# ARTICLES

## Gelation of β-Lactoglobulin Treated with Limited Proteolysis by Immobilized Trypsin†

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The gelation of β-lactoglobulin treated by limited proteolysis with immobilized trypsin was studied by dynamic rheometry. Gelation conditions were (1) 80 °C for 3 h with 7% protein (w/v) in 20 mM $CaCl_2$ at pH 7 and (2) 60 °C for 12 h with 7% (or 15%) protein (w/v) in 20 mM $CaCl_2$ (or 100 mM NaCl) at pH 7. Thermal transition temperatures of the different components in the limited proteolysis mixture were determined by differential scanning calorimetry to be 54.0, 58.8, 64.0, and 81.8 °C, which are believed to correspond to fragments of the β-barrel domain and native β-lactoglobulin. The partially hydrolyzed β-lactoglobulin had a lower gel point and gelled more rapidly than native β-lactoglobulin at 80 °C. Hydrolyzed β-lactoglobulin, heated at 60 °C in 20 mM $CaCl_2$, formed a weak gel at 7% (w/v) protein and a strong gel at 15% (w/v). By comparison, very weak gels were formed with native β-lactoglobulin at both 7% and 15% (w/v) protein at 60 °C.

### INTRODUCTION

β-Lactoglobulin is considered to be the protein primarily responsible for gel formation in whey proteins (Kuhn and Foegeding, 1991; Mulvihill and Kinsella, 1988). A gel is defined as a continuous network of macroscopic dimensions immersed in a liquid medium and exhibiting no steady-state flow (Ziegler and Foegeding, 1990). Under most conditions, β-lactoglobulin gel formation is thermally irreversible. Schmidt (1981) proposed a three-step gelation mechanism for formation of thermally irreversible gels: (1) unfolding of the protein; (2) formation of soluble aggregates; and (3) formation of either insoluble aggregates or a gel. Specific ion and temperature conditions during gel formation can have a major influence on gelation and the final gel rheology (Kinsella, 1976; Wu et al., 1991; Ziegler and Foegeding, 1990).

Limited proteolysis has been applied to many food proteins as a way of changing functionality. Limited proteolysis of $\alpha_{s1}$-casein ($\alpha_{s1}$-CN) with pepsin (Shimizu et al., 1986) or chymosin (Kaminogawa et al., 1980; Creamer et al., 1982) yields $\alpha_{s1}$-CN fragments f1–23 and f25–199 with concomitant changes in curd properties. At pH 4.0, a very limited hydrolysis of ovalbumin is catalyzed by pepsin, resulting in specific cleavage of the His22–Ala23 bond, yielding a large domain: ovalbumin(f23–385). As a result of this limited proteolysis, transparent thermally induced gels are formed rather than the opaque gels normally obtained with ovalbumin (Kitabatake et al., 1988).

Limited proteolysis of β-lactoglobulin with immobilized trypsin produced several fractions of the central β-barrel core domain (Chen et al., 1993; Chen, 1992). Fractionation of the hydrolysate by ion-exchange chromatography yielded a major fraction containing 7.9- and 8.6-kDa peptides, as determined by mass spectrometry, representing fragments of the central core domain. These domain fragments retained their native structure; however, the structural stability was less than that of the intact protein. In light of these characteristics, the gelation properties of limited hydrolysates of β-lactoglobulin are of interest since, potentially, such hydrolysates could easily be produced.

Salts have a large impact on gelation behavior; their effects on β-lactoglobulin gels have been investigated in several laboratories. Effects of sodium chloride and calcium chloride on the gelation of β-lactoglobulin indicated that gels did not form unless salts were added (Mulvihill and Kinsella, 1988). A maximum hardness was obtained with 200 mM sodium chloride or 10 mM calcium chloride. Foegeding et al. (1992) determined the effects of salt on gelation and showed that β-lactoglobulin solutions with 20 mM $CaCl_2$ had lower gel points and gelled more rapidly than those that form in 100 mM NaCl.

Our studies were performed to examine the effects of limited hydrolysis on the gelation properties of β-lactoglobulin. They also give insight into the relationship between structure and function under various gelation conditions.

### MATERIALS AND METHODS

**Immobilization of Trypsin.** Controlled pore glass beads (CPG-2000 Å, 120/200 mesh size, Electronucleonics, Inc., Fairfield, NJ) were cleaned with concentrated nitric acid in a steam bath for 1 h. The beads were washed with distilled water and dried at 110 °C overnight. Cleaned beads were silanized by reaction with at least 4 volumes of a 10% solution of (γ-aminopropyl)triethoxysilane reagent (Silar Laboratories, Inc., Scotia, NY) in toluene at 70 °C for 4 h. The silanized beads were washed extensively with acetone. Amino groups on beads were qualitatively assayed with 2,4,6-trinitrobenzenesulfonate (Sigma Chemical Co., St. Louis, MO) as described by Taylor (1979). The aminopropyl-glass beads were succinylated by treatment with 4 volumes of 10% (w/v) succinic anhydride (Sigma) and 1% triethylamine (Sigma) in acetone at room temperature for 5 min (DuVal et al., 1984). The derivatized beads were extensively washed with acetone and water and then dried at 120 °C overnight.

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The sequential activation/immobilization procedure described by Janolino and Swaisgood (1982) was employed for enzyme immobilization. Succinaminopropyl-glass beads were activated by reacting with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (Sigma) for 20 min in 50 mM sodium phosphate buffer at pH 4.75. Excess reagent was removed by washing with 250 mL of sodium phosphate buffer at pH 7 prior to circulation of trypsin solution. A 10-mL solution of trypsin (6 mg/mL) dissolved in 50 mM sodium phosphate at pH 7 containing 0.02% $NaN_3$ was circulated through 0.5 g of activated beads at 4 °C for 24 h. After circulation, the enzyme solution was drained and noncovalently bound trypsin was removed by washing with 250 mL of 4 M urea in 0.05 M phosphate buffer at pH 7, followed by 250 mL of the same buffer without urea. The immobilized trypsin beads were stored in 50 mM sodium phosphate buffer at pH 7 containing 0.02% $NaN_3$ to prevent microbial growth. The amount of immobilized trypsin per gram of glass beads was 6.4 mg, as determined by an *o*-phthalaldehyde assay (Thresher, 1989) of an acid hydrolysate of the beads. Since the total amount of amino groups of the protein's constituent amino acids is measured in the complete acid hydrolysate, this assay is analogous to a Kjeldahl nitrogen determination.

**Trypsin Activity Assay.** Immobilized trypsin (15 μL of beads) was assayed at 25 °C in a microreactor (Taylor and Swaisgood, 1980) by recirculation of 3.0 mL of 1.0 mM *p*-tosyl-L-arginine methyl ester in 0.04 M Tris buffer, pH 7.5, containing 0.01 M $CaCl_2$. The absorbance change was monitored at 247 nm.

**Limited Proteolysis by Immobilized Trypsin.** β-Lactoglobulin (5 mg/mL) (A + B, 1× or 3× crystallized, Sigma) was dissolved in 20 mM Tris-HCl buffer at pH 8. The solution was cooled for an hour at 5–10 °C before digestion. Digestion was performed by mixing the β-lactoglobulin solution (12 mL) with the immobilized trypsin beads (2 mL) using a stir bar for a period of 20 min. The hydrolysis products were filtered through a 0.45-μm syringe filter, desalted with a prepacked PD-10 column (Bio-Rad), and dried by lyophilization.

**SDS–PAGE.** Samples were analyzed by SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) with an LKB 2050 Midget electrophoresis unit. All procedures were followed according to the LKB Laboratory Manual with a 0.75 mm thick 15% acrylamide slab gel. Samples were reduced with dithiothreitol. Protein bands were visualized by silver staining using a kit obtained from Bio-Rad Laboratories (Richmond, CA) following the manufacturer's protocol.

**Protein Concentration Determination.** β-Lactoglobulin and hydrolyzed β-lactoglobulin concentrations were determined spectrophotometrically. The absorbance was measured at 278 nm with a Gilford spectrophotometer (System 2600, Gilford Instrument Laboratories Inc., Oberlin, OH). The concentration (percent w/v) was calculated by using an extinction coefficient of 0.955 $cm^2\ mg^{-1}$ for the mixture of A and B variants (Bell and McKenzie, 1967).

**Differential Scanning Calorimetry.** A Perkin-Elmer DSC-4 (Perkin-Elmer Corp., Norwalk, CT) differential scanning calorimeter was used to assess the thermal denaturation of each major component resulting from limited proteolysis of β-lactoglobulin. The buffer consisted of 50 mM TES and 20 mM $CaCl_2$ at pH 7. A 60-μL sample containing 6 mg of protein was sealed in a small stainless steel capsule. As a reference, a pan containing 54 μL of buffer was used. The scanning temperature was raised from 20 to 90 °C at a rate of 10 °C/min.

**Dynamic Rheology.** A Bohlin VOR rheometer (Bohlin Reologi AB, Lund, Sweden) was used for heating and rheological measurements. A concentric cylinder-fixed bob and rotating cup (C14) measuring cell attached to a 103 or 11.22 g cm torsion bar were used. Protein solution (2 mL) was added to the cell, and a few drops of corn oil were placed on top to prevent water evaporation. Solutions were heated from 25 to 80 °C or from 25 to 60 °C at a rate of 1 °C/min, held at 80 °C for 3 h or at 60 °C for 12 h, and then cooled to 25 °C at a rate of 1 °C/min. Storage moduli ($G'$), loss moduli ($G''$), and phase angles were determined at a frequency of 0.05 Hz and maximum strain of 0.1. The gel time, which is the point (GP) when an infinite network is formed, was measured by extrapolating the rapidly rising $G'$ back to the time axis (Steventon et al., 1991).

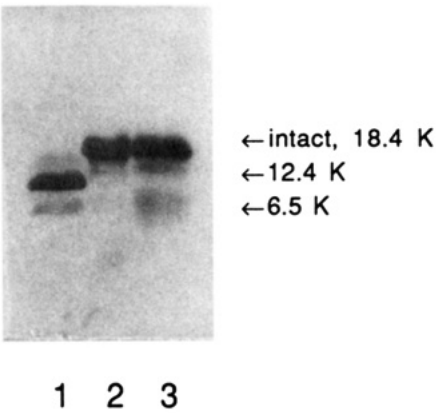


**Figure 1.** SDS–PAGE patterns for digestion of β-lactoglobulin by immobilized trypsin. Digestion was performed in 20 mM Tris buffer, pH 8, at 5-10 °C using 500 units of activity/g of β-lactoglobulin for 20 min. Samples were run on a 15% (w/v) polyacrylamide gel. Protein bands were visualized by silver staining. (Lane 1) Molecular weight marker; (lane 2) native β-lactoglobulin; (lane 3) hydrolyzed β-lactoglobulin.

## RESULTS AND DISCUSSION

**Properties of Hydrolyzed β-Lactoglobulin.** The SDS–PAGE pattern of β-lactoglobulin after limited proteolysis is shown in Figure 1. There are three observable bands in the hydrolysate: native β-lactoglobulin on the top, a 13.8-kDa domain, and broad band that represents a mixture of two fragments of the domain with estimated molecular masses of 9.6 and 6.7 kDa and another peptide with a molecular mass of 3.5 kDa. A number of small peptides are also present, which are not shown on the SDS–PAGE patterns but are observed by ion-exchange chromatography. The actual sizes of the domain fragments were estimated by adding the mass of the C-terminal peptide attached through a disulfide bridge, determined by mass spectrometry, to the weight of fragments determined by SDS–PAGE (Chen, 1992; Chen et al., 1993). The molecular masses are 15.5, 11.3, and 8.4 kDa, corresponding to large portions of the core β-barrel domain and a β-lactoglobulin C-terminal peptide connected by a disulfide bond. The total amount of domain fragments in the hydrolysate was estimated from the peak areas by ion-exchange chromatography to be 13 ± 3% (w/w), and the total amount of hydrolysis was 35 ± 4% as determined from reduction of peak area for the intact protein (Chen, 1992; Chen et al., 1993). Properties of the 8.4-kDa domain fragment have been extensively studied (Chen, 1992; Chen et al., 1993). It is more hydrophobic and less stable and binds to retinal in a pH-dependent manner when domain solutions are exposed to immobilized retinal beads.

Differential scanning calorimetry reveals four peaks in the 25–90 °C temperature range (Figure 2). Limited proteolysis converts β-lactoglobulin from a unicomponent to a multicomponent system. The observed transition temperatures are 54.0, 58.8, 64.0, and 81.8 °C, the latter corresponding to that of intact protein (Table 1). All of the peptide domain structures were shown to be much less stable than native β-lactoglobulin. On the basis of the transition temperature of the purified domain (Chen, 1992; Chen et al., 1993), the transition at 54 °C corresponds to the transition temperature of the 8.4-kDa domain fragment. Since fragments of the intact protein apparently have less stability, we suggest that the transition at 58.8 °C may correspond to the transition temperature of the 11.3-kDa domain fragment and that at 64 °C to the transition temperature of the 15.5-kDa domain fragment. The presence of transitions in small peptides would be

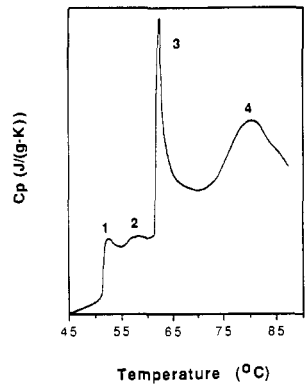


**Figure 2.** Temperature dependence of the heat capacity ($C_p$) of hydrolyzed β-lactoglobulin in 50 mM TES, 50 mM $CaCl_2$, pH 7; protein concentration, 100 mg/mL; heating rate 10 °C/min. (1-3) Thermal transitions of the domain fragments; (4) thermal transition of native β-lactoglobulin.

**Table 1. Effect of Limited Proteolysis on Stability As Measured by Differential Scanning Calorimetry**

| peak | $T_0$[a] (°C) | $T_{max}$[b] (°C) | width at half-peak height (Δ°C) |
|---|---|---|---|
| 1 | 53.2 ± 1.4 | 54.0 ± 0.4 | 5.3 ± 0.2 |
| 2 | 57.7 ± 0.8 | 58.8 ± 0.6 | 2.9 ± 0.4 |
| 3 | 63.4 ± 0.5 | 64.0 ± 0.3 | 2.7 ± 0.1 |
| 4 | 76.4 ± 0.7 | 81.8 ± 0.1 | 7.6 ± 0.5 |

[a] Temperature of onset. [b] Temperature at peak maximum.

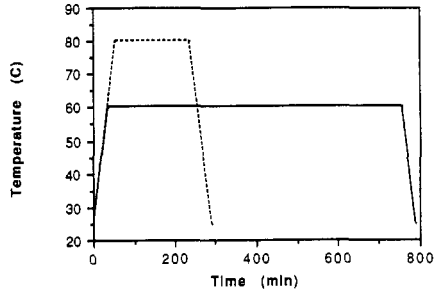


**Figure 3.** Temperature history of the β-lactoglobulin and hydrolyzed β-lactoglobulin during gelation in two experiments with different thermal treatments. Solid line represents holding at 60 °C. Dashed line represents holding at 80 °C.

undetected due to the lack of tertiary structures. The measured transition temperature for pure β-lactoglobulin was 78.5 °C, and the enthalpy change for the transition was calculated to be 221 ± 14 kJ/mol. The observed transition temperature was slightly higher than the reported value for similar conditions, but the enthalpy was about the same (75.3 °C and 230 ± 23 kJ/mol, respectively) (Foegeding et al., 1992). This difference might be due to a different proportion of each genetic variant in the sample. At pH 6.8, denaturation temperatures of variant B were 3.0, 3.3, and 5.5 °C higher than those of variant A in simulated milk ultrafiltrate, sodium phosphate, and sodium–potassium phosphate buffers, respectively (Imafidon et al., 1991).

**Effect of Limited Proteolysis on Gelling Properties at 80 °C.** Rheological transitions during thermally induced gelation can be monitored by small strain oscillatory experiments (Hamann, 1991). Temperature profiles recorded during the gelation and rheological measurements are shown in Figure 3. Changes in storage modulus ($G'$, elastic element) (Figure 4A) and phase angle (Figure 4B) were recorded for the 80 °C gelation regime. The $G'$ values for both β-lactoglobulin and the β-lactoglobulin hydrolysate increased during the holding and cooling period. Due to equipment sensitivity, phase angle measurements did

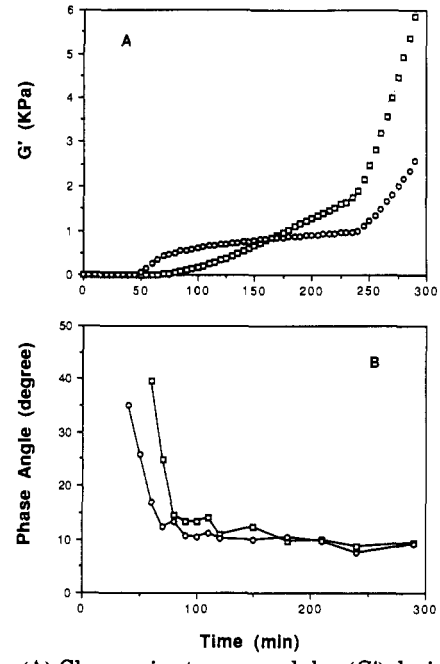


**Figure 4.** (A) Changes in storage modulus ($G'$) during cooking at 80 °C. (B) Changes in phase angle during cooking at 80 °C. Suspensions were 7% (w/v) in β-lactoglobulin (open square) or hydrolyzed β-lactoglobulin (open circle) containing 20 mM $CaCl_2$ at pH 7. The temperature history is shown as a dashed line in Figure 3.

**Table 2. Rheological Transitions during Gelation[a]**

| | GP | | | |
|---|---|---|---|---|
| β-lactoglobulin | temp (°C) | time (min) | $t_{50\%G'}$ (min) | $t_{90\%G'}$ (min) |
| intact 7% | 80 | 38 ± 1 | 110 ± 4 | 164 ± 1 |
| hydrolyzed 7% | 80 | 7 ± 1 | 15 ± 1 | 113 ± 5 |
| intact 7% | 60 | 755 ± 15 | 508 ± 25 | 675 ± 8 |
| hydrolyzed 7% | 60 | 152 ± 22 | 386 ± 10 | 630 ± 4 |
| intact 15% | 60 | 343 ± 21 | 527 ± 7 | 688 ± 4 |
| hydrolyzed 15% | 60 | 116 ± 5 | 388 ± 5 | 639 ± 2 |

[a] Mean value ± standard deviation of three replications for the gel point (GP) and time to achieve 50% ($t_{50\%G'}$) or 90% ($t_{90\%G'}$) of the final $G'$ value after holding at 80 or 60 °C.

not stabilize prior to initiation of the transition. The gel point measures the phase transition from a viscoelastic fluid to a viscoelastic solid (Winter, 1987). A gel time value of 7 min after reaching 80 °C was observed for hydrolyzed β-lactoglobulin, whereas the value was 38 min at 80 °C for native β-lactoglobulin (Table 2). However, the $G'$ for 7% β-lactoglobulin was about twice that obtained with gels from hydrolyzed β-lactoglobulin (Table 3).

The phase angle indicates the relative amounts of viscous and elastic properties of the gels. Phase angles were low in both cases, indicating an elastic structure. The phase angle transition for hydrolyzed β-lactoglobulin occurs earlier than that of β-lactoglobulin (Figure 4B), which is consistent with the gel point data as previously discussed. The times to achieve 50% ($t_{50\%G'}$) and 90% ($t_{90\%G'}$) of the final $G'$ value after holding were found to be greater with intact β-lactoglobulin than with hydrolyzed β-lactoglobulin (Table 2). These changes in the gelation process may be attributed to structural stability changes in the proteolytic fragments, reflecting mixed molecular populations ranging from intact β-lactoglobulin to central core β-barrel domain fragments of progressively lower stability.

The hydrolyzed β-lactoglobulin mixture may be considered a multiprotein system. Several possible mecha-

**Table 3. Rheological Properties of β-Lactoglobulin Gels**

| | G′ (Pa) | | | phase angle | | |
|---|---|---|---|---|---|---|
| β-lactoglobulin | 80 °C | 60 °C | 25 °C | 80 °C | 60 °C | 25 °C |
| intact 7% | 1733 ± 196 | | 5843 ± 795 | 9.9 ± 1.5 | | 10.2 ± 0.8 |
| hydrolyzed 7% | 963 ± 225 | | 2573 ± 294 | 9.4 ± 0.1 | | 9.0 ± 1.6 |
| intact 7% | | 12.9 ± 0.1 | 26.1 ± 0.6 | | 9.9 ± 0.7 | 7.3 ± 0.1 |
| hydrolyzed 7% | | 176 ± 16 | 329 ± 28 | | 11.3 ± 0.7 | 9.1 ± 0.2 |
| intact 15% | | 68.4 ± 15 | 140 ± 30 | | 10.5 ± 1.0 | 8.2 ± 0.4 |
| hydrolyzed 15% | | 4913 ± 103 | 8523 ± 212 | | 9.2 ± 0.5 | 8.4 ± 0.3 |

[a] Mean value ± standard deviation of three replications for the storage modulus (G′) and phase angle after 3 h at 80 °C or after 12 h at 60 °C and after cooling to 25 °C from 80 or 60 °C.

nisms for formation of various types of mixed gels have been described (Ziegler and Foegeding, 1990). (1) Filled gels are obtained when additional components are interspersed throughout the primary gel network. The filler remains soluble for a single-phase gel. (2) A "nongelling" component may associate with the primary network in a random fashion via nonspecific interaction. (3) Two or more proteins may copolymerize to form a single heterogeneous network. (4) An interpenetrating polymer network can be formed, in which networks of two gelling components are continuous throughout the sample. Since hydrolyzed β-lactoglobulin probably contains multiple protein fragments, the mechanism may be a combination of several of these mechanisms. However, the lower gel point of the hydrolyzed protein suggests a copolymerized matrix.

The faster rate observed for gelation of hydrolyzed β-lactoglobulin is attributed to a lower stability of some protein fragments (domains) as shown by DSC. However, the lower storage modulus may result from concentration effects or property differences corresponding to different gel microstructures. Although the total concentrations of β-lactoglobulin and hydrolyzed β-lactoglobulin were the same, the concentration of both intact β-lactoglobulin and domain fragments was obviously less in solutions of hydrolyzed β-lactoglobulin due to the presence of small peptides which may be unable to gel. The effect of concentration on gel stiffness can usually be modeled as a power law: $G' \approx c^n$, where $n$ is typically 2–3 and $c$ is concentration (Paulsson et al., 1990).

**Effect of Gelation Temperature.** Another set of experiments was carried out under the same conditions, except that the temperature was held at 60 °C for 12 h. The progress curves of G′ (Figure 5A) and phase angle (Figure 5B) for gel formation are shown. Note that G′ increased during the holding period for hydrolyzed β-lactoglobulin but did not noticeably increase for β-lactoglobulin. However, since phase angle transitions were observed for both hydrolyzed β-lactoglobulin and β-lactoglobulin, both began to gel during the holding period (Figure 5B). Although the storage moduli for these β-lactoglobulin gels were lower, they were still quite elastic, as indicated by the phase angle (Table 3). The storage modulus was about 10-fold stronger for hydrolyzed β-lactoglobulin than for intact β-lactoglobulin (Table 3), which was the opposite of the results obtained in the 80 °C gelation experiment. Also, the rate of gelation was faster for hydrolyzed β-lactoglobulin than for β-lactoglobulin, as shown by the $t_{50\%G'}$ and $t_{90\%G'}$ values (Table 2) and phase angle transition (Figure 5B). For intact β-lactoglobulin, the final G′ (26.1 Pa) and phase angle (7.3) after cooling for the gel formed at 60 °C showed that the gel was much less rigid than that formed at 80 °C: final G′, 5843 Pa; phase angle, 10.2. Also, for the hydrolyzed β-lactoglobulin, the final G′ after the gel formed at 60 °C cooled (329 Pa) would indicate a weak gel compared with the gel

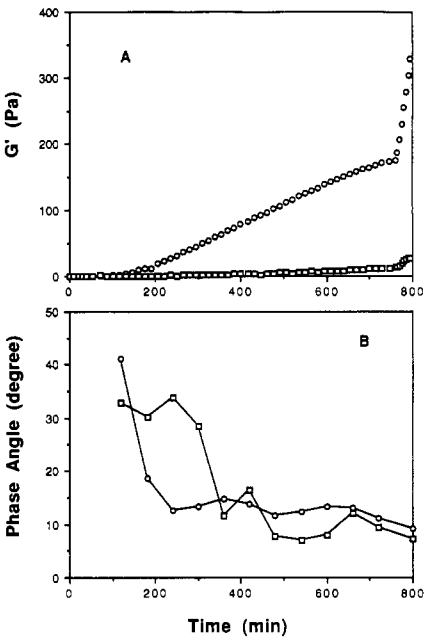


**Figure 5.** (A) Changes in storage modulus (G′) during cooking at 60 °C. (B) Changes in phase angle during cooking at 60 °C. Suspensions were 7% (w/v) in β-lactoglobulin (open square) or hydrolyzed β-lactoglobulin (open circle) containing 20 mM $CaCl_2$ at pH 7. The temperature history is shown as a solid line in Figure 3.

formed at 80 °C with a final G′ of 2573 Pa. Furthermore, the rates of gelation for both samples were much slower at 60 °C than at 80 °C. These results show that the gelation temperature has a major effect on the gelation properties of the protein and hydrolysate preparations.

Gelation of β-lactoglobulin at 60 °C has never been reported. This temperature is considered to be the lowest temperature possible for gelation of whey protein (Ziegler and Foegeding, 1990). The onset temperature for thermal unfolding of β-lactoglobulin, as measured by DSC, is around 70–75 °C. However, the first stage in thermal denaturation, measured by IR, occurs at about 60 °C (Casal et al., 1988). Formation of a gel at 72 °C has been reported by Foegeding et al. (1992). From a kinetic point of view, the gelation rate increases with rising temperature. This expectation is consistent with observations that the rate of β-lactoglobulin gelation is slower at 72 °C than at 80 °C (Foegeding et al., 1992) and that hydrolyzed β-lactoglobulin gels at 60 °C, yet much slower than it does at 80 °C. However, the slow rate of gelation of β-lactoglobulin at 60 °C is not due to a lower rate of intermolecular association but is the result of a large thermodynamic barrier to unfolding. Thus, gelation requires that partial unfolding occur prior to intermolecular association. However, there is no temperature at which both the unfolding and aggregation processes are greatly favored (Wu et al., 1991). Therefore, both formation rate and the final equilibrium

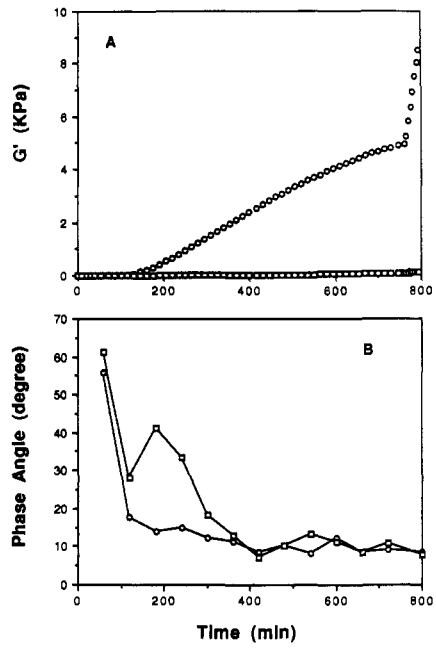


**Figure 6.** (A) Changes in storage modulus ($G'$) during cooking at 60 °C. (B) Changes in phase angle during cooking at 60 °C. Suspensions were 15% (w/v) in β-lactoglobulin (open square) or hydrolyzed β-lactoglobulin (open circle) containing 20 mM $CaCl_2$ at pH 7. The temperature history is shown as a solid line in Figure 3.

modulus depend upon temperature. In contrast to its behavior at 80 °C, intact β-lactoglobulin is not a good gelling agent at 60 °C; consequently, the mechanism of gelation at 80 °C for hydrolyzed β-lactoglobulin must be different from the one at 60 °C. The low $G'$ of 7% hydrolyzed β-lactoglobulin at 60 °C may be due to the fact that β-lactoglobulin is mostly folded at this temperature, thus lowering the concentration of molecules which can undergo intermolecular interactions.

**Effect of Concentration on Gel Properties.** The effects of protein concentration are very dramatic (Tables 2 and 3). The results in Figure 6A and Table 3 show that 15% hydrolyzed β-lactoglobulin is capable of forming a very strong gel at 60 °C. The $G'$ values for the hydrolyzed β-lactoglobulin gel did not reach a plateau, indicating that an equilibrium gel matrix had not formed completely. In contrast, $G'$ values for 15% β-lactoglobulin changed very little, although the data indicate that a very weak gel formed after cooling (140 Pa; Table 3). Thus, the gel formed by 15% hydrolyzed β-lactoglobulin was 61 times more rigid than that of intact β-lactoglobulin gels formed at 60 °C. Comparison of 7% and 15% hydrolyzed β-lactoglobulin gels formed at 60 °C shows that the final $G'$ for the 15% protein gel was 29 times stronger than for the 7% gel. However, the time course of gelation was the same, as indicated by the $t_{50\%G'}$ and $t_{90\%G'}$ values (Table 2) for both hydrolyzed and intact β-lactoglobulin. Also, the phase angle transitions occurred at an early holding period for both hydrolyzed and intact β-lactoglobulin (Figure 6B). Apparently, concentration only affects the rigidity and not the rate constants of gelation, as would be expected if the rate-limiting step is unfolding rather than intermolecular association. Rate constants that were independent of concentration were also observed by Wu et al. (1991) with myosin gelation.

Since intact β-lactoglobulin only forms very weak gels at 60 °C, the gel formed by 15% (w/v) hydrolyzed β-lactoglobulin could be attributed to a primary network formed by the domain fragments or an interaction between

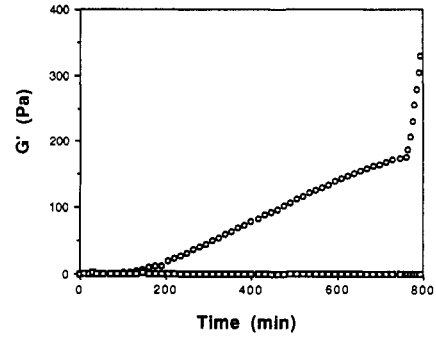


**Figure 7.** Changes in storage modulus ($G'$) during cooling at 60 °C. Suspensions were 7% (w/v) in hydrolyzed β-lactoglobulin, pH 7, containing 20 mM $CaCl_2$ (open circle) or 100 mM NaCl (open square). The temperature history is shown as a solid line in Figure 3.

domains and β-lactoglobulin. In the hydrolyzed β-lactoglobulin mixture, roughly 13% of the total protein is present as β-barrel domains; the rest is present as a mixture of folded and unfolded intact β-lactoglobulin. Therefore, the concentration of gelling agents is about 2% (w/v) (=13% of total protein) domains and an unknown amount of unfolded β-lactoglobulin. Both the type of protein and the environment determine the critical concentration required for gelation. Paulsson et al. (1986) found that 2% β-lactoglobulin was required to detect transitions in a complex modulus during heating. However, Matsudomi et al. (1991) reported that a minimum of 5% β-lactoglobulin was required for gelation in 100 mM Tris-HCl buffer, pH 8, at 90 °C for 15 min. Our results indicate that about 1% (w/v) of domains (present in a 7% hydrolyzed β-lactoglobulin mixture) and partly unfolded β-lactoglobulin form a very weak gel, whereas about 2% (w/v) of domains (in 15% hydrolyzed β-lactoglobulin) and partly unfolded β-lactoglobulin form a very strong gel. Although intact β-lactoglobulin gels were very weak after 12 h at 60 °C, the increase of $G'$ for hydrolyzed β-lactoglobulin may be due to intact protein associating with the network formed by partially unfolded β-lactoglobulin domains or unfolded intact β-lactoglobulin may become part of the network. This may be one reason why 15% hydrolyzed β-lactoglobulin gels have such high $G'$ values.

**Effect of $CaCl_2$ and NaCl on Gelation.** All previous results were obtained in 20 mM $CaCl_2$ and 50 mM TES buffer. If the protein is partially unfolded and above a critical concentration, strong and elastic gels can be formed. Effects of NaCl and $CaCl_2$ on gelation and gel properties have been reported by Mulvihill and Kinsella (1988), Kuhn and Foegeding (1991), and Foegeding et al. (1992). Although some differences were observed in the presence of NaCl vs $CaCl_2$, gels were formed with either or both of these salts. Our results indicate that 7% hydrolyzed β-lactoglobulin will only form gels with 20 mM $CaCl_2$ and not with 100 mM NaCl at 60 °C (Figure 7). Furthermore, no precipitate was present in the protein solution with 100 mM NaCl. Therefore, the presence of $Ca^{2+}$ is critical for gelation when the concentrations of gelling agents are low. According to Foegeding et al. (1992), two potential explanations may be offered for the specific effects of divalent cations on gelation: (1) the cations may alter the denatured state and thus stabilize a structure that is more prone to aggregation; or (2) the protein structures denature in a similar manner, and divalent cations mediate the association process in a different way from monovalent cations.

**Conclusions.** Limited proteolysis of β-lactoglobulin has a dramatic effect on gelation of solutions of this

partially hydrolyzed protein. Although only about 15% of the protein is hydrolyzed to yield core β-barrel domains, the thermal stability of the mixture is reduced and gelation occurs at lower temperatures than with the intact protein. The intact protein gels at 80 °C, and the $G'$ is less for the hydrolyzed protein. However, at 60 °C, strong gels are obtained with 15% hydrolyzed protein solutions, whereas only very weak gels are formed with intact protein.

These results illustrate how limited proteolysis of β-lactoglobulin affects gelling functionality. We have previously shown that treatment with immobilized trypsin beyond 20 min resulted in a rapid disappearance of the β-barrel domains (Chen et al., 1993). We suggest that the existence of structured domains with lower thermal stability can explain the altered gelation characteristics.

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