Properties of Gels Induced by Heat, Protease, Calcium Salt, and Acidulant from Calcium Ion-Aggregated Whey Protein Isolate

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ABSTRACT

Gelation that was induced by heat, protease, calcium salt, or acidulant from a solution of Ca^{2+}-aggregated whey protein was investigated by analyses of the rheological, textural, and microstructural properties of the gel. The addition of 40 mM $CaCl_2$ to 18% whey protein solution resulted in aggregation during 4 h of incubation at 45°C. The occurrence of aggregation was determined as increases in turbidity and in mean aggregate size. Hydrolysis by a protease from *Bacillus licheniformis* (1% enzyme to protein, wt/wt), a decline in pH by glucono-δ-lactone (1.5%, wt/vol), an increase in ionic strength with $CaCl_2$ (60 mM), or heat treatment (80°C for 30 min) all led to gelation of the aggregated whey protein solutions within 40 min. The gels formed differed widely in texture and rheological properties. The heat-induced gel was over 20 times stronger than the gels that were induced by protease from *Bacillus licheniformis*, glucono-δ-lactone, and $CaCl_2$. The heat-induced gel also showed significantly highest adhesiveness. The gels induced by $CaCl_2$ and glucono-δ-lactone had significantly higher cohesiveness than the gels induced by heat or enzyme.

The micrograph of the aggregated whey protein solution showed loose, irregular aggregates, which were reflected in gels induced by $CaCl_2$ or glucono-δ-lactone. The aggregates in the gels that were induced by heat or enzyme were larger than the parent aggregates. This difference may be due to fusion or to further aggregation of the parent aggregates during the inducement of gelation.

(**Key words**: aggregation of whey proteins, gelation, microstructure, calcium)

Abbreviation key: **BLP** = *Bacillus licheniformis* protease, **G'** = storage modulus, **GDL** = glucono-δ-lactone, **WP** = whey proteins, **WPS** = WP solution.

INTRODUCTION

The microstructures of whey protein (**WP**) or β-LG gels are assembled from either fine-stranded or particulate aggregates. The type of aggregate depends on the presence of salt or on pH value during thermal gelation (15, 16, 20). Particulate gels were formed at high ionic strength or at pH ranging from 4 to 6, and fine-stranded gels were formed at low ionic strength or outside this pH range (12, 16). However, WP or β-LG in solution could form aggregates at high ionic strength or at pH 4 to 6 without heat treatment (13, 22, 23).

The protein aggregates are the basic building materials for a gel network (21). The existence of protein aggregates in solution should facilitate gelation and affect the properties of the gel formed. Such external factors as enzymes, salts, and acids can cause heat-induced aggregates to form gels at low temperatures (20 to 40°C) (1, 9, 10, 17). However, it is not clear whether these factors can promote gelation of Ca^{2+}-induced WP aggregates. The objectives of this research were to investigate the gelation of the Ca^{2+}-aggregated WP that was induced by heat, enzyme, acid, or salt and to determine the textural, rheological, and microstructure properties of the resultant gels.

MATERIALS AND METHODS

Materials

Commercial whey protein isolate (BiPro, Davisco Intl. Inc., Le Sueur, MN) was used to prepare an 18% WP solution (**WPS**). The reagent grade $CaCl_2$ and the acidulant glucono-δ-lactone (**GDL**) were from Sigma Chemical Company (St. Louis, MO). The protease from *Bacillus licheniformis* (**BLP**) was from Novo Nordisk A/S (Bagsvaerd, Denmark).

Aggregation of WPS

A 100-ml portion of 18% WPS was centrifuged at $10,000 \times g$ for 1 h. The supernatant was incubated at

45°C with 40 mM CaCl$_2$. Samples were taken at fixed intervals during incubation to monitor changes in turbidity and development of aggregate size.

Turbidity

Each sample was diluted 50 times with distilled water, and turbidity was determined as the apparent optical density at 420 nm (18).

Aggregate Size

The development of CaCl$_2$-induced aggregate sizes was monitored by dynamic light scattering using a Microtrac Ultrafine Particle Analyzer (Leeds and Northrup Instruments, St. Petersburg, FL). Samples were diluted 50 times with distilled water. Each sample was then analyzed by dynamic light scattering for 3 min. The experimental determinations of turbidity and aggregate size were replicated twice.

Preparations of Various Gels

The aggregation of the proteins in 18% WPS was allowed to proceed for 60 min at 45°C in the presence of 40 mM CaCl$_2$. The aggregated solution was used as a raw material for heat, BLP, GDL, and CaCl$_2$ to induce gelation. Fifteen-milliliter samples were placed in 25-ml beakers, and BLP at a 1% ratio of enzyme to protein, 1.5% (wt/vol) GDL, or 60 mM CaCl$_2$ was added. Immediately after the additions, the samples were incubated in a 45°C water bath for 1 h for gelation. One 15-ml aliquot was also heated at 80°C for 30 min for gelation. Formed gels were immediately cooled to room temperature with tap water and stored at 5°C overnight before analysis. The experiments were replicated three times. Aggregated WPS (15 ml) without additives was incubated at 45°C up to 10 h as a control.

Transmission Electron Microscopy

The CaCl$_2$-aggregated 18% WPS was gelled by adding an equal volume of 4% molten agar at 45°C (7). The agar-gelled samples and four gels induced by heat, BLP, GDL, or CaCl$_2$ were examined by transmission electron microscopy according to the procedure of Otte et al. (19). Ultrathin sections (60 to 70 nm) were imaged and photographed using a JEOL 1200 EXII transmission electron microscopy (Peabody, MA) under an accelerating voltage of 80 kV.

Color of Gels

The surface reflectance of freshly prepared gels was measured with a Minolta CR-200 colorimeter (Ramsey, NJ) using the standard CIE (Paris, France) color system (L = whiteness, a = red to green, and b = yellow to blue). The sample in the beaker was placed on a black surface, and the reflectance of the circular surface was recorded in triplicate for each of three experiments.

Gel Hardness

Gel hardness was determined by a texture analyzer (model TA XT-2; Texture Technologies Corporation, Scarsdale, NY). The gels formed in the beaker were penetrated with a cylindrical probe of 12-mm diameter. A force-time curve was obtained at a crosshead speed of 60 mm/min for a 15-mm displacement. The resulting force-time curves were analyzed using texture profile analysis (2).

Rheological Properties

The formation of the four gels made of CaCl$_2$-induced aggregates was monitored using a dynamic oscillatory shear spectrometer (RFS II; Rheometrics, Inc., Piscataway, NJ). Rhios software (Rheometrics, Inc.) was used to program the instrument in a dynamic time sweep mode. Temperature was controlled at 45°C with an environment control circulator (Rheometrics, Inc.). Immediately after the addition of BLP, GDL, or CaCl$_2$ to the aggregated WPS, 2 ml of the mixture were placed in a cuvette, and 0.5 ml of mineral oil was applied to the surface of the sample to prevent evaporation. Oscillatory measurements were made every 2 min for 4 h at a strain of 1% and at a fixed frequency of 1 rad/s. For heat-induced gelation, the temperature was set to increase from 45 to 80°C at a rate of 5°C/min and to hold at 80°C for 23 min. The experiments were replicated twice.

RESULTS AND DISCUSSION

Ca^{2+}-Induced Aggregation

Turbidity and size of aggregates. The Ca^{2+}-induced aggregation of 18% WPS, determined as increase of turbidity and aggregate size, is shown in Figure 1. Both turbidity and aggregate size increased as incubation time increased after the addition of CaCl$_2$ (40 mM) at 45°C, indicating that the aggregation was a time-dependent process. Rapid increases in turbidity and mean aggregate size (from 8 nm to 1.35 μm) occurred during the initial 60 min. At the end of incubation (4 h), the mean aggregate size approached 1.84 μm. Although the determined mean aggregate size was large, no sedimentation was observed during the incubation or during the subse-

quent 2 d of storage at room temperature (21°C), suggesting that the formed aggregates were stable hydrocolloids. This phenomenon was also reported by Zhu and Damodaran (24) for CaCl$_2$-induced aggregation of WPS at room temperature (21°C). Changes in turbidity and aggregate size were not detected in the control WPS (without addition of CaCl$_2$) during a 4-h incubation at 45°C.

Microstructure of aggregates. Electron microscopy showed 150 to 500 nm of aggregate size from the WPS that was incubated with 40 mM CaCl$_2$ for 60 min (Figure 2); the light scattering method detected 1.35 μm of mean aggregate size for the same aggregated WPS (Figure 1). The micrograph of Ca^{2+}-induced aggregated WPS revealed that the Ca^{2+}-induced aggregates had loose and irregular structures. Some aggregate clumps also appeared in the image. The microstructure of Ca^{2+}-induced WP aggregates at low temperature has not been previously reported. However, Parris et al. (20) revealed a similar microstructure of dissolved aggregates from heated WPS in the presence of Ca^{2+}.
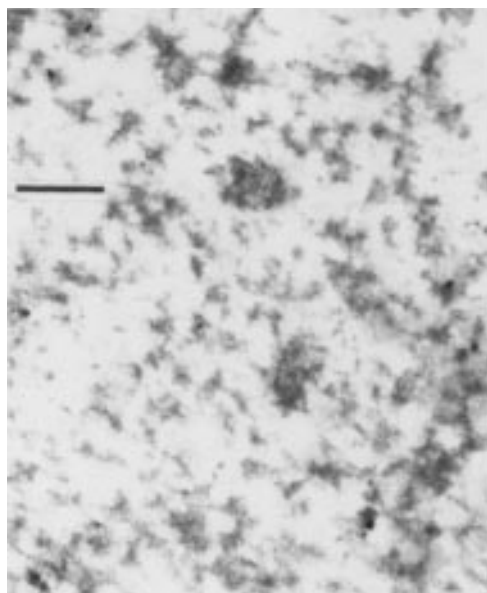
Appearance of Various Gels

Heat treatment or the addition of BLP, GDL, or CaCl$_2$ to the aggregated WPS all resulted in gelation.

Figure 2. Transmission electron micrograph of a solution of 18% Ca^{2+}-aggregated whey protein isolate. Bar = 500 nm.

The control sample of aggregated WPS without additives did not gel even after 10 h of incubation at 45°C.

The gels induced by heat, BLP, GDL, or CaCl$_2$ all were visibly shiny, white, and homogenous. The color readings revealed that the four gels had positive high L (whiteness) and b (yellow to blue) values and low negative a values (red to green; Table 1). The gels that were induced by heat treatment or BLP demonstrated higher ($P < 0.05$) L, a, and b values than did the gels induced by GDL or CaCl$_2$. This result suggests that the gels that were induced by heat or enzyme treatment reflected more light and had greater opacity. The gels that were induced by GDL and CaCl$_2$ did not differ ($P < 0.05$) in L, a, and b values, indicating that these two gels had similar opacity.

Textural Properties of Gels

From the same Ca^{2+}-aggregated WPS (18% WP), heat-induced gels were more than 20 times harder than the gels induced by BLP, CaCl$_2$, or GDL (Table 2). The enzyme-induced gel was harder ($P < 0.05$) than the gels induced by addition of salt or acid (Table 2). The heat-induced gel also showed the highest ($P < 0.05$) adhesiveness, but the lowest ($P < 0.05$) cohesiveness (Table 2). The gels induced by acid or salt were more cohesive ($P < 0.05$) than were the two other gels.

Differences in gel hardness may reflect the difference of interaggregate linkages, such as disulfide
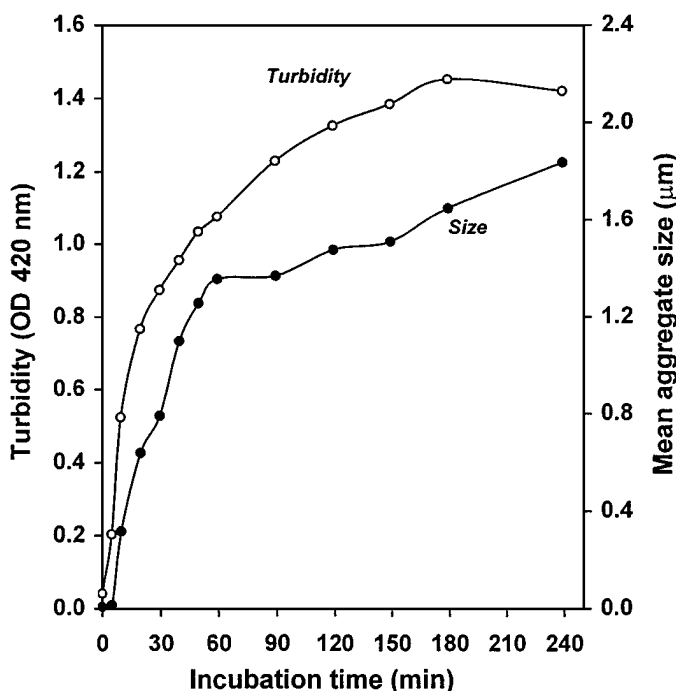

Figure 1. Turbidity (optical density at 420 nm) and mean aggregate size during a 4-h incubation of an 18% whey protein isolate solution with 40 mM CaCl$_2$ at 45°C.

TABLE 1. Color index of gels induced by heat, *Bacillus licheniformis* protease (BLP), $CaCl_2$, or glucono-δ-lactone (GDL) from a solution of 18% $CaCl_2$-aggregated whey protein isolate.

Color index[1]	Heat		BLP		$CaCl_2$		GDL	
	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE
L	88.74[a]	0.13	82.27[b]	1.07	70.03[c]	0.57	68.59[c]	0.69
a	−1.64[b]	0.27	−0.67[a]	0.08	−3.38[c]	0.05	−3.10[c]	0.02
b	10.62[a]	2.08	11.67[a]	0.32	5.42[b]	0.19	6.69[b]	0.11

[a,b,c]Means within a row followed by no common superscript differ ($P < 0.05$).

[1]As determined using the CIE (Paris, France) color system (L = whiteness, a = red to green, and b = yellow to blue).

crosslink, hydrogen bonds, and polar and hydrophobic interactions (11). Heat could promote the formation of covalent disulfide bonds, but enzyme, salt, or acid generally favor the formation of weak, noncovalent bonds between protein molecules or protein aggregates (11).

Rheological Properties

The gelation induced by enzyme, acid, or salt from $CaCl_2$-aggregated WPS was evidenced by an increase of storage modulus (G') and a change of phase angle (Figure 3). By definition of the gel point (or gel time), the aggregated protein changed suddenly from a viscous liquid to an elastic gel. Gel times, which were estimated by linear extrapolation of the rapidly rising G' to the intercept with the time axis (5), were approximately 5, 16, and 40 min for gelation induced by $CaCl_2$, BLP, or GDL (Figure 3A), respectively. The control aggregated WPS without additives showed a horizontal line during the 4 h of incubation (Figure 3A), indicating that no gel was formed.

The G' of the gel continuously increased with incubation time after the addition of $CaCl_2$ (60 mM) to the Ca^{2+}-aggregated WPS (Figure 3A), reflecting an increase in the elastic rigidity of the gel. The storage modulus of gelation that was induced by BLP increased more rapidly than those of gelation induced

by GDL and $CaCl_2$ (Figure 3). Finally, the enzyme hydrolysis resulted in a higher elastic modulus or a harder gel than the gels formed by the actions of $CaCl_2$ and GDL. Acidulation by GDL slowly increased G', which might have resulted from the release of Ca^{2+} and the disassociation of the Ca^{2+}-induced aggregates upon lowering pH, a phenomenon that is similar to the acidification of casein micelles (23). The rheological analysis (Figure 3) showed the same order of hardness as the texture analysis for the three gels (Table 2).

The phase angle (tan δ) is the ratio of G''/G' (where $G'' =$ loss modulus), which reflects the relative importance of viscous and elastic effects in the sample. The percentage change (Figure 3B) of tan δ showed a transition from a viscoelastic fluid to a viscoelastic gel. The gelation induced by BLP and $CaCl_2$ demonstrated a continuous decrease of tan δ, suggesting that the viscous component in the samples gradually decreased and that the elastic component increased with incubation time. The gelation that was induced by GDL showed a different pattern of transition from sol to gel; the tan δ initially increased and then slowly decreased with incubation time.

The addition of BLP resulted in rapid gelation (16 min) of the aggregated WPS, but 150 min of gel time were required for BLP-induced gelation from unaggregated WPS at the same protein concentration. The

TABLE 2. Texture parameters of gels induced by heat, *Bacillus licheniformis* protease (BLP), $CaCl_2$, or glucono-δ-lactone (GDL) from a solution of 18% $CaCl_2$-aggregated whey protein isolate.

Texture parameter	Heat		BLP		$CaCl_2$		GDL	
	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE	$\overline{X}$	SE
Hardness, kg	1.983[a]	0.113	0.097[b]	0.012	0.067[c]	0.014	0.058[c]	0.011
Cohesiveness	0.495[c]	0.073	0.536[b]	0.059	0.665[a]	0.098	0.615[a]	0.151
Adhesiveness	0.852[a]	0.034	0.123[b]	0.019	0.073[c]	0.021	0.044[c]	0.009

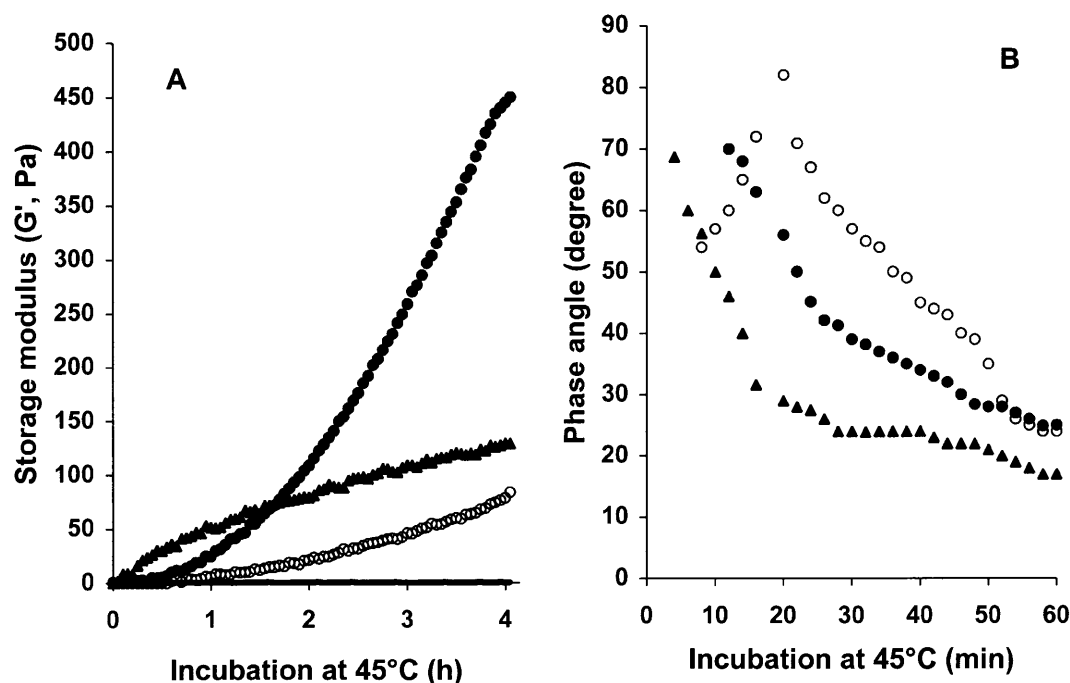[a,b,c]Means within a row followed by no common superscript differ ($P < 0.05$).

Figure 3. Changes in storage modulus (G') (A) and phase angle (B) during gelation induced by incubation of 18% CaCl$_2$-aggregated whey protein isolate (WPI) at 45°C for 60 min with 1% (enzyme to protein, wt/wt) *Bacillus licheniformis* protease (●), 60 mM CaCl$_2$ (▲), or 0.8% (wt/vol) glucono-δ-lactone (○). The modulus of a control aggregated WPI solution is shown as a solid line.

role of enzyme at this stage may have been to connect existing aggregates rather than starting to induce aggregates first (9).

Heat treatment of the aggregated WPS resulted in a rapid increase in storage modulus (Figure 4), reflecting the formation of an extremely rigid gel, which was consistent with our analysis of the texture (Table 2). As the temperature increased from 45 to 80°C at a rate of 5°C/min, the aggregated WPS gelled at 5.5 min (Figure 4), indicating that gelation occurred at 72.5°C, which was near the transition temperatures of WP (62 to 78°C, at pH 6.7; 15). The tan δ (percentage) rapidly decreased at an early stage and finally decreased to 18%, indicating the formation of a more elastic and less viscous gel (Figure 4).

The heat-induced gel (Figure 4) showed G' values approximately 80 times higher than those of gels induced by BLP, GDL, or CaCl$_2$ (Figure 3). This difference was three times higher than that determined by texture analysis, probably because these gels were sensitive to the regular shearing of the probe in the Rheometrics machine. The shear might frequently disrupt development of gel network cross-linking (18), which suggests that rheological analysis may not reflect the true elasticity and rigidity properties of these gels.

Microstructures of Gels

The TEM was used to study the three-dimensional structure of the aggregates in each gel and to determine how the aggregates were related to the parent aggregates.

Heat-induced gel. Aggregates in the heat-set gels (Figure 5A) were elliptical with regular edges, and size was around 250 to 450 nm. Similarly shaped and sized aggregates were reported in heat-induced 10% β-LG gel containing 15 mM CaCl$_2$ (16). These elliptical aggregates were tightly linked or fused together in clusters. A similar phenomenon was also reported for heat-coagulated gels of casein micelles (23). The closely joined aggregates led to big pores (voids) in the microstructure. The aggregates in the heat-set gel differed from the parent aggregates in size and shape (Figure 2), suggesting that the parent aggregates underwent fusion or further aggregation during heating.

BLP-induced gel. Aggregates in the enzyme-induced gel were loose, had irregular edges, and were less dense than were heat-induced aggregates (Figure 5B). Mean size (500 nm) was larger than those in the heat-induced gel and the parent solution. The aggregates were loosely connected to one another. Pores in micrographs were small and unclear. The
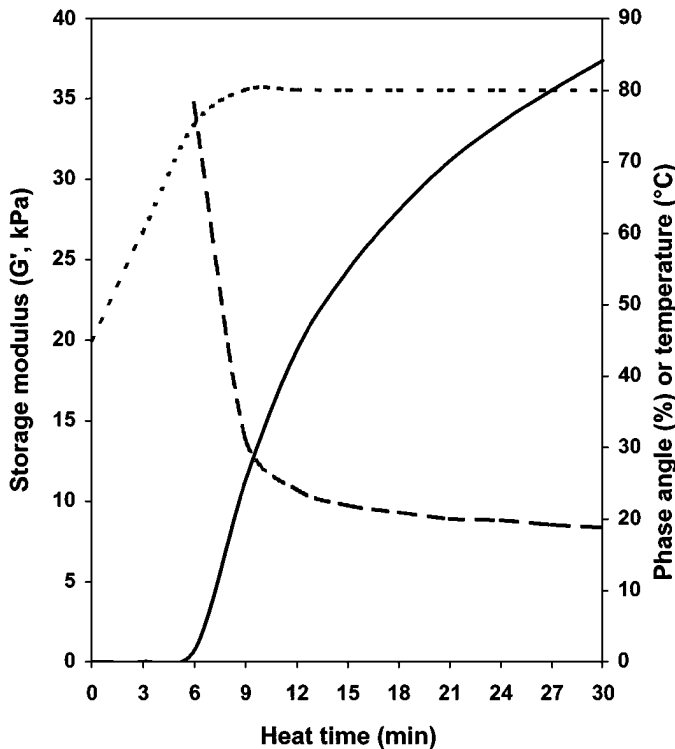
Figure 4. Changes in storage modulus (G'; solid line) and phase angle (dashed line) during gelation induced by heat treatment of CaCl$_2$-aggregated whey protein isolate (18%) from 45 to 80°C at a rate of 5°C/min. Dotted line indicates temperature.

three-dimensional structures of these aggregates were much different from those of BLP-induced aggregates, which were smaller (200 nm) and had a rigid structure (9).

CaCl$_2$-induced gel. Micrographs of the CaCl$_2$-induced gel (Figure 5C) were not stained because the whole image appeared totally dark after staining. The micrograph without staining, however, clearly shows the microstructure of aggregates (Figure 5C). The aggregates appeared spherical and were of relatively consistent sizes (200 to 300 nm). Interestingly, many small dark strips appeared on the edges of aggregates or on connected points of aggregates. These strips might be crystals of CaCl$_2$. Their appearance may be related to the CaCl$_2$ that had been added to induce gelation because the micrograph of the CaCl$_2$-aggregated protein solution did not reveal any such strips, nor did the three other gels. The crystal strips were probably involved in the formation of CaCl$_2$-induced gel.

GDL-induced gel. This gel was formed as a result of lowering the pH of aggregated WPS (from pH 6.6

to 5.2) via lactone hydrolysis. Micrographs of the pH-induced gel were composed of consistently sized and shaped aggregates (Figure 5D). The aggregates were irregular, and size was around 250 nm. The edges of the aggregates appeared to have protruding hairs, which might have been a result of a release of CaCl$_2$ from aggregates during pH-induced gelation (6).

The aggregates were loosely joined by strands. This microstructure differed from that of gels induced by heat treatment of the WP or β-LG solution at pH close to pI (12) in which aggregates appeared smooth, spherical, and larger.

Moreover, the three-dimensional structure of the aggregates in the four gels varied from elliptical to spherical and irregular. The aggregates in the microstructure of the four gels differed from the parent aggregates and reflected various modifications during inducements of gelation as was observed for casein micelles from gels or curds induced by heat, acid, enzyme, or salt (3, 6, 7, 8, 14, 23).

The formation of aggregates was an essential step for the gelation. Protease, acid, salt, or heat can independently induce WP to form aggregates (9, 18, 24). These factors also induced gelation from denatured WPS containing heat-induced aggregates. Any action, if it can induce the aggregation of WP molecules, possesses the potential to induce gelation from various WP aggregates by promoting bond formation among WP aggregates (4). This gelation process or phenomenon may be depicted as following formula:

$$P \xrightarrow{\text{i factor}} (P_A)_i \xrightarrow{\text{j factor}} (P_G)_{ij}.$$
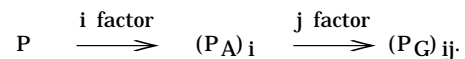
The mechanism of WP gelation supposes that native protein molecules (P) first form protein aggregates (P$_A$) under the action of the i factor. The protein aggregates could form a gel (P$_G$) via promotion of j factors. The i or j factors could be heat, enzyme, salt, acid, or other chemicals. Properties and microstructure of the formed gel are related to the factors inducing the aggregates and the gel. The protein aggregates were considered to be basic building materials for the gel network.

CONCLUSIONS

The addition of CaCl$_2$ (40 mM) to 18% WPS can lead to the formation of stable protein aggregates at 45°C. The aggregated WPS could be gelled by heat treatments, hydrolysis, lowered pH, or increased ionic

strength such as the coagulation (reactions) of casein micelles under the similar treatments. The formed gels differed greatly in rheological and textural properties. Heat-induced gels were more than 20 times stronger than the gels induced by the enzyme, acidulant, or calcium salt. The gels induced by enzyme were stronger ($P < 0.05$) than those induced by pH or salt ions. The gels induced by pH or cation ions had higher adhesive properties.

The microstructure of the Ca^{2+}-aggregated WPS was composed of irregular aggregates with a wide range of sizes (150 to 500 nm). The aggregates in the microstructures of the gels that were induced by GDL or $CaCl_2$ were similar to the aggregates in parent solution. The gels induced by heat or BLP were composed of larger aggregates than the parent aggregates, possibly as a result of fusion or hydration of the parent aggregates during the gelation.
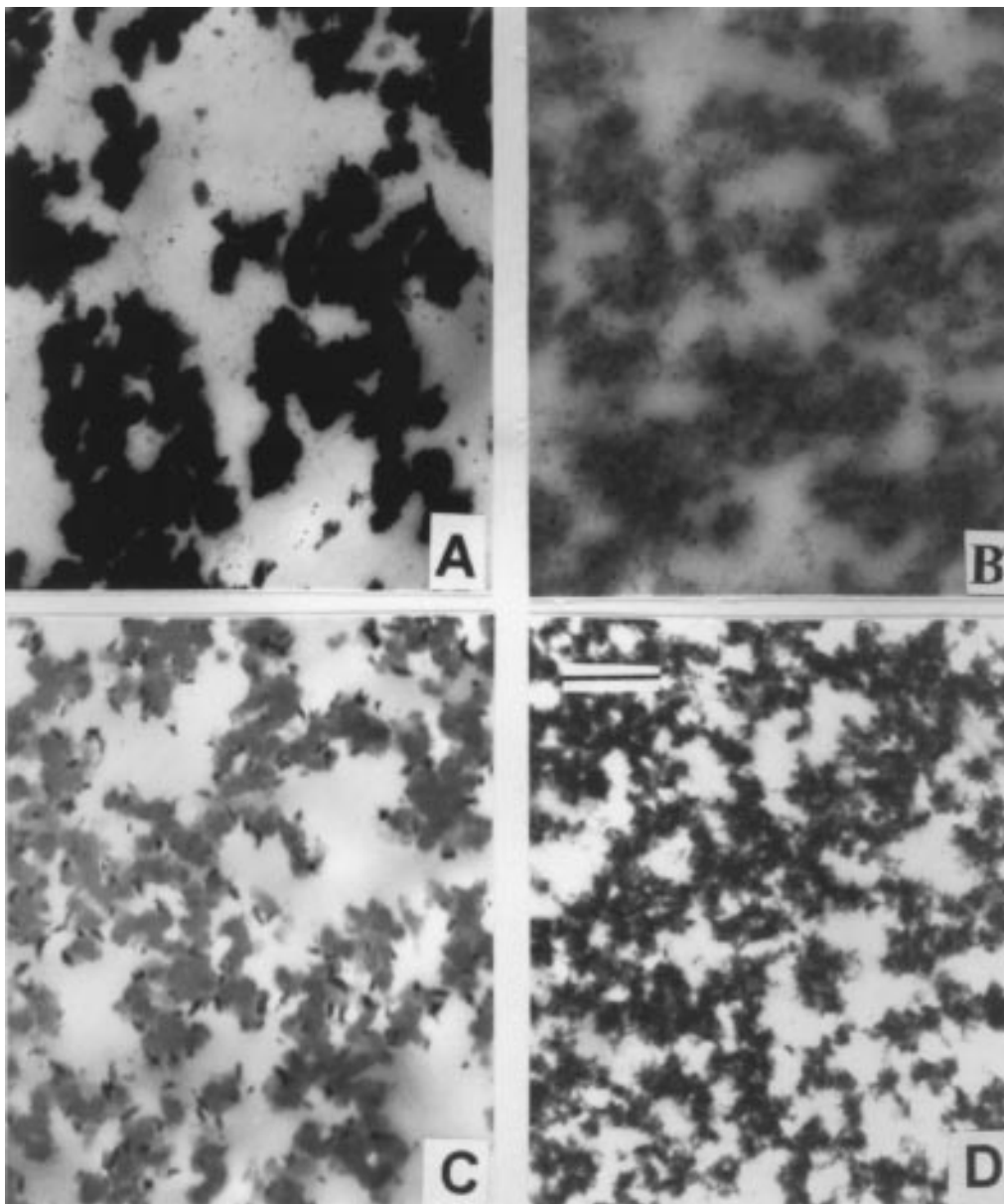

Figure 5. Transmission electron micrographs of four gels induced from a solution of 18% Ca^{2+}-aggregated whey protein isolate by heat treatment at 80°C for 30 min (A) and by incubation at 45°C for 60 min with 1% (enzyme to protein, wt/wt) *Bacillus licheniformis* protease (B), 60 mM $CaCl_2$ (C), or 0.8% (wt/vol) glucono-δ-lactone (D). Bar = 500 nm.

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