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Moisture-Induced Aggregation of Whey Proteins in a Protein/Buffer Model System

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Moisture-induced protein aggregation in a dry or intermediate-moisture food matrix can contribute to the loss of product acceptability. The present study evaluated the molecular mechanisms and controlling factors for moisture-induced whey protein aggregation in a premixed protein/buffer model system. Insoluble aggregates rapidly formed during the first 3 days of storage at 35 °C with a slower rate afterward. Evaluation of the insoluble aggregates by solubility tests in solutions containing SDS/urea/guanidine HCl/dithiothreitol and gel electrophoresis showed that the formation of intermolecular disulfide bonds was the main mechanism for protein aggregation, and all major whey proteins were involved in the formation of insoluble aggregates. Effects of various factors on aggregation were also investigated, including moisture content, medium pH, and the addition of NaCl. The dependence of aggregation on moisture content was bell-shaped, and the maximal extent of aggregation was achieved at a moisture content of around 70−80% on a dry weight basis.

KEYWORDS: Whey proteins; moisture; aggregation; disulfide bond; non-covalent interaction

INTRODUCTION

In foods, protein works not only as a nutrient but also as a class of building blocks to induce some global texture. The aggregation of protein molecules in a food matrix can result in dramatic changes in its functionalities. It is well-known that protein aggregation can be induced through exposure to thermal treatment (*1, 2*), high pressure (*3–6*), low pH (*7, 8*), and salts (*9, 10*).

Liu and others (*11*) made the seminal observation that in the solid amorphous state, moisture-induced protein aggregation can also occur as a function of moisture content and storage temperature. In their study, they suggested that moisture-induced protein aggregation of bovine serum albumin (BSA) could occur mainly by the formation of disulfide bonds through thiol−disulfide interchange as well as by non-covalent interactions. A following study on insulin from the same group (*12*) suggested that this moisture-induced protein aggregation could also result from the formation of intermolecular disulfide bonds by intermolecular thiol-catalyzed disulfide interchange following β-elimination of an intact disulfide bridge.

This moisture-induced aggregation can be influenced by many factors, such as protein sources, moisture content, storage temperature, pH, salt type, and content (*11–13*). The protein source itself has a significant effect on moisture-induced aggregation. At the same moisture content and storage temperature, different proteins could vary in molecular mobility and show different aggregation rates (*11, 13*). In addition, protein sources would also affect the possible mechanisms causing this aggregation, due to the variation in amino acid composition and tertiary structures (*11, 12, 14*). Previous studies on moisture-induced protein aggregation have been mainly focused on pharmaceutical proteins in the dry to intermediate-moisture state, due to their importance in dry inhalation drug formulations and delivery systems (*14, 15*). Stote and Feldman (*16*) in a review of solution and powder inhalation drug delivery systems for insulin noted that the only dry formulation so far approved by the U.S. FDA has led to abnormal lung function, possibly due to this aggregation. This lung abnormality disappears after discontinuance of the drug. Because of this the other protein inhalation drugs in phase III trials are also being tested to see if similar problems develop.

Whey proteins, in the form of whey protein isolates, whey protein concentrate, or whey protein hydrolysates, have been widely used as major ingredients for many foods, functional foods, and dietary supplement products. Whey proteins contain several component proteins, including β-lactoglobulin (LG), α-lactalbumin (LA), and BSA. LG is the main whey protein (about 50% of total whey proteins) and contains 1 free thiol group and 2 disulfide groups per monomer; LA represents about 20% of whey proteins and contains 4 disulfide groups; BSA represents about 10% of whey proteins and contains 1 free thiol group and 17 disulfide groups (*17*). The moisture-induced aggregation of whey proteins would possibly occur during the storage of a high-protein-containing food matrix such as a nutritional bar and affect the stability and shelf life. However,

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Table 1. Manufacturing Characteristics (Davisco Inc.) of Whey Protein Isolate (WPI)

sample	protein (% dry basis)	lactose (% dry basis)	fat (% dry basis)	pH[a]	a_w[b]	moisture[b] (g of H_2O/100 g of solid)
WPI	97.4	<0.1	0.3	7.2	0.133 ± 0.002	5.20 ± 0.17

[a] The pH was determined as a 10% protein solution at 20 °C. [b] a_w (by AquaLab 3TE water activity meter) and moisture (by Karl Fisher) were measured in the laboratory.

Table 2. Chemical Properties of Major Whey Proteins [a]

	MW	SH	S−S	p*I*
LG	18,300	1	2	5.2
LA	14,100	0	4	4.8
BSA	66,300	1	17	4.7

[a] Data from ref *17*. MW, molecular weight; SH, free thiol group; S−S, intramolecular disulfide bonds; LG, β-lactoglobulin; LA, α-Lactalbumin; BSA, bovine serum albumin.

little research has explored this moisture-induced effect on food proteins or the resulting chemical changes in the food matrix (*13*). Thus, the objectives of the present study were to investigate the moisture-induced whey protein aggregation in a protein/buffer model system and to evaluate its molecular mechanisms and controlling factors.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI, BioPRO) was obtained from Davisco Foods International, Inc. (Eden Prairie, MN). The characteristics of WPI were obtained from Davisco and are shown in **Table 1**, and the properties of three major whey proteins are shown in **Table 2**. The initial moisture content in WPI powder was determined by the Karl Fischer method using the Aquatest cma Karl Fischer Coulometric Titrator (Photovolt Co., Indianapolis, IN), and water activity was determined using the AquaLab 3TE water activity meter (Decagon Devices, Inc., Pullman, WA). The WPI powder is lactose-free and contains a very small amount of fat, so protein aggregation caused by the Maillard reaction or oxidation should be minimized. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Preparation of Premixed Protein/Buffer Model System. To investigate the moisture-induced aggregation in protein powders, one method was to incubate the protein powders in chambers using saturated-salt solutions to control the relative humidities (*11, 13*). However, for many protein powders such as LG, a high moisture content was needed to achieve the aggregation (*11*), which required a high environmental relative humidity and a long storage time for the protein powder to reach the equilibrium state (*18, 19*). In addition, given the high relative humidity, the growth of microorganisms on protein powders may be possible. Thus, in the present study, we used a premixed protein/buffer dough model system to achieve the high moisture content and ensure equilibrium protein hydration, with 0.05% sodium azide added to inhibit the growth of microorganisms.

The whey protein/buffer model system contained WPI and phosphate buffer, with sodium azide (0.05%, wet weight basis) being added to control microbial growth. To investigate protein aggregation and its mechanism, 4 g of phosphate buffer (10 mM, pH 7) was added into 6 g of WPI powder and mixed until a uniform dough texture was achieved. The model system was placed in a plastic water activity sample cup (Decagon Device, Inc.), tightly covered with the lid, and further double sealed with Parafilm completely around the cup/lid junction to avoid moisture loss. The sample cups were then placed into a sealed glass jar and equilibrated at room temperature for 30 min before being placed into an incubator at 35 °C.

Determination of Protein Aggregates. To determine protein aggregation as a function of storage time, the model system (with WPI/buffer ratio at 3:2) was stored at 35 °C and samples were taken at appropriate intervals (**Figure 1**). The solubility of the sample in phosphate buffer (10 mM, pH 7) was determined by adding 60 mg of sample into 10 mL of phosphate buffer. The suspension was stirred at room temperature at a speed of 400 rpm for 60 min and then centrifuged at 20000*g* for 15 min. The concentration of soluble proteins in the supernatant was then determined using the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL). The decrease in the amount of soluble fraction would suggest the denaturation of proteins and possible formation of insoluble protein aggregates, so the amount of insoluble protein aggregates formed during storage (percentage of total proteins) was calculated by using the total protein in the sample (grams) on the basis of the formula minus the protein content (grams) in the soluble protein fraction (converted to percentage of total proteins). The buffer soluble fractions were further examined by gel electrophoresis (native-PAGE and SDS-PAGE) on a PhastGel Gradient 8-25 (Amersham Biosciences, Piscataway, NJ) using PhastSystem (Amersham Biosciences) to determine whether there was an increase of high molecular weight fractions, which would be a sign of soluble aggregates.

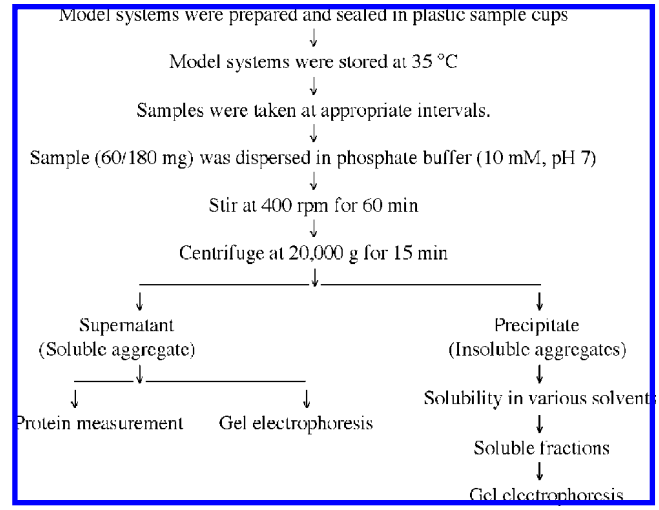

Figure 1. Outline of experimental steps in measuring moisture-induced aggregation.

Molecular Mechanisms of Aggregation. To determine the aggregation mechanisms, a 180 mg sample (after storage for 3 weeks at 35 °C) was dissolved in 10 mL of buffer, followed by stirring and centrifugation. The insoluble aggregates were dissolved in various solutions: phosphate buffer solution alone (works as a control); buffer solution with strong denaturants (0.1% SDS, 6 M guanidine HCl, or 8 M urea, to determine the non-covalent interactions including hydrophobic interaction and hydrogen bonding); buffer solution with reducing regents [10 mM dithiothrietol (DTT), to determine intermolecular disulfide bond formation)], and buffer solution with both strong denaturants and reducing regents (0.1% SDS and 10 mM DTT). The suspension was stirred at a speed of 400 rpm for 60 min and then centrifuged at 20000*g* for 15 min. The protein concentration dissolved in the different solutions was determined using the BCA Protein Assay Kit, and the BCA assay in the presence of DTT was conducted according to the modified method of Hill and Straka (*20*) to minimize the influence of reducing reagent. The soluble fractions of protein aggregates in different solvents were further investigated by gel electrophoresis on a PhastGel Gradient 8-25 (Amersham Biosciences).

Controlling Factors of Moisture-Induced Protein Aggregation. To determine the effect of moisture content on protein aggregation, model systems with different WPI/buffer ratio (5:3, $a_w = 0.967$; 3:2, $a_w = 0.978$; 4:3, $a_w = 0.981$; 5:4, $a_w = 0.983$; 10:9, $a_w = 0.985$; 1:1, $a_w = 0.986$; or 5:6, $a_w = 0.988$) were prepared. The water activity (a_w) of model systems was determined using the AquaLab 3TE water activity meter. To determine the effect of buffer pH on protein aggregation, model systems (the ratio of WPI/buffer was 3:2) made

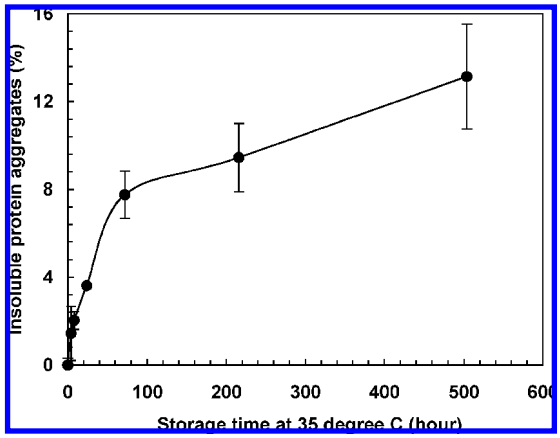

Figure 2. Formation of insoluble protein aggregates as a function of time during storage at 35 °C.

from 10 mM buffers with various pH values (4, 4.5, 5, 5.5, 6, 7, 7.5, or 8) were prepared. To determine the effect of NaCl on protein aggregation, model systems (the ratio of WPI/buffer was 3:2) made from buffers with various concentrations of NaCl (0, 0.05, 0.15, 0.3, or 0.45 M) at pH 4, 5.5, or 7 were prepared.

All model systems were stored at 35 °C, and samples were tested after 1 week of storage. The solubility of samples in phosphate buffer was measured to determine the formation of insoluble aggregates; and the solubility of protein aggregates in various solutions (buffer, buffer with SDS, and buffer with DTT) was also measured. The soluble fractions of protein aggregates in different solvents were further investigated by gel electrophoresis on a PhastGel Gradient 8-25 (Amersham Biosciences).

RESULTS AND DISCUSSION

Moisture-Induced Whey Protein Aggregation. Protein aggregates formed during storage may include two types: soluble aggregates and insoluble aggregates. **Figure 2** shows the formation of insoluble aggregates in the whey protein/buffer model system (the ratio of protein/buffer was 3:2, $a_w = 0.978$) as a function of storage time. During storage at 35 °C, the formation of insoluble aggregates was much faster during the first 3 days (72 h), and the total amount of insoluble aggregates continued to increase during 3 weeks of storage (504 h).

In dilute solutions, both soluble aggregates and insoluble aggregates form (*2, 5, 6*). To investigate the formation of soluble aggregates in the model system, the soluble fractions in phosphate buffer were determined by native-PAGE (**Figure 3A**), SDS-PAGE without added reducing reagent (**Figure 3B**), and SDS-PAGE with reducing reagent (**Figure 3C**). In native-PAGE, all three major protein bands (BSA, LG, and LA) were detected, and the intensity of these bands decreased over storage time. There were no clear protein aggregate bands detected in native-PAGE, and it is either because there was no significant amount of soluble aggregates formed during storage or because the soluble aggregates may be too big to get into the gel matrix. Similar protein patterns were found in the SDS-PAGE without any added reducing reagent, except that a protein band (the one labeled with *) with a molecular mass of around 200 kDa was found (**Figure 3B**). This band (the one labeled with *) was also found in the soluble fraction of the WPI control (**Figure 3B**) and disappeared in the presence of reducing reagent (**Figure 3C**), which suggests that it was not a result of aggregation during the storage of the model system but rather was formed during manufacturing of the isolate.

Molecular Mechanism of Aggregation. At least two possible mechanisms were proposed for moisture-induced protein aggregation: (1) formation of intermolecular covalent bonds and/or (2) formation of non-covalent interactions (*14*). **Table 3** shows the solubility of the separated protein aggregates in different solvents. After the additon of 0.1% SDS, 6 M guanidine HCl, or 8 M urea, the solubility of aggregates increased slightly compared with the control phosphate buffer. This suggested that neither hydrophobic interactions nor hydrogen bond formation was the major factor causing protein aggregation. After the

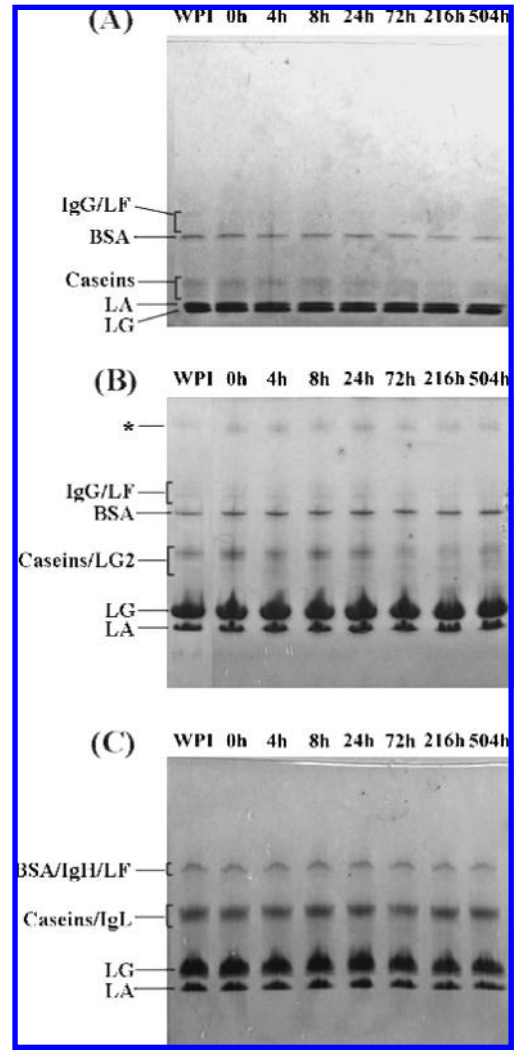

Figure 3. Gel electrophoresis of soluble fractions in a protein/buffer system stored at 35 °C: (**A**) native-PAGE; (**B**) SDS-PAGE without reducing; (**C**) SDS-PAGE with reducing. WPI is the native whey protein isolate powder, which serves as a control; 0−504 h on the top of gel pictures represents the storage time at 35 °C. Protein bands: LG, β-lactoglobulin; LG2, the dimer of β-lactoglobulin; LA, α-lactalbumin; BSA, bovine serum albumin; IgG, immunoglobulin G; IgH, immunoglobulin G heavy chain; IgL, immunoglobulin G light chain; LF, lactoferrin.

Table 3. Solubility of WPI Aggregates[a] in Various Solutions with Denaturing or Reducing Chemicals

solution	protein aggregate solubility (%)
buffer (10 mM, pH 7)	4.4 ± 0.6
buffer with 0.1% SDS	8.2 ± 0.8
buffer with 6 M guanidine HCl	10.9 ± 0.7
buffer with 8 M urea	11.6 ± 1.7
buffer with 10 mM DTT	92.2 ± 0.9
buffer with 0.1% SDS and 10 mM DTT	97.1 ± 1.7

[a] The insoluble aggregates refer to those formed after storage at 35 °C for 3 weeks (504 h).

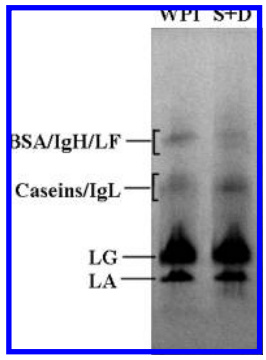

Figure 4. SDS-PAGE of the fractions of protein aggregates (after 3 weeks of storage at 35 °C) that dissolved in solution containing denaturing and reducing chemicals. WPI is the native WPI powder that directly dissolved in buffer containing 0.1% SDS and DTT, which serves as a control, and S+D is the soluble fractions of the formed insoluble protein aggregates (after 3 weeks of storage at 35 °C) in buffer containing 0.1% SDS and DTT. Protein bands: LG, β-lactoglobulin; LA, α-lactalbumin; BSA, bovine serum albumin; IgH, immunoglobulin G heavy chain; IgL, immunoglobulin G light chain; LF, lactoferrin.

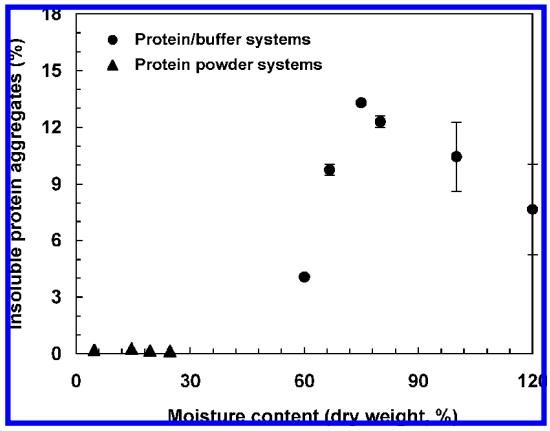

Figure 5. Effect of moisture content on moisture-induced whey protein aggregation. The data developed in protein/buffer system were obtained after storage of the model systems at 35 °C for 1 week, whereas the data developed in protein powder systems were obtained after storage of the whey protein isolate powders at different relative humidities for 2 weeks at 35 °C (*13*).

addition of 10 mM DTT, the solubility of the protein aggregates increased to >90%, which indicates that the formation of intermolecular disulfide bonds played an important role in protein aggregation. After combination of 0.1% SDS with 10 mM DTT, nearly all of the aggregates were dissolved. The dissolved fraction of insoluble protein aggregates in the buffer containing both SDS and DTT was further investigated by SDS-PAGE (**Figure 4**). In **Figure 4**, the first lane (WPI) is the native WPI powder directly dissolved in phosphate buffer containing SDS and DTT, which served as a control; the second lane (S+D) is the soluble fractions of protein aggregates of the stored model system (35 °C for 3 weeks) after dissolving in buffer containing SDS and DTT. If most of the protein aggregates that formed during storage dissolved in the buffer containing SDS and DTT (**Table 3**), the protein patterns in lane S+D should illustrate the protein fraction pattern of the aggregates. The bands of all three major whey proteins (LG, LA, and BSA) were observed in the SDS-PAGE (**Figure 4**) and had a similar density ratio compared with the WPI control in lane 1, suggesting that all three major proteins participated in moisture-induced aggregation.

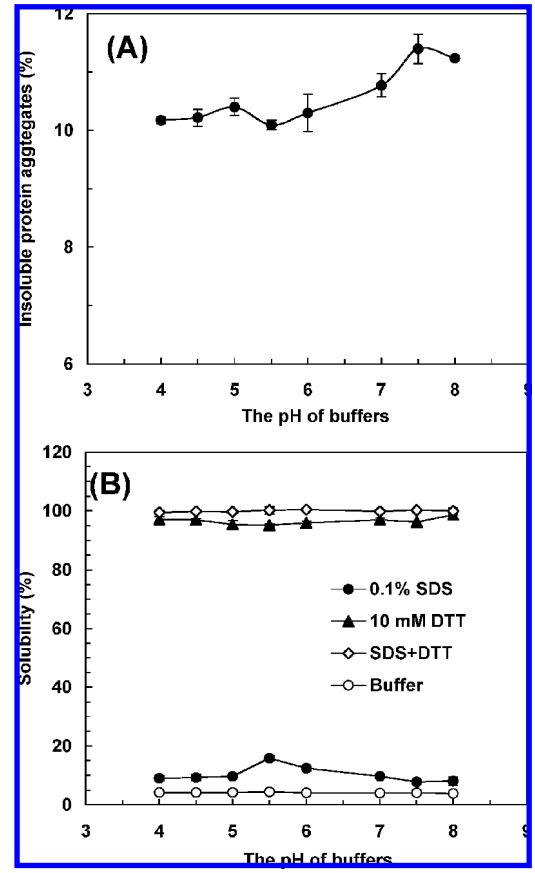

Figure 6. Effect of buffer pH on moisture-induced whey protein aggregation after storage at 35 °C for 2 week (168 h): (**A**) formation of insoluble aggregates in model systems made with buffers at various pH values; (**B**) solubility of the formed protein aggregates in solutions containing denaturing and/or reducing chemicals.

The literature suggests that the molecular mechanisms responsible for moisture-induced aggregation vary among proteins. Proteins such as BSA and LG undergo moisture-induced aggregation predominantly via intermolecular thiol−disulfide exchange, whereas other proteins such as ovalbumin and glucose oxidase formed aggregates mainly through non-covalent interactions (*11*). In some cases, the formation of covalent bonds was not via thiol−disulfide exchange but either by the reaction of lysine with the carbonyl group of asparagine or glutamine or the reaction with dehydroalanine derivatives formed after β-elimination of the disulfide groups (*21, 22*).

Effect of Moisture Content on Aggregation. The moisture content was found to be a very important factor for the moisture-induced aggregation (**Figure 5**). Our previous study on whey protein powder suggested that below 30% moisture content (dry weight basis, $a_w \sim 0.84$), no protein aggregation would be observed at temperatures at ≤35 °C (*13*). By increasing the moisture content in the protein/water system, the mobility of protein molecules increases and whey proteins began to form aggregates during storage (**Figure 5**). The maximal extent of aggregation in the model system was reached at a moisture content of around 70−80% (dry weight basis), but above this moisture range, the dilution effect of water becomes more important, and protein aggregation was slowed. In fact, the amount of water required to achieve the maximal extent of aggregation varies from protein to protein. For BSA powder, the maximal extent of aggregation was reached at 30% (dry weight basis) moisture content, but for lyophilized LG powder, it was reached at around a 1:1 ratio of protein/water (*11*).

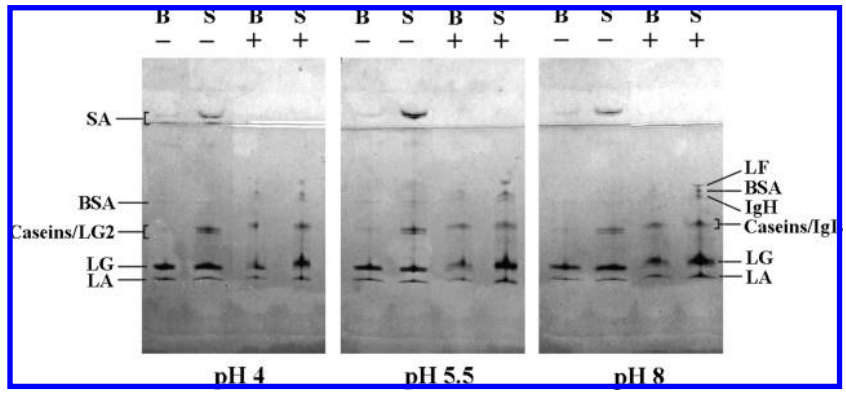

Figure 7. Effect of buffer pH on the SDS-PAGE pattern of soluble fractions of protein aggregates (formed after storage at 35 °C for 1 week) in buffer control and buffer containing 0.1% SDS. B, soluble fraction in control buffer; S, soluble fraction in buffer containing 0.1% SDS; −, SDS-PAGE without reducing reagent; +, SDS-PAGE with reducing reagent. The pH value on the bottom of gel pictures represents the buffer pH value of the model systems. Protein bands: SA, large polymers in soluble fractions; LG, β-lactoglobulin; LG2, dimer of β-lactoglobulin; LA, α-lactalbumin; BSA, bovine serum albumin; IgG, immunoglobulin G; IgH, immunoglobulin G heavy chain; IgL, immunoglobulin G light chain; LF, lactoferrin.

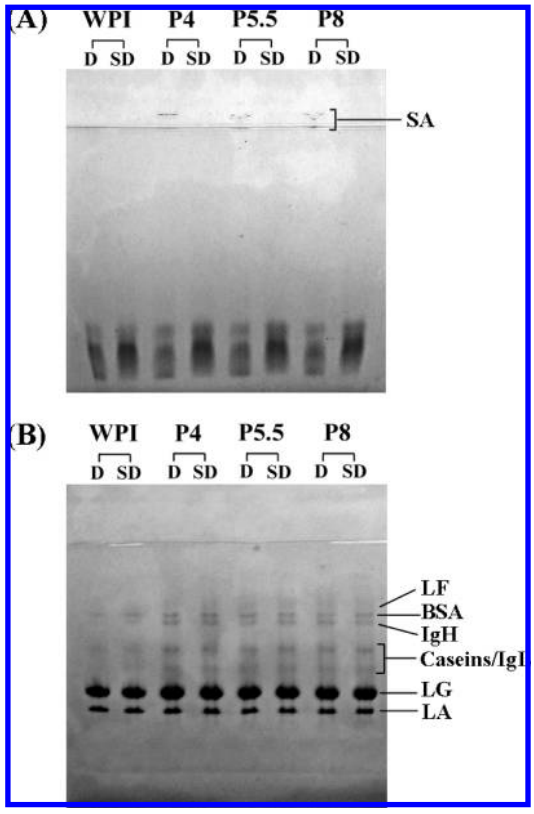

Figure 8. Effect of buffer pH on the gel electrophoresis pattern of soluble fractions of protein aggregates (formed after storage at 35 °C for 1 week) in buffer containing DTT and buffer containing both DTT and SDS: (**A**) native-PAGE; (**B**) SDS-PAGE. WPI, native whey protein isolate powder; P4, P5.5, P8, protein aggregates formed in the model systems with buffer pH at 4, 5.5, or 8; D, soluble fractions of protein aggregates in buffer containing 10 mM DTT; SD, soluble fractions of protein aggregates in buffer containing 0.1% SDS and 10 mM DTT. Protein bands: SA, large polymers in soluble fractions; LG, β-lactoglobulin; LA, α-lactalbumin; BSA, bovine serum albumin; IgH, immunoglobulin G heavy chain; IgL, immunoglobulin G light chain; LF, lactoferrin.

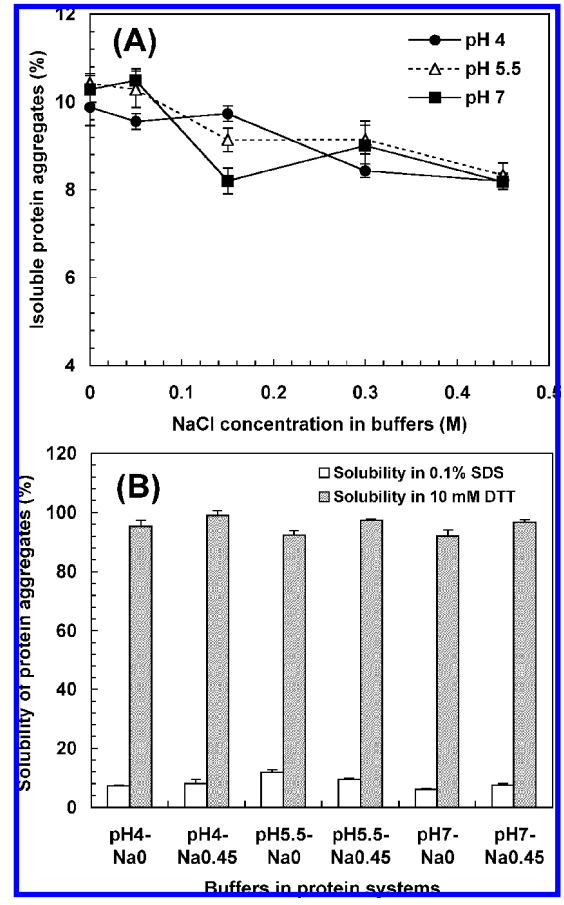

Figure 9. Effect of the addition of NaCl on the moisture-induced whey protein aggregation after storage at 35 °C for 1 week: (**A**) formation of insoluble aggregates in model systems containing buffers with various concentrations of NaCl at different buffer pH values; (**B**) solubility of aggregates (formed at model systems with different buffer pH values and NaCl concentrations) in different solutions. pH 4, pH 5.5, pH7, buffer pH at 4, 5.5 and 7; Na0, Na0.45, NaCl concentration in buffer at 0 and 0.45 M.

Effect of pH on Aggregation. Figure 6A shows the formation of insoluble aggregates in model systems made with buffers at different pH values and suggests that the amount of aggregates increased slightly when the buffer pH increased above 7. During the thiol−disulfide interchange, the thiolate anion instead of the thiol group is the reactive form. Below pH 7, the ratio of thiolate anion to thiol group (reactive form to nonreactive form) was low, which slowed the formation of intermolecular disulfide bonds and resulted in less formation of aggregates during storage (**Figure 6A**). Liu and others (*11*) have also shown that the pH of protein aqueous solutions from which the protein powders was lyophilized was critical for the

aggregation of BSA during storage. It was observed that at high pH (around 10), insulin formed aggregates mainly through the formation of free thiols from existing disulfide bonds, followed by intermolecular disulfide exchange participated in by these newly formed free thiols (*12*). However, at neutral pH (around 7), the aggregation of insulin occurred mainly via non-covalent interactions (*12*). The effect of pH on the changes in whey protein aggregation mechanisms is shown in **Figure 6B**. The buffer pH in the investigated model system ranged from 4 to 8. For all of these systems, >90% of the aggregates dissolved in the solvent containing DTT, suggesting the formation of disulfide bonding was the major driving force causing protein aggregation no matter what the buffer pH was. When the buffer pH of model system was at 5−6 (around the p*I* values of major whey proteins, **Table 2**), the non-covalent interactions played a more important role in protein aggregation than those at other pH values, and about 16% of the insoluble aggregates dissolved after adding SDS, compared with only 8% at other pH values.

Although after the addition of SDS or DTT, some aggregates became soluble (**Figure 6B**), it was not clear whether these soluble fractions still contained some large polymers that formed by intermolecular covalent bonds or non-covalent interactions. Therefore, we further determined the soluble fractions of aggregates by gel electrophoresis (**Figures 7** and **8**). **Figure 7** shows the protein patterns of soluble fraction of aggregates in the control buffer and buffer containing 0.1% SDS. It is suggested that in the SDS-PAGE nonreducing analysis, the soluble fractions in the control were mainly LG and LA bands (lane B−), whereas those in 0.1% SDS showed a similar amount of LG and LA but more of large polymers (SA band in line S−). However, in SDS-PAGE with reducing reagent, the large polymer band disappeared, and the single-protein bands (LG, LA, BSA, and etc.) in 0.1% SDS soluble fractions (line S+) had a larger amount of protein compared to those in the buffer control (lane B+). The above results suggest that the soluble fractions of aggregates in SDS solvent still contained a significant amount of large polymers that were formed by intermolecular disulfide bonds.

Figure 8 shows the protein patterns of the soluble fraction of the aggregates in buffer containing DTT and buffer containing both DTT and SDS. In native-PAGE (**Figure 8A**), a large polymer band (SA) was observed in the DTT soluble fractions (lane D), but not in the SDS+DTT soluble fraction (lane SD). In SDS-PAGE (**Figure 8B**), the polymer band in DTT soluble fractions that was observed in the native-PAGE disappeared, which suggests that those large polymers formed mainly by non-covalent interactions. These results suggest that both intermolecular disulfide bonding and non-covalent interactions contribute to whey protein aggregation. The formation of intermolecular disulfide bonds was the major driving force for this moisture-induced whey protein aggregation at the experimental conditions, with the non-covalent interactions playing a minor role. It is possible in some cases that the non-covalent interactions first form non-covalent linked aggregates, which might further initiate disulfide bond formation in these protein aggregates.

Effect of Adding NaCl on Aggregation. Figure 9 shows the formation of insoluble aggregates in model systems made from buffer with various NaCl concentrations at different buffer pH values. The amount of aggregates decreased with increases in NaCl concentration at all three pH values (**Figure 9A**). A storage study of BSA also suggested that adding NaCl would slow aggregation (*11*). The formation of intermolecular disulfide bonds was the main mechanism causing protein aggregation, independent of the NaCl concentration (**Figure 9B**). Buffer with DTT could dissolve most of the protein aggregates, whereas only <15% of aggregates could be dissolved in buffer with SDS.

In conclusion, whey proteins tended to form insoluble aggregates in the protein/buffer model system. The formation of intermolecular disulfide bonds was the main driving force for aggregation. Factors such as moisture content, buffer pH, and the addition of NaCl affect the degree of aggregation, but in this model system, the changes in molecular mobility resulting from moisture content had a more significant effect than the medium pH and the presence of NaCl. Future studies are needed to determine changes in the structure and texture of model systems resulting from moisture-induced protein aggregations and possible methods to reduce this moisture-induced deleterious effect on food proteins.

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