Comparison of the Gelation Properties of β-Lactoglobulin Genetic Variants A and B†

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The gelation of β-lactoglobulin genetic variants A and B (β-Lg A and β-Lg B) was examined with 7% protein solutions at pH 7. Both variants formed viscoelastic solid gels exhibiting low phase angles (<8°) and dramatically increasing storage moduli during the cooling period as measured by small-strain dynamic rheology. However, β-Lg A had a lower gelation point (72.3 °C) and a higher initial gelling rate (15.4 Pa/min) than β-Lg B (7.6 min at 80 °C and 2.9 Pa/min, respectively). Furthermore, the β-Lg A gel had different viscoelastic properties than the B variant gel, as indicated by a shorter relaxation time (60 s) than that of the B variant (156 s). Thus, the subtle change in the primary structure involving two amino acid residues results in a very significant change in functionality.

INTRODUCTION

Protein gels, defined as a continuous network of macroscopic dimensions immersed in a liquid medium and exhibiting minimal steady-state flow (Ziegler and Foegeding, 1990), can be induced either by heat treatment or by chemical treatment or by a combination of the two. A two-stage mechanism has been proposed (Ferry, 1948) to describe the formation of thermally induced gels, *viz.*, initial conformational alteration of the native structure induced by heat, followed by interactions between the conformationally altered protein to form a three-dimensional network. It is well-known that gelation properties depend upon the characteristics of the protein, particularly the structural stability. Environmental factors that influence heat stability and protein interactions such as heating temperature, heating and cooling rates, pH, type of ions present, and ionic strength all affect gelation (Foegeding *et al.*, 1986; Kinsella and Whitehead, 1989).

The major genetic variants of β-lactoglobulin present in bovine milk are A and B (β-Lg A and β-Lg B). Their primary structures differ only at residues 64 (A/B: D64G) and 118 (A/B: V118A) (Swaisgood, 1982). Nevertheless, this minor change in primary structure is manifested in differences in thermal stability (Gough and Jenness, 1962; Imafidon *et al.*, 1991; Huang *et al.*, 1993), structural stability in urea (Alexander and Pace, 1971), and structural flexibility as measured by susceptibility to proteolysis (Huang *et al.*, 1993). Consequently, these differences in structural stability may result in different gelling characteristics since partial denaturation of a protein is a prerequisite for gelation. There is a wealth of information on gelation of pooled β-lactoglobulin containing all genetic variants (Mulvihill and Kinsella, 1987, 1988; Paulsson *et al.*, 1990; Foegeding *et al.*, 1992); however, the behavior of individual variants has not been examined.

Variability of functionality of whey protein concentrates (WPC) or whey protein isolates (WPI) obtained from different sources has limited the utilization of these protein ingredients (Morr and Foegeding, 1990). As the major protein component in WPC or WPI, β-Lg is principally responsible for the functionality of these products (Mulvihill and Kinsella, 1987, 1988). Consequently, if the gelation characteristics of β-Lg A and β-Lg B are significantly different, the ratio of these variants in a particular product could be important and possibly contribute to the variation in functionality observed. Moreover, if a particular variant is more desirable for an application, the possibility of genetic selection for that variant exists.

In this study, we compared the gelling characteristics of β-Lg A and B and found that the minor difference in primary structure resulted in a significant difference in gelation temperature and rheological properties of the gel formed.

MATERIALS AND METHODS

Materials. β-Lactoglobulins A and B were purified from the milks of homozygous cows according to the method of Fox *et al.* (1967). After removal of the caseins, 3% trichloroacetic acid was added and the resulting precipitate removed by centrifugation at 12000*g* for 30 min at 4 °C. The supernatant was adjusted to pH 2–3 with 1 M NaOH, concentrated, and dialyzed against distilled–deionized water using a Millipore concentrator/dialyzer with a 10-kDa cutoff membrane until the pH of the solution reached 6–7. The concentrated protein solution was lyophilized and stored at −22 °C in a desiccator until use.

Solutions of β-Lg A or B were prepared at a concentration of 7% (w/v) in 50 mM TES buffer, pH 7.0, containing 50 mM NaCl. After stirring for 1 h, the solutions were aspirated for 1 h at room temperature (23 °C) and the pH was adjusted to pH 7.0 with 1 M HCl or 1 M NaOH. Protein concentration was checked before and after aspiration by measuring the absorbance at 280 nm to confirm that a change in concentration had not occurred.

All chemicals used were of reagent grade.

Determination of Rheological Characteristics. A Bohlin VOR dynamic oscillation rheometer (Bohlin Reologi, Inc., Cranbury, NJ) was used to measure rheological properties of protein solutions and gels during heating, holding at isothermal conditions, and cooling. In all experiments, a Bohlin C-14 concentric cylinder fixture with a 41.349 g-cm torsion bar was used with 2.1 mL of protein solution covered by a 5-mm-thick layer of vegetable oil. Thermal scanning experiments were performed by heating from 25 to 80 °C at a rate of 1 °C/min, holding at 80 °C for 3 h, followed by cooling to 25 °C at a rate of 1 °C/min. Storage moduli (G', elastic element), loss moduli (G'', viscous element), and phase angles were determined at a frequency of 0.05 Hz and an amplitude of 10%, which produced a strain of 0.02. The gel point and initial gelation rate were determined by the method of Hines and Foegeding (1993). A linear regression of G' against time for the first 7–10 points above

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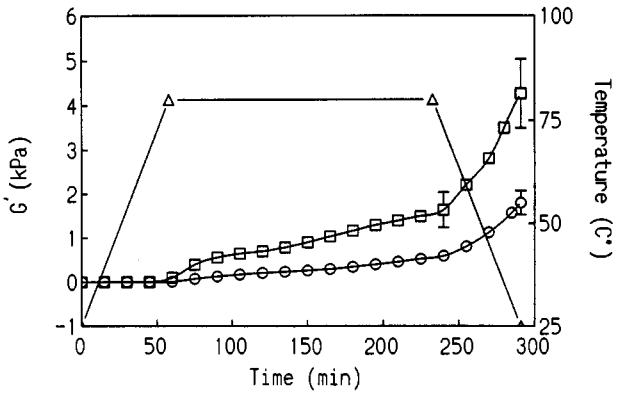

Figure 1. Storage modulus (G') development with 7% (w/v) solutions of β-Lg A (□) and β-Lg B (○) during a temperature program (△) of heating (25–80 °C at 1 °C/min), holding (80 °C for 3 h), and cooling (80–25 °C at 1 °C/min). Protein solutions were in 50 mM TES buffer, pH 7.0, containing 50 mM NaCl. Each data point is the average of three replications of samples with a standard error bar.

1% of the torque bar range was used to calculate the initial gelation rate (Pa/min) and time (min) or temperature (°C) of gel formation as obtained from the slope and intercept, respectively.

Stress relaxation characteristics were obtained with the Bohlin VOR rheometer at 25 °C. The strain rise time was set at 0.1 s, and a constant normal shear strain of 0.0213 was used for all measurements. The data were analyzed according to the method of Purkayastha and Peleg (1986) by the equation

$$tG_0/(G_0 - G) = K_1 + K_2t \quad (1)$$

where t is the time (min), G_0 is the initial modulus obtained from the first stable reading after compression, and G is the modulus at time t. The values for K_1 and K_2 were obtained from the intercept and slope, respectively, of a linear regression of the left side of the equation vs time.

The equilibrium modulus, G_0, was obtained from the equation

$$G_0/(G_0 - G_e) = K_2 \quad (2)$$

and the relaxation modulus, G_r, from the relationship

$$G_r = G_e + (1/e)(G_0 - G_e) \quad (3)$$

The relaxation time, τ, is the time at which the fraction $1/e$ of the stress decay remained to be accomplished.

Statistical Analysis. All measurements were replicated three times. In each replicate, freshly prepared protein solutions were used. Statistical comparisons were made by using the unpaired t-test of the Graphpad Instat program (Graphpad software, San Diego, CA). Differences were considered to be significant at $P < 0.05$ throughout this study.

RESULTS

Gelation properties of genetic variants β-Lg A and B were compared using dynamic rheometry with a small strain (0.02) and deformation to prevent disruption of the gel structure. Profiles of the rheological changes observed with the two variants were recorded during heating (25–80 °C), holding (80 °C for 3 h), and cooling (80–25 °C), and the results are shown in Figure 1. The observed increase in storage moduli and small phase angles (Table 1) for both variants indicate formation of viscoelastic gels in both cases. Also, a dramatic increase in G' values was observed for both variants during the cooling period, perhaps due to increase H-bonding as the temperature is lowered. Low phase angles (all <8°) and loss modulus values (G'') suggest that the gels formed with both β-Lg A and B were more elastic than viscous. A similar pattern of the gelation process has been observed for mixtures of β-Lg A and B by Paulsson *et al.* (1990) and Foegeding *et al* (1992).

Although the overall gelation patterns are similar for both variants and mixtures of the two, the specific gelation characteristics of the two variants are quite different. The storage modulus value (G') and the loss modulus (G'') obtained with β-Lg A were nearly 3-fold larger than those for β-Lg B (Figure 1; Table 1), indicating that a more rigid viscoelastic gel was formed (Hamann, 1991) by the A variant. Furthermore, their gelation points (GP) are different, as are their initial gelation rates (IGR), as can be noted from the values listed in Table 2. β-Lg A gelled at a lower temperature and exhibited a higher IGR than β-Lg B, which did not gel until well after the 80 °C holding temperature was reached. The difference between β-Lg A and B in gelation point is clearly illustrated in Figure 2, which depicts a logarithmic plot of G' values. Early onset of gelation of β-Lg A is indicated by a rapid increase in the storage modulus accompanied by a rapid decrease in phase angle which occurs upon conversion from a viscoelastic fluid to a viscoelastic gel (Hamann, 1991). The time required to attain 50% or 90% of the final G' values determined at the end of the holding period at 80 °C was calculated from the gelation time course, and the values are listed in Table 2. Although the times required to reach 90% G'_{80} were similar for both variants, a shorter time appeared to be required to reach 50% G'_{80} with β-Lg A, reflecting a more rapid initial gelling phase.

The above results imply that β-Lg A must have relatively less thermal stability than β-Lg B so that early denaturation or conformational alteration occurs, resulting in a lower gelation point. This conclusion is consistent with our previous observation of a 5 °C lower onset temperature and maximum temperature of thermal unfolding for β-Lg A obtained by differential scanning calorimetry (Huang *et al.*, 1993).

In the stress relaxation experiments, an instantaneous strain was applied and the decay in shear stress was measured as a function of time. Results of stress relaxation measurements with both β-Lg A and B gels are shown in Figure 3. Both protein gels exhibited a typical viscoelastic solid pattern (Hamann and MacDonald, 1992); however, the gels differed in their rheologic properties, as can be noted from the data given in Table 3. β-Lg A gels appeared to have higher initial stress values (G_0) and larger ΔG values ($G_0 - G_e$) than did β-Lg B gels, suggesting that the A gels were more resistant to strain and that, upon continued application of strain, the structural components of β-Lg A gels readjusted, resulting in a greater relaxation of stress than β-Lg B gels (Hamann and MacDonald, 1992). Moreover, the relaxation time for the β-Lg A gel was 4-fold lower than that of the B gel, indicating a lower viscous constant to elastic constant ratio. Also, a higher G_e value for the β-Lg A gel suggests that this gel may be more elastic than the B gel. These results imply different gel matrix structures for the two genetic variants resulting from different molecular interactions between the partially unfolded chains.

DISCUSSION

Gelation has been recognized as an important functionality of food protein ingredients, involving a dramatic change in rheological behavior and immobilization of a considerable volume of water. The gelation characteristics of many food proteins such as those in dough (Glucklich and Shelef, 1962), surimi (Hamann and MacDonald, 1992), and whey (Mulvihill and Kinsella, 1988; Paulsson *et al.*,

Table 1. Rheological Properties of β-Lactoglobulin Gels[a]

protein	G′ (kPa)		G″ (kPa)		δ[b] (deg)	
	80 °C	25 °C	80 °C	25 °C	80 °C	25 °C
β-Lg A	1.55[a] ± 0.33	4.27[a] ± 0.08	0.20[a] ± 0.06	0.06[a] ± 0.012	7.3[a] ± 0.6	8.0[a] ± 0.1
β-Lg B	0.56[b] ± 0.09	1.80[b] ± 0.27	0.05[a] ± 0.02	0.25[b] ± 0.04	5.2[a] ± 1.1	7.7[a] ± 0.2

[a] Mean value ± standard error of three replications for storage modulus (G′), loss modulus (G″), and phase angle after 3 h at 80 °C and after cooling to 25 °C. Means in a column which are not followed by the same letter are significantly different ($P < 0.05$). [b] δ, phase angle.

Table 2. Rheological Transitions during β-Lactoglobulin Gelation[a]

protein	gel point		IGR (Pa/min)	$T_{50\%G'}$ (min)	$T_{90\%G'}$ (min)
	temp (°C)	time (min)			
β-Lg A	72.3 ± 2.2	–[b]	15.4[a] ± 3.9	68.3[a] ± 19.7	151.3[a] ± 2.7
β-Lg B	80	7.61 ± 1.5	2.9[b] ± 0.1	93.3[a] ± 13.7	162.3[a] ± 3.7

[a] Mean ± standard error of three replications for gel point (GP), initial gel rate (IGR), and time to achieve 50% ($T_{50\%G'}$) or 90% ($T_{90\%G'}$) of the final G′ value after holding at 80 °C. Means in a column which are not followed by the same letter are significantly different ($P < 0.05$). [b] Gelation occurred during heating from 25 to 80 °C at 1 °C/min.

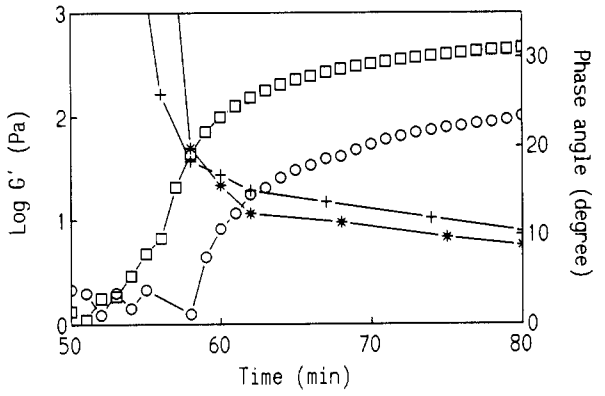

Figure 2. Changes in storage moduli (log G′) of β-Lg A (□) and β-Lg B (○) and phase angles of β-Lg A (+) and β-Lg B (*). The conditions were the same as in Figure 1.

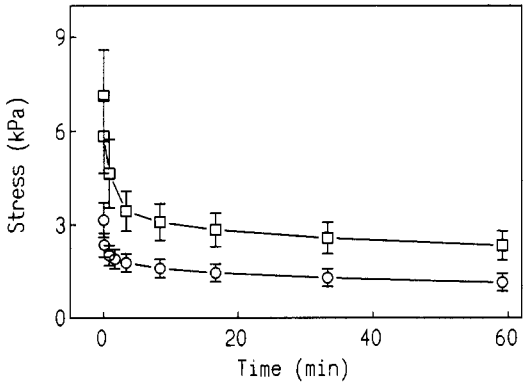

Figure 3. Stress relaxation pattern of β-Lg A (□) and β-Lg B (○). The tests were performed at 25 °C, right after the oscillation tests. Each data point is the average of triplicate measurements, and bars show the standard error.

1990; Foegeding *et al.*, 1992) have been studied extensively. Collectively, the results demonstrate that different proteins from different sources form gels which have greatly different rheological properties. This conclusion is not unexpected since these proteins would have greatly different surface charges, hydrophobicities, and structural stabilities, resulting in different thermal stabilities and molecular interactions between partially unfolded forms. However, little is known of the molecular mechanism of the gelation process or the effect of a subtle change in the primary structure of a protein on the formation and structure of gels. Results of this study show that changes in two amino acid residues of the 162-residue β-Lg molecule produce significant change in the rheological behavior. Such differences may afford the opportunity in future studies to obtain more direct information on the molecular mechanism of gelation processes.

Table 3. Rheological Properties Determined from Stress Relaxation[a]

protein	G_0 (kPa)	G_e (kPa)	τ (s)
β-Lg A	7.14[a] ± 1.45	2.37[a] ± 0.47	40[a] ± 6
β-Lg B	3.16[a] ± 0.54	1.17[a] ± 0.28	156[b] ± 34

[a] Mean value ± standard error of three replications for the initial modulus (G_0), equilibrium modulus (G_e), and relaxation time (τ) at 25 °C. Means in a column which are not followed by the same latter are significantly different ($P < 0.05$). However, G_0 and G_e were significantly different at $P < 0.10$.

Comparison of the molecular properties of recombinant proteins produced using the technique of site-directed mutagenesis and differing in only one or two residues has revealed that significant changes in these properties result from such substitutions. It is already known that the genetic variants of β-Lg have slightly different molecular properties in the native state. For example, the denaturation temperature of β-Lg A is roughly 5 °C lower than that of the B variant (Imafidon *et al.*, 1991; Huang *et al.*, 1993) as determined by differential scanning calorimetry, and β-Lg A is more susceptible to proteolysis (Huang *et al.*, 1993). Furthermore, when a mixture of variants A and B was subjected to proteolysis with immobilized trypsin, the major peptides initially released were identified as fragments of the central core β-barrel domain from β-Lg A (Chen *et al.*, 1993). All of these observations are consistent with the conclusion that the structure of β-Lg A is more flexible and less stable than that of variant B, and therefore, β-Lg A would be expected to initiate gel formation at a lower temperature and the IGR would be expected to be higher since the concentration of partially unfolded forms should be greater.

The rheological parameters for the gel formed with β-Lg A and B are also different, suggesting that there may be differences in the gel matrix. There are no discernible differences in the three-dimensional structures of native forms of β-Lg A and B (Monaco *et al.*, 1987). However, differences in gel structure may result from differences in the structure of the partially unfolded forms that interact to produce gels. Such partially unfolded precursors of gel structure may represent the "molten globule" state of protein structure. These structures, containing appreciable amounts of secondary structure but lacking tertiary structure (Kuwajima, 1989), exhibit strong intramolecular (as in protein folding) or intermolecular (as in formation of inclusion bodies) interactions, the latter being prevented in protein biosynthesis by binding to chaperonins (Hendrick and Hartl, 1993). Evidence for the formation of

such structures in thermally unfolded β-Lg was obtained by CD spectral studies of fragments of the central core β-barrel domain (Chen *et al.*, 1993). Furthermore, the argument that the structures and subsequent interactions of partially unfolded (molten globule) β-Lg A and B are different is supported by the apparently contradictory conclusion reached by Sawyer (1967) and Gough and Jenness (1962) that β-Lg A is more stable than β-Lg B. In both cases, products of the interactions of the molten globule were analyzed rather than measuring directly the formation of the molten globule. Thus, interactions of the molten globule of β-Lg B may have more rapidly formed larger aggregates (turbidity), the aggregates may be less soluble at pH 5, and their sulfhydryl groups may be less accessible to reagents or more available for oxidation to disulfides.

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