collector. The presence of κ-CN in the eluted fractions was confirmed using reduced samples on alkaline-urea PAGE.

Dialysis of pooled fractions

Selected fractions were pooled and then dialysed against 0.005 M NaCl and then against pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) exhaustively and stored frozen at -21 °C. Before heat treatment or any measurement, some soybean trypsin inhibitor (SBTI, Type I-S: from soybean lyophilised and chromatographically prepared, T 9003, Sigma Chemical Co., St. Louis, MO, USA) was added to the κ-CN sample (1 % w/w of κ-CN).

4.2.3. Results and discussion

4.2.3.1. Alkaline-urea PAGE

The alkaline-urea PAGE results (Fig. 4.2.3.1.A (non-reduced) and B (reduced)) indicate that the first centrifugation at 8,000 *g* for 1 h removed most of the α_{s1} and β-caseins. The middle layer containing small casein micelles and most of the whey proteins was selected.

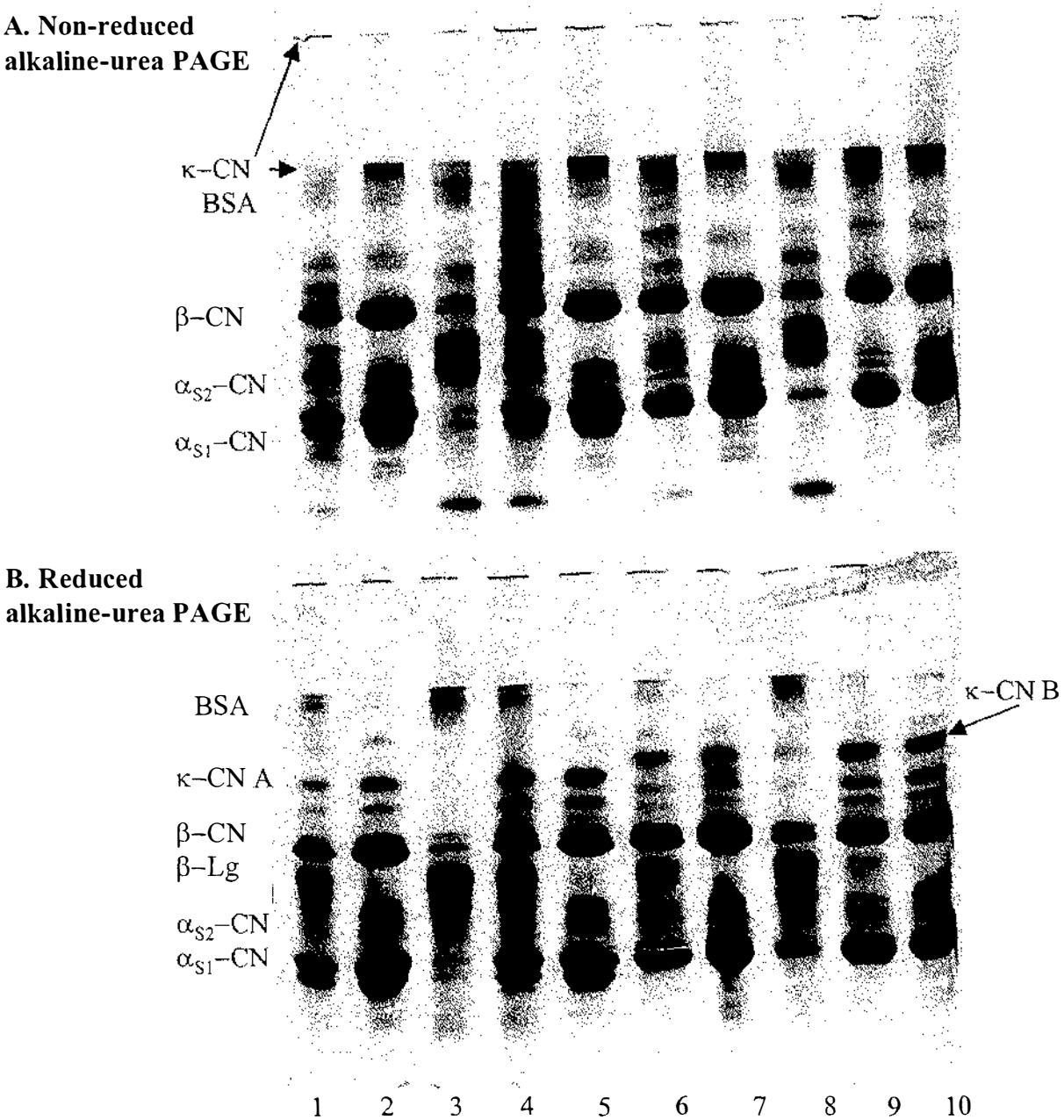

Figure 4.2.3.1. Alkaline-urea PAGE electropherograms of bovine milk protein fractions obtained when κ-CN A and B were prepared. (A) Non-reduced and (B) reduced alkaline-urea PAGE. Gel lanes contain (1) middle layer and (2) pellet after first centrifugation; (3) middle layer, (4) thick liquid layer and (5) pellet after second centrifugation for κ-CN A. Gel lanes 6-10 show the same order for κ-CN B. The measurements were made using the alkaline-urea PAGE method described in Section 3.2.1.4.

This middle layer was centrifuged again at 90,000 *g* for 1 h. The heavy thick liquid layer and the pellet were retained, as they contained the major portion of κ-CN compared with the middle layer.

4.2.3.2. Size-exclusion chromatography

κ-CN was further purified by size-exclusion chromatography (Fig. 4.2.3.2) and then alkaline-urea PAGE was used to identify the eluted fractions (Fig. 4.2.3.3.A, B). As shown in Fig. 4.2.3.2, the thick liquid layer solution contained more of the κ-CN than the pellet solution. In each run, eluate fractions containing pure κ-CN were identified and pooled. There were usually three such fractions (Lanes 1 to 3 in Fig. 4.2.3.3.A, B). Although many other fractions also contained κ-CN, they were contaminated with other proteins.

The yields of κ-CN B obtained from each size-exclusion FPLC run were about 44 % and 21 % from the thick liquid layer and the pellet, respectively. The yields of κ-CN A were lower than those of κ-CN B, i.e. 20 % and 34 %, respectively. After chromatography, the average κ-CN concentration in the three 3 mL fractions collected was 2.3 and 1.9 mg/mL for κ-CN B and κ-CN A, respectively.

4.2.3.3. Addition of soybean trypsin inhibitor

Even though purified κ-CN showed as one band in alkaline-urea PAGE, other proteins such as BSA were revealed when using SDS-PAGE (Fig. 4.2.3.3.C, Lanes 1-3). Furthermore, under SDS-PAGE reducing conditions, para-κ-CN was revealed (Fig. 4.2.3.3.C, Lanes 4-6). Therefore, 1 % (w/w) soybean trypsin inhibitor (SBTI) was added to the κ-CN sample as soon as it came off the column in order to suppress plasmin activity that occurs in the absence of urea (Fig. 4.2.3.3.C, Lanes 7-9).

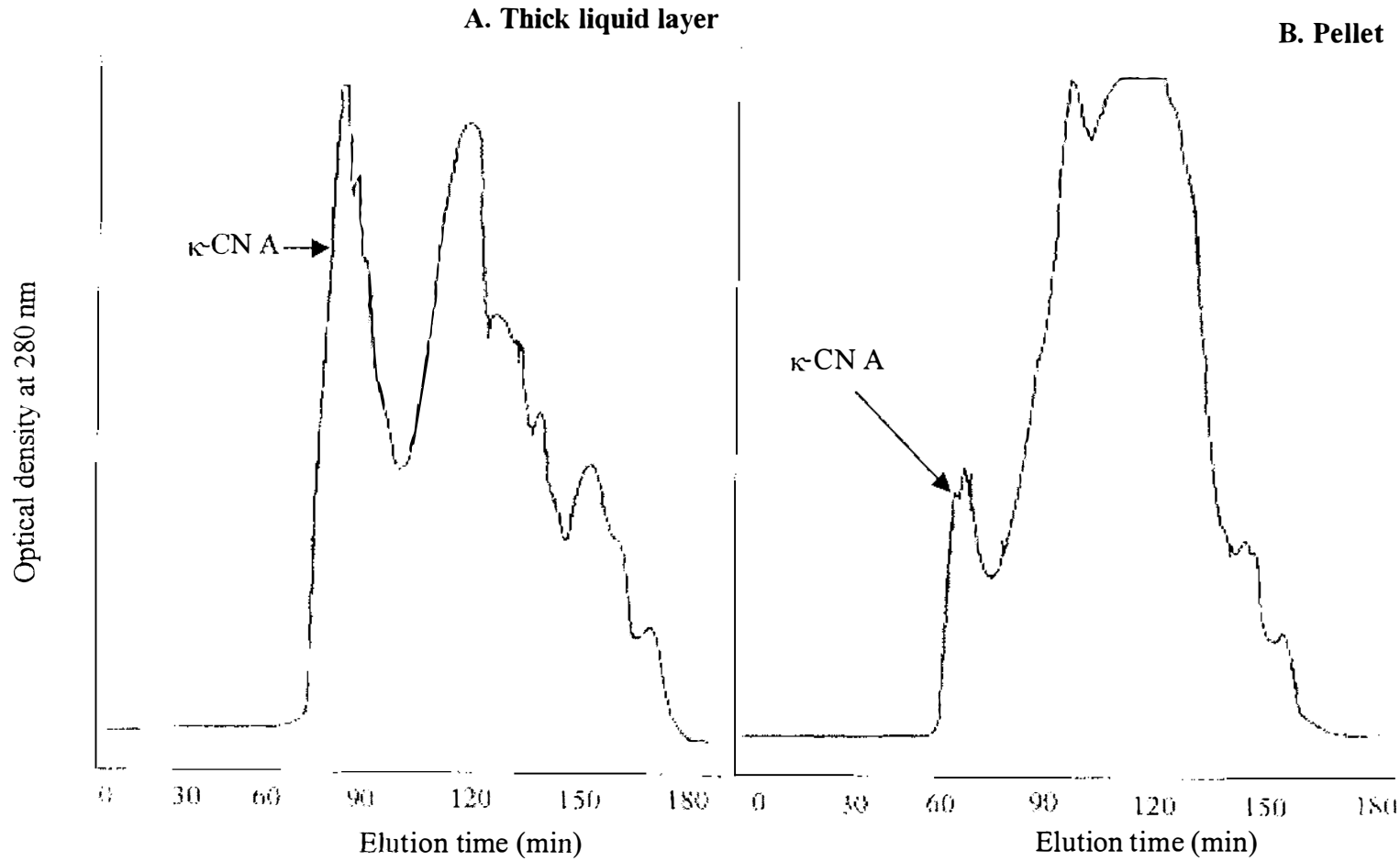

Figure 4.2.3.2. Size-exclusion FPLC chromatograms of κ-CN A from (A) the thick liquid layer and (B) the pellet, separated on a Sephacryl S-300 HR column equilibrated with pH 6.3 TCU buffer (0.005 M Tris-citrate buffer containing 6 M urea). The measurements were made using the FPLC system described in Section 4.1.2.3.

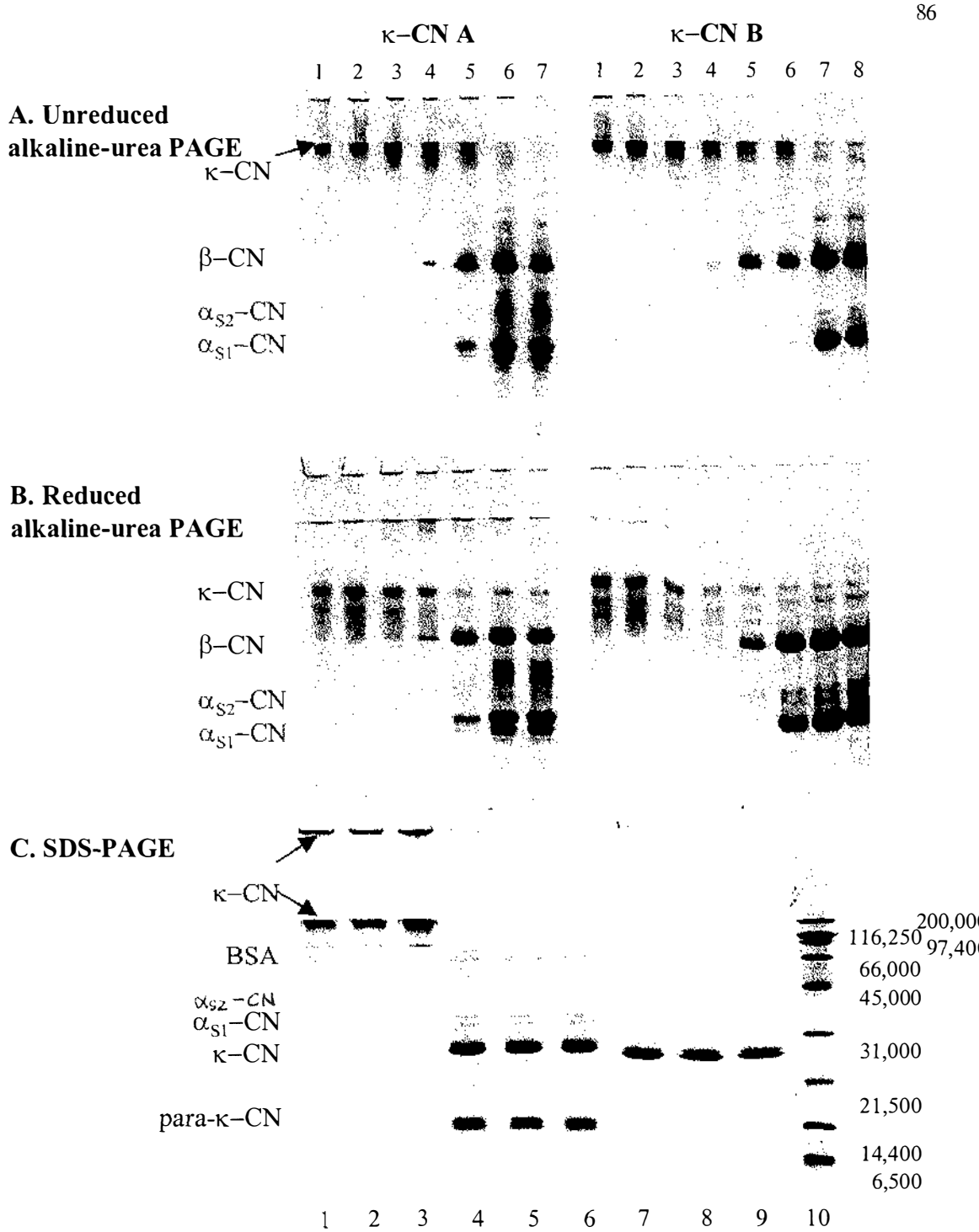

Figure 4.2.3.3. Electropherograms of eluted fractions obtained when crude preparations of κ-CN A and B were further purified by size-exclusion chromatography. (A) Non-reduced and (B) reduced alkaline-urea PAGE. Fractions are shown in order of increasing elution time from left to right. The measurements were made using the alkaline-urea PAGE method described in Section 3.2.1.4.

(C) SDS-PAGE electropherogram of κ-CN B. Lanes contain κ-CN B heated at 80 °C for (1) 0 min, (2) 15 min and (3) 45 min in non-reduced SDS-PAGE. The same samples were applied to lanes 4-6 without and to lanes 7-9 with adding SBTI in reduced SDS-PAGE. Lane 10 contains the molecular standard.

CHAPTER 5.

EFFECT OF HEAT TREATMENT ON THE STRUCTURE AND LIGAND-BINDING PROPERTIES OF BOVINE β-LACTOGLOBULIN AND PORCINE β-LACTOGLOBULIN

5.1. INTRODUCTION

β-Lg is the major whey protein in the milk of ruminant and some non-ruminant mammals, such as pigs and horses, but is absent from human and rodent milk (Hambling et al., 1992).

Bovine β-lg has a monomer molecular weight of approximately 18,300 Da and is associated into dimers between about pH 3 and pH 7.5 (McKenzie, 1971; Hambling et al., 1992). Each monomer comprises 162 amino acids and contains two disulphide bridges (Cys66-Cys160 and Cys106-Cys119) and one free thiol (Cys-121), which is buried beneath the α-helix and is unavailable for reaction in the native protein (Papiz et al., 1986).

Porcine β-lg is significantly different from the bovine protein. It is reported to be monomeric irrespective of the pH and devoid of a free –SH group (Kessler and Brew, 1970), and to have a molecular weight of approximately 17,800 Da (Alexander and Beattie, 1992) with only one tryptophan side chain (Trp-19, Alexander and Beattie, 1992) (bovine β-lg: Trp-19 and Trp-61, Eigel et al., 1984).

The ruminant β-lgs have similar properties but the non-ruminant β-lgs, e.g. equine or porcine, show greater differences in the amino acid sequence which is reflected in the physico-chemical properties of the proteins (Godovac-Zimmermann, 1988). Cats, horses and donkeys express distinct forms of two or three β-lgs and cat II, horse II and donkey II appear to be more closely related to the β-lg pseudogenes in cow and goat (Sawyer, 2000). The sequence of mature porcine β-lg shows 67 % to the bovine β-lg A variant (Alexander and Beattie, 1992) and 65 % to the β-lg B variant, and it is two amino acids shorter that bovine β-lg. The different characteristics include the conformational transition at about pH 7.2 that bovine β-lg undergoes (Tanford et al., 1959), but which has not been reported for

equine or porcine β-lg. The heat-induced disulphide-bonded aggregation of bovine β-lg, which contains a Cys residue at position 121 (ruminant proteins), has been reported by McSwiney et al. (1994a, b) and Manderson et al. (1998). Bovine β-lg has been shown to bind a variety of hydrophobic substances *in vitro* which include retinol (Futterman and Heller, 1972), retinoic acid and long chain fatty acids (Spector and Fletcher, 1970). However, porcine β-lg does not form aggregates during heat treatment (Gallagher et al., 1996) and binds with only fatty acids but not retinol, as measured using tryptophan fluorescence enhancement (Frapin et al., 1993). Gallagher et al. (1996) also found that the T_{max} for porcine β-lg is 81.0 ± 0.1 °C, whereas that for bovine β-lg is 74.8 ± 0.9 °C as measured by DSC.

In order to throw more light on the role of Cys-121 in bovine β-lg changes during heat treatment, the binding of ligands to bovine β-lg and porcine β-lg and the effect of heat treatment on each protein were investigated in this study.

It is known that bovine β-lg can bind fatty acids (Spector and Fletcher, 1970) and retinol (Fugate and Song, 1980) *in vitro* and that fatty acids compete with retinol for binding (Puyol et al., 1991). In this study, a CD spectrometer-based method was used to investigate ligand binding by bovine β-lg B and porcine β-lg. Palmitic acid and PnA, which are long chain fatty acids, and retinol, which is a conjugated tetraene system similar to that of the PnA, were used as ligands. Their structures are shown in Fig. 5.1.1.

5.2. EXPERIMENTAL PROTOCOL

5.2.1. Preparation of porcine β-lactoglobulin

The lyophilised whey provided by D. Gallagher (Department of Food Chemistry, University College Cork, Ireland) was prepared by the acid precipitation of caseins from skim milk at pH 4.5 (Gallagher et al., 1996). Porcine β-lg was purified from the lyophilised acid whey using the procedure of Dalgalarrondo et al. (1992).

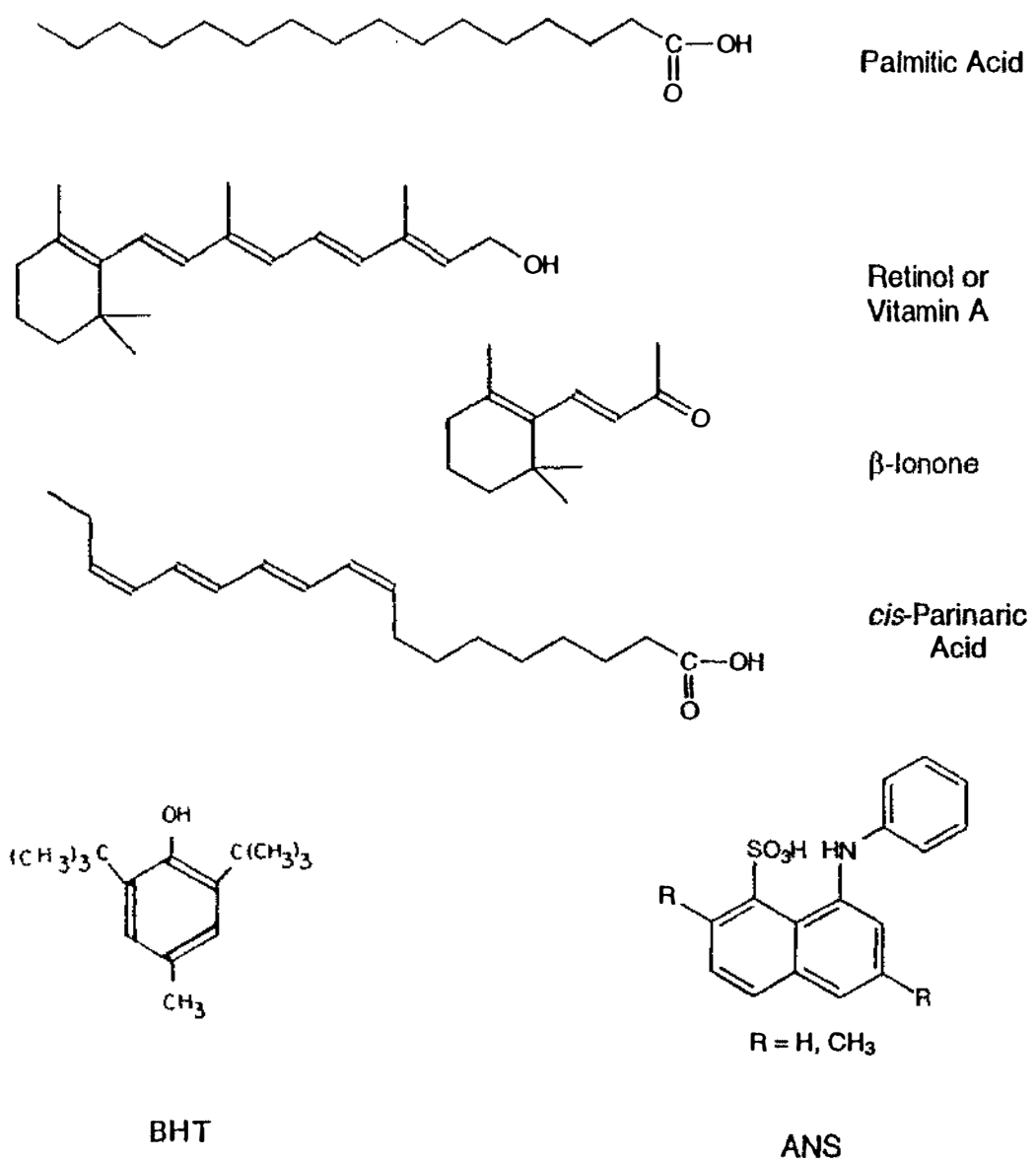

Figure 5.1.1.1. The structures of ligands referred to in the text. BHT, butylated hydroxytoluene; ANS, 1,8-anilinonaphthalene sulphonate.

The porcine β-lg was further purified by Gavin Manderson (New Zealand Dairy Research Institute, Palmerston North, New Zealand) using the following procedure. The porcine β-lg sample was concentrated fourfold by stirred cell ultrafiltration, using apparatus supplied by Amicon, Inc. (Beverly, MA, USA). It was purified further by size-exclusion chromatography on a Superdex 75 column (50 mm × 600 mm) using the Pharmacia FPLC system (see Section 4.1.2.3). The column was equilibrated with pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) prior to chromatography. After checking the purity of each fraction by alkaline-PAGE (see Section 3.2.1.4), selected fractions were pooled and stored frozen at -21 °C.

5.2.2. Measurement protocols

Sample treatments

Frozen solutions of porcine or bovine β-lg were thawed at 4 °C, dialysed against pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) or pH 7.7 Tris-phosphate buffer (13 mM Tris, 13 mM sodium phosphate and 68 mM NaCl) and then filtered using a membrane filters (0.45 μm, Millipore Corporation, Bedford, MA, USA). The concentration of protein was determined using 280 nm absorbance and extinction coefficients of 5.65 (10.0 mg/mL of porcine β-lg) (Kessler and Brew, 1970) and 9.6 (10.0 mg/mL of bovine β-lg) (Bell and Mckenzie, 1967) so that they could be diluted to appropriate concentrations with buffer for each set of experiments.

Retinol, PnA and palmitic acid were made to 1 mg/mL in boiled and degassed ethanol and stored under oxygen-free nitrogen in the dark. On occasions, equimolar concentrations of BHT were mixed into these solutions. This reagent does not compete with ANS, PnA or retinol for binding to bovine β-lg (unpublished result, L. K. Creamer, 1995). However, it does provide some protection against oxidative degradation of retinol and PnA.

CD measurements

The porcine and bovine β-lg concentrations were adjusted to 1.0 mg/mL with pH 6.7 phosphate buffer. For investigation of ligand binding, the molar ratio β-lg:ligand was kept between 1.0:1.1 and 1.0:1.2 throughout the experiment. The β-lg solutions were placed in a water-jacketed 10 mm path length CD cell (Jasco,

Ishikawa-cho, Hachioji city, Tokyo, Japan) which was connected to a Neslab model RTE-100 water bath (Neslab Instruments Inc., Newington, NH, USA). Each β-lg solution was held at 20 °C for 20 min prior to measuring the spectrum. Because of the difficulty in obtaining good temperature control in the short path length cells, far-UV CD measurements were not made.

The temperature of the water bath was then increased to 40 °C and held for 20 min, and then another spectrum was run. This procedure was repeated using 20 or 10 °C intervals until a temperature of 80 °C was reached. The water bath temperature was then decreased by 20 °C increments to 20 °C. A calibration run was done to determine the relationship between cell contents and water bath temperatures, after the water bath had been calibrated against a secondary temperature standard (Manderson, 1998). The baseline correction is described in Section 3.2.2.

Ligand fluorescence spectroscopy

Fluorescence measurements were made on a 3.0 mL volume of the unheated 1.0 mg/mL stock solution of porcine β-lg or bovine β-lg B was put into a 10 mm × 10 mm fluorimeter cell. The cell was then placed in the temperature-controlled cell holder of a Perkin-Elmer MPF-2A fluorescence spectrophotometer (Norwalk, CT, USA), which was connected to the Neslab water bath, and given 20 min to attain thermal equilibrium at a water bath temperature setting of 20 °C. Measurements were then made using excitation and emission slit widths of 8 nm, at a scan speed of 25 nm/min.

For ANS fluorescence measurement, an aliquot (60 μL) of ANS (1.41 mM) was added to the protein solution in the fluorimeter cell and mixed by inversion, after which the fluorescence spectrum of the ANS was determined. After ANS addition, the mixture was excited at 370 nm and the emission was scanned from 375 to about 520 nm.

For retinol fluorescence measurement, the molar ratio β-lg:ligand was kept between 1.0:1.0 and 1.0:1.2 and the fluorescence spectrum of the retinol was determined as above. The mixture was excited at 350 nm and the emission was scanned from 310 to about 510 nm.

The temperature of the water bath was increased to 40 °C and then increased stepwise by 4 °C increments to 88 °C; after each increase, the fluorescence emission

spectrum was recorded as described above. After measurements had been made at 88 °C, the temperature of the water bath was decreased stepwise by 10 °C increments to 20 °C and, after each decrease and a 20 min holding period, the emission spectrum was recorded as described above. During all holding periods, the β-lg solution was shielded from the light source of the fluorimeter.

For both ANS and retinol fluorescence measurements, the peak position, λ_{max}, and the peak heights at λ_{max} (I_{ANS} and I_{RET}) were measured from the recorder chart. β-Lg and ANS or retinol concentrations were such that the absorbances of the solutions were less than 0.35 at the excitation wavelengths. Because the measurements were of a comparative nature, corrections for inner filter effects were not made. Solutions of 20 μM NATA were used as standards.

5.3. RESULTS AND DISCUSSION

5.3.1. Structural changes in bovine and porcine β-lactoglobulin during heating and subsequent cooling

5.3.1.1. Unheated bovine and porcine β-lactoglobulin

CD is the difference between the absorption of the left and right components of circularly polarised light by a chiral component. Bands in the near-UV CD spectra of proteins are due to the aromatic side chains of tryptophan, tyrosine and phenylalanine. Absorption involves the transition of an electron from a filled π orbital to an empty higher energy π* orbital (Strickland, 1974). Because the aromatic rings possess several π* orbitals, a number of transitions occur (Strickland, 1974), which are usually categorised according to the properties of the excited state.

The intensities and polarities of the CD bands are dependent on the conformation of the chiral groups and the bands are usually broad and may represent the superposition or cancellation of the bands of several chiral groups (Strickland, 1974).

The spectrum of a tryptophan side chain fixed in a particular conformation usually exhibits two intense bands with the same sign between approximately 285 and 293 nm. A pair of small sharp bands with the same sign and located between 255 and 270 nm is usually observed in the spectrum of a phenylalanine side chain fixed in a

particular conformation. For a tyrosine side chain in a fixed conformation, a band is usually observed between 275 and 282 nm (Strickland, 1974).

Residue Trp-19 in bovine β-lg is one of the invariant residues throughout the lipocalin superfamily which has two characteristic features: a binding ability for small hydrophobic molecules and the unique β-barrel three-dimensional structure. Several studies have indicated that Trp-19 of β-lg was essential for the binding of retinol (Fugate and Song, 1980; Papiz et al., 1986). However, Katakura et al. (1994) investigated the relationship between Trp-19 and maintaining the molecular structure or binding ability of bovine β-lg using site-directed mutagenesis and concluded that Trp-19 of β-lg is not essential for binding retinol, but is relevant to stabilising bound retinol and maintaining the β-lg structure.

The near-UV CD spectra of native bovine and porcine β-lg are shown in Fig. 5.3.1.1. The bovine β-lg spectrum appears to be similar to those obtained by Townend et al. (1967) and Manderson et al. (1999b). When aromatic side chains are packed against other side chains, they usually produce strong CD bands, because they are located in chiral environments (Strickland, 1974). Therefore, the strong troughs observed at 293 and 285 nm in bovine β-lg suggest that the tryptophan side chain is located in a chiral environment. According to the crystal structure of bovine β-lg (Brownlow et al., 1997; Qin et al., 1998b), Trp-19 is packed against Thr-18, Tyr-20, Leu-46, Leu-103 and Arg-124, whereas Trp-61 is solvent exposed. Therefore, the troughs at 293 and 285 nm in the native β-lg B spectrum may be ascribed to Trp-19 alone. There are another two sharp bands at 260 and 267 nm, probably due to phenylalanine side chains, and small peaks centred at 277 nm, probably tyrosine bands. All these observations are in agreement with the results of Manderson (1998).

In contrast, the spectrum of porcine β-lg shows a series of overlapping bands spread between 255 and 300 nm. Unlike bovine β-lg, porcine β-lg lacks the fine structure and maximum negativity ellipticity observed at about 285 nm.

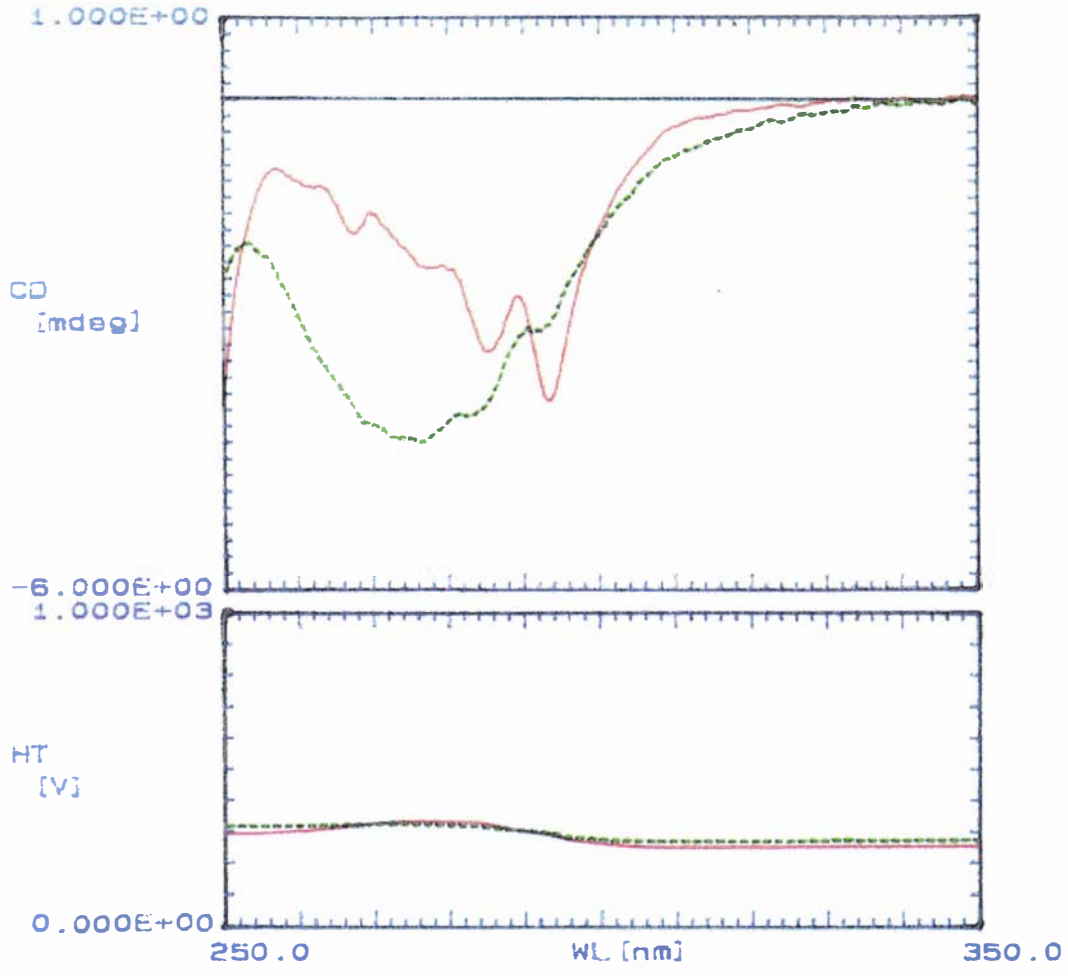

Figure 5.3.1.1. Near-UV CD spectra of bovine β-lg B (red solid) and porcine β-lg (green dotted) at concentrations of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at 20 °C. Protein CD spectra were acquired at 20 °C in stoppered water-jacketed 10 mm path length cells using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 50 nm/min, using a spectral band width of 1.0 nm, at a time constant of 2 s and a step resolution of 0.2 nm. In each case, the final spectrum is the average of five scans of the solution. See Section 5.2.2 for experimental detail.

5.3.1.2. Heat-treated bovine and porcine β-lactoglobulin

Bovine β-lactoglobulin. The changes in the near-UV CD spectrum of bovine β-lg B as the protein solution was heated (A) and then cooled (B) were recorded at several temperatures as shown in Fig. 5.3.1.2. The CD spectrum of the solution heat treated at 40 °C was not significantly different from that of the unheated solution. However, as the heat treatment temperature was increased above 60 °C, the intensities of both the 285 and 293 nm tryptophan CD bands decreased by about 45 %, although there was no discernible wavelength shift (Fig. 5.3.1.2.A). The intensity of the main trough at 293 nm decreased to almost zero at 80 °C. Most of the other bands, which were clearly observed in unheated bovine β-lg, also decreased. These results are consistent with earlier observations by Matsuura and Manning (1994) and Manderson et al. (1999b). As the temperature was decreased, the band intensity at 293 nm increased slightly, although much of this change was caused by the shifts in baseline position (Fig. 5.3.1.2.B). From these results, the large spectral change in bovine β-lg during heat treatment was largely irreversible.

Porcine β-lactoglobulin. The near-UV spectra of porcine β-lg recorded under the same conditions as for bovine β-lg are shown in Fig. 5.3.1.3. As the heating temperature was increased, the band intensity at 285 nm, which originated from Trp-19, decreased. Subsequent cooling of the solution caused the spectrum to return to the original spectrum. It seems that Trp-19 of porcine β-lg is in a lower chirality environment at elevated temperatures. Clearly the effect of heat treatment on the structural changes of porcine β-lg was relatively small and the structural changes were essentially reversible.

The decrease in the intensity of the tryptophan CD bands with heat treatment is indicative of a decrease in the chirality of the environment of Trp-19. The results in Figs 5.3.1.2.A and 5.3.1.3.A suggest that both bovine β-lg and porcine β-lg show a decrease in the chirality of the environment of Trp-19 with increasing temperature. However, Trp-19 in the porcine protein is not affected irreversibly by heat treatment.

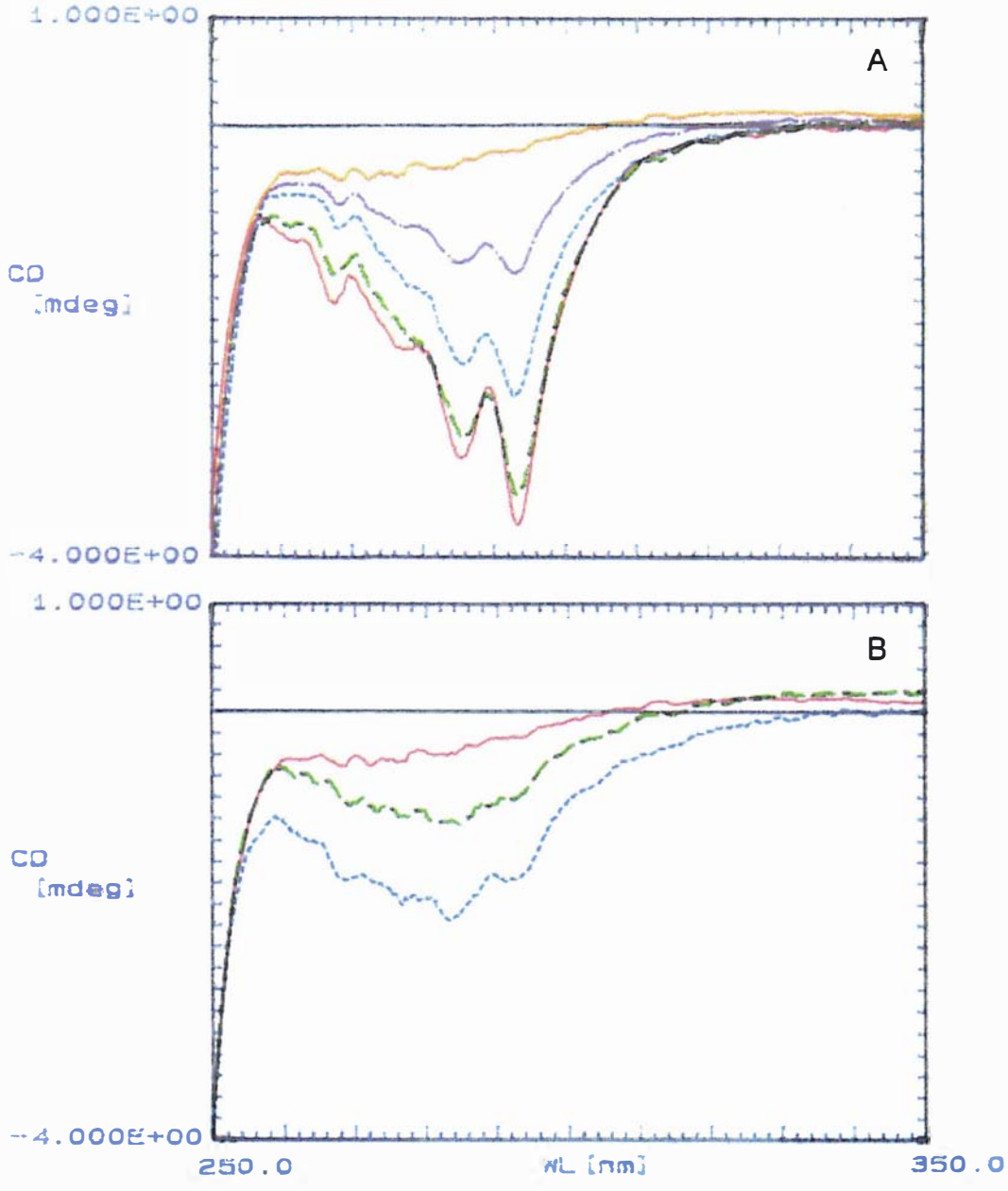

Figure 5.3.1.2. Near-UV CD spectra of bovine β-lg B at a concentration of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at various temperatures.

(A) Bovine β-lg B at 20 °C (red solid), heated to 40 (green dashed), 60 (blue dotted), 70 (purple centre) and 80 °C (orange solid).

(B) Bovine β-lg B at 80 °C (red solid) and then cooled to 60 (green dashed) and 40 °C (blue dotted). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

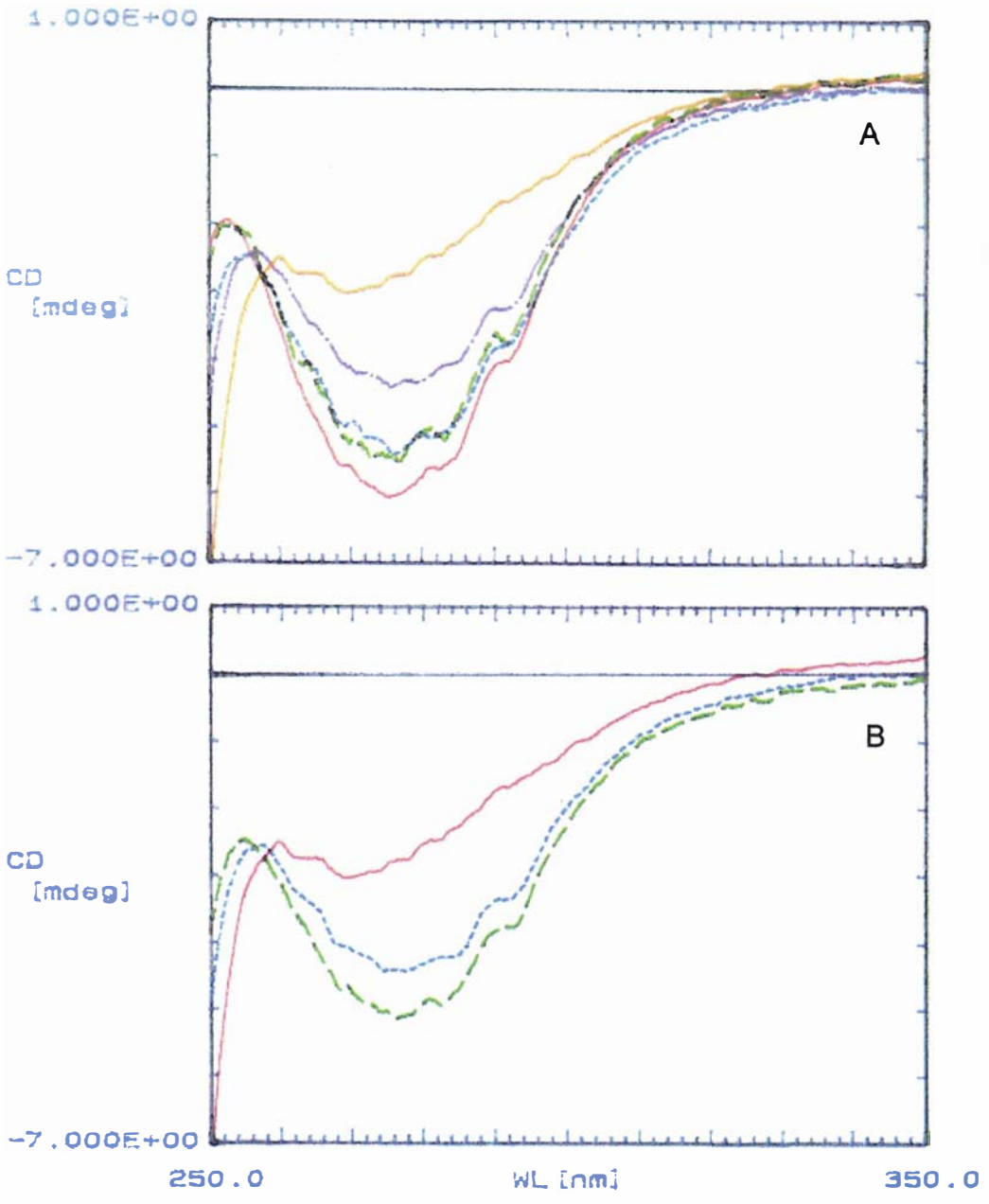

Figure 5.3.1.3. Near-UV CD spectra of porcine β-lg at a concentration of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at various temperatures.

(A) Porcine β-lg at 20 °C (red solid), heated to 40 (green dashed), 60 (blue dotted), 70 (purple centre) and 80 °C (orange solid).

(B) Porcine β-lg B at 80 °C (red solid) and then cooled to 60 (blue dotted) and 40 °C (green dashed). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

5.3.2. Binding properties of bovine and porcine β-lactoglobulin during heating and subsequent cooling

5.3.2.1. Near-UV CD

Results from preliminary experiments showed that, when ethanol solutions of retinol, PnA and palmitic acid were diluted into buffer solutions, none of the solutions showed any significant bands in the near-UV range. However, in the presence of bovine β-lg, characteristic induced CD spectra of retinol and PnA were present (Figs 5.3.2.1 and 5.3.2.4). The induced CD signals from both retinol and PnA were pH dependent and, whereas retinol showed a mid-point pH of about 7.2, PnA had a mid-point pH of 5.1 (Creamer et al., 2000). Therefore, pH 7.7 was selected to observe the maximum effect of the ligand-binding property of β-lg.

(i) Retinol

Bovine β-lactoglobulin. Addition of retinol to bovine β-lg B in pH 7.7 Tris-phosphate buffer solution induced new near-UV CD bands at about 250 and 345 nm (Fig. 5.3.2.1.A). A new peak centred on 255 nm and a new broad trough centred at about 350 nm were observed. The intensities of the tryptophan side chain bands at 285 and 293 nm were also increased by adding retinol. Increasing retinol concentration increased the intensity of all these bands, which reached a maximum at close to a 1:1 molar ratio of retinol to β-lg (Fig. 5.3.2.1.A). Above this ratio, the trough between 345 and 355 nm intensified more slowly with increasing retinol addition.

A mixture of 90 μL of retinol (1.0 mg/mL, 101.94 μmoles) and 3 mL of bovine β-lg B (1.55 mg/mL, 84.2 μmoles) was subjected to heat treatment and CD spectral changes were observed (Fig. 5.3.2.2). The intensities of the bands at 293 and 350 nm reached a maximum at 40 °C and then started to decrease at 60 °C. Further significant decreases in the bands were observed as the heating temperature was increased and these bands had essentially vanished at 80 °C (Fig. 5.3.2.2.A). Subsequent cooling of the heated protein solution did not alter the spectra significantly (Fig. 5.3.2.2.B). This suggests that, as the heating temperature was increased, bovine β-lg lost the ability to bind retinol in a chiral environment and lost the chiral environment of Trp-19. These changes were not restored by subsequent cooling (Fig. 5.3.2.2.B).

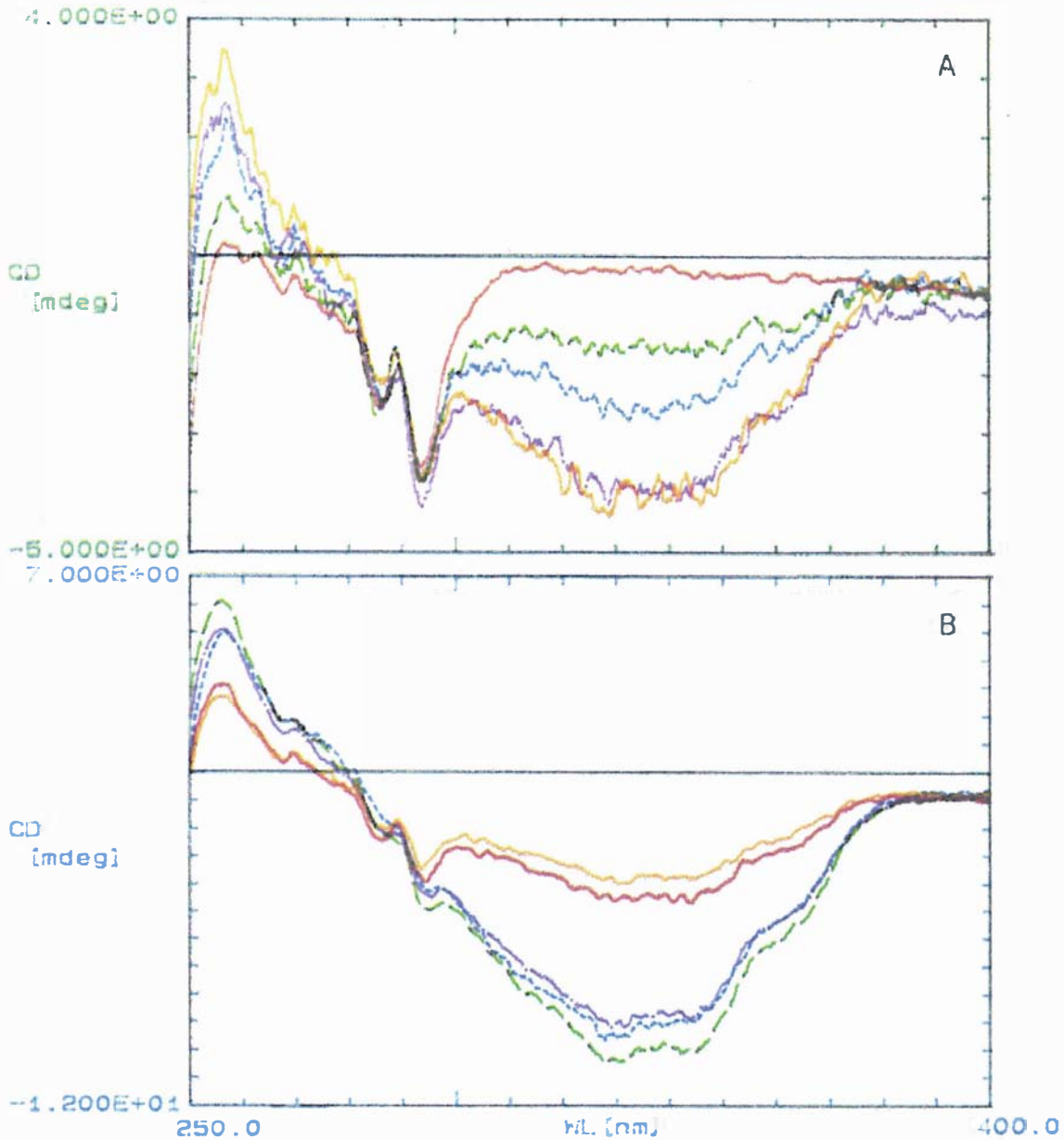

Figure 5.3.2.1. Near-UV CD spectra of:

(A) Increasing amounts of retinol (1.0 mg/mL) added to bovine β-lg B at a concentration of 1.55 mg/mL (84.2 μmoles) in pH 7.7 Tris-phosphate buffer. Retinol added: 0 μL (red solid), 30 μL (green dashed), 60 μL (blue dotted), 90 μL (purple centre) and 100 μL (orange solid).

(B) Mixture of retinol (90 μL of 1.0 mg/mL solution = 101.94 μmoles) and bovine β-lg B (3 mL of 1.55 mg/mL solution = 84.2 μmoles) at 20 °C (red solid), heated to 40 °C (green dashed) and 60 °C (blue dotted) and then cooled to 40 °C (purple centre) and 20 °C (orange solid). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

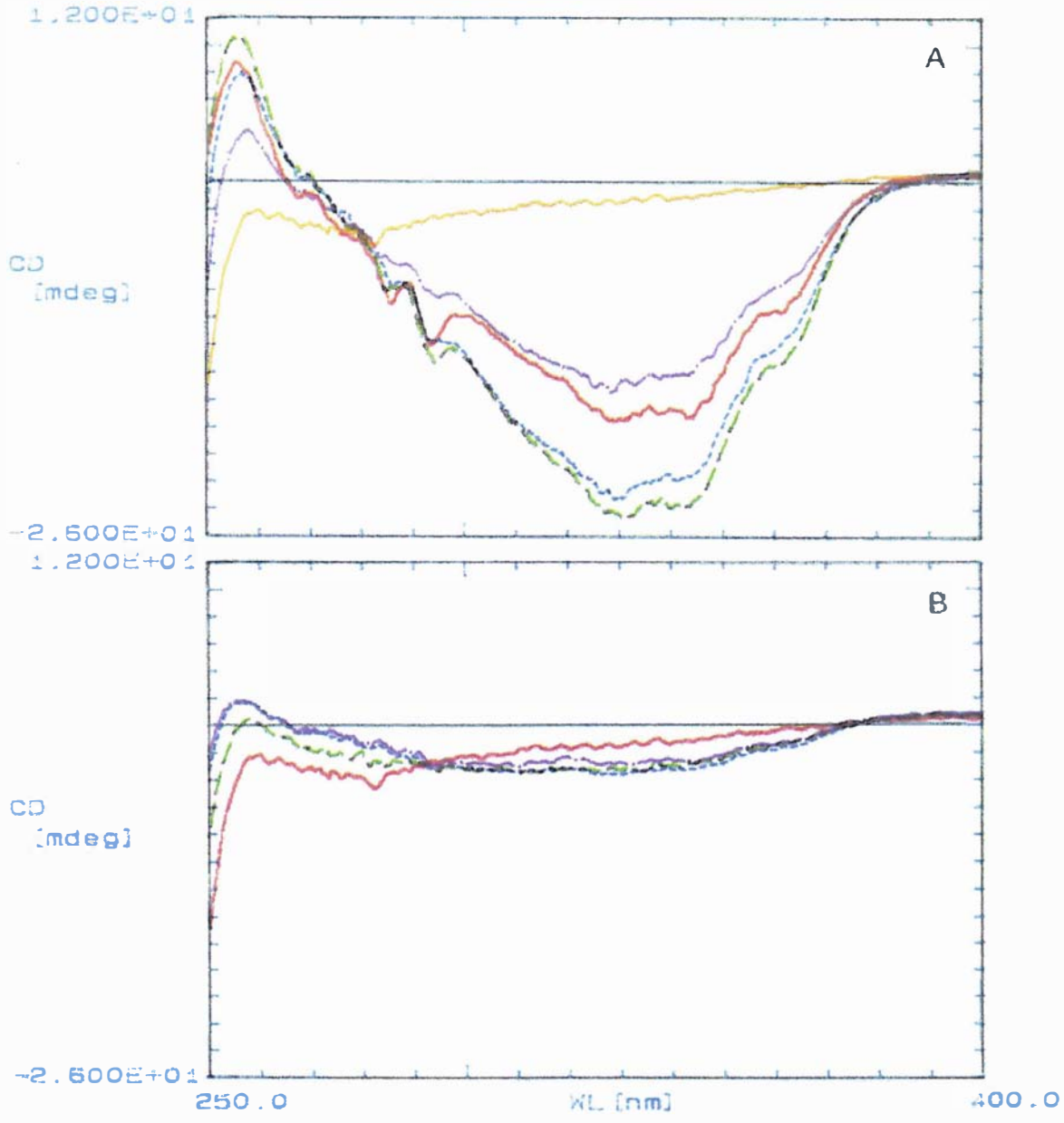

Figure 5.3.2.2. Near-UV CD spectra of mixtures of retinol (90 μL of 1.0 mg/mL solution = 101.94 μmoles) and bovine β-lg B at a concentration of 1.55 mg/mL (84.2 μmoles) in pH 7.7 Tris-phosphate buffer at various temperatures.

(A) Bovine β-lg B at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

(B) Bovine β-lg B at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

However, heating a mixture of β-lg and retinol to 60 °C and subsequent cooling to 20 °C did not affect the CD spectrum of the mixture. This indicated the almost complete renaturation of β-lg and the chiral retinol-binding site (Fig. 5.3.2.1.B). The spectrum of a mixture of bovine β-lg and retinol at 60 °C showed less intense bands than the unheated spectrum. This suggests that the retinol-binding ability of β-lg can be restored upon cooling if β-lg does not lose its native structure completely during the 60 °C heat treatment, e.g. the chiral environment of the side chain of Trp-19. Manderson et al. (1999b) also showed strong correlation of native protein content of heated β-lg solutions with CD band at 293 nm.

Porcine β-lactoglobulin. A mixture of 90 μL of retinol (1.0 mg/mL, 101.94 μmoles) and 3 mL of porcine β-lg (1.55 mg/mL, 87.1 μmoles) was subjected to heat treatment and CD spectral changes were observed (Fig. 5.3.2.3). Unlike the result with bovine β-lg, the addition of retinol to porcine β-lg did not change the near-UV CD spectrum of porcine β-lg appreciably and no induced CD bands for retinol were observed. As the heating temperature was increased, the spectrum of porcine β-lg diminished, especially the trough at 265-285 nm (Fig. 5.3.2.3.A). Subsequent cooling (after heating up to 80 °C) of the mixture largely restored the spectrum (Fig. 5.3.2.3.B). This result is very similar to that for porcine β-lg by itself (Fig. 5.3.1.3) and indicates that retinol did not bind within a chiral site of porcine β-lg. However, the binding of retinol to porcine β-lg might be different, e.g. binding may occur within a non-chiral environment. This is in disagreement with Frapin et al. (1993) who reported that porcine β-lg binds retinol at neutral pH (pH 7.0), on the basis of tryptophan fluorescence results. However, their results depend on changes in the quenching of tryptophan fluorescence signals, whereas CD results are based on the chirality of the protein structure.

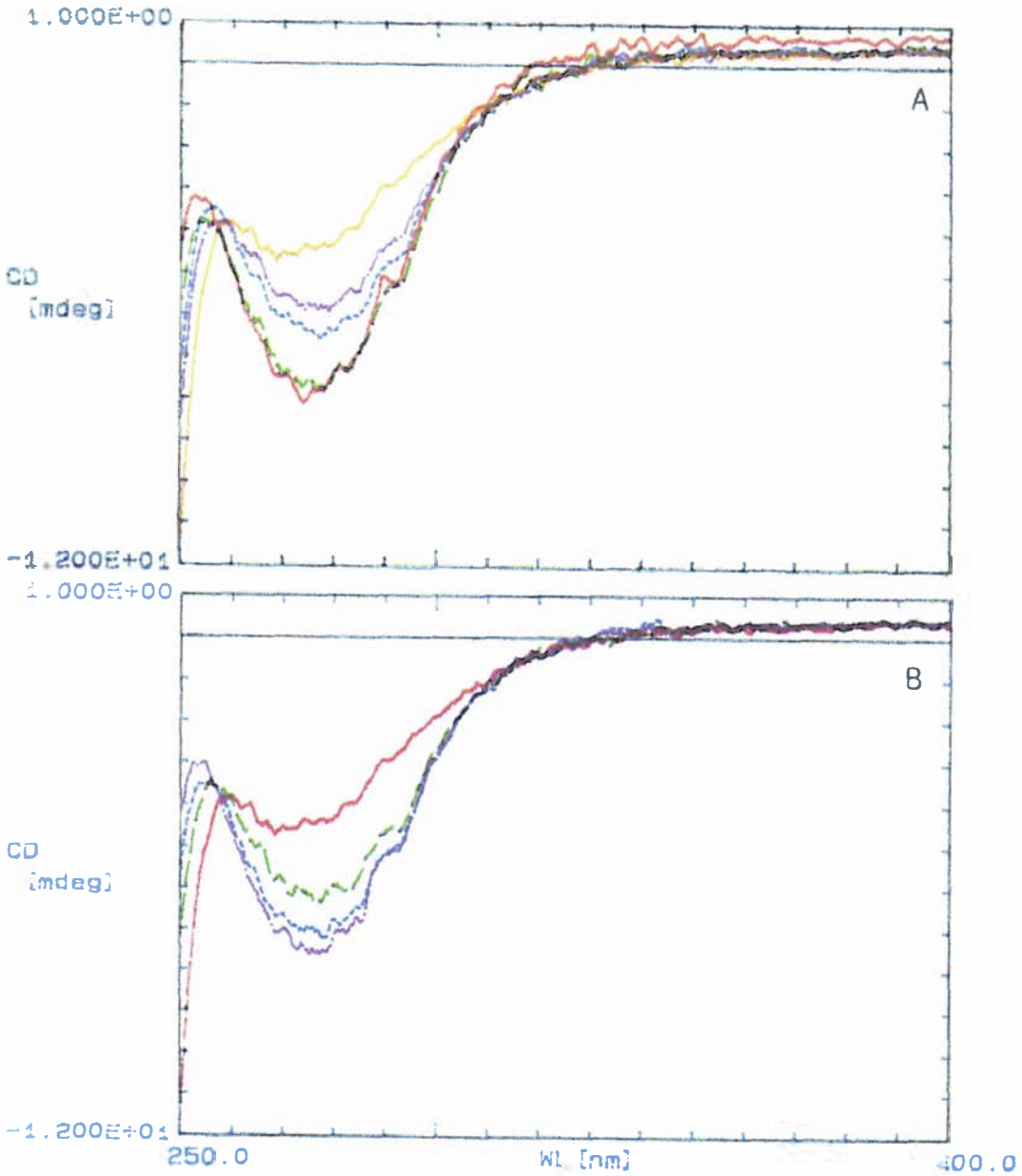

Figure 5.3.2.3. Near-UV CD spectra of mixtures of retinol (90 μL of 1.0 mg/mL solution = 101.94 μmoles) and porcine β-lg at a concentration of 1.55 mg/mL (87.1 μmoles) in pH 7.7 Tris-phosphate buffer at various temperatures.

(A) Porcine β-lg at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

(B) Porcine β-lg at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

(ii) cis-Parinaric acid

Bovine β-lactoglobulin. Addition of PnA to bovine β-lg created four deep troughs at 285, 293, 310 and 325 nm (Fig. 5.3.2.4) and similar induced near-UV CD bands from binding of PnA to BSA was reported by Sklar et al. (1977a). The troughs at 285 and 293 nm overlapped with and deepened the tryptophan near-UV CD bands of bovine β-lg. As the concentration of PnA increased, the intensities of all four bands increased. The intensities of the bands reached a maximum at close to a 1:1 molar ratio (Fig. 5.3.2.4.A). This result is similar to that obtained with retinol and β-lg (Fig. 5.3.2.1.A). Frapin et al. (1993) also reported that a maximum change in intrinsic fluorescence intensity was observed for 1:1 stoichiometries with several fatty acids and suggested that bovine β-lg has only one high affinity fatty-acid-binding site.

A mixture of 90 μL of PnA (1.0 mg/mL, 93.09 μmoles) and 3 mL of bovine β-lg (1.55 mg/mL, 84.2 μmoles) was subjected to heat treatment and CD spectral changes were observed (Fig. 5.3.2.5). Increasing the temperature diminished the intensities of these bands and the band intensities approached zero at 80 °C (Fig. 5.3.2.5.A), but subsequent cooling of mixtures of PnA and bovine β-lg allowed partial restoration of the four CD bands (Fig. 5.3.2.5.B). This result is similar to that obtained for the mixtures of retinol and bovine β-lg, although there was a greater extent of reversibility for the PnA mixture than the retinol mixture.

Heating the mixtures of β-lg and PnA up to 60 °C and then cooling them showed almost complete reversibility of the spectral changes (Fig. 5.3.2.4.B). A similar result was obtained for the retinol/β-lg mixture (Fig. 5.3.2.1.B).

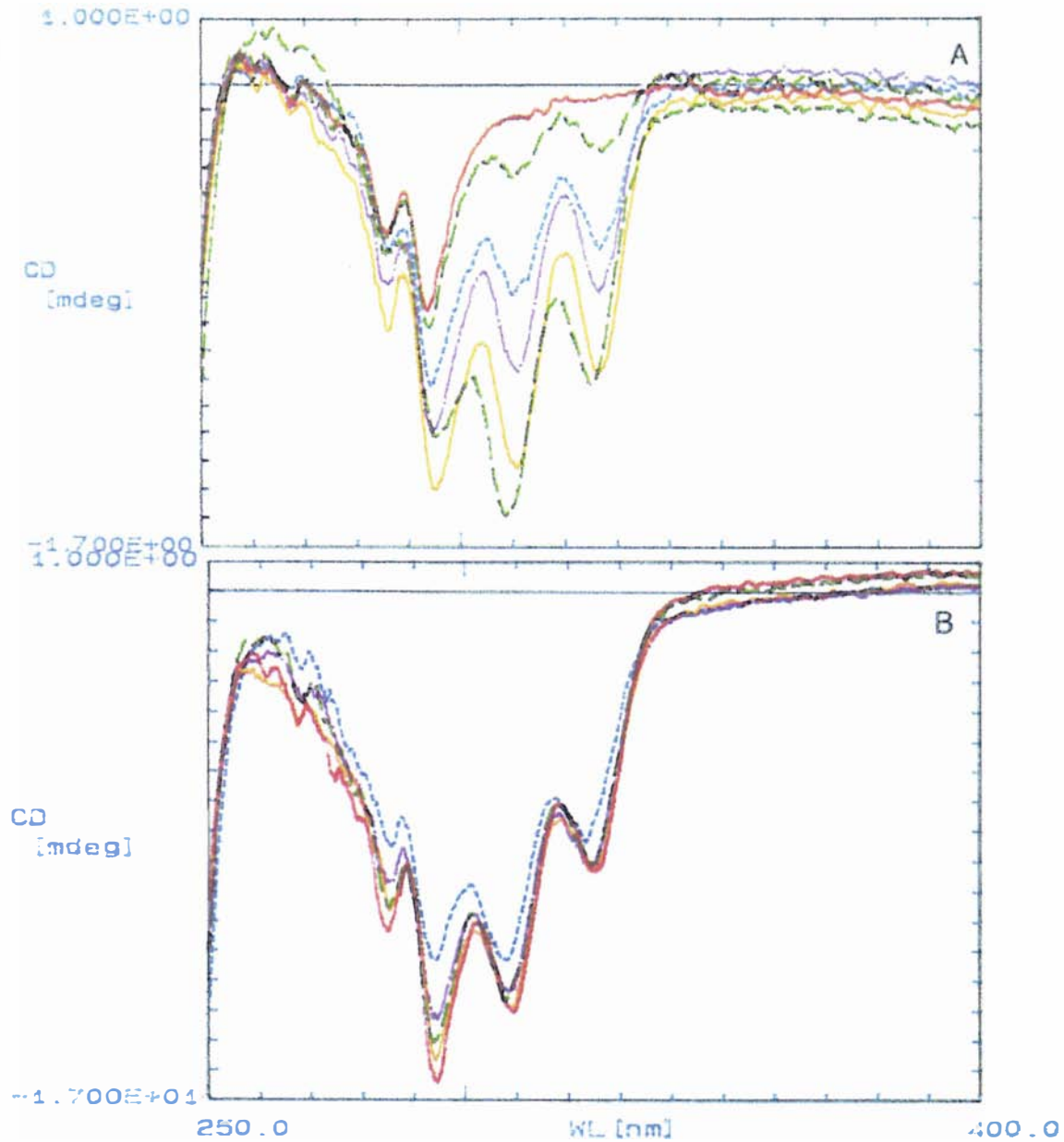

Figure 5.3.2.4. Near-UV CD spectra of:

(A) Increasing amounts of PnA (1.0 mg/mL) added to bovine β-lg B at a concentration of 1.55 mg/mL (84.2 μmoles) in pH 7.7 Tris-phosphate buffer. PnA added: 0 μL (red solid), 30 μL (green dashed), 60 μL (blue dotted), 70 μL (purple centre), 90 μL (orange solid) and 100 μL (green dashed).

(B) Mixture of PnA (90 μL of 1.0 mg/mL solution = 93.09 μmoles) and bovine β-lg B (3 mL of 1.55 mg/mL solution = 84.2 μmoles) at 20 °C (red solid), heated to 40 °C (green dashed) and 60 °C (blue dotted) and then cooled to 40 °C (purple centre) and 20 °C (orange solid). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

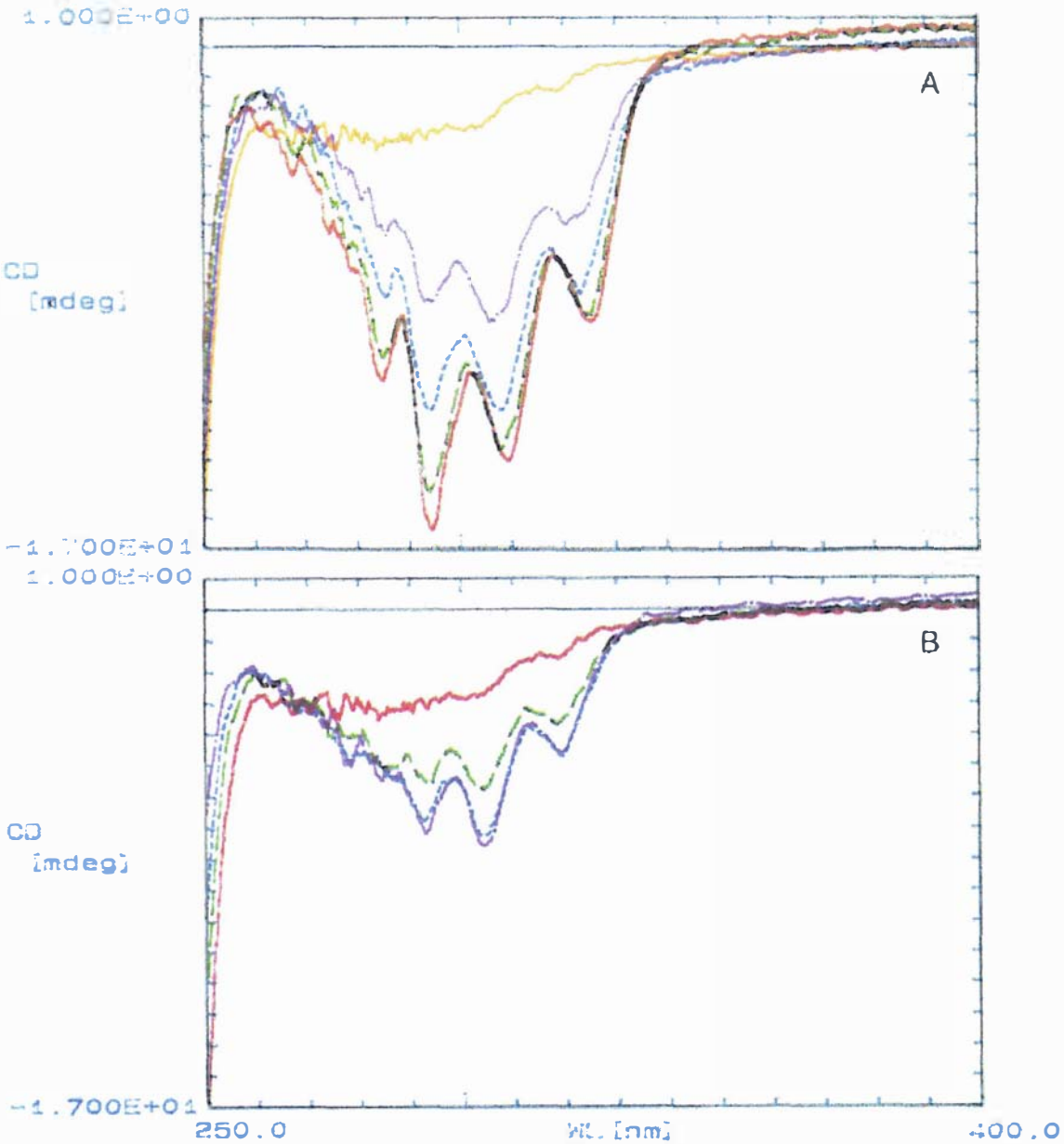

Figure 5.3.2.5. Near-UV CD spectra of mixtures of PnA (90 μL of 1.0 mg/mL solution = 93.09 μmoles) and bovine β-lg B at a concentration of 1.55 mg/mL (84.2 μmoles) in pH 7.7 Tris-phosphate buffer at various temperatures.

(A) Bovine β-lg B at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

(B) Bovine β-lg B at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

Porcine β-*lactoglobulin.* An increasing PnA concentration added to a porcine β-lg solution gave rise to two low intensity peaks at 308 and and 324 nm (spectra not shown). A mixture of 90 μL of PnA (1.0 mg/mL, 93.09 μmoles) and 3 mL of porcine β-lg (1.55 mg/mL, 87.1 μmoles) was subjected to heat treatment and CD spectral changes were observed (Fig. 5.3.2.6). Addition of PnA to porcine β-lg gave rise to two low intensity peaks at 308 and 324 nm (Fig. 5.3.2.6) at 20 °C, which corresponded to the troughs at 310 nm and 325 nm of the bovine β-lg mixture, respectively. As the temperature increased, the intensity of the spectrum (the trough at 265-285 nm) of the mixture of porcine β-lg and PnA diminished and the peaks at 308 and 324 nm were not apparent (Fig. 5.3.2.6.A). The spectrum at 80 °C was very similar to that of the mixture of porcine β-lg and retinol (Fig. 5.3.2.3) and porcine β-lg itself (Fig. 5.3.1.3) at the same temperature. After subsequent cooling of the mixture (Fig. 5.3.2.6.B), the spectrum of the mixture of porcine β-lg and PnA was almost the same as the initial spectrum of the mixture except that the peaks at 308 and 324 nm, which were induced by adding PnA, were absent (Fig. 5.3.2.6.A).

The overall spectrum of porcine β-lg was essentially reversible during heating and cooling, but the peaks at 308 and 324 nm were not reversible. This suggests that an irreversible structural change, which was not evident from the near-UV CD, occurred during heating. It has been reported that there is easy oxidation and photo bleaching of PnA (Thumser and Wilton, 1994), and this could have been responsible for the disappearance of the bands at 308 and 324 nm. But Dufour et al. (1992) examined the binding of PnA to bovine β-lg and found that large complexes were formed and that the particles were up to 95 nm in diameter.

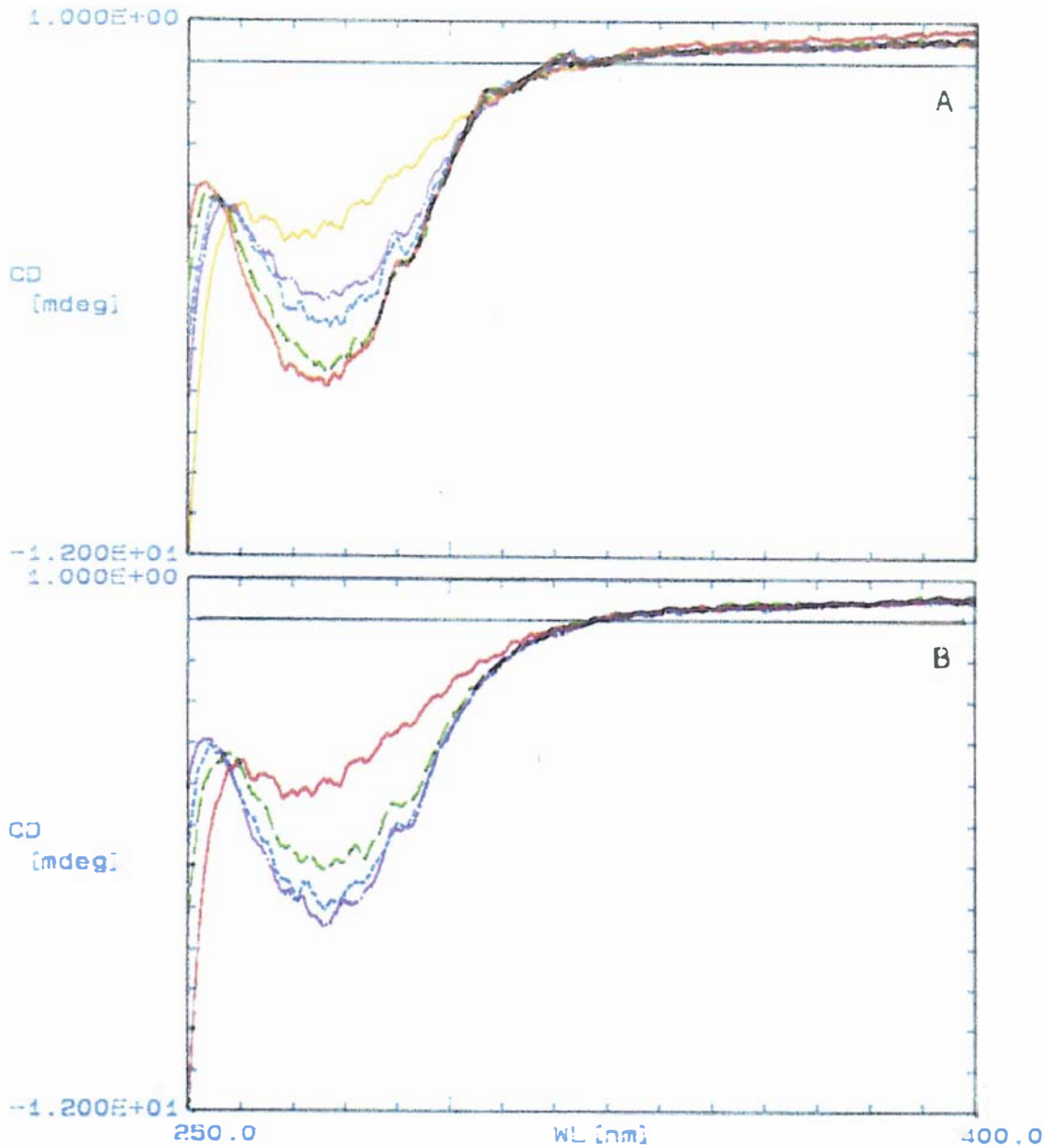

Figure 5.3.2.6. Near-UV CD spectra of mixtures of PnA (90 μL of 1.0 mg/mL solution = 93.09 μmoles) and porcine β-lg at a concentration of 1.55 mg/mL (87.1 μmoles) in pH 7.7 Tris-phosphate buffer at various temperatures.

(A) Porcine β-lg at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

(B) Porcine β-lg at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

(iii) Palmitic acid

Bovine β-lactoglobulin. A mixture of 90 μL of palmitic acid (1.0 mg/mL, 113.59 μmoles) and 3 mL of bovine β-lg B (1.55 mg/mL, 84.2 μmoles) was subjected to heat treatment and CD spectral changes were observed (Fig. 5.3.2.7). Adding palmitic acid to bovine β-lg did not obviously alter the CD spectrum, so that no induced near-UV CD bands were observed. Increasing the heating temperature decreased the band intensities at 285 and 293 nm, which was also observed during the heating of bovine β-lg itself (Fig. 5.3.1.2.A). It seems that palmitic acid did not affect the chiral environment of Trp-19 of bovine β-lg even during heat treatment of the mixture.

However, the mixture of bovine β-lg with palmitic acid showed considerably improved stability (Fig. 5.3.2.7.A) compared with bovine β-lg itself during heating (Fig. 5.3.1.2.A). Even at 80 °C, the troughs at 285 and 293 nm were observed clearly (with lower intensity), whereas these were not shown when bovine β-lg was heated by itself (Fig. 5.3.1.2). During subsequent cooling, the spectral intensity returned to about 65 % of that of the unheated samples (Fig. 5.3.2.7.B). In contrast, decreasing the temperature of β-lg solutions in the absence of palmitic acid did not restore the spectrum (Fig. 5.3.1.2.B).

Puyol et al. (1993) reported that the binding of fatty acids to bovine β-lg increased its conformational stability to trypsin degradation, whereas β-lg with or without bound retinol and treated with trypsin showed no differences. They suggested that this reduction in the digestibility of β-lg with bound fatty acids might be due to steric hindrance of the protein produced by the binding of the fatty acid.

Puyol et al. (1994) investigated the effects of binding retinol or palmitic acid to bovine β-lg on its resistance to thermal denaturation. The results indicated that the binding of fatty acids to β-lg may be an important factor in the stabilisation of the β-lg structure; β-lg with bound retinol showed lower stability than β-lg with bound palmitic acid. Hattori et al. (1995) reported that β-lg protects compounds related to β-ionone (Fig. 5.1.1.1) (retinol and β-carotene) from degradation by heating, oxidation and irradiation. They suggested that β-lg could be useful as a food additive to protect unstable β-ionone related compounds.

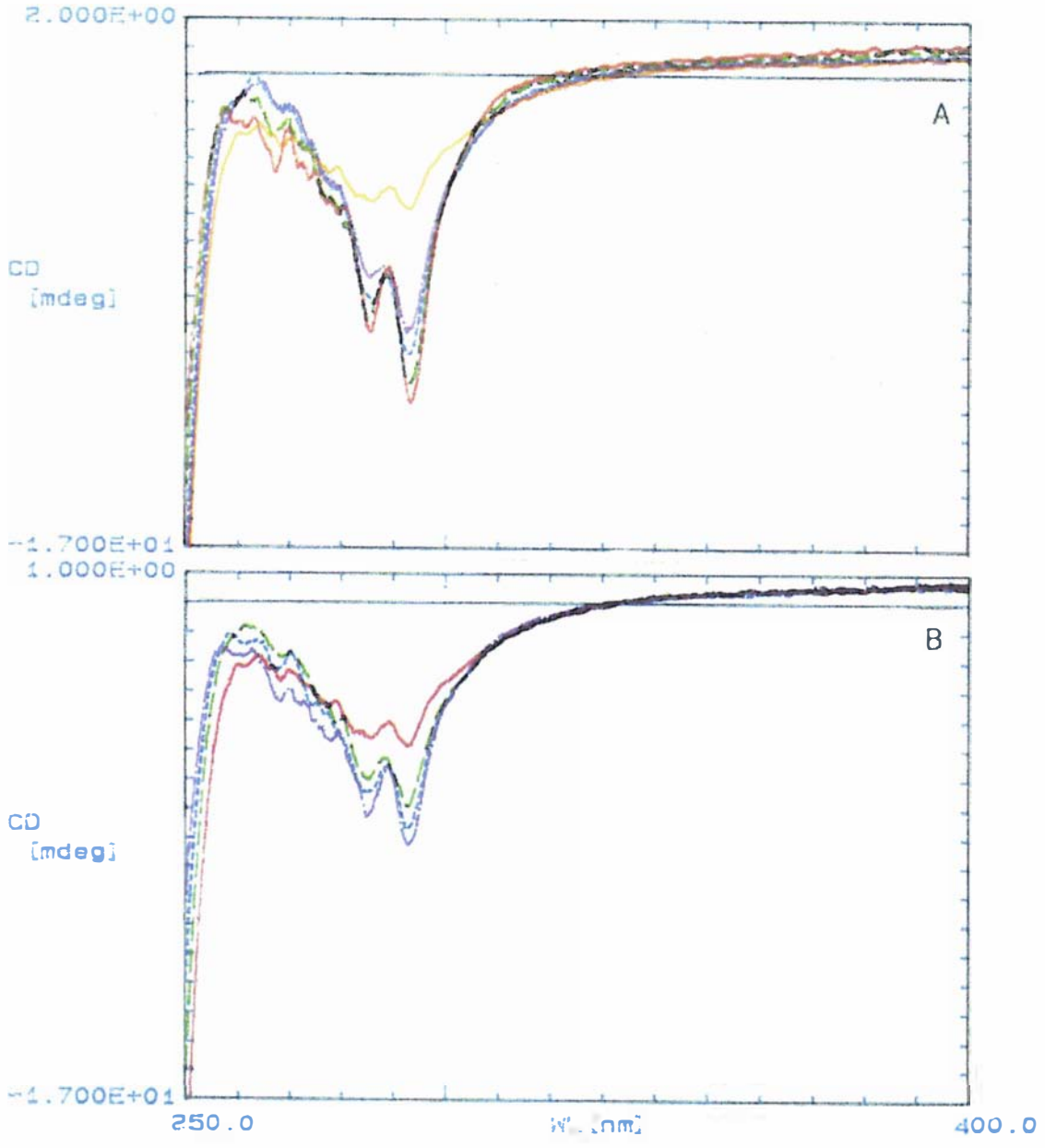

Figure 5.3.2.7. Near-UV CD spectra of mixtures of palmitic acid (90 μL of 1.0 mg/mL solution = 113.59 μmoles) and bovine β-lg B at a concentration of 1.55 mg/mL (84.2 μmoles) in pH 7.7 Tris-phosphate buffer at various temperatures.

(A) Bovine β-lg B at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

(B) Bovine β-lg B at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

Porcine β-lactoglobulin. A mixture of 90 μL of palmitic acid (1.0 mg/mL, 113.59 μmoles) and 3 mL of porcine β-lg (1.55 mg/mL, 87.1 μmoles) was subjected to heat treatment and CD spectral changes were observed (Fig. 5.3.2.8). Mixing palmitic acid with porcine β-lg also did not change the CD spectrum of porcine β-lg and there were no induced near-UV bands (Fig. 5.3.2.8). When the mixture was heated, the band intensities at 275 and 293 nm decreased, but cooling restored the spectrum completely. Even though the heating did not cause a permanent change in the tertiary structure of porcine β-lg, comparison with the spectrum of porcine β-lg itself (Fig. 5.3.1.3) at high temperature (80 °C) indicates that palmitic acid affected the spectrum of porcine β-lg.

5.3.2.2. Ligand fluorescence

Fluorescence is observed when the electrons in excited states in certain compounds return to the ground state by emitting photons. Compounds that fluoresce generally possess delocalised electrons formally present in conjugated double bonds, and may also contain electron-donating and electron-withdrawing groups. The intensity and λ_{max} (the wavelength of maximum intensity) of fluorescence emissions are dependent on temperature, solvent polarity, the existence of quenching groups and radiationless (or resonance) energy transfer (RET) phenomena (Lakowicz, 1983).

Several fluorophores are found naturally in proteins: tryptophan, tyrosine and phenylalanine side chains. The fluorescence of these side chains is collectively known as intrinsic protein fluorescence. Furthermore, emission λ_{max} values increase with increasing solvent polarity. The fluorescence emission spectra of native protein are usually dominated by the contributions from tryptophan side chains (Lakowicz, 1983).

Tryptophan can be excited at wavelengths between approximately 250 and 305 nm and λ_{max} can be as low as 325 nm and as high as 350 nm. In contrast, tyrosine can be excited at wavelengths between approximately 250 nm and 290 nm and λ_{max} is usually at about 300 nm (Lakowicz, 1983). The emission intensity from tyrosine side chains is usually small and often undetectable in native proteins that contain tryptophan because of RET.

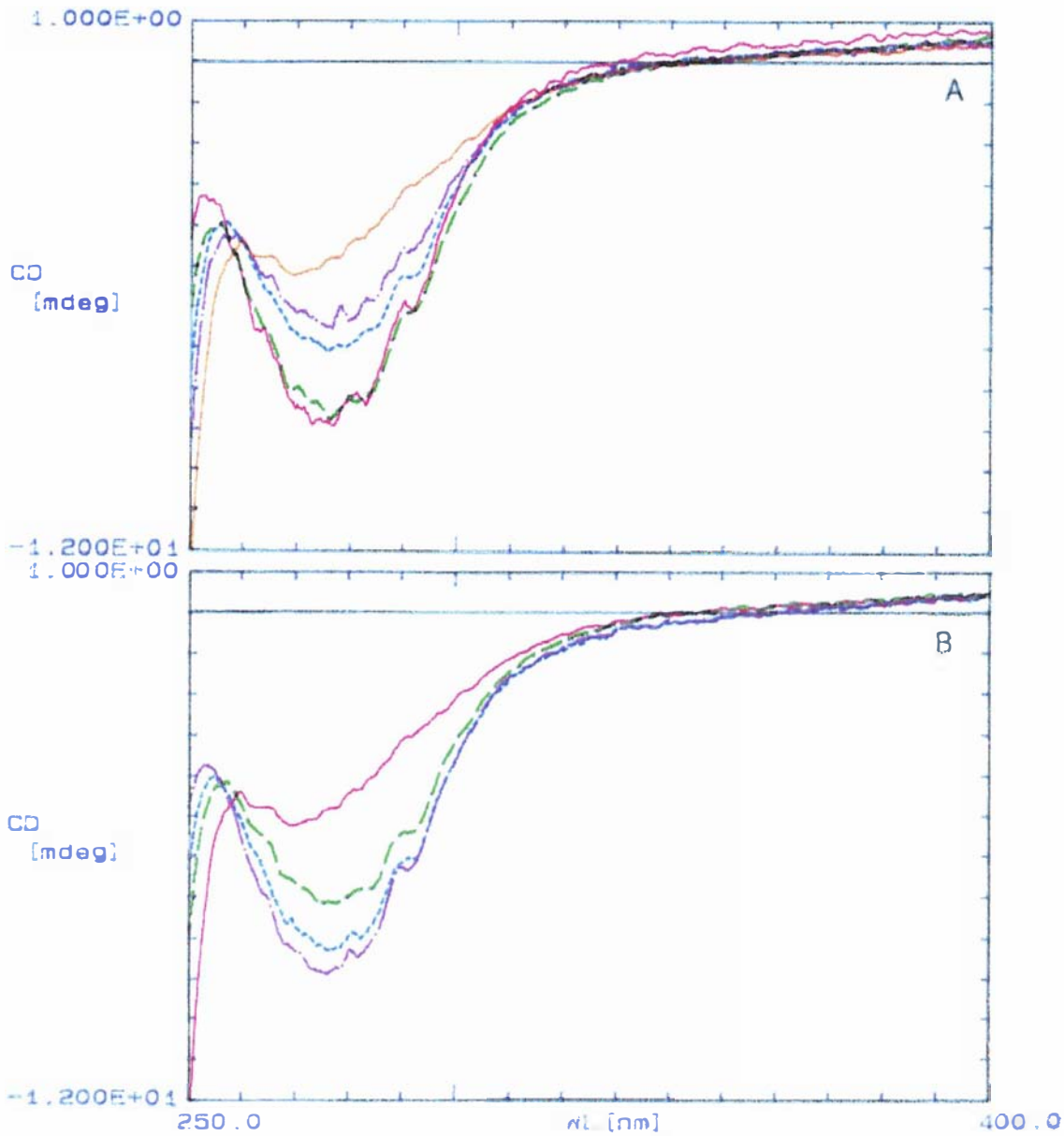

Figure 5.3.2.8. Near-UV CD spectra of mixtures of palmitic acid (90 μL of 1.0 mg/mL solution = 113.59 μmoles) and porcine β-lg at a concentration of 1.55 mg/mL (87.1 μmoles) in pH 7.7 Tris-phosphate buffer at various temperatures.

(A) Porcine β-lg at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

(B) Porcine β-lg at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

Intrinsic protein fluorescence can provide information on the structures of proteins in the vicinities of tryptophan and tyrosine side chains. In addition, protein structure and structural change can be studied by measuring the fluorescence from bound probes. Manderson et al. (1999a) measured and compared the results of intrinsic Trp fluorescence, ANS fluorescence and thiol availability to study the differences in heat-induced structural changes in three β-lg variants. They suggested that Trp intensity increased from consequential disulphide bond reorganisation during heat treatment and not from the initial unfolding reaction of β-lg and that creation of an ANS binding site on the β-lg molecule was linked to the irreversible exposure of a thiol group and the loss of native β-lg but preceded the decrease in Trp-61 fluorescence quenching.

Characterisation of the binding site(s) at the molecular level seems particularly interesting. Fluorescence spectroscopy is an ideal technique for such an investigation, provided the system has well-defined fluorophores (Dufour and Haertle, 1990). Porcine β-lg, which has only one tryptophan residue in a position conserved in all β-lg sequences, seemed to be quite appropriate for such a study.

In this study, the changes in bovine and porcine β-lg structures, which occurred as a consequence of heat treatment, were examined using extrinsic fluorescence spectra of the probes ANS and retinol by following changes in the emission intensity and the emission λ_{max} from the molecules. ANS and retinol fluorescences were used to monitor structural changes in the ligand-binding site(s) of the β-lg molecule during heat treatment. The ideal experimental system for examining the binding sites of β-lg using extrinsic fluorescence techniques would be one in which all the components were soluble, the ligand solutions were transparent at the protein excitation wavelengths and various ligands gave markedly different emission spectra. Although the ANS system is soluble, ANS has strong excitation bands that overlap with those of tryptophan so that RET cannot be interpreted unambiguously. In contrast, retinol has strong fluorescence excitation peaks at 325-330 nm and emission peaks at 470-490 nm (Ball, 1988).

(i) ANS fluorescence

ANS is a sensitive fluorophore that is often used to probe the hydrophobicity of proteins and to investigate the structural properties of protein molecules (Stryer,

1965; Brand and Gohlke, 1972; Slavik, 1982; Uversky et al., 1998; Creamer, 1995; Manderson et al., 1999a). When a mixture of ANS and bovine β-lg B was excited at 370 nm and scanned from 310 to 510 nm, a tryptophan emission peak was observed at approximately 330 nm and an ANS emission peak was observed at about 477-480 nm (Fig. 5.3.2.9).

Bovine β-lactoglobulin. Addition of bovine β-lg B to a solution of ANS increased I_{ANS} (emission intensity at λ_{max}) markedly and decreased λ_{max} from 510 to 480 nm. Increasing the temperature (between 20 and 48 °C) of the mixture did not alter I_{ANS} or λ_{max} (Fig. 5.3.2.10). Above this temperature, I_{ANS} increased gradually and reached a maximum at 64 °C and decreased at higher temperatures (Fig. 5.3.2.10.A). The I_{ANS} at 88 °C was lower than that of the unheated mixture of bovine β-lg with ANS. λ_{max} decreased from 480 to 475 nm as the temperature was increased to 56 °C and then steadily increased to reach 492 nm at 88 °C (Fig. 5.3.2.10.B). This suggests that changes in the polarity of the environment within the ANS-binding site may precede changes that affect the emission intensity, e.g. proximity or orientation of the quencher. Other possibilities include the loss of the ANS-binding site in unheated β-lg prior to the formation of a single higher affinity site as a consequence of the structural changes, e.g. disulphide-bonded dimer formation, caused by heat treatment (Manderson et al., 1999a).

Subsequent cooling of the mixture showed steadily increasing values for I_{ANS} (Fig. 5.3.2.10.A) and steadily decreasing values for λ_{max} (Fig. 5.3.2.10.B). In both cases, the values at 40 °C indicated that the ANS molecules were in a more constrained and hydrophobic environment than they were in the unheated β-lg mixture.

Cairoli et al. (1994) and Iametti et al. (1995) used the fluorescence emission intensity from β-lg/ANS mixtures to study heat-induced structural changes in bovine β-lg AB. They suggested that the increase in I_{ANS} observed at 70-75 °C reflects "protein swelling", and that the subsequent decrease in I_{ANS} at higher temperatures reflects the collapse of the swollen species. They suggested that, during the collapse phase, the hydrophobic groups that had become exposed as a consequence of temperature increase, were re-buried, producing a more compact species than the "heat-swollen" species.

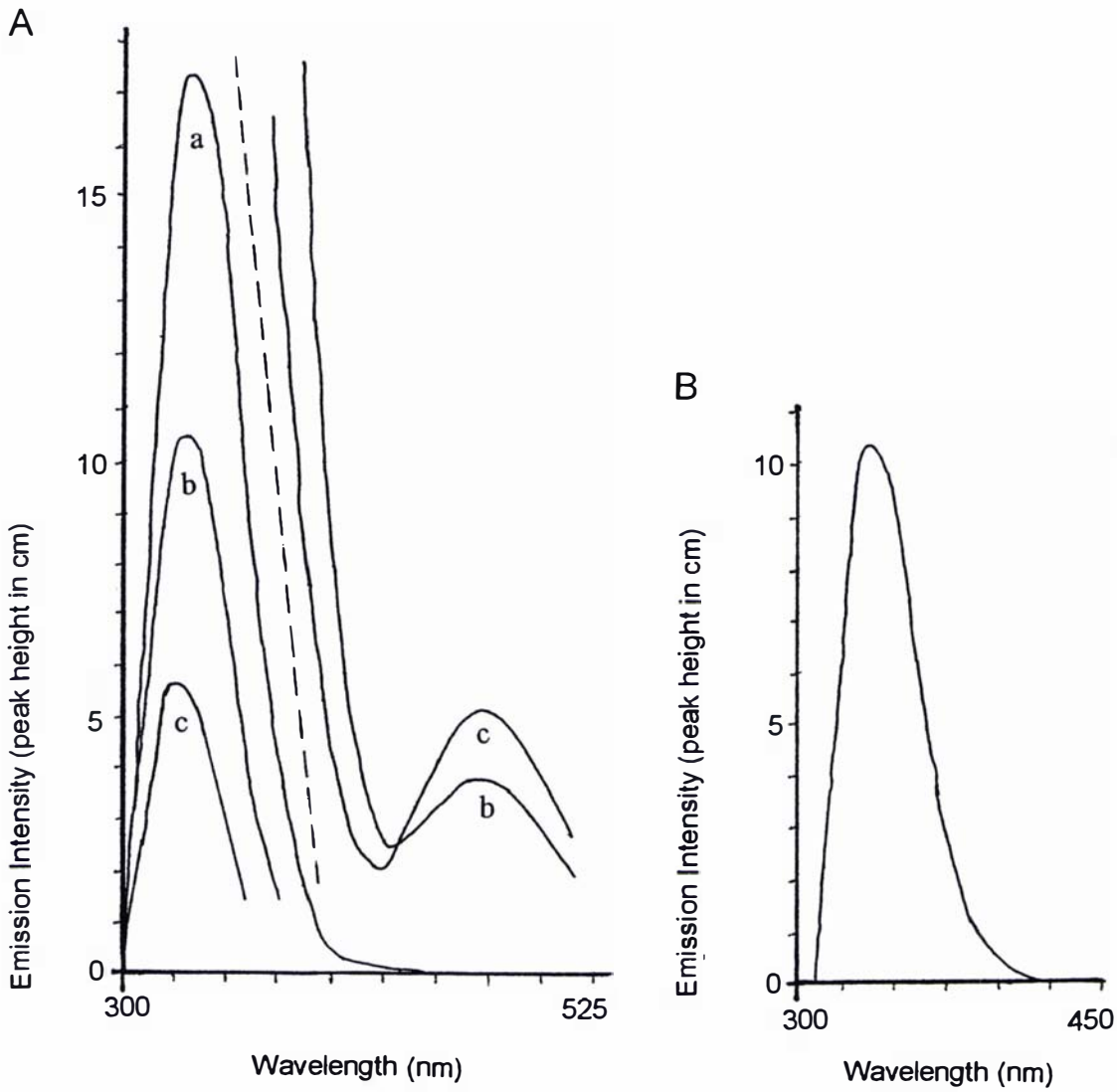

Figure 5.3.2.9. ANS fluorescence emission spectra of a mixture of ANS and (A) β-lg B, molar ratio β-lg B:ANS is (a) 1:0, (b) 1:0.5 and (c) 1:1; (B) NATA, molar ratio NATA:ANS = 1:1. The spectra were acquired from a 1.0 mg/mL solution of β-lg B and a 0.2 μM NATA solution in pH 7.7 Tris-phosphate buffer at 20 °C in a 10 mm × 10 mm path length cell using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The emission spectra were recorded between 310 and 510 nm, at an excitation wavelength of 370 nm and using excitation and emission slit widths of 8 nm at a scan speed of 25 nm/min and a chart speed of 1 cm/min. Experimental details are given in Section 5.2.2.

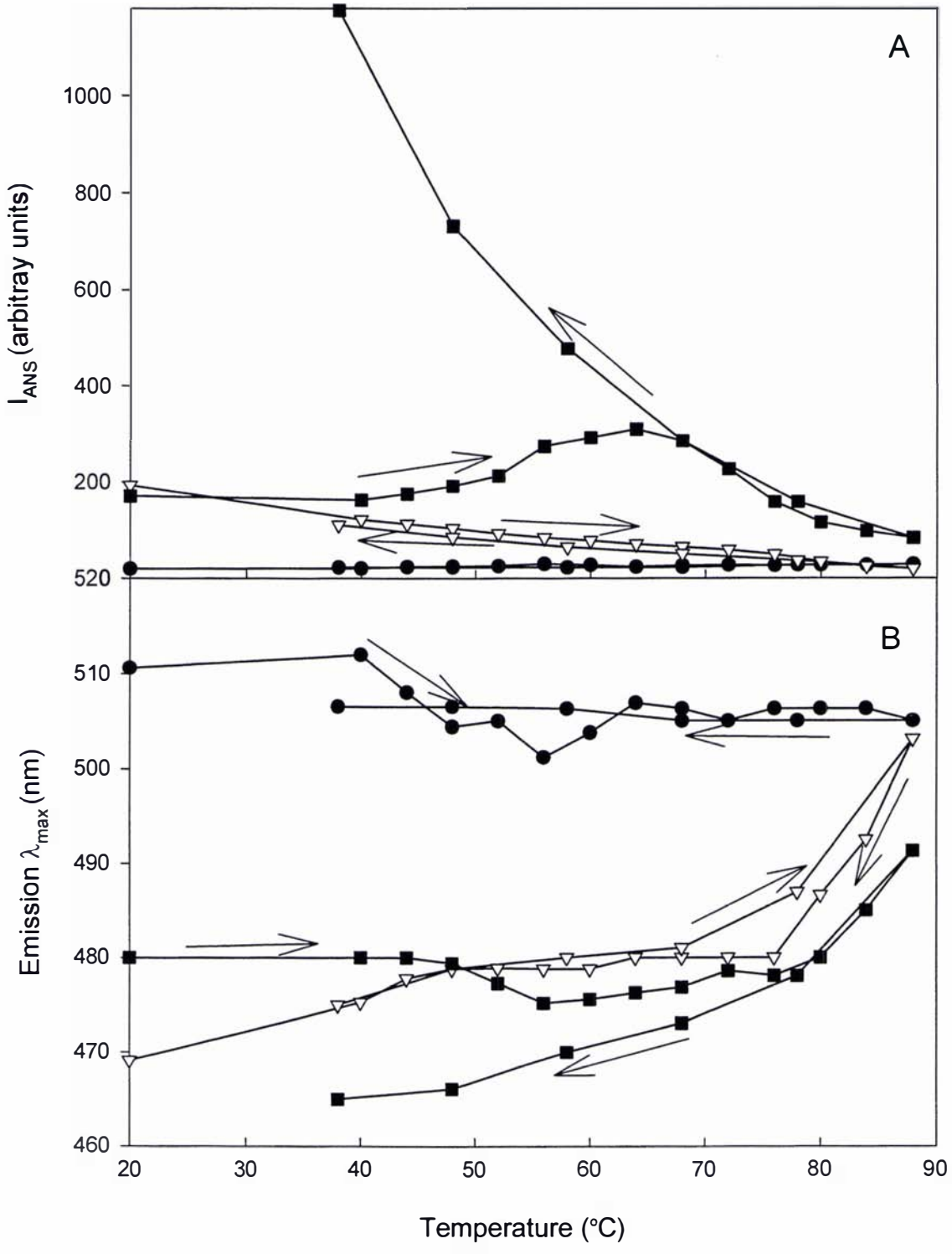

Figure 5.3.2.10. Effect of temperature on (A) I_{ANS} and (B) λ_{max} of ANS mixed with bovine β-lg (■), porcine β-lg (▽) and NATA (●). The spectra were obtained from a 1.0 mg/mL solution of each protein in pH 7.7 Tris-phosphate buffer at 20 °C. The solutions were excited at 370 nm and scanned from 340 to 550 nm. The spectra were acquired as described in Fig. 5.3.2.9. Experimental details are given in Section 5.2.2.

The preferable interaction of ANS with the molten globule states in relation to the native and completely unfolded proteins has been established (Semisotnov et al., 1991). The general properties of the intermediates are the presence of secondary structure and high compactness without rigid packing inside a molecule, and substantial increases in fluctuations of the side chains as well as of the larger part of the molecule (Cairoli et al., 1994). The "preferable interaction" of ANS with the molten globule was shown to be accompanied by a considerable increase in the fluorescence intensity of ANS between 44 and 64 °C (Fig. 5.3.2.10.A). Such a property was used for visualisation of the formation of the molten globule state in the course of protein folding (Uversky et al., 1998).

The ANS emission intensity increases with decreasing solvent polarity (Lakowicz, 1983); the observed increase in I_{ANS} (Fig. 5.3.2.10.A) may indicate that the ANS-binding site of heat-treated β-lg is more hydrophobic than that of the corresponding unheated species. Furthermore, the emission intensity from ANS is maximal when its two aromatic rings are coplanar, allowing electrons to pass freely between donor and acceptor groups. Therefore, the observed increase in I_{ANS} may also indicate that ANS is forced to assume a more planar conformation in the binding site of heat-treated β-lg compared with that when bound to the corresponding unheated species (Manderson et al., 1999a).

Mills (1976) reported that the fluorescence emission spectrum of bovine β-lg was red shifted from 328 to 338 nm and that the emission peak at half height or the emission intensity at λ_{max} (I_{Trp}) increased (Hayakawa and Nakai, 1985; Cairoli et al., 1994; Manderson et al., 1999a) as the heat treatment temperature increased. As the crystal structure (Brownlow et al., 1997; Qin et al., 1998a) indicated that Trp-61 is close to the disulphide bond Cys66-Cys160, the extent of quenching of fluorescence from Trp-61 is expected to be greater than that from Trp-19, which is located in a more hydrophobic environment. Mills (1976) suggested that the heat-induced increase in tryptophan emission is consistent with an increase in the solvent accessibility of tryptophan side chains. Furthermore, reactions that involve the thiol group of β-lg occur at elevated temperatures (Gough and Jenness, 1962; Watanabe and Klostermeyer, 1976). Because the thiol group of Cys-121 is solvent inaccessible in native β-lg, these results suggest that structural change in the vicinity of this side

chain, which is a considerable distance from both Trp-19 and Trp-61, occurs during heat treatment.

Porcine β-*lactoglobulin.* Using the same heating conditions as with a mixture of ANS and bovine β-lg, the changes in the values of λ_{max} and I_{ANS} of porcine β-lg are also shown in Figure 5.3.2.10. Both λ_{max} and I_{ANS} changed with temperature but were essentially independent of whether the temperature was increasing or decreasing. The initial value of λ_{max} was 470 nm at 20 °C and increased as the heating temperature was increased. The λ_{max} was about 505 nm at 88 °C, which was much higher than for the bovine β-lg mixture and close to that obtained for ANS in buffer solution or in the presence of NATA (Fig. 5.3.2.10.B).

Subsequent cooling restored the λ_{max} and I_{ANS} values of porcine β-lg to those before heat treatment, indicating that no irreversible change was detectable using ANS fluorescence.

Binding of ANS was almost equally weak for both native bovine β-lg and native porcine β-lg. The results for ANS bound to bovine β-lg indicate irreversible topological changes during heat treatment. The secondary structure of bovine β-lg changed irreversibly but this did not happen for porcine β-lg, and the initial values of λ_{max} and I_{ANS} were restored as a consequence of cooling.

(ii) Retinol fluorescence

Retinol is a weakly fluorescing compound in aqueous solution, but shows a markedly enhanced fluorescence intensity when bound to bovine β-lg (Futterman and Heller, 1972). The enhancement is considered to result from binding to β-lg, increasing the conformational rigidity of the retinol molecule, especially the β-ionone ring (Fig. 5.1.1.1) rather than the polyene chain moeity (Katakura et al., 1994).

Bovine β-*lactoglobulin.* A mixture of bovine β-lg and retinol was excited at 350 nm and the maximum fluorescence emission intensity was observed at about 482 nm. As the temperature was increased, λ_{max} did not change significantly up to a temperature of about 72 °C. λ_{max} then decreased to 470 nm and remained almost constant during further heat treatment (Fig. 5.3.2.11.B).

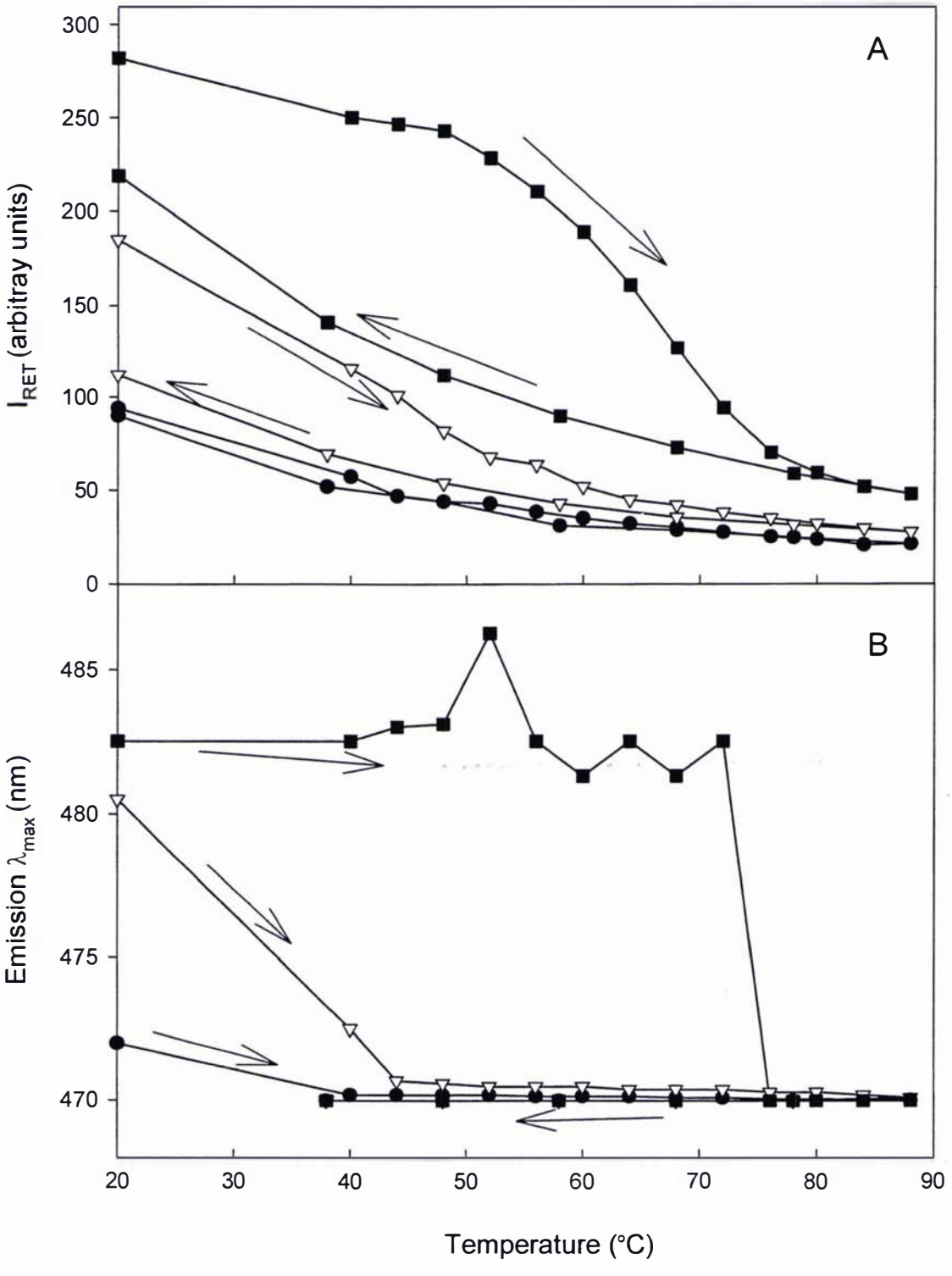

Figure 5.3.2.11. Effect of temperature on (A) I_{RET} and (B) λ_{max} of retinol mixed with bovine β-lg (■), porcine β-lg (∇) and NATA (●). The spectra were obtained from a 1.0 mg/mL solution of each protein in pH 7.7 Tris-phosphate buffer at 20 °C. The solutions were excited at 350 nm and scanned from 340 to 550 nm. The spectra were acquired as described in Fig. 5.3.2.9. Experimental details are given in Section 5.2.2.

I_{RET} decreased gradually as temperature increased, but decreased more rapidly between 60 and 76 °C. Above 76 °C, I_{RET} remained almost constant. During subsequent cooling, I_{RET} increased steadily and λ_{max} remained constant at 470 nm (Fig. 5.3.2.11.A).

These results indicate that there is a distinct and irreversible change in the way that retinol interacts with bovine β-lg at temperatures at or above 75 °C. When these results are taken in conjunction with the changes in the induced CD spectra of retinol with heat treatment (Fig. 5.3.2.2), it is clear that, as bovine β-lg loses native-like character, the particular retinol-binding site that gives rise to the induced CD spectrum is also responsible for a proportion of the retinol fluorescence. As Laligant et al. (1991) noted, the number and the strength of retinol binding sites are altered after heat treatment.

Porcine β-lactoglobulin. Porcine β-lg increased retinol fluorescence less than the bovine protein and I_{RET} decreased steadily with increasing solution temperature (Fig. 5.3.2.11.A). The value of λ_{max} decreased rapidly with temperatures up to 44 °C. At this point it decreased to 470 nm and remained at that wavelength up to 88 °C (Fig. 5.3.2.11.B). Decreasing the temperature of the solutions caused I_{RET} to increase steadily whereas λ_{max} did not change. In the case of the bovine protein, I_{RET} was less at all temperatures, whereas, for the porcine protein mixture, the I_{RET} vs. temperature curve was only slightly lower than the curve obtained during the temperature-increase phase. These results suggest that retinol binds to porcine β-lg and that there is a temperature-induced change in the character of the binding site such that λ_{max} is lowered by the heat treatment.

5.3. GENERAL DISCUSSION

The near-UV CD spectrum of bovine β-lg showed strong troughs at 293 and 285 nm, whereas that of porcine β-lg showed a series of overlapping bands spread between 255 and 300 nm. Furthermore, the effect of heat treatment on the structural changes of bovine β-lg was largely irreversible, but the structural changes of porcine β-lg were relatively small and essentially reversible.

Addition of retinol and PnA to bovine β-lg B at pH 7.7 induced new near-UV CD bands. As the solution temperature was increased, bovine β-lg lost the ability to bind retinol and PnA in a chiral environment and the chiral environment of Trp-19. Palmitic acid did not affect the chiral environment of Trp-19 of bovine β-lg even when the mixture was heat treated. Comparison of the CD spectra among mixtures cooled to 20 °C showed that palmitic acid was most effective in stabilising bovine β-lg against thermal denaturation followed by PnA (Fig. 5.3.3.1.A).

Unlike bovine β-lg, porcine β-lg did not show significantly induced CD bands when mixed with retinol, but a PnA/β-lg mixture showed two low intensity induced CD bands. Mixing palmitic acid with porcine β-lg also did not change the CD spectrum of porcine β-lg and there were no induced near-UV bands. When the mixture was heated, the band intensities at 275 and 293 nm decreased, but cooling restored the spectrum completely (Fig. 5.3.3.1.B). Even though the heating did not cause a permanent change in the tertiary structure of porcine β-lg, palmitic acid did affect the spectrum of porcine β-lg.

The results of ANS fluorescence for bovine β-lg suggest that there are irreversible heat-induced changes in the polarity of the environment within the ANS-binding site. Using the same heating conditions, λ_{max} and I_{ANS} of porcine β-lg changed with temperature but were essentially independent of whether the temperature was increasing or decreasing. Subsequent cooling restored the λ_{max} and I_{ANS} values of porcine β-lg to those before heat treatment, indicating that no irreversible change was detectable using ANS fluorescence.

The results of retinol fluorescence indicate that there is a distinct and irreversible change in the way that retinol interacts with bovine β-lg at temperatures at or above 75 °C. In the case of the bovine protein, I_{RET} was less at all temperatures, whereas, for the porcine protein mixture, the I_{RET} vs. temperature curve was only slightly lower than the curve obtained during the temperature-increase phase. These results suggest that retinol binds to porcine β-lg and that there is a temperature-induced change in the character of the binding site such that λ_{max} is lowered by the heat treatment.

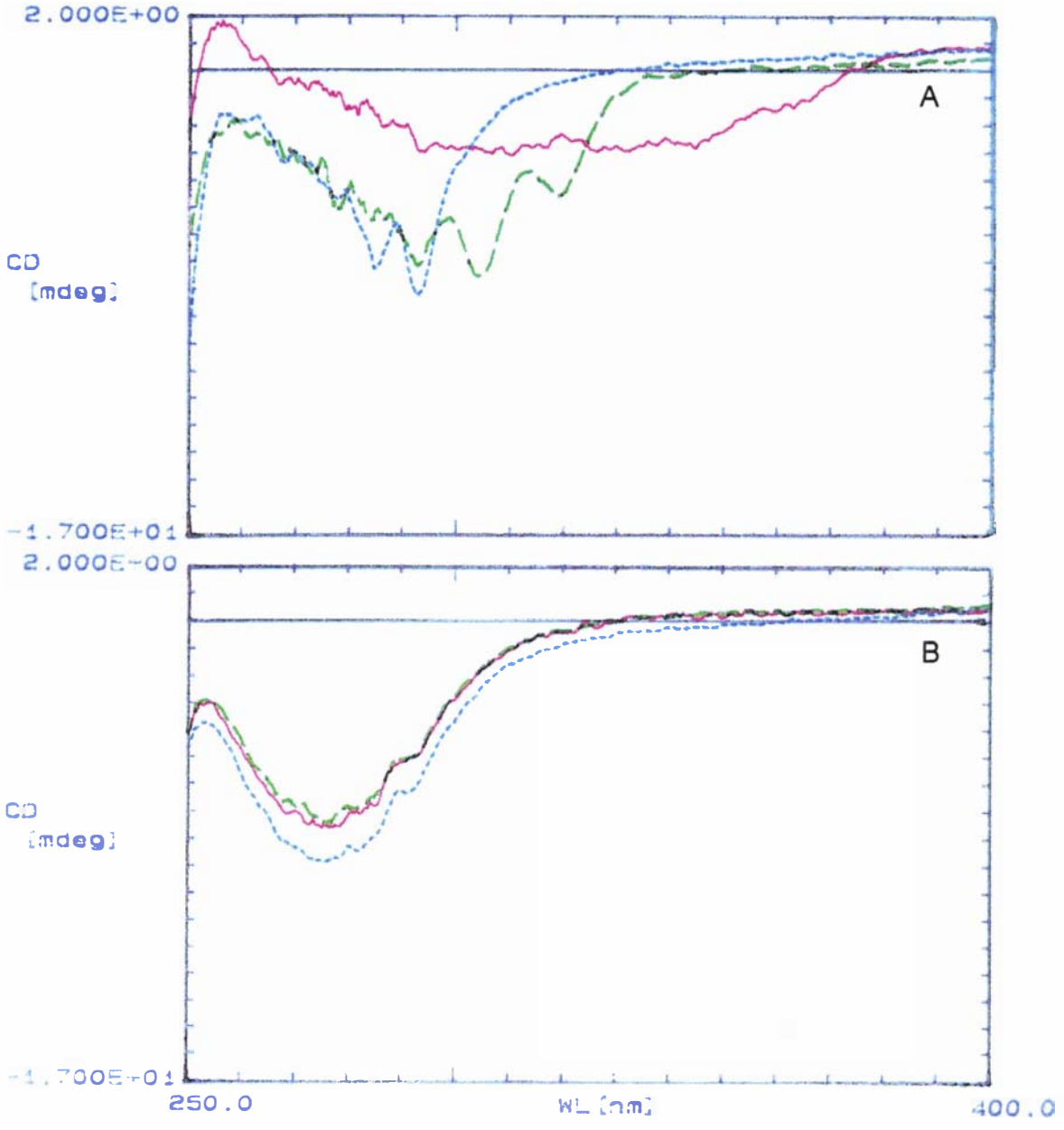

Figure 5.3.3.1. Near-UV CD spectra of bovine β-lg B and porcine β-lg at a concentration of 1.55 mg/mL in pH 7.7 Tris-phosphate buffer, cooled at 20 °C after heating up to 80 °C.

(A) Mixture of bovine β-lg B with retinol (red solid), PnA (green dashed) and palmitic acid (blue dotted).

(B) Mixture of porcine β-lg with retinol (red solid), PnA (green dashed) and palmitic acid (blue dotted). The spectra were acquired as described in Fig. 5.3.1.1. Experimental details are given in Section 5.2.2.

The ligand-binding properties of β-lg have been well studied but there is still no agreement about the biological function of β-lg (Sawyer, 2000). Qin et al. (1998a) reported that the carboxylate head group of 12-bromododecanoic acid lies at the surface of the molecule and that the lid (loop EF) to the calyx is open at the pH of crystallisation (pH 7.3), consistent with the conformation observed in ligand-free bovine β-lg at pH 7.1 and pH 8.2 (Qin et al., 1998b). They reported that the ligand binding cavity is exclusively hydrophobic in the crystal, except for two lysine residues, Lys-60 and Lys-69, at the opening of the cavity. Lys-60 and Lys-69 form hydrogen bonds with the carboxyl end group of the ligand and there is extensive interaction of the hydrocarbon tail of the ligand with the host β-lg. Wu et al. (1999) also observed binding of the carboxyl group of palmitic acid to both Lys-60 and Lys-69 at the entrance of the cavity of β-lg.

The above findings suggest that the presence of a positively charged residue such as Arg or Lys in the binding site would be likely to enhance the binding of fatty acids or fatty acid analogues to β-lg. Cho et al. (1994) showed that, by changing Lys-70 in bovine β-lg to an Ala, retinoic acid binding was diminished which suggests that Lys-70 might act as a positively charged centre that can interact with anionic ligands.

The failure of porcine β-lg to bind fatty acids and retinol (Figs 5.3.2.3 and 5.3.2.6) may be due to the substitution of Lys-69 by glutamate as suggested earlier (Frapin et al., 1993; Perez et al., 1993). In other non-ruminant β-lgs that are reported not to bind fatty acids, such as donkey II and horse II (Perez et al., 1993), a basic amino acid (Lys or Arg) is maintained at position 69, but instead a glutamate is substituted for Lys-60. Qin et al. (1998a) suggested that the presence of a carboxyl residue at either position 60 or position 69 can hinder the binding of a fatty acid in bovine β-lg. Frapin et al. (1993) reported that porcine β-lg binds retinol at neutral pH, but that palmitic acid does not appear to be bound. Comparison of the sequences of bovine and porcine β-lgs shows that Glu-35, Lys-70 and Glu-120 of the bovine protein are Lys, Val and Gln, respectively, in the porcine protein (Fig. 5.3.3.2). As the cationic site function could have been taken by Lys-35 in the porcine protein, the lack of fatty acid binding to porcine β-lg may be related to other changes within the β-barrel. However, only the acquisition of high resolution crystal or NMR structures of porcine β-lg is likely to resolve these questions.

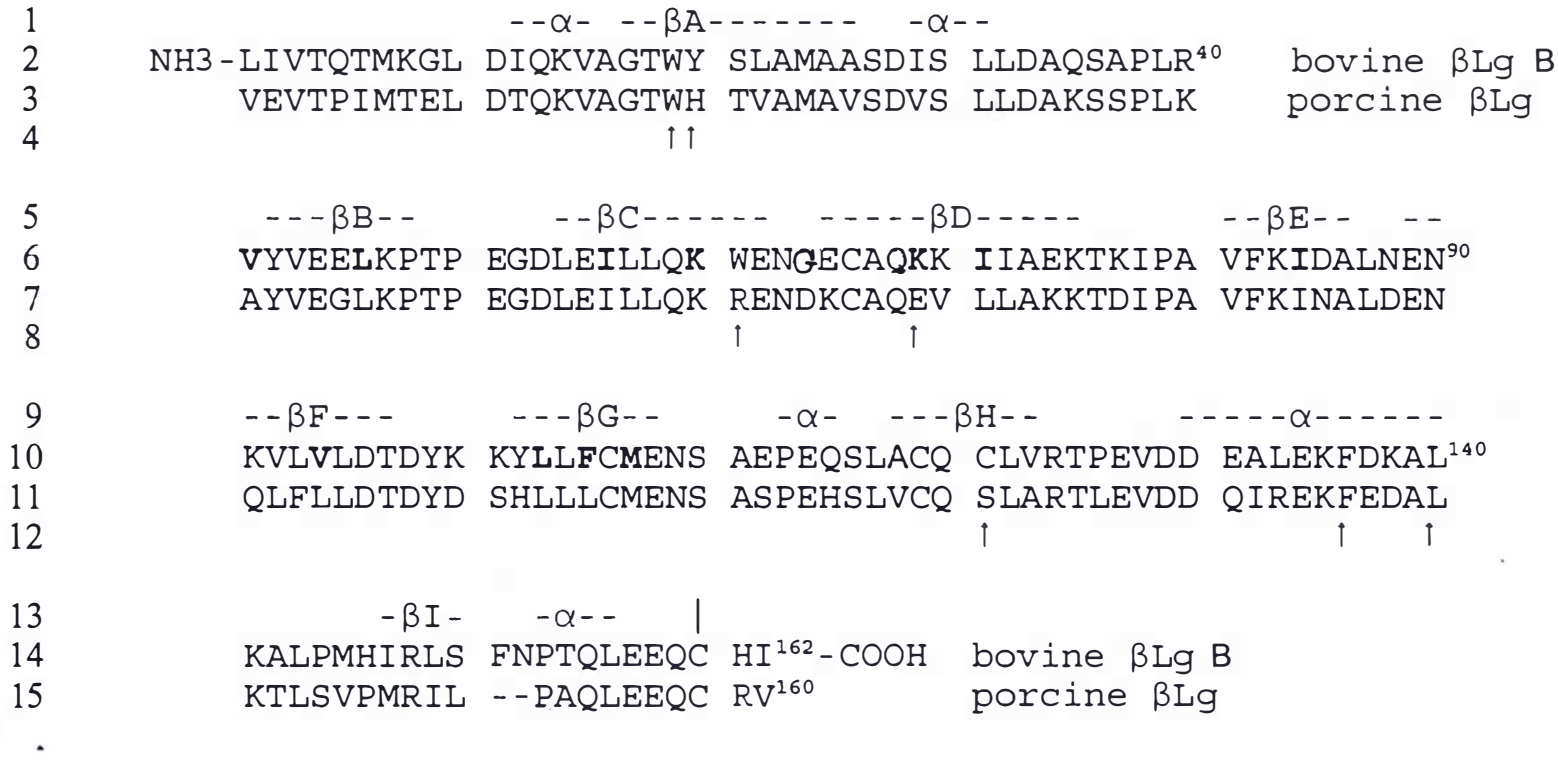

Figure. 5.3.3.2. Comparative sequences of bovine and porcine β-lg. The shaded areas indicate which residues are conserved between the two proteins. The residues shown in bold are those with side chains that interact with palmitic acid or 12-bromododecanoic acid that is co-crystallised with bovine β-lg (Qin et al., 1998a; Wu et al., 1999). The small arrows indicate the residues that are discussed in the text and notations above the sequences indicate the positions of the identified secondary structures in the Y lattice form of β-lg AB (Brownlow et al., 1997).

Frapin et al. (1993) found differences of the C-termini when comparing porcine β-lg and bovine β-lg sequences, whereas this region is highly conserved in all ruminant β-lg sequences (Godovac-Zimmermann, 1988). They suggested that the deletion of residues 151 and 152 in porcine β-lg, as well as the replacement of His-146, may explain the monomeric state of porcine β-lg. They also suggested that the fatty-acid-binding site of bovine β-lg is close to the region involved in dimer formation, i.e. strand I, helix 130-140, and strand A.

The induced CD signals from both retinol and PnA were pH dependent and, whereas retinol showed a mid-point pH of about 7.2, PnA had a mid-point pH of 5.1 (Creamer et al., 2000). The mid-point pH of retinol binding coincides with the pH of the Tanford transition, which marks the ready accessibility of the central lipid-binding cavity of β-lg. Hambling et al. (1992) also indicated that the binding affinity of palmitic acid to bovine β-lg increases over the pH range 6.5-8.5, suggesting a possible correlation with the Tanford transition.

The mid-point pH of PnA is close to the pK_a for a carboxyl group (and is also the pI of β-lg) and suggests that the interaction between Lys-69 and/or Lys-60 and the carboxyl group of PnA is critical for the binding of this ligand (Creamer et al., 2000). Bovine β-lg is expected to bind PnA through the interaction between Lys-69 and/or Lys-60 and the carboxyl group of PnA, but porcine β-lg has no Lys-69 or Lys-60 as previously discussed.

Summary of spectral changes of bovine β-lg and porcine β-lg during heating and subsequent heating.

Spectrometer	Ligand	Bovine β-lg	Porcine β-lg
CD	Retinol	Induced CD bands, Irreversible spectral change	No induced CD bands, Reversible spectral change
	PnA	Induced CD bands, Irreversible spectral change	Induced CD bands of low intensity, Reversible spectral change
	Palmitic acid	No induced CD bands, Irreversible spectral change	No induced CD bands, Reversible spectral change
Fluorescence	ANS	Irreversible spectral change	Reversible spectral change
	Retinol	Irreversible spectral change	Irreversible spectral change

CHAPTER 6.

EFFECT OF UREA CONCENTRATION ON THE UNFOLDING OF DIFFERENT GENETIC VARIANTS OF β-LACTOGLOBULIN AND LIGAND-BOUND β-LACTOGLOBULIN

6.1. INTRODUCTION

Virtually all studies of the protein folding and unfolding reactions use heat, pH or a chemical denaturant (e.g. urea or guanidine hydrochloride) in an aqueous protein solution in order to perturb the protein structure (Dunbar et al., 1997). The unfolding of β-lg can be induced by various treatments, e.g. alkali, heat or organic compounds (Hambling et al., 1992; Sawyer, 2000). The detailed unfolding mechanisms resulting from these treatments are not clear, although the initial stages of reversible unfolding by alkali and by heat at neutral pH seem to follow the same path (Casal et al., 1988).

The thermal denaturation of whey proteins has been studied by many researchers because whey proteins undergo significant changes during processing, especially when heat treatment or shearing is involved. β-Lg is the major protein in whey and therefore tends to dominate the thermal behaviour of the total whey protein. It has been assumed that the first step in the thermal aggregation is dimer dissociation (Roefs and de Kruif, 1994; Cairoli et al., 1994; Iametti et al., 1995; Qi et al., 1995, 1997; Hoffmann and van Mil, 1999; Manderson et al., 1999a, b). After dimer dissociation, which corresponds to a structural change in the β-lg monomer, it has been suggested that β-lg is converted to a molten-globule-like state at elevated temperature (McSwiney et al., 1994b; Iametti et al., 1996; Qi et al., 1997). This state appears to possess no α-helix and slightly less β-sheet than native β-lg (Qi et al., 1997) and may be more hydrophobic than the corresponding native species (Iametti et al., 1996). Heat treatment also leads to thiol exposure and changes in the environments of the aromatic side chains, suggesting a loss of tertiary structure (Iametti et al., 1996; Manderson et al., 1999a, b).

Urea denaturation is believed to occur via a two-state process. At pH 3.5, where β-lg tends to be monomeric, the first step is the first-order, reversible unfolding

of the polypeptide chain to a random-coiled structure (Greene and Pace, 1974). However, at pH 5.2, this step follows more complex kinetics; upon addition of NEM, which dissociates dimeric β-lg, the kinetics revert to first-order kinetics (McKenzie and Ralston, 1973). This indicates that the first stage involves both dissociation and unfolding. The second stage involves the unfolded protein undergoing SH-SS exchange reactions (and possibly -SH oxidation) which are irreversible.

The denaturants interact directly with the protein. Most of the denaturant molecules form hydrogen bonds between different portions of the protein and start decreasing the hydrophobic effect, which is usually postulated to be the dominant force stabilising the protein structure (Dunbar et al., 1997). Hedwig et al. (1991) suggested that urea and guanidine hydrochloride unfold proteins by migrating into the interior of the protein and forming hydrogen bonds with the atoms in the polypeptide backbone. The main advantage of these denaturants is that the extent of unfolding is generally greater than can be achieved by most other denaturants under isothermal conditions (Greene and Pace, 1974). Urea has been shown to destabilise or perturb the hydrophobic interactions and hydrogen bonds in proteins (Lapanje, 1978) and leads to a random-coiled structure.

In this chapter, the effects of genetic variants and added ligands on the reversible unfolding of β-lg by urea are examined. These results are compared with the results obtained by other workers on the thermal unfolding of β-lg and Chapter 5.

6.2. EXPERIMENTAL PROTOCOL

Measurement protocols

Protein solutions were dialysed against pH 6.7 phosphate buffer (26 mM sodium phosphate, 68 mM NaCl) or pH 5.0 buffer (26 mM glutaric acid, 68 mM NaCl). A series of urea solutions was made from weighed quantities of urea in phosphate buffer. Retinol, retinoic acid and palmitic acid were dissolved (1 mg/mL) in ethanol boiled and degassed in a Buchner flask with rapid stirring and stored under oxygen-free nitrogen in the dark. Occasionally equimolar concentrations of BHT to retinol or retinoic acid were added.

CD measurements

For near-UV CD measurement, 400 μL of a 15 mg/mL β-lg solution was added to 3 mL of buffered urea solution, which was no more than 12 h old. For far-UV CD spectra, 150 μL of a 15 mg/mL β-lg solution was added to a 2 mL aliquot of buffered urea solution. The spectrum of each solution was run about 10 min after protein addition, and then appropriate volumes of palmitic acid (or retinol or retinoic acid) solution (15 or 20 μL increment) were added to the mixture. After about 2 h, the spectrum of each mixture was redetermined.

Data analysis

The CD results were normalised by taking the value of the parameter in the absence of urea as zero and the value of the parameter at the highest urea concentration as 100 % and scaling the intermediate values between 0 and 100 %. In some cases, the maximum value of the parameter was obtained at a concentration below that of the maximum urea concentration used, and thus normalised values could be > 100 %. The value of the midpoint urea concentration was measured by near- or far-UV CD parameters in the urea-induced structural change (i.e. the urea concentration at which the band intensity at 293 nm for near-UV CD or at 220 nm for far-UV CD was exactly half-way between the values for the band intensities at 293 or 220 nm on the low and high urea concentration sides of the transition).

Modification of free thiol group of β-lactoglobulin

Bovine β-lg (5.28 mg/mL) in pH 7.4 phosphate buffer was mixed with a 10:1 molar excess of DTNB. After the DTNB had dissolved, the β-lg solution was diluted 1:1 with a freshly prepared 11 M urea solution. The 5.5 M urea solution would have been sufficient to induce partial unfolding of β-lg, i.e. enough to partially expose the thiol group but not enough to induce complete unfolding. The mixture was then left in the dark at 20 °C for 3 h and then the urea and excess DTNB were dialysed out using five changes of pH 6.7 phosphate buffer (26 mM sodium phosphate, 68 mM NaCl).

The thiol group in β-lg was also blocked with NEM using the same procedure as for DTNB.

6.3. RESULTS AND DISCUSSION

6.3.1. Effect of urea concentration on the unfolding of β-lactoglobulin

6.3.1.1. Near-UV CD

The near-UV CD spectra of bovine β-lg A, B and C in pH 6.7 phosphate buffer were all similar to one another in general outline (Figs 4.1.3.8 and 6.3.1.1.A) and to those obtained earlier (Timasheff et al., 1966, 1967; Townend et al., 1967; Creamer, 1995; Manderson et al., 1999b). The bands at 293 and 285 nm can be ascribed to tryptophan vibrational fine structure and the bands at 277 and 265 nm can be ascribed to tyrosine (Fig. 6.3.1.1) (Woody, 1985; Towell and Manning, 1994).

The two deep troughs at 285 and 293 nm (Figs 4.1.3.8 and 6.3.1.1.A) are believed to be a consequence of one or both of the β-lg tryptophan residues being in a chiral environment (Kahn, 1979). Qin et al. (1998a, b, 1999) showed that Trp-61 is on the surface of β-lg and has considerable rotational freedom. On the other hand, Papiz et al. (1986) showed that the indole side chain of Trp-19 is within the hydrophobic binding cavity of β-lg, which has been confirmed in recent high resolution X-ray crystallographic structures (Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998a, b, 1999; Wu et al., 1999). In the crystal structure of Brownlow et al. (1997), Trp-19 is surrounded by a greater number of side chains than Trp-61. Therefore, the contribution from Trp-19 to the near-UV CD spectrum is considered to be greater than that from Trp-61 (Creamer, 1995; Manderson et al., 1999b).

Increasing the urea concentration decreased the intensities of β-lg B CD at 285 and 293 nm (Fig. 6.3.1.1.A), which suggests that the environment of the tryptophan and tyrosine residues became less chiral (McKenzie and Ralston, 1973; Creamer, 1995). The reversibility was examined by dialysing urea from sample and original spectrum was regained. The β-lg A and β-lg C variants showed similar changing patterns for the overall spectrum to those of β-lg B, but with different intensities (spectra not shown). It has been reported that the intensities of the spectral bands, particularly those at 285 and 293 nm, also decrease with increasing temperature. However, even after heating at 90 °C for 10 min (Manderson et al., 1999b), $\Delta\varepsilon$ (molar CD extinction coefficient, $M^{-1}cm^{-1}$) between 260 and 320 nm was greater than that of β-lg in 7.3 M urea solution at pH 6.7 (Creamer, 1995) or in 4 M guanidine hydrochloride solution at pH 3.2 (Kuwajima et al., 1996).

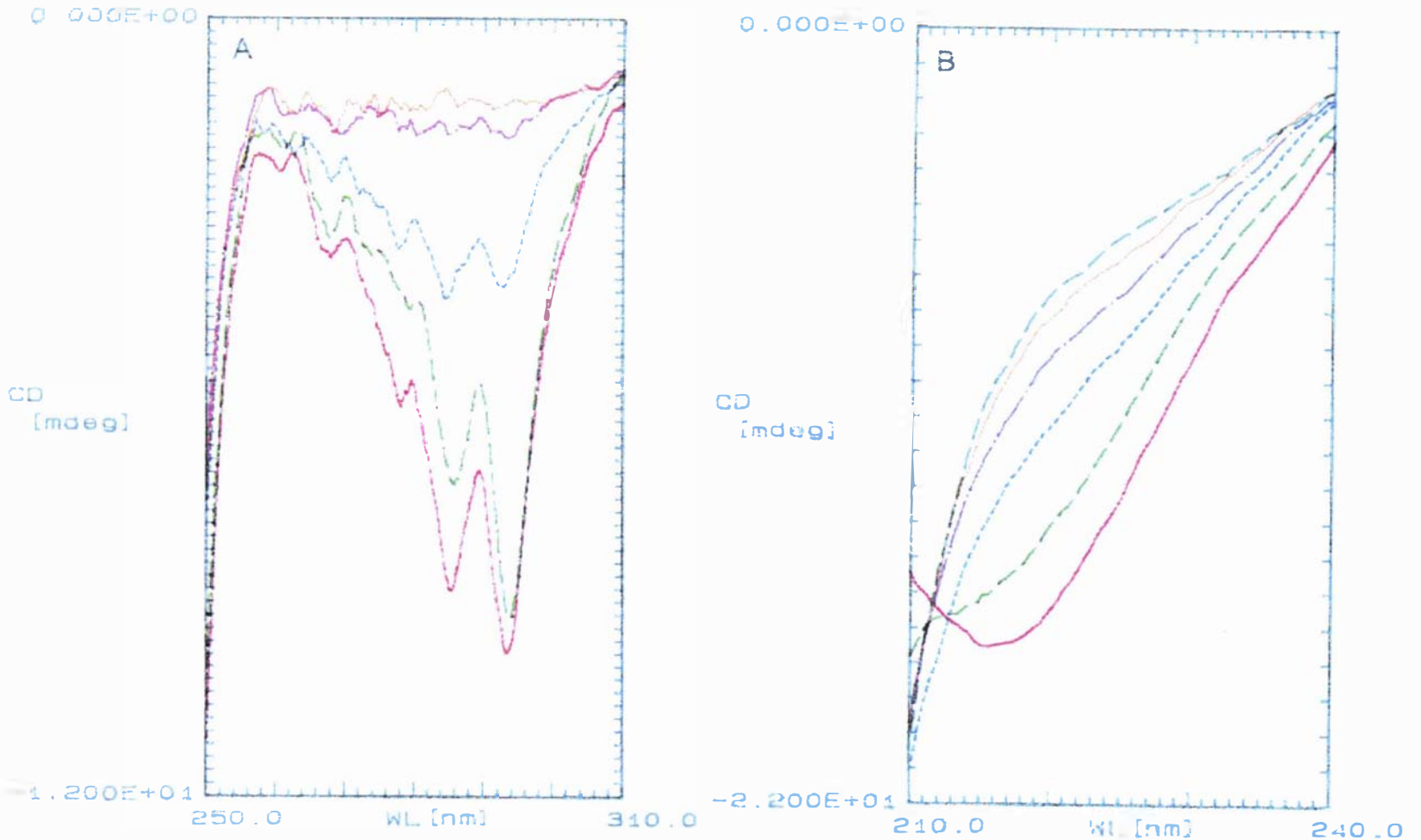

Figure 6.3.1.1. Effect of urea concentration on (A) near-UV and (B) far-UV CD spectra of β-lg B. The purified proteins were dissolved (1.3 mg/mL for near-UV or 0.13 mg/mL for far-UV) in pH 6.7 phosphate buffer in the presence of 0 M (red solid), 4 M (green dashed), 5 M (blue dotted), 6 M (purple centre) and 8 M (orange solid) urea (for far-UV, 7 M (orange solid) and 8 M (green dashed) urea were included). Protein CD spectra were acquired at 20 °C in 10 mm (1 mm for far-UV) path length cells using a Jasco J-720 spectropolarimeter. Measurements were made at a scan speed of 50 nm/min, using a spectral band width of 1.0 nm, a time constant of 2 s and a step resolution of 0.2 nm. In each case, the final spectrum is the average of five scans of the solution. See Section 6.2 for experimental detail.

The CD spectral patterns of β-lg in urea are in agreement with earlier observations. The spectra of β-lg between 0 and 4 M urea concentration showed no significant changes. It is also known that between pH 2.0 and pH 5.0, no significant conformational changes take place (Townend et al., 1967; Uhrínová et al., 1998). In 8 M urea, β-lg is randomly coiled except for the constraints imposed by the disulphide bonds and urea promotes β-lg dimer dissociation to the monomer, which is complete at urea concentrations above 3 M at pH 3.2 (Pace and Tanford, 1968). Creamer (1995) suggested that the β-sheet structure of β-lg B breaks down simultaneously with the loss of the hydrophobic sites and exposure of the tryptophan residues to the environment, based on CD and fluorescence measurements conducted at neutral pH in 7.3 M urea.

Manderson et al. (1999b) used the changes in CD at 270 nm as an index of significant alteration to intermolecular and intramolecular disulphide bond dihedral angles. Disulphide bonds give broader bands near 260 nm that seem to be related to the dihedral angle of the disulphide bond (Woody, 1973). With increasing urea concentration, the band intensity at 270 nm showed a gradual decrease (Fig. 6.3.1.1.A).

Manderson et al. (1999b) reported that an increasing intensity at 270 nm occurred as the temperature was increased and that these changes occurred at higher temperatures than those for the changes at 293 nm. This indicates that significant changes in protein tertiary structure preceded extensive disulphide bond interchange reactions during heat treatment. Normally the disulphide bond dihedral angle is approximately 90° and changes in this angle result in splitting into two broad bands at higher and lower wavelengths (Woody, 1973, 1995; Strickland, 1974). However, the decreasing band intensity at 270 nm was observed as urea concentration increased based on the near-UV CD result in this study, which may be caused either by different changing patterns of the disulphide bond dihedral angles or by loss of chiral environment of aromatic residues in the presence of urea.

Genetic variant effects

The changes in band intensity at 293 nm were plotted against the urea concentration in Fig. 6.3.1.2.A for the three variant proteins in pH 6.7 phosphate buffer. The values of the midpoint urea concentration were in the order β-lg B < β-lg A < β-lg C (i.e. 5.26 M for β-lg A, 5.13 M for β-lg B and 5.57 M for β-lg C). Similar changes were observed for the urea denaturation of β-lg at pH 5.0, but both the midpoint urea concentration values and the slopes were steeper (Fig. 6.3.1.2.B). Manderson et al. (1999a, b) also reported that the thermal stability was in the order β-lg B < β-lg A < β-lg C in either pH 6.7 or pH 7.4 phosphate buffer based on the midpoint temperatures obtained from the near-UV CD intensity at 293 nm ($\Delta\varepsilon_{293}$). Their samples were heated at temperatures between 40 and 94 °C for 12.5 min (Fig. 6.3.1.3). Alexander and Pace (1971) showed that the midpoint concentrations for the urea denaturation of bovine β-lg A, bovine β-lg B and goat β-lg at 25 °C at pH 3 were 5.3, 5.19 and 4.86 M. These values showed that β-lg A was 1 kJ/mol more stable than β-lg B and 4 kJ/mol more stable than goat β-lg. At pH 3, β-lg would usually remain as a monomer, which can create differences in the unfolding process.

Variant C was noticeably more stable than variants B and A to both thermal denaturation (Manderson et al., 1999a, b) and urea denaturation. Variant A resembled variant B in stability at low temperatures and low urea concentrations, but at higher temperatures and higher urea concentrations it resembled variant C (Figs 6.3.1.2.A and 6.3.1.3 - Manderson et al., 1997). This result indicates that the substitution of Val (β-lg A) for Ala (β-lg B or β-lg C) at position 118 altered the entropic contribution to the unfolding of the protein, as discussed by Qin et al. (1999). Therefore, urea-induced changes to the native structure of β-lg A may follow a different pathway from those of either the B variant or the C variant, which was also suggested by Manderson et al. (1999a, b) for heat-induced denaturation.

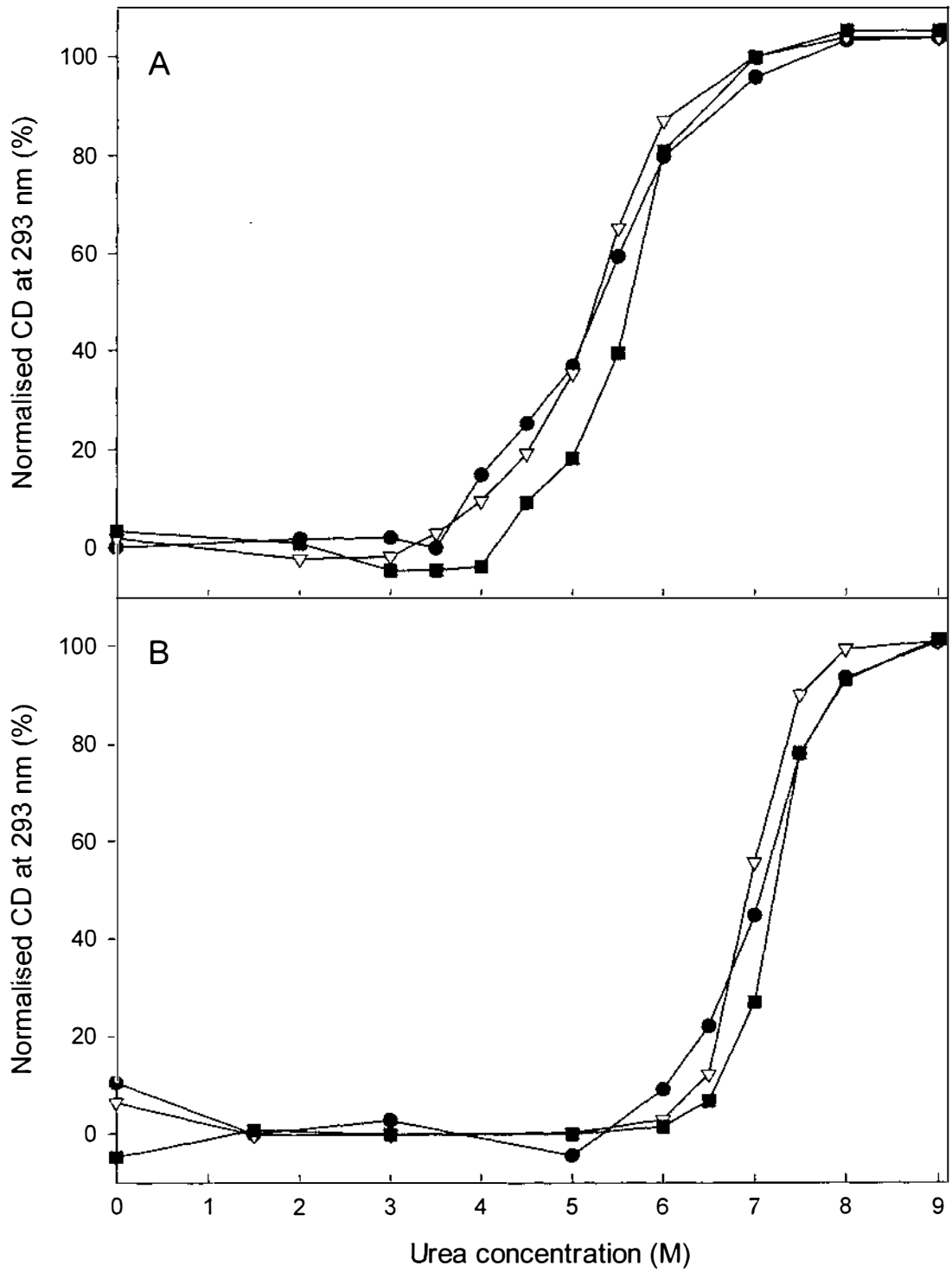

Figure 6.3.1.2. Effect of urea concentration on the CD band intensity at 293 nm of β-lg in (A) pH 6.7 phosphate buffer and (B) pH 5.0 glutaric acid buffer. The results were obtained from samples of β-lg A (●), β-lg B (▽) and β-lg C (■) mixed with urea solution and were plotted versus urea concentration. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section 6.2.

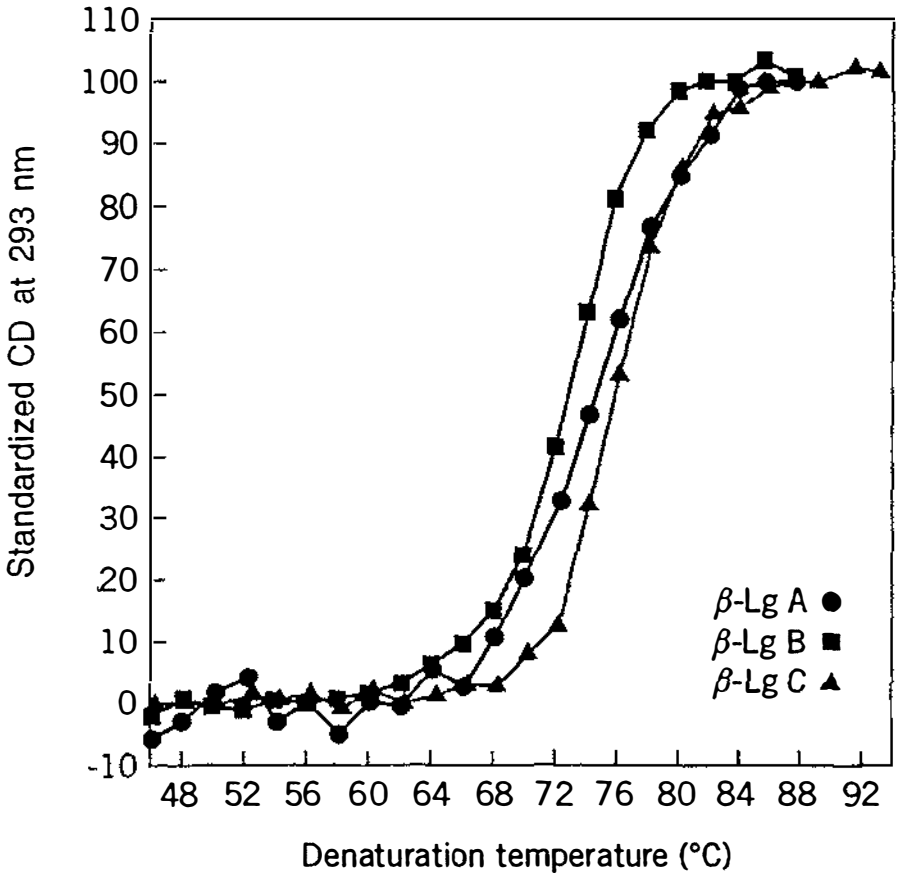

Figure 6.3.1.3. Thermal denaturation of bovine β-lg A (●), β-lg B (▲) and β-lg C (■) in pH 6.7 phosphate buffer. The percentage denaturation obtained from the near-UV CD ellipticity that occurs for a fixed period (12.5 min) of heating is plotted as a function of temperature. Taken from Manderson et al. (1997).

pH effects

Comparison of the results at pH 6.7 and pH 5.0 shows that the midpoint urea concentrations and the rates of intensity change at 293 nm were higher at pH 5.0 than pH 6.7 (Fig. 6.3.1.2.A, B). The three variants of β-lg showed the same order of stability based on the midpoint urea concentration for both pHs (7.05 M for β-lg A, 6.8 M for β-lg B and 7.2 M for β-lg C in pH 5.0 glutaric acid buffer).

The isoelectric points of β-lg variants A, B and C differ slightly, with p*I* values of 5.26, 5.34 and 5.33, respectively, in 0.1 M KCl at room temperature (McKenzie, 1971) and, as the protein in solution approaches its isoelectric pH, it becomes less charged and more stable. It has been shown that urea facilitates extensive

intermolecular SH-SS interchange with concomitant network formation by causing protein unfolding and by exposing free thiol groups (Monahan et al., 1995). Monahan et al. (1995) also reported that, at pHs close to and lower than the p*I* of whey, thiol oxidation and disulphide-mediated polymerisation are limited. As the pH is increased above the p*I*, the propensity of the whey proteins to undergo irreversible thermal denaturation involving SH-SS interchange increases. The greater change in the midpoint urea concentration at pH 5.0 is probably related to the slight net positive charge on the protein as the pH approaches the isoelectric point, which would lead to a higher stability of the β-lg solutions at pH 5.0 (Fig. 6.3.1.2.B). The reason for the changing slopes of the lines is less apparent, but suggests a higher co-operativity in the unfolding reaction and a steeper decrease of the water concentration at pH 5.0.

6.3.1.2. Far-UV CD

The far-UV CD spectrum largely reflects the secondary structure of the protein from which it was derived and arises from the peptide bond absorption bands and the inherent chirality of the polypeptide chain (Johnson, 1990; Woody, 1995). Analysis of the spectra obtained can give relative proportions of helical, sheet etc. structure. Tryptophan side chains and disulphide bonds can also contribute to the far-UV spectra (Woody, 1995).

The far-UV CD spectra of the three variants of β-lg in pH 6.7 phosphate buffer were very similar to one another (Figs 4.1.3.11 and 6.3.1.1.B) and to those obtained earlier (Timasheff et al., 1967; Creamer, 1995; Manderson et al., 1999b). Both β-sheet and α-helix secondary structure show CD peaks at about 200 nm and troughs in the 210-230 nm region whereas random and turn structures give rise to deep troughs near 200 nm (Johnson, 1990). Earlier analyses (Timasheff et al., 1967; Creamer et al., 1983; Griffin et al., 1993; Qi et al., 1997) consistently indicate that the native protein has about 40% β-sheet and 10% α-helix; these values are supported by X-ray crystal structures (Bewley et al., 1997; Brownlow et al., 1997; Qin et al., 1998a, b, 1999) and NMR structures (Uhrínová et al., 1998, 2000). However, in this experiment, because of the noise caused by the presence of urea, the spectrum was measured between 210 and 240 nm. As the urea concentration increased, the β-lg troughs between 210 and 230 nm disappeared (Fig. 6.3.1.1.B) and a deep trough developed below 210 nm, which was not observed as a consequence of the noise caused by the presence of urea.

This indicated most of the helical and sheet secondary structure of β-lg was lost with increasing urea concentration.

In thermal denaturation of β-lg, as the temperature was increased from approximately 40 to 90 °C, the trough at 216 nm was reported to be gradually broadened and deepened so that the minimum shifted to lower wavelengths, as observed by Griffin et al. (1993), Prabakaran and Damodaran (1997) and Manderson et al. (1999b). Casal et al. (1988) and Qi et al. (1997) also reported progressive loss of β-sheet and α-helix structure and an increase in random structure with increasing temperature based on the results of CD and FTIR (Fourier transform infrared) spectroscopy. On the other hand, Matsuura and Manning (1994) and Sawyer et al. (1971) reported a more intense trough at 216 nm and Lapanje and Poklar (1989) reported a decrease in the trough at 216 nm as the temperature was increased without shifting minimum wavelength.

Genetic variant effects

The normalised CD signal at 220 nm decreased slightly up to a urea concentration of about 3 M and then increased with a further increase in the urea concentration in pH 6.7 phosphate buffer. The midpoint urea concentration values were in the order β-lg A < β-lg B ≤ β-lg C (Fig. 6.3.1.4.A), which was different from the midpoint urea concentration values for the band at the 293 nm transition (Fig. 6.3.1.2.A). However, the differences in the slopes at the midpoint urea concentration for the results at pH 6.7 fitted the pattern expected on the basis of the results obtained in the near-UV CD. Similar results were obtained from the urea denaturation of β-lg at pH 5.0, but both the midpoint urea concentration values and the slopes were steeper (Fig. 6.3.1.3.B).

pH effects

The slopes of normalised CD signal at 220 nm as a result of increasing urea concentration were generally greater at pH 5.0 than at pH 6.7 (Fig. 6.3.1.4), which is in agreement with the results of near-UV CD. The midpoint urea concentration values at pH 5.0 were 6.5 M for β-lg A, 6.57 M for β-lg B and 6.87 M for β-lg C, whereas those at pH 6.7 were 4.75 M for β-lg A, 4.96 M for β-lg B and 5.07 M for β-lg C.

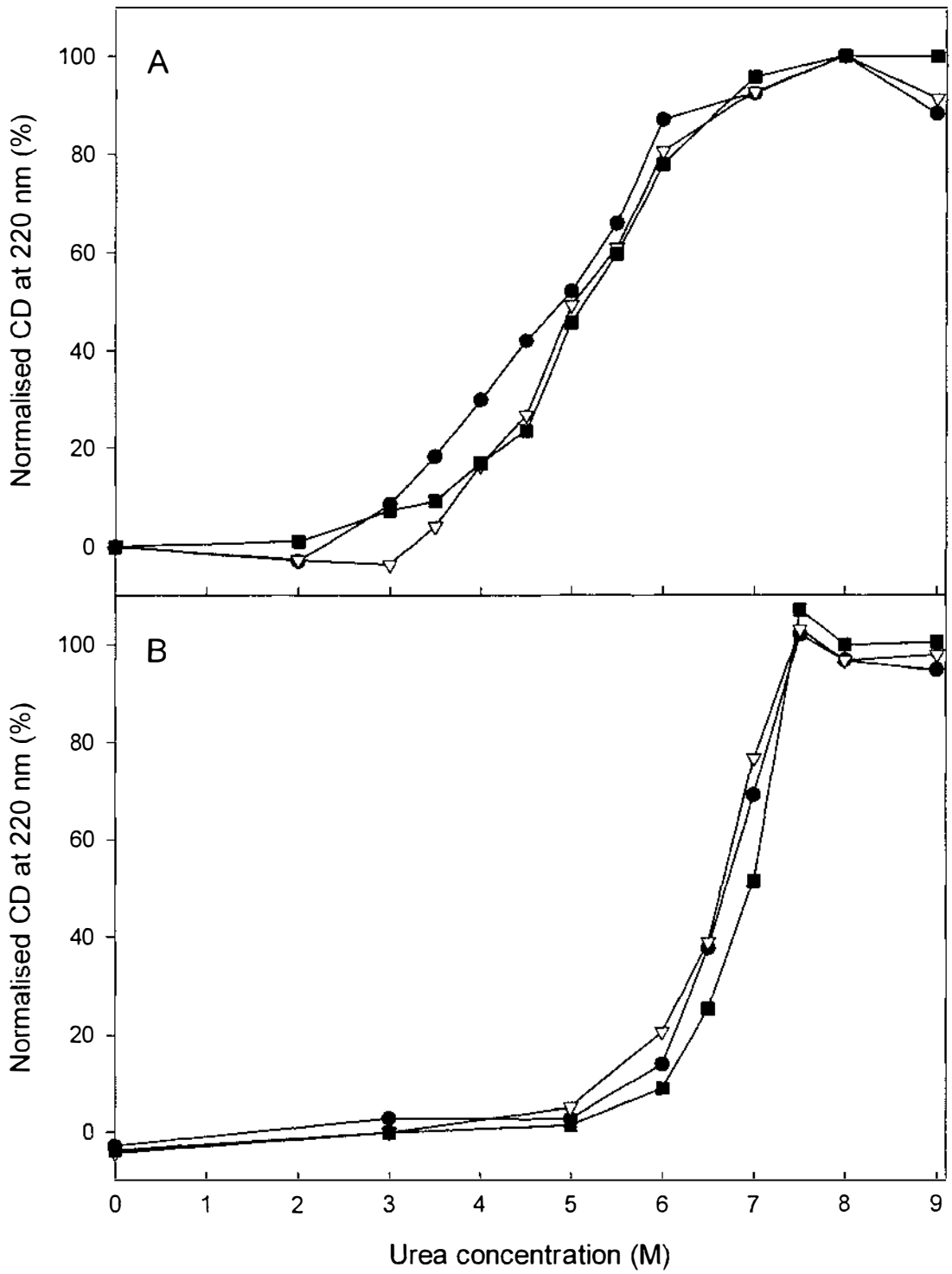

Figure 6.3.1.4. Effect of urea concentration on the CD band intensity at 220 nm of β-lg in (A) pH 6.7 phosphate buffer and (B) pH 5.0 glutaric acid buffer. The results were obtained from samples of β-lg A (●), β-lg B (▽) and β-lg C (■) mixed with urea solution and were plotted versus urea concentration. The spectra were acquired as described in Fig. 6.3.1.1. Experimental are given in Section 6.2.

The derived midpoint urea concentration values increased as the pH decreased from 6.7 to 5.0 for all variants (Fig. 6.3.1.4.A, B). The midpoint urea concentration values at 220 nm were always less than those at 293 nm. This probably suggested that the loss of helical and sheet structure with increasing urea denaturation was preceded the changing in a chiral environment of Trp-19.

6.3.2. The role of the thiol group in the urea-induced unfolding of β-lactoglobulin

Many proteins that contain cysteine and cystine are capable of undergoing sulphydryl oxidation reactions or disulphide interchange reactions which lead to the formation of aggregated species (Sawyer et al., 1963). Brownlow et al. (1997) and Qin et al. (1999) determined the three-dimensional structure of three variants of β-lg by X-ray crystallography and indicated that the single free sulphydryl group in β-lg belongs to Cys-121; it is located on the outside of the β-barrel, but is largely buried beneath the α-helix. Exposure of the sulphydryl group, as may occur on heating or adding denaturants, would require the α-helix to roll back a little. In the native protein, Cys-121 is also too far (7 Å) from Cys-119 to undergo disulphide interchange without major conformational change of the molecule (Bewley et al., 1997); again, however, heating could give sufficient flexibility for this to occur. It is worth noting that neither the position nor the environment of Cys-121 and its free sulphydryl group is changed significantly in any of the variants (Bewley et al., 1997).

Modification of the sulphydryl group with reagents such as IAM (iodoacetamide) and PHMB (*p*-hydroxymercuribenzoate) is known to prevent the irreversible aggregation of β-lg at pH values even above pH 8 (McKenzie and Sawyer, 1967). Similarly, the addition of NEM to urea solutions of β-lg at pH 5.2 was found to prevent the irreversible aggregation of the denatured protein (Ralston, 1972; McKenzie et al., 1972). Hoffmann and van Mil (1997) reported that the decrease in thiol content, and the subsequent decrease in disulphide bond formation in the presence of NEM, may increase molecular flexibility and enhance interactions via non-specific bonding (Xiong et al., 1993), and suggested that NEM favours aggregation by non-covalent interactions.

6.3.2.1. Thiol-blocked β-lactoglobulin

Within native β-lg, a free SH group is buried between the helix and sheet of the folded protein (Fig. 2.2.2.2) (Bewley et al., 1997) and conformation-steric constraints protect the SH group from the external solvent, rendering it unreactive, for example, toward DTNB. However, quaternary or tertiary structure changes, arising from the exposure of β-lg to pH $\geq$ 6.7 (Kella and Kinsella, 1988), denaturants (Iametti et al., 1996), high temperatures (Iametti et al., 1996; Qi et al., 1997; Manderson et al., 1999a) and high pressures (Tanaka et al., 1996), increase the SH group reactivity.

In the work discussed in the following section, the products of β-lg thiol blocked using DTNB and NEM are characterised by size-exclusion chromatography and alkaline- and SDS-PAGE, and β-lg thiol blocked using DTNB treated with urea to elucidate the role of the free thiol group in β-lg denaturation.

(i) β-Lactoglobulin blocked by DTNB

FPLC. The chromatograms of the β-lg thiol blocked using DTNB using the Superdex 75 column are shown in Fig. 6.3.2.1. The absorbance of the products was monitored at 280 and 305 nm. The chromatograms of β-lg A (Fig. 6.3.2.1.A) and β-lg C (Fig. 6.3.2.1.C) showed a major peak after 88.3 min and a small peak at about 96.8 min. The major peak of β-lg B (Fig. 6.3.2.1.B) was eluted after 95 min and a small shoulder was observed at about 82 min. The retention times of β-lg dimer at pH 6.7 and β-lg monomer at pH 2.0 were checked for comparison and the dimer and monomer were found to elute at about 87 and 100 min, respectively.

Hoffmann and van Mil (1999) reported that, in the reaction of DTNB with the thiol group of native β-lg, two steps can be distinguished: (1) diffusion of DTNB into the β-lg molecule to the thiol group and (2) reaction of TNB (thio-nitrobenzoic acid) with the thiolate anion as shown in Fig. 6.3.2.2. At neutral pH, the thiol group in native β-lg is well shielded and sufficient time was allowed during preparation to complete the reaction between DTNB and β-lg (Section 6.2).

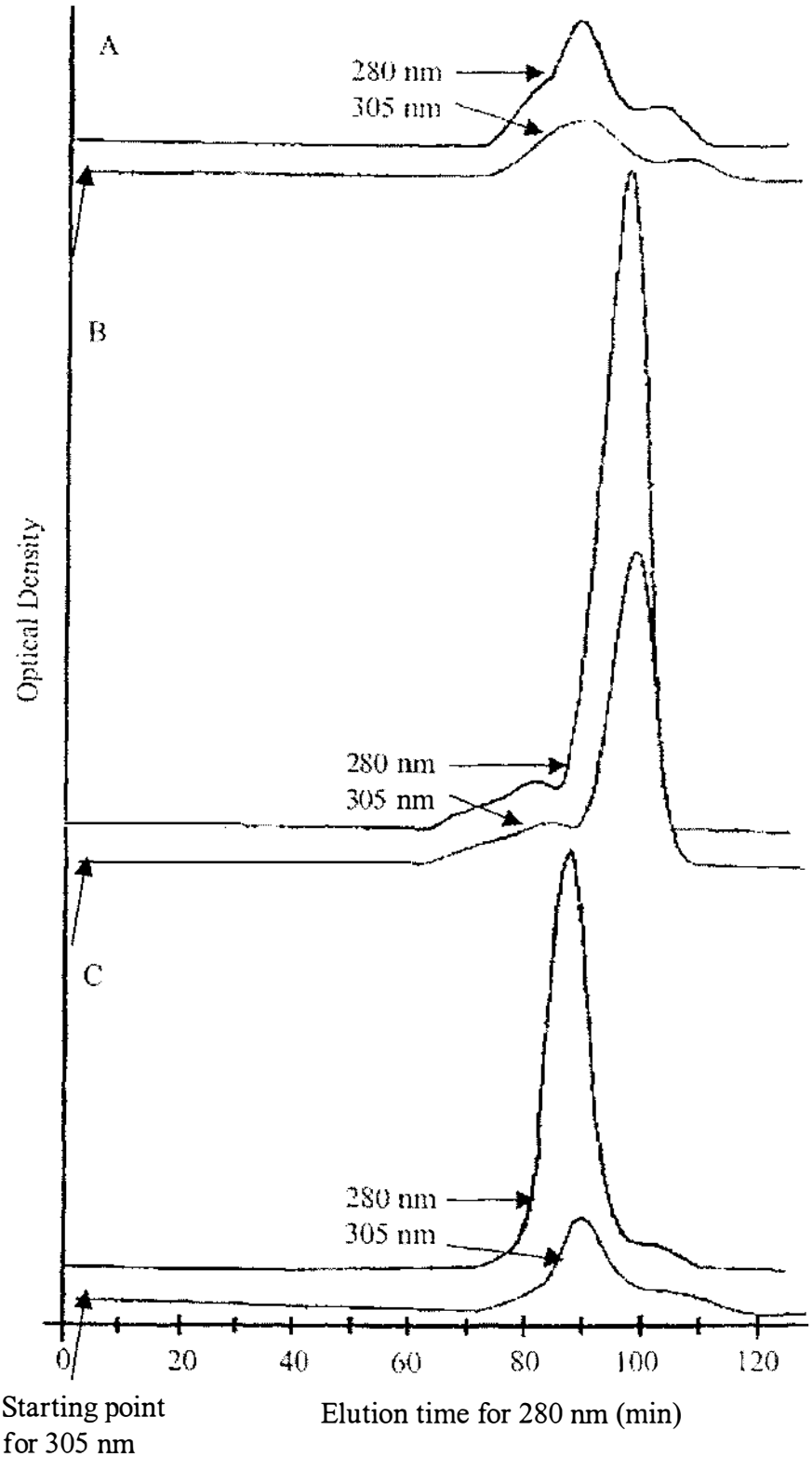

Figure 6.3.2.1. Chromatograms of (A) β-lg A (1.3 mg/mL), (B) β-lg B (2.1 mg/mL) and (C) β-lg C (1.6 mg/mL) thiol blocked using DTNB in pH 6.7 phosphate buffer. Protein solutions were separated on a Superdex 75 column equilibrated with pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl). The measurements were made using the FPLC system described in Section 4.1.2.3.

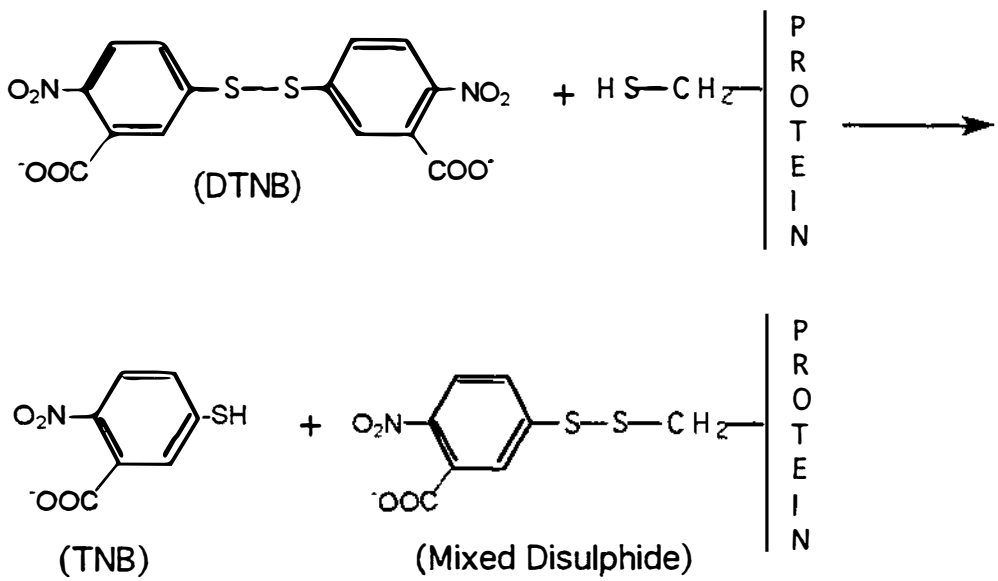

Figure 6.3.3.2. The reaction of DTNB with protein thiol group (Manderson, 1998).

During the preparation of the β-lg B derivative, the solution became a vivid yellow whereas the β-lg A and β-lg C solutions turned a cloudy yellow. The progressive formation of the yellow thiolate anion from the –SH reagent indicated time-dependent exposure of a reacting –SH group (Iametti et al., 1996). Therefore, there may be a difference in affinity to DTNB among the three β-lg variants and the B variant showed higher reactivity than the variants A and C.

On the other hand, it has been reported that β-lg A and β-lg B reacted with Ellman's reagent at identical rates, whereas β-lg C reacted at about one-tenth the rate of β-lg A and β-lg B (Phillips et al., 1967). Thresher (1997) also found that β-lg C reacted with Ellman's reagent at a slower rate than β-lg A and β-lg B. However, the latter study also showed a difference in the reactivity of the A variant compared with that of the B variant. Thresher (1997) reported that the relative rate of reaction of the β-lg variants with Ellman's reagent in simulated milk ultrafiltrate pH 6.6 followed the order β-lg A > β-lg B > β-lg C.

Urea was found to promote dissociation of dimer to the monomer, which is complete at a urea concentration above 3 M at pH 2 (Pace and Tanford, 1968), without effect of heat treatment (Kella and Kinsella, 1988; Creamer, 1995). Therefore urea was preferred over heat treatment for the partial unfolding of β-lg in the preparation of thiol-blocked β-lg in this experiment.

Gel electrophoresis. The β-lgs modified using DTNB were separated using size-exclusion chromatography and the collected fractions (fractions 6-11) were subjected to alkaline-PAGE (Fig. 6.3.2.3.A-C) and SDS-PAGE (Fig. 6.3.2.3.D-F). The alkaline-PAGE results indicate that there were two separate species for β-lg B and β-lg C and three species for β-lg A, whereas the SDS-PAGE results showed these bands appeared as one band. This suggests that intramolecular disulphide exchange (Manderson et al., 1998) had probably taken place by adding DTNB.

Although the free thiol group in β-lg may be accessible for reaction with DTNB by partial urea-induced unfolding, it appears that not all the thiol groups reacted with DTNB based on the results of gel electrophoresis. This may be because some thiol groups may have disappeared via oxidation, thiol-disulphide exchange followed by oxidation, inaccessible -SH group by thiol-disulphide exchange, the hydrolysis-off protein or detachment of DTNB during storage.

(ii) β-Lactoglobulin blocked by NEM

It has been reported that, at pH 6.5-7.5, maleimides bind rapidly and specifically to the SH group (Hoffmann and van Mil, 1997). Hoffmann and van Mil (1997) reported that, in the presence of NEM, all the thiol groups of β-lg were blocked and no disulphide-linked aggregates formed at a molar ratio NEM:β-lg monomer of 1. They also reported that, at pH 8.0, the aggregation of β-lg occurred solely by sulphydryl-disulphide interaction and that, when β-lg was heated in the presence of NEM, the formation of disulphide-linked aggregates was prevented. At this pH, as aggregation via non-covalent interactions is very unfavourable, NEM results in a retardation of the decrease in the concentration of native β-lg and consequently the formation of aggregates. NEM, another widely used sulphydryl blocking reagent, was used in this study to try to obtain more efficiently thiol-blocked β-lg A and β-lg B.

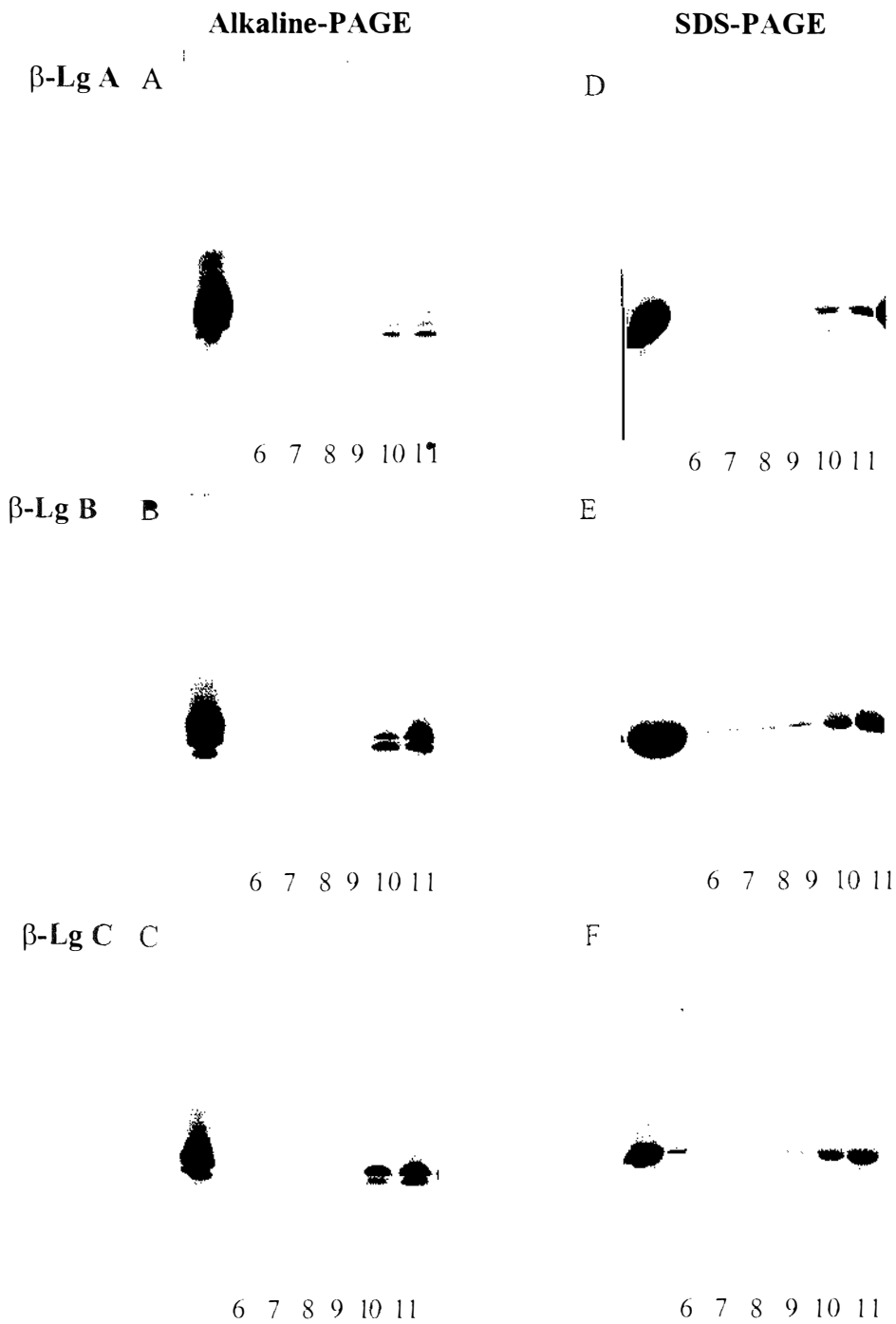

Figure 6.3.2.3. Electrophoretic patterns of eluted fractions 6-11 (elution time 80-105 min) obtained when β-lg thiol blocked using DTNB was further separated by size-exclusion chromatography. Alkaline-PAGE of (A) β-lg A, (B) β-lg B and (C) β-lg C and non-reduced SDS-PAGE of (D) β-lg A, (E) β-lg B and (F) β-lg C. Lane 1 contained protein prior to separation. All samples were diluted 1:1 with alkaline- or SDS-PAGE sample buffer, electrophoresed, stained/destained and photographed as described in Sections 3.2.1.2 and 3.2.1.1, respectively.

FPLC. The chromatograms of the β-lg A and β-lg B products of reaction are shown in Figs 6.3.2.4.A and B, respectively. They were monitored at 280 and 305 nm and separated using the Superdex 75 column. The NEM derivatives showed somewhat different chromatograms from the DTNB derivatives. Only one peak was eluted after 93.2 min in the β-lg A derivatives, whereas the β-lg B derivatives showed peaks after 86 and 94.1 min. Again the retention times of β-lg dimer at pH 6.7 and β-lg monomer at pH 2.0 were checked for comparison and were about 87 and 100 min, respectively.

The number of thiol groups remaining was determined by reaction of the NEM reaction products with DTNB using the method of Manderson (1998). DTNB reacts with thiol compounds to produce 1 mole of *p*-nitrophenol anion per mole of thiol and the absorbance at 412 nm was recorded as a function of time. No thiol group was detected in the NEM derivatives produced.

Gel electrophoresis. The NEM derivatives, separated using size-exclusion chromatography (fractions 1-15), were analysed by alkaline- and SDS-PAGE. The alkaline-PAGE results indicate that two species co-existed in the β-lg A product (Fig. 6.3.2.5.A) and that three co-existed in the β-lg B product (Fig. 6.3.2.5.B). However, analysis of the same samples by SDS-PAGE showed one thick band (Fig. 6.3.2.5.C, D). These results were similar to those for the DTNB derivatives (Fig. 6.3.2.2), indicating that both DTNB and NEM caused the intramolecular disulphide exchange reaction in β-lg molecules.

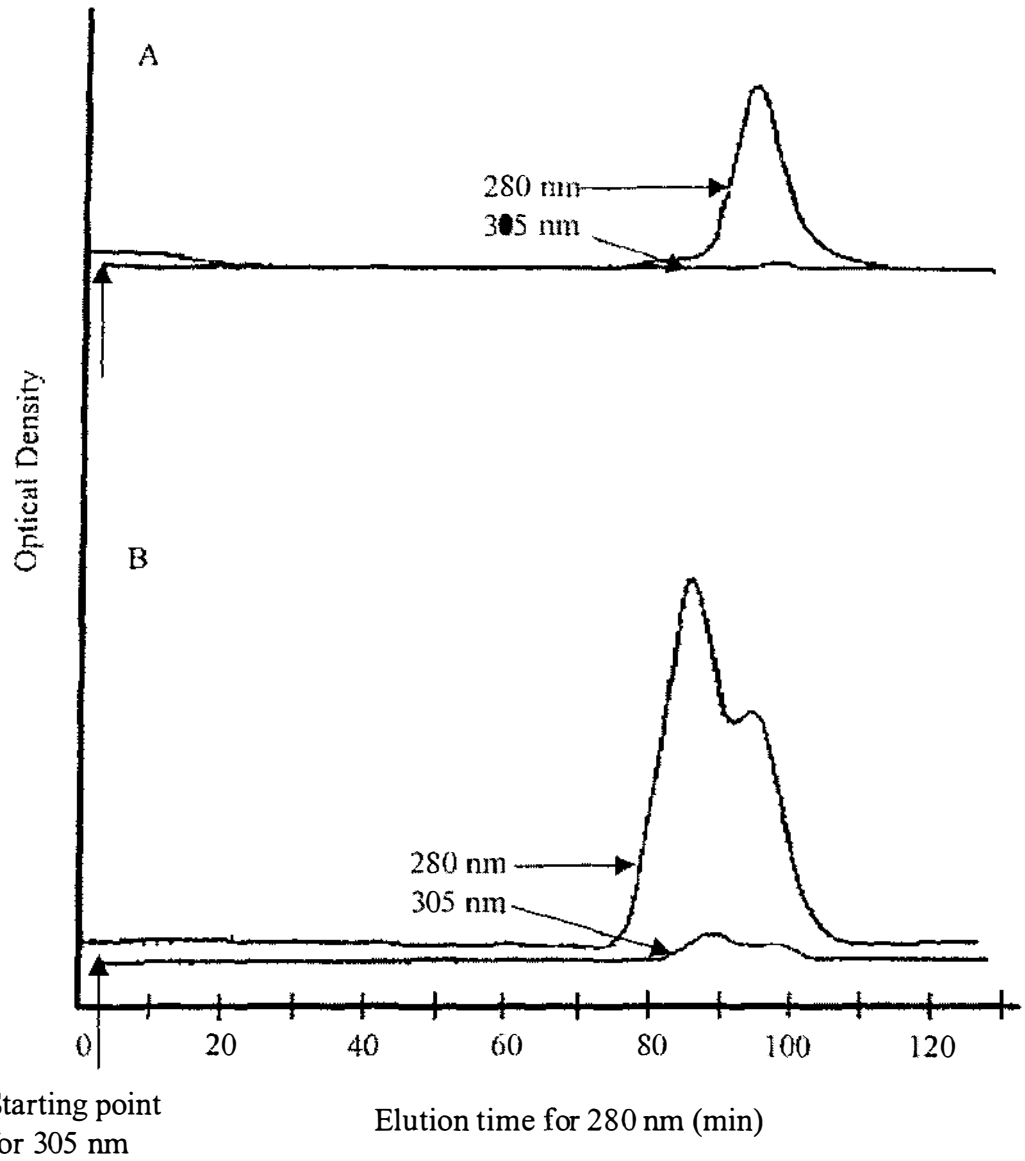

Figure 6.3.2.4. Chromatograms of (A) β-lg A and (B) β-lg B (1.3 mg/mL) thiol blocked using NEM in pH 6.7 phosphate buffer. Protein solutions were separated on a Superdex 75 column equilibrated with pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl). The measurements were made using the FPLC system described in Section 4.1.2.3.

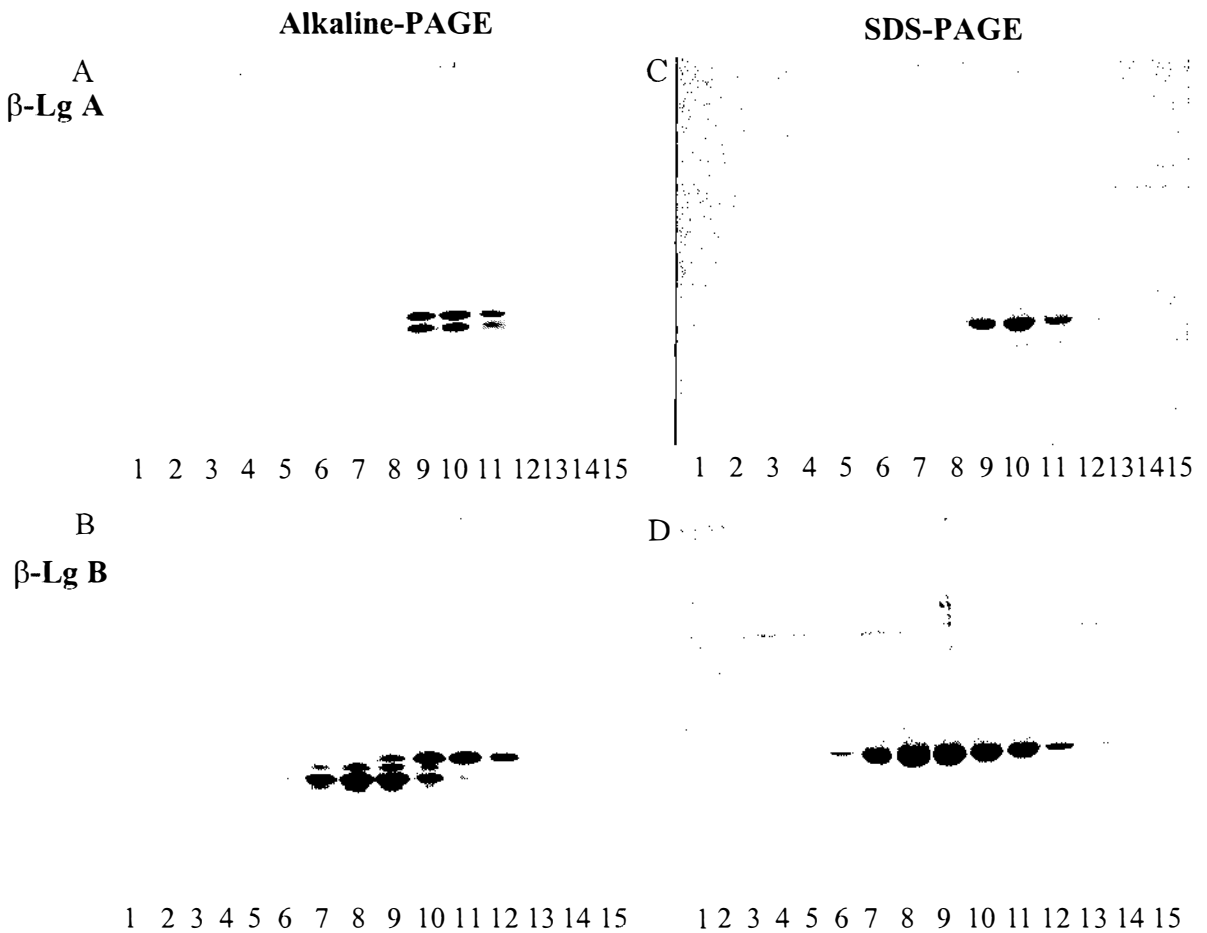

Figure 6.3.2.5. Electrophoretic patterns of eluted fractions 1-15 (elution time 75-110 min) obtained when β-lg thiol blocked using NEM was further separated by size-exclusion chromatography. Alkaline-PAGE of (A) β-lg A and (B) β-lg B and non-reduced SDS-PAGE of (C) β-lg A and (D) β-lg B. Lane 1 contained protein prior to separation. All samples were diluted 1:1 with alkaline- or SDS-PAGE sample buffer, electrophoresed, stained/destained and photographed as described in Sections 3.2.1.2 and 3.2.1.1, respectively.

6.3.2.2. Effect of urea concentration on the unfolding of thiol-blocked β-lactoglobulin

Dissociation of non-covalently linked β-lg dimers and the subsequent initiation reaction will lead to the exposure of the reactive thiol group. To understand this in more detail, monomer β-lg, stable at neutral pH, was prepared by trapping the exposed thiol group in transiently dissociated monomers using DTNB. As shown for other studies using this sulphydryl reagent (Iametti et al., 1996, 1998), the covalently modified β-lg monomer did not reassociate. Therefore, urea-induced unfolding of thiol-blocked β-lg was investigated in this work to get further insight into the role of the thiol group of β-lg in isothermal urea denaturation.

Dunnill and Green (1965) reported that the thiol group became more exposed at higher pH. Whether the pH-dependent reactivity of the Cys residues results from the Tanford (N ↔ R) conformational change or dissociation is not clear (Hambling et al, 1992). Therefore, changes in the CD spectra of the β-lg derivatives were also examined at different pH values.

Near-UV CD

Effect of pH. Figure 6.3.2.6 compares features in the near-UV region of the CD spectrum of the three variants of DTNB-blocked β-lg at three different pHs (pH 6.7, 7.7 and 8.7). In addition to the troughs at 285 and 293 nm from the tryptophan residue of β-lg, a new peak centred at about 350 nm was observed, which was not observed in the NEM-blocked β-lg (spectrum not shown).

The spectrum of blocked β-lg showed that it retained a chiral environment of aromatic residues (285 and 293 nm) that characterises the tertiary structure of native β-lg (Fig. 4.1.3.8). The strong UV absorbance of the protein-bound nitrobenzoate induced a broad peak centred at 350 nm for the β-lg A and β-lg B variants and at 310 nm for the β-lg C variant in pH 6.7 phosphate buffer. As the pH of the blocked β-lg increased up to pH 8.7, the band intensities at 285 and 293 nm decreased whereas the peak intensity at 350 nm increased. For β-lg C, the peak centre moved from 310 to 350 nm as the pH increased to 7.7 (Fig. 6.3.2.6). β-Lg B showed the highest band intensity at 350 nm among three variants, which depended on the proportion of thiol-blocked β-lg monomers.

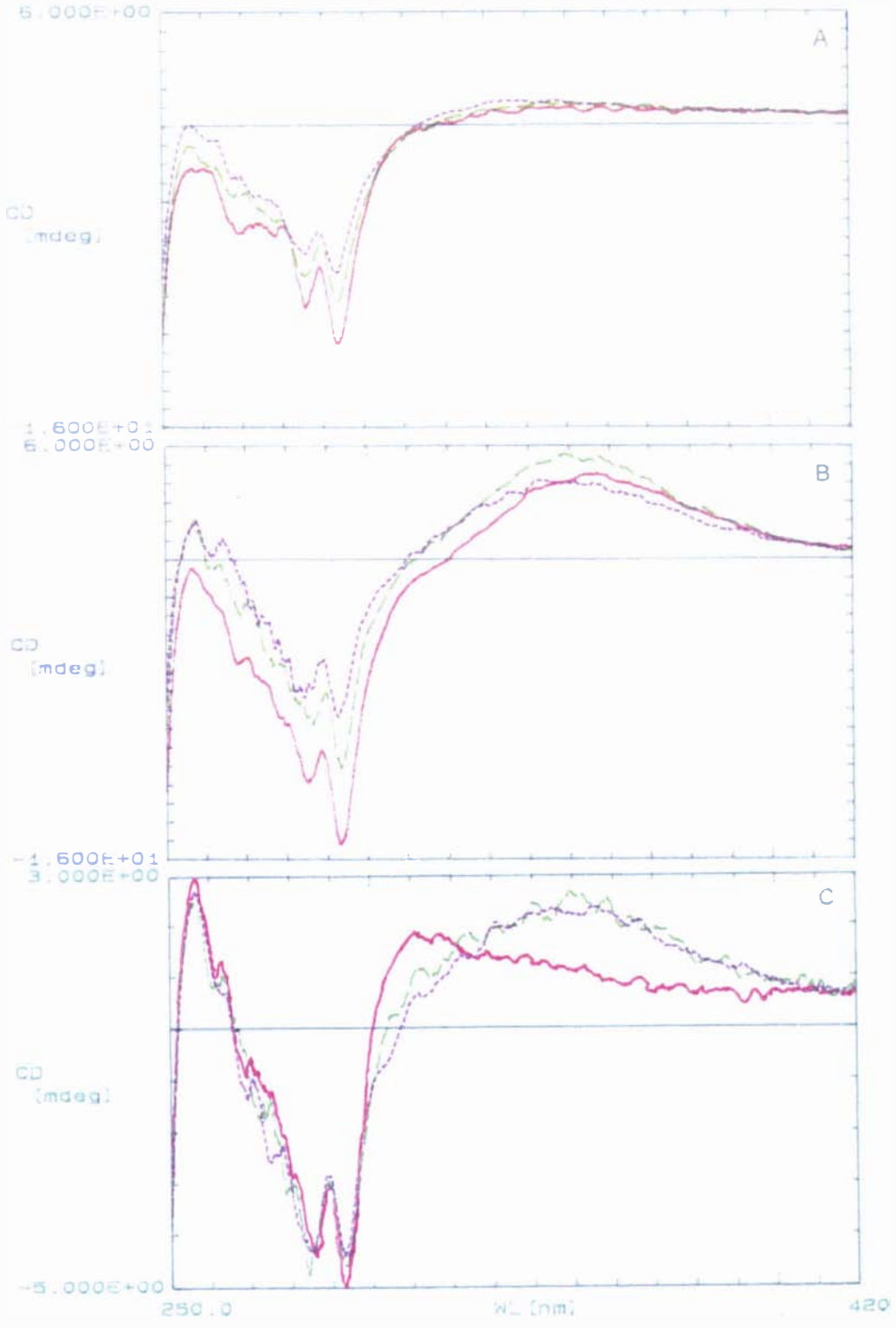

Figure 6.3.2.6. Near-UV CD spectra of (A) β-lg A, (B) β-lg B and (C) β-lg C (1.3 mg/mL) thiol blocked using DTNB at pH 6.7 (red solid), pH 7.7 (green dashed) and pH 8.7 (purple dotted). The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section 6.2.

High pH facilitates the dissociation and gives band intensities decreasing at 293 nm (lost dimer characteristics) and increasing at 350 nm (increasing bound DTNB absorbance) as shown in Fig. 6.3.2.6.

Cairoli et al. (1994) reported that the near-UV CD spectra of thiol-blocked monomers are similar to those recorded for native β-lg heated at 60-65 °C at neutral pH. Qi et al. (1997) reported a decrease in β-sheet between 40 and 60 °C where, under the conditions of the CD experiment, the dimer is expected to have dissociated into monomer (Josh and Ralston, 1996). At 60-65 °C, lower than the temperature required for the occurrence of irreversible thermal modifications, a significant fraction of the β-lg molecules is present in solution as dissociated monomers (Iametti et al., 1996), and the observed spectroscopic changes are fully reversible upon cooling (Cairoli et al., 1994). On this basis, Iametti et al. (1998) suggested that the tertiary structure of the blocked monomer is representative of that of monomers obtained through thermal dissociation, which is in agreement with the results of present study (Fig. 6.3.2.6).

Burova et al. (1998) showed that modification of Cys-121 in β-lg results only in tertiary and quaternary structure changes and reported that, at neutral pH, the equilibrium dimer-monomer of β-lg shifted to the monomeric form by modification. However, blocking the sulphydryl group of β-lg may have an effect on the conformation and stability of the native protein because of the introduction of the bulky group into the compact structure of β-lg. Ralston (1972) reported that the introduction of the modifying group perturbs the structure of the protein, either by steric hindrance of neighbouring groups or by disrupting non-covalent interactions. It is also known (Sawyer et al., 1963; Hoffmann and van Mil, 1997) that blocking the thiol group of β-lg with NEM changes the course of heat-induced reactions of bovine β-lg.

Effect of urea concentration. The recorded CD spectra of thiol-blocked β-lg B in the presence of urea in the near-UV region are presented in Fig. 6.3.2.7.A. With increasing urea concentration, the band intensities at 293 and 350 nm decreased and the band centred at around 350 nm blue shifted to 345 nm.

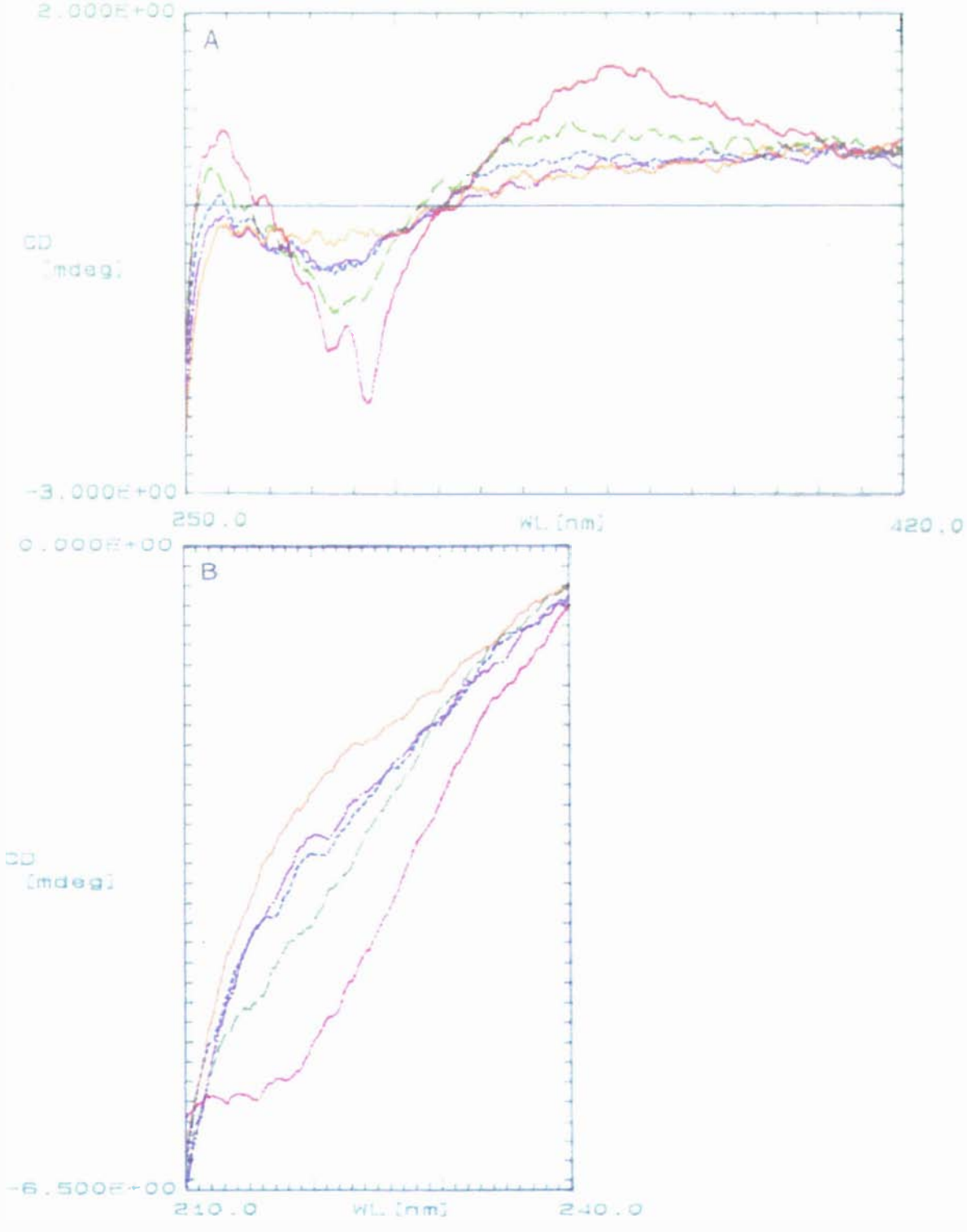

Figure 6.3.2.7. (A) Near-UV CD and (B) far-UV CD spectra of β-lg B thiol blocked using DTNB (1.3 mg/mL) in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) in the presence of 0 M (red solid), 4 M (green dashed), 5 M (blue dotted), 6 M (purple centre) and 8 M (orange solid) urea. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section 6.2.

The band intensities at 293 and 350 nm were plotted against urea concentration (Fig. 6.3.2.8.A, B). The midpoints of the urea concentration values of the thiol-blocked β-lgs were 4.43 M for β-lg A, 3.65 M for β-lg B and 4.85 M for β-lg C, which were lower than those of the unmodified β-lg observed in Section 6.3.1.1 (i.e. 5.26 M for β-lg A, 5.13 M for β-lg B and 5.57 M for β-lg C) in pH 6.7 phosphate buffer. The values of the midpoint urea concentration followed the same order β-lg B < β-lg A < β-lg C in both cases, but β-lg became less stable with the blocking of the thiol group. Other than the initial increase in the band intensity at 350 nm, the decreasing intensity probably indicates that changing in a chiral environment of DTNB bound β-lg with increasing urea concentration.

The plots of the normalised CD band at 293 nm against normalised CD band at 350 nm of β-lgs modified by DTNB during urea denaturation are shown in Fig. 6.3.2.9 and the correlation coefficients of β-lg A, B and C were 0.53, 0.94 and 0.94, respectively. This suggests that the loss of a chiral environment of Trp-19 in β-lg B and β-lg C is proportional to the decrease of a chiral environment of TNB during urea denaturation. Whereas the plot of β-lg A modified by DTNB did not show the same pattern, and this suggests that the orientation of DTNB in the β-lg A may be different from β-lg B and β-lg C. This may be caused by the destabilising cavity created by the Val118Ala (A⇒B and C) substitution or by different helix position in protein.

The decrease in the heat stability of β-lg on blocking of the thiol group with NEM was also noticed by Ralston (1972) using optical rotation measurements, Hoffmann and van Mil (1997) using DSC and Burova et al. (1998) who showed substantial destabilisation of the tertiary structure of the protein by modification of Cys-121. The destabilisation can be interpreted in terms of steric interference and disruption of non-covalent interactions, due to introduction of the thiol-modifying group. Disruption of non-covalent interactions by means of this modification would then favour the production of the non-native-like state and so would increase the overall rate of unfolding.

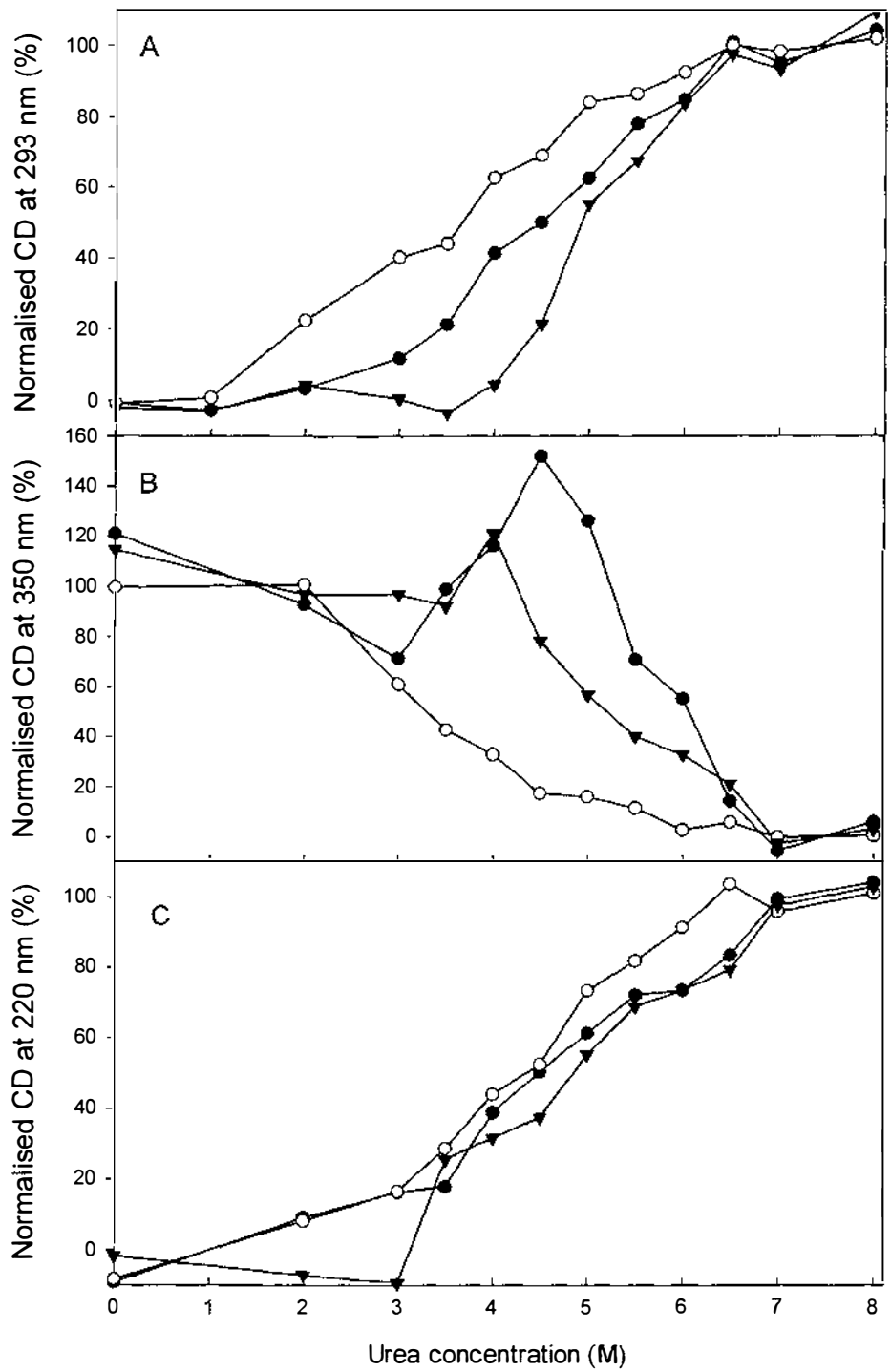

Figure 6.3.2.8. Effect of urea concentration on the CD band intensity at (A) 293 nm, (B) 350 nm and (C) 220 nm. The results were obtained from samples of β-lg A (●), β-lg B (○) and β-lg C (▼) thiol-blocked using DTNB. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section 6.2.

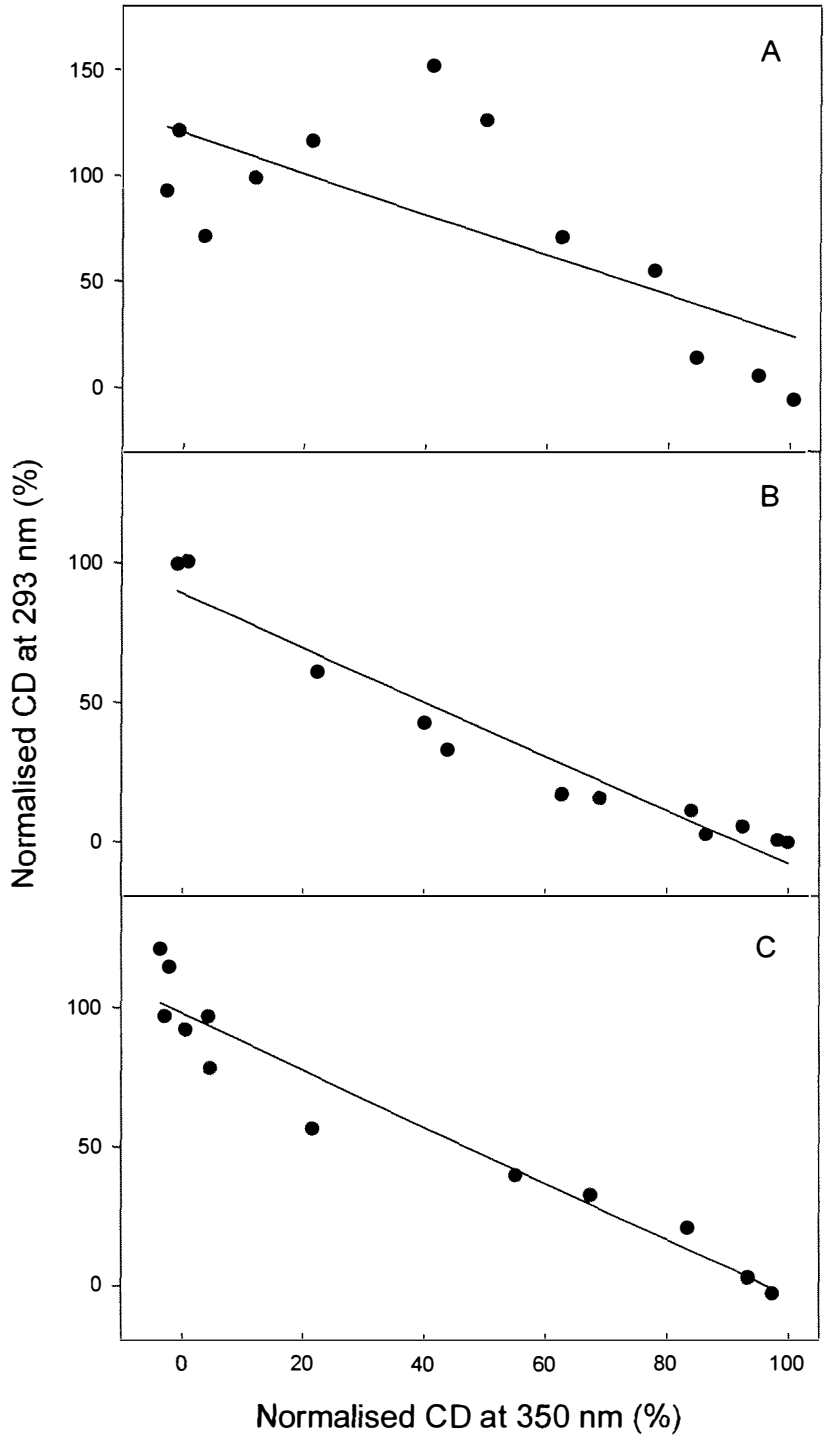

Figure 6.3.2.9. Plots of normalised CD band intensity at 293 nm versus normalised CD band intensity at 350 nm. The results were obtained from samples of (A) β-lg A, (B) β-lg B and (C) β-lg C thiol-blocked using DTNB during urea denaturation. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section 6.2.

Far-UV CD

The effect of increasing urea concentrations on the far-UV CD spectra of thiol-blocked β-lg B is shown in Fig. 6.3.2.7.B. The change in the CD spectrum of thiol-blocked β-lg B with increasing urea concentration between 210 and 240 nm is plotted in all figures so that direct comparison is possible. Because of solvent absorption, CD measurements below 210 nm are not feasible in urea solutions. The far-UV CD spectrum of the thiol-blocked protein shows a minimum near 216 nm, which is typical of β-sheet and in agreement with native β-lg in pH 6.7 phosphate buffer (Fig. 4.1.3.11) and literature data (Townend et al., 1967; Su and Jirgensons, 1977; Manderson et al., 1999b).

With increasing urea concentration, the CD spectrum of β-lg became less negative at wavelength above 212 nm and more negative below 212 nm, as shown in Fig. 6.3.2.7.B. It is known that the CD spectrum of a random coil displays a single intense negative band near 200 nm (Towell and Manning, 1994; Woody, 1995). Therefore, the effect of urea on the secondary structure of thiol-blocked β-lg is consistent with an increase in random coil content. Similar results were obtained in Section 6.3.2.1 using native β-lg, i.e. addition of urea diminishes the broad trough centred on 216 nm as a consequence of the loss of most of the helical and sheet secondary structure. Pace and Tanford (1968) also reported that β-lg is randomly coiled in 8 M urea except for the constraint imposed by the disulphide bonds, indicating some conversion of α-helix and β-sheet to aperiodic structure (Prabakaran and Damodaran, 1997).

The band intensity at 220 nm was plotted against urea concentration (Fig. 6.3.2.8.C). The midpoints of the urea concentration values of the thiol-blocked β-lgs were 4.43 M for β-lg A, 4.23 M for β-lg B and 4.72 M for β-lg C, which were lower than those of the unmodified β-lg observed in Section 6.3.1.1. The order of the midpoint urea concentration among three variants followed β-lg B < β-lg A < β-lg C, which is in agreement with the near-UV CD results (Fig. 6.3.2.8.A, B).

Blocking the thiol group with DTNB did not allow simple explanations because the bound group gave rise to CD signals itself. Also there were reactions other than the simple blocking of the -SH group, as shown by chromatography and alkaline- and SDS-PAGE.

6.3.3. Effect of urea concentration on the unfolding of ligand-bound β-lactoglobulin

6.3.3.1. Near-UV CD

(i) Retinol

Addition of retinol to β-lg in pH 6.7 phosphate buffer induced bands at about 255 and 350 nm as previously described in Section 5.3.2.1 and shown in Fig. 5.3.2.2. β-Lg A induced a deeper trough than β-lg B or β-lg C, which gave similar patterns to each other, in the presence of 2 M urea concentration (Fig. 6.3.3.1.A).

The decreases in the near-UV CD intensity at 293 nm were plotted against increasing urea concentration (Fig. 6.3.3.2.A-C), and the stability was in the order β-lg C > β-lg A ≥ β-lg B based on the midpoints of the urea concentration values (i.e. 5.45 M for β-lg A, 5.41 M for β-lg B and 5.72 M for β-lg C). The midpoints of the urea concentration for all three variants were higher than those for each β-lg itself (Section 6.3.1.1), indicating the improved stability of retinol-bound β-lg. The overall band intensity became more intense until the urea concentration was about 2 M and then started to decrease as the urea concentration increased (Fig. 6.3.3.2.A-C). With further increasing urea concentration the tryptophan side chain troughs at 285 and 293 nm as well as the induced band at 350 nm decreased (spectrum not shown). This is similar to the effects observed during thermal denaturation in Section 5.3.2.1, which showed increased band intensities at 293 and 350 nm up to 40 °C and then decreased band intensities during further heat treatment (Fig. 5.3.2.2.A).

(ii) Retinoic acid

The addition of retinoic acid to β-lg in pH 6.7 phosphate buffer induced a broad band between 330 and 370 nm with less intensity (Fig. 6.3.3.1.B) than the mixture of retinol and β-lg.

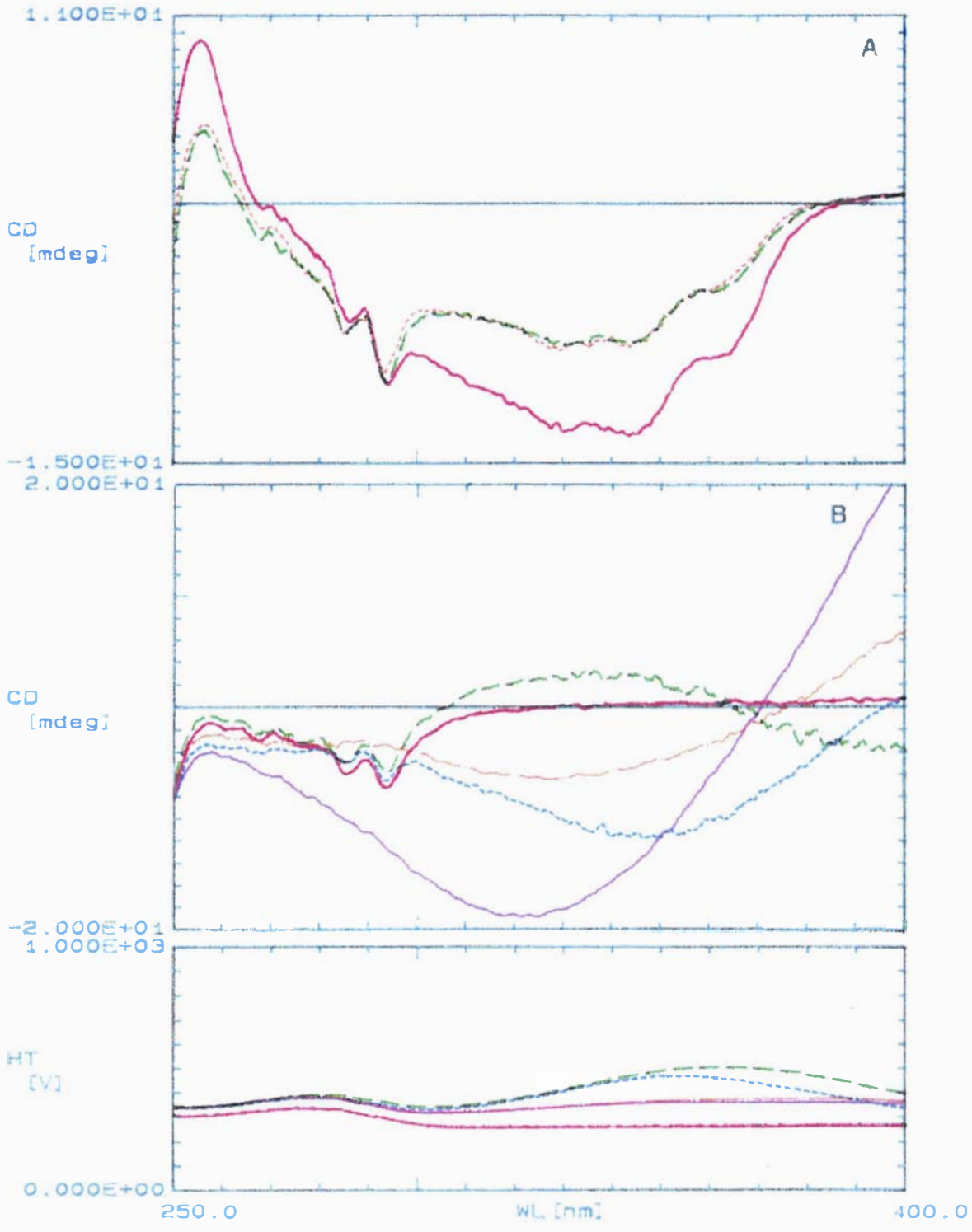

Figure 6.3.3.1. Near-UV CD spectra of (A) retinol-bound β-lg A (red solid), β-lg B (green dashed) and β-lg C (orange dotted) in the presence of 2 M urea and (B) the effect of urea concentration on retinoic-acid-bound β-lg. The spectrum of β-lg B (1.5 mg/mL) in the absence of retinoic acid (red solid), and in the presence of retinoic acid (20 μL) containing 0 M (green dashed), 4.5 M (blue dotted), 6.5 M (purple solid) and 7.5 M (orange centre) urea. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section 6.2.

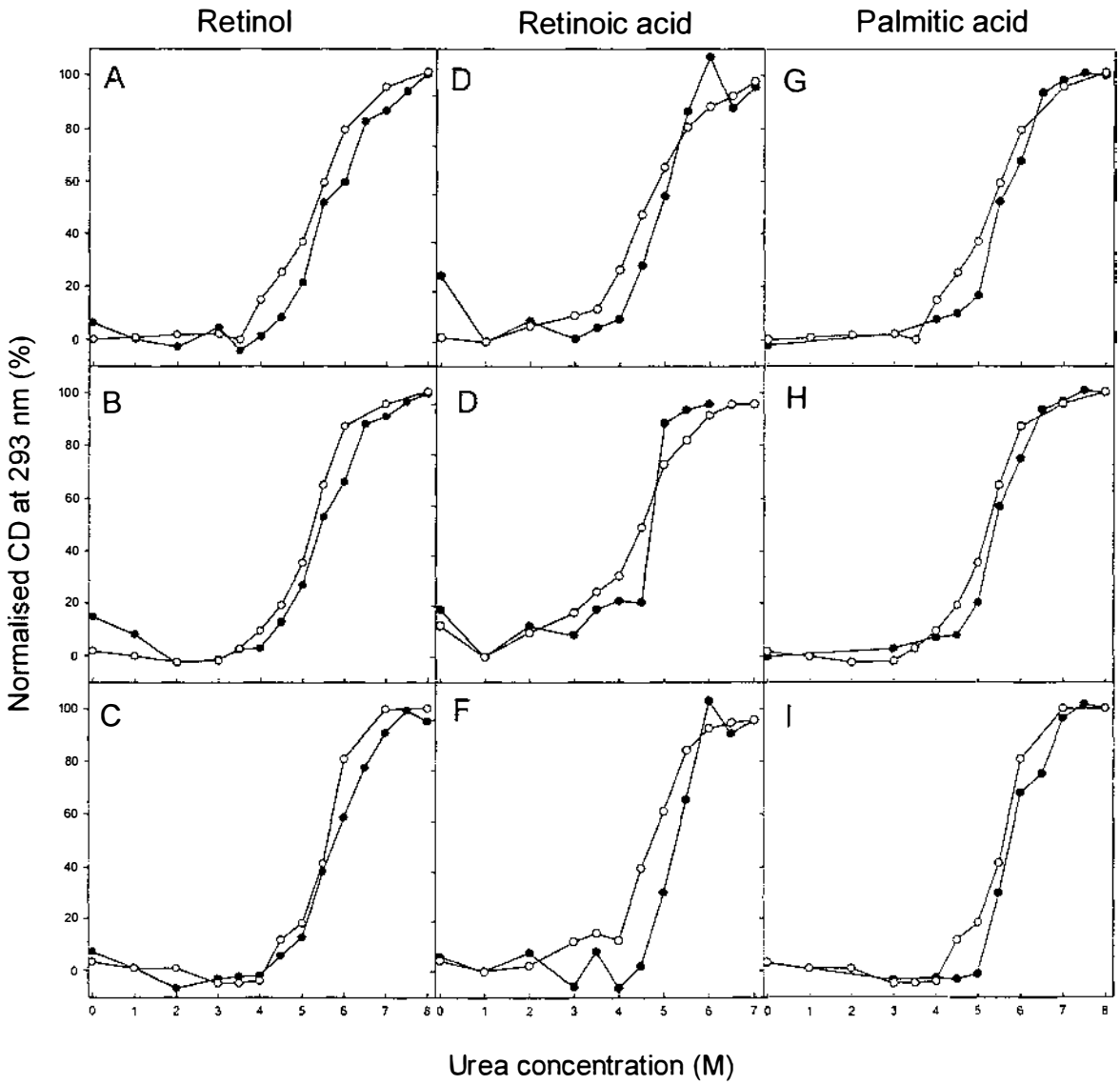

Figure 6.3.3.2. Effect of urea concentration on the CD band intensity at 293 nm of the mixture of retinol and (A) β-lg A, (B) β-lg B or (C) β-lg C; the mixture of retinoic acid and (D) β-lg A, (E) β-lg B or (F) β-lg C; the mixture of palmitic acid and (G) β-lg A, (H) β-lg B or (I) β-lg C in the absence (O) and the presence (●) of ligand. The results were obtained from β-lg samples (1.5 mg/mL) mixed with urea solution and then with the addition of ligand in a 1:1.1-1.2 molar ratio (β-lg:ligand) and plotted versus urea concentration. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details and data analyses are given in Section 6.2.

However, with increasing urea concentration, the mixture of retinoic acid and β-lg lost the broad band centred at 350 nm as well as the tryptophan bands at 285 and 293 nm in all three variants of β-lg. This suggests that the reaction of the carboxyl group of retinoic acid with urea seems to destroy all the ordered tertiary structure of the mixture of retinoic acid with β-lg, especially when the urea concentration exceeds 4 M. Above 4 M urea concentration, one very broad band centred at 318 nm developed. The decreases in the near-UV CD intensity at 293 nm were plotted against increasing urea concentration up to 6-7 M (Fig. 6.3.3.2.D-F); above that urea concentration, the band intensities at 293 nm decreased dramatically in all three variants and so these points were ignored. However, these plots showed some enhanced stability (Fig. 6.3.3.2.D-F), compared with each β-lg itself, in all three variants of β-lg.

(iii) Palmitic acid

The presence of palmitic acid in the β-lg solution (pH 6.7) did not alter the near-UV CD spectra markedly (Fig. 5.3.2.7). Decreases in the intensity at 293 nm were observed and plotted against urea concentration (Fig. 6.3.3.2.G-I). The mixture of palmitic acid and β-lg seemed to be more stable than β-lg itself against urea denaturation in all three variants. The stability was in the order β-lg C > β-lg B ≥ β-lg A, based on the midpoints of the urea concentration values (i.e. 5.32 M for β-lg A, 5.33 M for β-lg B and 5.79 M for β-lg C), i.e. the same order as in the presence of retinol (Fig. 6.3.3.2.A-C) and for β-lg without ligands (Fig. 6.3.1.2). It is hard to compare the effects of the three ligands on the unfolding of β-lg because of the unexpected values of retinoic-acid-bound β-lg at higher urea concentrations; however, retinol-bound β-lg and palmitic-acid-bound β-lg showed very similar enhanced stability based on the midpoints of the urea concentration.

In addition to the enhanced stability of β-lg against urea denaturation by ligand binding (Creamer, 1995), many compounds bind to β-lg and a consequent enhancement in the stability of β-lg has been found over many years. Futterman and Heller (1972) showed that β-lg enhanced the fluorescence lifetime of retinol and reduced its susceptibility to oxidation. Shimoyamada et al. (1996) also reported that

β-lg and retinol or retinoic acid complexes showed increased stability against tryptic hydrolysis, heat-induced oxidation and light-induced oxidation.

6.3.3.2 Far-UV CD

(i) Retinol

The addition of retinol to β-lg solution in pH 6.7 phosphate buffer did not change its far-UV CD spectrum greatly (spectrum not shown). The trough, with a minimum at 216 nm, gradually diminished and the minimum shifted to lower wavelength with increasing urea concentration, as a consequence of the loss of most of the helical and sheet secondary structure. However, the shape of the spectrum was very similar to that of β-lg itself (Fig. 6.3.3.1). The band intensity at 220 nm was plotted against increasing urea concentration (Fig. 6.3.3.3.A-C). The mixture of retinol and β-lg showed higher midpoint values than β-lg itself in all three variants. The stability of the retinol/β-lg mixture was in the order β-lg C > β-lg B ≥ β-lg A based on the midpoints of the urea concentration (i.e. 5.16 M for β-lg A, 5.24 M for β-lg B and 5.37 M for β-lg C), which is in agreement with the results of near-UV CD (Section 6.3.3.1.(i)).

(ii) Retinoic acid

Unlike the results of near-UV CD, the secondary structure of the mixture of retinoic acid and β-lg changed similarly to that of the retinol mixture. As the urea concentration increased, the band intensity at 220 nm in the retinoic acid/β-lg mixture in pH 6.7 phosphate buffer gradually decreased up to 4.5 M urea concentration (Fig. 6.3.3.3.D-F). Further increase in the urea concentration induced a rapid decrease in the band intensity at 220 nm up to 7 M urea and then it increased slightly in the presence of 8 M urea. This pattern was observed in all three variants of β-lg and it was assumed that the carboxyl group of retinoic acid affected the urea unfolding of β-lg differently.

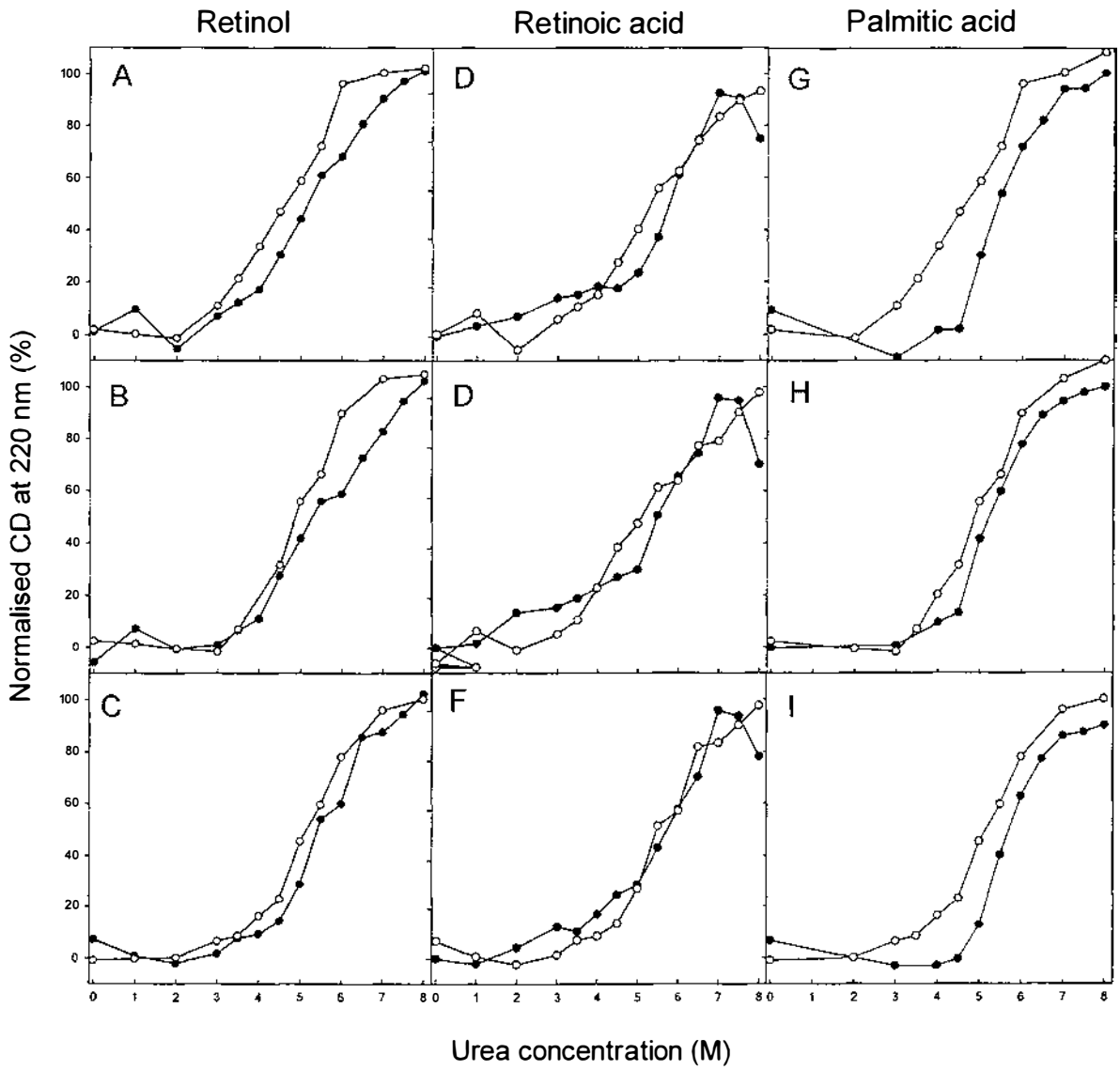

Figure 6.3.3.3. Effect of urea concentration on the CD band intensity at 220 nm of the mixture of retinol and (A) β-lg A, (B) β-lg B and (C) β-lg C; the mixture of retinoic acid and (D) β-lg A, (E) β-lg B and (F) β-lg C; the mixture of palmitic acid and (G) β-lg A, (H) β-lg B and (I) β-lg C in the absence (O) and the presence (●) of ligand. The results were obtained from β-lg samples (1.5 mg/mL) mixed with urea solution and then with the addition of ligand in a 1:1.1-1.2 molar ratio (β-lg:ligand) and plotted versus urea concentration. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details and data analyses are given in Section 6.2.

The stability was in the order β-lg C ≥ β-lg A > β-lg B based on the midpoints of the urea concentration values (i.e. 5.62 M for β-lg A, 5.45 M for β-lg B and 5.69 M for β-lg C), but barely showed the enhancement in stability. At lower urea concentration (< 4.0 M), retinoic-acid-bound β-lg showed faster urea-induced denaturation than β-lg itself.

(iii) Palmitic acid

The addition of palmitic acid to β-lg in pH 6.7 phosphate buffer also showed a shift of the minimum to lower wavelengths in the far-UV CD spectrum with increasing urea concentration (spectrum not shown). The band intensity at 220 nm was plotted against urea concentration as shown in Fig. 6.3.3.3.G-I. The order of stability was β-lg C > β-lg A > β-lg B based on the midpoints of the urea concentration values (i.e. 5.35 M for β-lg A, 5.18 M for β-lg B and 5.71 M for β-lg C); the mixture of palmitic acid and β-lg showed higher midpoint values than β-lg itself, which is in agreement with near-UV CD results. This result is also in agreement with Creamer (1995) who demonstrated enhanced stability of β-lg to urea denaturation when complexed with either palmitic acid or a low concentration of SDS.

6.3.4. General discussion

Many studies have been carried out on the structural changes of β-lg caused by urea or guanidine hydrochloride at acidic pH (Greene and Pace, 1974; McKenzie and Ralston, 1973; Raymond et al., 1974; Azuaga et al., 1992; Civera et al., 1996). β-Lg is stable at acid pH values, but, as the pH increases, it self-associates and undergoes conformational changes, to finally denature under alkaline conditions (Azuaga et al., 1992).

In this study, the urea-induced unfolding of β-lg at neutral pH (6.7) was investigated using different genetic variants, and thiol-blocked and ligand-bound proteins. It seems that urea, which causes protein dissociation and unfolding, exposing free thiol groups, facilitated extensive intermolecular SH-SS interchanges at high pH. Variant C was noticeably more stable than variants B and A to urea denaturation as well as thermal denaturation (Manderson et al., 1999a, b). The differences in the denaturation curves of β-lg A, B and C can be attributed to the

structural differences within the proteins which give rise to an interplay of enthalpic and entropic effects as a consequence of a salt bridge involving His-59 (β-lg C), a destabilising cavity created by the Val118Ala (A⇒B) substitution and a changed charge distribution within the CD loop caused by the Asp64Gly (A⇒B) substitution (Qin et al., 1998b; Manderson et al., 1999b).

The urea unfolding of the three variants of β-lg was slightly different (Fig. 6.3.1.1) but not as different as either with thermal denaturation (Fig. 6.3.1.3) or the effect of palmitic acid on any of the variants (i.e. β-lg A, B and C+ palmitic acid > β-lg C, Fig. 6.3.3.2.G-I). Retinol, retinoic acid and palmitic acid all stabilised β-lg against urea denaturation. However, the results of near-UV CD were not useful for retinoic acid, probably because too many conflicting signals were produced as retinoic acid moved around several binding sites of β-lg.

In contrast, thiol-blocked β-lg was significantly destabilised during urea-induced unfolding (Section 6.3.2.2), in agreement with the findings of Hoffmann and van Mil. (1997) who reported that blocking of the sulphydryl group can cause a significant decrease in both kinetic and thermodynamic stability of the protein.

The effect of the concentration of ammonium sulphate on the binding of bovine β-lg A with ligands (retinol, retinyl acetate and PnA) was also investigated in the Appendix 1 using near-UV CD. Addition of retinol to β-lg A in pH 7.1 and pH 8.5 and retinyl acetate in pH 8.5 Tris-phosphate buffer induced a peak at 255 nm and a broad trough centred at about 350 nm and increased the intensity of the tryptophan CD band at 293 nm (Fig. A1.3.1 and A1.3.2.B). An increasing ammonium sulphate concentration restored the spectrum to that of β-lg before the addition of retinol and retinyl acetate, probably suggesting detachment of ligand from the β-lg molecules. This provides an explanation of the difficulty in co-crystallising β-lg and bound ligand. Addition of PnA to β-lg A gave rise to four deep troughs at 286, 293, 310 and 325 nm. As the concentration of ammonium sulphate added to β-lg increased, the intensities of these bands decreased at both pH values. However, unlike retinol and retinyl acetate, with increasing ammonium sulphate the spectrum was not restored to that of β-lg alone (i.e. without PnA). This explains why co-crystallisation of fatty acid/β-lg was achieved earlier than that of retinoid/β-lg.

Urea denaturation is believed to occur via a two-state process by destabilisation or perturbation of the hydrophobic interactions and hydrogen bonds in proteins (Lapanje, 1978). The first stage involves both reversible dimer dissociation and unfolding of the polypeptide chain and the second stage involves the unfolded protein undergoing irreversible SH-SS exchange reactions (and possibly -SH oxidation), which is similar with the mechanism of thermal denaturation (Fig. 6.3.5.1).

Although the blocking of thiol group of β-lg would prevent the second stage of denaturation, the addition of thiol-blocking agent (e.g. DTNB), which promotes dissociation of dimeric β-lg, causes significant destabilisation during urea-induced denaturation (Section 6.3.2.2). This may suggest the importance of initial stage of unfolding of β-lg for further denaturation. Furthermore, the stabilisation effect of ligands during thermal (Chapter 5) and urea denaturation of β-lg may be involved in shift of equilibrium position between native and non-native β-lg.

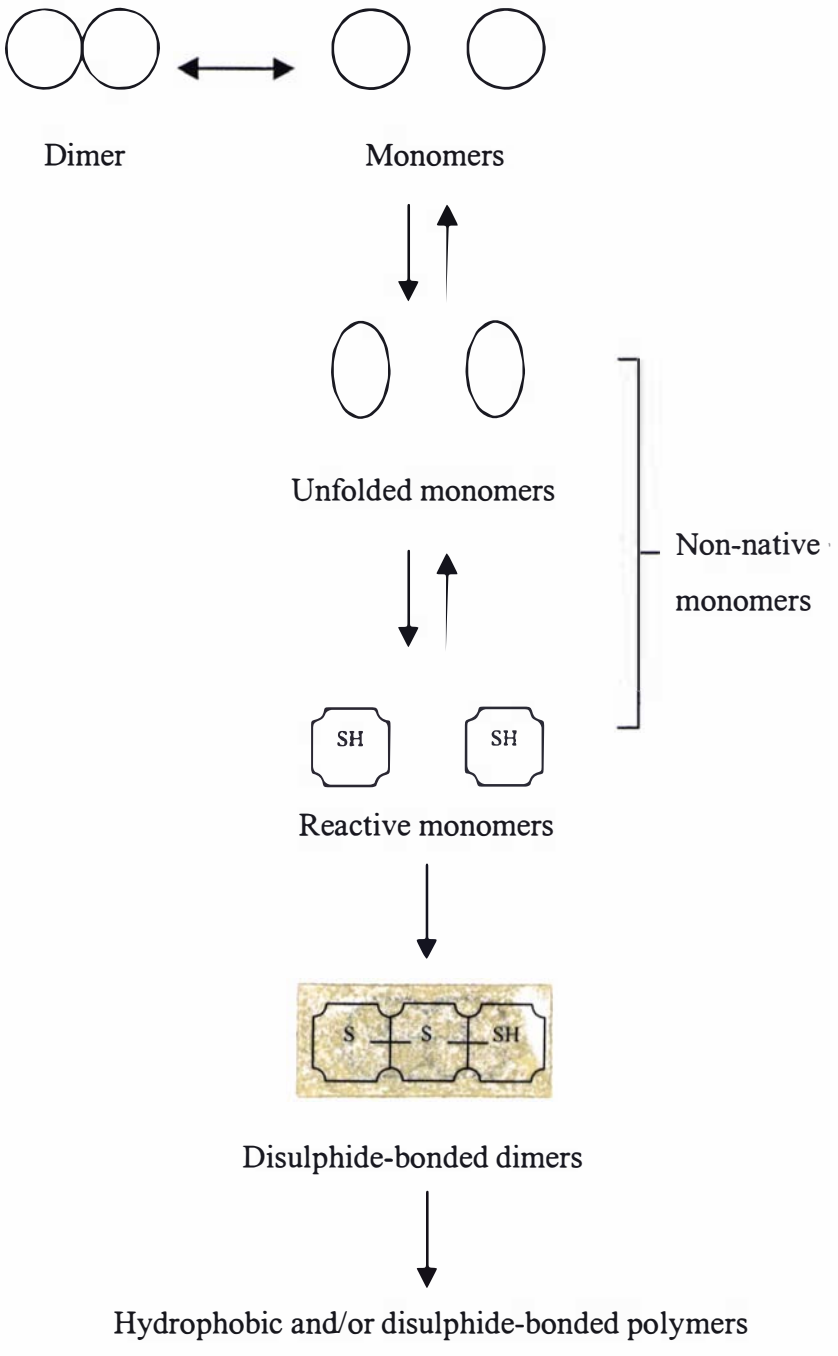

Figure 6.3.5.1. Schematic presentation of possible mechanism of β-lg denaturation.

CHAPTER 7.

CONCLUSIONS - *Part I*

Role of ligands and free cysteine in β-lactoglobulin denaturation

In the first part of this thesis, it was possible to build on some earlier results and expand our understanding of how β-lg behaves in solution and the factors that are important in the denaturation pathway.

In Chapter 5, the characteristics and ligand-binding properties of bovine β-lg and porcine β-lg were compared using thermal denaturation. The near-UV CD spectrum of bovine β-lg showed typical strong troughs at 293 and 285 nm, whereas that of porcine β-lg showed a series of overlapping bands spread between 255 and 300 nm. It was found that the heat-induced structural change was irreversible for bovine β-lg B, but reversible for porcine β-lg. The results of ANS fluorescence also indicated irreversible secondary structural changes for bovine β-lg B during heat treatment, but reversible changes for porcine β-lg where the initial values were restored as a consequence of cooling. In both cases, the values at 40 °C indicated that the ANS molecules were in a more constrained and hydrophobic environment, compared with the unheated β-lg solution. The results of retinol fluorescence indicated that there was a distinct and irreversible change in the way that retinol interacted with bovine β-lg at temperatures at or above 75 °C. In the case of the bovine protein, I_{RET} (the emission intensity at λ_{max}) was less at all temperatures, whereas, for the porcine protein mixture, the I_{RET} versus temperature curve was only slightly lower than the curve obtained during the temperature-increase phase. These results suggest that retinol may bind to porcine β-lg and that there is a temperature-induced change in the character of the binding site such that λ_{max} is lowered by the heat treatment. Overall, the effect of heat treatment on the structural changes of bovine β-lg was largely irreversible, but the structural changes of porcine β-lg were relatively small and essentially reversible.

The presence of retinol or PnA in bovine β-lg gave rise to new bands in the near-

UV CD spectra, because of binding to a chiral site of β-lg. Upon heating of the protein solutions, these induced CD bands had essentially vanished at 80 °C. Subsequent cooling of the retinol/bovine β-lg mixture did not restore the spectra, but the PnA/bovine β-lg mixture allowed partial restoration of the CD bands. This suggests that, during heating, bovine β-lg loses the ability to bind retinol and PnA in a chiral environment. However, the partial restoration of the chiral structure of β-lg on cooling indicates that adding the ligand to β-lg enhances the stability of β-lg against heat denaturation. Palmitic acid did not affect the chiral environment of Trp-19 of bovine β-lg even during heat treatment of the mixture. However, the palmitic acid/bovine β-lg mixture showed considerably improved stability compared with bovine β-lg itself during heating. In contrast, when retinol/bovine β-lg and PnA/bovine β-lg mixtures were heated up to 60 °C, subsequent cooling restored the CD spectrum of the mixtures. This suggests that loss of the chiral structure of β-lg is a prerequisite for loss of the ligand-binding property of β-lg.

The addition of retinol or palmitic acid to porcine β-lg did not show any induced CD bands, which indicates that retinol and palmitic acid did not bind within a chiral site of porcine β-lg. However, PnA induced low intensity bands, which disappeared as the temperature rose to 40 °C. Frapin et al. (1993) reported that porcine β-lg binds retinol at neutral pH (pH 7.0), on the basis of tryptophan fluorescence results. Because their results depended on changes in the quenching of tryptophan fluorescence signals, they cannot be related to structural changes of the binding site of β-lg. When the mixtures of palmitic acid and porcine β-lg were heated, the band intensities at 275 and 293 nm decreased, but cooling restored the spectrum completely. Even though the heating did not cause a permanent change in the tertiary structure of porcine β-lg, palmitic acid affected the spectrum of porcine β-lg. In addition to the lack of a free thiol group in porcine β-lg, the sequence differences between the porcine and bovine β-lgs are also likely to affect the behaviour of these β-lgs during heat treatment and when binding ligands.

In Chapter 6, the urea-induced unfolding of β-lg at neutral pH (6.7) was investigated using different genetic variants, and thiol-blocked β-lg and ligand-bound proteins using near-UV and far-UV CD spectrophotometry.

The urea unfolding of the three variants of β-lg was slightly different, but not as different as during heat-induced denaturation (Manderson et al., 1999b, Fig. 6.3.1.2.). Variant C is more stable than variants A and B in urea-induced denaturation and heat-induced denaturation (Manderson, 1998) and tryptic hydrolysis (Hill et al., 1996; Nilsson et al., 2000). The differences in the denaturation curves of β-lg A, B and C can be attributed to the structural differences within the proteins which give rise to an interplay of enthalpic and entropic effects as a consequence of a salt bridge involving His-59 (β-lg C), a destabilising cavity created by the Val118Ala (A⇒B) substitution and a changed charge distribution within the CD loop caused by the Asp64Gly (A⇒B) substitution (Qin et al., 1998b; Manderson et al., 1999b).

Decreasing the pH of the protein solutions to 5.0, which is close to the isoelectric point of β-lg, increased the stabilities of all three variants of β-lg, but did not change the stability order between them (β-lg C > β-lg B ≥ β-lg A). It seems that urea, which causes protein dissociation and unfolding, exposing free thiol groups, facilitated extensive intermolecular SH-SS interchanges more easily at high pH. However, at a pH close to and lower than the p*I* of the protein, thiol oxidation and disulphide-mediated polymerisation seemed to be limited, although they did occur.

The urea-induced denaturation of thiol-blocked β-lg using DTNB did not allow simple explanations and bound DTNB gave rise to a CD signal at about 350 nm. Thiol-blocked β-lg was destabilised significantly during the urea-induced unfolding and there was also an increase in the random coil content in the secondary structure. These results are in agreement with the findings of Hoffmann and van Mil (1997) who reported that blocking the sulphydryl group caused a significant decrease in both kinetic and thermodynamic stability of the protein. McKenzie (1971) reported that, in contrast to the behaviour of unmodified bovine β-lg, but like that of thiol-modified derivatives, porcine β-lg underwent immediate changes in optical rotation when placed in urea solution at pH 3.5, 5.2 or 8.9. The results in Chapter 5, together with the results of thiol-blocked β-lg in Chapter 6, confirm that the sulphydryl group plays an important role in the unfolding of bovine β-lg. It has been reported that blocking the sulphydryl group can cause a

significant decrease in both kinetic and thermodynamic stability of the protein (Hoffmann and van Mil, 1997).

Retinol, retinoic acid and palmitic acid all stabilised the three variants of β-lg against urea denaturation. However, the results of near-UV CD were not useful for retinoic acid, probably because too many conflicting signals were produced as retinoic acid moved within the binding site of β-lg.

CHAPTER 8.

HEAT-INDUCED INTERACTIONS OF β-LACTOGLOBULIN A AND κ-CASEIN B

8.1. INTRODUCTION

It has long been recognised that heat treatment of milk above 70 °C causes denaturation of the whey proteins, some of which complex with the casein micelles. This involves the formation of a complex between β-lg and κ-CN, which determines many characteristics of milk and milk products (Singh and Creamer, 1992; Singh, 1995). The degree of interaction between β-lg and κ-CN depends on the time and temperature of heating, concentrations of the proteins, pH and the presence and concentrations of the milk salts (Singh, 1995).

Complex formation between β-lg and κ-CN has been investigated in κ-CN model systems (Zittle et al., 1962; Long et al., 1963; Sawyer et al., 1963; Purkayastha et al., 1967; Tessier et al., 1969; Euber and Brunner, 1982; Haque et al., 1987), casein micelle model systems (Smits and van Brouwershaven, 1980; Singh and Fox, 1987; Jang and Swaisgood, 1990) and milk (Tessier et al., 1969; Parnell-Clunies et al., 1988).

In many studies, the involvement of sulphydryl-disulphide interchange in the complex formation between β-lg and κ-CN was shown by treatment with thiol-blocking reagents (Sawyer et al., 1963; Purkayastha et al., 1967), by zonal electrophoresis with and without reduction of disulphide bonds (Pukayastha et al., 1967; Smits and van Brouwershaven, 1980; Parnell-Clunies et al., 1988) or by using immobilised β-lg which was exposed to κ-CN (Euber and Brunner, 1982; Jang and Swaisgood, 1990).

Sawyer (1968) suggested that the interaction between β-lg and κ-CN involved a sulphydryl-disulphide interchange mechanism but that non-covalent interactions, e.g. hydrophobic interactions, may also be involved. Doi et al. (1981) and Haque and Kinsella (1988) reported that hydrophobic bond formation was involved in the complexation in addition to sulphydryl-disulphide interactions, especially in the initial phase of the reaction.

Despite the extensive research in the area, the mechanism of this interaction is not clear. Therefore, the objective of this study was to elucidate the mechanism of interactions between β-lg and κ-CN, by examining the effects of κ-CN on the established heat-induced denaturation pathway of β-lg using the recently developed PAGE techniques of Havea et al. (1998) and Manderson et al. (1998).

The effect of heat treatment on mixtures of β-lg and κ-CN at four different ratios was investigated. To gain direct evidence for the participation of thiol groups in the complex formation, a two-dimensional (2D) gel electrophoresis technique was used. κ-CN was added to native β-lg or pre-heated β-lg, and the mixture was heated and analysed using PAGE. β-Lg A and κ-CN B were selected for this work because the A and B genetic variants of both proteins are commonly found in bovine milk and β-lg A and κ-CN B have been shown to be one of the most important factors in the fouling of UHT plants (FitzGerald and Hill, 1997; Hill et al., 1997b). To preserve the natural disulphide bonding patterns of κ-CN, it was not subjected to a reduction step at any point during purification (Section 4.2.2) and consequently it was polymeric and in a native state.

In the results from alkaline- and SDS-PAGE, the terms used to differentiate the various forms of protein that occur as a consequence of heat-induced interactions have been defined by Havea et al. (1998) and are shown in Fig. 8.1.1.1. When heated samples are analysed using alkaline-PAGE, they give protein bands that have the same mobilities as unheated β-lg; these bands are referred to as "alkaline-monomeric" β-lg. SDS-PAGE gels of the heated samples give more intense protein bands, because covalently linked aggregates appear on the SDS gels. The band that migrates at the same rate as native β-lg in the SDS-PAGE system is referred to as "SDS-monomeric" β-lg. Analysis of heated samples by SDS-PAGE in the presence of a reducing reagent gives an even more intense band. This is called "total reducible" β-lg. Thus, the total reducible β-lg is made up of several species: alkaline-monomeric β-lg, SDS-monomeric β-lg that is not native β-lg, and many aggregates that are held together by intermolecular disulphide bonds.

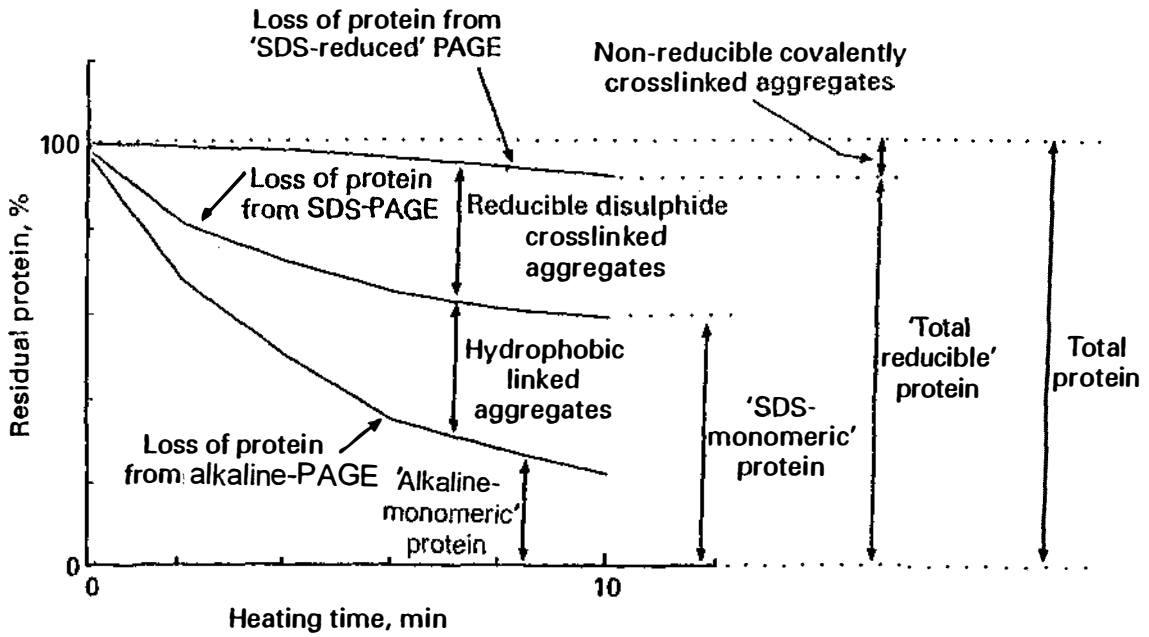

Figure 8.1.1.1. Schematic representation of the relationship between the relative amounts of different forms of proteins (e.g. "alkaline-monomeric", "SDS-monomeric", "total reducible") in a heated mixture of β-lg and κ-CN (Havea et al., 1998).

8.2. EXPERIMENTAL PROTOCOL

Heat treatment of samples

Samples of β-lg or κ-CN were dialysed against phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) and adjusted to pH 6.7 and to appropriate protein concentrations. Aliquots of β-lg A and/or κ-CN B were heated in microcentrifuge tubes at 80 °C for various times between 2.5 and 60 min in a Neslab model RTE-100 temperature-controlled water bath (Neslab Instruments Inc., Newington, NH, USA). The time taken for the samples to attain 80 °C was 15 s. Tubes and samples were removed after heating and immediately placed in ice water for 5 min and left at room temperature for 2 h. The above sets of experiments were carried out in duplicate.

Four ratios of sample mixtures between β-lg A and κ-CN B were prepared by mixing an equal volume of each protein solution: 2 mg/mL β-lg:1 mg/mL κ-CN = 2:1; 2 mg/mL β-lg:2 mg/mL κ-CN = 2:2; 2 mg/mL β-lg:3 mg/mL κ-CN = 2:3; 2 mg/mL β-lg:4 mg/mL κ-CN = 2:4. For comparison, 2 mg/mL of β-lg A and 2 mg/mL of κ-

CN B were mixed with the same volume of phosphate buffer, respectively, and then heated individually under the same conditions.

To detect and characterise the appearance and disappearance of the intermediate aggregates formed between β-lg and κ-CN more clearly, pre-heated β-lg A was used for a further study. β-Lg A solution (2 mg/mL), that had been heated for 5, 10, 15 and 30 min at 80 °C, cooled to room temperature and held for 2 h, was then mixed with κ-CN B solution (2 mg/mL β-lg A:2 mg/mL κ-CN B = 2:2, w/w) and reheated immediately for various times at 80 °C.

Solutions set aside for alkaline-PAGE and SDS-PAGE analysis were diluted with appropriate sample buffer and PAGE analyses carried out as described in Sections 3.2.1.2 and 3.2.1.1, respectively.

8.3. RESULTS AND DISCUSSION

8.3.1. Structural changes during heating and cooling of β-lactoglobulin A and κ-casein B

The changes in the near-UV CD spectrum of bovine β-lg A in pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) as the protein solution was heated (A) and then cooled (B) were recorded at several temperatures and are shown in Fig. 8.3.1.1. For the solution heat treated at 60 °C, the CD spectrum did not change significantly compared with that of the unheated solution. However, as the heat treatment temperature was increased above 70 °C, the intensities of both the 285 and 293 nm tryptophan CD bands decreased to about half of the original intensities, although there was no discernible wavelength shift (Fig. 8.3.1.1.A). The intensity of the main trough at 293 nm was diminished to almost zero at 80 °C. Most of the other bands, which were clearly observed in unheated bovine β-lg, were also diminished. These results are consistent with earlier observations in this thesis with the β-lg B variant (Fig. 5.3.1.2). Decreasing temperature again did not cause much change in the CD spectrum of β-lg A (Fig. 8.3.1.1.B).

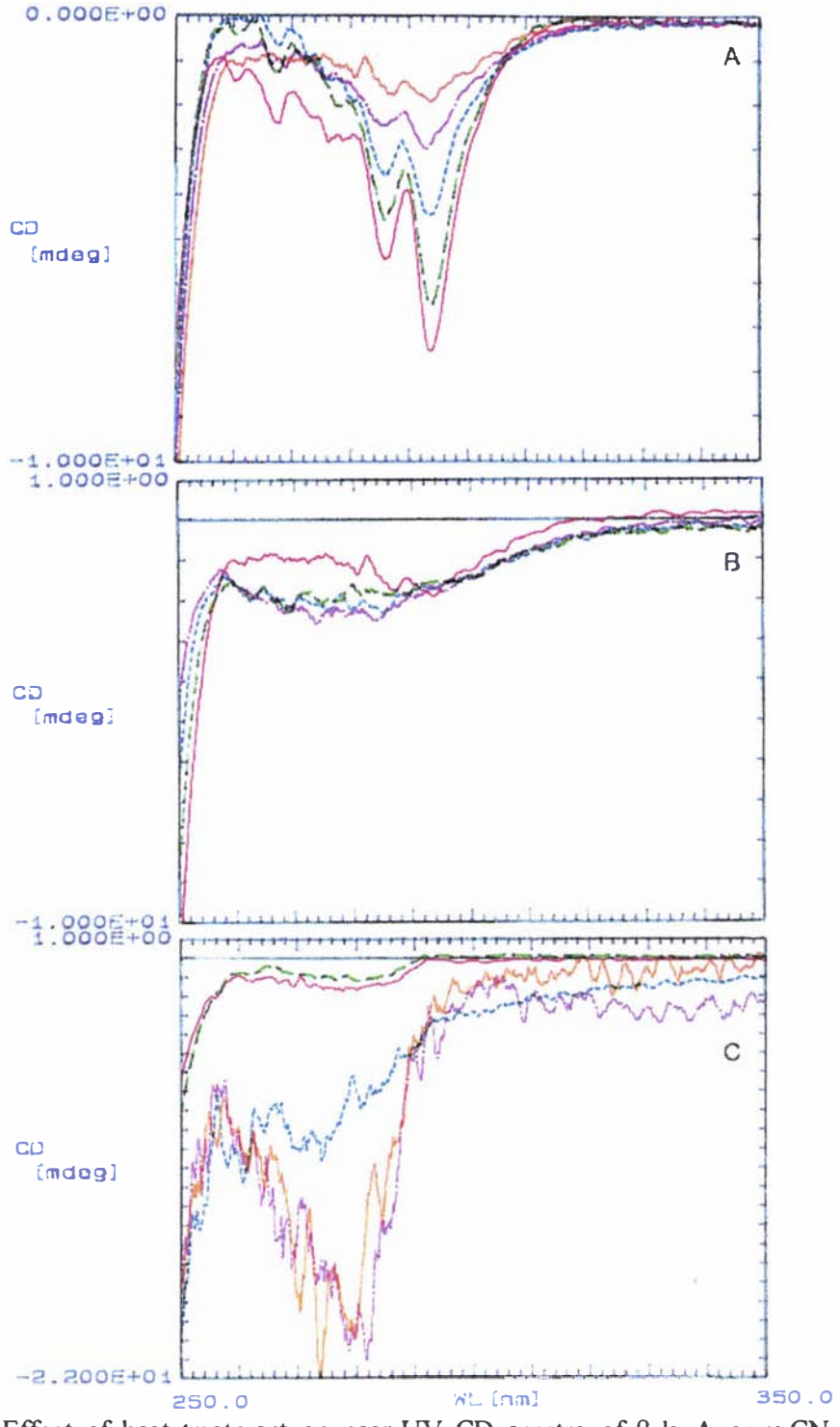

Figure 8.3.1.1. Effect of heat treatment on near-UV CD spectra of β-lg A or κ-CN B at a concentration of 1.0 mg/mL in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at various temperatures.

A. β-Lg A at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

B. β-Lg A at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre).

C. κ-CN B at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

As the heating temperature was increased up to 40 °C, the CD spectrum of κ-CN B in pH 6.7 phosphate buffer did not change significantly compared with that obtained using an unheated sample. However, as the temperature was increased above 60 °C, a broad deep trough centred at 270 nm started to develop, and increased with further heat treatment (Fig. 8.3.1.1.C). It has been reported that disulphide bonds give broad bands that seem to be related to the dihedral angle of the disulphide bond (Woody, 1973). Normally this angle is approximately 90° and gives rise to a broad band near 260 nm, but changes in this angle result in splitting into two broad bands at higher and lower wavelengths (Woody, 1973, 1995; Strickland, 1974; Kahn, 1979). Kuwajima et al. (1996) suggested that the shorter wavelength band is not distinguishable from the intense peptide and amide bond bands, whereas the long wavelength band appears as a broad band at 270-280 nm. Manderson et al. (1999b) also reported the intensity increase in CD at 270 nm, an index of significant alteration to the disulphide bond dihedral angle, with increasing temperature of β-lg solutions (2.9-5.4 mg/mL). Further experimental support arises from similar CD spectra obtained from heat-treated β-lg solutions at high concentrations (50-60 mg/mL), which probably involved a high proportion of sulphydryl-disulphide interactions between the molecules (G. A. Manderson and L. K. Creamer, 1998, unpublished result). Pure κ-CN has also been reported to aggregate with heat treatment to form high molecular weight polymers, primarily via sulphydryl-disulphide interactions (Groves et al., 1998). Consequently, the increase in band intensity at 270 nm in Fig. 8.3.1.1.C was probably caused by a reorganisation of the disulphide bonding within the large aggregates and/or intermolecular sulphydryl-disulphide interactions of κ-CN.

However, during cooling, the trough diminished and a white precipitate developed; therefore the spectrum became noisy and no further measurements were made.

In Fig. 8.3.1.2.A, the effect of mixing the two protein solutions is shown. The typical strong troughs observed at 293 and 285 nm in the near-UV CD spectrum of β-lg, which suggest that the tryptophan side chain is located in a chiral environment, were reduced and the overall spectrum was shifted. The reduction in the band intensity at 293 and 285 nm in the mixture can be explained by the dilution effect caused by the κ-CN solution. The near-UV CD band for κ-CN B centred at 270 nm disappeared.

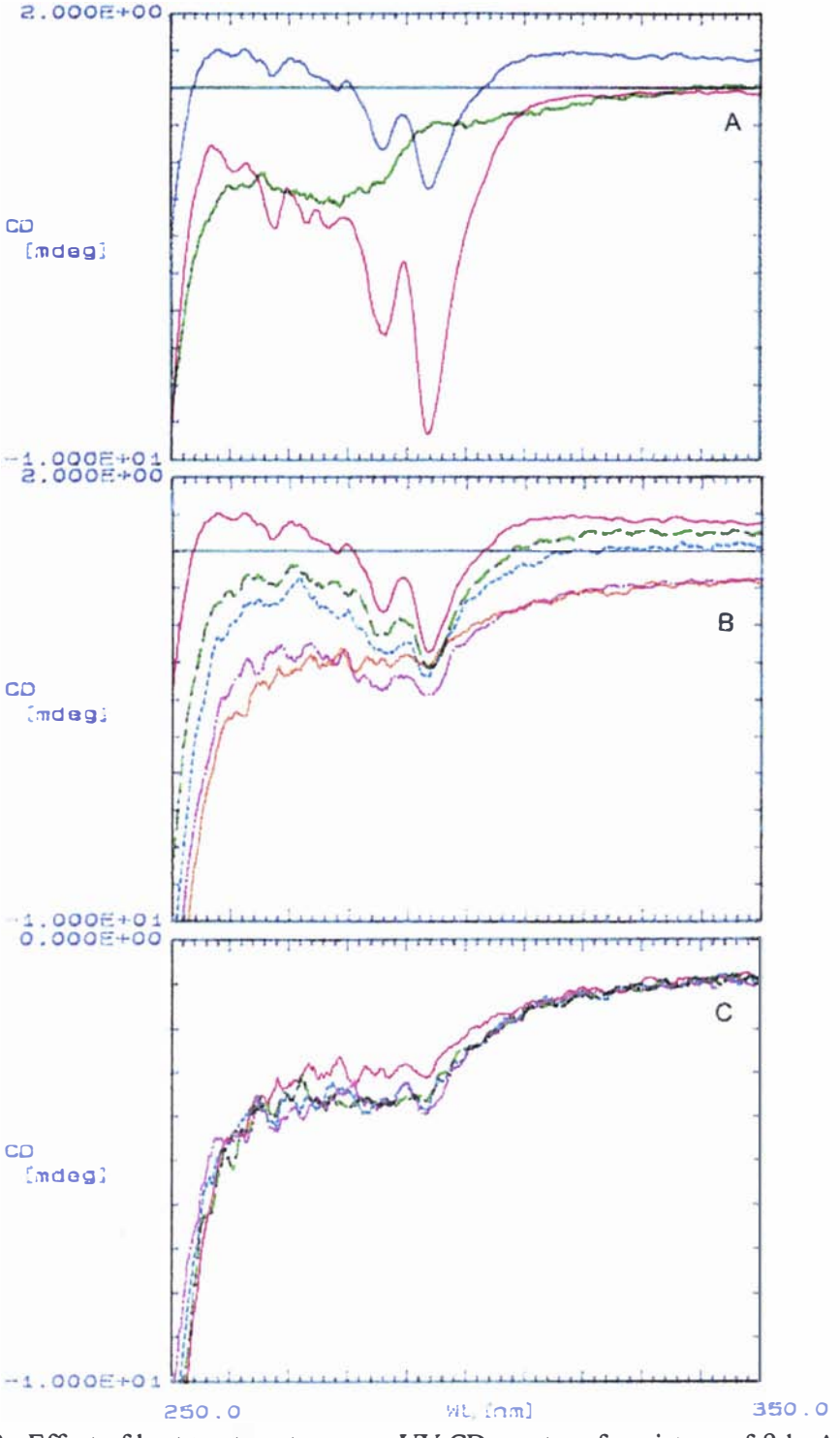

Figure 8.3.1.2. Effect of heat treatment on near-UV CD spectra of a mixture of β-lg A and κ-CN B at a total protein concentration of 1.0 mg/mL (1:1, w/w) in pH 6.7 phosphate buffer (26 mM, with 68 mM NaCl) at various temperatures.

A. β-Lg A (red), κ-CN B (green) and mixture of β-lg A and κ-CN B (purple) at 20 °C.

B. Mixture of β-lg A and κ-CN B at 20 °C (red solid), heated to 40 °C (green dashed), 60 °C (blue dotted), 70 °C (purple centre) and 80 °C (orange solid).

C. Mixture of β-lg A and κ-CN B at 80 °C (red solid) and then cooled to 60 °C (green dashed), 40 °C (blue dotted) and 20 °C (purple centre).

The changes in the near-UV CD spectrum of a mixture of β-lg A and κ-CN B in pH 6.7 phosphate buffer as the protein solution was heated (B) and then cooled (C) were recorded at several temperatures and are shown in Fig. 8.3.1.2. As the temperature was increased, the CD spectrum of the mixture became more negative compared with that of the unheated solution (Fig. 8.3.1.2.B). As the temperature was increased above 70 °C, the intensities of both the 285 and 293 nm tryptophan CD bands diminished and the band intensity at 270 nm increased. Cooling the protein solution did not cause any change in the CD spectrum (Fig. 8.3.1.2.C). From these results, it appears that the heat-induced spectral changes for the mixture of β-lg A and κ-CN B were essentially caused by irreversible disulphide bond changes that involved κ-CN, and the chiral environment of Trp-19 of β-lg essentially disappeared, cf. Fig. 8.3.1.1.B for β-lg A alone and Fig. 8.3.1.2.C for β-lg A and κ-CN B mixture.

8.3.2. Effect of heat treatment on β-lactoglobulin A and κ-casein B

8.3.2.1. Effect of heat treatment on β-lactoglobulin A

Alkaline-PAGE

The alkaline-PAGE gel of heat-treated β-lg A in pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) is shown in Fig. 8.3.2.1.A. At pH 8.8, the pH at which alkaline-PAGE gels are run, native dimers do not form and β-lg denatures (Groves et al., 1951; Casal et al., 1988). Therefore, only "native-like" alkaline-monomeric β-lg was observed.

As the heating time at 80 °C increased, β-lg A aggregated to give rise to a number of bands and diffuse regions of stained protein (Fig. 8.3.2.1.A). Lane 2 in Fig. 8.3.2.1.A shows a series of bands in the resolving gel and these are referred to as alkaline-monomeric β-lg (m), non-native monomer (nm), dimer (d), trimer (t) and tetramer (tet) bands, which have also previously been characterised by Manderson et al. (1998) under similar conditions.

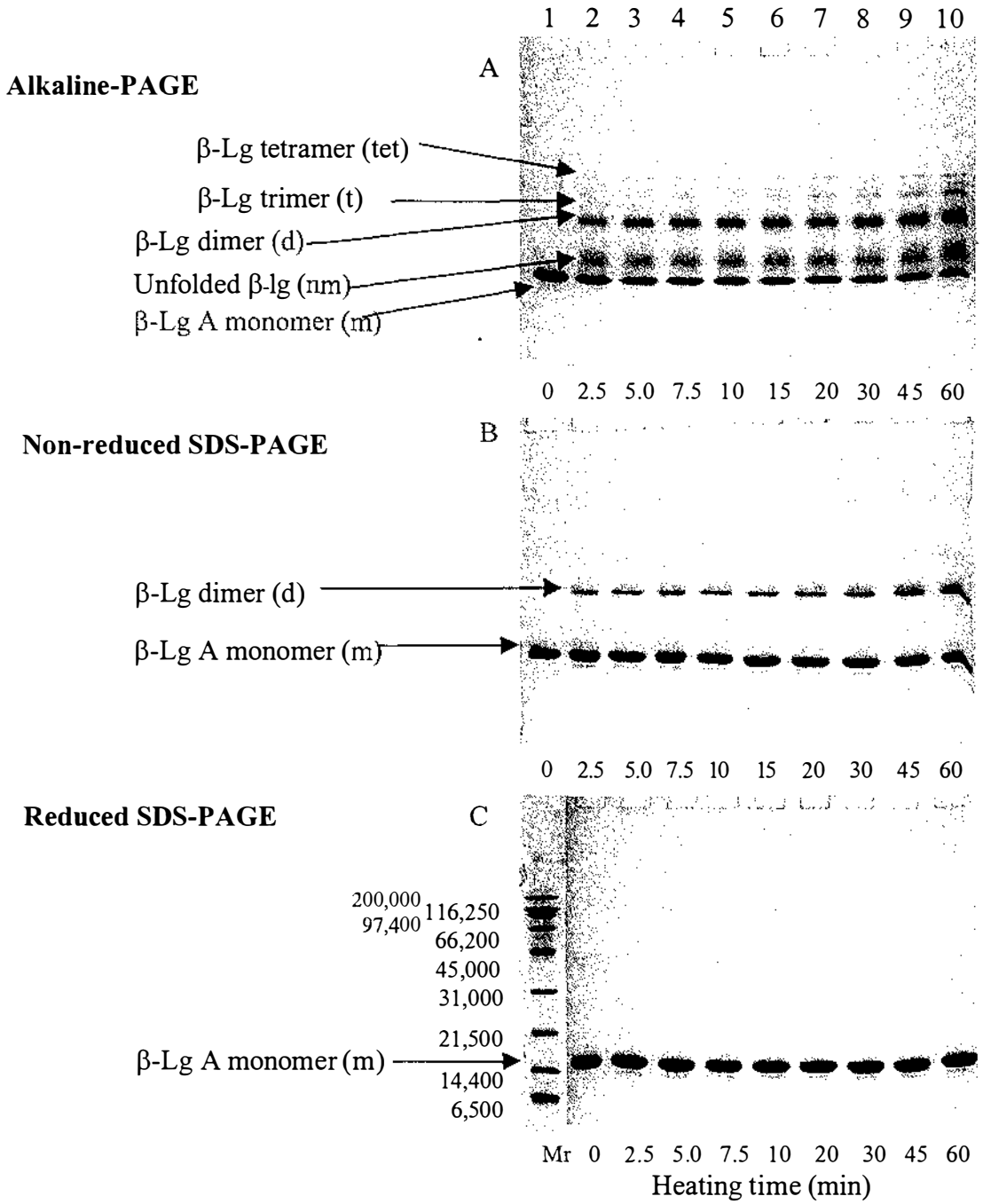

Figure 8.3.2.1. Effect of heat treatment on β-lg A. Electrophoretic patterns of (A) alkaline- and (B) non-reduced SDS-PAGE of β-lg A (1 mg/mL) heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. (C) Reduced SDS-PAGE of β-lg A (1 mg/mL) heated at 80 °C for: lane 1, an attached M_w standard; lane 2, 0 min; lane 3, 2.5 min; lane 4, 5.0 min; lane 5, 7.5 min; lane 6, 10 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min.

SDS-PAGE

The non-reduced SDS-PAGE patterns of heat-treated β-lg A in pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) are shown in Fig. 8.3.2.1.B. As the heating time at 80 °C increased, β-lg A gave rise to a number of bands (Fig. 8.3.2.1.B). In non-reduced SDS-PAGE, β-lg monomer (m) and dimer (d) bands were present in the resolving gel, in order of decreasing mobility. The non-native monomer bands that were observed between the monomer and dimer bands in the alkaline-PAGE gel (Fig. 8.3.2.1.A) could not be seen in the SDS-PAGE gel (Fig. 8.3.2.1.B). It has been reported that an appreciable proportion of the partially unfolded monomeric β-lg A (non-native monomer), which resolved in the alkaline-PAGE gel, was stabilised by non-native intramolecular disulpide bonds and was characterised as a monomer band in SDS-PAGE (Manderson et al., 1998). In the reduced SDS-PAGE gel, all polymeric β-lg A bands resolved as monomeric β-lg A bands (Fig. 8.3.2.1.C).

8.3.2.2. Effect of heat treatment on κ-casein B

Alkaline-PAGE

The alkaline-PAGE gel for heat-treated κ-CN B in pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) did not show any bands in the resolving gel as the heating time at 80 °C increased (Fig. 8.3.2.2.A). It seems that native κ-CN stayed as disulphide-linked polymers (Groves et al., 1991, 1998), as all of the stained protein was present on the top of the resolving and stacking gels.

SDS-PAGE

The non-reduced SDS-PAGE gel for heat-treated κ-CN B at pH 6.7 also did not show any changes as the heating time at 80 °C increased (Fig. 8.3.2.2.B), which was similar to the result for alkaline-PAGE analysis (Fig. 8.3.2.2.A). Whereas the reduced SDS-PAGE gel for heat-treated κ-CN B at pH 6.7 (Fig. 8.3.2.2.C) showed monomeric κ-CN B. Also traces of BSA, α_{s1}-, α_{s2}-caseins and para-κ-CN were present in the sample resolved using reduced SDS-PAGE.

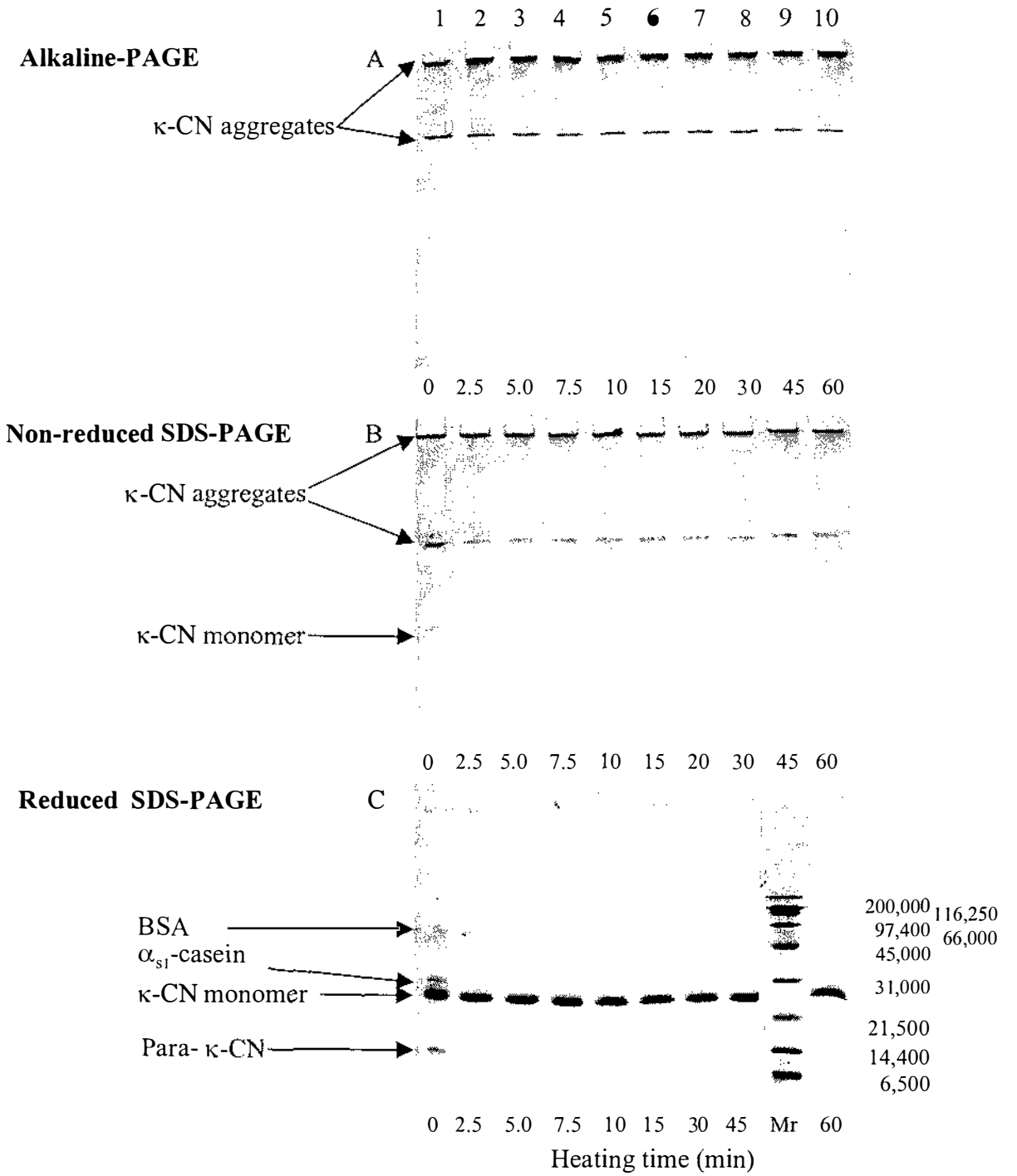

Figure 8.3.2.2. Effect of heat treatment on κ-CN B. Electrophoretic patterns of (A) alkaline- and (B) non-reduced SDS-PAGE of κ-CN B (1 mg/mL) heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. (C) Reduced SDS-PAGE of κ-CN B (1 mg/mL) heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 30 min; lane 8, 45 min; lane 9, an attached M_w standard; lane 10, 60 min.

In this study, no intermediate-sized aggregates were observed in non-reduced SDS-PAGE (Fig. 8.3.2.2.B) and in reduced SDS-PAGE (Fig. 8.3.2.2.C), the intermediate-sized aggregates resolved as monomeric κ-CN B. The result of this study is in agreement with that of Groves et al. (1998) who reported that heating purified κ-CN samples caused a significant increase in the proportion of high molecular weight polymers, as seen by electrophoresis and by analytical ultracentrifugation, but an apparent decrease in the polymeric distribution. Also they reported that polymerisation appeared to be driven by either one or both of the two cysteines in κ-CN.

8.3.3. Effect of heat treatment on β-lactoglobulin A and κ-casein B mixtures

8.3.3.1. Alkaline-PAGE

The alkaline-PAGE patterns for the mixture of β-lg A and κ-CN B in pH 6.7 phosphate buffer heated at 80 °C for up to 60 min are shown in Fig. 8.3.3.1. Lane 1 shows the alkaline-PAGE gel of the β-lg A and κ-CN B mixture before heating. The mixture was composed of alkaline-monomeric β-lg A and κ-CN B aggregates, which could not enter the resolving and/or stacking gel. Using SDS-PAGE without reducing agents, Groves et al. (1991) and Farrell et al. (1998) reported that the purified form of κ-CN occurs as complexes with a molecular weight range from monomer to octomer and above. Vreeman et al. (1981) reported that the self-association of κ-CN is somewhat insensitive to temperature and that the complex has an average aggregation number of about 30 and a radius of 11 nm.

The intensities of the monomeric β-lg A bands decreased as the heating time increased, which occurred at all four different ratios of the β-lg A and κ-CN B mixtures (Fig. 8.3.3.1). At a β-lg A:κ-CN B ratio of 2:1, heat treatment resulted in a series of bands, i.e. unfolded β-lg, β-lg dimer, etc. When the sample contained higher proportions of κ-CN, monomeric β-lg A and these bands disappeared rapidly (Fig. 8.3.3.1.D). As the ratio of β-lg A to κ-CN B decreased, high molecular weight aggregates, which could not enter the resolving and/or stacking gels, were formed more rapidly (Fig. 8.3.3.1.A-D).

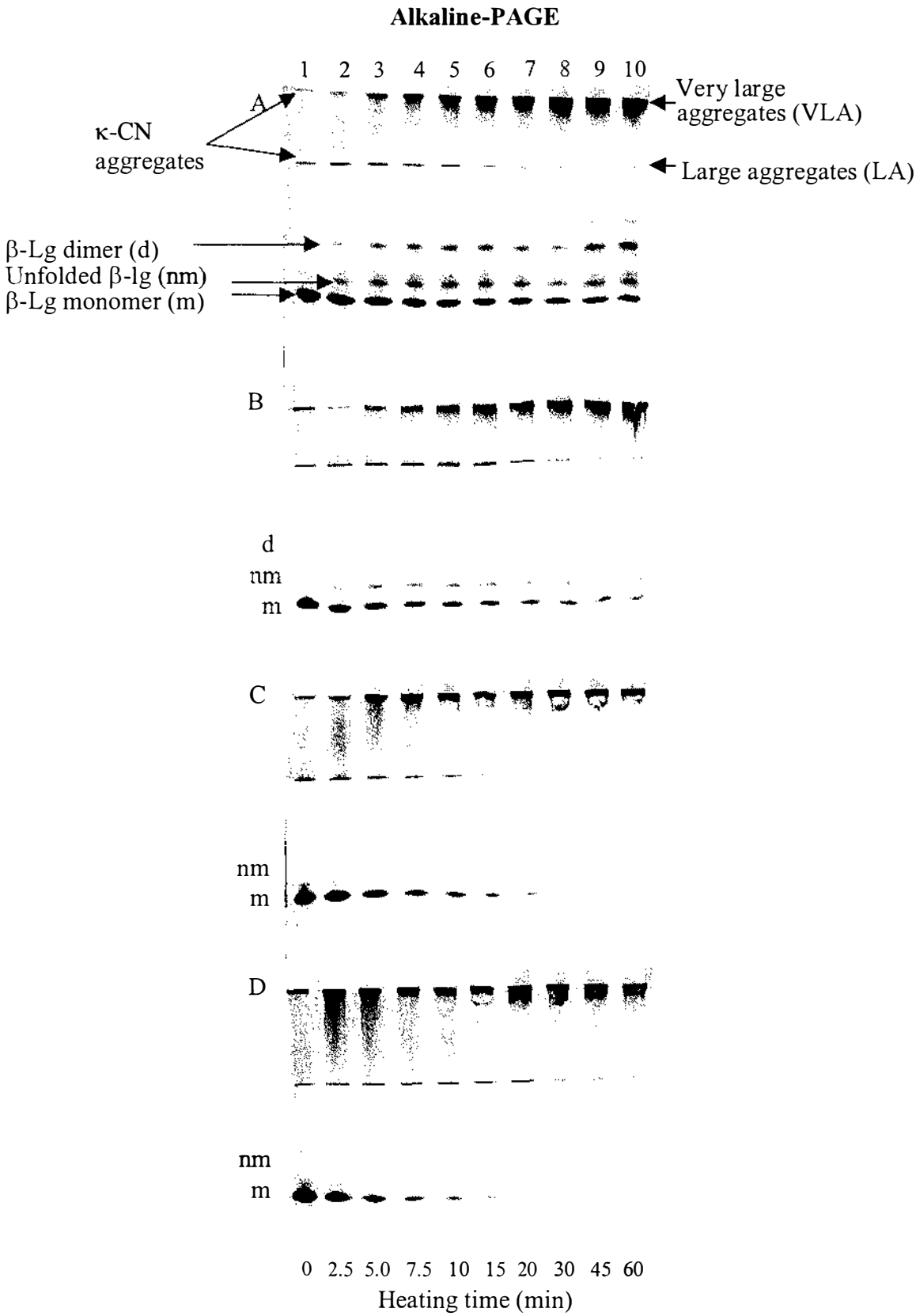

Figure 8.3.3.1. Alkaline-PAGE patterns of β-lg A (2 mg/mL) and κ-CN B mixtures heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. (A) 2:1, (B) 2:2, (C) 2:3 and (D) 2:4 (β-lg A:κ-CN B, w/w).

By comparison of these patterns (Fig. 8.3.3.1) with those shown in Figs 8.3.2.1.A and 8.3.2.2.A, which showed the series of protein bands produced by β-lg A and κ-CN B, respectively, it was possible to evaluate the effect of β-lg A on κ-CN B aggregation and vice versa. As the heating time increased, alkaline-PAGE of β-lg A (Fig. 8.3.2.1.A) showed increasing band intensities of several β-lg polymers in the resolving gel, whereas the β-lg A/κ-CN B mixtures did not show significant increases in intermediate-sized aggregates, although the decrease in the alkaline-monomeric β-lg A band still occurred. This suggests rapid aggregation between β-lg A and κ-CN B, which quickly surpassed the stage of intermediate-sized aggregates and appeared as large aggregates shown on the top of the alkaline-PAGE gel.

8.3.3.2. SDS-PAGE

When the mixtures of β-lg A and κ-CN B were analysed by non-reduced SDS-PAGE, a band corresponding to SDS-monomeric β-lg A was observed, and κ-CN B showed some bands at the top of the resolving gel as well as material on the top of the stacking gel (Fig. 8.3.3.2). Fewer bands (only disulphide-linked aggregates) were observed in the non-reduced SDS-PAGE gel (Fig. 8.3.3.2) than in the alkaline-PAGE gel (Fig. 8.3.3.1) during heating at 80 °C. The decreases in the band intensities of SDS-monomeric β-lg A, observed in the non-reduced SDS-PAGE patterns with heating time, were similar to the results obtained using alkaline-PAGE. The SDS-monomeric β-lg A band disappeared more rapidly from mixtures containing a higher concentration of κ-CN B (Fig. 8.3.3.2.D) and more aggregates were observed on the top of the gel. As the heating time increased, β-lg and/or κ-CN formed large aggregates, via disulphide bonds, which could not pass through the stacking gel. SDS-PAGE confirmed the accelerated aggregate formation between β-lg A and κ-CN B, as compared with β-lg heated alone under the same conditions (Fig. 8.3.2.1.B).

In the samples with the lowest concentration of κ-CN B, the band intensity of SDS-dimeric β-lg A increased as the heating time increased (Fig. 8.3.3.2.A). In contrast, the band intensity of SDS-dimeric β-lg A was lower when the samples contained a higher proportion of κ-CN B (Fig. 8.3.3.2.B-D).

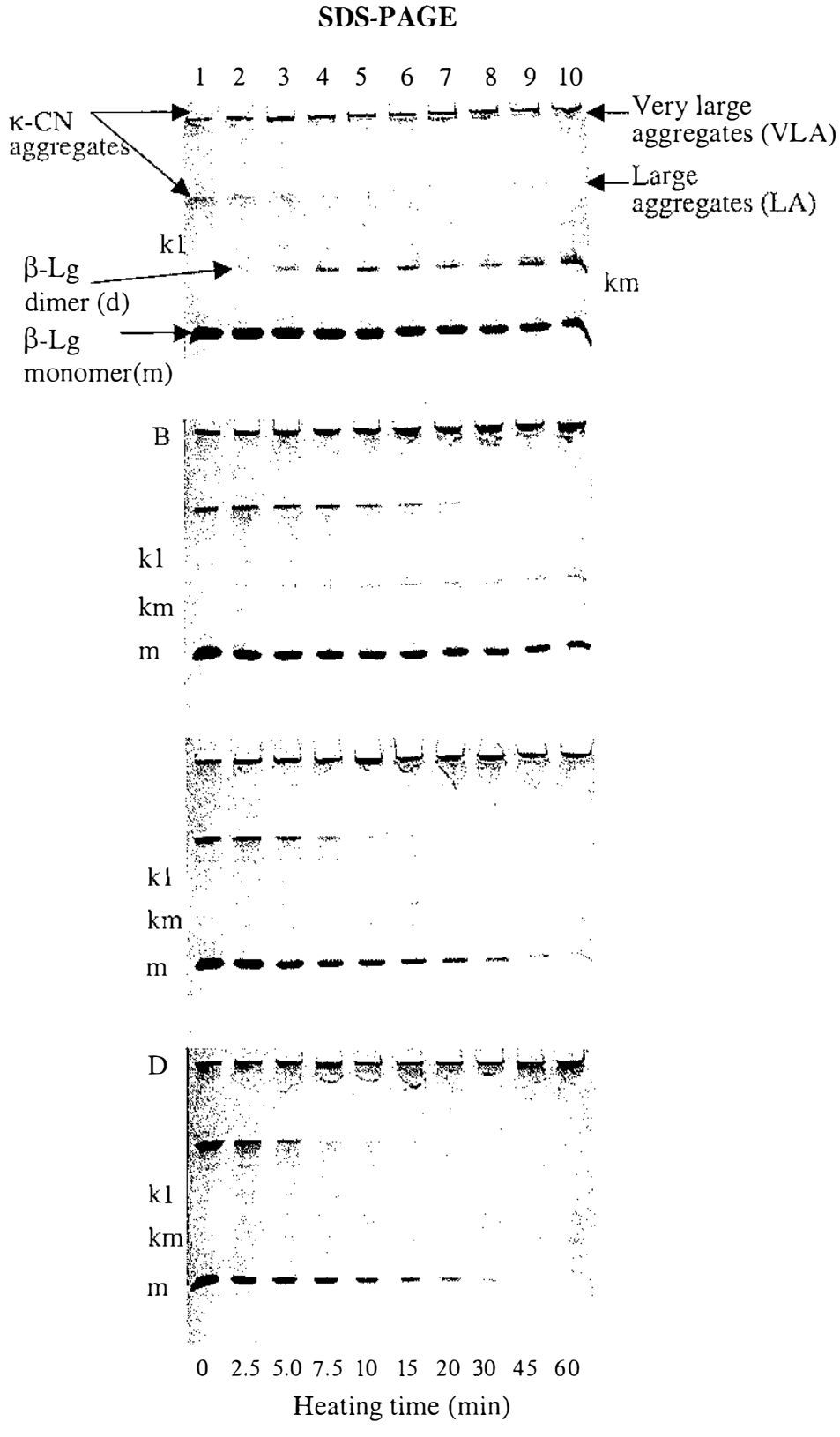

Figure 8.3.3.2. SDS-PAGE patterns of β-lg A (2 mg/mL) and κ-CN B mixtures heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. (A) 2:1, (B) 2:2, (C) 2:3 and (D) 2:4 (β-lg A:κ-CN B, w/w).

In the SDS-PAGE patterns, two faint bands labelled as km and k1, respectively, were observed (Fig. 8.3.3.2); these bands were not seen in the alkaline-PAGE patterns (Fig. 8.3.3.1) nor were they seen on unheated mixtures at high concentration (Fig. 8.3.3.2.D, lane 1). The band intensity of k1 diminished rapidly as the heating time increased, but the km band was observed in all heated samples.

8.3.3.3. 2D-PAGE

To determine the composition of the intermediate aggregate bands formed during heating at 80 °C, a 2D-PAGE technique was used. The aggregates observed in one-dimensional non-reduced SDS-PAGE were characterised (Fig. 8.3.3.2) using 2D-PAGE, which used reduced SDS-PAGE two-dimensionally, as shown in Fig. 8.3.3.3 (non-reduced SDS and then reduced SDS). The mixture of β-lg A and κ-CN B (1:1, w/w) was heated at 80 °C for 10 min.

The SDS-PAGE in the presence of reducing agent carried out in lane 1 showed monomeric β-lg A and monomeric κ-CN B bands (Fig. 8.3.3.3). After reduction, the material caught on the top of the stacking or resolving gels in SDS-PAGE was resolved and was determined to be β-lg A and κ-CN B aggregates linked by disulphide bonds.

The bands discernible in 1D SDS-PAGE (Fig. 8.3.3.2) were also resolved. The bands km and k1 were found to be κ-CN B monomer and the 1:1 β-lg A/κ-CN B disulphide-linked aggregate, respectively (Fig. 8.3.3.3).

In many studies, involvement of sulphydryl-disulphide interchange in complex formation was inferred from the observed inhibition of β-lg complexation with κ-CN by treatment with thiol reagent (Sawyer et al., 1963; Purkayastha et al., 1967). However, structural changes resulting from disulphide bonding could prevent the interaction between β-lg and κ-CN or thiol modification *per se* could alter the site of interaction. Therefore, more direct evidence for intermolecular disulphide bond formation between the two proteins was sought and was obtained by zonal electrophoresis with and without reduction of the disulphide bond (Purkayastha et al., 1967; Smits and van Brouwershaven, 1980; Parnell-Clunies et al., 1988). However, it was difficult to distinguish aggregated β-lg from β-lg/κ-CN complex in the resulting complicated electropherograms.

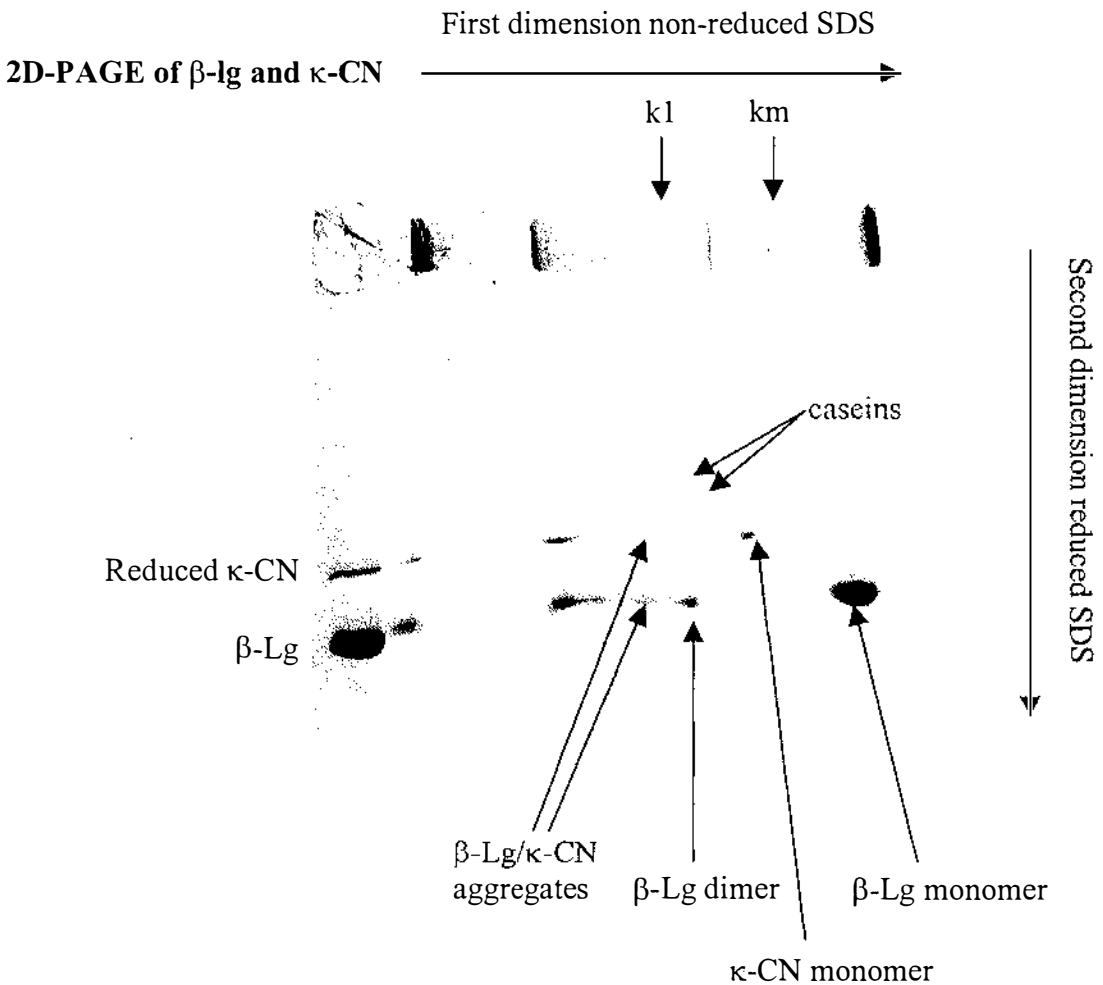

Figure 8.3.3.3. 2D electrophoretic pattern (non-reduced and then reduced SDS) of a β-lg A and κ-CN B mixture (2:2, w/w) heated at 80 °C for 10 min.

Euber and Brunner (1982) provided evidence of β-lg/κ-CN intermolecular disulphide bond formation using thermally denatured immobilised β-lg and κ-CN solutions. The κ-CN bound to immobilised β-lg was released only by reduction of the disulphide bond. Jang and Swaisgood (1990) used the same techniques, but used κ-CN in the casein micelles instead of the purified form of κ-CN, to prove that an intermolecular disulphide complex can form between β-lg and κ-CN in casein micelles under conditions similar to those of milk. Furthermore, from the results of the 2D-PAGE in this study (Fig. 8.3.3.3), more direct evidence for heat-induced intermolecular disulphide bond formation between β-lg and κ-CN has been established.

8.3.3.4. Quantitative analysis

The changes in the concentration of monomeric and dimeric species of β-lg A with heating time at 80 °C were determined by measuring the intensities of the appropriate bands on the gels, shown in Figs 8.3.2.1, 8.3.2.2, 8.3.3.1 and 8.3.3.2, using a laser densitometer.

The effect of increasing the heating time at 80 °C on the band intensities of monomeric and dimeric β-lg A in the mixtures of β-lg A and κ-CN B on alkaline- or SDS-PAGE gels is shown in Fig. 8.3.3.4. The band intensities of both alkaline-monomeric β-lg A (Fig. 8.3.3.4.A) and SDS-monomeric β-lg A (Fig. 8.3.3.4.C) decreased rapidly for the first 10 min, but the decreases in the SDS-monomeric β-lg A intensities were more gradual. The difference in the band intensity values between alkaline- and SDS-PAGE (without reducing reagent) corresponds to the concentration of non-covalently linked aggregates in the heat-treated β-lg samples (Fig. 8.1.1.1). Furthermore, the difference in the band intensity values of SDS-PAGE with and without reducing reagents corresponds to the concentration of disulphide-linked aggregates. Different PAGE techniques have been used previously by Havea et al. (1998) and Manderson et al. (1998) to study the formation of disulphide-linked and non-covalently linked aggregates.

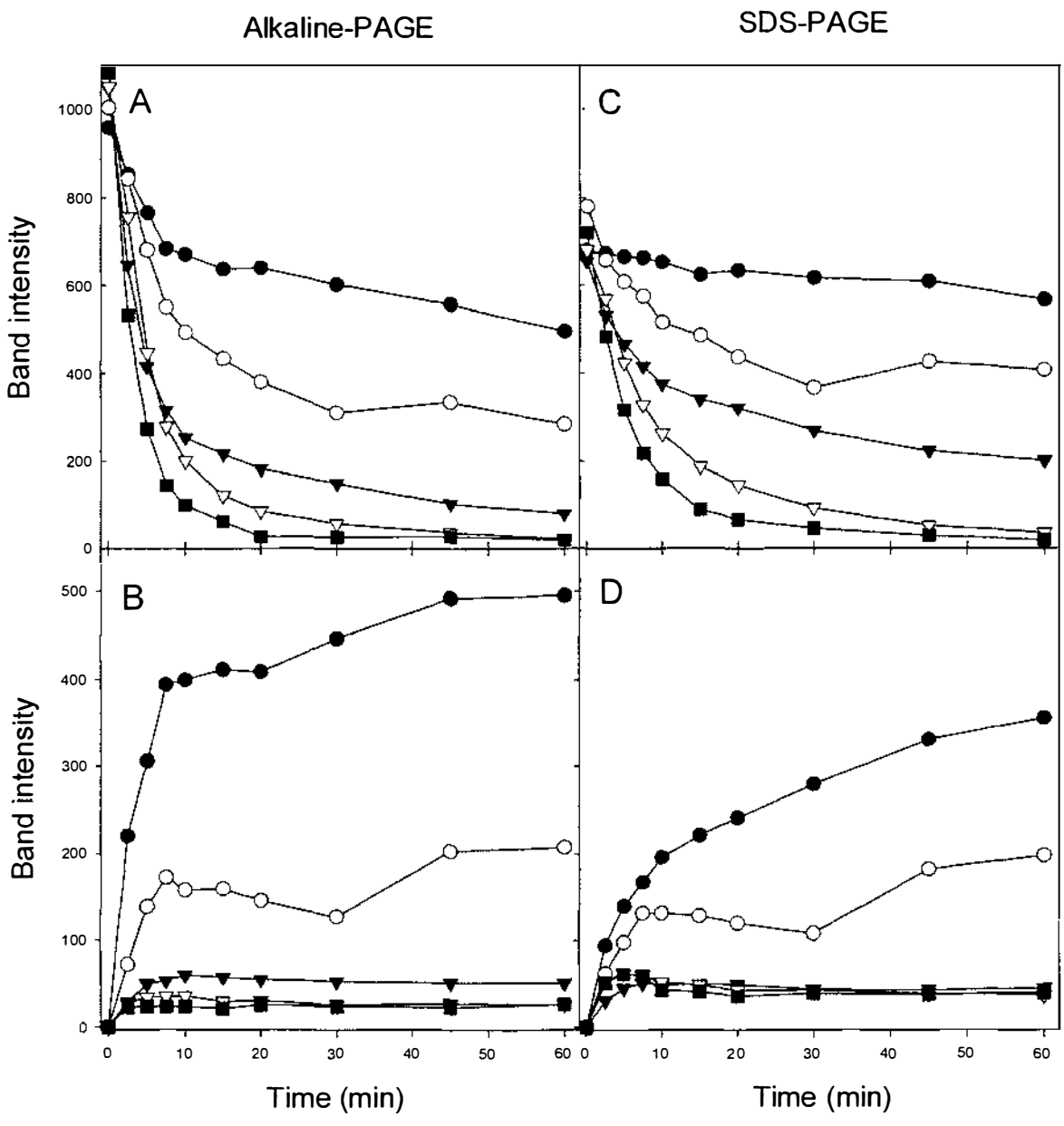

Figure 8.3.3.4. Changes in the quantities of (A) alkaline-monomeric, (B) alkaline-dimeric, (C) SDS-monomeric and (D) SDS-dimeric β-lg obtained from heat-treated mixtures with different ratios β-lg:κ-CN (w/w): 2 mg/mL β-lg:0 mg/mL κ-CN = 2:0 (●), 2 mg/ml β-lg:1 mg/mL κ-CN = 2:1 (○), 2 mg/ml β-lg:2 mg/mL κ-CN = 2:2 (▼), 2 mg/mL β-lg:3 mg/mL κ-CN = 2:3 (▽) and 2 mg/mL β-lg:4 mg/mL κ-CN = 2:4 (■).

The intensities of both alkaline- and SDS-dimeric β-lg A bands increased for the first 10 min at 80 °C, but slightly decreased or remained relatively constant thereafter when samples containing κ-CN (Fig. 8.3.3.4.B, D). The initial rapid decrease in monomeric β-lg band intensity in the alkaline and SDS gels from the mixtures suggested that κ-CN acts as a catalyst in the early stages of β-lg denaturation. In the case of samples containing lower κ-CN content, the denaturation of β-lg seemed to precede aggregate formation between β-lg and κ-CN, as more polymeric β-lg bands were observed (Figs 8.3.3.1 and 8.3.3.2). Sawyer (1969) suggested that the primary thermodenaturation of β-lg itself involves disulphide aggregation and it may be that only the denatured form is capable of association with κ-CN.

As the proportion of κ-CN B increased, the decreases in the monomeric and dimeric β-lg A bands accelerated (Fig. 8.3.3.4). Tessier et al. (1969) and Long et al. (1963) found that the content of β-lg in the associated complex increased with increasing β-lg:κ-CN concentration ratios and the maximum ratios reported were 2.2:1 and 3:1 in studies with pure β-lg and κ-CN. Haque et al. (1987) reported that an equimolar mixture of β-lg and κ-CN interacted to form a β-lg/κ-CN complex apparently composed of three molecules of β-lg and one molecule of κ-CN at 70 °C.

On the other hand, Dalgleish et al. (1997) reported that the quantity of β-lg associated with the casein micelles is limited in the temperature range 75-90 °C (β-lg:κ-CN (w/w) < 0.6) in skim milk. The low molar ratio (< 1) is in agreement with the knowledge that κ-CN itself is likely to be in the form of disulphide-linked oligomers on the micellar surface (Rasmussen et al., 1992; Farrell et al., 1998). This also implies that there is sufficient β-lg present in milk to saturate the available sites for disulphide bond formation between β-lg and casein micelles (with κ-CN and α_{s2}-casein) and that not all of the casein is available for this reaction. Alternatively, non-disulphide interactions are also possible (Doi et al., 1981), but there is strong evidence for the final interaction being via disulphide bonds (Jang and Swaisgood, 1990). The state of self-association of κ-CN, which has unique disulphide bonding pattern (Groves et al. 1998), may play an important role in the interaction. Alterations in pH, concentration, ionic strength or temperature could bring about changes in the state of self-association of κ-CN.

8.3.4. Heat-induced interaction between pre-heated β-lactoglobulin A and native κ-casein B

Heat treatment of a mixture of unheated β-lg A and unheated κ-CN B at 80 °C for up to 60 min suggested that the monomeric β-lg→ unfolded β-lg → dimeric β-lg --→ trimeric β-lg disulphide aggregation may be partially displaced by a monomeric β-lg --→ unfolded β-lg ⇒ β-lg-ss-κ-CN reaction (Section 8.3.3). To observe intermediate polymers more closely, pre-heated β-lg A (at 80 °C for 5, 10, 15 and 30 min) was mixed with κ-CN B and the mixtures were heated again. Alkaline- and SDS-PAGE techniques were used to observe the band intensity changes during heating.

8.3.4.1. Alkaline-PAGE

The alkaline-PAGE patterns of mixtures of pre-heated β-lg A and κ-CN B are shown in Fig. 8.3.4.1 (2 mg/mL β-lg A:2 mg/mL κ-CN B = 2:2, w/w).

Lane 2 in Figs 8.3.4.1.A, B, C and D contained β-lg A that had been pre-heated at 80 °C for 5, 10, 15 and 30 min, respectively. As the pre-heating time for β-lg A increased, more polymeric β-lg A bands, including non-native monomer (nm), dimer (d), trimer (t) and tetramer (tet) etc., appeared (lane 2 in Fig. 8.3.4.1.A-D). Two intense bands were observed between the interface of the stacking and resolving gels and the bottom of the sample loading cell. These bands were assumed to represent the disulphide-linked aggregates with M_w values greater than 200,000 Da (the M_w cut-off of the resolving gel) and 500,000 Da (the M_w cut-off of the stacking gel), respectively. All aggregates with M_w values greater than approximately 200,000 Da are subsequently referred to as large aggregates (LA) and those with M_w values greater than approximately 500,000 Da are referred to as very large aggregates (VLA).

However, upon mixing with κ-CN B and reheating at 80 °C, all the polymeric bands of β-lg A diminished rapidly. As the reheating time of the mixture of pre-heated β-lg A and κ-CN B increased, the intensity of alkaline-monomeric and dimeric β-lg bands decreased, as shown in Fig. 8.3.4.1. But native β-lg A lost quite slowly and non-native monomer seemed stable. The polymeric bands of β-lg A, which formed during pre-heating, quickly disappeared as the reheating time increased and disappeared more rapidly in the presence of higher concentrations of κ-CN B (Fig. 8.3.4.1.D).

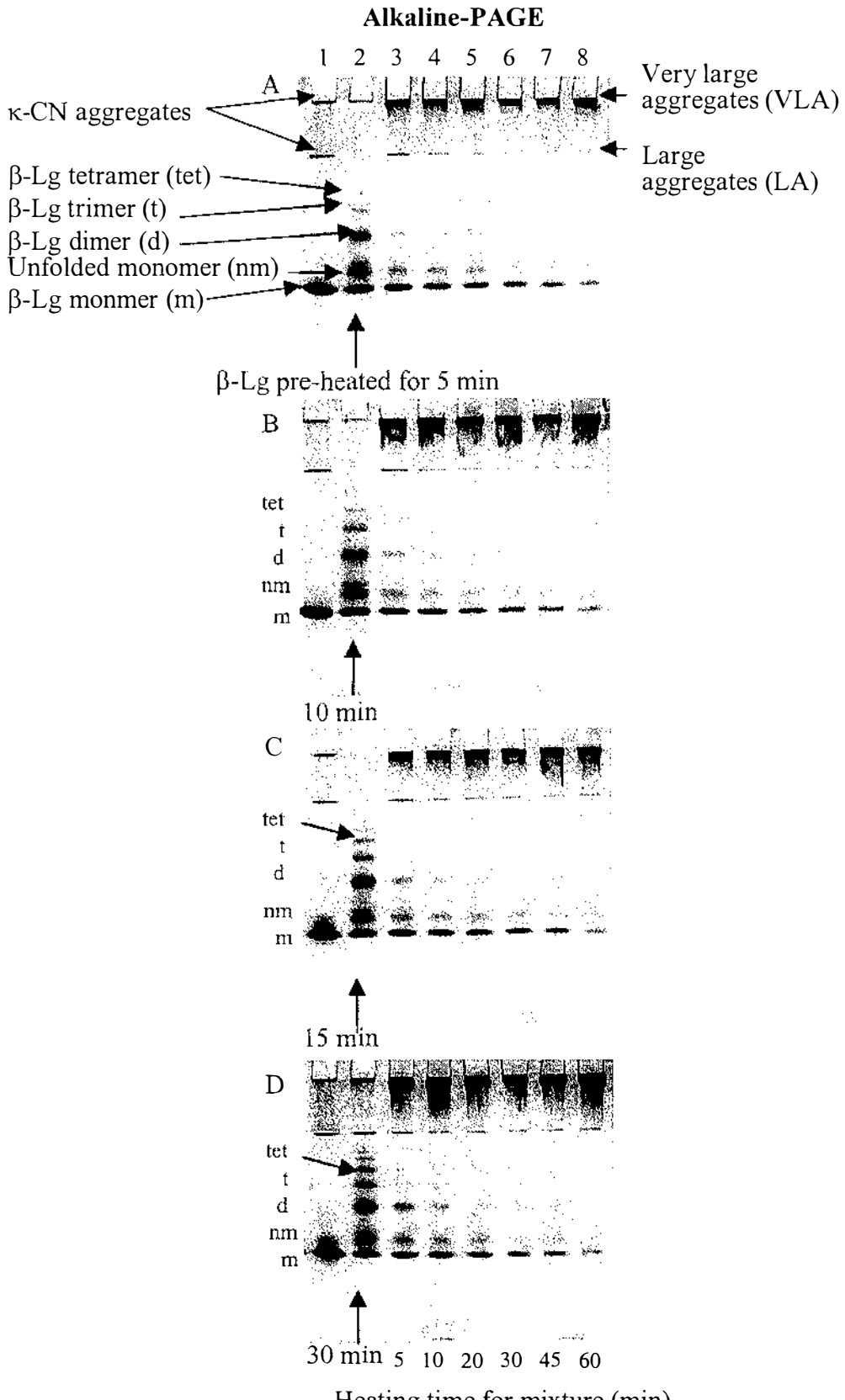

Figure 8.3.4.1. Alkaline-PAGE patterns of the mixture of pre-heated β-lg A (2 mg/mL) and κ-CN B (2 mg/mL) (2:2, w/w) heated at 80 °C for: lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 45 min; lane 8, 60 min. Lane 1, mixture of unheated β-lg and unheated κ-CN; lane 2, pre-heated β-lg A at 80 °C for (A) 5 min, (B) 10 min, (C) 15 min and (D) 30 min.

8.3.4.2. SDS-PAGE

The same samples were subjected to SDS-PAGE analysis (Fig. 8.3.4.2). Lane 1 contained the M_w standard, lane 2 contained the mixture of unheated β-lg A and unheated κ-CN B and lane 3 contained pre-heated β-lg A at 80 °C for 5 (gel A), 10 (gel B), 15 (gel C) and 30 min (gel D) (2 mg/mL β-lg A:2 mg/mL κ-CN B = 2:2, w/w).

The protein species with an M_w value of about 36,000 Da in lane 3 suggested that disulphide-linked dimeric β-lg A species were formed as a consequence of the heat treatment. As the pre-heating time of β-lg A increased, the band intensity of monomeric β-lg A decreased and more polymeric β-lg A bands (dimer, trimer and tetramer etc.) were observed in lane 3 of the SDS resolving gels.

The bands labelled km and k1 (Fig. 8.3.4.2), which also appeared in Fig. 8.3.3.2, did not appear to have counterparts in the alkaline-PAGE gels. Band km had an M_w value between 21,500 and 31,000 Da and k1 had an M_w value slightly less than 45,000 Da.

As the reheating time at 80 °C increased up to 60 min, SDS-monomeric and dimeric β-lg decreased (Fig. 8.3.4.2), as expected from the results shown using alkaline-PAGE (Fig. 8.3.4.1). At longer pre-heating times of β-lg A, more polymeric aggregates of β-lg A were present and these polymeric aggregates of β-lg A reacted with κ-CN B more efficiently than monomeric β-lg A during subsequent heating.

Sawyer (1969) and McKenzie et al. (1971) also interpreted the restriction of β-lg self-aggregation during thermal denaturation in the presence of κ-CN as evidence for κ-CN complexing with intermediate species of aggregated β-lg. Long et al. (1963) presented evidence indicating that primary denaturation of β-lg precedes its interaction with κ-CN. Although it was not demonstrated that the same products were formed, it was shown that only β-lg needed to be heated for an interaction to occur. On the other hand, Euber and Brunner (1982) reported that aggregation of β-lg was not a prerequisite for the interaction, and that the β-lg/κ-CN complex was stabilised by intermolecular disulphide bonds.

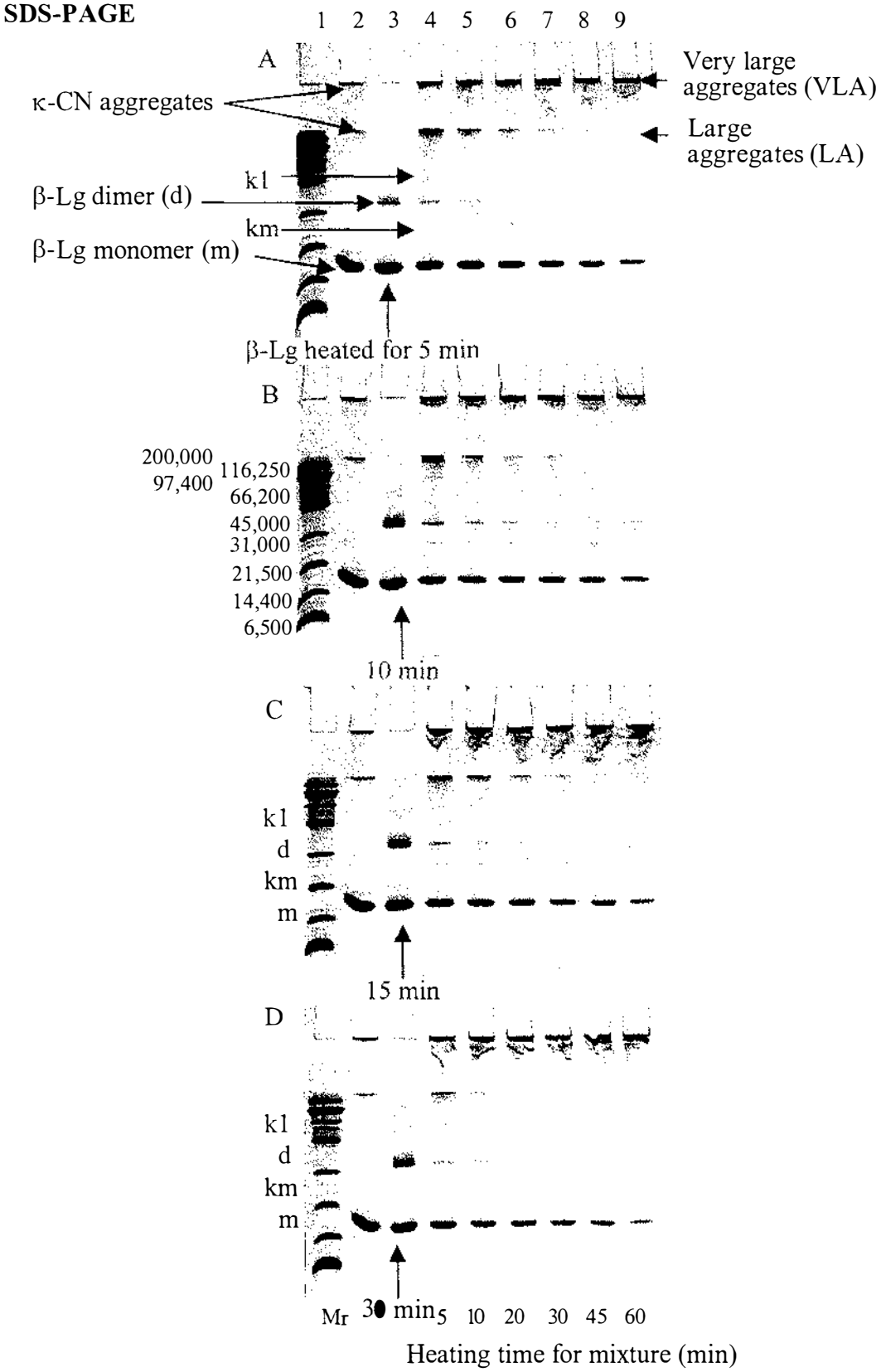

Figure 8.3.4.2. SDS-PAGE patterns of the mixture of pre-heated β-lg A (2 mg/mL) and κ-CN B (2 mg/mL) (2:2, w/w) heated at 80 °C for: lane 4, 5 min; lane 5, 10 min; lane 6, 20 min; lane 7, 30 min; lane 8, 45 min; lane 9, 60 min. Lane 1, M_w standard; lane 2, mixture of unheated β-lg and unheated κ-CN; lane 3, pre-heated β-lg A at 80 °C for (A) 5 min, (B) 10 min, (C) 15 min and (D) 30 min.

8.3.4.3. High molecular weight SDS-PAGE

In an attempt to observe the intermediate-sized aggregates and the large aggregates (M_w values between 200,000 and 500,000 Da) formed during heating mixtures of β-lg and κ-CN, which could not enter the resolving and stacking gels of a gel made with a standard recipe, the concentration of acrylamide was reduced to 9 % for the resolving gel and to 3 % for the stacking gel, instead of the usual 16 % acrylamide for the resolving gel and 4 % for the stacking gel.

The samples discussed in Section 8.3.4 were used, i.e. pre-heated β-lg A mixed with aliquots of κ-CN B in the ratio of 2:2 (w/w) and then reheated another 5-60 min at 80 °C (Fig. 8.3.4.3).

Unfortunately, as the concentration of acrylamide was reduced, the resolution of the PAGE also decreased, so that clear separation of the stained protein was not obtained. However, several bands around M_w values between 42,000 and 65,000 Da, which could be dimers or trimers of β-lg and κ-CN aggregates, were observed.

8.3.5. General discussion

The central role of β-lg in heat-induced aggregation is connected with the presence of a free -SH group in the β-lg monomer. β-Lg, which exists as a dimer at low temperatures, dissociates to monomers and then begins to unfold at high temperatures, leading to increased reactivity of its -SH group (Sawyer, 1968). The -SH group functions as a catalyst in the heat-induced formation of intermolecular S-S bonds through S-S interchange reactions (Smits and van Brouwershaven, 1980). However, aggregation of β-lg might not be a prerequisite for interaction with κ-CN, although pre-heated β-lg was apparently more reactive in aggregate formation.

A model for the heat-induced interaction between β-lg A and κ-CN B, based on the models of Manderson (1998) and Schokker et al. (1999) for β-lg heat denaturation, is shown in Fig. 8.3.5.1. Aggregation may be initiated by reversible structural changes in β-lg monomers, with increasing thiol group exposure, by producing non-native β-lg molecules (①, ②). Heat-induced unfolded monomers (⑤, ⑥, ⑦) and polymers of β-lg complex (⑧, ⑨) with κ-CN via thiol oxidation or thiol-disulphide interchange reactions. Some monomeric κ-CN is found (④) during reaction with β-lg, confirming the disulphide bond interchanges between β-lg and κ-CN (⑥).

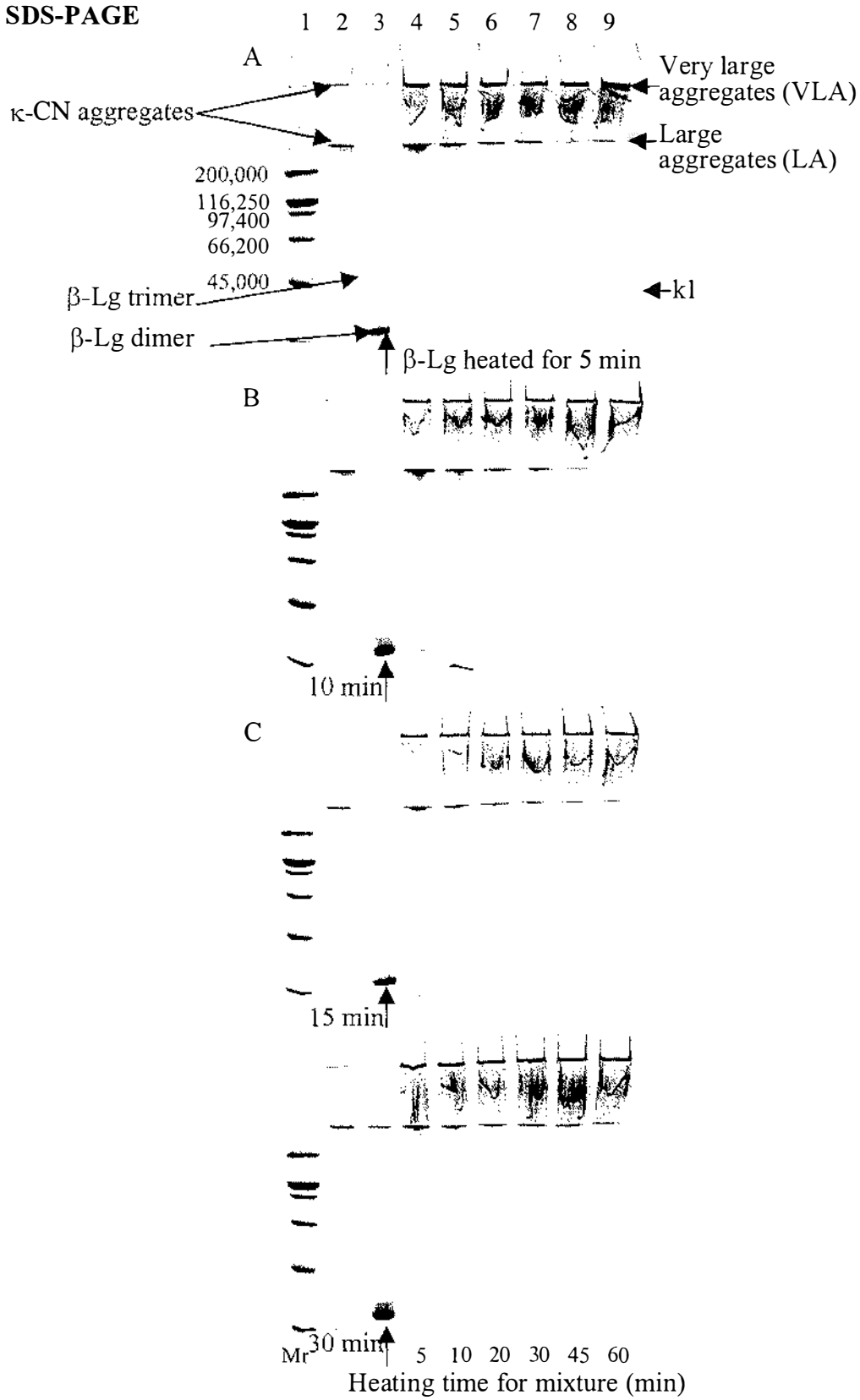

Figure 8.3.4.3. High molecular weight SDS-PAGE (9 % acrylamide) patterns of the mixture of pre-heated β-lg A (2 mg/mL) and κ-CN B (2 mg/mL) (2:2, w/w) heated at 80 °C for: lane 4, 5 min; lane 5, 10 min; lane 6, 20 min; lane 7, 30 min; lane 8, 45 min; lane 9, 60 min. Lane 1, M_w standard; lane 2, mixture of unheated β-lg and unheated κ-CN; lane 3, pre-heated β-lg A at 80 °C for (A) 5 min, (B) 10 min, (C) 15 min and (D) 30 min.

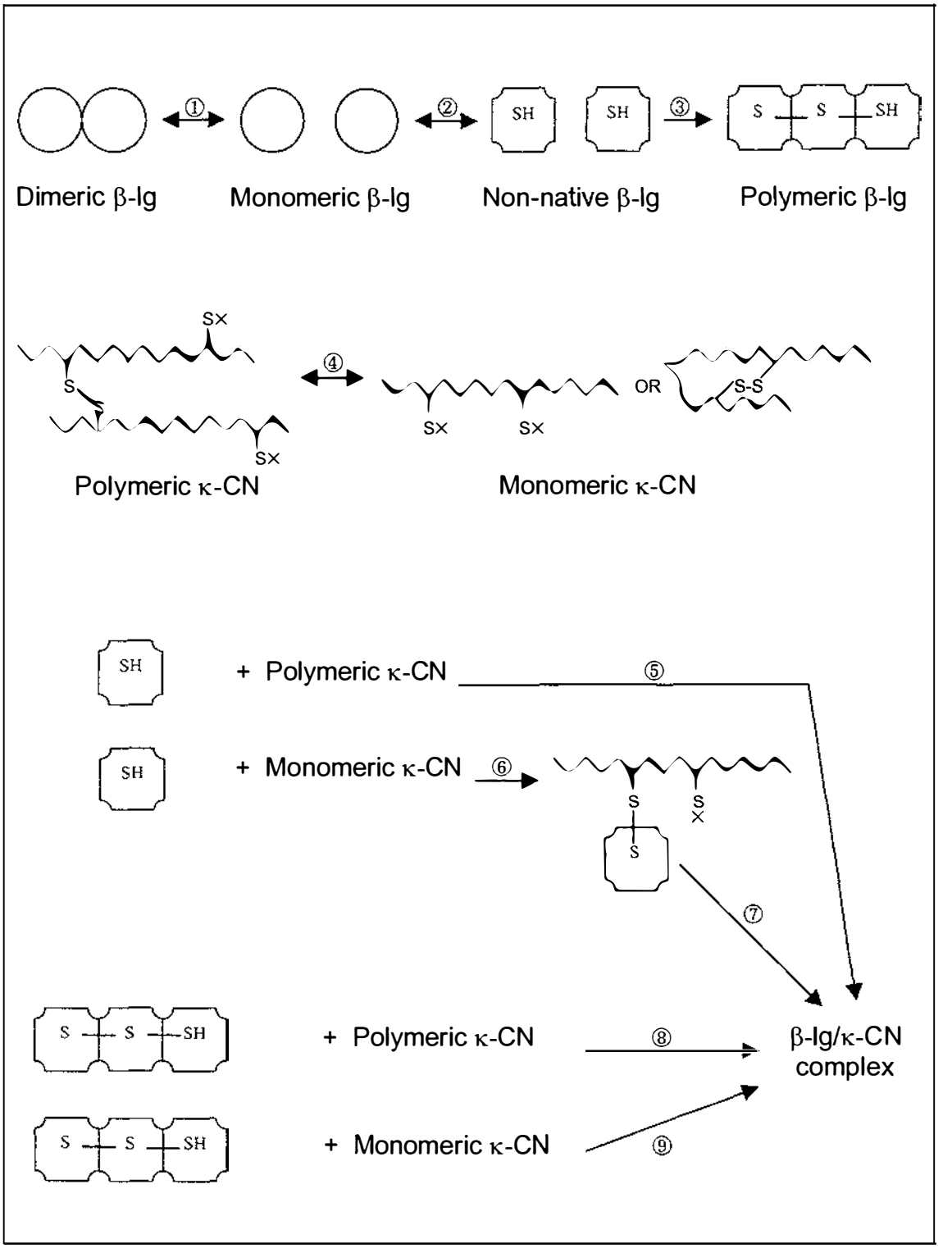

Figure 8.3.5.1. Proposed model for the heat-induced interactions between β-lg and κ-CN. The cysteine residues of κ-CN are oxidised in the native protein and could bond to small molecules, shown as ×, or form disulphide bonds.

The monomeric κ-CN also participates in the interaction between the two proteins and forms complexes (⑥, ⑨).

It is possible that the step ② become faster by the presence of κ-CN, i.e. equilibrium between native β-lg and denatured β-lg shifted rapidly probably because reversible steps in the pathway became less reversible in the presence of κ-CN. Also it seems that unfolded monomeric β-lg prefers to react with κ-CN, i.e. ⑤ and ⑥ > ③, consequently intermediate sized aggregates immediately joined to form large aggregates.

Interaction between β-lg A and κ-CN B during heat treatment at different protein concentrations (different ratios between β-lg A and κ-CN B) was investigated in this chapter. Bovine β-lg and κ-CN exhibit genetic polymorphism and their different heat susceptibilities have been reported. To extend this study, the effect of genetic variants on the interaction between β-lg and κ-CN is investigated in Chapter 9. The research considers how the stabilities and structural changes of β-lg A, B and C variants differ from each other and with κ-CN A and B variants during heat treatment.

CHAPTER 9.

EFFECT OF GENETIC VARIANTS ON THE HEAT-INDUCED INTERACTIONS BETWEEN β-LACTOGLOBULIN AND κ-CASEIN

9.1. INTRODUCTION

Genetic polymorphism has been detected in all major bovine milk proteins except α-la and has been shown to affect milk yields, milk composition (McLean et al., 1984; Ng-Kwai-Hang et al., 1986), milk properties such as cheese making capacity (Schaar, 1984; van den Berg et al., 1992; Jakob abd Puhan, 1992; FitzGerald and Hill, 1997) and the heat stability of evaporated milk (McLean et al., 1987). The genetic variants differ from each other in terms of amino acid substitution(s) or deletion(s), which result in different physico-chemical properties.

Thirteen genetic variants of β-lg have been identified (Sawyer, 2001) with A and B dominating and occurring at almost equal frequency (Hambling et al., 1992). The primary sequences of the A, B and C variants vary at residues 59, 64 and 118: β-lg A (Gln-59, Asp-64 and Val-118), β-lg B (Gln-59, Gly-64 and Ala-118) and β-lg C (His-59, Gly-64 and Ala-118) – see Fig. 2.2.2.2. Extensive studies of the thermal properties of the A and B variants of β-lg have shown that β-lg B is more heat sensitive than β-lg A in skim milk, whole milk or buffered solution (McKenzie et al., 1971; Dannenberg and Kessler, 1988a; Parnell-Clunies et al., 1988; Manderson et al., 1998). However, this is not the case in cheese whey, where β-lg A is less stable than β-lg B at temperatures above 90 °C (Hillier et al., 1979), or for 10 % β-lg in simulated milk ultrafiltrate (SMUF) as measured by DSC (Imafidon et al., 1991a, b). However, β-lg C is more stable than both the A and B variants in model systems (Sawyer, 1969; Manderson et al., 1999a, b).

There are two common variants of κ-CN in New Zealand: the A and B variants (Creamer and Harris, 1997). The κ-CN B variant differs from the A variant by having isoleucine and alanine at positions 136 and 148, respectively, instead of threonine and aspartic acid, in the 169 amino acid sequence (Eigel et al., 1984) – see Fig. 2.5.2.2. It

has been demonstrated that κ-CN B would be more desirable than κ-CN A because the former is associated with a higher casein content in milk, a higher cheese-yielding capacity, a more favourable cheese composition and better coagulating properties in terms of rennet clotting time and curd firmness (Ng-Kwai-Hang et al., 1986; Jakob and Puhan, 1992; Puhan, 1997; Lodes et al., 1996; FitzGerald and Hill, 1997). Feagan et al. (1972) and McLean et al. (1987) reported that κ-CN BB skim milk had greater heat stability than κ-CN AA skim milk. In the case of concentrated milk, it is generally considered that the B variant is associated with higher heat stability (McLean et al., 1987), although van den Berg et al. (1992) reported that the κ-CN AA phenotype gave higher heat stability in condensed skim milk.

The heat-induced interactions between β-lg A and κ-CN B were investigated and it was shown that 1:1 complexes could be identified (Chapter 8). In the work described in this chapter, these studies were extended to the six combinations between the β-lg A, B and C and κ-CN A and B variants to elucidate the effect of genetic variants on the heat-induced interaction between β-lg and κ-CN.

9.2. EXPERIMENTAL PROTOCOL

All the analytical techniques and the ratios between β-lg and κ-CN were as in Chapter 8 so that direct comparison could be made among the different combinations of β-lg and κ-CN variants (Section 8.2).

9.3. RESULTS AND DISCUSSION

9.3.1. Effect of genetic variants and β-lactoglobulin:κ-casein ratios on the heat-induced interactions between β-lactoglobulin and κ-casein

The arrangement of the various concentrations of the three variants of β-lg and the six sets of β-lg/κ-CN variant combinations analysed using two different PAGE techniques is shown in table 9.3.1.1.

Table 9.3.1.1. Results shown in Figs 9.3.1.4 to 9.3.1.9

Section I. Content of the figures

	Alkaline-PAGE	SDS-PAGE
β-lg A	Fig. 9.3.1.4	Fig. 9.3.1.7
β-lg B	Fig. 9.3.1.5	Fig. 9.3.1.8
β-lg C	Fig. 9.3.1.6	Fig. 9.3.1.9

Section II. Contents of each figure

Samples across each row have same total protein content

β-Lg	β-Lg + κ-CN A*	β-Lg + κ-CN B*
A. β-lg 1.5 mg/mL	**E.** β-lg 2.0 mg/mL + κ-CN A 1.0 mg/mL (2:1, w/w); β-lg 1.0 mg + κ-CN A 0.5 mg/mL	**I.** β-lg 2.0 mg/mL + κ-CN B 1.0 mg/mL (2:1, w/w); β-lg 1.0 mg + κ-CN B 0.5 mg/mL
B. β-lg 2.0 mg/mL	**F.** β-lg 2.0 mg/mL + κ-CN A 2.0 mg/mL (2:2, w/w); β-lg 1.0 mg + κ-CN A 1.0 mg/mL	**J.** β-lg 2.0 mg/mL + κ-CN B 2.0 mg/mL (2:2, w/w); β-lg 1.0 mg + κ-CN B 1.0 mg/mL
C. β-lg 2.5 mg/mL	**G.** β-lg 2.0 mg/mL + κ-CN A 3.0 mg/mL (2:3, w/w); β-lg 1.0 mg + κ-CN A 1.5 mg/mL	**K.** β-lg 2.0 mg/mL + κ-CN B 3.0 mg/mL (2:3, w/w); β-lg 1.0 mg + κ-CN B 1.5 mg/mL
D. β-lg 3.0 mg/mL	**H.** β-lg 2.0 mg/mL + κ-CN A 4.0 mg/mL (2:4, w/w); β-lg 1.0 mg + κ-CN A 2.0 mg/mL	**L.** β-lg 2.0 mg/mL + κ-CN B 4.0 mg/mL (2:4, w/w); β-lg 1.0 mg + κ-CN B 2.0 mg/mL

* Each mixture contained equal volumes of β-lg and κ-CN solutions. The final concentration of β-lg was always 1 mg/mL.

9.3.1.1. Heat treatment of β-lactoglobulin variant solutions at various concentrations

β-Lg samples containing the same total protein concentration as mixtures of β-lg and κ-CN, in pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl), were heat treated at 80 °C for various times (0-60 min). These treatments provided the control data in measuring the different behaviours of β-lg caused by the presence of κ-CN. The concentrations of the β-lg samples were 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL and analyses for the β-lg A, B and C variants were carried out separately using alkaline- and SDS-PAGE. The PAGE patterns for samples containing 1.5 and 2.5 mg/mL of β-lg A, B and C are presented in Figs 9.3.1.1 and 9.3.1.2 and the results of quantitative PAGE analyses are presented in Figs 9.3.1.3 and 9.3.1.4-9.3.1.9.A to D.

The bands in the resolving gel were characterised as "alkaline- and SDS-" monomeric (m), dimeric (d), trimeric (t) and tetrameric (tet) β-lg in alkaline-PAGE gels (Fig. 9.3.1.1) and SDS-PAGE gels (Fig. 9.3.1.2). In addition, non-native monomer (nm) bands positioned between the "alkaline" monomer and dimer bands were observed in alkaline-PAGE. All aggregates shown on the top of the resolving gel, with M_w values greater than approximately 200,000 Da, are referred to as large aggregates (LA) and those shown on the top of the stacking gel, with M_w values greater than approximately 500,000 Da, are referred to as very large aggregates (VLA).

Alkaline-PAGE

The alkaline-PAGE gels for heat-treated samples of β-lg A, B and C are shown in Fig. 9.3.1.1. With increasing heating time, an increase in polymeric β-lg bands (dimer, trimer and tetramer etc.) and a decrease in monomeric β-lg bands were observed for all three variants. However, the heat-treated β-lg B samples also showed a decrease in all polymeric bands during the later stages of heating (i.e. after 7.5 min in Fig. 9.3.1.1.B and after 5 min in Fig. 9.3.1.1.E). As the heating time increased, large aggregates appeared on the top of the resolving gels, and were more clearly observed for the heat-treated β-lg B samples (Fig. 9.3.1.1.B, E).

Alkaline-PAGE

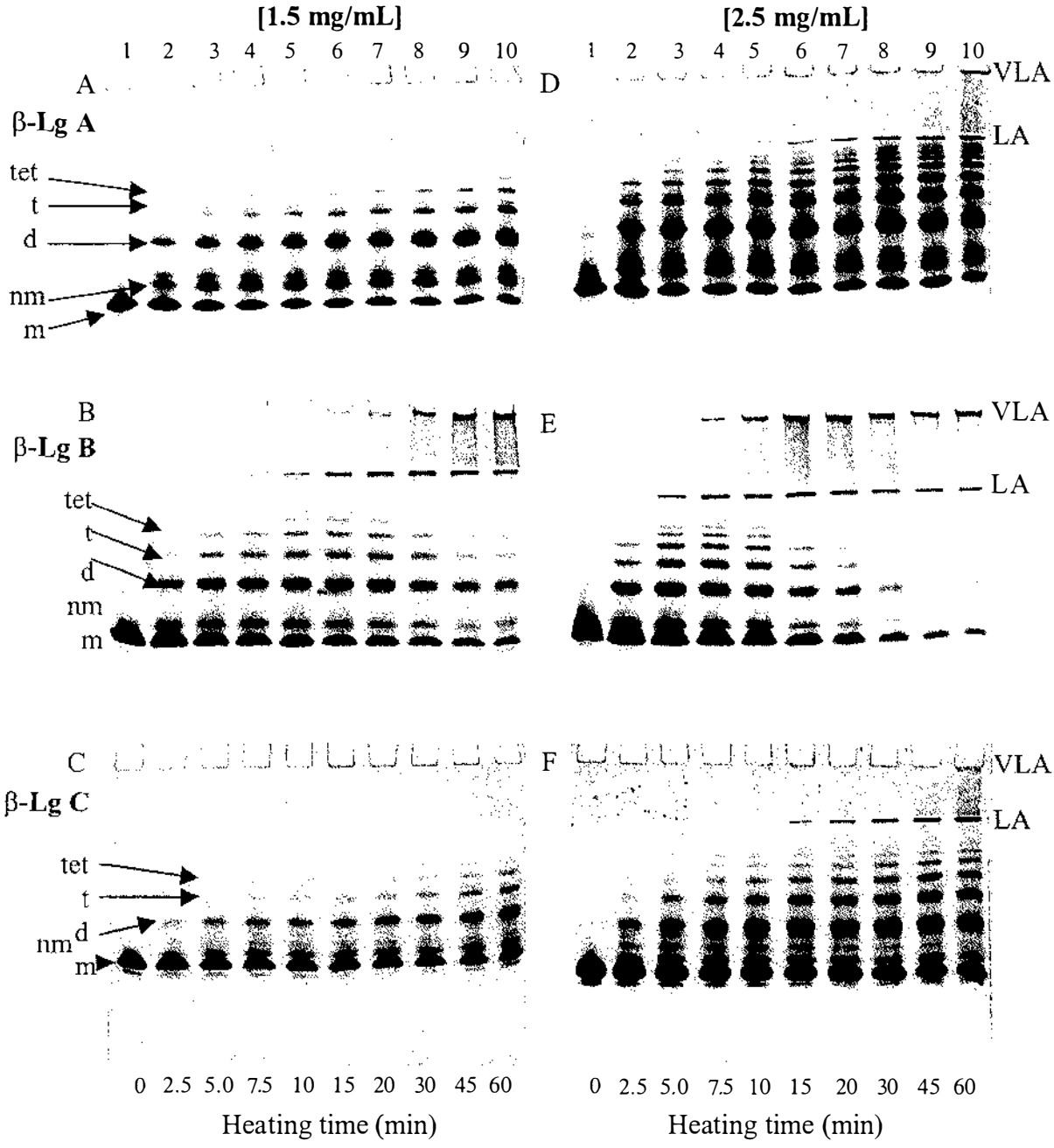

Figure 9.3.1.1. Alkaline-PAGE patterns of β-lg heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. (A) β-Lg A, (B) β-lg B and (C) β-lg C (1.5 mg/mL); (D) β-lg A, (E) β-lg B and (F) β-lg C (2.5 mg/mL).

The β-lg A, B and C samples heated at 80 °C showed similar gel patterns (Fig. 9.3.1.1). There were fewer protein bands on the resolving gel of β-lg C (Fig. 9.3.1.1.C, F) than on those of β-lg A (Fig. 9.3.1.1.A, D) and β-lg B (Fig. 9.3.1.1.B, E) at the same heating time. The non-native monomer protein bands were more pronounced in heated solutions of β-lg A than in those of either β-lg B or β-lg C and two separate non-native monomer bands were observed for β-lg A. These results are in agreement with those of Manderson et al. (1998) who showed, using alkaline-PAGE, that heat-treated solutions of β-lg A contained higher concentrations of stable unfolded monomeric protein species than equivalently treated solutions of β-lg B and β-lg C.

Although similar protein band patterns were observed among the three variants, the rates of aggregation were considerably different. Samples of heated β-lg B showed more polymeric bands during the initial stages of heating because of faster aggregation to form large aggregates on the top of the resolving gel, which were observed even when the concentration was just 1 mg/mL (not shown). In contrast, for β-lg A and β-lg C, large aggregates only appeared when the protein concentration was above 2 mg/mL, i.e. 2.5 mg/mL or 3.0 mg/mL (Fig. 9.3.1.1.D to F and some gels not shown).

The band intensities of large aggregates and very large aggregates for the β-lg solutions containing 2.5 mg/mL of protein (Fig. 9.3.1.1.D to F) were greater than those for the samples containing 1.5 mg/mL of protein (Fig. 9.3.1.1.A to C) in all three variants indicating that the rate of polymer formation was greater for β-lg solutions containing 2.5 mg/mL of protein. Also samples containing a higher protein concentration gave rise to a number of bands with lower mobilities, i.e. above tetrameric β-lg, during earlier stages of the heating compared with samples containing a lower protein concentration.

SDS-PAGE

When the same samples were analysed using SDS-PAGE (Fig. 9.3.1.2), there were bands corresponding to a number of the bands observed in alkaline-PAGE. However, fewer polymer bands were observed in SDS-PAGE, probably because the polymers linked through non-covalent bonds resolved in the presence of SDS, as indicated by the lower band intensities of large aggregates in SDS-PAGE as compared with alkaline-PAGE.

SDS-PAGE

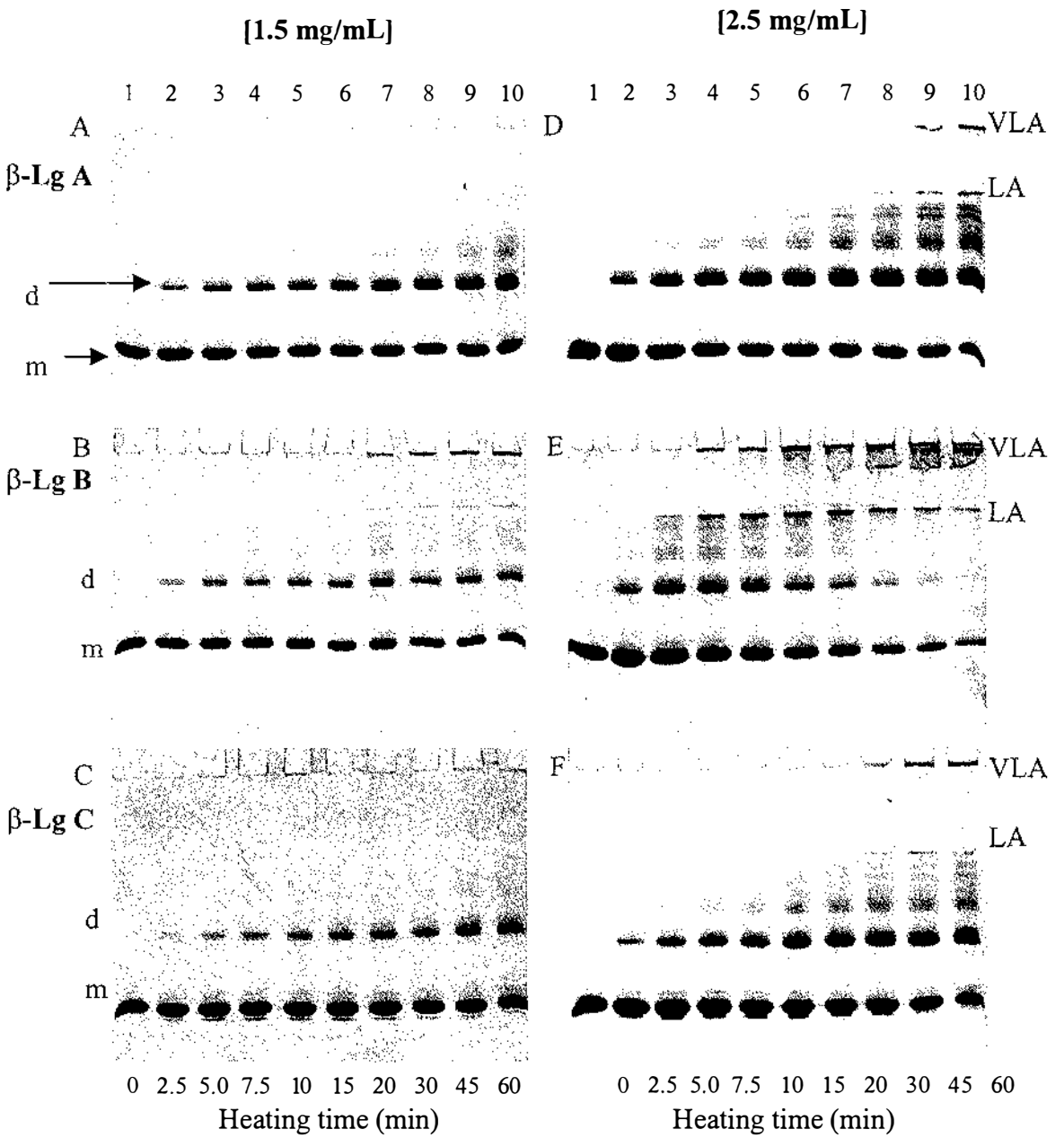

Figure 9.3.1.2. SDS-PAGE patterns of β-lg heated at 80 °C for: lane 1, 0 min; lane 2, 2.5 min; lane 3, 5.0 min; lane 4, 7.5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. (A) β-Lg A, (B) β-lg B and (C) β-lg C (1.5 mg/mL); (D) β-lg A, (E) β-lg B and (F) β-lg C (2.5 mg/mL).

This result supports earlier findings, which showed that SDS was able to dissociate monomeric β-lg from heat-induced aggregates of β-lg formed at protein concentrations > 50 mg/mL (McSwiney et al., 1994a, b) as well as from mixtures of β-lg and other proteins (Gezimati et al., 1996, 1997; Havea et al., 1998). Furthermore, Manderson et al. (1998) used 2D-PAGE to show that disulphide-bonded dimers could be dissociated from trimers and tetramers in heat-treated β-lg solutions in the presence of SDS.

In agreement with the alkaline-PAGE results, the results of non-reduced SDS-PAGE analysis indicated that the extent of conversion of monomeric β-lg to large and very large aggregate species increased with increasing heat treatment and total protein concentration. Also, as the initial concentration of β-lg increased, the rate of polymer formation increased (compare Fig. 9.3.1.2.A to C (1.5 mg/mL) with Fig. 9.3.1.2.D to F (2.5 mg/mL)).

Quantitative analysis of alkaline-PAGE.

Changes in the concentrations of alkaline- and SDS- monomeric, non-native monomeric and dimeric β-lgs and large aggregates were examined quantitatively by measuring the intensities of the appropriate bands on the gels. The band intensity of very large aggregates could not be measured because its loss during long destaining process (20 h). The band intensities obtained using alkaline- and SDS-PAGE were normalised relative to one another using the band intensity of the unheated β-lg sample, which was run on each gel (lane 1) as control samples.

Changes in the band intensities in alkaline- and SDS-PAGE, obtained from samples containing 1 mg/mL of β-lg, carried out for β-lg A, B and C separately, are presented in Fig. 9.3.1.3. When β-lg was mixed with κ-CN, the concentration of β-lg in the mixtures was always 1 mg/mL, so that Fig. 9.3.1.3 could be used for comparison with Figs 9.3.1.4-9.3.1.9.A to D.

Quantitative analyses of alkaline gels were carried out for heat-treated β-lg A, B and C samples (Figs 9.3.1.4-9.3.1.6.A to D). The losses in band intensities of samples containing 1.5, 2.0, 2.5 and 3.0 mg/mL of β-lg and are presented in parts A, B, C and D, respectively (Table 9.3.1.1).

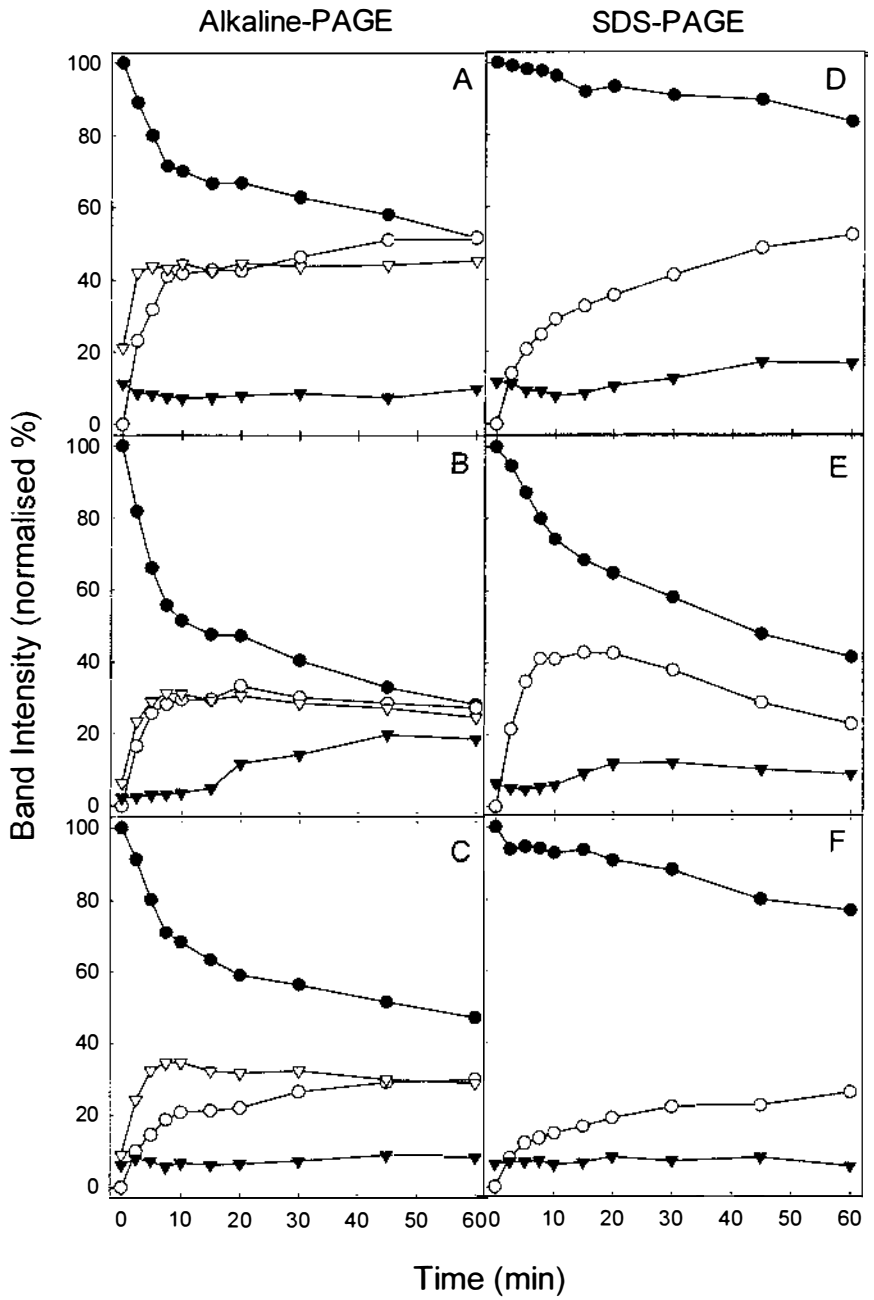

Figure 9.3.1.3. Changes in the band intensities for samples containing 1 mg/mL of (A) β-lg A, (B) β-lg B and (C) β-lg C in alkaline-PAGE and 1 mg/mL of (D) β-lg A, (E) β-lg B and (F) β-lg C in SDS-PAGE heated at 80 °C for various times. Monomer (●), non-native monomer (∇), dimer (○) and large aggregates (▼) in alkaline-PAGE and monomer (●), dimer (○) and large aggregates (▼) in SDS-PAGE.

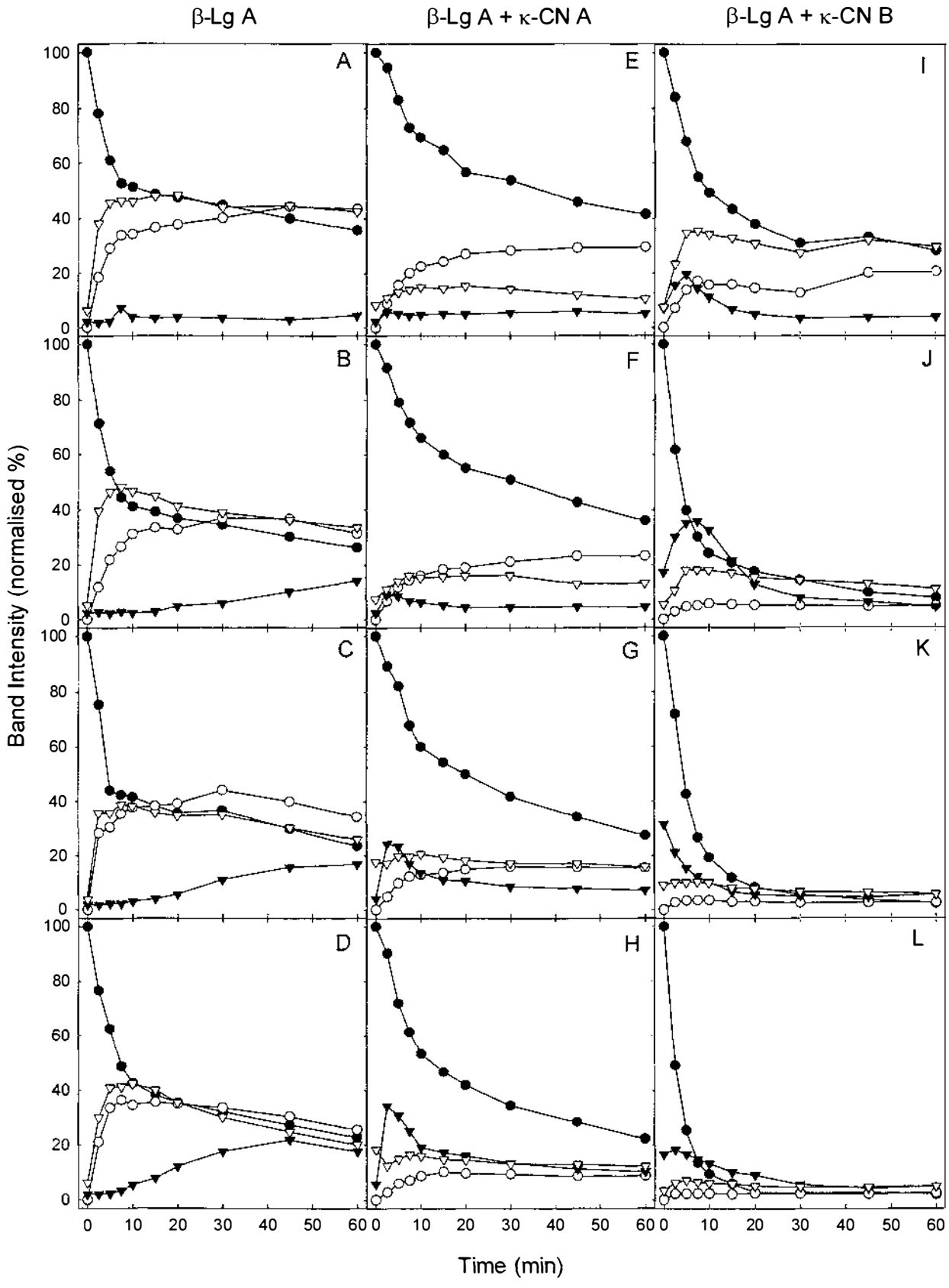

Figure 9.3.1.4. Changes in the band intensities for samples of β-lg A containing (A) 1.5, (B) 2.0, (C) 2.5 and (D) 3.0 mg/mL of β-lg A; mixture of β-lg A and κ-CN A containing (E) 1.0, (F) 2.0, (G) 3.0 and (H) 4.0 mg/2 mL of κ-CN A; mixture of β-lg A and κ-CN B containing (I) 1.0, (J) 2.0, (K) 3.0 and (L) 4.0 mg/2 mL of κ-CN B in alkaline-PAGE heated at 80 °C for various times. Monomer (●), non-native monomer (∇), dimer (O) and large aggregates (▼).

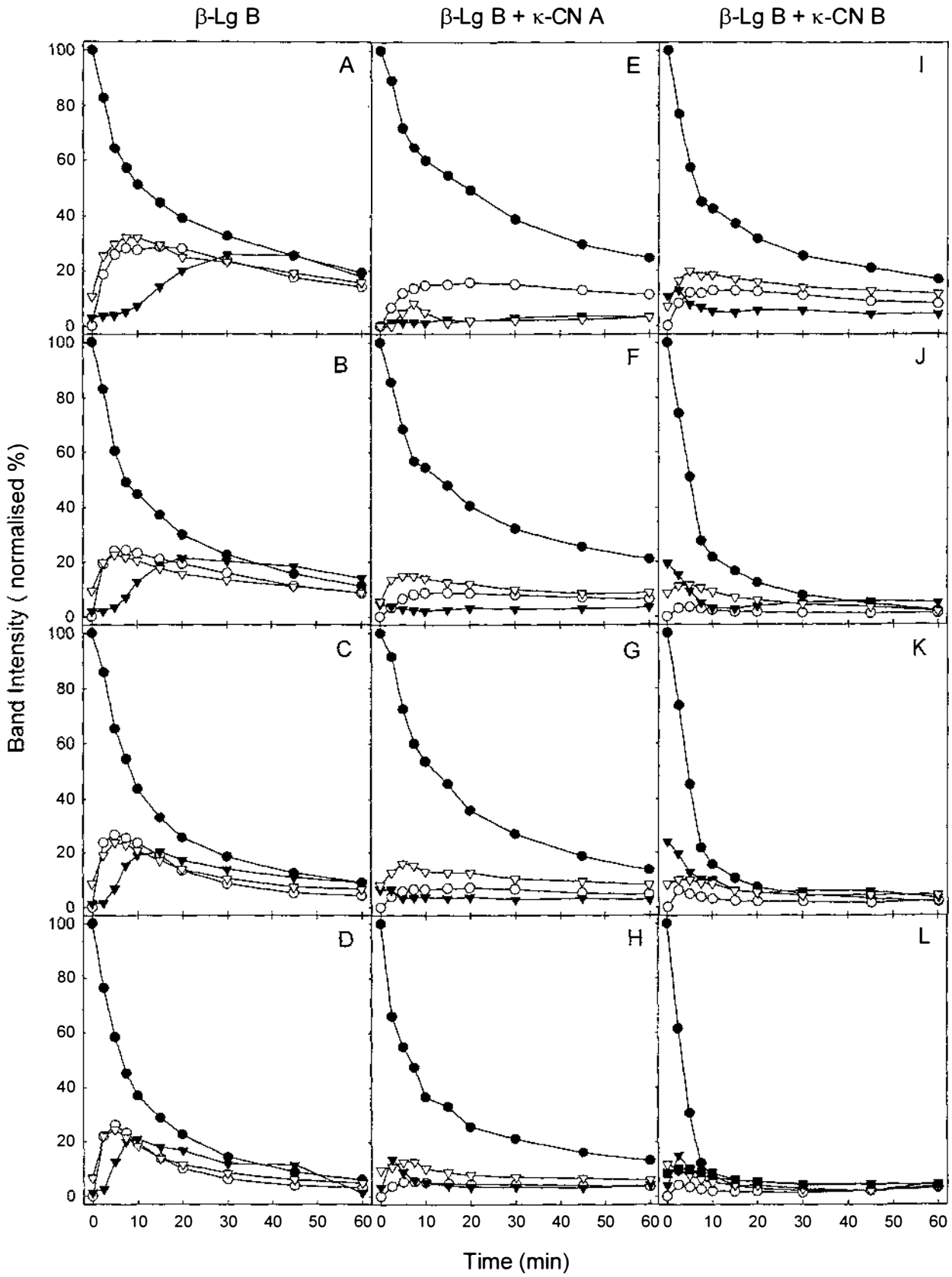

Figure 9.3.1.5. Changes in the band intensities for samples of β-lg B containing (A) 1.5, (B) 2.0, (C) 2.5 and (D) 3.0 mg/mL of β-lg B; mixture of β-lg B and κ-CN A containing (E) 1.0, (F) 2.0, (G) 3.0 and (H) 4.0 mg/2 mL of κ-CN A; mixture of β-lg B and κ-CN B containing (I) 1.0, (J) 2.0, (K) 3.0 and (L) 4.0 mg/2 mL of κ-CN B in alkaline-PAGE heated at 80 °C for various times. Monomer (●), non-native monomer (▽), dimer (○) and large aggregates (▼).

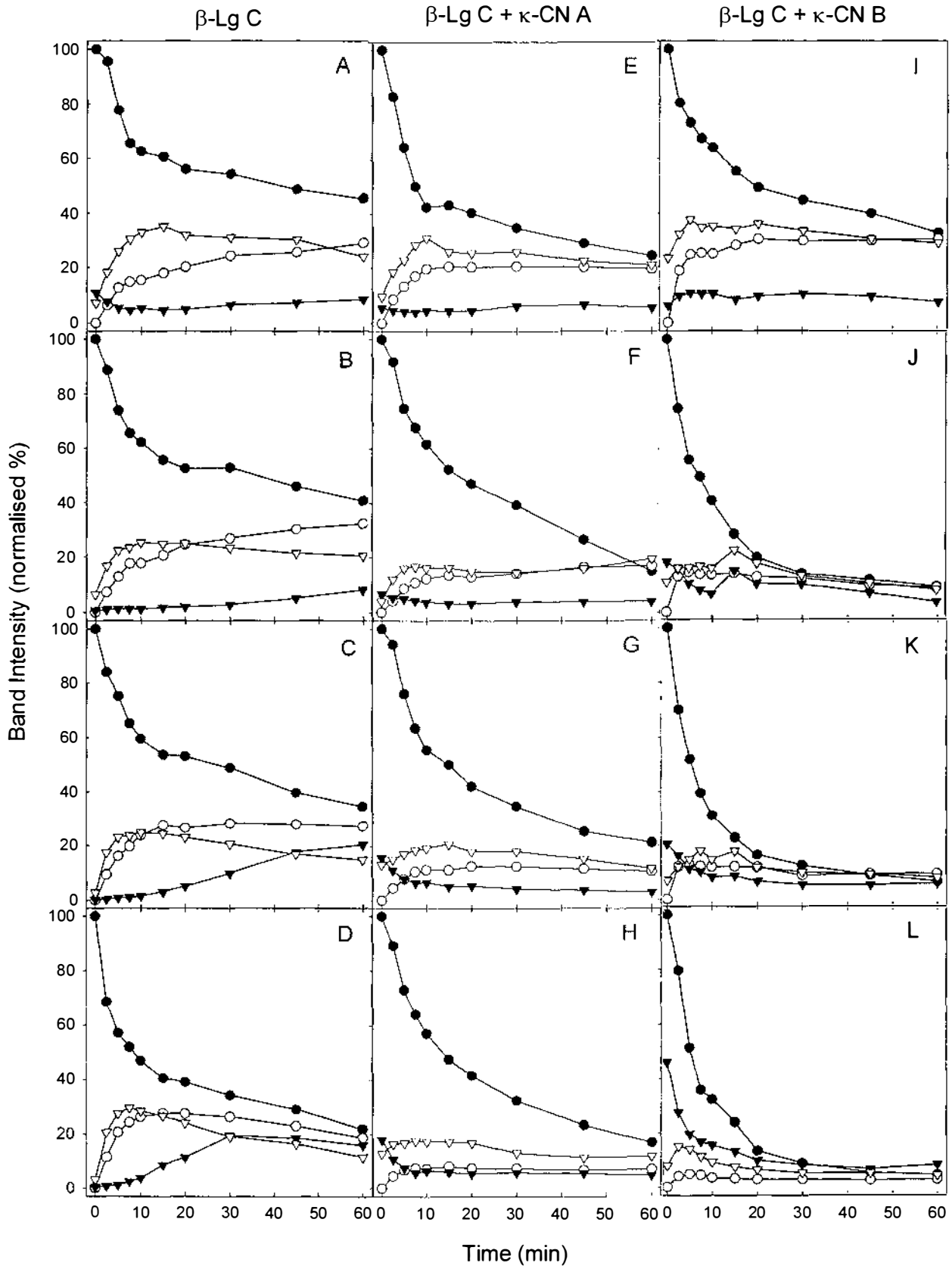

Figure 9.3.1.6. Changes in the band intensities for samples of β-lg C containing (A) 1.5, (B) 2.0, (C) 2.5 and (D) 3.0 mg/mL of β-lg C; mixture of β-lg C and κ-CN A containing (E) 1.0, (F) 2.0, (G) 3.0 and (H) 4.0 mg/2 mL of κ-CN A; mixture of β-lg C and κ-CN B containing (I) 1.0, (J) 2.0, (K) 3.0 and (L) 4.0 mg/2 mL of κ-CN B in alkaline-PAGE heated at 80 °C for various times. Monomer (●), non-native monomer (▽), dimer (○) and large aggregates (▼).

The normalised intensity values of the alkaline-monomeric β-lg band decreased with increasing heating time for all three β-lg variants. The monomer bands decreased more rapidly during the first 10 min than during the rest of the heating (Figs 9.3.1.3.A to C and 9.3.1.4-9.3.1.6.A to D).

The concentration of non-native monomer was greater than the concentration of dimer in β-lg A samples during the initial stage of heating (< 10 min), but, as the heating time increased, the band intensity of non-native monomer remained relatively constant or decreased slightly in alkaline-PAGE (Fig. 9.3.1.4.A to D). Similar patterns were observed for β-lg C (Fig. 9.3.1.6.A to D), but for β-lg B, only samples containing lower protein concentration (1.5 mg/mL of β-lg B, Fig. 9.3.1.5.A) showed greater non-native monomer concentration than dimer concentration during the initial stage of the heating.

Changes in the large aggregate band intensity were also measured quantitatively in alkaline-PAGE (Figs 9.3.1.4-9.3.1.6.A to D). As the heating time increased, the concentration of large aggregates in the β-lg A samples increased, particularly at higher concentrations of β-lg. A similar pattern was observed for the β-lg C samples. However, the β-lg B samples showed an increase in the concentration of large aggregates during the initial stage of heating and then the concentration remained relatively constant or decreased during the rest of the heating. The decrease in the concentration of large aggregates indicated further aggregation with increasing heating time, which resulted in an increasing concentration of very large aggregates.

The concentration of non-native monomer bands was greater for β-lg A than for β-lg B or β-lg C. The concentration of large aggregates was higher during the initial stage of heating in the β-lg B samples than in the β-lg A or β-lg C samples. This suggests that monomers and non-native monomers of β-lg A or β-lg C were incorporated into aggregates more slowly than those of β-lg B and that aggregates were formed more rapidly for β-lg B than for β-lg A or β-lg C.

Quantitative analysis of SDS-PAGE.

Quantitative analyses of SDS gels were carried out for heat-treated β-lg A, B and C samples and the results are shown in Figs 9.3.1.7-9.3.1.9.A to D. Similar trends were observed to those obtained with alkaline-PAGE.

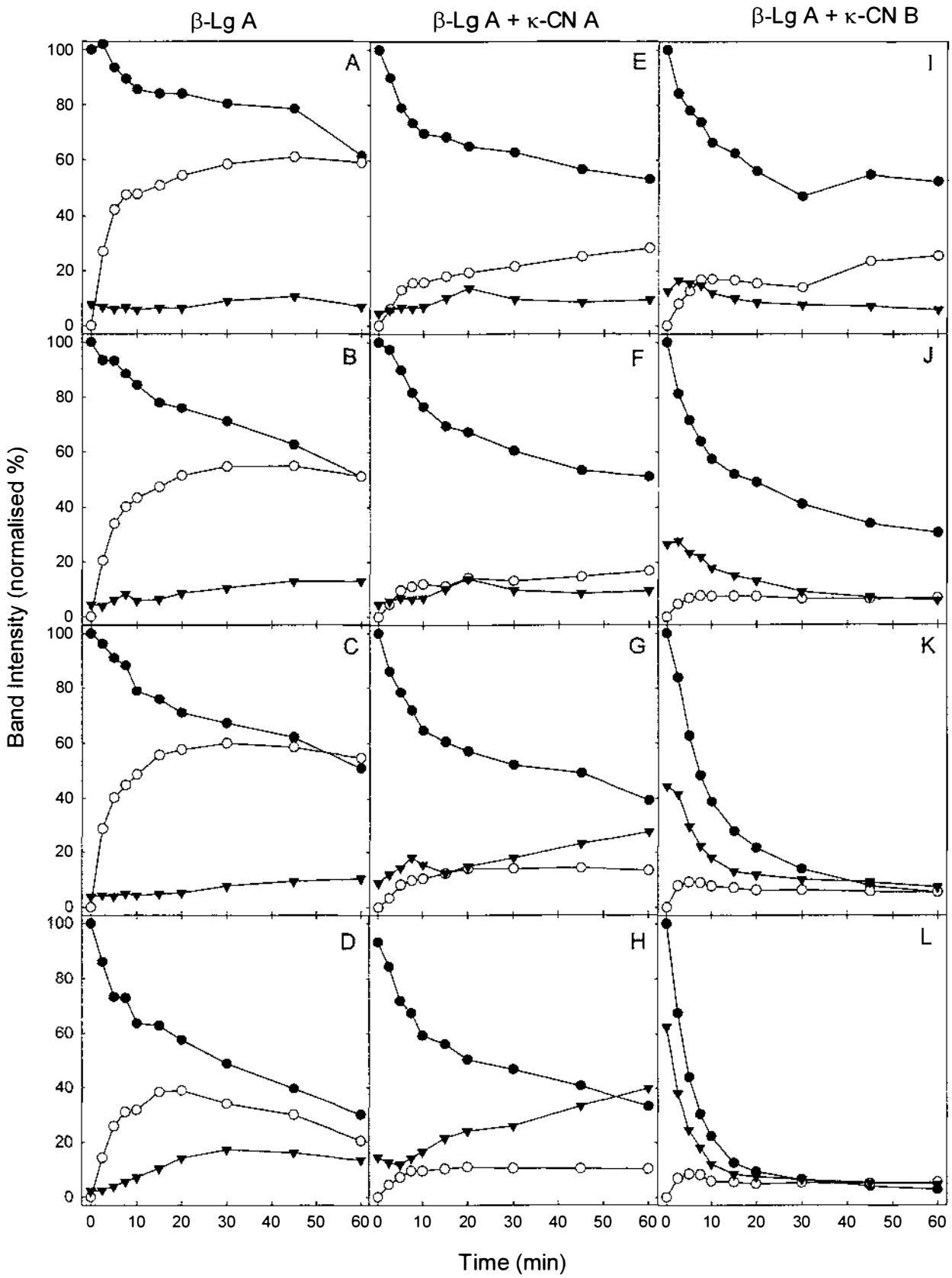

Figure 9.3.1.7. Changes in the band intensities for samples of β-lg A containing (A) 1.5, (B) 2.0, (C) 2.5 and (D) 3.0 mg/mL of β-lg A; mixture of β-lg A and κ-CN A containing (E) 1.0, (F) 2.0, (G) 3.0 and (H) 4.0 mg/2 mL of κ-CN A; mixture of β-lg A and κ-CN B containing (I) 1.0, (J) 2.0, (K) 3.0 and (L) 4.0 mg/2 mL of κ-CN B in SDS-PAGE heated at 80 °C for various times. Monomer (●), dimer (○) and large aggregates (▼).

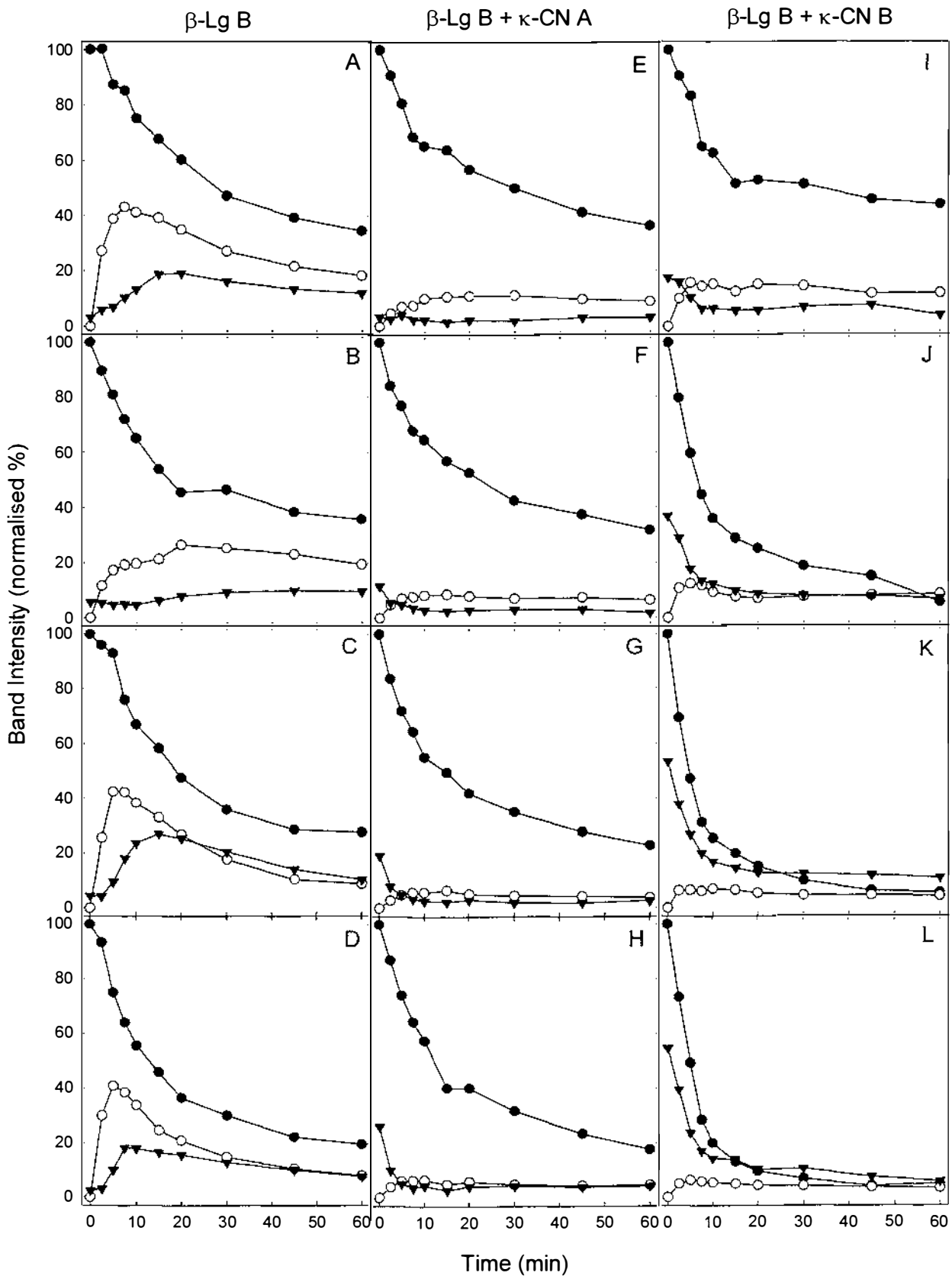

Figure 9.3.1.8. Changes in the band intensities for samples of β-lg B containing (A) 1.5, (B) 2.0, (C) 2.5 and (D) 3.0 mg/mL of β-lg B; mixture of β-lg B and κ-CN A containing (E) 1.0, (F) 2.0, (G) 3.0 and (H) 4.0 mg/2 mL of κ-CN A; mixture of β-lg B and κ-CN B containing (I) 1.0, (J) 2.0, (K) 3.0 and (L) 4.0 mg/2 mL of κ-CN B in SDS-PAGE heated at 80 °C for various times. Monomer (●), dimer (○) and large aggregates (▼).

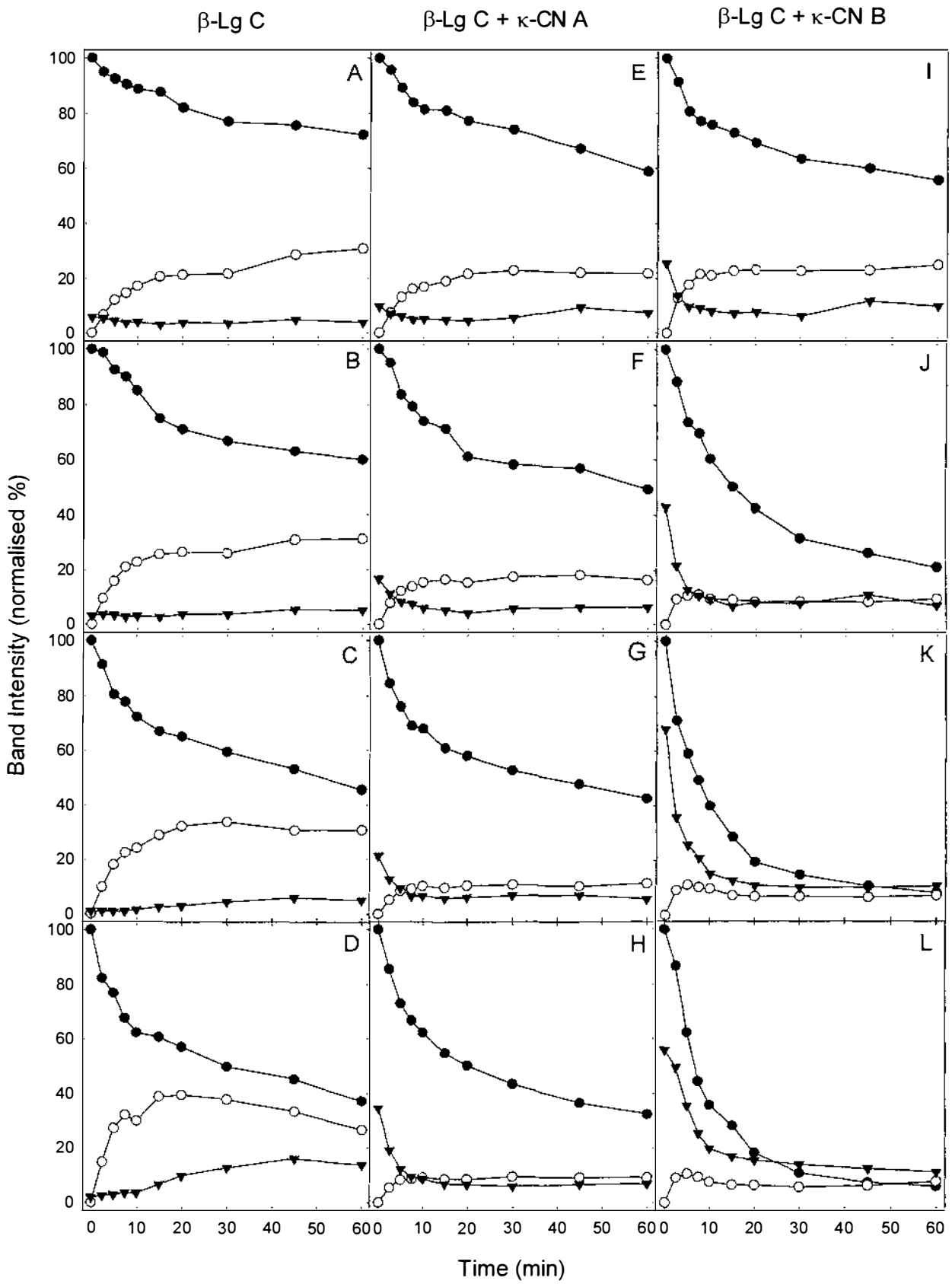

Figure 9.3.1.9. Changes in the band intensities for samples of β-lg C containing (A) 1.5, (B) 2.0, (C) 2.5 and (D) 3.0 mg/mL of β-lg C; mixture of β-lg C and κ-CN A containing (E) 1.0, (F) 2.0, (G) 3.0 and (H) 4.0 mg/2 mL of κ-CN A; mixture of β-lg C and κ-CN B containing (I) 1.0, (J) 2.0, (K) 3.0 and (L) 4.0 mg/2 mL of κ-CN B in SDS-PAGE heated at 80 °C for various times. Monomer (●), dimer (○) and large aggregates (▼).

The normalised intensity values of the SDS-monomeric β-lg band decreased with increasing heating time for all three β-lg variants. The monomer bands decreased more rapidly during the first 10 min than during the rest of the heating, which were similar with the results of alkaline-PAGE, but this was more apparent in the alkaline-PAGE gels (Figs 9.3.1.4-9.3.1.6.A to D) than in the SDS-PAGE gels (Figs 9.3.1.7-9.3.1.9.A to D).

The concentration of dimer in β-lg A samples increased during the initial stage of heating (< 20 min) and then remained relatively constant or decreased slightly during the rest of the heating (Fig. 9.3.1.7.A to D). A similar pattern was observed for the β-lg C samples, but the β-lg B samples showed greater decrease of the concentration of dimer during later stages of heating.

As the heating time increased, the concentration of large aggregates in the β-lg A samples increased, particularly at high concentrations. A similar pattern was observed for the β-lg C samples. However, the β-lg B samples showed an increase in the concentration of large aggregates during the initial stage of heating and then the concentration remained relatively constant or decreased during the rest of the heating. These trends are similar to those obtained from alkaine-PAGE results.

As the concentration of β-lg increased, the decreases in the band intensities of SDS-monomeric β-lg and the increases in those of large aggregates became greater for all three β-lg variants.

The differences in the band intensities on the alkaline- and SDS-PAGE gels, which indicated that the proportion of aggregates stabilised only by non-covalent associations, was greater for β-lg A than for β-lg B and β-lg C. With the higher concentration of non-native monomer and lower concentration of large aggregates of β-lg A obtained from alkaline-PAGE results, this suggests that β-lg A favours the formation of hydrophobically driven association and non-native monomers as intermediates in the aggregation pathway compared to β-lg B or β-lg C, in agreement with the results of Manderson et al. (1998).

Based on the results of alkaline- and SDS-PAGE, the heat stability of three variants of β-lg was in the order β-lg C > β-lg A > β-lg B, which is consistent with results of Manderson et al. (1999a, b). They used the midpoint temperatures from the results of CD spectra or thiol availability tests and suggested that the β-lg A variant may denature by a different mechanism from that for β-lg B or β-lg C. Manderson et

al. (1998) suggested that the differences among the β-lg variants probably reflect the greater net negative charge on the β-lg A molecule than on the β-lg B and β-lg C molecules at pH 6.7 and specific amino acid substitutions.

9.3.1.2. Heat treatment of β-lactoglobulin and κ-casein variant mixtures at various concentrations

Plots of normalised values of monomeric, non-native monomeric and dimeric β-lgs and large aggregates obtained from heat-treated mixtures of β-lg and κ-CN at various concentrations versus heating time (0-60 min) are shown in Figs 9.3.1.4-9.3.1.9.E to L. Table 9.3.1.1 gives the overall arrangement of the variant combinations between β-lg and κ-CN.

Quantitative analysis of alkaline-PAGE.

In the mixtures of β-lg A and κ-CN A, as the concentration of κ-CN A increased from 1 to 4 mg/2 mL (Fig. 9.3.1.4.E to H), the heat-induced loss of alkaline-monomeric β-lg accelerated and decreases in the concentration of β-lg dimer were also observed.

The trends found were similar to those in the samples containing only β-lg A, which showed an increase in the heat-induced loss of monomeric β-lg and decreased proportions of non-native monomers and dimers with increasing β-lg concentration in alkaline-PAGE (Fig. 9.3.1.4.A to D). However, the proportion of each band present in the resolving gels became quite different in the presence of κ-CN A (Figs 9.3.1.4-9.3.1.6.E to H), i.e. less loss of alkaline-monomeric β-lg and about 50 % less non-native monomers and dimers of β-lg when the samples contained κ-CN A.

When the samples contained κ-CN B (Fig. 9.3.1.4.I to L), alkaline-monomeric β-lg A decreased more rapidly compared with other samples containing the same protein concentration (Fig. 9.3.1.4.A to D and E to H). The proportion of each band was different, i.e. greater loss of alkaline-monomeric β-lg A compared with both β-lg A itself (Fig. 9.3.1.4.A to D) and the β-lg A and κ-CN A mixture (Fig. 9.3.1.4. E to H), and 70 % less dimeric β-lg A in the β-lg A and κ-CN B mixture compared with β-lg A itself.

Similar trends were observed in the mixtures of β-lg B and κ-CN (Fig. 9.3.1.5.E to H) and β-lg C and κ-CN (Fig. 9.3.1.6.E to H). The mixture of β-lg B and κ-CN B showed the most rapid loss of alkaline-monomeric β-lg B and the lowest proportion of dimeric β-lg.

Quantitative analysis of SDS-PAGE.

The above mixtures of β-lg and κ-CN were also analysed using non-reduced SDS-PAGE. As the concentration of κ-CN A increased, more rapid decreases in the band intensities of monomeric β-lg A and smaller proportions of dimeric β-lg bands were observed in the mixtures of β-lg A and κ-CN A (Fig. 9.3.1.7.E to H). The greater increase in the large aggregate band intensity compared with that for β-lg A itself (Fig. 9.3.1.7.A to D) suggested that reactive (denatured) forms of β-lg easily aggregated with κ-CN, which resulted in increasing band intensities of large aggregates on the top of the resolving gel.

In the presence of κ-CN A, the loss of monomeric β-lg A slightly increased and less dimeric β-lg was observed (Fig. 9.3.1.7.E to H) compared with β-lg A samples containing the same protein concentration (Fig. 9.3.1.7.A to D). Similar trends were observed for the mixtures of β-lg A and κ-CN B (Fig. 9.3.1.7.I to L), which showed more rapid loss of SDS-monomeric β-lg A.

Unlike β-lg A itself (Fig. 9.3.1.7.A to D) and the mixtures of β-lg A and κ-CN A (Fig. 9.3.1.7.E to H), which showed gradual increases in the concentration of large aggregates, the mixtures of β-lg A and κ-CN B (Fig. 9.3.1.7.I to L) showed decreases in the concentration of large aggregates as the heating time increased. Because the native κ-CN used was not subjected to a reduction step at any point during purification, it was polymeric and therefore positioned between the resolving and stacking gels both in alkaline-PAGE (Fig. 8.3.2.2.A) and in non-reduced SDS-PAGE (Fig. 8.3.2.2.B). Apparent decreases in the large aggregate band intensity were also observed for samples containing higher total protein concentrations (> 1.5 mg/mL), i.e. a high concentration of κ-CN B. It is possible that κ-CN A may have existed in a more aggregated form than κ-CN B, so that it could not enter and be seen on the stacking gel.

Similar trends were observed in the mixtures of β-lg B and κ-CN (Fig. 9.3.1.8.E to H and I to L) and β-lg C and κ-CN (Fig. 9.3.1.9.E to H and I to L). The mixture of β-lg B and κ-CN B showed the most rapid loss of SDS-monomeric β-lg B and the lowest proportion of dimeric β-lg (Fig. 9.3.1.8.E to H). The concentration of large aggregates in the mixtures of β-lg B or β-lg C and κ-CN decreased as the heating time increased.

Based on the results of alkaline- and SDS-PAGE in this study, β-lg B is the most reactive and β-lg C is the least reactive during thermal aggregation in the presence and in the absence of κ-CN. Qin et al. (1999) suggested the decreased thermal stability of β-lg B compared to β-lg A arises from the reduced hydrophobic contact that result from Val118Ala substitution. Alternatively, relative to the unfolded structure in water, the removal of larger hydrophobic valine moiety from water, compared to the smaller alanine moiety, is entropically more favourable. Variant C has a histidine at position 59 in place of the glutamine found for β-lg A and β-lg B. The replacement of a weak hydrogen bond between Glu-44 and Gln-59 in β-lg A and β-lg B with a salt bridge between Glu-44 and His-59 in β-lg C, leads to enhanced rigidity of this region (Bewley et al., 1997). Manderson et al. (1998) suggested that the differences among the β-lg variants probably reflect the greater net negative charge on the β-lg A molecule than on the β-lg B and β-lg C molecules at pH 6.7 and specific amino acid substitutions. In addition, the proximity of residue 64, which is an Asp in β-lg A and a Gly in β-lg B and β-lg C, to the side chain Glu-65 and the disulphide bond Cys66-Cys160 probably affects the aggregation behaviour of β-lg. Namely, at pH 6.7, both Asp-64 and Glu-65 will carry a negative charge and the side chain of Glu-65 will be repelled towards the disulphide bond Cys66-Cys160. The negative charge close to the disulphide bond Cys66-Cys160 will therefore be greater for β-lg A than for β-lg B and β-lg C, making it less likely that molecules of β-lg A will participate in intermolecular thiol-disulphide interchange reactions with thiolate anions. Furthermore, the response of β-lg A, B and C variants to denaturation by the chaotrope urea in Chapter 6 is the same as that to thermal denaturation.

The presence of κ-CN affects the rates of heat-induced interactions of β-lg, but κ-CN did not affect the relative reactivity among the β-lg variants. It seems that the

genetic variants of β-lg had greater involvement in the heat-induced complex formation than the genetic variants of κ-CN.

9.3.2. 2D-PAGE

Heat-treated β-lg/κ-CN mixtures were separated in 1D-non-reduced SDS-PAGE and then characterised by reduced SDS-PAGE in the second dimension (Figs 9.3.2.1 and 9.3.2.2). Each sample was selected from among the heat-treated mixtures of the six variant combinations of β-lg and κ-CN (2:2, w/w) from the SDS gels and was heated at 80 °C for 10 min.

Bands with different mobilities, which were resolved in non-reduced SDS-PAGE, were resolved in the reduced SDS-PAGE second dimensionally. By comparing the relative mobilities of the bands in the reduced sample or with the M_w standards, it was possible to estimate the M_w values of the species in the reduced SDS dimension of the 2D gels, as shown in Figs 9.3.2.1 and 9.3.2.2.

In Chapter 8, 2D-PAGE revealed that β-lg A and κ-CN B aggregated through disulphide bonds and bands, km and k1, corresponding to κ-CN B monomer and a 1:1 complex of β-lg A/κ-CN B aggregate, respectively, were identified (Fig. 8.3.3.3).

All the spots identified in Fig. 8.3.3.3 were observed again in the 2D gels of the various mixtures of β-lg and κ-CN. Traces of para-κ-CN was also observed after reduction in the first lane. The km bands corresponding to κ-CN monomers were observed in all six sets. However, the k1 bands corresponding to β-lg/κ-CN aggregates were identified only in the presence of κ-CN B and in the β-lg C/κ-CN A mixture. The k1 bands were very faint in the presence of κ-CN A, because a slower or lesser degree of interaction was observed in the presence of κ-CN A (Section 9.3.1.2).

SDS-PAGE in the present study showed the dissociation of κ-CN, which was held together by disulphide bonds, to monomer during the heating of β-lg/κ-CN mixtures. As observed earlier, dissociation of κ-CN was not observed during the heating of κ-CN itself (Fig. 8.3.2.2). This agrees with several investigations using different systems. Aoki et al. (1975) and Aoki and Kako (1983) reported the formation of soluble casein containing approximately 40% κ-CN on heating serum-protein-free casein micelle dispersions above 110 °C.

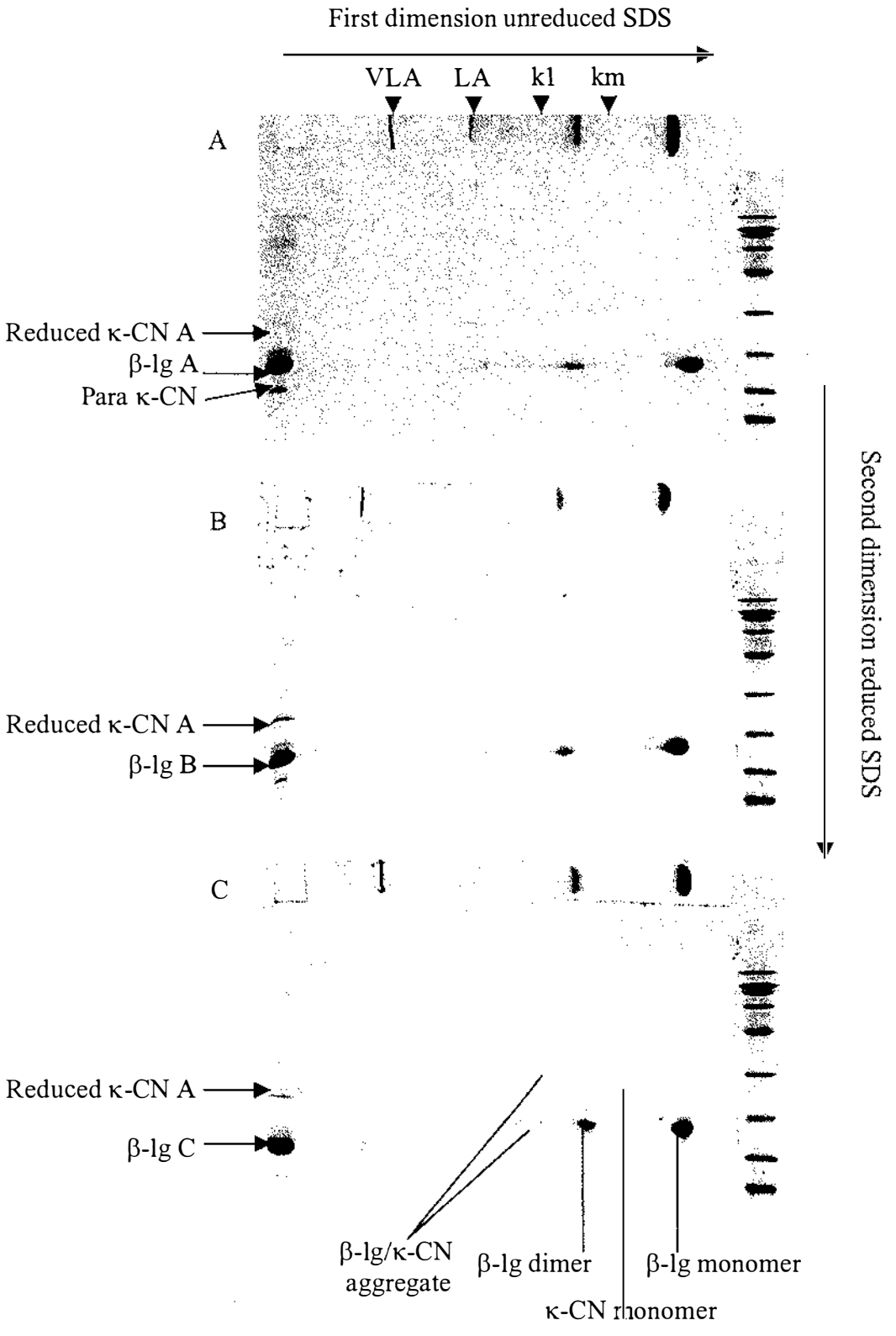

Figure 9.3.2.1. 2D electrophoretic patterns (non-reduced and then reduced SDS-PAGE) of mixture of κ-CN A and (A) β-lg A, (B) β-lg B and (C) β-lg C (2:2, w/w) heated at 80 °C for 10 min.

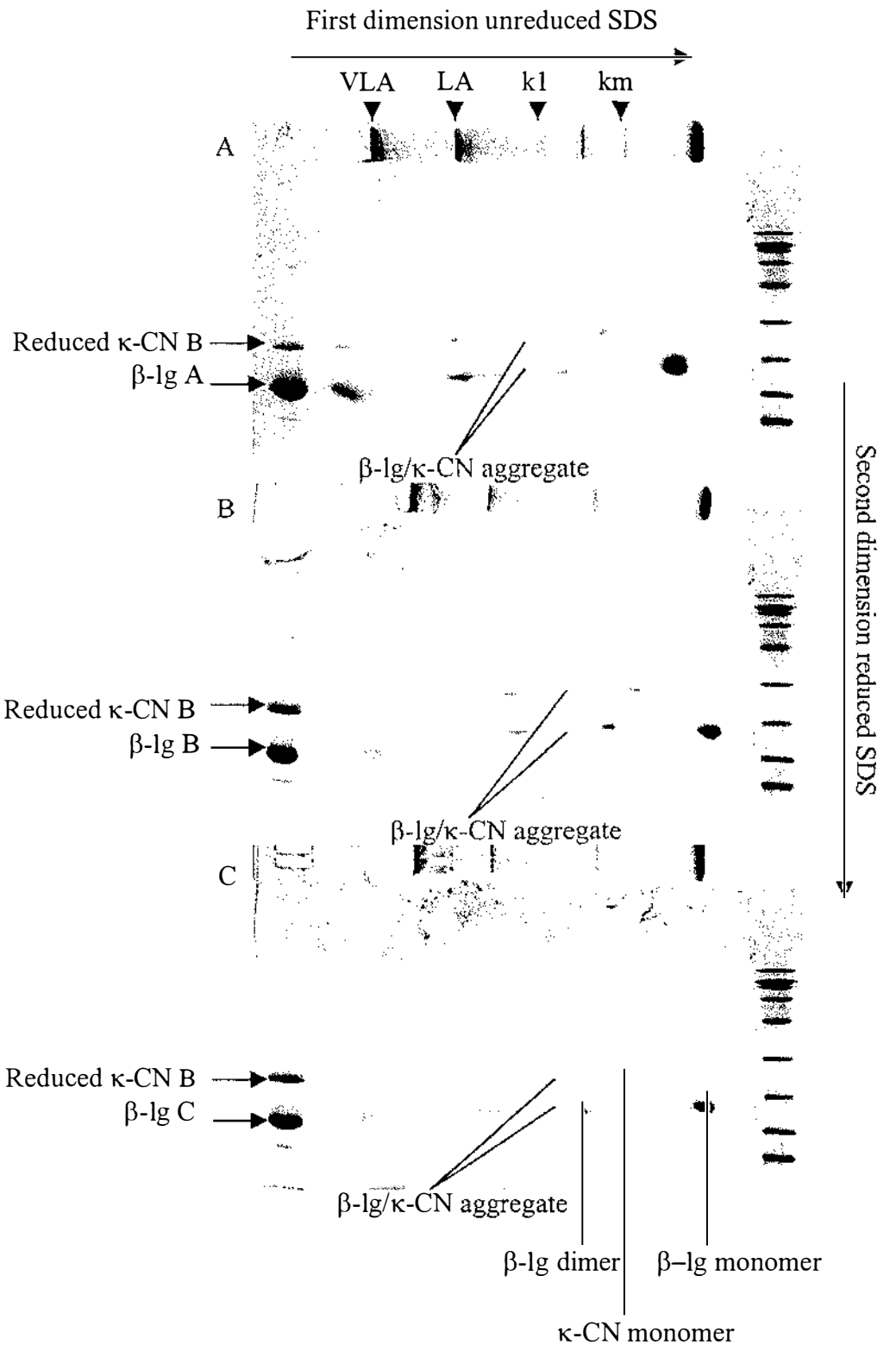

Figure 9.3.2.2. 2D electrophoretic patterns (non-reduced and then reduced SDS-PAGE) of mixture of κ-CN B and (A) β-lg A, (B) β-lg B and (C) β-lg C (2:2, w/w) heated at 80 °C for 10 min.

Singh and Fox (1985, 1986) confirmed that non-sedimentable κ-CN-rich protein dissociated from the micelles on heating milk at temperatures ≥ 90 °C.

Sawyer (1969) suggested that the level of κ-CN dissociation might be a consequence of the ratio of β-lg to κ-CN in the milk. He suggested that a higher β-lg:κ-CN ratio, such as that found in β-lg A milk when compared with β-lg B milk, gave a higher degree of κ-CN dissociation. However, the degree of κ-CN dissociation was dependent on the κ-CN variant in the present study, i.e. κ-CN B showed more dissociation (Fig. 9.3.2.2) than κ-CN A (Fig. 9.3.2.1).

9.3.3. Reaction kinetics of heat-induced interactions between β-lactoglobulin and κ-casein

There are numerous reports of kinetic studies on the denaturation of the major whey proteins in buffer systems (Gough and Jenness, 1962; Harwalkar, 1980; Park and Lund, 1984), whey (Hillier and Lyster, 1979; Kessler and Beyer, 1991), skim milk (Gough and Jenness, 1962; Manji and Kakuda, 1986; Dannenberg and Kessler, 1988b) and reconstituted whole milk (Anema and McKenna, 1996). In this section, to gain a better understanding of the relationship between heat treatment and the denaturation of β-lg/κ-CN mixtures with different variant combinations, the kinetics of the thermal denaturation of alkaline-monomeric β-lg were studied.

Kinetic evaluation of the heat-induced loss of β-lactoglobulin

The results of the heat-induced loss of monomeric β-lg at 80 °C, obtained using alkaline-PAGE, were used to evaluate the kinetics of the interaction and the effect of κ-CN and κ-CN A/B on the loss of native β-lg at pH 6.7. Irreversible denaturation and/or aggregation lead to a decrease in the concentration of native protein molecules. The rate of β-lg concentration is quantified in reaction kinetic terms by the following equation (Dannenberg and Kessler, 1988a, b):

$$v = -(dC_t/dt) = k_n C_t^n \qquad (1)$$

where v is the reaction rate [$(g/L)s^{-1}$], C_t (g/L) is the concentration of native protein at time t, n is the order of the reaction and k_n [$(g/L)^{n-1}s^{-1}$] is the reaction rate constant,

which is a function of temperature and solvent composition. Integration of the general rate equation yields

$$(C_t/C_0)^{1-n} = 1 + (n-1)k_n C_0^{n-1} t \qquad (2)$$

for $n \neq 1$, and

$$\ln(C_t/C_0) = -k_n C_0^{n-1} t = -k_1 t \qquad (3)$$

(where $k_1 = k_n C_0^{n-1}$)

when $n = 1$.

Equations (2) and (3) were used to analyse the results to obtain the overall order n for the heat-induced loss of monomeric β-lg. Linear relationships obtained when $\ln(C_t/C_0)$ was plotted against t would indicate that the denaturation of this protein followed first-order reaction kinetics. Reaction orders of 1.5 and 2.0 were obtained when plots of $(C_t/C_0)^{-0.5}$ and $(C_t/C_0)^{-1.0}$, respectively, against t yielded straight lines. The rate constants, k, were determined from the slopes of the straight lines.

The data of the loss of alkaline-monomeric β-lg were fitted to equation (2) with $n = 1.5$ and $n = 2$ and to equation (3) with $n = 1$. However, the results show that the whole data set could not be described by any reaction order between 1.0 and 2.0. The plots of reaction orders did not yield a single straight line but two lines with different slopes at about 7.5-10 min heating time. Therefore, the data for the loss of alkaline-monomeric β-lg before and after 7.5 or 10 min heating time were fitted to the equations separately and reasonable fits were obtained. Examples of these graphs with $n = 1.5$ are shown in Fig. 9.3.3.1. The best fit was determined by linear regression analysis, and the coefficient values (r^2) were compared (Appendix 2, Tables A2.3.1-A2.3.3) and the changes in the slopes of the graphs were observed at 7.5 or 10 min heating. Figures 9.3.1.4-9.3.1.6 also indicated changes in the rates of the loss of monomeric β-lg and changes in the increases in non-native monomeric and dimeric β-lg at around 7.5-10 min. This probably indicated that the different reaction rates occurred before and after the reversible early steps of thermal denaturation of β-lg.

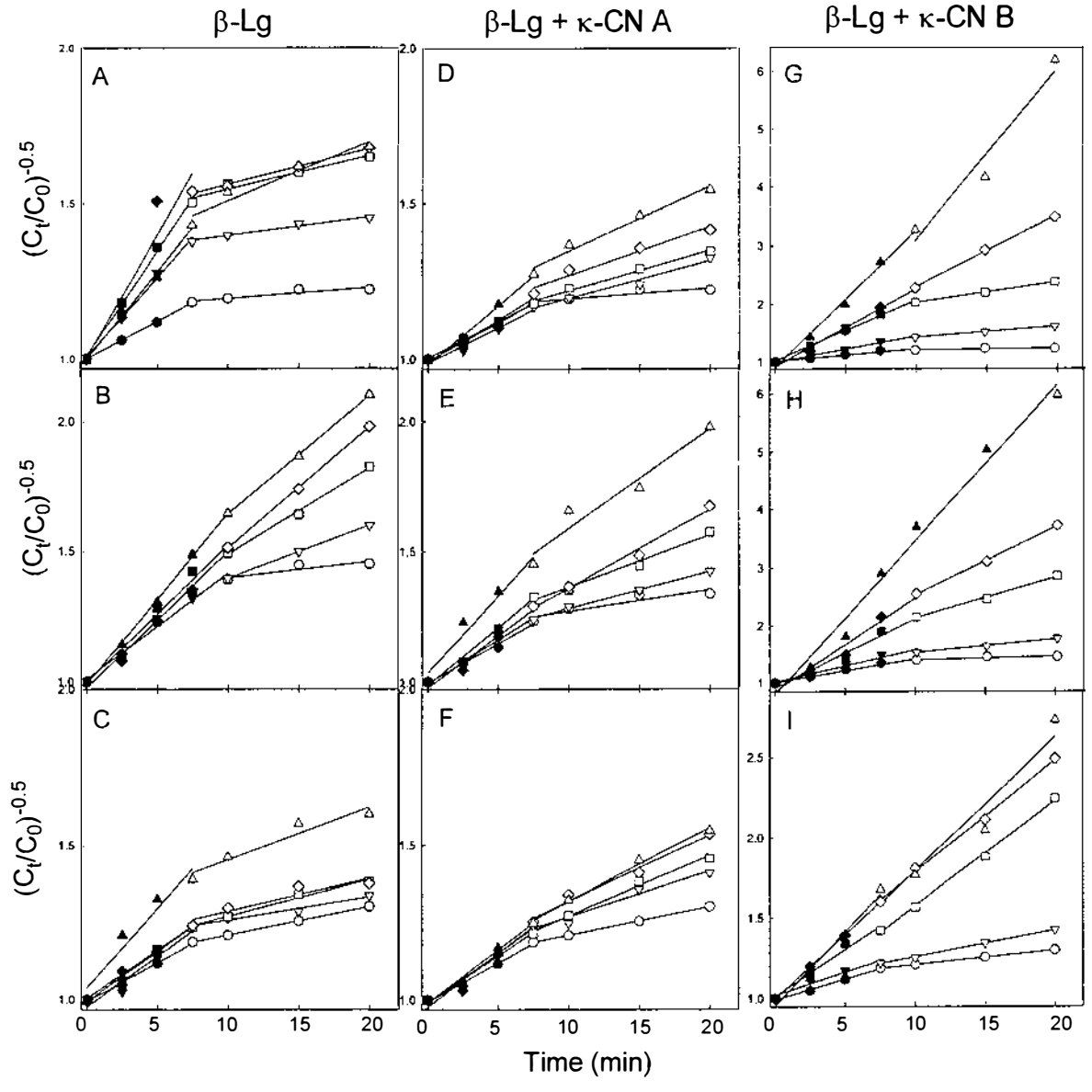

Figure 9.3.3.1. Loss of alkaline-monomeric β-lg at 80 °C from (A) β-lg A, (B) β-lg B and (C) β-lg C; mixture of κ-CN A and (D) β-lg A, (E) β-lg B and (F) β-lg C; mixture of κ-CN B and (G) β-lg A, (H) β-lg B and (I) β-lg C estimated at n = 1.5. The total protein concentrations were 1 (●, ○), 1.5 (▼, ▽), 2.0 (■, □), 2.5 (♦, ◊) and 3.0 (▲, Δ) mg/mL, and filled symbols and open symbols indicated the initial (< 7.5-10 min) and the rest of (< 20 min) of the heating time, respectively.

The initial reaction rate constants (< 7.5-10 min) were plotted against the total protein concentration as shown in Fig. 9.3.3.2. Similar trends were observed with n = 1, 1.5 and 2; the initial rate constants increased with increasing total protein concentration. As the β-lg A concentration increased, the rate constants increased until the total protein concentration exceeded 2.5 mg/mL (Fig. 9.3.3.2.A). The rate constants decreased in the presence of κ-CN A, but increased in the presence of κ-CN B. The effect of κ-CN A on the rate constants depended on the β-lg variant. Unlike β-lg A, β-lg B and β-lg C showed similar rate constants for mixtures of β-lg B and β-lg C with κ-CN A to those for β-lg B and β-lg C alone. Of the three variants of β-lg, the mixtures of β-lg C and κ-CN B showed the least increase in rate constants.

The reaction order for the denaturation of β-lg is not always in agreement. Several studies (Hillier and Lyster, 1979; Park and Lund, 1984; Manji and Kakuda, 1986) have reported second-order kinetics for the thermal denaturation of both β-lg A and β-lg B. In contrast, Gough and Jenness (1962) found that the denaturation of β-lg followed first-order reaction kinetics, whereas McKenzie et al. (1971) and Harwalkar (1980) suggested that the denaturation reaction followed a series of consecutive first-order reactions. Dannenberg and Kessler (1988a, b) and Anema and McKenna (1996) reported a reaction order of 1.5 for the denaturation of both β-lg A and β-lg B. Oldfield et al. (1998), using non-linear regression analysis, reported reaction orders from 1.0 to 1.6 for β-lg denaturation in heated skim milk. On the other hand, Allmere et al. (1998) reported that the denaturation of β-lg in skim milk did not follow true first- or second-order kinetics, varying among the different genetic combinations between β-lg (AA, AB and BB) and κ-CN (AB and BB).

The discrepancies among these studies may be explained by the concentration or composition of the protein solution, the medium of the protein solution, the method and conditions of heat treatment, the method of assay to determine the residual native protein concentration and insufficient data to accurately determine the reaction order (Anema and McKenna, 1996).

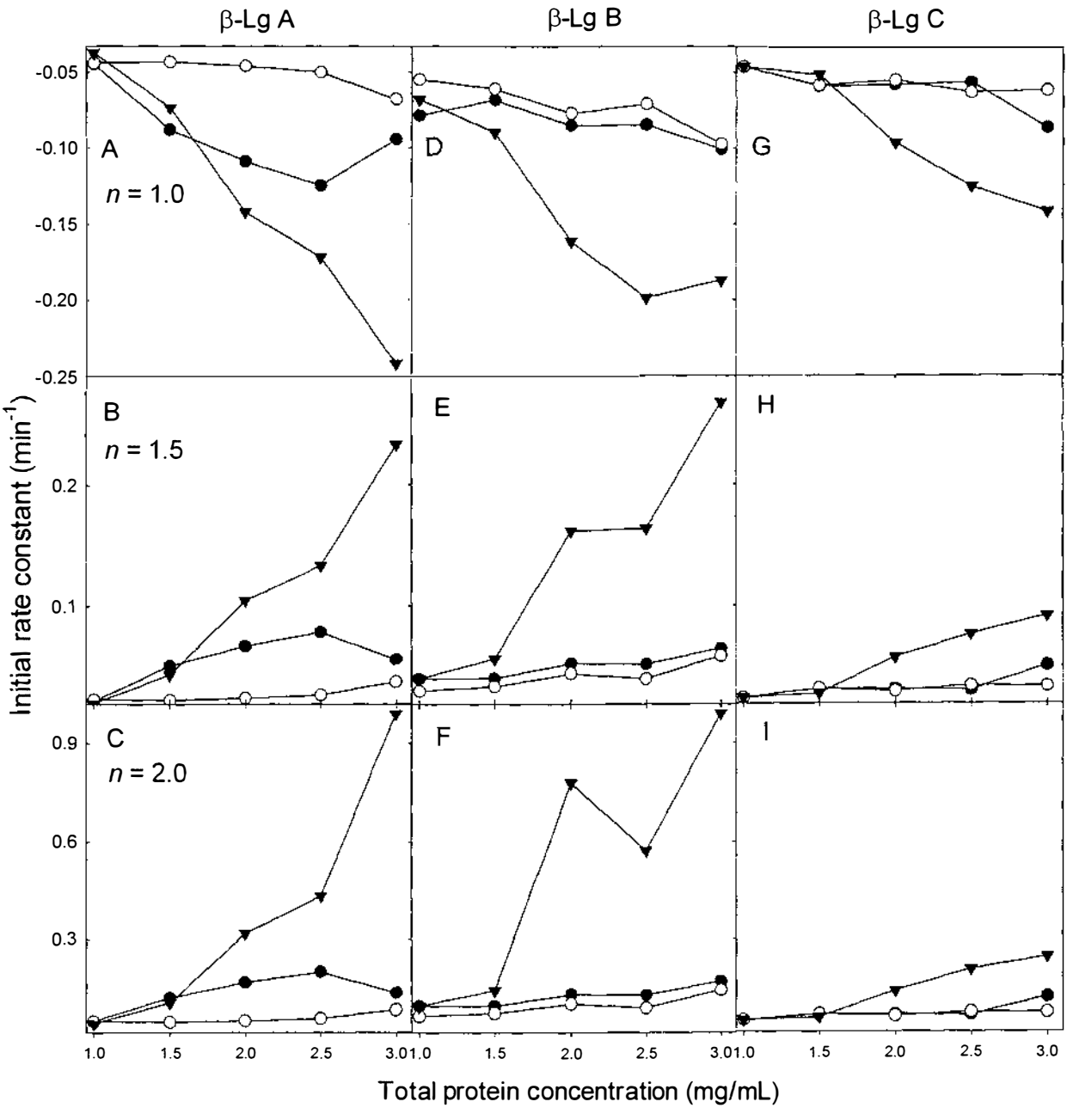

Figure 9.3.3.2. The initial reaction rate constants (< 7.5-10 min) versus total protein concentration obtained from β-lg A when (A) n = 1, (B) n = 1.5 and (C) n = 2; β-lg B when (D) n = 1, (E) n = 1.5 and (F) n = 2; β-lg C when (G) n = 1, (H) n = 1.5 and (I) n = 2. Loss of alkaline-monomeric β-lg obtained from β-lg (●), mixture of β-lg and κ-CN A (○) and mixture of β-lg and κ-CN B (▼).

9.3.4. Heat-induced interactions between pre-heated β-lactoglobulin and native κ-casein

To elucidate the composition of the aggregates formed during heating of a β-lg and κ-CN mixture, pre-heated β-lg (80 °C for 10 min), which contained more visible polymer bands in the resolving gels, and κ-CN were mixed, heat treated and analysed using alkaline- and SDS-PAGE.

Alkaline-PAGE

The alkaline-PAGE gels of the heat-treated (at 80 °C for up to 60 min) mixtures of pre-heated β-lg (at 80 °C for 10 min) and κ-CN are shown in Fig. 9.3.4.1. Each lane 1 contained a β-lg sample heated for 10 min and lane 2 contained the mixture of pre-heated β-lg and unheated native κ-CN before reheat treatment. The disappearance of some of the β-lg bands formed from previous heating was observed by just adding unheated κ-CN solution (lane 2 of each gel), and was more clearly shown in the presence of κ-CN B (Fig. 9.3.4.1.D to F) than κ-CN A. As the reheating time increased, polymeric β-lg bands disappeared rapidly and large aggregates started to form on the top of the resolving gels.

SDS-PAGE

The same samples were analysed using SDS-PAGE and the results are shown in Fig. 9.3.4.2. As the reheating time increased, the monomeric and dimeric β-lg band intensities decreased. Bands km (κ-CN monomer) and k1 (disulphide-linked β-lg/κ-CN aggregate) were found in the SDS-PAGE gels and the disappearance of the k1 band was observed.

The monomeric and polymeric β-lg bands of the mixture of β-lg B and κ-CN B in lane 10, which was obtained after reheating at 80 °C for 60 min, almost vanished compared with the other sets. This result agrees with previous results (without pre-heating of β-lg), viz. the B variant of κ-CN was the more reactive and the B variant of β-lg was the most reactive among the six genetic variant combinations.

Alkaline-PAGE

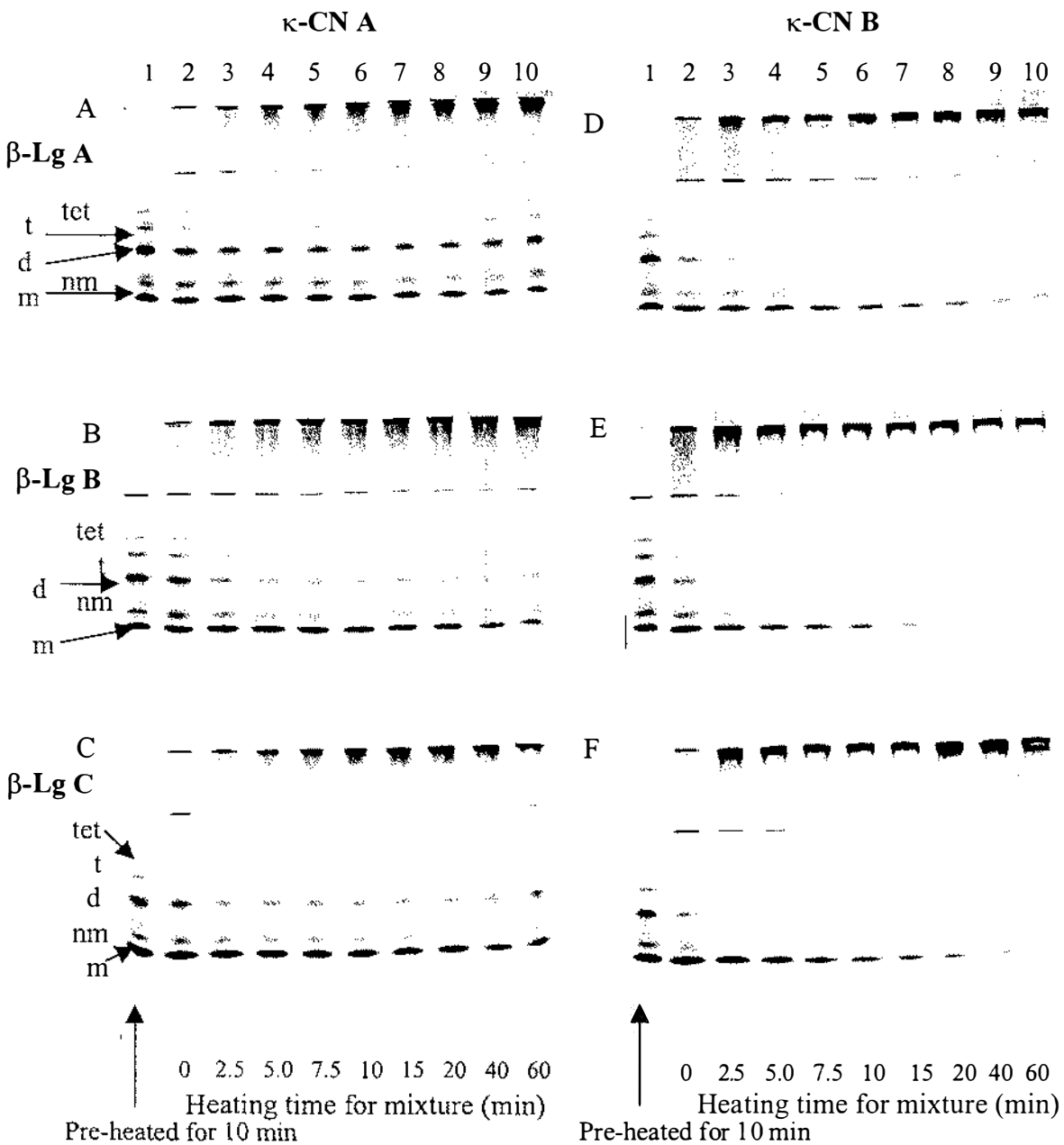

Figure 9.3.4.1. Alkaline-PAGE patterns of a pre-heated β-lg and κ-CN mixture (2:2, w/w) heated at 80 °C for: lane 2, 0 min; lane 3, 2.5 min; lane 4, 5 min; lane 5, 7.5 min; lane 6, lane 10 min; lane 7, 15 min; lane 8, 20 min; lane 9, 40 min; lane 10, 60 min. Lane 1, β-lg pre-heated for 10 min. Mixture of κ-CN A and (A) β-lg A, (B) β-lg B and (C) β-lg C; mixture of κ-CN B and (D) β-lg A, (E) β-lg B and (F) β-lg C.

SDS-PAGE

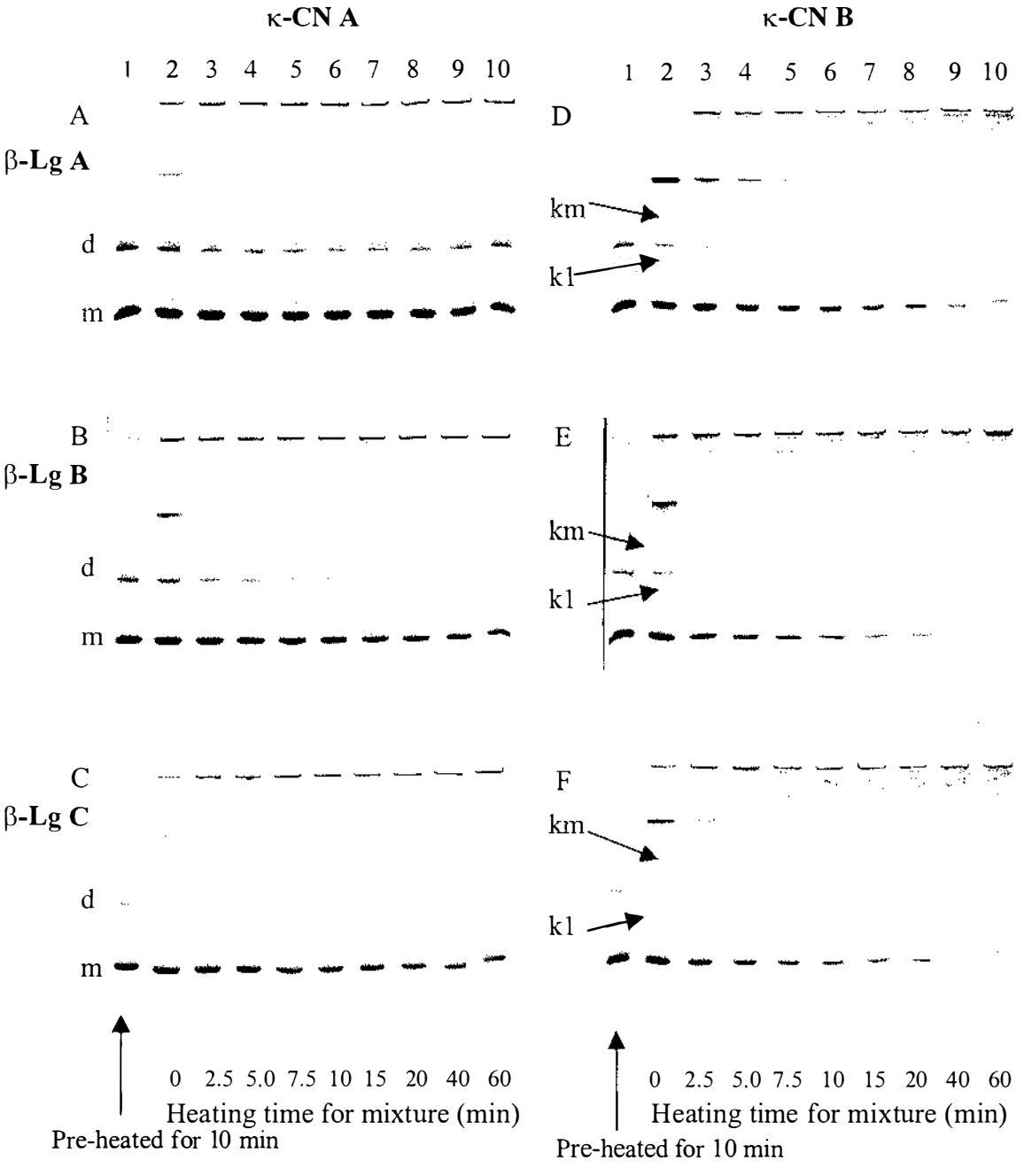

Figure 9.3.4.2. SDS-PAGE patterns of a pre-heated β-lg and κ-CN mixture (2:2, w/w) heated at 80 °C for: lane 2, 0 min; lane 3, 2.5 min; lane 4, 5 min; lane 5, 7.5 min; lane 6, lane 10 min; lane 7, 15 min; lane 8, 20 min; lane 9, 40 min; lane 10, 60 min. Lane 1, β-lg pre-heated for 10 min. Mixture of κ-CN A and (A) β-lg A, (B) β-lg B and (C) β-lg C; mixture of κ-CN B and (D) β-lg A, (E) β-lg B and (F) β-lg C.

Quantitative analysis of alkaline-PAGE

The heat-induced changes in the band intensities obtained from mixtures of pre-heated β-lg and native κ-CN in the six variant combinations were compared using alkaline-PAGE (Fig. 9.3.4.3). In the presence of κ-CN A (Fig. 9.3.4.3.A to C), the band intensities of monomers and non-native monomers of pre-heated β-lg slightly increased just after mixing with κ-CN A and then decreased as the reheating time increased. This can be interpreted as the dissociation of β-lg aggregates caused by the mixing with κ-CN A. In contrast, in the case of κ-CN B (Fig. 9.3.4.3.D to F), the concentration of β-lg monomers increased with mixing for β-lg B and β-lg C, but the concentrations of β-lg non-native monomer and β-lg dimer decreased. This suggests that β-lg non-native monomer and β-lg dimer were more reactive than monomers in the interaction with κ-CN and that the B variant of κ-CN was more reactive as shown by the instant decrease in non-native monomers. The decrease in the concentration of large aggregate with increasing heating time, after the instant increase caused by the presence of κ-CN, confirmed the participation of κ-CN in the aggregation and the formation of very large aggregates during heating.

Quantitative analysis of SDS-PAGE

The same samples were analysed using SDS-PAGE and the results are shown in Fig. 9.3.4.4. Similar trends were observed to those obtained with alkaline-PAGE, except that there was a decrease in β-lg monomer concentration after mixing with κ-CN B.

Complex formation occurred to a limited extent when the heated and then cooled β-lg solution was mixed with κ-CN solution. Previously Long et al. (1963) noted that mixing of heated β-lg with unheated κ-CN at room temperature was sufficient to initiate some complex formation. They found that no interaction occurred when unheated β-lg was added to heated or unheated κ-CN, which was also observed in Section 8.3.3. Using electrophoresis, Zittle et al. (1962) also showed that heating β-lg before mixing with κ-CN brought about interaction at 20-25 °C. They suggested that the β-lg was activated by heating, perhaps by the exposure of –SH groups so that interaction occurred at the lower temperature. Also heating κ-CN alone did not lead to interaction when subsequently mixed with β-lg.

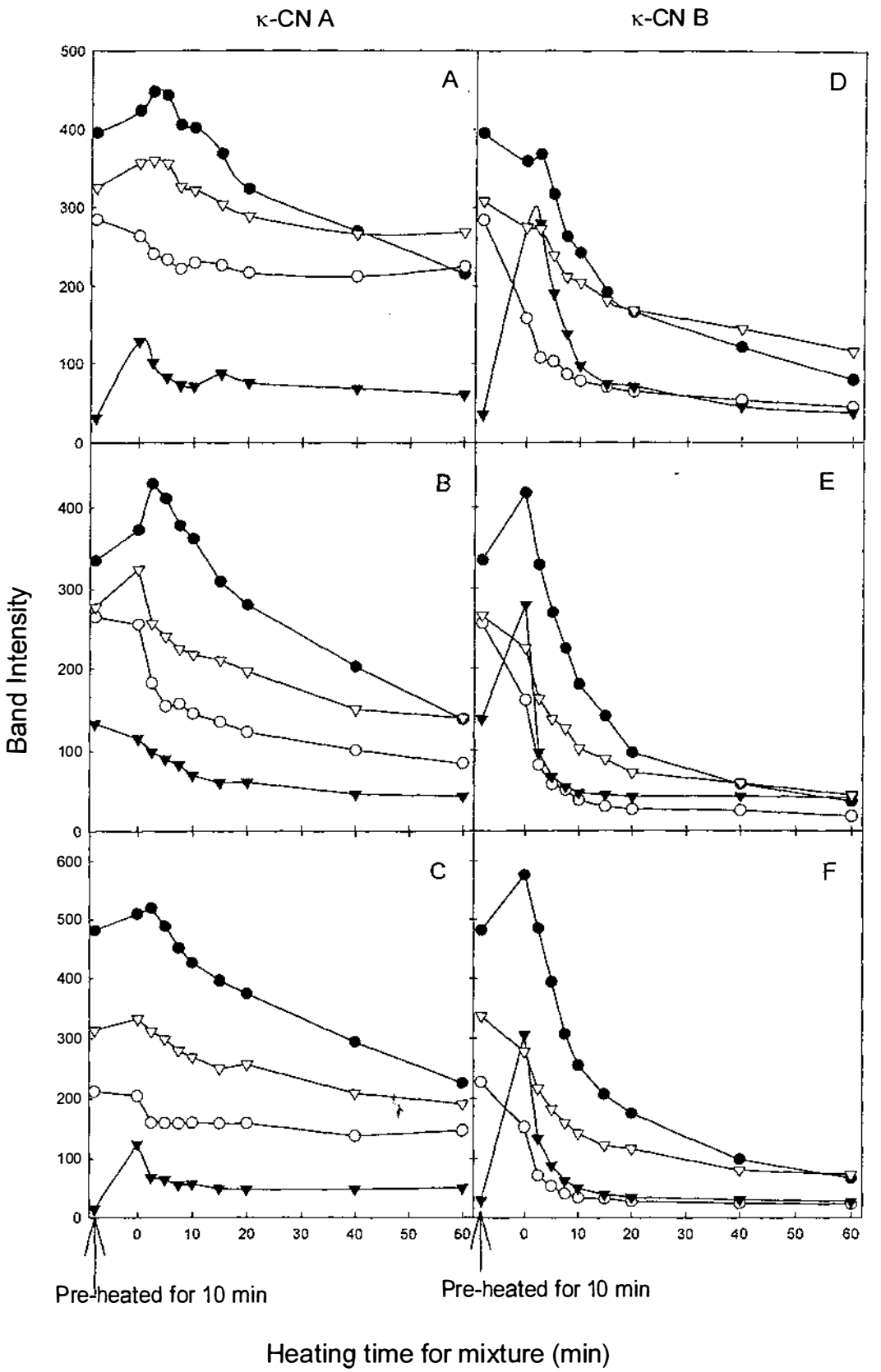

Figure 9.3.4.3. Changes in the band intensities for samples of mixture (2:2, w/w) of κ-CN A and (A) β-lg A, (B) β-lg B and (C) β-lg C; mixture of κ-CN B and (D) β-lg A, (E) β-lg B and (F) β-lg C heated at 80 °C for various times in alkaline-PAGE. Monomer (●), non-native monomer (▽), dimer (○) and large aggregates (▼).

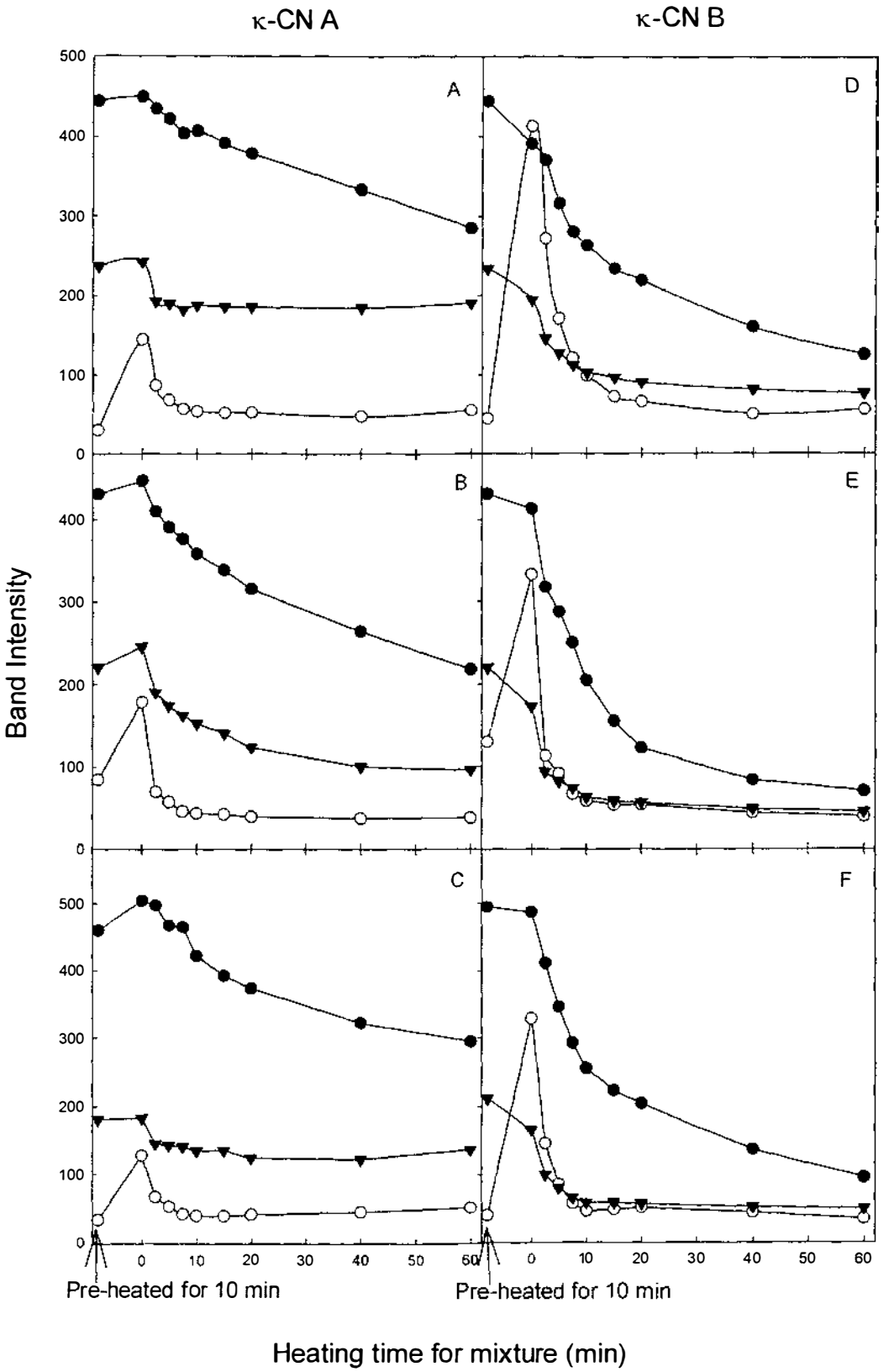

Figure 9.3.4.4. Changes in the band intensities for samples of mixture (2:2, w/w) of κ-CN A and (A) β-lg A, (B) β-lg B and (C) β-lg C; mixture of κ-CN B and (D) β-lg A, (E) β-lg B and (F) β-lg C heated at 80 °C for various times in SDS-PAGE. Monomer (●), dimer (○) and and large aggregates (▼).

The structure of κ-CN is altered by heating as shown in Fig. 8.3.1.1.C. However, it is noted that heated κ-CN solutions frequently retain their ability to stabilise calcium-sensitive caseins (Zittle et al., 1962).

9.3.5. General discussion

Sawyer (1969) suggested that the primary thermodenaturation of β-lg itself involves disulphide aggregation and it may be that only the aggregated form is capable of association with κ-CN in a non-specific manner. Alternative interactions (non-disulphide) are also possible (Haque et al., 1987; Parris et al., 1991), but there is strong evidence for the final interaction being via disulphide bonding (Jang and Swaisgood, 1990). Noh et al. (1989) reported that whey proteins denaturing in milk during heat treatment used κ-CN as a nucleation site for the formation of heat-induced complexes involving disulphide bonding. They also showed that these heat-induced complexes, like κ-CN itself, remained associated with the external surface of the casein micelle and continued to enlarge with additional heat treatment. The results of the present study (Section 9.3.4) suggested that such behaviour may be attributed to the preferential binding of the aggregated form of β-lg to κ-CN.

The self-aggregation of β-lg molecules would be concentration dependent (McSwiney et al., 1994a, b). Thus, at high mixing ratios (i.e. high β-lg concentrations), the aggregated form of β-lg would be favoured and the amount of β-lg/κ-CN complex would increase. However, as shown in Sections 8.3.3 and 9.3.1, the rate of interaction increased with increasing concentration of κ-CN. Therefore, the interaction between β-lg and κ-CN was affected by not only the denaturation of β-lg but also the availability of the disulphide bond of κ-CN, i.e. the state of self-association of κ-CN may play an important role in the interaction. This could explain the different reactivities of the κ-CN A and B variants which can be caused by different structures (pattern of disulphide bonds or aggregated form) or the degree of glycosylation.

In all cases, the B variant of κ-CN was the more reactive and the B variant of β-lg was the most reactive. It was noted that κ-CN reacted more rapidly with β-lg that had been unfolded by prior heat treatment than with native β-lg. In the presence of κ-

CN, the band intensities of non-native monomers and dimers of pre-heated β-lg decreased just after mixing and then decreased continuously.

The reaction kinetics could not be described by any reaction order between 1.0 and 2.0. Plots of reaction orders did not yield a single straight line but two lines with different slopes at about 7.5-10 min heating time, which coincided with the changes in the rates of the loss of monomeric β-lg and the changes in the rates of the increase in non-native monomeric and dimeric β-lg concentration. This probably suggests that the reaction rates between β-lg and κ-CN changed about when the reversible step monomer-dimer of β-lg reached. The initial reaction rate constants were affected by variants of both κ-CN and β-lg.

At temperatures above 30 °C, β-lg (native dimer) dissociates into the monomer form. Above 55 °C, it begins to unfold and lose its globular structure (Gough and Jenness, 1962); its thiol and disulphide groups become more reactive and the molecules undergo aggregation reactions. The heat activation of endogenous sulphydryl and disulphide groups, which is also accompanied by partial denaturation, may be sufficient for the interaction with κ-CN to proceed. It was concluded that β-lg probably denatured (unfolded) independently and either simultaneously, or consequently, reacted via disulphide interchange reactions with κ-CN.

The observed differences in the heat stability and other qualitative characteristics found in this study may be related to a number of factors, an important factor being the thiol reactivity of β-lg. Sawyer et al. (1963) reported the importance of thiol or disulphide groups in the formation of the β-lg/κ-CN complex. They suggested that β-lg unfolds and aggregates through a series of parallel and consecutive steps, some of which involve thiol-disulphide bond interchange, whereas others involve hydrophobically driven association reactions between β-lg molecules (Gezimati et al., 1997; Manderson et al., 1998). Sawyer (1969) proposed that the thiol groups are more important for the initial aggregation of β-lg itself than for the subsequent β-lg/κ-CN interaction, and this probably has a great importance also for the reaction in total. It is known that the thiol group in native β-lg C is less reactive toward DTNB than the thiol group in the A and B variants (Purkayastha et al., 1967; Manderson et al., 1998). This probably explains the high heat stability of the β-lg C variant.

The size and shape of the κ-CN polymers and the available κ-CN per unit mass of β-lg can be important factors in addition to the pattern of disulphide bonds. The self-association of κ-CN may depend on the available monomers of this protein in solution and their genetic type (Imafidon et al., 1991a). It could be assumed that the κ-CN AA samples in this investigation may be more polymeric and consequently show lower reaction reactivity than the κ-CN BB samples and this was confirmed in this study.

From all six possible combinations of the homozygous phenotypes of β-lg (AA, BB and CC) and κ-CN (AA and BB), it can be concluded that the reaction rate was influenced highly by the genotype of β-lg, but the κ-CN B variant showed considerably higher reactivity than κ-CN A. In conclusion, the loss of native β-lg in β-lg/κ-CN solutions during heating at 80 °C was shown to be significantly influenced by the genotypes of both β-lg and κ-CN. The double homozygote β-lg BB/κ-CN BB was also found to have the highest reaction rate.

CHAPTER 10.

CONCLUSIONS - *Part II*

Heat-induced interactions between β-lactoglobulin and κ-casein

The heat-induced interactions of β-lg with κ-CN were investigated in Chapters 8 and 9. It was shown (Fig. 8.3.1.2) that the near-UV CD spectral changes in the β-lg A and κ-CN B mixture during heat treatment were mostly irreversible. With increasing temperature, the decreases of band intensities at both the 285 and 293 nm (tryptophan CD bands) and the increase of band intensity at 270 nm (the indication of a reorganisation of the disulphide bonding) were observed. Subsequent cooling of the protein solution did not cause any change in the CD spectrum (Fig. 8.3.1.2.C), which indicated that the heat-induced spectral changes for the mixture of β-lg A and κ-CN B were essentially caused by irreversible disulphide bond changes that involved κ-CN. However, the instability of the κ-CN solution and precipitation of κ-CN during heating made quantitative measurement difficult.

The effect of κ-CN on the established heat-induced unfolding and aggregation pathway of β-lg was examined by adding κ-CN A or κ-CN B to native or pre-heated β-lg A, B or C and heating the mixture at 80 °C. In the presence of κ-CN, the loss of monomeric β-lg increased and less non-native monomer and dimer were observed compared to β-lg alone. It is possible that κ-CN preferred to interact with denatured (unfolded) β-lg that has higher sulphydryl reactivity than native β-lg, i.e. κ-CN accelerated the aggregation of β-lg, probably because reversible steps in the pathway became less reversible in the presence of κ-CN. It was also concluded that κ-CN was not a catalyst, but a reactant during the aggregation process. After the initial stage of aggregation between β-lg and κ-CN, the intermediate sized aggregates immediately joined to form large aggregates. Heat-induced unfolding of both β-lg and κ-CN can permit higher sulphydryl reactivity that can lead to disulphide interchange and aggregation, although aggregation can also occur without the involvement of the sulphydryl group.

During reaction with β-lg, some monomeric κ-CN was found by SDS-PAGE, probably as a result of disulphide bond interchanges between κ-CN and β-lg and some stability in the disulphide bond of κ-CN monomer. 2D-PAGE also revealed disulphide-bonded β-lg/κ-CN aggregates. Heating of β-lg and κ-CN mixtures apparently causes rapid disulphide interchange, resulting in more monomeric species and more polymeric species in both β-lg and κ-CN.

It was also noteworthy that κ-CN reacted more rapidly with β-lg that had been unfolded by prior heat treatment than with native β-lg. The aggregation of β-lg does not seem to be a prerequisite for its interaction with κ-CN; heat-induced unfolding of β-lg may be sufficient for the interaction to proceed. It was concluded that β-lg probably denatured (unfolded) independently and either simultaneously or consequently and contained higher sulphydryl reactivity than native β-lg, which lead to the reactions with κ-CN via disulphide bond interchange (Fig. 8.3.5.1).

The results of the heat-induced loss of monomeric β-lg at 80 °C obtained from alkaline-PAGE were used to evaluate the kinetics of the interaction between β-lg and κ-CN at pH 6.7. Plots of reaction orders did not yield a single straight line but two lines with different slopes at about 7.5-10 min heating time. This indicates that the interaction between β-lg and κ-CN cannot be described by any reaction order between 1.0 and 2.0. The slopes changing at about 7.5-10 min heating time corresponded to intensity changes in the alkaline-monomeric, non-native monomeric and dimeric β-lg bands, which increased during the first 10 min of heating and then slightly decreased or remained relatively constant for the rest of the heating. This probably indicated that the different reaction rates occurred before and after the reversible early steps of thermal denaturation of β-lg.

In all cases, the B variant was the more reactive κ-CN and the B variant was the most reactive β-lg. The observed differences in the heat stability and other qualitative characteristics of heat-treated β-lg and κ-CN solutions may be partly explained by the genetic polymorphism of β-lg and κ-CN. κ-CN B may reduce the surface charge and increase the hydration of the β-lg/κ-CN complex more than κ-CN A because of its less negatively charged nature, i.e. κ-CN B (Ile-136 and Ala-148) is less net negatively

charged than κ-CN A (Thr-136 and Asp-148) at pH 6.7. Also the state of self-association of κ-CN may play an important role in the interaction, which can be altered by changes in pH, concentration, ionic strength and temperature. From all six possible combinations of the homozygous phenotypes of β-lg (AA, BB and CC) and κ-CN (AA and BB), it can be concluded that the reaction rate was influenced highly by the genotype of β-lg, which showed a consistent heat stability order (β-lg C > β-lg A > β-lg B) with/without κ-CN. But the κ-CN B variant showed considerably higher reactivity than κ-CN A. In conclusion, the reaction rates of β-lg/κ-CN solutions during heating at 80 °C were shown to be significantly influenced by the genotypes of both β-lg and κ-CN. The double homozygote β-lg BB/κ-CN BB was also found to have the highest reaction rate.

Interactions between proteins and various components that were absent from the buffers used in this study must be recognised when applying these results to whole milk. Studies of this model system do not prove that intermolecular disulphide complexes form between β-lg and κ-CN in micelles under conditions similar to those in milk.

In terms of future studies, determining the disulphide bonding pattern in native and heated κ-CN, heated β-lg and heated mixtures of β-lg and κ-CN would provide a clearer understanding and may elucidate the pathway during the interactions between β-lg and κ-CN. Further characterisation of κ-CN A and B variants (e.g. the degree of aggregation and glycosylation in the native protein) could be important factors that determine the greater reactivity of κ-CN B.

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APPENDIX 1. The effect of ammonium sulphate concentration on the unfolding of ligand-bound β-lactoglobulin

A1.1. INTRODUCTION

Co-crystallisation of a ligand and bovine β-lg is a significant problem. There are two methods of introducing a ligand into a protein crystal. The first is to equilibrate the protein solution with an excess of the ligand and then grow the crystals. The second method relies upon the significant amount of space available within the crystals such that a ligand can percolate through the lattice to the binding sites on every molecule. The latter method is generally preferred, because the problem of crystallisation is overcome (Sawyer et al., 1999). Before X-ray diffraction data are collected, samples are usually subjected to ammonium sulphate crystal screening. In general, β-lg will produce crystals in the lattice Z form in 2-4 days over an ammonium sulphate concentration range of 2.2-2.8 M (Qin et al., 1998b).

The effect of the concentration of ammonium sulphate on the binding of bovine β-lg A with ligands was investigated using near-UV CD in the work described in this appendix. The molar ratio of β-lg to ligand was 1:1.1-1.2 for three different ligands and the pHs of the protein solutions were 7.1 and 8.5.

Two types of experiment were performed: (i) mixing ammonium sulphate into β-lg solution first and then adding the appropriate amount of ligand solution (molar ratio β-lg:ligand = 1:1.1-1.2); or (ii) adding ammonium sulphate solution to a ligand-bound β-lg solution. There were no differences between the results obtained by the two methods.

A1.2. EXPERIMENTAL PROTOCOL

Measurement protocols

Protein solutions were dialysed against pH 7.1 or pH 8.5 Tris-phosphate buffer and were diluted to 1.5 mg/mL with buffer. Retinol, retinyl acetate and PnA were made to 1 mg/mL in boiled and degassed ethanol and stored under oxygen-free nitrogen in the dark. On occasions, equimolar concentrations of BHT were mixed into these solutions.

CD measurements

For investigation of ligand binding, the molar ratio β-lg:ligand was kept between 1:1.1 and 1:1.2 throughout the experiment. The β-lg solutions were placed in a water-jacketed 10 mm path length CD cell (Jasco, Ishikawa-cho, Hachioji city, Tokyo, Japan) which was connected to a Neslab model RTE-100 water bath (Neslab Instruments Inc., Newington, NH, USA). Each β-lg solution was held at 20 °C for 20 min prior to measuring the spectrum.

The temperature of the water bath was then increased to 40 °C and held for 20 min, and then another spectrum was run. This procedure was repeated at 10 or 20 °C intervals until a temperature of 80 °C was reached. The water bath temperature was then decreased by 20 °C increments to 20 °C. The baseline correction has been described in Section 3.2.2.

A1.3. RESULTS AND DISCUSSION

A1.3.1. Retinol

The final concentration of β-lg A in pH 7.1 or pH 8.5 Tris-phosphate buffer was 1 mg/mL after mixing with ammonium sulphate and ligand solution. As the pH increased from 7.1 to 8.5, the band intensities at 285 and 293 nm decreased slightly and a decreasing band intensity below 280 nm was also observed although the overall spectra at the two pHs were quite similar.

Addition of retinol to β-lg A in pH 7.1 Tris-phosphate buffer induced a peak at 255 nm and a broad trough centred at about 350 nm and increased the intensity of the tryptophan CD band at 293 nm (Fig. A1.3.1.A). At pH 8.5, the effect of retinol addition was magnified and more intense bands were observed at 255 and 340 nm, as shown in Fig. A1.3.1.B. It has been reported that loop EF (residues 85-90) of bovine β-lg forms a lid to the calyx, which is closed in β-lg A in crystals formed at pH 6.2 and open at pH 7.1 and 8.2 (Qin et al., 1998b). This conformational change accounts for the physical and chemical pH-dependent properties of β-lg and has functional implications for the reversible binding and release of ligands. It seems that the equilibrium in the opening and closure of loop EF shifted with an increase in the pH of the protein solution.

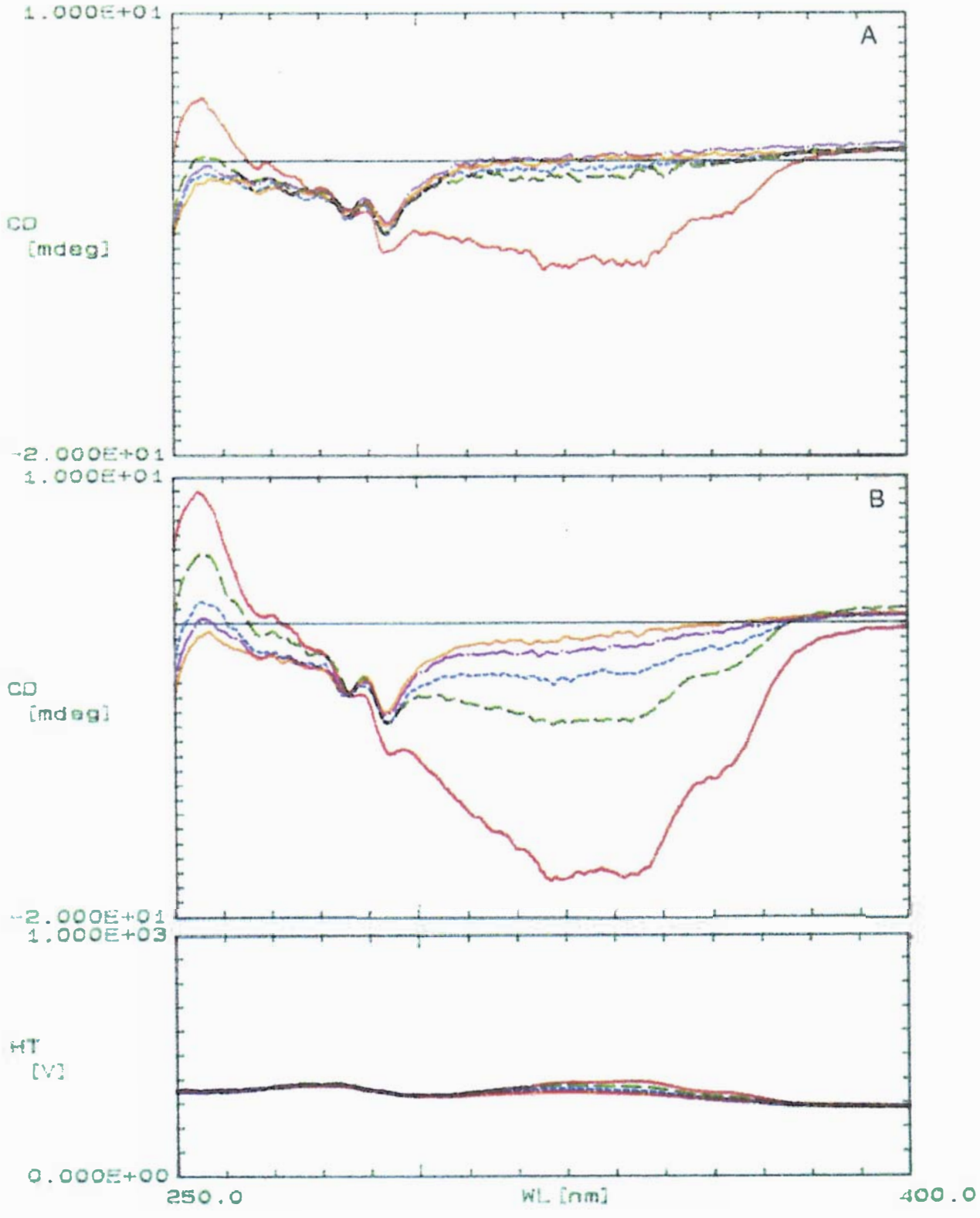

Figure A1.3.1. Near-UV CD spectra of mixtures of retinol (90 μL of 1 mg/mL = 101.94 μM) and β-lg at a concentration of 1.5 mg/mL (84.2 μM) in 0.02 M Tris-phosphate buffer at (A) pH 7.1 and (B) pH 8.5 at 0 M (red solid), 0.5 M (green dashed), 1.0 M (blue dotted), 1.5 M (purple centre) and 2.2 M (orange solid) ammonium sulphate. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section A1.2.

As the pH of the β-lg solution increased, more open-calyx-formed β-lg would be expected and this may have caused the more intense induced bands in the near-UV CD spectrum.

With increasing ammonium sulphate concentration, the CD bands at 255 and 350 nm in retinol/β-lg mixtures rapidly disappeared at both pH 7.1 and pH 8.5 (Fig. A1.3.1). Almost total absence of induced CD signals at 255 nm and 350 nm in retinol/β-lg mixtures was observed when about 1.5 M ammonium sulphate was added at both pHs, probably suggesting detachment of retinol from the β-lg molecules. This provides an explanation of the difficulty in co-crystallising β-lg and bound ligand.

A1.3.2. Retinyl acetate

The same procedure was carried out with retinyl acetate instead of retinol. Addition of retinyl acetate to β-lg A in pH 7.1 Tris-phosphate buffer gave rise to a very noisy spectrum with bands above 300 nm that were induced by retinyl acetate. As the concentration of ammonium sulphate increased, the bands above 300 nm disappeared (Fig. A1.3.2.A).

However, the induced peak at 255 nm and the trough at 380 nm were clearly observed at pH 8.5 (Fig. A1.3.2.B) and the spectrum was very similar to that of the retinol/β-lg mixture at pH 7.1 (Fig. A1.3.1.A) rather than to the retinyl acetate/β-lg mixture at pH 7.1. The intensity of the tryptophan CD band at 293 nm was slightly increased by the addition of retinyl acetate at pH 8.5 (Fig. A1.3.2.B). An increasing ammonium sulphate concentration restored the spectrum to that of β-lg before the addition of retinyl acetate.

The different behaviors at pH 7.1 and pH 8.5 appeared to be caused by the acetyl group of retinyl acetate, which can cause a drop in the pH of the mixture.

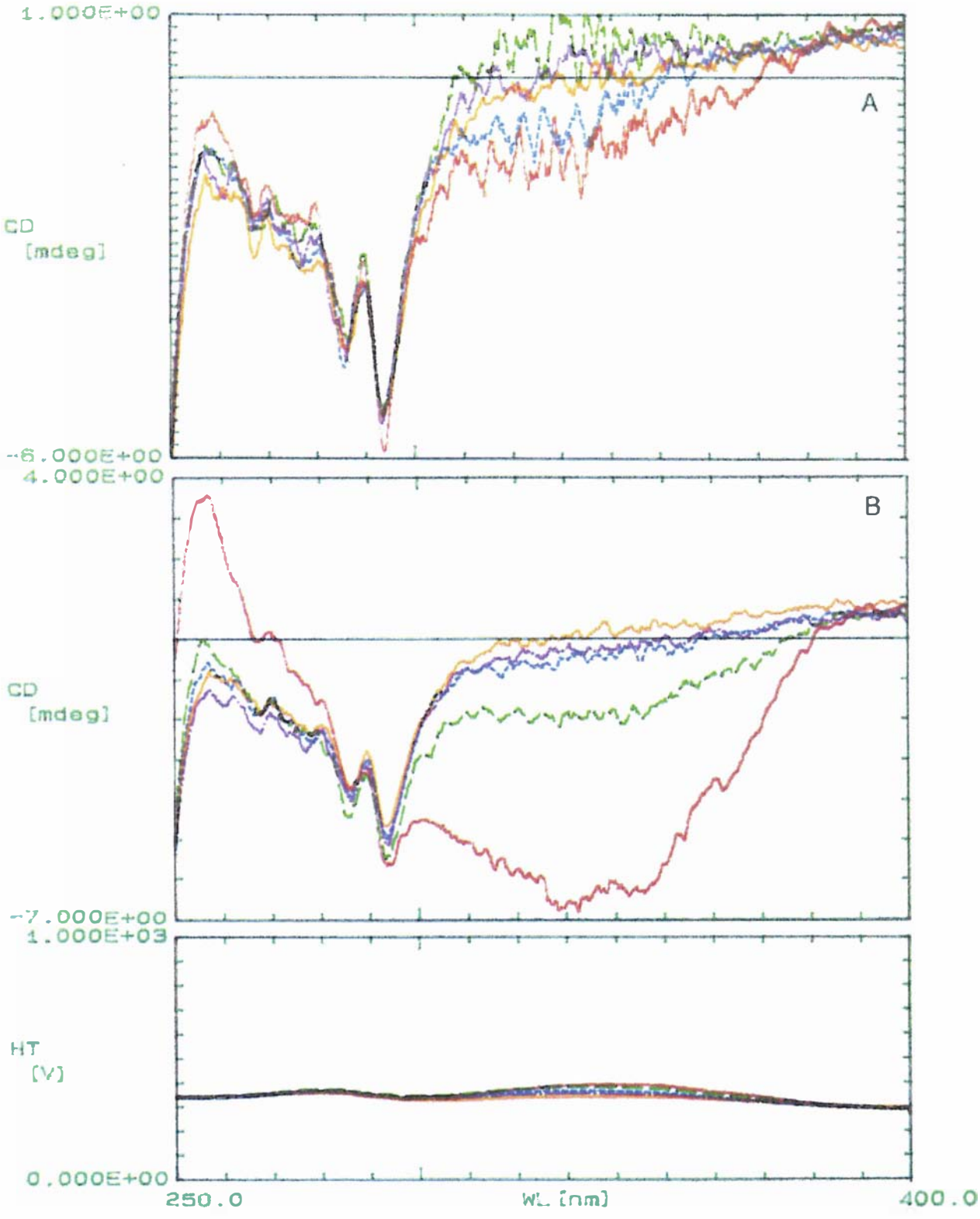

Figure A1.3.2. Near-UV CD spectra of mixtures of retinyl acetate (90 μL of 1 mg/mL = 88.66 μM) and β-lg at a concentration of 1.5 mg/mL (84.2 μM) in 0.02 M Tris-phosphate buffer at (A) pH 7.1 and (B) pH 8.5 at 0 M (red solid), 0.5 M (green dashed), 1.0 M (blue dotted), 1.5 M (purple centre) and 2.2 M (orange solid) ammonium sulphate. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section A1.2.

A 1.3.3. cis-Parinaric acid

The intensities of the induced signals of the PnA/β-lg mixtures were similar at both pH 7.1 and pH 8.5 (Fig. A1.3.3.A, B). Addition of PnA to β-lg A gave rise to four deep troughs at 286, 293, 310 and 325 nm. As the concentration of ammonium sulphate added to β-lg increased, the intensities of these bands decreased at both pH values, but the effect was more apparent at pH 7.1. Similar absorption spectra of PnA were reported by Sklar et al. (1977b) using a strong near-UV transition with vibronic structure.

However, unlike retinol and retinyl acetate, with increasing ammonium sulphate the spectrum was not restored to that of β-lg alone (i.e. without PnA). This explains why co-crystallisation of fatty acid/β-lg was achieved earlier than that of retinoid/β-lg.

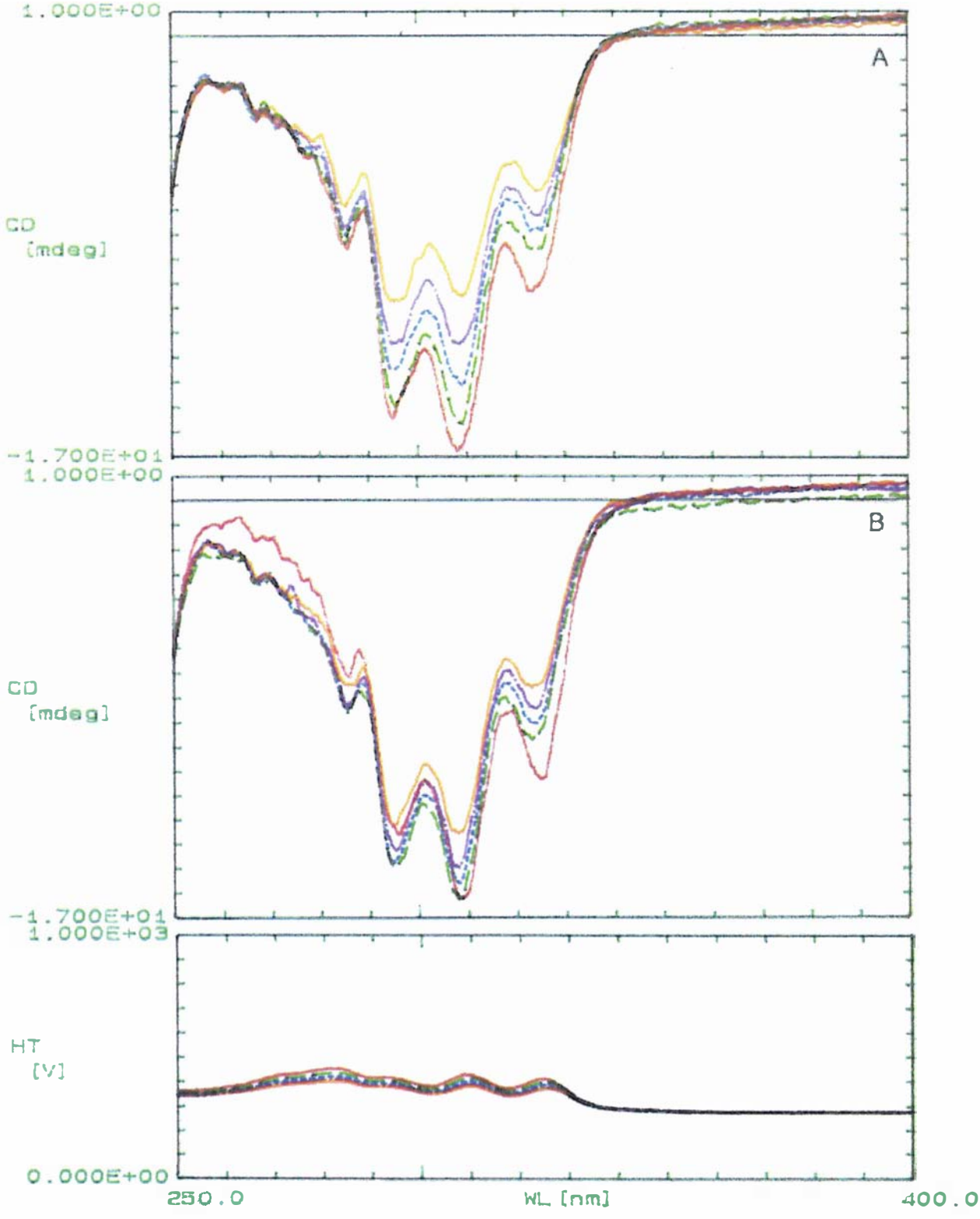

Figure A1.3.3. Near-UV CD spectra of mixtures of PnA (90 μL of 1 mg/mL = 93.09 μM) and β-lg at a concentration of 1.5 mg/mL (84.2 μM) in 0.02 M Tris-phosphate buffer at (A) pH 7.1 and (B) pH 8.5 at 0 M (red solid), 0.5 M (green dashed), 1.0 M (blue dotted), 1.5 M (purple centre) and 2.2 M (orange solid) ammonium sulphate. The spectra were acquired as described in Fig. 6.3.1.1. Experimental details are given in Section A1.2.

APPENDIX 2. Kinetic evaluation of the heat-induced interactions between β-lactoglobulin and κ-casein at various concentrations

A2.1. INTRODUCTION

To gain a better understanding of the relationship between heat treatment and the denaturation of β-lg/κ-CN mixtures with different variant combinations, the kinetics of the thermal denaturation of alkaline-monomeric β-lg were studied.

A2.2. EXPERIMENTAL PROTOCOL

Four ratios of sample mixtures between β-lg and κ-CN in pH 6.7 phosphate buffer (26 mM sodium phosphate and 68 mM NaCl) were prepared by mixing an equal volume of each protein solution: 2 mg/mL β-lg:1 mg/mL κ-CN = 2:1; 2 mg/mL β-lg:2 mg/mL κ-CN = 2:2; 2 mg/mL β-lg:3 mg/mL κ-CN = 2:3; 2 mg/mL β-lg:4 mg/mL κ-CN = 2:4. This was done for the six variant combinations between β-lg A, B, C and κ-CN A, B. For comparison, 1.5, 2, 2.5 and 3 mg/mL samples of β-lg A, B or C alone were heated at 80 °C under the same conditions.

The losses of alkaline-monomeric β-lg were obtained from the results of Section 9.3.1.

A2.3. RESULTS

The data for the loss of alkaline-monomeric β-lg were fitted to equation (2) with n = 1.5 or 2, and to equation (3) with n = 1 (Section 9.3.3). The plots of reaction orders did not yield straight lines but curved at about 7.5-10 min heating time, indicating that the data could not be described by any reaction order between 1.0 and 2.0. Therefore, the data for the loss of alkaline-monomeric β-lg before and after 7.5 or 10 min heating time were separately fitted to the equations and showed reasonable fits. An example of these graphs for β-lg A/κ-CN B with n = 1, 1.5 and 2 is shown in Fig. A2.3.1. The best fit was determined by linear regression analysis, and the coefficient values (r^2) and reaction constants (k_n) are presented in Tables A2.3.1-A2.3.3.

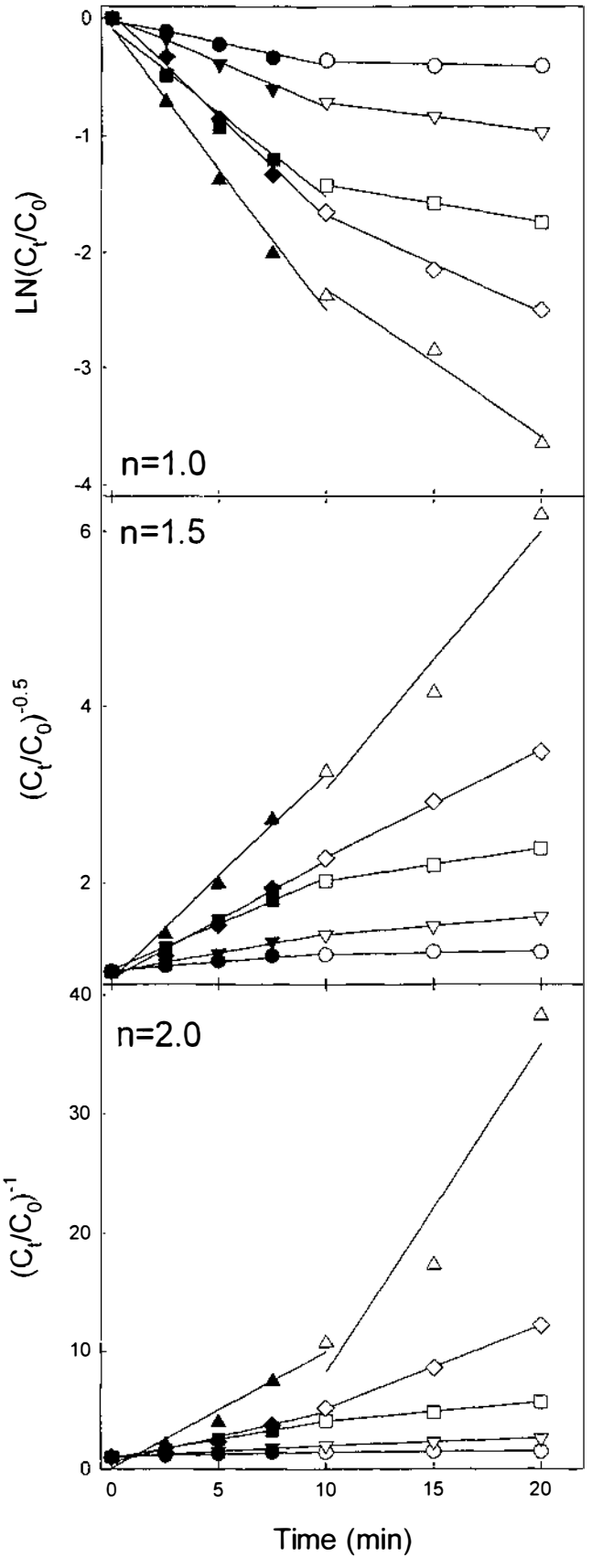

Figure A2.3.1. Loss of alkaline-monomeric β-lg at 80 °C obtained from the mixture of β-lg A and κ-CN B, estimated reaction order of (A) n = 1, (B) n = 1.5 and (C) n = 2. The total protein concentrations were 1 (●, ○), 1.5 (▼, ▽), 2.0 (■, □), 2.5 (♦, ◇) and 3.0 (▲, △) mg/mL, and filled symbols and open symbols indicated 0-10 min and 10-20 min of heating time, respectively.

Table A2.3.1. The effect of altering the ratio of β-lg A to κ-CN A or κ-CN B on the rate constant (k_n) for the loss of alkaline-monomeric β-lg A at 80 °C, with n = 1, 1.5 or 2

		β-Lg A				β-Lg A and κ-CN A				β-Lg A and κ-CN B			
Total protein concentration (mg/mL)		From 0 to 7.5 min		From 7.5 to 20 min		From 0 to 7.5 min		From 7.5 to 20 min		From 0 to 10 min		From 10 to 20 min	
		k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2
n = 1	1.0	-0.0448	0.99	-5.76E-03	0.85	-0.0440	0.99	-5.76E-03	0.85	-0.0374	0.96	-4.76E-03	0.72
	1.5	-0.0879	0.99	-8.46E-03	0.98	-0.0433	0.97	-0.0194	0.98	-0.0734	0.99	-0.0262	1.00
	2.0	-0.1089	0.99	-0.0137	0.95	-0.0459	0.99	-0.0205	0.98	-0.1420	0.97	-0.0325	1.00
	2.5	-0.1246	0.92	-0.0144	0.99	-0.0500	0.97	-0.0233	0.95	-0.1720	0.99	-0.0850	0.99
	3.0	-0.0941	0.99	-0.0243	0.93	-0.0679	0.98	-0.0291	0.96	-0.2413	0.99	-0.1275	0.98
n = 1.5	1.0	0.0224	0.99	3.48E-03	0.85	0.0240	0.99	3.48E-03	0.85	0.0206	0.97	2.88E-03	0.72
	1.5	0.0514	0.99	5.99E-03	0.98	0.0234	0.97	0.0121	0.97	0.0442	0.99	0.0199	0.99
	2.0	0.0674	0.99	0.0107	0.96	0.0250	0.99	0.0129	0.99	0.1045	0.99	0.0358	1.00
	2.5	0.0786	0.92	0.0116	0.99	0.0275	0.96	0.0154	0.96	0.1332	0.99	0.1029	1.00
	3.0	0.0566	0.99	0.0189	0.94	0.0384	0.98	0.0205	0.97	0.2335	0.99	0.2914	0.95
n = 2	1.0	0.0529	0.99	8.38E-03	0.84	0.0524	1.00	8.38E-03	0.85	0.0454	0.97	6.96E-03	0.71
	1.5	0.1225	0.99	0.0170	0.98	0.0507	0.96	0.0545	0.97	0.1071	0.99	0.0607	1.00
	2.0	0.1690	0.99	0.0340	0.96	0.0545	0.99	0.0328	0.99	0.3181	1.00	0.1582	1.00
	2.5	0.2011	0.91	0.0371	0.99	0.0609	0.95	0.0405	0.97	0.4322	0.96	0.6980	1.00
	3.0	0.1375	0.99	0.0592	0.95	0.0871	0.97	0.0582	0.98	0.9906	0.96	2.7547	0.92

Table A2.3.2. The effect of altering the ratio of β-lg B to κ-CN A or κ-CN B on the rate constant (k_n) for the loss of alkaline-monomeric β-lg B at 80 °C, with n = 1, 1.5 or 2

		β-Lg B				β-Lg B and κ-CN A				β-Lg B and κ-CN B			
Total protein concentration (mg/mL)		From 0 to 10 min		From 10 to 20 min		From 0 to 7.5 min		From 7.5 to 20 min		From 0 to 10 min		From 10 to 20 min	
		k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2
n = 1	1.0	-0.0787	1.00	-0.0127	0.84	-0.0550	0.99	-0.0127	0.84	-0.0683	0.98	-8.50E-03	0.81
	1.5	-0.0685	0.97	-0.0266	1.00	-0.0611	0.98	-0.0210	0.99	-0.0898	0.96	-0.0292	1.00
	2.0	-0.0853	0.97	-0.0400	1.00	-0.0774	0.99	-0.0272	0.99	-0.1619	0.98	-0.0569	1.00
	2.5	-0.0846	0.99	-0.0537	1.00	-0.0711	0.97	-0.0401	0.99	-0.1990	0.98	-0.0751	1.00
	3.0	-0.1007	1.00	-0.0489	1.00	-0.0971	0.94	-0.0445	0.94	-0.1874	0.93	-3.88E-03	0.94
n = 1.5	1.0	0.0407	0.98	6.05E-03	0.81	0.0306	0.99	8.18E-03	0.85	0.0407	0.98	6.05E-03	0.81
	1.5	0.0409	0.98	0.0199	1.00	0.0341	0.98	0.0140	1.00	0.0567	0.97	0.0241	0.99
	2.0	0.0528	0.98	0.0331	1.00	0.0447	0.99	0.0196	0.98	0.1617	0.97	0.0708	0.99
	2.5	0.0522	0.99	0.0467	1.00	0.0404	0.97	0.0296	0.99	0.1637	0.96	0.1164	1.00
	3.0	0.0654	1.00	0.0456	1.00	0.0591	0.96	0.0381	0.95	0.2682	0.99	-0.0118	0.86
n = 2	1.0	0.0980	0.99	0.0172	0.81	0.0683	0.98	0.0211	0.85	0.0980	0.99	0.0172	0.81
	1.5	0.0982	0.99	0.0597	1.00	0.0766	0.98	0.0375	1.00	0.1449	0.98	0.0798	0.99
	2.0	0.1320	0.98	0.1099	0.99	0.1040	0.98	0.0569	0.97	0.7780	0.85	0.1412	0.86
	2.5	0.1304	0.98	0.1630	1.00	0.0922	0.96	0.0878	0.98	0.5722	0.92	0.7296	0.99
	3.0	0.1728	0.99	0.1707	1.00	0.1457	0.98	0.1315	0.95	0.9906	0.96	0.6802	0.84

Table A2.3.3. The effect of altering the ratio of β-lg C to κ-CN A or κ-CN B on the rate constant (k_n) for the loss of alkaline-monomeric β-lg C at 80 °C, with n = 1, 1.5 or 2

		β-Lg C				β-Lg C and κ-CN A				β-Lg C and κ-CN B			
Total protein concentration (mg/mL)		From 0 to 7.5 min		From 7.5 to 20 min		From 0 to 7.5 min		From 7.5 to 20 min		From 0 to 7.5 min		From 7.5 to 20 min	
		k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2	k_n	r^2
n = 1	1.0	-0.0462	0.99	-0.0147	1.00	-0.0462	0.99	-0.0147	1.00	-0.0462	1.00	-0.1473	1.00
	1.5	-0.0591	0.95	-0.0115	0.97	-0.0583	0.99	-0.0222	0.96	-0.0515	0.94	-0.0255	1.00
	2.0	-0.0580	0.99	-0.0181	0.98	-0.0550	0.97	-0.0291	0.99	-0.0963	0.97	-0.0729	1.00
	2.5	-0.0561	0.99	-0.0162	0.87	-0.0627	0.96	-0.0307	0.98	-0.1247	1.00	-0.0691	0.99
	3.0	-0.0860	0.92	-0.0230	0.92	-0.0613	0.99	-0.0343	0.99	-0.1413	0.99	-0.0773	0.96
n = 1.5	1.0	0.0251	0.99	9.11E-03	1.00	0.0251	0.99	9.11E-03	1.00	0.0251	0.99	9.11E-03	1.00
	1.5	0.0327	0.95	7.42E-03	0.97	0.0325	0.98	0.0147	0.96	0.0286	0.95	0.0168	1.00
	2.0	0.0323	0.99	0.0118	0.98	0.0303	0.97	0.0194	0.99	0.0580	0.98	0.0658	1.00
	2.5	0.0313	1.00	0.0106	0.87	0.0350	0.95	0.0214	0.98	0.0770	1.00	0.0424	0.98
	3.0	0.0512	0.94	0.0172	0.93	0.0343	0.99	0.0240	0.99	0.0919	0.97	0.0598	0.96
n = 2	1.0	0.0549	0.99	0.0227	1.00	0.0549	0.99	0.0227	1.00	0.0549	0.99	0.0227	1.00
	1.5	0.0729	0.94	0.0191	0.97	0.0726	0.98	0.0388	0.97	0.0636	0.97	0.0444	1.00
	2.0	0.0722	0.99	0.0309	0.99	0.0671	0.97	0.0520	0.99	0.1411	0.99	0.2413	0.99
	2.5	0.0701	1.00	0.0278	0.88	0.0785	0.94	0.0599	0.98	0.2071	0.99	0.2532	1.00
	3.0	0.1228	0.96	0.0517	0.93	0.0770	0.98	0.0672	1.00	0.2442	0.95	0.3780	0.99