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Heat-Induced Whey Protein Gels: Protein−Protein Interactions and Functional Properties

PALATASA HAVEA,* PHILIP WATKINSON, AND BARBARA KUHN-SHERLOCK

Fonterra Research Centre (formerly New Zealand Dairy Research Institute), Private Bag 11029, Palmerston North, New Zealand

Heat-induced gelation (80 °C for 30 min or 85 °C for 60 min) of whey protein concentrate (WPC) solutions was studied using small deformation dynamic rheology, small and large deformation compression, and polyacrylamide gel electrophoresis (PAGE). The WPC solutions (15% w/w, pH 6.9) were prepared by dispersing WPC powder in water (control), 1% (w/w) sodium dodecyl sulfate (SDS) solution, and *N*-ethylmaleimide (NEM) solution at a protein/NEM molar ratio of 1:1 or in 10 mM dithiothreitol (DTT) solution. PAGE analyses showed that the heat treatment of control solutions contained both disulfide and non-covalent linkages between denatured protein molecules. Only disulfide linkages were formed in heated SDS−WPC solutions, whereas only non-covalent linkages were formed in DTT−WPC and NEM−WPC solutions during heating. In heated NEM−WPC solutions, the pre-existing disulfide linkages remained unaltered. Small deformation rheology measurements showed that the storage modulus (*G′*) values, compared with those of the control WPC gels (∼14000 Pa), were 3 times less for the SDS−WPC gels (∼4000 Pa), double for the NEM−WPC gels (∼24000 Pa), and even higher for the DTT−WPC gels (∼30000 Pa). Compression tests suggested that the rubberiness (fracture strain) of the WPC gels increased as the degree of disulfide linkages within the gels increased, whereas the stiffness (modulus) of the gels increased as the degree of non-covalent associations among the denatured protein molecules increased.

KEYWORDS: Disulfide bonds; dithiothreitol; gelation; non-covalent bonding; sodium dodecyl sulfate; whey protein; functional properties

INTRODUCTION

The industrial manufacture and utilization of whey protein products have progressed significantly in the past two decades, because of extensive research and interest in understanding whey protein products as functional ingredients. The most common products are the whey protein concentrates (WPCs), which are used in a wide range of food applications not only because of their nutritional value but also because of their functional properties, such as their ability to form heat-induced gels. They are used as functional ingredients in many foods, such as processed meat, bakery products, and dairy products. When WPCs are used for these applications, they are required to deliver consistent functional properties. However, this is not easily achieved because the functional properties of WPCs can be easily affected by many factors that can cause variations in the composition and quality of the final products.

Studies on the use of WPCs as functional ingredients (see, e.g., refs *1*−*3*) have highlighted the formation of two main types of heat-induced gels by globular proteins including whey proteins: particulate gels and fine-stranded gels. The formation of one or the other type is determined by a complex combination of factors including protein composition, mineral environment, pH, and others. The interplay of these factors can result in differences between the rate of protein denaturation and the rate of aggregation of denatured proteins (*4*) and the relative proportions of protein−protein attractive and repulsive interactions (*5*, *6*).

The relationships between these variables and the functional properties of heat-induced whey protein gels are not fully understood. To improve the use of WPC products as functional ingredients, a better understanding of how these factors relate to the functional and textural properties of heat-induced WPC gels is required.

When WPC solutions (e.g., >8% w/w protein, pH 6.9) are heated at a sufficiently high temperature (e.g., 75 °C), the protein molecules unfold and interact to form intermediate aggregates prior to the formation of a gel network (*3*, *7*). The formation of intermediate aggregates involves two broad types of bonding: covalent bonding and non-covalent bonding. The former consists of inter- and intramolecular disulfide bonds (*8*) formed via sulfhydryl−disulfide interchange or sulfhydryl oxidation reactions (*9*). The latter are relatively weak interactions, such as hydrophobic, hydrogen bonding, ionic, and other weak interactions that also contribute to the formation of aggregates and a

* Address correspondence to this author at Ingredient Functionality, Fonterra Research Centre, Private Bag 11029, Palmerston North, New Zealand (telephone 64 6 350 4649; fax 64 6 350 4663; e-mail palatasa.havea@fonterra.com).

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gel network (*7, 10*). Although these studies showed that the heat-induced gelation of whey proteins involves these two types of bonds, the relative contribution of each type of bond to the overall functional properties of heat-induced WPC gels remains unknown.

In a recent publication (*11*) we proposed that disulfide bonds in heat-induced whey protein gels were responsible for the rubbery (high fracture strain) characteristics of the gel, whereas the non-covalent bonding was responsible for the brittleness (low fracture strain) and hardness (high storage modulus) characteristics of the gels. The results suggested that the role of non-covalent protein interactions in the formations of gel network is probably more important than previously understood. This paper is a continuation of the same work, wherein more data are presented and discussed, with specific emphasis on the impact of the degree of disulfide linkages on the functional properties of heat-induced WPC gels.

MATERIALS AND METHODS

Materials. A commercial spray-dried WPC powder, derived from mineral acid whey, was obtained from NZMP, Fonterra Co-operative Group (formerly Anchor Products), Edgecumbe, New Zealand. This WPC was typical of a standard commercial product.

The chemicals used for the preparation of the electrophoresis buffers (obtained from Bio-Rad Laboratories, Richmond, CA) were of analytical grade.

WPC Composition. The total protein content of the WPC powder was determined using the Kjeldahl method (*12*), with a nitrogen conversion factor of 6.38. The fat content was determined using the Soxhlet extraction method, as described by Russell et al. (*13*). The moisture content was determined by oven-drying preweighed duplicate samples at 102 °C for 5 h, cooling in a desiccator for 2 h, and reweighing the samples. The mineral analyses were carried out at the New Zealand Pastoral Agricultural Research Laboratory, Palmerston North, by inductively coupled argon-plasma emission spectrometry using the method described by Lee et al. (*14*).

Preparation of WPC Solutions. Appropriate quantities of WPC powder were dissolved in water (purified using a Milli-Q system, Millipore Corp., Bedford, MA) so that the final solutions contained WPC concentrations of 15.0% (w/w), that is, control samples. Other solutions were prepared by dissolving the same quantity of WPC powder in 1% sodium dodecyl sulfate (SDS) solution (SDS−WPC solutions), *N*-ethylmaleimide (NEM) solution at a 1:1 molar ratio of protein to NEM (NEM−WPC solutions; this level of NEM was slightly higher than that required to block all of the sulfhydryl groups in the system), or 10 mM DL-dithiothreitol (DTT−WPC solutions). The protein molarity of the NEM−WPC solutions was estimated by assuming that the total protein in the system consisted of β-lactoglobulin only. The protein concentration in the 15% WPC solutions was approximately 12.5% (w/w). The solutions were stirred for 2 h at room temperature using a magnetic stirrer, and the pH was adjusted to 6.9 using 0.1 M NaOH or 0.1 M HCl. The WPC solutions were then used for dynamic rheological measurements or for heat treatment to obtain WPC gels for further functional analyses.

The purpose of heating these whey protein solutions in the presence of SDS, NEM, or DTT was to control the protein interactions in each system. It was expected that heating the control whey protein solutions would result in the formation of both disulfide and non-covalent linkages. In the heated SDS−WPC systems denatured protein molecules were expected to interact via disulfide only. In the presence of DTT all of the disulfide linkages within the system, including intradisulfide linkages or any new disulfide linkages formed during heating, were expected to be reduced. Any interactions would be restricted to non-covalent linkages only. In the NEM−WPC systems formation of new heat-induced disulfide linkages was prevented. The disulfide linkages present before heating were expected to remain unaltered.

Polyacrylamide Gel Electrophoresis (PAGE). Another set of solutions was prepared by diluting the above 15% WPC solutions with water so that a set of 5% WPC solutions was obtained, and 5 mL aliquots of each in 10 mL glass test tubes were heated by placing the test tubes in a thermostatically controlled (85 ± 0.5 °C) water bath for 60 min. Samples (0.25 mL) of the solutions were then removed, diluted with appropriate buffer, and analyzed using either alkaline- or SDS-PAGE as described by Havea et al. (*7*). After preparation of the appropriate gel, 10 μL samples of 0.01% (w/w) protein solution were injected into the sample wells, and then the proteins were separated using electrophoresis. After the gels had been stained with Amido black dye and destained, they were photographed, as described by Havea et al. (*7*).

Dynamic Rheological Measurement. A set of WPC solutions (15% w/w, pH 6.9) was prepared and then heated in the 25 mm cup-and-bob configuration of a Paar Physica US200 rheometer. The gap between the cup and the bob was 1 mm. A 19 mL sample of each WPC solution was placed in the cup. The top of the solution was about 1 mm above the top of the bob. A layer of paraffin oil was placed on top of the solution to avoid evaporation during heating. The changes in the viscoelastic properties of the WPC solutions were monitored during heating using the rheometer in oscillatory mode at a frequency of 1 Hz and a shear stress of 1%. The solutions were heated from 20 to 80 °C at a rate of 1 °C min^{-1}, held at 80 °C for 30 min, cooled from 80 to 20 at 1 °C min^{-1}, and finally held at 20 °C for 20 min. The storage modulus, G', of the heated WPC solutions was measured every minute during the heating cycle. At the end of the 20 min of holding at 20 °C, a frequency sweep (from 10 to 0.1 Hz) followed by a strain sweep (from 1 to 100%) was conducted on the WPC gels. The frequency sweep and strain sweep data showed that the conditions of measurement were within the range of viscoelastic behavior of these whey protein gels (results not shown). Each WPC solution was tested in duplicate, at least.

Preparation of Heat-Induced WPC Gels. The WPC solutions were placed in 400 cm long, 30 mm diameter, medium-walled polycellulosic plastic tubes, and the ends were closed off using rubber bands to make stiff "sausages". These sausages were then placed in a thermostatically controlled water bath (85 ± 0.5 °C) for 60 min. It took approximately 44 s to heat the middle of each tube to 84.8 °C. After heating, the tubes were removed from the water bath and immediately placed in an ice bath (~0 °C) for 30 min before being stored at 4 °C overnight. The gels were then analyzed using a range of techniques.

The gels made from the control WPC solutions are referred to as "control WPC gels" in the text. The gels made from WPC solutions containing SDS, NEM, or DTT are referred to as "SDS−WPC", "NEM−WPC", or "DTT−WPC" gels, respectively.

Compression Test. Each of the WPC gel types was cut to give four to six cylindrical slices, 25 mm in length, using a wire cutter and a template. Each slice was wrapped with plastic film to prevent moisture loss, placed in a sealed container, and then left in a thermostatically controlled (20 °C) room for 2 h to equilibrate before testing. The samples were placed between the upper 85 mm diameter Teflon plate and the lower 95 mm × 105 mm Teflon plate of a TA.HD texture analyzer (Stable Micro Systems, Godalming, Surrey, U.K.). The surfaces of the plates were lubricated with paraffin oil to minimize friction. The samples were then compressed to 80% of their original height at a rate of 0.83 mm s^{-1} using a load cell of 500 N. The testing conditions and the calculation of modulus of deformability, fracture stress, and fracture strain were based on the technical specification for protocols for cheese testing and analysis developed by the International Standards Organization (ISO) and the International Dairy Federation (IDF) (*15*). Modulus of deformability (hereafter abbreviated to modulus) is measured by the maximum slope at the initial strain part of the stress versus strain curve. Fracture stress is measured by the local maximum in stress as a function of strain. Fracture strain is the strain corresponding to the fracture stress (*15*).

The results were used to describe the textural nature of each gel according to the method described by Hamann and MacDonald (*16*) and Truong and Daubert (*17*), including the use of fracture strain and fracture stress to broadly characterize texture.

Statistical Analysis. One-way analysis of variance (ANOVA) followed by Tukey's simultaneous tests (Minitab release 15.1) was performed to test for the effect of WPC gel on fracture strain, fracture stress, and modulus. A p value of <0.05 was declared to be significant.

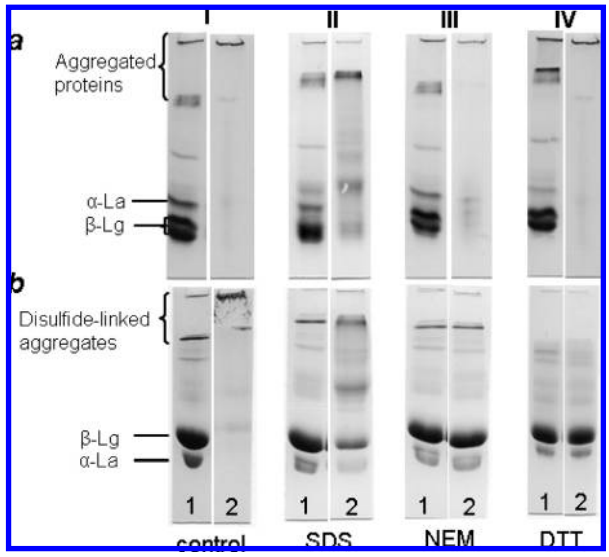

Figure 1. (**a**) Alkaline-PAGE and (**b**) SDS-PAGE patterns of (I) control, (II) SDS−WPC, (III) NEM−WPC, and (IV) DTT−WPC solutions (5% w/w, pH 6.9), heated at 85 °C for 0 min (lane 1) or 60 min (lane 2). β-Lg, β-lactoglobulin; α-La, α-lactalbumin.

RESULTS

WPC Composition. Analysis showed that the WPC powder had 85% (w/w) total protein, 5.5% fat, and 4.2% moisture. Mineral analyses showed that the powder contained 1.7, 13.8, 8.0, and 2.5 g kg^{-1} of calcium, potassium, sodium, and phosphorus, respectively. According to the manufacturer's specification, the WPC had a lactose content of 2.3% relative to the total solids.

PAGE Analyses of Heated WPC Solutions. Analysis of heated WPC solutions using PAGE reveals information about the nature of the protein−protein interactions formed during heating. For the PAGE analyses, as it was necessary to obtain samples of the heated WPC solutions before they gelled (*7*), only 5% w/w (pH 6.9) heated (85 °C) WPC solutions were used. When the WPC solutions were heated, the DTT−WPC and NEM−WPC solutions formed thick pastes or weak gels even after heating for 5 min at 85 °C, whereas the control and SDS−WPC solutions remained liquid. It was still possible to sample the DTT−WPC and NEM−WPC solutions for PAGE analyses even though the heated samples were more viscous. **Figure 1** shows the PAGE patterns of the unheated and heated (60 min) WPC samples for each WPC solution. In this figure, each pair of lanes shows the PAGE patterns of the unheated sample (lane 1) and those of the same sample heated at 85 °C for 60 min (lane 2).

PAGE Analyses of Control WPC Solutions. When the unheated and heated (85 °C) control WPC solutions (i.e., WPC dispersed in water, 5% w/w, pH 6.9) (**Figure 1**-Ia) are compared, it is clear that native-like monomeric proteins that were present in the unheated sample (lane 1) disappeared completely from the heated sample (lane 2) and that some material was caught on top of the stacking gel. When the same samples were run on an SDS-PAGE gel (**Figure 1**-Ib), similar trends were observed, except that some of the material caught on top of the stacking gel of the alkaline-PAGE gel dissociated to give SDS−monomeric proteins in the SDS-PAGE gel (lane 2). Considerable amounts of aggregated material appeared to partially migrate into the stacking gel, which indicated that they were small low molecular weight disulfide-linked aggregates (*7*). There were faint bands corresponding to SDS−monomeric β-lactoglobulin and α-lactalbumin in lane 2 (**Figure 1**-Ib) that were not present in the alkaline-PAGE gel (**Figure 1**-Ia), indicating that some of the aggregated proteins dissociated under SDS-PAGE conditions. These results showed that heat treatment of the control WPC solutions resulted in the formation of both disulfide bonding and non-covalent bonding between the denatured proteins, confirming previously reported results (*7*, *11*).

PAGE Analyses of SDS−WPC Solutions. The unheated SDS−WPC solution (**Figure 1**-IIa, lane 1) showed an alkaline-PAGE pattern containing smears of monomeric protein bands with intensities similar to those of the unheated control (**Figure 1**-Ia, lane 1). Significant amounts of monomeric proteins remained as smears within the lane after heating for 60 min (**Figure 1**-IIa, lane 2), indicating that either native or non-native monomeric proteins (*18*) were still present in the SDS−WPC solutions. Using densitometry, it was shown that the intensities of the monomeric protein bands in the alkaline-PAGE gel (**Figure 1**-IIa, lane 2) were similar to those of the corresponding bands in the SDS-PAGE gel (**Figure 1**-IIb, lane 2), indicating that there was no aggregated material that was resolved by SDS. The results suggested that the protein aggregates present in the heated SDS−WPC solutions were formed by disulfide bonding only. The formation of disulfide bonding between denatured proteins is a relatively slow reaction because of the limited number of reactive sites per protein molecule (*7*).

PAGE Analyses of NEM−WPC Solutions. Heat treatment of the NEM−WPC solutions (protein/NEM molar ratio of 1:1) resulted in complete loss of native-like monomeric proteins after heating for 60 min (**Figure 1**-IIIa, lane 2). Running the same heated NEM−WPC sample on an SDS-PAGE gel (**Figure 1**-IIIb, lane 2) resolved most of the aggregated proteins to give monomeric bands that were the same as those in the control unheated sample (**Figure 1**-IIIb; compare lanes 1 and 2). Disulfide-linked protein aggregates remained on top of the stacking gel (lane 1), even after heating in the presence of NEM. Fresh commercial WPC products usually contain ∼8−10% denatured and aggregated proteins, some of which could be disulfide linked (*7*). The fact that the heated NEM−WPC sample showed the same SDS-PAGE pattern as that of the unheated sample suggests that all of the protein aggregates formed during heating (**Figure 1**-IIIa, lane 2) were formed by non-covalent associations, with no further disulfide-linked aggregates being formed. When the same sample was run on an SDS-PAGE gel, these aggregates were resolved, leaving the heated solution with the same disulfide-linked aggregates as were present in the original unheated WPC solution. This point is further illustrated in **Figure 2**.

PAGE Analyses of DTT−WPC Solutions. When the heated DTT−WPC sample was run on an alkaline-PAGE gel, the native-like monomeric proteins were completely lost (**Figure 1**-IVa, lane 2) after heating for 60 min. Some protein aggregates were caught on top of the stacking gel. When the same heated DTT−WPC sample was run on an SDS-PAGE gel (**Figure 1**-IVb, lane 2), all of the aggregated material resolved to give monomeric protein bands and the SDS-PAGE pattern was virtually the same as that of the unheated sample (**Figure 1**-IVb, lane 1). This showed that all of the aggregated proteins were linked by non-covalent associations, with no disulfide-linked aggregates being formed. Unlike the WPC solution heated in the presence of NEM, all of the disulfide-linked aggregates present in the initial unheated DTT−WPC solution (**Figure 1**-IVb, lane 2) had been reduced by DTT.

Difference between the Effects of NEM and DTT on Protein Interactions. To further illustrate the difference between the

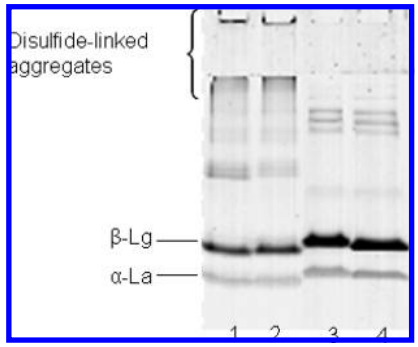

Figure 2. SDS-PAGE patterns of a WPC solutions (1%, w/w) prepared from a denatured WPC powder (the powder contained 81% protein; ~65% of the protein was denatured). The samples were heated at 85 °C for 4 min, in the presence of SDS (lane 1), NEM (lane 2), DTT (lane 3), or 2-mercaptoethanol (lane 4). See text for more details on sample preparations.

effects of NEM and DTT on protein interactions during heating, a denatured WPC powder sample, which was reported to contain 65% denatured proteins, was obtained. A reconstituted solution (1% w/w) of this powder was prepared, heated at 85 °C for 4 min in the presence of 1% SDS, NEM (protein/NEM molar ratio of 1:1), 10 mM DTT, or 2-mercaptoethanol (1 mL/L), and then analyzed using SDS-PAGE. On comparison of lane 1 (heated in the presence of SDS) and lane 2 (heated in the presence of NEM, **Figure 2**), it is clear that no further disulfide-linked aggregates were formed when the WPC solution was heated in the presence of NEM. It is also clear that the sample heated in the presence of DTT or 2-mercaptoethanol, lanes 3 and 4, respectively (**Figure 2**), contained monomeric proteins only. All of the disulfide-linked aggregates had been reduced to monomeric proteins.

Overall, the results showed that, during heating, all of the proteins were denatured and aggregated. In the control WPC solution (**Figure 1**-I), the aggregated proteins were formed both by disulfide bonds (i.e., those that remained under SDS-PAGE conditions) and by non-covalent associations (i.e., those that resolved under SDS-PAGE conditions). In the SDS−WPC solutions (**Figure 1**-II), the aggregates were formed only by disulfide bonds, with no contribution from non-covalent associations. In the NEM−WPC solutions (**Figure 1**-III), the aggregates were formed only by non-covalent associations (**Figure 2**). The disulfide aggregates present in the heated solutions were there prior to heat treatment. In the DTT−WPC solutions (**Figure 1**-IV), all of the aggregates formed during heating were linked by non-covalent associations only and all of the disulfide-linked aggregates present in the original solutions were reduced to give monomeric proteins. Under SDS-PAGE conditions, the aggregated material was completely resolved to give monomeric proteins.

Development of WPC Gels during Heating. The formation of WPC gels during heating was observed by recording the continuous changes in the viscoelastic properties of the WPC solutions at small deformation, thus avoiding the problems associated with gel fracture during measurement. The storage modulus, G', which is a measure of the gel rigidity, was recorded every minute during the heating cycle (**Figure 3**). The initial liquid systems typically have a G' of zero (*19, 20*). At the gel point, G' begins to increase, indicating the formation of a gel network. Therefore, the gel point is defined as the time or temperature at which a measurable value of G' is achieved. The

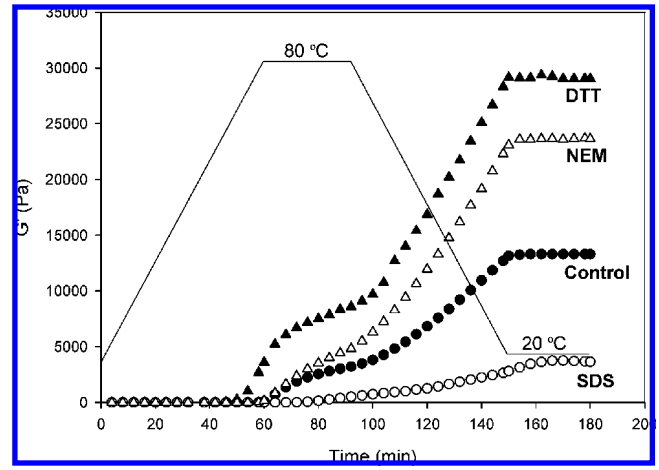

Figure 3. Changes in the rheological properties of the WPC solutions during heating: ●, control WPC solution; ○, SDS-WPC solution; ▲, NEM−WPC solution; △, DTT−WPC solution. The WPC solutions (15% w/w, pH 6.9) were heated from 20 to 80 at 1 °C min^{-1}, held at 80 °C for 30 min, cooled from 80 to 20 at 1 °C min^{-1}, and held at 20 °C for 20 min. G' values were measured every minute (the plotted data are representative only, i.e., every fifth point). Measuring conditions: oscillatory mode, frequency of 1 Hz, strain of 1%, cup-and-bob configuration.

Table 1. Rheological Properties of WPC Solutions during Heating ($n = 3$)

property	control	SDS	NEM	DTT
gel point (temperature, °C)	78 ± 0.5[a]	80 ± 0.5[b]	70 ± 0.5[a]	63 ± 0.5[b]
gel point (time, min)	60 ± 0.5	84 ± 0.5	58 ± 0.5	50 ± 0.5
G'_{80}[c] (Pa)	3200 ± 20	80 ± 7	5000 ± 50	9000 ± 100
G'_f[d] (Pa)	13500 ± 300	4000 ± 100	24000 ± 200	29000 ± 100

[a] These WPC solutions started to form gels before 80 °C was reached. [b] The SDS−WPC solutions started to form gels only after holding at 80 °C for a period. [c] G'_{80} are the values of G' at the end of the 30 min of holding at 80 °C. [d] G'_f are the values of G' at the end of the heating cycle (i.e., after the final holding at 20 °C).

changes in G' during the heating−cooling cycles are shown in **Figure 3**, with specific points of interest summarized in **Table 1**.

When the control WPC solutions were heated from 20 to 80 °C (**Figure 3**), G' started to increase (i.e., the gel point, **Table 1**) when the temperature reached 78 °C. During the 30 min of holding at 80 °C, G' increased at a slow rate, reaching ~3200 Pa. On cooling from 80 to 20 °C, G' continued to increase, reaching a value of ~13500 Pa when the temperature reached 20 °C. During the second holding phase (20 °C), there were no measurable changes in G'.

When the SDS−WPC solution was heated from 20 to 80 °C, there were no measurable changes in G' (**Figure 3**). Unlike the control solution, when the solution was held at 80 °C, G' showed no measurable changes until after ~24 min of holding (i.e., the gel point, **Table 1**), when it started to increase, reaching ~80 Pa at the end of the 30 min of holding. G' continued to increase during the cooling phase, reaching ~4000 Pa, and then remained constant during the final holding phase.

When the NEM−WPC solutions were heated from 20 to 80 °C, G' started to increase when the temperature reached 70 °C (**Figure 3**; **Table 1**). It continued to increase, reaching ~5000 Pa at the end of the 30 min of holding at 80 °C. G' then increased steadily at a faster rate, reaching ~24000 Pa at the end of the cooling phase, and remained constant during the second holding phase (20 °C).

When the DTT−WPC solutions (**Figure 3**) were heated, G' started to increase when the temperature reached 63 °C (i.e.,

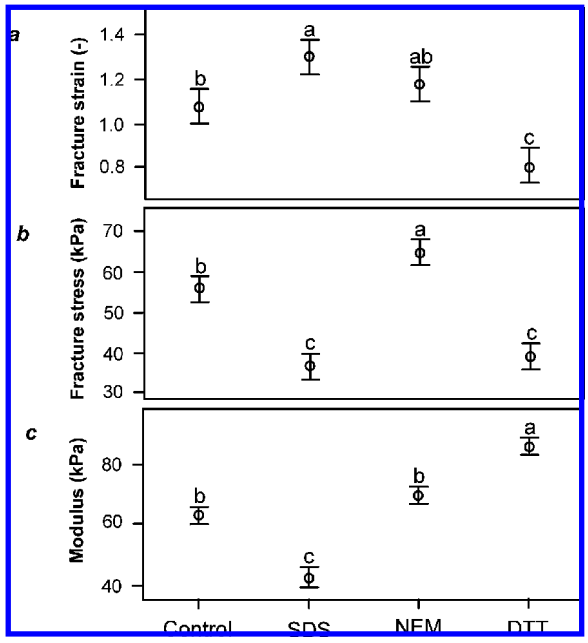

Figure 4. Effect of various treatments [heat treatment in water (i.e., control) or in the presence of SDS, NEM, or DTT] on the texture properties of the heat-induced WPC gels: (**a**) fracture strain; (**b**) fracture stress (kPa); (**c**) modulus (kPa).The WPC solutions (15% w/w, pH 6.9) were heated at 85 °C for 60 min. Each point is a mean of five or six measurements of gels from two different experiments. The error bars indicate 95% confidence intervals of the mean. The labels a−c denote the ranking of the gels for each parameter. The gels were not significantly different when the labels are the same.

the gel point, **Table 1**), reaching ∼3500 Pa at the end of the heating phase. It continued to increase, reaching ∼9000 Pa (**Table 1**) at the end of the holding phase at 80 °C. The G' value increased to ∼29000 Pa during the cooling phase and then remained constant during the second holding phase.

Overall, the four WPC systems exhibited different gelation behaviors. The control gels had G' values that were about 4 times (∼13500 Pa) higher than those of the SDS−WPC gels (∼4000 Pa) after the heating cycle. In contrast, the NEM−WPC and DTT−WPC gels had G' values (∼24000 and ∼29000 Pa, respectively) that were about double or more those of the control WPC gels and many times higher than those of the SDS−WPC gels. The storage modulus (G') values of the heat-induced WPC gels followed the order DTT−WPC > NEM−WPC > control ≫ SDS−WPC gels.

The gel point of the control WPC solutions occurred just before 80 °C was reached, whereas that of the WPC solutions containing NEM and DTT occurred much earlier (70 and 63 °C, respectively) and that of the WPC solutions containing SDS occurred at a much later stage (after 24 min at 80 °C).

In all of the heated WPC solutions, the increases in the G' values occurred at faster rates (steeper slopes of the graphs) during the cooling phase and there were no measurable changes during the holding at 20 °C.

Compression Tests. Heat-induced WPC gels (15% w/w, pH 6.9, 85 °C for 60 min) were also analyzed using compression tests (*11*, *15*). When a WPC gel is compressed using this technique, a constant displacement rate is applied, and the sample eventually fractures, shown by a local maximum in the calculated stress. The results obtained from these analyses (**Figure 4**) showed that the fracture strain, fracture stress, and modulus were significantly different ($p = 0.05$) between the WPC gels. The fracture strain values for the WPC gels followed the order DTT−WPC < control < NEM−WPC < SDS−WPC gels (**Figure 4a**). All of the pairwise comparisons of the fracture strain values of the gels were significant (at $p = 0.05$) except that the fracture strain of the NEM−WPC gel was not different from that of either the control gel or the SDS−WPC gel. Fracture strain is a measure of the relative "longness" or "rubberiness" of the gels. The higher the fracture strain value (arbitrary), the more resistant to crumbling or the more rubbery is the gel (*21*). It is clear that the SDS−WPC gels were significantly ($p = 0.05$) "more long" than the control WPC gels, whereas the NEM−WPC gels were somewhat intermediate between these gels. The DTT−WPC gels were significantly "less long" than any of the other three gels (**Figure 4a**).

The fracture stress values followed the order SDS−WPC = DTT−WPC < control < NEM−WPC gels. Fracture stress is a measure of the firmness of the gels. It is an indication of the force during compression that is required to crack the gel upon compression (*21*). It is interesting to note that the SDS−WPC gels (consisting of disulfide linkages only) and the DTT−WPC gels (consisting of non-covalent interactions only) had similarly low fracture stress values compared with those of the NEM−WPC gels or the control gels.

The modulus followed the order SDS−WPC < control < NEM−WPC < DTT−WPC gels. Modulus is a measure of the stiffness or "the resistance to dent the surface" of the gels (*21*). The modulus from compression had a strain rate that increased with strain; it was compared with the storage modulus (G') measured using oscillatory shear. It is interesting to note that the compression modulus (**Figure 4c**) correlated well ($R^2 = 0.94$) with the storage modulus (G') (**Figure 3**; **Table 1**), even though the test mode and the strain rates were not the same.

DISCUSSION

The electrophoresis analyses (**Figures 1** and **2**) served largely to demonstrate that the heat-induced protein−protein interactions in the heated WPC systems were different. The existence of both disulfide and non-covalent interactions in heated control WPC systems (**Figure 1**-I) has previously been reported in a number of publications (*7*, *11*, *22*). Restriction of the protein interactions to disulfide linkages only in SDS−WPC gels (**Figure 1**-II) and to non-covalent associations only in DTT−WPC gels (**Figure 1**-IV) has also been reported previously (*11*). The difference between the NEM−WPC gels (**Figure 1**-III) and the DTT−WPC gels (**Figure 1**-IV) was that the disulfide bonds in the NEM−WPC gels that were present in the original WPC solutions prior to heating were not affected. These included small amounts of intermolecular (disulfide-linked aggregates) and mostly intramolecular disulfide linkages (**Figure 2**). In the DTT−WPC gels, all disulfide bonds, both intermolecular and intramolecular, would have been reduced, leaving long peptide chains with many reactive sites that could freely interact solely via non-covalent associations.

Ranking the WPC gels in terms of the degree of disulfide linkages should follow the order SDS−WPC > control > NEM−WPC > DTT−WPC gels. Thus, the rubberiness (fracture strain) of the WPC gels increased as the degree of disulfide linkages within the gels increased (**Figure 4a**). It is difficult to determine if all of the reactive sites within a molecule had effectively formed a disulfide bond either intramolecularly or with other denatured molecules. We only know that, in the presence of SDS, non-covalent interactions were absent and the formation of the gel was by disulfide linkages only (**Figure 1**). Because of the presence of non-covalent interactions in the control WPC gels, it is reasonable to assume that these gels

contained fewer disulfide linkages than the SDS−WPC gels. The formation of non-covalent interactions restricted the mobility of the denatured molecules, and hence the chances of effectively forming a disulfide bond with another molecule were reduced. **Figure 2** demonstrates the presence of mostly intramolecular disulfide bonds in the NEM−WPC gels and their complete absence in the DTT−WPC gels. Therefore, it is logical to assume that the degree of disulfide linkages among the WPC gels followed the above order.

In a previous publication (*11*), we discussed the early gel formation of WPC solutions heated in the presence of DTT, consistent with our current results (**Figure 3**; **Table 1**). We suggested that it was unlikely that the early gel formation was due to the denaturation of bovine serum albumin (denaturation temperature = 64 °C) and α-lactalbumin (denaturation temperature = 62 °C) (*23*) in the heated system, because a heated control did not show the same behavior, but rather to a reduction in the intramolecular disulfide bonds that maintained the globular structure of the protein molecules (including β-lactoglobulin, etc.). Therefore, the protein molecules would have been partially denatured, possibly similar in structure to the molten globule state (*24*), and held together by non-covalent interactions. As the heating progressed, the protein molecules would have unfolded readily, exposing the hidden hydrophobic residues and forming intermolecular linkages with each other. Because of the numerous reactive sites on the unfolded molecules, and the lack of restriction of movement by disulfide bonds, the chance of forming intermolecular linkages upon collision with another molecule would have been high. Consequently, gel formation occurred quickly, and strong gel networks were formed (**Figures 3** and **4**).

In contrast, the presence of SDS delayed gel formation (**Figure 3**; **Table 1**) and produced weak gels at the end of the heating cycle (**Figure 4**), consistent with previously reported results (*11*). It was also reported that the proteins were denatured almost completely after 10 min of heating at 85 °C but that it took much longer for the solutions to form a gel network. In the absence of non-covalent associations and because of the limited number of reactive sites (−SH and −S−S−), the interactions between the denatured proteins would have been relatively slow. Protein molecules would have had to collide in a certain way to effectively form intermolecular disulfide bonds. Furthermore, the presence of SDS effectively charged the proteins negatively. The negative net charges of the proteins resulted in repulsive forces between them, hence slowing the formation of a gel network. The current results are consistent with the previously reported data.

Furthermore, we also explored the impact of NEM on the formation of heat-induced WPC gels. If a solution of whey protein was heated sufficiently in the presence of NEM, the unfolded β-lactoglobulin molecules would be expected to adopt a polypeptide chain with two loops, a small loop linked by a disulfide bond between Cys106 and Cys119, and a bigger loop linked by another disulfide bond between Cys66 and Cys160 (*25*). The presence of these loops probably restricted the formation of non-covalent associations, yielding a network with less than the maximum possible number of linkages. In addition, small amounts of disulfide-linked aggregates were present. The question now is how much influence did these loops, and pre-existing disulfide aggregates, have on the functional properties of the WPC gels? Apparently, there were some disulfide linkages in the NEM system, but they would be expected to have an insignificant impact on the overall functional properties of the final heat-induced gels, because only a few pre-existing disulfide bonds were present for each molecule. It should be noted that the NEM was still associated with the protein in the heated system. The involvement of the NEM in the protein system is virtually unknown (Dr. Skelte Anema, personal communication).

It is clear from the results that the NEM−WPC system produced heat-induced gels that were significantly different from the control, SDS−WPC, or DTT−WPC gels. Heat treatment of a WPC system in the presence of NEM provided us with a system with an intermediate degree of disulfide linkages, between those of the control and DTT−WPC systems. It is interesting to note that the stiffness or the rigidity of the gels (G', **Figure 3**; and modulus, **Figure 4**) followed the reverse order of the degree of disulfide linkages, that is, SDS−WPC < control < NEM−WPC < DTT−WPC. Thus, the stiffness (modulus) of the gels increased as the degree of non-covalent associations among the denatured protein molecules increased (**Figure 4c**). This reinforces our previous notion that the formation of heat-induced whey protein gels is probably dominated by non-covalent interactions (*11*). It is also important to note that the presence of intramolecular disulfide bonds has a significant impact on the properties of heat-induced whey protein gels. In the complete absence of disulfide linkages (as in the DTT−WPC gels), the gels were the most stiff (**Figure 4c**) but required the least deformation to break them (**Figure 4a**). The DTT−WPC gels had a high resistance to small deformations but broke at lower deformations than the other gels. In the presence of predominantly intramolecular disulfide bonds (as in the NEM−WPC gels), the gels were significantly less stiff (**Figure 4c**) and required more deformation to break them (**Figure 4a**). The presence of the intramolecular disulfide bonds probably restricted the degree of unfolding of the protein molecules so that some of the reactive hydrophobic sites were not fully exposed to allow the formation of intermolecular non-covalent interactions with other molecules, resulting in less stiffness. Because of the presence of disulfide linkages, the NEM−WPC gels probably needed more deformation to break them than the DTT−WPC gels.

The current work is another attempt to describe how heat-induced protein−protein interactions relate to the textural properties of whey protein gels. On the basis of the results presented here, we have reason to suggest that, under most conditions, the heat-induced gelation of whey proteins is dominated by non-covalent associations of the denatured proteins. A heat-induced gel that is formed predominantly by non-covalent associations between the denatured whey protein molecules is expected to be relatively stiff and brittle. A heat-induced gel that is formed predominantly by disulfide bonding is expected to be relatively more rubbery (high fracture strain) and less stiff (low modulus). The rubberiness of a whey protein gel is expected to increase as the degree of disulfide linkages within the gel increases. The presence of intramolecular disulfide bonds is expected to make a gel significantly more rubbery than a gel formed solely by non-covalent associations.

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