

## Characterization of heat-induced aggregates of $\beta$ -lactoglobulin, $\alpha$ -lactalbumin and bovine serum albumin in a whey protein concentrate environment

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**SUMMARY.** Bovine  $\beta$ -lactoglobulin ( $\beta$ -lg),  $\alpha$ -lactalbumin ( $\alpha$ -la) and bovine serum albumin (BSA), dispersed in ultrafiltration permeate, that had been prepared from whey protein concentrate solution (100 g/kg, pH 6.8), were heated at 75 °C. The consequent protein aggregation was studied by one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). When 100 g  $\beta$ -lg/kg permeate solution was heated at 75 °C, cooled and examined, large aggregates were observed. These aggregates were partially dissociated in SDS solution to give monomers, disulphide-bonded dimers, trimers and larger aggregates. When mixtures of  $\beta$ -lg and  $\alpha$ -la or BSA were heated, homopolymers of each protein as well as heteropolymers of these proteins were observed. These polymer species were also observed in a heated mixture of the three proteins. Two-dimensional PAGE of mixtures demonstrated that these polymer species contained disulphide-bonded dimers of  $\beta$ -lg,  $\alpha$ -la and BSA, and 1:1 disulphide-bonded adducts of  $\alpha$ -la and  $\beta$ -lg, or BSA. These results are consistent with a mechanism in which the free thiols of heat-treated  $\beta$ -lg or BSA catalyse the formation of a range of monomers, dimers and higher polymers of  $\alpha$ -la. It is likely that when whey protein concentrate is heated under the present conditions, BSA forms disulphide-bonded strands ahead of  $\beta$ -lg and that  $\alpha$ -la aggregation with  $\beta$ -lg and with itself is catalysed by the heat-induced unfolded BSA and  $\beta$ -lg.

**KEYWORDS:**  $\beta$ -Lactoglobulin,  $\alpha$ -lactalbumin, bovine serum albumin, heat-induced aggregation.

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Whey proteins are highly functional food proteins and are extensively used in foods as gelling ingredients. Consequently, the heat-induced gelation of whey protein isolates (WPI) and concentrates (WPC) has been studied extensively over the last

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few years (Tang *et al.* 1993, 1995; Aguilera, 1995; Havea *et al.* 1998). The major protein components of whey include  $\beta$ -lactoglobulin ( $\beta$ -lg; 50%),  $\alpha$ -lactalbumin ( $\alpha$ -la; 20%) and BSA (5%), and it is generally agreed that the characteristics of the major protein,  $\beta$ -lg, dominate the behaviour of WPC and WPI. The heat-induced protein-protein interactions that precede gelation have been studied extensively in different buffers at low protein concentrations using a wide range of techniques (Griffin *et al.* 1993; Elofsson *et al.* 1996; Boye *et al.* 1997; Hoffmann & van Mil, 1997) with some studies also at higher protein concentrations (Matsuura & Manning, 1994; Hollar *et al.* 1995; Qi *et al.* 1995). It is generally accepted that thiol-disulphide interchange reactions, leading to the formation of inter-molecular disulphide bonds, play a major role in the heat-induced aggregation and gelation of  $\beta$ -lg. McSwiney *et al.* (1994a, b) showed that in addition to the intermolecular disulphide bonds, stable non-covalently linked aggregates were also formed prior to gel formation in heated solutions of 100 g  $\beta$ -lg/kg solvent.

Since whey proteins are a heterogenous group of proteins, it is likely that the gelation behaviour of any individual protein would be altered by the presence of the others. A number of studies have shown that soluble aggregates of whey proteins are formed during the early stages of heat-induced gel formation, and that subsequent polymerization results in the formation of a rigid gel network (Baer *et al.* 1976). It is possible that gel properties may be influenced by the formation of specific soluble aggregates between different whey proteins in the early stages of heating. For example, addition of BSA or  $\alpha$ -la to a solution of  $\beta$ -lg increases the rigidity of the resultant gels and disulphide-bonded complexes are formed between  $\beta$ -lg and BSA or  $\alpha$ -la (Matsudomi *et al.* 1992, 1993, 1994; Gezimati *et al.* 1996a, b 1997). Recent studies on the aggregation and gelation of mixtures of  $\beta$ -lg and BSA (Gezimati *et al.* 1996a) or  $\alpha$ -la (Gezimati *et al.* 1996b; 1997; Dalgleish *et al.* 1997) indicated that non-covalently linked aggregates were also formed in these mixtures at the early stages of gel formation.

Havea *et al.* (1998) have shown that when WPC solutions were heated and then examined by one-dimensional (1D) and two-dimensional (2D)-PAGE, there was evidence for the formation of specific intermediate aggregates between various whey proteins. More recently we (Havea *et al.* 2000) investigated the effect of heat on the model system of BSA and  $\alpha$ -la in WPC permeate and concluded that BSA aggregates catalysed the formation of disulphide-bonded  $\alpha$ -la dimers. The present investigation extends that study by examining in greater detail the intermediate protein aggregates formed during the interactions between  $\beta$ -lg and BSA, or  $\alpha$ -la, or both in the early stages of gel formation in permeate prepared from whey protein concentrate solutions.

## MATERIALS AND METHODS

### *Materials*

$\beta$ -Lg (product L-2506),  $\alpha$ -la (product L-5385), BSA (product A-4378), molecular weight standards and gel buffer salts were obtained from Sigma (St. Louis, MO 63178, USA). The extent of the changes to the proteins in these samples was assessed by alkaline PAGE and the most appropriate samples selected for further examination. In all cases, there were small quantities of protein impurities, which were usually other whey or blood proteins and polymers of  $\beta$ -lg,  $\alpha$ -la or BSA. A commercial WPC powder, derived from acid whey, was obtained from the New Zealand Dairy Board (Wellington, New Zealand). Most of the reagents used for the

preparation of electrophoresis gels were obtained from Bio-Rad Laboratories (Hercules, CA 94547, USA). Water was purified using a MilliQ-system (Millipore Corp., Bedford, MA 01730, USA).

#### *Sample preparation*

WPC solutions (100 g/kg) were prepared by dissolving WPC powder in water, and the mixtures were then stirred for 2 h at room temperature using a magnetic stirrer, and the pH was adjusted to pH 6.8 using 0.1 M-NaOH or HCl. WPC permeate was obtained using an ultrafiltration unit equipped with a SO-HF-131-VIV filter and was then used to dissolve  $\beta$ -lg,  $\alpha$ -la and BSA to give 100 g/kg solutions of the proteins. Analyses of the WPC permeate showed that it contained 4.3 g lactose/kg, no protein, and 4.2, 36, 4.0 and 0.5 mmol/kg Ca, K, Na and Mg respectively. Mixtures of  $\beta$ -lg and  $\alpha$ -la or BSA at a ratio of 2:1 were prepared by dissolving 6.65 g  $\beta$ -lg and 3.35 g  $\alpha$ -la or BSA in WPC permeate and made up to 100 g. Mixtures of  $\beta$ -lg,  $\alpha$ -la and BSA were prepared by dissolving 5.0 g  $\beta$ -lg and 2.5 g  $\alpha$ -la and 2.5 g BSA in WPC permeate and made up to 100 g. The pH was re-adjusted to 6.8. Aliquots (0.4 ml) were transferred into 2-ml round-bottomed Beckman polyallomer centrifuge tubes (0.35 mm wall thickness, 11 mm internal diameter, 34 mm height) which were closed with appropriately fitted lids, and heated for up to 10 min in a water-bath thermostatically controlled at 75 °C (It took 10 s for the samples to reach 74.9 °C). The tubes and heated WPC solutions were immediately placed in ice water for 5 min and then held at room temperature ( $\sim 20$  °C) for 2 h. The heated solutions (plus unheated controls) were then analysed using 1D-PAGE.

#### *Polyacrylamide gel electrophoresis*

The protein samples were analysed using the Mini-Protein II dual slab cell system (Bio-Rad Laboratories) using discontinuous PAGE system for both the 1D and 2D separations. The methods described by Havea *et al.* (1998) and Manderson *et al.* (1998) were used for the 1D and 2D alkaline- and SDS-PAGE. The 1D PAGE was either run on alkaline- (Andrews, 1983) or SDS-PAGE (Laemmli, 1970). After preparing the appropriate gel, 10  $\mu$ l samples of 0.1 g protein/kg solutions, diluted with appropriate sample buffer, were injected into the sample wells and then electrophoresed to separate the proteins. For the alkaline 2D-PAGE, a strip from the alkaline gel, containing the protein bands that had been separated in a first dimensional run, was cut and rinsed in SDS sample buffer. This strip was placed on top of a SDS gel and run in a second dimension. For the SDS-reduced 2D-PAGE, a strip from a SDS gel, containing the protein bands that had been separated in a first-dimensional run, was cut, placed in hot (94 °C) solution of SDS buffer containing 2-mercaptoethanol (5 ml/l SDS sample buffer) for 30 s. The gel strip was then rinsed with water to remove excess 2-mercaptoethanol, then used for a second dimensional run on another SDS gel (Havea *et al.* 1998; Manderson *et al.* 1998).

## RESULTS

#### *Heat treatment of $\beta$ -lg solutions*

The alkaline- and SDS-PAGE patterns of the unheated  $\beta$ -lg (100 g/kg) samples showed intense monomer bands, and faint bands corresponding to dimers and other impurities (Fig. 1a & b). When the samples were heated at 75 °C (Fig. 1a), the intensity of the native-like  $\beta$ -lg band and the dimer band (which had molecular

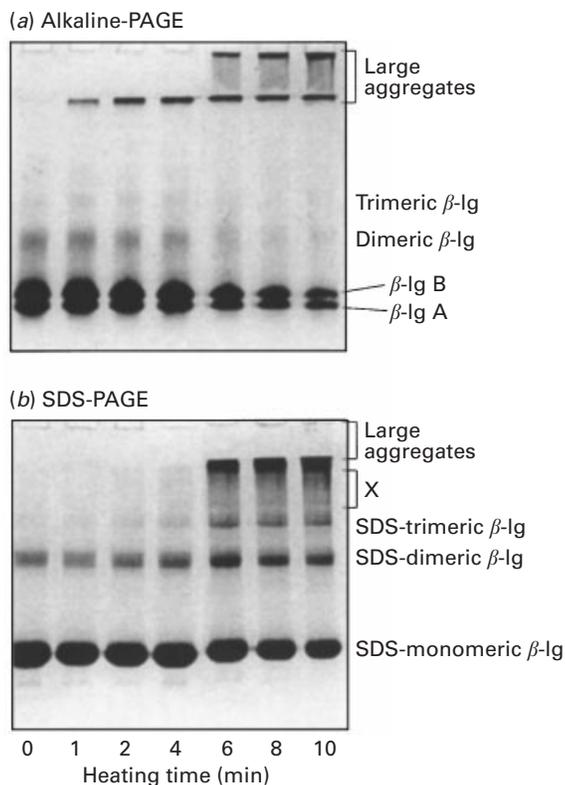


Fig. 1. Typical electrophoretic patterns of  $\beta$ -lactoglobulin ( $\beta$ -lg) solutions (100 g/kg) heated at 75 °C for various times obtained using (a) alkaline-PAGE and (b) SDS-PAGE. Direction of mobility is from top to bottom.

weight of  $\sim 38$  kDa) diminished with heating time with corresponding increases in the intensities of the material caught on top of the resolving gel and within the stacking gels (Fig. 1a).

SDS-PAGE patterns of heated samples (Fig. 1b) showed that the intensity of monomeric  $\beta$ -lg band decreased with heating time at 75 °C. The samples that were heated for  $\sim 4$  min showed a series of bands close to the beginning of the resolving gel (labelled as region X). These bands corresponded to various disulphide-linked aggregates of  $\beta$ -lg. The material caught on top or within the stacking gel of the alkaline-PAGE (Fig. 1a) had migrated into the resolving gel in the SDS-PAGE system (Fig. 1b). This suggests that the material caught within the stacking gel of the alkaline-PAGE (Fig. 1a) consisted of large protein aggregates that are dissociated to smaller aggregates in SDS solutions, in agreement with the results of McSwiney *et al.* (1994a).

The protein aggregate bands observed in SDS-PAGE (i.e. disulphide-bonded aggregates) were further characterized using 2D PAGE. The identifications made for the bands in Fig. 1b have been transferred to Fig. 2 containing the 2D gel patterns. These patterns showed that most of the material caught in the sample well travelled into the gel (Fig. 2). A series of spots, corresponding to monomer, dimer, trimer and greater molecular aggregates before mercaptoethanol treatment could be observed

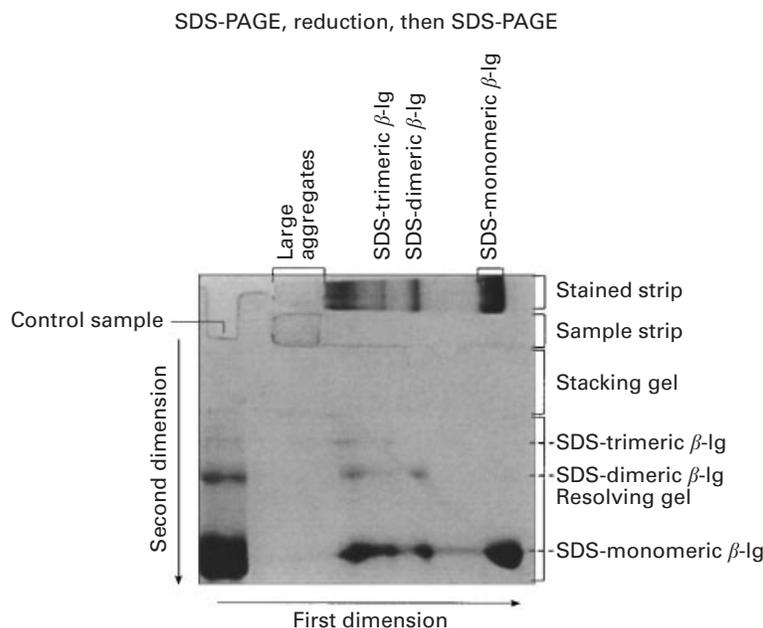


Fig. 2. Two-dimensional electrophoretic patterns of a heat-treated ( $75^{\circ}\text{C}$  for 8 min) solution of  $\beta$ -lactoglobulin (100 g/kg solution) in permeate prepared from a solution of whey protein concentrate powder in water (100 g/kg). First dimension, SDS-PAGE without treatment with 2-mercaptoethanol; second dimension, SDS-PAGE of samples within a gel strip that was treated with 2-mercaptoethanol. At the top of each gel a stained gel strip shows the protein bands from the first dimension separation running from left to right. Immediately below this strip is a second strip, which had contained the same protein bands that were used as samples for the second-dimension separation. On the left-hand side, a portion of the corresponding sample was run from top to bottom, to help identify the protein bands.

(Fig. 2). There were also a number of faint spots that had mobilities corresponding to  $\beta$ -lg dimer after reduction, indicating that after heat treatment a proportion of the  $\beta$ -lg existed as dimers and trimers linked by non-disulphide covalent bonds. Alternatively, dimerization of the monomeric protein may have occurred through oxidation of thiol groups during sample manipulation or electrophoresis. Non-disulphide bonded  $\beta$ -lg has also been observed by Gezimati *et al.* (1996b) in mixtures of  $\beta$ -lg and  $\alpha$ -la heated at  $75^{\circ}\text{C}$  using 1D-PAGE.

#### Heat treatment of mixtures of $\beta$ -lg and $\alpha$ -la

The alkaline-PAGE patterns (Fig. 3a) of the unheated 2:1 mixture of  $\beta$ -lg and  $\alpha$ -la (100 g/kg solvent) showed intense monomer bands and a faint band, corresponding to  $\beta$ -lg dimer. Heating the mixture at  $75^{\circ}\text{C}$  gave patterns in which the intensity of these bands decreased, and the amounts of material in the stacking gel area or that did not enter the stacking or resolving gels increased with heating time (Fig. 3a).

The SDS-PAGE patterns of the heated mixtures of  $\beta$ -lg and  $\alpha$ -la (Fig. 3b) showed that there was more material caught on top and within the stacking gel than when  $\beta$ -lg was heated alone and examined by SDS-PAGE (Fig. 1b). There was a series of very faint bands of lower mobility than  $\beta$ -lg monomer (labelled as Y), corresponding to various disulphide-linked aggregates of  $\beta$ -lg (cf. Fig. 1b) and mixed aggregates of

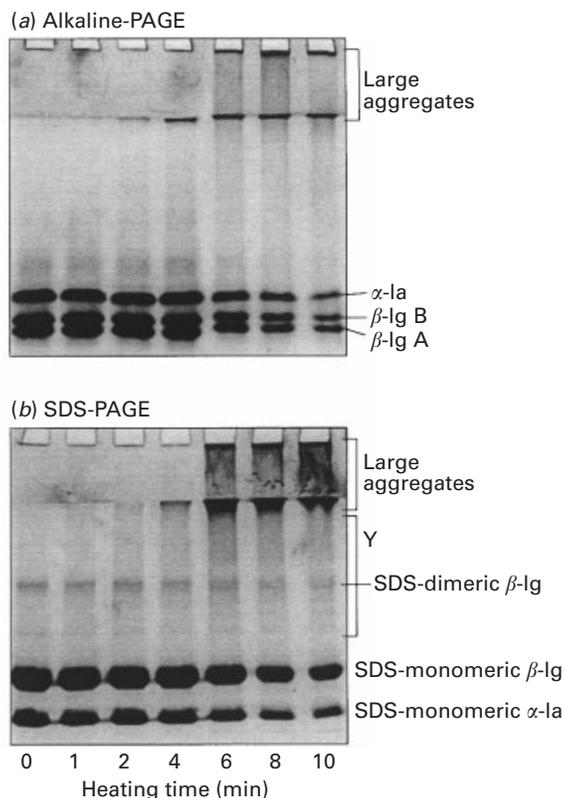


Fig. 3. Typical electrophoretic patterns of 2:1 mixtures of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin ( $\alpha$ -la) solutions (100 g/kg) heated at 75 °C for various times obtained using (a) alkaline-PAGE and (b) SDS-PAGE. See Fig. 1 for direction of protein mobility and symbols.

both  $\alpha$ -la and  $\beta$ -lg. The  $\beta$ -lg dimer band appeared to diminish slowly with heating time (Fig. 3b), in contrast to the increasing intensity observed for this band in the solution of  $\beta$ -lg heated alone (Fig. 1b). These results suggest that heating a solution of  $\beta$ -lg alone resulted in the formation of higher proportions of smaller aggregates (e.g. dimers and trimers) than with heat-treated mixtures of  $\beta$ -lg and  $\alpha$ -la.

The SDS 2D-PAGE (Fig. 4) patterns showed that the large aggregates that were caught within the stacking gel were solubilized by mercaptoethanol treatment to give a long smear of material corresponding to various bands in the sample strip. The intermediate aggregates resolved to give various spots corresponding to dimers and trimers of either proteins and possibly adducts of the two proteins.

#### *Heat treatment of mixtures of $\beta$ -lg and BSA*

Both of the alkaline- and SDS-PAGE patterns of the heated mixtures of  $\beta$ -lg and BSA (results not shown) showed typical faster loss of native BSA during heating, compared with  $\beta$ -lg. In addition, a fine band with lower mobility than BSA monomer appeared after heating for 30 s and increased in intensity with heating time up to 90 s, and then diminished in intensity with heating time. The SDS 2D-PAGE patterns of the heated mixtures (Fig. 5) showed that this band dissociated after treatment with mercaptoethanol to give spots corresponding to both monomeric

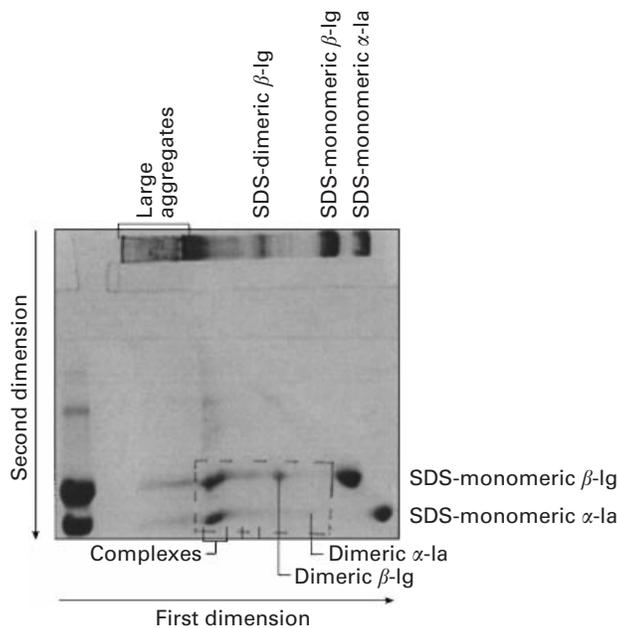


Fig. 4. Two-dimensional electrophoretic patterns of heat-treated (75 °C for 8 min) solution of a 2:1 mixture of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin (100 g/kg) in permeate prepared from a solution of whey protein concentrate powder in water (100 g/kg). See Fig. 2 for further details.

proteins. This band, which was not present in the unheated sample, corresponded to a disulphide-bonded 1:1  $\beta$ -lg-BSA adduct formed during heating. In addition, there were spots dissociated from the sample gel strip, corresponded to  $\beta$ -lg dimer and trimer.

#### Heat treatment of mixtures of $\beta$ -lg, $\alpha$ -la and BSA

The alkaline- and SDS-PAGE patterns of the unheated (0 heating time) and heated 2:1:1 mixture of  $\beta$ -lg,  $\alpha$ -la and BSA (100 g/kg) are shown in Fig. 6a, and Fig. 6b, respectively. The intermediate aggregates that were observed in heated  $\beta$ -lg (Fig. 1) or mixtures of  $\beta$ -lg and  $\alpha$ -la (Fig. 3) or BSA (results not shown) were less apparent in these mixtures. Some fine bands (labelled as M) with lower mobility than BSA bands became apparent after heating for 2 min in both alkaline- and SDS-PAGE patterns, and then decreased in intensity with heating time.

The SDS 2D-PAGE pattern of the heated mixture of the three proteins (Fig. 7) showed that the aggregate bands in the sample gel strip dissociated to give spots corresponding to the monomeric proteins. The positions of these spots indicated the formation of various aggregates during heating. These included homopolymers of each proteins as well as adducts (Fig. 7b). The monomeric spots of  $\alpha$ -la or  $\beta$ -lg arising from dimer and trimers after reduction could be clearly identified. There were three pairs of monomeric spots that arose from resolving adducts of the two proteins. The most intense pair of spots (labelled 'I', Fig. 7b) arose from large aggregates caught on top of the resolving gel or which travelled short distances into the gel, indicating that these aggregates consisted of disulphide-bonded polymers of  $\beta$ -lg and  $\alpha$ -la. The spots, labelled II, arose from reduction of a band corresponding to a molecular weight of  $\sim 62$  kDa, which was consistent with the molecular weight of a  $\beta$ -lg- $\alpha$ -la

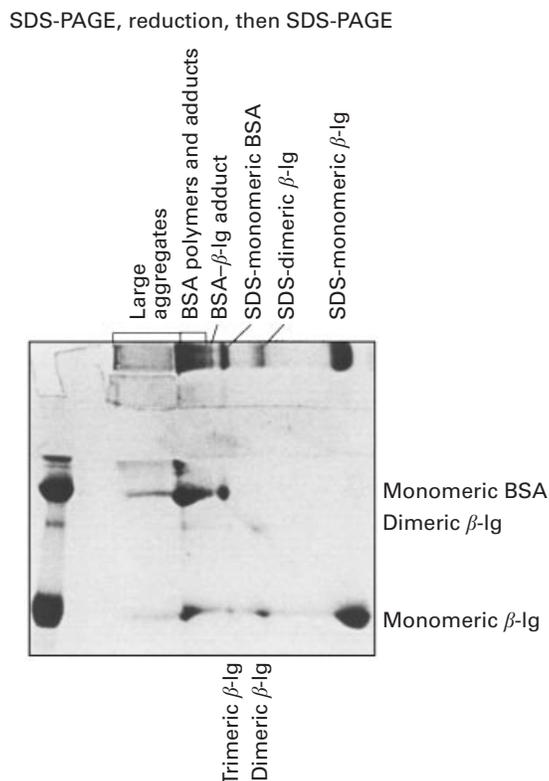


Fig. 5. Two-dimensional electrophoretic patterns of heat-treated ( $75^{\circ}\text{C}$  for 8 min) solution of a 2:1 mixture of  $\beta$ -lactoglobulin and BSA (100 g/kg) in permeate prepared from a solution of whey protein concentrate powder in water (100 g/kg). See Fig. 2 for further details.

complex tetramer. The other pair of spots (labelled 'III' Fig. 7b), solubilized from a band corresponding to a molecular weight of  $\sim 32$  kDa, representing  $\beta$ -lg- $\alpha$ -la disulphide bonded adduct formed. These two spots ran just ahead of the  $\beta$ -lg monomer band that was dissociated from  $\beta$ -lg dimer (36 kDa). There were two other spots that corresponded to only  $\alpha$ -la; one was solubilized from disulphide-bonded  $\alpha$ -la trimers (42 kDa), whereas the other was from  $\alpha$ -la dimers ( $\sim 28$  kDa). Two spots of  $\beta$ -lg, that were monomeric after reduction, arose from  $\beta$ -lg dimers and trimers.

#### *Development of aggregates during heat treatment of protein solutions*

The development of heat-induced aggregates in mixtures of  $\beta$ -lg,  $\alpha$ -la and BSA was followed using SDS 2D-PAGE (Fig. 8). After heating at  $75^{\circ}\text{C}$  for 1 min, a few non-diagonal spots corresponding to BSA were seen (Fig. 8a), indicating that BSA had started to aggregate with itself during the early stages of heating. After heating for 2 min, BSA spots became more intense (Fig. 8b) and faint spots corresponding to  $\beta$ -lg and  $\alpha$ -la appeared. After heating for 4 min (Fig. 8c), a similar spot pattern was observed but the series of spots corresponding to reduced monomeric  $\beta$ -lg and  $\alpha$ -la were more intense. Heating for 6 min (Fig. 8d), resulted in more intense and sharply defined spots corresponding to  $\beta$ -lg,  $\alpha$ -la and BSA.

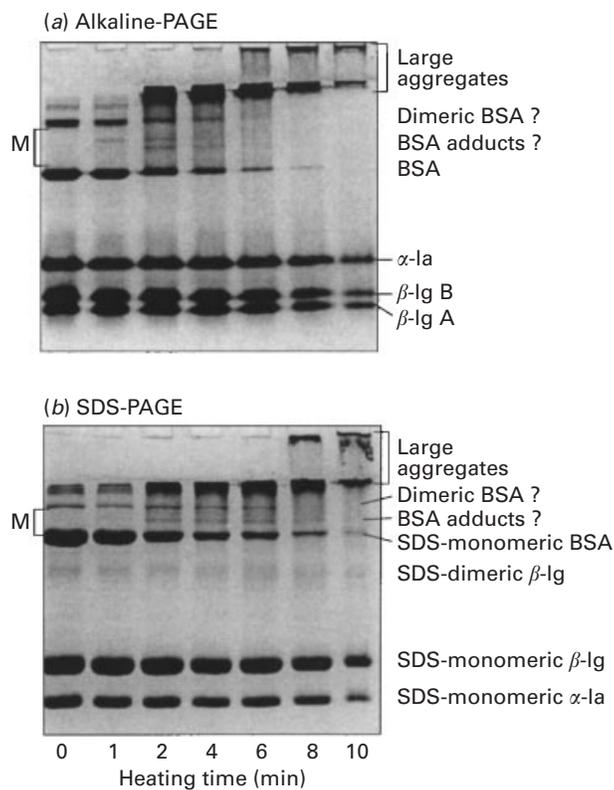


Fig. 6. Typical electrophoretic patterns of 2:1:1 mixtures of  $\beta$ -lactoglobulin, BSA and  $\alpha$ -lactalbumin solutions (100 g/kg) heated at 75 °C for various times obtained using (a) alkaline-PAGE and (b) SDS-PAGE. See Fig. 1 for further details.

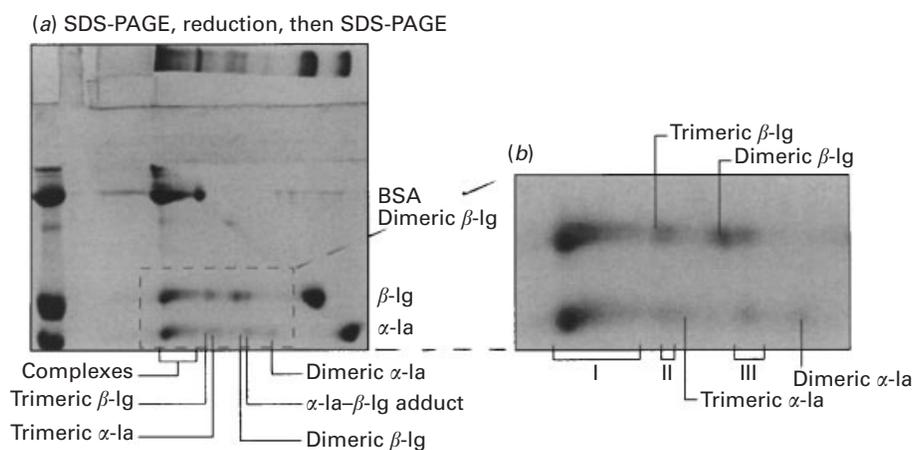


Fig. 7. Two-dimensional electrophoretic patterns of heat-treated (75 °C for 6 min) 2:1:1 mixtures of  $\beta$ -lactoglobulin, BSA and  $\alpha$ -lactalbumin solutions (100 g/kg) in permeate prepared from a solution of whey protein concentrate powder in water (100 g/kg). (b) is a magnification of the highlighted box in (a). See Fig. 2 for further details.

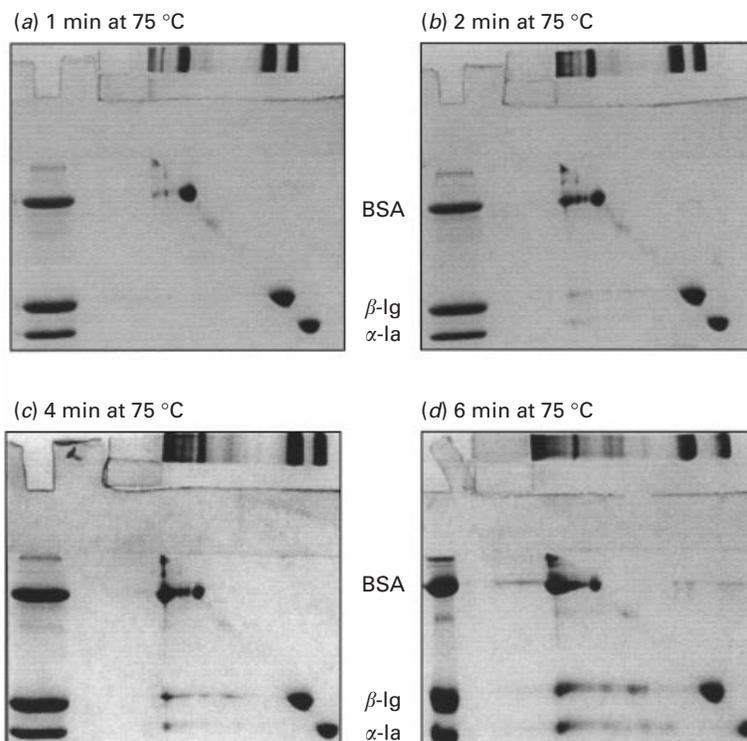


Fig. 8. Two-dimensional electrophoretic patterns of 2:1:1 mixtures of  $\beta$ -lactoglobulin, BSA and  $\alpha$ -lactalbumin solutions (100 g/kg) in permeate prepared from a solution of whey protein concentrate powder in water (100 g/kg) heat-treated at 75 °C for (a), 1 (b), 2; (c), 4; (d), 8 min. First dimension SDS-PAGE without treatment with 2-mercaptoethanol; second dimension, SDS-PAGE of samples within gel strip that were treated with 2-mercaptoethanol.

#### DISCUSSION

In our earlier work (Havea *et al.* 1998) we examined the protein aggregates formed in heated commercial WPC solutions and found evidence of formation of various intermediates and complexes between different whey proteins. In order to gain a better understanding of the nature and type of these complexes and intermediates in this study we used pure protein model systems, in which the ratios of proteins, dissolved in WPC permeate (free of casein, macro-peptides and other minor whey proteins, such as Ig and lactoferrin), were altered. The advantage of using WPC permeate is that the proteins are in the neutral pH, calcium-loaded form that would be expected to exist in a WPC system.

The heat-induced aggregation of  $\beta$ -lg has been the subject of numerous studies in recent years (e.g. Foegeding *et al.* 1995; McSwiney *et al.* 1994; Manderson *et al.* 1998). Recent studies using CD spectroscopy (Prabakaran & Damodaran, 1997) and  $^1\text{H}$  NMR and deuterium exchange (Belloque & Smith, 1998) support the proposal (Qi *et al.* 1995, 1997; Morgan *et al.* 1999) that the initiation reaction in heat-induced aggregation of  $\beta$ -lg involved critical conformational changes in  $\beta$ -lg to form reactive monomers which then react with each other via sulphydryl-disulphide exchange reactions to form dimers and other aggregates. Initially, disulphide-bonded dimers formed and these reacted further leading to the formation of higher molecular weight polymers (Prabakaran & Damodaran, 1997). Manderson *et al.* (1998) also noted the

relatively high concentration of disulphide-bonded dimers present in heated solutions of  $\beta$ -lg and that some trimers and tetramers dissociated to give monomers and disulphide-bonded dimers and suggested that these dimers could be important intermediates in the further aggregation of  $\beta$ -lg. These results were recently confirmed by Schokker *et al.* (1999) using a combination of size-exclusion chromatography and multi-angle light scattering.

Heat treatment of  $\beta$ -lg under the conditions of this study gave rise to the formation of disulphide linked dimers, trimers and higher molecular weight aggregates (Figs 1 & 2), confirming the earlier results (e.g. Gezimati *et al.* 1996a, b). However, Manderson *et al.* (1998), using similar techniques, reported a larger number of protein products of intermediate mobility in alkaline PAGE, a faster loss of native-like  $\beta$ -lg from solution and a lower dissociation of monomer and dimer from the large aggregates by SDS. There are three differences between these studies; a difference in protein concentration (3.5 g/l *v.* 100 g/kg), a difference in sources of  $\beta$ -lg (prepared from raw milk *v.* Sigma product), and a difference in buffer systems (phosphate buffer *v.* permeate from whey protein concentrate).

A protein concentration effect on the size distribution of aggregates and the degree of dissociation by SDS has been noted previously (Hoffmann & van Mil, 1997; Havea *et al.* 1998) and increased salt and/or calcium concentration is known to increase aggregation and gelation in heated  $\beta$ -lg solutions (Foegeding *et al.* 1995). The results of Havea *et al.* (1998) indicate that the protein concentration effect is very significant and sufficient to explain the relatively small number of intermediate products (dimers, trimers etc.) in the present results.

When the mixture of  $\beta$ -lg and  $\alpha$ -la was heated at 75 °C, disulphide-linked aggregates were formed between the two proteins (Figs 3 & 4), which is in agreement with previous observations (Matsudomi *et al.* 1992, 1993; Hines & Foegeding, 1993; Dalgleish *et al.* 1997; Gezimati *et al.* 1997). In addition, we observed the formation of intermediate disulphide-linked homopolymers (especially dimers and trimers) of  $\beta$ -lg or  $\alpha$ -la. The formation of dimers of  $\alpha$ -la is particularly interesting. It could arise from a reaction that involves interaction between a disulphide bond of  $\alpha$ -la molecule with the exposed thiol of  $\beta$ -lg, followed by thiol-catalysed disulphide exchange with a second  $\alpha$ -la molecule and finally thiol exchange back to the  $\beta$ -lg as outlined for enhanced  $\alpha$ -la polymer formation in heat-treated solutions of mixtures of  $\alpha$ -la and BSA (Havea *et al.* 2000). The intensity of the spots corresponding to  $\alpha$ -la homopolymers (dimer and trimer) formed in heated  $\alpha$ -la and  $\beta$ -lg mixture (Fig. 4), was less than in  $\alpha$ -la and BSA mixtures (Havea *et al.* 2000), suggesting that BSA was more effective than  $\beta$ -lg in catalysing the formation of  $\alpha$ -la polymers. This can be explained by the differences between the thermal transition temperatures of the two proteins. BSA denatures at 64 °C, compared with 78 °C for  $\beta$ -lg, whereas  $\alpha$ -la unfolds at  $\sim$  62 °C (Ruegg *et al.* 1977; de Wit, 1984; Kinsella & Whitehead, 1989). Because BSA and  $\alpha$ -la denature at relatively similar temperatures, the availability of a free thiol group in a denatured BSA readily catalyses the formation of disulphide bond between two thermally altered  $\alpha$ -la molecules before re-naturation can occur.

When the mixtures of  $\beta$ -lg and BSA were heated at 75 °C, the intermediate polymers of  $\beta$ -lg (dimer and trimer) formed were similar to those formed when  $\beta$ -lg was heated by itself (Fig. 2). It appears that the intermediate aggregates formed in heated mixtures of BSA and  $\beta$ -lg were mostly homopolymers of each protein, which confirms the results of Gezimati *et al.* (1996a). This can be explained by the thermal transition temperature of BSA being lower than that of  $\beta$ -lg (Ruegg *et al.* 1977) and the majority of the BSA molecules would be denatured and aggregated during the

very early stage of heating ( $\sim 1$  min) at  $75^\circ\text{C}$ . When  $\beta$ -lg molecules begin to undergo conformational changes to form reactive monomers (e.g.  $\sim 2$  min), there would only be a limited number of reactive monomeric BSA molecules available to react with  $\beta$ -lg to form complexes between the two proteins. Alkaline-PAGE results suggest that BSA molecules react very rapidly to give high molecular weight aggregates and some of these aggregates were dispersed into a mixture of disulphide-linked intermediates in the SDS-PAGE system. Some fine and lightly stained bands observed in 2D gels (Fig. 5) may have been BSA and  $\beta$ -lg complexes, but the quantities of these complexes were very small. In contrast to our results, Matsudomi *et al.* (1994) observed that  $\beta$ -lg interacted with BSA to form soluble aggregates through thiol-disulphide interchange reaction after heating 2 g protein/l solution at  $80^\circ\text{C}$  for 30 min.

Our results clearly show that when the mixture of the three major whey protein components ( $\beta$ -lg,  $\alpha$ -la, and BSA) is heated at  $75^\circ\text{C}$ , the composition of the aggregates and various complexes changes with heating time. The 2D-PAGE patterns of the heated mixture of the three proteins (Fig. 7) clearly demonstrate the presence of various disulphide homopolymers of each protein as well as various adducts of the three proteins. Initial aggregates are formed predominantly by polymerization of BSA (Fig. 8) with itself while the aggregates involving  $\beta$ -lg and  $\alpha$ -la are generated at a later stage and appear to be in proportion to the quantity of monomeric protein present in the unheated sample.

A schematic presentation of the behaviour of whey proteins in our model protein system during heat-induced aggregation is shown in Fig. 9. Because of the differences in thermal stability of the three proteins, during the initial stages of heating, BSA molecules will begin to unfold and aggregate (mainly via inter-molecular thiol-disulphide exchange, and to a lesser extent, non-covalent interactions) before  $\beta$ -lg. The exposed thiol groups of BSA molecules/aggregates could also react via thiol-disulphide interchange with one of the disulphide bonds of  $\alpha$ -la. Consequently,  $\alpha$ -la dimers (as described earlier) and  $\alpha$ -la/BSA adducts could be generated (Havea *et al.* 2000). At later stages of heating,  $\beta$ -lg will unfold to expose a thiol-group, which would react with disulphide bonds of another  $\beta$ -lg molecule and/or with  $\alpha$ -la resulting in dimers, trimers and higher molecular weight polymers of  $\beta$ -lg or  $\alpha$ -la, as well as mixed aggregates of the two proteins. Under the high protein concentration used in our study, these interactions would continue, with increase in the intensity of heating resulting in the formation of a self-supporting gel. Therefore, the heat-induced gels formed in these whey protein solutions can be visualized as a single heterogeneous network, consisting of gel strands made up largely of disulphide-linked co-polymers of  $\beta$ -lg and  $\alpha$ -la with the BSA aggregates being embedded in the spaces between the gel stands. BSA aggregates, provided they have free thiol available, can potentially interact with  $\beta$ -lg and  $\alpha$ -la aggregates and strands. Because  $\beta$ -lg and  $\alpha$ -la constitute 75% of the total protein in these solutions, the gel strands consist mainly of these proteins. BSA aggregates, being formed prior to other aggregates, would become embedded in the main gel strands.

The ratio of the major whey proteins ( $\beta$ -lg: $\alpha$ -la:BSA) in a commercial WPC (about 10:4:1) is different from the ratio of the same proteins (2:1:1) in the model systems used in this study. As mentioned earlier, there is a concentration effect on the size distribution of the intermediate aggregates in these heated solutions. This may give rise to different quantities of various intermediate aggregates being formed at the early stage of heating of a WPC solution. We have reported, in our earlier work (Havea *et al.* 1998), the presence of some of these aggregates (e.g.  $\beta$ -lg and  $\alpha$ -la

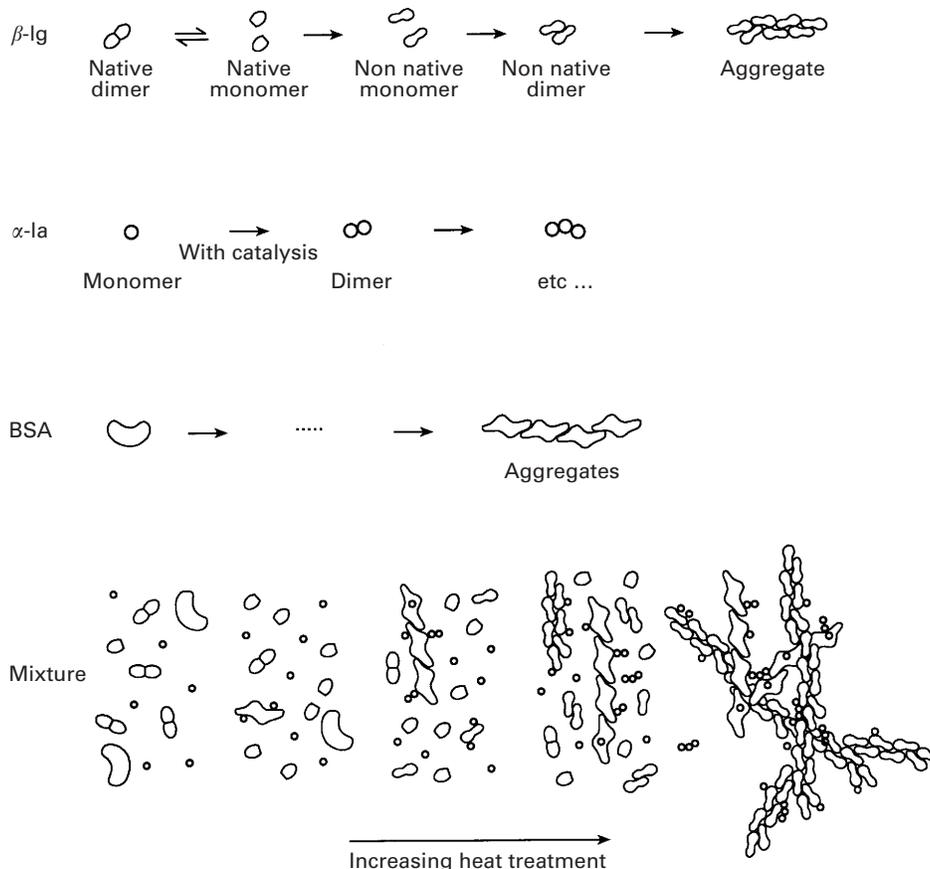


Fig. 9. Diagrammatic representation of the formation of gel networks in heated solutions of mixtures of BSA,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, with the initial formation of strands of aggregated BSA followed by the catalysis of  $\alpha$ -lactalbumin aggregation and the formation of  $\beta$ -lactoglobulin aggregates and  $\beta$ -lactoglobulin/ $\alpha$ -lactalbumin aggregates. The aggregation pathways of individual proteins are also shown. There is no differentiation between hydrophobically associated aggregates and disulphide-linked aggregates.

dimers) in heated WPC solutions (75 °C, 120 g/kg, pH 6.9), and there was some indication of the presence of others (e.g. heteropolymers). Based on the present results obtained using higher ratios of  $\alpha$ -la/ $\beta$ -lg and BSA/ $\beta$ -lg than in a standard WPC, we conclude that in a heated WPC solution the same aggregate species (i.e. various homopolymers and heteropolymers of these proteins) are formed prior to gel formation.

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# Characterization of heat-induced aggregates of beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin in a whey protein concentrate environment

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