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Acid-Induced Gelation of Enzymatically Modified, Preheated Whey Proteins

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Low-pH whey protein gels are formulated using a sequential protocol of heat treatment, enzyme incubation, and cold-set acidification. The heat-induced disulfide and enzyme-catalyzed ϵ-(γ-glutamyl)-lysine linkages, both at neutral pH, produce a polymerized protein *solution*. The molecular weights of these samples show an exponential increase with protein concentration. The additional enzyme-catalyzed cross-links cause little change in molecular weight from that of heat-treated samples at low protein concentrations, indicating predominant intramolecular cross-linking. Enzyme treatment at higher protein concentration however causes increase in molecular weight, possibly due to formation of intermolecular cross-links. Acidification of the polymerized protein solutions through glucono-δ-lactone acid leads to *gel formation* at pH 4. The elastic (G') and viscous (G'') moduli of gels with and without enzyme treatment show similar frequency dependence, indicating comparable microstructures, consistent with all samples exhibiting similar fractal dimensions of $\sim$2 obtained independently using rheology and confocal microscopy. A substantial increase in fracture strain and stress of the gel is achieved by enzyme treatment. However, the elastic modulus (G') is only slightly larger after enzyme treatment compared with heat-treated samples. These results indicate that factors responsible for fracture properties may not be apparent in the gel microstructure and linear viscoelastic properties.

INTRODUCTION

Whey proteins have become an important ingredient for many food products because of their high nutritional value, easy digestibility, and capacity to impart functional characteristics that include emulsification, stabilization, foaming, and gelation (*1*−*3*). The ability of whey proteins to form gels is particularly desirable as gelation plays an essential part in dictating the texture of the final food products (*4*−*7*). Typically, gelation, which involves formation of a three-dimensional polymeric network, is achieved through heat treatment of whey proteins at high or moderate ionic strengths and/or close to the isoelectric point (*5*, *7*−*12*). These gels are often referred to heat-set gels, as the single heat treatment step leads to gel formation through protein denaturation and polymerization (disulfide cross-links) and subsequent physical bond formation. Gels can also be produced using a two-step process that involves heat treatment at low ionic strength and/or far from the isoelectric point, followed by increase in ionic strength and/or adjustment in pH (*13*−*15*). These gels are labeled as cold-set gels, as the initial heat treatment produces a polymerized solution with gelation occurring during the subsequent cold-set conditions through screening of the repulsive forces. Such an approach is commensurate with some food applications in which it is not desirable to heat the products to high temperatures to induce gelation, and it becomes advantageous to induce gelation at ambient or near ambient temperatures (*16*).

An area of importance in which whey protein gels are having limited use is in low-pH food products. This is because heat-set whey protein gels under acidic conditions (pH $<$4.6) exhibit poor rheological properties, such as brittleness and weakness, due to the reduction of the strong disulfide bonds under such acidic conditions and pH-associated effects on the denaturation and aggregation reactions (*17*). The rheological properties of such gels can however be improved by introducing additional intermolecular chemical cross-links. A viable approach in this regard is to use enzymes (*18*−*22*). For instance, transglutaminase (TG, EC2.3.2.13) can be used to cross-link whey proteins (*22*−*29*) through cross-linking glutamine and lysine residues as it catalyzes the acyl transfer reaction and produces ϵ-(γ-glutamyl)lysine bonds.

Previous and current work in our laboratory has focused on enzyme-facilitated gelation of whey proteins at low pH (*25*). In our first approach, we developed cold-set whey protein gels at low pH with enhanced rheological properties by cross-linking the proteins with transglutaminase enzyme (henceforth referred to as TG) under alkaline conditions. The alkaline conditions were chosen to partially denature the protein and enable cross-linking by TG. However, TG activity decays more rapidly under alkaline conditions than at neutral pH. In addition, the maximum temperature used in the process (50 °C) may not have allowed formation of all possible disulfide linkages.

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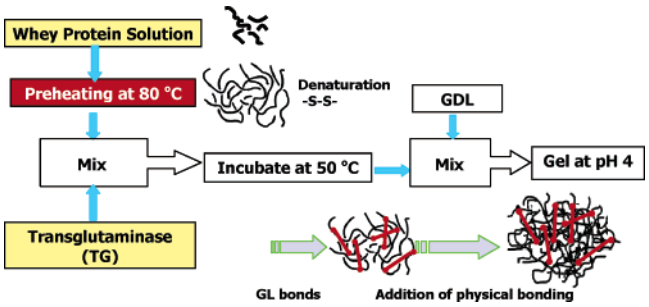

Figure 1. Schematic diagram for low-pH whey protein (WP) gel preparation combining preheat, enzymatic treatment, and cold-set gelation. The enzyme-catalyzed GL bonds are shown in red.

To circumvent these issues as well as introduce new functionality, we examine in this study a different approach for modifying whey protein gels. In this protocol, as illustrated in **Figure 1**, whey protein solution at pH 7.0 is preheated at 80 °C for 1 h, followed by TG treatment at 50 °C. The ensuing polymerized solution is gelled through slow (room temperature) acidification to pH 4 by adding glucono-δ-lactone (GDL) acid. The distinguishing features of this approach are the use of neutral pH and the preheating step to eliminate the drawbacks of our prior work. The disulfide linkages formed during the preheating step also enables us to (i) tailor gels with both disulfide and TG-catalyzed bonds and (ii) understand the added functionality of the ϵ-(γ-glutamyl)lysine bonds in cold-set gels in reference to the traditional cold-set gels containing only disulfide linkages. As such, we examine in detail the gelation process and gel properties for formulations with and without TG. Linear and nonlinear rheological experiments are conducted to characterize and understand gel behavior. This is supplemented with confocal laser scanning microscopy (CSLM) to directly probe gel microstructure and obtain fractal dimensions that can be compared with that acquired through rheology.

MATERIALS AND METHODS

Materials. Whey protein (WP) isolate was obtained from Davisco Food International (LeSueur, MN) and used as received. A commercial version of transglutaminase enzyme (1% TG and 99% maltodextrin, by weight) was provided by Ajinomoto Co. (Tokyo, Japan). Sodium azide, glucono-δ-lactone (GDL), sodium dodecyl sulfate (SDS), and urea were obtained from Sigma Chemical Co. (St. Louis, MO). Deionized water (>15 MΩ) was used in all the experiments.

Preparation of Protein Solutions. The stock protein solution was prepared by dissolving WP powder in deionized water to the desired final concentration at pH 7 and stirring for 1 h to ensure complete solubility. The resulting solution was deaerated for 30 min under vacuum of 30 in. Hg at room temperature to eliminate trapped air bubbles. Protein concentrations mentioned in this work are expressed as weight of protein per weight of total solution. Protein volume fraction is defined as the volume of protein molecules per volume of the solution/gel.

Polymerization of the protein solution was carried out by preheating the solution at 80 °C for 1 h to denature the protein and induce disulfide bonds. In the case of samples treated with TG, enzyme was added at 10 units/g of protein after the preheating step. The samples were then stirred for about 20 min and incubated at 50 °C for 10 h. Each unit of TG is defined in terms of its activity, which corresponds to the amount of the enzyme necessary to catalyze the reaction of benzyloxycarbonyl-L-glutaminylglycine and hydroxylamine to yield 1 μmol of hydroxamic acid/min at 37 °C.

Preparation of Gels. WP gels at pH 4 were obtained by adding GDL to the heat- or heat+TG-treated WP solutions (0.2 g of GDL/g of protein) at room temperature and stirring for 15 min. For in situ measurements of gelation and subsequent gel properties, the solutions were then poured into a rheometer cup for rheological measurements.

Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed using a Mini-Protean 3 electrophoresis cell (Bio-Rad, Hercules, CA). Protein solutions (5%) were mixed with 40 volumes of sample buffer (40% glycerol, 8% SDS, and the balance being deionized water). β-Mercaptoethanol was added (50 μL per 950 μL of sample buffer) when reducing conditions were needed. The mixture was heated for 5 min at 95 °C and then subjected to electrophoresis in 5−15% polyacrylamide gels using the discontinuous system. These gels were then stained with 0.1% Coomassie blue and then destained by deionized water for 2 h. The gels were subsequently immersed in drying solution (containing 20% ethanol and 10% glycerol in deionized water) for 30 min and fixed in drying frames for 24 h. The dry gels were finally scanned and the bands analyzed in terms of molecular weights.

Rheological Measurements. Dynamic rheological measurements were conducted on an ARES rheometer (TA Instruments, New Castle, DE) at 25 °C, using couette geometry. In these experiments, the samples were subjected to a sinusoidal deformation as either a function of increasing strain amplitude or frequency of oscillation, and the corresponding elastic (G') and viscous (G'') moduli were measured. The strain sweep experiments, which were conducted at a constant frequency of 1 rad/s, served two purposes. First, they provided the limit of linear viscoelasticity (LVE) that could be used in the frequency experiments. Second, as discussed later, they provided a method to examine yield and fracture stress/strain of the samples (*30*, *31*). The frequency spectrum of the elastic and viscous modulus, on the other hand, provided a signature of the state (e.g., liquid or gel) of the samples (*32*). In all experiments conducted, the samples were covered with *n*-hexadecane to prevent evaporation.

Size Exclusion Chromatography (SEC). A Waters 2690 separation module (Waters, Milford, MA) size exclusion chromatography (SEC) unit attached to a DAWN-DSP laser photometer (Wyatt Technology, Santa Barbara, CA) multiangle laser light scattering (MALLS) instrument was used for analyzing molecular weights and aggregate sizes of the protein samples. A series of three columns (Waters Ultrahydrogel 120, 500, and 2000) was used to separate molecular weights in the range of 10−7000 kDa. Interferometric refractometer (Optilab DSP), and an ultraviolet detector (Waters 966 Photodiode Array Detector) were used as concentration detectors. The eluting solvent consisted of 10 mM Tris buffer (pH 7), 100 mM NaCl, and 0.02% sodium azide. The value of d*n*/d*c* (where *n* is refractive index and *c* is the protein concentration) was taken as 0.19 mL/mg (*33*). Flow rate through the SEC was maintained at 0.5 mL/min in all the runs. Molecular weight was determined using Astra software and the Zimm detector fit method, with values of d*n*/d*c* and the refractometer calibration constant as known quantities. Fifteen out of the 18 light scattering detectors between the angles of 30° and 142° were used.

Confocal Microscopy. A confocal laser scanning system (Leica TCS SP) attached to an inverted microscope (Leica DM IBRE, Leica Microsystems, Wetzlar, Germany) was used to obtain confocal images of the gel microstructure. Samples dyed with Rhodamine B (0.01%) were viewed with a 100× numerical aperture 1.4 oil immersion objective. A spacer of 0.12 mm thickness was adhered on the microscope slide. Immediately after acidulation with GDL, 25 μL of protein solution was poured onto a microscope slide fitted with a 0.12 mm spacer. The sample was then covered with a glass cover and viewed after 24 h of acidification.

Data Analysis. Samples for rheological characterization were run in triplicate. Mean values and standard deviations are shown in the graphs or tables. In size exclusion chromatography (SEC) experiments, samples were run in duplicates. In the determination of the fractal dimension by confocal microscopy, five layers through the sample thickness were analyzed by Image J software using the fractal box count method. Average fractal dimension and standard deviation were obtained.

RESULTS AND DISCUSSIONS

Polymerization of Whey Proteins by Disulfide Interchange and Enzymatic Cross-Linking. Previous work (*25*) in our laboratory revealed that whey proteins (WP) cannot be cross-linked by TG in the native state at pH 7. However, heating the

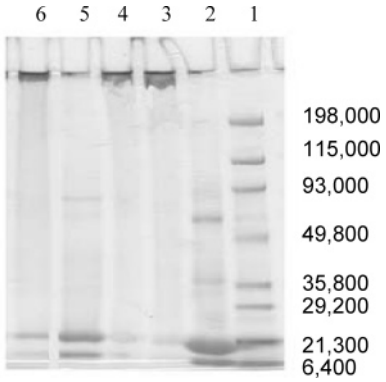

Figure 2. SDS–PAGE analysis for WP isolate samples. Lane 1 gives molecular weight markers (corresponding values shown in daltons). Lane 2 corresponds to native WP isolate sample. Lanes 3 and 5 are WP samples (pH 7) preheated at 80 °C for 1 h (lane 3 is under nonreducing conditions, whereas lane 5 is under reducing conditions). Lanes 4 and 6 are WP samples (pH 7) preheated at 80 °C for 1 h and then incubated with transgluatminase for 10 h at 50 °C (lane 4 is under nonreducing conditions, whereas lane 6 is under reducing conditions).

WP to a temperature higher than the denaturation temperature enables TG-catalyzed cross-linking to occur. This is illustrated in the SDS−PAGE analysis of **Figure 2**, which shows the effects of heat treatment and TG incubation on whey protein molecular weight. Three samples are portrayed in the figure, a native WP sample, a WP sample heated to 80 °C for 1 h (lanes 3 and 5), and a WP sample heated to 80 °C for 1 h and then incubated with transglutaminase at 50 °C for 10 h (lanes 4 and 6). Under nonreducing conditions, i.e., without the presence of β-mercaptoethanol (lanes 3 and 4), high molecular weight aggregates are noticed on the top of the gel, with and without TG. However, under reducing conditions (lanes 5 and 6), we observe only monomeric bands in the absence of the TG (lane 5), corresponding mainly to β-lactoglobulin and α-lactalbumin. The disulfide bonds created in the preheating step have been cleaved by the β-mercaptoethanol used in the electrophoresis run. In the presence of TG (lane 6), on the other hand, we clearly notice the presence of high molecular weight aggregates at the top of the gel. These aggregates can be attributed to polymerization through TG-catalyzed ϵ-(γ-glutamyl)lysine bonds, since the disulfide bonds have been cleaved by the reducing agent. The results obtained in **Figure 2** correspond to samples containing 5% protein; however, a similar outcome was observed for a 3% protein sample.

To investigate in more details the molecular weight distribution, samples were examined using size exclusion chromatography. **Figure 3** shows chromatograms of native whey proteins and proteins subjected to heat or combined heat and TG treatments. The native whey proteins exhibit three peaks, 1, 2, and 3, corresponding to bovine serum albumin (BSA), β-lactoglobulin (β-lg), and α-lactalbumin (α-lac), respectively. Preheating the protein at 80 °C for 1 h produces higher molecular weight aggregates (polymers) from disulfide interchange polymerization (*34*−*47*), as seen in the peaks eluting earlier than the native peaks. Upon treatment with TG, we expect an increase in molecular weight due to formation of additional cross-links. However, the chromatograms in **Figure 3** show that the effect of TG is highly dependent on whey protein concentration. At 3% (curve b), the large aggregate peak (eluting at ∼43 min) is not affected by TG treatment, whereas at 7.5% (curve c), the large aggregate peak moves to earlier elution time with TG treatment. We also observe another peak eluting at ∼51 min for 3% and 7% protein concentration prior to TG treatment. This peak was found to have an average degree of polymerization of 3 (based on a β-lg monomer of 18 600 Da). After TG treatment, this peak moves to a slightly lower elution time (∼49 min) and the average degree of polymerization increases to 5, suggesting formation of intermolecular cross-links. It is not clear, however, why these oligomers are undergoing intermolecular cross-linking by TG while the larger aggregates at 3% are not. Further investigation is needed to explain this phenomenon.

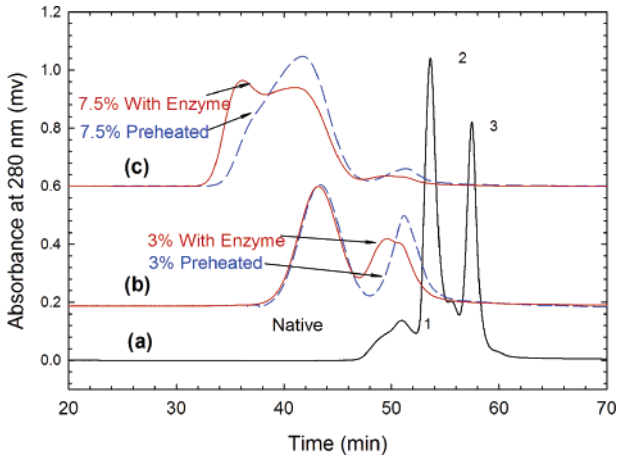

Figure 3. Chromatograms of WP samples subjected to different treatments: (a) native WP; (b) 3% WP sample preheated at 80 °C, with and without TG treatment; (c) 7.5% WP sample preheated at 80 °C, with and without TG treatment.

We observe in **Figure 3** that heat treatment of a 7.5% WP solution results in the appearance of a small shoulder to the left of the main aggregate peak, indicating onset of a bimodal distribution. At the same protein concentration but with additional TG treatment, we find the bimodal distribution of molecular weight to become more evident with the presence of separate peaks. The bimodality at higher WP concentrations may be attributed to a retarded termination rate in what is known as the Trommsdorff effect (*48*). In this case, an increase in solution viscosity causes autoacceleration of polymerization (*48*), slowing down diffusion of the growing polymers and causing a drop in the termination rate. The Trommsdorff effect is expected to take place mainly in the disulfide interchange polymerization, which is a typical chain growth polymerization. Enzymatic cross-linking takes place after the disulfide interchange polymerization and causes further separation of the two aggregate peaks. For clarity, throughout the paper we will use the terms "primary aggregates" to account for the protein chains that are polymerized via disulfide bonding and "secondary aggregates" to indicate primary aggregates that are further cross-linked by transglutaminase.

Effect of Concentration on Molecular Weight. Figure 4a shows the weight-average molecular weight of the protein aggregates as a function of protein concentration. At low protein concentrations (<5%), the weight-average molecular weight shows very little change after TG treatment; however, at higher concentrations, TG treatment increases molecular weight considerably. The fact that TG cross-linking does not increase the molecular weight at low concentrations can be explained as follows. At low protein concentrations, especially in a repulsive environment, TG tends to produce intramolecular cross-links of the primary aggregates. At higher WP concentrations, the primary aggregates tend to overlap extensively and increase the chance of intermolecular cross-linking.

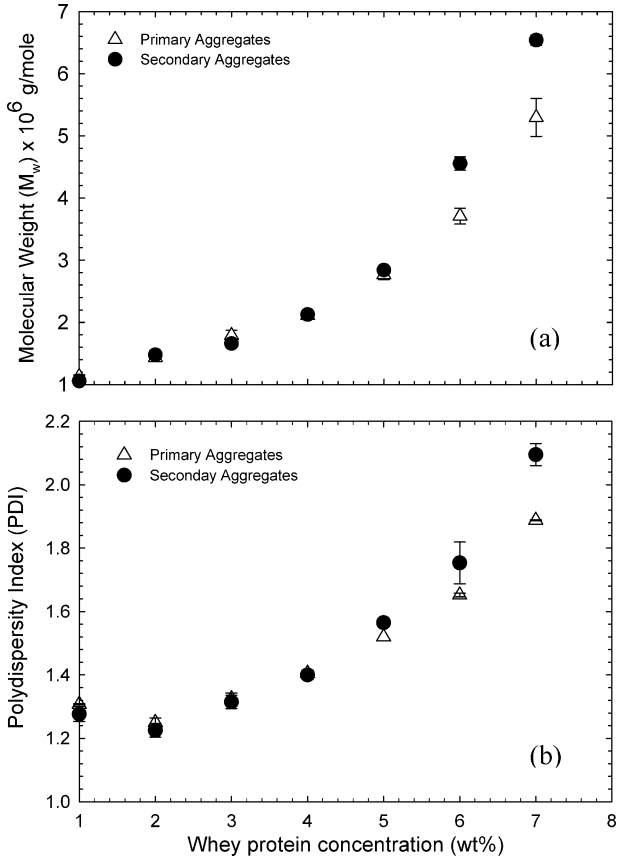

Figure 4. Weight-average molecular weight (M_w) (a) and polydispersity index (PDI) (b) of primary and secondary aggregates at different protein concentrations. Primary aggregates correspond to WP molecules polymerized via disulfide interchange, whereas secondary aggregates refer to primary aggregates that were further polymerized by TG.

Figure 4b shows the polydispersity index (PDI) of the primary and secondary aggregates. PDI falls between 1 and 1.5 at low protein concentrations, as expected in a typical chain growth polymerization. At higher concentrations, ~5% and higher, the PDI exceeds 1.5 due to the Trommsdorff effect mentioned earlier. Subsequent polymerization by TG cross-linking increases the PDI further, with the effect being more pronounced at higher protein concentrations.

Rheological Characterization of Cold-Set Gelation by GDL. Primary as well as secondary aggregates were slowly acidified by adding GDL and allowing them to set at 25 °C. The amount of GDL needed was found to be linearly dependent on protein concentration (0.2 g GDL/g of protein). **Figure 5** shows the evolution of the elastic (G') modulus with time for samples with and without TG treatment. From the inset in the figure, which shows the evolution of both the elastic (G') and viscous (G'') moduli during the initial time period, we observe G'' to be larger than G' immediately after GDL addition. This is consistent with the fact that the sample following heat and TG treatment is a solution and not a gel. However, both moduli increase rapidly with time, with G' crossing over G'' as elasticity starts to dominate. We notice a maximum in G' for all samples shown in **Figure 5** at ~2 h corresponding to the isoelectric point of the protein at pH ~5 (*25*, *26*). While this feature is common to both heat and combined heat and TG treated samples, several distinctive differences can be observed between these two types of samples. First, the elastic modulus is modestly higher for samples with TG treatment. Second, the reduction in G' with time (after reaching its maximum) is smaller for samples with

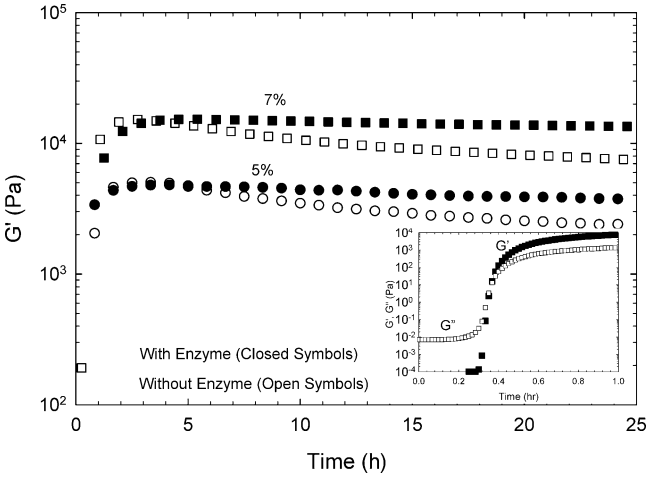

Figure 5. Evolution of the elastic (G') modulus of WP samples upon the addition of GDL (0.2 g/g of protein) at 25 °C. All samples were preheated at 80 °C for 1 h and some samples were incubated with TG following heat treatment (as noted in figure) prior to GDL addition. The inset reveals evolution of the elastic (G') and viscous (G'') moduli in the first hour for a 7% WP sample without TG. Squares and circles represent 7 and 5% (by wt) samples, respectively.

Table 1. Values of G'_{24h}/G'_{max} (%) of WP Gels (pH 4) at 5% and 7%, with and without TG Treatment

	heat + TG		heat only	
	5%	7%	5%	7%
G'_{24h}/G'_{max} (%)	78.3	88.6	47.8	49.8

TG treatment than their counterparts with only heat treatment. Finally, this lower sensitivity of G' to pH reduction or time is more pronounced for samples with higher WP concentrations. The last two features are observed clearly when one compares the value of G' after 24 h with respect to the maximum (G'/G'_{max}) as a percentage (**Table 1**). We find that G' after 24 h (at pH 4) is less than 50% of its maximum value when the sample has been heat treated only. In contrast, gels prepared with additional TG treatment retain 78 and 89% of their maximum G' values for WP concentrations of 5 and 7%, respectively. The reasons behind these observations may be explained as follows. As the pH decreases below the isoelectric point, the chains start to repel each other electrostatically because of the net positive charge on the molecules. Consequently, the network, which is held together through physical bonds, is weakened as some bonds dissociate. This leads to a decrease in G'. The use of TG produces additional cross-links that keep the three-dimensional network more stable against electrostatic repulsion. The effect is more pronounced at higher protein concentration, as there are more TG cross-links.

Gel Rheology. Figure 6 shows the frequency spectrum of G' and G'' for samples at pH 4. We find both heat- and heat+TG-treated samples to exhibit gel-like features with $G' > G''$ and both moduli exhibiting very weak frequency dependence. While the TG-treated gels exhibit slightly larger G', it is interesting to note that the mechanical spectra of both gels look very similar, with G' scaling as $\sim\omega^{0.1}$.

Figure 7 shows the effect of whey protein concentration (or volume fraction) on the elastic modulus of gels. We find G' of the TG-treated samples to be consistently higher than the samples without TG treatment. In both cases, though, G' exhibits a power-law behavior with protein volume fraction ϕ, $G' \sim \phi^n$, with the power-law exponent n varying between 4.4 and 4.6

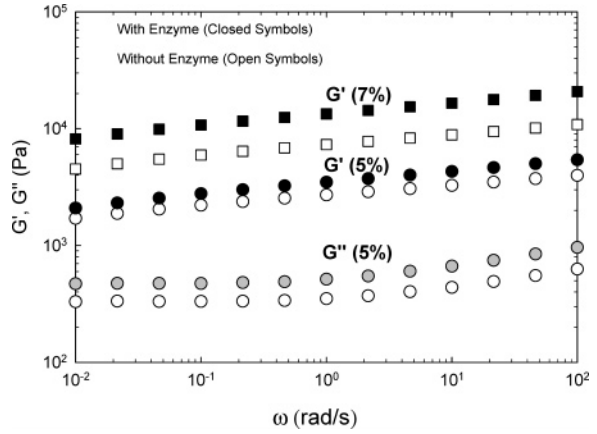

Figure 6. Elastic and viscous moduli of WP gels (pH 4) as a function of frequency, measured at 25 °C. Squares and circles represent 7% and 5% (by wt) samples, respectively. All samples were preheated at 80 °C for 1 h.

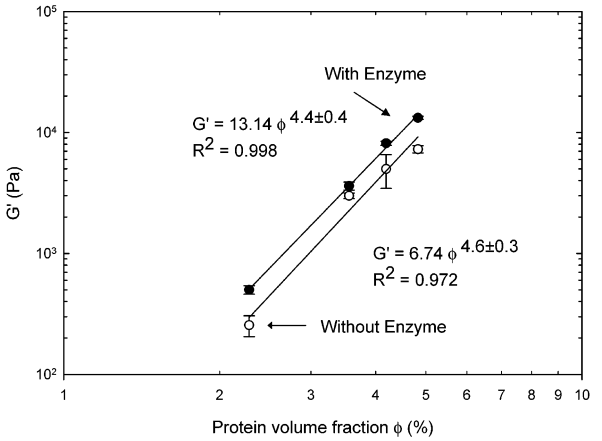

Figure 7. Elastic modulus of WP gels (pH 4) as a function of protein volume fraction. Data for samples with and without TG treatment are shown.

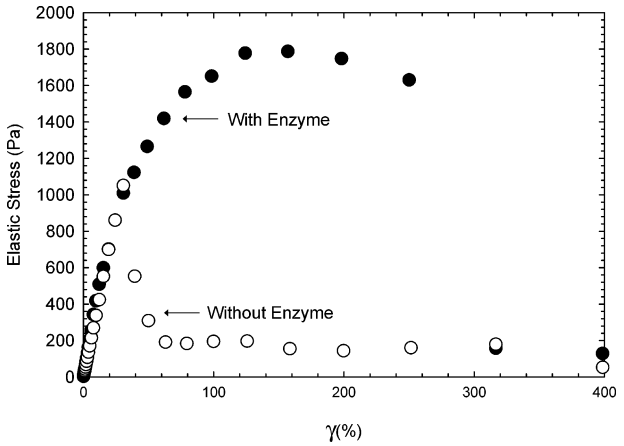

Figure 8. Elastic stress of 5% WP gels (pH 4), with and without TG treatment, as a function of strain, measured at 25 °C and 1 rad/s. Both samples were preheated to 80 °C for 1 h.

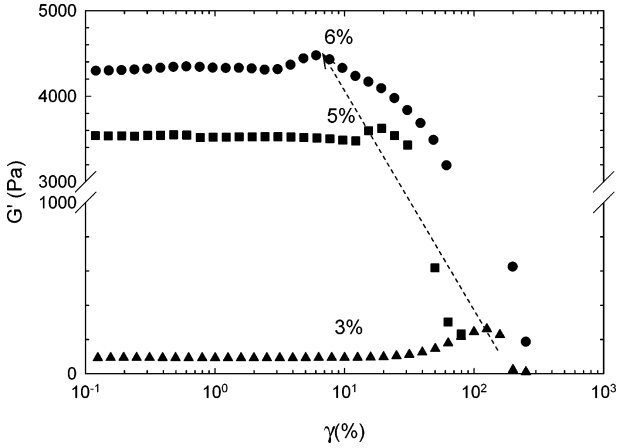

Figure 9. Elastic modulus of WP gels (pH 4) at different concentrations obtained without TG treatment, as a function of strain. The limit of linearity shifts to lower strain values as concentration increases.

for the two types of gels. The power-law dependence of G', together with the similar values for n observed for both types of gels, suggests that the gels are fractal in nature and that they possess similar microstructures (*49*−*51*). We examine these issues in more detail in the next section.

The large strain behavior of the TG-treated gel is, however, substantially different than the gel which has been exposed to heat treatment only. This is illustrated in **Figure 8**, which compares the elastic stress (product of shear stress and strain) as a function of increasing strain of the two types of gels. (*25*, *30*, *31*). The maximum in the plots corresponds to the fracture of the gel and has been visually observed as well. We find the gel treated with TG to fracture at higher stress and strain values. In fact, the fracture strain is about an order of magnitude higher while the fracture stress is 3-fold larger. The higher facture strain value (i.e., more deformability of the gel) may be due to the additional ϵ-(γ-glutamyl)lysine bonds created by the TG. These additional cross-links not only affect the fracture values but also alter the shape of the elastic stress curve. In the case of the gel without TG treatment, we observe only two regions: an initial linear regime up to the maximum and then a very steep decrease in stress following the linear regime. In contrast, gels with TG-catalyzed ϵ-(γ-glutamyl)lysine bonds reveal three regimes: an initial linear regime, an intermediate nonlinear regime leading to the elastic stress maximum, and gradual decrease in elastic stress following the maximum.

Fractal Dimension and Microstructure of Gels. We examine first the fractal dimensions of the gels using a rheological approach suggested by Shih and co-workers (*52*). According to these authors, we can view the gels as made of flocs consisting of aggregated macromolecules. These flocs then interconnect to form a three-dimensional gel network. The first step toward determination of the fractal dimension is to examine whether a gel is strong-linked or weak-linked. In strong-linked gels, the links between flocs are stronger than the links within the flocs. As a result, failure under deformation occurs through breaking of the intrafloc linkages. This manifests itself as a decrease in the linear viscoelastic region with increasing sample concentration. The limit of linearity is defined in the literature as the value of the strain at which there is an appreciable deviation in the elastic modulus from the linear viscoelastic plateau value. This value ranges from 5% to 30% (*52*, *53*). In the case of weak-linked gels, the intrafloc bonds are more rigid than the interfloc links, and failure occurs in the interfloc links. In this case, the limit of linearity increases with concentration. To evaluate the category of our gels, we plot in **Figure 9** the elastic modulus (G') as a function of strain for different protein concentrations. We notice quite clearly that the onset of nonlinearity (regardless of how one defines it) shifts to lower values with increasing concentration, indicating our gels to be

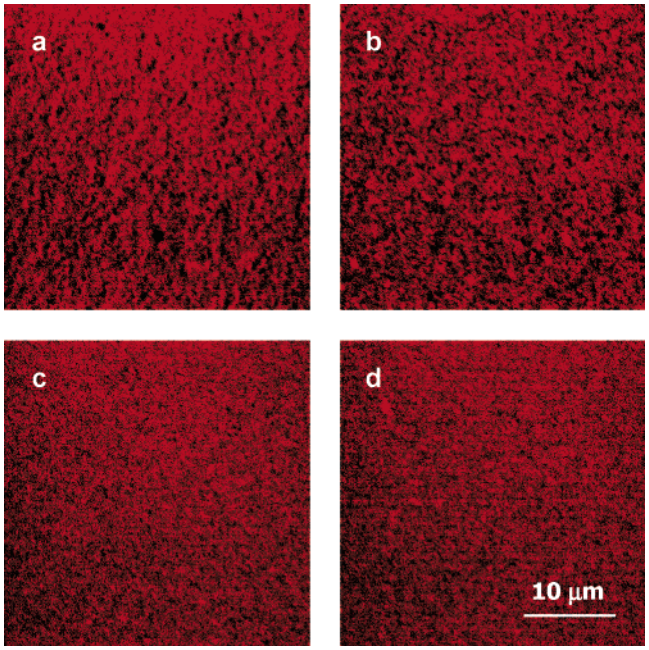

Figure 10. Confocal images of WP gels (pH 4). **A** and **B** correspond to 5% WP samples without and with TG treatment, respectively. **C** and **D** represent 7% WP samples obtained without and with TG treatment, respectively.

strong-linked. The same trend was observed for the gels with TG treatment (data not shown).

In a strong-linked gel, G' scales as $G' \sim \phi^{(d+x)/(d-D)}$, where x is the backbone fractal dimension of the flocs, d is the Euclidean dimension, and D is the gel fractal dimension. The backbone fractal dimension ranges from 1 to 1.3 for colloidal gels (*54*). We find from **Figure 7** that $G' \sim \phi^n$ for our gels with n corresponding to 4.4 ± 0.4 and 4.6 ± 0.3 for samples with and without TG treatment, respectively. Equating the theoretical and experimental power-law exponents, and substituting $x = 1.15$ and $d = 3$, we find $D \cong 2.1$ for both types of gels. The same value of the fractal dimension suggests very similar structures for both types of gels. It is important to emphasize that the arbitraryily chosen value of $x = 1.15$ has a negligible effect on the final value of D. A difference of 0.3 in the value of x (which accounts for the maximum change of x from 1 to 1.3) changes the value of D by only ~2%. The value of the fractal dimension approximating 2.0 suggests reaction-limited aggregation (RLA) (*52*). Such a mechanism is expected for the slow aggregation processes. This agrees with the slow acidulation in our case with GDL. An interesting point to note in **Figure 9** is the presence of strain hardening behavior prior to failure/fracture. Such behavior has been observed in the literature (*55*) and is attributed to simultaneous creation and loss of junctions between molecules, with the rate of creation being slightly higher than the loss. We refrain from a detailed discussion of the strain hardening behavior of our system, as this is beyond the scope of this work.

An alternative approach for determining the fractal dimension, as well as directly visualizing microstructure, is through use of confocal microscopy (*56*). **Figure 10** shows confocal images of the heat- and heat+TG-treated gels for two different WP concentrations. We observe all gels to exhibit fine stranded structures. More importantly, we find the microstructures of the gels to be independent of the preparation method. The fractal dimensions of the gels were calculated from the images by the box counting technique using Image J software. In this technique, the fractal dimension can be obtained from the scaling relation $N \propto r^{-D}$, where N is the number of boxes filled with protein flocs, r is the size of the square box, and D is the fractal dimension of the protein network. **Table 2** provides values of D obtained using both confocal microscopy and rheology. We find the fractal dimension of all gels obtained via confocal microscopy, regardless of treatment type or concentration, to have the same value of ~2.0. This is in excellent agreement with that obtained through rheology.

Table 2. Fractal Dimensions of WP Gels (pH 4) at 5%, and 7%, with and without TG Treatment, As Obtained by Rheology and Confocal Microscopy

	heat + TG		heat only	
	5%	7%	5%	7%
by confocal microscopy	1.96 ± 0.02	1.98 ± 0.03	1.96 ± 0.03	1.98 ± 0.02
by rheology	2.05 ± 0.11		2.09 ± 0.09	

It is interesting to note that although the microstructure as well as fractal properties of both types of gels are similar, the fracture stress and strain are considerably different. A similar trend has been reported by Errington and Foegeding (*17*), who found WP gels prepared under different conditions to exhibit similar microstructure and close values of modulus, but different fracture properties. The observation from our gels suggests that factors responsible for fracture properties may not be apparent in the microstructure, at least not in the length scales we are probing through confocal microscopy.

CONCLUSIONS

In this study, we examined the role of transglutaminase enzyme in modulating the properties of low-pH (~4) whey protein gels. A multistep approach involving preheating at neutral pH, subsequent TG treatment at 50 °C, and final cold-set gelation through acidulation with glucono-δ-lactone was adopted. The new contribution presented in this work eliminates the drawbacks of cross-linking under alkaline conditions and couples both disulfide and TG-catalyzed bonds to produce acid-induced cold-set gels at low pH. The disulfide and ϵ-(γ-glutamyl)lysine bonds formed through heat and TG treatment respectively produced polymerized whey protein solutions. The molecular weight of these samples increased with protein concentration with the role of additional TG cross-linking manifesting as higher molecular weights only at higher protein concentration. This seemed to suggest that TG cross-linking was predominantly intramolecular at lower protein concentrations.

During acidulation, vis a vis, the gelation process, the elastic modulus of the samples increased by several orders of magnitude and exhibited a maximum at the isoelectric point. The reduction in modulus as pH fell below the isoelectric point could be attributed to the development of electrostatic repulsion between the chains. This caused the network held together by physical bonds to weaken as some bonds dissociated. The use of TG produced additional cross-links that retarded the modulus decrease and made the network more stable against electrostatic repulsion.

The elastic modulus of the final gels showed a modest increase for samples treated with TG. In contrast, the fracture strain and stress revealed significant increase with TG treatment. Interestingly, the microstructures of all gels, with and without TG treatment, were found to be similar with a fractal dimension of ~2. This common value was obtained independently using rheology and confocal microscopy. These results taken together

suggest that the nonlinear fracture/yield properties are not reflected in the microstructure of the gels, at least not in the length scales probed using confocal microscopy.

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