JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 6825–6832 6825
DOI:10.1021/jf1009796

# Gelling Properties of Microparticulated Whey Proteins

Muditha Dissanayake,[†] Alan L. Kelly,[‡] and Todor Vasiljevic*[,†,§]

[†]School of Biomedical and Health Sciences, and [§]Institute for Sustainability and Innovation, Faculty of Health, Engineering and Science, Victoria University, Werribee Campus, Melbourne, Victoria 8001, Australia, and [‡]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Subjecting whey proteins to high-pressure shearing with or without heating, commonly termed microparticulation, results in novel ingredients with modulated functionalities. Gelling properties of microparticulated whey proteins (MWP) were specifically assessed in this study. MWP powders were produced from commercial cheese whey retentate, standardized to 10% (w/w) protein, and subjected to microfluidization (MFZ) at 140 MPa either with or without prior heat-induced denaturation, followed by spray-drying. Gels were created from aqueous MWP dispersions either by heating at 90 °C for 20 min or by allowing gels to form at ambient temperature through addition of glucano-$\delta$-lactone and/or NaCl. MWP powders produced from unheated WP dispersions created firm gels upon heating, whereas those produced from denatured WP gave only cold-set gels. Covalent and noncovalent protein−protein interactions were involved during both heat- and cold-induced gelation. Hydrophobic interactions were more pronounced during aggregation of bovine serum albumin. In conclusion, microparticulation of WP resulted in heat- and cold-set gels with different molecular and physical characteristics from those of untreated controls.

**KEYWORDS: Whey proteins; microfluidization; cold gelation; heat gelation; polyacrylamide gel electrophoresis; *N*-ethylmaleimide; sodium dodecyl sulfate; Tween 20**

## INTRODUCTION

The term gel defines a range of substances that exhibit solid-like properties in which a great excess of solvent is present (*36*). The ability of whey proteins (WP) to form gels by immobilizing large amounts of water and other ingredients is important in processed food formulations and product development, as this phenomenon can be used to improve textural attributes (*22*, *29*, *38*, *43*). Also, WP (mainly α-lactalbumin (α-LA), $\beta$-lactoglobulin ($\beta$-LG), bovine serum albumin (BSA), and immunoglobulins) possess superior nutritional attributes compared to other gel-forming agents such as pregelatinized starches and similar hydrocolloids (*38*). However, functional properties of WP are highly dependent on their heat sensitivity as well as compositional variability (*15*), all of which greatly affect their utility in food systems. Although a number of approaches have been suggested to improve their application (*6*, *7*, *11*, *31*), much remains to be resolved before WP can gain widespread commercial importance.

Gelation is a phase transition of polymers from a liquid state with "disconnected monomers" to a well-connected network or gel (*35*). The sol−gel transition involves linking of the basic structural units via physical and/or chemical bonds, resulting in formation of a continuous network, leading to solid-like properties. Protein gelation can be induced by many extrinsic factors, such as heat, acids, pressure, enzymes, and salts (*2*, *6*, *20*, *23*, *25*, *26*). Depending on the balance between attractive and repulsive forces among denatured protein molecules, different types of gel networks are formed (*13*). Protein aggregates are the basic building material for a gel network (*9*, *26*), and the size, shape, and spatial arrangement of the aggregates can have an impact on rheological behavior, sensory quality, and water-holding capacity of resulting gels. Also, depending on the macroscopic nature of the gels, they can be described as "true gels", with the ability to "free stand", or "weak gels", which show less clear solid nature and flow when subjected to a yield stress (*36*).

Gelling of WP upon heating provides textural benefits in many food applications. On the other hand, cold-set WP gels are also important in preparations of heat-sensitive food products with delicate texture and flavor. This can be achieved at lower temperatures and is thus suitable for food applications where a postprocessing preservation step by heating is not required. The general prerequisite for the cold gelation process is the activation of protein molecules by denaturation, at a protein concentration below the critical gelation concentration and at low ionic strength and/or far from the isoelectric point, followed by the addition of salts or adjustment of pH to screen repulsive forces at cold-set conditions (*3*, *41*).

Microparticulation is a recent approach to improving or modulating WP functionality and can also be achieved by dynamic high-pressure shearing or microfluidization (MFZ) (*24*). As we showed earlier (*14*), MFZ, combined with heat, produces MWP products with different functional properties compared to the use of MFZ alone. Improvements included enhanced heat stability and emulsifying activity of these particles. The particle sizes of WP aggregates induced by heating are greatly reduced (*14*, *24*) by the forces of mechanical shearing. Conformational changes of native WP induced by microparticulation may thus be important in modulating their physical functionalities. Moreover, gelling

*Corresponding author: phone, +61 3 9919 8062; fax, +61 3 9919 8284; e-mail, todor.vasiljevic@vu.edu.au.


Published on Web 05/17/2010

properties of MWP may also be important and introduce novel ingredients in the food industry.

The objective of the current study was to establish gelling behavior of MWP induced by either heating (heat-set gels) or changing the solvent quality (cold-set gels) in order to enhance MWP usage as functional agents in processed foods. One of the research foci was also to assess different fundamental interactions driving gelation of these MWPs.

## MATERIALS AND METHODS

**Materials, Treatments, and Proximate Composition.** All chemicals used in the study were of analytical grade (Sigma-Aldrich, St. Louis, MO; BDH Chemicals, Australia Pt. Ltd., Kilsyth, Australia), and gels for polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA) and NuSep (French Fores, Australia).

The study was performed using two different batches of WP retentate containing around 30% total solids and their corresponding whey permeates, kindly supplied (on separate occasions) by Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). The WP retentates were diluted using corresponding whey permeates and adjusted to 10% (w/w) protein at pH 7.0 using NaOH. They were subjected to either high-pressure shearing by MFZ at 140 MPa or to heat denaturation followed by MFZ (at 140 MPa). This resulted in five different types of samples. Two samples were microfluidized only (Model 110Y; Microfluidics, Newton, MA) with either 1 or 5 passes (N1 and N5). Two further samples were first heated at 90 °C for 20 min for complete protein denaturation (*16*) and then microfluidized using 1 or 5 passes (H1 and H5). The untreated sample served as a control (C). Finally, all preparations were spray-dried using a pilot-scale spray dryer (SL-10 mini-maxi pilot spray dryer; Saurin Enterprises Pty. Ltd., Melbourne, Australia). Powders were collected, stored in airtight containers in a dry and cool place, and used for gelation studies when required. Proximate analysis (*4*) showed that all five powders had a similar gross composition of 64.6% protein, 6.1% moisture, 19.6% lactose, 3.9% fat, 4.4% ash, 0.74% calcium, and 0.42% phosphorus.

**Preparation of Heat-Induced Gels.** Heat-induced gelation was carried out using approximately 12% (w/w) WP dispersions, following an established method (*31*) with some modifications. The dispersions were prepared with Milli-Q water and 0.03 M $CaCl_2$, and sample pH was adjusted to 7.0 by dropwise addition of 1 M NaOH. The mixtures were used either for rheological studies or for preparation of gels for further analysis. Gels were prepared by allowing the WP dispersions to sit for 15 min at room temperature prior to heating at 90 °C for 30 min (10 min come-up time) in a water bath, followed by immediate cooling in an ice bath for 30 min. Subsequently, the gels were aged at 4 °C overnight.

**Preparation of Cold-Set Gels.** Cold-set gelation was performed to assess the rheological properties of such gels using microparticulated, heat-treated (H1 and H5) and unheated (N1 and N5) WP powders with or without glucano-$\delta$-lactone (GDL) and NaCl. These powders were fully hydrated as 12% (w/w) WP dispersions in Milli-Q water with 0.03 M $CaCl_2$. These samples were divided as per MFZ treatment (1 or 5 passes), and each set contained six different samples. These included two control samples, heat-treated (HT) and unheated (NHT) controls without GDL or NaCl addition, and four others mixed with GDL and/or NaCl to achieve GDL or NaCl concentrations in their final mixtures of 2% GDL, 0.1 M NaCl, 0.5 M NaCl, or 2% GDL with 0.1 M NaCl.

**Flow Behavior of Microparticulated WP Dispersions.** Whey protein samples were tested by a CS/CR rheometer (MCR 301; Anton Paar, GmbH, Germany) equipped with a proprietary software (Rheoplus/32 v2.81; Anton Paar) and a double-gap-cylinder measuring system (DG26.7- SN7721; Anton Paar). About 3.9 g of 12% (w/w) WP dispersion was introduced into the measuring unit, and a thin layer of low-density, low-viscosity oil was placed on top of the sample to prevent evaporation. Prior to analysis, the mixture was presheared for 5 s at a controlled rate of 500 $s^{-1}$ at 20 °C and held for 30 s to equilibrate. The flow behavior of WP samples was investigated using shear rate sweep measurements over the range of 0.1−100 $s^{-1}$ for 5 min at the same temperature. The flow curves were fitted to two rheological models, namely, the power law (Ostwald) and Herschel−Bulkley, presented by the equations:

$$\text{Ostwald model}: \quad \sigma = k\dot{\gamma}^n \tag{1}$$

$$\text{Herschel}-\text{Bulkley model}: \quad \sigma = \sigma_0 + k'\dot{\gamma}^{n'} \tag{2}$$

where $k$ ($k'$), $n$ ($n'$), $\sigma$, $\sigma_0$, and $\dot{\gamma}$ are consistency index (Pa·$s^n$), flow behavior index (dimensionless), shear stress (Pa), yield stress (Pa), and shear rate (1/s), respectively (*40*).

**Viscoelastic Properties of Heat- and Cold-Set Gels.** *In situ* thermal gelation was also assessed using the same rheological system by applying dynamic small amplitude oscillatory measurements at a constant strain of 1% and frequency of 1 Hz. During heat gelation, the samples were heated from 20 to 90 °C at a heating rate of 1 °C $min^{-1}$ for approximately 70 min, held at 90 °C for 10 min, and cooled from 90 to 4 °C at a rate of 1 °C $min^{-1}$ over 86 min, followed by holding at 4 °C for 60 min for aging.

*In situ* cold-set gelation was examined using a cone-and-plate measuring system (CP 50-1; Anton Paar). This measuring system was specifically selected to avoid possible sedimentation of WP occurring in the double-gap system via gravitational forces due to their larger particle size. During cold gelation, the 12% WP mixture was immediately introduced to the measuring system, and the process was monitored using the same parameters as described above, at 20 °C for 150 min. After this period, the frequency sweep from 1 to 10 Hz at 1% shear strain was used to ascertain viscoelastic properties of created gels. The pH change of the GDL-acidified WP mixtures was simultaneously recorded every 15 min using a pH meter (Model 8417; Hanna Instruments, Singapore). An additional mechanistic study assessing the prevalence of protein−protein interactions during cold-set gelation was also carried out *in situ* using the same cone-and-plate measuring system. Gelation was achieved as described previously (*18*, *19*) using H1 and H5 protein dispersions mixed with 2% GDL or 0.1 M NaCl in the presence of either 20 mM NEM or 1% SDS.

**Polyacrylamide Gel Electrophoresis (PAGE).** Heat- and cold-set gels were created from the samples as described under preparation of "heat-induced gels" and "cold-set gels". Only the mixtures that created gels were used in PAGE analysis. All gel samples were dissolved in appropriate buffers before performing either native (with or without 0.45% polyoxyethylene sorbitan monolauarate, i.e., Tween 20) or reducing/nonreducing sodium dodecyl sulfate (SDS) PAGE (*18*).

**Gel Permeability.** The permeability coefficient ($B$) of heat-set WP gels was determined as described previously (*27*) with slight variations. The WP mixtures, prepared for heat gelation, were placed in 50 mL Falcon tubes (Falcon, Blue Max; Becton Dickinson and Co., Franklin Lakes, NJ). Open-ended glass tubes (inner diameter 3.7 mm and length of 25.0 cm) were then immersed in the mixtures with the aid of rubber stoppers. The tubes were further sealed with parafilm to prevent evaporation of the solvent, and gelation was induced by heating at 90 °C for 30 min. Afterward, the glass tubes with the gels were removed from the Falcon tubes, and the heights of the gels were measured. The glass tubes with gels were then immersed in 6% (w/w) whey solutions. Due to the osmotic pressure gradient between the top of gels and the surface of whey in the Falcon tubes, the whey diffuses through the gels and collects on the surface. The height of the whey on the gel surface was measured after two different time intervals. The experiment was conducted at 20 °C, and the density of the whey was 1.0033 g/mL. The permeability coefficient ($B$) was determined using the equation:

$$B = -\left[\ln\left(\frac{h_\infty - h_{t2}}{h_\infty - h_{t1}}\right)\right]\frac{\eta H}{\rho g(t_2 - t_1)}$$

where $B$ is the permeability coefficient ($m^2$), $h_\infty$ is the height of the whey in the reference tube (m), and $h_{t1}$ and $h_{t2}$ are the heights of whey (m) in the gel tube at time $t_1$ (s) and $t_2$ (s), respectively. The value $\eta$ is the viscosity of whey (Pa·s), $\rho$ is the density of whey (kg/$m^3$), $g$ is acceleration due to gravity ($ms^{-2}$), and $H$ is the length of the gel (m). The reference glass tube was included with the absence of gels but supplying all other similar experimental conditions.

**Water-Holding Capacity of Gels.** Water-holding capacity (WHC) of heat-set WP gels was examined using an established method (*34*). After overnight storage at 4 °C, the gels were centrifuged (Model RT7, Sorvall; DuPont, Newtown, CT) at 700*g* at 8 °C for 10 min. The supernatant

**Table 1.** Rheological Parameters of Whey Protein Dispersions Obtained by Fitting the Experimental Data to Power Law and Herschel−Bulkley Models

| | power law | | | Herschel−Bulkley | | | |
|---|---|---|---|---|---|---|---|
| treatment[a] | $k$[b] (mPa·s$^n$) | $n$[c] | $R^2$[d] | $\tau_0$[e] (Pa) | $k'$[b] (mPa·s$^{n'}$) | $n'$[c] | $R^{2'}$[d] |
| control | 15 c | 0.61 ab | 0.93 | 2.26 bc | 14 c | 0.76 a | 0.98 |
| N1 | 17 c | 0.57 a | 0.91 | 5.62 d | 12 bc | 0.70 a | 0.98 |
| N5 | 14 bc | 0.63 b | 0.93 | 2.91 c | 9 ab | 0.75 a | 0.99 |
| H1 | 9 a | 0.82 c | 0.98 | 0.57 a | 7 a | 0.89 b | 0.99 |
| H5 | 10 ab | 0.80 c | 0.98 | 0.95 ab | 9 ab | 0.84 b | 0.99 |
| SEM[f] | 1 | 0.02 | 0.009 | 0.53 | 1 | 0.02 | 0.002 |

[a] Treatments: native control; N1, non-heat-treated, 1 MFZ pass; N5, non-heat-treated, 5 MFZ passes; H1, heat-treated, 1 MFZ pass; H5, heat-treated, 5 MFZ passes. [b] $k$ and $k'$, consistency indexes. [c] $n$ and $n'$, flow behavior indexes. [d] $R^2$ and $R^{2'}$, correlation ratios. [e] $\tau_0$, yield stress. [f] SEM, pooled standard error of the mean, $P < 0.05$. Means present the average of at least eight independent observations ($n = 8$). The different lowercase letters in a column indicate significant difference ($P < 0.05$).

(whey) was carefully decanted, and the WHC of the gel was expressed as a percentage, taking into account the weight of gel after whey was expelled, relative to the initial weight of gel.

**Color of Gels.** Color of heat-induced gels was measured with a Minolta Chromameter (CR-300; Minolta Corp., Ramsey, NJ) using CIE 1976 ($L^*$, whiteness; $a^*$, red to green; $b^*$, yellow to blue) color system. Five determinations were carried out for each gel.

**Statistical Analysis.** The study was organized as a randomized block, full factorial design with treatment (heat, no heat) and number of MFZ passes (1 or 5) as the major factors and the replications as a block. All experiments were replicated at least once with subsequent subsampling ($n \geq 4$). Results were analyzed using a general linear model (*39*). The level of significance was preset at $P = 0.05$.

## RESULTS AND DISCUSSION

**Flow Behavior of WP Dispersions. Table 1** presents the rheological parameters of 12% (w/w) WP dispersions obtained by fitting the data to the power law and Herschel−Bulkley models. The results show that the combined effect of heat and shearing significantly ($P < 0.05$) increased the $n$ value (H1 and H5 compared to unheated samples), while heat treatment markedly ($P < 0.05$) decreased the $k$ values and yield stress of the H1 sample compared to unheated samples. The viscosity behavior of proteins is a result of several factors, including the size, shape, and polydispersity of protein molecules and their aggregates, protein−solvent interactions, hydrodynamic volume, and molecular flexibility of proteins in their hydrated state (*12*, *36*). **Figure 1** and **Table 1** show the higher yield stress of N1 compared to all other samples and also the relatively prominent shear-thinning nature of unheated preparations (i.e., higher slopes) compared to heat-treated species. Shear-thinning behavior of protein solutions can arise due to the orientation of the major axes of protein molecules in the direction of flow, as well as dissociation of weakly held dimers and oligomers into monomers (*12*). The effect of hydrodynamic pressure on globular WP is distinctly different from that of hydrostatic pressure. During application of dynamic high-pressure shearing (MFZ), the collision, shearing, and flowing of molecules may occur leading to contraction and, more importantly, elongation and flowing of molecules (*1*). In addition to quaternary and tertiary structures, the secondary conformations of WP, i.e., $\beta$-sheets and α-helixes, are also more prone to be affected as a result of dynamic high-pressure shearing due to perturbation of comparatively weaker interactions such as hydrogen bonds between protein strands (*1*).

The particle size distribution pattern, presented in our previous study (*14*), showed a decrease in average particle size of MWP. The significantly ($P < 0.05$) higher yield stress of N1 compared to the control is also indicative of the existence of greater protein−protein and protein−solvent interactions. However,

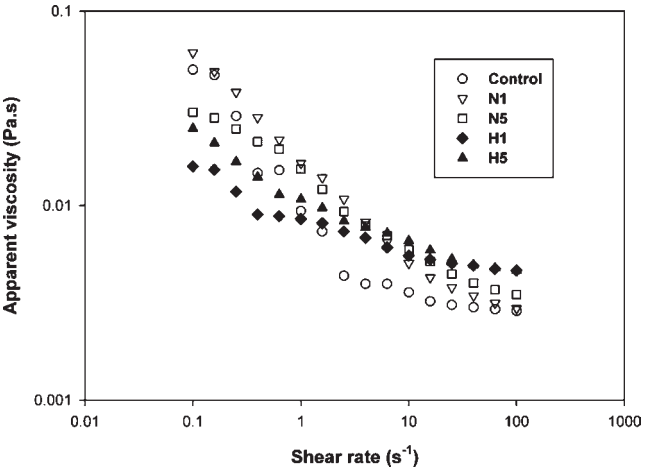


**Figure 1.** Apparent viscosity of 12% (w/w) WP dispersions at 20 °C during a controlled shear rate sweep (0.1−100 s$^{-1}$). N1 = native, 1 pass; N5 = native, 5 passes; H1 = denatured, 1 pass; H5 = denatured, 5 passes.

extensive shearing (N5) may have caused further stretching of protein molecules, possibly leading to exposure of individual protein strands in their secondary structures. This may result in partial denaturation and likely subsequent aggregation via newly exposed reactive sites. This situation may be further explained by significantly increased $n$ values and reduced $k$ and $\tau_0$ values of N5 samples and widened particle size distribution pattern of this sample (*14*). Interestingly, although apparent viscosity of heat-treated samples was lower at low shear rates than their unheated counterparts, it was even higher than that of unheated preparations at high shear rates (**Figure 1**). Additional conformational rearrangements of heat-treated MWP samples may have occurred at higher shear rates leading to greater particle−particle and particle−solvent attractive interactions and thus increase in apparent viscosity.

**Heat-Induced Gelation.** During heat-induced gelation, conformational changes of proteins occur with protein unfolding and exposure of reactive sites such as thiol groups and hydrophobic groups followed by intermolecular aggregation and progressive development of an infinitely cross-linked and self-supporting network created from protein−protein and protein−solvent interactions (*22*, *28*, *29*, *38*, *44*). In addition, ionic interactions, hydrogen bonding, and calcium bridges are equally important in formation of infinite viscoelastic gel network (*7*).

**Figure 2** shows the changes in storage modulus ($G'$) of 12% (w/w) unheated and heat-treated WP dispersions, including an untreated control, during heating, holding, cooling, and aging at constant strain and frequency. Only unheated (N1 and N5) preparations, which could unfold and aggregate via various molecular interactions as described above, formed heat-induced gels. Also, the $G'$ value increased with the number of microfluidizing passes. Formation of heat-induced gels from heat-treated (H1 and H2) WP is unlikely even if they were subjected to high-pressure shearing. This is mainly due to the unavailability or insufficient number of required reactive sites for cross-linking, such as thiol groups and hydrophobic groups in denatured samples.

MFZ may have a disruptive effect on protein conformation by affecting different inter- and intramolecular interactions. Therefore, dissociation, reversible changes of the conformation and unfolding of WP molecules, and activation of the thiol group may occur under MFZ conditions, with generation of higher numbers of activated molecules available for further heat-induced aggregation and, thus, a greater solid-like behavior compared to untreated controls, as observed. Furthermore, the elasticity of the

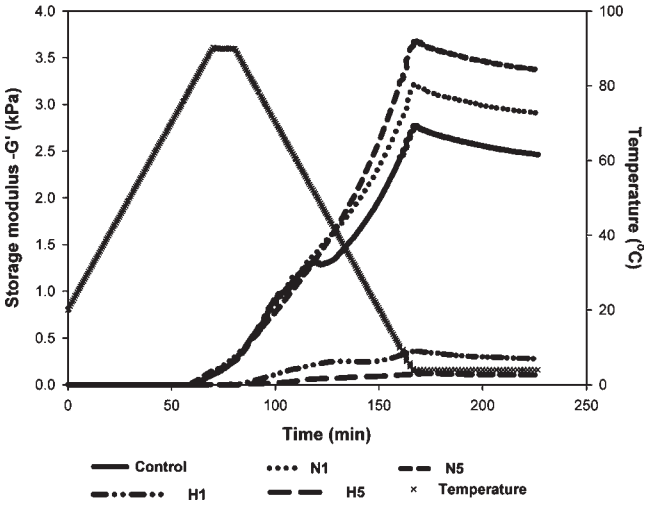


**Figure 2.** Changes in storage modulus ($G'$) of 12% (w/w) native (N) or denatured (H) WP dispersions microparticulated by either 1 or 5 MFZ passes, during heating (20−90 °C), holding (90 °C/10 min), cooling (90−4 °C), and aging (4 °C/approximately 60 min) at constant strain (1%) and frequency (1 Hz).

gel is directly proportional to the density of cross-linking in the network (*33*). Therefore, MFZ may have changed the conformation of proteins, which in turn may have facilitated covalent chain reactions and attractive noncovalent (hydrogen, hydrophobic, van der Waals forces) protein interactions, enhancing the extent of intermolecular cross-linking (*2*). This was reflected by increased $G'$ value of gels, especially in the extensively microfluidized sample (N5). During heat-induced gelation, hydrogen bonds present in the native form of proteins are disrupted (*12*). However, upon cooling, these bonds may again reestablish, as observed by increased $G'$ values of the gels. In addition, the slight decrease of $G'$ again during gel aging may have resulted from reversibility or disruption of some hydrophobic interactions, which are generally less favored at low temperatures (*12*).

**Cold Gelation. Figure 3** shows the development of storage modulus ($G'$) during cold gelation of 12% (w/w) heat-treated WP dispersions and nonheated controls and WP dispersions microparticulated by 1 MF pass (**A**) or 5 passes (**B**) at 20 °C, at constant strain (1%) and frequency (1 Hz).

In general, cold gelation involves initial controlled thermal denaturation of proteins. In the second step, the solvent quality is changed by salt addition or acidification, inducing the gelation process at low temperatures (*2*, *10*). In the current study, a slightly different approach involved utilizing MWP powders for preparation of cold-set gels. In contrast to heat-induced gels, heat-treated MWP samples created cold-set gels as shown in **Figure 3**.

During cold gelation, even samples H1 and H5 gelled without GDL or salt addition although the gel strength of these samples was comparatively low. It is quite possible for heat-treated WP at a sufficiently high concentration, such as 12% as used in this study, to form a viscoelastic gel network via intermolecular protein−protein as well as protein−water interactions through exposed reactive sites (*36*). In comparison, unheated N1 and N5 samples did not form gels under the same conditions since they were not unfolded WP molecules for the subsequent aggregation. In the presence of GDL or salts, H1 and H5 samples created stronger gels compared to heat-treated controls, as indicated by higher $G'$ values. In general, increasing the number of MF passes increased the $G'$ values of cold-set gels, indicating the existence of comparatively higher intermolecular interactions in the viscoelastic system. As clearly shown by **Figure 3**, stronger WP gels

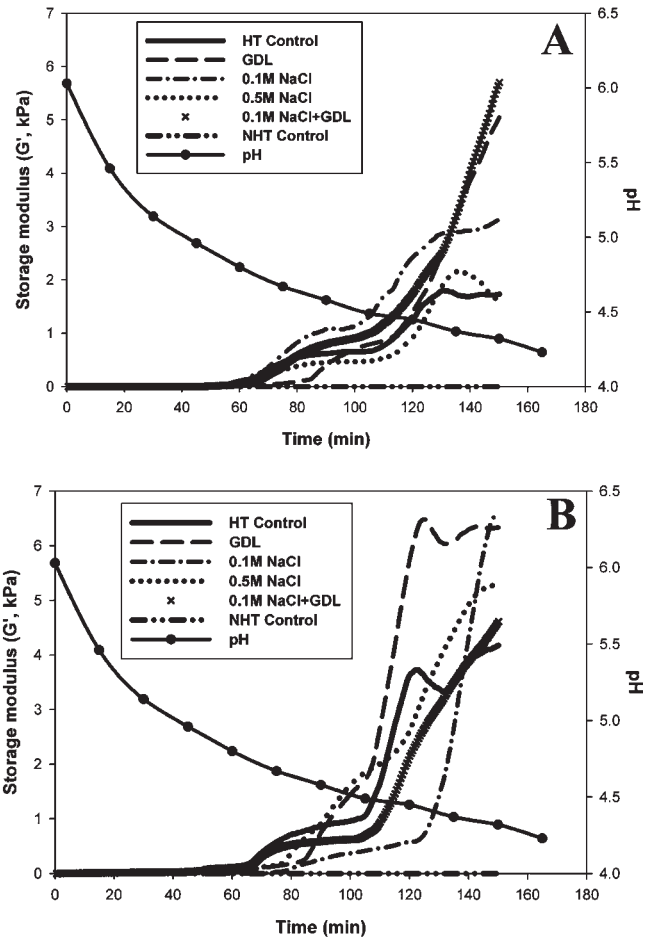


**Figure 3.** Changes of storage modulus ($G'$) during cold (GDL, salt) gelation of 12% (w/w) denatured WP dispersions microparticulated by 1 MFZ pass (**A**) or 5 MFZ passes (**B**). Measurements were performed at 20 °C at constant strain (1%) and frequency (1 Hz).

could be produced with 0.1 M NaCl in both instances (H1 and H5) than with 0.5 M NaCl. As described by Verheul and Roefs (*42*), monovalent salts primarily interact with charges on the protein at up to 0.1−0.2 M NaCl, increasing intermolecular interactions, but beyond that, charges are saturated and NaCl concentration affects the solvent properties (*7*). Therefore, this may have happened during cold gelation of MWP at higher salt concentration (0.5 M NaCl) in our study, which had a negative effect on gel strength compared to the results obtained at lower salt concentration. In addition, H5 samples with either 0.1 M NaCl or GDL produced the strongest gels, indicating the equal importance of shielding of charges as well as acidification in reducing repulsion between proteins during the gelation process. When both 0.1 M NaCl and GDL were present in the medium, the strength of H5 gels was lower compared to that of H1. These differences were likely due to the conformational changes of proteins obtained under varying high-pressure shearing conditions, which may have affected all types of molecular interactions. In addition, the elongation of protein molecules during shearing likely played a role in the alignment of molecules, thus enhancing their interactions. The different effects of the extent of MFZ are further revealed by the $G'$ values of H1 and H5 gels produced with GDL alone. Although the maximum $G'$ value of H5 gel created with GDL is fairly higher than that of H1 gel, further acidification has made the H5 gel brittle (as shown by the graph), possibly due to enhancement of repulsive positive charges on proteins. The nature of these gels is further explained by **Figure 4**, irrespective of

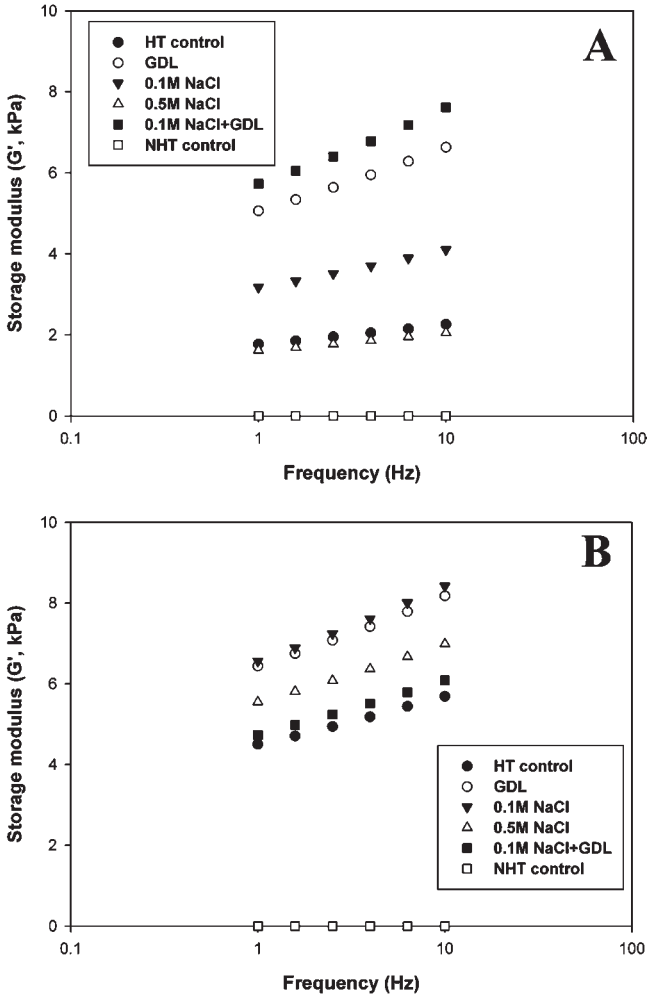


**Figure 4.** Viscoelastic properties, expressed as storage modulus ($G'$), of 12% (w/w) denatured WP preparations microparticulated by 1 MFZ pass (**A**) or 5 MFZ passes (**B**) obtained by a frequency sweep (1−10 Hz) at a constant strain (1%) and 20 °C.

their high $G'$ values. The direct dependency of $G'$ on frequency indicates that these gels were weaker or more brittle compared to stronger gel networks with an elastic plateau (*35*).

**Electrophoretic Analysis.** Immediately after thermal and cold-set gelation, WP samples were analyzed by native PAGE and SDS−PAGE to determine the nature of interactions among proteins. **Figure 5A** shows the results of native PAGE (with or without Tween 20), and **Figure 5B** shows the SDS−PAGE (reducing or nonreducing) patterns of C (control), N1, and N5 WP samples during heat-induced gelation. The additional control sample was prepared directly from untreated, spray-dried WP powder and contained no Tween 20 in the buffer (i.e., control). As indicated by the native PAGE patterns without Tween 20 (**Figure 5A**), most of the protein bands originally present in the control sample disappeared in C, N1, and N5 samples, indicating protein aggregation during heating. Very faint bands of monomeric α-LA and $\beta$-LG were still present, indicating the presence of low levels of their native forms under these conditions. The presence of these bands could also be attributed to higher lactose content in these WP preparations, which can improve the heat stability of WP (*30*).

In contrast, BSA bands were absent from this PAGE gel, which indicated their complete denaturation. These bands, however, reappeared in the presence of Tween 20 (**Figure 5A**), indicating the importance of hydrophobic interactions during the aggregation of

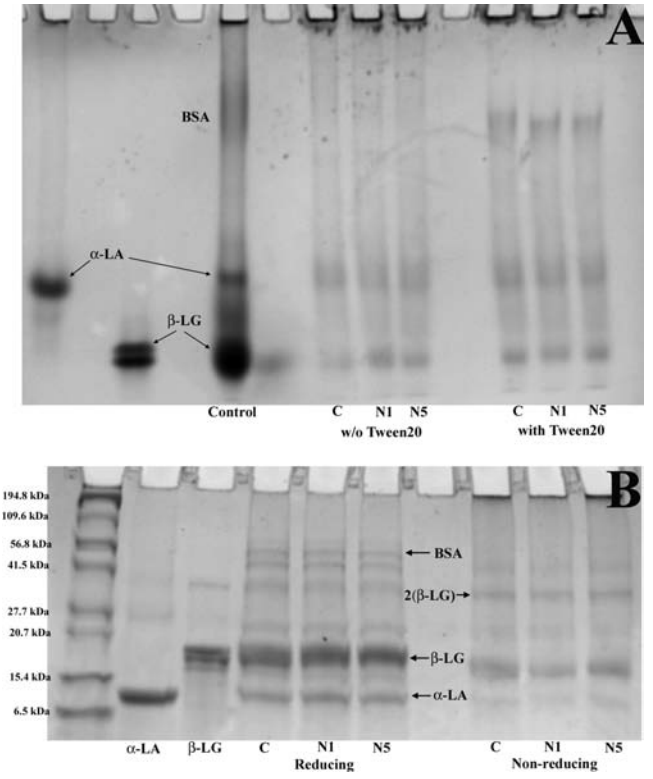


**Figure 5.** Electrophoretograms of WP dispersions analyzed by (**A**) native PAGE, with or without Tween 20, and (**B**) SDS−PAGE (reducing or nonreducing) (C, native control; N1, native, 1 MFZ pass; N5, native, 5 MFZ passes) after heat-induced gelation. Control was untreated native powder which was not gelled.

BSA, as Tween 20 is a weak nonionic surfactant which affects hydrophobic interactions (*5*). As shown by **Figure 5B**, the protein bands on nonreducing SDS−PAGE are relatively less prominent than the bands on reducing PAGE, indicating that heat-induced protein aggregation has mostly occurred via covalent bonding (*32*). In addition, some disulfide-linked small aggregated proteins, possibly dimers of $\beta$-LG, were also visible without resolving under nonreducing SDS conditions.

The PAGE analysis of cold-set gel samples revealed very little (results not shown) since the size of generated aggregates was too large, which prevented a proper resolution in native or nonreducing SDS−PAGE gels. However, all proteins did appear in SDS-reducing PAGE gels (data not shown). These unresolved aggregates with covalent disulfide bonds have most probably been formed due to the heat treatment at pH 7 when MWP powders were prepared. Nevertheless, further reduction of the particle size of WP aggregates may be achieved by microparticulation at a lower pH (*24*).

**Figure 6** shows the changes in storage modulus ($G'$) during cold gelation (either with 2% GDL or 0.1 M NaCl) of 12% (w/w) MWP preparations (H1 and H5, respectively) in the presence of SDS or NEM and two additional samples of H1 and H5 without SDS and NEM. As shown in **Figure 6A**, GDL-acidified H1 gels (without SDS or NEM) were stronger than those produced with 0.1 M NaCl. NEM is a thiol blocker (*17*) and in its presence, formation of new covalent bonds is prevented (*2*), whereas SDS is an ionic surfactant which blocks noncovalent interactions (*19*). The strength of acid or salt gels, as indicated by $G'$ values, in the presence of NEM was always higher than that of those prepared with SDS, supporting the observations (*2*) that noncovalent interactions prevailed during cold gelation processes of H1 samples rather than thiol-mediated covalent interactions. This observation

was even confirmed by the strength of H1 gels produced with either GDL or GDL with NEM almost being equal. Although SDS inhibits the noncovalent bond formation, weak attractive molecular interactions such as van der Waals forces could still be present among protein molecules.

In addition, as **Figure 6B** indicates, when acid gelation of H5 samples was carried out in the presence of either NEM or SDS, the strength of these gels was found to be comparable. This trend was also observed in salt-induced gels of these H5 samples. This may indicate equal contribution of both covalent and noncovalent interactions during the cold-gelation process. Apparently, hydrodynamic high-pressure shearing altered the protein conformation and existing interactions among WP by disrupting certain associations and also promoting their reactivity by exposing buried reactive sites or creating new reactive sites which may affect both covalent and noncovalent interactions and, thus, the final gel characteristics.

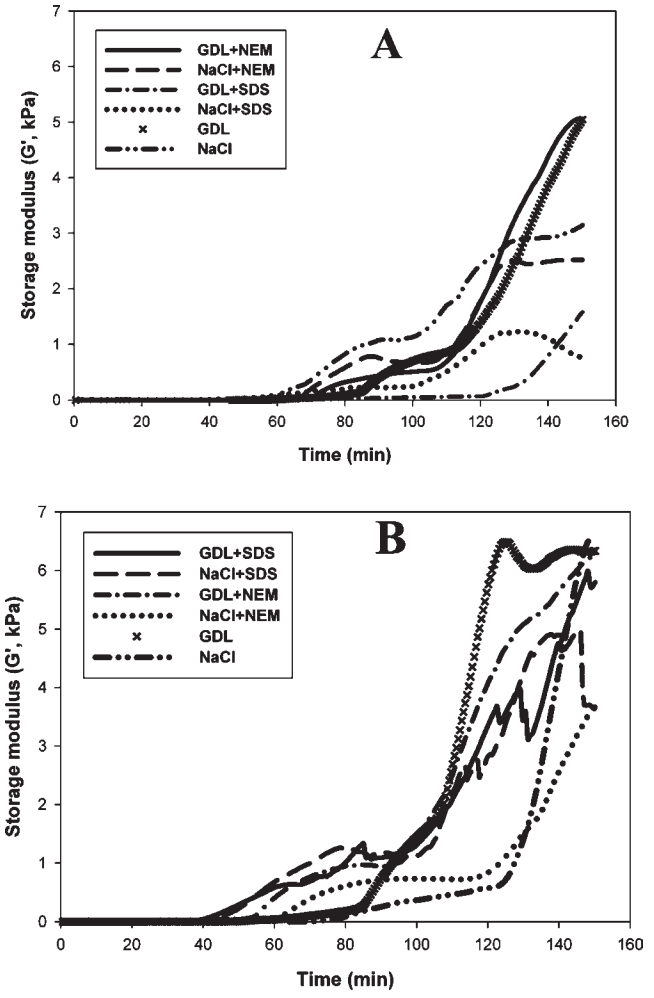


**Figure 6.** Changes in storage modulus ($G'$) during cold gelation of 12% (w/w) denatured WP preparations (H1 and H5) with GDL or 0.1 M NaCl and in the presence of SDS or NEM at constant strain (1%) and frequency (1 Hz).

**Water-Holding Capacity, Permeability, and Surface Reflectance.** As shown in **Table 2**, heat-set gels had almost 100% water-holding capacity (WHC). WHC quantitatively indicates water retention within a protein matrix under defined conditions and reflects the pore size of the gels (*21*). The extensive hydrodynamic shearing significantly ($P < 0.05$) increased the WHC of heat-set gels, which may be related to increased charge distribution on proteins, resulting in enhanced water−protein interactions (*12*).

This positive effect of MFZ on the compactness of gels was further supported by permeability coefficients of heat-set gels. Permeability coefficients of heat-set gels (N1 and N5 samples) were significantly ($P < 0.05$) reduced compared to the untreated control (**Table 2**), reflecting their relative compactness. The permeability coefficient ($B$) is positively correlated with the particle size (*8*) and the pore size (*27*) and lower $B$ values of microfluidized samples are indicative of a more compact network with smaller pores. Hydrogen-bonding and hydrophobic interactions are the main contributors to network formation except when multivalent ions are involved in cross-linking (*12*). In addition, the possible formation of disulfide bonds in MWP gels may also have contributed to the creation of a stronger network with greater cross-linking.

The appearance of heat-induced gels as indicated by color parameters, i.e., $L^*$ (lightness), $a^*$ (red to green), and $b^*$ (yellow to blue), is provided in **Table 2**. Microparticulation significantly ($P < 0.05$) changed the color of heat-set gels, compared to the control gel samples as indicated by these parameters. Increased lightness of gels with extensive MFZ (5 passes) also reveals higher opacity of these gels and greater compactness. Attractive hydrophobic interactions and repulsive electrostatic interactions fundamentally control the gelation mechanism and the gel appearance (*12*). Thus, hydrodynamic shearing may result in exposure of buried reactive sites which may lead not only to enhanced hydrophobic interactions and covalent bonding but also to increased ionic interactions and, finally, creation of a stronger protein−protein network and greater protein−solvent interactions, producing highly opaque gels.

In conclusion, MFZ of unheated or heat-treated WP resulted in ingredients with distinguishably different gelling behavior. The unheated MWP produced more compact heat-set gels compared to the untreated controls. Although heat-set gels were mainly created via covalent interactions, high-pressure shearing likely changed the protein conformation, thus affecting all other molecular interactions, which may have been reflected by facilitated reactivity of proteins during heat gelation. Meanwhile, heat-treated MWP created cold-set gels which were formed primarily via noncovalent associations under a reduced number of

**Table 2.** Physical Characteristics of Heat-Set Gels

| | | | | | | color | | |
|---|---|---|---|---|---|---|---|---|
| treatment[a] | $G'$ [b] ($_{90\,°C}$) (kPa) | $G'$ [b] ($_{4\,°C}$) (kPa) | $G'$ [b] (GDL)[c] (Pa) | permeability coeff ($m^2$) | WHC[d] (%) | $L^*$ [e] | $a^*$ [f] | $b^*$ [g] |
| control | 2.8 | 2.5 | ND[h] | $6.61 \times 10^{-17}$ b | 98.8 a | 91.1 a | −2.58 a | 11.8 c |
| N1 | 3.2 | 2.9 | ND | $3.32 \times 10^{-17}$ a | 99.3 a | 92.4 b | −3.15 b | 9.8 b |
| N5 | 3.7 | 3.4 | ND | $3.95 \times 10^{-17}$ a | 99.0 a | 96.0 c | −3.46 c | 8.8 a |
| SEM[i] | 0.2 | 0.2 | | $5.92 \times 10^{-18}$ | 1.51 | 0.32 | 0.08 | 0.27 |

[a] Treatments: N1, non-heat-treated, 1 MFZ pass; N5, non-heat-treated, 5 MFZ passes; H1, heat-treated, 1 MFZ pass; H5, heat-treated, 5 MFZ passes. [b] $G'$ represents the average value of gel storage modulus. [c] GDL, glucano-$\delta$-lactone. [d] WHC, water-holding capacity. [e] $L^*$, lightness. [f] $a^*$, positive $a^*$ is red and negative $a^*$ is green. [g] $b^*$, positive $b^*$ is yellow and negative $b^*$ is blue. [h] ND, not determined. [i] SEM, pooled standard error of the mean, $P < 0.05$. Means present the average of at least eight independent observations ($n = 8$). The different lowercase letters in a column indicate significant difference ($P < 0.05$).

microfluidizing passes. Extensive MFZ has increased the prevalence of interactions between proteins since under these circumstances both covalent and noncovalent interactions were found to be involved with increasing the strength of both acid and salt gels. The disruption of aggregates which were created during heating and thereby the reduction of particle size with creation of new reactive sites as well as exposure of buried reactive sites may have apparently governed the increased reactivity of MWP.

Most notably, the size of aggregates appeared to play an important role in creation of gels, as stronger gels were formed by smaller particles. Slight process modifications, including simultaneous heating and hydrodynamic high-pressure shearing under controlled environmental conditions (pH, ionic strength) during microparticulation, may lead to further particle size reduction. For example, the average particle size of WP aggregates formed after heating at 80 °C for 1 h at pH 3.35 was $>10$ $\mu$m (*37*). Further, the average particle size of hydrated WP aggregates, which were prepared after heat-treating the protein dispersions at acidic pH and then freeze-drying and grinding the formed gels, was 25.3 $\mu$m (*22*). However, the average particle size of MWP produced at pH 7 in our study (*14*) was also around 10 $\mu$m. Further reduction of the particle size thus appears achievable at a lower pH at which the reactivity of thiol groups is suppressed. Newly formed species would thus have smaller particle size and likely greater reactivity in forming gels under different conditions, all of which would greatly enhance usage of MWP in the food industry.

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**Received for review March 14, 2010. Revised manuscript received April 26, 2010. Accepted May 06, 2010. The authors thankfully acknowledge the Faculty of Health, Engineering and Science, Victoria University, Melbourne, for financial and technical support and the Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia) for the provision of materials used in this research. We also thank Dairy Innovation of Australia Limited for partial financial assistance in the initial stages of the project.**