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Heat-induced interactions of β-lactoglobulin A and κ-casein B in a model system

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The interaction of κ-casein and β-lactoglobulin is fundamental to all heat-induced modifications of milk product functionality, such as the heat stability of concentrated milks. Purified native κ-casein B and β-lg A solutions were heated at 80 °C at pH 6·7 separately and in a mixture. The circular dichroism spectra in the near UV indicated irreversible changes in the disulphide bonding patterns involving both proteins. Alkaline- and SDS-PAGE of heated samples showed that, in the presence of κ-casein, less β-lg was converted into β-lg polymers and the rate of loss of native β-lg was greater. When κ-casein was added to previously heated β-lg and the mixture was heated, the κ-casein reacted with the heat-induced β-lg polymers more readily than with the β-lg native monomers. The formation of β-lg dimers, trimers etc. was diminished. It was concluded that, when β-lg and κ-casein were heated together, β-lg formed thiol-exposed monomers, which reacted with each other or with the native κ-casein depending on the relative concentrations of β-lg and κ-casein. The products of these reactions included some disulphide-bonded 1 : 1 β-lg : κ-casein complexes, some monomer κ-casein and a range of large aggregates held together by either or both disulphide bonds and hydrophobic association.

Keywords: κ-Casein–β-lactoglobulin complex, heat-induced aggregation, disulphide bonding, aggregation pathway, β-lactoglobulin aggregation.

It has long been recognized that heat treatment of milk above 70 °C causes denaturation of the whey proteins, some of which complex with the casein micelles and this determines many characteristics of milk and milk products (Singh & Creamer, 1992; Singh, 1995). The major whey protein is β-lactoglobulin and early studies indicated that κ-casein, which resides on the casein micelle surface, is the casein involved in this reaction (Sawyer, 1969). The degree of interaction between β-lg and κ-casein depends on the time and temperature of heating, the concentrations of the proteins, pH and the presence and concentrations of milk salts (Singh, 1995).

Complex formation between β-lg and κ-casein has been investigated in pure protein model systems (Zittle et al. 1962; Long et al. 1963; Sawyer et al. 1963; Purkayastha et al. 1967; Tessier et al. 1969; McKenzie et al. 1971; Euber & Brunner, 1982; Haque et al. 1987), casein micelle model systems (Smits & van Brouwershaven, 1980; Singh & Fox, 1987; Jang & Swaisgood, 1990) and milk (Tessier et al. 1969; Creamer et al. 1978; Parnell-Clunies et al. 1988).

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

Long et al. (1963) presented evidence indicating that primary denaturation of β-lg precedes its interaction with κ-casein. Although it was not demonstrated that the same products were formed, it was shown that only β-lg needed to be heated for an interaction to occur. Sawyer (1969) suggested that the interaction between β-lg and κ-casein involved a sulphydryl–disulphide interchange mechanism but that non-covalent interactions, e.g. hydrophobic interactions, may also be involved. Sawyer (1969) and McKenzie et al. (1971) also interpreted the restriction of β-lg self-aggregation, during thermal denaturation in the presence

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of κ-casein, as evidence for κ-casein forming complexes with intermediate species of aggregated β-lg. In contrast, Euber & Brunner (1982) reported that aggregation of β-lg was not a prerequisite for the interaction, and that the β-lg/κ-casein complex was stabilized by intermolecular disulphide bonds. The findings of Doi et al. (1981) and Haque & Kinsella (1988) supported this notion. However, despite extensive research in the area, the detailed mechanism of this interaction remains unclear.

Recent studies (e.g. Iametti et al. 1996; Manderson et al. 1998; Schokker et al. 1999) have clarified the steps involved in the heat-induced aggregation of β-lg and we have now extended these studies to include the effects of κ-casein on the heat-induced denaturation pathway of β-lg using the techniques developed by Havea et al. (1998) and Manderson et al. (1998).

Milk containing the A variant of β-lg fouls heated surfaces more readily than milk containing either the B or the C variant protein (FitzGerald & Hill, 1997; Hill et al. 1997). Similarly, milk containing the B variant of κ-casein fouls more readily than milk containing the A variant protein (GR Paterson, MJ Boland & JP Hill, personal communication, 2000). Consequently, it was expected that any observable effect would be pronounced if the combination of β-lg A and κ-casein B were selected for this work.

In untreated milk, κ-casein is either on the surface of the micelle or, at low temperature, present in the serum phase. In addition, κ-casein exists as a mixture of disulphide-bonded polymers with a wide range of molecular weights (Farrell et al. 1998; Groves et al. 1998). In order to have a model system that was close to the milk system, κ-casein was not subjected to any treatment that could modify the primary structure of the protein polymers, such as heat treatment, high pH or reduction, at any point during purification.

Materials and Methods

Materials

The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Hercules, CA 94547, USA). 2-Mercaptoethanol was obtained from Sigma Chemical Co. (St. Louis, MO 63178, USA). All other chemicals were analytical grade from BDH Chemicals Ltd. (Palmerston North, New Zealand). Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and deionization using Milli-Q apparatus (Millipore Corporation, Bedford, MA 01730, USA). β-Lg A was prepared as described by Manderson et al. (1998).

Preparation of κ-casein B

Fresh whole milk, known to be homozygous for κ-casein B by alkaline-urea-PAGE (Cho, 2000), was centrifuged at 8000 ***g*** at 20±5 °C for 1 h in a temperature-controlled centrifuge (Sorvall RC5B, DuPont Company, Wilmington, DE 19898, USA). After centrifugation, three fractions were obtained, i.e. top (cream) layer, middle layer and pellet. The opaque middle layer, which contained the small casein micelles and most of the whey proteins, was collected and centrifuged at 90 000 ***g*** at 20±5 °C for 1 h in a temperature-controlled centrifuge (Sorvall RC28S, DuPont Company). A dense viscous liquid layer was observed in addition to the top (cream) layer, middle layer and pellet. This time, both the dense liquid layer and the pellet were retained and stored at −21 °C.

Size-exclusion chromatography was used to separate disulphide-bonded κ-casein from the other casein species using a procedure similar to those described earlier (Yaguchi et al. 1968; Rasmussen & Petersen, 1991). The frozen thick liquid layer and the pellet were thawed at 4 °C and either sample was dissolved in pH 6·3 TCU buffer (0·005 M-Tris-citrate buffer containing 6 M-urea; Yaguchi et al. (1968) used pH 8·6 TCU buffer). Both solutions were filtered using a 0·45-μm filtering unit (Millipore Corporation). Proteins were separated on a column (50 mm × 400 mm) of Sephacryl S-300 HR equilibrated with TCU buffer using the Pharmacia FPLC system. The absorbance of the eluate from each run was monitored at 280 nm by a VWM 2141 detector and recorded using an REC 102 multi-channel chart recorder. Fractions containing polymeric κ-casein were collected using a Frac-200 fraction collector. Purity of the κ-casein in the eluted fractions was determined using reduced samples on alkaline-urea-PAGE.

Selected fractions were pooled, dialysed against 0·005 M-NaCl and then exhaustively against pH 6·7 phosphate buffer (26 mM-sodium phosphate and 68 mM-NaCl), and the κ-casein concentration was determined from UV absorbance measurements (absorbance of a 1% solution was taken as 9·5 (Swaisgood, 1992)). Soybean trypsin inhibitor (Type I-S: chromatographically prepared from soybean and lyophilized, T 9003, Sigma) was added at a ratio of 10 mg/g (κ-casein) to the κ-casein sample and the mixture was stored frozen at −21 °C.

Circular dichroism (CD) measurements

Solutions of β-lg, κ-casein and 1 : 1 (w/w) mixtures, at total protein concentrations of 1·0 mg/ml in phosphate buffer, pH 6·7, were placed in a water-jacketed 10-mm path length CD cell (Jasco, Ishikawa-cho, Hachioji city, Tokyo, Japan) which was connected to a Neslab model RTE-100 water bath (Neslab Instruments Inc., Newington, NH 03801, USA). Each solution was held at 20 °C for 20 min prior to measuring the initial spectrum. The temperature of the water bath was then increased to 40 °C and held for 20 min, and a second spectrum was run. This procedure was repeated using 20 or 10 °C intervals until a temperature of 80 °C was reached. Water bath temperature was then decreased by 20 °C increments to 20 °C. A calibration run was done to determine the relationship between cell contents and water bath temperatures, after the water bath had been calibrated

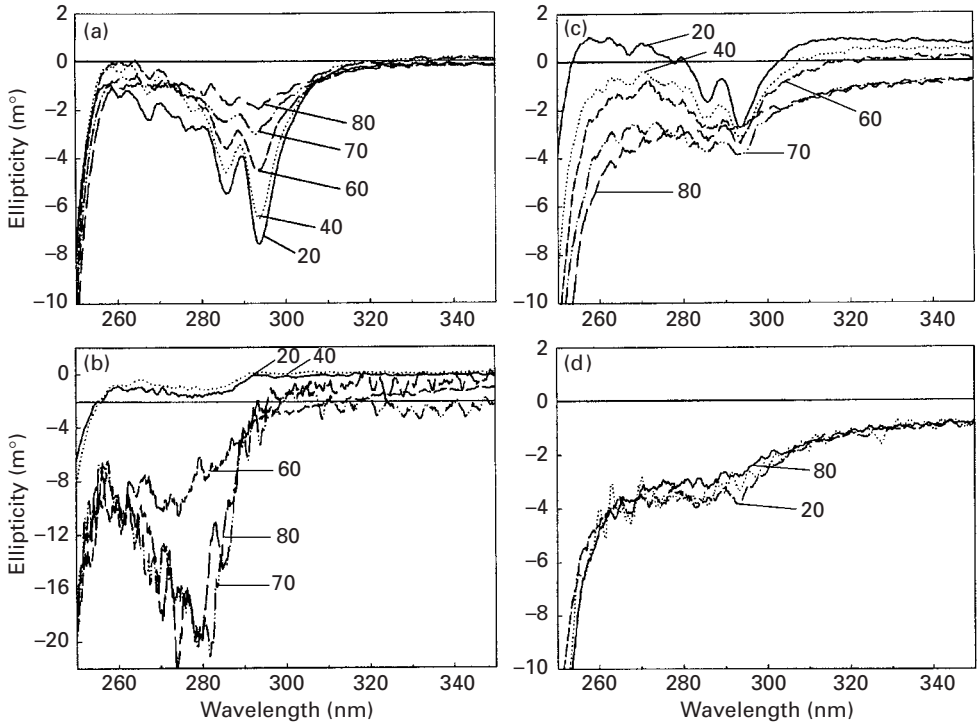

Fig. 1. Effect of heat treatment on near-UV CD spectra of β-lactoglobulin A and/or κ-casein B at a total protein concentration of 1·0 mg/ml in phosphate buffer (26 mM, with 68 mM-NaCl), pH 6·7 at various temperatures. (a) β-Lg A at 20 °C, and then heated to 40, 60, 70 and 80 °C; (b) κ-casein B at 20 °C, and then heated to 40, 60, 70 and 80 °C; (c) mixture of β-lg A and κ-casein B at 20 °C, and then heated to 40, 60, 70 and 80 °C; (d) mixture of β-lg A and κ-casein B at 80 °C, and then cooled to 60, 40 and 20 °C.

against a secondary temperature standard (Manderson, 1998). The baseline correction was made by subtraction of the phosphate buffer spectrum from all protein spectra.

Polyacrylamide gel electrophoresis

The various protein samples were analysed using alkaline- and SDS-PAGE as described by Manderson et al. (1998) and using 2 D PAGE (non-reduced SDS-PAGE and then reduced SDS-PAGE) as described by Havea et al. (1998).

Heat treatment of samples

Samples of β-lg or κ-casein were dialysed against phosphate buffer, pH 6·7 and diluted to appropriate protein concentrations and pH was readjusted to 6·7. Aliquots of β-lg A and/or κ-casein B were heated in microcentrifuge tubes at 80 °C for various times between 2·5 and 60 min in the Neslab temperature-controlled water bath (time taken for the samples to attain 80 °C was 15 s). Tubes and samples were removed after heating and immediately placed in an ice–water mixture for 5 min and left at room temperature for 2 h. The above sets of experiments were carried out in duplicate.

Sample mixtures all contained 1 mg β-lg A/ml and 0·5, 1·0, 1·5 or 2·0 mg κ-casein B/ml. For comparison, 2 mg/ml solutions of β-lg A and of κ-casein B were prepared. Aliquots of every solution were heated individually under the same conditions.

In a second set of experiments, β-lg A solutions (2 mg/ml), which had been heated for 5, 10, 15 or 30 min at 80 °C, were cooled to room temperature, held for 2 h, and then mixed with κ-casein B solution (2 mg/ml). Aliquots of each mixture were promptly reheated for various times at 80 °C. The appropriate samples were then analysed by PAGE.

Results and Discussion

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

Changes in the near-UV CD spectrum of bovine β-lg A, in phosphate buffer, pH 6·7, as the protein solution was heated from 20 to 80 °C are shown in Fig. 1a. The CD spectrum of the solution at 40 °C was not significantly different from that of the unheated solution. However, the intensities of both the 286 and 293-nm tryptophan CD bands decreased to about half of the original intensities when the temperature was increased to 70 °C, and decreased to almost zero at 80 °C. Decreasing the temperature from 80 to 20 °C did not change the CD spectrum (Cho, 2000); this supports the findings of Iametti et al. (1996) and Manderson et al. (1999).

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The near-UV CD spectrum of κ-casein B in phosphate buffer, pH 6·7 at 20 °C did not show any strong features (Fig. 1b) even though the protein contains one Trp, nine Tyr and four Phe residues. Absence of CD signals between 280 and 330 nm could indicate low level of tertiary structure near Trp^{76} and the Tyr residues.

The CD spectrum of κ-casein B did not change significantly between 20 and 40 °C (Fig. 1b), but the intensity at 270 nm increased markedly at 60 °C. Spectra were quite similar at 70 and 80 °C and contained intense broad bands near 280 nm. These spectra were very noisy, probably because of large κ-casein aggregates in the solution. However, during cooling, the 265–280 nm trough intensity diminished and a white precipitate developed.

It has been reported that disulphide bonds give broad bands in the CD spectrum that seem to be related to the dihedral angle of the disulphide bond (Woody, 1973). Normally this angle is approximately 90° and the disulphide bond then gives rise to a broad band near 260 nm, but changes in this angle result in the splitting of this band into two broad bands at higher and lower wavelengths (Woody, 1973, 1995; Strickland, 1974; Kahn, 1979). This effect has been demonstrated by Matsuura & Manning (1994) for β-lg during heat-induced gelation and has also been observed by Kuwajima et al. (1996) and Manderson et al. (1999) at 270–280 nm.

Pure κ-casein has been reported (Groves et al. 1998) to aggregate during heat treatment to form high molecular weight polymers, primarily via sulphydryl–disulphide interactions. Consequently, the increase in band intensity at 270 nm in Fig. 1b was probably caused by a reorganization of the disulphide bonding patterns within the large aggregates and/or intermolecular sulphydryl–disulphide interactions of κ-casein. However, it is possible that the heat treatment also caused changes to the chirality of the environment of one or more of the aromatic residues of κ-casein.

The unheated mixture of β-lg A and κ-casein B showed the expected CD spectrum (Fig. 1c), with the β-lg 293 and 286 nm troughs as the only prominent features. Heating the mixture to 80 °C (Fig. 1c) gradually decreased the intensity of these troughs although there was a general increase in the CD signal from 250 to 350 nm. Cooling the solution again (Fig. 1d) did not alter the spectrum appreciably. Clearly the pattern of the heat-treated mixture at 20 or 80 °C did not appear to be intermediate between the patterns of the heat-treated individual proteins (Figs 1a, b). This result supports the proposition that β-lg and κ-casein had interacted with one another via disulphide-bond interchange reactions.

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

The various protein bands that are seen in the alkaline (native)- and SDS-PAGE patterns are described using the terms defined by Havea et al. (1998). For example, when heated samples were analysed, protein bands in the alkaline-PAGE system that had the same mobility as unheated β-lg are referred to as 'alkaline-monomeric' or 'native-like' β-lg and the equivalent band in the SDS-PAGE system is referred to as 'SDS-monomeric' β-lg.

β-Lactoglobulin alone. The alkaline-PAGE patterns of heat-treated β-lg A in phosphate buffer, pH 6·7, are shown in Fig. 2a (lanes 1–3). At pH 8·9, the pH at which alkaline-PAGE gels are run, native dimers dissociate to monomers (Groves et al. 1951; Casal et al. 1988) and only native-like β-lg was observed in the unheated sample (lane 1).

After heating the β-lg solution at 80 °C, β-lg aggregated to give rise to a number of bands and diffuse regions of stained protein (Fig. 2a, lanes 2 and 3) and, by comparison with earlier results (Havea et al. 1998; Manderson et al. 1998), these were identified in order of decreasing mobility as native-like monomeric, non-native monomeric, dimeric, trimeric and tetrameric β-lg.

SDS-PAGE patterns of heat-treated β-lg A are shown in Fig. 2a, lanes 4–6 (unless specifically stated, all SDS-PAGE samples were not treated with reducing agents). As the heating time at 80 °C increased, β-lg A gave rise to a group of bands (Fig. 2a, lanes 5 and 6) that were identified as (SDS-) dimeric β-lactoglobulins, supporting the findings of Manderson et al. (1998).

The non-native monomer bands that had mobilities between the monomer and dimer bands in the alkaline-PAGE gel (Fig. 2a, lane 2 or 3) could not be seen in the SDS-PAGE gel (Fig. 2a, lane 5 or 6). Manderson et al. (1998) and Schokker et al. (1999), used 2 D PAGE to show that an appreciable proportion of the partially unfolded monomeric β-lg A (non-native monomer) and a proportion of the larger aggregates were present in the SDS-monomeric fraction, which explains the markedly higher band intensity for the SDS-monomeric bands than the native-like β-lg bands in any sample (Manderson et al. 1998). In the 'reduced' SDS-PAGE gel (Fig. 2a, lanes 7–9), all polymeric β-lg bands seen in the alkaline- and SDS-PAGE patterns were condensed into single SDS-monomeric β-lg A bands (Fig. 2a, lanes 7–9). Consequently, the native-like β-lg band was less intense than the SDS-monomeric β-lg band, which was less intense than the SDS-monomeric β-lg band in the reduced protein samples (cf. monomeric β-lg bands in lanes 3, 6 and 9 in Fig. 2a).

κ-Casein B alone. The alkaline-PAGE gel for unheated κ-casein B (Fig. 2b, lane 1) showed only large aggregates with the stained protein present at the top of the resolving gel, through the stacking gel and at the top of the stacking gel. The molecular weights of these aggregates must have been at least 250 kDa. Heat treatment (Fig. 2b, lanes 2 and 3) did not alter the pattern appreciably, and thus the κ-casein probably stayed as disulphide-linked or hydrophobically associated polymers (Groves et al. 1992, 1998).

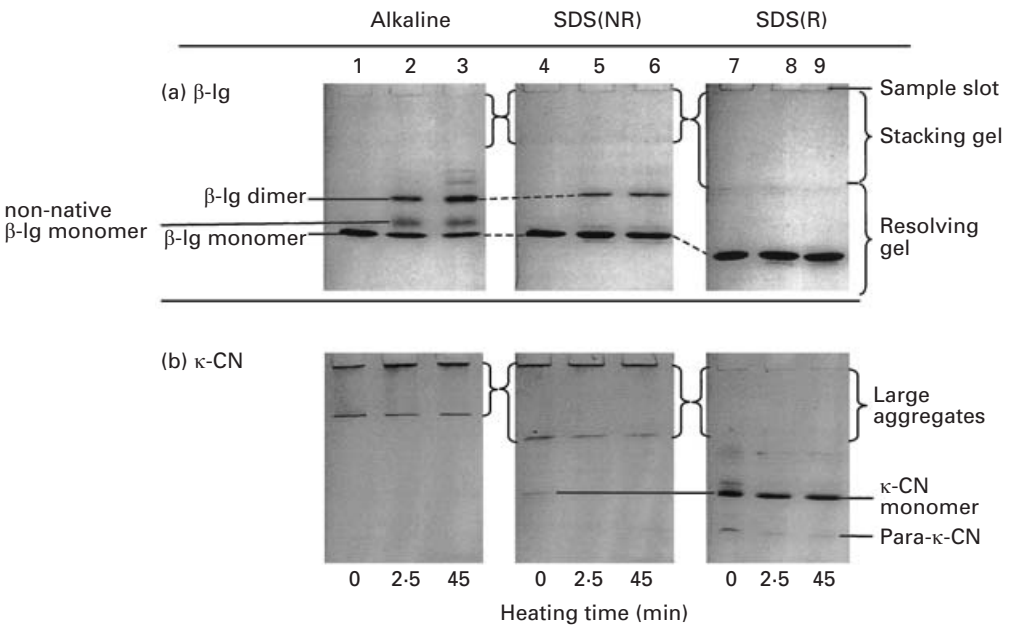

Fig. 2. Electrophoretic patterns of (a) β-lactoglobulin A and (b) κ-casein B (1 mg/ml) heated at 80 °C. Alkaline-PAGE: lane 1, 0 min; lane 2, 2·5 min; lane 3, 45 min. Non-reduced SDS-PAGE (NR): lane 4, 0 min; lane 5, 2·5 min; lane 6, 45 min. Reduced SDS-PAGE (R): lane 7, 0 min; lane 8, 2·5 min; lane 9, 45 min.

SDS-PAGE patterns for κ-casein and heat-treated κ-casein (Fig. 2b, lanes 4–6) showed only minor differences from the alkaline-PAGE patterns (Fig. 2b, lanes 1–3). However, a small band of monomeric κ-casein was observable in the unheated sample, indicating that some monomer protein was hydrophobically associated with the large aggregates. The heat treatment altered the relative intensities of the bands at the top of the stacking and resolving gels and removed the monomer, indicating that disulphide-bond interactions had taken place. Reduction of the sample indicated that there was a low level of para-κ-casein and possibly α_{S2}-caseins in all samples (e.g. Fig. 2b, lane 7).

Although Groves et al. (1998) reported that heat treatment altered the distribution of κ-casein polymers, the present study (Fig. 2b, lanes 4–6) did not show the wide range of intermediate-sized aggregates that were observed by Groves et al. (1998). This is a likely consequence of the intentional exclusion of the lower molecular weight species from our starting material (Fig. 2b, lane 4).

Nevertheless, the present PAGE (Fig. 2b) and CD (Fig. 1) results both support the proposition (Groves et al. 1998) that the heat-induced κ-casein polymers can be different from the native polymers.

Mixtures of β-lactoglobulin A and κ-casein B. β-Lg A solution (2 mg/ml) and κ-casein B solutions (1–4 mg/ml) were mixed 1 : 1 to give solutions containing 1 mg β-lg/ml and 0·5 to 2 mg κ-casein/ml. These were heated at 80 °C in phosphate buffer, pH 6·7, for up to 60 min and analysed by alkaline- (Figs 3a–3d) and SDS- (Figs 3e–3h) PAGE. Control samples, containing 1 mg β-lg/ml, were also heated and analysed and are included in Fig. 5, but not in Fig. 3.

Each of the various bands in the PAGE patterns could be identified by comparison with the patterns shown in Fig. 2 and previously published PAGE results (Havea et al. 1998, 2000, 2001; Manderson et al. 1998; Schokker et al. 1999). This approach was successful for the alkaline-PAGE patterns but there were some bands in the SDS-PAGE patterns (Figs 3e–3h) that were not present in either Fig. 2a or Fig. 2b. Consequently, the sample from lane 5 (10 min heating at 80 °C) in Fig. 3f (2 mg β-lg/ml and 2 mg κ-casein/ml) was analysed by 2 D SDS-PAGE and the resultant electrophoretic pattern is shown in Fig. 4.

All the spots that lie on the diagonal represent proteins that have conformations in SDS buffer and, putatively, molecular weights that are not greatly affected by treatment with the reducing agent, mercaptoethanol. These were clearly SDS-monomeric β-lg, monomeric κ-casein and a few casein impurities. The high-mobility horizontal stripe in Fig. 4 contains reduced SDS-monomeric β-lg and the lower mobility horizontal stripe contains reduced monomeric κ-casein. The large spot at the right-hand end of the high-mobility stripe corresponds to SDS-monomeric β-lg in the unreduced sample and the next, rather ovoid, spot to the left corresponds to (SDS-) dimeric β-lg in the unreduced sample. The protein material between these spots is possibly some of the various modified monomer species. The faint spot to the left of the dimer spot has a corresponding

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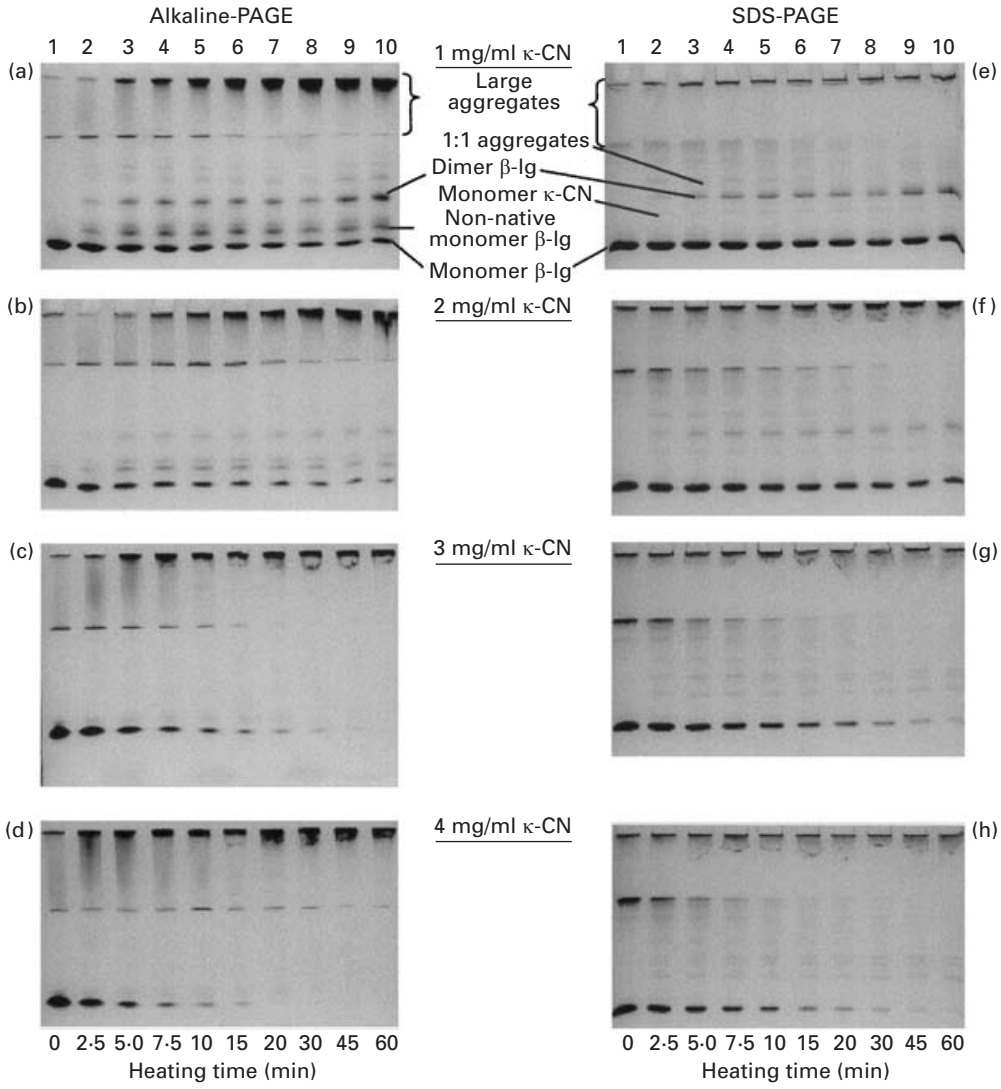

Fig. 3. (a–d) Alkaline- and (e–h) SDS-PAGE patterns of β-lactoglobulin A (1 mg/ml) and κ-casein B (0·5, 1, 1·5, and 2 mg/ml). The mixtures were mixtures heated at 80 °C for: lane 1, 0 min; lane 2, 2·5 min; lane 3, 5·0 min; lane 4, 7·5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; lane 8, 30 min; lane 9, 45 min; lane 10, 60 min. Ratios (w/w) β-lg A : κ-casein B were (a, e) 2 : 1, (b, f) 2 : 2, (c, g) 2 : 3 and (d, h) 2 : 4.

spot in the κ-casein stripe and these probably constitute the disulphide-bonded 1 : 1 adduct of β-lg and κ-casein. The remaining sections of the β-lg and κ-casein stripes correspond to various other disulphide-bonded complexes.

Each lane 1 of the PAGE gels (Fig. 3) shows the pattern of the unheated mixture and clearly these exhibit the features of the patterns for the individual proteins (lanes 1 and 4 of Figs 2a and 2b). Increasing the heating time at 80 °C (lanes 2–10) decreased the concentration of native-like (Figs 3a–3d) or SDS-monomeric (Figs 3e–3h) β-lg; this is shown graphically in Fig. 5a and Fig. 5c, respectively. As the concentration of κ-casein increased, i.e. Figs 3a–3d and Figs 3e–3h, the rate of both native-like and SDS-monomeric β-lg disappearance increased (Figs 5a and 5c, respectively).

The quantity of dimeric β-lg increased with heating time (up to 10 min) when the concentration of β-lg was higher than the concentration of κ-casein (e.g. Figs 3a, 5b, 3e and 5d) but levelled off or decreased slightly with further increases in heating time (e.g. Figs 3c–d and 3g–h) when the κ-casein concentration was greater than the β-lg concentration (Figs 5b and 5d).

The appearance of the large aggregate region changed with heating time and with κ-casein concentration (Fig. 3). In general, the high dye intensity region shifted from the top of the resolving gel to the top of the stacking gel, indicating an increase in the overall size (and molecular weight) of the heat-induced protein aggregates and complexes. The total quantity of protein in the largest aggregates increased with increasing κ-casein concentration in the sample.

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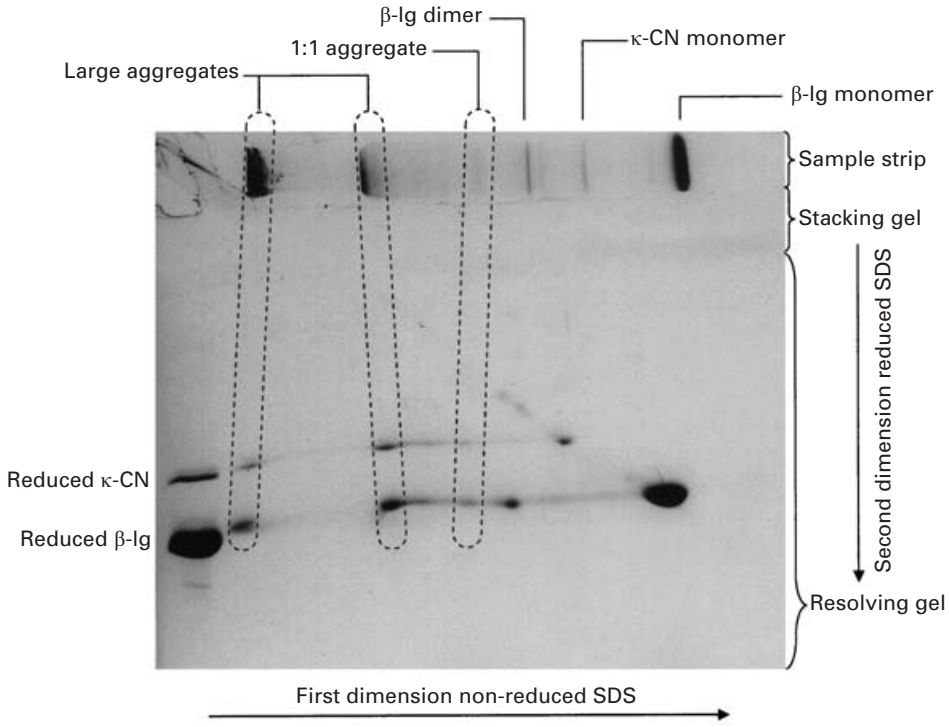

Fig. 4. 2 D electrophoretic pattern (non-reduced and then reduced SDS) of a β-lactoglobulin A and κ-casein B mixture (2 : 2, w/w) heated at 80 °C for 10 min.

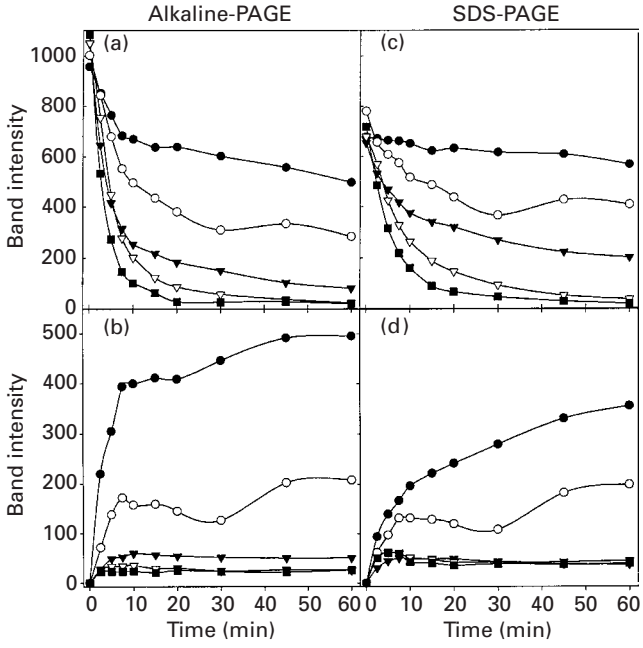

Fig. 5. Changes in the band intensities of (a) alkaline-monomeric, (b) alkaline-dimeric, (c) SDS-monomeric and (d) SDS-dimeric β-lactoglobulin obtained from heat-treated mixtures with different β-lg:κ-casein ratios (w/w): 2 mg/ml β-lg : 0 mg/ml κ-casein = 2 : 0 (●), 2 mg/ml β-lg : 1 mg/ml κ-casein = 2 : 1 (○), 2 mg/ml β-lg : 2 mg/ml κ-casein = 2 : 2 (▼), 2 mg/ml β-lg : 3 mg/ml κ-casein = 2 : 3 (▽) and 2 mg/ml β-lg : 4 mg/ml κ-casein = 2 : 4 (■).

Earlier studies (McSwiney et al. 1994; Iametti et al. 1996; Manderson et al. 1998; Schokker et al. 1999) using purified β-lg showed that heat treatments caused the protein to form, possibly reversibly, a non-native monomer that could then form a disulphide-bonded dimer by sulphydryl-catalysed disulphide-bond interchange reactions. The non-native monomers and dimers then interacted to form a series of β-lg aggregates held together by a combination of covalent bonds and non-covalent association to give a wide range of aggregated species that were clearly observable by size-exclusion chromatography multiangle laser light scattering and PAGE techniques (Schokker et al. 1999). As the heat treatment intensified, the proportion of disulphide bonds increased and the aggregation became essentially irreversible. Addition of proteins such as α-lactalbumin did not alter this pattern of behaviour greatly (Schokker et al. 2000; Havea et al. 2001; Hong & Creamer, 2002). In contrast, the present results (Fig. 3) show that addition of κ-casein to β-lg prior to heat treatment at a 1 : 1 weight (also approximately molar) ratio (Figs 3b and 3f) suppressed the formation of intermediate aggregated species and partially suppressed the formation of non-native monomer and dimer.

To determine whether this was because of a very high reactivity of the intermediate-sized aggregates towards κ-casein or the reactivity of the non-native monomer, we mixed native κ-casein with samples of heat-treated β-lg and determined the effect this had on the resultant aggregates.

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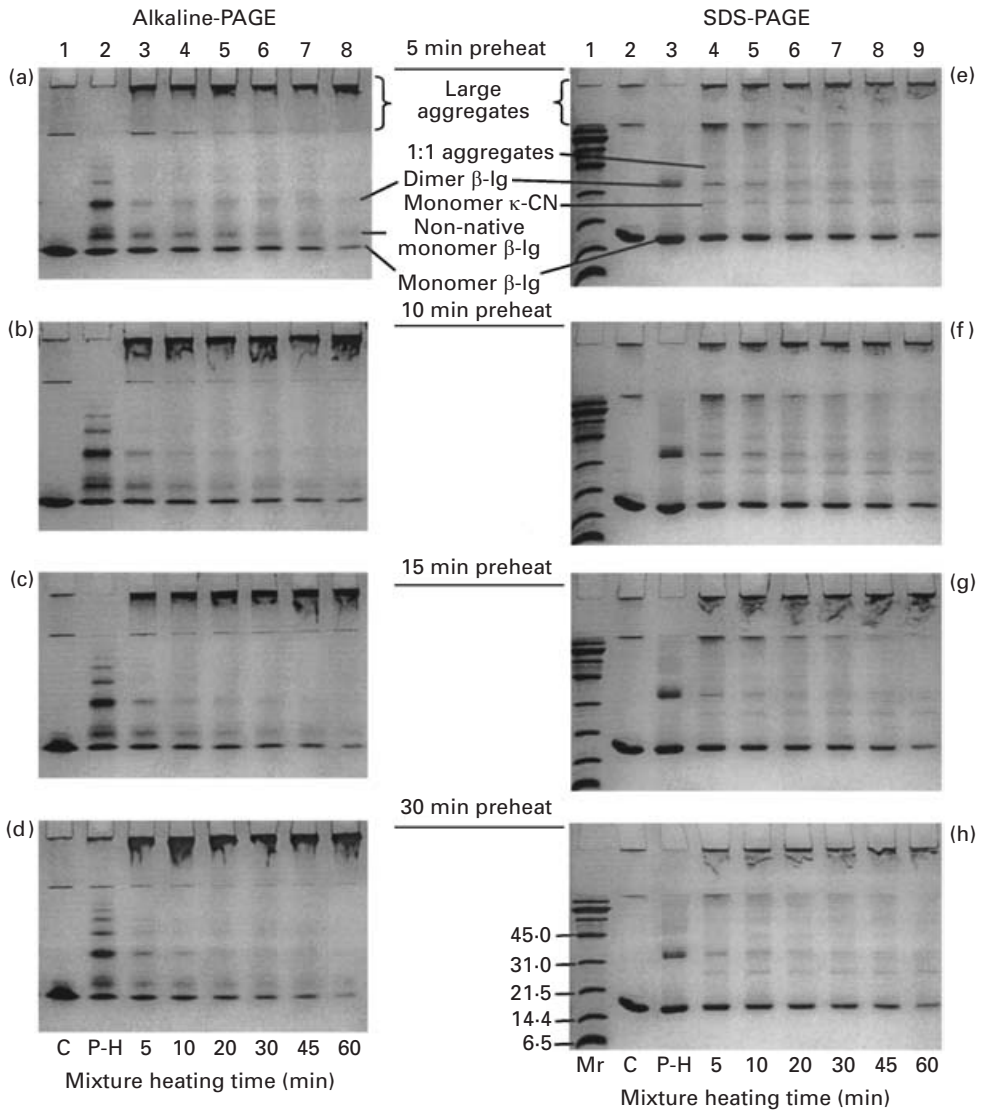

Fig. 6. Alkaline-PAGE (a–d) patterns of the mixture of preheated β-lactoglobulin A (2 mg/ml) and κ-casein B (2 mg/ml) (2 : 2, w/w) heated at 80 °C for: lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 45 min; lane 8, 60 min. Lane 1 (C, control), mixture of unheated β-lg and unheated κ-casein; lane 2 (P-H, preheated), β-lg A preheated at 80 °C for (a) 5 min, (b) 10 min, (c) 15 min and (d) 30 min.

SDS-PAGE (e–h) patterns of the mixture of preheated β-lg A (2 mg/ml) and κ-casein B (2 mg/ml) (2 : 2, w/w) heated at 80 °C for: lane 4, 5 min; lane 5, 10 min; lane 6, 20 min; lane 7, 30 min; lane 8, 45 min; lane 9, 60 min. Lane 1 (Mr, relative mass), molecular weight standard; lane 2, mixture of unheated β-lg and unheated κ-casein; lane 3, β-lg A preheated at 80 °C for (a) 5 min, (b) 10 min, (c) 15 min and (d) 30 min.

The molecular weight standard (Sigma Chemical Co., St. Louis, MO 63 178, USA) contained the following proteins: myosin (M_w 200 000), β-galactosidase (M_w 11 6250), phosphorylase b (M_w 97 000), bovine serum albumin (M_w 66 200), ovalbumin (M_w 45 000), carbonic anhydrase (M_w 31 000), soybean trypsin inhibitor (M_w 21 500), lysozyme (M_w 14 400) and aprotinin (M_w 6500).

Heat-induced interaction between preheated β-lactoglobulin A and native κ-casein B

When samples of preheated (at 80 °C for 5, 10, 15 and 30 min) β-lg A were mixed with κ-casein B and the mixtures were heated again and the products were analysed using alkaline- and SDS-PAGE, the patterns shown in Fig. 6 were obtained.

The unheated samples run in lanes 1 in the PAGE patterns shown in Fig. 3 were run in lanes 1 of Figs 6a–6d and lanes 2 of Figs 6e–6h. The preheated β-lg samples were run in lanes 2 of Figs 6a–6d and lanes 3 of Figs 6e–6h. Apart from the molecular weight standards in lanes 1 of the SDS-PAGE patterns, the remainder of the lanes of the gels shown in Fig. 6 showed the patterns after further heat treatment.

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It is clear that the number of observable β-lg species seen in the alkaline-PAGE patterns of the preheated samples (lanes 2 of Figs 6a–6d) increased with heating time. It is also quite clear that, even at the minimum reheating time (5 min), the reduction in intensity of the dimer and trimer bands was greater than that of either the native-like monomer or the non-native monomer. SDS-PAGE patterns (Figs 6e–6h) show that there was a very rapid production of both monomeric κ-casein and the 1 : 1 adduct, particularly at lower preheat treatments (cf. Fig. 6e and Fig. 6h).

After about 30 min reheating, all of the patterns were essentially the same for either alkaline-PAGE (Figs 6a–6d, lanes 6–8) or SDS-PAGE (Figs 6e–6h, lanes 7–9) and they were not greatly different from the patterns for the same β-lg : κ-casein ratio with no preheat treatments (Figs 3b and 3f for alkaline- and SDS-PAGE, respectively). This indicated that the most important factor in controlling the relative concentrations of the various reaction products was the ratio of β-lg to κ-casein in the initial mixture. The very rapid loss of the polymeric aggregates indicated that the slow step might be conversion of the native and non-native monomer β-lg into a species that could react with polymeric κ-casein.

Possible mechanism of aggregation

The central role of β-lg in the heat-induced reactions involving the casein micelles and the whey proteins is connected with the presence of a Cys residue in the β-lg monomer that is uncovered at higher temperatures and consequently can give rise to a reactive sulphydryl group after heat treatment. Recent studies with β-lg alone (Manderson et al. 1998; Schokker et al. 1999; Havea et al. 2001; Hong & Creamer, 2002) suggested that a non-native monomer may be the first stable entity in the pathway from the native protein to the large, disulphide-bonded β-lg aggregates. The present results, showing that the loss of the native-like character of β-lg was faster in the presence of κ-casein (Fig. 3), suggest that the first steps could involve an equilibrium step as well as irreversible reaction steps. The more rapid loss of β-lg polymers (Fig. 6) indicates that each larger aggregate is more reactive than either native or non-native monomeric β-lg. The simple explanation of this observation would be that each β-lg molecule that is in the heat-induced aggregate contributes a free thiol group and, as it takes only a single thiol interchange reaction to join a κ-casein aggregate to a β-lg aggregate, the reaction would be expected to be faster for the larger aggregates on a statistical basis alone. Nevertheless, the loss of native-like β-lg was faster at higher κ-casein concentrations (Fig. 3). This could be a protein concentration effect or it could be that the κ-casein reacts only with non-native β-lg. However, if there was a reverse reaction of non-native β-lg to native β-lg, albeit slower than the forward reaction, then, *a priori*, there is no reason to believe the disulphide bond shuffling excludes the native disulphide bond species from being one of the several stable 'non-native' β-lg species.

These ideas can be put into equation form as follows:

$$(\beta\text{-lg})_2 \rightleftharpoons (\beta\text{-lg}) \tag{1}$$

$$(\beta\text{-lg}) \rightleftharpoons \beta\text{-lg}^* \rightleftharpoons \{\beta\text{-lg}^*\} \tag{2}$$

where $(\beta\text{-lg})_2$ is native β-lg dimer, (β-lg) is native β-lg monomer, β-lg* is native-like thiol-exposed β-lg and {β-lg*} is non-native thiol-exposed β-lg.

$$\beta\text{-lg}^* + \beta\text{-lg}^* \rightleftharpoons \{\beta\text{-lg}_2^*\} \tag{3}$$

$$\{\beta\text{-lg}^*\} + \{\beta\text{-lg}^*\} \rightleftharpoons \{\beta\text{-lg}_2^*\} \tag{4}$$

where $\{\beta\text{-lg}_2^*\}$ is non-native disulphide-bonded dimeric β-lg.

$$\beta\text{-lg}^* + \{\beta\text{-lg}_2^*\} \rightarrow \{\beta\text{-lg}\}\{\beta\text{-lg}_2^*\} \;\&\; \{\beta\text{-lg}_3^*\} \rightarrow \text{etc} \rightarrow \{\beta\text{-lg}_n^*\} \tag{5}$$

where $\{\beta\text{-lg}\}\{\beta\text{-lg}_2^*\}$ is a β-lg trimer comprising a non-native monomer and a non-native disulphide-bonded dimer, $\{\beta\text{-lg}_3^*\}$ is a disulphide-bonded β-lg trimer and $\{\beta\text{-lg}_n^*\}$ is a large disulphide-bonded β-lg aggregate.

Native κ-casein exists as a mixture of polymers, including monomers, some of which have exposed thiols (Groves et al. 1998). Heating the monomer-free mixture in the present study alters the size distribution to give some monomeric κ-casein.

$$(\kappa\text{-CN})_n + (\kappa\text{-CN})_{n-x} + (\kappa\text{-CN})_{n+x} + \ldots \rightleftharpoons (\kappa\text{-CN}) + (\kappa\text{-CN})_n + (\kappa\text{-CN})_{n-x} + (\kappa\text{-CN})_{n+x} + \ldots \tag{6}$$

where $(\kappa\text{-CN})_n$ is the large native, or non-native, disulphide-bonded κ-casein and x is any small number.

$$(\kappa\text{-CN})_n^{<n} + \{\beta\text{-lg}_n^*\} \rightarrow \text{large aggregates} \tag{7}$$

where $(\kappa\text{-CN})_n$ is any of the κ-casein entities.

The principal question is: how does κ-casein affect this unfolding and aggregation reaction of β-lg? The results clearly show that κ-casein preferentially reacts with the various aggregate species by disulphide-bond interchange (Fig. 6). The effect of κ-casein concentration on the rates of loss shows (Figs 3 and 5) that the concentration of dimer, particularly in relation to the concentration of monomer (cf. open circle plots of Figs 5b and 5a), increases after the first 30 min heating. This indicates that, once a ratio of about 1 : 1 denatured β-lg to κ-casein is attained, the β-lg unfolding and aggregation pathway is restored. This does not preclude the further reaction of the β-lg disulphide-bonded dimers, for example, with the κ-casein–β-lg aggregate. These results, which indicate that a ratio of approximately 1 : 1 κ-casein to β-lg generates the maximum quantity of the disulphide-bonded complexes, confirm and clarify the early results from heat stability studies of Rose (1962) and Tessier & Rose (1964).

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