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Mechanism of Urea-Induced Whey Protein Gelation

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Whey protein isolate (WPI) at an 11.0% concentration spontaneously formed a gel at 25 °C in 6 M urea. As the pH was increased from 7 to 10, gel formation, from a viscous sol to an elastic network, was accelerated. Addition of *N*-ethylmaleimide up to 6 mM inhibited gelation. The sulfhydryl (SH) content of WPI decreased during urea incubation, especially with increasing pH. Electrophoretic analyses revealed the progressive disappearance of α-lactalbumin, β-lactoglobulin, and serum albumin during the gelation process with concomitant formation of polymers of these proteins. The spontaneous formation of gels in 6 M urea resulted from protein–protein cross-linkages via oxidation of thiol groups and SH–disulfide interchange reactions.

INTRODUCTION

Protein gelation is an important functional property in food systems. Protein gels may be defined as a three-dimensional matrix or network in which polymer–polymer and polymer–solvent interactions occur in an ordered manner resulting in the immobilization of large amounts of water by a small proportion of protein (Ferry, 1948; Flory, 1974; Clark and Ross-Murphy, 1987). The ability of gels to act as a matrix for holding water, lipids, sugars, flavors, and other ingredients is useful in food applications and in new product development (Kinsella, 1984).

Heat-induced gelation of whey proteins has been extensively studied (Kinsella, 1976; Schmidt et al., 1978; Hillier et al., 1980; Schmidt, 1981; Mulvihill and Kinsella, 1988; Zirbel and Kinsella, 1988). These studies have demonstrated that the properties of whey protein gels are dictated by intrinsic factors such as the composition and concentration of the proteins and by extrinsic factors such as heating temperature, pH, ionic strength, and divalent cations. Although it has been proposed that gelation is a two-stage process, i.e., an initial denaturation or conformational change followed by the association of activated molecules to form an ordered gel matrix (Ferry, 1948), the mechanisms underlying the formation of the three-dimensional network characteristic of protein gels have not been elucidated. Limited research has been done to determine the forces involved in whey protein gel network formation. Hydrogen bonding, electrostic (ionic bonds), and hydrophobic interactions may be involved in the thermally induced protein network formation (Clark and Ross-Murphy, 1987; Kinsella and Whitehead, 1989). In the presence of calcium chloride, a whey protein concentrate and β-lactoglobulin (β-Lg) formed gels through protein–Ca^{2+}–protein cross-bridges (Schmidt et al., 1978; Mulvihill and Kinsella, 1988). Furthermore, intermolecular disulfide linkages may contribute to the gelation. The buried free thiol groups in the whey proteins can be exposed upon unfolding of the protein molecules during heat treatment, so that new intermolecular disulfide bonds can be formed in the protein gel network structure (Schmidt et al., 1978; Hillier et al., 1980; Zirbel and Kinsella, 1988).

The principal effect of heat treatment during protein gelation is to unfold the native structure of the protein molecules and to promote hydrophobic interactions. Some exposed functional groups resulting from the structural unraveling can interact with each other to form intermolecular linkages. A logical question is whether approaches other than heat treatment would also induce protein gelation by altering the conformation of the protein molecules. One possible alternative is urea as a protein denaturant. Kella and Kinsella (1988) have shown that urea is effective in changing the structure of β-Lg, thereby enhancing the availability and reactivity of SH originally occluded in the protein dimer. It has been shown that egg ovalbumin and bovine serun albumin (BSA) can form gels in concentrated urea (e.g., 8 M) in the temperature range 30–40 °C (Huggins et al., 1951; Frensdorf et al., 1953). Recently, it was observed that both WPI and β-Lg

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spontaneously formed gels in the presence of 8 M urea at 25 °C (Katsuta and Kinsella, 1990). However, the exact mechanisms for the gelation were not determined.

This study was conducted to investigate the role of urea in WPI gelation and to explore the mechanisms involved in the urea-induced gelation.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI) (>95% protein) prepared by ion-exchange chromatography was obtained from Le Sueur Isolates (Le Sueur, MN). The WPI was extensively dialyzed in distilled water containing 0.02% EDTA and NaN_3 to remove calcium while inhibiting potential microbial contamination. The WPI was further purified by dialysis in pure distilled water and lyophilized. The dried WPI was stored in a vacuum desiccator for a minimum of 7 days before the use. Certified ACS grade urea was purchased from Fisher Scientific (Springfield, NJ). The chemicals used for electrophoresis were obtained from Bio-Rad (Richmond, CA) and were of electrophoretic grade. Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-Ethylmaleimide (NEM) and other chemicals used in this study (all of reagent grade) were obtained from Sigma Chemical Co. (St. Louis, MO).

Gel Preparation. The lyophilized WPI was suspended in distilled water to produce a 27.1% (by weight) protein concentration. To the protein suspension, 1.46 volumes of the 10.1 M urea was added so that 11.0% (w/v) WPI in 6 M urea was prepared. The mixture was constantly stirred while 1 N NaOH or HCl was added to bring the solution to a specific pH. Immediately after the pH adjustment, the solution was poured into culture tubes [11.5 (i.d.) × 10 mm], deaerated by centrifugation at 1000*g* for 1 min, and sealed with parafilm. Gels were formed spontaneously when the WPI–urea mixture was held at 25 °C. Three replicated experiments were conducted for each of the treatments reported in this study.

Mechanical Spectrometry. The development and continuing formation of the gel network was monitored by using a dynamic mechanical spectrometer, Model RDS-7700 (Rheometrics, Inc., Piscataway, NJ). A 0.2-mL aliquot of WPI–urea mixture was placed on the gap (0.1-mm clearance) between a plate and a cone (0.10 rad) attached to the mechanical spectrometer. The exposed perimeter of the solution on the plate was covered with a layer of paraffin oil to prevent dehydration of the sample during testing. The sample material was subjected to a 2.0% sinusoidal strain at an oscillating frequency of 0.6309 rad/s (100 mHz). The shear storage modulus (G') and loss modulus (G'') were measured at a measurement time of 1 s, integrated over a 200-s period, and averaged. The measurement delay time was set at 10 s to allow the computer sufficient time to record the output results. The loss tangent (tan δ) was calculated by using the equation (Ferry, 1980) $\tan \delta = G''/G'$.

Effect of *N*-Ethylmaleimide (NEM). The effect of blocking thiol groups on WPI–urea gelation was studied by using NEM. NEM was dissolved in 50% ethanol to attain 0–4.0 M NEM. Twenty microliters of the NEM solutions was mixed with 6.50 mL of WPI (27.1% by weight) which was preadjusted to pH 7.0. At pH 6.5–7.5, maleimides bind rapidly and specifically to SH (Partis et al., 1983). The mixture was swirled in a mechanical shaker at 60 rpm for 30 min at 25 °C to permit the completion of the SH–NEM binding reaction. The WPI solution was subsequently mixed with urea to make gels as described above.

Determination of Free SH Concentration. The sulfhydryl content of WPI was continuously monitored during gel network formation. After specific incubation times, 1.0 mL of the pregel WPI solution was diluted in 24 mL of distilled water, yielding a 0.44% protein concentration. The diluted solution was immediately subjected to SH analysis according to the method of Ellman (1959) using DTNB. The SH assay was conducted at 25 °C, and the assay solution contained 0.088% protein in 6 M urea, 0.07 mM DTNB, and 20 mM potassium phosphate, pH 8.0. The absorbance developed at 412 nm was recorded against a blank by using a Cary 219 double-beam spectrophotometer (Varian Associates Inc., Palo Alto, CA). A molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} was used for the conversion of absorbance to SH concentration (Ellman, 1959).

Electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was conducted according to the method of Laemmli (1970) in a miniature slab gel electrophoresis apparatus (Hoefer Scientific Instrument, San Francisco, CA). A 4% stacking and 10% resolving polyacrylamide gel with a 1.5-mm thickness was used, and 15 μg of protein was loaded into each slot. The gel was fixed and stained in 50% methanol and 12% acetic acid containing 0.1% Coomassie brilliant blue R250 and destained in 18% ethanol and 8% acetic acid. A mixture of α-lactalbumin (molecular weight 14 200), β-lactoglobulin (18 300), bovine serum albumin (66 300), and immunoglobulin G (~160 000) was employed as protein standards for the identification and estimation of the molecular weight of the WPI components.

Densitometry. The relative concentration of the individual proteins in WPI samples was estimated by scanning the protein bands of the SDS–PAGE gels at 600 nm, using a video densitometer, Model 620 (Bio-Rad). The peak areas of the protein bands obtained from the densitrometric measurement were determined with computer software. The percentage of individual proteins was calculated from the peak area corresponding to each protein band.

RESULTS AND DISCUSSION

The WPI spontaneously formed gels in 6 M urea. The dynamic viscoelastic behavior of the proteins during the initial stage of network formation is shown in Figure 1. The shear storage modulus (G') increased linearly with the incubation time (Figure 1A). Since G' is a measure of the elastic energy stored and recovered per cycle of sinusoidal deformation (Ferry, 1980), the elasticity and rigidity of the gel was apparently enhanced during the gel structure formation. Van Kleef et al. (1978) and Ziegler and Rizvi (1989) have shown that the development in G' can be correlated to the number of intermolecular cross-links. The network formation was quite dependent upon the pH as indicated by gel onset time, i.e., point at which the elastic property was initially detected by the mechanical spectrometer. Gels were formed more readily and the rigidity developed more rapidly as the pH was increased from 7.0 to 10.0. However, in the absence of urea, no gel was formed in this pH range. The loss modulus (G'') (Figure 1B) represents the energy dissipated in viscous flow or lost as heat per sinusoidal cycle (Ferry, 1980). The rate of development in G'' was enhanced with time, indicating the formation of a viscous matrix. However, the increase in G'' was slower than that in G' as illustrated by the changes in the loss tangent (tan δ) (Figure 1C). The abrupt decrease in loss tangent is evidence of a transition from a viscous sol to an elastic gel network. The reduction in tan δ became slower during prolonged incubation, and this may be indicative of an attenuation in the viscous attributes.

Since urea destabilizes or perturbs the hydrophobic interactions and hydrogen bonds in proteins (Lapanje, 1978), the involvement of these bonds in the gelation was unlikely. Thus, gelation may be attributed to other forces. To test if formation of intermolecular disulfide bonds was involved in formation of the gel network, the sulfhydryl (SH) concentration of WPI at different stages of incubation in 6 M urea was analyzed. In 6 M urea, the SH concentration decreased with the incubation time (Figure 2), presumably because of the oxidation in the thiol groups, whereas in the absence of urea the absorbance remained unchanged, reflecting a constant thiol content. The maximum absorbance was reached within 80 min of SH assay, indicating complete binding between the exposed SH and DTNB. Longer incubation of WPI in urea decreased the rate and extent of reaction with DTNB. This

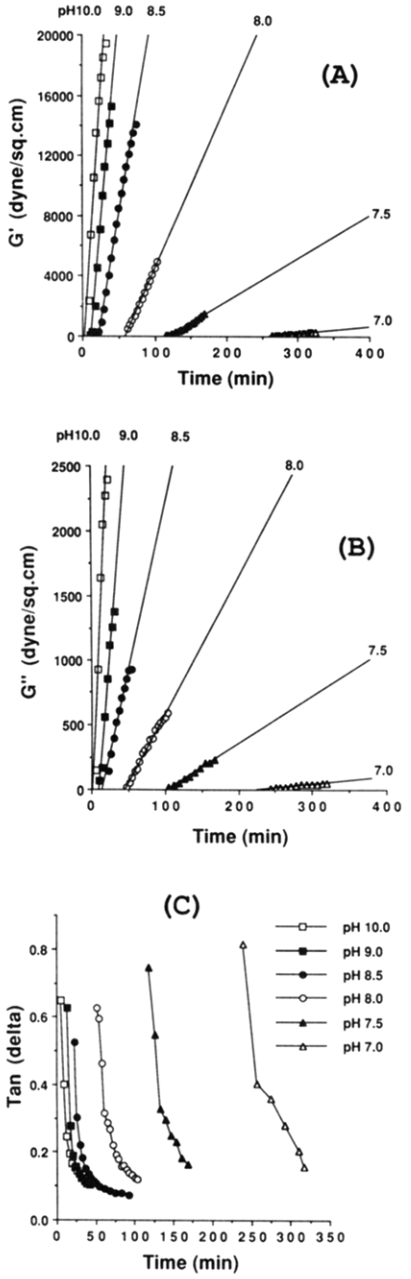

Figure 1. Changes in shear storage modulus (G') (A), loss modulus (G'') (B), and loss tangent (tan δ) (C) during gel formation of WPI (11.0%) in 6 M urea at various pH values and 25 °C.

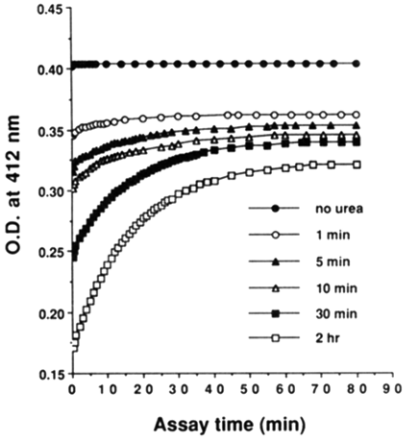

Figure 2. Assay for sulfhydryl content of WPI preincubated with 6 M urea for various times. Conditions: 11% whey protein, 6 M urea at pH 7.0, incubated at 25 °C for up to 2 h.

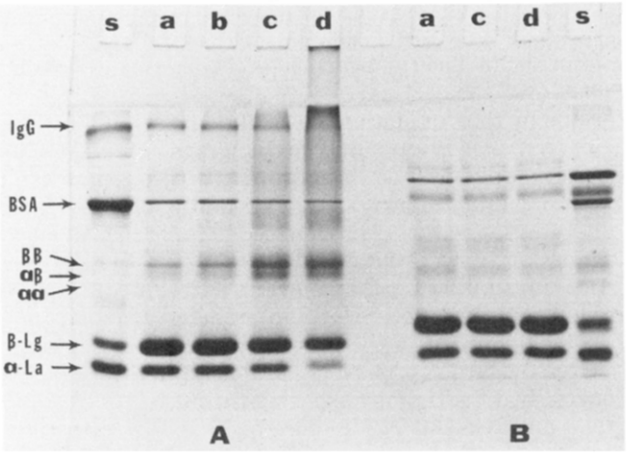

Figure 3. SDS–PAGE of WPI incubated in 6 M urea at pH 7.0 and 25 °C for different times. SDS–PAGE was performed without (A) and with (B) 5% β-mercaptoethanol in the sample buffer. Lanes represent protein standard (s), original WPI (a), and WPI incubated in 6 M urea for 1 (b), 30 (c), and 120 (d) min. IgG, immunoglobulin G; BSA, bovine serum albumin; $\beta\beta$, β-lactoglobulin dimer; $\alpha\beta$, α-lactalbumin and β-lactoglobulin complex; $\alpha\alpha$, α-lactalbumin dimer; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

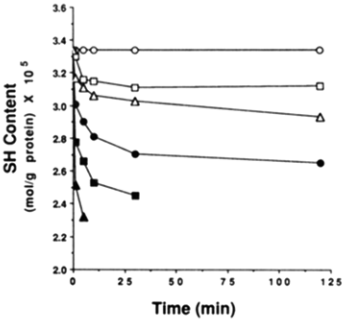

Figure 4. Effect of pH on the oxidation of thiol groups of WPI at different incubation times and 25 °C. The sulfhydryl (SH) content was determined at the end of 80 min of the SH assay. pH values were 7.0 (○, ●), 8.0 (□, ■), and 9.0 (△, ▲) in the absence of urea (open symbols) and in 6 M urea (filled symbols).

may have been caused by the rapid formation of polymers which structurally hindered the SH–DTNB binding.

SDS–PAGE revealed marked changes in the protein composition of WPI following incubation in 6 M urea (Figure 3A). The concentrations or β-lactoglobulin (β-Lg) and serum albumin (BSA) gradually decreased, while α-lactalbumin (α-La) almost disappeared within 2 h of incubation. Concomitantly, there was a progressive formation in the dimers of α-La ($\alpha\alpha$) and β-Lg ($\beta\beta$) and the α-La and β-Lg complex ($\alpha\beta$), all tentatively identified on the basis of their molecular weight and their relative migation rate in the polyacrylamide gel. Furthermore, more polymers were produced with time as manifested by the increased band intensity in the high molecular weight regions. The exact composition of the WPI and the compositional changes during incubation are given in Table I. There were reductions of 80% and 60% in the amount of α-La and β-Lg, respectively, as the WPI was incubated in the urea for 2 h. During the same period, the concentration of polymers increased 6-fold. When the WPI samples were treated with 5% β-mercaptoethanol after urea incubation, the polymerized protein complexes of WPI disappeared (Figure 3B). In fact, gels were readily resolubilized in 5% β-mercaptoethanol (data not shown). These results indicate that in 6 M urea, WPI formed gels via intermolecular disulfide (SS) linkages, ostensibly

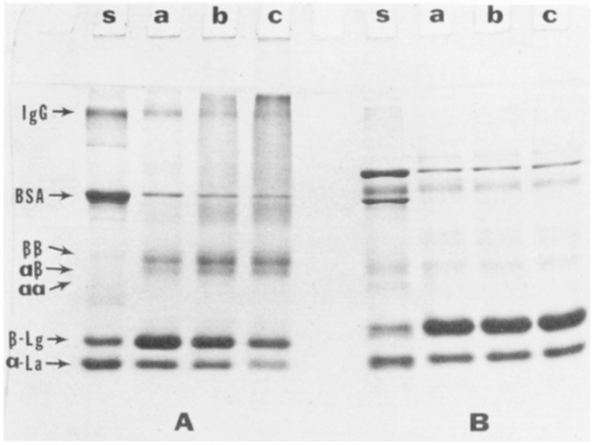

Figure 5. SDS–PAGE of WPI incubated for 5 min at 25 °C in 6 M urea at pH 7.0 (lane a), pH 8.0 (lane b), and pH 9.0 (c). SDS–PAGE was performed without (A) and with (B) 5% β-mercaptoethanol in the sample buffer. Lane s, protein standard; IgG, immunoglobulin G; BSA, bovine serum albumin; ββ, β-lactoglobulin dimer; αβ, α-lactalbumin and β-lactoglobulin complex; αα, α-lactalbumin dimer; β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

formed by thiol oxidation to disulfide bonds and by SH–SS interchange reactions. The changes in SDS–PAGE gel patterns appeared to be more marked for incubation beyond 1 min (Figure 3A), whereas the change in SH concentration was more pronounced in 1 min than at longer incubation times (Figure 2). This suggests that initially SH oxidation was a major event but that during the prolonged incubation SH–SS interchange reactions may become dominant in the protein–protein cross-linking.

Urea apparently caused unfolding of the proteins, especially β-Lg, thereby exposing the buried thiol group. Kella and Kinsella (1988) showed that the β-Lg dimer dissociated in 3 M urea resulting in an enhanced reactivity of SH. In 6 M urea, the tertiary and secondary structure of most of the whey proteins may be disrupted, and this promotes the interactions between the free thiol groups, such as those in β-Lg and BSA, and catalyzes SH–SS interchanges possibly according to the reactions

$$RSH + R'SH \rightarrow RSSR' \text{ (intermolecular)}$$

$$RSH + R''SSR''' \text{ (intramolecular)} \rightarrow R''SH + RSSR''' \text{ (intermolecular)}$$

Rate of SH oxidation increased with pH (Figure 4). In the absence of urea, some SH oxidation occurred at pH 8 and 9. However, this oxidation did not result in any gelation. The addition of 6 M urea facilitated the SH oxidation and induced gelation which was more pronounced at increasing pH. The thiol groups generally tend to be more reactive via deprotonation to form mercaptide ion species (S^-) as the pH rises. The increased reactivity of SH at an elevated pH was also observed by other researchers (Schmidt, 1981; Kella and Kinsella, 1988). The pH effect on the rate of SH oxidation corresponded to the rate of gelation (Figure 1), namely, both SH oxidation and gel network development accelerated with increasing pH. This was consistent with the idea that thiol groups participated in forming the WPI gels. Electrostatic interactions may also be involved in the gelation. However, because of the increased electronegativity, ionic bonding is expected to be trivial compared to the covalent disulfide bonds. In fact, whey protein gel strength was impaired by increasing pH from 7 to 10, presumably due to the repulsion between like charges (Hillier et al., 1980; Schmidt, 1981). Thus, it seems that the pH change from 7 to 10 had a major effect in promoting the formation of disulfide bonds.

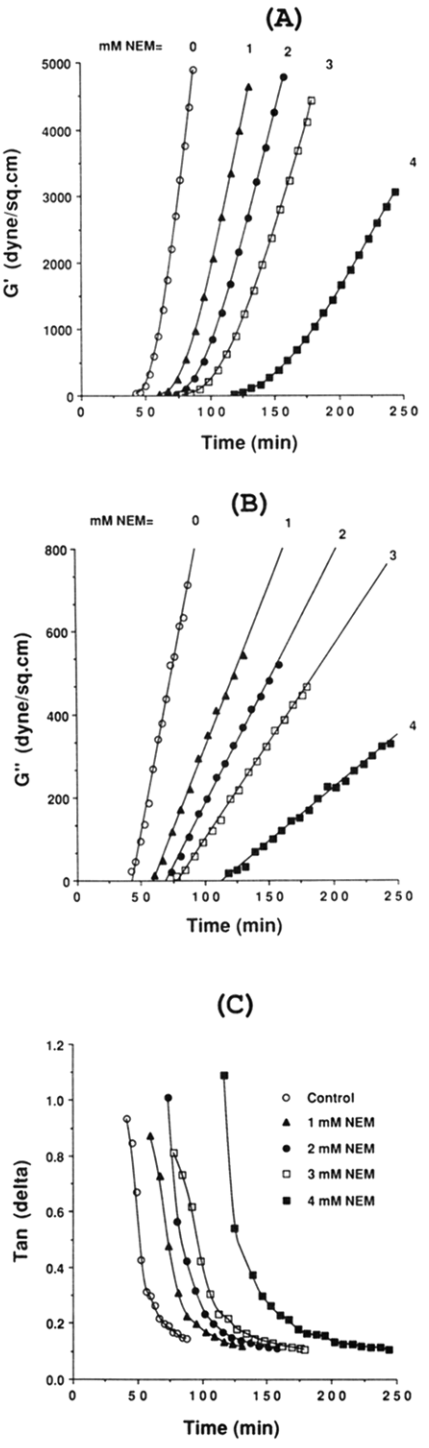

Figure 6. Effect of N-ethylmaleimide (NEM) on the changes in shear storage modulus (G') (A), loss modulus (G'') (B), and loss tangent (tan δ) (C) of WPI during incubation in 6 M urea at 25 °C (11.0% protein, pH 8.0).

SDS–PAGE showed similar protein compositions for WPI samples incubated in 6 M urea for 5 min at pH 7 (a), 8 (b) and 9 (c), although the relative content of the individual proteins varied among these samples (Figure 5). It is evident that the concentration of α-La and β-Lg was less, whereas more polymers were formed at higher pH values. This correlated with the results of SH analyses (Figure 4). Since the samples at the different pH values yielded identical electrophoretic patterns in 5% β-mercaptoethanol (Figure 5B), other non-disulfide covalent bonds, which potentially might form at high pH values, were unlikely to be involved in the gel network structure.

To further verify that sulfhydryl oxidation and SH–SS

Table I. Percentage Distribution of Protein Components in Whey Protein Isolate (WPI) Incubated in 6 M Urea at pH 7.0 and 25 °C for Various Times

WPI component[a]	incubation time, min				
	0	1	10	30	120
α-La	20.5 (0.1)[b]	18.8 (1.2)	14.9 (0.8)	10.7 (2.0)	4.6 (0.4)
β-Lg	52.0 (3.3)	47.5 (3.2)	39.6 (0.8)	32.9 (0.8)	20.4 (3.1)
αα	0.4 (0.3)	0.5 (0.7)	0.4 (0.1)	0.4 (0.6)	0.2 (0.3)
αβ	1.6 (0.5)	2.9 (0.6)	4.6 (1.7)	6.0 (1.3)	4.4 (0.8)
ββ	4.9 (0.4)	7.9 (1.9)	10.6 (2.3)	11.2 (0.8)	9.0 (1.5)
BSA	6.1 (0.5)	5.9 (0.7)	4.9 (0.4)	4.8 (0.8)	3.6 (0.2)
IgG	5.6 (1.0)	5.6 (1.1)	4.8 (0.8)	4.9 (0.2)	4.6 (0.7)
polymers and other proteins	8.9 (2.0)	11.0 (3.2)	20.6 (2.2)	29.1 (9.5)	53.2 (5.3)

[a] For symbol notations, see Figure 3 legend. [b] Percentage distribution. Mean values and standard errors (in parentheses) from three replicates.

interchange were primarily responsible for the gel matrix formation, the WPI was treated with a thiol blocking agent, *N*-ethylmaleimide (NEM). The dynamic viscoelastic properties, G', G'', and tan δ, of the gelling solution were significantly influenced by NEM (Figure 6). In general, the gel onset time was delayed, and the rate of the structure development (G' and G'') was decreased with an increasing amount of NEM; above 5 mM NEM, no gel was formed. Similar results were obtained in the case of thermally induced whey protein gelation (Hillier et al., 1980). Ostensibly, because of binding to NEM, fewer SH groups became available for the SH–SS interchange, and therefore, fewer cross-links were formed. These results provided further evidence that urea-induced WPI gels were indeed the product of sulfhydryl oxidation and SH–SS interchange reactions.

Thus, urea, by causing protein dissociation and unfolding and by exposing free thiol groups, facilitated extensive intermolecular SH–SS interchange with concomitant network formation and gelation. These results suggest the importance of covalent disulfide bonds in gelation of whey proteins. Zirbel and Kinsella (1988) reported that when the thiol groups were completely blocked, whey proteins did not form gels upon heat treatment, and Hillier et al. (1980) showed that oxygen was required for the heat-induced gelation of whey proteins, indicating the necessity of thiol oxidation. Therefore, disulfide linkages are of a primary importance in whey protein gelation even though electrostatic and hydrophobic interactions have also been implicated (Kinsella and Whitehead, 1989).

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