Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions

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Summary. Whey protein concentrate (WPC) solutions containing 10, 30, 60 and 120 g dry powder/kg were heated at 75 °C and whey protein aggregation was studied by following the changes in the distribution of β-lactoglobulin, α-lactalbumin and bovine serum albumin, using one dimensional and two dimensional PAGE. The one dimensional PAGE results showed that a minimal quantity of large aggregates was formed when 10 g WPC/kg solutions were heated at 75 °C for up to 16 min whereas appreciable quantities were formed when 30, 60 and 120 g WPC/kg solutions were similarly treated. The two dimensional PAGE analysis showed that some disulphide-linked β-lactoglobulin dimers were present in heated 10 g WPC/kg solution, but very little was present in heated 120 g WPC/kg solution. By contrast, SDS was able to dissociate monomeric protein from high molecular mass aggregates in heated WPC solution of 120 g/kg but not in 10 g WPC/kg solution heated for 30 min. The rates of loss of native-like and SDS-monomeric β-lactoglobulin, α-lactalbumin and bovine serum albumin during heating increased as the WPC concentration was increased from 10 to 120 g/kg. In 120 g WPC/kg solution heated at 75 °C, the amounts of SDS-monomeric β-lactoglobulin in each sample were greater than the quantities of native-like protein. However, in WPC solutions of 10, 30 and 60 g/kg, the differences between the amounts of native-like and SDS-monomeric proteins were slight. The loss of the native-like or SDS-monomeric proteins was consistent with a first or second order reaction. In each case, the apparent reaction rate constant appeared to be concentration-dependent, suggesting a change of aggregation mechanism in the more concentrated solutions. Overall, these results indicate that in addition to disulphide-linked aggregates, hydrophobic aggregates involving β-lactoglobulin, α-lactalbumin and bovine serum albumin were formed in heated WPC solution at high protein concentration, as suggested by model studies using binary mixtures of these proteins.

Whey protein concentrate (WPC) products are used in a wide range of food applications, not only because of their nutritional value but also because they have desirable functional properties, such as the ability to form heat-induced gels. Consequently the heat-induced gelation of WPC solutions has been the subject of

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many studies in recent years (Mangino *et al.* 1987; Tang *et al.* 1993, 1995; Karleskind *et al.* 1995). These have shown that the gelation properties of WPC are affected by many factors including sodium, calcium and protein concentrations, pH, heating temperature and the presence of other components.

It has been suggested that the whey proteins undergo heat-induced gelation through a series of steps involving denaturation, aggregation, strand formation and network formation (Aguilera, 1995) to give a translucent slightly rubbery gel or an opaque compressible gel. The former type has also been called fine stranded and the latter type particulate, on the basis of microscopic examination (Stading & Hermansson, 1991; Langton & Hermansson, 1992). The type of gel formed is determined by pH, mineral composition and protein concentration and type (Tang *et al.* 1993, 1995; Bowland & Foegeding, 1995).

The gel-forming properties of the protein components of WPC have been investigated with particular emphasis on β-lactoglobulin, the major protein present. Paulsson & Dejmek (1989) reported that the shear modulus of β-lactoglobulin gels was proportional to the protein concentration. McSwiney *et al.* (1994*a*) observed that the loss of native-like β-lactoglobulin during heating was faster than the formation of disulphide-linked aggregates, which was faster than gel formation. These results supported the hypothesis that hydrophobically associated protein aggregates were formed before a self-supporting gel network (McSwiney *et al.* 1994*a*, *b*). Paulsson *et al.* (1986) reported that a bovine serum albumin (BSA) concentration of $\sim$ 20 g/l was required for gel formation during heating compared with $\sim$ 50 g/l for β-lactoglobulin, whereas α-lactalbumin did not form a gel even up to 200 g/l. In a recent study (Gezimati *et al.* 1996*a*), it was found that when a mixture of BSA and β-lactoglobulin in a buffer that mimicked the WPC environment was heated at 70 °C there was no obvious interaction between the two proteins under some gelling conditions and they formed two separate homogeneous aggregates, strands and gel systems which intertwined to give the final gel. However, when mixtures of β-lactoglobulin and α-lactalbumin were heated, it was concluded that these proteins interacted to form heterogeneous aggregates which formed the final gel structure (Gezimati *et al.* 1997).

The mechanisms of aggregation and gel formation in commercial WPC could be quite different from those in these model systems which often do not contain glycomacropeptide (GMP), minor proteins, uncharacterized and diverse high molecular mass material, lactose or different levels of mineral components. Consequently, the present study examined the effects of heating WPC solutions of 10, 30, 60 and 120 g/kg at 75 °C on the aggregation of the whey proteins.

Materials and methods

Materials

A commercial spray-dried rennet WPC powder was obtained from the New Zealand Dairy Board, Wellington, New Zealand. The chemicals used for the preparation of electrophoresis buffers (obtained from Bio-Rad Laboratories, Richmond, CA 94804, USA) were of analytical grade.

Whey protein concentrate composition

The total protein content of the WPC powder was determined using the Kjeldahl method (Association of Official Analytical Chemists, 1984), with an N conversion factor of 6·38. The fat content was determined using the Soxhlet extraction method as described by Russell *et al.* (1980). The moisture content was determined by oven-

drying preweighed duplicate samples at 105 °C for 24 h, cooling in a desiccator for 2 h and then reweighing the samples. The GMP content was determined by cation-exchange FPLC as described by Léonil & Mollé (1991). The mineral analyses were carried out at the New Zealand Pastoral Agricultural Research Laboratory, Palmerston North, by inductively coupled argon-plasma emission spectrometry using the method described by Lee *et al.* (1986).

Sample preparation

Appropriate quantities of WPC powder were dissolved in water (purified using a Milli-Q system; Millipore Corporation, Bedford, MA 01730, USA) so that the final solutions contained WPC (dry weight) concentrations of 10, 30, 60 or 120 g/kg. The protein concentrations in these solutions were 8·2, 24·1, 48·9 and 97·8 g/kg respectively. The solutions were stirred for 2 h at room temperature using a magnetic stirrer and the pH adjusted to 6·9 using 0·1 M-NaOH or 0·1 M-HCl. At each concentration, samples (1·5 ml) were transferred into a set of 14 preweighed 2 ml round bottomed Beckman polyallomer centrifuge tubes (0·35 mm wall thickness, 11 mm i.d., 34 mm high). The tubes containing the samples were then reweighed, closed with fitted lids, and placed in a water bath at 75 °C. Tubes and samples were removed after heating for 2, 4, 6, 8, 10, 12, 14, 16 or 30 min (the time taken for the samples to attain 74·9 °C was 20 s). The tubes and heated WPC solutions were immediately placed in ice water for 5 min and then held at room temperature (~ 20 °C) for 2 h. Samples of solutions at each concentration were also heated at 75 °C for 30, 8 or 4 min, for WPC solutions of 10, 60 or 120 g/kg respectively, and used for two dimensional (2D) PAGE analysis.

The above sets of experiments were repeated at least in triplicate.

Polyacrylamide gel electrophoresis

One dimensional PAGE. The samples were analysed by PAGE using Mini Protean II equipment (Bio-Rad). The WPC solutions were diluted (1 : 7·5, 1 : 22·5, 1 : 45 or 1 : 85 for WPC solutions of 10, 30, 60 or 120 g/kg respectively) with the appropriate sample buffer and analysed using native-PAGE as described by Andrews (1983) and SDS-PAGE as described by Laemmli (1970). After the gels had been stained with Coomassie blue and destained, they were photographed using a 35 mm camera fitted with both a green (XI) and an orange (G) Hoya filter (to minimize the stray light) on to 100 ASA Kodak T-max film. The protein bands on the resultant gels were identified by concurrent electrophoresis of samples of BSA, α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B and acid casein. Some of the other bands were identified by comparing the results with reported patterns (Basch *et al.* 1985; N. Haggarty, pers. comm.).

The gels were scanned using an Ultrascan XL laser densitometer and the results were analysed using an LKB 2400 GelScanXL software program (LKB Produkter AB, S-161 26 Bromma, Sweden) to obtain quantitative results. The peak area of each protein band was reported as a percentage of the corresponding band in the unheated control WPC samples.

Two dimensional PAGE. 2D PAGE was used to identify the components in the protein aggregates in heated WPC solutions. The WPC solutions of 10, 60 and 120 g/kg were diluted 1 : 2·5, 1 : 15 and 1 : 30 respectively, with native or SDS sample buffer prior to electrophoresis on native or SDS gels, as described above. Each gel had quadruplicate samples of a heated WPC, which had been selected on the basis of the extent of the loss of native-like β-lactoglobulin. After electrophoresis, the gel

was removed from between the glass plates and cut so that each strip contained all the protein bands from each sample, including the material that did not migrate into the stacking gel. Two of the strips were stained to confirm that the separation in the 'first dimension' was satisfactory. The strip of native gel was placed on one of the glass plates and perpendicular to the spacers (0·75 mm), the second plate was placed over it and the two plates were assembled into the gel setting equipment. SDS resolving gel solution (3·3 ml) was then carefully poured between the plates, leaving a space of ~ 12 mm between the top of the gel solution and the bottom of the gel strip. The gel solution was then overlaid with water in the usual fashion, taking care not to wet the gel strip. After the resolving gel had set, the water was removed, the equipment was flushed with a little stacking gel solution and the space was filled with stacking gel solution, with the equipment tilted at ~ 30 °C, so that no bubbles were trapped beneath the gel strip. A standard well-forming comb, which had most of the teeth removed, was then inserted so that wells formed were level with the centre of the gel strip and at least 4 mm distant from the end of the gel strip. After loading the appropriate chamber buffer and the control sample, the proteins were subjected to electrophoresis in the 'second dimension'. The resultant gel was then stained, destained and photographed as described above.

For the SDS 2D gels, 'first dimension' SDS gels were treated with 2-mercaptoethanol to reduce the protein components. After the first dimension separation (described above for SDS-PAGE), the sample wells were dried using strips of filter paper and filled with SDS stacking gel solution, and this was allowed to set (~ 30 min). The gel was then removed and cut into strips as described above. One of the unstained strips was placed in a test tube containing a solution of 5 ml 2-mercaptoethanol/l SDS sample buffer, and held in a water bath thermostatically controlled at 94 °C. After 2 min, the strip was removed from the test tube and washed with a little water to remove excess 2-mercaptoethanol solution, and the surplus water was blotted from the strip surface with filter paper strips. The gel strip was then held at room temperature for 30 min, to allow evaporation of excess 2-mercaptoethanol from the surface of the strip (the 2-mercaptoethanol would have inhibited the acrylamide polymerization). The strip was then fitted into the gel apparatus and the SDS gels were set and run as described above for the native-SDS 2D gel.

Reaction kinetic evaluation

The rates of the losses of native-like and SDS-monomeric β-lactoglobulin, α-lactalbumin and BSA at 75 °C, as measured by one dimensional PAGE, were determined using the general reaction kinetics equations described by Dannenberg & Kessler (1988). Each set of results was fitted to the appropriately derived equation for $n = 1$ or $n > 1$, and the reaction rate constants were calculated using the FigP statistical program (Biosoft, Ferguson, MO 63135, USA). The coefficients of determination (r^2) were used to indicate how well each data set fitted the equations.

RESULTS

Nomenclature

Based on the PAGE results, some key terms used to differentiate the various forms of protein in heated WPC systems were defined by Gezimati *et al.* (1996*a*) and the relationships between these different forms of protein and their likely changes during the heat treatment of WPC solutions are shown in Fig. 1. Analysis of heated WPC solutions by native-PAGE (pH ~ 8·3) gave protein bands that coincided with

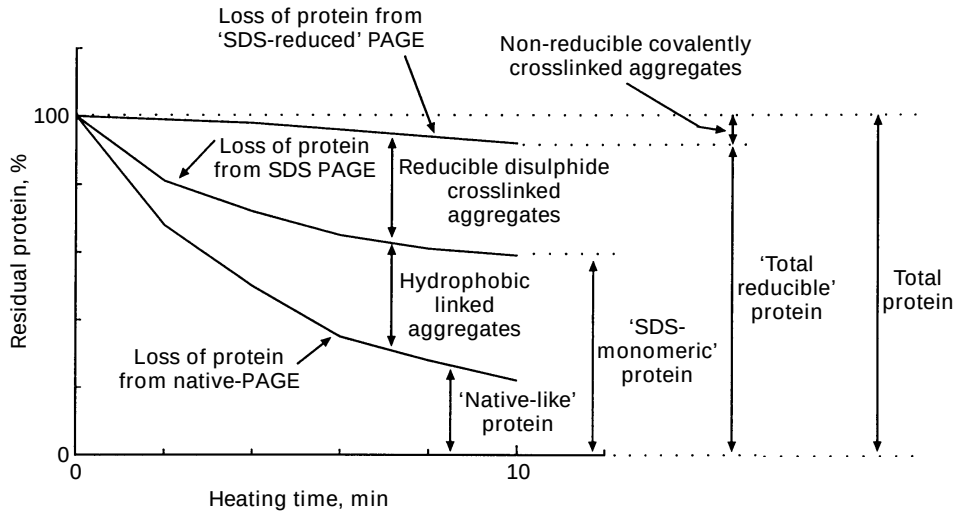

Fig. 1. Schematic representation of the relationship between the relative amounts of different forms of proteins (e.g. 'native-like', 'SDS-monomeric', 'total reducible') in heated whey protein concentrate solutions.

the native proteins in the unheated samples; these bands could be referred to as 'native-like' proteins. SDS-PAGE (pH ~ 8·3, 1 g SDS/l) analysis of heated samples gave more intense protein bands that ran as though they were monomeric; these could be referred to as 'SDS-monomeric' proteins. Analysis of heated samples by SDS-PAGE in the presence of a reducing agent, which converts disulphide bonds into free sulphydryl groups, gave even more intense bands that migrated as though they were monomers. These could be called 'total reducible' proteins.

Whey protein concentrate composition

Analysis showed that the WPC powder contained 941 g dry matter/kg of which 813 g was protein, 157 g GMP (also measured as part of the protein), 65 g fat and 47 g lactose. GMP contents of ~ 150 g/kg are quite common for WPC derived from rennet or cheese whey (M. Pritchard, pers. comm.).

Identification of protein components in unheated whey protein concentrate solutions

One dimensional PAGE. The unheated WPC solutions gave native-PAGE patterns (Fig. 2*a*, lanes 1 and 2) with bands that could be identified as β-lactoglobulin A and B, α-lactalbumin and BSA. There was also a band close to the beginning of the resolving gel, another that indicated that some proteins had not entered the gel, and a slightly diffuse region between the α-lactalbumin and BSA bands. Similarly, the major protein bands in unheated WPC solutions were identified in the SDS gels (Fig. 2*b*, lanes 1 and 2). Again, there was material that did not enter the resolving gel, that only travelled a short distance into the gel and that ran between the β-lactoglobulin and BSA bands. When the samples were reduced prior to electrophoresis (Fig. 2*c*), the light and heavy immunoglobulin (IgL, IgH) and lactoferrin bands could be identified by comparison with previously reported patterns (Basch *et al*. 1985). There was no material that did not enter the resolving gel, although there was a light band near the top of the resolving gel and some diffuse staining between the β-lactoglobulin and BSA bands.

Two dimensional PAGE. The bands that could be identified on the native and SDS

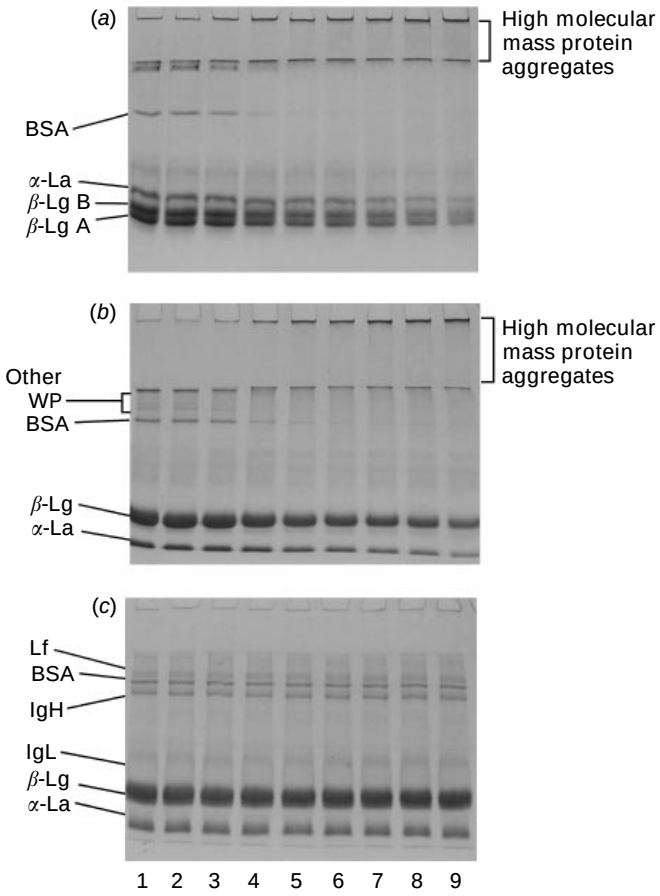

Fig. 2. Typical electrophoretic patterns of whey protein concentrate solutions (60 g/kg) heated at 75 °C for: lanes 1 and 2, 0; lane 3, 2; lane 4, 4; lane 5, 6; lane 6, 8; lane 7, 10; lane 8, 12 and lane 9, 16 min obtained using (*a*) native-PAGE, (*b*) SDS-PAGE and (*c*) SDS-PAGE of the reduced samples. BSA, bovine serum albumin; WP, whey proteins, α-La, α-lactalbumin; β-Lg, β-lactoglobulin; Lf, lactoferrin; β-Lg A, β-Lg B, β-lactoglobulins A and B; IgH, IgL, heavy and light immunoglobulin fractions.

one dimensional gels as β-lactoglobulin A and B, α-lactalbumin and BSA were readily identified as the various spots on the native-SDS 2D gel (Fig. 3*a*). Similarly, the spots for these proteins, lactoferrin and the corresponding reduced proteins, as well as reduced IgL and IgH, were identified on the SDS 2D gel (Fig. 3*b*). Some other spots were identified in this way. The spot labelled X in Fig. 3(*a*) was probably glyco-α-lactalbumin (N. Haggarty, pers. comm.) whereas the spots labelled Y1 and Y2 in Fig. 3(*b*) were likely to be α_{s1}- and β-caseins respectively. There were a number of spots corresponding to BSA, lactoferrin, IgH and IgL, which after reduction were resolved from the material caught at the top of the stacking and resolving gels. There were other spots for which the proteins were not identified. It was not possible to identify GMP in any of these gel systems because of the staining techniques used.

Formation of whey protein aggregates during heat treatment

When the WPC solutions were heated for various times at 75 °C and examined by PAGE, the intensities of all the whey protein bands diminished for the non-reduced samples with corresponding increases in the material in the stacking gel and caught

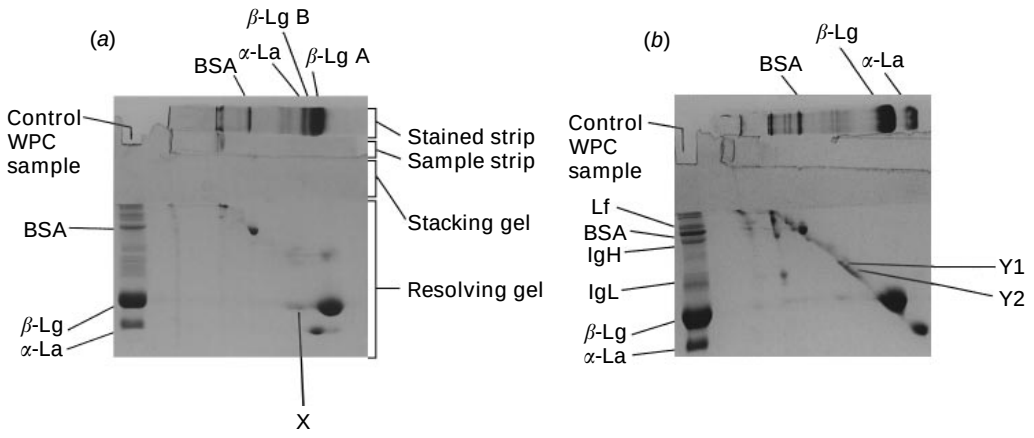

Fig. 3. Two dimensional electrophoretic patterns of an unheated control whey protein concentrate sample (120 g/kg): (*a*) first dimension, native-PAGE; second dimension, SDS-PAGE; (*b*) first dimension, SDS-PAGE without treatment with 2-mercaptoethanol; second dimension, SDS-PAGE of samples within gel strip that were treated with 2-mercaptoethanol. At the top of each gel there is a stained strip showing 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 to help identify the protein bands. BSA, bovine serum albumin; WPC, whey protein concentrate, α-La, α-lactalbumin; β-Lg, β-lactoglobulin; IgH, IgL, heavy and light immunoglobulin fractions; X, glyco-α-lactalbumin; Y1, α_{s1}-casein; Y2, β-casein.

at the top of the resolving gel (Fig. 2*a*, *b*). Reduction of these WPC samples resulted in complete disappearance of the material within the stacking gel and that caught at the top of the resolving gel, and all the bands were essentially the same from one lane to another (Fig. 2*c*). The minor whey protein components were the most heat sensitive and their concentration decreased faster than that of the major proteins (Fig. 2*a*, *b*).

Quantitative PAGE showed that when 10 g WPC/kg solutions were heated, there were only slight decreases in the quantities of β-lactoglobulin and α-lactalbumin with heating time (Fig. 4). However, the quantities of the native-like and SDS-monomeric BSA (Fig. 4*c*), Ig and lactoferrin decreased more with heating time. The loss of native-like and SDS-monomeric β-lactoglobulin and α-lactalbumin with heating time was greater from the 60 g/kg solution and greatest from the 120 g/kg solution (Fig. 4); the loss from the WPC solution of 30 g/kg was intermediate between those from the 10 and 60 g/kg solutions (results not shown). The results for the loss of native-like β-lactoglobulin from the WPC solutions of 30 and 60 g/kg indicated a slower initial stage, followed by more rapid loss. However, the initial stage was undetectable in 120 g WPC/kg solution. In this solution, the amounts of SDS-monomeric β-lactoglobulin and α-lactalbumin present in each heated sample were considerably greater than the quantities of native-like β-lactoglobulin and α-lactalbumin. However, the loss of SDS-monomeric β-lactoglobulin from WPC solutions of 30 and 60 g/kg was only slightly lower than that of the native-like proteins.

The rates of depletion of native-like β-lactoglobulin, α-lactalbumin and BSA could be adequately described by equations for either first or second order reactions, although a first order reaction had the higher correlation (results not shown). For both native-like and SDS-monomeric proteins, the first and second order reaction rate constants increased with WPC concentration, the extent of increase being

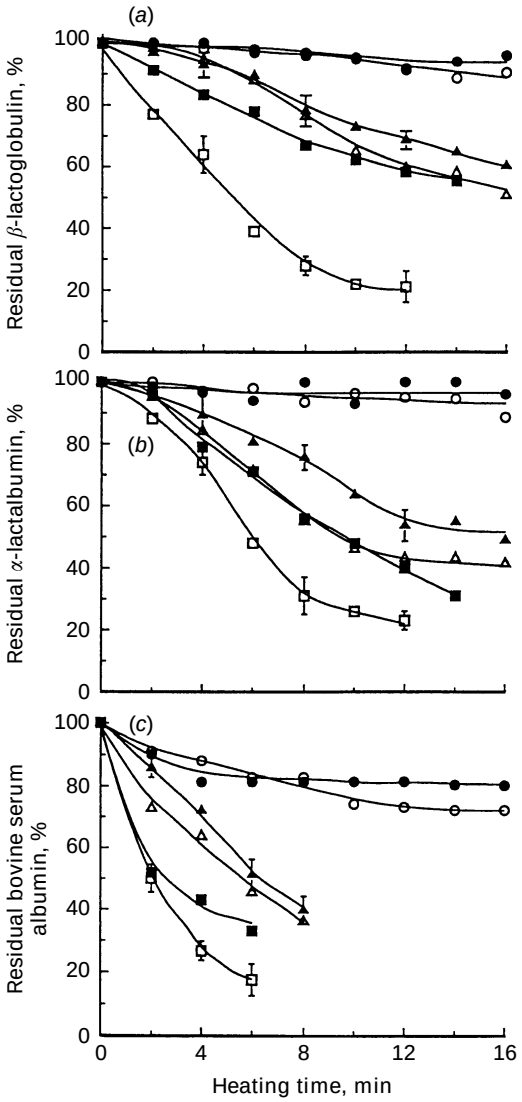

Fig. 4. Loss of ○, △, □, 'native-like'; and ●, ▲, ■, 'SDS-monomeric' forms of (*a*) β-lactoglobulin; (*b*) α-lactalbumin and (*c*) bovine serum albumin on heating at 75 °C whey protein concentrate solutions of ○, ●, 10; △, ▲, 60 and □, ■, 120 g/kg. Each point represents a mean of three replicates with typical SD indicated by vertical bars for some points.

greater for the native-like proteins. This suggests that the loss of protein did not occur via simple first or second order processes despite the high correlation. Differences in protein and mineral concentrations in the WPC solutions may result in a change in reaction mechanism.

Native-like α-lactalbumin was lost (in terms of proportion of original protein) from the WPC solutions of 30 and 60 g/kg more quickly than native-like β-lactoglobulin, although the losses of the two proteins were comparable from the 120 g WPC/kg solution (Fig. 4). The rate of loss of SDS-monomeric α-lactalbumin appeared to be faster than that of β-lactoglobulin at all WPC concentrations. The observation that the loss of native α-lactalbumin was faster than that of

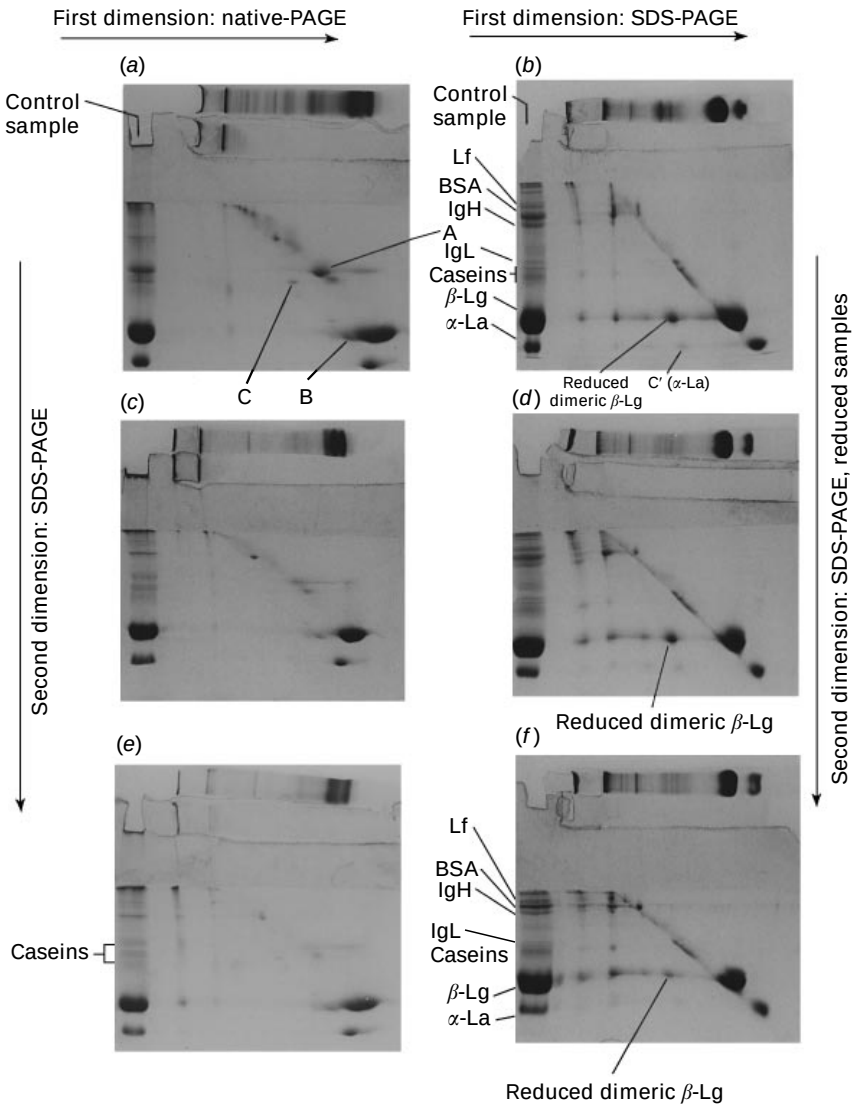

Fig. 5. Two dimensional electrophoretic patterns of whey protein concentrate solutions of (*a*, *b*) 10, (*c*, *d*), 60 and (*e*, *f*) 120 g/kg heated at 75 °C for 30, 8 and 4 min respectively. Samples were diluted with sample buffer and analysed in two dimensions by the same procedure as for the samples in Fig. 3. BSA, bovine serum albumin; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; IgH, IgL, heavy and light immunoglobulin fractions; A, β-lactoglobulin dimers; B, partly unfolded forms of β-lactoglobulin; C, C′, α-lactalbumin dimers and/or β-lactoglobulin–α-lactalbumin dimers?

β-lactoglobulin was in agreement with the results of Hollar *et al.* (1995) obtained by heating 160 g WPC/kg solutions at 71 °C.

Characterization of heat-induced whey protein aggregates

The aggregates observed using native- and SDS-PAGE (Fig. 2*a*, *b*) were characterized using 2D PAGE (Fig. 5). Comparison of the native-SDS 2D PAGE (Fig. 5*a*) of the 10 g WPC/kg sample heated at 75 °C for 30 min with the pattern for the control sample (Fig. 3*a*) indicated a number of new spots. Examination of the 'sample' gel strip showed that the material caught in the sample well and that at the

top of the resolving gel had not moved out of the sample strip. The material that had moved a few millimetres into the gel was only partly eluted from the sample gel. The region labelled A had a moderately well defined spot which corresponded to the bands in the region between BSA and α-lactalbumin. A distinct spot, labelled C, was observed in the native-SDS 2D gel (Fig. 5*a*) and had slightly lower molecular mass than β-lactoglobulin dimer (A). Upon reduction, this spot resolved to give monomeric α-lactalbumin (labelled C′, in Fig. 5*b*) and a faint β-lactoglobulin spot, indicating the presence of disulphide-linked α-lactalbumin dimers and/or disulphide-linked α-lactalbumin β-lactoglobulin dimer complex in the heated 10 g WPC/kg solution. There was also a faint horizontal comet tail of intensity between the β-lactoglobulin spot on the 2D gel and the β-lactoglobulin band at the left hand side.

Comparison of the SDS 2D PAGE pattern (Fig. 5*b*) with the control pattern (Fig. 3*b*) showed that most of the material caught in the sample well travelled into the gel and that all the other material in the sample gel travelled into the 2D gel. After reduction, the material caught in the sample well resolved into a series of spots corresponding to many of the proteins in the reduced sample in the left hand channel. The relative band intensities were different, the most notable being the absence of α-lactalbumin, and the presence of little β-lactoglobulin and BSA, which was less intense than IgH and lactoferrin. The material that had been caught at the top of the resolving gel also gave a number of spots. In this case, the BSA spot was more intense than any other. Between the β-lactoglobulin spot and the β-lactoglobulin band in the left hand channel there were a number of spots and an almost continuous horizontal line up to the point corresponding to the material caught at the top of the resolving gel. An intense spot (Fig. 5*b*) could well correspond to a disulphide-bonded β-lactoglobulin dimer, based on the position of the unreduced protein on the SDS gel. Also apparent were a number of faint α-lactalbumin spots across the gel (i.e. between the α-lactalbumin spot and the corresponding band in the left hand channel) indicating the presence of disulphide-bonded α-lactalbumin in the aggregates.

Comparison of the 2D PAGE results from the heated 120 g WPC/kg sample (Fig. 5*e*, *f*) with those from the 10 g/kg sample (Fig. 5*a*, *b*) showed some interesting differences. For the native-SDS 2D PAGE pattern (Fig. 5*e*), BSA, casein, β-lactoglobulin, α-lactalbumin and glyco-α-lactalbumin were all present but the dimeric spot (A in Fig. 5*a*) was markedly diminished in intensity. Most of the material that had been caught in the sample slot and at the top of the resolving gel did not travel into the SDS gel, as was the case for the 10 g WPC/kg sample (Fig. 5*a*). However, some of this material travelled down the SDS gel, indicating that some of it was dissociated from high molecular mass aggregates by the SDS buffer. It is quite clear that SDS dissociated monomeric β-lactoglobulin from a number of aggregates to show up as a faint line and a series of spots between the β-lactoglobulin spot and the β-lactoglobulin band in the left hand channel. A number of faint spots, which were apparently of slightly lower mobility than β-lactoglobulin, were observed in the region labelled B (Fig. 5*a*). We could not identify the protein components that corresponded to these spots. Comparable spots were also apparent for BSA.

Comparison of the non-reduced and the reduced SDS 2D PAGE pattern (Fig. 5*f*) with that of the equivalent 10 g WPC/kg pattern (Fig. 5*b*) showed substantial differences in the way that β-lactoglobulin formed disulphide-bonded aggregates at the two different concentrations, with less dimer at the higher concentration. A number of α-lactalbumin spots across the gel were also found in heated 120 g/kg solution, although the intensities of these spots appeared to be much lower than those observed in heated 10 g WPC/kg solutions (Fig. 5*b*).

The patterns for the heated 60 g WPC/kg solution (Fig. 5*c*, *d*) were intermediate between the 10 and 120 g WPC/kg patterns.

DISCUSSION

In this study, the possible protein interactions leading to aggregation and gelation during the heating of WPC were examined by 2D electrophoretic methods. The differentiation of peptides or proteins crosslinked by disulphide bonds from other peptides has been conducted using 2D SDS-PAGE with an intermediate reduction step (Zeece *et al.* 1989), but we are not aware of the use of combinations of native- and SDS-PAGE systems to distinguish hydrophobically associated protein aggregates from disulphide-bonded aggregates in a heat-treated mixture of proteins.

The present studies showed that, when WPC solutions were heated at 75 °C, aggregates consisting of β-lactoglobulin, α-lactalbumin, BSA, caseins and minor proteins were formed and that both hydrophobic interactions and disulphide crosslinkages were involved in forming these aggregates. The extent of aggregation as well as the nature of stabilizing forces involved in the formation of the aggregates were affected by the WPC concentration. The amount of hydrophobically associated β-lactoglobulin (the protein that is stable under the conditions of separation in native-PAGE, i.e. $\sim$ 20 °C and $\sim$ pH 8·5, but not in SDS) increased with concentration, especially between 60 and 120 g/kg (Fig. 4*a*). However, the quantity of hydrophobically associated α-lactalbumin increased to a smaller extent with concentration. It is estimated that, when 120 g WPC/kg was heated at 75 °C for 12 min, $\sim$ 80 % of total β-lactoglobulin was aggregated, of which $\sim$ 50 % was associated hydrophobically (Fig. 4*a*). By comparison, $\sim$ 78 % of total α-lactalbumin was present as aggregates, of which 25 % was linked by hydrophobic interactions (Fig. 4*b*).

When the WPC solutions were heated at 75 °C, disulphide-linked α-lactalbumin and β-lactoglobulin dimers were observed predominantly in the WPC solutions of 10 and 60 g/kg (Fig. 5*b*, *d*), but not in 120 g/kg solutions (Fig. 5*f*). The various spots observed in region B (Fig. 5*a*) may be attributable to various partly unfolded forms of β-lactoglobulin. Such forms of β-lactoglobulin have been implicated in heated solutions of pure protein using differential scanning calorimetry (Qi *et al.* 1995) and light-scattering techniques (Griffin *et al.* 1993).

From analyses of the WPC used in this study the 'whey protein' content was probably 650 g/kg. The β-lactoglobulin content of the WPC was probably $\sim$ 600 g/kg, i.e. $\sim$ 72 g/kg solution for the 120 g WPC/kg solution. This compares with concentrations of $\sim$ 100 g/l for many of the other studies, e.g. McSwiney *et al.* (1994*a*, *b*), involving β-lactoglobulin alone. When Gezimati *et al.* (1996*a*, *b*, 1997) examined mixtures of β-lactoglobulin and BSA or α-lactalbumin, they found that in addition to disulphide-linked aggregates β-lactoglobulin and BSA, but not α-lactalbumin, were able to form stable hydrophobic aggregates when the pure proteins were heated at 75 °C in a buffer that simulated the WPC environment. When α-lactalbumin was heated with β-lactoglobulin (and probably BSA) it formed stable hydrophobic aggregates (Gezimati *et al.* 1996*b*, 1997). The results from the one dimensional PAGE experiments, which confirmed the differences between the loss of native-like proteins and the loss of SDS-monomeric proteins from the heated 120 g WPC/kg solutions (Fig. 4*a*, *b*), were as expected from the earlier results involving mixtures of pure proteins (Gezimati *et al.* 1996*a*, *b*, 1997). Therefore, it is likely that the protein–protein interactions that led to the formation of aggregates in heated

120 g WPC/kg solutions were similar to those observed in the model systems used by Gezimati *et al.* (1996*a*, *b*, 1997).

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