Factors Determining Fracture Stress and Strain of Fine-Stranded Whey Protein Gels

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Whey proteins form translucent heat-induced gels at low and high pH that differ in fracture properties. Gels formed at pH 7.0 and 6.5 were strong (fracture stress of 59−92 kPa) and rubbery (fracture strain of 1.7−1.2). Gels formed at pH 3.0 and 2.5 were weak (fracture stress of 17−19 kPa) and brittle (fracture strain of 0.33−0.39). Altering disulfide bond formation changed gel texture. Addition of a disulfide reducing agent (dithiothreitol) at concentrations ≥ 25 mM, at pH 7.0, caused the gel to weaken (>46% reduction in fracture stress) and become brittle (fracture strain of 0.30−0.32). Increasing the number of disulfide bonds in gels formed at pH values ≤3.5 increased fracture strain to 2.0 while causing a minor increase in fracture stress. Formation of disulfide bonds appears to be essential to the deformability of heat-induced whey protein gels.

Keywords: *Gelation; fracture properties; disulfide bonding; whey proteins*

INTRODUCTION

Whey protein ingredients are used in a number of foods including beverages, confectionery, convenience foods, desserts, and dairy and meat products (deWit, 1989). These proteins provide structure and desirable texture in foods by a variety of mechanisms including heat-induced gelation. With the need to produce shelf-stable foods at a pH <4.6, understanding the molecular mechanisms of gelation under acidic conditions will allow for a greater utilization of this ingredient.

Protein−protein interactions that produce heat-induced gels are noncovalent (hydrogen bonds, hydrophobic, and electrostatic interactions) and covalent (disulfide bonds) (Bezrukov, 1979; Schmidt, 1981). Environmental conditions (pH, ionic strength, and temperature) will affect the availability of these bonding groups for stabilization of tertiary structure and intermolecular interactions. Since pH affects molecular conformation and intermolecular interactions, it is not surprising that it influences gel network structure and rheological characteristics. Several investigations on whey protein gelation have found that opaque gels are formed at pH 4−6, while above and below this range gels are translucent (Stading and Hermansson, 1991; Langton and Hermansson, 1992; Foegeding, 1993). Translucent whey protein gels have fine-stranded microstructures and the rheological properties are pH-dependent (Stading and Hermansson, 1991; Langton and Hermansson, 1992; Foegeding, 1993). Fine-stranded gels formed at pH <4 are weak (low values for fracture stress) and brittle (low values for fracture strain). In contrast, fine-stranded gels formed at pH >6 are strong and rubbery, with high fracture stress and strain values (Stading and Hermansson, 1991; Foegeding, 1993; Tang et al., 1995).

The mechanism responsible for the weak/brittle texture of fine-stranded gels at low pH is not understood. A factor that may influence gel formation at low pH is the decrease in sulfhydryl oxidation and thiol/disulfide exchange (Mangino et al., 1987; Shimada and Cheftel, 1988; Xiong, 1992). Sulfhydryl oxidation and thiol/disulfide exchange alters rheological properties of gels made at pH 8.0. Gel hardness (force at 20% or 70% compression) decreases when disulfide reducing agents (dithiothreitol or mercaptoethanol) or a sulfhydryl blocking agent (*N*-ethylmaleimide) are added prior to heat-induced gelation (Matsudomi et al., 1991; Mulvihill et al., 1991). In contrast, thiol/disulfide exchange does not appear to alter rheological properties of gels made at pH 2.5. Shimada and Cheftel (1988) found that, at pH 2.5, gel firmness and elasticity were not affected by the addition of a sulfhydryl blocking agent, *N*-ethylmaleimide (NEM). This showed that disulfide bonds do not contribute to rheological properties of gels formed at pH 2.5.

The weak/brittle texture of fine-stranded gels formed at pH ≤4.0 could be due to (1) inhibition of sulfhydryl oxidation and thiol/disulfide exchange, (2) pH-associated effects on denaturation and aggregation reactions, or (3) a combination of both mechanisms. The objective of this research was to determine the mechanism(s) responsible for the weak/brittle texture of fine-stranded whey protein gels formed at pH ≤4.0.

MATERIALS AND METHODS

Protein Solutions. Whey protein isolate (WPI) (Davisco Foods International, LeSueur, MN) solutions were prepared at 20% (w/v) protein. The protein content of WPI powders was determined by microKjeldahl analysis with a conversion factor of 6.38*N* = % protein (AOAC, 1984).

Effect of Dispersion pH. Protein solutions were hydrated for 1 h by slow stirring, degassed under vacuum for 1 h, and then brought to volume by dilution with 2 M NaCl and deionized water. The pH was adjusted to 7.0, 6.5, 3.0, and 2.5 by slow addition of 1 or 5 M HCl and then degassed under vacuum for 30 min. The solutions were adjusted to final concentrations of 14% (w/v) protein and 50 mM NaCl. Gels

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were made from three lots of WPI and each lot was replicated twice, producing a total of 6 replications.

Effect of Dithiothreitol at pH 7.0. A 16% (w/v) WPI solution was prepared as above. The solution was diluted to 14% (w/v) with 2 M NaCl, deionized water, and 1 M dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO), to produce solutions containing 50 mM NaCl and 0, 2.5, 5, 10, 25, 50, or 100 mM DTT. The pH of the suspensions was maintained at pH 7.0. Gels were made from two lots of WPI and each lot was replicated once, producing a total of 2 replications.

One-Step Heat-Induced Gelation. Protein solutions at different pH values or containing DTT were poured into stoppered glass tubes, 19 mm in diameter. Glass tube interiors were coated with Sigmacote (Sigma Chemical Co., St. Louis, MO) to prevent adhesion of the gel to the glass. Each tube was covered with aluminum foil and heated at 80 °C for 30 min in a water bath. Gels were cooled in an ice water bath for 30 min and held in the tubes overnight at 4 °C prior to analysis.

Two-Step Heat-Induced Gelation. Two hundred milliliters of a 10% (w/v) WPI solution was prepared without the addition of salt. The solution was degassed for 1 h, brought to volume with deionized water, and then degassed again for 30 min. The solution was split into two aliquots. The first was used as a control and held at room temperature, while the other was heated for 30 min at 80 °C, producing "predenatured" WPI. After cooling to room temperature in an ice water bath, 50 mM NaCl and 10% (w/v) glucono-δ-lactone (GDL) (Sigma Chemical Co., St. Louis, MO) were added to the predenatured WPI dispersion. The unheated control had the same concentration of NaCl but no GDL. Solutions were stirred for 2 min and then poured into tubes precoated with Sigmacote and stoppered at one end. The tubes were covered with aluminum foil and placed in a 90 °C water bath for 30 min. All tubes were cooled in an ice water bath for 30 min and held overnight in the tubes at 4 °C. Gels were made from three lots of WPI and each lot was replicated twice, producing a total of 6 replications.

Uniaxial Compression to Fracture. Gel cylinders (19 mm diameter, 28.7 mm height) were compressed to the point of fracture using a universal testing machine (Model 1122, Instron Engineering Corp., Canton, MA) controlled with TestWorks 3.01 software (MTS Sintech Inc., Research Triangle Park, NC). A 50 kg load cell and 100 mm/min compression rate were used for all samples. Six gel cylinders were tested for each replication of each treatment. From the sample dimensions, true shear stress (σ_s), true shear strain (γ_s), and fracture modulus (G_f) were calculated on the basis of the concepts of Hamann (1982) using

$$\lambda_z = L_f/L_i \quad (1)$$

$$\epsilon_z = -\ln \lambda_z \quad (2)$$

$$\gamma_s = (3/2)\epsilon_z \quad (3)$$

$$\sigma_z = (F\lambda_z)/A_i \quad (4)$$

$$\sigma_s = (3/4)\sigma_z \quad (5)$$

$$G_f = \sigma_s/\gamma_s \quad (6)$$

where λ_z (dimensionless) is the change in length ratio; L_f is the sample length at fracture (millimeters); L_i is the initial sample length (millimeters); ϵ_z is Hencky's true axial strain (dimensionless); σ_z is axial stress at fracture (pascals); F is force (neutrons), which includes gravitational force, and A_i is the initial area of contact (square meters).

Sulfhydryl Measurement. A method to measure *reactive sulfhydryls* in unheated dispersions and *total sulfhydryls* in heated and unheated dispersions was developed based on the methods of Beveridge et al. (1974) and Patrick and Swaisgood (1976) using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid). A 10% WPI solution was prepared as above without the addition of NaCl. The solution was split into three aliquots for determination of (1) reactive sulfhydryl groups, (2) total sulfhydryl groups in the unheated solution, and (3) total sulfhydryl groups in a heated sample. The aliquot for the heated sample was heated in a water bath at 80 °C for 30 min, cooled in an ice water bath for 30 min, and allowed to return to room temperature.

A 1.0 mL aliquot was taken from each of the three treatments and placed in glass test tubes. The sample used to measure the reactive sulfhydryl groups was diluted to 8.0 mL with phosphate buffer (pH 8.0, 0.1 ionic strength), while the samples for measurement of total sulfhydryl groups, both heated and unheated, were diluted with 6.86 mL of 7 M guanidine hydrochloride (GuHCl) (Boehringer Mannheim Corp., Indianapolis, IN) and 0.14 mL of phosphate buffer. This resulted in a final solution of 6 M GuHCl that unfolds the protein molecules, exposing sulfhydryl groups. After adjustment of each sample to pH 8.0 with 0.25 M NaOH, 1.0 mL of a 2 mM DTNB solution in pH 7.0 phosphate buffer was added and held for 40 min at room temperature. The solutions were adjusted to a final volume of 11.0 mL with phosphate buffer for the measurement of reactive groups or with 7 M GuHCl for total sulfhydryls and then passed through a sterile Acrodisc 0.45 μm filter (Gelman Sciences, Ann Arbor, MI). The absorbance at 412 nm of the filtered solutions was determined with a spectrophotometer (Model UV160U, Shimadzu Corp., Kyoto, Japan). Dilutions were done as needed, using the phosphate buffer stock solution for the unheated samples and the GuHCl stock solution for the heated sample. In all cases, the spectrophotometric blank was treated the same as the samples with omission of the whey protein solution. Sulfhydryl groups were calculated as micromoles of SH per gram of protein based on a molar absorptivity of 13 600 for nitrothiobenzoate (Ellman, 1959). Solutions were made from three lots of WPI and each lot was replicated twice, producing a total of 6 replications.

Table 1. Effect of pH on Fracture Properties of 14% (w/v) Protein Whey Protein Isolate Gels[a]

pH	true shear stress σ_s (kPa)	true shear strain γ_s	G_f (kPa)
7.0	74.5 ± 5.2	1.25 ± 0.09	59.7 ± 0.9
6.5	58.5 ± 3.5	1.15 ± 0.01	60.0 ± 3.4
3.0	18.5 ± 0.3	0.39 ± 0.01	47.8 ± 2.1
2.5	17.7 ± 0.5	0.33 ± 0.02	53.5 ± 2.6

[a] Mean values ± standard deviation for 6 replications.

RESULTS AND DISCUSSION

Effect of pH on Fracture Rheological Properties. The appearance of all whey protein isolate gels was translucent. This indicated a fine-stranded microstructure was formed (Clark et al., 1981; and Doi, 1993).

Gels formed at pH 7.0 and 6.5 were strong (fracture stress of 59−75 kPa) and deformable (fracture strain of 1.15−1.25), while those formed at pH 3.0 and 2.5 were weak (fracture stress of 18−19 kPa) and brittle (fracture strain of 0.33−0.39) (Table 1). These trends are in agreement with fracture stress and strain values for 10% (w/v) protein whey protein isolate gels formed from solutions containing 100 mM NaCl (Foegeding, 1993) and 12% (w/v) β-lactoglobulin gels (Stading and Hermansson, 1991). The relationships among fracture properties and terms used to describe gel texture are seen in Figure 1.

The fracture modulus (G_f) of weak/brittle gels was ~15% lower than the G_f values for strong/rubbery gels, in contrast to the major decreases in fracture stress (~73% decrease) and fracture strain (~70% decrease) (Table 1). A modulus, being a ratio of stress to strain, is independent of the absolute values of stress and

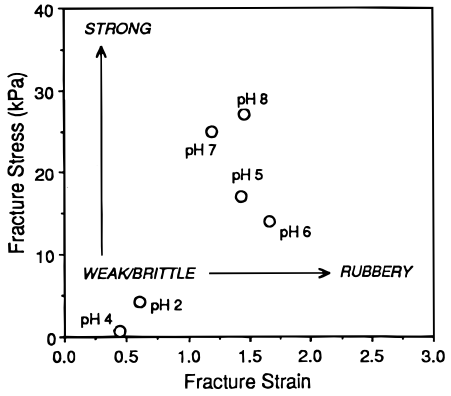

Figure 1. Fracture properties of whey protein isolate gels. Data are from Foegeding (1993) for whey protein isolate gels formed by heating 10% (w/v) protein solutions, containing 100 mM NaCl, at 80 °C for 30 min. Gel pH is indicated.

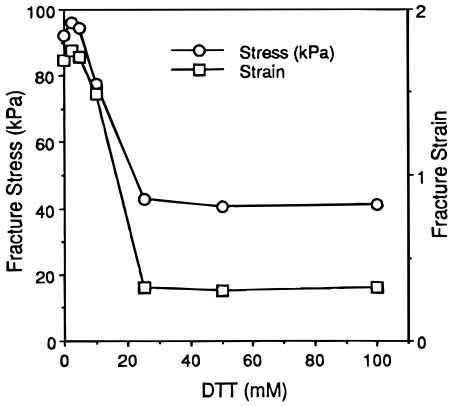

Figure 2. Effect of dithiothreitol on fracture stress and strain of 14% (w/v) protein whey protein isolate gels. Gels were formed from pH 7.0 solutions containing 50 mM NaCl and 0−100 mM DTT.

strain. The small difference in G_f values indicated that the force−deformation relationships for the two types of fine-stranded gels were quite similar. It is possible that the differences in G_f reflect the small changes seen in microstructure, whereas factors responsible for absolute values of fracture stress and strain are not apparent in gel matrix structure. Langton and Hermansson (1992) showed that fine-stranded β-lactoglobulin gels formed at pH >6 and pH <4 had some microstructural differences. Gels made at pH 3.5 have networks of short thin strands (∼4 nm in diameter) with several strands intersecting at one junction, while gel networks formed at pH 7.0 and 6.5 are composed of longer strands of equal or greater thickness than those formed at pH 3.5 (Langton and Hermansson, 1992). Additional investigations are needed to determine the links between microstructure and rheological properties of fine-stranded gels.

Effects of Disulfide Reduction at pH 7.0. Heat-induced gelation of whey proteins involves structural changes and protein−protein interactions that are altered by pH. Sulfhydryl−disulfide exchange and sulfhydryl oxidation are reactions that can produce inter- and intramolecular covalent cross-links. The pK_a of cysteine is 9.0−9.5 (Creighton, 1993) and one would expect minimal sulfhydryl oxidation and sulfhydryl−disulfide exchange at pH 3.0. We therefore made the simple hypothesis that the weak/brittle texture in gels formed at pH 3.0 was due to the prevention of intermolecular disulfide bonding. This hypothesis was tested by adding various amounts of a sulfhydryl reducing agent, dithiothreitol (DTT), to pH 7.0 WPI dispersions and determining the changes in fracture properties of heat-induced gels.

Addition of 2.5−5.0 mM DTT caused a slight increase in fracture stress and strain (Figure 2). This could be due to an increase in protein solubility caused by reduction of intermolecular disulfide bonds formed during processing or to a partial unfolding caused by reduction of intramolecular disulfide bonds. Matsudomi et al. (1991) observed that addition of 2 mM DTT caused an increase in bovine serum albumin and β-lactoglobulin gel hardness (force at a fixed level of compression). Gels containing 0−10 mM DTT were highly deformable, with strain values ranging from 1.8 to 1.5 (Figure 2). Gels that contained 25−100 mM DTT had brittle textures with strain values never exceeding 0.32. The transition from rubbery to brittle structures coincided with a decrease in fracture stress (Figure 2). The overall changes in fracture properties caused by addition of DTT were similar to changes caused by decreasing pH from 7.0 to 3.0 and 2.5 (Table 1 and Figure 1).

It is interesting to note that fracture strain values were 0.30−0.39 for brittle gels formed by decreasing pH or reducing sulfhydryls at pH 7.0. This suggests that the formation of disulfide bonds is key to the mechanism determining fracture strain. In contrast, fracture stress values for brittle gels formed at pH 3.0 and 2.5 were 25% of the values for pH 7.0 gels (Table 1), while brittle gels formed at pH 7.0 by adding DTT had stress values that represented 44% of the values for gels without DTT (Figure 2). This suggests that the interactions and matrix structures which determine strain at fracture for brittle gels are due to sulfhydryl/disulfide reactions which could be inhibited by lowering the pH or adding ≥25 mM DTT. In contrast, other factors are involved with fracture stress. There are several plausible reasons for the differences in fracture stress values. One is that the proteins have different structures or "states" when denatured under acid and neutral conditions (Darwin et al., 1991). This would change the intermolecular interactions and thereby alter the stress required to fracture the matrix. It is also possible that there is a critical minimum concentration of disulfide bonds required to alter fracture strain, whereas fracture stress is sensitive to lower concentrations of disulfide bonds.

Heat-induced gels were formed at all DTT concentrations and gels containing 25−100 mM DTT had similar rheological properties. This indicated that disulfide bonds were not essential to formation of heat-induced whey protein gels but contributed greatly to the textural properties. This is in agreement with previous research, which showed that bovine serum albumin and β-lactoglobulin can form gels from solutions containing 50 mM DTT or *N*-ethylmaleimide (Matsudomi et al., 1991). The similar rheological properties of gels made with 25−100 mM DTT indicated that some level of saturation was achieved. While the formation of disulfide bonds was not essential to gelation, it is clear that disulfide bonds had a major contribution to the rheological properties. The reducible disulfide bonds accounted for 82% of fracture strain and 50% of fracture stress (Figure 2).

The fracture modulus (G_f) is an indication of the overall firmness or stiffness of the gel. Addition of DTT caused a "statelike" shift in the fracture modulus (Figure 3). At 0−5 mM DTT the modulus does not

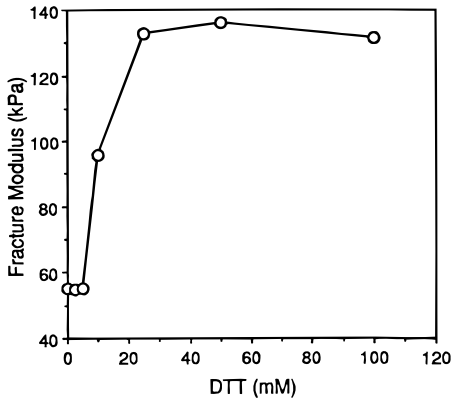

Figure 3. Effect of dithiothreitol on fracture modulus of 14% (w/v) protein whey protein isolate gels. Gels were formed from pH 7.0 solutions containing 50 mM NaCl and 0−100 mM DTT.

change; then there is a shift to a much higher, and again constant, modulus at 25−100 mM DTT.

Reducing the disulfide bonds in gels formed at pH 7.0 resulted in a gel with rheological properties similar to those of gels formed at pH 3.0 and 2.5. At low pH values intermolecular disulfide bonding would be suppressed and this would support our hypothesis that brittle textures are due to the loss of disulfide bond formation. If disulfide bond formation is the only critical reaction for the change from a brittle to a rubbery texture, then rubbery textures should be possible at pH ≤4.0 if intermolecular disulfide bonds are formed prior to decreasing pH and gelation.

There are several lines of evidence showing that intermolecular disulfide bond formation precedes the fluid to solid transformation in heat-induced whey protein gelation. Disulfide-linked polymers of β-lactoglobulin A are formed when pH 7.0 solutions are heated at temperatures ≥ 75 °C (Watanabe and Klostermeyer, 1976). Shimada and Cheftel (1989) showed that in a 9% WPI solution a rapid decrease in free sulfhydryl groups occurred within the first 3 min of heating. They proposed a two-step gelation mechanism with the first step involving the formation of initial junction zones by intermolecular disulfide bonds and a second step of strengthening by hydrophobic interactions (though not excluding hydrogen-bond formation and electrostatic interactions). Roefs and De Kruif (1994) have modeled heat-induced β-lactoglobulin aggregation based on the initiation reaction being that which exposes the sulfhydryl so it becomes reactive and forms a disulfide-linked dimer by sulfhydryl−disulfide exchange.

One way to increase disulfide bonding is to heat under conditions that slow the aggregation process and delay gelation. Whey protein isolate can be heat-denatured in low ionic strength, pH 7.0, dispersions and remain fluid. This "predenaturation" converts the proteins to states that forms gels at ambient temperature when salts are added (Barbut and Foegeding, 1993; Roff and Foegeding, 1996; Sato et al., 1995). Disulfide bond formation caused by predenaturation appears to be a key element of this gelation mechanism because decreasing intermolecular disulfide bonding by blocking sulfhydryl groups inhibits salt-induced gelation (Sato et al., 1995).

A two-step heating method was developed with the intent of first causing disulfide bond formation at neutral pH and then inducing gelation by heating after the solution pH was lowered to <3.5. Protein dispersions contained 19.3 ± 1.2 μmol of total sulfhydryl groups/g of soluble protein, of which only 3.6 ± 0.36 μmol/g of soluble protein were reactive in the unheated solutions (Table 2). Heating (80 °C for 30 min) reduced the total sulfhydryl content to 10.0 ± 1.06 μmol/g of soluble protein. Although some of the 48% decrease in total sulfhydryl groups may have been due to formation of oxidized states other than disulfides, under these mild heating conditions it was assumed that the major decrease was due to the formation of intra- and intermolecular disulfide bonds.

Table 2. Sulfhydryl Content of Whey Protein Isolate Solutions[a]

	sulfhydryls (μmol/g of protein)		
		total	
	reactive	unheated	heated (80 °C, 30 min)
mean	3.6	19.3	10.0
standard deviation	0.36	1.24	1.06
range	3.2−4.0	18.9−21.7	8.5−11.5

[a] Values for 6 replications.

Table 3. Fracture Properties of 10% (w/v) Protein Gels Formed by Single-Step (pH 7.0) or Two-Step (pH 3.1) Heating[a]

pH	true shear stress σ_s (kPa)	true shear strain γ_s	G_f (kPa)
7.00 ± 0.09	26.0 ± 2.7	1.70 ± 0.31	15.5 ± 1.5
3.14 ± 0.18	9.8 ± 0.9	1.98 ± 0.28	5.0 ± 0.8

[a] Mean values ± standard deviation for 6 replications.

The two-step heating mechanism produced gels at pH 3.14 ± 0.18 with fracture strain values exceeding gels formed at pH 7.0 (Table 3). The formation of a deformable, rubbery gel was not due to forming a matrix during the pH decrease because WPI solutions that were gelled by addition of glucono-δ-lactone and then heated at 90 °C for 30 min (i.e., no predenature heating) formed brittle gels (data not shown).

The G_f of gels formed by the two-step heating was much lower than that of gels formed by the single-step heating (Table 3). This indicated that the overall firmness of the pH 3.14 gel was less than that of the pH 7.0 gels. The deformable gels formed at pH 3.14 ± 0.18 had fracture stress values that represented 38% of the values for rubbery gels formed at neutral pH (Table 3). This decrease in stress, relative to rubbery gels formed at neutral pH, was intermediate between the effect of decreasing the pH to 2.5−3.0 (those gels had stress values that were 25% of the pH 7.0 gels) and forming brittle gels by adding DTT (those gels had stress values that were 44% of the values for elastic gels). The lower fracture stress for gels formed at pH 2.5 and 3.0 could be due to a combination of inhibiting sulfhydryl oxidation, altered thiol/disulfide exchange, and pH-associated effects on the denaturation and aggregation reactions. However, the formation of rubbery gels by two-step heating when viewed in combination with the effects of low pH and addition of DTT on gel deformability, strongly suggests that disulfide bond formation determines shear strain at fracture in fine-stranded whey protein gels.

CONCLUSIONS

Whey protein isolate gels formed at pH 2.5, 3.0, and 7.0 are brittle when disulfide bonding is inhibited.

Brittle gels are characterized by strain values ranging from 0.3 to 0.35. Whey protein isolate gel texture is changed from brittle to rubbery by increasing the amount of disulfide bonds.

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