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Polymerization and Gelation of Whey Protein Isolates at Low pH Using Transglutaminase Enzyme

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Dynamic and steady shear rheology is used to examine the synthesis of low-pH (~4) whey protein gels obtained through a two-step process. The first step involves cross-linking of whey proteins at pH 8 and 50 °C using transglutaminase enzyme, while the second step entails cold-set acidification of the resulting solution using glucono-δ-lactone (GDL) acid. During the first step, the sample undergoes enzyme-catalyzed ϵ-(γ-glutamyl)lysine bond formation with a substantial increase in viscosity. Acidification in the second step using GDL acid leads to a rapid decrease in pH with a concomitant increase in the elastic (G') and viscous (G'') moduli and formation of a gelled network. We examine the large strain behavior of the gel samples using a relatively new approach that entails plotting the product of elastic modulus and strain ($G'\gamma$) as a function of increasing dynamic strain and looking for a maximum, which corresponds to the yield or fracture point. We find the enzyme-catalyzed gels to have significantly higher yield/fracture stress and strain compared to cold-set gels prepared without enzyme or conventional heat-set gels. In addition, the elastic modulus of the enzyme-catalyzed gel is also higher than its non-enzyme-treated counterpart. These results are discussed in terms of the gel microstructure and the role played by the enzyme-induced cross-links.

KEYWORDS: Rheology; gel; whey protein; enzyme; transglutaminase; yield stress; cold set

INTRODUCTION

Whey proteins are important food ingredients that are used in a number of food products that includes dairy, confectionary, and dessert items. A major coproduct of the cheese industry, whey protein production (dry) has been exceeding 1 000 000 lb annually in the United States in recent years (*1*). In addition to their nutritional values, incorporation of whey proteins in food products can promote emulsification, stabilization, foaming, and gelation properties. In this regard, whey protein gelation is of considerable interest because it can provide new food products with unique functional performance and favorable textural properties (*2*−*4*). Moreover, food gelling systems are potential alternatives in replacing fats in foods (*5*). Whey protein gels can substitute the role of fats in enhancing textural properties of foods. Such gels are most commonly produced using heat treatment (*2*,*3*,*6*−*10*), although other factors such as salt addition (*11*), acidification (*12*,*13*), and enzyme treatment (*14*−*19*) can cause gelation of whey proteins with or without heating. In many food applications, however, 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 (*20*).

Whey protein gels, prepared by heating acidic whey protein solution (pH < 4.6), are weak and brittle (*21*), limiting their use in food products under such conditions. Such poor rheological properties occur mainly due to the absence of the strong disulfide bonds at these acidic conditions and pH-associated effects on the denaturation and aggregation reactions (*21*). However, disulfide bonds can presumably be introduced through a two-step procedure that involves creating chemical cross-links at neutral pH by heating the whey protein solution (step 1), followed by acidifying the polymerized protein solution to the required pH (step 2) (*21*).

A powerful and alternative approach for producing permanent bonds in whey proteins is by using enzymes (*14*,*17*,*22*,*23*). The specificity of enzyme-catalyzed cross-links combined with its ability to work at different (low) pH and low temperature makes this a viable route for developing whey protein gels. In this regard, earlier work in our laboratory focused on direct cross-linking of whey proteins at low pH. Burke et al. (*23*) treated whey proteins at pH 4 with transaminase using a combined enzyme and heat treatment protocol (incubation with enzyme at 40 °C for 40 min, followed by incubation at 80 °C for 20 min). The resulting gels exhibited higher elasticity and strength than that produced through conventional treatment. In the present study, we take a different approach to producing whey protein gels at low pH using transglutaminase enzyme. Transglutaminase (TG, EC2.3.2.13), which has been used to cross-link whey protein (*14*,*17*,*23*−*27*), links glutamine and lysine residues as it catalyzes the acyl transfer reaction, and produces ϵ-(γ-glutamyl)lysine bonds. Although cross-linking by TG has been studied for some proteins such as α_{s1}-casein, κ-casein, β-casein, *11s*, and *7s* soy globulins (*28*−*36*), cross-linking of whey proteins using TG is still not well developed. Most of the previous work used denaturant (such as dithiotheritol) to

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denature the protein in order to facilitate cross-linking (*15*,*27*,*37*,*38*). However, the use of such reagents is not allowed in food applications. Moreover, the cold-set whey protein gelation using TG has not been studied. In particular, the role of TG-induced bonds in fortifying the whey protein network at low pH has not been discussed.

In this study, we adopt a two-step strategy to develop whey protein gels using enzymatic treatment at alkaline conditions followed by cold setting using glucono-δ-lactone acid to low pH (4). While a two-step procedure has previously been used (*21*), it has been for heat-treated samples at high temperatures and without any enzymes that can lead to new bond formation and microstructure. The distinctive feature of our work involves the combined use of enzyme (TG) with cold-setting conditions, monitoring the initial enzyme-catalyzed polymerization step both rheologically and biochemically, and evaluating the gelation characteristics in-depth. In this regard, we use a new dynamic rheological approach to explore the large strain behavior of samples and identify yield/fracture points. We investigate the different conditions affecting cross-linking of whey proteins by TG. Gels produced by enzymatic treatment at alkaline conditions followed by cold setting using GDL acid to low pH are compared to gels produced with no enzyme but cold set and those prepared with conventional heat treatment. Our results reveal enzymatic treatment to produce gels with superior rheological properties. These results are discussed in terms of the microstructure and role of enzyme-induced cross-links in the gel network.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) was obtained from Davisco Food International (LeSueur, MN) and used as received. A commercial version of transglutaminase enzyme (1% enzyme and 99% maltodextrin, by weight) was provided by Ajinomoto Co., Japan. Enzyme activity was determined using the hydroxamate method as described by Folk (*39*). The activity unit is defined as the amount of the enzyme producing 1 μmol of hydroxamic acid per minute at 37 °C. Glucono-δ-lactone (GDL), *N*-ethylmaleimide, was purchased from Fisher Scientific (Pittsburgh, PA). Sodium chloride (>99%) was purchased from Sigma Chemicals (St. Louis, MO). Deionized water (>15 MΩ) was used in all the experiments.

Sample Preparation. Protein solutions were prepared by dissolving WPI powder in deionized water to obtain a final concentration of 7.5% w/w and stirring for about 1 h to ensure complete solubility. The pH was adjusted by using either NaOH (1 N) or HCl (1 and 0.1 N). Solutions were deaerated for about 15 min under vacuum of 25 in. Hg at room temperature to eliminate trapped air bubbles.

Samples with enzyme were prepared by adding the requisite amount of powdered enzyme to WPI solutions contained in vials and mixing for 20 min at room temperature. The vials were closed immediately after enzyme addition to prevent enzyme deactivation through air−enzyme contact. Samples were then incubated for 5 h at 50 °C to induce enzymatic reaction. Samples without enzyme were heated at 50 °C for 5 h to mimic the thermal treatment of the samples with enzyme. *N*-Ethylmaleimide (NEM) was added to some samples to prevent disulfide bonding.

To study the effect of ionic strength on cross-linking of protein by enzyme, NaCl was dissolved in the WPI solution at pH 8 before enzyme treatment. The molar concentration of NaCl ranged from 0 to 200 mM.

WPI gels at pH 4 were obtained by adding 1.85% GDL to the WPI solution (no salt added, originally at pH 8) at 25 °C and stirring for about 10 min. The solution was then poured into a rheometer cup. All rheological measurements were conducted at 25 °C, unless otherwise mentioned. Conventional heat-treated gels were obtained by heating WPI solution (7.5% w/w, pH 4) at 80 °C for 5 h.

Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) under reducing conditions was performed on whey protein samples using Mini-PROTEAN 3 Electrophoresis Cell (BIO-RAD, Hercules, CA) to obtain information on their molecular weights. Protein solutions (7.5%) were diluted 40 times (i.e., ratio of protein to buffer was 1:40) by Laemelli sample buffer (25% glycerol, 2% SDS, and 0.01% Brumophenol Blue), mixed with 50 μL of β-mercaptoethanol/mL of solution and heated for 5 min at 95 °C and then subjected to electrophoresis in gradient gels of 4−15% polyacrylamide using the discontinuous system (*40*). These gels were stained with 0.1% Coomassie blue and destained using first a solution of high destaining power (40 wt % ethanol and 10 wt % acetic acid in deionized water) for 2 h and later a solution of low destaining power (10 wt % ethanol and 10 wt % acetic acid in deionized water) for approximately 12 h. The gels were subsequently immersed in a 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 examined to determine the different molecular weight bands. Gels under nonreducing condition were prepared the same way as above but without the addition of β-mercaptoethanol.

Rheological Measurements. Steady and dynamic shear rheological experiments were conducted on a TA Instruments AR2000 rheometer using a couette geometry (cup diameter = 30 mm, and bob diameter = 28 mm). In the steady shear experiments samples were heated to 50 °C and maintained at this temperature. Viscosity was measured at 5-min intervals while subjecting the sample to a continuous shear rate of 50 s^{-1}. In the dynamic experiments the samples were subjected to a sinusoidal deformation as either a function of increasing stress 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, it provided the limit of linear viscoelasticity (LVE) that could be used in the frequency experiments. Second, as discussed later, it provided a method to examine yield and fracture stress/strain of the samples (*41*,*42*). 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 (*43*). In all experiments conducted, the samples were covered with *n*-hexadecane to prevent evaporation.

Field Emission Scanning Electron Microscopy (FESEM). FESEM (JEOL 6400F) was used with an accelerating voltage of 5 kV and a magnification of 10 000× to investigate the microstructure of whey protein gels obtained under different conditions. Small pieces of gels were fixed with 2.5% (w/w) glutaraldehyde in water for 1 h and rinsed with water 3 times. The samples were then soaked in 0.2% (w/w) osmium tetraoxide for 12 h. The gels were initially dehydrated using a graded ethanol series (*44*) and completely dried using critical point drying with CO_2 (*44*). The dried samples were gently fractured and coated with Au/Pd by diode sputter coating prior to scanning.

RESULTS AND DISCUSSION

Enzyme Activity at Different pH. To develop the two-step gelation process, it is essential to (i) determine the conditions in which the enzyme will have sufficient activity and subsequently (ii) examine which of these conditions are most conducive for the enzyme-catalyzed polymerization of whey protein. In this regard, we first explored the effect of pH on enzyme activity at 50 °C (**Figure 1**). We chose this temperature as it had been found to be the optimal temperature for transglutaminase use (Ajinomoto Data Sheet). From **Figure 1**, which shows relative activity as a function of time, it is apparent that enzyme activity decays rapidly for pH < 5 or > 8. This behavior can be explained in terms of protonation and deprotonation of enzyme active site (cysteine) at low and high pH, respectively (*45*), or due to disruption of the active site conformation resulting from enzyme denaturation. We also find enzyme activity to be fairly stable for pH 5−7 and to have lower degree of stability at pH 8. On the basis of these results, we undertook enzymatic treatment of WPI samples at pH 6, 7, and 8, as detailed in the next section. Studies of enzymatic polymerization of WPI at pH 4 and 9 were not undertaken

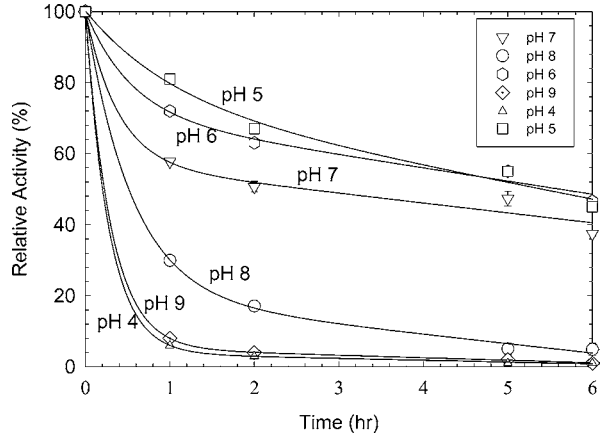

Figure 1. Effect of pH on the relative activity of transglutaminase enzyme at 50 °C.

because of the rapid decay of enzyme activity under these conditions. Enzymatic polymerization at pH 5 was also excluded because native (not subjected to any treatment) whey protein precipitates out of solution immediately after the solution pH is lowered to this value.

Whey Protein Polymerization with Enzyme. The effect of pH on enzyme treatment of whey proteins was examined by incubating samples at 50 °C for 5 h with 100 U of enzyme/g of protein at pH 6, 7, and 8. **Figure 2** shows the molecular weight of various samples following enzyme treatment obtained using reducing SDS−PAGE analysis. In **Figure 2a** the two bands appearing on the lowest (bottom) part of the gel correspond to α-lactalbumin and *β*-lactoglobulin, as evident from the SDS−PAGE analysis of these individual components (**Figure 2b**). We find the *β*-lactoglobulin band to remain effectively unchanged upon enzyme treatment at pH 6 and 7 but to become quite faint at pH 8. On the other hand, the α-lactalbumin band disappears at all pH following enzyme treatment. These results indicate that α-lactalbumin can be readily cross-linked (polymerized) at different pH, consistent with earlier reports (*15*,*46*). However, *β*-lactoglobulin requires slightly alkaline medium to be cross-linked. The difference in behavior between the two proteins can be attributed to the molten globular state of α-lactalbumin that facilitates cross-linking at all pH due the disordered tertiary structure (*26*). In contrast, *β*-lactoglobulin is believed to get partially denatured at a high pH of 8, which then facilitates its cross-linking by the enzyme (*47*). To study the individual behavior of α-lactalbumin and *β*-lactoglobulin, we incubated pure fractions of α-lactalbumin and *β*-lactoglobulin with enzyme (**Figure 2b**). We can clearly observe that at pH 8, *β*-lactoglobulin molecules are cross-linked with each other in the absence of α-lactalbumin (lane 9). We can also observe from the same figure that pure fractions of α-lactalbumin are able to polymerize irrespective of the pH (lanes 3, 4, and 5), whereas *β*-lactoglobulin needs alkaline pH to polymerize. On the basis of these results, pH 8 represents a viable environment for the cross-linking of whey proteins by transglutaminase. Any enzymatic treatment reported in the rest of this study has therefore been conducted at a pH of 8.

To examine the relative portion of enzyme versus disulfide linkages formed when whey proteins are treated at 50 °C for 5 h, SDS−PAGE analysis was undertaken for both reducing and nonreducing conditions. **Figure 2c** shows these results for whey protein samples (pH 8) subjected to various types of treatment. Lane 2, which corresponds to native WPI, reveals that there is almost no polymerization occurring. This indicates that although whey proteins are partially denatured, heat treatment is needed

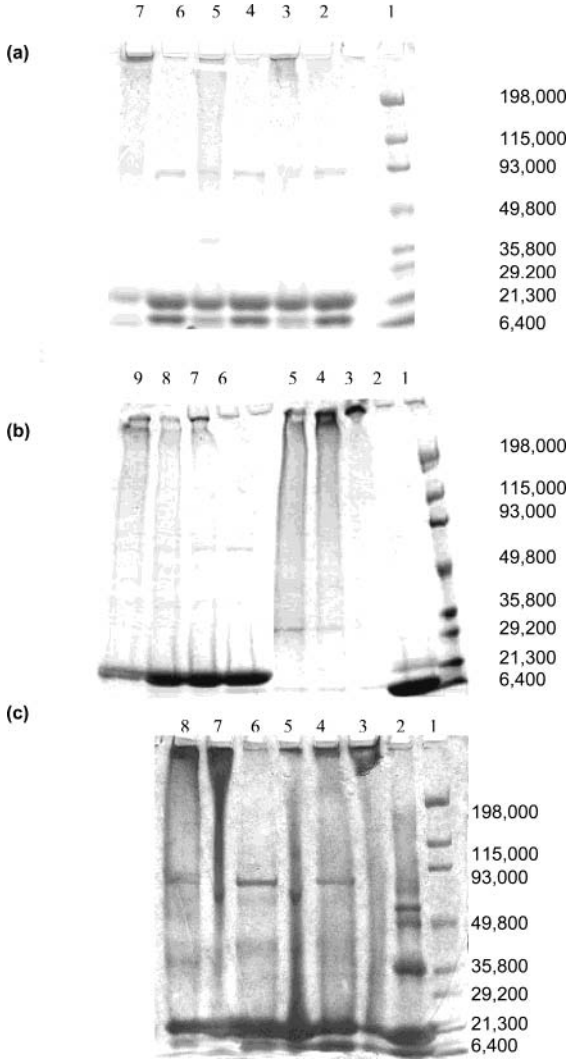

Figure 2. SDS–PAGE bands for (a,c) whey protein isolate samples, (b) α-lactoglobulin and *β*-lactoglobulin samples. (a) Lane 1: molecular weight markers in Daltons. Lanes 2, 4, and 6 are native (not subjected to any treatment) whey protein isolate samples. Lanes 3, 5, and 7 are whey protein sample treated with enzyme at pH 6, 7, and 8, respectively. Heating for all samples was conducted at 50 °C for 5 h. (b) Lane 1: molecular weight markers. Lanes 2, 3, 4, and 5 are α-lactalbumin samples: lane 2 is native; lanes 3, 4, and 5 are incubated with enzyme for 5 h at pH 6, 7, and 8, respectively. Lanes 6, 7, 8, and 9 are *β*-lactoglobulin samples: lane 6 is native, lanes 7, 8, and 9 are incubated with enzyme for 5 h at pH 6, 7, and 8, respectively. (c) Lane 1: molecular weight markers. Lanes 2–8 are whey protein isolate samples at pH 8. Lanes 3, 5, and 7 were analyzed under nonreducing conditions, while lanes 2, 4, 6, and 8 were analyzed under reducing conditions. Lane 2: unheated. Lanes 3 and 4: heated for 1 h at 80 °C. Lanes 5 and 6: heated for 5 h at 50 °C. Lanes 7 and 8: incubated with enzyme for 5 h at 50 °C.

to initiate polymerization. Treatment of WPI at 80 °C for 1 h (lane 3) reveals extensive polymerization via disulfide bonding (note the effect of reducing conditions in decreasing the molecular weight in lane 4). Heating the sample at 50 °C for 5 h (lane 5) produces a weak high molecular weight band and some aggregates with molecular weight less than 93 kDa (i.e., tetramers and lower). In contrast, the presence of enzyme (lane 7) under similar conditions reveals a large proportion of high molecular weight aggregates that remain unabated even under reducing conditions (pH 8). This suggests that disulfide bonds

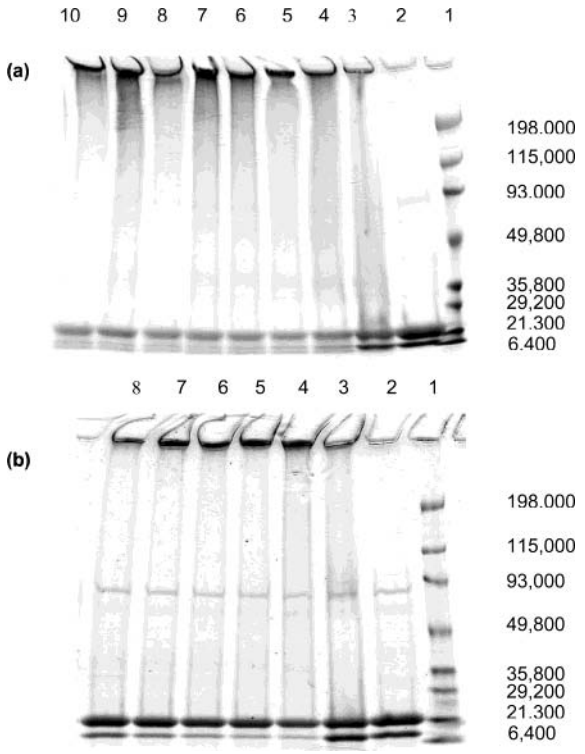

Figure 3. SDS–PAGE bands for enzymatic polymerization of whey protein isolate samples at different (a) enzyme and (b) NaCl concentrations. All experiments were conducted at pH 8. (a) Lane 1: molecular weight marker. Lane 2 is native whey protein isolate sample. Lanes 3–10 are whey protein isolate samples treated with enzyme at 50 °C for 5 h at different concentrations as follows: lane 3 [1 U/g of protein], lane 4 [5 U/g of protein], lane 5 [10 U/g of protein], lane 6 [20 U/g of protein], lane 7 [30 U/g of protein], lane 8 [40 U/g of protein], lane 9 [50 U/g of protein], and lane 10 [100 U/g of protein]. (b) Lane 1: molecular weight marker. Lane 2 is native whey protein isolate sample. Lane 3 is sample heated at 50 °C for 5 h without enzyme. Lanes 4–8 are samples treated with 10 U/g of enzyme at 50 C for 5 h: lane 4 [no salt added], lane 5 [20 mM NaCl], lane 6 [50 mM NaCl], lane 7 [100 mM NaCl], lane 8 [200 mM NaCl].

form in small quantity at pH 8 at 50 °C, while the overwhelming aggregation occurs via the enzyme-catalyzed ϵ-(γ-glutamyl)-lysine cross-linking.

The effects of enzyme concentration and solution ionic strength on whey protein polymerization are displayed in **Figure 3**. SDS−PAGE analysis following incubation with different concentrations of enzyme at pH 8 and 50 °C for 5 h is shown in **Figure 3a**. We find no further effect on polymerization for enzyme concentrations higher than 10 U/g of protein. This can be surmised from the constant intensity of the monomeric bands of α-lactalbumin and β-lactoglobulin from 10 to 100 units (lanes 5−10). **Figure 3b** shows the effect of salt concentration on the extent of polymerization. These samples (pH 8) were incubated with enzyme for 5 h at 50 °C. It is apparent that an increase in ionic strength decreases the extent of polymerization of whey protein chains. At larger salt concentrations, darker (or heavier) bands of α-lactalbumin and β-lactoglobulin, indicating a higher concentration of unpolymerized fraction of protein, is noticed. This behavior can be explained on the basis of electrostatic interactions. At higher ionic strength, these interactions between protein chains are screened and the chains collapse. This collapse prevents the "induced fit" required for enzyme action. According to the induced fit concept, the substrates (α-lactalbumin and β-lactoglobulin in our case) cause the three-dimensional structure of the enzyme to change. This change in the structure brings

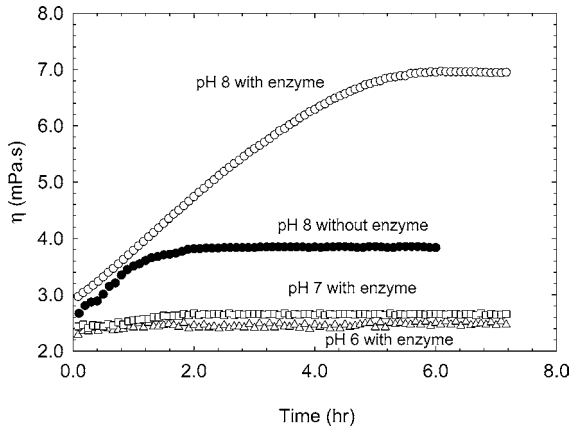

Figure 4. Apparent viscosity (at a shear rate of 50 s^{-1}) of whey protein isolate samples as a function of incubation time with enzyme. Results are shown for different pHs.

the catalytic groups (glutamine and lysine) into proper alignment and induce the (ϵ-(γ-glutamyl)lysine bonds. The collapsed chains of the α-lactalbumin and β-lactoglobulin molecules at high ionic strength fail to produce the proper enzyme conformation.

To investigate the effect of the enzyme treatment on rheological properties, steady shear viscosity was measured at a fixed shear rate (50 s^{-1}) as a function of incubation time (**Figure 4**). No increase in viscosity is observed when incubating the enzyme with the whey protein solution at 50 °C at pH 6 or 7. This indicates that the few cross-links (mainly between α-lactalbumin molecules) have little effect on solution viscosity. On the other hand, there is an appreciable increase in viscosity at pH 8 due to the polymerization of both β-lactoglobulin and α-lactalbumin molecules, consistent with our SDS page analysis. It should be noted that heating whey protein at 50 °C and pH 8 induces some disulfide formation. This is also exhibited in **Figure 4**, in which we observe a modest increase in viscosity for a sample containing no enzyme.

Cold-Set Acidic Gels following Enzyme Treatment. After enzyme treatment at pH 8, the protein solution was acidified by GDL (1.85 wt %), a slow release food-grade acidulant, to pH 4. The use of GDL over other strong acids (e.g., HCl) thereby allowed the time scale of sample pH change to be long enough to produce homogeneous gelation throughout the sample. Please note that minimal, if any, enzyme cross-linking occurred during acidification as the enzyme had negligible activity (**Figure 1**) following the initial polymerization step. **Figure 5**a shows the change in protein pH following GDL addition. We observe two distinct regimes consisting of a large initial decrease in pH to approximately 4.7 in about 2 h followed by a much slower decrease to pH 4 spanning 48 h. In **Figure 5b** we examine the corresponding change in elastic (G') and viscous (G'') modulus upon GDL addition. For comparison purposes, results of a sample without any enzyme are also included. This sample was incubated at 50 °C for 5 h to mimic the procedure that the enzyme-treated samples received. Gelation of both samples was monitored in situ in the rheometer at a constant strain amplitude (0.1%) and frequency (1 rad/s).

Several features are apparent from **Figure 5b**. First, there is a substantial increase in moduli with acidification as repulsion between protein chains starts to diminish with a decrease in pH. Most of this increase occurs within the first 2 h, consistent with the initial pH decay to 4.7 observed in **Figure 5a**. Second, following the initial increase in moduli, we observe a slight decrease in their value with time. The maximum in moduli may

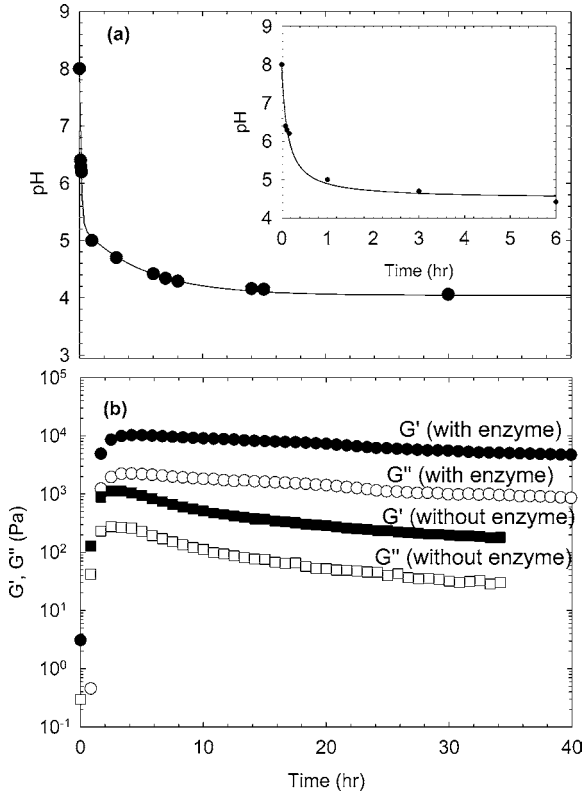

Figure 5. Changes in whey protein properties during cold-set acidification through addition of 1.85% glucono-δ-lactone (GDL) acid. The original sample was enzyme treated at pH 8. (a) Change in pH with time upon addition of GDL. Inset at the upper right corner is an enlarged view of the initial pH decrease with time. (b) Evolution of the elastic (G') and viscous (G'') moduli of the enzyme-treated whey protein sample during decrease of its pH.

be attributed to the sample passing through the isoelectric point. Third, the elastic (as well as the viscous) modulus of the enzyme-treated sample is an order of magnitude higher than that of the sample containing no enzyme. Finally, the elastic modulus of both samples is larger than their corresponding viscous modulus, suggesting presence of a gelled network.

It is pertinent to verify the presence of this gelled network upon acidification, since the previous results were monitored at a single frequency. **Figure 6** shows the frequency spectrum of the elastic and viscous modulus of both samples (circles corresponding to enzyme treated and squares without enzyme treatment) after 48 h of acidification. For both cases, we find G' and G'' to exhibit a very weak frequency dependence, with G' larger than G'' over the entire frequency ranged observed. Such features are characteristics of a gel. In addition, the sample treated with enzyme has a higher elastic modulus compared to the untreated sample. Since G' is related to the degree of cross-linking; this suggests the presence of additional cross-links in the enzyme-treated sample.

An important issue to consider when examining gel modulus is the extent of disulfide linkages formed upon heating the sample at 50 °C for 5 h and its impact on gel rheology. To examine this, samples were prepared in which *N*-ethylmaleimide (NEM) was added to the whey protein solution (pH 8) prior to heating at 50 °C for 5 h. NEM is believed to prevent disulfide bonding by blocking the free sulfhydryl groups. The samples were then cold set by GDL to pH 4. The frequency spectrum of the dynamic moduli of such a sample is shown in **Figure 6** (triangles). We find both G' and G'' of the samples with and without NEM to be essentially identical, thereby demonstrating

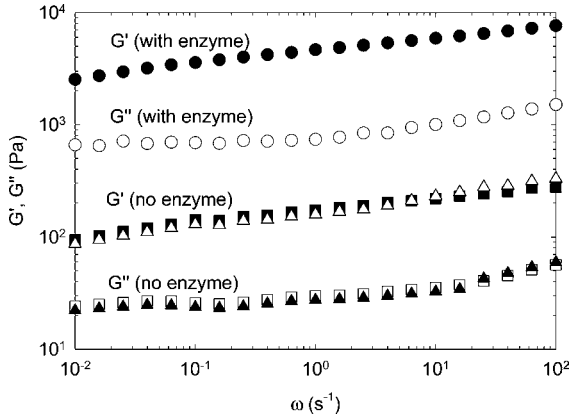

Figure 6. Dynamic frequency spectrum of the elastic (G') and viscous (G'') moduli of acidic (pH 4) cold-set gels. Results are compared for a sample that has been enzyme treated prior to acidification with samples without enzyme pretreatment. Circles represent sample with enzyme treatment. Squares represent sample incubated at 50 °C for 5 h without enzyme. Triangles represent sample treated with NEM (to prevent disulfide linkages) and incubated at 50 °C for 5 h without enzyme (△, G'; ▲, G'')

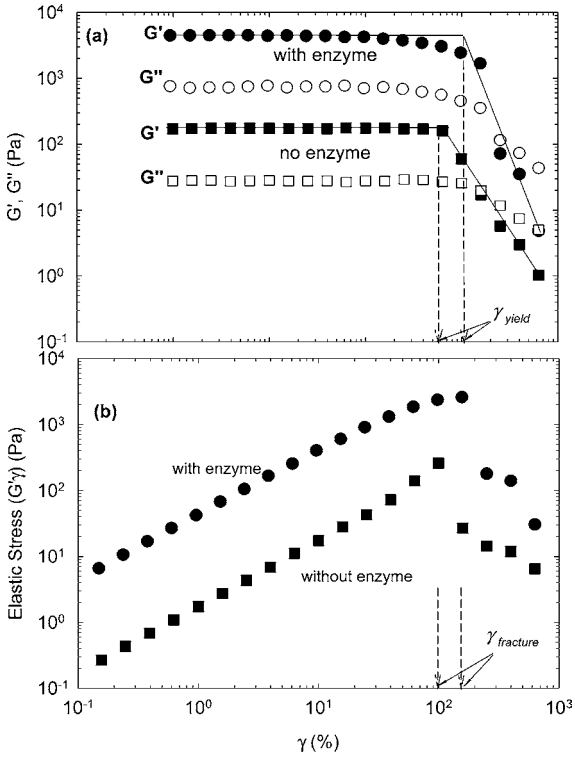

Figure 7. Effects of increasing strain amplitude on the (a) dynamic moduli and (b) elastic stress (product of elastic modulus and strain) of cold-set acidic gels. The intersection of the asymptotes in a corresponds to the "yield" point. All experiments were conducted at a frequency of 1 rad/s.

several important points. First, the number of disulfide linkages formed during the polymerization step is few, consistent with our SDS−PAGE results (**Figure 2c**). Second, these disulfide linkages have a negligible effect on gel rheology, with physical interactions being the primary mechanism for gel formation (without enzyme). Finally, the enhanced modulus observed upon enzyme treatment can be directly attributed to the additional enzyme-catalyzed bonds formed.

Large Strain and Yield Behavior. Figure 7a shows the dynamic moduli of the enzyme-treated and untreated samples as a function of increasing strain amplitude. At low strains, both G' and G'' are flat, reminiscent of materials in the linear

Table 1. Yield and Fracture Strains and Stresses of Acidic Gels

	cold set without enzyme treatment	cold set with enzyme treatment	conventional heat treatment (1 h)	conventional heat treatment (5 h)
yield strain (%)	60	100	5	10
fracture strain (%) (at max elastic stress)	100	157	6.5	26
fracture stress (Pa) (max elastic stress)	262	2678	40	810

viscoelastic regime. With increasing strain, however, nonlinearity sets in with a slow decrease of both G' and G''. Subsequently, G' and G'' tend to cross over as the material microstructure gets disrupted. Eventually at large strains, G'' becomes larger than G' and both decrease rapidly as the material yields and/or fractures. Qualitatively, we find the yield strain of the enzyme-treated sample to be larger than the untreated sample. However, it is difficult to pinpoint the yield (or critical strain) exactly. One approach to do this has been to draw asymptotes through the low and large strain values of G' (as shown in **Figure 7a**) and call the intersection point the yield point (*41*,*42*). However, such an approach can produce errors because of the variation possible in drawing the asymptotic lines. It is interesting to note that reversible "healing" of sample microstructure is possible in the immediate vicinity of the yield strain regime (data not shown) if the material is left to relax.

An alternative and precise way to determine fracture/yield strain or stress is to plot the elastic stress, defined as a product of the elastic modulus and strain ($G'\gamma$), as a function of increasing strain (*41*,*42*,*48*). Such an approach has been used effectively for particulate (*41*) and polysaccharide (*42*) gels to locate the yield point, which corresponds to the maximum in the plot. **Figure 7b** shows the elastic stress as function of increasing strain for enzyme-treated and untreated samples. In both cases, we observe a sharp maximum that corresponds to the yield/fracture stress and strain. In our samples, this point possibly corresponds to fracture rather than yield as samples beyond this strain showed no reversibility (data not shown). In fact, a fracture surface was visually identified in the rheometer geometry at/beyond the point of maximum stress. In addition, the strain values corresponding to the maximum stress in this figure are higher than those obtained from the yield point in **Figure 7a**.

We find from **Figure 7b** that the fracture stress of the enzyme-treated sample is about an order of magnitude higher than the untreated sample whereas the fracture strain is larger by about 50%. The same result is also obtained if we plot of the total stress (instead of the elastic stress) as a function of strain (data not shown). This is because the dominant component of the total stress is the elastic stress. **Table 1** summarizes the values for the yield and fracture strains and stresses. The higher values of the yield/fracture strain and stress, as well as the elastic modulus, in the case of enzymatic treatment can be attributed to the presence of chemical cross-links (ϵ-(γ-glutamyl)lysine bonds) that strengthen the network. In contrast, the absence of such chemical cross-links in the untreated sample causes the gel network to yield/fail at lower strain and stress values.

Cold-Set, Enzyme-Treated versus Conventional Heat-Treated Gels. Protein gels are commonly prepared though heat treatment, typically at 80 °C. This produces complete heat denaturation of protein molecules, leading to hydrophobic, hydrogen, and electrostatic interactions together with disulfide bond formation (*49*−*51*). Conventional gels at pH 4 were obtained by heating whey protein solution (7.5% w/w, pH 4) at 80 °C for specified time intervals. **Figure 8** compares the frequency spectra (at 25 °C) of both conventional heat-set gels and an enzyme-treated cold-set gel. In this regard, heat-set gels obtained using two different heating times, 1 and 5 h, are shown. We find conventional gels obtained after 1 h of heating to have a lower G' than the enzyme-treated gels, whereas that heated for 5 h exhibits a slightly higher G' than the enzyme-treated sample. The latter can be a result of the denaturation of most of the protein chains in the conventional gels, which are strengthened by physical interactions with prolonged heating, whereas only part of the protein chains in the cold-set gel denatures. The higher degree of denaturation allows more protein chains to be part of the network forming the gel, thereby leading to a larger modulus. In fact, the inset in **Figure 8** shows G' of conventional gels (at 80 °C) as a function of heat treatment time. We find that the elastic modulus of these gels increases with heating time and asymptotes out after about 4 h.

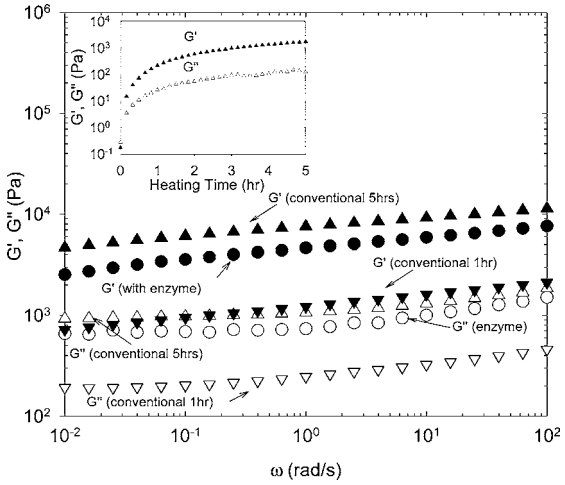

Figure 8. Comparison of the frequency spectrum of the dynamic moduli of an acidic cold-set gel (enzyme pretreated) with conventional gels; all measured at 25 °C. The conventional gels were prepared at the same pH (of 4) of the acidic, cold-set gel through heat treatment at 80 °C for different time intervals. The inset in the upper left corner shows the evolution of G' and G'' (at 80 °C, $\omega = 1$ rad/s)) as a function of heating time for the conventional gel.

Figure 9 compares the large strain behavior of the two conventional gels (1 and 5 h heat treated) to an enzyme-treated one. In **Figure 9a**, which shows G' for the three samples, we find nonlinearity and yielding of the conventional gels to precede that of the enzyme-treated one. This seems to hold true regardless of the heat treatment time and initial modulus of the conventional gels. **Figure 9b** plots the elastic stress as a function of increasing strain for these three samples. We not only find a substantially larger fracture strain for the enzyme-treated gel but also a higher fracture stress. A comparison of the yield stress and strain values of a cold-set sample without enzyme, a cold-set sample with enzyme, and conventional heat-treated gels is given in **Table 1**. The fracture stress of the enzyme-treated sample is larger than both the untreated sample and heat-treated sample (5 h) by about a factor of 10 and 3, respectively. The fracture strain of the enzyme-treated sample is 1.5 and 6 times larger than the untreated sample and heat-treated sample,

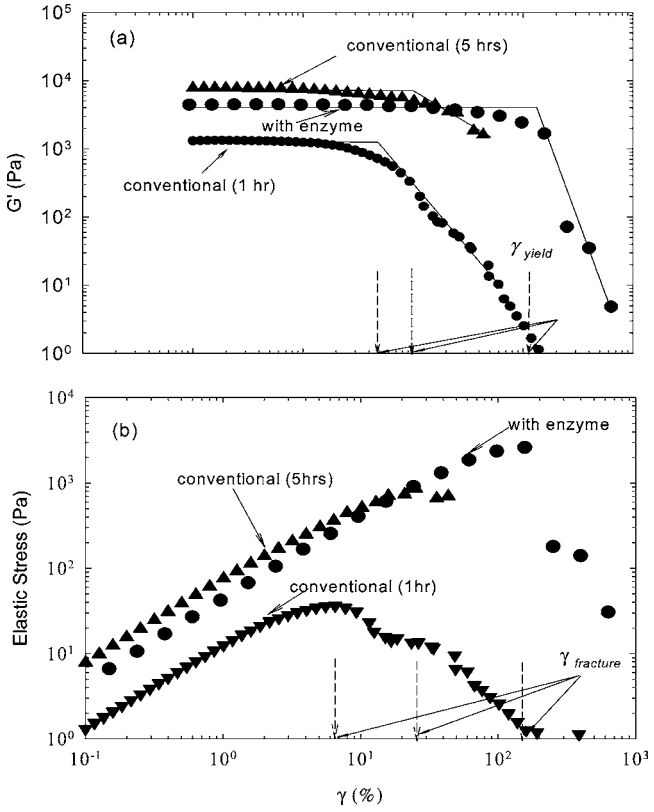

Figure 9. Comparison of the large strain behavior of a cold-set acidic gel (enzyme pretreated) with conventional, heat-treated gels at the same pH (4): (a) elastic modulus (G') as a function of strain amplitude; (b) elastic stress ($G'\gamma$) showing maximum with increasing strain amplitude.

respectively. It is interesting to note that although the conventional gel with 5 h of heat treatment has a higher elastic modulus, its yield and fracture properties are much lower in value compared to the enzyme-treated gel. These results highlight the fact that the elastic modulus alone is insufficient to characterize whey protein gels without knowledge of its yield and fracture properties. Interestingly, yield properties are of special importance in food applications as they mimic the chewing and biting of food.

Figure 10 shows SEM images of the microstructures of whey protein gels obtained under different conditions, conventional (5 h), cold set without enzyme treatment, and cold set with enzyme treatment. The conventional gel shows large voids and a nonhomogeneous particulate structure. These large voids in the structure may be the reason for the lower values of fracture stress and strain. This is because fracture occurs at the weakest parts of the gel network, which are expected to be the large void regions. On the other hand, both cold-set gels (with and without enzyme treatment) reveal a more homogeneous network structure. Although the cold-set gel without enzyme treatment appears more uniform and homogeneous than its enzyme-treated counterpart, it has lower fracture properties. This can be attributed to the presence of additional chemical cross-links (ϵ-(γ-glutamyl)lysine bonds) in the enzyme-treated gel, making it capable of carrying larger stresses with higher reversible extensibility (rubberiness).

CONCLUSIONS

In this study, we examined the rheological characteristics of a low-pH whey protein gel prepared using a new enzyme-catalyzed two-step process. The first step involved enzymatic

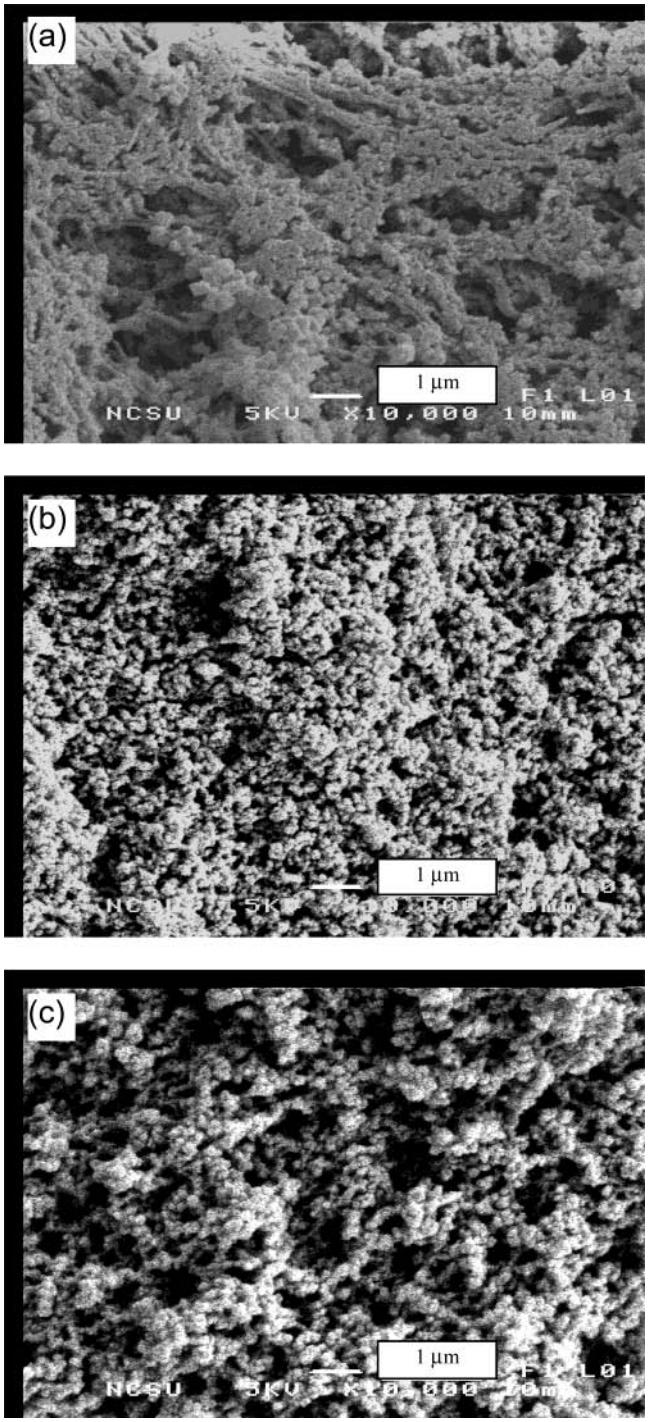

Figure 10. SEM micrographs of acidic gels (pH 4) obtained under different conditions: (a) conventional, (b) cold set without enzyme, (c) cold set with enzyme. All samples are shown at a magnification of 10 000×. Bars correspond to 1 μm.

polymerization at pH 8 and 50 °C using transglutaminase. The second step entailed formation of gels at pH 4 through acidification of the polymerized whey using GDL under cold-set conditions. The alkaline conditions for the first step were chosen based on the relative activity of enzyme under different pH and its ability to polymerize β-lactoglobulin, the major component of whey protein, at this pH. Enzyme catalysis during this process led to the formation ϵ-(γ-glutamyl)lysine bonds and a substantial increase in molecular weight and sample viscosity. The low-pH gels obtained through the second acidification step exhibited substantially higher fracture/yield stress and strain

compared to cold-set gels with no enzyme and conventional heat-set gels at 80 °C. In this regard, we used a new approach to obtain yield/fracture properties from dynamic rheological experiments. The elastic modulus of the enzyme-catalyzed gel was also higher than the one without enzyme. Interestingly, the modulus of the heat-treated sample was a function of heating time and lower than the enzyme-treated sample except for samples heated for a prolonged period (~5 h). Even these gels had comparable or slightly higher modulus than the enzyme-treated sample but considerably lower fracture stress/strain. These results taken together indicate the use of transglutaminase enzyme as a viable approach to produce low-pH gels with enhanced rheological characteristics.

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