Location of the Free Thiol Group in Bovine β-Lactoglobulin A, B and C

A thesis presented in partial fulfilment of the requirements
for the degree of
Master of Science in Biochemistry,
Massey University,
New Zealand.

Helen Brittan
1997

Abstract

Under non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) conditions unheated samples of β-lactoglobulin (β-LG) A, B and C all run as a single band, the A variant having a slightly lower mobility than the B and C variants. Following heating of these samples to 110°C, two bands are seen in the monomer region of SDS-PAGE gels run under non-reducing conditions. As heat can induce disulphide exchange, the individual bands forming the doublet may represent species of the same molecular size but having different arrangements of the disulphide bonds. The band formed in the A variant as a result of heating appears to have the same mobility as the unheated B and C variants, while the band formed in the B and C variants as a result of heating appears to have the same mobility as the unheated A variant.

Under reducing conditions only a single band was seen in both heated and unheated samples, and the mobility of this band is the same in all three variants. This indicates that the difference in mobility between variants seen in non-reduced samples involves disulphide bonding. If the difference in the mobility of the two bands seen in heated samples is due to a difference in the position of a disulphide bond, and thus the free thiol, then it is possible that the position of the free thiol group in the A variant is different to that of the B and C variants even in unheated samples of this protein. A difference in the distribution of the thiol could explain observed differences in the reactivity of this group.

The purpose of this study was to determine whether the observed differences in the mobility of unheated samples of purified bovine β-LG A, B and C under non-reducing SDS-PAGE conditions is due to a difference in the location of the free thiol group within the primary sequence of these variants. This was achieved by reacting the β-LG variants with a radioactively labelled thiol-reactive reagent [1,4-^{14}C] N-ethylmaleimide (^{14}C-NEM), thereby attaching a radioactive marker to the free thiol group. Following labelling of the protein, carried out under conditions that did not induce band splitting,

the protein was hydrolysed and the resulting labelled peptide was purified and sequenced.

The free thiol group was found to be at residue 121 in β-LG A, B and C. Therefore differences in mobility during non-reduced SDS PAGE of β-LG A, B and C are not due to a difference in the location of the thiol group. However, results indicate that it is possible that, particularly in the B and C variants, there is a tendency for disulphide exchange to occur, even under relatively mild conditions.

In establishing the conditions under which band splitting did not occur, the effect of exposure to various conditions on the mobility of purified β-LG variants on native-PAGE and SDS-PAGE was studied. The mobilities of caprine β-LG and porcine β-LG were also studied in order to further characterise the factors within the primary sequence of β-LG that have an influence on band splitting.

With bovine β-LG A, B and C, band splitting was found to be both temperature- and pH-dependent. Protein concentration and the ionic strength of the buffer also appeared to effect band splitting. Heating also induced the formation of aggregated species, visible on both native and SDS-PAGE gels. The presence of aggregated material on SDS-PAGE gels indicates that disulphide bonding is involved in the formation of these species.

On native-PAGE, material that ran as a smear between the monomer band and dimer band was observed following heating. The protein present in this region may represent monomeric β-LG that has been sufficiently denatured for its mobility under native-PAGE to be retarded. Comparisons of the amount of material present in monomeric forms under native and non-reduced SDS-PAGE suggest that multiple monomeric species of β-LG are present in heated samples.

Storage at -18°C in SDS-PAGE sample buffer was also shown to induce changes in the mobility of bovine β-LG A, B and C, and of caprine β-LG, on SDS-PAGE. Storage under these conditions caused the aggregation of β-LG but did not induce band splitting. The banding pattern in the dimer region of the stored samples differed

between the variants, with the A variant showing a banding pattern that was markedly different to that of the B and C variants and the caprine protein, which showed similar patterns. The bovine β-LG B and C and caprine β-LG showed similar tendencies to form aggregates, and had a greater tendency to form these high molecular weight species than β-LG A. These differences may be due to a difference in the reactivity of the free thiol group under these conditions, influenced by the substitution at position 118.

Purified, unheated caprine β-LG ran as a single band in non-reduced SDS gels, and appeared to have the same mobility as the unheated bovine B and C variants under these conditions. Heating of caprine β-LG also induced the formation of a second band with a similar mobility to that of unheated β-LG A. Caprine β-LG has an Asp at position 64 (as found in bovine β-LG A) and an Ala at position 118 (as found in bovine β-LG B and C). The fact that in non reducing SDS-PAGE caprine β-LG runs as a band with a similar mobility to bovine β-LG B and C and a slightly higher mobility than bovine β-LG A suggests that the substitution at position 118 in the primary protein sequence may somehow be causing the mobility difference. Aggregated material was also seen in caprine β-LG following heating.

Unheated samples of porcine β-LG ran as two bands under non-reduced SDS-PAGE. Heating the porcine β-LG did not appear to induce any change in the appearance of the two bands, and there was no evidence of aggregation of this protein. Bovine β-LG A, B and C and caprine β-LG all contain a free cysteine residue in their protein sequence. Porcine β-LG does not contain a free Cysteine and thus the lack of heat-induced changes to the banding pattern in porcine β-LG when compared with the bovine variants and caprine β-LG is possibly due to the absence of this potentially reactive thiol group. The presence of a free thiol group appears to be required both to induce band splitting and for the formation of higher molecular weight aggregates following heating. Band splitting is thus probably a consequence of disulphide interchange reactions, the interchange reaction in β-LG A causing a second band to run in the position of β-LG Band C, and the interchange reaction in β-LG B and C causing a second band to run in the position of β-LG A.

Acknowledgements

I would like to thank the following people for their assistance in the completion of this project:

My supervisors, Jeremy Hill (Food Science section, NZDRI), Trevor Kitson (Department of Chemistry, Massey University) and Gill Norris (Department of Biochemistry, Massey University).

All the members of the Food Science section, NZDRI, for their help and encouragement during my time in this section. I would particularly like to thank:
Ruth Lowe, who did the initial work on which this project was based, and Richard Burr, Christina Coker, Gavin Manderson, Lawrie Creamer, Skelte Anema and Wayne Thresher, for their practical assistance and invaluable advice.

Nicky White (Cheese Technology section, NZDRI).
Rose Motion (Biological Sciences section, NZDRI).
Garnett Davy and Kim Hubbard (Graphics Unit, NZDRI).

Jo Mudford (Department of Biochemistry, Massey University) for peptide sequencing.

This project was supported in part by funding from the Foundation for Research, Science and Technology, [redacted]

Table of Contents

Part 2 Identification of the free thiol group in β-Lactoglobulin A, B and C

List of Figures

List of Tables

Appendix Tables

Introduction

β-Lactoglobulin (β-LG) is the major whey protein in bovine milk. Numerous genetic variants of bovine β-LG have been identified, but in New Zealand herds the A and B variants predominate, with the C variant occurring at low levels in Jersey and related breeds (Paterson *et al.*, 1995). The primary sequence of bovine β-LG has been determined, and the sequences of these three variants vary at residues 59, 64, 118 (Figure 1).

Included in the 162 residue sequence are five cysteine residues, four of which form two disulphide bridges. One disulphide bridge, between Cys 66 and Cys 160, has been unambiguously identified by a variety of methods, but the position of the other bridge and the location of the free thiol has given rise to some controversy (Hambling *et al.*, 1992). The free cysteine occurs in the sequence -Cys-Gln-Cys- (119-121), and has been reported as Cys 119, Cys 121, or an equal mixture of the two, with the other cysteine in this sequence forming a disulphide bridge with Cys 106. The reactivity of the thiol group varies between variants (Thresher and Hill, 1997), and it is possible that the amino acid substitution at position 118 in the variants (see Figure 1) is responsible for this difference. As the thiol group of β-LG is involved in both the interaction between β-LG and κ-casein (Sawyer, 1969) and the gelation of whey protein solutions containing β-LG (Monahan *et al.*, 1995) ,the reactivity of the thiol group can have a major influence on the chemical and physical properties of milk and milk products. The tertiary structure of β-LG has an 8-stranded β-barrel with a three-turn α-helix on the surface of the barrel (Papiz *et al.*, 1986, Monaco *et al.*, 1987, Brownlow *et al.*, 1997). The cysteine residues at positions 106, 119 and 121 are all close in the tertiary structure, and are located at the sheet-helix interface (Figure 2).

Figure 1 Primary sequence of bovine β-LG A, B and C. Locations of the variant substitutions are shown in yellow. Locations of the cysteine residues are shown in red. Residues shown in purple correspond to the α-helix, and residues shown in blue correspond to the β-sheets and helical turn, as shown in Figure 2.

Figure 2 Tertiary structure of β-LG A as determined by Sawyer (personal communication). Cysteine residues 106, 119 and 121 are shown in red. Residue 118, valine in β-LG A, is shown in yellow. β-sheets G and H, and the α-helical turn joining these sheets (residues 102-123) are shown in blue. Residues 130-140 are shown in purple.

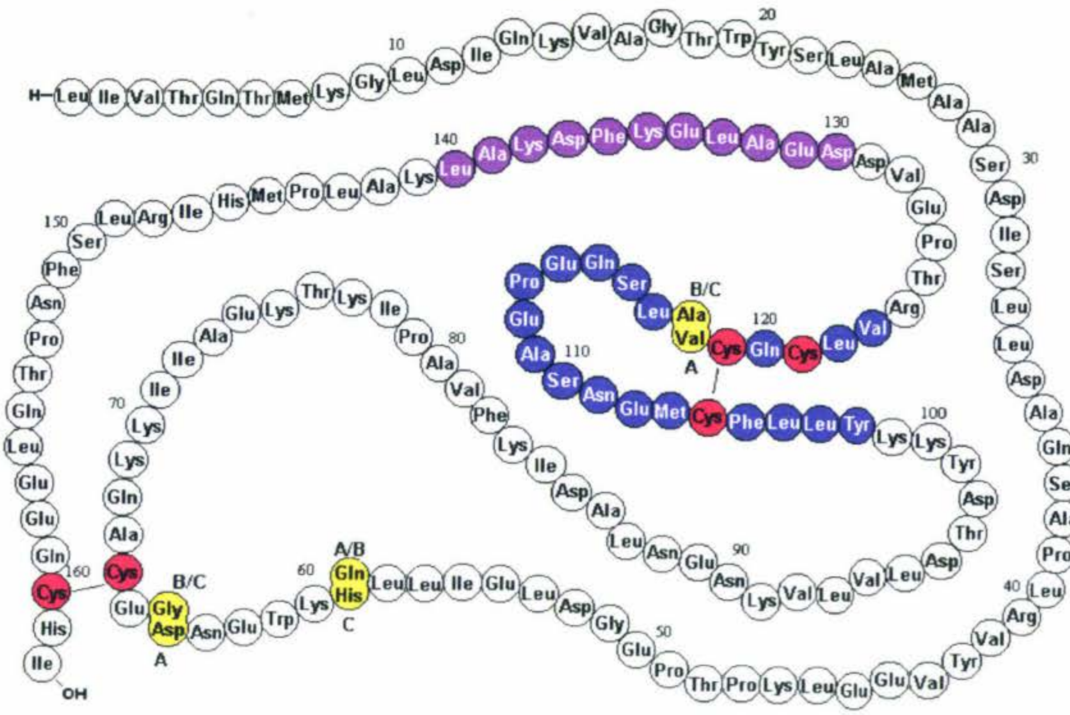


Figure 1

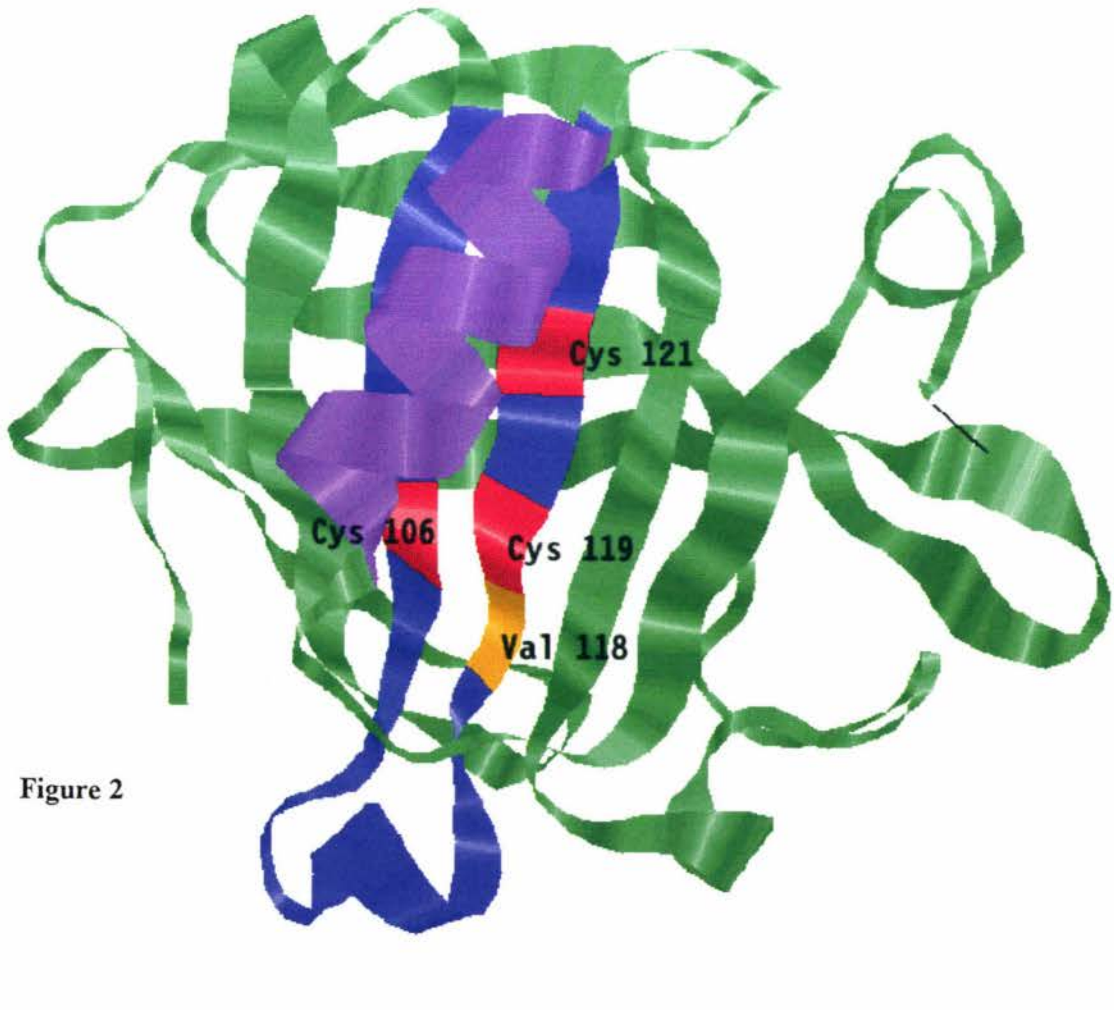


Figure 2

McKenzie (1971) reported that the free thiol occurred at position 119 in the B variant. In reaching this conclusion it was assumed that the thiol would be at one position only. It was later concluded that the alternative positions of the free thiol (119 and 121) occurred in equal proportions in each of the variants studied (McKenzie *et al.*, 1972). This was determined by diagonal peptide mapping using a method for quantitatively determining the ratio of multiple positions of a free thiol if such exist. McKenzie *et al.* (1972) labelled β-LG using [^{14}C]-iodoacetamide in the presence of 8 M urea. However, 8 M urea can disrupt disulphide bridges (McKenzie *et al.*, 1972), and the distribution of the disulphide bond position between 106-119 and 106-121 seen in this study could have been due to disulphide exchange induced by the presence of urea.

The results of McKenzie *et al.* (1972) conflict with those of Mainferme *et al.* (1971), Martial *et al.* (1971), Pérez-Gómez *et al.* (1971), and of Phelan and Malthouse (1994). Mainferme *et al.* (1971) labelled the free thiol of β-LG A with NEM at pH 8.2. Martial *et al.* (1971) used similar conditions and an NEM derivative to label β-LG B. Pérez-Gómez *et al.* (1971) also investigated the position of the free thiol group in β-LG B, using iodoacetamide, carrying out the labelling reaction at pH 10.2 for one minute, and then at pH 8.3. None of these studies used urea, and all determined the free thiol to be at position 121.

McKenzie *et al.* (1972) suggest that the conclusions of Mainferme *et al.* (1971), Martial *et al.* (1971) and Pérez-Gómez *et al.* (1971) were made on the assumption that the free thiol was present only at a single position. Martial *et al.* (1971) noted an excess of 0.8 residue of glutamic acid in a peptide containing Cys 121 but did not investigate this further. Differences between these results and those of McKenzie *et al.* (1972) could arise from the different methods of cleavage and separation resulting in preferential isolation of different sets of peptides. Assuming a single position, the composition of one purified labelled fragment would be considered sufficient to define the system. It is also possible that the long reaction times could result in a specific -SH/-SS- interchange, or there could be preferential labelling of a more exposed or

reactive form of the thiol group in the absence of considerable unfolding such as occurs in urea.

Phelan and Malthouse (1994) cyanylated β-LG and used C-n.m.r. to identify the free thiol. A mixture of β-LG A and β-LG B gave two signals from thiocyanate carbons, one apparently located in a hydrophilic environment and one apparently located in a hydrophobic environment. It was not possible to determine whether these were due to a single thiocyanate carbon being present on each of the cyanylated A and B variants, or if they resulted from each protein having two thiocyanate carbons with different chemical shifts. It was found that the same signals were detected when the A and B variants were cyanylated separately, and it was therefore concluded that one signal was due to a denatured cyanylated β-LG while the other was due to an intact cyanylated β-LG.

Recombinant ovine β-LG has been expressed and secreted by *Kluyvermyces lactis* (Rocha *et al.* 1996). The recombinant protein is virtually indistinguishable by antibodies, CD, native-PAGE and SDS-PAGE from β-LG purified from ovine milk. Site-directed mutagenesis techniques were used to replace selected cysteine residues with serine. While the C121S mutant was expressed and secreted, the C119S mutant was synthesised but was not secreted. Rocha *et al.* (1996) suggested that correct disulphide bond formation is required for secretion.

Basch *et al.* (1985) observed the splitting of mixed variant β-LG into two bands under non-reducing conditions in a discontinuous SDS-PAGE system. The β-LG A, B and C variants were all found to show this behaviour, the reason for this being unclear. Shimada and Cheftel (1989) found that, following heating of whey protein isolate for four minutes heating at 85°C (pH 7.5), an additional, faster-migrating band appeared following SDS-PAGE carried out under non-reducing conditions. This extra band was poorly separated from the β-LG A+B band but was visibly detectable. It was suggested that this new fraction may represent partially denatured monomeric β-LG with different pairs of disulphide bonds, due to intramolecular disulphide exchange reactions.

Hill *et al.* (1997b) found that heating β-LG to 110°C promoted the splitting of β-LG into two bands under non-reducing SDS-PAGE conditions, whereas unheated β-LG ran as a single band (Figure 3). Under reducing conditions only a single band was seen in both heated and unheated samples, indicating that the band splitting may involve disulphide bonding. This effect was seen with purified β-LG A, B and C. The individual bands of the doublet may represent species of approximately the same molecular size but having different arrangements of the disulphide bonds. The distribution of protein between the two bands was not even and was different in the three variants. These differences may be due to differences in the distribution of the free thiol group in the heated protein. The similarity of the band distributions in the B and C variants and the striking difference to that of the A variant suggest that these effects may be due to the variant substitution at position 118.

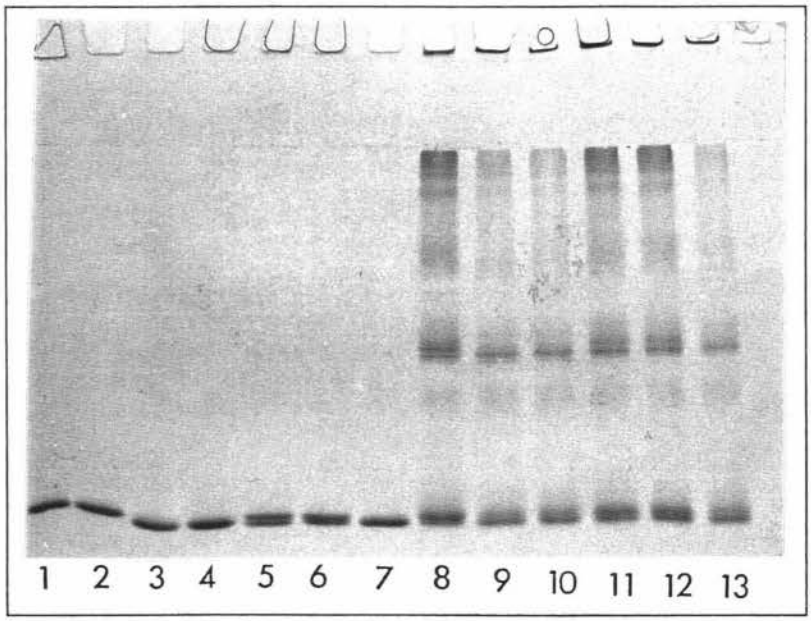


Figure 3 Band splitting of β-LG A, B and C variants on polyacrylamide gels following heating to 110°C. Unheated samples lanes 2-7, heated samples lanes 8-13, β-LG A (lanes 2 and 8), β-LG B (lanes 3 and 9), β-LG C (lanes 4 and 10), β-LG AB (lanes 5 and 11), β-LG AC (lanes 6 and 12), β-LG BC (lanes 7 and 13).

Kubo (1995) observed that, following the heating of α-amylase in the presence of SDS, two species with slightly different mobilities were seen under SDS-PAGE conditions. It was found that the two species differed in the locations of one disulphide and one thiol group.

Under non-reducing SDS-PAGE conditions unheated β-LG A has a slightly lower mobility than β-LG B and C, possibly running in a position corresponding to the upper of the two bands in the heated samples (Hill *et al.*, 1997b). If the difference in the mobility of the two bands seen in heated samples is due to a difference in the position of the free thiol, then it is possible that the position of the free thiol group in the A variant is different to that of the B and C variants even in unheated samples of this protein. A difference in the distribution of the thiol could explain observed differences in the reactivity of this group.

The objectives of this study were:

1. To establish conditions of pH and temperature under which β-LG may be incubated without inducing band splitting, as seen under non-reducing SDS-PAGE conditions, or irreversible denaturation, as seen in native gels.

2. To establish conditions for labelling the free thiol group in β-LG with a radioactive label. These conditions must be within the limits defined in 1 above.

3. To establish conditions for the hydrolysis of the labelled protein.

4. To establish a protocol for the purification of the labelled peptide.

5. To sequence the purified peptide and identify the labelled residue and, in doing so, to determine whether the location of the free thiol is the same in β-LG A, B and C.

Literature Review

1 Milk Composition.

Milk is a complex mixture of proteins, lipids, carbohydrates, minerals, vitamins, and salts dissolved, colloidly dispersed and emulsified in water. Some of the constituents of milk are synthesised in the mammary gland, while others are transferred from the blood stream. All milks so far analysed contain two protein groups: an acid precipitable fraction, commonly known as casein, and an acid soluble fraction, known as the whey proteins (Ng-Kwai-Hang and Grosclaude, 1992). The caseins, along with the major whey proteins β-LG and α-lactalbumin (α-LA), are unique to lactation and are synthesised from free amino acids under genetic control by the highly specialised secretory cells of the mammary gland (Larson, 1979). Some milk fatty acids are synthesised in the cell, as is lactose, the principal carbohydrate in milk. Lactose is synthesised in the Golgi apparatus from uridine diphosphogalactose and glucose through the action of the lactose synthase complex. α-LA interacts with a membrane-bound galactosyltransferase to form the complex. The function of α-LA in lactose synthase is to of promote the binding of glucose to galactosyltransferase, allowing lactose to be synthesised at physiological concentrations of glucose (Brew and Grobler, 1992).

The exact composition of milk varies from species to species. Differences are seen in the ratios of fat, protein and lactose, in the proportions of casein and whey proteins, and in the amounts of individual components. Milks important to humans in Western societies, other than human milk itself, are bovine, caprine, and, to a lesser extent, ovine milks. The gross composition (fat + protein + lactose + ash) of milks from these species is shown in Table 1. Large differences within species are reported by various workers, and not all represent true averages due to inadequate sampling.

Species	Composition (g/100g)					
				Whey		
	Water	Fat	Casein	Protein	Lactose	Ash
Human (Homo sapiens)	87.1	4.5	0.4	0.5	7.1	0.2
Cow (Bos taurus)	87.3	3.9	2.6	0.6	4.6	0.7
Zebu (Bos indicus)	86.5	4.7	2.6	0.6	4.7	0.7
Goat (Capra hircus)	86.7	4.5	2.6	0.6	4.3	0.8
Sheep (Ovis aries)	62.0	7.2	3.9	0.7	4.8	0.9

Table 1 Gross Composition of Milks of Various Species (Jenness, 1988).

1.1 Bovine Milk

Normal bovine milk contains 30-35 g/L of protein (Figure 4), of which 76-86% is casein.

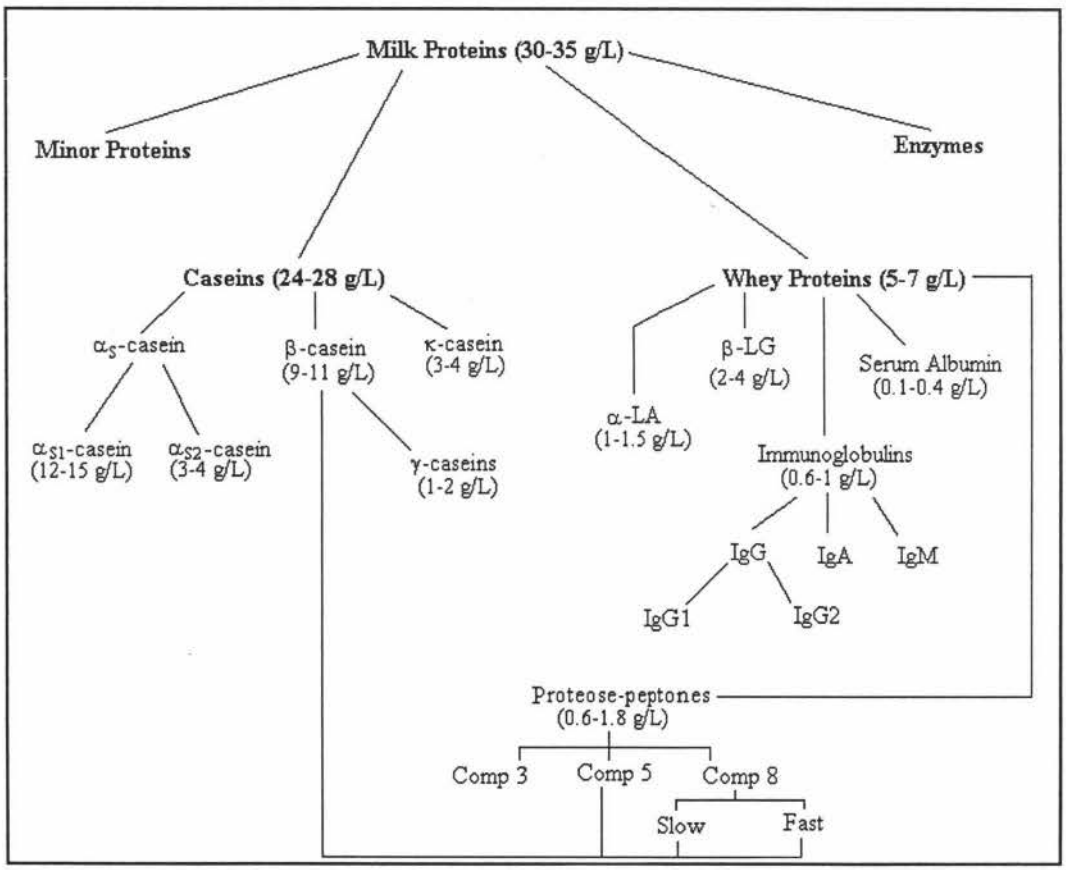


Figure 4 Distribution of major milk proteins and peptides in bovine milk (Swaisgood, 1992).

Caseins represent four gene products, α_{s1}-, α_{s2}-, β- and κ-casein. Some additional heterogeneity arises from post-translational processing, such as phosphorylation, glycosylation and limited proteolysis, e.g. the proteolytic action of plasmin on β-casein, yielding γ-caseins and proteose-peptones (Swaisgood, 1992). Almost all of the caseins are present in milk as large spherical protein complexes, called micelles, resulting from interaction of all the four individual casein components (92% total wt), and calcium phosphate, together with small amounts of magnesium, sodium, potassium and citrate (8%). The presence of calcium phosphate is essential for the integrity of the micelles, and magnesium and citrate play an important role in stabilizing the micellar structure. In addition, κ-casein plays a key role in the stability of casein micelle suspensions.

The major whey proteins in bovine milk are β-LG, α-LA, bovine serum albumin and immunoglobulins. Additional minor proteins and enzymes are found associated with caseins, in the whey fraction, and associated with the membrane which surrounds milk fat globules. These proteins possess a host of immunological, enzymatic and other functional properties (Larson, 1979).

The lipids of bovine milk are composed of 98% triacylglycerols, with smaller amounts of other lipid components. Diacylglycerols, monoacylglycerols and free fatty acids in quantities greater than traces are the products of lipolysis. Freshly drawn milk which is promptly pasteurised contains little of these components (Jenness, 1988). The lipids in milk are almost entirely present in the form of globules and their protective membrane, the milkfat globule membrane (MFGM). The MFGM is composed mainly of proteins and lipids (> 90% dry weight), although the exact composition varies. At least 26 enzymes have been found in the protein fraction of the MFGM, many with very low activities. The enzyme plasmin, which can catalyse proteolytic degradation of the caseins, is also present in the MFGM (Marshall, 1993).

The principal salts present in bovine milk are sodium potassium, calcium, and magnesium phosphates, citrates, chlorides and carbonates (Jenness, 1988). 65% of the calcium salts are associated with casein micelles (Swaisgood, 1992).

Lactose is the principal carbohydrate in bovine milk. Carbohydrates other than lactose include monosaccharides and small amounts of oligosaccharides, and glycosyl groups bound to proteins and lipids are also present in milk. A host of other constituents, including trace elements and vitamins have also been detected in milk. The exact composition is influenced by a number of environmental and genetic factors (Jenness, 1988).

2 β-LG

β-LG is the major whey protein in the milk of ruminants, and is found in the milk of some other mammals (Hambling *et al.*, 1992). It is absent in the milks of rodents and humans. All known ruminant β-LGs are dimers at physiological pH (Brownlow *et al.*, 1997), while most non-ruminant proteins are monomeric. Bovine β-LG has a molecular weight of about 18300 and an isoelectric point around pH 5.3. For reviews on the properties of β-LG solutions see McKenzie (1971) and Hambling *et al.* (1992).

2.1 Conformational transitions

β-LG undergoes three pH-dependent conformational transitions with increasing pH, which can be observed by optical rotational dispersion (ORD), sedimentation and titration studies. These changes can be summarised as:

$$Q \leftrightarrow N \leftrightarrow R \rightarrow S \qquad \text{(Hambling } \textit{et al.}\text{, 1992)}$$

The Q↔N transition occurs between pH 4 and 6. An increase in the sedimentation coefficient correlates with contraction of the protein. The N↔R, or Tanford, transition occurs at around pH 7.4. The structural basis of the change is not yet known, but no large change in the tertiary structure of the protein is evident. A refolding of the protein chain and rearrangement of side chains within each subunit appears to be an important part of the change. The changes are dramatic when observed by ORD, but much less so by other techniques. A small decrease in the sedimentation coefficient is seen, titration curves show that one carboxyl group per subunit becomes exposed and

ionised, and the reactivity of the thiol group to specific reagents is increased. At low pH the cysteine side chain may be shielded by other parts of the protein, and as the pH increases refolding may expose the thiol group to the external medium. The pK_a of the thiol group is predicted to be around 8.5, well removed from the pH at which this transition occurs. Thus it is unlikely that the ionisation of this group causes the change. However, as this change occurs at physiological pH, it may be functionally significant. Titration data, sedimentation data and specific rotation data at pH values between the end of the Tanford transition and the onset of alkali denaturation are consistent with the R form being a rigid, compact molecule (Dunnill and Green, 1966). The R→Q transition represents the irreversible alkali-induced denaturation of the protein. Alkali-induced denaturation becomes significant above pH 8, and is time-dependent. Complete irreversible denaturation occurs at pH 9.5-10, depending on the speed with which measurements are made.

2.2 Crystallisation Studies

The structures of four different crystal forms of β-LG were determined at 6Å resolution by Green *et al.* (1979). Two of these lattices, K and X, were crystallised at pH 6.5, corresponding to the N state in solution. The X crystals were formed from native protein, while the K crystals were formed from carboxypeptidase A-treated β-LG, from which two amino acid residues had been cleaved from the C-terminus. The other two crystal forms, Y and Z, were crystallised at pH 7.5, corresponding to the R state in solution. At 6 Å resolution, no clear distinction could be made between the structures. All four structures showed certain features suggestive of α-helices and β-pleated sheets, but resolution was insufficient to trace the entire course of the polypeptide chain. Studies of the free thiol using a heavy atom derivative initially gave results consistent with a single site, but, on use of a second derivative, results consistent with the presence of a second, minor site were obtained. However, the minor site was at about noise level, and the second derivative was not able to be refined to the same resolution as the first derivative. A difference in the position of the site of the free thiol was detected in the X lattice relative to the Y and Z lattices, consistent with a movement of the thiol group as a result of the Tanford transition.

Papiz *et al.* (1986) studied the orthorhombic crystal (lattice Y), formed at pH 7.6 and refined to 2.8 Å. The molecule was shown to consist of 9 strands of antiparallel β-sheet, 8 of which wrap around to form the flattened conical barrel, closed at one end, and a three-turn α-helix on the outer surface of the barrel. The free thiol, at position 121, is buried at the sheet-helix interface, and the disulphide bond at 106-119 is clearly resolved. The other disulphide, 66-160, is not clear, but appears to be on the outer surface, linking strand D to the C-terminal region.

Monaco *et al.* (1987) refined a trigonal crystal (lattice Z) to 2.5 Å and compared the structure to that of the orthorhombic form. Significant differences, mainly due to different packing between lattices, were seen between residues 56 and 68, resulting in displacement of the 66-160 disulphide, and in residues 1-14. However, the β-barrel itself did not appear to be significantly altered. In the trigonal form, the species crystallised does not appear to be dimeric, but a linear polymer with tight intermolecular contacts. Monaco *et al.* (1987) also found the free thiol to be at position 121 with a disulphide bridge between 106 and 119. In this structure, the second disulphide bridge is clearly visible, joining the flexible 56-68 loop to the C-terminal region of the molecule.

Brownlow *et al.* (1997) redetermined the structure of lattice Z to 3Å and, using this lattice as a model, solved the structure of the triclinic crystal (lattice X), grown at pH 6.5, to 1.8Å. Although some parts of the polypeptide chain are repositioned in this study, the basic fold of the polypeptide remains unchanged. The 66-160 bridge was shown to be exposed in an extremely mobile region, while the other disulphide, between residues 106 and 119, is buried in a hydrophobic channel at the sheet-helix interface, along with the free cysteine. Crystallisation above and below the transition pH shows there are different crystal forms either side of the transition point. Although the relationship between monomers differs significantly in the different lattices, the conformation of the monomers remains very similar.

None of these last three crystallography studies showed any evidence of the Cys 106 and 119/121 exchange suggested by McKenzie *et al.* (1972). All clearly show a

106-119 disulphide and a free thiol at position 121. Since this region of the molecule is a well-resolved area of tertiary structure, and the distance between the S_γ atoms of Cys 119 and 121 is 10.5Å (Papiz *et al.*, 1986), any interchange would require significant conformational flexibility. What is not ruled out by the crystallography is the selection by crystallisation of the Cys106-119 isomer, although the two crystal forms which grow simultaneously in the same solutions at pH 7.8 both have this cystine (Hambling *et al.*, 1992).

2.3 Biological Function

Although β-LG is the most abundant whey protein in ruminant milk, its exact physiological role is still unclear. The β-barrel structure seen in β-LG identifies it as a member of the lipocalin family, a diverse and widely distributed family of small, predominantly extracellular transport proteins which show high binding affinity for small, hydrophobic molecules (Sansom *et al.* 1994). Family members share limited sequence homology, but all have a central 8-stranded β-barrel with an N-terminal helical turn and at least one C-terminal helix. The common core of the β-barrel is conserved between lipocalins, while the loop regions are variable. Another member of the lipocalin family is retinol binding protein (RBP), a protein that transports retinol in plasma. RBP and β-LG show limited sequence homology, but when backbone C_α coordinates were superimposed, the two structures were shown to be closely aligned (Papiz *et al.* 1986). The β-barrel and α-helix were conserved, and the greater length of the RBP chain was accommodated in larger surface loops. A related group of proteins, the fatty-acid binding proteins (FABPs), show a similar 3D structure, being made up of a 10-stranded β-barrel (Sansom *et al.*, 1994). FABPs often bind the same ligands as lipocalins but are almost exclusively intracellular.

β-LG is stable under acidic conditions, and is resistant to gastric proteolysis, allowing it to pass intact through the stomach of animals. This suggests that its function may be in the intestine. β-LG is known to bind a variety of hydrophobic molecules, including retinol and fatty acids, and may be involved in the transport or uptake of these molecules within the gut. The fact that retinol binds to β-LG with a greater affinity

than to RBP lead to speculation that biological function of β-LG is involved with vitamin A transport. Papiz *et al.* (1986) modelled a retinol molecule into the calyx of the β-LG molecule by analogy with RBP, but a study of the β-LG-retinol complex (Monaco *et al.*, 1987), suggested that this was not the case. The retinol binding site appeared to be in a superficial hydrophobic pocket. Other experimental evidence supports the hypothesis that the binding of retinol is quite different in β-LG and RBP; for example the retinol molecule is fully protected against oxidation by liver alcohol dehydrogenase when bound to RBP, but not when bound to β-LG (Papiz *et al.*, 1986). Specific receptors for the β-LG-retinol complex have been found in the small intestine of week-old calves but were absent in six-month old animals (Papiz *et al.*, 1986), suggesting a role in transport and/or uptake of vitamins in neonate calves. Alternatively, just as there are extra- and intra-cellular retinol binding proteins of related but distinct topology, β-LG might be the extracellular fatty acid binding protein that is the equivalent of FABPs. In this regard it is interesting that two species which have no β-LG, the rat and humans, have an intestinal FABP (Brownlow *et al.*, 1997). Ruminant β-LGs isolated by non-denaturing methods have been found to have lipids, mainly long chain fatty acids, bound to them under physiological conditions (Perez and Calvo, 1995). It is possible that β-LG could participate in the digestion of milk lipids during the neonatal period by enhancing the activity of ruminant pregastric lipase through removal of the fatty acids that inhibit this enzyme (Perez *et al.*, 1992).

2.4 Denaturation of β-LG

The denaturation of β-LG involves the dissociation of the dimer into monomer, the unfolding of the polypeptide, and aggregation of the unfolded polypeptides. The denaturation of β-LG is complex, and can depend on the denaturant, the environment of the protein (such as buffer type used or whether the measurements are made in whey or milk), the pH at which the measurements are made, the temperature range over which denaturation is investigated and the way in which denaturation is monitored.

2.4.1 Thermal Denaturation

The dissociation of dimeric β-LG into monomers occurs at temperatures between 30-55°C (Hambling *et al.*, 1992). As temperature increases, unfolding of the polypeptide chain occurs, resulting in an increase in the reactivity of the thiol group, and aggregation occurs. Briggs and Hull (1945) described two types of reaction occurring during the thermal denaturation of β-LG, and termed them "primary" and "secondary" denaturation reactions. Sawyer (1968) studied the products of these reactions, and determined the products of the primary reactions to be small aggregates arising from the formation of intermolecular disulphide bonds. The products of the secondary reaction gave rise to large aggregates that did not involve formation of disulphides.

2.4.2 Urea Denaturation

The first step of urea denaturation appears to involve both dissociation and unfolding (Hambling *et al.*, 1992). McKenzie and Ralston (1973) identified two types of product resulting from this process. The first consisted of protein molecules that had undergone conformational change without any aggregation or change in covalent linkages. Most (or all) of this material should revert to the original native conformation on removal of the urea. The second product described by McKenzie and Ralston (1973) had undergone intramolecular covalent linkage changes, usually via -SH/-SS- interchange. On removal of the urea these species could not revert to their original -SH and -SS- positions, although there may be partial reversion to the native conformation. The second step of urea denaturation involves the aggregation of the unfolded protein through the formation of intermolecular disulphide bonds (Hambling *et al.*, 1992). On removal of the urea these products retain their respective sizes (dimers, trimers, etc.) but may recover part of their original conformation (McKenzie and Ralston, 1973).

Hill *et al.* (1997b), using non-reduced SDS-PAGE, identified two monomeric species of β-LG in heated samples. It was suggested that these were a result of intramolecular disulphide reactions, and, if so, would correspond to the species described by McKenzie and Ralston (1973) which formed as a result of the first stage of urea denaturation.

2.4.3 SDS Denaturation

Binding of SDS to β-LG appears to occur in three stages (Jones and Wilkinson, 1976). At low SDS concentrations β-LG binds 2 surfactant anions/mol dimeric protein (Hillquist Damon and Kresheck, 1982). The second stage involves the binding of 22 anions; there are no intermediate complexes between β-LG with 2 bound anions and β-LG with 22 bound anions (Jones and Wilkinson, 1976). The third stage involves the formation of a micellar-like complex, with ~110 anions bound per mol dimeric protein (Hillquist Damon and Kresheck, 1982). Binding of SDS to β-LG has been shown to increase the amount of secondary structure in β-LG (Hillquist Damon and Kresheck, 1982), and SDS concentrations of 0.1-0.5% w/v were found to cause an increase in the thermal stability of whey proteins (Donovan and Mulvihill, 1987).

2.5 Renaturation of β-LG

Denatured β-LG that has not undergone aggregation has been shown to refold on cooling. The renatured protein showed the same retinol binding activity as native β-LG and physiochemical studies indicated that almost complete refolding had occurred (Kaminogawa *et al.*, 1989). However, local structural differences were identified using antibodies that recognise different regions of the protein, with some regions of the β-LG molecule having greater heat stability than others (Kaminogawa *et al.*, 1989). A similar result was obtained when guanidine hydrochloride was used as a denaturant (Hattori *et al.*, 1989), where, through measuring changes in tryptophan environment, were able to identify long term changes in the conformation of β-LG following removal of guanidine hydrochloride compared with the native structure (Subramaniam *et al.*, 1996).

2.6 Gelation of β-LG

If β-LG concentration is high enough and pH and salt concentrations are appropriate, β-LG can form heat-induced gels (Creamer and MacGibbon, 1996). As with irreversible thermal denaturation, the formation of disulphide bonds between the

protein molecules is essential for gelation. Porcine β-LG, which lacks a free thiol, has been shown not to form gels (Gallagher *et al.*, 1996).

2.7 β-LG-κ-casein complex formation

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. Almost all milk undergoes some form of heat treatment at some stage during its processing. During heat treatment of milk, β-LG and κ-casein interact, through the formation of disulphide bonds, to form a complex (Sawyer, 1968). This reaction is a major determining factor in the heat stability of milk (Singh and Creamer, 1992, Fox and Morrissey, 1977), and is influenced by other factors, including pH, concentration of salt, protein concentration and processing factors.

3 Polymorphism of milk proteins

Genetic polymorphism in milk proteins was first reported by Aschaffenburg and Drewry in 1955, with the identification of two distinct forms of β-LG. Since then, polymorphism of all the major mammary-synthesised proteins of bovine milk has been shown. Milk protein polymorphism has also been shown in other species, but studies have mainly centred on bovine milk (Ng-Kwai-Hang and Grosclaude, 1992). Genetic variants differ in amino acid sequence, and are distinct from variations due to post-translational modifications.

Currently there are 5 known variants of α_{s1}-casein, 4 variants of α_{s2}, at least 7 variants of β-casein and 4 κ-casein variants. The pattern of inheritance of the four casein polymorphs indicates that their loci are closely linked (Ng-Kwai-Hang and Grosclaude, 1992). Numerous variants of bovine β-LG have been identified, and genetic variants of β-LG have also been identified in other species (see below). Three genetic variants of bovine α-LA have been identified, but only the B variant is observed in Western breeds (Whitney, 1988). The genes controlling the synthesis of these proteins are codominant, so individual cows can express two different variants. Amino acid differences between

two genetic variants may produce significant changes in their properties, and genetic variants of all the major milk proteins have been shown to influence the composition and/or technological behaviour of milk (Ng-Kwai-Hang and Grosclaude, 1992, Jacob and Puhan, 1992, Jacob, 1993, Fitzgerald and Hill, 1997).

4 β-LG polymorphisms

Genetic variants of β-LG have been identified in some non-bovine species (e.g. three ovine variants), while other non-bovine species are monovariant (e.g. caprine β-LG). A comparison of the sequences of some of the bovine variants, as well as of caprine β-LG and the ovine variants, is given in Table 2. β-LG has also been identified some in non-ruminant species (e.g. porcine β-LG, equine β-LG).

β-Lactoglobulin	*Variable amino acid positions*																		
	1	*11*	*20*	*28*	*45*	*50*	*53*	*59*	*64*	*78*	*84*	*87*	*118*	*129*	*130*	*148*	*150*	*158*	*162*
Bovine A	V	D	Y	D	E	P	D	Q	D	I	I	L	V	D	D	R	S	E	I
Bovine B	V	D	Y	D	E	P	D	Q	G	I	I	L	A	D	D	R	S	E	I
Bovine C	V	D	Y	D	E	P	D	H	G	I	I	L	A	D	D	R	S	E	I
Bovine D	V	D	Y	D	Q	P	D	Q	G	I	I	L	A	D	D	R	S	E	I
Bovine E	V	D	Y	D	E	P	D	Q	G	I	I	L	A	D	D	R	S	G	I
Bovine F	V	D	Y	D	E	S	D	Q	G	I	I	L	A	D/Y		R	S	G	I
Bovine G	V	D	Y	D	E	P	D	Q	G	M	I	L	A	D	D	R	S	G	I
Bovine Dr	V	D	Y	N	E	P	D	Q	D	I	I	L	V	D	D	R	S	E	I
Yak	V	N/D	Y	D	E	P	D	Q	G	I	L	I	A	D	D	R	S	G	I
Water buffalo	I	D	Y	D	E	P	D	Q	D	I	I	L	A	D	D	R	S	E	V
Goat	I	D	Y	D	E	P	N	Q	D	I	I	L	A	D	K	R	A	G	V
Sheep A	I	D	Y	D	E	P	N	Q	D	I	I	L	A	D	N	R	A	G	V
Sheep B	I	D	H	D	E	P	N	Q	D	I	I	L	A	D	N	R	A	G	V
Sheep C	I	D	Y	D	E	P	N	Q	G	I	I	L	A	D	N	Q	A	G	V

Table 2 Comparison of the amino acid sequences of ruminant β-lactoglobulins.

Studies on the effect of genetic polymorphism on the properties of bovine β-LG has largely centred on the predominant A and B variants, with a few studies including the C variant. The A, B and C variants differ in amino acid sequence at three positions, representing relatively small changes in the primary structure. No significant structural differences have been seen between crystal structures of β-LG A and B variants at 6Å (Green *et al.* 1979) or 2.5Å (Monaco *et al.* 1987), or between β-LG A, B and C (refined to 1.85 Å, 1.95 Å and 1.8 Å respectively, Bewley *et al.*, 1997), although, this latter study detected some structural differences between the A and B variants around

residues 43-40 (Baker, personal communication). Structural differences were not detected by Fourier transform infrared spectroscopy or circular dichroism (CD) spectroscopy (Dong *et al.*, 1996). However, significant differences are seen in the physicochemical properties of the variants. The rate of hydrogen-deuterium exchange is greater for β-LG A than for β-LG B, suggesting that the A variant has a greater conformational mobility (Dong *et al.*, 1996). The substitutions at residues 64 and 118 may alter the hydrogen-bonding in the core structure. Genetic differences in B-LG have been shown to influence denaturation-related characteristics, such as the heat stability of milk and the gelation properties of β-LG solutions (Hill *et al.*, 1996). β-LG variants have also been shown to be associated with changes in both the composition and the technological properties of milk and other systems containing these variants (Jacob and Puhan, 1992).

4.1 Physicochemical properties of β-LG variants

4.1.1 Thermal Denaturation of purified β-LG

The majority of studies on the heat denaturation of bovine β-LG have focused on the A and B variants. A large number of studies on the denaturation characteristics of these two variants have been carried out (for review see Hill *et al.*, 1997b). Studies on the thermal stability of individual variants in purified β-LG solutions at temperatures below 90-95°C generally agree that the B variant is less stable than the A variant, but above 95°C results are conflicting (Jacob and Puhan, 1992).

Relatively few studies have been performed which include β-LG C. Sawyer (1968) found that the susceptibility of the β-LG variants to denaturation was C > B > A. A study of the heat denaturation of β-LG A, B and C, using circular dichroism at 293 nm (spatial orientation of tryptophan residues), 216 nm (proportion of β-sheet structure) and 205 nm (proportion of random structure) to probe changes in the secondary structure of β-LG (Manderson *et al.*, 1997a), indicated that the denaturation midpoint for β-LG C is 2°C higher than that of β-LG A, which in turn is 2°C higher than the denaturation midpoint for β-LG B.

The irreversible loss of native β-LG A, B and C in the ultrafiltrate permeate from whey protein concentrate at 80°C, measured using SDS-PAGE under non-reducing and reducing conditions (Hill and Lowe, personal communication), indicated that, under these conditions, the loss of native β-LG B was more rapid than the loss of both native β-LG A and C, which showed similar denaturation characteristics. The formation of disulphide linked aggregates of β-LG followed the order B > C > A.

4.1.2 Dissociation, association and aggregation behaviour of β-LG

Using sedimentation equilibrium experiments, McKenzie and Sawyer (1972) found that the tendency of β-LG variants to dissociate from dimers into monomers followed the order A > B > > C.

Using gel permeation and analytical affinity chromatography (Thresher *et al.*, 1994), examined the subunit interactions of the β-LG A and B variants in simulated milk ultrafiltrate (SMUF). The interaction of immobilised β-LG B with soluble β-LG B was stronger than the interaction of immobilised β-LG A with soluble β-LG A, with the interaction between β-LG A and β-LG B being the weakest.

At pH values between 3.7 and 6.5 dimers of the β-LG A variant will form octamers. This behaviour is not shown by dimers of the β-LG B or C variants (Hambling *et al.*, 1992). There is evidence that the caxboxyl group of the Asp-64 residue, which is only found in the β-LG A variant, together with the carboxyl group of Asp-28 is important in determining the ability of β-LG to form octamers.

Parris *et al.* (1993) observed that two types of aggregate, soluble and insoluble, were formed upon the heating of whey proteins in sweet whey samples prepared from both bulk whole milk and milk containing only β-LG A or β-LG B. An increase in the relative amount of insoluble aggregate was observed with increasing concentrations of calcium. Sweet whey prepared from milk containing β-LG B had a tendency to form a greater proportion of soluble than insoluble aggregates, whilst the reverse was seen in

sweet whey prepared from milk containing β-LG A. A possible reason for this difference in the aggregation properties of the β-LG A and B wheys is the lower charge on the β-LG B variant due to the amino acid substitution at residue 64 (Asp in the A variant, Gly in the B) leading to a reduction in calcium binding and therefore the formation of fewer insoluble aggregates. An alternative explanation is that a difference in the reactivity of thiol groups between these variants could be responsible for the observed difference in the proportion of soluble and insoluble aggregates that form in the β-LG A and B type wheys.

4.1.3 Heat-induced gelation

The heat-induced gelation characteristics of β-LG A and B have been found to be different. Huang *et al.* (1994a) found that although both these variants formed gels, the gelation point was lower and initial gelation rate higher for β-LG A, and the gels exhibited different rheological properties in stress relaxation experiments. Huang *et al.* (1994a) concluded that due to these different rheological properties, the gel matrix structures formed from β-LG A and B must involve different molecular interactions between the partially unfolded chains of β-LG A and B. McSwiney *et al.* (1994) found that the gel strength of β-LG A type gels was greater than that of β-LG B type gels in imidazole-NaCl buffer, particularly at protein concentrations above 5%. This study also found that the gel strength of β-LG B type gels was markedly affected by pH, whereas the gel strength of β-LG A type gels appeared to be independent of pH.

4.1.4 Reactivity of the free thiol group

β-LG A and B were found to react with Ellman's reagent at identical rates, whereas β-LG C reacted at about 1 tenth the rate of A and B (Phillips *et al.*, 1967). Thresher and Hill (1997) also found that the free thiol group of β-LG C reacted with Ellman's reagent at a slower rate than that of β-LG A and B. However this latter study also showed a difference in the reactivity of the A variant compared to that of the B variant. The relative rates of reaction of the β-LG variants with Ellman's reagent in SMUF at pH 6.6 at 60.9, 61.2 and 62.5°C followed the order A > B > C.

4.1.5 Reaction of β-LG with κ-casein

McKenzie *et al.* (1971) and Parnell-Clunies *et al.* (1988) have shown that the reaction between β-LG B and κ-casein is faster than that of β-LG A and κ-casein. However Thresher (1997) found that the reaction of the β-LG A, B and C variants with activated κ-casein followed the order A > B > C.

4.1.6 Hydrolysis

β-LG A has been shown to be hydrolysed by trypsin at a faster rate than β-LG B in a number of studies (Monnot, 1964, Chen *et al.*, 1993, Huang *et* al., 1994b, van Willige and Fitzgerald, 1995). A similar result was obtained using chymotrypsin (van Willige and Fitzgerald, 1995) and papain (Schimdt and van Markwijk, 1993). The study by van Willige and Fitzgerald (1995) found that the differences in the rates of hydrolysis between the β-LG A and B variants disappeared if the hydrolysis was conducted at high pressure, suggesting that a conformational difference between these two variants was responsible for the differences in their susceptibility to hydrolysis, and that these conformation differences are removed at high pressure due to unfolding of the protein. Huang *et al.* (1994b) also concluded that the differences in the rates of hydrolysis of β-LG A and B were due to differences in conformation between the two molecules. However, Schimdt and van Markwijk (1993) proposed that the more rapid hydrolysis of β-LG A by papain was possibly due to the Gly (β-LC B) to Asp (β-LG A) substitution at position 64 and that the first bond to be cleaved in the protein occurs in an external loop containing this substitution.

Relatively few reports have included studies on the hydrolysis of β-LG C. Kalan *et al.* (1965) found that the release of C-terminal amino acids by carboxypeptidase from β-LG C was slower than from β-LG A or B. Motion and Hill (1994) examined the hydrolysis of the β-LG A, B and C variants by trypsin and chymotrypsin using size exclusion chromatography. The rate of hydrolysis of the β-LG variants was found to follow the order A > B > C. Comparison of the intermediates and products of the hydrolysis indicated that the orders of bond cleavage are the same in β-LG A and B,

but that these bonds were more rapidly hydrolysed in the β-LG A variant. This conclusion differed from that of Huang *et al.* (1994c), who found that the products of β-LG hydrolysis by immobilised trypsin were different for β-LG A and B, and suggested that the susceptibilities of the tryptic hydrolysis sites were altered due to differences in conformation between the variants.

4.2 Effect of β-LG polymorphism on milk composition

The effect of β-LG phenotype on milk protein content is still controversial, with some studies showing no effect, while others link β-LG polymorphism to differences in yield, not only of the protein itself, but also of other milk proteins and of milkfat (Jacob 1993, Hill *et al.*, 1997b). Studies in New Zealand have shown that bulk milk collected from β-LG AA phenotype cows had a composition that was markedly different to that of β-LG BB phenotype cows. The β-LG AA phenotype was associated with higher whey protein concentrations, and lower casein, fat and total solid concentrations than the β-LG BB phenotype (Hill 1993, Hill *et al.*, 1993, Hill, 1994, Hill and Paterson 1994, Hill and MacGibbon, 1994, Hill *et al.*, 1995b). The studies on New Zealand herds showed the higher whey protein content and lower casein content in the milk produced by β-LG AA phenotype cows to be a consequence of higher levels of β-LG in this milk type (Hill, 1993, Hill *et al.*, 1995b), and this has also been shown to be the case in many other studies (for review see Hill *et al.*, 1997b). Relatively few studies have looked at the effect of β-LG C on the composition of milk. However, Paterson *et al.* (1995) found that the variants of β-LG affect the level of β-LG in milk in the order A> B> C. Feagan *et al.* (1972) report a similar result.

The relationship between β-LG polymorphism and the total protein content of milk is unclear but appears to minor, with the higher whey protein content in the milks produced by β-LG AA phenotype cows being balanced by a decrease in the casein content in this milk type, with the opposite being true for the milk produced by β-LG BB phenotype cows (Hill *et al.*, 1997b).

van den Berg *et al.* (1992) found no relationship between β-LG polymorphism and the calcium content of milk, and various New Zealand studies (Hill, 1993, Hill *et al.*, 1993, Hill, 1994, Hill and Paterson, 1994, Hill *et al.*, 1995b) have shown little correlation between β-LG polymorphism and the levels of a variety of milk minerals and lactose. An exception was sodium, which was found at higher levels in the milk produced by β-LG AA phenotype cows than the milk from β-LG BB phenotype cows.

A higher milk yield was also associated with the β-LG AA phenotype (Hill *et al.*, 1997b). β-LG polymorphism has also been related to differences in the levels of proteose peptones in milk (Scharr, 1985), and to the size of casein micelles (Lodes, 1995).

The composition of milk is important in determining its nutritional value, processing properties and properties of manufactured dairy products. The higher casein content of milk produced by β-LG BB phenotype cows is important when one considers the manufacture of casein rich milk products (see below).

4.3 Effect of β-LG polymorphism on the technological properties of milk

Differences between the variants of β-LG are of significant commercial importance to the dairy industry, as they have been found to influence the processing properties of milk:

4.3.1 Heat stability of milk

Because the heat stability of milk is dependent upon a variety of different factors, including κ-casein phenotype, the effect of β-LG variants on this property has not always produced consistent results. Comparisons of heat coagulation time-pH (HCT-pH) curves for milks containing β-LG A and B variants (Rose 1962, Feagan *et al.*, 1971, McLean *et al.*, 1987) all showed that the minimum of the curve was affected by β-LG phenotype following the general trend AA > AB > BB. However, Robitaille (1995) found that the effect of β-LG phenotype on HCT-pH was only significant (AA > BB) in milks which were of the κ-casein phenotype AA.

Feagan *et al.* (1971) found that β-LG AA, AB, AC and CC phenotype milks always gave HCT-pH profiles which had a marked maximum at around pH 6.7-6.8 and minimum at around pH 6.8-7.0 (Type A curves), whereas β-LG BB and BC phenotype milks generally gave HCT-pH profiles which increased steadily over the range pH 6.4-7.2 (Type B curves). Other studies (Rampilli *et al.*, 1988, Robitaille, 1995) found that the type of curve (A or B) was strongly influenced by κ-casein phenotype.

In a recent study on the heat stability of pooled β-LG AA and BB phenotype milks throughout a New Zealand dairy season (Paterson, Anema and Hill, personal communication) it was found that β-LG AA phenotype milks gave Type A HCT-pH curves. β-LG BB phenotype milk gave curves which did not correspond to either those of Type A or B, but instead had a relatively high heat stability that was unaffected by pH. It was also found that the apparatus used for HCT-pH measurement could markedly influence the results from these types of experiments.

4.3.2 Fouling properties of milk

Fouling, the deposition of material on heat exchange surfaces of milk processing plant during pasteurisation and ultra-high-temperature (UHT) processes, is a significant problem in milk processing (Fryer *et al.*, 1995). Hill *et al.* (1997a) found that recombined and reconstituted β-LG AA type milk powders rapidly fouled UHT plant, while recombined and reconstituted β-LG BB type milk powders gave very low fouling of UHT plant, except for those powders manufactured at the beginning of the season. Recombined evaporated milk heat stability for β-LG BB type milk powders was shown to be superior and that of β-LG AA type milk powders to be inferior to standard mixed variant milk (Hill *et al.*, 1997c). There was also evidence that it may be more difficult to manufacture milk powders from β-LG AA type milk, and that BB milk is more suitable for milk powder manufacturing than standard mixed variant milk.

4.3.3 Heat stability of condensed milk

van den Berg *et al.* (1992) found that the maximum heat stability of condensed milk manufactured from skim milk was significantly lower in the product produced from the milk of β-LG BB phenotype cows when compared with that produced from the milk of β-LG AA phenotype cows. Product made from the milk supplied from β-LG AB phenotype cows had an intermediate heat stability.

4.3.4 Rennet coagulation properties

In a study of the rennet coagulation properties of pooled β-LG AA and BB phenotype milks throughout a New Zealand dairy season, β-LG BB phenotype milk was found to have a significantly faster curd firming rate than β-LG AA phenotype milk upon rennet coagulation, but β-LG phenotype did not affect gelation time in raw or pasteurised milk (Hill *et al.*, 1995a). van den Berg *et al.* (1992) also found that β-LG variant does not affect gelation time.

A number of studies on the relationship between β-LG variants and curd firmness found β-LG BB phenotype milk to give firmer curds when compared with other β-LG phenotype milks (Sherbon *et al.*, 1967, Feagan *et al.*, 1972, Mariani *et al.*, 1982, Rahli and Menard, 1991). However Marziali and Ng-Kwai-Hang (1986) found that β-LG AA phenotype milk gave firmer curds, and a number of other studies (Trevala *et al.*, 1983), Aaltonen and Antila, 1987, Pagnacco and Caroli, 1987, Politis and Ng-Kwai-Hang, 1988) found no correlation between β-LG phenotype and curd firmness.

Hill *et al.* (1995a) found that the milk supplied from β-LG BB phenotype cows was more suitable for the manufacture of cheddar cheese, giving increased yields without any apparent detrimental effects on cheese properties. β-LG BB phenotype milk has also been shown to give higher yields of Svezia cheese (Schaar *et al.*, 1985), Parmesan cheese (Aleandri *et al.*, 1990), Camembert cheese (Rahali and Menard, 1991) and Gouda cheese (van den Berg *et al.*, 1992).

Milk supplied from β-LG BB phenotype cows was also found to be more suitable for the manufacture of rennet and lactic caseins as it contains more casein and gave a higher yield of product with lower processing losses than the milk supplied from β-LG AA phenotype cows and standard factory supply milk (β-LG AB) (Hill *et al.*, 1995c). The increase in casein yield associated with the processing of the milk supplied from β-LG BB phenotype cows did not have any negative effects on the physical or functional properties of the rennet or lactic caseins produced from that milk type.

Experimental

Part 1 Investigation of the mobilities of various β-LGs under native-PAGE, non-reducing SDS-PAGE and reducing SDS-PAGE conditions, and factors affecting the mobility of bovine β-LG A, B and C under these electrophoresis conditions.

Part 2 Determination of conditions under which β-LG can be reacted with NEM (Section 4), conditions under which the labelled protein can be hydrolysed (Section 5), and identification of the position of the labelled residue within the amino acid sequence of the β-LG A, B and C variants (Section 6).

Materials

β-LG

Bovine β-LG A, B and C variants were prepared from milk collected from individual β-LG AA, BB and CC cows. Caprine β-LG was prepared from milk obtained from Dairy Goat Co-operative (NZ).

A freeze dried sample of purified bovine β-LG D was provided by Ingo Krause of the Institute for Dairy Science and Food and Process Engineering, Technical University Munich, Germany.

Purified porcine β-LG was provided by Gavin Manderson (Food Science Section, NZDRI). The sample was prepared from acid whey, provided by Dan Gallagher (Department of Food Chemistry, University College Cork, Ireland). Whey was separated from porcine casein at pH ~ 4.5, dialysed and freeze dried, and β-LG was purified by NaCl salting out as described by Dalgalarrondo *et al.* (1992). Dialysis and gel filtration was performed as described for bovine β-LG A, B and C (see following).

Chemicals

Sodium dihydrogen orthophosphate-1-hydrate ($NaH_2PO_4.H_2O$) [AnalaR]
Di-sodium hydrogen orthophosphate-2-hydrate ($Na_2HPO_4.2H_2O$) [AnalaR]
Sodium Chloride (NaCl) [AnalaR]
Tris(hydroxymethyl)methylamine [AnalaR]
Ethylenediaminetetra-acetic acid disodium salt (Na_2EDTA) [AnalaR]
Sodium dodecyl sulphate (SDS) [Biochemical]
2-Mercaptoethanol (2-ME) [AnalaR]
Urea [AnalaR]
Glycine [AnalaR]
Glycerol [AnalaR]
Acetonitrile (CH_3CN) [HiperSolv For HPLC]

Trifluoroacetic acid (TFA) [HiperSolv For HPLC]
BDH Laboratory Supplies, Poole, England

Acrylamide/Bis 37.5:1 (2.6%C)
Amonium persulphate (APS)
N,N,N′,N′-Tetramethylethylenediamine (TEMED)
Bromophenol Blue
Coomassie Brilliant Blue R-250
Bio-Rad Laboratories, Hercules, California, USA

Amido Black 10B (Merck Art. 1167)
Iodoacetamide
Merck-Schuchardt, Schuchardt, Hohenbrunn bei Munchen, Germany

N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES)
N-Ethyl maleimide (NEM)
5,5'-Dithio-bis(2-nitrobenzoic Acid) (DTNB)
2-Mercaptopyridine
2,2'-Dithiopyridine (2,2'-dipyridyl disulfide) (DPDS)
DL-Dithiothreitol (DTT)
N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated Trypsin
Sigma Chemical Co, St. Louis, Missouri, U.S.A.

2,5-Diphenyloxazole (DPO)
Park Scientific Ltd., Northhampton, England

[1,4-^{14}C] N-ethyl maleimide (^{14}C-NEM)
American Radiolabeled Chemicals Inc., St. Louis, Missouri, USA

Milli-Q deionised water
Millipore Corp., Bedford, Massachusetts, USA

β-LG Purification

Bovine A, B and C variants.

Fresh milk was heated to 30°C and passed through an Elecrem 80 separator (Vertrieb + Kundendienst, HÄKA, Buttermaschinen CmbH, Stutensee) to remove fat. Whey was prepared from skim milk by acid precipitation at pH 4.6 (pH adjusted using ~3 M HCl), at 40°C, followed by filtration through cheesecloth to remove preciptated caseins. The whey was freeze concentrated and the concentrated whey was centrifuged (5000 x g for 15 minutes) to remove any remaining fat, casein and precipitated lactose. β-LG was purified from concentrated acid whey by NaCl precipitation using a modification of the method described by Mailliart and Ribadeau-Dumas (1988). To precipitate all whey proteins except β-LG, the pH of the whey was adjusted to 3.0 (~3 M HCl). NaCl (food grade) was added to give a concentration of 7% (w/w) and the pH adjusted to 2.00 (~3 M HCl). After 20 minutes at room temperature the solution was centrifuged (10,000 x g for 20 minutes) and the supernatant retained. Additional NaCl was added to give final concentration of 30% (w/w) and the pH readjusted to 2.0 (5 M NaOH). After 20 minutes at room temperature the solution was centrifuged (10,000 x g for 20 minutes) and the resulting pellet resuspended in Milli-Q water, giving a pH of ~5.8. The protein solution was dialysed (10,000 MW cutoff) against Milli-Q water (2x) and buffer (20 mM $NaH_2PO_4.H_2O$/30 mM NaCl, pH adjusted to 6.0 with 5 M NaOH) (3x).

The protein was further purified by gel filtration using a Superdex G75 26/60 column on a Pharmacia BioPilot system (Pharmacia, Uppsala, Sweden). Proteins were eluted in 20 mM $NaH_2PO_4.H_2O$/30 mM NaCl (pH 6.0). The protein peaks were monitored by following absorbance at 280 nm, and fractions were collected over the major peak. The purity of the fractions was analysed by native-PAGE, and those containing only β-LG were pooled and stored frozen at ~4 mg/mL. Samples were thawed and concentrated to 40-60 mg/mL before use, using an Amicon stirred cell with a YM10 membrane (Amicon, Inc., Beverly, Massachusetts, U.S.A).

Caprine β-LG

Caprine β-LG was prepared as described for the bovine β-LG A, B and C variants with the following exceptions:
Caseins were precipitated at pH 4.2 (Richardson *et al.*, 1973).
Whey was not freeze concentrated before salt precipitation.

PAGE methods

SDS-PAGE and native-PAGE were performed using the Mini-Protean II Dual Slab Cell system (Bio-Rad Laboratories, hercules, California, USA) and the methods described by Creamer (1991). A standard procedure using the following methods was followed:

SDS-PAGE

Stock solutions of Acrylamide/Bis (30% w/v in Milli-Q water), SDS-PAGE resolving gel buffer (1.5 M Tris-HCl, pH 8.8), SDS-PAGE stacking gel buffer (0.5 M Tris-HCl, pH 6.8) APS (10% w/v in Milli-Q water) and SDS (10% w/v in Milli-Q water) were made. Resolving gel (16%T, 2.6%C) and stacking gel (3.87%T, 2.6%C) were made as described in Appendix A. Gels were left overnight to polymerise.

Native-PAGE

Stock solutions of Acrylamide/Bis (30% w/v in Milli-Q water), native-PAGE resolving gel buffer (3 M Tris-HCl, pH 8.8), native-PAGE stacking gel buffer (0.5 M Tris-HCl, pH 6.8) and APS (10% w/v in Milli-Q water) were made. Resolving gel (15%T, 2.6%C) and stacking gel (3.75%T, 2.6%C) were made as described in Appendix B. Gels were left overnight to polymerise.

Sample Preparation

SDS-PAGE sample buffer was prepared by mixing 125 mL SDS-PAGE stacking gel buffer, 25 mL Bromophenol Blue (0.4% w/v in 0.1 M NaOH), 100 mL glycerol, 200 mL 10% SDS and 500 mL Milli-Q water, to give a final pH of 6.8. Native sample buffer was prepared by mixing 200 mL native-PAGE stacking gel buffer, 20 mL Bromophenol Blue (0.4% w/v), 80 mL glycerol and 600 mL Milli-Q water to give a pH of 6.8.

Samples were diluted to the required concentration with the appropriate sample buffer. Just prior to loading, samples for reduced SDS-PAGE were reduced by 2-mercaptoethanol (2-ME). 2-ME (20 μL) was added to 1 mL of sample and samples were heated in a boiling water bath for 4 minutes.

Aliquots of each sample (2-10 μL) were loaded into gel lanes, and gels were run using the Bio-Rad 1000/500 power supply, with the voltage set at ≤ 210 V, current ≤ 70 mA, power ≤ 3.25 W/gel, time ≤ 2.0 hours, until 20 minutes after the Bromophenol Blue tracer dye seeped from the bottom of the gel. Electrode buffer consisted of 25 mM Tris-HCl, 0.2 M glycine, 3.5 mM SDS, pH 8.6 ± 0.2 (SDS-PAGE) or 25 mM Tris-HCl, 0.2 M glycine, pH 8.3 (native-PAGE).

Staining

Coomassie Blue staining solution was prepared by dissolving 2.5 g of dye in 1250 mL isopropanol, 500 mL glacial acetic acid and 3250 mL Milli-Q water. The mixture was stirred overnight and then filtered through Whatman No 1 filter paper under vacuum. Destain consisted of 500 mL isopropanol, 500 mL glacial acetic acid and 4 L Milli-Q water.

Amido Black staining solution was prepared by dissolving 1 g of dye in 250 mL isopropanol, 100 mL glacial acetic acid and 650 mL Milli-Q water. The destaining solution was a 10% (v/v) solution of glacial acetic acid in Milli-Q water.

Gels were stained for 1 hour with either Coomassie Blue or Amido Black and then destained with the appropriate destain solution for a total of 20 hours, the destain solution being changed after 1 hour. After destaining, gels were scanned using Molecular Dynamics - Series 300 - Personal Densitometer (Molecular Dynamics Inc., Sunnyvale, California, U.S.A.) and the resulting scan quantified using an IBM compatible computer installed with ImageQuant (Molecular Dynamics Inc.) and Microsoft Excel software (Microsoft Corp., Redmond, Washington, U.S.A.).

PAGE Protein Standards

Standards for PAGE were prepared from freeze dried samples of β-LG A, B and C. Solutions of approximately 1 mg/g (in Milli-Q water) were made and the absorbance of each was measured at 280 nm. The purity of each sample was calculated using the equation:

$$\% \text{ purity} = (A_{280}/9.7) \times (1000/\text{concentration } \beta\text{-LG (mg/g)}) \times 100$$

where $9.6 = A_{280}^{1\%,1cm}$ (Bell and McKenzie, 1967). A stock solution of each variant was made by dissolving freeze dried protein in Milli-Q water. From each stock solution a series of dilutions was made using the appropriate PAGE sample buffers, giving samples with concentrations ranging from 0.02 to 0.7 mg/mL. Ten µl of standard solutions were loaded on to gels where quantification was required.

Part 1. Polyacrylamide Gel Electrophoresis of β-lactoglobulin

1 Qualitative Study

1.1 Band Splitting

1.1.1 Methods

Concentrated β-LG was diluted to approximately 1 mg/mL in 20 mM phosphate buffer ($NaH_2PO_4.H_2O$, adjusted to the required pH with 5 M NaOH).1 mL samples of β-LG solutions at pH values of 6.0 or 7.0 were heated for ten minutes in a Julabo 19 circulating waterbath set at 90 or 95°C, or in an oil bath filled with silicon oil set at 110°C and with continuous shaking. Samples were analysed using SDS-PAGE under non-reducing and reducing conditions.

1.1.2 Results

1.1.2.1 Bovine β-LG A, B and C

Purified, unheated β-LG ran as a single band under non-reducing SDS-PAGE conditions, the A variant having a slightly lower mobility than the B and C variants. Heating in 20 mM phosphate buffer, pH 6.0, at 95°C for ten minutes promoted the splitting of β-LG into two bands under non-reducing SDS-PAGE conditions, as shown in Figure 5(i). This behaviour was observed with all three variants. In the A variant the band formed after heating had a higher mobility than the original band, whilst in the B and C variants this band had a lower mobility than the original band. Heating also induced the formation of aggregated material which appeared as higher molecular weight bands on the gel. Under reducing conditions only a single band was seen in both heated and unheated samples, as shown in Figure 5(ii). No difference in mobility between the variants was seen in reduced samples. This result is the same as that obtained by Hill *et al.* (1997b) under similar conditions.

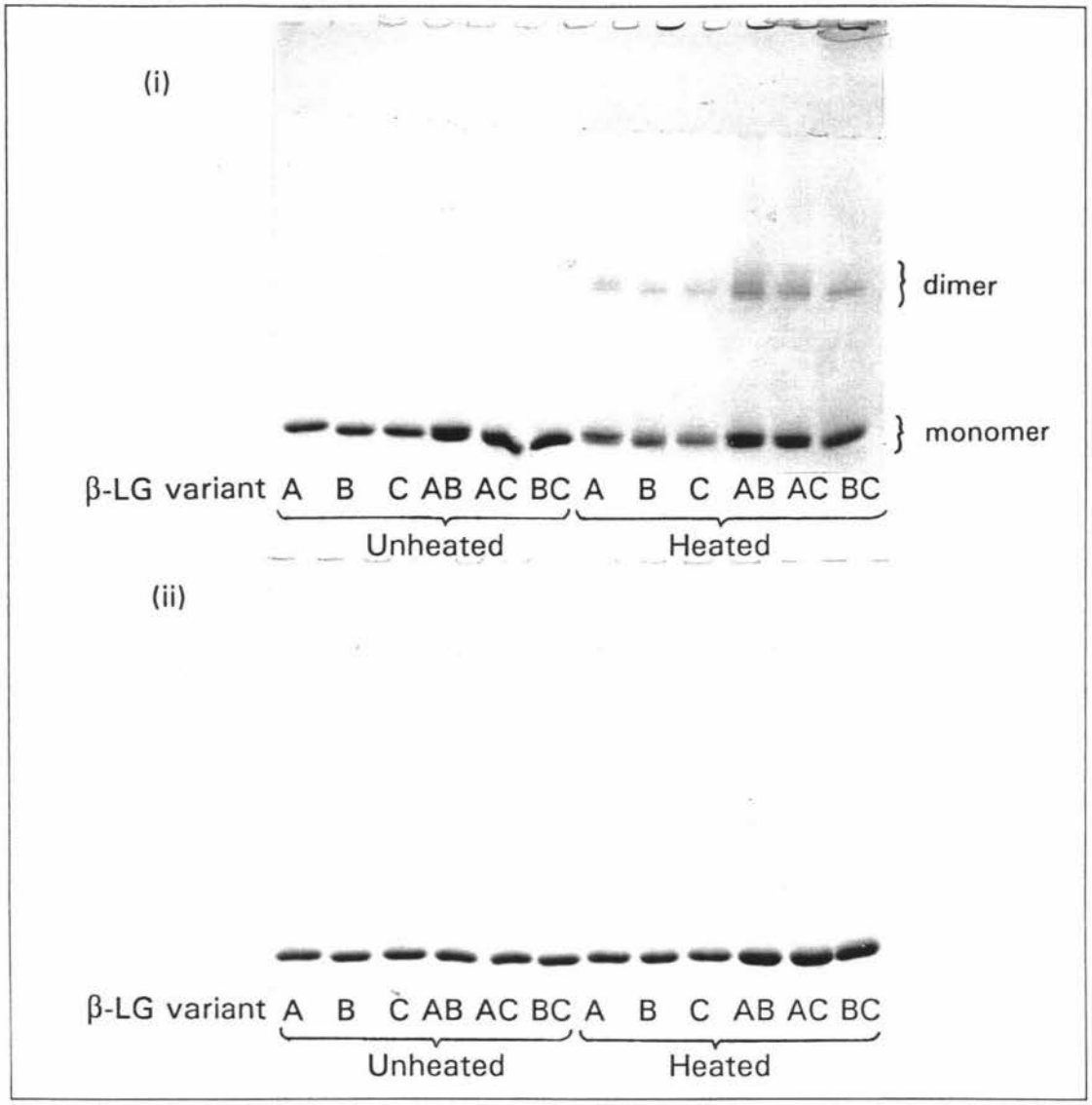


Figure 5 SDS-PAGE of purified bovine β-LG A, B and C after heating at 95°C. (i) Under non-reducing conditions. (ii) Following reduction of samples with 2-ME.

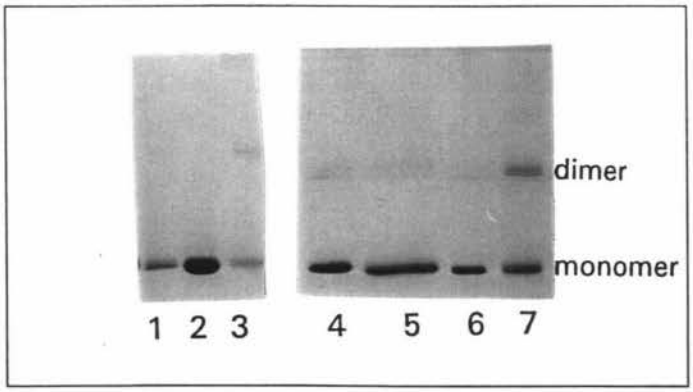


Figure 6 SDS-PAGE of purified bovine β-LG A, B and D under non-reducing conditions. Lanes 1 and 5, β-LG D. Lanes 2 and 6, β-LG B. Lane 4, β-LG A. Lanes 3 and 7, β-LG D following heating at 95°C.

1.1.2.2 Bovine β-LG D

The relative mobilities of unheated and heated bovine β-LG variants A, B and D under non-reducing SDS-PAGE conditions were compared. Two bands were seen in the unheated sample of β-LG D, as shown in Figure 6. Band splitting had probably occurred either during purification or storage. The major band was at the bottom and appeared to have a similar mobility to that of the B variant, i.e. slightly higher than that of the A variant. After heating in 20 mM phosphate buffer, pH 6.0, at 95°C, the relative distribution between these two bands was more equal (Figure 6). It is possible that the lower of the two bands represents the original position of the monomeric protein. Heating also resulted in the formation of aggregated material which appeared as higher molecular weight bands on the gel.

1.1.2.3 Caprine β-LG

The relative mobilities of unheated and heated bovine β-LG variants A and B and caprine β-LG under non-reducing SDS-PAGE conditions were compared. The mobility of unheated caprine β-LG. under non-reducing SDS-PAGE conditions appeared to be the same as that of the bovine B variant, slightly higher than that of the A variant (Figure 7). After heating in 20 mM phosphate buffer, pH 7.0, at 90°C, a second band of slightly lower mobility was seen in caprine β-LG, but under these conditions a large percentage of the protein formed aggregates that did not enter the gel (Figure 7). In bovine β-LG A and B, heating under these conditions induced band splitting and aggregation as seen previously at pH 6.0. Under reducing conditions caprine β-LG ran as a single band and appeared to have a slightly higher mobility than the bovine variants (Figure 7).

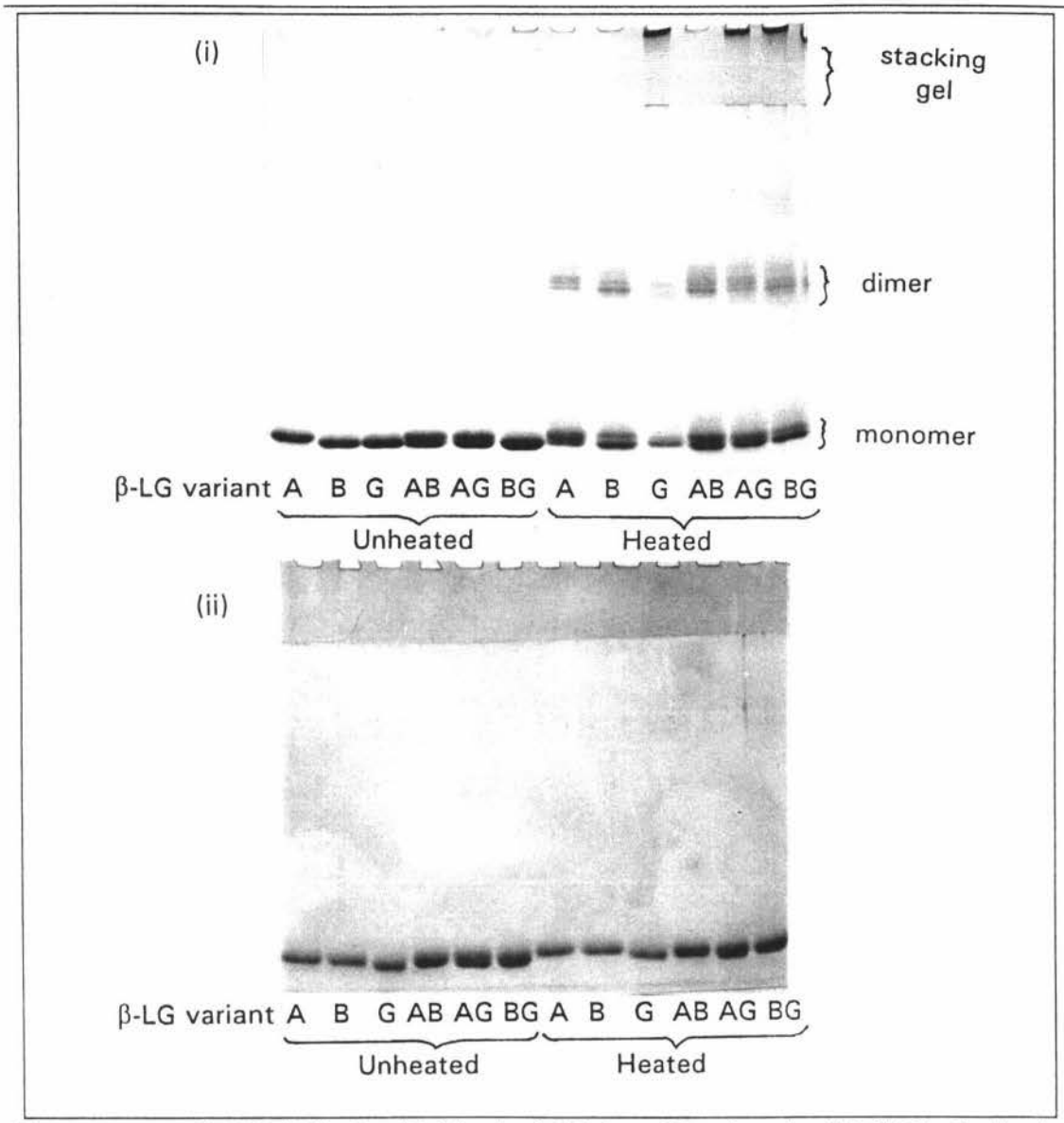


Figure 7 SDS-PAGE of purified bovine β-LG A and B and caprine β-LG (G) after heating at 90°C. (i) Under non-reducing conditions. (ii) Following reduction of samples with 2-ME.

1.1.2.4 Porcine β-LG

Porcine β-LG ran as two bands under non-reducing SDS-PAGE, with one of the bands having higher mobility and being more dense in appearance than the other (Figure 8). Heating the porcine β-LG did not appear to induce any change in the appearance of the two bands. There was also no evidence of aggregation of this protein as shown by the lack of higher molecular weight bands on the gel. It is possible that the minor of the two bands in porcine β-LG is a contaminant, which is to be investigated as part of future work.

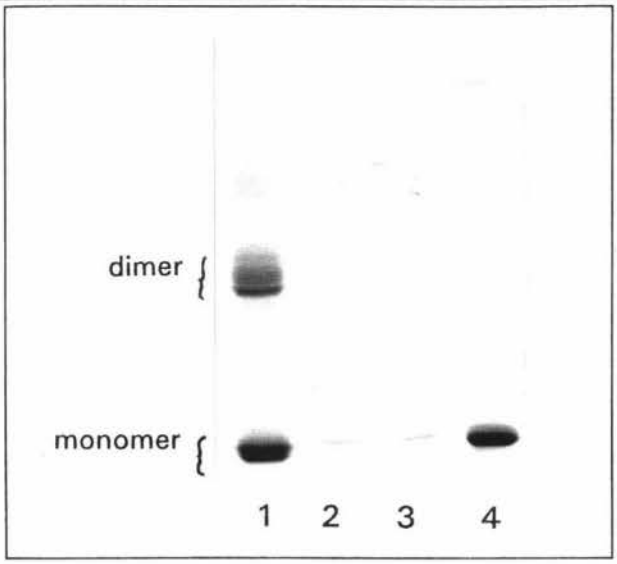


Figure 8 Non-reduced SDS-PAGE of bovine β-LG A and porcine β-LG before and after heating at 110°C. Unheated samples: lanes 2 (porcine β-LG) and 4 (β-LG A). Heated samples: lanes 1 (porcine β-LG) and 3 (β-LG A).

1.2 Conditions affecting band splitting in bovine A, B and C variants

1.2.1 Methods

1.2.1.1 Heat treatment of β-LG

Concentrated β-LG was diluted to approximately 1 mg/mL or 10 mg/mL in either 20 mM phosphate solution ($NaH_2PO_4.H_2O$, adjusted to the required pH with 3 M Hcl or 5 M NaOH) or 0.2 M phosphate solution ($NaH_2PO_4.H_2O/Na_2HPO_4.2H_2O$, adjusted to the required pH by changing the ratio of the mono- to di-sodium hydrogen phosphate components). Samples (1 mL) of β-LG at pH values of 5.0-8.4 were heated for ten minutes in a Julabo 19 circulating waterbath set at temperatures ranging from 50-95°C, or in an oil bath filled with silicon oil set at temperatures ranging from 100-110°C and with continuous shaking.

1.2.1.2 Urea treatment of β-LG

β-LG was freeze dried, and 100-129 mg of freeze dried β-LG (A, B or C) was dissolved in 0.1 M Tris-HCl/0.027 M Na_2EDTA, pH 7.5. Iodoacetamide (52 mg/mL in 0.1 M Tris-HCl/0.027 M Na_2EDTA, pH 7.5) and urea (10 M in milliQ water/0.1 M Tris-HCl/0.027 M Na_2EDTA, pH 7.5) were added as follows:

Method (i): 100 μL of iodoacetamide solution was added to the protein solution, followed by 8 mL of urea solution to give a final urea concentration of 8 M. The mixture was incubated at 25°C for 10 minutes, After which 10 mL of sodium acetate (0.5 M, pH 5.0) was added and the resulting solution dialysed against 5% acetic acid (v/v) then Milli-Q water (3 x 2 L) for 14 hours.

Method (ii): 10 mL of urea solution was added to the protein solution and the mixture incubated at 25°C for 1.5 hrs. Iodoacetamide solution (100 μL) was added and the mixture incubated for a further 10 minutes. Sodium acetate (10 mL) was added and the solution dialysed as before.

1.2.2 Results

1.2.2.1 Temperature

In 0.2 M phosphate buffer, pH 6.0, band splitting was not observed after heating for 10 minutes at temperatures below 85°C. At higher temperatures the proportion of material in the heat induced band of the doublet increased. Increasing temperatures also resulted in increasing amounts of aggregated material, particularly in the A variant (Figure 9).

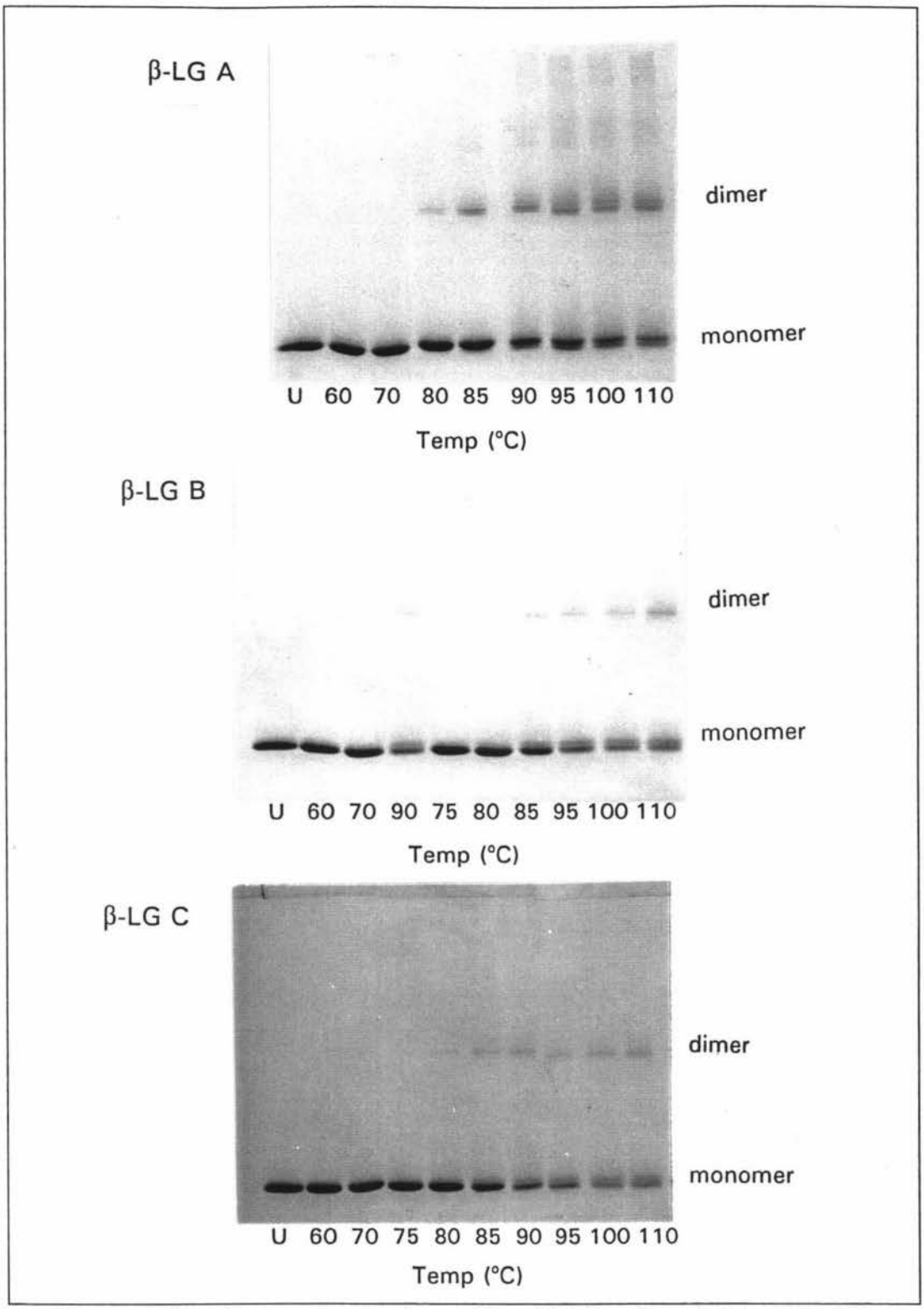


Figure 9 Effect of temperature on the mobility of β-LG variants A, B and C under non-reducing SDS-PAGE conditions. U refers to unheated samples. Heated samples were incubated for 10 minutes at the temperatures shown.

1.2.2.2 pH

In 20 mM phosphate buffer, increasing the pH at which heating was performed promoted band splitting. At pH 5.0 no band splitting was seen. A precipitate formed at 80°C in the A variant and at 85°C in the B and C variants. At pH 6.0 band splitting was seen at 85°C, at pH 7.0 band splitting was seen at 70°C, and at pH 8.0 and 8.4 band splitting was seen at 60°C (results not shown). Under these conditions no difference in the tendency to show band splitting was seen between the three variants.

1.2.2.3 Ionic Strength

Increasing the ionic strength of the buffer from 20 mM to 0.2 M had no effect on the temperature at which protein precipitated at pH 5.0. At this ionic strength heating at 110°C resulted in band splitting with all three variants at pH 6.0, but only in the A variant at pH 7 (Figure 10) and pH 8.2 (results not shown). More higher molecular weight aggregated material was seen in heated samples of β-LG at pH 7.0 than at pH 6.0.

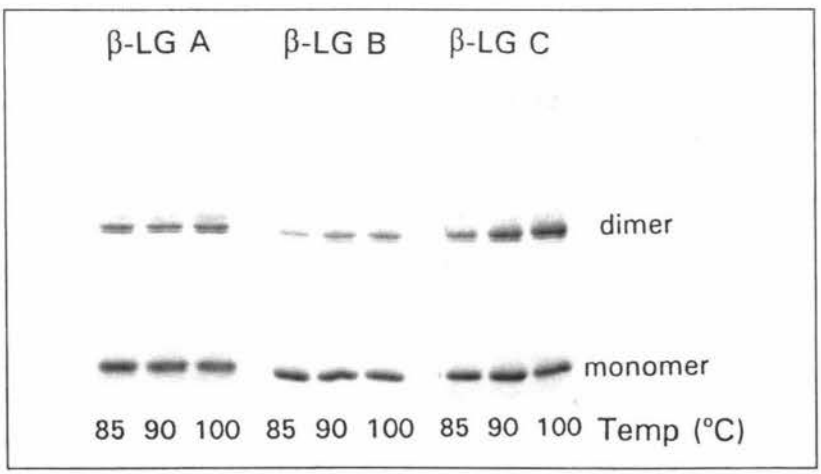


Figure 10 Effect of ionic strength on the mobility of β-LG variants A, B and C under non-reducing SDS-PAGE conditions after heating at pH 7.0. Samples were heated for 10 minutes in 0.2 M phosphate buffer at the temperatures shown.

1.2.2.4 Protein Concentration

At 110°C in 20 mM phosphate buffer, pH 7.0, no band splitting was observed with β-LG C upon increasing the protein concentration from 1 mg/ml to 10 mg/ml. However,

band splitting was still observed in β-LG A and B under these conditions. There was an increase in the amount of higher molecular weight aggregated material that formed upon heating at higher protein concentration in all three variants (Figure 11).

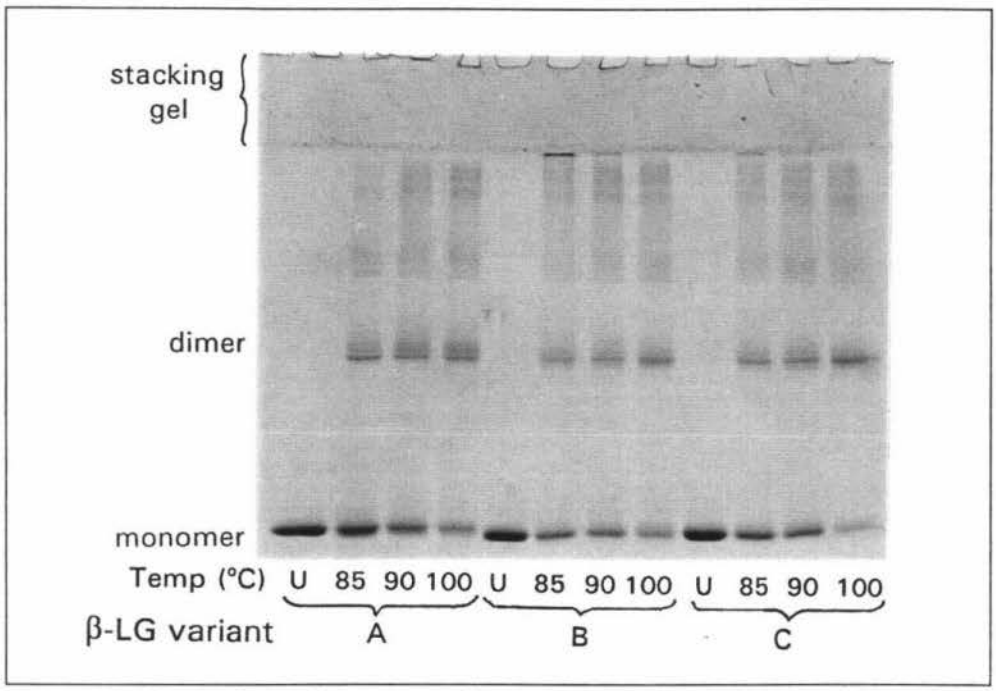


Figure 11 Effect of protein concentration on the mobility of β-LG variants A, B and C under non-reducing SDS-PAGE conditions after heating at pH 7.0. U refers to unheated samples. Heated samples were incubated for 10 minutes at the temperatures shown.

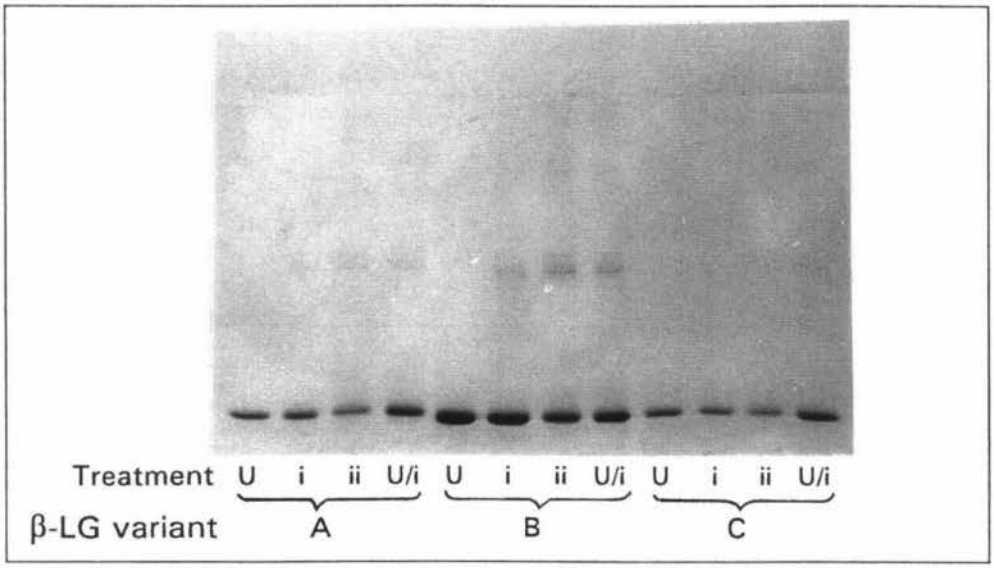


Figure 12 Effect of urea on the mobility of β-LG variants A, B and C under non-reducing SDS-PAGE conditions. U represents untreated β-LG samples. (i) and (ii) represent samples treated as described in methods (i) and (ii).

1.2.2.5 Urea

McKenzie *et al.* (1972) labelled β-LG with iodoacetamide under conditions similar to those described in method (i), and, as a control to demonstrate the amount of disulphide exchange induced by high concentrations of urea, β-LG was labelled with iodoacetamide under conditions similar to those described in method (ii). No band splitting was observed following either of these treatments, although some aggregation had occurred (Figure 12).

1.3 Discussion and Conclusions

Purified, unheated bovine β-LG variants A, B and C ran as a single band in both non-reduced and reduced SDS gels. The mobility of bovine β-LG A under non-reducing SDS-PAGE conditions is slightly lower than that of bovine β-LG B and C, and bovine β-LG D probably also has the same mobility as the B and C variants. As mobility in SDS is due to the SDS coating and not the charge on the protein, a difference in the mobility between proteins of almost identical molecular weight indicates that the structures of these proteins could be different. The mobilities of β-LG A, B and C under reducing SDS-PAGE conditions appear to be the same. This suggests that the difference in mobility between variants seen in non-reduced samples involves disulphide bonding.

Purified, unheated caprine β-LG also ran as a single band in both non-reduced and reduced SDS gels, and appeared to have the same mobility as the bovine B and C variants under non-reducing SDS-PAGE conditions but a slightly higher mobility than the bovine variants under reducing SDS-PAGE conditions. Caprine β-LG has an Asp at position 64 (as found in bovine β-LG A) and an Ala at position 118 (as found in bovine β-LG B and C) (Hambling *et al.*, 1992). The fact that in non reducing SDS-PAGE caprine β-LG runs as a band with a similar mobility to bovine β-LG B and C and a slightly higher mobility than bovine β-LG A suggests that the substitution at position 118 in the primary protein sequence may somehow be causing the mobility difference. At present this suggestion can only be speculative as caprine β-LG also differs from bovine β-LG at other positions in the primary protein sequence (Hambling *et al.*, 1992).

Heating promoted the appearance of a number of new bands in bovine β-LG A, B, C and D, and in caprine β-LG under non-reduced SDS-PAGE conditions. After heating two clearly resolved bands were seen in the monomer region of each lane, a phenomenom described here as band splitting. In the A variant the band formed after heating had a slightly higher mobility than the original band, apparently corresponding to the mobility of the unheated B and C variants. In the heated samples of the B and C variants the band formed on heating had a lower mobility than the original band, corresponding to the band seen in the unheated sample of the A variant. Induction of band splitting was temperature- and pH-dependent, occuring at lower temperatures as pH increased. Protein concentration and the ionic strength of the buffer also appeared to affect band splitting. Caprine β-LG also split into two bands on heating. Heating also induced the formation of aggregated material which appeared as higher molecular weight bands or as material that did not enter the gel. Aggregated material was seen in the bovine β-LG A, B, C and D variants, and in caprine β-LG. Reduction of heated samples of bovine β-LG A, B, C and caprine β-LG resulted in a single band with the same mobility as the unheated, reduced samples. Heating did not appear to affect the appearance of the bands in porcine β-LG.

Bovine β-LG A, B, C and D and caprine β-LG all contain a free thiol in their protein sequence (Phillips *et al.*, 1967). Porcine β-LG does not contain a free thiol (Kessler and Brew, 1970) and thus the lack of heat-induced changes to the banding pattern in porcine β-LG when compared with the bovine variants and caprine β-LG is possibly due to the absence of this potentially reactive SH group. The free thiol appears to be required both to induce band splitting and for the formation of higher molecular weight aggregates following heating. Only those β-LGs containing a free thiol in their sequence exhibit both band splitting and aggregation. Band splitting is thus probably a consequence of disulphide interchange reactions, the interchange reaction in β-LG A causing a second band to run in the position of β-LG B and C, and the interchange reaction in β-LG B and C causing a second band to run in the position of β-LG A.

Possible reasons for the observed differences in mobility under non-reducing SDS-PAGE conditions in unheated samples of the β-LGs studied are:

1. There is a difference in the position of a disulphide bond in β-LG A compared to β-LG B and C in the native protein, the position of the bond being directed by the substitution at position 118. Of the two disulphide bridges present in bovine β-LG, one has been shown by a variety of methods to be between residues Cys 66 and Cys 160 (Hambling *et al.*, 1992), and therefore close to the Asp 64 (β-LG A) → Gly 64 (β-LG B and C) variant substitution. The position of the second disulphide bridge has been debated, and has been reported to be between residues Cys 106 and Cys 121, with a free thiol at residue Cys 119 (McKenzie, 1971), between Cys 106 and Cys 119 with a free thiol at residue Cys 121 (Mainferme *et al.*, 1971, Martial *et al.*, 1971, Pérez-Gómez *et al.*, 1971, Phelan and Malthouse, 1994), or as an equal mixture of these two possible arrangements (McKenzie *et al.*, 1972). While it seems unlikely that the position of this bond differs within any one variant, it is possible that there is a difference in the position between different variants. Adjacent to Cys 119 is a second variant substitution, with Val 118 in the A variant becoming Ala 118 in the B and C variants. Thus it is possible that this substitution directs the position of the disulphide bridge in the native protein. This possibility was investigated as part of this current study (see Section 6). This would also imply that bovine β-LG D and caprine β-LG have a free thiol in the same position as the B and C variants, and different to that of the A.

2. The disulphide bonds are in the same position in all the variants studied, but the substitution at position 118 causes enough of a difference in structure under non-reducing SDS-PAGE conditions to cause a difference in mobility. It appears unlikely that the substitution at position 64 is responsible for the proposed difference in structure between bovine β-LG A when compared with B and C. This is because caprine β-LG exhibited similar behaviour to β-LG B and C even though this protein contains an Asp at position 64 (i.e. as in bovine β-LG A). However, like bovine β-LG B and C, caprine β-LG contains an Ala at position 118.

3. Although unlikely, it is possible that the substitution at position 118 causes a difference in the binding of SDS to the protein.

2. Quantitative Study

The focus of this chapter was to examine the various heat-induced protein species that form upon heating the A, B and C variants of bovine β-LG and to look for relationships between these protein species.

2.1 Methods

2.1.1 Precipitation at pH 5.0

Concentrated β-LG variants A, B and C were diluted to ~2 mg/ml in a 0.2 M phosphate solution ($NaH_2PO_4.H_2O/Na_2HPO_4.2H_2O$), pH 5.0. Aliquots (1 mL) were heated for ten minutes at temperatures ranging from 60-95°C as described in Section 1.1.1. Samples for SDS- and native-PAGE were prepared by adding 0.5-1 mL of the appropriate sample buffer to 75-200 μL of each protein solution. The weight of the protein solution was recorded, as was the total weight of the sample. These weights were used for calculating dilution factors. Solutions in which a precipitate formed during heating were vortexed, spun in a bench top centrifuge to remove large aggregates, and samples for PAGE were prepared from the supernatant.

Each sample was run, with three standards of known concentration on each gel. Gels were stained with Amido Black and, after destainig was complete, each gel was scanned. Laser densitometry was used to obtain a measure of the total volume of each defined band. This volume was directly proportional to the amount of protein present in the band. The volumes obtained for the standards were plotted against the concentration of the standard and a linear regression was performed using a spread sheet programme (Quattro Pro, Borland International, Inc., Scotts Valley, California, USA.). The resulting equation was used to determine the amount of protein present in each band, and correction for the dilution factors gave the concentration (mg/ml) in the original sample of the β-LG species represented by each band. Concentrations calculated from each band were converted into a percentage of the protein present in the unheated sample.

2.1.2 Quantification of monomeric species.

Concentrated β-LG variants A, B and C were diluted to ~1 mg/ml in 0.2 M phosphate buffer ($NaH_2PO_4.H_2O/Na_2HPO_4.2H_2O$), pH 6.0. Aliqots (1 ml) were heated for ten minutes at temperatures ranging from 60-110°C as described in Section 1.1.1, and samples for SDS- and native-PAGE were prepared as described above. Each sample was run in triplicate, with three standards of known concentration run on each gel. Of each set of three repeats, one was stained in Coomassie Blue, the other two in Amido Black. After destaining was complete each gel was scanned and laser densitometry was used to obtain a measure of the total volume of each defined band or region. Concentrations were calculated as described above.

The concentration of protein present in the "SDS-monomeric" region (Figure 13) was calculated from SDS gels, and the concentration of protein present in the "native" band and the "smear" region (Figure 13) were calculated from native gels. Quantification of the "smear" region was performed only on the native gels that had been stained in Coomassie Blue. The concentration of protein present in the "smear" region was added to the concentration obtained for the "native" band to give the concentration of the "native+smear" region.

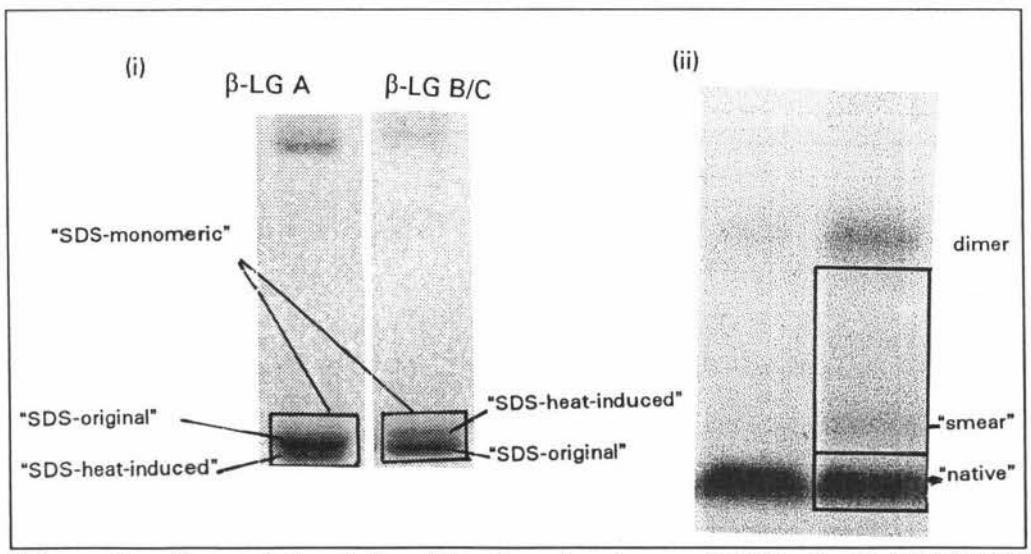


Figure 13 Location and description of bands and regions on PAGE gels. (i) SDS-PAGE. (ii) Native-PAGE.

To determine the percentage of "SDS-monomeric" protein that was present in the band with the same mobility as the unheated protein ("SDS-original", Figure 13), four methods were used (Figure 14). Where possible, individual bands of the doublet were defined, the concentration was calculated as described above, and the resulting values were converted into percentages. The other measurements were made from a line graph generated using ImageQuant. Values obtained from the four methods were averaged within each gel.

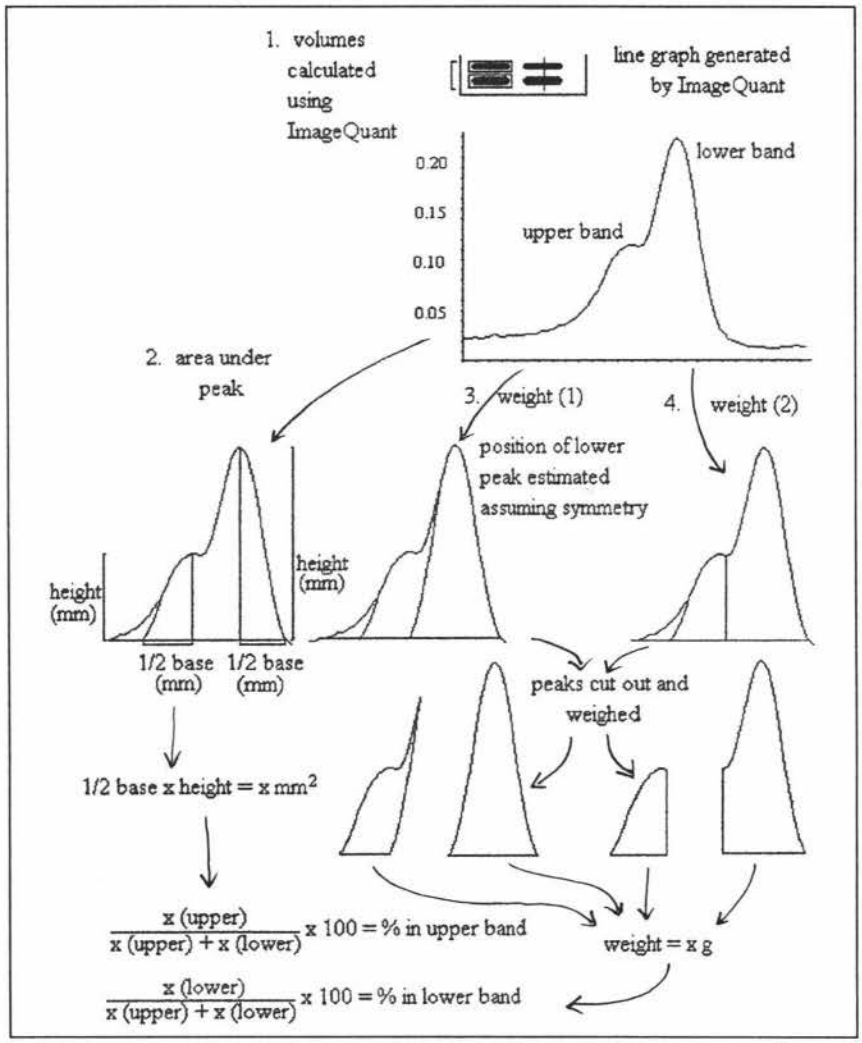


Figure 14 Methods used to determine the percentage of each band following band splitting of the "SDS-monomeric" species.

Protein concentrations obtained for the "SDS-monomeric" and "native" regions from each repeat were averaged for each variant. The concentrations obtained for these regions, and the concentrations of protein in the "native+smear" and "smear" regions were calculated as a percentage of the protein present in the unheated sample.

Percentages obtained for the "SDS-original" band from each repeat were averaged for each variant. Concentrations obtained for the "SDS-monomeric" region were multiplied by the percentage of protein present in the original band, giving the concentration of "SDS-original". This value was subtracted from the concentration of "SDS-monomeric" to give the concentration of "SDS-heat induced". These concentrations were also calculated as a percentage of the protein present in the unheated sample, and the values for the four regions were compared.

2.1.3 Aggregation as seen by native-PAGE

Bovine β-LG variants A, B and C were heated under the following conditions:

1. 0.2 M phosphate buffer ($NaH_2PO_4.H_2O/Na_2HPO_4.2H_2O$), pH 6.0, ~1 mg/ml, 60-110°C
2. 0.2 M phosphate buffer, pH 7.0, ~1 mg/ml, 60-100°C
3. 20 mM phosphate buffer ($NaH_2PO_4.H_2O$, pH adjusted with NaOH), pH 7.0, ~10 mg/ml, 60-100°C
4. 20 mM phosphate buffer, pH 8.4, ~1 mg/ml 50-80°C (A & B variants only)
5. 0.2 M phosphate buffer, pH 8.2, ~1 mg/ml 50-80°C

Samples were analysed using native-PAGE.

2.2 Results

The data used to support the results shown in this section can be found in Appendix C.

2.2.1 Precipitation at pH 5.0

A white precipitate was observed at 80°C with the A variant and with 85°C in the B and C variants. This was also observed at protein concentrations of 1 mg/ml in 20 mM phosphate (see Section 1.2.2.2). No band splitting was observed in SDS gels. The C variant appeared to have the highest stability under these conditions, remaining

detectable in solution at 90°C (Figure 15), while the A variant had the lowest stability. Results from native gels showed the same trends as seen in SDS gels, and no high molecular weight aggregates were observed (results not shown).

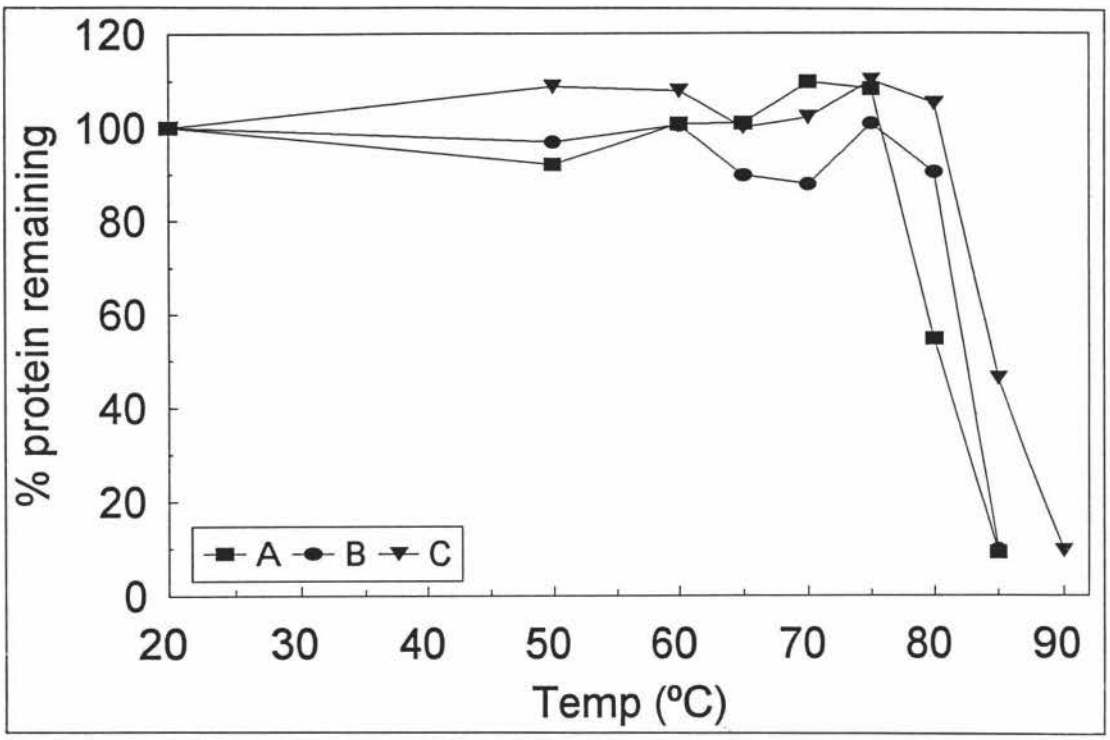


Figure 15 Effect of heat on β-LG A, B and C at pH 5.0. The percentage of protein remaining following heat treatment (10 mins) at each temperature was determined using non-reducing SDS-PAGE. Data and calculations are shown in Appendix C, Tables 5 and 6.

2.2.2 Quantification of monomeric species.

The results for the "SDS-monomeric" region and the "native" band calculated from individual gels were generally consistent within each set of repeats, with the exception of a single point in one SDS gel of the B variant. This point was not used in the calculations. Averages of these values were calculated (Figure 16). In each case the concentrations obtained from the SDS-PAGE were ~0.3 mg/ml higher than the concentrations obtained from native-PAGE. This is discussed in Section 3.

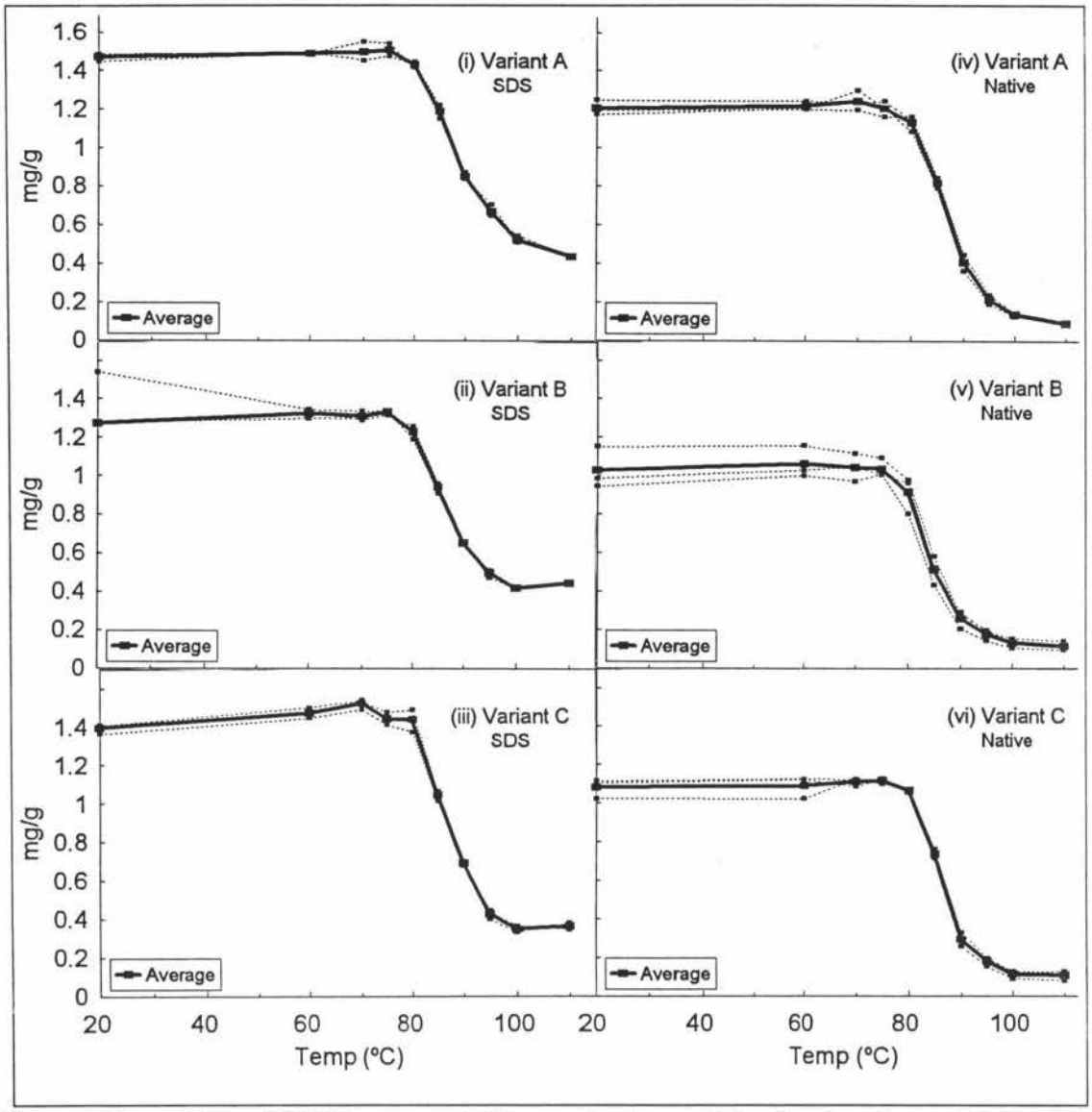


Figure 16 Loss of "SDS-monomeric" (non-reducing conditions) and native protein bands following heating for 10 minutes at the temperatures indicated. Each experiment was performed in triplicate and an average value given for each variant. Data and calculations are shown in Appendix C, Tables 7 to 18.

The percentage of "SDS-monomeric" protein present in each of the two bands, as determined by the four methods described in Figure 14, are shown in Appendix C, Table 19 (method 1) and Table 20 (methods 2-4). At 85°C it was not always possible to quantify the individual bands by all the methods, and variation between results was high at this temperature. At the other temperatures measured there was generally good agreement between the results obtained by the different methods and between the results obtained from each gel (Appendix C, Table 21), although in some cases the result obtained by one method was significantly different to the other three. In all

cases, the average was calculated from all values obtained. With all three variants, the amount of material in the heat-induced band of the doublet increased with increasing temperature, moving towards a distribution of ~60% in the original band and ~40% in the heat-induced band (Table 3).

Temp. (°C)	Variant A		B		C	
	% upper	% lower	% upper	% lower	% upper	% lower
85	84.26	15.74	26.48	73.52	34.49	65.51
90	74.92	25.08	31.47	68.53	27.50	72.50
95	67.50	32.50	32.38	67.62	31.70	68.30
100	62.94	37.06	38.30	61.70	35.80	64.20
110	62.02	37.98	42.57	57.43	41.11	58.89

Table 3 The distribution of protein in the two bands following band splitting as determined by the four methods used.

If the protein present in the "smear" region represents monomeric β-LG that has been sufficiently denatured for its mobility under native-PAGE to be retarded, then all the species present within the "SDS-monomeric" and "native+smear" regions are monomeric forms of β-LG. Comparison of the results obtained for the four regions studied showed that "native+smear" was not equivalent to either "SDS-monomeric" or "SDS-original", and "SDS-original" was not equivalent to "native" (Figure 17). This suggests that multiple monomeric species of β-LG are present in heated samples.

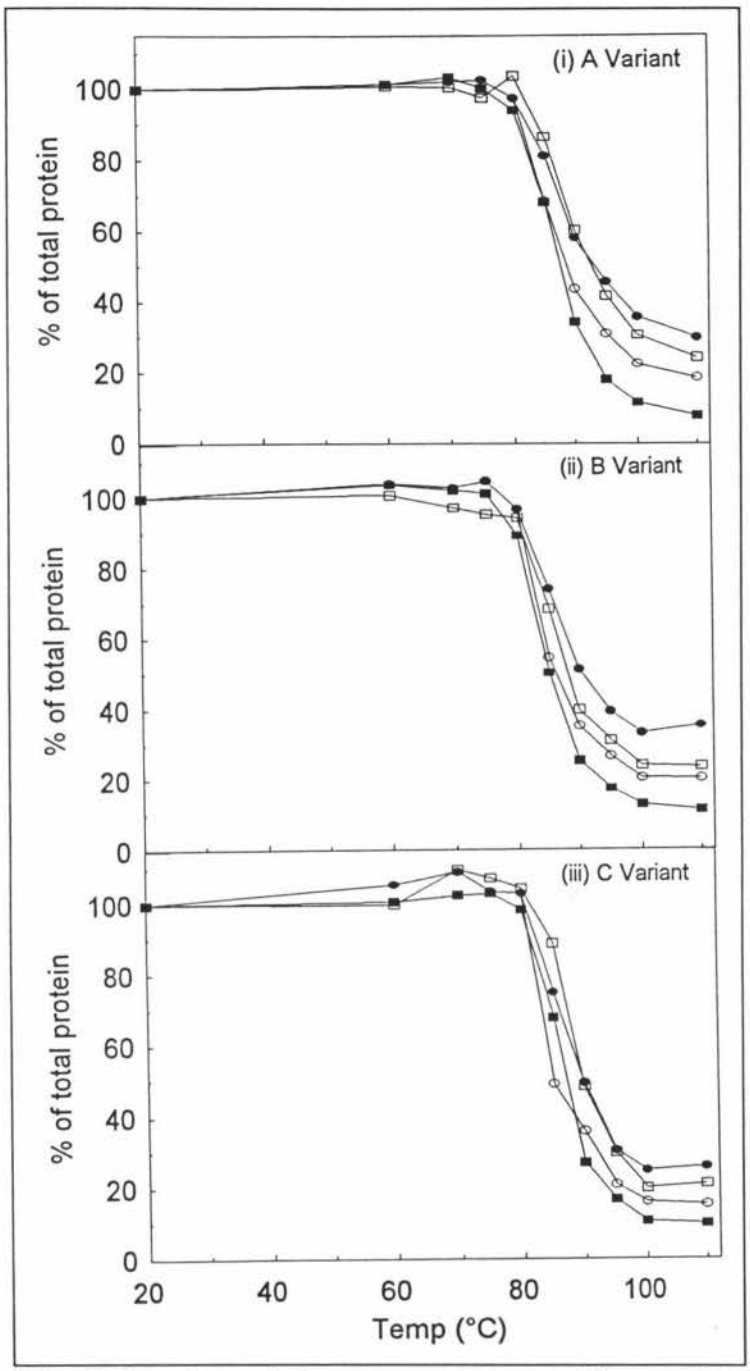


Figure 17 Distribution of protein between non-aggregated/monomeric species following heating. The percentage of the protein in unheated samples that ran as "SDS-monomeric" (⊸) and "SDS original" (⊸) were calculated using non-reduced SDS-PAGE. The percentage of the material that ran as "native" (⊸) and "native+smear" (⊸) was calculated using native-PAGE. Data and calculations are shown in Appendix C, Tables 22 to 26.

Comparison of the percentage of the total protein present in the "smear" region and the percentage of the total protein present in the heat-induced band of the doublet

showed these regions to represent a similar amount of material in the B and C variants. However, in the A variant, a significantly higher percentage of protein was present in the smear region (Figure 18).

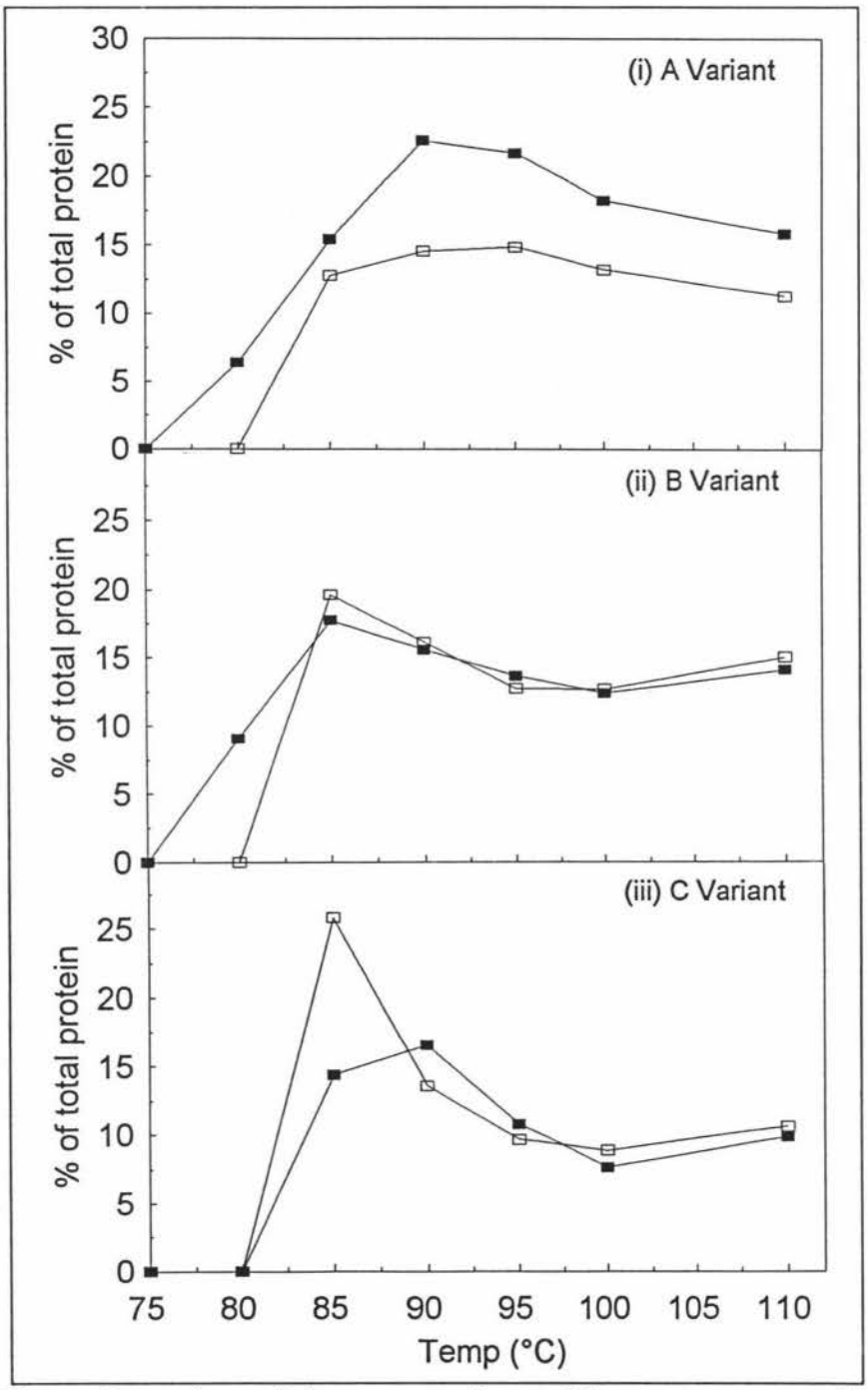


Figure 18 Comparison of the amount of material present as non-native monomeric protein under non-reduced SDS-PAGE and native-PAGE. The amount of material present in the "SDS-heat-induced" band (⊟) was calculated using non-reduced SDS-PAGE. The amount of material present in the "smear" region (■) was calculated using native-PAGE. Data and calculations are shown in Appendix C, Tables 24 to 26.

2.2.3 Aggregation as seen by native-PAGE

As temperature increased there was a decrease in the intensity of the band with the same mobility as unheated β-LG (i.e. the "native" band) and an increase in the amount and intensity of bands with lower mobility. This was seen with all three variants under all the conditions examined. In some cases, particularly in the B and C variants, a band with slightly higher mobility than unheated β-LG was seen. This band may not be obvious in the A variant, as β-LG A has a higher mobility than β-LG B and C.

2.2.3.1 pH 6.0

There was a higher number of aggregated species visible in the A variant than in the B and C variants. Material that was unable to enter the resolving gel was seen in the A variant but not in the B or C variants (Figure 19a).

2.2.3.2 pH 7.0

Following heating in 0.2 M phosphate buffer and with a protein concentration of ~1 mg/ml, material that was unable to enter the stacking gel was seen with the B and C variants, but not with the A variant, at both 85 and 90°C, but not at 100°C (Figure 19b (i)). There appeared to be an increase in intensity of the dimer band at 100°C in these two variants. Material unable to enter the resolving gel was seen with all three variants; the B variant appeared to have more of this material than the C variant, with the A variant having less than the B or C variants. In both the B and C variants the amount of material unable to enter the resolving gel decreased at temperatures above 85°C.

Following heating in 20 mM phosphate buffer and with a protein concentration of ~10 mg/ml, material unable to enter the stacking gel was not seen with any of the variants (A, B and C), even at high protein loadings (A and C variants only)(Figure 19b (ii)). Material unable to enter the resolving gel showed the same trends as was seen in 0.2 M phosphate buffer at pH 7.0. The two bands in the region between the "native" band and the dimer band were more obvious at higher protein loadings.

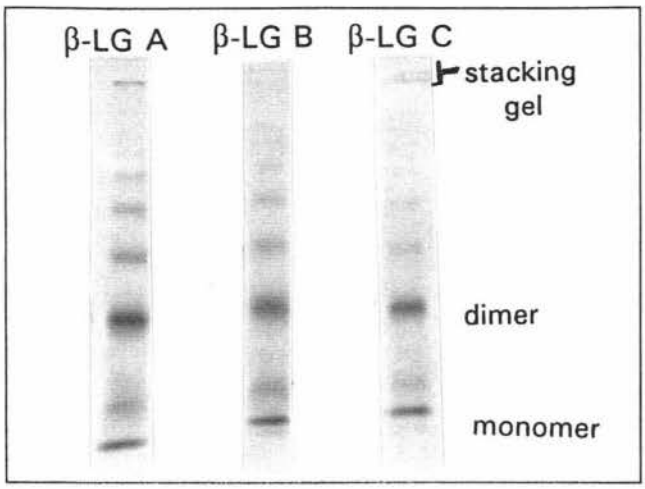


Figure 19 Heat-induced aggregation of purified β-LG A, B and C as seen following native-PAGE.

Figure 19a Heat-induced aggregation following heating at 100°C in 0.2 M phosphate buffer, pH 6.0, and at a protein concentration of ~1 mg/ml.

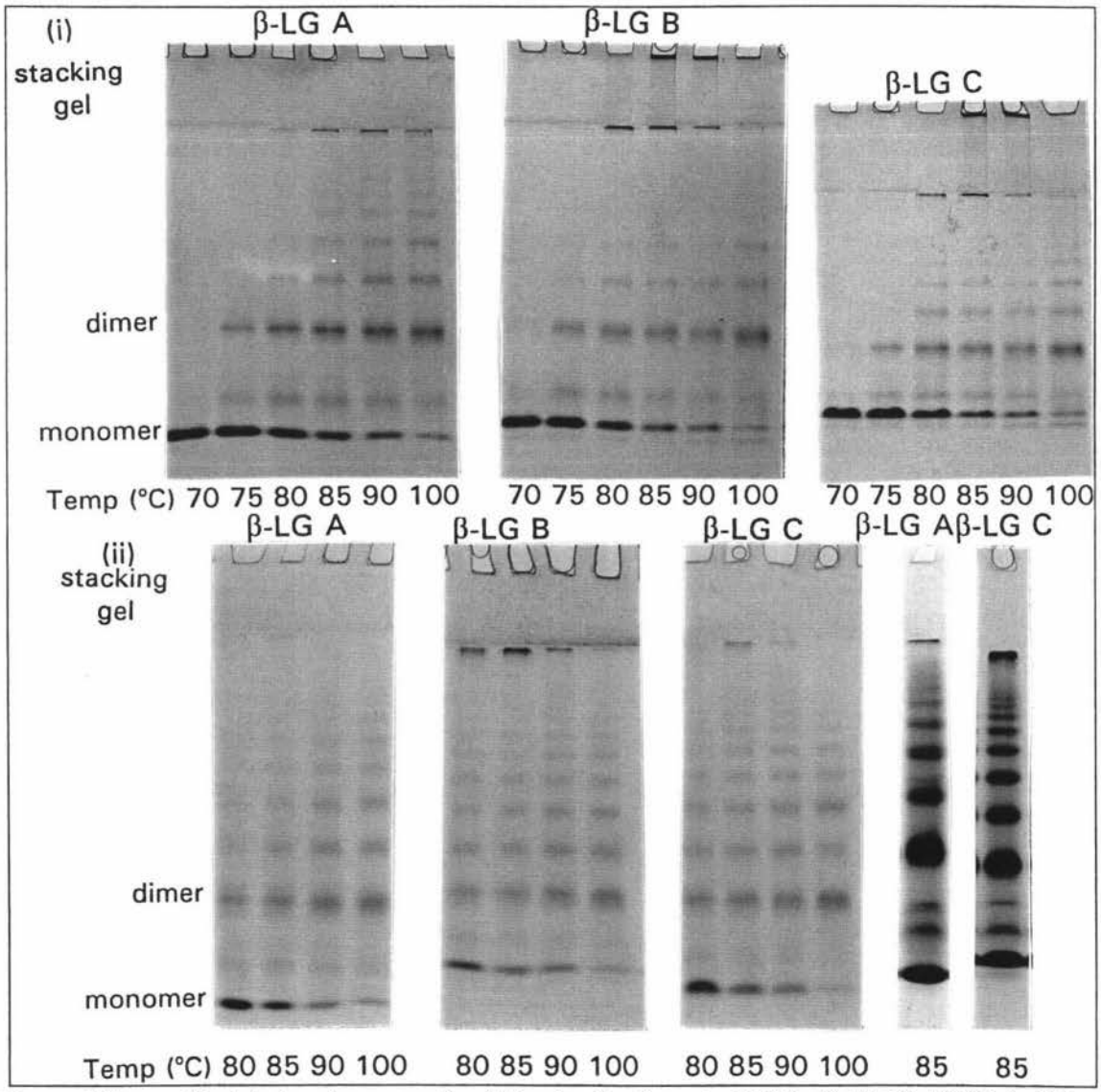


Figure 19b Heat-induced aggregation following heating at pH 7.0. (i) At 70-100°C, in 0.2 M phosphate buffer, and at a protein concentration of ~1 mg/ml (ii) At 80-100°C, in 20 mM phosphate buffer, and at a protein concentration of ~10 mg/ml.

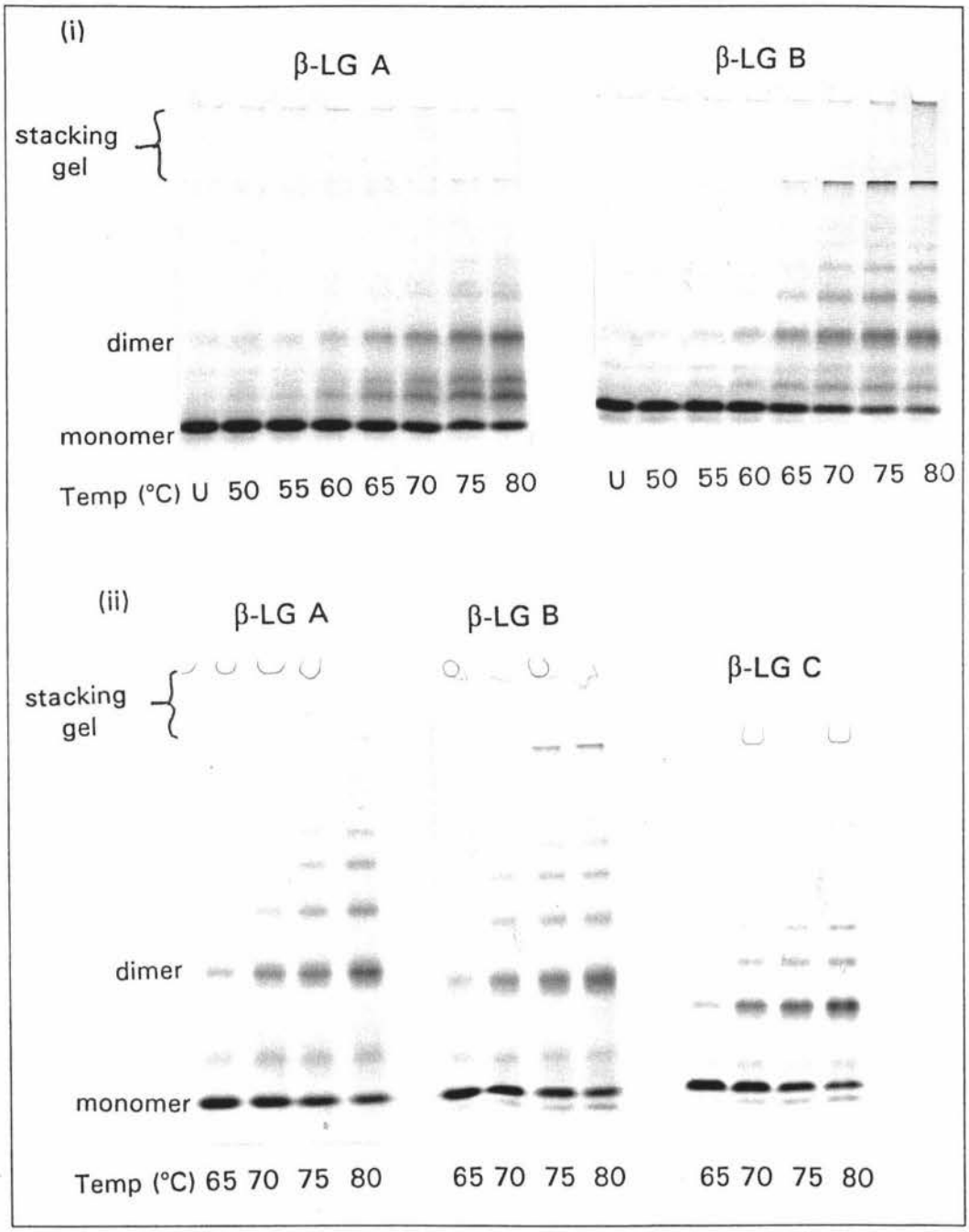


Figure 19c Heat-induced aggregation following heating in (i) 20 mM phosphate buffer, pH 8.4, at 50-80°C, and at a protein concentration of ~1 mg/ml, or (ii) 0.2 M phosphate buffer, pH 8.2, at 65-80°C, and at a protein concentration of ~1 mg/ml.

2.2.3.3 pH 8.4

The two bands in the region between the "native" and dimer bands were more obvious at this pH. Material unable to enter the stacking gel was only seen with the B variant,

while material unable to enter the resolving gel was seen with both the A and B variants, although more was seen with the B variant (Figure 19c (i)).

2.2.3.4 pH 8.2

Material unable to enter the stacking gel was not seen under these conditions with any of the variants. There was more material unable to enter the resolving gel with the B variant than with the C variant. This material was not seen with the A variant (Figure 19c (ii)).

2.3 Discussion and Conclusions

Precipitation of β-LG at pH 5.0 was probably due to the lack of electrostatic repulsion between molecules at pH values close to the isoelectric point of β-LG, calculated to be 5.26 (β-LG A), 5.34 (β-LG B) or 5.33 (β-LG C) (McKenzie, 1971).

The absence of high molecular weight aggregates suggests that, under these conditions, disuphide-linked complexes cannot form. This is probably due to the free thiol group being in the protonated form under acidic conditions.
Heating β-LG A, B and C at pH 6.0 for 10 minutes at temperatures between 85 and 110°C caused the formation of a number of protein species which could be observed using native-PAGE and non-reduced SDS-PAGE.

On native-PAGE the material that ran as a smear between the monomer band and dimer band was equivalent in amount to the heat-induced band of the doublet observed by non-reduced SDS-PAGE in the case of β-LG B and C, but not in the case of β-LG A. This result would suggest that it is possible that in the case of β-LG B and C the heat-induced smear material observed by native-PAGE (which must be some form of modified monomeric β-LG) is the material giving rise to the heat-induced band in the doublet observed by non-reduced SDS-PAGE. However, this cannot be the case with β-LG A.

Recent work using 2D-PAGE (Manderson *et al.*, 1997b) has shown that, following heating, even the band found in the position of native monomeric protein by native-PAGE gives rise to the original and heat-induced band in the doublet observed by non-reduced SDS-PAGE. It was also found that the material in the smear observed in native-PAGE gave rise to both bands in the doublet observed by non-reduced SDS-PAGE. Moreover, each of the higher molecular weight bands (dimer, trimer etc) gave rise to some material that ran as monomer on non-reduced SDS-PAGE.

Upon heating β-LG at 110°C for 10 minutes the heat-induced band in the doublet observed by non-reduced SDS-PAGE accounted for 40% of the total amount of doublet material in all three variants. This result is similar to that observed by Hill *et al.* (1997b) with β-LG B and C, but slightly different to that observed with β-LG A where the heat-induced band accounted for only 26% of the material in the doublet. It is possible that the difference between these current results with those of Hill *et al.* (1997b) is a consequence of the slightly different conditions used for heating and/or differences in the methods used to quantify the bands. In some samples it was difficult to quantify the individual bands of the doublet accurately, particularly in the B and C variants where tailing of the faster-migrating original band may result in higher values being obtained for the minor, heat-induced band.

Clearly these results show that there is a complex relationship between the various protein species observed by native-PAGE and non-reduced SDS-PAGE. It appears to be only coincidental that in the case of β-LG B and C the heat-induced band of the doublet observed by non-reduced SDS-PAGE corresponds in quantity to the smear material observed by native-PAGE.

The heat-induced aggregation behaviour of bovine β-LG, as seen following native-PAGE, depends on the variant and on the conditions under which it is heated. At pH 6.0, the A variant appears to have a higher tendency to form high molecular weight aggregates than the B or C variants. At pH 7.0, 8.2 and 8.4 the B and C variants appear to have a higher tendency to form high molecular weight aggregates than the A variant, with the B variant having a slightly higher tendency to form these species than

the C variant. At pH 7.0, there appears to be a maximum temperature for the formation of very high molecular weight species; above this temperature these species were observed to decrease in concentration. The differences seen in the formation of very high molecular weight species above pH 8 may be due to the higher buffer concentration stabilising β-LG against aggregation, or, as most of these aggregates involve intermolecular disulphide bonding, small changes in pH may have a large effect on the formation of these bonds as the pH approaches the ionisation pH of the thiol group (predicted to be ~8.5).

3 Effect of Storage

On SDS gels stained in Coomassie Blue, bands in the dimer region were visible in unheated β-LG A, B and C following storage at -18°C in SDS-PAGE sample buffer. With β-LG B and C, but not β-LG A, a band was also seen in the trimer region (Figure 20). The banding pattern in the dimer region of the stored, unheated samples was different to that of the heated samples, and differed between the variants. In the B and C variants three clear bands were seen, but in the A variant only two bands were observed, although there was evidence of some smeared material above these two bands. A small amount of material was also seen in the dimer region in samples of β-LG B and C on native gels at high protein loadings. These samples had been stored in native-PAGE sample buffer at -18°C. The stored, unheated β-LG samples were prepared from the same stock solutions of β-LG as the unheated samples which had not been stored under these conditions, where there was no evidence of higher molecular weight bands.

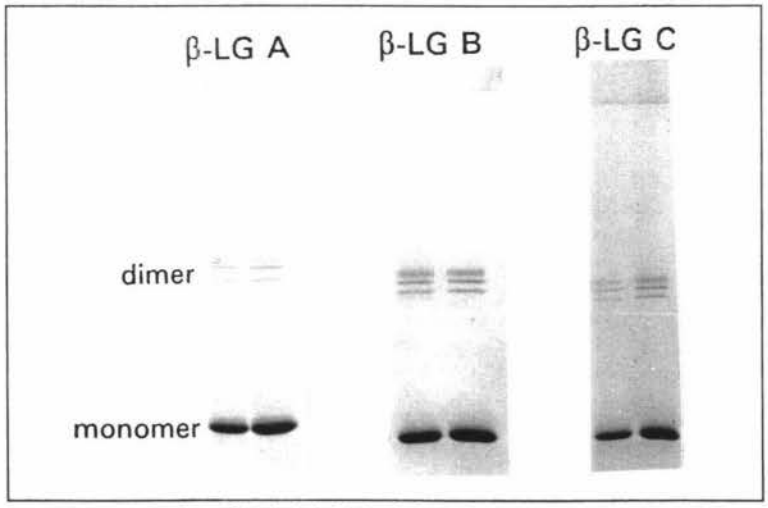


Figure 20 Non-reducing SDS-PAGE of β-LG A, B and C following storage at -18°C in SDS-PAGE sample buffer.

When converted to mg/mL, the concentrations in unheated samples obtained from SDS-PAGE gels at pH 6.0 were ~0.3 mg/ml higher than the concentrations obtained from native-PAGE gels. This was seen with all three variants (Figure 21, i-iii). When the concentrations were recalculated from SDS gels stained in Coomassie Blue, taking into account the material in the dimer region, the difference in concentration between the native and SDS results was reduced. In the A variant the concentrations were

essentially the same, while in the B and C variants some differences were still seen. (Figure 21, iv-vi). These differences may reflect differences in tendency to form trimers and higher molecular weight species, the A variant having the lowest tendency and the C having the highest tendency.

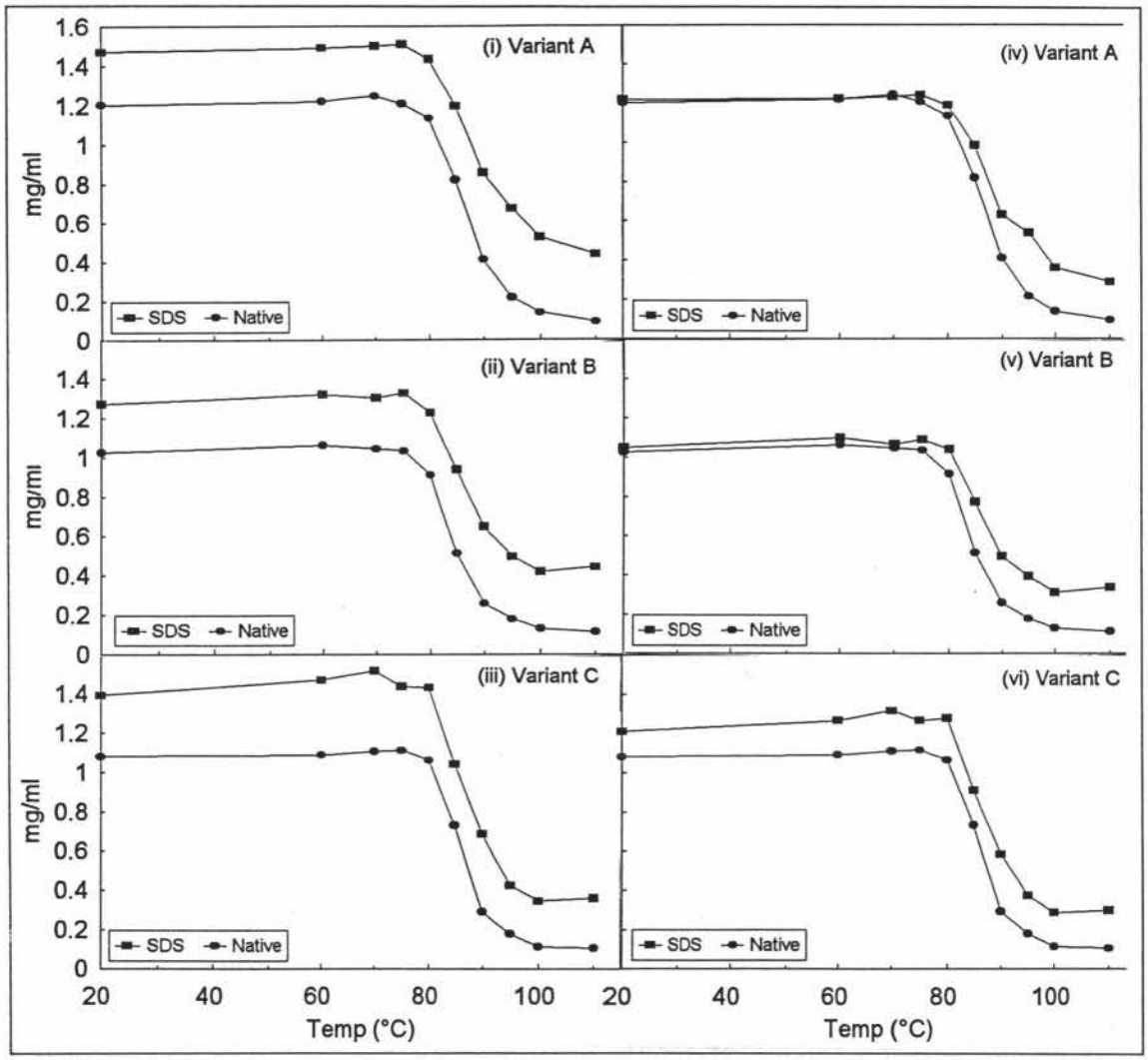


Figure 21 Apparent concentration of "SDS-monomeric" β-LG, as determined by non-reducing SDS-PAGE, and "native" β-LG, as determined by native-PAGE, following heating for 10 minutes at temperatures from 20-110°C. The concentration of protein that ran as "SDS-monomeric" (-■-) and "native" (-●-) was calculated using a regression equation in which the concentration of protein in the standard was the independent value and the band volume obtained for the standard using ImageQuant was the dependent value. (i-iii) Plots determined using a regression equation for SDS-PAGE calculated using the volume of the monomeric band. (iv-vi) Plots determined using a regression equation for SDS-PAGE calculated using the combined volumes of the monomeric and dimeric bands. Data and calculations are shown in Appendix D.

The differences in the tendency of the β-LG variants to form aggregated species upon storage is more obvious in the series of gels shown in Figure 22. The samples represented here are the same as those represented in Figure 20, but had subsequently been refrozen and stored for longer. Relatively little material is present in higher molecular weight bands in the A variant, while in the B and C variants bands are clearly visible in both the dimer region and between the top of the resolving gel and the trimer region. Quantification of the material present as monomer, dimer and trimer in the B and C variants showed a slightly higher proportion of material to be present as dimer and trimer in the C variant (Table 4).

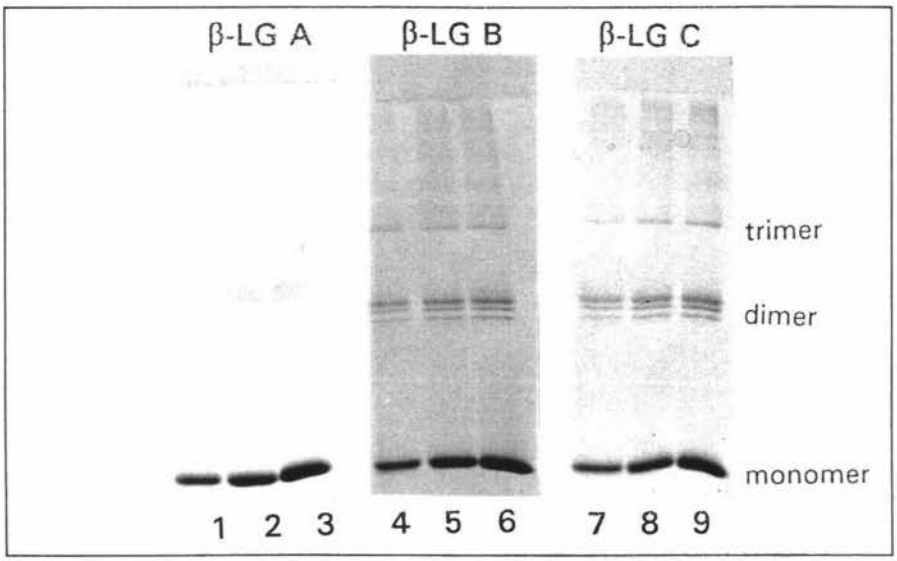


Figure 22 Non-reducing SDS-PAGE of β-LG A, B and C following storage at -18°C in SDS-PAGE sample buffer. The relative distributions of protein between monomer, dimer and trimer in lanes 5, 6, 8 and 9 are shown in Table 4.

Variant	Lane	% monomer	% dimer	% trimer
B	5	78.43	20.87	0.71
	6	83.03	15.04	1.93
C	8	74.25	23.85	1.90
	9	71.49	23.47	5.04

Table 4 Distribution of material between monomer, dimer and trimer regions following SDS-PAGE of β-LG B and C. ImageQuant was used to obtain volumes for the individual bands seen in Figure 22, lanes 5, 6, 9 and 10. The three values (monomer, dimer and trimer) for each lane were added to give a lane total, and the percentage of the total was calculated for each band.

Thus, under these conditions, β-LG B and C have a greater tendency to aggregate than β-LG A, and β-LG C appears to have a slightly greater tendency to aggregate than β-LG B.

A comparison of caprine β-LG with bovine β-LG A and B, stored in SDS-PAGE sample buffer at -18°C for six days, showed that β-LG B and caprine β-LG had a similar tendency to form aggregates (not quantified). These bands were too faint to be seen in a photograph, but were visible when the gel was scanned. The banding pattern seen in the caprine β-LG was similar to that seen in the bovine B and C variants (Figure 23).

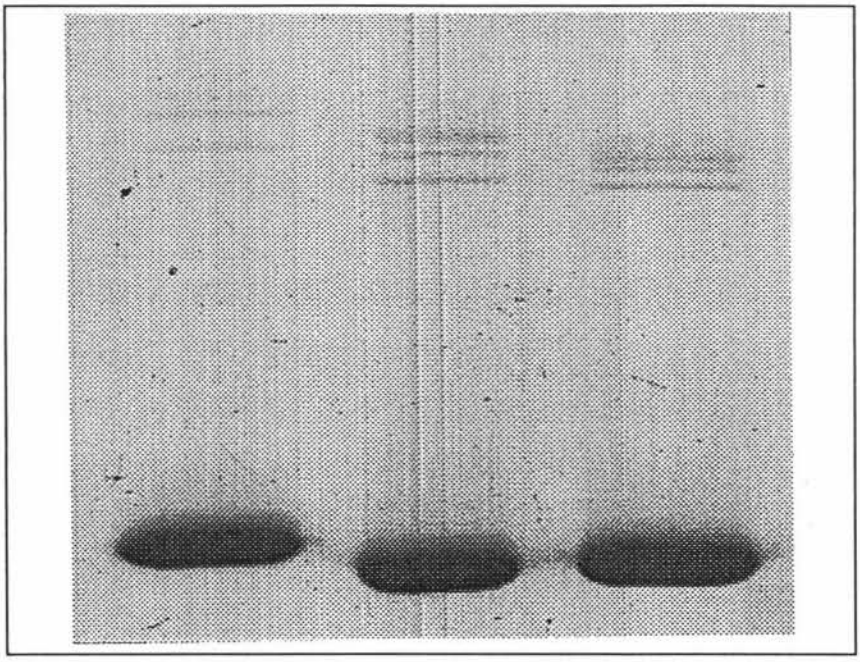


Figure 23 Non-reducing SDS-PAGE of bovine β-LG A and B and caprine β-LG following storage at -18°C in SDS-PAGE sample buffer.

The samples in which aggregated bands were seen had been freeze dried and stored in SDS-PAGE sample buffer. To confirm that the aggregation observed is caused by storage in buffer and not freeze drying, fresh samples were made from the same freeze-dried material that had been used for the storage experiments in the SDS-PAGE sample buffer. This material had been stored in the dry form at -18°C. No bands of higher molecular weight were observed (Figure 24).

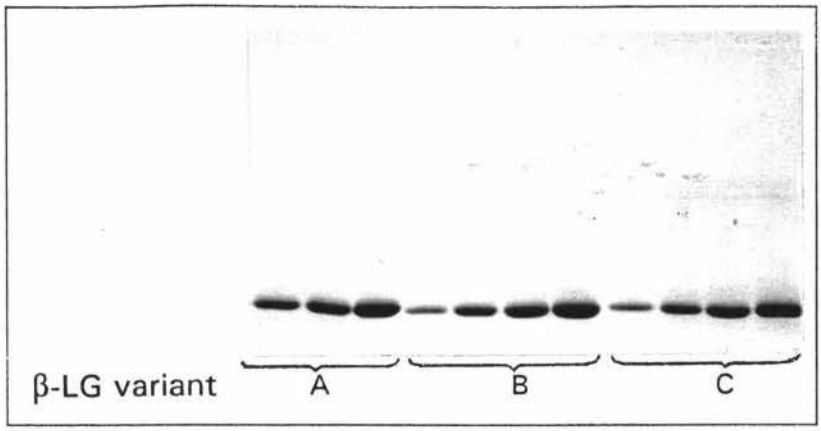


Figure 24 Non-reducing SDS-PAGE of β-LG A, B and C following freeze drying and storage at -18°C.

Formation of disulphide-linked aggregates is a result of intermolecular thiol/disulphide interchange, and requires the free thiol to be exposed and reactive. Band splitting is probably a result of intramolecular thiol/disulphide interchange reactions, and so for band splitting to occur the thiol group must be reactive, but may not need to be exposed to the external environment of the protein. However, it is likely that some molecular movement within the protein facilitates exchange via the reactive thiolate group.

Storage in SDS-PAGE sample buffer causes the aggregation of β-LG but does not induce band splitting. Why, under these conditions, the thiolate group is only able to promote intermolecular thiol/disulphide exchange and not intramolecular exchange (band splitting) is unclear. It is possible that in the open extended structure in SDS-PAGE sample buffer it is not possible for the thiol to attack the intramolecular disulphide bonds, but that the disulphide bonds of adjacent β-LG molecules are free to attack. In the absence of SDS, i.e. for samples stored in native-PAGE sample buffer, aggregation did not occur to the same extent, and was not observed with β-LG A.

Differences between the variants in their tendency to aggregate under equivalent conditions suggests that the thiol group of bovine β-LG A is either less exposed and/or less reactive than that of bovine β-LG B and C and caprine β-LG. In the presence of high concentrations of SDS, as in SDS-PAGE sample buffer, most tertiary structure is lost. However, it has been shown that in the presence of SDS the amount of secondary

structure increases with β-LG B (Hillquist Damon and Kresheck, 1982). It is possible that there is a difference in SDS-induced secondary structure in β-LG A compared to β-LG B, C and caprine β-LG, this difference being a result of the amino acid substitution at position 118. This structural difference may result in a decrease in the reactivity and/or the exposure of the thiol group in β-LG A.

Part 2. Identification of the Free Thiol Group in β-lactoglobulin A, B and C

4 Labelling of β-LG with NEM

NEM has been widely used for the study of thiol groups in proteins. It is water soluble, reacts rapidly at neutral pH without inducing a change in pH (Cohen, 1968), and the reaction is irreversible. Although non-specific reactions with the imidizole group of histidine and the α-amino groups of peptides and amino acids can occur (Smythe *et al.*, 1964), under the conditions in which NEM is usually used for modification of thiol groups (pH 6-7 and without a large excess of NEM to protein thiol groups), alkylation of amino groups is rare, and the reaction is relatively specific for thiol groups (Brewer and Riehm, 1967). Above pH 7, NEM the reaction loses specificity (Riordan and Vallee, 1967).

Because the thiol group of β-LG is buried in the native protein at neutral pH, the reaction of native β-LG with NEM is slow at pH 7.0, but rapid when the protein is denatured in urea or guanidine hydrochloride (McKenzie, 1971). The effect of SDS as a denaturant when labelling the thiol group of β-LG is dependant on the SDS concentration used. Franklin and Leslie (1968) found that decreasing SDS concentrations from 1% to 0.5% resulted in an increase in the rate constant for the reaction between β-LG and NEM. However, at SDS concentrations of less than 0.3% the rate of reaction was slower and the reaction did not go to completion, presumably due to incomplete denaturation of the protein. Phillips *et al.* (1967) also found that the half time for the reaction between β-LG and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in the presence of SDS increased and then subsequently decreased with increasing SDS concentration.

NEM has an absorbance peak between 300 and 305 nm (Lunderblad and Noyes, 1984, Jocelyn, 1972, Riordan and Vallee, 1967), with an extinction coefficient of 620 M^{-1} cm^{-1} at this absorbance peak. The adduct formed on reaction with thiols does not absorb at this wavelength, allowing quantitative spectrophotometric determination of the number of groups reacting. However, some proteins absorb significantly at 305 nm (Riordan and Vallee, 1967) and, in the case of β-LG, spectral effects arising out of

changes in the environment of tyrosine and tryptophan groups during the denaturation reaction can affect results. Monitoring the reaction at another λmay therefore give clearer results (McKenzie, 1971). The loss of absorbance during the reaction may also be due to hydrolysis of NEM. NEM is unstable in aqueous solution (Jocelyn, 1972), and at pH values above 7.0 hydrolytic cleavage of the imide ring occurs, resulting in the formation of N-ethylmaleamic acid and the loss of the 300 nm peak. The rate of hydrolysis increases when the pH is increased (Torchinski, 1981).

By determining the absorbance spectra of both β-LG and NEM at concentrations suitable for following the reaction spectrophotometrically (1 mM NEM, sufficient protein to give an NEM:SH ratio of about 1:1.2 (Riordan and Vallee, 1967)), a wavelength at which the reaction can be followed with minimum interference from the protein can be determined. Following the reaction of β-LG and NEM under different conditions can be used to determine the conditions where a reasonable level of reaction between β-LG and NEM occurs but no band splitting is seen and the chances of disulphide exchange occurring during labelling is minimised.

Previously (Part 1) it was shown that no irreversible denaturation occurs at pH 5.0, 70°C, or at pH 6.0, 60°C, and that SDS does not cause band splitting in unheated samples of β-LG. SDS is thus a useful agent to promote protein unfolding and denaturation. However, heating α-amylase in the presence of SDS has been reported to induce a restricted, single thiol/disulphide exchange reaction (Kubo, 1995), therefore, if heat is used in conjunction with SDS, both temperature and SDS concentration must be kept to a level which does not induce such a reaction.

Because the extinction coefficient is relatively low (620 $M^{-1} cm^{-1}$), the sensitivity of this method of quantifying the reaction is low, and there are likely to be large errors in measuring the small changes in absorbance. Disappearance of reagent rather than formation of product is monitored, so evidence for reaction with protein is only inferential and conclusions based solely on such data can be in doubt. Because quantifying the reaction by following the disappearance of NEM may not give a true indication of the extent of reaction, the number of thiol groups remaining unreacted after incubation with NEM can be determined by reaction with the highly specific thiol

reagents DTNB and 2,2'-dithiopyridine (2,2'-dipyridyl disulfide) (DPDS) under conditions where most thiol groups should be available for reaction.

4.1. Absorbance spectra of β-LG and NEM

4.1.1. Methods

To determine the absorbance spectra of NEM and β-LG at the required concentrations, NEM was dissolved in 0.1 M phosphate buffer (pH 6.0) to give a final concentration of 1 mM NEM, and 0.1 M phosphate buffer (pH 6.0) was added to purified, concentrated β-LG A to give a concentration of ~18 mg/ml (~1 mM). A wavelength scan (200-400 nm) of the β-LG and NEM solutions was performed on a Hewlett Packard 8452A Diode Array Spectrophotometer running with HP 89531A MS-DOS UV/VIS operating software (Copyright (1989) H-P company), and the resulting scans were compared.

To determine the absorption coefficients of NEM around the 300 nm peak, the absorbance of 1 mM NEM in phosphate buffer was recorded at individual wavelengths between 280 and 335 nm (blanked against phosphate buffer) on a Shimazu UV-260 UV-visible spectrophotometer. Absorbance values were multiplied by 1000 to give the value of ε at each wavelength.

4.1.2 Results and Discussion

NEM has absorbance maxima at approximately 220 nm and between 300-305 nm, whereas β-LG absorbs strongly in the region 200-310 nm (Figure 25). However, at 320 nm there is little absorbance by the protein relative to the NEM, but this wavelength is close enough to the peak that changes in the concentration of NEM should still be measurable. The absorbance data for NEM (1 mM solution) can be found in Appendix E, Table 30. The extinction coefficient for NEM at 320 nm was calculated to be 455. This value will be used to calculate the percentage of thiol groups reacting under each of the conditions tested.

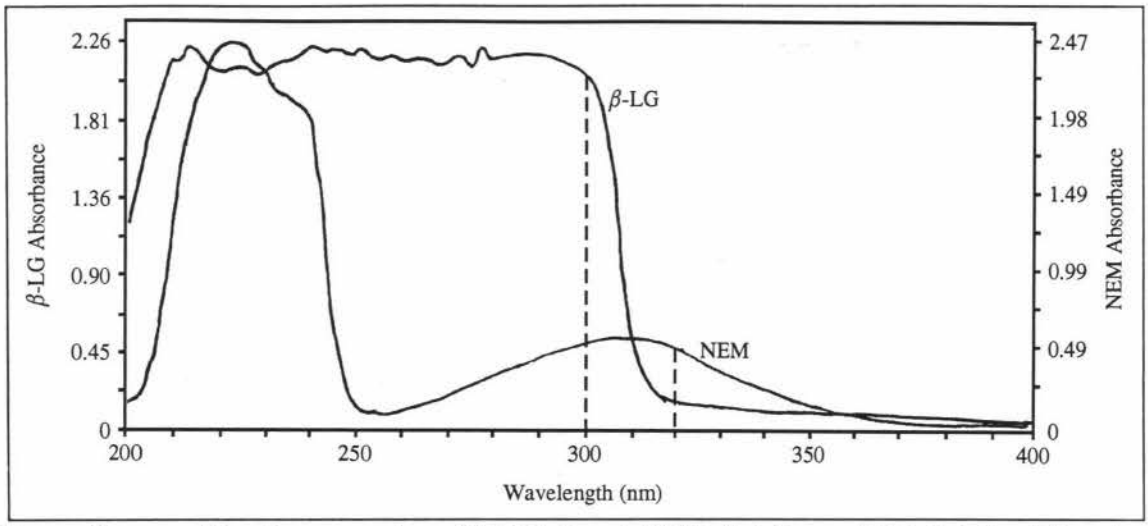


Figure 25 Absorbance spectra of β-LG A and NEM. Solutions of β-LG (~1 mM) and NEM (1 mM) in 0.1 M phosphate buffer were used.

4.2. Test conditions

4.2.1. Methods

0.1 M phosphate solutions ($NaH_2PO_4.H_2O$, pH adjusted with 3 M HCl or 5 M NaOH), with or without NEM, were made up as shown below and added to concentrated β-LG A (25.9 mg/ml) or C (30.3 mg/ml) and SDS solution (10% w/v) in 3 mL quartz cuvettes as shown in Tables 5 and 6. In the experiments shown in Table 5, 3 mL solution II or IV was used as a blank, and in the experiments shown in Table 6, 3 mL buffer IV with 80 μL SDS solution added was used as a blank.

Phosphate solutions used:

solution I: 0.1M phosphate, 0.002M NEM, pH 5.0
solution II: 0.1M phosphate, pH 5.0
solution III: 0.1M phosphate, 0.002M NEM pH 6.0
solution IV: 0.1M phosphate, pH 6.0
solution V: 0.1M phosphate, 0.001M NEM pH 6.0

conditions		cuvette #1: protein + NEM	cuvette #2: protein	cuvette #3: NEM
pH 5.0	70°C,60 min	1.7 mL β-LG A 1.5 mL solution I	1.7 mL β-LG A 1.5 mL solution II	
pH 6.0	60°C,120 min 50°C,90 min 40°C,90 min	1.7 mL β-LG A 1.5 mL solution III	1.7 mL β-LG A 1.5 mL solution IV	3 mL solution V 120 min (60°C,40°C)

Table 5 Conditions used to examine the effect of pH and temperature on the reaction between β-LG and NEM.

conditions (pH 6.0) (code)	cuvette #1: protein + NEM	cuvette #2: protein
40°C 0.03% SDS (w/v) (SDS-10,40)	1.5 mL β-LG C 1.5 mL solution III 10 μL SDS	1.6 mL β-LG C 1.3 mL solution IV 80 μL SDS
40°C 0.13% SDS(w/v) (SDS-40,40)	1.5 mL β-LG C 1.5 ml solution III 40 μL SDS	1.6 mL β-LG C 1.3 mL solution IV 80 μL SDS
25°C 0.26% SDS(w/v) (SDS-80,25)	1.5 mL β-LG C 1.5 mL solution III 80 μL SDS	
40°C 0.27% SDS(w/v) (SDS-80,40)	1.6 mL β-LG A 1.3 mL solution III 80 μL SDS	1.6 mL β-LG A 1.3 mL solution IV 80 μL SDS

Table 6 Conditions used to examine the effect of SDS concentration and temperature on the reaction between β-LG and NEM at pH 6.0

The cuvettes were placed in a temperature-controlled cell block, within the Shimazu spectrophotometer, that had previously been equilibrated to the required temperature. Solutions were incubated for 60-120 minutes and loss of absorbance at 320 nm was followed.

To test the effect of high pH on hydrolysis, 5 M NaOH was added to solution 5 to raise the pH to 8.12, and loss of absorbance at 320 nm was followed at 25°C for two hours.

The theoretical total thiol content of each sample that contained protein was calculated using 18300 as the molecular weight of β-LG. The percentage of SH groups that had reacted under each set of conditions was calculated from the loss of absorption at 320

nm. In samples where no protein was present, the percentage of NEM that had hydrolysed was determined.

Samples from cuvettes 1 and 2 from experiments shown in Table 5 and experiment SDS-80,40 (Table 6) were dialysed extensively against solution IV to remove excess NEM. After dialysis, the protein concentration of each sample was determined using the extinction coefficient ($A_{280}^{1\%} = 9.7$). Samples were analysed for band splitting and denaturation using native-PAGE and non-reduced SDS-PAGE as described previously (Section 1). To determine the percentage of SH groups still available for reaction after incubation with NEM, the dialysed samples from pH 6.0, 60°C and SDS-80,40 were reacted with DTNB and DPDS as follows:

Reaction with DTNB was performed in 0.1 M phosphate buffer ($NaH_2PO_4.H_2O$), pH 7.0 (adjusted with 5 M NaOH). Aliquots of each sample to be tested were diluted with buffer to give a final volume of 2.7 mL and the required protein concentration. A 1 mM solution of DTNB in buffer was made and 300 µL of the DTNB solution was added to each sample in a 3 mL cuvette. Samples were incubated in the temperature controlled heating block of the Shimazu spectrophotomer for 150 min at 50°C, and the increase in absorbance at 412 nm was monitored. The spectrophotometer was blanked against 300 µL DTNB solution in 2.7 mL of phosphate buffer.

Reaction with DPDS was performed in 0.2 M phosphate buffer ($Na_2HPO_4.2H_2O/NaH_2PO_4.H_20$), pH 7.5. Aliquots of each sample to be tested were diluted with buffer to give a final volume of 2.6 mL and the required protein concentration. A 1 mM solution of DPDS in buffer was made and 400 µL of the DPDS solution was added to each sample in a 3 mL cuvette. Samples were incubated in the temperature controlled heating block of the Shimazu spectrophotomer for 180 min at 50°C, and the increase in absorbance at 343 nm was monitored. The spectrophotometer was blanked against 400 µL DPDS solution in 2.7ml of phosphate buffer. The pH of each sample was recorded after 180 minutes. To determine the molar extinction coefficient (ε) at 343nm for the product of the reaction, 2-mercaptopyridine was made up to 1 mM in 0.2 M phosphate buffer (pH 7.5) and absorbance at 343 nm was recorded.

The theoretical total thiol content of each sample was determined using the calculated concentration of β-LG and 18300 as the molecular weight of the protein. The percentage of SH groups that had reacted under each condition was calculated from the peak absorbance value in the reactions with DTNB and DPDS. The difference between the values obtained from samples with and without NEM gave the percentage of SH groups that had reacted with NEM.

4.2.2 Results and Discussion

The data used to support the results shown in this section can be found in Appendix E.

4.2.2.1 pH 5.0

At pH 5.0 and 70°C, an increase in absorbance at 320 nm is seen in both the presence and absence of NEM (Figure 26). This is probably due to changes in particle size as heat-induced protein aggregation starts to occur. Hydrolysis of NEM in the absence of protein under these conditions was not tested for, but as no loss of absorbance is seen in the sample containing NEM, NEM may be stable under these conditions. These conditions were unsuitable for this labelling study, as there was no evidence that a reaction between β-LG and NEM had occurred.

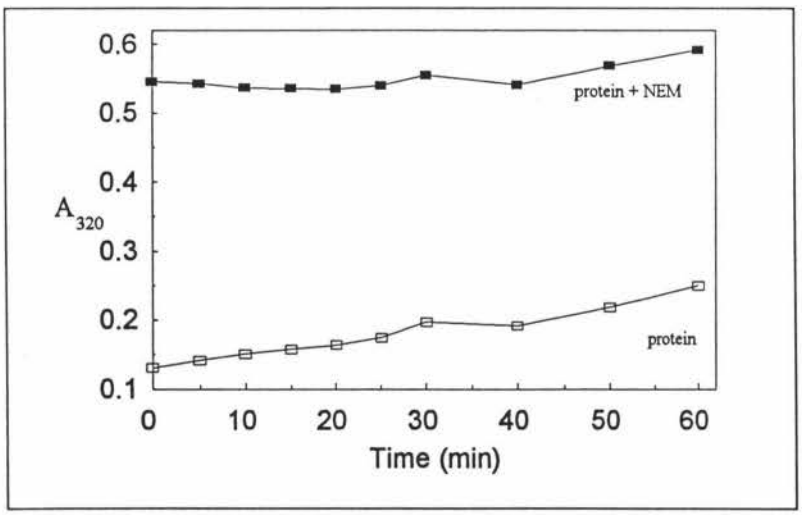


Figure 26 Change in absorbance at 320 nm of solutions of β-LG A and β-LG A/NEM during heating at pH 5.0, 70°C. Data is shown in Appendix E, Table 31.

4.2.2.2 pH 6.0

4.2.2.2.1 Reaction of β-LG with NEM

There was little change in the absorbance of β-LG in the absence of NEM under any of the temperatures and SDS concentrations tested at pH 6.0. The absorbance of the protein was subtracted from the absorbance of the protein/NEM solution at each point. Loss of absorbance at 320 nm is seen under all conditions tested. Assuming that loss of absorbance in solutions containing both β-LG and NEM is due only to specific reaction between NEM and protein thiol groups, the highest reaction seen is at 60°C, in the absence of SDS. Reducing the reaction temperature or the SDS concentration resulted in reduced reaction rates under all the conditions tested (Figure 27).

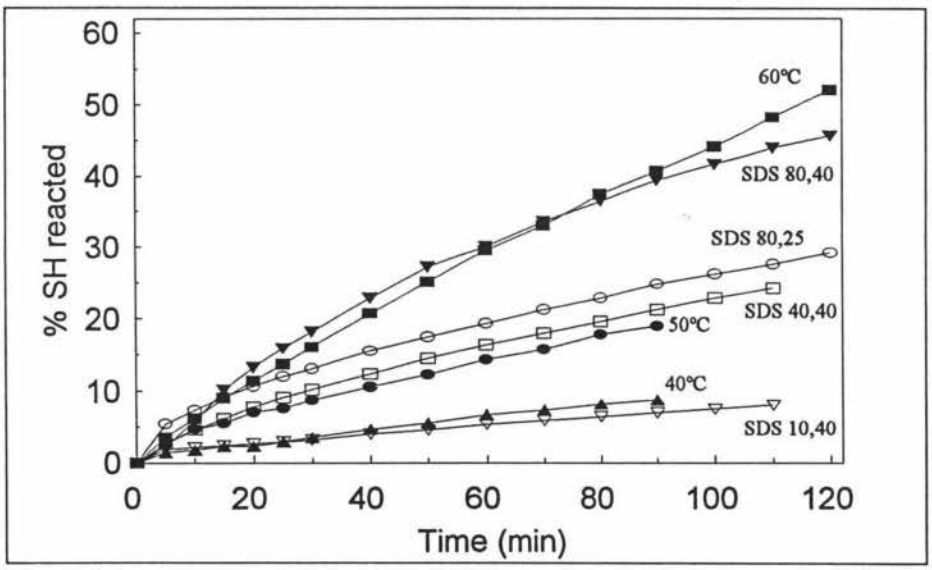


Figure 27 Reaction of NEM with β-LG at pH 6.0. The percentage thiol groups reacted was determined by monitoring the reaction at 320 nm. Data and calculations are shown in Appendix E, Tables 32-38.

Hydrolysis of NEM in the absence of protein was assumed to be unaffected by the presence of SDS. Subtraction of the moles of NEM that hydrolysed in the absence of protein at 25°C, 40°C and 60°C resulted in a small decrease in the estimated degree of reaction at 25°C and 40°C, and a large reduction in the estimated degree of reaction at 60°C. The possibility that the hydrolysis product had reacted with β-LG was not considered in these results, as this reaction could not be quantified. Assuming that the difference between the total moles of NEM reacted and the moles of NEM that have

hydrolysed represents the reaction between β-LG and NEM, the highest percentage of reaction was seen in SDS-80,40 (Figure 28).

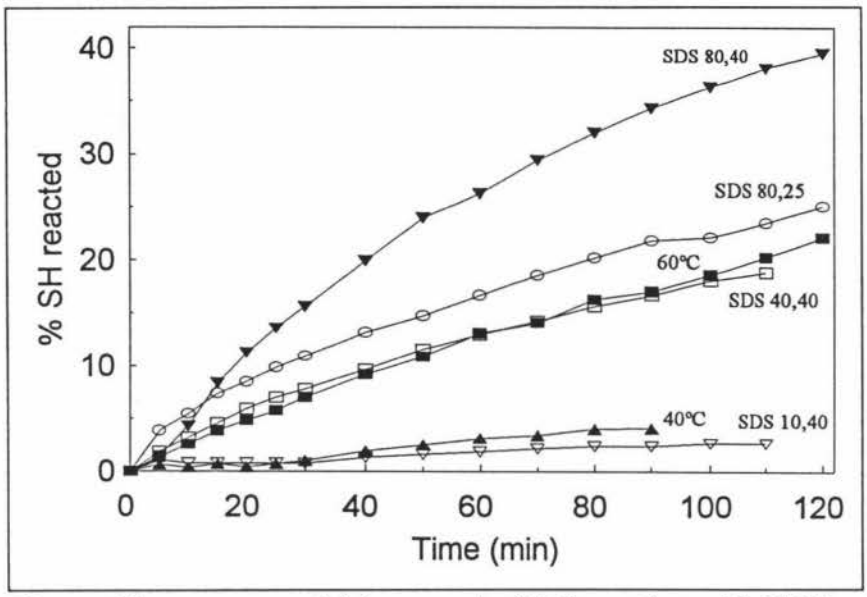


Figure 28 The percentage thiol groups in β-LG reacting with NEM at pH 6.0. The percentage thiol groups reacted was determined at 320 nm and corrected for the hydrolysis of NEM under the same conditions. Data and calculations are shown in Appendix E, Tables 32 and 34-47.

4.2.2.2.2 PAGE

No band splitting was seen following non-reduced SDS-PAGE with any of the reaction conditions tested (Figure 29 (i)). No denaturation was seen by native PAGE (Figure 29 (ii)), but a second band with slightly lower mobility was seen in the sample SDS-80,40 following incubation with NEM (Figure 29 (ii), lane 10). Binding of NEM has been reported to effect the electrophoretic mobility of β-LG (Donovan and Mulvihill, 1987), and the second band seen in this sample may represent the β-LG-NEM complex. If this were the case, then this is the only sample in which a significant amount of reaction between β-LG and NEM has occurred. This band is unlikely to be due to extra SDS binding to the protein, as the sample incubated in the presence of SDS but the absence of NEM (Figure 29 (ii), lane 9) shows only one band on native-PAGE gels.

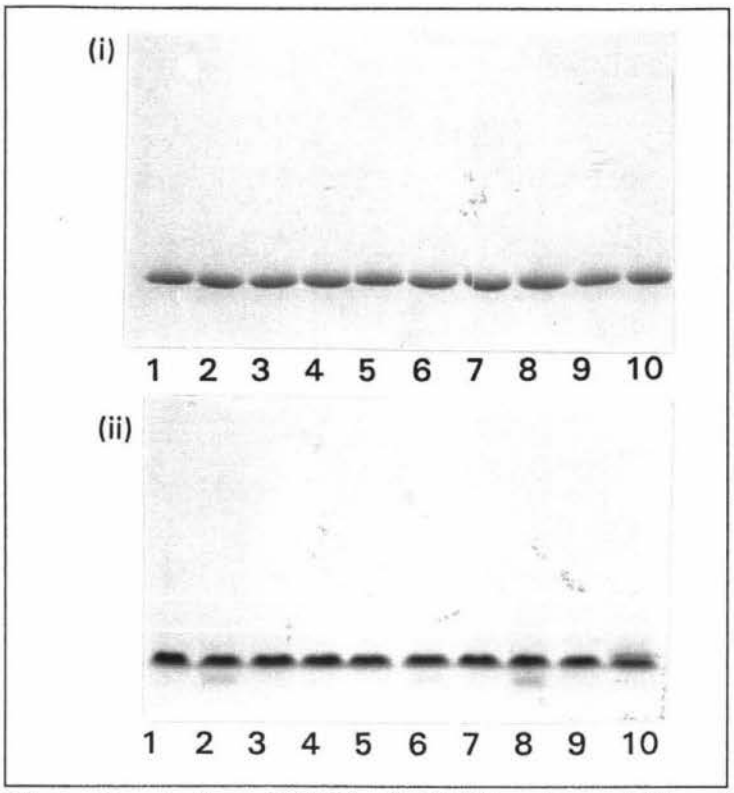


Figure 29 Effect of incubation of β-LG with NEM on the mobility of β-LG (i) under non-reducing SDS-PAGE conditions and (ii) under native-PAGE conditions. Lanes 1 and 2, β-LG incubated at pH 5.0 and 70°C in the absence of NEM (lane 1) or in the presence of NEM (lane 2). Lanes 3-10, β-LG incubated at pH 6.0 under the following conditions: lane 3, 40°C in the absence of NEM; lane 4, 40°C in the presence of NEM; lane 5, 50°C in the absence of NEM; lane 6, 50°C in the presence of NEM; lane 7, 60°C in the absence of NEM; lane 8, 60°C in the presence of NEM; lane 9, 40°C in the presence of 0.27% SDS and the absence of NEM; lane 10, 40°C in the presence of 0.27% SDS and in the presence of NEM.

4.2.2.2.3 DTNB and DPDS

The measured pH of the solutions after 180 minutes incubation with DPDS was 7.1-7.4. The extinction coefficient for 2-mercaptopyridine at 343 nm was calculated to be 7837 M^{-1} cm^{-1}. The literature value of ε_{max} for DTNB (13,600 at 412 nm, Jocelyn, 1972) was used. The effect of the modification of β-LG with NEM on the absorbance of β-LG at 280 nm is not known, and thus, any difference in the extinction coefficient of β-LG and that of the β-LG/NEM complex could not be taken into account.Therefore the calculated protein concentrations, and hence thiol content, of each sample used in the following calculations is only an estimate.

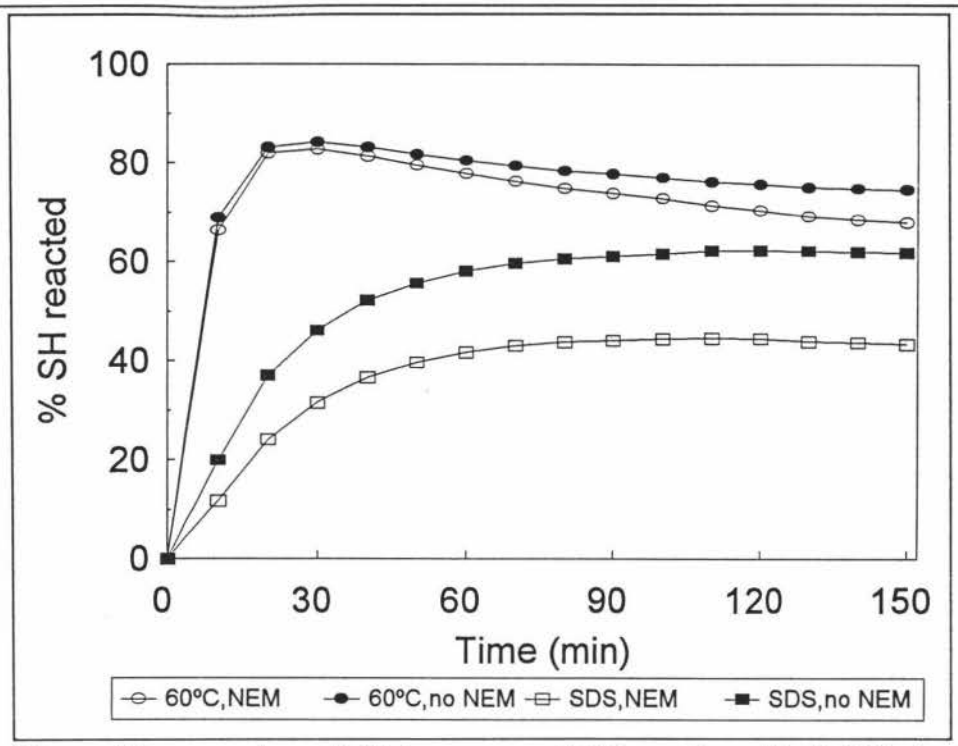


Figure 30 The percentage of thiol groups on β-LG reacting with DTNB at pH 7.0. Protein was previously incubated for two hours at pH 6.0, in the presence or absence of NEM, at 60°C, or at 40°C and in the presence of 0.27% SDS. Data and calculations are shown in Appendix E, Tables 48-51.

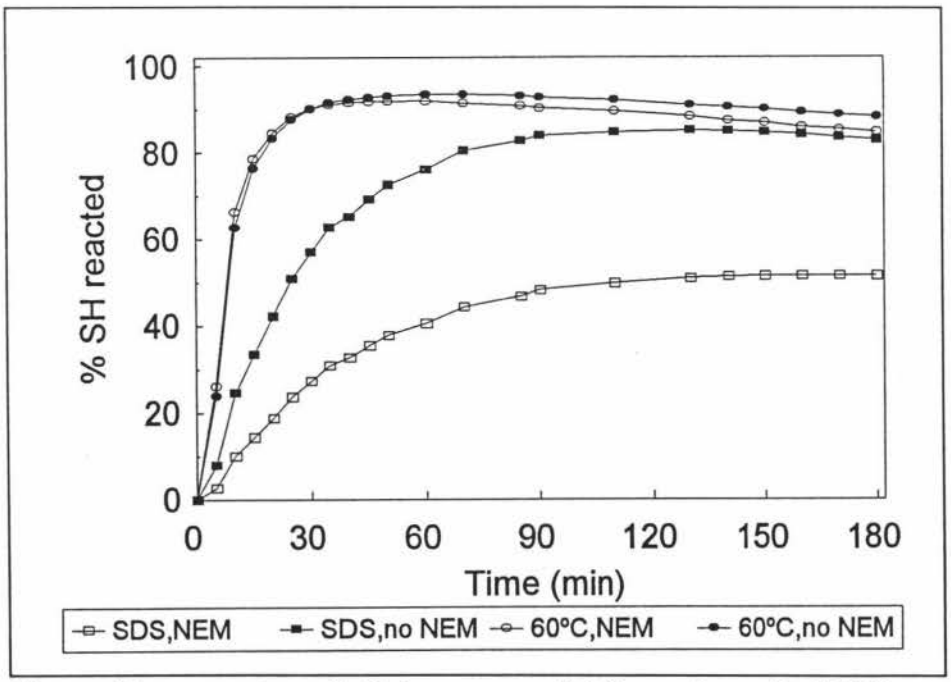


Figure 31 The percentage of thiol groups on β-LG reacting with DPDS at pH 7.5. Protein was previously incubated for two hours at pH 6.0, in the presence or absence of NEM, at 60°C, or at 40°C and in the presence of 0.27% SDS. Data and calculations are shown in Appendix E, Tables 52-55.

During the reaction between DTNB and β-LG that had been incubated at 60°C there was an initial increase in absorbance, but absorbance decreased again (Figure 30), probably due to the reoxidation of the product. This was less noticable with DPDS (Figure 31), possibly due to the higher stability of product. In both cases there was little difference in the number of thiol groups available between the sample that had been incubated in NEM and the untreated protein. This suggests that there has been little reaction between the thiol groups of β-LG and NEM.

β-LG samples treated at 40 °C in the presence of SDS showed a significant difference in the thiol availability between the sample that had been incubated in NEM and the untreated protein with both DTNB (Figure 30) and DPDS (Figure 31), indicating that reaction between NEM and the thiol groups had occurred. Both samples that had been treated in the presence of NEM showed relatively low levels of reaction with DTNB. This may be due to extra SDS binding to the protein, as the presence of detergents results in a reduction of the extinction coefficient of the reaction product (Jocelyn, 1972).

The exact percentage of thiol groups that have reacted with NEM after 2 hours' incubation at 60°C cannot be determined from these results, but the results obtained from experiments with DTNB and DPDS suggest that it is considerably less than the initial result obtained by monitoring loss of absorbance at 320 nm (52%), and probably less than the value obtained after correction for hydrolysis (22%). If the second band seen in the native gel in the sample SDS-80,40 does represent β-LG with bound NEM, this is further evidence for the lack of a significant amount of reaction with NEM under these conditions.

Results obtained for samples SDS-80,40 are relatively consistent between the methods used. If loss of absorbance at 320 nm is taken as a quantitative measure of the reaction, 47% of the thiol groups present can be considered to have reacted after two hours under these conditions. If this value is corrected for the hydrolysis of NEM that occurs under these conditions, this value is reduced to 40%. If the percentage of thiol groups unavailable for reaction with DTNB (calculated from the peak absorbance values

recorded) is considered to be the maximum value for the percentage of thiol groups that have reacted with NEM, then a value of 55% is obtained. The same calculation for the reaction with DPDS gives a value of 46%. If the difference between the percentage of groups available for reaction in samples not treated with NEM and the percentage available for reaction in samples treated with NEM is considered the minimum value for the percentage of thiol groups that have reacted with NEM, then a value of 18% is obtained from results with DTNB and a value of 39% is obtained from results with DPDS.

4.2.2.3 pH 8.12

At pH 8.12 NEM hydrolysed rapidly, even at 4°C. Mainferme *et al.* (1971) used this pH and 25°C, conditions under which NEM rapidly hydrolysed (Figure 32). However, the product of the hydrolysis reaction will react slowly with thiol groups, and long reaction times were used by this group.

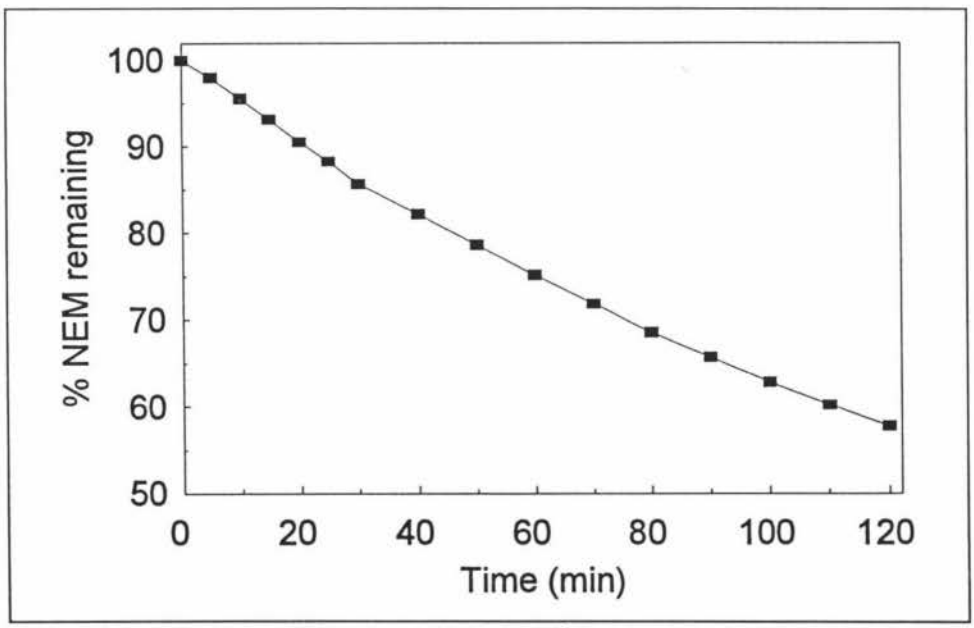


Figure 32 Hydrolysis of NEM at pH 8.12, 25°C. The loss of NEM was determined by monitoring changes in absorbance at 320 nm. Data and calculations are shown in Appendix E, Table 56.

4.3 Conclusions

NEM is unstable in aqueous solution, and both heat and pH affect stability. Therefore monitoring the reaction of NEM with protein by following the loss of absorbance at or around 300 nm does not always give a true indication of the percentage of the sulfhydryl groups that have reacted.

Use of a specific reagent such as DTNB or DPDS as a method of determining the amount of free thiol groups remaining after treatment with NEM also presented problems. Determination of the end point of the reacton was difficult where reoxidation had occurred, though this could have been minimised by the addition of EDTA to the reaction mixture (Jocelyn, 1972). Optimisation of the reaction conditions may have provided a more accurate result. A second source of error in this method is the calculation of the protein concentration after dialysis.

Despite the difficulties in quantifying the exact degree of reaction which had occurred under the conditions tested, two hours incubation at 40 °C in the presence of 0.27% SDS and at pH 6.0 would appear to give a level of reaction that was adequate for the purposes of this study, and in particular conditions which did not induce band splitting, aggregation or irreversible denaturation of β-LG.

5. Determination of Digestion Conditions

In order to get the smallest peptide possible for sequencing, this current study requires the use of conditions that give the maximum amount of digestion possible.

Optimum conditions for the hydrolysis of β-LG by trypsin and analysis of the peptide products of this hydrolysis were established during an extensive study on this subject (Motion, personal communication). Motion found that a high rate of digestion occurred at 50°C in 100mM Hepes buffer, pH 8.0, with a trypsin to β-LG ratio of 1%. Under these conditions only 5% of β-LG A and 10% of β-LG B remained undigested after one hour. Four peaks that formed and then disappeared during the course of the digestion were identified. These were termed transient peaks and were named T1-T4 (Figure 33). A further 10% of β-LG A and 15% of β-LG B was present as transient peptides T1-T3 after one hour incubation under the conditions described above. Motion also found that undigested β-LG eluted from the size exclusion column with an apparent molecular weight of 21000 under the conditions used (Figure 33).

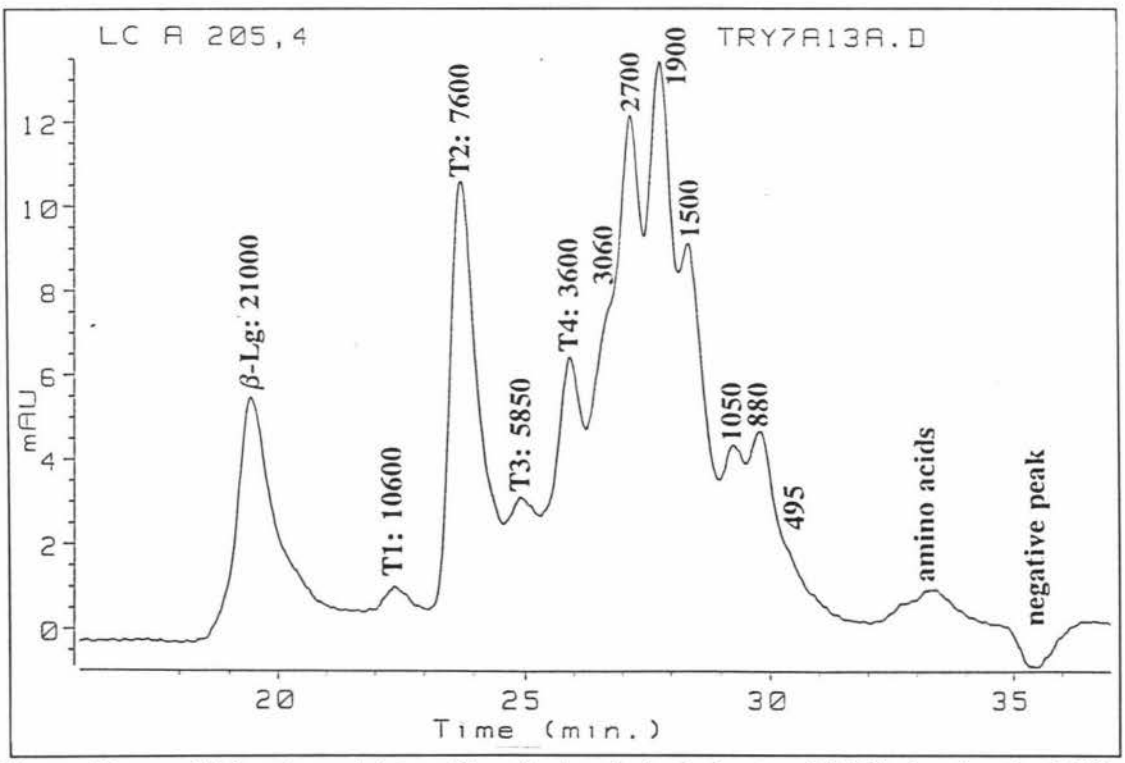


Figure 33 Molecular weight profile of a tryptic hydrolysate of β-LG showing the MWs assigned to peptide peaks.

The conditions used by Motion were adopted as reference conditions for this current study.

5.1 Digestion of β-LG under reference conditions

5.1.1 Methods

β-LG C, labelled with NEM under the optimum conditions described in Section 4, was dialysed and freeze dried. Labelled protein (27 mg) was dissolved in 2.6 ml HEPES buffer (100 mM, pH adjusted to 8.0 with 5 M NaOH) and the solution was equilibrated to 50°C in a circulating waterbath. N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated-Trypsin (Sigma) (100 μL at 2.7 mg/ml in HEPES buffer) was added, giving a final β-LG concentration of 10 mg/ml and a trypsin to β-LG ratio of 1%.

A 200 μl sample taken immediately after addition of the enzyme and mixed with 4.8 mL of 36% acetonitrile, 0.1% TFA in Milli-Q water, to stop the reaction immediately. The hydrolysis mixture was incubated at 50°C for 4 hours, after which a 200 μL sample was taken and reaction stopped as before. Samples were analysed by size exclusion (SE)-HPLC using a modification of a method developed by Motion (personal communication), which is based on the method of Swergold and Rubin (1983), and a Waters HPLC system (Waters Associates, Millipore Corp., Waters Chromatography Division, Massachussetts, USA) comprised of a Waters 600E system controller and a Waters 700 Satellite WISP autoinjector connected to a Hewlett-Packard 1040A multiwavelength detection system (Hewlett-Packard Company, Camas, Washington, USA) linked to a Hewlett-Packard ChemStation for data collection and analysis. A silica TSK-GEL G2000-SW_{XL} column (7.5 mm I.D. x 30cm) (Tosoh Corporation, Tokyo, Japan) with a TSK-GEL SW guard column (7.5 mm I.D. x 7.5 cm) was used with a mobile phase comprised of 36% acetonitrile and 0.1% TFA in Milli-Q water. The column was equilibrated with the mobile phase at a flow rate of 0.4 ml/min. The sample (50 μL) was injected onto the column at a flow rate of 0.4 mL/min and elution was complete after 45 min. The absorbance was monitored at 205, 210, 220, 280 and 295 nm.

5.1.2 Results

After incubation of the labelled β-LG/enzyme mixture for four hours ~50% of the peak corresponding to the unhydrolysed β-LG remained (Figure 34).

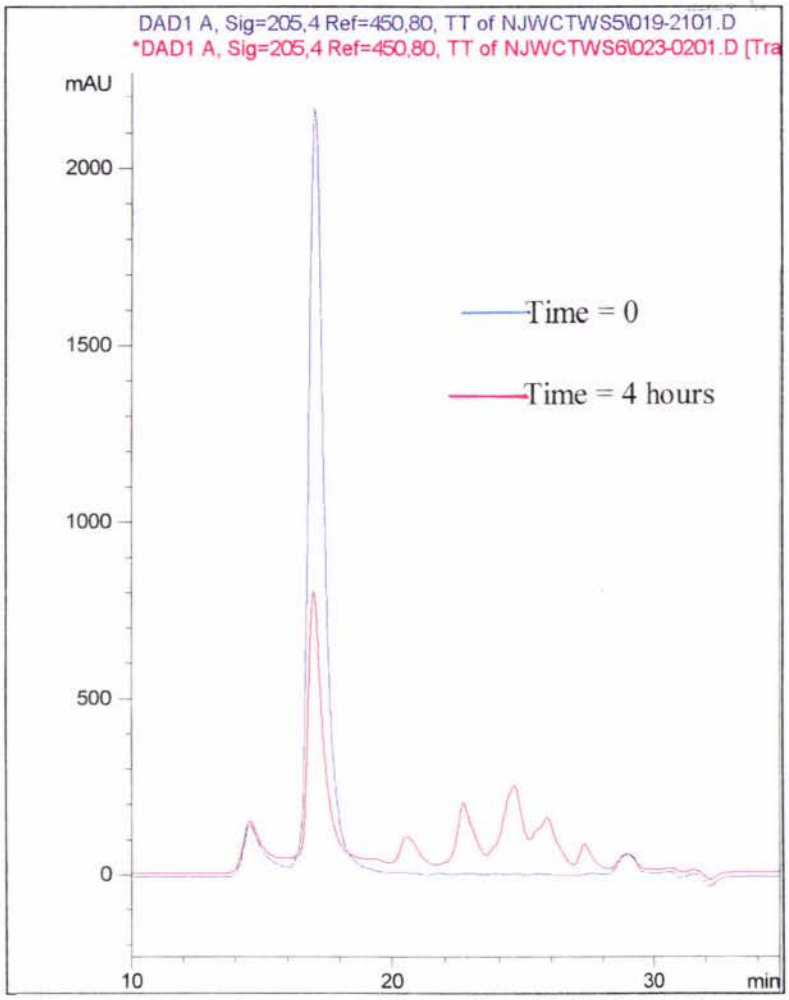


Figure 34 Size exclusion chromatography profile of β-LG C following hydrolysis for four hours by TPCK-trypsin. Instrument printouts for individual samples can be found in Appendix F.

5.1.3 Discussion and Conclusions

The degree of hydrolysis seen under these conditions was lower than was expected, even allowing for the greater resistance of β-LG C to tryptic hydrolysis (Hill *et al.*, 1997b). This could be due to the effect of SDS and/or NEM molecules bound to the protein. The experiment was repeated, increasing the enzyme concentration and/or the

incubation time, and the effect of SDS and NEM on the hydrolysis of β-LG by trypsin was investigated.

5.2 Effect of SDS and NEM, and enzyme concentration

5.2.1 Methods

NEM (10 mM in 100 mM HEPES, pH 8.0, containing 10 mM DTT, and SDS solution were added to 300 µl β-LG B (36.7 mg/mL) as shown in Table 7. Samples were made up to 1.1 mL with 100 mM HEPES/10 mM DTT buffer. TPCK-trypsin (1.1 mg/mL in 100 mM HEPES/10 mM DTT buffer) was added as shown in Table 7. Samples were incubated overnight at 50°C, after which 200 µl samples were taken and the enzyme reaction stopped as previously described. Non-reduced SDS-PAGE was used to monitor the loss of native protein.

Lane	µl SDS	µl NEM	µl enz
1	-	-	-
2	30	-	-
3	60	-	100
4	30	-	100
5	15	-	100
6	8	-	100
7	3	-	100
8	-	110	100
9	-	50	100
10	-	10	100
11	30	110	200
12	30	110	100
13	-	110	-

Table 7 Reagent volumes used during hydrolysis experiments as shown in Figure 35.

5.2.2 Results and Discussion

The presence of SDS clearly affects the hydrolysis of β-LG by trypsin, however the presence of NEM appears not to affect the action of the enzyme (Figure 35).

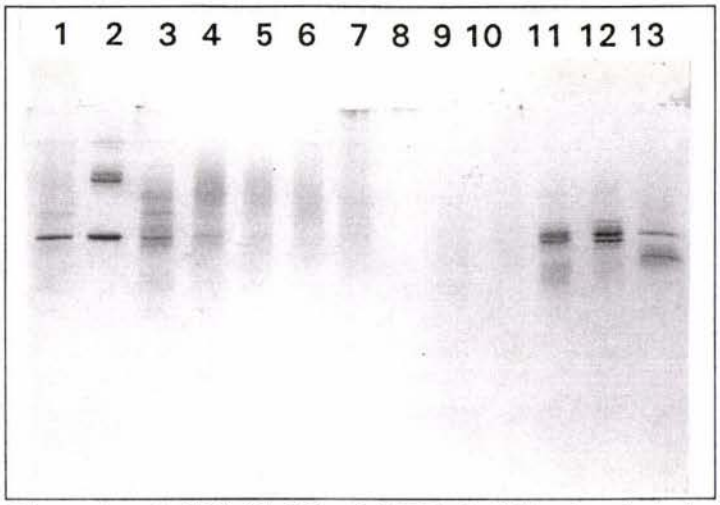


Figure 35 Non-reduced SDS-PAGE of β-LG B following hydrolysis overnight with TPCK-trypsin under the conditions shown in Table 7.

In all the samples where enzyme and SDS are present and NEM absent (Figure 35, lanes 3-7), high molecular weight products are seen. These are probably mainly disulphide linked peptides. The presence of these peptides would indicate that the reducing conditions used are not adaquate to prevent disulphide aggregation when incubation is continued for extended times. These aggregates are not seen to the same degree in the presence of NEM (Figure 35, lanes 11 and 12) and do not appear to be present in the absence of SDS (Figure 35, lanes 8-10). Even though it is likely that the NEM has hydrolysed under these conditions, the hydrolysis product appears to prevent disulphide-mediated aggregation. As expected, increasing the enzyme concentration to 2% (Figure 35, lane 11) increased the amount of β-LG digestion.

Addition of 110 μL of NEM solution and 30 μL of SDS solution gave concentrations approximately equivalent to those under which labelling was carried out. However, dialysis before digestion should remove almost all the unreacted NEM, as well as at least some of the SDS.

5.2.3 Conclusions

To reduce the formation of disulphide linked aggregates during the hydrolysis reaction, it may be preferable to carry out the digestion at lower temperature and pH. In the study previously refered to (Motion, personal communication) it was found that β-LG

was hydrolysed by trypsin at pH 6.0 in 100 mM phosphate buffer, although hydrolysis occurred at a slower rate. Higher ionic strength buffer will further reduce the rate of reaction (Motion, personal communication), as will lowering the temperature, but increasing the trypsin to β-LG ratio to 5% and using long reaction times may compensate for this and allow the same buffer solution to be used for labelling and tryptic hydrolysis.

5.3 Effect of increased enzyme concentration

5.3.1 Methods

SDS (30 μL), 110 μL NEM (10 mM in 0.2 M phosphate buffer, pH 6.0) and 560 μL of 0.2 M phosphate buffer (pH 6.0), was mixed with 300 μL β-LG B (36.7 mg/ml) and the mixture incubated for 2 hours at 40°C. TPCK-trypsin (100 μL, 5.5 mg/ml in 0.2 M phosphate buffer) was added and the mixture incubated overnight at 40°C. Samples were taken 5 minutes and 14.5 hours after addition of the enzyme and the reaction stopped as described previously. SDS-PAGE was used to monitor loss of native protein.

5.3.2 Results and Discussion

Even after 5 minutes digestion a significant amount of hydrolysis had occurred (Figure 36, lanes 2 and 5). After 14 hours there was no intact β-LG remaining (Figure 36, lanes 3 and 6). Some disulphide-mediated aggregation had occurred, but these aggregates were dissociated when the samples were reduced.

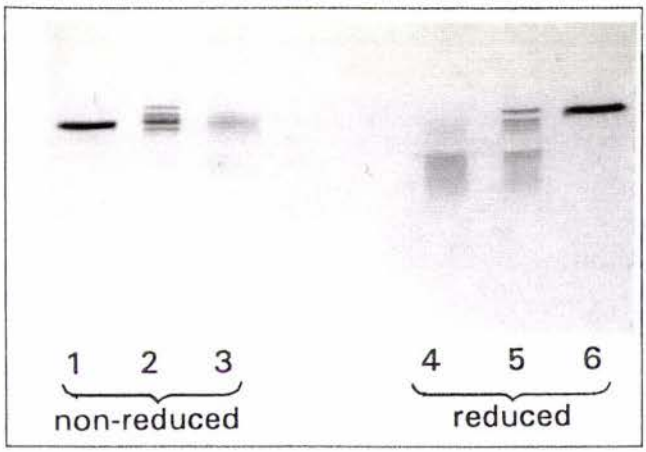


Figure 36 SDS-PAGE of β-LG B following hydrolysis with TPCK-trypsin. Lanes 1 and 4, unhydrolysed β-LG; lanes 2 and 5, following 5 minutes hydrolysis; lanes 3 and 6, following 14.5 hours hydrolysis.

5.3.3 Conclusions

After labelling and removal of excess NEM, digestion can be carried out in the same buffer as used for labelling (0.2 M phosphate, pH 6.0). An enzyme:substrate ratio of 5% should be used, and the digest incubated overnight (14 hours) at 40°C. After digestion 2-ME should be added to the digest to reduce any disulphide-linked peptide aggregates.

6 Identification of the position of the free thiol group in bovine β-LG

The free thiol in β-LG was labelled under the optimum conditions determined in Section 4 and the protein hydrolysed using the optimum conditions determined in Section 5. After labelling and hydrolysis, the labelled peptide was purified using HPLC methods and sequenced.

6.1 Methods

A stock solution of NEM was prepared from 7 μmoles of [1,4-^{14}C] NEM (in 1 mL hexane) (American Radiolabeled Chemicals Inc., St. Louis, Missouri) and 33 μmoles of unlabelled NEM (Sigma) dissolved in 5 mL of hexane. Aliquots (300 μL, 0.002 moles NEM) were taken and the hexane evaporated to near dryness just prior to use. Phosphate buffer (0.2 M, pH 6.0), SDS (10% w/v) and protein solution were added as shown in Table 8.

		mL	mg/mL	mg	moles	ratio
β-LG A	buffer	1.150			0.002 NEM	1.65
	SDS	0.054	100	5.4	0.019	15.48
	protein	0.500	44.28	22.14	0.0012	1
β-LG B	buffer	1.380			0.002 NEM	1.65
	SDS	0.054	100	5.4	0.019	15.48
	protein	0.560	39.52	22.13	0.0012	1
β-LG C	buffer	1.580			0.002 NEM	1.65
	SDS	0.054	100	5.4	0.019	15.48
	protein	0.363	61.00	22.14	0.0012	1

Table 8 Relative proportion of reagents used for labelling of β-LG with NEM.

Solutions were incubated for two hours at 40°C, after which unreacted NEM was removed by ultrafiltration in a 50 mL Amicon stirred cell using a YM10 membrane (Amicon, Inc., Beverly, Massachussetts, USA). The solution was diluted with phosphate buffer to give ~50 mL volume and then concentrated down to ~10 mL a total of four times, giving a final dilution factor (with respect to the volume of the digestion solution) of approximately 650-fold and a final volume of ~10 mL. TPCK-

trypsin (200 μL, 5.5 mg/mL in phosphate buffer) was added and the solutions were incubated at 40°C for 14 hours. 2-ME (300 μL) was added and peptides were reduced by incubating in a circulating waterbath set at 80 °C for one hour.

Peptides were separated by SE-HPLC followed by reverse phase (RP)-HPLC using a Spectra-Physics HPLC system comprised of a SP8800 ternary HPLC pump and a SP8490 variable wavelength detector. The change in absorbance at 220 nm was monitored.

SE-HPLC was carried out using a BIOSEP SEC-S2000, 7.80 mm I.D. x 30 cm column, a BIOSEP SEC-S2000, 7.80 mm I.D. x 3.5 cm guard column (Phenomenex, Torrance, California, USA) and a prefilter, and a mobile phase, flow rate and run time as described in Section 5.1.1. Aliquots of the reduced hydrolysate (40-60 μL, depending on the final concentration after ultrafiltration) were injected directly onto the column and peaks were collected. Fractions were tested for radioactivity using liquid scintillation counting. A sample (100 μL) of each fraction was added to 10 mL of scintillation liquid (4 g 2,5-diphenyloxazole in 666 mL distilled toluene and 333 mL Triton X-100) and was the mixture was counted (counts per minute (cpm) for ^{14}C) for 50 min using a Beckman LS800 liquid scintillation counter. The fraction containing the labelled peptide was freeze-dried.

The freeze dried fraction was redissolved in Milli-Q water and separated into individual peptides by RP-HPLC using a Alltech/Applied Science Vydac 218TP C18 column (10μ, 4.6 mm I.D. x 25 cm), a water/acetonitrile gradient and a flow rate of 1 mL/min. The gradient consisted of 100% solvent A (H_2O/0.1% TFA) for 10 min and 0-50% solvent B (acetonitrile/0.08% TFA) over 30 min. Between runs the column was cleaned and reequilibrated using a gradient of 50-80% solvent B over 10 min, 80-0% solvent B over 10 min, and 100% solvent A for 5 min. Absorbance was monitored at 220 nm, peaks were collected, and the labelled peptide was identified and freeze dried as before.

The purified peptide was sequenced using an Applied Biosystems Protein Sequencer Model 476A, linked to model 610A Version 1.2.2 (Applied Biosystems software) for

data analysis. The effluent from each cycle of the Edman degradation was collected, and fractions from cycles 1 (β-LG A), 4-7 and 14-23 were freeze dried, resuspended in 100 μL 36% acetonitrile, 0.1% TFA in Milli-Q water, and tested for radioactivity as before.

6.2 Results

6.2.1 Size Exclusion HPLC

A typical trace is shown in Figure 37. Some variation was seen in the appearance of the peaks labelled A-D, depending on the amount of hydrosylate injected on to the column. The distribution of radioactivity between the peaks is shown in Table 9. In each variant fraction E was significantly higher in radioactivity than the other fractions. A second smaller peak in radioactivity was seen for fraction G in all three variants.

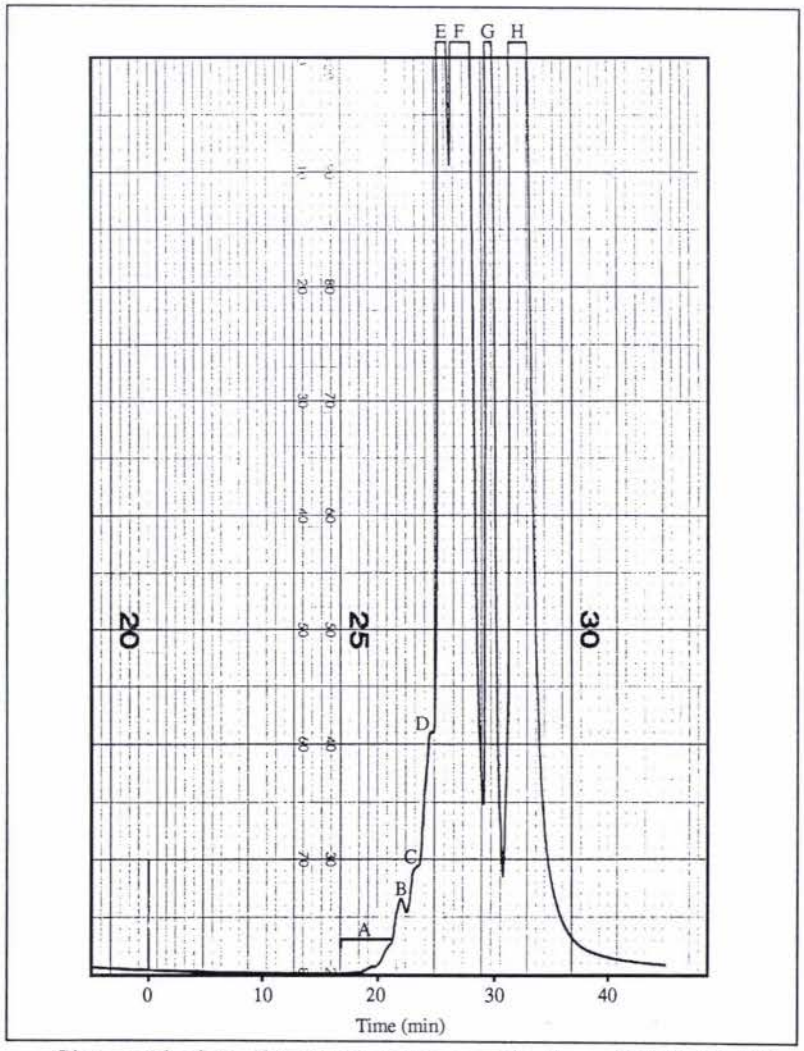


Figure 37 Size exclusion chromatography profile for β-LG B following 14 hours hydrolysis with TPCK-trypsin. Changes in absorbance were monitored at 220 nm.

Fraction	cpm
A	31
B	36
C	69
D	245
E	1057
F	137
G	296
H	45
Background	20

Table 9 Radioactivity (cpm) associated with fractions collected from SE-HPLC.

6.2.2 Reverse Phase HPLC

A typical trace is shown in Figure 38. The distribution of radioactivity between the peaks is shown in Table 10. The percentage CH_3CN at which the labelled peptide eluted varied slightly (±3%) between runs, but the overall shape of the elution profile and the position of the labelled peptide within the elution profile was constant.

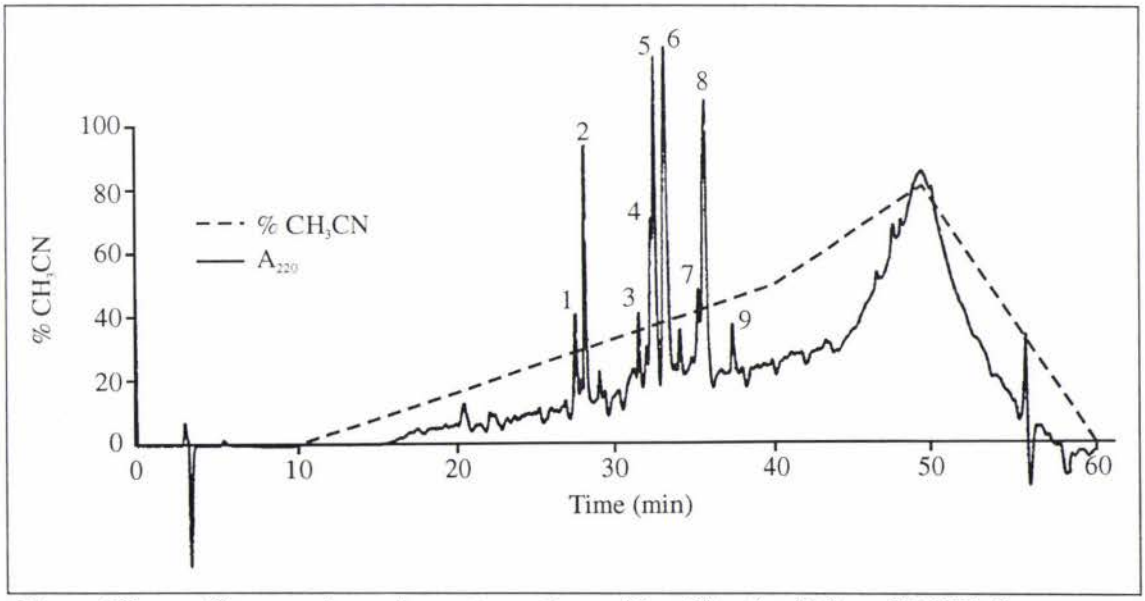


Figure 38 Reverse-phase chromatography profile of fraction E from SE-HPLC.

Fraction	cpm
1	26
2	25
3	53
4	47
5	31
6	30
7	61
8	283
9	29
Background	20

Table 10 Radioactivity (cpm) associated with fractions separated by RP-HPLC.

6.2.3 Sequencing

Each of the β-LG variants was labelled and sequenced twice. In each variant the peptide Tyr 102-Arg 124 (for sequence see Hambling *et al.*, 1992) was found to contain radioactivity. The first time this procedure was performed there was an insufficient amount of the labelled peptide purified to give a significant difference between background radioactivity and radioactivity associated with specific amino acids, in particular Cys 106, 119 and 121 (Figure 39, i-iii). However, results from this first run would indicate that both Cys 119 and Cys 121 were associated with a peak in radioactivity with β-LG B and C. With the A variant more radioactivity was associated with Cys 121. When larger amounts of labelled peptide were sequenced, a peak in radioactivity was associated only with Cys 121 with all three variants (Figures 39, iv-vi). The high degree of similarity in the distribution of label between the variants suggests that the location of the free thiol is in the same position in β-LG A, B and C, and that the position of this group is residue Cys 121.

No peak in radioactivity associated with Cys 106 was observed in any of the experiments.

Differences in the heights of various peaks in Figure 39 are probably due to differences in the amount of peptide loaded on to the sequencer.

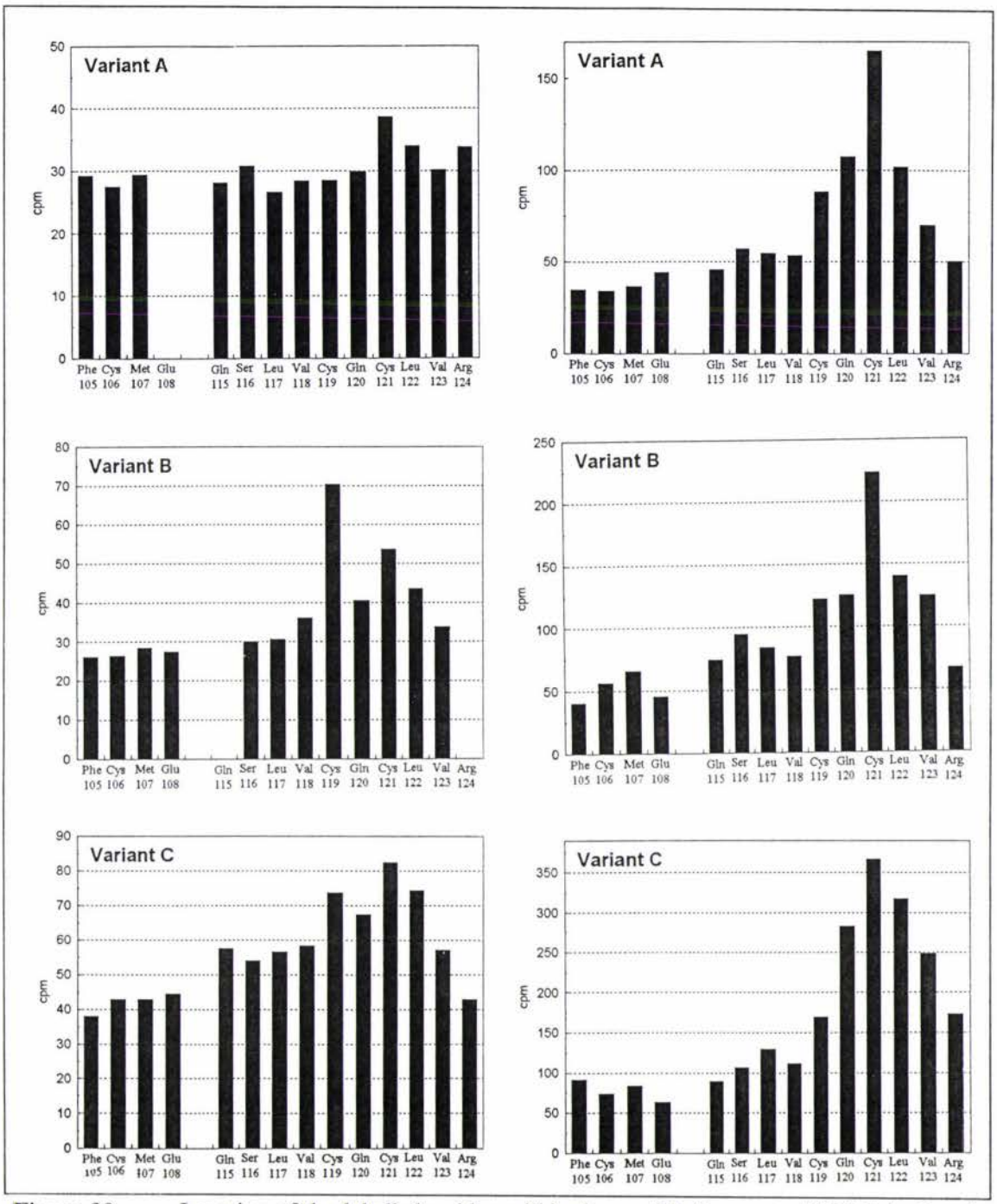


Figure 39 Location of the labelled residue within the peptide fragment of β-LG A, B and C. (i-iii) radioactivity (cpm) of amino acid residues following sequencing at low peptide loadings. (iv-vi) radioactivity (cpm) of amino acid residues following sequencing at high peptide loadings. Data is shown in Appendix G.

6.3 Discussion and Conclusions

The free thiol group would appear to be at residue 121 in both β-LG A and B, confirming the findings of previous experiments (Mainferme *et al.*, 1971, Martial *et al.*, 1971, Pérez-Gómez *et al.*, 1971, Phelan and Malthouse, 1994, Papiz *et al.*, 1986, Monaco *et al.*, 1987, Brownlow *et al.*, 1997). The free thiol also appears to be found at residue 121 in β-LG C. Therefore differences in mobility (structure) during non-reduced SDS-PAGE of β-LG A, B and C are not due to a difference in the location of the thiol group. However, it is possible that, particularly in the B and C variants, there is a tendency for disulphide exchange to occur, even under relatively mild conditions. If this is the case, then the absence of a peak in radioactivity associated with Cys 106 in β-LGs B and C where radioactive peaks are associated with both Cys 119 and Cys 121 could suggest that disulphide exchange is occurring via a mechanism that never allows Cys 106 to be available for reaction (Figure 40). i.e. the thiolate at Cys 121 always attacks the Cys 106-Cys 119 disulphide bond to create a new disulphide (Csy 106-Cys 121), with the same being true for the thiolate at Cys 119. It is possible that the goemetry or structure of β-LG dictates this effect.

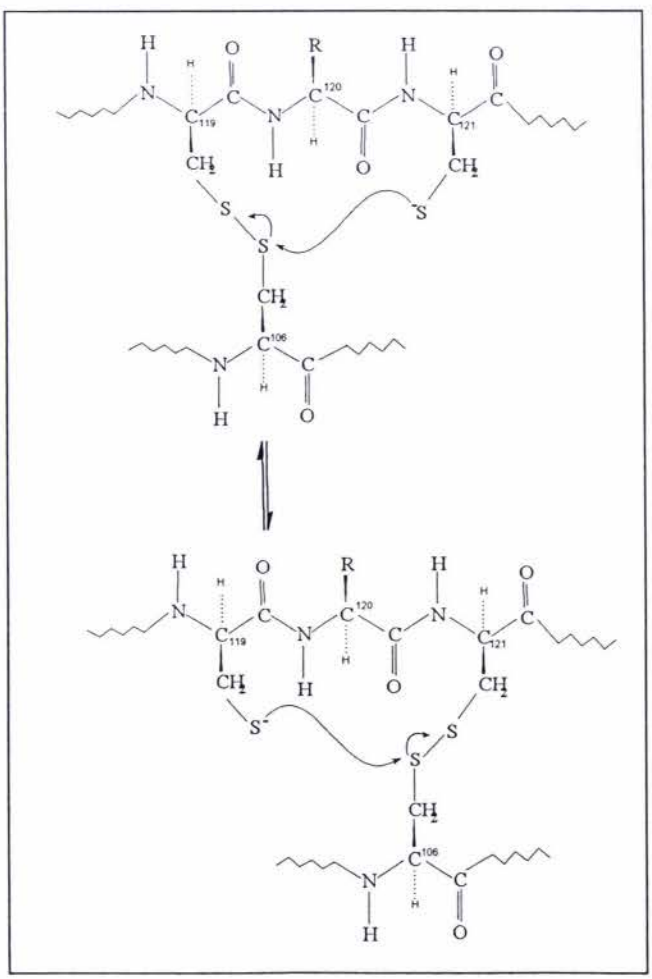


Figure 40 A possible mechanism of disulphide exchange in β-LG. If this is the mechanism whereby the position of the free thiol group switches between position 119 and 121, then Cys 106 will never be reactive towards thiol reagents.

Summary

Three possible reasons for the observed differences in the mobility of β-LG A and B/C under non-reducing SDS-PAGE conditions in unheated samples of the β-LGs studied were:

1. There is a difference in the position of a disulphide bond in β-LG A compared to β-LG B and C in the native protein.

2. The disulphide bonds are in the same position in all the variants studied, but the substitution at position 118 causes enough of a difference in structure under non-reducing SDS-PAGE conditions to cause a difference in mobility.

3. Although unlikely, it is possible that the substitution at position 118 causes a difference in the binding of SDS to the protein.

The purpose of this study was to determine whether the observed differences in the mobility of unheated samples of purified bovine β-LG A, B and C under non-reduced SDS-PAGE is due to a difference in the location of the free sulphydryl group within the primary sequence of these variants (as suggested in 1 above). As the free thiol group was found to be at residue 121 in all three variants, these differences in mobility (structure) are not due to a difference in the location of the thiol group.

Although no significant structural differences between variants have been detected in native β-LG (Bewley *et al.*, 1997), during SDS-PAGE the presence of SDS may induce the formation of secondary structures, and these structures may be different in β-LG A compared to β-LG B and C. The difference seen in the aggregation behaviour of the variants when stored at -18°C in SDS-PAGE sample buffer may be due to structural differences in the variants causing differences in either the availability or the reactivity of the thiol group under these conditions. Thus, the substitution at position 118 may cause enough of a difference in structure under non-reducing SDS-PAGE conditions to cause a difference in mobility (as suggested in 2 above).

It seems likely that heating of β-LG does induce disulphide exchange, and that such a reaction can occur under relatively mild conditions. It would also appear that the mechanism by which this exchange occurs does not generate a free thiol at Cys 106. Thus, it is possible that the distribution of the free thiol between residues 119 and 121 observed by McKenzie *et al.* (1972) was indeed due to exchange occurring during the labelling reaction.

This study also examined the factors affecting the induction of band splitting. It was shown that a number of factors had an influence on this phenomenon, but it was outside the scope of the investigation to examine these factors in detail. A more detailed study of the effect of these variables may shed more light on the nature of the products formed during heating of β-LG solutions. A similar study on the products formed following denaturation of β-LG with urea would also be of interest, in light of the result obtained by McKenzie *et al.* (1972).

Heated samples did not show a single band in the dimer region under either native PAGE or SDS-PAGE. In all ruminant β-LGs studied, a number of bands are seen in this region. These may be due to the heat-induced formation of a number of disulphide-linked dimers, differing in the cysteine residues contributing to the intramolecular bond. For instance, if only residues 119 and 121 form these bonds, three products are possible (119-119, 119-121 and 121-121). If intramolecular disulphide exchange occurs before intramolecular disulphide exchange, the reactivity of the free thiol present may change. Thus, the potential products, both monomeric and aggregated, and their tendancy to form, could be extremely complex.

Results of this study and those of Manderson *et al.* (1997b) indicate that there are also a large number of monomeric products formed when β-LG is heated.

Aaltonen, M. L. and Antila, V. (1987).
Milk Renneting Properties and Genetic Variants of Proteins.
Milchwissenschaft **42**, 490-492.

Aleandri, R., Buttazzoni, L. G., Schneider, J. C., Caroli, A. and Davoli, R. (1990).
The Effect of Milk Protein Polymorphisms on Milk Components and Cheese-Producing Ability.
Journal of Dairy Science **73**, 241-255.

Aschaffenberg, R. and Drewry, J. (1955).
Occurrence of Different Beta-Lactoglobulins in Cow's Milk.
Nature **176**, 218-219.

Basch, J. J., Douglas, F. W. Jr., Procino, L. G., Holsinger, V. H. and Farrell, H. M. Jr. (1985).
Quantitation of Caseins and Whey Proteins of Processed Milks and Whey Protein Concentrates, Application of Gel Electrophoresis, and Comparison with Harland-Ashworth Procedure.
Journal of Dairy Science **68**, 23-31.

Bell, K. and McKenzie, H. A. (1967).
Isolation and Properties of Beta-lactoglobulin C.
Biochemica et Biophysica Acta **147**, 109-122.

Bewley, M. C., Qin, B. Y., Jameson, G. B., Sawyer, L. and Baker, E. N. (1997).
β-lactoglobulin and Its Variants: A Three-Dimensional Perspective. In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press).

Brew, K. and Gobler, J. A. (1992).
α-Lactalbumin. In: *Advanced Dairy Chemistry - Volume 1.*
Elsevier Science Publishers Ltd., London. pp 191-230.

Brewer, C. F. and Riehm, J. P. (1967).
Evidence for Possible Non-specific Reactions Between N-ethylmaleimide and Proteins.
Analytical Biochemistry **18**, 248-255.

Briggs, D. R, and Hull, R. (1945).
Studies on Protein Denaturation. I. Electrophoretic Study Kinetics at Neutrality of Heat Denaturation of β-lactoglobulin.
Journal of the American Chemical Society **67**, 2007-2041.

Brownlow, S., Morais Cabral, J. H., Cooper, R., Flower, D. R., Yewdall., S. J., Polikarpov, I., North, A. C. T. and Sawyer, L. (1997).
Bovine β-lactoglobulin at 1.8 Å Resolution - Still an Enigmatic Lipocalin.
Structure (in press).

Chen, S. X., Hardin, C. C. and Swaisgood, H. E. (1993).
Purification and Characterization of β-structural Domains of β-lactoglobulin Liberated by Limited Proteolysis.
Journal of Protein Chemistry **12**, 613-625.

Cohen, L. A. (1968).
Group Specific Reagents in Protein Chemistry.
Annual Review of Biochemistry **37**, 695-726.

Creamer, L.K. (1991).
Electrophoresis of Cheese.
Bulletin of the International Dairy Federation **261**, 14-28.

Creamer, L.K. and MacGibbon A. K. H. (1996).
Some Recent Advances in the Basic Chemistry of Milk Proteins and Lipids.
International Dairy Journal **6**, 539-568.

Dalgalarrondo, M., Dufour, E., Bertrand-Harb, C., Chobert, J. M. and Haertlé, T. (1992).
Purification and Characterization of Two Porcine β-Lactoglobulin variants by NaCl Salting-out and Reversed Phase-HPLC.
Lait **72**, 35-42.

Dong, A., Matsuura, J., Allison, S. D., Chrisman, E., Manning, M. C. and Carpenter, J. F. (1996).
Infrared and Circular Dicroism Spectroscopic Characterization of Structural Differences Between β-Lactoglobulin A and B.
Biochemistry **35**, 1450-1457.

Donovan, M. and Mulvihill, D. M. (1987).
Effects of Chemical Modification and Sodium Dodecyl Sulphate Binding on the Thermostability of Whey Proteins.
Irish Journal of Food Science and Technology **11**, 77-85.

Dunnill P and Green D W. (1966).
Sulphydryl Groups and the N↔R Conformational Change in β-Lactoglobulin.
Journal of Molecular Biology **15**, 147-151.

Feagan, J. T., Bailey, L. F., Hehir, A. F., McLean, D. M. and Ellis, N. J. S. (1972).
Coagulation of Milk Proteins 1. Effect of Genetic Variants of Milk Proteins on Rennet Coagulation and Heat Stability of Milk.
Australian Journal of Dairy Technology **27**, 129-134.

Fitzgerald, R. J. and Hill, J. P. (1997).
The Relationship Between Milk Protein Polymorphism and the Manufacture of Dairy Products.
In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press)

Fox, P. F. and Morrissey, P. A. (1977).
Reviews of the Progress of Dairy Science: The Heat Stability of Milk.
Journal of Dairy Research **44**, 627-646.

Franklin, J. G. and Leslie, J. (1968).
The Kinetics of the Reaction of N-ethylmaleimide With Denatured β-lactoglobulin and Ovalbumin.
Biochimica et Biophysica Acta **160**, 333-339.

Freedman, R. B., Bullied, N. J., Hawkins, H. C. and Paver, J. L. (1989).
Role of Protein Disulphide-Isomerase in the Expression of Native Proteins.
Biochemical Society Symposium **55**, 167-192.

Fryer, P. J., Belmar-Beiny, M. T. and Schreier, P. J. R. (1995).
Fouling and Cleaning in Milk Processing. In: *Heat Induced Changes in Milk.*
International Dairy Federation, Brussels, Belgium. pp 364-388.

Gallagher, D. P., Lynch, M. G. and Mulvihill, D. M. (1996).
Porcine β-Lactoglobulin Does Not Undergo Thermally-Induced Gelation.
Journal of Dairy Research **63**, 479-482.

Green, D. W., Aschaffenburg, R., Camerman, A., Coppola, J. C., Dunnill, P., Simmons, R. M., Komorowski, E. S., Sawyer, L., Turner, E. M. C., and Woods, K. F. (1979)
Structure of Bovine β-lactoglobulin at 6Å Resolution
Journal of Molecular Biology **131**, 375-397.

Hambling, S. G., McAlpine, A. S. and Sawyer, L. (1992).
β-lactoglobulin. In: *Advanced Dairy Chemistry - Volume 1.*
Elsevier Science Publishers Ltd., London, pp. 141-190.

Hattori, M., Ametani, A., Katakura, Y., Shimizu, M. and Kaminogawa, S. (1993).
Unfolding/Refolding Studies on Bovine β-Lactoglobulin with Monoclonal Antibodies as Probes.
Journal of Biological Chemistry **268**(30) 22414-22419

Hill, J.P. (1993).

The Relationship Between β-Lactoglobulin Phenotypes and Milk Composition in New Zealand Dairy Cattle.

*Journal of Dairy Sci*ence **76**, 281-286.

Hill, J. P. (1994).

The Effect of Season and β-lactoglobulin Phenotypes on Milk Composition in New Zealand Friesian Dairy Cattle.

Brief Communication of the XXIV International Dairy Congress, Melbourne 18-22 eptember 1994. Australian National Committee of the International Dairy Federation, 1601 Malvern Road, Glen I7is, Victoria 3146, Australia. p 59.

Hill, J. P., Boland, M. J. and Creamer, L. K. (1993).

The Alteration of Bulk Milk Composition Through the Selection of β-lactoglobulin Phenotypes.

Livestock Production Science **35**, 193.

Hill J. P. and MacGibbon A. K. H. (1994).

The Relationship Between β-lactoglobulin Phenotypes With the Composition and Yield of Milkfat.

Brief Communication of the XXIV International Dairy Congress, Melbourne 18-22 eptember 1994. Australian National Committee of the International Dairy Federation, 1601 Malvern Road, Glen Iris, Victoria 3146, Australia. p 61.

Hill, J. P. and Paterson, G. R. (1994).

The Variation in Milk Composition From Individual β-lactoglobulin AA and BB Phenotype Cows.

Proceedings of the New Zealand Society of Animal Production **54**, 293-295.

Hill, J. P., Paterson, G. R., Lowe, R. and Johnston, K. A. (1995a).

Effect of β-Lactoglobulin Variants on Curd Firming Rate, Yield, Maturation and Sensory Properties of Cheddar Cheese.

International Dairy Federation Seminar - Implications of Genetic Polymorphism of Milk Proteins on Production and Processing of Milk, Zurich, Switzerland, 28-29 March 1995. pp 18-19.

Hill, J. P., Paterson, G. R., Lowe, R. and Wakelin, M. (1995b).
The Effect of β-lactoglobulin Phenotype on Milk Composition.
Proceedings of the New Zealand Society of Animal Production **55**, 94-96.

Hill, J. P., Thompson, C. J. and Elston, P. D. (1995c).
Effect of β-Lactoglobulin Variants on the Yield and Properties of Rennet and Lactic Casein.
International Dairy Federation Seminar - Implications of Genetic Polymorphism of MilkProteins on Production and Processing of Milk, Zu71ch, Switzerland, 28-29 March 1995. pp 19-20

Hill, J. P., Boland, M. J., Creamer, L. K., Anema, S. G., Otter, D. E., Paterson, G. R., Lowe, R., Motion, R. L. and Thresher, W. C. (1996).
Effect of Bovine β-lactoglobulin Phenotype on the Properties of Beta-lactoglobulin, Milk Composition, and Dairy Products. In: *Macromolecular Interactions in Food Technology.*
Ed: Parris, N. and Creamer, L. K. Pub: American Chemical Society. Washington, DC, USA. ACS Symposium Series; no. 650 pp 281-294

Hill, J. P., Boland, M. J. and Smith, A.F. (1997a).
The Effect of β-lactoglobulin Variants on Milk Powder Manufacture and Properties. In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press).

Hill , J. P., Thresher, W. C., Boland, M. J., Creamer, L. K., Anema, S. G., Manderson, G., Otter, D. E., Paterson, G. R., Lowe, R., Burr, R. G., Motion, R. L., Winkelman, A. and Wickham, B. (1997b).
The Polymorphism of the Milk Protein β-lactoglobulin. A Review.
Biotechnology for Dairy Production. Proceedings of the OECD Conference, Wellington, New Zealand 7-9 February 1996. CAB International. (in press).

Hill, J. P., Boland, M. J. and Smith, A.F. (1997c).
The Influence of β-lactoglobulin Variants on the Properties of Recombined Evaporated Milk.
In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press).

Hillquist Damon, A. J. and Kresheck G. C. (1982).
Influence of Surfactants on the Conformation of β-Lactoglobulin B Using Circular Dichroism.
Biopolymers **21**, 895-908.

Huang, X. L., Catignani, G. L., Foegeding, E. A. and Swaisgood, H. E. (1994a).
Comparison of the Gelation Properties of β-lactoglobulin Genetic Variants A and B.
Joumal of Agricultural and Food Chemistry **42**, 1064-1067.

Huang, X. L., Catignani, G. L., and Swaisgood, H. E. (1994b).
Relative Structural Stabilities of β-lactoglobulins A and B as Determined by Proteolytic Susceptibility and Differential Scanning Calorimetry.
Joumd of Agricultural and Food Chemistry **42**, 1276-1280.

Huang, X. L., Catignani, G. L., and Swaisgood, H. E. (1994c).
Comparison of the Size and Rate of Formation of Peptides Released by Limited Proteolysis of β-lactoglobulin A and B with Immobilized Trypsin.
Journal of Agricultural and Food Chemistry **42**, 1281-1284.

Jacob, E. (1994).
Genetic Polymorphism of Milk Proteins.
Bulletin of the International Dairy Federation **298**, 17-27.

Jacob, E. and Puhan, Z. (1992).
Technological Properties of Milk as Influenced by Genetic Polymorphism of Milk Proteins - A Review.
International Dairy Journal **2**, 157-178.

Jenness, R. (1988).
Composition of Milk. In: *Fundamentals of Dairy Chemistry.*
Van Nostrand Reinhold Company Inc., New York. pp 1-38.

Jocelyn, P. C. (1972).
In: *Biochemistry of the SH Group.*
Academic Press Inc. (London) Ltd., London. pp 63-93.

Jones, M. N. and Wilkinson A. (1976).
The Interaction Between β-lactoglobulin and Sodium n-Dodecyl Sulphate.
Biochemical Journal **153**, 713-718.

Kalan, E., Greenberg, R. and Walter, M. (1965).
Studies on β-lactoglobulins A, B, and C. I. Comparisons of Chemical Properties.
Biochemistry **4**, 991-997.

Kaminogawa, S., Shimizu, M., Ametani, A., Hattori, M., Ando, O., Hachimura, S., Nakamura, Y., Totsuka, M. and Yamauchi, K. (1989).
Monoclonal Antibodies as Probes For Monitoring the Denaturation Process of Bovine β-Lactoglobulin.
Biochimica et Biophysica Acta **998**, 50-56

Kessler, E. and Brew, K. (1970).
The Whey Proteins of Pig's Milk. Isolation and Characterisation of a β-lactoglobulin.
Biochimica et Biophysica Acta, **200**, 449-458.

Kubo, K. (1995).
Variability in Heat-Induced Fragmentation of a Protein in the Presence of Dodecyl Sulphate: The Role of an Intramolecular Sulfhydryl/Disulfide Exchange.
Journal of Biochemistry **118**, 1112-1117.

Larson, B. L. (1979).
Biosynthesis and Secretion of Milk Proteins: A Review.
Journal of Dairy Research **46**, 161-174.

Lodes, A. (1995).
Beziehungen Zwischen Zusammensetzung und Labgerinnungseigenschaften der Milch und Genetischen Varianten der Milchproteine.
Ph.D thesis, Institute for Chemistry and Physics, Technical University of Munich.

Lunderblad, R. L. and Noyes, C. M. (1984).
In: *Chemical Reagents for Protein Modification. Vol I.*
CRC Press Inc., Boca Raton, Florida. pp 55-94.

Mailliart, P. and Ribadeau-Dumas, B. (1988).
Preparation of β-lactoglobulin and β-lactoglobulin-Free Proteins From Whey Retentate by NaCl Salting Out at Low pH.
Journal of Food Science **53**(3), 743-745,752.

Mainferme, F., Prèaux, G. and Lontie, R. (1971).
Location of the Disulfide Bridges in the Sequence of β-lactoglobulin A with CNBr and Thermolysin.
Archives Internationales de Physiologie et de Biochimie **79**, 840-841.

Manderson, G. A., Creamer, L. K. and Hardman, M. J. (1997a).
Spectroscopic Examination of the Heat-Induced Changes in β-lactoglobulin A, B and C. In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press).

Manderson G. A., Hardman, M. J. and Creamer, L. K. (1997b).
Electrophoretic Examination of the Heat-Induced Changes in β-lactoglobulin A, B and C. In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press).

Mariani, P., Morini, D., Castagnetti, G. B. and Losi, G. (1982).
La Cosistenza del Coagulo in Latte Caratterizzati dalle Varianti A e B della Beta-lattoglobulina.
L' Industria del Latte **18**, 9-12.

Marshall, R. J. (1993).
Milk. Physical and Chemical Properties. In: *Encyclopaedia of Food Science, Food Technology and Nutrition.*
Academic Press Ltd., London. pp 3075-3080.

Martial, J., Prèaux, G. and Lontie, R. (1971).
Location of the Thiol Group and of the Disulfide Bridges in the Sequence of β-lactoglobulin B.
Archives Internationales de Physiologie et de Biochimie **79**, 842-843

Marziali, A. S. and Ng-Kwai-Hang, K. F. (1986).
Effects of Milk Composition and Genetic Polymorphism on Coagulation Properties of Milk.
Journal of Dairy Science **69**, 1793-1798.

McKenzie, H. A. (1971).
β-lactoglobulin. In: *Milk Proteins. Chemistry and Molecular Biology.*
Ed: McKenzie, H. A. Pub: Academic Press, New York. Vol II pp 257-330.

McKenzie, G. H., Norton, R. S. and Sawyer, W. H. (1971).
Heat-Induced Interaction of β-lactoglobulin and κ-casein.
Journal of Dairy Research **38**, 343-351.

McKenzie, H. A., Ralston, G. B. and Shaw, D. C. (1972).
Location of Sulphydryl and Disulfide Groups in Bovine β-lactoglobulins and Effects of Urea.
Biochemistry **11**(24), 4539-4547.

McKenzie, H. A. and Sawyer W. H. (1972).
On the Dssociation of Bovine β-lactoglobulins A, B and C Near pH 7.
Australian Journal of Biological Science **25**, 949-961.

McKenzie, H. A. and Ralston, G. B. (1973).
Nature of Products Formed by the Action of Urea on Bovine β-Lactoglobulins.
Australian Journal of Biological Science **26**, 859-870.

McLean, D. M., Graham, E. R. B., Ponzoni, R. W. and McKenzie, H. A. (1987).
Effects of Milk Protein Genetic Variants and Composition on Heat Stability of Milk.
Journal of Dairy Research **54**, 219-235.

McSwiney, M., Singh, H., Campanella, 0. and Creamer, L. K. (1994).
Thermal Gelation and Denaturation of Bovine β-lactoglobulin A and B.
Journal of Dairy Research **61**, 221-232.

Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L. and Eliopoulos, E. E. (1987).
Crystal Structure of the Trigonal Form of Bovine Beta-lactoglobulin and of its Complex with Retinol at 2.5 Å Resolution.
Journal of Molecular Biology **197**, 695-706.

Monahan, F. J., German, J. B. and Kinsella, J. E. (1995).
Effect of pH and Temperature on Protein Unfolding and Thiol/Disulphide Reactions During Heat-Induced Gelation of Whey Proteins.
Journal of Agricultural and Food Chemistry **43**, 46-52.

Monnot, M. (1964)
Etude Cinetique Comparee de l'Hydrolyse Trypsique des Lactoglobulines A et B.
Biochemica et Biophysica Acta **93**, 31-39.

Motion, R. L. and Hill, J. P. (1994)
A Comparison of the Tryptic and Chymotryptic Hydrolysis of β-lactoglobulin A, B and C Variants.
Brief Communication of the XXIV International Dairy Congress, Melbourne 18-22 September 1994. Australian National Committee of the International Dairy Federation, 1601 Malvem Road, Glen Ids, Victotia 3146, Australia. p 122.

Ng-Kwai-Hang, K. F. and Grosclaude, F. (1992).
Genetic Polymorpism of Milk Proteins. In: *Fox, P. F. Advanced Dairy Chemistry - Volume* 1. Elsevier Science Publishers Ltd., London, pp. 405-455.

Pagnaco, G. and Caroli, A. (1987).
Effect of Casein and β-lactoglobulin Genetic Variants on Renneting Properties in Milk.
Journal of Dairy Research **54**, 479-485.

Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E. and Kraulis, P. J. (1986).
The Structure of β-lactoglobulin and Its Similarity to Plasma Retinol-Binding Protein.
Nature **324**, 383-385.

Parnell-Clunies, E., Kakuda, Y. and Irvine, D. (1988).
Heat Induced Changes in Milk Processed by Vat and Continuous Heating Systems.
Journal of Dairy Science **71**, 1472-1483.

Parris, N., Anema, S. G., Singh H. and Creamer L. K. (1993).
Aggregation of Whey Proteins in Heated Sweet Whey.
Journal of Agricultural and Food Chemistry **41**, 460-464.

Paterson, G. R.., Otter, D. E. and Hill, J. P. (1995).
Application of Capillary Electrophoresis in the Identification of Phenotypes Containing the β-lactoglobulin C Variant.
Journal of Dairy Science **78**, 2637-2644.

Perez, M. D., Sanchez, L., Aranda, P., Ena, J. M., Oria, R., and Calvo, M. (1992).
Effect of β-lactoglobulin on the Activity of Pregastric Lipase. A Possible Role for this Protein in Ruminant Milk.
Biochimica et Biophysica Acta **1123**, 151-155.

Pèrez, M. D. and Calvo, M. (1995).
Interaction of β-Lactoglobulin with Retinol and Fatty Acids and Its Role as a Possible Function For This Protein: A Review.
Journal of Dairy Science **78**, 978-988.

Pèrez Gómez, G., Prèaux, G. and Lontie, R. (1971).
Location of the Disulfide Bridges in β-lactoglobulin B with Pepsin and Trypsin After CNBr Cleavage.
Archives Internationales de Physiologie et de Biochimie **79**, 842-843.

Phelan, P. and Malthouse, J. P. G. (1994).
C-n.m.r. of the Cyanylated β-lactoglobulins : Evidence That Cys-121 Provides the Free Thiol Group of β-lactoglobulins A and B.
Biochemical Journal **302**, 511-516.

Phillips, N. I., Jenness, R. and Kalan, E. B. (1967).
Reactivity of Sulphydryls in Several β-Lactoglobulins.
Archives of Biochemistry and Biophysics **120**, 192-197.

Politis, I. and Ng-Kwai-Hang, K. F. (1988).
Effects of Somatic Cell Count and Milk Composition on the Coagulation Properties of Milk.
Journal of Dairy Science **71**, 1740-1746.

Rahali, V. and Menard, J. L. (1991).
Influence des Variants Genetiques de la β-lactoglobuline et la κ-caseine sue la Composition du Lait et son Aptitude Fromagere.
Lait **71**, 275-297.

Rampilli, M., Cattanco, T., Caroli, A., San Martino, A. and Sciocco Saita, A. (1988). Study of Heat Stability and Coagulation Properties of Milks and Different Protein Genotype. In: *Milk Proteins. Nutritional, Clinical, Functional and* Technological *Aspects.*
Ed: Barth, C. A. and Schlimme, E. Pub; Springer-Verlag New York. pp 251-255.

Richardson, B. C., Creamer, L. K. and Munford, R.E. (1973).
Comparative Micelle Structure. I. The Isolation and Chemical Characterization of Caprine κ-Casein.
Biochimica et Biophysica Acta **310**, 111-117.

Riordan, J. F. and Vallee, B.L. (1967).
Reactions with N-Ethylmaleimide and p-Mercuribenzoate. In *Methods in Enzymology. XI Enzyme Structure.*
Academic Press, Inc., New York. pp 541-548.

Robitaille, G. (1995).
Influence of κ-casein and β-lactoglobulin Genetic Variants on the Heat Stability of Milk.
Journal of Dairy Research **62**, 593-600.

Rocha, T. L., Paterson, G., Crimmins, K., Boyd, A., Sawyer, L. and Fothergill-Gilmore, L. A. (1996).
Expression and Secretion of Recombinant Ovine β-lactoglobulin in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.
Biochemical Journal **313**, 927-932.

Rollema, H. S. (1992).
Casein Association and Micelle Formation In: *Advanced Dairy Chemistry - Volume 1.*
Elsevier Science Publishers Ltd., London. pp.111-140.

Rose, D. (1962).
Factors Affecting the Heat Stability of Milk.
Journal of Dairy Science **44**, 1305-1311.

Sansom, S. E., North, A. C. T. and Sawyer, L. (1994).
Structural Analysis and Classification of Lipocalins and Related Proteins Using a Profile-Search Method.
Biochimica et Biophysica Acta **1208**, 247-255.

Sawyer, W. H. (1968).
Heat Denaturation of Bovine β-lactoglobulins and Relevance of Disulfide Aggregation.
Journal of Dairy Science **51**(3), 323-329.

Schaar, J. (1985).
Plasmin Activity and Proteose-Peptone Content of Individual Milks.
Journal of Dairy Research **52**, 369-378.

Schaar, J., Hansson, B. and Petterson, H. E. (1985).
Effect of Genetic Variants of κ-casein and β-lactoglobulin on Cheesemaking.
Journal of Dairy Research **52**, 429-437.

Sawyer, W. H. (1969).
Complex Between β-lactoglobulin and κ-casein. A Review.
Journal of Dairy Science **52**(9), 1347-1355.

Schmidt, D. G. and van Markwijk, B. W. (1993).
Enzymatic Hydrolysis of Whey Proteins. Influence of Heat Treatment of α-lactalbumin and β-lactoglobulin on Their Proteolysis by Pepsin and Papain.
Netherlands Milk Dairy Journal **47**, 15-22.

Sherbon, J. W., Ledford, R. A., Regenstein, J. and Thompson, M. P. (1967).
Variants of Milk Proteins and Their Possible Relation to Milk Properties.
Journal of Dairy Science **50**, 951.

Shimada, K. and Cheftel, J. C. (1989).
Sulphydryl Group/Disulfide Bond Interchange Reactions During Heat-Induced Gelation of Whey Protein Isolate.
Journal of Agricultural and Food Chemistry **37**, 161-168.

Singh, H and Creamer, L. K. (1992).
Heat Stability of Milk. In: Fox, P. F. *Advanced Dairy Chemistry - Volwne 1.*
Elsevier Science Publishers Ltd., London. pp. 621-656.

Smythe, D. G., Blumenfeld, O.O. and Konigsberg, W. (1964).
Reactions of N-ethylmaleimide with Peptides and Amino Acids.
Biochemistry **91**, 589-595.

Subramaniam, V., Steele, D. G. and Gafni, A. (1996).
In Vitro Renaturation of Bovine β-Lactoglobulin A Leads to a Biologically Active But Incompletely Refolded State.
Protein Science **5**, 2089-2094.

Swaisgood, H. E. (1992).
Chemistry of the Caseins. In: *Advanced Dairy Chemistry - Volume 1.*
Elsevier Science Publishers Ltd., London. pp. 63-110.

Swergold, G. D. and Rubin, C. S. (1983).
High Performance Gel Permeation Chromatography of Polypeptides in a Volatile Solvent: Rapid Resolution and Molecular Weight Estimation of Proteins and Peptides on a Column of TSK G3000 PW.
Analytical Biochemistry **131**, 295-300.

Tanford, C., Bunville, L. G. and Nozaki, Y. (1959).
The Reversible Transformation of β-Lactoglobulin at pH 7.5.
Journal of the American Chemical Society **81**, 4032-4035.

Thresher, W. C., Knighton, D. R., Otter, D. E. and Hill, J. P. (1994).
Subunit Interactions of β-lactoglobulins: Effect of Ligand Binding and pH.
Brief Communication of the XXIV International Dairy Congress, Melbourne 18-22 September 1994. Australian National Committee of the International Dairy Federation, 1601 Malvern Road, Glen I;Is, Victoria 3146, Australia. p 101.

Thresher, W. C. and Hill, J. P. (1997).
Thermodynamic Characterization of β-lactoglobulin A, B and C Subunit Interactions Using Analytical Affinity Chromatography. In: *Milk Protein Polymorphism.*
International Dairy Federation, Brussels, Belgium. (In Press)

Torchinski, Y. M. (1981).
In: *Sulfur in Proteins.*
Pergamon Press Ltd., Oxford. 294 pp

Trevala, H. L., Antila, V., Syvajarvi, J. and Lindstrom, U. B. (1983).
Variations of the Renneting Properties of Milk.
Meijeritieteellinen Aikakauskirja **41**, 24-33.

van den Berg, G., Escher, J. T. M., de Koning, P. J. and Bovenhuis, H. (1992).
Genetic Polymorphism of κ-casein and β-lactoglobulin in Relation to Milk Composition and Processing.
Netherlands Milk Dairy Joumal **46**, 145-168.

van Willige, R. W. G. and FitzGerald, R. J. (1995).
Tryptic and Chymotryptic Hydrolysis of β-lactoglobulin A, B and AB at Ambient and High Pressure.
Milchwissenschaft **50**, 183-186.

Whitney, R. M (1988).
Proteins of Milk In: *Fundamentals of Dairy Chemistry*.
van Nostrand Reinhold Company Inc., New York. pp 81-170

Appendix A

SDS-PAGE gel preparation

Milli-Q water, SDS-PAGE resolving gel buffer (1.5 M Tris-HCl) and acrylamide-Bis solution, in the volumes shown in Table 1 A, were degassed under vacuum for 15 minutes. Following degassing, SDS solution, N,N,N′,N′-Tetramethylethylenediamine (TEMED) and APS solution, in the volumes shown in Table 1 B, were added and 3.3 mL of the resulting solution was pipetted into a sandwich clamp assembly (prepared as described in Mini PROTEAN II Dual Slab Cell Instruction Manual - Bio-Rad). Milli-Q water (2 x 200 µL) was pipetted on to the top of the gel solution.

Milli-Q water, SDS-PAGE stacking gel buffer (0.5 M Tris-HCl) and acrylamide-Bis solution, in the volumes shown in Table 2 A, were degassed as described above. Following degassing, SDS solution, TEMED and APS solution, in the volumes shown in Table 2 B, were added. The water was removed from the top of the resolving gel, and stacking gel solution was added until the gel sandwich overflowed. A comb was slotted into the top of the assembly and the gel left overnight to polymerise.

REAGENTS	2 GELS	4 GELS
A		
Milli-Q water	2.00 mL	3.00 mL
1.5 M Tris-HCl buffer	2.50 mL	3.75 mL
Acrylamide/Bis (30%T)	5.30 mL	7.95 mL
B		
10% SDS stock	100 µL	150 µL
TEMED	5 µL	7.5 µL
APS (10%)	50 µL	75.0 µ L

Table 1 Volumes of reagents used for SDS-PAGE resolving gels.

REAGENTS	2 GELS	4 GELS
A		
Milli-Q water	3.05 mL	6.10 mL
0.5 M Tris-HCl buffer	1.25 mL	2.50 mL1.3
Acrylamide/Bis (30%T)	0.65 mL	mL
B		
10% SDS stock	50 µL	100 µL
TEMED	5 µL	10 µL
APS (10%)	25 µL	50 µL

Table 2 Volumes of reagents used for SDS-PAGE stacking gels.

Appendix B

Native gel preparation

Milli-Q water, native-PAGE resolving gel buffer and acrylamide-Bis solution, in the volumes shown in Table 3 A, were degassed as previously described. Following degassing, TEMED and APS solution, in the volumes shown in Table 3 B, were added and 3.3 mL of the resulting solution was pipetted into a sandwich clamp assembly. Milli-Q water (2 x 200 μL) was pipetted on to the top of the gel solution.

Milli-Q water, native-PAGE stacking gel buffer and acrylamide-Bis solution, in the volumes shown in Table 4 A, were degassed as previously described. Following degassing, TEMED and APS solution, in the volumes shown in Table 4 B, were added. The water was removed from the top of the resolving gel, and stacking gel solution was added until the gel sandwich overflowed. A comb was slotted into the top of the assembly and the gel left overnight to polymerise.

REAGENTS	2 GELS	4 GELS
A		
Milli-Q Water	3.75 mL	6.00 mL
Resolving Gel Buffer	1.25 mL	2.00 mL
Acrylamide/Bis (30% T)	5.00 mL	8.00 mL
B		
TEMED	5 μL	8 μL
APS	50 μL	80 μL

Table 3 Volumes of reagents used for native-PAGE resolving gels.

REAGENTS	2 GELS	4 GELS
A		
Milli-Q Water	3.15 mL	5.00 mL
Stacking Gel Buffer	1.25 mL	2.00 mL
Acrylamide/Bis (30% T)	625 μL	1.00 mL
B		
TEMED	5 μL	8 μL
APS 10%	25 μL	40 μL

Table 4 Volumes of reagents used for native-PAGE stacking gels.

Appendix C Data used in Section 2

Table 5 ImageQuant volumes determined by laser densitometry analysis of β-LG standards following SDS-PAGE. (i) β-LG A, (ii) β-LG β, (iii) β-LG C. Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

(i) SDS5.0A2

lane	conc.	vol.
11	0.064	75.08
12	0.162	204.4
13	0.257	326.9

Regression Output:

Constant	-7.95335
Std Err of Y Est	1.1862
R Squared	0.99996
No. of Observations	3
Degrees of Freedom	1
X Coefficient(s)	1304.84
Std Err of Coef.	8.69156

(ii) SDS5.0B2

lane	conc.	vol.
11	0.0598	80.88
12	0.15	219.6
13	0.241	334.9

Regression Output:

Constant	1.16799
Std Err of Y Est	10.019
R Squared	0.9969
No. of Observations	3
Degrees of Freedom	1
X Coefficient(s)	1401.68
Std Err of Coef.	78.1951

(iii) SDS5.0C2

lane	conc.	vol.
11	0.1616	137.7
12	0.2416	262.9
13	0.404	488.6

Regression Output:

Constant	-90.828
Std Err of Y Est	7.52451
R Squared	0.9991
No. of Observations	3
Degrees of Freedom	1
X Coefficient(s)	1439.15
Std Err of Coef.	43.0778

Table 6 ImageQuant volumes of β-LG samples analysed by SDS-PAGE following heating (50-90°C) for 10 minutes at pH 5.0. (i) β-LG A, (ii) β-LG β, (iii) β-LG C.

Concentrations were determined using the following formula:

$$x = \frac{(\text{band volume} - \text{constant})}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 5.

(i) Variant A Gel: SDS5.0A2 formula: x=y+7.953/1304.843

Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	conc. (mg/g)	%
1	unheated	351.9	0.27578	0.6183	0.0689	2.47484	100
2	50	322.9	0.25356	0.6154	0.0685	2.27795	92.0443
3	60	349	0.27356	0.621	0.0682	2.49092	100.65
4	65	352	0.27586	0.6188	0.0684	2.49564	100.84
5	70	383.1	0.29969	0.6176	0.0682	2.71394	109.661
6	75	375.4	0.29379	0.6196	0.068	2.67697	108.167
7	80	235.5	0.18658	0.6407	0.0883	1.35379	54.7021
8	85	32.8	0.03123	0.6326	0.0862	0.2292	9.26139

(ii) Variant B Gel: SDS5.0B2 formula: x=y-1.168/1401.677

Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	conc. (mg/g)	%
1	unheated	513.9	0.36591	0.6178	0.0687	3.2905	100
2	50	496.6	0.35356	0.6166	0.0684	3.18725	96.8622
3	60	512.2	0.36469	0.6171	0.0681	3.30474	100.433
4	65	457.2	0.32546	0.6156	0.0679	2.95067	89.6723
5	70	441.1	0.31397	0.626	0.0681	2.88612	87.7107
6	75	513.9	0.36591	0.621	0.0685	3.3172	100.811
7	80	461.1	0.32824	0.6134	0.0677	2.97402	90.382
8	85	63.11	0.0443	0.6346	0.0878	0.32019	9.73061

(iii) Variant C Gel: SDS5.0C2 formula: x=y+90.828/1439.153

Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	conc. (mg/g)	%
1	unheated	261.7	0.24496	0.5863	0.0477	3.01084	100
2	50	290.1	0.26469	0.5953	0.0481	3.27587	108.802
3	60	297	0.26948	0.5886	0.0489	3.24372	107.735
4	65	252.6	0.23863	0.608	0.0482	3.01013	99.9763
5	70	269.7	0.25051	0.5876	0.0479	3.07311	102.068
6	75	299.5	0.27122	0.5861	0.048	3.31172	109.993
7	80	274.5	0.25385	0.5973	0.0479	3.16543	105.134
8	85	185.3	0.19187	0.6325	0.0871	1.3933	46.2762
9	90	18.28	0.07581	0.7475	0.1965	0.2884	9.57878

Table 7 ImageQuant volumes determined by laser densitometry analysis of β-LG A standards following SDS-PAGE. Samples were run on four separate gels (SDS6.0A1, SDS6.0A2, SDS6.0A3 and SDS6.0A4). Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

gel	lane	conc	vol	Regression Output:	
SDS6.0A1	11	0.064	62.27	Constant	-16.2702
	12	0.162	190	Std Err of Y Est	4.07111
	13	0.257	304	R Squared	0.99943
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1252.75
				Std Err of Coef.	29.83
SDS6.0A2	11	0.064	120.6	Constant	13.1975
	12	0.162	309.7	Std Err of Y Est	13.437
	13	0.257	460.6	R Squared	0.99689
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1762.54
				Std Err of Coef.	98.4558
SDS6.0A3	11	0.064	63.53	Constant	-17.9026
	12	0.162	187	Std Err of Y Est	0.66734
	13	0.257	308.3	R Squared	0.99999
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1268.19
				Std Err of Coef.	4.88978
SDS6.0A4	11	0.064	66.63	Constant	-12.5747
	12	0.162	183.2	Std Err of Y Est	2.56975
	13	0.257	302.4	R Squared	0.99976
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1221.44
				Std Err of Coef.	18.8291

Table 8 ImageQuant volumes of β-LG A samples analysed by SDS-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) Results from gel SDS6.0A1. (ii) Results from gel SDS6.0A2. (iii) Results from gels SDS6.0A3 and SDS6.0A4. (iv) Averages of concentration values obtained in (i-iii).

Concentrations were determined using the following formula:

$$x = \frac{\text{band volume - constant}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 7.

(i)

Gel: SDS6.0A1			formula: x=y+16.2702/1252.755		stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	202.8	0.17487	0.6219	0.0732	1.48568
2	60	206.8	0.17806	0.6192	0.0737	1.49603
3	70	201.9	0.17415	0.6221	0.0744	1.45618
5	75	201.2	0.17359	0.6282	0.0737	1.47967
6	80	196.8	0.17008	0.6214	0.0737	1.43404
7	85	156	0.13751	0.6218	0.0739	1.15705
4	90	109.1	0.10008	0.6219	0.0738	0.84332
8	95	108.7	0.09976	0.6496	0.0986	0.65722
9	100	81.68	0.07819	0.6446	0.0984	0.51219
10	110	68.01	0.06728	0.6433	0.0973	0.4448

(ii)

Gel:SDS6.0A2			formula: x=y-13.197/1762.542		stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	320.9	0.17458	0.6219	0.0732	1.4832
2	60	325.2	0.17702	0.6192	0.0737	1.48724
3	70	328.1	0.17866	0.6221	0.0744	1.49391
5	75	325	0.17691	0.6282	0.0737	1.50789
6	80	316	0.1718	0.6214	0.0737	1.44852
7	85	270	0.1457	0.6218	0.0739	1.22593
4	90	190	0.10031	0.6219	0.0738	0.8453
8	95	203.2	0.1078	0.6496	0.0986	0.71021
9	100	152.1	0.07881	0.6446	0.0984	0.51626
10	110	129.9	0.06621	0.6433	0.0973	0.43777

(iii)

gel: SDS6.0A3/A4			formula-A3: x=y+17.903/1268.194 formula-A4: x=y+12.575/1221.437			stain: imido black	
Lane	Temp (°C)	Gel	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	A3	198.1	0.17032	0.6219	0.0732	1.44705
2	60	A3	207.3	0.17758	0.6192	0.0737	1.49194
3	70	A3	217.7	0.18578	0.6221	0.0744	1.55339
5	75	A3	211.8	0.18113	0.6282	0.0737	1.54387
6	80	A4	193.2	0.16847	0.6214	0.0737	1.42045
7	85	A4	161.9	0.14284	0.6218	0.0739	1.2019
4	90	A3	114.1	0.10409	0.6219	0.0738	0.87712
8	95	A4	108.3	0.09896	0.6496	0.0986	0.65198
9	100	A4	89.82	0.08383	0.6446	0.0984	0.54916
10	110	A4	67.49	0.06555	0.6433	0.0973	0.43338

(iv)

Lane	Temp. (°C)	Conc (mg/g)
1	20	1.47198
2	60	1.49174
3	70	1.50116
5	75	1.51048
6	80	1.43433
7	85	1.19496
4	90	0.85525
8	95	0.67314
9	100	0.52587
10	110	0.43865

Table 9 ImageQuant volumes determined by laser densitometry analysis of β-LG B standards following SDS-PAGE. Samples were run on four separate gels (SDS6.0B1, SDS6.0B2, SDS6.0B3 and SDS6.0B4). Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

gel	lane	conc.	vol	Regression Output:	
SDS6.0B1	11	0.0598	73.55	Constant	-15.281
	12	0.15	200	Std Err of Y Est	4.08619
	13	0.241	337.625	R Squared	0.99952
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1457.45
				Std Err of Coef.	31.8914
SDS6.0B2	11	0.0598	119.4	Constant	-10.2612
	12	0.15	321.4	Std Err of Y Est	3.48184
	13	0.241	516.625	R Squared	0.99985
				No. of Observations	3
	lane	conc.	vol	Degrees of Freedom	1
				X Coefficient(s)	2192.12
				Std Err of Coef.	27.1746
SDS6.0B3	11	0.0598	62.52	Constant	-23.8194
	12	0.15	185.7	Std Err of Y Est	3.82159
	13	0.241	319.375	R Squared	0.99956
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1417.6
				Std Err of Coef.	29.8263
SDS6.0B4	11	0.0598	64.89	Constant	-24.0139
	12	0.15	192.1	Std Err of Y Est	3.73391
	13	0.241	329.625	R Squared	0.9996
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1461.08
				Std Err of Coef.	29.142

Table 10 ImageQuant volumes of β-LG B samples analysed by SDS-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) Results from gel SDS6.0B1. (ii) Results from gel SDS6.0B2. (iii) Results from gels SDS6.0B3 and SDS6.0B4. (iv) Averages of concentration values obtained in (i-iii).

Concentrations were determined using the following formula:

$$x = \frac{\text{band volume - constant}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 9.

(i)

Gel: SDS6.0B1			formula: x=y+15.281/1457.449		stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	205.2	0.15128	0.6197	0.0734	1.27721
2	60	203.6	0.15018	0.6369	0.0736	1.2996
3	70	207.4	0.15279	0.6223	0.0733	1.29714
5	75	215	0.158	0.6231	0.0735	1.33948
6	80	197.7	0.14613	0.6252	0.0737	1.23965
7	85	144.1	0.10936	0.6382	0.0732	0.95343
4	90	93.08	0.07435	0.6384	0.0736	0.6449
8	95	97.48	0.07737	0.651	0.0986	0.51082
9	100	78.17	0.06412	0.6504	0.0987	0.42253
10	110	82.13	0.06684	0.6498	0.0978	0.44407

(ii)

Gel: SDS6.0B2			formula: x=y+10.2612/2192.122		stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	319.9	0.15061	0.6197	0.0734	1.27159
2	60	326	0.1534	0.6369	0.0736	1.32741
3	70	322.7	0.15189	0.6223	0.0733	1.28951
5	75	329.7	0.15508	0.6231	0.0735	1.31473
6	80	315.5	0.14861	0.6252	0.0737	1.26063
7	85	231.8	0.11042	0.6382	0.0732	0.96273
4	90	154.3	0.07507	0.6384	0.0736	0.65115
8	95	160.2	0.07776	0.651	0.0986	0.51341
9	100	129.8	0.06389	0.6504	0.0987	0.42103
10	110	138.9	0.06804	0.6498	0.0978	0.4521

(iii)

Gel: SDS6.0B3/B4			formula- C3: x=y+23.819/1417.598 formula- C4: x=y+24.0139/1461.084			stain: imido black	
Lane	Temp (°C)	Gel	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	B3	235	0.18258	0.6197	0.0734	1.54145
2	60	B3	196.5	0.15542	0.6369	0.0736	1.34491
3	70	B3	199.2	0.15732	0.6223	0.0733	1.33563
5	75	B3	200	0.15789	0.6231	0.0735	1.33849
6	80	B4	181.3	0.14052	0.6252	0.0737	1.19205
7	85	B4	128.5	0.10438	0.6382	0.0732	0.91008
4	90	B3	83.96	0.07603	0.6384	0.0736	0.65947
8	95	B4	80.77	0.07172	0.651	0.0986	0.4735
9	100	B4	69	0.06366	0.6504	0.0987	0.4195
10	110	B4	73.8	0.06695	0.6498	0.0978	0.4448

(iv)

Lane	Temp. (°C)	Conc (mg/g)
1	20	1.2744
2	60	1.32397
3	70	1.30742
5	75	1.3309
6	80	1.23078
7	85	0.94208
4	90	0.65184
8	95	0.49925
9	100	0.42102
10	110	0.44699

Table 11 ImageQuant volumes determined by laser densitometry analysis of β-LG C standards following SDS-PAGE. Samples were run on four separate gels (SDS6.0C1, SDS6.0C2, SDS6.0C3 and SDS6.0C4). Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

gel	lane	conc.	vol.	Regression Output:	
SDS6.0C1	11	0.0476	55.55	Constant	-9.96833
	12	0.0909	120	Std Err of Y Est	2.58639
	13	0.2	270.9	R Squared	0.99973
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1407.25
				Std Err of Coef.	23.2881
SDS6.0C2	11	0.0476	112.2	Constant	9.17728
	12	0.0909	217	Std Err of Y Est	5.91039
	13	0.2	454.8	R Squared	0.99943
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	2234.77
				Std Err of Coef.	53.2177
SDS6.0C3	11	0.0476	56.92	Constant	-10.3882
	12	0.0909	119	Std Err of Y Est	0.45439
	13	0.2	273.4	R Squared	0.99999
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1419.45
				Std Err of Coef.	4.09138
SDS6.0C4	11	0.0476	67.83	Constant	1.68754
	12	0.0909	113.4	Std Err of Y Est	7.7839
	13	0.2	262.8	R Squared	0.99709
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1296.8
				Std Err of Coef.	70.0869

Table 12 ImageQuant volumes of β-LG C samples analysed by SDS-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) Results from gel SDS6.0C1. (ii) Results from gel SDS6.0C2. (iii) Results from gels SDS6.0C3 and SDS6.0C4. (iv) Averages of concentration values obtained in (i-iii).

Concentrations were determined using the following formula:

$$x = \frac{\text{(band volume - constant)}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 11.

(i) Gel: SDS6.0C1 formula: x=y+9.96833/1407.253 stain: imido black

Lane	Temp. (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	224.5	0.16661	0.6227	0.0736	1.40966
2	60	238.3	0.17642	0.6316	0.0744	1.49768
3	70	241.8	0.17891	0.6301	0.0732	1.54002
5	75	223.9	0.16619	0.642	0.074	1.44179
6	80	228.4	0.16939	0.6244	0.0731	1.44684
7	85	163.4	0.1232	0.6254	0.073	1.05544
4	90	106.1	0.08248	0.6278	0.0738	0.70163
8	95	81.68	0.06513	0.6575	0.0981	0.43649
9	100	68.35	0.05565	0.6473	0.0984	0.3661
10	110	62.88	0.05177	0.6514	0.0978	0.34479

(ii) Gel: SDS6.0C2 formula: x=y-9.177/2234.766 stain: comassie blue

Lane	Temp. (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	381.7	0.16669	0.6227	0.0736	1.41033
2	60	396.8	0.17345	0.6316	0.0744	1.47247
3	70	406.9	0.17797	0.6301	0.0732	1.53196
5	75	388.8	0.16987	0.642	0.074	1.47375
6	80	398.3	0.17412	0.6244	0.0731	1.48731
7	85	287.3	0.12445	0.6254	0.073	1.0662
4	90	190.7	0.08123	0.6278	0.0738	0.69098
8	95	157.2	0.06624	0.6575	0.0981	0.44394
9	100	126.2	0.05236	0.6473	0.0984	0.34447
10	110	129.9	0.05402	0.6514	0.0978	0.3598

(iii) Gel: SDS6.0C3/C4 formula - C3: x=y+10.3882/1419.453 formula - C4: x=y-1.6875/1296.802 stain: imido black

Lane	Temp. (°C)	Gel	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	C3	218.5	0.16125	0.6227	0.0736	1.36428
2	60	C3	231.2	0.1702	0.6316	0.0744	1.44485
3	70	C3	235.2	0.17302	0.6301	0.0732	1.48931
5	75	C3	220.4	0.16259	0.642	0.074	1.41057
6	80	C4	210.3	0.16087	0.6244	0.0731	1.37408
7	85	C4	154.9	0.11815	0.6254	0.073	1.01217
4	90	C3	102.75	0.07971	0.6278	0.0738	0.67804
8	95	C4	78.75	0.05943	0.6575	0.0981	0.39829
9	100	C4	67.25	0.05056	0.6473	0.0984	0.33258
10	110	C4	75.61	0.057	0.6514	0.0978	0.37967

Average:

Lane	Temp. (°C)	Conc (mg/g)
1	20	1.39476
2	60	1.47167
3	70	1.52043
5	75	1.44204
6	80	1.43608
7	85	1.0446
4	90	0.69021
8	95	0.42624
9	100	0.34772
10	110	0.36142

Table 13 ImageQuant volumes determined by laser densitometry analysis of β-LG A standards following native-PAGE. Samples were run on three separate gels (Nat6.0A1, Nat6.0A2 and Nat6.0A3). Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

gel	lane	conc	vol	Regression Output:		
Nat6.0A1	11	0.051	68.49	Constant		-3.88018
	12	0.211	325.6	Std Err of Y Est		10.9878
	13	0.424	636.4	R Squared		0.99925
				No. of Observations		3
				Degrees of Freedom		1
				X Coefficient(s)	1519.14	
				Std Err of Coef.	41.5204	
Nat6.0A2	11	0.051	61.78	Constant		-9.52369
	12	0.211	308.7	Std Err of Y Est		8.48661
	13	0.424	613.1	R Squared		0.99953
				No. of Observations		3
				Degrees of Freedom		1
				X Coefficient(s)	1475.44	
				Std Err of Coef.	32.0689	
Nat6.0A3	11	0.051	128.8	Constant		-2.47234
	12	0.211	552.4	Std Err of Y Est		4.29964
	13	0.424	1104	R Squared		0.99996
				No. of Observations		3
				Degrees of Freedom		1
				X Coefficient(s)	2613.14	
				Std Err of Coef.	16.2473	

Table 14 ImageQuant volumes of β-LG A samples analysed by native-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) Results from gel Nat6.0A1. (ii) Results from gel Nat6.0A2. (iii) Results from gel Nat6.0A3. (iv) Averages of concentration values obtained in (i-iii). Concentrations were determined using the following formula:

$$x = \frac{\text{(band volume - constant)}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 13.

(i)

Gel: Nat6.0A1		formula: x=y+3.880/1519.141			stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	371.6	0.24717	0.7041	0.1483	1.1735
2	60	391.9	0.26053	0.6981	0.15	1.2125
3	70	422	0.28034	0.6929	0.1494	1.3002
4	75	392.4	0.26086	0.6922	0.1489	1.21267
5	80	349.9	0.23288	0.6938	0.1487	1.08657
6	85	254.7	0.17021	0.6983	0.15	0.79241
7	90	115.4	0.07852	0.6952	0.1486	0.36733
8	95	74.9	0.05186	0.7468	0.1994	0.19422
9	100	47.97	0.03413	0.7549	0.1996	0.12909
10	110	32.75	0.02411	0.7594	0.2048	0.08941

(ii)

Gel: Nat6.0A2		formula: x=y+9.524/1475.439			stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	378.3	0.26285	0.7041	0.1483	1.24798
2	60	385.6	0.2678	0.6981	0.15	1.24635
3	70	383.5	0.26638	0.6929	0.1494	1.23543
4	75	385.7	0.26787	0.6922	0.1489	1.24526
5	80	354.3	0.24659	0.6938	0.1487	1.15052
6	85	248.8	0.17508	0.6983	0.15	0.81507
7	90	121.4	0.08874	0.6952	0.1486	0.41513
8	95	75.72	0.05778	0.7468	0.1994	0.21638
9	100	45.54	0.03732	0.7549	0.1996	0.14115
10	110	28.44	0.02573	0.7594	0.2048	0.09541

(iii)

gel: Nat6.0A3		formula: x=y+2.472/2613.144			stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	655.5	0.25179	0.7041	0.1483	1.19547
2	60	673.2	0.25857	0.6981	0.15	1.20337
3	70	673.7	0.25876	0.6929	0.1494	1.20009
4	75	652.4	0.25061	0.6922	0.1489	1.16501
5	80	649.8	0.24961	0.6938	0.1487	1.16463
6	85	474.9	0.18268	0.6983	0.15	0.85044
7	90	249.8	0.09654	0.6952	0.1486	0.45164
8	95	164.9	0.06405	0.7468	0.1994	0.23988
9	100	99.72	0.03911	0.7549	0.1996	0.1479
10	110	67.26	0.02669	0.7594	0.2048	0.09895

(iv)

Lane	Temp. (°C)	Conc (mg/g)
1	20	1.20565
2	60	1.22074
3	70	1.24524
4	75	1.20764
5	80	1.13391
6	85	0.81931
7	90	0.41137
8	95	0.21683
9	100	0.13938
10	110	0.09459

Table 15 ImageQuant volumes determined by laser densitometry analysis of β-LG B standards following native-PAGE. Samples were run on three separate gels (Nat6.0B1, Nat6.0B2 and Nat6.0B3). Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

gel	lane	conc.	vol	Regression Output:	
Nat6.0B1	11	0.0702	113.7	Constant	-13.6355
	12	0.268	470.9	Std Err of Y Est	0.66871
	13	0.466	830.1	R Squared	1
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1810.01
				Std Err of Coef.	2.38934
Nat6.0B2	11	0.0702	92.37	Constant	-43.9142
	12	0.268	430.2	Std Err of Y Est	19.4344
	13	0.466	816	R Squared	0.99856
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1828.29
				Std Err of Coef.	69.4401
Nat6.0B3	11	0.0702	200.2	Constant	-21.1449
	12	0.268	761.8	Std Err of Y Est	26.1278
	13	0.466	1388	R Squared	0.99903
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	3001.04
				Std Err of Coef.	93.356

Table 16 ImageQuant volumes of β-LG B samples analysed by native-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) Results from gel Nat6.0B1. (ii) Results from gel Nat6.0B2. (iii) Results from gel Nat6.0B3. (iv) Averages of concentration values obtained in (i-iii). Concentrations were determined using the following formula:

$$x = \frac{\text{(band volume - constant)}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 15.

(i)

Gel: Nat6.0B1		formula: x=y+13.636/1810.006			stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	344.8	0.19803	0.7108	0.149	0.9447
2	60	369.8	0.21184	0.6999	0.1482	1.00046
3	70	360.8	0.20687	0.6996	0.1488	0.97262
4	75	374.8	0.2146	0.6993	0.1488	1.00855
5	80	295.2	0.17063	0.7015	0.1492	0.80224
6	85	152.6	0.09184	0.7045	0.1485	0.43571
7	90	64.52	0.04318	0.7058	0.1482	0.20564
8	95	60.59	0.04101	0.7736	0.2194	0.14459
9	100	37.03	0.02799	0.7554	0.1992	0.10615
10	110	31.74	0.02507	0.7503	0.1988	0.09461

(ii)

Gel: Nat6.0B2		formula: x=y+43.914/1828.292			stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	333.5	0.20643	0.7108	0.149	0.98477
2	60	354.9	0.21813	0.6999	0.1482	1.03018
3	70	362.8	0.22246	0.6996	0.1488	1.0459
4	75	347	0.21381	0.6993	0.1488	1.00484
5	80	330.2	0.20462	0.7015	0.1492	0.96209
6	85	158.4	0.11066	0.7045	0.1485	0.52497
7	90	69.23	0.06189	0.7058	0.1482	0.29473
8	95	55.58	0.05442	0.7736	0.2194	0.19188
9	100	30.56	0.04073	0.7554	0.1992	0.15447
10	110	24.26	0.03729	0.7503	0.1988	0.14073

(iii)

Gel: Nat6.0B3		formula: x=y+21.145/3001.038			stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	703.1	0.24133	0.7108	0.149	1.15126
2	60	714.6	0.24516	0.6999	0.1482	1.15783
3	70	691.8	0.23757	0.6996	0.1488	1.11694
4	75	677.4	0.23277	0.6993	0.1488	1.09391
5	80	603.5	0.20814	0.7015	0.1492	0.97863
6	85	347.9	0.12297	0.7045	0.1485	0.58339
7	90	153.9	0.05833	0.7058	0.1482	0.27779
8	95	149.9	0.057	0.7736	0.2194	0.20096
9	100	84.5	0.0352	0.7554	0.1992	0.13349
10	110	66.46	0.02919	0.7503	0.1988	0.11017

(iv)

Lane	Temp. (°C)	Conc (mg/g)
1	20	1.02691
2	60	1.06282
3	70	1.04516
4	75	1.03577
5	80	0.91432
6	85	0.51469
7	90	0.25939
8	95	0.17915
9	100	0.13137
10	110	0.11517

Table 17 ImageQuant volumes determined by laser densitometry analysis of β-LG C standards following native-PAGE. Samples were run on three separate gels (Nat6.0C1, Nat6.0C2 and Nat6.0C3). Linear regression was performed as described in Section 2.1.1.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

gel	lane	conc.	vol.	Regression Output:	
Nat6.0C1	11	0.09	150.1	Constant	1.04828
	12	0.287	484.9	Std Err of Y Est	3.61359
	13	0.583	976.8	R Squared	0.99996
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1675.68
				Std Err of Coef.	10.2969
Nat6.0C2	11	0.09	156	Constant	-22.9774
	12	0.287	499.3	Std Err of Y Est	20.5003
	13	0.583	1078.38	R Squared	0.99903
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	1877.72
				Std Err of Coef.	58.4156
Nat6.0C3	11	0.09	272.9	Constant	-31.7134
	12	0.287	905	Std Err of Y Est	14.664
	13	0.583	1900	R Squared	0.99984
				No. of Observations	3
				Degrees of Freedom	1
				X Coefficient(s)	3305.25
				Std Err of Coef.	41.785

Table 18 ImageQuant volumes of β-LG C samples analysed by native-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) Results from gel Nat6.0C1. (ii) Results from gel Nat6.0C2. (iii) Results from gel Nat6.0C3. (iv) Averages of concentration values obtained in (i-iii). Concentrations were determined using the following formula:

$$x = \frac{\text{band volume - constant}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 17.

(i)

Gel: Nat6.0C1		formula: x=y-1.048/1675.682			stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	396.2	0.23582	0.7063	0.1493	1.11558
2	60	401.4	0.23892	0.7013	0.149	1.12452
3	70	396	0.2357	0.7045	0.1482	1.12043
4	75	396.9	0.23623	0.7046	0.1488	1.11862
5	80	381.3	0.22692	0.7083	0.1499	1.07225
6	85	256	0.15215	0.7062	0.1483	0.72453
7	90	92.35	0.05449	0.7052	0.1497	0.25667
8	95	68.84	0.04046	0.7494	0.1989	0.15243
9	100	41.16	0.02394	0.7521	0.1991	0.09042
10	110	36.28	0.02103	0.7524	0.1985	0.0797

(ii)

Gel: Nat6.0C2		formula: x=y+22.977/1877.716			stain: imido black	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	415.875	0.23372	0.7063	0.1493	1.10565
2	60	425.25	0.23871	0.7013	0.149	1.12353
3	70	405.125	0.22799	0.7045	0.1482	1.0838
4	75	423.25	0.23764	0.7046	0.1488	1.12529
5	80	395	0.2226	0.7083	0.1499	1.05181
6	85	258.8	0.15006	0.7062	0.1483	0.7146
7	90	94.53	0.06258	0.7052	0.1497	0.2948
8	95	72.81	0.05101	0.7494	0.1989	0.1922
9	100	40.45	0.03378	0.7521	0.1991	0.1276
10	110	40.19	0.03364	0.7524	0.1985	0.12751

(iii)

Gel: Nat6.0C3		formula: x=y+31.713/3305.25			stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. mg/g
1	unheated	683.75	0.21646	0.7063	0.1493	1.02403
2	60	686.25	0.21722	0.7013	0.149	1.02239
3	70	748.625	0.23609	0.7045	0.1482	1.12231
4	75	735	0.23197	0.7046	0.1488	1.09842
5	80	715.75	0.22614	0.7083	0.1499	1.06857
6	85	496.5	0.15981	0.7062	0.1483	0.76101
7	90	198.1	0.06953	0.7052	0.1497	0.32754
8	95	140.8	0.05219	0.7494	0.1989	0.19665
9	100	78.56	0.03336	0.7521	0.1991	0.12603
10	110	69.14	0.03051	0.7524	0.1985	0.11566

(iv)

Lane	Temp. (°C)	Conc (mg/g)
1	20	1.08175
2	60	1.09015
3	70	1.10885
4	75	1.11411
5	80	1.06421
6	85	0.73338
7	90	0.293
8	95	0.18043
9	100	0.11468
10	110	0.10762

Gel	Temp (°C)	Top Band						Bottom Band					
		Vol (IQ)	x	total wt(g)	sample wt(g)	mg/g	% total	Vol (IQ)	x	total wt(g)	sample wt(g)	mg/g	% total
SDS6.0A1	90	64.16	0.064202657	0.6219	0.0738	0.541024832	66.99702791	23.35	0.031626455	0.6219	0.0738	0.266510739	33.00297209
	95	74.28	0.072280853	0.6496	0.0986	0.476203267	62.19944443	38.76	0.043927344	0.6496	0.0986	0.289403679	37.80055557
	100	51.36	0.053985177	0.6446	0.0984	0.353646798	59.08095018	30.57	0.037389753	0.6446	0.0984	0.24493328	40.91904982
	110	35.13	0.04102973	0.6433	0.0973	0.271268506	55.71209316	24.59	0.032616274	0.6433	0.0973	0.215642846	44.28790684
SDS6.0A2	90	116.7	0.05872344	0.6219	0.0738	0.494852406	66.6833038	64.91	0.029339743	0.6219	0.0738	0.247241008	33.3166962
	95	118.9	0.059971638	0.6496	0.0986	0.395107259	59.16901928	86.14	0.041384846	0.6496	0.0986	0.272653106	40.83098072
	100	94.86	0.046332246	0.6446	0.0984	0.303513882	63.09150705	60.97	0.027104336	0.6446	0.0984	0.177555435	36.90849295
	110	76.14	0.035711223	0.6433	0.0973	0.236105135	60.13135316	54.93	0.023677467	0.6433	0.0973	0.156543828	39.86864684
SDS6.0A3/4	90	66.37	0.066450874	0.6219	0.0738	0.559970172	59.23627141	40.09	0.045728493	0.6219	0.0738	0.3853462	40.76372859
	95	70.5	0.068013905	0.6496	0.0986	0.448091609	59.44547884	44.1	0.046400019	0.6496	0.0986	0.305694243	40.55452116
	100	45.55	0.047587145	0.6446	0.0984	0.31173449	52.89381869	39.19	0.042380164	0.6446	0.0984	0.277624528	47.10618131
	110	37.21	0.040759122	0.6433	0.0973	0.269479377	53.79838209	30.18	0.035003606	0.6433	0.0973	0.231426722	46.20161791
SD6.0SB1	90	28.34	0.029929692	0.6384	0.0736	0.259607548	35.38597573	64.37	0.054650969	0.6384	0.0736	0.474037752	64.61402427
	95	37.06	0.035912749	0.651	0.0986	0.237111557	40.69049692	61.01	0.052345571	0.651	0.0986	0.345608181	59.30950308
	100	31.9	0.032372316	0.6504	0.0987	0.213322741	43.72173623	45.45	0.041669383	0.6504	0.0987	0.274587299	56.27826377
	110	33.7	0.033607351	0.6498	0.0978	0.223293013	44.65321081	45.43	0.04165566	0.6498	0.0978	0.276767361	55.34678919
SDS6.0B2	90	67.62	0.035527767	0.6384	0.0736	0.308164763	42.73964123	94.08	0.047598263	0.6384	0.0736	0.412863196	57.26035877
	95	55.65	0.030067305	0.651	0.0986	0.198517397	35.8851956	107.5	0.053720185	0.651	0.0986	0.354683982	64.1148044
	100	54.22	0.029414969	0.6504	0.0987	0.193834809	43.64138924	73.01	0.037986572	0.6504	0.0987	0.250318808	56.35861076
	110	56.68	0.030537169	0.6498	0.0978	0.202894197	44.08016731	74.66	0.038739267	0.6498	0.0978	0.257390346	55.91983269
SDS6.0B3/4	85	30.97	0.037632265	0.6382	0.0732	0.328099883	29.49814858	107.4	0.089942741	0.6382	0.0732	0.784172915	70.50185142
	90	44.43	0.048144396	0.6384	0.0736	0.417600308	46.26401714	57.69	0.055920057	0.6384	0.0736	0.485045708	53.73598286
	95	32.07	0.03838513	0.651	0.0986	0.253435294	41.00965356	56.66	0.055215101	0.651	0.0986	0.364554062	58.99034644
	100	30.42	0.037255832	0.6504	0.0987	0.245503476	44.20208887	44.7	0.047029397	0.6504	0.0987	0.309908004	55.79791113
	110	35.7	0.040869587	0.6498	0.0978	0.271544558	48.79870358	38.64	0.042881792	0.6498	0.0978	0.284913991	51.20129642
SDS6.0C1	90	35.69	0.032445005	0.6278	0.0738	0.276002356	35.91562148	71.5	0.057891744	0.6278	0.0738	0.492472042	64.08437852
	95	28.42	0.027278911	0.6575	0.0981	0.182832664	37.87823891	52.99	0.044738459	0.6575	0.0981	0.299852564	62.12176109
	100	23.43	0.023732996	0.6473	0.0984	0.15612163	38.23215082	43.99	0.03834302	0.6473	0.0984	0.25223005	61.76784918
	110	23.91	0.024074086	0.6514	0.0978	0.160346214	42.64823068	35.59	0.032373944	0.6514	0.0978	0.215627681	57.35176932
SDS6.0C2	90	53.16	0.01968113	0.6278	0.0738	0.167422945	26.11716106	133.6	0.05567595	0.6278	0.0738	0.473622783	73.88283894
	95	54.62	0.020334442	0.6575	0.0981	0.136288438	30.83675521	111.1	0.045607782	0.6575	0.0981	0.305679067	69.16324479
	100	50.97	0.018701162	0.6473	0.0984	0.123020954	37.92255627	77.59	0.030612923	0.6473	0.0984	0.201379524	62.07744373
	110	47.44	0.017121578	0.6514	0.0978	0.114038812	36.27298706	76.4	0.030080429	0.6514	0.0978	0.200351651	63.72701294
SDS6.0C3/4	85	40.25	0.029736614	0.6254	0.073	0.254757235	25.15902789	116.4	0.088457991	0.6254	0.073	0.757830517	74.84097211
	90	41.33	0.036437651	0.6278	0.0738	0.309966903	40.23151884	66.45	0.054132261	0.6278	0.0738	0.460490967	59.76848116
	95	24.55	0.017629908	0.6575	0.0981	0.118161718	30.9754996	52.63	0.039285745	0.6575	0.0981	0.263306602	69.0245004
	100	27.2	0.019673397	0.6473	0.0984	0.129416561	40.03007322	39.91	0.029473145	0.6473	0.0984	0.193881776	59.96992678
	110	32.19	0.023522609	0.6514	0.0978	0.156673084	43.37085308	41.52	0.030713375	0.6514	0.0978	0.20456741	56.62914692

Table 19 Calculation of the percentage of monomeric protein present in each band of the doublet seen following SDS-PAGE of heated samples of β-LG A, B and C using ImageQuant volumes. Volumes were obtained for each band of the doublet and concentrations calculated using the equations shown in Table 8 (β-LG A), Table 10 (β-LG B) and Table 12 (β-LG C). Percentages were calculated using the following formulae:

$$\frac{\text{mg/g top band}}{\text{mg/g top band + mg/g bottom band}} \times 100 = \% \text{ top band} \quad \text{and} \quad \frac{\text{mg/g bottom band}}{\text{mg/g top band + mg/g bottom band}} \times 100 = \% \text{ top band}$$

			Temp (°C)									
			85		90		95		100		110	
Gel	method		upper	lower	upper	lower	upper	lower	upper	lower	upper	lower
SDS6.0A1	weight 1	weight (g)	0.1663	0.0362	-	-	0.1914	0.0837	0.1653	0.1143	0.1689	0.0956
		%	82.12	17.88	-	-	69.57	30.43	59.12	40.88	63.86	36.14
	area	area (mm)	2294	361	2240	800	3225	1104	2698	1479	2240	1344
		%	86.40	13.60	73.68	26.32	74.50	25.50	64.59	35.41	62.50	37.50
	weight 2	weight (g)	-	-	0.1998	0.0437	0.1641	0.0678	0.1369	0.0655	0.1150	0.0623
		%	-	-	82.05	17.95	70.76	29.24	67.64	32.36	64.86	35.14
SDS6.0A2	weight 1	weight (g)	-	-	0.1772	0.0340	0.1480	0.0831	0.1251	0.0938	0.1001	0.0857
		%	-	-	83.90	16.10	64.04	35.96	57.15	42.85	53.88	46.12
	area	area (mm)	-	-	2880	432	2673	1160	2280	1176	1950	960
		%	-	-	86.96	13.04	69.74	30.26	65.97	34.03	67.01	32.99
	weight 2	weight (g)	-	-	0.2009	0.0529	0.2217	0.0900	0.2153	0.0938	0.2121	0.0766
		%	-	-	79.16	20.84	71.13	28.87	69.65	30.35	73.47	26.53
SDS6.0A3/4	weight 1	weight (g)	-	-	0.1669	0.0846	0.2002	0.1040	0.1899	0.1341	0.1665	0.1236
		%	-	-	66.36	33.64	65.81	34.19	58.61	41.39	57.39	42.61
	area	area (mm)	-	-	3382	945	3600	1280	3510	1610	2765	1426
		%	-	-	78.16	21.84	73.77	26.23	68.55	31.45	65.97	34.03
	weight 2	weight (g)	-	-	0.1724	0.0388	0.1421	0.0612	0.1242	0.0559	0.1215	0.0634
		%	-	-	81.63	18.37	69.90	30.10	68.96	31.04	65.71	34.29
SDS6.0B1	weight 1	weight (g)	0.0781	0.1403	0.0597	0.1677	0.0819	0.1537	0.0978	0.1573	0.1024	0.1767
		%	35.76	64.24	26.25	73.75	34.76	65.24	38.34	61.66	36.69	63.31
	area	area (mm)	594	2392	1120	2322	1118	2001	1664	2241	2257	2268
		%	19.89	80.11	32.54	67.46	35.84	64.16	42.61	57.39	49.88	50.12
	weight 2	weight (g)	-	-	0.0530	0.1748	0.0609	0.1886	0.0791	0.1720	0.1013	0.1734
		%	-	-	23.27	76.73	24.41	75.59	31.50	68.50	36.88	63.12
SDS6.0B2	weight 1	weight (g)	0.0646	0.1460	0.0780	0.1640	0.0802	0.1720	0.0855	0.1798	0.1156	0.1774
		%	30.67	69.33	32.23	67.77	31.80	68.20	32.23	67.77	39.45	60.55
	area	area (mm)	858	2525	-	-	-	-	1820	2328	2442	2425
		%	25.36	74.64	-	-	-	-	43.88	56.12	50.17	49.83
	weight 2	weight (g)	-	-	0.0865	0.2278	0.0811	0.2189	0.1032	0.2074	0.1204	0.2186
		%	-	-	27.52	72.48	27.03	72.97	33.23	66.77	35.52	64.48
SDS6.0B3/4	weight 1	weight (g)	0.0407	0.1509	0.0345	0.1121	0.0705	0.1763	0.0695	0.1507	0.0855	0.1542
		%	21.24	78.76	23.53	76.47	28.57	71.43	31.56	68.44	35.67	64.33
	area	area (mm)	525	2093	627	1476	1222	2700	1300	1794	1736	1850
		%	20.05	79.95	29.81	70.19	31.16	68.84	42.02	57.98	48.41	51.59
	weight 2	weight (g)	-	-	0.0431	0.1366	0.0561	0.1607	0.0648	0.1333	0.0928	0.1353
		%	-	-	23.98	76.02	25.88	74.12	32.71	67.29	40.68	59.32
SDS6.0C1	weight 1	weight (g)	0.0606	0.1174	0.0605	0.1442	0.0601	0.1276	0.0576	0.1225	0.0803	0.1188
		%	34.04	65.96	29.56	70.44	32.02	67.98	31.98	68.02	40.33	59.67
	area	area (mm)	-	-	800	2001	1036	1716	984	1827	1680	1518
		%	-	-	28.56	71.44	37.65	62.35	35.01	64.99	52.53	47.47
	weight 2	weight (g)	-	-	0.0401	0.1621	0.0575	0.1574	0.0683	0.1366	0.0683	0.1372
		%	-	-	19.83	80.17	26.76	73.24	33.33	66.67	33.24	66.76
SDS6.0C2	weight 1	weight (g)	0.0872	0.1312	0.0679	0.1707	0.0735	0.1710	0.0970	0.1850	0.1172	0.1811
		%	39.93	60.07	28.46	71.54	30.06	69.94	34.40	65.60	39.29	60.71
	area	area (mm)	-	-	1026	2470	1408	2604	2146	2520	2405	2548
		%	-	-	29.35	70.65	35.09	64.91	45.99	54.01	48.56	51.44
	weight 2	weight (g)	-	-	0.0493	0.1918	0.0505	0.1632	0.0867	0.1704	0.0911	0.1735
		%	-	-	20.45	79.55	23.63	76.37	33.72	66.28	34.43	65.57
SDS6.0C3/4	weight 1	weight (g)	0.0737	0.1442	0.0492	0.1397	0.0800	0.1189	0.0647	0.1203	0.0786	0.0950
		%	33.82	66.18	26.05	73.95	40.22	59.78	34.97	65.03	45.28	54.72
	area	area (mm)	-	-	825	1968	728	1632	748	1488	928	1300
		%	-	-	29.54	70.46	30.85	69.15	33.45	66.55	41.65	58.35
	weight 2	weight (g)	-	-	0.0249	0.1308	0.0369	0.1145	0.0445	0.1011	0.0576	0.1039
		%	-	-	15.99	84.01	24.37	75.63	30.56	69.44	35.67	64.33

Table 20 Calculation of the percentage of monomeric protein present in each band of the doublet seen following SDS-PAGE of heated samples of β-LG A, B and C using line graphs generated by ImageQuant and methods 2, 3 and 4 as shown in Section 2.1.2, Figure 15.

		Temp (°C)									
		85		90		95		100		110	
Gel	Method	upper	lower	upper	lower	upper	lower	upper	lower	upper	lower
SDS6.0A1	wt 1	82.12	17.88	-	-	69.57	30.43	59.12	40.88	63.86	36.14
	mm	86.40	13.60	73.68	26.32	74.50	25.50	64.59	35.41	62.50	37.50
	wt 2	-	-	82.05	17.95	70.76	29.24	67.64	32.36	64.86	35.14
	IQ	-	-	67.00	33.00	62.20	37.80	59.08	40.92	55.71	44.29
	Average	84.26	15.74	74.24	25.76	69.26	30.74	62.61	37.39	61.73	38.27
SDS6.0A2	wt 1	-	-	83.90	16.10	64.04	35.96	57.15	42.85	53.88	46.12
	mm	-	-	86.96	13.04	69.74	30.26	65.97	34.03	67.01	32.99
	wt 2	-	-	79.16	20.84	71.13	28.87	69.65	30.35	73.47	26.53
	IQ	-	-	66.68	33.32	59.17	40.83	63.09	36.91	60.13	39.87
	Average	-	-	79.17	20.83	66.02	33.98	63.97	36.03	63.62	36.38
SDS6.0A3/4	wt 1	-	-	66.36	33.64	65.81	34.19	58.61	41.39	57.39	42.61
	mm	-	-	78.16	21.84	73.77	26.23	68.55	31.45	65.97	34.03
	wt 2	-	-	81.63	18.37	69.90	30.10	68.96	31.04	65.71	34.29
	IQ	-	-	59.24	40.76	59.45	40.55	52.89	47.11	53.80	46.20
	Average	-	-	71.35	28.65	67.23	32.77	62.26	37.74	60.72	39.28
SDS6.0B1	wt 1	35.76	64.24	26.25	73.75	34.76	65.24	38.34	61.66	36.69	63.31
	mm	19.89	80.11	32.54	67.46	35.84	64.16	42.61	57.39	49.88	50.12
	wt 2	-	-	23.27	76.73	24.41	75.59	31.50	68.50	36.88	63.12
	IQ	-	-	35.39	64.61	40.69	59.31	43.72	56.28	44.65	55.35
	Average	27.83	72.17	29.36	70.64	33.93	66.07	39.04	60.96	42.02	57.98
SDS6.0B2	wt 1	30.67	69.33	32.23	67.77	31.80	68.20	32.23	67.77	39.45	60.55
	mm	25.36	74.64	-	-	-	-	43.88	56.12	50.17	49.83
	wt 2	-	-	27.52	72.48	27.03	72.97	33.23	66.77	35.52	64.48
	IQ	-	-	42.74	57.26	35.89	64.11	43.64	56.36	44.08	55.92
	Average	28.02	71.98	34.16	65.84	31.57	68.43	38.24	61.76	42.31	57.69
SDS6.0B3/4	wt 1	21.24	78.76	23.53	76.47	28.57	71.43	31.56	68.44	35.67	64.33
	mm	20.05	79.95	29.81	70.19	31.16	68.84	42.02	57.98	48.41	51.59
	wt 2	-	-	23.98	76.02	25.88	74.12	32.71	67.29	40.68	59.32
	IQ	29.50	70.50	46.26	53.74	41.01	58.99	44.20	55.80	48.80	51.20
	Average	23.60	76.40	30.90	69.10	31.65	68.35	37.62	62.38	43.39	56.61
SDS6.0C1	wt 1	34.04	65.96	29.56	70.44	32.02	67.98	31.98	68.02	40.33	59.67
	mm	-	-	28.56	71.44	37.65	62.35	35.01	64.99	52.53	47.47
	wt 2	-	-	19.83	80.17	26.76	73.24	33.33	66.67	33.24	66.76
	IQ	-	-	35.92	64.08	37.88	62.12	38.23	61.77	42.65	57.35
	Average	34.04	65.96	28.47	71.53	33.57	66.43	34.64	65.36	42.19	57.81
SDS6.0C2	wt 1	39.93	60.07	28.46	71.54	30.06	69.94	34.40	65.60	39.29	60.71
	mm	-	-	29.35	70.65	35.09	64.91	45.99	54.01	48.56	51.44
	wt 2	-	-	20.45	79.55	23.63	76.37	33.72	66.28	34.43	65.57
	IQ	-	-	26.12	73.88	30.84	69.16	37.92	62.08	36.27	63.73
	Average	39.93	60.07	26.09	73.91	29.91	70.09	38.01	61.99	39.64	60.36
SDS6.0C3/4	wt 1	33.82	66.18	26.05	73.95	40.22	59.78	34.97	65.03	45.28	54.72
	mm	-	-	29.54	70.46	30.85	69.15	33.45	66.55	41.65	58.35
	wt 2	-	-	15.99	84.01	24.37	75.63	30.56	69.44	35.67	64.33
	IQ	25.16	74.84	40.23	59.77	30.98	69.02	40.03	59.97	43.37	56.63
	Average	29.49	70.51	27.95	72.05	31.60	68.40	34.75	65.25	41.49	58.51

Table 21 Summary of the results shown in Tables 19 and 20.

Table 22 Determination of the percentage of protein present in the band corresponding to that of unheated β-LG under native-PAGE conditions following heating (60-110°C) for 10 minutes at pH 6.0. Concentrations are as determined in Table 14(iv) (β-LG A), Table 16(iv) (β-LG B) and Table 18(iv) (β-LG C).

conc = concentration

Temp	Native A		Native B		Native C	
	Conc		Conc		Conc	
(°C)	(mg/g)	%	(mg/g)	%	(mg/g)	%
unheated	1.21	100	1.03	100	1.08	100
60	1.22	101.25	1.06	103.50	1.09	100.78
70	1.25	103.28	1.05	101.78	1.11	102.50
75	1.21	100.17	1.04	100.86	1.11	102.99
80	1.13	94.05	0.91	89.04	1.06	98.38
85	0.82	67.96	0.51	50.12	0.73	67.80
90	0.41	34.12	0.26	25.26	0.29	27.09
95	0.22	17.98	0.18	17.45	0.18	16.68
100	0.14	11.58	0.13	12.79	0.11	10.60
110	0.09	7.85	0.12	11.22	0.11	9.95

Table 23 ImageQuant volumes of β-LG samples analysed by native-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (I) β-LG A. (ii) β-LG B. (iii) β-LG C. The volumes given are the sum of the volumes obtained for the "native" band and the "smear" region, as defined in Section 2.1.2, Figure 14.

Concentrations were determined using the following formula:

$$x = \frac{\text{band volume - constant}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 13 (gel Nat6.0A3), Table 15 (gel Nat6.0B3) and Table 17 (gel Nat6.0C3).

(i)

gel: Nat6.0A3			formula: x=y+2.472/2613.144		stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. (mg/g)
1	unheated	655.5	0.25179	0.7041	0.1483	1.19547
2	60	673.2	0.25857	0.6981	0.15	1.20337
3	70	673.7	0.25876	0.6929	0.1494	1.20009
4	75	652.4	0.25061	0.6922	0.1489	1.16501
5	80	692.49	0.26595	0.6938	0.1487	1.24086
6	85	578.3	0.22225	0.6983	0.15	1.03465
7	90	400.6	0.15425	0.6952	0.1486	0.72162
8	95	345.6	0.1332	0.7468	0.1994	0.49887
9	100	250.52	0.09682	0.7549	0.1996	0.36616
10	110	200.86	0.07781	0.7594	0.2048	0.28852

(ii)

Gel: Nat6.0B3			formula: x=y+21.145/3001.038		stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. (mg/g)
1	unheated	703.1	0.24133	0.7108	0.149	1.15126
2	60	714.6	0.24516	0.6999	0.1482	1.15783
3	70	691.8	0.23757	0.6996	0.1488	1.11694
4	75	677.4	0.23277	0.6993	0.1488	1.09391
5	80	670.26	0.23039	0.7015	0.1492	1.08323
6	85	476.8	0.16592	0.7045	0.1485	0.78716
7	90	266.5	0.09585	0.7058	0.1482	0.45648
8	95	283.3	0.10145	0.7736	0.2194	0.3577
9	100	196.9	0.07266	0.7554	0.1992	0.27553
10	110	194.76	0.07194	0.7503	0.1988	0.27152

(iii)

Gel: Nat6.0C3			formula: x=y+31.713/3305.25		stain: comassie blue	
Lane	Temp (°C)	Vol (IQ)	x value	total wt (g)	sample w (g)	Conc. (mg/g)
1	unheated	683.75	0.21646	0.7063	0.1493	1.02403
2	60	686.25	0.21722	0.7013	0.149	1.02239
3	70	748.625	0.23609	0.7045	0.1482	1.12231
4	75	735	0.23197	0.7046	0.1488	1.09842
5	80	715.75	0.22614	0.7083	0.1499	1.06857
6	85	598.9	0.19079	0.7062	0.1483	0.90854
7	90	317.1	0.10553	0.7052	0.1497	0.49714
8	95	237.75	0.08153	0.7494	0.1989	0.30717
9	100	147.38	0.05418	0.7521	0.1991	0.20468
10	110	157.4	0.05722	0.7524	0.1985	0.21687

(i)

	Temp. (°C)	nat + smear conc (mg/g)	native conc (mg/g)	smear conc (mg/g)
Variant A	unheated	1.20	1.20	0
	60	1.20	1.20	0
	70	1.20	1.20	0
	75	1.17	1.17	0
	80	1.24	1.16	0.08
	85	1.03	0.85	0.18
	90	0.72	0.45	0.27
	95	0.50	0.24	0.26
	100	0.37	0.15	0.22
	110	0.29	0.10	0.19
Variant B	Temp. (°C)	nat + smear conc (mg/g)	native conc (mg/g)	smear conc (mg/g)
	unheated	1.15	1.15	0
	60	1.16	1.16	0
	70	1.12	1.12	0
	75	1.09	1.09	0
	80	1.08	0.96	0.10
	85	0.79	0.58	0.20
	90	0.46	0.28	0.18
	95	0.36	0.20	0.16
	100	0.26	0.13	0.14
	110	0.27	0.11	0.16
Variant C	Temp. (°C)	nat + smear conc (mg/g)	native conc (mg/g)	smear conc (mg/g)
	unheated	1.02	1.02	0
	60	1.02	1.02	0
	70	1.12	1.12	0
	75	1.10	1.10	0
	80	1.07	1.07	0
	85	0.91	0.76	0.15
	90	0.50	0.33	0.17
	95	0.31	0.20	0.11
	100	0.20	0.13	0.08
	110	0.22	0.12	0.10

(ii)

	Temp. (°C)	nat + smear %	nat %	smear %
Variant A	unheated	100	100	0
	60	100.28	100.28	0
	70	100.01	100.01	0
	75	97.08	97.08	0
	80	103.40	97.05	6.36
	85	86.22	70.87	15.35
	90	60.14	37.64	22.50
	95	41.57	19.98	21.56
	100	30.51	12.33	18.19
	110	24.04	8.25	15.80
Variant B	Temp. (°C)	nat + smear %	nat %	smear %
	unheated	100	100	0
	60	100.68	100.68	0
	70	97.13	97.13	0
	75	95.12	95.12	0
	80	94.19	85.10	9.10
	85	68.45	50.73	17.72
	90	39.69	24.16	15.54
	95	31.10	17.48	13.63
	100	23.96	11.61	12.35
	110	23.51	9.58	14.03
Variant C	Temp. (°C)	nat + smear %	nat %	smear %
	unheated	100	100	0
	60	100.23	100.23	0
	70	110.03	110.03	0
	75	107.69	107.69	0
	80	104.76	104.76	0
	85	89.07	74.61	14.463768
	90	48.74	32.11	16.627731
	95	30.11	19.28	10.834833
	100	20.07	12.36	7.7110676
	110	21.26	11.34	9.9231076

Table 24 Determination of (i) the concentration of protein present in the "smear" region (as defined in Section 2.1.2, Figure 14) of native-PAGE gels following analysis of β-LG samples that had been heated (60-110°C) for 10 minutes at pH 6.0, and (ii) the percentage of total protein present in the "native" band, the "smear" region, and the combined area ("native + smear").

The concentrations given for the "native + smear" region are as determined in Table 23 (i) (β-LG A), (ii) (β-LG B) and (iii) (β-LG C. The concentrations given for the "native" band are as determined in Table 14(iii) (β-LG A), Table 16(iii) (β-LG B) and Table 18(iii) (β-LG C). The concentrations given for the "smear" region are the difference between "native + smear" and "native".

conc = concentration

nat + smear = native + smear

Variant A

Temp. (°C)	monomeric (mg/g)	% SDS original	original (mg/g)	heat-induced (mg/g)
unheated	1.47	100	1.47	0
60	1.49	100	1.49	0
70	1.50	100	1.50	0
75	1.51	100	1.51	0
80	1.43	100	1.43	0
85	1.19	84.26	1.01	0.19
90	0.86	74.92	0.64	0.21
95	0.67	67.50	0.45	0.22
100	0.53	62.94	0.33	0.19
110	0.44	62.02	0.27	0.17

Variant B

Temp. (°C)	monomeric (mg/g)	% SDS original	original (mg/g)	heat-induced (mg/g)
unheated	1.27	100	1.27	0
60	1.32	100	1.32	0
70	1.31	100	1.31	0
75	1.33	100	1.33	0
80	1.23	100	1.23	0
85	0.94	73.52	0.69	0.25
90	0.65	68.53	0.45	0.21
95	0.50	67.62	0.34	0.16
100	0.42	61.70	0.26	0.16
110	0.45	57.43	0.26	0.19

Variant C

Temp. (°C)	monomeric (mg/g)	% SDS original	original (mg/g)	heat-induced (mg/g)
unheated	1.39	100	1.39	0
60	1.47	100	1.47	0
70	1.52	100	1.52	0
75	1.44	100	1.44	0
80	1.44	100	1.44	0
85	1.04	65.51	0.68	0.36
90	0.69	72.50	0.50	0.19
95	0.43	68.30	0.29	0.14
100	0.35	64.20	0.22	0.12
110	0.36	58.89	0.21	0.15

Table 25 Determination of the concentration of protein present in the "SDS-original" and "SDS-heat-induced" bands (as defined in Section 2.1.2, Figure 14) of SDS-PAGE gels following analysis of β-LG samples that had been heated (60-110°C) for 10 minutes at pH 6.0. The concentrations given for the "SDS-monomeric" region are as determined in Table 8(iv) (β-LG A), Table 10 (iv) (β-LG B) and Table 12 (iv) (β-LG C). The values given for the percentage of "SDS-monomeric" protein present in the band with the same mobility as unheated protein (% SDS-original) are as determined in Section 2.2.2, Table 2. The concentration of protein present in this band was calculated by multiplying the concentration of "SDS-monomeric" protein by % SDS-original. The concentrations given for the "SDS-heat-induced" band is the difference between "SDS-monomeric" and "SDS-original".

conc = concentration

Variant A

Temp. (°C)	% SDS monomeric	% SDS original	% SDS heat-induced
unheated	100	100	0
60	101.48	101.48	0
70	102.12	102.12	0
75	102.75	102.75	0
80	97.57	97.57	0
85	81.29	68.49	12.79
90	58.18	43.59	14.59
95	45.79	30.91	14.88
100	35.77	22.52	13.26
110	29.84	18.51	11.33

Variant B

Temp. (°C)	% SDS monomeric	% SDS original	% SDS heat-induced
unheated	100	100	0
60	104.25	104.25	0
70	102.95	102.95	0
75	104.80	104.80	0
80	96.91	96.91	0
85	74.18	54.54	19.64
90	51.33	35.17	16.15
95	39.31	26.58	12.73
100	33.15	20.45	12.70
110	35.20	20.21	14.98

Variant C

Temp. (°C)	% SDS monomeric	% SDS original	% SDS heat-induced
unheated	100	100	0
60	105.88	105.88	0
70	109.38	109.38	0
75	103.74	103.74	0
80	103.31	103.31	0
85	75.15	49.23	25.92
90	49.66	36.00	13.66
95	30.66	20.94	9.72
100	25.02	16.06	8.96
110	26.00	15.31	10.69

Table 26 Determination of the percentage of total protein present in the "SDS-monomeric" region and the "SDS-original" and "SDS-heat-induced" bands following heating (60-110°C) for 10 minutes at pH 6.0. Percentages were determined using the concentrations shown in Table 25.

Appendix D Data used in Section 3

(i)

Temp. (°C)	SDS gels A conc (mg/g)	B conc (mg/g)	C conc (mg/g)
20	1.47	1.27	1.39
60	1.49	1.32	1.47
70	1.50	1.31	1.52
75	1.51	1.33	1.44
80	1.43	1.23	1.44
85	1.19	0.94	1.04
90	0.86	0.65	0.69
95	0.67	0.50	0.43
100	0.53	0.42	0.35
110	0.44	0.45	0.36

(ii)

Temp. (°C)	Native gels A conc (mg/g)	B conc (mg/g)	C conc (mg/g)
20	1.21	1.03	1.08
60	1.22	1.06	1.09
70	1.25	1.05	1.11
75	1.21	1.04	1.11
80	1.13	0.91	1.06
85	0.82	0.51	0.73
90	0.41	0.26	0.29
95	0.22	0.18	0.18
100	0.14	0.13	0.11
110	0.09	0.12	0.11

Table 27 Concentration values obtained for β-LG A, B and C using (i) SDS-PAGE and (ii) native-PAGE. Values shown in (i) are those calculated in Table 8(iv) (β-LG A), Table 10(iv) (β-LG B) and Table 12(iv) (β-LG C). Values shown in (ii) are those calculated in Table 14(iv) (β-LG A), Table 16(iv) (β-LG B) and Table 18(iv) (β-LG C).

Gel:SDS6.0A2			
lane	11	12	13
conc	0.064	0.162	0.257
vol bottom	132.1	328.6	480
vol top	29.05	43.17	48.94
vol total	161.15	371.77	528.94
Regression Output:			
Constant			46.93776
Std Err of Y Est			19.48615
R Squared			0.994425
No. of Observations			3
Degrees of Freedom			1
X Coefficient(s)		1906.929	
Std Err of Coef.		142.7797	

Gel:SDS6.0B2			
lane	11	12	13
conc	0.0598	0.15	0.1928
vol bottom	117.6	317.1	413.4
vol top	41.19	71.02	71.93
vol total	158.79	388.12	485.33
Regression Output:			
Constant			12.7832
Std Err of Y Est			6.295607
R Squared			0.999295
No. of Observations			3
Degrees of Freedom			1
X Coefficient(s)		2468.68	
Std Err of Coef.		65.56871	

Gel:SDS6.0C2			
lane	11	12	13
conc	0.0476	0.0909	0.2
vol bottom	114.1	216.9	459.2
vol top	17.91	37.32	66.83
vol total	132.01	254.22	526.03
Regression Output:			
Constant			14.42237
Std Err of Y Est			8.129107
R Squared			0.999188
No. of Observations			3
Degrees of Freedom			1
X Coefficient(s)		2567.187	
Std Err of Coef.		73.19515	

Table 28 ImageQuant volumes determined by laser densitometry analysis of β-LG standards following SDS-PAGE. Volumes obtained for the band corresponding to monomeric β-LG were added to the volumes obtained for the region corresponding to dimeric β-LG to give a total volume for each lane. Linear regression was performed as described in Section 2.1.1, using the total volume for each lane as the dependant value.

conc = concentration (mg/g).

vol = volume of β-LG band as determined by ImageQuant.

(i)

Gel:SDS6.0A2			formula: x=y-46.938/1906.929			
Lane	Temp (°C)	vol (IQ)	x value	total wt (g)	sample wt (g)	conc (mg/g)
1	unheated	320.9	0.14367	0.6219	0.0732	1.22058
2	60	325.2	0.14592	0.6192	0.0737	1.22598
3	70	328.1	0.14744	0.6221	0.0744	1.23285
5	75	325	0.14582	0.6282	0.0737	1.2429
6	80	316	0.1411	0.6214	0.0737	1.18966
7	85	270	0.11697	0.6218	0.0739	0.98423
4	90	190	0.07502	0.6219	0.0738	0.6322
8	95	203.2	0.08194	0.6496	0.0986	0.53987
9	100	152.1	0.05515	0.6446	0.0984	0.36126
10	110	129.9	0.04351	0.6433	0.0973	0.28764

(ii)

Gel: SDS6.0B2			formula: x=y-12.783/2468.68			
Lane	Temp (°C)	vol (IQ)	x value	total wt (g)	sample wt (g)	conc (mg/g)
1	unheated	319.9	0.12441	0.6197	0.0734	1.05033
2	60	326	0.12688	0.6369	0.0736	1.09793
3	70	322.7	0.12554	0.6223	0.0733	1.0658
5	75	329.7	0.12838	0.6231	0.0735	1.08831
6	80	315.5	0.12262	0.6252	0.0737	1.04022
7	85	231.8	0.08872	0.6382	0.0732	0.7735
4	90	154.3	0.05732	0.6384	0.0736	0.49723
8	95	160.2	0.05971	0.651	0.0986	0.39426
9	100	129.8	0.0474	0.6504	0.0987	0.31235
10	110	138.9	0.05109	0.6498	0.0978	0.33943

(iii)

Gel: SDS6.0C2			formula: x=y-14.422/2567.187			
Lane	Temp. (°C)	vol (IQ)	x value	total wt (g)	sample wt (g)	conc (mg/g)
1	unheated	381.7	0.14307	0.6227	0.0736	1.21043
2	60	396.8	0.14895	0.6316	0.0744	1.26446
3	70	406.9	0.15288	0.6301	0.0732	1.316
5	75	388.8	0.14583	0.642	0.074	1.26519
6	80	398.3	0.14953	0.6244	0.0731	1.27727
7	85	287.3	0.10629	0.6254	0.073	0.91064
4	90	190.7	0.06867	0.6278	0.0738	0.58412
8	95	157.2	0.05562	0.6575	0.0981	0.37276
9	100	126.2	0.04354	0.6473	0.0984	0.28642
10	110	129.9	0.04498	0.6514	0.0978	0.29961

Table 29 ImageQuant volumes of β-LG samples analysed by SDS-PAGE following heating (60-110°C) for 10 minutes at pH 6.0. (i) β-LG A (ii) β-LG B (iii) β-LG C

Concentrations were determined using the following formula:

$$x = \frac{\text{band volume - constant}}{\text{x coefficient}}$$

The values of the constant and the x coefficient were determined using the regressions shown in Table 28.

Appendix E Data used in Section 4

Wavelength (nm)	Absorbance	Wavelength (nm)	Absorbance
280	0.439	308	0.581
285	0.501	309	0.574
290	0.551	310	0.566
291	0.56	311	0.558
292	0.569	312	0.55
293	0.576	313	0.54
294	0.582	314	0.529
295	0.586	315	0.518
296	0.591	316	0.506
297	0.594	317	0.493
298	0.598	318	0.481
299	0.601	319	0.468
300	0.604	320	0.455
301	0.605	321	0.441
302	0.604	322	0.428
303	0.602	323	0.414
304	0.599	324	0.4
305	0.595	325	0.386
306	0.592	330	0.319
307	0.587	335	0.253

Table 30 Absorbance values for a 1 mM solution of NEM at wavelengths between 280 and 335 nm (inclusive).

Time (min)	A320	
	BLG+NEM	BLG
0	0.546	0.131
5	0.543	0.142
10	0.537	0.151
15	0.536	0.158
20	0.535	0.164
25	0.54	0.175
30	0.555	0.197
40	0.541	0.192
50	0.569	0.219
60	0.592	0.25

Table 31 Change in absorbance at 320 nm of solutions of β-LG A and β-LG A/NEM during heating at pH 5.0, 70°C.

Time	A320			To-T	A/E	moles SH	moles	% SH
(min)	(t)	(c)	t-c	(A)	M SH	reacted	BLG	reacted
0	0.553	0.121	0.432	0	0	0	2.4E-06	0
5	0.546	0.126	0.42	0.012	2.6E-05	8.4E-08	2.4E-06	3.5077
10	0.539	0.128	0.411	0.021	4.6E-05	1.5E-07	2.4E-06	6.13847
15	0.531	0.13	0.401	0.031	6.8E-05	2.2E-07	2.4E-06	9.06155
20	0.523	0.13	0.393	0.039	8.6E-05	2.7E-07	2.4E-06	11.4
25	0.515	0.13	0.385	0.047	0.0001	3.3E-07	2.4E-06	13.7385
30	0.507	0.13	0.377	0.055	0.00012	3.9E-07	2.4E-06	16.077
40	0.491	0.13	0.361	0.071	0.00016	5E-07	2.4E-06	20.7539
50	0.477	0.131	0.346	0.086	0.00019	6E-07	2.4E-06	25.1385
60	0.463	0.132	0.331	0.101	0.00022	7.1E-07	2.4E-06	29.5231
70	0.451	0.132	0.319	0.113	0.00025	7.9E-07	2.4E-06	33.0308
80	0.437	0.133	0.304	0.128	0.00028	9E-07	2.4E-06	37.4154
90	0.426	0.133	0.293	0.139	0.00031	9.8E-07	2.4E-06	40.6308
100	0.415	0.134	0.281	0.151	0.00033	1.1E-06	2.4E-06	44.1385
110	0.403	0.136	0.267	0.165	0.00036	1.2E-06	2.4E-06	48.2309
120	0.391	0.137	0.254	0.178	0.00039	1.3E-06	2.4E-06	52.0309

Table 32 Change in absorbance at 320 nm of solutions of β-LG A and β-LG A/NEM during heating at pH 6.0, 60°C, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

(t) = β-LG A/NEM solution, (c) = β-LG A solution

To = the value of (t) - (c) at Time = 0, T = the value of (t) - (c) at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455), M SH = moles SH/L

Time	A320			To-T	A/E	moles SH	moles	% SH
(min)	(t)	(c)	t-c	(A)	M SH	reacted	BLG	reacted
0	0.574	0.142	0.432	0	0	0	2.4E-06	0
5	0.57	0.147	0.423	0.009	2E-05	6.3E-08	2.4E-06	2.63077
10	0.564	0.148	0.416	0.016	3.5E-05	1.1E-07	2.4E-06	4.67693
15	0.56	0.147	0.413	0.019	4.2E-05	1.3E-07	2.4E-06	5.55386
20	0.555	0.147	0.408	0.024	5.3E-05	1.7E-07	2.4E-06	7.0154
25	0.553	0.147	0.406	0.026	5.7E-05	1.8E-07	2.4E-06	7.60001
30	0.549	0.147	0.402	0.03	6.6E-05	2.1E-07	2.4E-06	8.76925
40	0.542	0.146	0.396	0.036	7.9E-05	2.5E-07	2.4E-06	10.5231
50	0.536	0.146	0.39	0.042	9.2E-05	3E-07	2.4E-06	12.2769
60	0.529	0.146	0.383	0.049	0.00011	3.4E-07	2.4E-06	14.3231
70	0.524	0.146	0.378	0.054	0.00012	3.8E-07	2.4E-06	15.7846
80	0.517	0.146	0.371	0.061	0.00013	4.3E-07	2.4E-06	17.8308
90	0.511	0.144	0.367	0.065	0.00014	4.6E-07	2.4E-06	19

Table 33 Change in absorbance at 320 nm of solutions of β-LG A and β-LG A/NEM during heating at pH 6.0, 50°C, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

(t) = β-LG A/NEM solution, (c) = β-LG A solution

To = the value of (t) - (c) at Time = 0, T = the value of (t) - (c) at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455), M SH = moles SH/L

Time	A320			To-T	A/E	moles SH	moles	% SH
(min)	(t)	(c)	t-c	(A)	M SH	reacted	BLG	reacted
0	0.559	0.134	0.425	0	0	0	2.4E-06	0
5	0.556	0.136	0.42	0.005	1.1E-05	3.5E-08	2.4E-06	1.46154
10	0.554	0.135	0.419	0.006	1.3E-05	4.2E-08	2.4E-06	1.75385
15	0.552	0.135	0.417	0.008	1.8E-05	5.6E-08	2.4E-06	2.33847
20	0.551	0.134	0.417	0.008	1.8E-05	5.6E-08	2.4E-06	2.33847
25	0.549	0.134	0.415	0.01	2.2E-05	7E-08	2.4E-06	2.92308
30	0.548	0.135	0.413	0.012	2.6E-05	8.4E-08	2.4E-06	3.5077
40	0.545	0.136	0.409	0.016	3.5E-05	1.1E-07	2.4E-06	4.67693
50	0.542	0.136	0.406	0.019	4.2E-05	1.3E-07	2.4E-06	5.55386
60	0.539	0.137	0.402	0.023	5.1E-05	1.6E-07	2.4E-06	6.72309
70	0.537	0.137	0.4	0.025	5.5E-05	1.8E-07	2.4E-06	7.3077
80	0.535	0.138	0.397	0.028	6.2E-05	2E-07	2.4E-06	8.18463
90	0.533	0.138	0.395	0.03	6.6E-05	2.1E-07	2.4E-06	8.76925

Table 34 Change in absorbance at 320 nm of solutions of β-LG A and β-LG A/NEM during heating at pH 6.0, 40°C, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

(t) = β-LG A/NEM solution, (c) = β-LG A solution

To = the value of (t) - (c) at Time = 0, T = the value of (t) - (c) at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455), M SH = moles SH/L

Time	A320			To-T	A/E	moles SH	moles	% SH
(min)	(t)	(c)	t-c	(A)	M SH	reacted	BLG	reacted
0	0.513	0.11	0.403	0	0	0	2.3E-06	0
5	0.499	0.104	0.395	0.008	1.8E-05	5.2E-08	2.3E-06	2.3138
10	0.487	0.104	0.383	0.02	4.4E-05	1.3E-07	2.3E-06	5.78451
15	0.472	0.104	0.368	0.035	7.7E-05	2.3E-07	2.3E-06	10.1229
20	0.46	0.103	0.357	0.046	0.0001	3E-07	2.3E-06	13.3044
25	0.451	0.103	0.348	0.055	0.00012	3.6E-07	2.3E-06	15.9074
30	0.443	0.103	0.34	0.063	0.00014	4.1E-07	2.3E-06	18.2212
40	0.426	0.102	0.324	0.079	0.00017	5.2E-07	2.3E-06	22.8488
50	0.411	0.102	0.309	0.094	0.00021	6.2E-07	2.3E-06	27.1872
60	0.4	0.101	0.299	0.104	0.00023	6.8E-07	2.3E-06	30.0794
70	0.388	0.101	0.287	0.116	0.00025	7.6E-07	2.3E-06	33.5501
80	0.379	0.102	0.277	0.126	0.00028	8.3E-07	2.3E-06	36.4424
90	0.369	0.102	0.267	0.136	0.0003	8.9E-07	2.3E-06	39.3346
100	0.361	0.102	0.259	0.144	0.00032	9.4E-07	2.3E-06	41.6484
110	0.353	0.102	0.251	0.152	0.00033	1E-06	2.3E-06	43.9622
120	0.346	0.101	0.245	0.158	0.00035	1E-06	2.3E-06	45.6976

Table 35 Change in absorbance at 320 nm of solutions of β-LG A and β-LG A/NEM during heating at pH 6.0, 40°C and in the presence of 0.27% SDS, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

(t) = β-LG A/NEM solution, (c) = β-LG A solution

To = the value of (t) - (c) at Time = 0, T = the value of (t) - (c) at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455), M SH = moles SH/L

Time (min)	A320	To-T (A)	A/E M SH	moles SH reacted	moles BLG	% SH reacted
0	0.604	0	0	0	2.5E-06	0
5	0.594	0.01	2.2E-05	6.7E-08	2.5E-06	2.69017
10	0.587	0.017	3.7E-05	1.1E-07	2.5E-06	4.57329
15	0.581	0.023	5.1E-05	1.5E-07	2.5E-06	6.18739
20	0.575	0.029	6.4E-05	1.9E-07	2.5E-06	7.80149
25	0.57	0.034	7.5E-05	2.3E-07	2.5E-06	9.14657
30	0.566	0.038	8.4E-05	2.5E-07	2.5E-06	10.2226
40	0.558	0.046	0.0001	3.1E-07	2.5E-06	12.3748
50	0.55	0.054	0.00012	3.6E-07	2.5E-06	14.5269
60	0.543	0.061	0.00013	4.1E-07	2.5E-06	16.41
70	0.537	0.067	0.00015	4.5E-07	2.5E-06	18.0241
80	0.531	0.073	0.00016	4.9E-07	2.5E-06	19.6382
90	0.525	0.079	0.00017	5.3E-07	2.5E-06	21.2523
100	0.519	0.085	0.00019	5.7E-07	2.5E-06	22.8664
110	0.514	0.09	0.0002	6E-07	2.5E-06	24.2115

Table 36 Change in absorbance at 320 nm of solutions of β-LG C and β-LG C/NEM during heating at pH 6.0, 40°C and in the presence of 0.13% SDS, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

To = absorbance at Time = 0, T = absorbance at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455), M SH = moles SH/L

Time (min)	A320	To-T (A)	A/E M SH	moles SH reacted	moles BLG	% SH reacted
0	0.549	0	0	0	2.5E-06	0
5	0.542	0.007	1.5E-05	4.6E-08	2.5E-06	1.86453
10	0.541	0.008	1.8E-05	5.3E-08	2.5E-06	2.1309
15	0.54	0.009	2E-05	6E-08	2.5E-06	2.39726
20	0.539	0.01	2.2E-05	6.6E-08	2.5E-06	2.66362
25	0.538	0.011	2.4E-05	7.3E-08	2.5E-06	2.92998
30	0.537	0.012	2.6E-05	7.9E-08	2.5E-06	3.19634
40	0.534	0.015	3.3E-05	9.9E-08	2.5E-06	3.99543
50	0.532	0.017	3.7E-05	1.1E-07	2.5E-06	4.52815
60	0.529	0.02	4.4E-05	1.3E-07	2.5E-06	5.32724
70	0.527	0.022	4.8E-05	1.5E-07	2.5E-06	5.85996
80	0.525	0.024	5.3E-05	1.6E-07	2.5E-06	6.39269
90	0.523	0.026	5.7E-05	1.7E-07	2.5E-06	6.92541
100	0.521	0.028	6.2E-05	1.9E-07	2.5E-06	7.45814
110	0.519	0.03	6.6E-05	2E-07	2.5E-06	7.99086

Table 37 Change in absorbance at 320 nm of solutions of β-LG C and β-LG C/NEM during heating at pH 6.0, 40°C and in the presence of 0.03% SDS, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

To = absorbance at Time = 0, T = absorbance at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455), M SH = moles SH/L

Table 38 Change in absorbance at 320 nm of solutions of β-LG C and β-LG C/NEM during heating at pH 6.0, 25°C and in the presence of 0.26% SDS, and the percentage of thiol groups in β-LG reacting with NEM as determined by changes in absorbance.

To = absorbance at Time = 0

T = absorbance at each given time

E = the molar extincion coefficient of NEM at 320 nm (=455)

M SH = moles SH/L

Time (min)	A320	To-T (A)	A/E M SH	moles SH reacted	moles BLG	% SH reacted
0	0.601	0	0	0	2.5E-06	0
5	0.581	0.02	4.4E-05	1.4E-07	2.5E-06	5.45113
10	0.574	0.027	5.9E-05	1.8E-07	2.5E-06	7.35903
15	0.567	0.034	7.5E-05	2.3E-07	2.5E-06	9.26692
20	0.562	0.039	8.6E-05	2.6E-07	2.5E-06	10.6297
25	0.557	0.044	9.7E-05	3E-07	2.5E-06	11.9925
30	0.553	0.048	0.00011	3.2E-07	2.5E-06	13.0827
40	0.544	0.057	0.00013	3.9E-07	2.5E-06	15.5357
50	0.537	0.064	0.00014	4.3E-07	2.5E-06	17.4436
60	0.53	0.071	0.00016	4.8E-07	2.5E-06	19.3515
70	0.523	0.078	0.00017	5.3E-07	2.5E-06	21.2594
80	0.517	0.084	0.00018	5.7E-07	2.5E-06	22.8947
90	0.51	0.091	0.0002	6.2E-07	2.5E-06	24.8026
100	0.505	0.096	0.00021	6.5E-07	2.5E-06	26.1654
110	0.5	0.101	0.00022	6.8E-07	2.5E-06	27.5282
120	0.494	0.107	0.00024	7.2E-07	2.5E-06	29.1635

Table 39 Change in absorbance at 320 nm of a solution of NEM during heating at pH 6.0, 60°C, and the moles of NEM that have hydrolysed as determined by changes in absorbance.

To = absorbance at Time = 0

T = absorbance at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455)

M NEM = moles NEM/L

Time (min)	A320	To-T (A)	A/E M NEM	moles NEM hydrolysed
0	0.354	0	0	0
5	0.346	0.008	1.8E-05	5.2747E-08
10	0.341	0.013	2.9E-05	8.5714E-08
15	0.335	0.019	4.2E-05	1.2527E-07
20	0.33	0.024	5.3E-05	1.5824E-07
25	0.325	0.029	6.4E-05	1.9121E-07
30	0.321	0.033	7.3E-05	2.1758E-07
40	0.312	0.042	9.2E-05	2.7692E-07
50	0.302	0.052	0.00011	3.4286E-07
60	0.294	0.06	0.00013	3.956E-07
70	0.285	0.069	0.00015	4.5495E-07
80	0.277	0.077	0.00017	5.0769E-07
90	0.268	0.086	0.00019	5.6703E-07
100	0.261	0.093	0.0002	6.1319E-07
110	0.252	0.102	0.00022	6.7253E-07
120	0.245	0.109	0.00024	7.1868E-07

Table 40 Change in absorbance at 320 nm of a solution of NEM during heating at pH 6.0, 40°C, and the moles of NEM that have hydrolysed as determined by changes in absorbance.

To = absorbance at Time = 0

T = absorbance at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455)

M NEM = moles NEM/L

Time (min)	A320	To-T (A)	A/E M NEM	moles NEM hydrolysed
0	0.354	0	0	0
5	0.351	0.003	6.6E-06	1.978E-08
10	0.349	0.005	1.1E-05	3.2967E-08
15	0.348	0.006	1.3E-05	3.956E-08
20	0.347	0.007	1.5E-05	4.6154E-08
25	0.346	0.008	1.8E-05	5.2747E-08
30	0.345	0.009	2E-05	5.9341E-08
40	0.344	0.01	2.2E-05	6.5934E-08
50	0.343	0.011	2.4E-05	7.2527E-08
60	0.341	0.013	2.9E-05	8.5714E-08
70	0.34	0.014	3.1E-05	9.2308E-08
80	0.339	0.015	3.3E-05	9.8901E-08
90	0.337	0.017	3.7E-05	1.1209E-07
100	0.336	0.018	4E-05	1.1868E-07
110	0.334	0.02	4.4E-05	1.3187E-07
120	0.333	0.021	4.6E-05	1.3846E-07

Table 41 Change in absorbance at 320 nm of a solution of NEM during heating at pH 6.0, 25°C, and the moles of NEM that have hydrolysed as determined by changes in absorbance.

To = absorbance at Time = 0

T = absorbance at each given time

E = the molar extincion coefficient of NEM at 320 nm (= 455)

M NEM = moles NEM/L

Time (min)	A320	To-T (A)	A/E M NEM	moles NEM hydrolysed
0	0.461	0	0	0
5	0.455	0.006	1.3E-05	3.956E-08
10	0.454	0.007	1.5E-05	4.6154E-08
15	0.454	0.007	1.5E-05	4.6154E-08
20	0.453	0.008	1.8E-05	5.2747E-08
25	0.453	0.008	1.8E-05	5.2747E-08
30	0.453	0.008	1.8E-05	5.2747E-08
40	0.452	0.009	2E-05	5.9341E-08
50	0.451	0.01	2.2E-05	6.5934E-08
60	0.451	0.01	2.2E-05	6.5934E-08
70	0.451	0.01	2.2E-05	6.5934E-08
80	0.451	0.01	2.2E-05	6.5934E-08
90	0.45	0.011	2.4E-05	7.2527E-08
100	0.446	0.015	3.3E-05	9.8901E-08
110	0.446	0.015	3.3E-05	9.8901E-08
120	0.446	0.015	3.3E-05	9.8901E-08

Table 42 Determination of the percentage of thiol groups in β-LG reacting with NEM during heating at pH 6.0, 60°C. The moles of SH that appear to have reacted (r) was determined in Table 31. The moles of NEM that have hydrolysed during heating at pH 6.0, 60°C (h) was determined in Table 38.

Time (min)	moles SH reacted (r)	moles NEM hydrolysed (h)	r-h	moles BLG	% SH reacted with NEM
0	0	0	0	2.4E-06	0
5	8.44E-08	5.274725E-08	3.2E-08	2.4E-06	1.3153869
10	1.477E-07	8.571429E-08	6.2E-08	2.4E-06	2.5759659
15	2.18E-07	1.252747E-07	9.3E-08	2.4E-06	3.8548143
20	2.743E-07	1.582418E-07	1.2E-07	2.4E-06	4.8230852
25	3.305E-07	1.912088E-07	1.4E-07	2.4E-06	5.791356
30	3.868E-07	2.175824E-07	1.7E-07	2.4E-06	7.0336659
40	4.993E-07	2.769231E-07	2.2E-07	2.4E-06	9.2442466
50	6.048E-07	3.428571E-07	2.6E-07	2.4E-06	10.88848
60	7.103E-07	3.956044E-07	3.1E-07	2.4E-06	13.080792
70	7.947E-07	4.549451E-07	3.4E-07	2.4E-06	14.12214
80	9.002E-07	5.076923E-07	3.9E-07	2.4E-06	16.314451
90	9.776E-07	5.67033E-07	4.1E-07	2.4E-06	17.063491
100	1.062E-06	6.131868E-07	4.5E-07	2.4E-06	18.652916
110	1.16E-06	6.725275E-07	4.9E-07	2.4E-06	20.278881
120	1.252E-06	7.186813E-07	5.3E-07	2.4E-06	22.160615

Table 43 Determination of the percentage of thiol groups in β-LG reacting with NEM during heating at pH 6.0, 40°C. The moles of SH that appear to have reacted (r) was determined in Table 33. The moles of NEM that have hydrolysed during heating at pH 6.0, 40°C (h) was determined in Table 39.

Time (min)	moles SH reacted (r)	moles NEM hydrolysed (h)	r-h	moles BLG	% SH reacted with NEM
0	0	0	0	2.4E-06	0
5	3.516E-08	1.978022E-08	1.5E-08	2.4E-06	0.6394242
10	4.22E-08	3.296703E-08	9.2E-09	2.4E-06	0.3836545
15	5.626E-08	3.956044E-08	1.7E-08	2.4E-06	0.694232
20	5.626E-08	4.615385E-08	1E-08	2.4E-06	0.420193
25	7.033E-08	5.274725E-08	1.8E-08	2.4E-06	0.7307705
30	8.44E-08	5.934066E-08	2.5E-08	2.4E-06	1.0413479
40	1.125E-07	6.593407E-08	4.7E-08	2.4E-06	1.9365418
50	1.336E-07	7.252747E-08	6.1E-08	2.4E-06	2.5394274
60	1.618E-07	8.571429E-08	7.6E-08	2.4E-06	3.1605823
70	1.758E-07	9.230769E-08	8.4E-08	2.4E-06	3.4711598
80	1.969E-07	9.89011E-08	9.8E-08	2.4E-06	4.0740454
90	2.11E-07	1.120879E-07	9.9E-08	2.4E-06	4.1105839

Table 44 Determination of the percentage of thiol groups in β-LG reacting with NEM during heating at pH 6.0, 40°C and in the presence of 0.27% SDS. The moles of SH that appear to have reacted (r) was determined in Table 34. The moles of NEM that have hydrolysed during heating at pH 6.0, 40°C (h) was determined in Table 39.

Time (min)	moles SH reacted (r)	moles NEM hydrolysed (h)	r-h	moles BLG	% SH reacted with NEM
0	0	0	0	2.3E-06	0
5	5.24E-08	1.978022E-08	3.3E-08	2.3E-06	1.4403029
10	1.31E-07	3.296703E-08	9.8E-08	2.3E-06	4.3286733
15	2.292E-07	3.956044E-08	1.9E-07	2.3E-06	8.3758857
20	3.013E-07	4.615385E-08	2.6E-07	2.3E-06	11.266197
25	3.602E-07	5.274725E-08	3.1E-07	2.3E-06	13.578058
30	4.126E-07	5.934066E-08	3.5E-07	2.3E-06	15.600694
40	5.174E-07	6.593407E-08	4.5E-07	2.3E-06	19.937131
50	6.156E-07	7.252747E-08	5.4E-07	2.3E-06	23.984344
60	6.811E-07	8.571429E-08	6E-07	2.3E-06	26.294264
70	7.597E-07	9.230769E-08	6.7E-07	2.3E-06	29.4738
80	8.252E-07	9.89011E-08	7.3E-07	2.3E-06	32.074887
90	8.907E-07	1.120879E-07	7.8E-07	2.3E-06	34.384806
100	9.431E-07	1.186813E-07	8.2E-07	2.3E-06	36.407442
110	9.955E-07	1.318681E-07	8.6E-07	2.3E-06	38.138911
120	1.035E-06	1.384615E-07	9E-07	2.3E-06	39.583096

Table 45 Determination of the percentage of thiol groups in β-LG reacting with NEM during heating at pH 6.0, 40°C and in the presence of 0.13% SDS. The moles of SH that appear to have reacted (r) was determined in Table 35. The moles of NEM that have hydrolysed during heating at pH 6.0, 40°C (h) was determined in Table 39.

Time (min)	moles SH reacted (r)	moles NEM hydrolysed (h)	r-h	moles BLG	% SH reacted with NEM
0	0	0	0	2.5E-06	0
5	6.681E-08	1.978022E-08	4.7E-08	2.5E-06	1.8937366
10	1.136E-07	3.296703E-08	8.1E-08	2.5E-06	3.2459
15	1.537E-07	3.956044E-08	1.1E-07	2.5E-06	4.5945236
20	1.938E-07	4.615385E-08	1.5E-07	2.5E-06	5.9431473
25	2.272E-07	5.274725E-08	1.7E-07	2.5E-06	7.0227541
30	2.539E-07	5.934066E-08	1.9E-07	2.5E-06	7.8333442
40	3.073E-07	6.593407E-08	2.4E-07	2.5E-06	9.7200015
50	3.608E-07	7.252747E-08	2.9E-07	2.5E-06	11.606659
60	4.076E-07	8.571429E-08	3.2E-07	2.5E-06	12.958822
70	4.476E-07	9.230769E-08	3.6E-07	2.5E-06	14.307446
80	4.877E-07	9.89011E-08	3.9E-07	2.5E-06	15.656069
90	5.278E-07	1.120879E-07	4.2E-07	2.5E-06	16.739216
100	5.679E-07	1.186813E-07	4.5E-07	2.5E-06	18.08784
110	6.013E-07	1.318681E-07	4.7E-07	2.5E-06	18.901969

Table 46 Determination of the percentage of thiol groups in β-LG reacting with NEM during heating at pH 6.0, 40°C and in the presence of 0.03% SDS. The moles of SH that appear to have reacted (r) was determined in Table 36. The moles of NEM that have hydrolysed during heating at pH 6.0, 40°C (h) was determined in Table 39.

Time (min)	moles SH reacted (r)	moles NEM hydrolysed (h)	r-h	moles BLG	% SH reacted with NEM
0	0	0	0	2.5E-06	0
5	4.631E-08	1.978022E-08	2.7E-08	2.5E-06	1.0681029
10	5.292E-08	3.296703E-08	2E-08	2.5E-06	0.8035107
15	5.954E-08	3.956044E-08	2E-08	2.5E-06	0.8043956
20	6.615E-08	4.615385E-08	2E-08	2.5E-06	0.8052805
25	7.277E-08	5.274725E-08	2E-08	2.5E-06	0.8061655
30	7.938E-08	5.934066E-08	2E-08	2.5E-06	0.8070504
40	9.923E-08	6.593407E-08	3.3E-08	2.5E-06	1.3406593
50	1.125E-07	7.252747E-08	4E-08	2.5E-06	1.6079063
60	1.323E-07	8.571429E-08	4.7E-08	2.5E-06	1.8760382
70	1.455E-07	9.230769E-08	5.3E-08	2.5E-06	2.1432851
80	1.588E-07	9.89011E-08	6E-08	2.5E-06	2.410532
90	1.72E-07	1.120879E-07	6E-08	2.5E-06	2.4123019
100	1.852E-07	1.186813E-07	6.7E-08	2.5E-06	2.6795488
110	1.985E-07	1.318681E-07	6.7E-08	2.5E-06	2.6813187

Table 47 Determination of the percentage of thiol groups in β-LG reacting with NEM during heating at pH 6.0, 25°C and in the presence of 0.26% SDS. The moles of SH that appear to have reacted (r) was determined in Table 37. The moles of NEM that have hydrolysed during heating at pH 6.0, 40°C (h) was determined in Table 40.

Time (min)	moles SH reacted (r)	moles NEM hydrolysed (h)	r-h	moles BLG	% SH reacted with NEM
0	0	0	0	2.5E-06	0
5	1.354E-07	3.956044E-08	9.6E-08	2.5E-06	3.8582671
10	1.828E-07	4.615385E-08	1.4E-07	2.5E-06	5.5006855
15	2.302E-07	4.615385E-08	1.8E-07	2.5E-06	7.4085809
20	2.64E-07	5.274725E-08	2.1E-07	2.5E-06	8.5058862
25	2.978E-07	5.274725E-08	2.5E-07	2.5E-06	9.8686686
30	3.249E-07	5.274725E-08	2.7E-07	2.5E-06	10.958895
40	3.858E-07	5.934066E-08	3.3E-07	2.5E-06	13.146426
50	4.332E-07	6.593407E-08	3.7E-07	2.5E-06	14.788844
60	4.806E-07	6.593407E-08	4.1E-07	2.5E-06	16.69674
70	5.28E-07	6.593407E-08	4.6E-07	2.5E-06	18.604635
80	5.686E-07	6.593407E-08	5E-07	2.5E-06	20.239974
90	6.16E-07	7.252747E-08	5.4E-07	2.5E-06	21.882392
100	6.498E-07	9.89011E-08	5.5E-07	2.5E-06	22.183266
110	6.837E-07	9.89011E-08	5.8E-07	2.5E-06	23.546049
120	7.243E-07	9.89011E-08	6.3E-07	2.5E-06	25.181388

Table 48 Change in absorbance at 412 nm of a solution containing DTNB and β-LG A during heating at pH 7.0, 50°C, and the percentage of thiol groups in β-LG reacting with DTNB as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 60°C.

E = the molar extincion coefficient of DTNB at 412 nm (= 13600)

M SH = moles SH/L

time (min)	A412	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	2.3E-07	0
10	0.715	5.3E-05	1.6E-07	2.3E-07	68.9509
20	0.863	6.3E-05	1.9E-07	2.3E-07	83.2233
30	0.874	6.4E-05	1.9E-07	2.3E-07	84.2841
40	0.863	6.3E-05	1.9E-07	2.3E-07	83.2233
50	0.848	6.2E-05	1.9E-07	2.3E-07	81.7768
60	0.835	6.1E-05	1.8E-07	2.3E-07	80.5231
70	0.824	6.1E-05	1.8E-07	2.3E-07	79.4624
80	0.813	6E-05	1.8E-07	2.3E-07	78.4016
90	0.807	5.9E-05	1.8E-07	2.3E-07	77.823
100	0.8	5.9E-05	1.8E-07	2.3E-07	77.1479
110	0.79	5.8E-05	1.7E-07	2.3E-07	76.1836
120	0.785	5.8E-05	1.7E-07	2.3E-07	75.7014
130	0.779	5.7E-05	1.7E-07	2.3E-07	75.1228
140	0.776	5.7E-05	1.7E-07	2.3E-07	74.8335
150	0.774	5.7E-05	1.7E-07	2.3E-07	74.6406

Table 49 Change in absorbance at 412 nm of a solution containing DTNB and β-LG A during heating at pH 7.0, 50°C, and the percentage of thiol groups in β-LG reacting with DTNB as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 60°C in the presence of NEM.

E = the molar extincion coefficient of DTNB at 412 nm (= 13600)

M SH = moles SH/L

time (min)	A412	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	2.3E-07	0
10	0.688	5.1E-05	1.5E-07	2.3E-07	66.3631
20	0.85	6.3E-05	1.9E-07	2.3E-07	81.9892
30	0.859	6.3E-05	1.9E-07	2.3E-07	82.8574
40	0.843	6.2E-05	1.9E-07	2.3E-07	81.314
50	0.825	6.1E-05	1.8E-07	2.3E-07	79.5778
60	0.807	5.9E-05	1.8E-07	2.3E-07	77.8416
70	0.791	5.8E-05	1.7E-07	2.3E-07	76.2982
80	0.776	5.7E-05	1.7E-07	2.3E-07	74.8514
90	0.766	5.6E-05	1.7E-07	2.3E-07	73.8868
100	0.756	5.6E-05	1.7E-07	2.3E-07	72.9222
110	0.74	5.4E-05	1.6E-07	2.3E-07	71.3789
120	0.73	5.4E-05	1.6E-07	2.3E-07	70.4143
130	0.718	5.3E-05	1.6E-07	2.3E-07	69.2568
140	0.711	5.2E-05	1.6E-07	2.3E-07	68.5816
150	0.705	5.2E-05	1.6E-07	2.3E-07	68.0028

Table 50 Change in absorbance at 412 nm of a solution containing DTNB and β-LG A during heating at pH 7.0, 50°C, and the percentage of thiol groups in β-LG reacting with DTNB as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 40°C in the presence of 0.27% SDS.

E = the molar extincion coefficient of DTNB at 412 nm (= 13600)

M SH = moles SH/L

time (min)	A412	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	2.3E-07	0
10	0.206	1.5E-05	4.5E-08	2.3E-07	19.9418
20	0.382	2.8E-05	8.4E-08	2.3E-07	36.9795
30	0.476	3.5E-05	1.1E-07	2.3E-07	46.0791
40	0.539	4E-05	1.2E-07	2.3E-07	52.1778
50	0.575	4.2E-05	1.3E-07	2.3E-07	55.6628
60	0.6	4.4E-05	1.3E-07	2.3E-07	58.0829
70	0.616	4.5E-05	1.4E-07	2.3E-07	59.6318
80	0.626	4.6E-05	1.4E-07	2.3E-07	60.5999
90	0.631	4.6E-05	1.4E-07	2.3E-07	61.0839
100	0.637	4.7E-05	1.4E-07	2.3E-07	61.6647
110	0.643	4.7E-05	1.4E-07	2.3E-07	62.2456
120	0.643	4.7E-05	1.4E-07	2.3E-07	62.2456
130	0.642	4.7E-05	1.4E-07	2.3E-07	62.1488
140	0.641	4.7E-05	1.4E-07	2.3E-07	62.0519
150	0.639	4.7E-05	1.4E-07	2.3E-07	61.8583

Table 51 Change in absorbance at 412 nm of a solution containing DTNB and β-LG A during heating at pH 7.0, 50°C, and the percentage of thiol groups in β-LG reacting with DTNB as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 60°C in the presence of NEM and 0.27% SDS.

E = the molar extincion coefficient of DTNB at 412 nm (= 13600)

M SH = moles SH/L

time (min)	A412	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	2.3E-07	0
10	0.122	9E-06	2.7E-08	2.3E-07	11.7258
20	0.251	1.8E-05	5.5E-08	2.3E-07	24.1245
30	0.328	2.4E-05	7.2E-08	2.3E-07	31.5252
40	0.381	2.8E-05	8.4E-08	2.3E-07	36.6192
50	0.413	3E-05	9.1E-08	2.3E-07	39.6949
60	0.434	3.2E-05	9.6E-08	2.3E-07	41.7132
70	0.448	3.3E-05	9.9E-08	2.3E-07	43.0588
80	0.456	3.4E-05	1E-07	2.3E-07	43.8277
90	0.459	3.4E-05	1E-07	2.3E-07	44.1161
100	0.462	3.4E-05	1E-07	2.3E-07	44.4044
110	0.464	3.4E-05	1E-07	2.3E-07	44.5966
120	0.463	3.4E-05	1E-07	2.3E-07	44.5005
130	0.458	3.4E-05	1E-07	2.3E-07	44.02
140	0.455	3.3E-05	1E-07	2.3E-07	43.7316
150	0.452	3.3E-05	1E-07	2.3E-07	43.4433

Table 52 Change in absorbance at 343 nm of a solution containing DPDS and β-LG A during heating at pH 7.5, 50°C, and the percentage of thiol groups in β-LG reacting with DPDS as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 60°C.

E = the molar extincion coefficient of DPDS at 343 nm (= 7837)

M SH = moles SH/L

time (min)	A343	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	3.8E-07	0
5	0.285	3.6E-05	1.1E-07	3.8E-07	28.5009
10	0.667	8.5E-05	2.6E-07	3.8E-07	66.7022
15	0.802	0.0001	3.1E-07	3.8E-07	80.2027
20	0.87	0.00011	3.3E-07	3.8E-07	87.0029
25	0.913	0.00012	3.5E-07	3.8E-07	91.303
30	0.936	0.00012	3.6E-07	3.8E-07	93.6031
35	0.952	0.00012	3.6E-07	3.8E-07	95.2031
40	0.958	0.00012	3.7E-07	3.8E-07	95.8032
45	0.963	0.00012	3.7E-07	3.8E-07	96.3032
50	0.967	0.00012	3.7E-07	3.8E-07	96.7032
60	0.97	0.00012	3.7E-07	3.8E-07	97.0032
70	0.97	0.00012	3.7E-07	3.8E-07	97.0032
85	0.967	0.00012	3.7E-07	3.8E-07	96.7032
90	0.963	0.00012	3.7E-07	3.8E-07	96.3032
110	0.957	0.00012	3.7E-07	3.8E-07	95.7032
130	0.946	0.00012	3.6E-07	3.8E-07	94.6031
140	0.941	0.00012	3.6E-07	3.8E-07	94.1031
150	0.936	0.00012	3.6E-07	3.8E-07	93.6031
160	0.929	0.00012	3.6E-07	3.8E-07	92.9031
170	0.924	0.00012	3.5E-07	3.8E-07	92.4031
180	0.918	0.00012	3.5E-07	3.8E-07	91.803

Table 53 Change in absorbance at 343 nm of a solution containing DPDS and β-LG A during heating at pH 7.5, 50°C, and the percentage of thiol groups in β-LG reacting with DPDS as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 60°C in the presence of NEM.

E = the molar extincion coefficient of DPDS at 343 nm (= 7837)

M SH = moles SH/L

time (min)	A343	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	3.8E-07	0
5	0.282	3.6E-05	1.1E-07	3.8E-07	28.2009
10	0.683	8.7E-05	2.6E-07	3.8E-07	68.3023
15	0.807	0.0001	3.1E-07	3.8E-07	80.7027
20	0.866	0.00011	3.3E-07	3.8E-07	86.6029
25	0.903	0.00012	3.5E-07	3.8E-07	90.303
30	0.922	0.00012	3.5E-07	3.8E-07	92.203
35	0.932	0.00012	3.6E-07	3.8E-07	93.2031
40	0.936	0.00012	3.6E-07	3.8E-07	93.6031
45	0.938	0.00012	3.6E-07	3.8E-07	93.8031
50	0.939	0.00012	3.6E-07	3.8E-07	93.9031
60	0.94	0.00012	3.6E-07	3.8E-07	94.0031
70	0.935	0.00012	3.6E-07	3.8E-07	93.5031
85	0.929	0.00012	3.6E-07	3.8E-07	92.9031
90	0.923	0.00012	3.5E-07	3.8E-07	92.3031
110	0.916	0.00012	3.5E-07	3.8E-07	91.603
130	0.904	0.00012	3.5E-07	3.8E-07	90.403
140	0.895	0.00011	3.4E-07	3.8E-07	89.503
150	0.889	0.00011	3.4E-07	3.8E-07	88.9029
160	0.88	0.00011	3.4E-07	3.8E-07	88.0029
170	0.874	0.00011	3.3E-07	3.8E-07	87.4029
180	0.867	0.00011	3.3E-07	3.8E-07	86.7029

Table 54 Change in absorbance at 343 nm of a solution containing DPDS and β-LG A during heating at pH 7.5, 50°C, and the percentage of thiol groups in β-LG reacting with DPDS as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 40°C in the presence of 0.27% SDS.

E = the molar extincion coefficient of DPDS at 343 nm (= 7837)

M SH = moles SH/L

time (min)	A343	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	3.2E-07	0
5	0.135	1.7E-05	5.2E-08	3.2E-07	16.0086
10	0.276	3.5E-05	1.1E-07	3.2E-07	32.7286
15	0.351	4.5E-05	1.3E-07	3.2E-07	41.6223
20	0.424	5.4E-05	1.6E-07	3.2E-07	50.2788
25	0.498	6.4E-05	1.9E-07	3.2E-07	59.0538
30	0.55	7E-05	2.1E-07	3.2E-07	65.2201
35	0.597	7.6E-05	2.3E-07	3.2E-07	70.7935
40	0.618	7.9E-05	2.4E-07	3.2E-07	73.2837
45	0.652	8.3E-05	2.5E-07	3.2E-07	77.3155
50	0.68	8.7E-05	2.6E-07	3.2E-07	80.6358
60	0.71	9.1E-05	2.7E-07	3.2E-07	84.1932
70	0.747	9.5E-05	2.9E-07	3.2E-07	88.5808
85	0.766	9.8E-05	2.9E-07	3.2E-07	90.8338
90	0.776	9.9E-05	3E-07	3.2E-07	92.0196
110	0.783	1E-04	3E-07	3.2E-07	92.8497
130	0.786	0.0001	3E-07	3.2E-07	93.2055
140	0.785	0.0001	3E-07	3.2E-07	93.0869
150	0.782	1E-04	3E-07	3.2E-07	92.7311
160	0.778	9.9E-05	3E-07	3.2E-07	92.2568
170	0.773	9.9E-05	3E-07	3.2E-07	91.6639
180	0.767	9.8E-05	2.9E-07	3.2E-07	90.9524

Table 55 Change in absorbance at 343 nm of a solution containing DPDS and β-LG A during heating at pH 7.5, 50°C, and the percentage of thiol groups in β-LG reacting with DPDS as determined by changes in absorbance. The β-LG had previously been incubated for two hours at 60°C in the presence of NEM and 0.27% SDS.

E = the molar extincion coefficient of DPDS at 343 nm (= 7837)

M SH = moles SH/L

time (min)	A343	A/E (M SH)	moles SH reacted	moles BLG	% SH reacted
0	0	0	0	5E-07	0
5	0.069	8.8E-06	2.6E-08	5E-07	5.25392
10	0.166	2.1E-05	6.4E-08	5E-07	12.6399
15	0.223	2.8E-05	8.5E-08	5E-07	16.9801
20	0.281	3.6E-05	1.1E-07	5E-07	21.3964
25	0.344	4.4E-05	1.3E-07	5E-07	26.1935
30	0.392	5E-05	1.5E-07	5E-07	29.8484
35	0.439	5.6E-05	1.7E-07	5E-07	33.4271
40	0.463	5.9E-05	1.8E-07	5E-07	35.2546
45	0.499	6.4E-05	1.9E-07	5E-07	37.9958
50	0.531	6.8E-05	2E-07	5E-07	40.4324
60	0.566	7.2E-05	2.2E-07	5E-07	43.0974
70	0.616	7.9E-05	2.4E-07	5E-07	46.9046
85	0.648	8.3E-05	2.5E-07	5E-07	49.3412
90	0.668	8.5E-05	2.6E-07	5E-07	50.8641
110	0.688	8.8E-05	2.6E-07	5E-07	52.3869
130	0.703	9E-05	2.7E-07	5E-07	53.5291
140	0.708	9E-05	2.7E-07	5E-07	53.9098
150	0.71	9.1E-05	2.7E-07	5E-07	54.0621
160	0.711	9.1E-05	2.7E-07	5E-07	54.1383
170	0.711	9.1E-05	2.7E-07	5E-07	54.1383
180	0.71	9.1E-05	2.7E-07	5E-07	54.0621

Time (min	A320	To-T	A/E (M NEM)	moles NEM hydrolysed	moles NEM	% NEM hydrolysed	% NEM remaining
0	0.409	0	0	0	3E-06	0	100
5	0.4	0.009	2E-05	5.93407E-08	3E-06	1.978022	98.022
10	0.389	0.02	4.4E-05	1.31868E-07	3E-06	4.3956044	95.6044
15	0.378	0.031	6.8E-05	2.04396E-07	3E-06	6.8131868	93.1868
20	0.368	0.043	9.5E-05	2.83516E-07	3E-06	9.4505495	90.5495
25	0.356	0.053	0.00012	3.49451E-07	3E-06	11.648352	88.3516
30	0.344	0.065	0.00014	4.28571E-07	3E-06	14.285714	85.7143
40	0.328	0.081	0.00018	5.34066E-07	3E-06	17.802198	82.1978
50	0.312	0.097	0.00021	6.3956E-07	3E-06	21.318681	78.6813
60	0.296	0.113	0.00025	7.45055E-07	3E-06	24.835165	75.1648
70	0.281	0.128	0.00028	8.43956E-07	3E-06	28.131868	71.8681
80	0.266	0.143	0.00031	9.42857E-07	3E-06	31.428571	68.5714
90	0.253	0.156	0.00034	1.02857E-06	3E-06	34.285714	65.7143
100	0.24	0.169	0.00037	1.11429E-06	3E-06	37.142857	62.8571
110	0.228	0.181	0.0004	1.19341E-06	3E-06	39.78022	60.2198
120	0.217	0.192	0.00042	1.26593E-06	3E-06	42.197802	57.8022

Table 56 Change in absorbance at 320 nm of a solution of NEM during heating at pH 8.12, 25°C, and the percentage of NEM that has hydrolysed as determined by changes in absorbance.

To = absorbance at Time = 0

T = absorbance at each given time

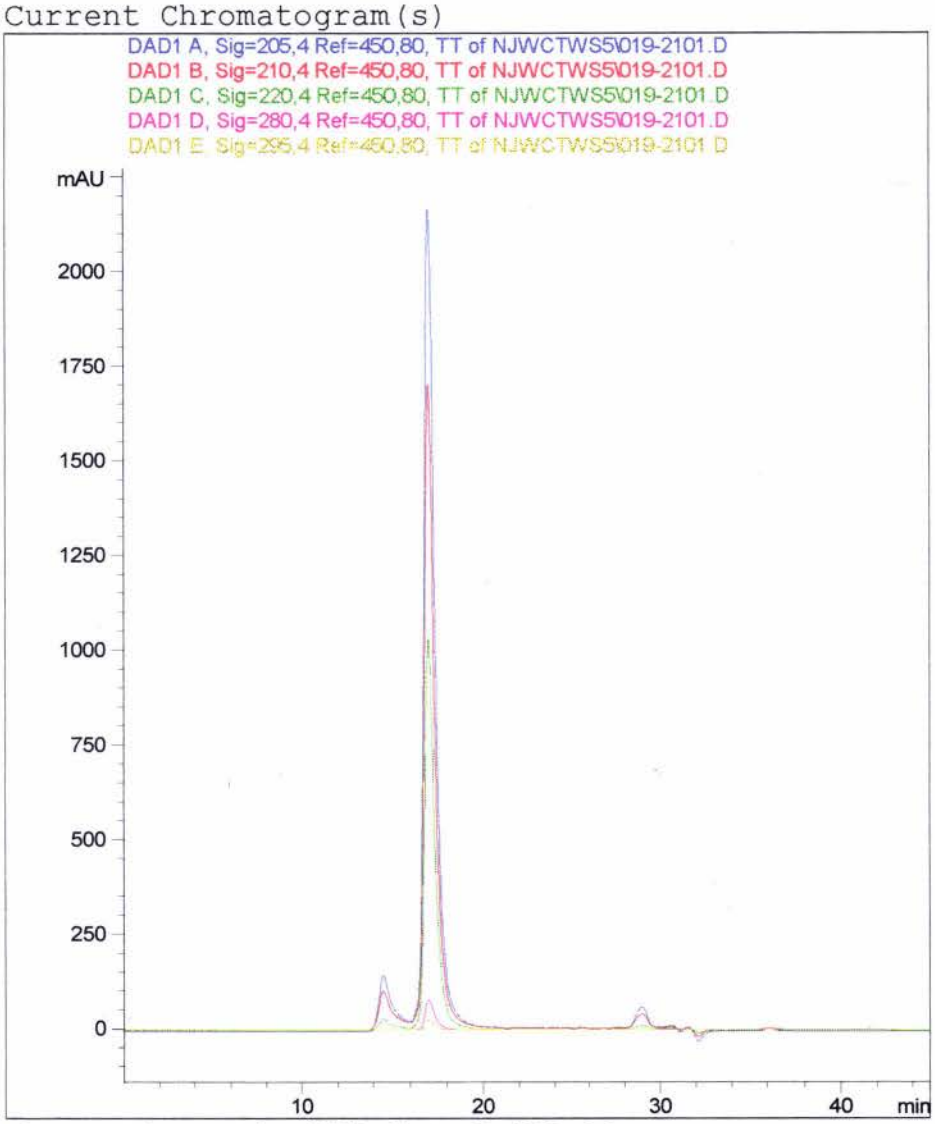
E = the molar extincion coefficient of NEM at 320 nm (= 455)

M NEM = moles NEM/L

Appendix F HPLC instrument printouts for results shown in Section 5

1 Sample taken immediately after addition of TPCK-trypsin to labelled β-LG.

Current Chromatogram(s)



Signal 1: DAD1 A, Sig=205,4 Ref=450,80, TT

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	14.549	BV	0.695	7821.92822	146.09686	7.0782
2	16.985	VV	0.649	94897.81250	2167.70923	85.8753
3	28.969	BV	0.868	4962.66455	71.26647	4.4908
4	30.633	VV	0.686	1541.81067	31.79090	1.3952
5	31.530	VV	0.560	1282.35510	30.72346	1.1604
Totals :				110506.57031	2447.58691	

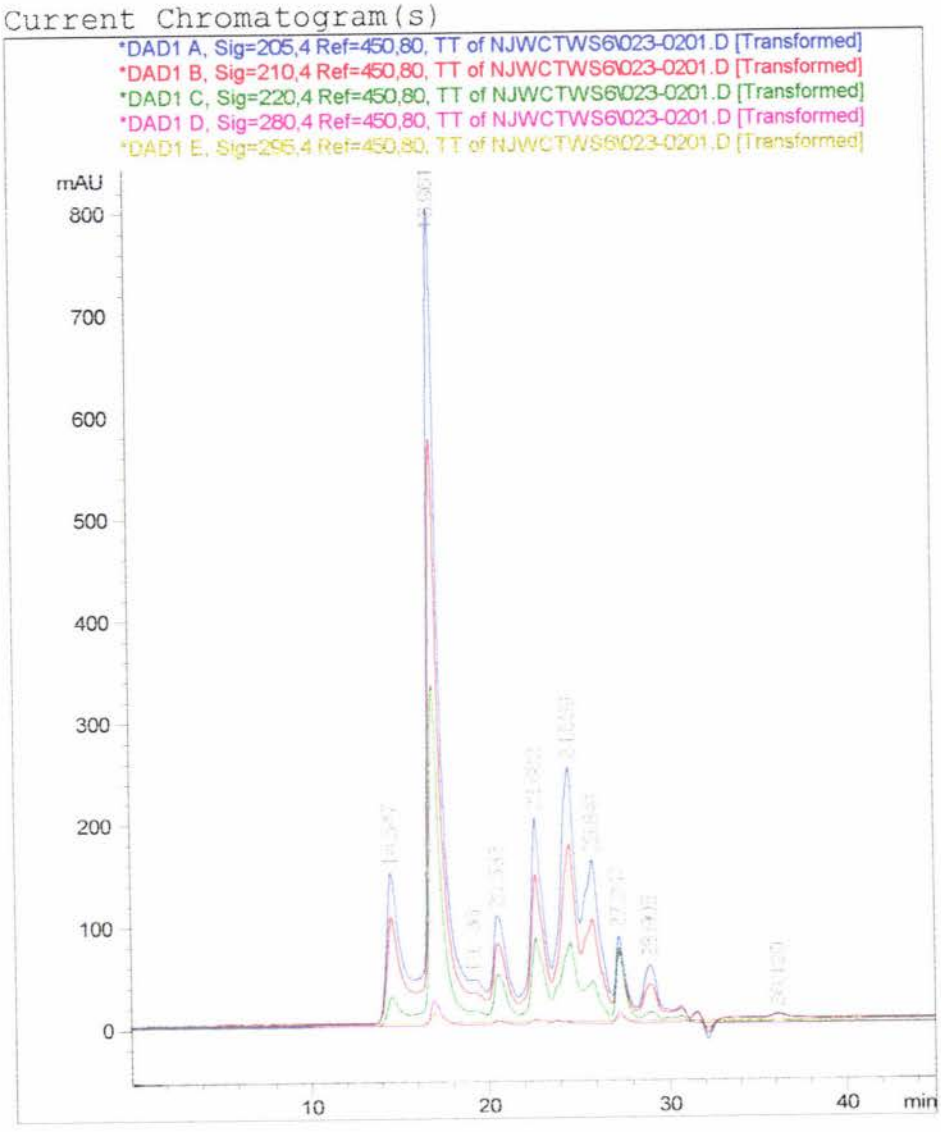
Signal 2: DAD1 B, Sig=210,4 Ref=450,80, TT

Signal 3: DAD1 C, Sig=220,4 Ref=450,80, TT

Signal 4: DAD1 D, Sig=280,4 Ref=450,80, TT

Signal 5: DAD1 E, Sig=295,4 Ref=450,80, TT

2 Sample taken four hours after addition of TPCK-trypsin to labelled β-LG.



Signal 1: DAD1 A, Sig=205,4 Ref=450,80, TT

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	14.547	BV	0.758	8394.48242	148.67995	7.866(
2	16.961	VV	0.680	38732.03906	802.29327	36.293
3	19.136	VV	0.692	2546.76074	47.59145	2.386
4	20.533	VV	0.828	6844.03320	111.44895	6.413
5	22.682	VV	0.727	11380.75684	208.87944	10.664
6	24.559	VV	0.885	15505.87988	259.97064	14.529
7	25.841	VV	0.857	11090.75195	170.46829	10.392
8	27.257	VV	0.645	4511.70654	96.72366	4.227
9	28.905	VV	1.011	4906.56445	69.68715	4.597
10	36.120	PB	4.407	2805.87402	7.74828	2.629
Totals :				106718.85156	1923.49109	

Signal 2: DAD1 B, Sig=210,4 Ref=450,80, TT

Signal 3: DAD1 C, Sig=220,4 Ref=450,80, TT

Signal 4: DAD1 D, Sig=280,4 Ref=450,80, TT

Signal 5: DAD1 E, Sig=295,4 Ref=450,80, TT

Appendix G Data for results shown in Section 6

		counts/minute (cpm)					
		low peptide loading			high peptide loading		
cycle	residue	A	B	C	A	B	C
1	102	25					
2	103	28					
3	104	26	27				
4	105	29	26	38	35	41	91
5	106	28	26	43	34	57	74
6	107	29	29	43	37	66	84
7	108		28	45	44	46	64
8	109		27				
14	115	28		58	46	75	90
15	116	31	30	54	57	95	107
16	117	27	31	57	55	84	130
17	118	28	36	58	54	77	112
18	119	28	70	74	88	122	170
19	120	30	41	67	107	125	283
20	121	39	54	82	165	223	367
21	122	34	44	74	102	140	317
22	123	30	34	57	70	124	249
23	124	34		43	51	67	173

Table 57 Radioactivity (cpm) associated with amino acid residues following sequencing of peptides generated by the tryptic hydrolysis of β-LG A, B and C.

New Zealand Dairy Research Institute

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of β-Lactoglobulin Variants A, B and C. Relative Mobilities of Heated and Unheated Samples

Helen Brittan[2], Ruth Lowe[1], Gill E Norris[2], Trevor M Kitson[3] and Jeremy P Hill[1]
[1] Food Science Section, New Zealand Dairy Research Institute, Private Bag 11 029, Palmerston North, New Zealand
[2] Department of Biochemistry, Massey University, Palmerston North, New Zealand
[3] Department of Chemistry, Massey University, Palmerston North, New Zealand

Introduction

Polyacrylamide gel electrophoresis (PAGE) is a common technique used in the analysis of milk proteins. Sodium dodecyl sulphate (SDS)-PAGE separates proteins on the basis of the molecular size of the protein-SDS complex. SDS disrupts non-covalent interactions within and between protein molecules, and reduction with 2-mercaptoethanol breaks disulphide bonds. Comparison between reduced and non-reduced samples can reveal information about the type of bonding in the sample.

Basch *et al.* (1985) observed the splitting of mixed variant β-lactoglobulin (β-LG) into two bands under non-reducing conditions in a discontinuous SDS-PAGE system. The β-LG A, B and C variants were all found to show this behaviour.

Shimada and Cheftal (1989) found that under non-reducing PAGE conditions, an additional, faster migrating band appeared after four minutes heating at 85°C (pH 7.5). This extra band was poorly separated from the A+B band but was visibly detectable. They suggested that this new fraction may represent partially denatured monomeric β-LG with different pairs of disulphide bonds, due to intramolecular disulphide exchange reactions.

In this current study we examined the relative mobilities of the β-LG A, B and C variants using SDS-PAGE under non-reducing and reducing conditions.

Methods

β-LG A, B and C was prepared by NaCl precipitation as described by Mailliart and Ribadeau-Dumas (1988) and further purified by gel filtration. Samples of purified β-LG A, B and C were heated for ten minutes in phosphate buffer (NaH_2PO_4/NaOH) to different temperatures (60-110°C) and at different pHs (5, 6, 7, 8 and 8.4). SDS-PAGE was performed under non-reducing and reducing conditions in a discontinuous system (resolving gel 16%T, 2.6%C, stacking gel 3.9%T, 2.6%C) on unheated and heated β-LG A, B and C variants.

Results

Purified, unheated β-LG ran as a single band (Figure 1 a). Under non-reducing SDS-PAGE conditions in unheated samples the A variant had a slightly lower mobility than the B and C variants (Figure 1 a). Heating to 110°C promoted the splitting of β-LG into two clearly resolved bands under non-reduced SDS-PAGE conditions (Figure 1 b). This behaviour was observed with all three variants. In the A variant the band formed after heating had a higher mobility than the original band, while in the B and C variants this band had a lower mobility than the original band (Figure 2). The temperature at which splitting occurred was pH dependant, occuring at lower temperatures as pH increased (Table 1). Under reducing conditions only a single band was seen in both heated and unheated samples (Figure 3).

Figure 1
SDS-PAGE of β-LG variants A, B, and C under non-reducing conditions.

Figure 1 (a)

Figure 1 (b)

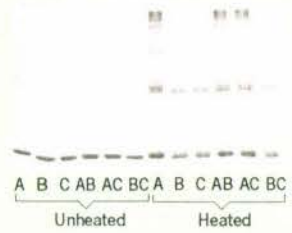


Figure 2
Effect of temperature on the mobility of β-LG variants A, B, and C in SDS-PAGE under non-reducing conditions.

Figure 2 (a) **β-LG A**

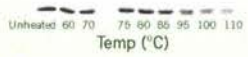


Figure 2 (b) **β-LG B**

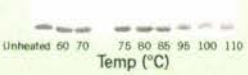


Figure 2 (c) **β-LG C**

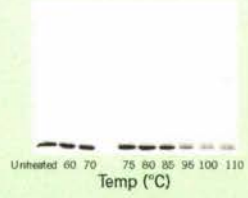


Figure 3
SDS-PAGE of β-LG variants A, B and C under reducing conditions.

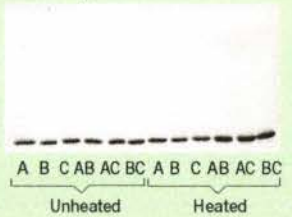


Table 1
Conditions where band splitting observed

pH	Temperature (°C)
5.0	no band splitting seen
6.0	85
7.0	70
8.0, 8.4	60

Conclusions

1. The mobility of β-LG A under non-reducing SDS-PAGE conditions is different to that of β-LG B and C, which have similar mobilities. As mobility in SDS is due to the SDS coating and not the charge on the protein, a difference in the mobility between proteins of almost identical molecular weight indicates that the structures of these proteins could be different.

2. The mobilities of β-LG A, B and C under reducing SDS-PAGE conditions appear to be the same. This suggests that the difference in mobility between variants seen in non-reduced samples involves disulphide bonding.

3. The mobility of the band formed on heating in the A variant appears to be the same as the mobility of the unheated B and C variants, while the mobility of the band formed in the B and C variants appears to be the same as that of the unheated A variant. As heat can induce changes in the intramolecular disulphide bonding, it is possible that there is a difference in the nature of the disulphide bonding in the A variant compared the B and C variants.

Acknowledgements

This work was supported by funding from the New Zealand Dairy Board and Foundation for Research, Science and Technology, Contract No. DRI 401. We gratefully acknowledge the services of the NZDRI Graphics Unit for preparing this poster.

References

Basch, J.J., Douglas, F.W. Jr., Procino, L.G., Holsinger, V.H. and Farrell, H.M. Jr. (1985) Quantitation of Caseins and Whey Proteins of Processed Milks and Whey Protein Concentrates, Application of Gel Electrophoresis, and Comparison with Harland-Ashworth Procedure.
J. Dairy Science **68** 23-31

Mailliart, P. and Ribadeau-Dumas, B. (1988) Preparation of β-Lactoglobulin and β-Lactoglobulin-Free Proteins from Whey Retentate by NaCl Salting Out at Low pH.
J. Food Science **53** (3) 743-745,752

Shimada, K and Cheftal, J.C. (1989) Sulfhydryl Group/Disulphide Bond Interchange Reactions during Heat-Induced Gelation of Whey Protein Isolate.
J. Agric. Food Chem **37** 161-168

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of β-Lactoglobulin Variants A, B and C. Relative mobilities of heated and unheated samples.

H. Brittan[2], R. Lowe[1], G.E. Norris[2], T. M. Kitson[3] and J.P. Hill[1].

[1] Food Science Section, New Zealand Dairy Research Institute, Palmerston North, New Zealand
[2] Department of Biochemistry, Massey University, Palmerston North, New Zealand
[3]Department of Chemistry, Massey University, Palmerston North, New Zealand

Abstract

The relative mobilities of purified A, B and C variants of bovine β-lactoglobulin was examined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions. Purified β-lactoglobulin variants ran as a single band under non-reducing conditions, the A variant having a slightly lower mobility than the B and C variants. Under reducing conditions there appeared to be no difference in the mobilities of the three variants. After heating a second band was seen under non-reducing conditions. In the A variant the new band had a slightly higher mobility than the original band, apparently corresponding to the mobility of the unheated B and C variant bands. In the heated samples of the B and C variants the band formed on heating had a lower mobility than the original band, corresponding to the band seen in the unheated sample of the A variant. Splitting was temperature- and pH-dependent. Reduction of heated samples gave a single band, all variants exhibiting a similar mobility.

Introduction

Polyacrylamide gel electrophoresis (PAGE) is a common technique used in the analysis of milk proteins. SDS-PAGE separates proteins on the basis of the molecular size of the protein-SDS complex. As different proteins bind SDS on the basis of molecular weight, once coated with SDS, the proteins are separated on the basis of molecular weight. SDS disrupts non-covalent interactions within and between protein molecules, and reduction with 2-mercaptoethanol breaks disulphide bonds. Comparison between reduced and non-reduced samples can reveal information about the type of bonding in the sample.

Basch *et al.* (1) observed the splitting of mixed variant β-lactoglobulin (β-LG) into two bands under non-reducing conditions in a discontinuous SDS-PAGE system. The β-LG A, B and C variants were all found to show this behaviour.

Shimada and Cheftel (3) found that under non-reducing PAGE conditions, an additional, faster migrating band appeared after four minutes heating at 85°C (pH 7.5). This extra band was poorly separated from the A+B band but was visibly detectable. They suggested that this new fraction may represent partially denatured monomeric β-LG with different pairs of disulphide bonds to those found in the native protein, due to intramolecular disulphide exchange reactions.

In this current study we examined the relative mobilities of the β-LG A, B and C variants using SDS-PAGE under non-reducing and reducing conditions.

Methods

Milk was collected from indvidual cows homozygous for β-LG A, B and C. Fat was removed with an Elecrem 80 separator, and acid whey was prepared from skim milk by precipitation with ~3 M HCl at 40°C. β-LG was prepared from acid whey by NaCl precipitation as described by Mailliart and Ribadeau-Dumas (2) and further purified by gel filtration (Sephadex G75 26/60 column, 0.1 M NaH_2PO_4/NaOH, pH 6.0). Samples of purified β-LG A, B and C were heated for ten minutes in phosphate buffer (NaH_2PO_4/NaOH) to different temperatures (60-110°C) and at different pHs (6, 7, 8 and 8.4), and in NaH_2PO_4 solution adjusted to pH 5.0. SDS-PAGE was performed in a discontinuous system (16% resolving gel (2.6% bis) and 3.9% stacking gel (2.6% bis)) on unheated and heated β-LG A, B and C variants under non-reducing and reducing conditions. Samples run under reducing conditions were treated with 2-mercaptoethanol just prior to loading.

Results

Purified, unheated β-LG ran as a single band in both non-reduced and reduced samples. Under non-reducing SDS-PAGE conditions in unheated samples the A variant had a slightly lower mobility than the B and C variants (Figure 1a).

Heating promoted the splitting of β-LG into two clearly resolved bands under non-reduced SDS-PAGE conditions (Figure 1b). This behaviour was observed with all three variants. In the A variant the band formed after heating had a higher mobility than the original band, whilst in the B and C variants this band had a lower mobility than the original band (Figure 2). The temperature at which splitting occurred was pH dependent, occuring at lower temperatures as pH increased (Table 1). At pH 5 the protein precipitated, precipitation occurring at 80°C in the A variant and 85°C in the B and C variants.

Under reducing conditions only a single band was seen in both heated and unheated samples (Figure 3). No difference in mobility between the variants was seen in reduced samples.

Discussion

The mobility of β-LG A under non-reducing SDS-PAGE conditions is different to that of β-LG B and C, which have similar mobilities. As mobility in SDS is due to the SDS coating and not the charge on the protein, a difference in the mobility between proteins of almost identical molecular weight indicates that the structures of these proteins could be different. The mobilities of β-LG A, B and C under reducing SDS-PAGE conditions appear to be the same. This suggests that the difference in mobility between variants seen in non-reduced samples involves disulphide bonding.

After heating, all three variants ran as a doublet under non-reducing conditions, but as a single band under reducing conditions. As heat can induce disulphide exchange, the band formed on heating may represent a species with different intramolecular disulphide bonding. Induction of bandsplitting is pH dependent, occuring at lower temperatures as pH increases.

Conclusions

The mobility of the band formed on heating in the β-LG A variant appears to be the same as the mobility of the unheated B and C variants, whilst the mobility of the band formed in the B and C variants appears to be the same as that of the unheated A variant. If the differences in the two bands in the heated samples is due to differences in intramolecular disulphide bonding, it is possible that there is a difference in the nature of the disulphide bonding in the A variant compared the B and C variants, even in unheated samples.

Acknowledgements

This work was supported by funding from the New Zealand Dairy Board and Foundation for Research, Science and Technology, Contract No. DRI 401.

References

1. Basch, J.J., Douglas, F.W. Jr., Procino, L.G., Holsinger, V.H. and Farrell, H.M. Jr. Quantitaton of Caseins and Whey Proteins of Processed Milks and Whey Protein Concentrates, Application of Gel Electrophoresis, and Comparison with Harland-Ashworth Procedure. *J. Dairy Science* 68 23-31(1985)

2. Mailliart, P. and Ribadeau-Dumas, B.
Preparation of β-Lactoglobulin and β-Lactoglobulin-Free Proteins from Whey Retentate by NaCl Salting Out at Low pH.
J. Food Science 53 743-745,752 (1988)

3. Shimada, K and Cheftel, J.C.
Sulfhydryl Group/Disulphide Bond Interchange Reactions during Heat-Induced Gelation of Whey Protein Isolate.
J. Agric. Food Chem 37 161-168 (1989)

Figure 1.

SDS-PAGE of β-LG A, B and C under non-reducing conditions.

Figure 2.

Effect of temperature on the mobility of β-LG A, B and C in SDS-PAGE under non-reducing conditions. U represents unheated protein.

Figure 3.

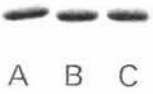
SDS-PAGE of β-LG A, B and C under reducing conditions.

Table 1. Conditions where bandsplitting observed.

pH	Temperature (°C)
5.0	No band splitting seen
6.0	85
7.0	70
8.0	60
8.4	60

Figure 1

(1a)



(1b)

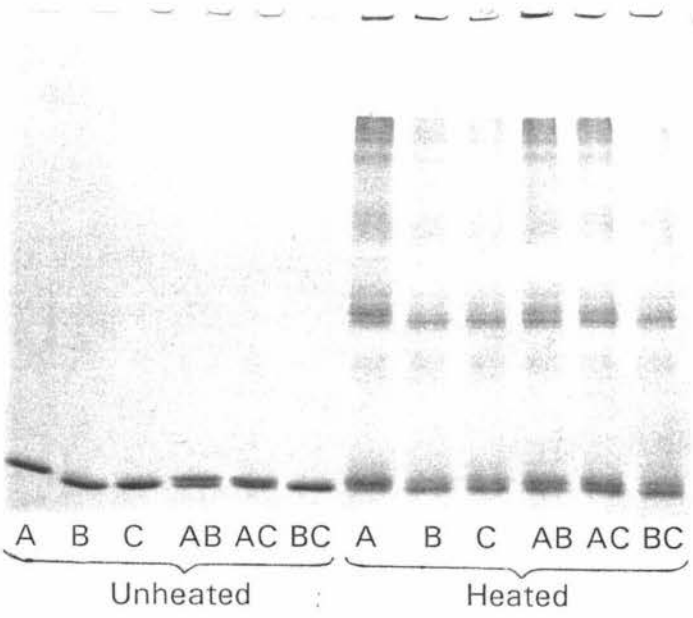
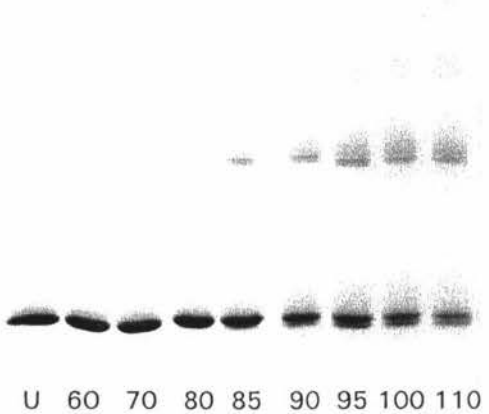
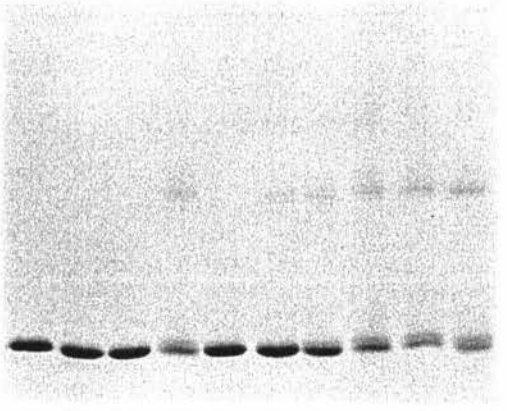


Figure 2 (2a) β-LG A



(2b) β-LG B



(2c) β-LG C

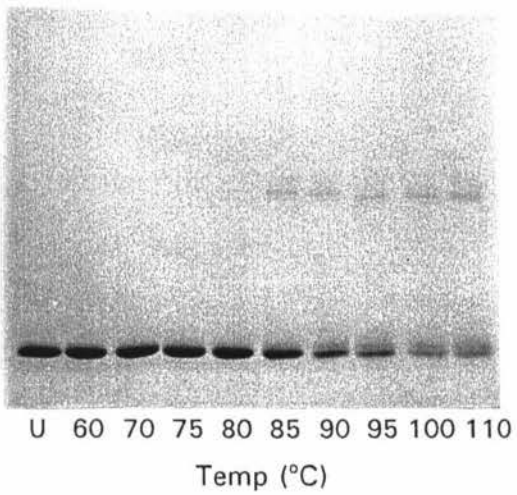
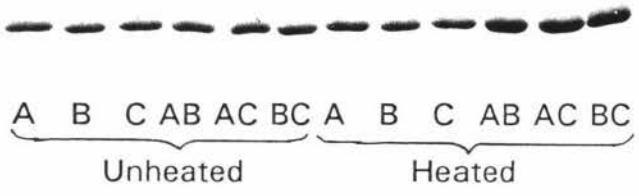


Figure 3



New Zealand Dairy Research Institute

Labelling the Free Sulphydryl Group in β-Lactoglobulin A, B and C

H. Brittan[1], J C Mudford[1], G E Norris[1], T M Kitson[2] and J P Hill[3]
[1] Department of Biochemistry, Massey University, Palmerston North, New Zealand
[2] Department of Chemistry, Massey University, Palmerston North, New Zealand
[3] Food Science Section, New Zealand Dairy Research Institute, Private Bag 11 029, Palmerston North, New Zealand

Introduction

Under some conditions there is evidence that the structures of the β-Lactoglobulin (β-LG) A, B and C variants are different, for example Hill *et al.* (2) found that β-LG A has a slightly lower mobility than the B and C variants during polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) under non-reducing conditions.

It is probable that the primary sequence differences between β-LG A, B and C are responsible for the differences in mobility/structure. The differences (Asp 64 and Val 118 in A→Gly 64 and Ala 118 in B and C) are found near the positions of the disulphide bonds Cys 66 - Cys 160 and Cys 106 - Cys 119, and the free thiol group at Cys 121 (1).

An early investigation (4) found that the free thiol group was found in equal proportions at positions Cys 119 and 121, with the disulphide bridge forming between Cys 106 and Cys 119 or Cys 121 in β-LG A, B and C. However, other studies using thiol labelling (5, 6, 8) or x-ray crystallography (7) found that the free thiol was located only at position 121 in β-LG A and B.

In this current study we examine the possibility that a difference in the position of the free thiol group is responsible for differences in the mobility/structure of the β-LG A, B and C variants. In labelling the free thiol, conditions were chosen to minimise the possibility of thiol-disulphide interchange occurring.

Methods

β-LG A, B and C was prepared by NaCl precipitation as described by Mailliart and Ribadeau-Dumas (3) and further purified by gel filtration. Purified protein was incubated with [1,4-^{14}C] N-ethyl maleimide in 0.2M phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 6.0, at 40°C for 2 hours in the presence of 0.27% SDS (w/v). Excess reagent was removed and the labelled protein was digested with TPCK-Trypsin (5% E:S) in 0.2M phosphate buffer, pH 6.0, for 14 hours at 40°C. The digest was reduced with 2-mercaptoethanol at 80°C for 1 hour. Peptides were purified by HPLC (gel filtration followed by reverse phase). The labelled peptide was identified and sequenced. Each cycle of the Edman degradation was collected and tested for radioactivity.

Results

The location of the free sulphydryl, Cys 121, is the same in all three variants (Figures 1-3).

Figures 1-3

Location of the labelled residue within the peptide fragment of the β-LG variants A, B and C.

Figure 1 β-LG A

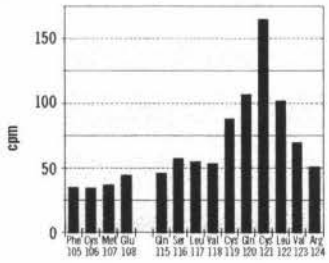


Figure 2 β-LG B

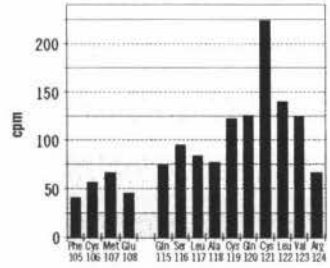
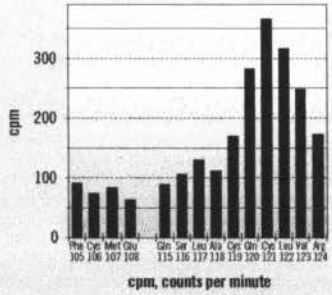


Figure 3 β-LG C



Conclusions

The free sulphydryl group is at residue 121 in β-LG A and B, confirming the findings of previous experiments (5, 6, 7, 8). The free thiol is also found at residue 121 in β-LG C. Therefore differences in mobility/structure observed between β-LG A, B and C are not due to a difference in the location of the free thiol group.

Acknowledgements

This work was supported by funding from the New Zealand Dairy Board and the Foundation for Research, Science and Technology, Contract No. DRI 401.
We gratefully acknowledge the services of the NZDRI Graphics Unit in preparing this poster.

References

1. Hambling, S. G., McAlpine, A. S. and Sawyer L. (1992). β-lactoglobulin. In: Advanced Dairy Chemistry - Volume 1. Elsevier Science Publishers Ltd., London, pp. 141-190.

2. Hill, J. P., Thresher, W. C., Boland, M. J., Creamer, L. K., Anema, S. G., Manderson, G., Otter, D. E., Paterson, G. R., Lowe, R., Burr, R. G., Motion, R. L., Winkelman, A. and Wickham, B. The Polymorphism of the Milk Protein β-lactoglobulin. A review. Biotechnology for Dairy Production. Proceedings of the OECD Conference, Wellington, New Zealand, 7-9 February 1996. CAB International (in press).

3. Mailliart, P. and Ribadeau-Dumas, B. (1988). β-lactoglobulin and β-lactoglobulin-Free Proteins from Whey Retentate by NaCl Salting Out at Low pH. *J. Food Science* **53** (3) 743-745,752.

4. McKenzie, H. A., Ralston, G. B. and Shaw, D. C. (1972). Location of Sulphydryl and Disulphide Groups in Bovine β-lactoglobulin and Effects of Urea. *Biochem.* **11**, 4539-4547.

5. Mainferme, F., Préaux, G and Lontie,R. (1971). Location of the Disulphide Bridges in the sequence of β-lactoglobulin A with CNBr and Thermolysin. *Arch. Internat Physiol. Biochim.*, **79** 840-841.

6. Martial, J., Préaux, G and Lontie, R. (1971). Location of the Thiol Group and of the Disulphide Bridges in the Sequence of β-lactoglobulin B. *Arch. Internat Physiol. Biochim.*, **79** 842-843.

7. Monaco, H.L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L. and Eliopoulos, E.E. (1987). Crystal Structure of the Trigonal Form of Bovine β-lactoglobulin and its Complex with Retinol at 2.5 Å *Resolution. J Mol. Biol.* **197** 695-706.

8. Péréz-Gómez, G., Préaux, G and Lontie, R. (1971). Location of the Disulphide Bridges in β-lactoglobulin B with Pepsin and Trypsin after CNBr Cleavage. *Arch. Internat Physiol. Biochim.*, **79** 843-844

Labelling the Free Sulphydryl Group in β-Lactoglobulin A, B and C.

H. Brittan[1], J.C. Mudford[1], G.E. Norris[1], T. M. Kitson[2] and J.P. Hill[3].

[1] Department of Biochemistry, Massey University, Palmerston North, New Zealand
[2] Department of Chemistry, Massey University, Palmerston North, New Zealand
[3] Food Science Section, New Zealand Dairy Research Institute, Palmerston North, New Zealand.

Abstract

In order to investigate the possibility of a difference in the location of the free sulphydryl group between the A, B and C variants of bovine β-Lactoglobulin (β-lg), each variant was labelled with [1,4-^{14}C] N-ethyl maleimide. The position of the free thiol was determined by digestion of the protein with trypsin, followed by sequencing of the resultant labelled peptide. In all three variants, the free sulphydryl group was found to be at position 121.

Introduction

Numerous genetic variants of bovine β-lg have been identified (3), the most common of which are A and B (3, 4). These two variants differ in amino acid sequence at positions 64 (Asp in A → Gly in B) and 118 (Val in A → Ala in B). The effect of these substitutions on the secondary and tertiary structure of the protein appears to be small. A comparison of crystals of the A and B variants (8) showed no significant conformational changes, and the CD spectra for the A and B variants (2) are essentially identical. β-lg C, which occurs at low frequency in Jersey and related breeds, differs from the B variant at position 59 (Gln in B → His in C). There have been relatively few studies involving β-lg C (4).

Under some conditions there is evidence that the structures of the β-lg A, B and C variants are different, for example Brittan *et al.* (1) found that β-lg A has a slightly lower mobility than the B and C variants during polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) under non-reducing conditions. This difference in mobility is not seen under reducing conditions, indicating that it is due to a difference in structure and not charge, and that this structure is lost following the loss of intramolecular disulphide bonding.

It is probable that the primary sequence differences between β-lg A, B and C are responsible for the differences in mobility/structure. The substitutions at positions 64 and 118 are found near the positions of the disulphide bonds (Cys 66 - Cys 160 and Cys 106 - Cys 119) and the free thiol group (Cys 121) (3). The substitution at position 59 which differentiates the β-lg B and C variants does not appear to affect mobility in SDS-PAGE.

In an early investigation, McKenzie *et al.* (5) found that the free thiol group was found in equal proportions at positions Cys 119 and 121, with the disulphide bridge forming between Cys 106 and Cys 119 or Cys 121 in β-lg A, B and C. Other studies using thiol labelling (6, 7, 9) found that the free thiol was located only at position 121 in β-lg A and B, suggesting that the conditions used by McKenzie *et al.* had induced disulphide exchange. Studies using X-ray crystallography (8) and ^{13}C-NMR (10) also found that the free thiol was located only at position 121 in both the A and B variants.

In this current investigation we determine the position of the free thiol group in the β-lg C variant and examine the possibility that a difference in the position of the free thiol group is responsible for differences in the mobility/structure of the β-lg A, B and C variants. In

labelling the free thiol, conditions were chosen to minimise the possibility of thiol-disulphide interchange occurring.

Methods

β-LG A, B and C was prepared as described by Brittan *et al.*(1). A stock solution of N-ethyl maleimide (NEM) was prepared from 7 μmoles of [1,4-^{14}C] NEM in 1ml hexane (American Radiolabeled Chemicals Inc., St. Louis, Mi.) and 33 μmoles of unlabelled NEM (Sigma Chemical Co., St. Louis, Mi.), dissolved in 5 ml of hexane. Three hundred μl alliquots were taken and the hexane evaporated under nitrogen to near dryness just prior to use. Purified protein was incubated with NEM and sodium dodecyl sulphate (SDS) (molar ratio βlg:NEM:SDS 1:1.65:15.5) in 0.2M phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 6.0), at 40°C for 2 hours. Excess reagent was removed by ultrafiltration in a 50ml Amicon stirred cell,using a YM10 membrane, following dilution of the sample 650-fold with phospate buffer and concentrated to 10ml. The labelled protein was digested with TPCK-Trypsin (Sigma Chemical Co., St. Louis, Missouri.) (5% E:S) in 0.2M phosphate buffer, pH 6.0, for 14 hours at 40°C. The digest was reduced with 2-mercaptoethanol at 80°C for 1 hour.

Peptides were purified by HPLC (Spectra-Physics SP8800 ternary HPLC pump, SP8490 detector). Fifty μl of the reduced digest were loaded directly onto a size exclusion column (BIOSEP SEC-S2000, 300 x 7.80mm Phenomenex, solvent: 36% acetonitrile, 0.1% TFA). Protein in the peaks eluting from the column were collected and freeze dried. Individual peptides were isolated from size exclusion fractions by reverse phase chromatography on a C18 column (Alltech/Applied Science, Vydac 218TP C18 10μ), 0-50% acetonitrile, 0.1-0.08% TFA gradient).

Peptides were examined for radioactivity using liquid scintilation counting in a Beckman LS8000 scintillation counter. The labelled peptide was then sequenced (Applied Biosystems Protein Sequencer Model 476A, data analysed model 610A Version 1.2.2, Applied Biosystems software). Each cycle of the Edman degradation was collected and tested for radioactivity as described above.

Results

The same peptide (Tyr 102-Arg 124) (for sequence see ref. 3) was found to be radioactive in each of the βlg variants, with a peak in radioactivity being associated with Cys 121 (Figures 1-3). Differences in the heights of the peaks are probably due to variations in the amount of peptide loaded onto the sequencer.

Conclusions

The free thiol group is at residue 121 in β-lg A and B, confirming the findings of previous experiments (6, 7, 8, 9, 10). The free thiol is also found at residue 121 in β-lg C. Therefore differences in mobility/structure observed between β-lg A, B and C are not due to a difference in the location of the free thiol group.

Acknowledgements

This work was supported by funding from the New Zealand Dairy Board and Foundation for Research, Science and Technology, Contract No. DRI 401.

References

1. Brittan, H., Lowe, R., Norris, G.E., Kitson, T.M. and Hill, J.P. (1997). Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis of β-lactoglobulin Variants A, B and C. Relative mobilities of heated and unheated samples. Milk Protein Polymorphism. Proceedings of the International Dairy Federation Seminar, Palmerston North, New Zealand, February 25-27, 1997, pp??

2. Dong, A., Matsuura, J., Allison, S. D., Chrisman, E., Manning, M. C. and Carpenter, J. F. (1996). Infrared and Circular Dichroism Spectroscopic Characterization of Structural Differences between β-lactoglobulin A and B. *Biochemistry* **35**, 1450-1457.

3. Hambling, S. G., McAlpine, A. S. and Sawyer L. (1992). β-lactoglobulin. In: *Advanced Dairy Chemistry - Volume 1*. Elsevier Science Publishers Ltd., London, pp. 141-190.

4. Hill, J. P., Thresher, W. C., Boland, M. J., Creamer, L. K., Anema, S. G., Manderson, G., Otter, D. E., Paterson, G. R., Lowe, R., Burr, R. G., Motion, R. L., Winkelman, A. and Wickham, B. The polymorphism of the milk protein β-lactoglobulin. A review. *Biotechnology for Dairy Production.* Proceedings of the OECD Conference, Wellington, New Zealand, 7-9 February 1996. CAB International (in press).

5. McKenzie, H. A., Ralston, G. B. and Shaw, D. C. (1972). Location of sulphydryl and disulphide groups in bovine β-lactoglobulin and effects of urea.
Biochem. **11**, 4539-4547.

6. Mainferme, F., Préaux, G and Lontie, R. (1971).
Location of the disulphide bridges in the sequence of β-lactoglobulin A with CNBr and Thermolysin.
Arch. Internat Physiol. Biochim., **79** 840-841

7. Martial, J., Préaux, G and Lontie, R. (1971).
Location of the thiol group and of the disulphide bridges in the sequence of β-lactoglobulin B.
Arch. Internat Physiol. Biochim., **79** 842-843

8. Monaco, H.L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L. and Eliopoulos, E.E. Crystal Structure of the trigonal Form of Bovine Beta-lactoglobulin and its Complex with Retinol at 2.5 Å Resolution. J Mol. Biol. 1987 197 695-706

9. Péréz-Gómez, G., Préaux, G and Lontie, R. (1971).
Location of the disulphide bridges in β-lactoglobulin B with Pepsin and Trypsin after CNBr Cleavage.
Arch. Internat Physiol. Biochim., **79** 843-844

10. Phelan, P. and Malthouse, J. P. G. (1994).
^{13}C-n.m.r. of the cyanylated β-lactoglobulins: evidence that Cys-121 provides the free thiol group in β-lactoglobulins A and B. *Biochem. J.* **302**, 511-516.

Figures 1-3.

Location of the labelled residue within the peptide fragment of the β-lg variants A, B and C. cpm - radioactivity in counts per minute.

Figure 1 **B-LG A**

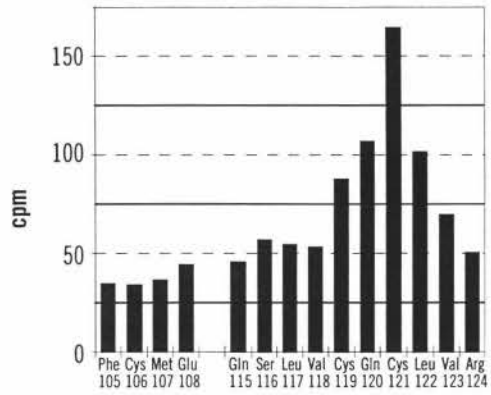


Figure 2 **B-LG B**

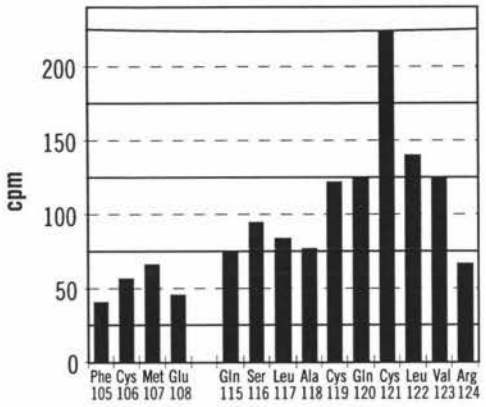
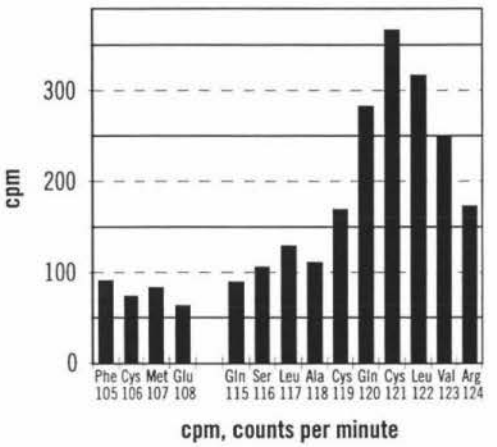


Figure 3 **B-LG C**



cpm, counts per minute