# Molecular and Microstructural Studies of Thermal Denaturation and Gelation of β-Lactoglobulins A and B

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The thermal properties of β-lactoglobulins (β-lg) A and B at pH 3, 5, 7, and 8.6 were studied by differential scanning calorimetry. Fourier transform infrared spectroscopy was used to monitor changes in the secondary structure of the proteins when heated from 25 to 95 °C. The microstructure of β-lg A and B gels made from 10% (w/v) protein solutions by heating at 90 °C for 30 min was studied by scanning and transmission electron microscopy. β-Lg B had greater thermal stability and required more energy to denature than β-lg A; denaturation of β-lg B was also more cooperative. Infrared spectroscopy showed that β-lg B had a higher proportion of β-sheet than the A variant at pH 3 and 5. At pH 7 and 8.6 the secondary structures of the two variants were similar. At all four pH values, aggregation bands (1682 and ~1622 $cm^{-1}$) were observed when the proteins were heated. Electron microscopy showed that the gel matrix of β-lg B at both acid and alkaline pH was made up of larger aggregate structures than β-lg A. The aggregates formed by both variants were large (1−2 μm) and globular at acid pH but much smaller (nanometer range) and amorphous at alkaline pH. This information provides a useful model for studying the relationship between protein structure and function.

**Keywords:** *β-Lactoglobulin; genetic variants; denaturation; gelation; differential scanning calorimetry; electron microscopy; infrared spectroscopy*

## INTRODUCTION

β-Lactoglobulin (β-lg) is the most abundant globular protein of milk and the major component in the whey fraction of milk, with concentrations ranging from 2 to 4 g/L (Phillips et al., 1994) with a mean value of approximately 3 g/L. The protein is made up of 162 amino acid residues and contains two disulfide groups and one free sulfhydryl group (Swaisgood, 1982). Genetic polymorphism is a characteristic of bovine β-lg; seven different variants have been identified, the most prevalent in commercial preparations being the A and B forms (Eigel et al., 1984). The primary structures of these two variants vary in positions 64 and 118, where the aspartic acid and valine of β-lg A are replaced by glycine and alanine in β-lg B. This seemingly minor difference in the primary structure of the A and B variants has significant effects on their thermal stability (Imafidon et al., 1991; Huang et al., 1994b) and structural flexibility as measured by susceptibility to proteolysis (Huang et al., 1994b). Glycine, valine, and alanine are aliphatic amino acids, while aspartic is a dicarboxylic acid; replacement of one amino acid with another from a different group could, therefore, have a profound effect on the physicochemical properties of the protein.

Physicochemical properties of β-lg play a significant role in the overall functionality of whey proteins, particularly the heat-induced gelation of whey proteins (Kinsella and Whitehead, 1989; Ziegler and Foegeding, 1990). Gelling properties of β-lg can be directly related to its amino acid composition and sequence, as well as its secondary and tertiary conformation in solution. As with other proteins, the gelation of β-lg has been viewed as a two-stage sequential process; the first phase involves heat-induced conformational changes in the protein, which may involve unfolding of some polypeptide segments followed by subsequent protein−protein interactions resulting in a progressive buildup of the gel matrix through aggregation and cross-linking interactions (Ferry, 1948; Bernal and Jelen, 1985). Gelation and aggregation of β-lg under a variety of conditions have been studied by several workers (Harwalkar, 1985; Foegeding et al., 1992; Li et al., 1994). Huang et al. (1994a) found that although both β-lg A and B formed viscoelastic gels when heated, β-lg A had a lower gelation point and a higher initial gelling rate than β-lg B. Under most conditions the storage modulus of β-lg A gels was higher than that of the B variant (McSwiney et al., 1994).

The above information clearly suggests that differences in the primary structure of the β-lg variants have marked effects on their physicochemical and functional characteristics. Very little is known about changes in the secondary structure of β-lg variants when subjected to heat treatments and the microstructure of their heat-induced gels. The objective of the present study was to monitor changes in the thermal properties and secondary structure of β-lg A and B as a function of pH using differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR), respectively. Electron microscopy was used to examine the micro-

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structure of gels made from β-lg variants at both acid and alkaline pH. The interrelationships among the differences in the molecular and microstructural properties are discussed.

## MATERIALS AND METHODS

**Materials.** β-Lg A (L-7880) and B (L-8005) (Sigma Chemical Co., St. Louis, MO) were used without further purification. Deuterium oxide ($D_2O$) (product 15,188-2, minimum 99.9 atom % D) was purchased from Aldrich (Milwaukee, WI). All other reagents were of analytical grade.

**Sample Preparation.** Solutions (10% w/v) of β-lg A and B were prepared by dispersing the proteins in phosphate buffers at pH 3, 5, 7, and 8.6 ($\mu = 0.2$) for the DSC study. The reagents used for the preparation of the phosphate buffers were $H_3PO_4$, $KH_2PO_4$, and $Na_2HPO_4$. For the FTIR study, $D_2O$ was used for making the phosphate buffers in which the proteins were dispersed because of its greater transparency in the region of interest (1600−1700 $cm^{-1}$). Aliquots (0.5 mL) of β-lg A and B solutions, prepared in phosphate buffer as described for the DSC study above, were placed in microcentrifuge tubes and heated at 90 °C for 30 min to form gels. The gels were allowed to equilibrate at 4 °C for 24 h, and their microstructures were examined by transmission and scanning electron microscopy. It should be mentioned that the preparation and storage history of the β-lg samples from Sigma are not known. Changes may occur in the secondary structural characteristics of the β-lg variants during their isolation and purification; the results reported in this study should, therefore, be compared with that of β-lg obtained by other separation processes to confirm the findings reported.

**DSC.** Thermal properties of the β-lg variants (A and B) were studied by DSC using a DuPont 1090 thermal analyzer equipped with a 910 DSC cell base and a high-pressure cell as described previously (Ma and Harwalkar, 1988). Aliquots (10 μL) of each solution were placed in preweighed DSC pans, hermetically sealed, and weighed accurately. A sealed empty pan was used as a reference. The samples were heated from 20 to 120 °C at a programmed heating rate of 5 °C/min. Indium standards were used for temperature and energy calibrations. Peak temperature ($T_m$) and heat of transition or enthalpy ($\Delta H$) were computed from the thermograms by the 1090 analyzer. The $\Delta H$ values were based on the actual protein content of the solution placed in the DSC pan. The width at half-peak height ($T_w$) was also measured. All DSC measurements were done in triplicate.

**FTIR.** Infrared spectra of the β-lg solutions were recorded with a 8210 Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector as described previously (Boye et al., 1995). A total of 512 scans were averaged at 4 $cm^{-1}$ resolution. Wavenumber accuracy was within ±0.01 $cm^{-1}$. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA). The samples were held in an IR cell with a 25 μm path length and $CaF_2$ windows. The temperature of the sample was regulated by placing the cell in a thermostat holder employing an Omega temperature controller (Omega Engineering, Laval, PQ). The temperature was increased in 5 °C increments and the cell allowed to equilibrate for 3 min prior to data acquisition. The reported temperatures are accurate to within ±0.5 °C. Deconvolution of the observed spectra was performed using the Nicolet FTIR Software, Omnic 1.2a. The deconvolution of the infrared spectra was done as described by Kauppinen et al. (1981). The signal to noise ratio was >20000:1, and the bandwidth used for deconvolution was 13 $cm^{-1}$ with a narrowing factor of 2.4 (Boye et al., 1996a). All FTIR experiments were done in duplicate.

**Electron Microscopy.** *Transmission Electron Microscopy (TEM).* The β-lg A and B gels were cut into 0.5 mm cubes, fixed in 2% aqueous glutaraldehyde for 24 h at 6 °C, and postfixed in 2% osmium tetraoxide in 0.05 M veronal−acetate buffer and 0.2 M imidazole (1:1 ratio of buffer to imidazole), adjusted to pH 6.75. Imidazole was used (Allan-Wojtas and Kalab, 1984) to prevent the development of "pepper artifact" by the incidence of minute black dots in the micrograph to which milk proteins are susceptible (P. Allan-Wojtas, personal communication). The samples were then washed with veronal−acetate buffer and dehydrated in a graded ethanol series (20%, 40%, 60%, 80%, 95%, and absolute ethanol). The dehydrated samples were embedded in medium-hard SPURR's low-viscosity medium (Spurr, 1969) and sectioned. Sections (90 nm thick), obtained using a diamond knife mounted on a Leica Ultracut microtome, were stained with uranyl acetate and lead citrate solutions (Reynolds, 1963). Each sample was embedded in duplicate, and six areas were examined in each block. Micrographs were taken on 35 mm film in a Zeiss EM 902 transmission electron microscope operated at 80 kV.

*Scanning Electron Microscopy (SEM).* The gels were cut into prisms (1 × 1 × 10 mm), fixed in 2% aqueous glutaraldehyde, washed in water, and dehydrated in a graded ethanol series (as described for the TEM above). The samples were returned into absolute ethanol, frozen in Freon 12, cooled to −150 °C with liquid nitrogen, and fractured under liquid nitrogen. The fragments were thawed in absolute ethanol at 20 °C and were critical point dried using carbon dioxide. Dry fragments were mounted on aluminum stubs, sputter-coated with gold (a layer approximately 20 nm thick) using a Technics Hummer II sputter coater, and examined in a Zeiss DSC 940 scanning electron microscope operated at 10 kV. Micrographs were taken on 35 mm film. All samples were analyzed in duplicate, and six areas were examined in each block.

## RESULTS AND DISCUSSION

**DSC.** Figure 1A shows the thermograms of β-lg A and B heated at different pH values. The effects of pH on peak temperature ($T_m$), width at half-peak height ($T_w$), and apparent enthalpy ($\Delta H$) are presented in Figure 1B. The highest $T_m$ (83.23 °C for variant A and 85.77 °C for variant B) was observed at pH 3 for both genetic variants (Figure 1B). The lowest $T_m$ (70.07 °C for variant A and 71.20 °C for variant B) was observed at pH 8.6. In general, the $T_m$ values at all four pH values were higher for variant B than for variant A, which suggests that β-lg B was more thermally stable than the A variant. These results confirm those reported by Imafidon et al. (1991) and Huang et al. (1994a,b) but contrast with those of Elofsson (1996), who reported that the peak temperatures for the two variants in phosphate buffer at pH 6.88 were very similar. Other studies (Gough and Jenness,1962; Hillier and Lyster, 1979; Dannenberg and Kessler,1988) also reported the denaturation of the B variant, in a variety of media, to be more rapid than that of the A variant. In a recent study, Nielsen et al. (1996) showed that the irreversible denaturation of β-lg was concentration dependent. This finding may provide an explanation for these contradictory results. In the study by Nielsen et al. (1996), it was observed that at concentrations of 1.25 or 2.5% (w/v), β-lg B was more sensitive to heat treatment than β-lg A, essentially in agreement with Gough and Jenness (1962), Hillier and Lyster (1979), and Dannenberg and Kessler (1988), who used protein concentrations ranging from 0.5 to 1.0% (w/v); at protein concentrations of 10 and 15% (w/v) (as was used in this study) they, however, found that the A variant denatured more rapidly than the B variant. Qi et al. (1995) also reported in an earlier study that both the scanning rate and protein concentration have an effect on the $T_m$ of β-lg, which further confirms the findings of Nielsen et al. (1996).

The differences in the thermal susceptibilities of the two β-lg variants (at the concentration at which they were analyzed in this study) may arise because of amino acid substitutions that result in weakened interactions

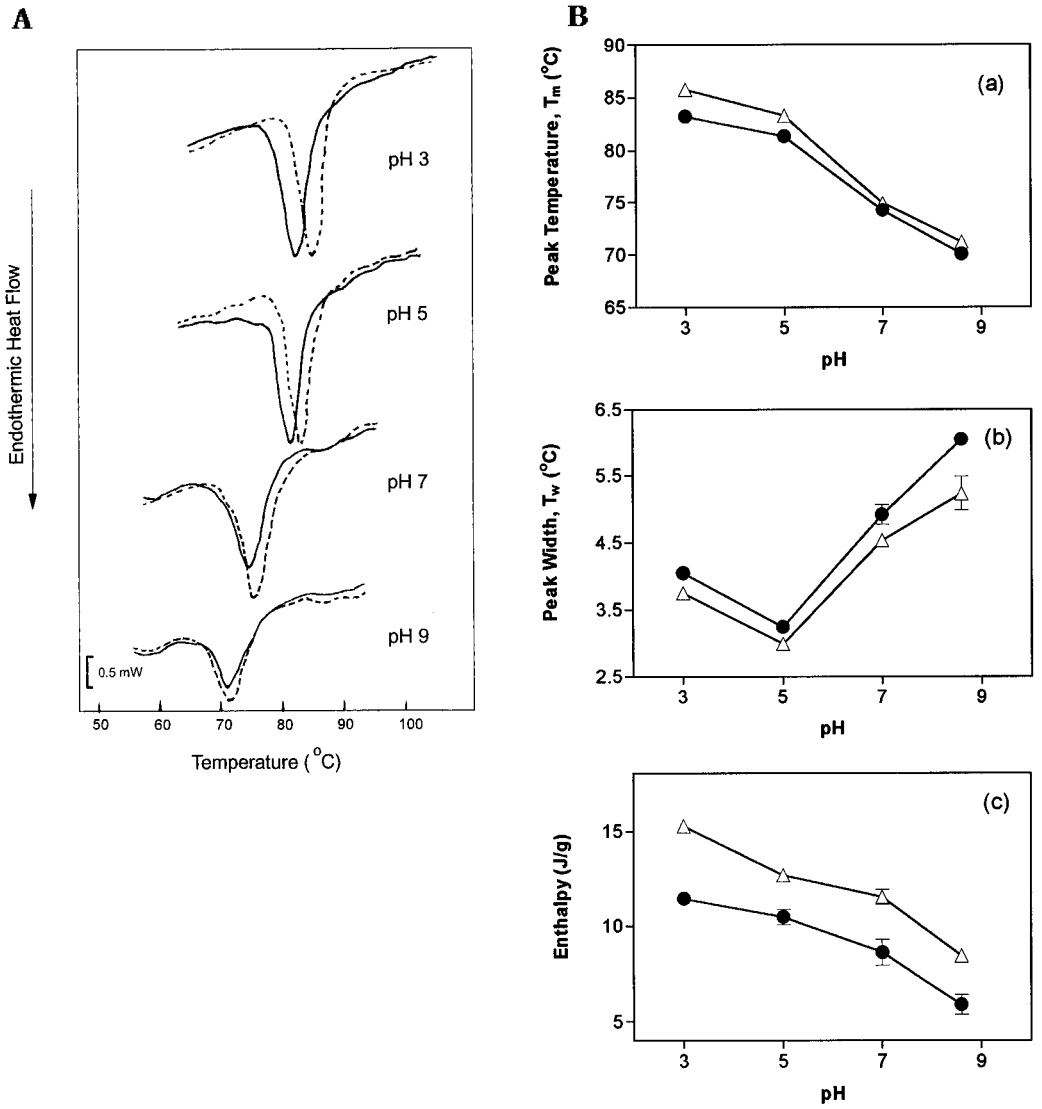


**Figure 1.** (A) Effect of pH on the DSC thermogram of $\beta$-lg A (—) and B (– –) (10% w/v in phosphate buffer; ionic strength = 0.2). Heating rate was 5 °C/min. (B) Effect of pH on the (a) peak temperature of denaturation ($T_m$), (b) width at half-peak height ($T_w$), and (c) enthalpy ($\Delta H$) of $\beta$-lg (10% w/v) A (●) and B (△) in phosphate buffer (ionic strength = 0.2). Heating rate was 5 °C/min.

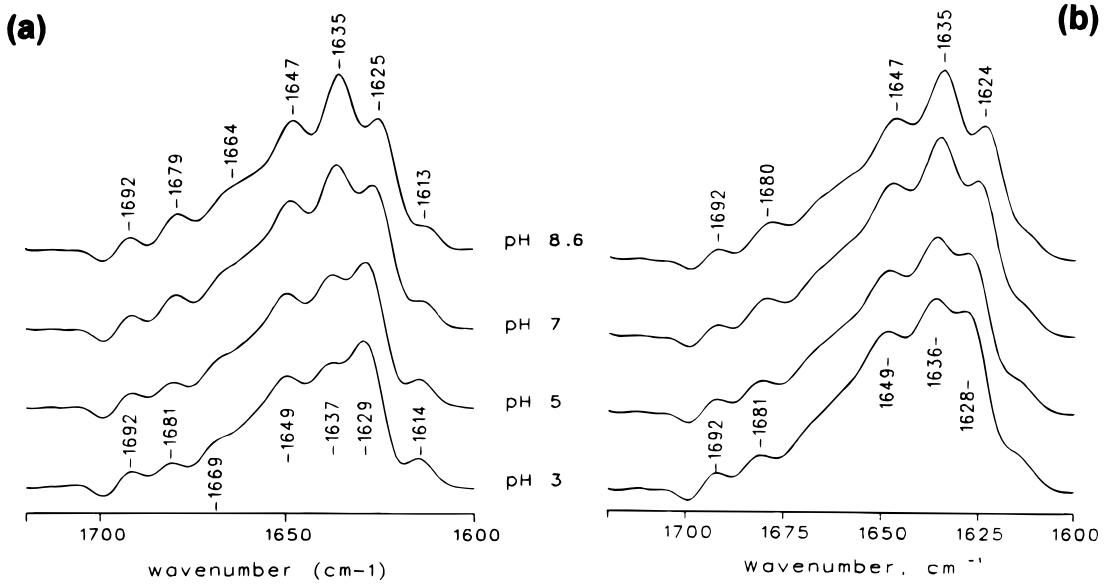


**Figure 2.** Deconvoluted infrared spectra of $\beta$-lg A (a) and B (b) (10% w/v, 25 °C) at pH 3, 5, 7, and 8.6.

critical for thermostability (Phillips et al., 1994). Stability of proteins to thermal denaturation is partially governed by the ratio of polar to nonpolar residues: the higher the proportion of nonpolar residues, the greater the resistance to thermal denaturation (Bigelow, 1967). $\beta$-Lg A has an extra aspartic acid residue at position

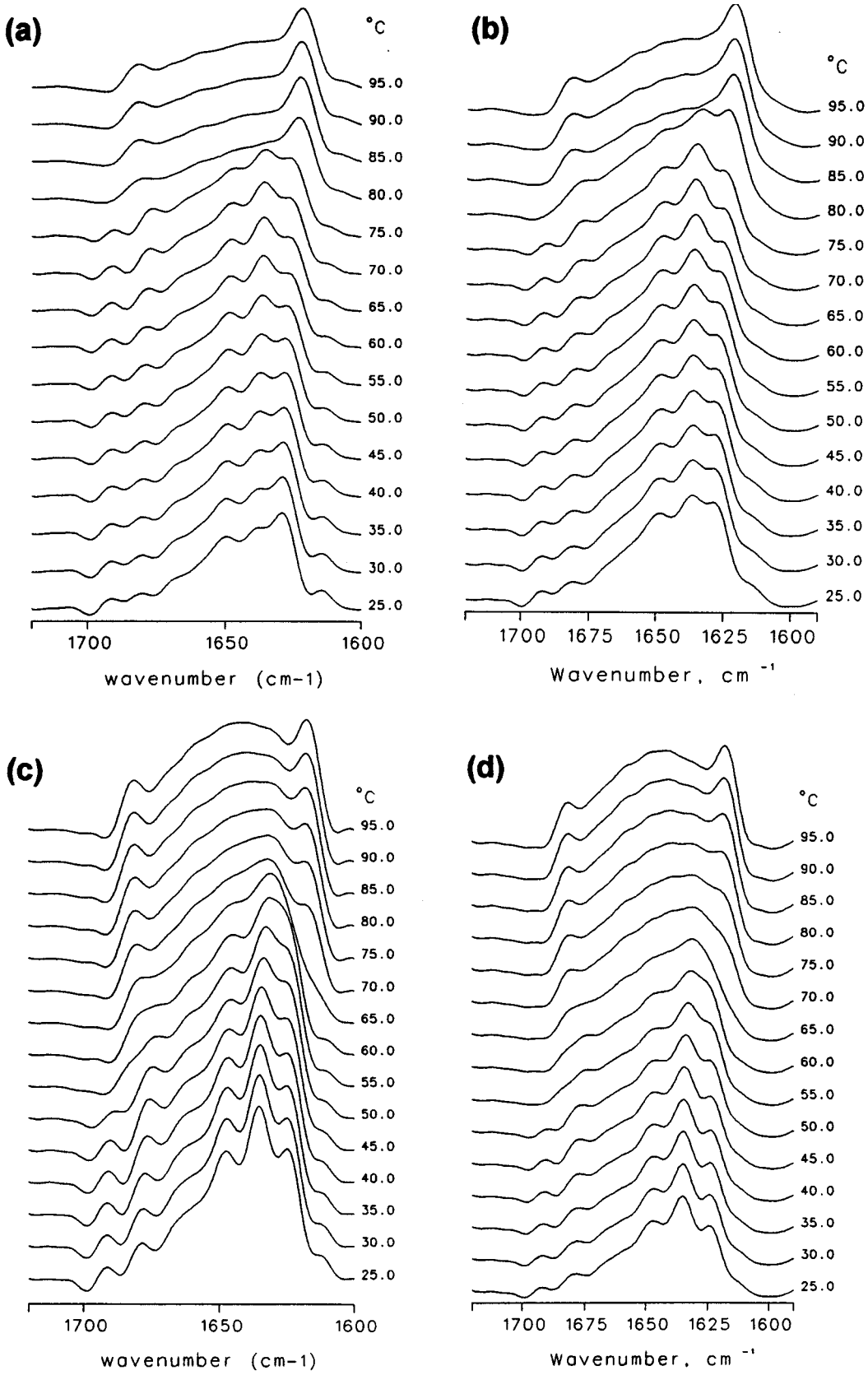


**Figure 3.** Stacked plot of deconvoluted infrared spectra of β-lg genetic variants (10% w/v) in deuterated phosphate buffer at pH 3 (a, b) and 8.6 (c, d): (a, c) β-lg A; (b, d) β-lg B.

64, which increases its relative polarity; this may explain its thermolability relative to the B variant.

The width of the DSC transition at half-peak height ($T_w$) has been used as an indication of the cooperativity of protein unfolding (Privalov et al., 1971). Progressive sharpening of the endothermic peak is indicative of an increase in cooperativity. The $T_w$ for both β-lg A and B was greater at pH 8.6 than at pH 3 (Figure 1Bb), which suggests that the denaturation of both β-lg variants was more cooperative at acid pH than at alkaline pH. Similar findings were reported for the AB variant (Boye et al., 1996). The $T_w$ of variant A was generally greater

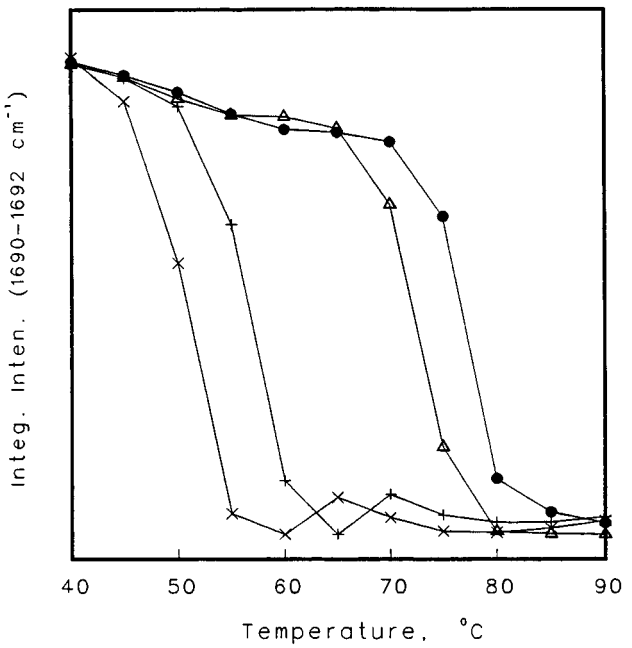


**Figure 4.** Plot of the integrated intensity of the 1690−1692 $cm^{-1}$ band in the infrared spectra of $\beta$-lg A (10% w/v) at pH 3 (●), 5 (△), 7 (+), and 8.6 (×) as a function of temperature.

than for variant B at both acid and alkaline pH, which may suggest that denaturation of $\beta$-lg A is less cooperative than for the B variant. The lowest $T_w$ for both variants was observed at pH 5. At this pH, which is close to the isoelectric pH (5.2) of $\beta$-lg (McKenzie, 1971), the protein molecules have the greatest number of attractive forces leading to precipitation (McSwiney et al., 1994). $\beta$-Lg is one of the few globular proteins remaining in solution near its isoelectric point (Hayakawa and Nakai, 1985). The molecular state of the proteins at this pH may explain the greater degree of cooperativity observed at pH 5.

The $\Delta H$ value represents the total amount of energy required to denature a protein. $\Delta H$ values observed for variant B at all four pH values were greater than for variant A (Figure 1Bc), which suggests that $\beta$-lg B, in addition to being more thermally stable, required a greater amount of energy to denature than the A variant. The $\Delta H$ values at pH 3 were 11.43 J/g for $\beta$-lg A and 15.27 J/g for $\beta$-lg B. These values decreased to 5.87 and 8.42 J/g at pH 8.6, respectively. Protein unfolding is augmented at alkaline pH values, because of excessive intramolecular charge repulsion (Casal et al., 1988). According to Waissbluth and Greiger (1974), the decrease in enthalpy of denaturation as the pH is raised is indicative of the $\beta$-lg molecule becoming more thermolabile and is consistent with the exposure of nonpolar groups to the solvent during heat-induced transition from the native to the unfolded state. Partial unfolding of protein molecules generally results in a lowering of $\Delta H$, since the partially denatured proteins require less heat to denature than native proteins (Ma and Harwalkar, 1988). The results therefore suggest that the proteins may have been partially denatured at pH 8.6 prior to heat treatment, and both variants were denatured to about the same extent.

**FTIR**. The deconvoluted infrared spectra of $\beta$-lg A and B at pH 3, 5, 7, and 8.6 are shown in Figure 2. The FTIR spectra of $\beta$-lg A at pH 3 (Figure 2a), showed seven bands at 1692 ($\beta$-type structure), 1681 ($\beta$-sheet), 1669 (turns), 1649 ($\alpha$-helix), 1636 ($\beta$-sheet), 1629 ($\beta$-strand), and 1614 $cm^{-1}$ (side-chain vibrations) (Chirgadze et al., 1975; Krimm and Bandekar, 1986; Casal et al., 1988; Susi and Byler, 1988; Boye et al., 1996). As the pH was increased to 8.6, a marked increase in the intensity of the band at 1637 $cm^{-1}$ was observed accompanied by a subtle increase in the 1681 $cm^{-1}$ band. The increase in the intensity of these bands suggests an increase in $\beta$-sheet formation with increasing pH. The 1681, 1669, 1649, 1636, and 1629 $cm^{-1}$ bands shifted by 2−5 $cm^{-1}$ to lower wavenumbers as the pH was increased from 3 to 8.6. Such shifting of the amide I bands to lower

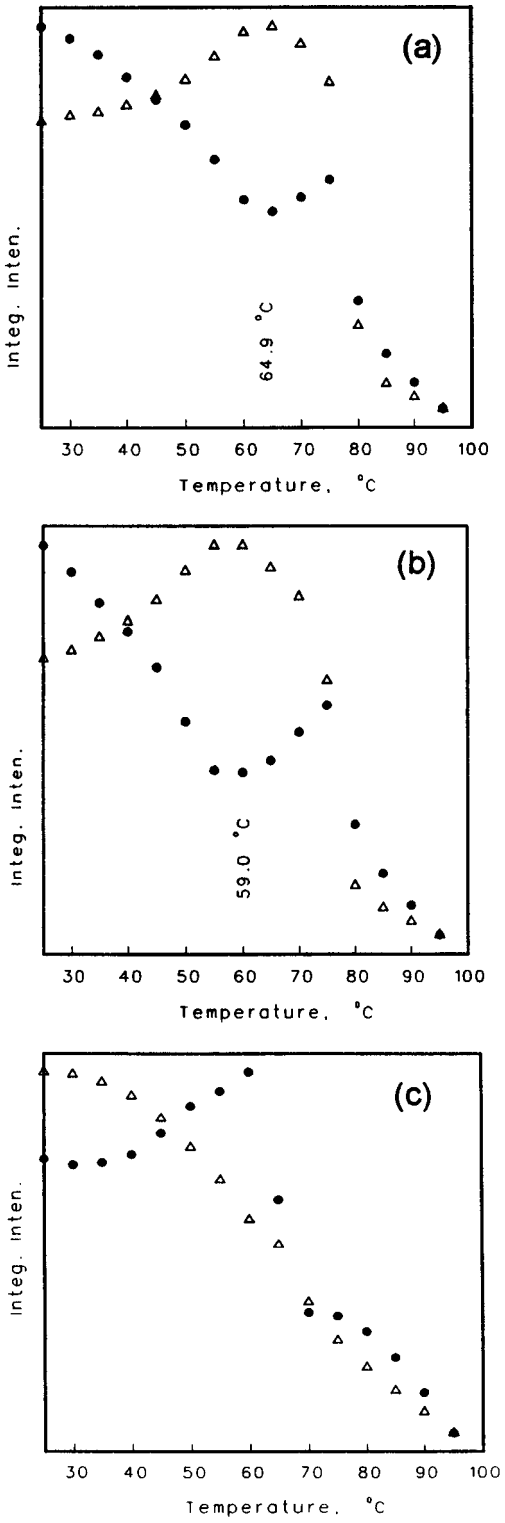


**Figure 5.** Plot of the integrated intensities of the 1628 −1629 $cm^{-1}$ (●) and 1635−1636 $cm^{-1}$ (△) bands in the infrared spectra of $\beta$-lg A at (a) pH 3, (b) pH 5, and (c) pH 8.6. [At pH 8.6, (●) represents integrated intensity of the 1625−1626 $cm^{-1}$ band.]

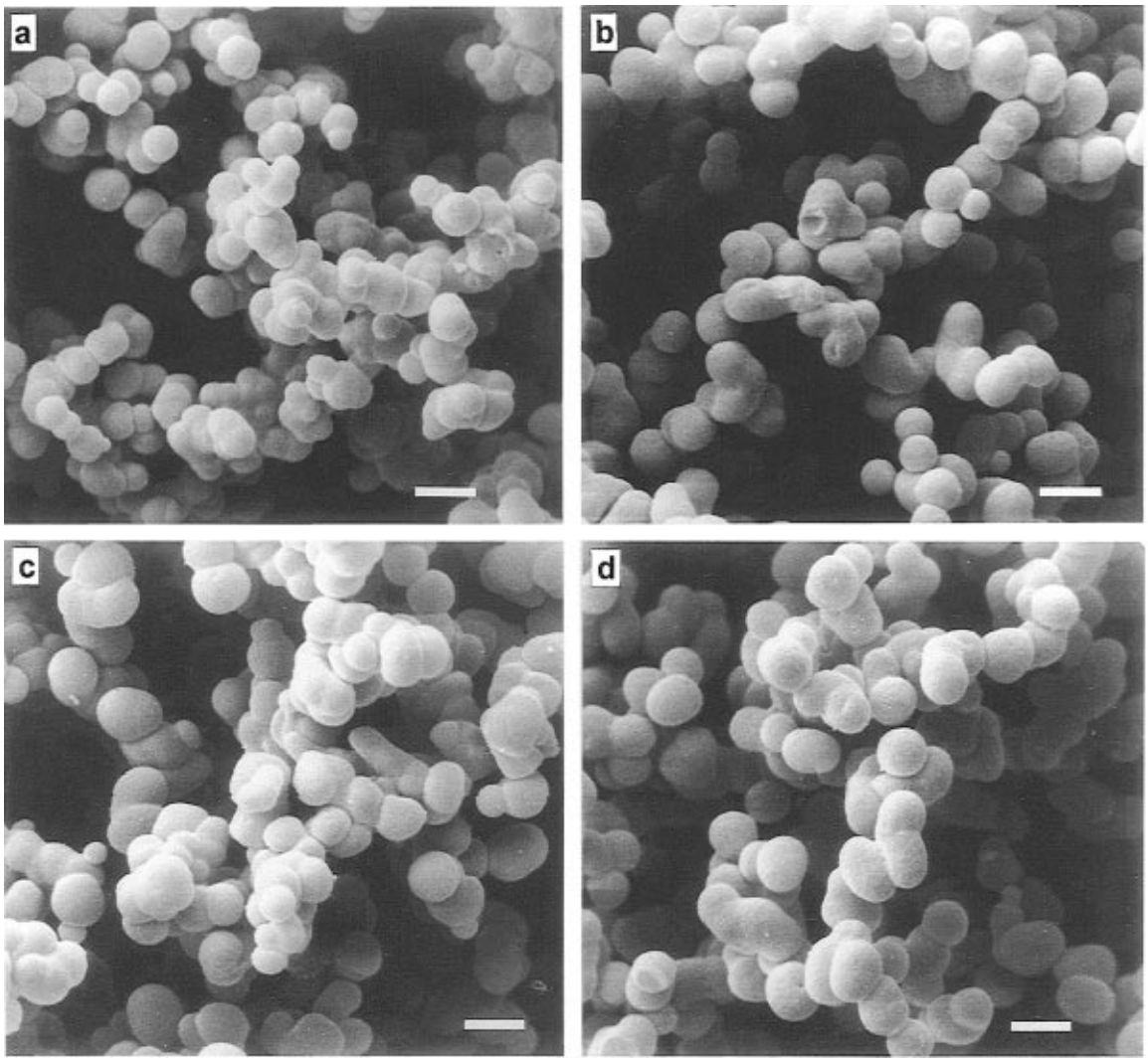


**Figure 6.** Microstructure (SEM) of β-lg A and B (10% w/v) gels produced by heating at 90 °C for 30 min at pH 3 and 5: (a) β-lg A, pH 3; (b) β-lg A, pH 5; (c) β-lg B, pH 3; (d) β-lg B, pH 5. Bar represents 2 μm.

wavenumbers may be attributed to a decrease in the strength of the C═O bond stretching vibration resulting from an increase in hydrogen bonding (Krimm and Bandekar, 1986). Partial unfolding of the protein as the pH was increased may have allowed more solvent within the protein structure and enhanced hydrogen bonding. In addition, the change in the relative intensities of the amide I bands, particularly those at 1625 and 1635 $cm^{-1}$, may be attributed to changes in the protein secondary structure. This confirms the findings of the DSC, which showed a lower $\Delta H$ value at alkaline pH indicative of partial denaturation.

The infrared spectra of the β-lg B variant at pH 3 (Figure 2b) showed five major bands at 1692, 1681, 1649, 1636, and 1628 $cm^{-1}$ and two shoulders at ca. 1669 and 1614 $cm^{-1}$. These bands are similar to those observed for the A variant. A major difference between the spectra of β-lg A and B variants at pH 3 was the relative ratio of the intensities of the bands at 1636/1637 to that at 1628/1629 $cm^{-1}$. In the IR spectra of the A variant, the intensity of the 1629 $cm^{-1}$ band (β-strand) was markedly higher than that of the 1637 $cm^{-1}$ band (β-sheet), which suggests that there is a greater proportion of β-strands in the A variant. In the spectra of the B variant, the intensity of the 1636 $cm^{-1}$ band was higher, suggesting a greater amount of β-sheet structure. The results suggest that β-lg B has a greater proportion of β-sheet relative to β-strands than the A variant at acid pH (3 and 5). Dong et al. (1996) also observed large differences in frequencies and intensities for the bands assigned to low wavenumber β-sheet components near 1633 and 1621 $cm^{-1}$ for β-lg A and B dispersed in $D_2O$. They observed that for any given time after hydrogen−deuterium exchange the 1621/1632 $cm^{-1}$ ratio was greater for variant A than for variant B. [The lower wavenumber assignments of these two bands (as with other bands reported in their study) may be due to the extended time allowed for hydrogen−deuterium exchange.]

At pH 7 and 8.6, however, the spectra of the β-lg variants were very similar, which suggests a similarity in secondary structure of the two β-lg genetic variants at these pH values. For both variants, there was an increase in β-sheet formation (1636/1637 $cm^{-1}$ band) relative to β-strands (1629/1628 $cm^{-1}$) as the pH was increased from 3 to 8.6. Huang et al. (1994b) found no differences in the secondary structure of β-lg A and B at pH 4 and 8 using circular dichroism. The protein concentration used in their study was, however, much lower (1 mg/mL) than that used in this study; as well, the buffer solutions used in the two studies are different, which may partially account for the different observations (Qi et al., 1995; Nielsen et al., 1996). Our results clearly show a difference in the secondary structure at acid pH but not at alkaline pH. Although the A and B forms differ at only two positions, the substitution of a

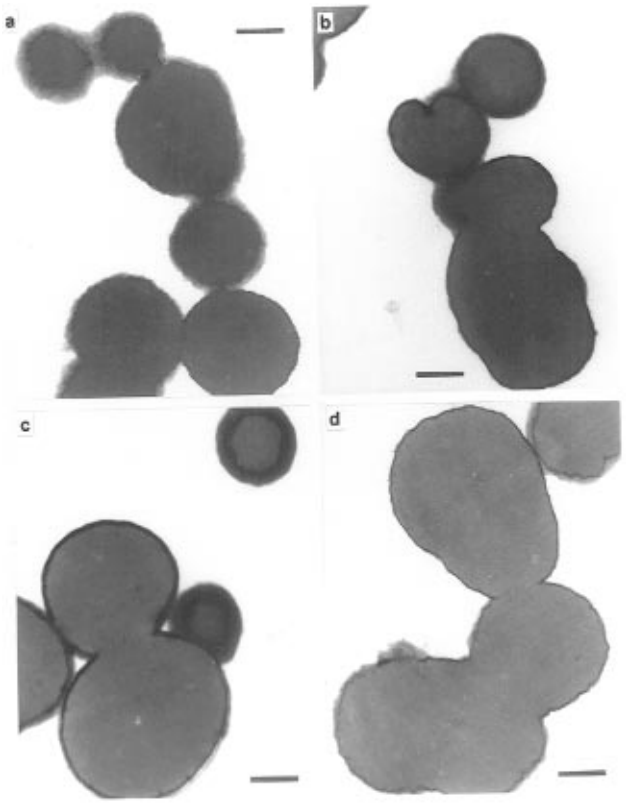


**Figure 7.** Microstructure (TEM) of $\beta$-lg A and B (10% w/v) gels produced by heating at 90 °C for 30 min at pH 3 and 5: (a) $\beta$-lg A, pH 3; (b) $\beta$-lg A, pH 5; (c) $\beta$-lg B, pH 3; (d) $\beta$-lg B, pH 5. Bar represents 0.5 $\mu$m.

Gly residue for an Asp residue at position 64 in the A form is significant because of the increased likelihood of an extra salt bridge forming between the carboxylic group of the Asp residue and any other basic group in the monomer unit (Phillips et al., 1994). At pH 3, the carboxyl group of the Asp residue is not ionized; this would tend to decrease self-association and may explain the decrease in the $\beta$-sheet content of the A variant at pH 3. At alkaline pH values, ionization of the carboxyl group of the Asp residue would tend to increase self-association, which might explain the increase in $\beta$-sheet content observed at alkaline pH for both variants.

The IR spectra of $\beta$-lg A and B heated from 25 to 90 °C at pH 3 and 8.6 are shown in Figure 3. At pH 3, heating both $\beta$-lg A and B to 80 °C resulted in the complete disappearance of the 1692 $cm^{-1}$ band (Figure 3a,b). The disappearance of this band in the IR spectra of $\beta$-lg AB has been previously associated with the onset of unfolding (Boye et al., 1996). For the A variant, the disappearance of the 1692 $cm^{-1}$ band at 80 °C was accompanied by the total breakdown in secondary structure (marked decrease in the intensities of the bands at 1682, 1648, 1636, and 1628 $cm^{-1}$), suggesting denaturation of the protein. For the B variant, however, the loss of secondary structure did not occur until the temperature was raised to 85 °C, suggesting that it required a higher temperature to completely denature. These findings are consistent with the DSC data, which showed that the B variant was more thermally stable than the A variant. The loss of the major bands in the spectra of the two $\beta$-lg variants resulted in the appearance of two new bands at 1682 and 1622 $cm^{-1}$. These two bands have been attributed to the formation of intermolecular hydrogen-bonded anti-parallel $\beta$-sheet structures associated with aggregate formation (Clark et al., 1981; Boye et al., 1996).

The infrared spectra of the $\beta$-lg A and B variants at pH 5 (spectra not shown) were similar to those at pH 3. The 1692 $cm^{-1}$ band, however, disappeared at 75 °C in the spectra for both variants, suggesting that the proteins were more thermally labile than at pH 3. Heating above 75 °C resulted in the disappearance of the bands at 1682, 1648, 1636, and 1628 $cm^{-1}$ (denaturation) and the rise of the bands at 1682 and 1620 $cm^{-1}$ (aggregation). At pH 7, the 1692 $cm^{-1}$ band disappeared at 60 °C in the spectra for both $\beta$-lg A and B (spectra not shown). Between 60 and 70 °C there was a gradual broadening of the amide I bands, and further heating resulted in the appearance of the two aggregation bands at 1682 and 1618 $cm^{-1}$. At pH 8.6 (Figure 3c,d), the 1692 $cm^{-1}$ band in the spectra of both genetic variants completely disappeared at 55 °C. This temperature represents a decrease of about 25 °C from that observed for pH 3. The results confirm that both $\beta$-lg A and B are less thermally stable at alkaline pH. This is true for most proteins; that is, the further one goes from the isoelectric point, the more readily denaturation occurs (Cheftel et al., 1985). Between 55 and 65 °C, at pH 8.6, the bands at 1647, 1635, and 1624 $cm^{-1}$ in the spectra of both variants become increasingly broad, suggesting a loss in secondary structure. Above 65 °C the two bands at 1682 and 1618 $cm^{-1}$ attributable to aggregate formation could be observed. At temperatures around 70 °C, polydisperse aggregates of $\beta$-lg form because of spontaneous interactions arising from the partial unfolding of the molecule, which releases previously committed hydrogen-bonded protein groups for alternative interactions (Sawyer et al., 1971; Elfgam and Wheelock, 1978). The formation of aggregate structures upon heat treatment of $\beta$-lg has also been attributed to hydrophobic and thiol−disulfide exchange reactions (Kella and Kinsella, 1988).

A plot of the decrease in the intensity of the 1692 $cm^{-1}$ band in the IR spectra of $\beta$-lg A at pH 3, 5, 7, and 8.6 is shown in Figure 4. (A similar plot was obtained for $\beta$-lg B; data not shown.) A comparison of the behavior of this band at different pH values can give valuable information on the flexibility of the proteins at different pH values. The data clearly show that at pH 3 and 5 the 1692 $cm^{-1}$ band was relatively stable until 70 and 65 °C, respectively, suggesting that the structure of the protein was tight and inflexible at acid pH, inhibiting unfolding. In striking contrast, there was very little stability in the band at pH 7 and 8.6 above 40 °C, which suggests that the structure of the proteins at neutral and alkaline pH was looser and more flexible, allowing the protein to unfold at a lower temperature.

Figure 5 shows the behavior of the 1636 and 1628 $cm^{-1}$ bands in the IR spectra of $\beta$-lg A at pH 3, 5, and 8.6 as the protein was heated. Closer examination of these bands was necessary because of their prominence in the IR spectra of both variants, which indicated a high amount of $\beta$-sheet/$\beta$-strand content in the secondary structure. The plot for $\beta$-lg A at pH 3 and 5 showed an initial increase in the intensity of the band at 1636 $cm^{-1}$ with heating, to a peak temperature (temperature of inflection), after which it decreased in intensity. The 1629 $cm^{-1}$ band, on the other hand, decreased in intensity with increasing temperature until the temperature of inflection and then increased again in intensity to a peak temperature and subsequently decreased to a minimum value. The temperature of inflection decreased from 64.9 °C at pH 3 to 59 °C at pH 5. Beyond this temperature the 1629 $cm^{-1}$ band

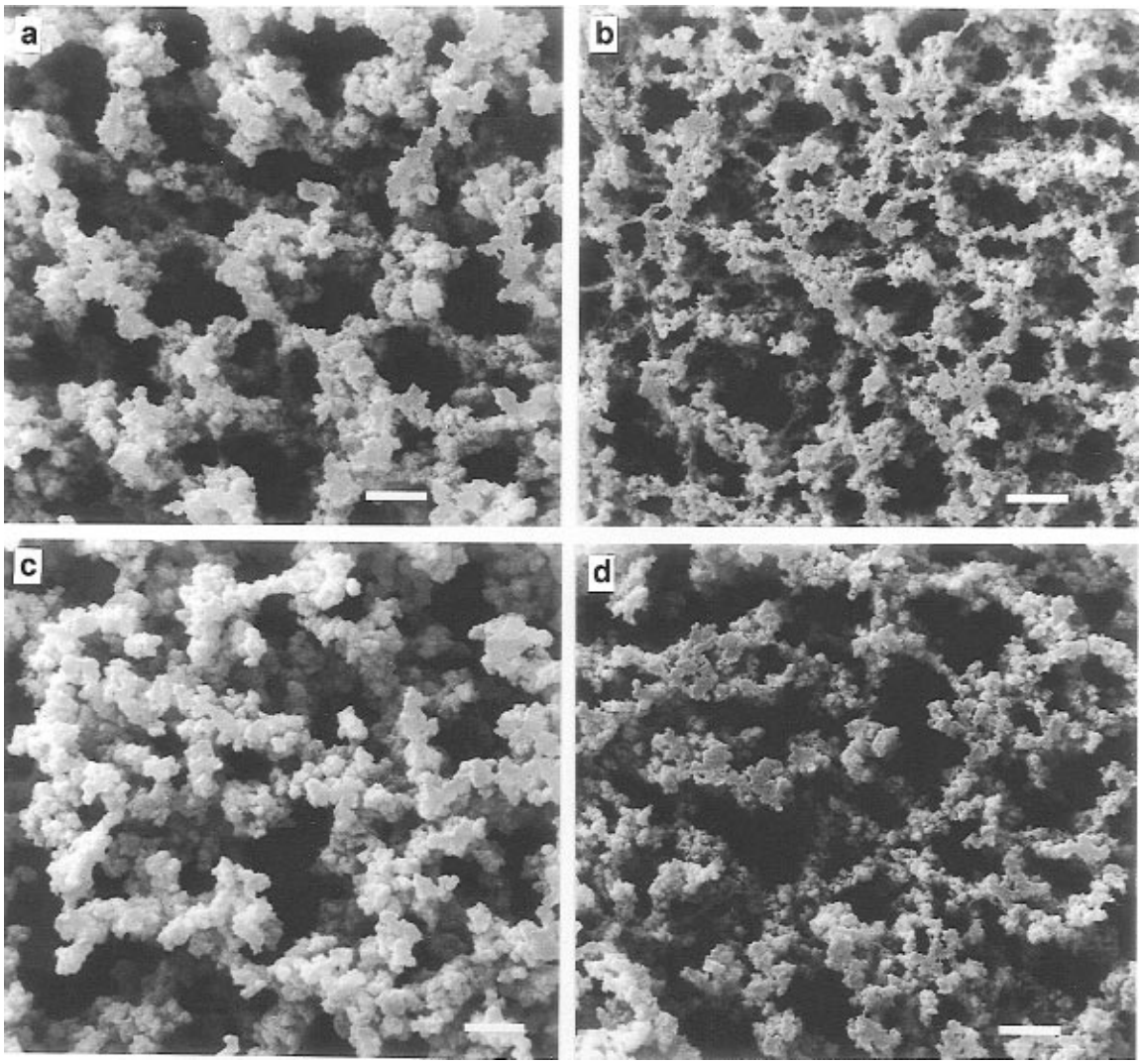


**Figure 8.** Microstructure (SEM) of $\beta$-lg A and B (10% w/v) gels produced by heating at 90 °C for 30 min at pH 7 and 8.6: (a) $\beta$-lg A, pH 7; (b) $\beta$-lg A, pH 8.6; (c) $\beta$-lg B, pH 7; (d) $\beta$-lg B, pH 8.6. Bar represents 2 $\mu$m.

increased to a maximum at 75 °C and then decreased abruptly. The behavior of the 1629 $cm^{-1}$ band in the spectra for the B variant was similar to that observed for the A variant (spectra not shown). The behavior of the 1636 $cm^{-1}$ band was, however, different from that observed for the A variant at pH 3, 5, and 7; the major difference was the absence of any initial increase in the intensity of the 1636 $cm^{-1}$ band between 25 and 45 °C for $\beta$-lg B. Thermal denaturation of $\beta$-lg follows a series of unfolding and association steps. The results obtained suggest three stages in the unfolding and subsequent aggregation of $\beta$-lg A and B at acid pH. The initial phase (between 25 and 60 °C) involves an increase in $\beta$-sheet formation (this was absent for the B variant) accompanied by a concomitant decrease in $\beta$-strand content possibly due to association of the protein molecules. Further heating results in partial dissociation of the $\beta$-sheet structure, possibly into $\beta$-strands. In the third phase (above 75 °C), there is further loss of both $\beta$-sheet and $\beta$-strand structures as new intermolecular anti-parallel $\beta$-sheet structures, attributable to aggregation, are formed. At pH 8.6 (Figure 5c), no initial increase in the intensity of the 1636 $cm^{-1}$ band was observed for $\beta$-lg A; in addition, there was no initial decrease in the intensity of the 1629 $cm^{-1}$ band, suggesting that the first phase of the denaturation process as speculated above is absent at pH 8.6 for both $\beta$-lg variants.

**Electron Microscopy.** Figures 6−9 show the scanning and transmission electron micrographs of gels formed from $\beta$-lg A and B at different pH values. Micrographs obtained using SEM correlated well with the micrographs obtained by TEM. The micrographs showed marked differences in the microstructure of the gels formed at acid and alkaline pH and also between the microstructures of the gels formed from the A and B variants. The greatest differences in gel microstructure were found between gels made at acidic pH (3 or 5) and gels made at neutral and alkaline pH (7 and 8.6 respectively). SEM showed compact spherical globules and their aggregates as structural components of gels made at acidic pH in Figure 6. Thin sections through such globules (TEM) reveal a densely staining uniform interior with a smooth boundary (Figure 7). It may be difficult to use SEM micrographs for an accurate evaluation of the globule dimensions; however, crude measurements of the globule diameters using a pair of calipers and a graduated rule (an average of 15 globules were measured of several micrographs of each individual gel obtained at the same magnification) suggested that the globules produced by variant A at pH 3 (Figure 6a) were in general smaller than those produced by variant B (Figure 6c). Formation of larger aggregate structures resulting from interactions of partially unfolded $\beta$-lg B has also been reported by Huang et al. (1994a) using rheological measurements. Both variants

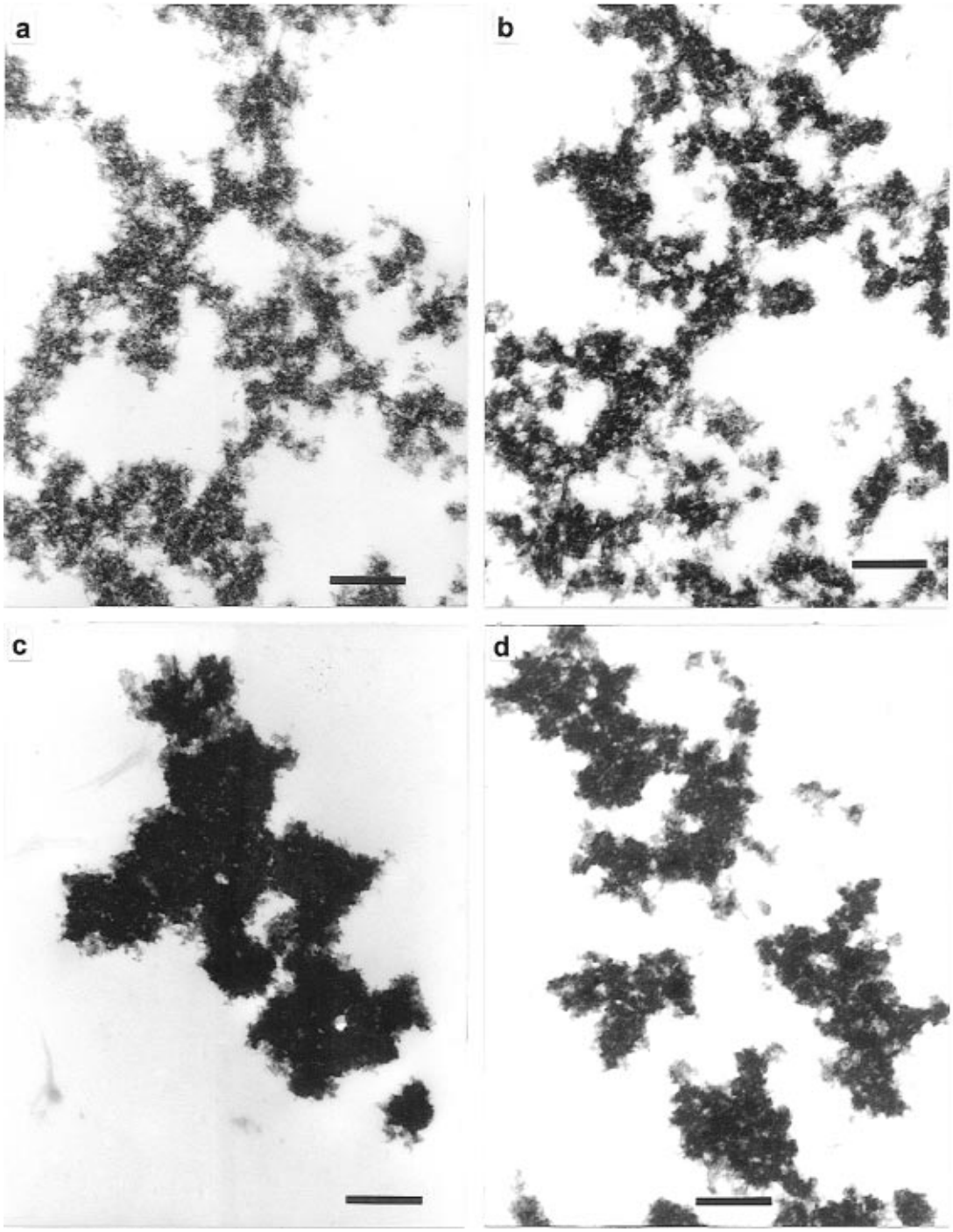


**Figure 9.** Microstructure (TEM) of $\beta$-lg A and B (10% w/v) gels produced by heating at 90 °C for 30 min at pH 7 and 8.6: (a) $\beta$-lg A, pH 7; (b) $\beta$-lg A, pH 8.6; (c) $\beta$-lg B, pH 7; (d) $\beta$-lg B, pH 8.6. Bar represents 0.5 $\mu$m.

produced somewhat larger globules (~2 $\mu$m) at pH 5 than at pH 3 (<2 $\mu$m). It should be noted, however, that the protein globules were sectioned at random and the cross sections do not reflect their true dimension. Statistical corrections would be required to convert such cross sections into true diameter values.

SEM also showed that the compact protein globules formed at acidic pH of 3 (Figure 6a,c) and 5 (Figure 6b,d) were either loosely aggregated or fused into chains or clusters of several globules. Aggregation of protein into these structures affected the initially uniform distribution of proteins and created large spaces void of proteins which were filled with the liquid phase of the gel. TEM micrographs further indicated that the globules consisted of uniformly compacted proteins (Figure 7).

Gels produced under neutral (pH 7.0) and alkaline (pH 8.6) conditions (Figures 8 and 9) had microstructural features completely different from those of the gels made under acidic conditions. In essence, the proteins were more evenly distributed in these gels than in the gels made at lower pH. Their clusters were connected through narrow bridges. Void spaces (between the clusters) were considerably smaller in these gels than in the acidic gels. Consequently, it may be anticipated that the neutral and alkaline gels would have better water holding capacity than the acidic gels. It was earlier reported that the water holding capacity of whey protein concentrate gels formed at pH 7 and 9 was higher than at pH 3 and 5 (Boye et al., 1997).

Coating the fixed and dehydrated gel samples with a

20 nm layer of gold during their preparation for SEM obscured the finest details in these porous gels; void spaces (within the interstitial spaces of each individual cluster) smaller than 40 nm would have been filled with gold (a 20 nm layer on one side and the same thickness on the opposite side would clog a 40 nm pore), and this would make the fine porous microstructure appear compact in the micrographs. TEM of thin sections (Figure 9) indeed shows that protein aggregates, particularly those of β-lg A (Figure 9a,b), were fluffy with pores much less than 40 nm in diameter. Porosity was decreased in gels made from β-lg B (Figure 9c,d), where the protein clusters were more robust. While the diameter of the spherical aggregates (globules) formed at pH 3 and 5 ranged from 1 to 2.5 μm, the aggregates formed at pH 7 and 8.6 were in the nanometer region. Furthermore, the sizes of the clusters formed by both variants at pH 7 (Figure 9a,c) were different from those formed at pH 8.6 (Figure 9b,d); in addition, clusters in the β-lg A gels (at both pH 7 and 8.6) were much smaller than in the β-lg B gels, resulting in finer strands within the gel network for the A variant when compared with the B variant (Figure 9).

The microstructural changes described above may be a reflection of the following physicochemical properties: β-lg is an acid stable molecule that unfolds at alkaline pH values (above pH 7.5); it has been speculated that the molecule undergoes specific structural transitions characterized by a tighter, less elastic conformation at acid pH compared with a highly flexible, more hydrophobic molecule at pH values above 7.5 (Shimizu et al., 1985; Kella and Kinsella, 1988; Reddy et al., 1988). Furthermore, decreased charge repulsion at pH 3 and 5 may have allowed the proteins to form larger more compact globules at acid pH. β-Lg has also been shown to form octamers between pH 3.5 and 5.5 (Hambling et al., 1992), which may also explain the larger aggregates formed. When the pH of β-lg is raised above 7.0, conformational transitions occur in the molecule characterized by an increase in reactivity of a buried carboxyl group, the free thiol group on Cys-121, and a change in the environment of a tyrosine residue (Tanford et al., 1959; Basch and Timasheff, 1967; Townend et al., 1969). The decrease in the thermostability of the β-lg molecule at alkaline pH, which causes the protein to easily unfold when heated, coupled with the increase in thiol−disulfide interchange reaction at this pH, may enhance gel network formation and inhibit the formation of large compact globular aggregates. This may explain the decrease in the size of the globules and the denser network structure formed at alkaline pH. Huang et al. (1994a) and McSwiney et al. (1994) both reported from viscoelastic studies that β-lg A formed stronger gels than β-lg B. This difference in gel properties is thought to result from different molecular interactions between the partially unfolded chains. Parris et al. (1993) observed that β-lg A formed larger, more insoluble aggregates than β-lg B when sweet whey was heated at temperature ranging from 70 to 80 °C. The pH used for their study, however, ranged only from 5.8 to 7. Our results suggest that at both acid and alkaline pH, the β-lg B variant formed larger aggregate structures. Various studies (Foegeding et al., 1992; Qi et al., 1995; Boye et al., 1995; Nielsen et al., 1996) have shown that protein concentration as well as the medium in which proteins are dissolved (eg., phosphate buffer, sweet whey, presence of salts, pH, etc.) can influence denaturation and subsequent aggregation of proteins. Our study examined the pure proteins in phosphate buffer, whereas the study by Parris et al. (1993) used modified sweet whey solutions. There is potential for markedly different interactions when sweet whey is heated, compared with a pure protein solution. Future studies on the aggregation behavior of the β-lg B variants at different pH values and protein concentrations and in different media should shed more light on these findings.

**Conclusion.** In this study, the genetic variants of β-lactoglobulin were found to behave differently when subjected to similar heat and pH treatments, indicating that relatively minor changes in primary structure may have profound influence on the physicochemical properties of proteins. This provides a valuable model for studying protein structure−function relation. Genetic polymorphism of milk proteins has been shown to affect composition and manufacturing properties of milk (Ng-Kwai-Hang, 1992). Detailed studies on the physicochemical and functional properties of individual milk protein genetic variants, such as β-lg A and B, without the interference of other milk components, will facilitate recommendations regarding the selection of particular variants for specific uses in food formulation.

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