

Cross-Linking and Rheological Changes of Whey Proteins Treated with Microbial Transglutaminase

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Modification of the functionality of whey proteins using microbial transglutaminase (TGase) has been the subject of recent studies. However, changes in rheological properties of whey proteins as affected by extensive cross-linking with TGase are not well studied. The factors affecting cross-linking of whey protein isolate (WPI) using both soluble and immobilized TGase were examined, and the rheological properties of the modified proteins were characterized. The enzyme was immobilized on aminopropyl glass beads (CPG-3000) by selective adsorption of the biotinylated enzyme on avidin that had been previously immobilized. WPI (4 and 8% w/w) in deionized water, pH 7.5, containing 10 mM dithiothreitol was cross-linked using enzyme/substrate ratios of 0.12–10 units of activity/g WPI. The reaction was carried out in a jacketed bioreactor for 8 h at 40 °C with continuous circulation. The gel point temperature of WPI solutions treated with 0.12 unit of immobilized TGase/g was slightly decreased, but the gel strength was unaffected. However, increasing the enzyme/substrate ratio resulted in extensive cross-linking of WPI that was manifested by increases in apparent viscosity and changes in the gelation properties. For example, using 10 units of soluble TGase/g resulted in extensive cross-linking of α -lactalbumin and β -lactoglobulin in WPI, as evidenced by SDS–PAGE and Western blotting results. Interestingly, the gelling point of WPI solutions increased from 68 to 94 °C after a 4-h reaction, and the gel strength was drastically decreased (lower storage modulus, G'). Thus, extensive intra- and interchain cross-linking probably caused formation of polymers that were too large for effective network development. These results suggest that a process could be developed to produce heat-stable whey proteins for various food applications.

KEYWORDS: Whey protein; immobilized transglutaminase; transglutaminase; protein cross-linking; electrophoresis; Western blots; rheology

INTRODUCTION

To be competitive in the food ingredient markets, the functionality of whey proteins must be continuously improved and designed for specific uses. Chemical and physical methods are commonly used for modifying whey proteins (1–3). More recently, enzymes including mammalian and microbial transglutaminase (TGase) have been applied (4–9). Transglutaminase (protein–glutamine: amine γ -glutamyltransferase, EC 2.3.2.13) catalyzes a protein cross-linking reaction through an acyl transferase mechanism involving protein-bound glutamyl residues (acyl donor) and primary amines (acyl acceptors), including the ϵ -amino group of lysine residues in certain proteins. The covalent cross-linking of proteins catalyzed by transglutaminase can cause dramatic changes in the size, conformation, stability, and other properties of proteins. The enzyme has been used for modifying the functionalities of various proteins including soy

proteins, myosin, gluten, globulin, casein, and whey proteins (4, 8, 10–13). Immobilization of mammalian TGase by selective adsorption of the biotinylated enzyme to avidin immobilized on a glass bead matrix was developed by Huang et al. (14). Wilcox and Swaisgood (15) applied this technology for immobilization of microbial TGase for modification of whey protein isolate (WPI). The immobilized form of the enzyme allowed for easy separation of the catalyst and substrate, and eliminated any requirements for a downstream inactivation step. These authors examined the effect of limited cross-linking on the rheological properties of the proteins. However, studies of changes in rheological properties of whey proteins as affected by extensive cross-linking with TGase, in either soluble or immobilized form, are limited.

This study examined the degree of cross-linking of WPI using TGase at varying enzyme/substrate ratios and characterized the rheological properties of the cross-linked proteins.

MATERIALS AND METHODS

Materials. WPI (~60% β -lactoglobulin and 20% α -lactalbumin) was obtained from Davisco International (Minneapolis,

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MN). Purified microbial transglutaminase was obtained from Ajinomoto Co. Inc. (Japan) and stored in the freezer ($-20\text{ }^{\circ}\text{C}$) until use. Controlled-pore glass was purchased from CPG Inc. (Lincoln Park, NJ). Immunopure avidin was purchased from Life Science Resources (Milwaukee, WI). Reagents for biotinylation and bicinehoninic acid (BCA) protein assays were purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. Immobilization of TGase. Purified TGase was immobilized on controlled-pore glass beads (CPG-3000) as previously described (15, 16). Briefly, the following steps were carried out: (1) CPG-3000 glass beads were cleaned with nitric acid, aminopropylated with 10% (3-aminopropyl)triethoxysilane, and biotinylated with *N*-hydroxysuccinimide long chain (NHS-LC) biotin reagent, also named succinimidyl 6-(biotinamide) hexanoate, and avidin was selectively bound by overnight recirculation of a 1 mg/mL avidin solution in 50 mM phosphate buffer, pH 6.0, containing 0.9% NaCl. (2) A 10 mg/mL TGase solution in 50 mM phosphate, pH 6.0, was dialyzed against the same buffer and centrifuged at 10 000 rpm for 20 min; the supernatant was mixed with 2 mg/mL of NHS-LC biotin in 50 mM bicarbonate, pH 8.5, and allowed to react overnight, and the excess reagent was removed by dialysis against 50 mM phosphate, pH 6.0. (3) Biotinylated TGase was recirculated over the prepared avidin beads for 12 h, after which the beads were washed with 1 M urea and 50 mM phosphate, pH 6.0, to remove nonselectively adsorbed protein. All steps involving centrifugation, recirculation, and dialysis were carried out at $4\text{ }^{\circ}\text{C}$.

Transglutaminase Activity. Enzyme activity measurements were performed at $37\text{ }^{\circ}\text{C}$ using *N*-carbobenzoxy (CBZ)-glutaminyl-glycine and hydroxylamine as substrates. Activities of soluble and immobilized TGase were assayed following the procedure described by Folk and Chung (17) and Swaisgood et al. (16), respectively. One unit of enzyme activity was equivalent to a change in absorbance of 0.29/min at 525 nm, corresponding to the formation of $1\text{ }\mu\text{mol}$ of hydroxamate/min at pH 6.0. In this study, WPI was treated with 10 mM DTT, a reducing agent, overnight prior to the addition of TGase for cross-linking. Reduction of the disulfide bonds partially unfolds globular proteins and increases enzyme access to NH_2 groups for more effective cross-linking (12, 18). Moreover, DTT maintains the active site sulfhydryl in the reduced state and enhances the activity of TGase by 10–25% (15). Substitution of DTT with a food-grade reducing agent such as sodium bisulfite (Na_2SO_3) was attempted, but enzymatic assays of TGase activity showed a decrease of ~60% in the presence of 50 mM Na_2SO_3 (data not shown).

Electrophoresis. All 4% WPI-treated samples were diluted 1:4 with distilled, deionized water, mixed 1:1 (v/v) with 8% SDS and 0.9 M Tris sample buffer (InVitrogen Inc., Carlsbad, CA) containing 5.0% β -mercaptoethanol, and heated at $100\text{ }^{\circ}\text{C}$ for 5 min prior to loading onto 10–20% Tris–Tricine gradient polyacrylamide gels (InVitrogen, Inc). After electrophoresis, the samples were either stained directly for visualization of proteins using a colloidal Coomassie Blue staining reagent (InVitrogen, Inc.) or transferred onto PDVF membranes (InVitrogen, Inc.) for Western blotting analyses.

Western Blotting. After transfer, the membranes were blocked overnight at room temperature using 3% gelatin (BioRad, Inc.) prepared with phosphate-buffered saline containing 0.02% Tween (PBS/Tween). Polyclonal rabbit antibodies, directed against either β -lactoglobulin (β -LG) or α -lactalbumin (α -LA) (Bethyl Laboratories, Montgomery, TX), were diluted 1:2000

with PBS/Tween containing 1% gelatin and incubated with the membrane for a minimum of 2–3 h at room temperature. After being washed three times with PBS/Tween, the membrane was incubated with a detection antibody solution. In this case, a goat-anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL) was diluted 1:2000 with PBS/Tween/1% gelatin and incubated for an additional 2–3 h at room temperature. Again, after the membrane was rinsed three times with PBS/Tween, the positive protein bands, either β -LG or α -LA, were visualized after addition of the HRP substrates, diaminobenzidine, and hydrogen peroxide (Pierce Inc.).

Cross-Linking of WPI. WPI was dissolved overnight at $4\text{ }^{\circ}\text{C}$ to protein concentrations of 4% and 8% (w/v) in deionized water containing 10 mM DTT, pH 7.5. The control solution also contained 10 mM DTT but was devoid of microbial transglutaminase activity. Immobilized TGase at enzyme/substrate ratios of 0.12 and 1.2 units/g WPI proteins and soluble TGase at 10–25 units/g WPI proteins were used in the cross-linking experiments. The reaction was carried out in a 1-L jacketed bioreactor for 8 h at $40\text{ }^{\circ}\text{C}$ with continuous circulation. Aliquots were taken after 0.5, 1, 2, 4, and 8 h and immediately transferred to a preset rheometer for rheological analyses. For electrophoresis, aliquots were immediately diluted and mixed 1:1 (v/v) with 8% SDS solutions containing 5% β -mercaptoethanol to stop the enzymatic reaction.

Rheological Analysis. Apparent viscosities of the WPI solutions were determined at $25\text{ }^{\circ}\text{C}$ using a stress-controlled rheometer (StressTech, Reologica Instruments AB, Lund, Sweden) equipped with a CC 25 concentric cylinder geometry. Dynamic oscillatory tests were performed with the Stress Tech rheometer using the same attachment. Calcium ions were added to all the samples at 2.5 mM prior to performing dynamic oscillatory tests. Temperature ramps were carried out by heating the samples from 45 to 85 or 95 $^{\circ}\text{C}$ at a rate of $0.5\text{ }^{\circ}\text{C}/\text{min}$, holding the sample at the targeted temperature for 30 min, and cooling to $25\text{ }^{\circ}\text{C}$ at the same rate, with a constant oscillation of 0.1 Hz. Frequency sweeps were performed from 0.01 to 20 Hz at $25\text{ }^{\circ}\text{C}$. Constant stresses of 0.25 and 2.00 Pa in the identified linear viscoelastic region of these samples were applied for all tests during heating, holding, and cooling, respectively. All rheological measurements were conducted at least in duplicate.

RESULTS AND DISCUSSION

The activity of the commercially available TGase used in this study was 1100 units per gram of the enzyme powder. Its purity was >90% based on its electrophoretic pattern, which was similar to that previously reported (15). The enzyme retained most of its activity throughout the immobilization process, resulting in a specific activity for the biocatalyst of 5.0 units/mL of beads. This activity is comparable with that of the immobilized TGase from guinea pig liver reported by Huang et al. (14). The immobilized TGase lost about 30% activity after 4 months of storage at $4\text{ }^{\circ}\text{C}$ in 50 mM phosphate, pH 6.0, containing 0.02% sodium azide. In the bioreactor, the stability of the immobilized TGase varied with concentration of the treated WPI solution. Wilcox and Swaisgood (15) reported that the immobilized TGase was stable in 1% WPI solutions but it lost about 50% of its activity after treatment of an 8% WPI solution. These authors speculated that the high protein concentration resulted in clogging of the pores and/or the formation of a monolayer of protein on the bead surface, resulting in an irreversible inactivation of the immobilized TGase. Activity loss is most likely not caused by dissociation of the enzyme from the support because of the high affinity of avidin for biotin ($K_D \approx 10^{-15}\text{ M}$). Moreover, no loss of activity was observed after

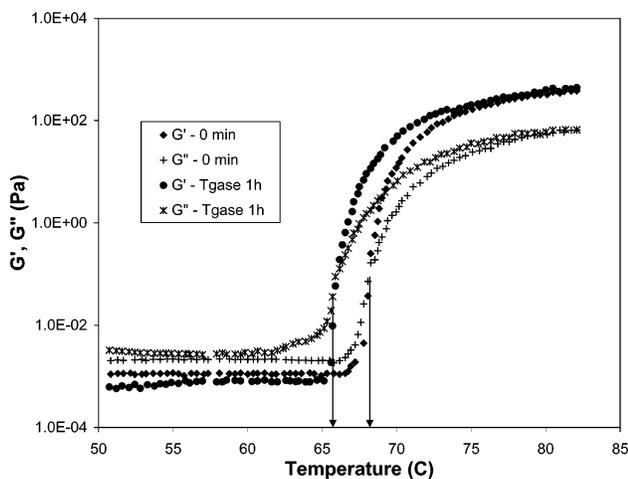


Figure 1. Gelation of 4% WPI incubated with immobilized TGase (0.12 unit/g WPI).

3 months of continuous operation of a bioreactor containing β -galactosidase immobilized using this technology (19). Also, no loss of transglutaminase activity immobilized by selective adsorption was observed by others during storage for several months (14, 15). It should also be noted that the immobilized enzyme was thoroughly washed with 1 M urea solution before use and that complete removal of the immobilized protein from the support requires washing with low-pH, 6 M guanidinium chloride solution.

To observe the effect of WPI concentration on the retained activity of the immobilized TGase, solutions of 4% WPI were used in the first part of this study. After circulation for 8 h at 40 °C in the bioreactor, the immobilized TGase lost about 70% of its activity, even after washing with regeneration agents such as 1–4 M urea. Apparently, factors in addition to protein concentration need to be investigated to effectively recover the activity of immobilized TGase after treatment of WPI solutions.

Cross-linking of 4% WPI solutions as affected by the enzyme/substrate ratio was investigated. At a ratio of 0.12 unit of immobilized TGase per gram of WPI protein (1 mL of beads/L of WPI solution), cross-linking in 4% WPI solutions was not clearly indicated by the electrophoretic pattern (data not shown). Dynamic oscillatory results with temperature ramps of the treated WPI solutions from 45 to 85 °C are shown in Figure 1. The crossover point of the storage modulus (G') and loss modulus (G'') curves was defined as the gel point temperature of the tested materials. This point represents the transition phase from what was perceived to be a liquid to a more solid-like material (20). The WPI sample treated with immobilized TGase for 1 h or longer exhibited a gel point temperature of 65.6 °C that was slightly lower than the 67.6 °C point of the untreated sample at zero time (Figure 1). This decrease in gelling point of WPI solutions treated with low immobilized enzyme/substrate ratios was in agreement with previous reports using either microbial or mammalian enzyme (15, 21). It was postulated that a network that was already partially formed during enzymatic cross-linking requires less heat for gelation than would be needed for the untreated WPI solutions. However, the slight changes in network formation and gelling point did not have a significant effect on the strength of the formed gels. As shown in Figure 1, there was no difference in G' and G'' values between the treated and untreated samples toward the end of the heating tests. Upon cooling to 25 °C, these samples exhibited similar mechanical spectra in the frequency sweep tests (data not shown).

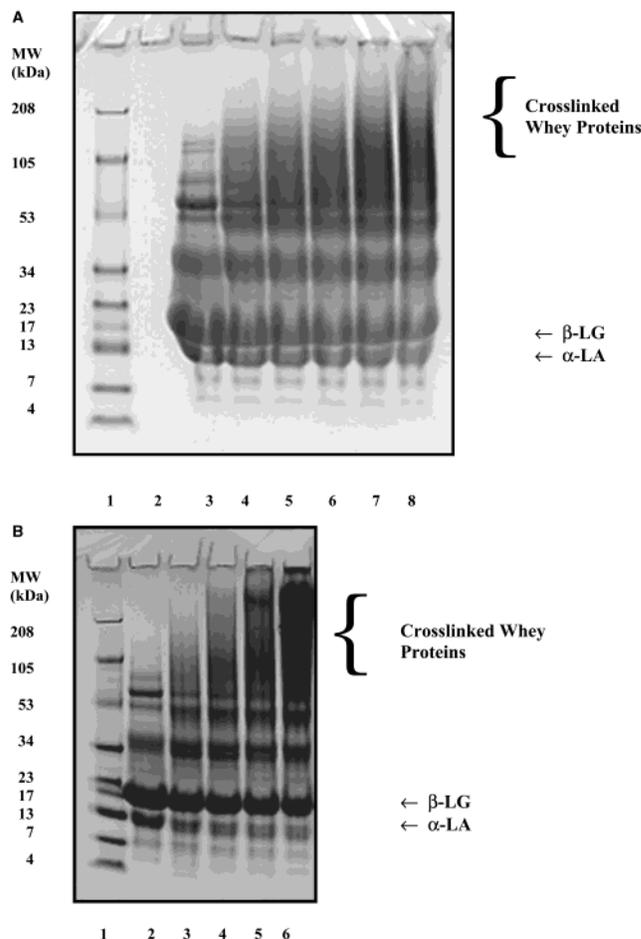


Figure 2. (A) SDS-PAGE of 4% WPI cross-linked using immobilized TGase (1.2 units/g WPI). Lane 1, protein standard markers; lane 2, blank; lane 3, 0 time; lane 4, 0.5 h; lane 5, 1 h; lane 6, 2 h; lane 7, 4 h; lane 8, 7 h. (B) SDS-PAGE of 4% WPI cross-linked using soluble TGase (10 units/g WPI). Lane 1, protein standard markers; lane 2, 0 time; lane 3, 0.5 h; lane 4, 1 h; lane 5, 2 h; lane 6, 4 h.

It was speculated that the enzyme/substrate ratio of 0.12 unit/g WPI was too low to create enough cross-linking to have a significant effect on the gel strength. As previously noted, electrophoretic analysis did not indicate significant cross-linking. Therefore, the amount of immobilized TGase was increased by 10-fold (1.2 units/g WPI) for cross-linking a 4% WPI solution under the same experimental conditions. As indicated in the SDS-PAGE patterns (Figure 2A), polymer formation due to cross-linking catalyzed by TGase was evident and increased with reaction time. The rheological results for gelation of these enzyme-treated 4% WPI samples during heating are shown in Figure 3. Unexpectedly, the TGase-induced cross-linking resulted in increasing the gel point temperatures of the treated WPI solutions from 67.6 °C for the untreated sample to 69.3 °C for 1–4 h reaction times. The G' values of the formed gels toward the end of the heating test decreased with TGase reaction time, indicating that intensive cross-linking under these experimental conditions resulted in decreases in gel strength. This trend of decreasing gel strength, as indicated by lower G' values, of the TGase-treated samples as compared to the control also held true during cooling to 25 °C (data not shown) and subsequent frequency sweeps, as shown in Figure 4. These observations of increasing gelling point and decreasing strength of the gels formed by TGase-induced cross-linking of WPI solutions led us to examine the effect of much higher enzyme/substrate ratios, 10–25 units of TGase/g WPI.

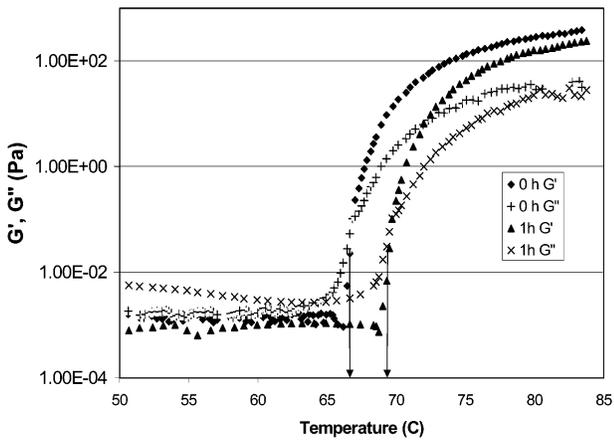


Figure 3. Gelation of 4% WPI incubated with immobilized TGase (1.2 units/g WPI).

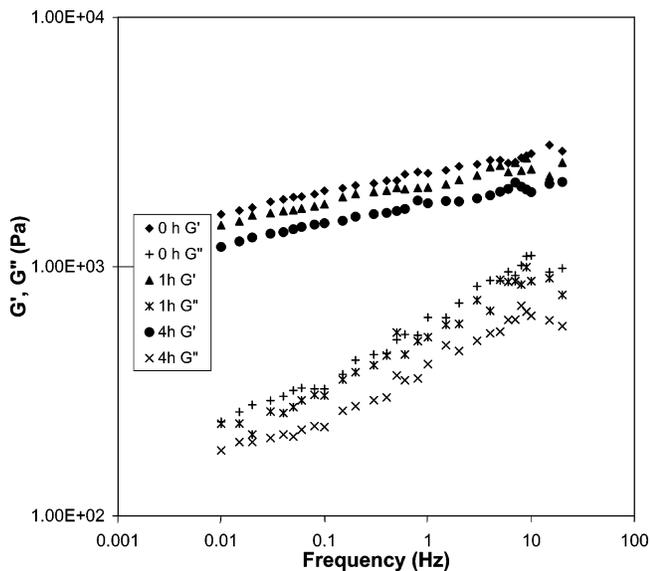


Figure 4. Mechanical spectra of the gels from 4% WPI treated with 1.2 units of immobilized TGase/g WPI. Frequency sweeps were performed on the gels cooled to 25 °C.

Due to a limited amount of available immobilized TGase and the large quantity of enzyme beads required, which are not practical for the circulating bioreactor, soluble TGase was used in these experiments for cross-linking of 4% and 8% WPI. In these experiments with soluble enzyme, TGase was heat-inactivated by immediately performing the rheology measurements (45°–95 °C) after enzymatic reaction. SDS–PAGE patterns of 4% WPI solution cross-linked by 10 units of TGase for each gram of WPI protein are shown in Figure 2B. Extensive cross-linking was evidenced by the high staining intensity of the bands for the large polymers formed during the prolonged reaction time (after 1 h) that could not enter the electrophoretic gels. This is in agreement with previous reports (22) that, unlike the guinea pig TGase-catalyzed systems, large polymers of whey proteins can be formed by microbial TGase in the late stage of reaction. Using light scattering techniques, Matsumura et al. (22) reported molecular weights of up to 4×10^7 for large polymers formed after 120–240 min of cross-linking of α -LA with microbial TGase.

Western blotting patterns in Figure 5 indicated that both major proteins of WPI, α -LA and β -LG, were involved in the TGase-induced cross-linking reaction. Similar to the SDS–PAGE patterns, the Western blot results also show the formation of

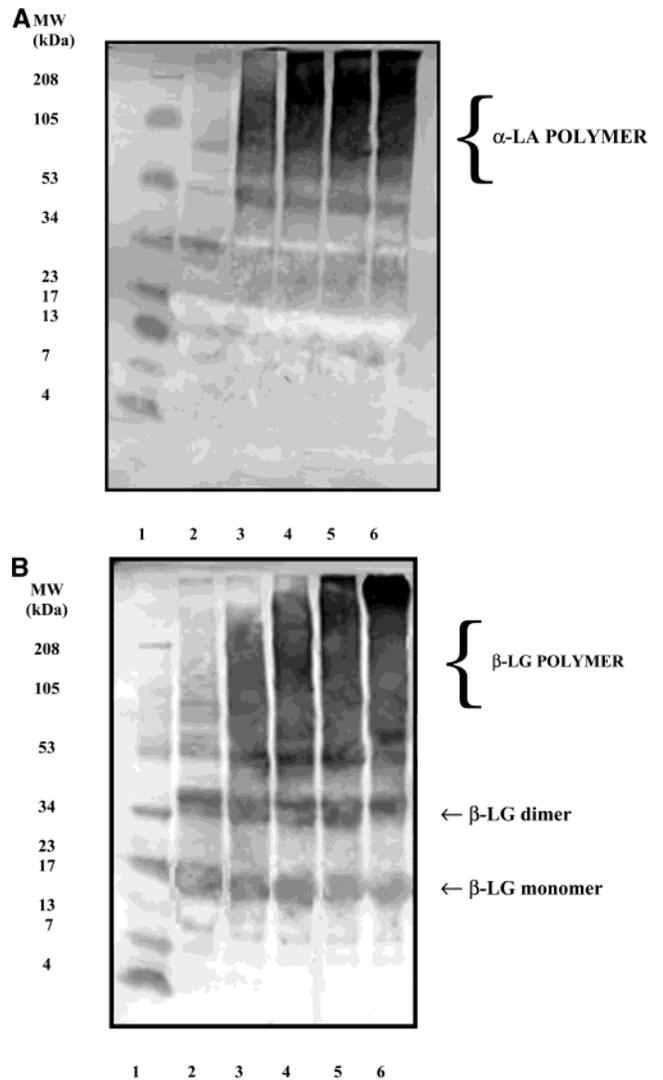


Figure 5. (A) Western analysis of 4% WPI cross-linked by soluble TGase (10 units/g WPI). Probe: anti- α -lactalbumin antiserum. Lane 1; protein standard markers; lane 2, 0 time; lane 3, 0.5 h; lane 4, 1 h; lane 5, 2 h; lane 6, 4 h. (B) Western analysis of 4% WPI cross-linked by soluble TGase (10 units/gWPI). Probe: anti- β -lactoglobulin antiserum. Lane 1, protein standard markers; lane 2, 0 time; lane 3, 0.5 h; lane 4, 1 h; lane 5, 2 h; lane 6, 4 h.

larger polymers with long incubation times. It should be noted that the reactivity and/or titer of the α -LA antiserum was lower than that of β -lactoglobulin. α -LA antibodies did react with monomeric subunits; however, this visualization was fairly weak (Figure 5A).

Of particular interest was the observation that α -LA appeared to serve as a more effective substrate than β -LG since there was a virtual absence of detectable α -LA monomeric subunits (Figure 5A). Also, it should be noted that the catalytic efficiency, k_{cat}/K_m , is 4–5-fold larger for α -casein as compared to β -LG, indicating the importance of chain flexibility for TGase activity (5). Abourmahmoud and Savello (4) reported similar findings based upon analogous cross-linking studies using α -LA, β -LG, α -casein, whey powder, modified whey powder, nonfat dry milk, and guinea pig liver TGase. In their studies, they observed that casein fractions were more susceptible to TGase cross-linking than whey protein fractions since casein is even more flexible than α -LA, serving as a better substrate for TGase. With microbial TGase, Sharma et al. (9) found that α -LA was effectively cross-linked with or without DTT, while β -LG was

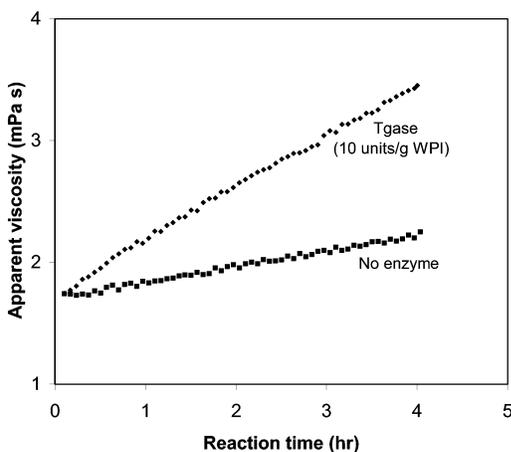


Figure 6. Viscosity of 8% WPI solution incubated with or without soluble Tgase (10 units/g WPI).

extensively cross-linked only when the proteins were partially denatured by DTT, cysteine, or alkali. However, they did not make a comparison of the degree of TGase-induced cross-linking of these two substrates, α -LA and β -LG, subjected to denaturation conditions. These differences between β -LG and α -LA suggest that factors in addition to chain flexibility, such as surface accessibility of the glutamyl residues, affect TGase activity.

The extensive cross-linking in 4% WPI solutions treated with 10 units of soluble TGase per gram of WPI, as evidenced by SDS-PAGE and Western blotting, had a pronounced effect on the rheological properties of the proteins. The 4% WPI sample cross-linked for 30 min did not exhibit a crossover point, defined as the gel point temperature, of the G' and G'' curves when it was heated to 95 °C in the dynamic oscillatory test (data not shown). Visual observation indicated that the heated solution became viscous upon cooling to 25 °C but did not gel. Frequency sweep tests at this temperature showed that the G' values of the untreated and 30-min-treated sample were 1750–3240 Pa and 15.7–33.5 Pa, respectively, over a frequency range of 0.001–20 Hz. This represents an approximately 100-fold decrease of the G' values. Apparently, extensive cross-linking by TGase resulted in increasing the heat stability of WPI. Further experiments were carried out to observe the rheological changes of higher protein concentration solutions (8% WPI) cross-linked with 10 units of TGase/g WPI. As shown in Figure 6, the apparent viscosity of the WPI solution steadily increased with cross-linking time, indicating polymerization of the proteins. Rheological behavior of the 8% WPI samples during the heating part of the dynamic oscillatory tests confirmed the previously described effects of the enzymatic cross-linking on the heat stability of the proteins. The gel point temperature increased from 67.6 °C for the untreated sample to 78 and 94 °C after 30 min and 4 h of cross-linking by TGase, respectively, and the strength (G' values) of the formed network during heating was drastically decreased (Figure 7). Upon cooling, G' values of all the samples increased, but the large decrease in the gel strength due to TGase-catalyzed polymerization was clearly shown (Figure 8). The G' values of these 8% WPI gels at 25 °C plotted against the oscillatory frequency (Figure 9) also demonstrated significant decreases in the network strength associated with extensive cross-linking of the proteins. The steep slopes of the curves of the TGase-treated samples represent typical frequency-dependent behavior of weak gel networks of liquid-like materials (20).

Our results differ from the common observations of other investigators that cross-linking of proteins by TGase increases

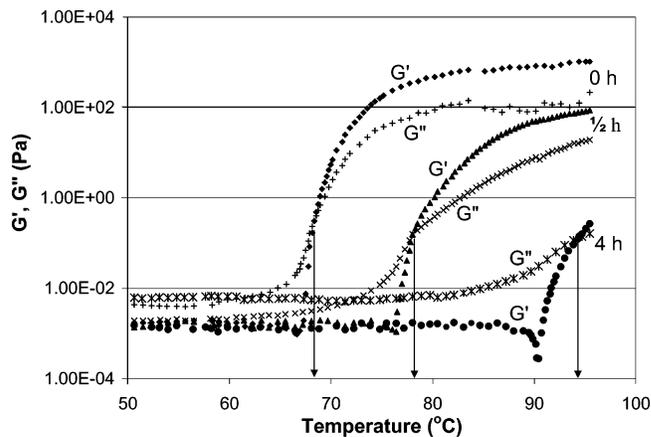


Figure 7. Gelation of 8% WPI cross-linked by soluble Tgase (10 units/g WPI).

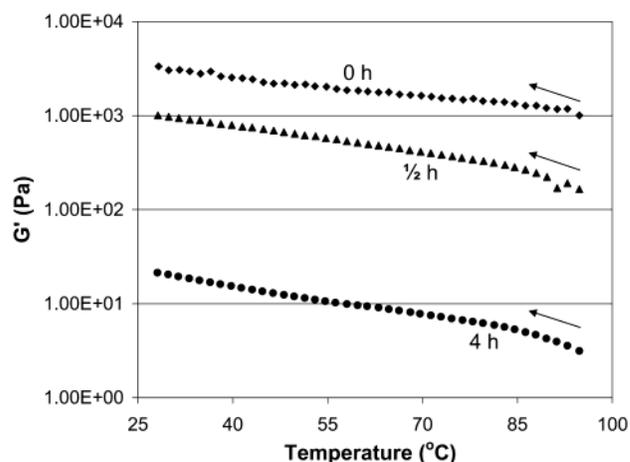


Figure 8. Gel strength increased upon cooling (8% WPI, 10 Tgase units/g WPI).

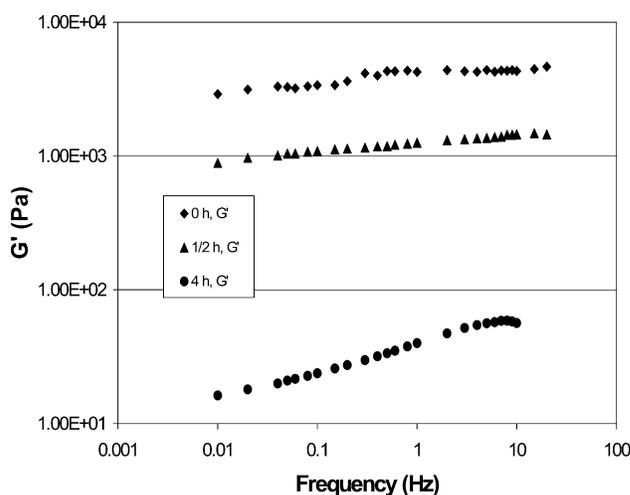


Figure 9. Mechanical spectra of gels from 8% WPI cross-linked by soluble Tgase (10 units/g WPI). Frequency sweeps were performed on the gels cooled to 25 °C.

the gel strength of various protein food materials (8). These contradictory results may be related to the degree of whey protein cross-linking. Limited cross-linking may result in higher gel strengths and lower gelling temperatures, as observed by Wilcox and Swaisgood (15, 21), whereas extensive cross-linking may have the opposite effect. It can be speculated that extensive intra- and interchain cross-linking caused formation of polymers

that were too large for effective unfolding for network development. Tanimoto and Kinsella (23) reported that extensive polymerization of 10% β -LG solution by guinea pig transglutaminase resulted in the formation of weak gels. At 5% β -LG, the enzyme-treated dispersions were heat stable and remained viscous at 100 °C. These authors suggested that the cross-linked proteins contained intramolecular bonds that impeded thermally induced unfolding of the molecules, thereby limiting network formation. Different effects induced by TGase cross-linking on rheological properties of other food proteins have also been observed. It is known that addition of microbial TGase can improve dough elasticity and may produce beneficial effects during bread making that are similar to those caused by oxidizing improvers (24). Recently, Basman et al. (25) reported that the effects of TGase could be beneficial or deleterious to bread making and quality, depending on the type of wheat flours and TGase addition levels. At high TGase addition levels, excessive cross-linking of gluten causes an overstrong dough, resulting in a decrease in loaf volume of the bread. For fish gels, incorporation of amidated low methoxyl pectin in the formulation increased the gel strength and water-holding capacity of the products. However, a destructive effect on the gel was observed when the pectin was added together with a high level of microbial TGase (26).

CONCLUSIONS

With high TGase/substrate ratios (1.2–10 units/g WPI), extensive cross-linking of the major components of WPI, α -LA and β -LG, was evident. The extensive intra- and interchain cross-linking probably caused formation of polymers that were too large and unfavorable for thermally induced unfolding for effective network development, resulting in an increase in gel point temperature and a severe decrease in gel strength. Previous studies have indicated that increased gel strength and reduced gelling temperatures result when the cross-linking is limited (15, 21). Thus, modified whey proteins with a wide range of gelling properties can be produced by varying the conditions of TGase-induced cross-linking. Results of the present study imply that a process could be developed to produce heat-stable whey proteins for various food applications, as was also suggested by the results of a previous report (27).

The use of immobilized enzymes has many advantages (28). In the case of transglutaminase, these include the ability to prepare concentrated solutions of proteins with a wide range of stable functional properties because the enzyme does not remain in the product. Also, because the cross-linking can be limited, possible negative effects on nutritional properties due to changes in bioavailability of lysine or digestibility would be limited. It should be noted that isopeptide bonds occur naturally, so enzymes exist that are capable of hydrolyzing this bond.

For the purposes of this study, DTT was used to reduce the disulfide bonds of β -lactoglobulin because its application for this purpose was previously well characterized. In future work, a food-grade reductant, such as cysteine, should be examined. Furthermore, the problem of TGase activity loss, observed as a result of enzyme immobilization on porous supports, could be addressed by using micrometer-size nonporous beads, such as ferromagnetic beads, as the matrix for the immobilized enzyme. The kinetic characteristics of an enzyme attached to small nonporous beads may approach that of the soluble enzyme.

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