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Effects of Moisture-Induced Whey Protein Aggregation on Protein Conformation, the State of Water Molecules, and the Microstructure and Texture of High-Protein-Containing Matrix

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Moisture-induced protein aggregation through intermolecular interactions such as disulfide bonding can occur in a high-protein-containing food matrix during nonthermal processing and storage. The present study investigated the effect of moisture-induced whey protein aggregation on the structure and texture of such high-protein-containing matrices using a protein/buffer model system. Whey proteins in the protein/buffer model systems formed insoluble aggregates during 3 months' storage at temperatures varying from 4 to 45 °C, resulting in changes in microstructure and texture. The level of aggregation that began to cause significant texture change was an inverse function of storage temperature. The protein conformation and the state of water molecules in the model system also changed during storage, as measured by differential scanning calorimetry and Fourier transform infrared spectroscopy. During storage, the model system that had an initially smooth structure formed aggregated particles (100−200 nm) as measured by scanning electron microscopy, which lead to an aggregation network in the high-protein-containing matrix and caused a harder texture.

KEYWORDS: Whey protein; moisture-induced aggregation; protein conformation; structure; texture

INTRODUCTION

Whey proteins have been widely used as a major ingredient for many food and dietary supplement products such as nutritional bars, infant formula, and protein and/or energy drinks. The major components of whey proteins belong to globular proteins with hydrophobic groups located inside the globular structure (*1*). During food processing and storage, whey proteins are liable to denaturation followed by aggregation (*2–11*). The aggregation of proteins in a food matrix can result in dramatic changes in the global structure and texture. In dilute solutions, the denaturation of whey proteins causes the formation of soluble (*2, 4, 8*) and insoluble aggregates (*2, 8*) through intermolecular disulfide bonding and/or noncovalent interactions (*7*) and will result in the increases in turbidity and precipitation in dairy beverages during processing and storage. At high protein concentrations, a gel network will form. The structure and texture of whey protein gels depend on the composition and concentration of whey proteins and other factors such as temperature, pressure, pH, and ionic strength (*3, 6, 9, 12*). In general, whey proteins form either a particulate gel network or a fine-stranded (or filamentous) gel network (*13, 14*). The particulate gels appear opaque and contain large aggregates (from several hundred to several thousand nanometers), while the fine-stranded gels are transparent and contain "flexible strand or more rigid fibrils" (*13, 14*).

During a study of the stability of dry protein powders for pharmaceutical applications, Liu and others (*15*) observed that in a solid amorphous state, moisture-induced protein aggregation could occur during storage at temperatures that are much below the protein denaturation temperature. It was postulated that this occurs mainly by the formation of both disulfide bonds through thiol−disulfide interchange and by noncovalent interactions (*15, 16*). This has resulted in chronic lung dysfunction for the only inhalation dry powder insulin drug on the market (*17*). This moisture-induced protein aggregation should also be very critical in the storage and handling of food protein powders (*15, 16, 18*) and may also be a cause of deleterious changes in product quality for high-protein-containing food products like soft-textured intermediate moisture ($a_w < 0.95$) protein nutrition bars.

In a former study, we investigated the mechanisms of whey protein aggregation in a protein/buffer model system and its controlling factors (*19*). The objectives of this study were to investigate the effects of moisture-induced whey protein aggregation on the changes in protein conformation, the states of water molecules, and the microstructure and texture of high-protein-containing food matrix using a whey protein/buffer model system.

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MATERIALS AND METHODS

Materials. Whey protein isolate (WPI, BioPRO) was obtained from Davisco Foods International, Inc. (Eden Prairie, MN). The WPI powder contained less than 0.1% of lactose (dry basis) and less than 0.3% of fat (dry basis), and it was placed in desiccators containing dry anhydrous calcium sulfate (W.A. Hammond Drierite Co. Ltd., Xenia, OH) for 2 weeks before use. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Preparation of Premixed Protein/Buffer Model System. The whey protein/buffer model system contained WPI and phosphate buffer (10 mM, pH 7), with sodium azide (0.05%, wet weight basis) being added to control microbial growth. Phosphate buffer (4 g) was combined with 6 g of dry WPI powder and mixed until a uniform dough texture was achieved. The premixed model system was placed in a plastic water activity sample cup (Decagon Device, Inc., Pullman, WA) that was tightly covered with the lid and further double sealed with Parafilm completely around the cup/lid junction to avoid moisture loss. The sample cup was then placed into a sealed glass jar and equilibrated at room temperature for 30 min before being placed into an incubator at the controlled test temperatures (4, 23, 34, and 45 °C).

Determination of Insoluble Protein. The formation of insoluble protein was determined by the solubility of sample in phosphate buffer (10 mM, pH 7). A 60 mg sample was added 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). Any decrease in the amount of soluble fraction would suggest the formation of insoluble protein.

Different Scanning Calorimeter (DSC). The measurements were done on duplicate samples using a Perkin-Elmer model DSC 7 (Perkin-Elmer, Norwalk, CT) that was calibrated using distilled water and indium for temperature and indium for energy. Two DSC scans were applied to each sample: The first scan was run to determine the melting of crystallized water molecules, and the second scan measured protein denaturation. All sample pans were weighed before and after experiments to confirm no losses of water. In the water-melting scan, samples were cooled rapidly to −40 °C and then heated from −40 to 10 °C at a heating rate of 2 °C/min (*20*). The melting temperature was determined as the onset temperature of the endothermic peak (note in all figures that the endotherm is up). For most samples, two melting peaks were found (**Figure 1A**). Melting peak 2 refers to water in state A, which melts at ∼0 °C, that is, what would be expected of bulk water with little to no colligative solutes in solution (very low molality). Melting peak 1 refers to water in state B, which is freezable at −40 °C but melts over the temperature range from −40 °C to just below 0 °C. Besides the water in states A and B, there may also be water that is nonfrozen (state C) at <−40 °C, mostly due to the strong hydrophilic interactions between the water and the hydrophilic groups on the protein molecules. To calculate the percentage of water in the various states, the endothermic enthalpy (J/g sample) of the ice-melting peaks was recorded. Given the total water content determined by the Karl Fischer method using the Aquatest cma Karl Fischer Coulometric Titrator (Photovolt Co., Indianapolis, IN), the endothermic enthalpy of melting peaks was further calculated to give a value as J/g water:

$$\Delta H\ (\text{J/g water}) = \Delta H\ (\text{J/g sample})/\text{water content}\ (\text{g water/g sample}) \quad (1)$$

The percentage of water in state A (W_A, %) or state B (W_B, %) was then calculated by dividing the endothermic enthalpy of melting peak 2 or 1 by the heat of fusion of ice (334 J/g water), and the percentage of water in state C (W_C, %) was calculated by subtracting W_A and W_B from 100%:

$$W_A\ (\%) = \Delta H \text{ of peak 2 (J/g water)}/334\ (\text{J/g water}) \times 100\% \quad (2)$$

$$W_B\ (\%) = \Delta H \text{ of peak 1 (J/g water)}/334\ (\text{J/g water}) \times 100\% \quad (3)$$

$$W_C\ (\%) = 100\% - W_A - W_B \quad (4)$$

In the protein denaturation scan, samples were cooled to 10 °C and then heated from 10 to 100 °C at a heating rate of 5 °C/min. The denaturation temperature was recorded as the peak temperature of the endothermic peak, and the total denaturation enthalpy (unit, J/g sample) was recorded (**Figure 1B**). Given the total protein content in samples, the endothermic enthalpy of denaturation peaks was further calculated to give a value as J/g dry protein:

$$\Delta H\ (\text{J/g dry protein}) = \Delta H\ (\text{J/g sample})/\text{protein content}\ (\text{g dry protein/g sample}) \quad (5)$$

Fourier Transform Infrared Spectroscopy (FTIR). FTIR spectra were collected from 1600 to 1700 cm^{-1} at a resolution of 2 cm^{-1} using a Nicolet 560 FTIR spectrometer (Nicolet instrument Corp., Madison, WI), with a ZnSe ATR crystal accessory (Thermo Spectra-Tech, Madison, WI), which was used for collecting the infrared spectra. Samples that were stored at various temperatures were transferred to 23 °C and equilibrated for 2 h before the measurements. The samples were stable and did not exhibit any obvious change during the examination period between sampling (at various temperatures) and subsequent incubation and FTIR measurements at 23 °C. A background spectrum of the ZnSe crystal was recorded before the sample measurement, and a total of 400 scans were accumulated to define each spectrum. The collected spectra were smoothed using a nine-point Savitsky−Golay method, and deconvolution of the spectra was then performed using the Nicolet FTIR OMNIC software (OMNIC E.S.P., Nicolet Instrument Corp.). The bandwidth used for deconvolution was 18 cm^{-1}, with a narrowing factor of 3. All FTIR experiments were done in triplicate.

Scanning Electron Microscopy (SEM). Changes in microstructure were determined using a Hitachi S3500N scanning electron microscope (Hitachi High-Technologies Corp.). Samples were quick frozen in liquid N_2, transferred to a cryo-prechamber (Emiteck K-1150, Houston, TX), and sputter coated with gold before scanning.

Texture Measurement. Changes in texture were measured using a TA-XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) with a cylindrical plunger (1/8 in. diameter) for 50% of deformation. Samples that were stored at the various temperatures were transferred to 23 °C and equilibrated for 2 h before the measurements. The samples were stable and did not exhibit any obvious change during the examination period between sampling (at various temperatures) and subsequent incubation and texture measurements at 23 °C. The cross-head speed of deformation was 1 mm/s, and the trigger force was 0.15 N. The maximum force (unit, N) during deformation was recorded as the hardness.

Statistical Analysis. The analysis of variance (ANOVA) using the general linear model procedure and the difference between means using the Duncan test were determined using SAS (SAS Institute Inc., Cary, NC), and all statistics were applied at an α level of 0.05.

RESULTS AND DISCUSSION

Formation of Insoluble Whey Protein Aggregates. Figure 2 shows the formation of insoluble protein in the protein/buffer model system as the function of storage temperature and time. It suggested that both temperature and time are critical factors for protein aggregation. Insoluble protein rapidly formed during the first 3 days of storage, followed by a slower rate afterward. The formation of insoluble protein at 45 °C was much faster than at lower temperatures. After storage for 100 days at 45 °C, almost two-thirds of the whey protein became insoluble.

Changes in Whey Protein Conformation. The changes in whey protein conformation after 3 months of storage at the various temperatures were determined with DSC (**Figure 3**). There were two endothermic peaks for the fresh-prepared control sample, which are related to the denaturation of the component proteins of whey proteins. The denaturation peak at the lower temperature (peak 1) corresponds to the denaturation of bovine serum albumin (BSA) and α-lactalbumin, and the second peak

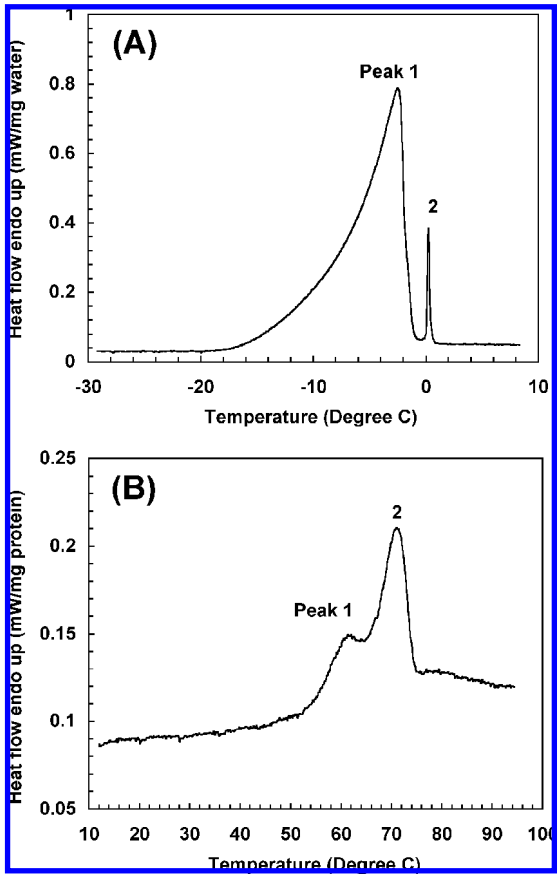

Figure 1. Typical DSC scans of crystallized water melting and protein denaturation in the protein/buffer model system right after preparation. (**A**) The crystallized water melting scan; (**B**) the protein denaturation scan.

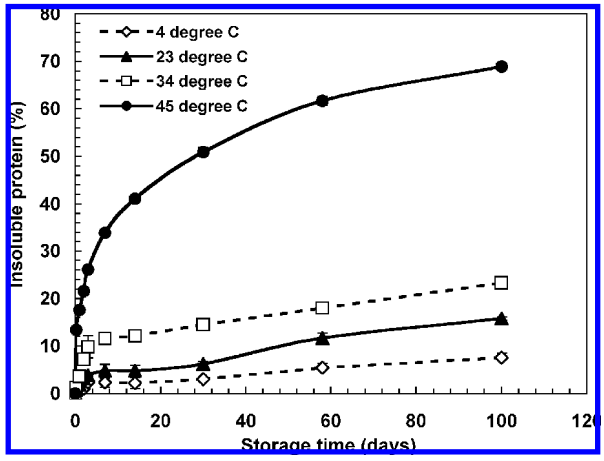

Figure 2. Formation of insoluble protein in protein/buffer model system as the functions of storage time and temperature (4, 23, 34, and 45 °C). The error bars represent standard deviation; $n = 2$.

at the higher temperature represents the denaturation of β-lactoglobulin (*21, 22*). The temperature of each endothermic denaturation peak and the total denaturation enthalpy are shown in **Table 1**. After storage at 4 or 23 °C for 3 months, there were no significant changes in whey protein denaturation temperatures and denaturation enthalpy (**Figure 3** and **Table 1**). However, after storage at 34 °C for 3 months, the temperatures of both endothermic denaturation peaks increased, but no significant change in total denaturation enthalpy was observed. The endothermic peak that corresponds to the denaturation of BSA and α-lactalbumin totally disappeared after

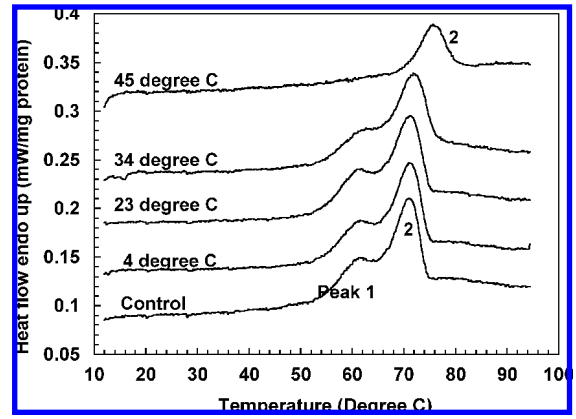

Figure 3. DSC scans of protein denaturation on protein/buffer model systems after storage for 3 months at various temperatures (4, 23, 34, and 45 °C). The control represents the fresh-prepared sample.

Table 1. Changes in Whey Protein Conformations during Storage by DSC Measurements (Mean ± Standard Deviation, $n = 2$)

	peak 1 (°C)[a]	peak 2 (°C)[a]	ΔH (J/g protein)[a,b]
control[c]	62.1 ± 0.0 B	71.8 ± 0.3 C	10.0 ± 0.2 A
C4[c]	62.1 ± 0.0 B	71.8 ± 0.0 C	10.0 ± 0.1 A
C23[c]	62.1 ± 0.1 B	71.9 ± 0.1 C	9.9 ± 0.6 A
C34[c]	62.8 ± 0.0 A	72.6 ± 0.0 B	9.7 ± 0.8 A
C45[c]		75.6 ± 1.2 A	3.7 ± 0.3 B

[a] Means in a column followed by different letters are significantly different ($P < 0.05$). [b] ΔH includes peak 1 and 2. [c] The control is fresh-prepared sample; C4, C23, C34, or C45 are samples after storing at 4, 23, 34, or 45 °C for 3 months.

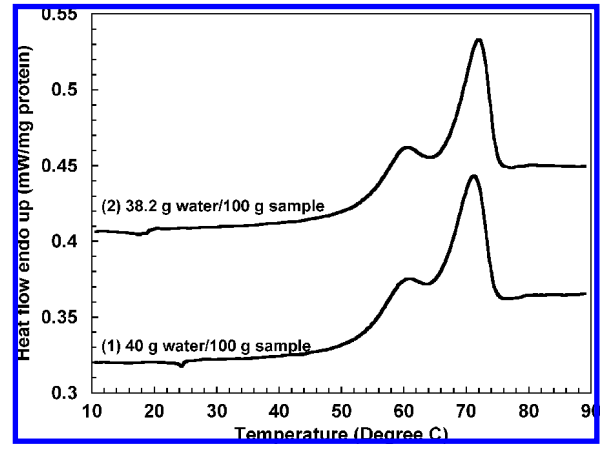

Figure 4. DSC scanning curves of protein denaturation on fresh-prepared protein/buffer model systems with different moisture contents (38.3 and 40%).

the 3 months of storage at 45 °C, while the denaturation temperature for β-lactoglobulin increased further and the total denaturation enthalpy decreased significantly. In our earlier research on the stability of whey protein powders (*18*), it was suggested that with an increase in storage temperature, the flexibility and mobility of protein peptides increase. Thus, whey proteins that are stored at higher temperature (such as 45 °C) would partly lose their tertiary structures, particularly for the component proteins that have lower denaturation temperatures (peak 1 in **Figure 3**). In addition, the increase in the accessibility of sulfhydryl group and disulfide bonds, due to the increases in chain mobility and the changes in tertiary structure, would contribute to the formation of more intermolecular disulfide bonds for samples that are stored at higher temperatures.

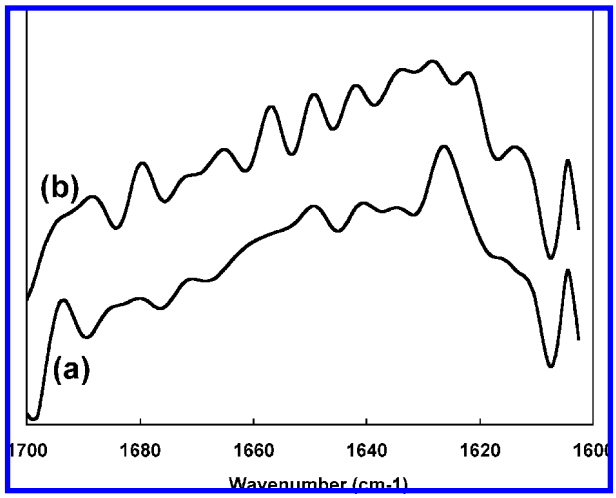

Figure 5. Deconvoluted spectra of protein/buffer model systems by FTIR: (**a**) freshly prepared control system; (**b**) sample after storage for 3 months at 45 °C.

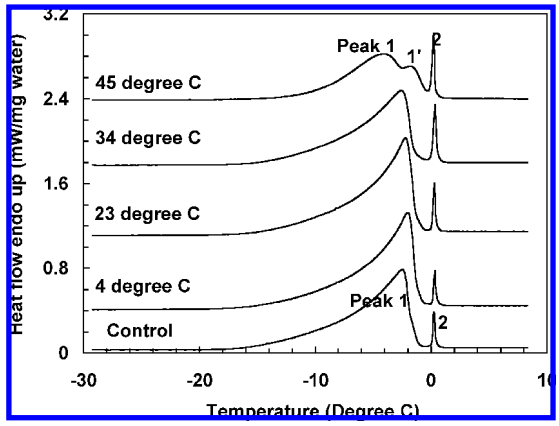

Figure 6. DSC scans of crystallized water melting in model systems after storage for 3 months at various temperatures (4, 23, 35, and 45 °C). The control represents the fresh-prepared sample.

Table 2. Changes in the States of Water Molecules during Storage as Determined by DSC Measurements (Mean ± Standard Deviation, $n = 2$)

		g/100 g water		
	total water (g/100 g sample)[a]	water in state A[a]	water in state B[a]	water in state C[a]
control[b]	40.2 ± 0.5 A	0.5 ± 0.4 C	35.8 ± 0.4 A	63.7 ± 1.9 C
C4[b]	39.8 ± 0.9 A	0.5 ± 0.3 C	36.5 ± 0.4 A	63.0 ± 1.7 C
C23[b]	40.2 ± 0.3 A	0.7 ± 0.2 C	36.1 ± 0.5 A	63.2 ± 1.3 C
C34[b]	40.0 ± 0.7 A	1.4 ± 0.2 B	30.4 ± 0.3 B	68.2 ± 1.4 B
C45[b]	38.3 ± 0.6 B	1.1 ± 0.2 B[c]	17.2 ± 1.9 C[c]	81.7 ± 1.7 A[c]
		5.2 ± 0.2 A[d]	16.5 ± 2.0 C[d]	78.3 ± 1.8 A[d]

[a] Means in a column followed by different letters (A, B, or C) are significantly different among samples ($P < 0.05$). [b] The control is fresh-prepared sample; C4, C23, C34, or C45 are samples after storing at 4, 23, 34, or 45 °C for 3 months. [c] The estimation of the states of water molecules in C45 based on the total water content of 38.3%. [d] The estimation of the states of water molecules in C45 based on the total water content of 40%. The lost water content of 1.7% (wet weight, equal to 40 − 38.3%) was treated as the water in state A (bulk water).

Former studies suggest that in the concentrated protein matrix, such as protein powder or protein dough, the denaturation temperature and enthalpy of whey proteins vary with the total water content in the system (*18*). The stored samples had no significant changes in water content during the 3 months of storage, except those stored at 45 °C, in which the total water content decreased from 40 to 38% (wet weight), probably due

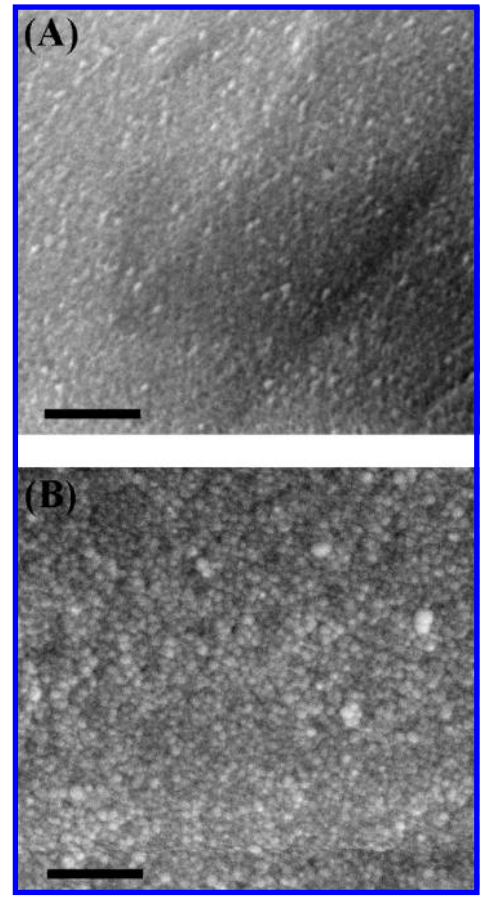

Figure 7. SEM of protein/buffer model systems: (**A**) freshly prepared control system; (**B**) sample after storage at 45 °C for 3 months. The bars in the graphs represent the 1 μm scale.

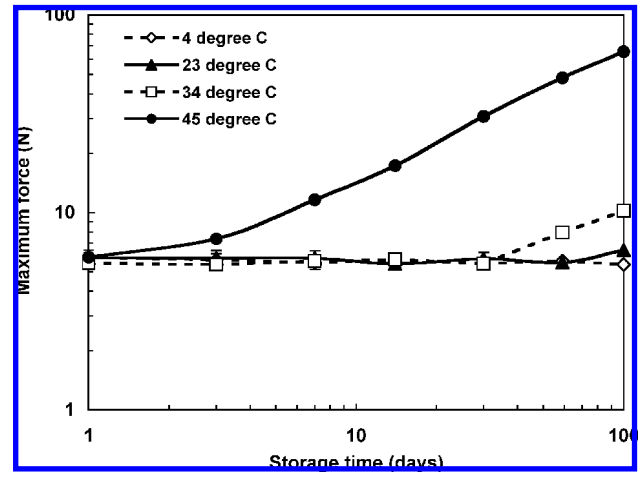

Figure 8. Changes in bar texture as the functions of storage time and temperature (4, 23, 34, and 45 °C). The error bars represent standard deviation; $n = 2$.

to the permeability of the plastic cup or some other reasons. Comparing the DSC curves of fresh prepared protein/buffer system containing 40% water with the one containing 38% water (**Figure 4**) shows that the loss of water in the model system increased the denaturation temperature of BSA/α-lactalbumin (peak 1) from 62.1 ± 0.0 to 62.2 ± 0.0 °C and β-lactoglobulin (peak 2) from 71.8 ± 0.3 to 72.7 ± 0.1 °C but not as much as what was observed in the sample after the storage at 45 °C for 3 weeks (peak 1, disappeared; peak 2, from 71.8 ± 0.3 to 75.6 ± 1.2 °C). In addition, the total denaturation enthalpy of whey

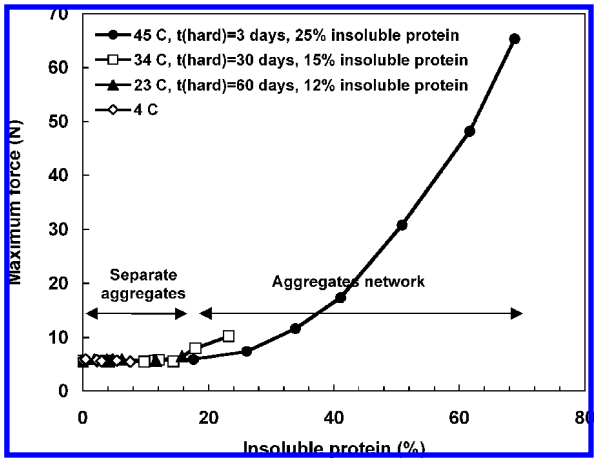

Figure 9. Relation between the formation of insoluble protein and hardening in texture.

proteins decreased by only 2% with the decrease in water content, that is, 10.0 ± 0.2 J/g protein at 40% water content, as compared with 9.8 ± 0.3 J/g protein at 38% water content. Thus, the changes in protein denaturation temperature and enthalpy of samples stored at 45 °C were mainly caused by the protein conformation changes during long-term storage.

FTIR was applied to further investigate the change in protein conformation (*12, 22–24*). **Figure 5** compares the FTIR spectra in the 1700−1600 cm^{-1} region for the fresh-prepared whey protein/buffer system to the sample that was stored at 45 °C for 3 months The bands at 1626 and 1634 cm^{-1} of fresh-prepared sample correspond to the β-sheet structure of whey proteins (*12, 23, 24*). After storage at 45 °C for 3 months, the peak at 1626 cm^{-1} was divided into two peaks at 1621 and 1628 cm^{-1}, which was also attributed to the β-sheets. The band at 1650 is attributed to an α-helix, and its intensity increased slightly after the 3 months of storage. A peak at 1681 cm^{-1} was observed after the 3 months of storage at 45 °C, which corresponds to the formation of intermolecular aggregation (*12*).

Changes in the State of Water Molecules. DSC can be used to monitor the global phase changes of water in biopolymer systems (*25*). **Figure 6** shows the typical DSC curve of the melting of crystallized water on protein/buffer model systems, from which the changes in the state of water molecules can be determined upon slowly warming a sample frozen to −40 °C (*19, 26*). For the fresh-prepared sample and those that were stored at 4, 23, or 34 °C, there are two melting peaks, suggesting the existence of two states of frozen water under the experimental conditions. The frozen water in melting peak 2 refers to water in state A, which melts at ∼0 °C and is similar to that of bulk water. The frozen water in melting peak 1 refers to water in state B, which is frozen at −40 °C but melts over a temperature range from −40 °C to just lower than 0 °C, due to the weak interaction between the water and the molecules in its surroundings (*27, 28*) . As noted previously, besides the water in states A and B, there are also water molecules that are nonfrozen at <−40 °C or lower (state C), mostly due to strong water−ion and possibly hydrogen-bonding and water−dipole interactions between the water and the protein molecules (*28*). In addition, for samples that were stored at 45 °C, the water molecules in state B had two endothermic peaks (peak 1 as above and 1′ as seen in **Figure 6**). This latter peak is at ∼−2 °C and is likely due to the changes in protein conformation and water−protein interactions occurring during storage (*28*).

The total water content in each sample was determined by the Karl Fischer method, and the sample stored at 45 °C for 3 months had a lower total water content as noted earlier than the fresh-prepared control sample and samples stored at 4−34 °C (**Table 2**).

The weight fraction of water molecules in each state was further calculated, using the heat of fusion of ice as 334 J/g water. It should be noted that the fusion enthalpy of water in state B is probably not exactly the same as that of bulk water (334 J/g water), due to the interaction between these water molecules and proteins (*25, 27–29*). However, using this value together with the results from DSC, we can provide an estimate of the amount of water molecules in different states. **Table 2** shows the changes in the states of water molecules after 3 months of storage at various temperatures. The percentage of water molecules in state B (frozen water but different from bulk water) only changes at 34 and 45 °C, where the amount of water in this state decreased significantly. Most of the water molecules lost from state B were transferred into state C

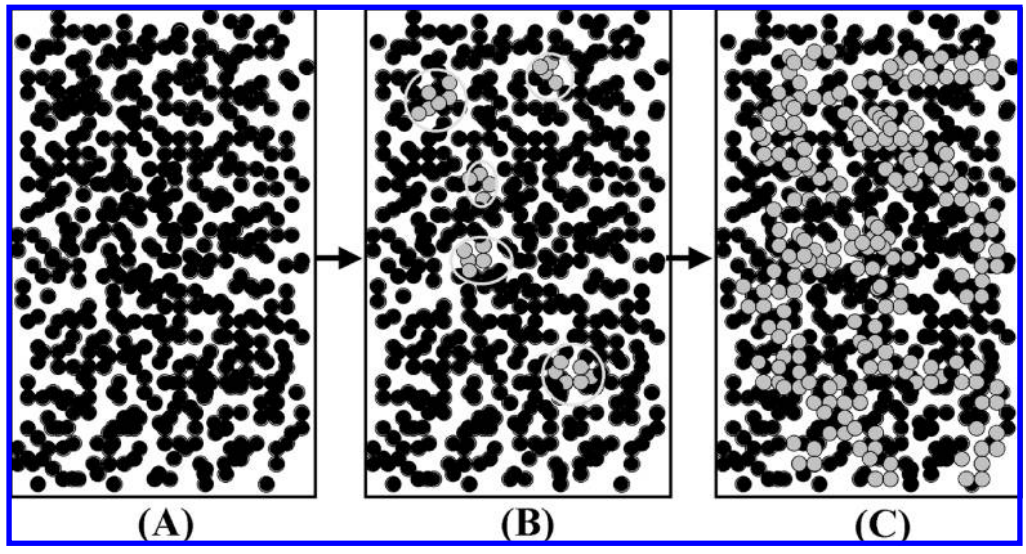

Figure 10. Possible mechanism of the formation of protein aggregates in model system and its effect on hardening. (**A**) Fresh-prepared model system; (**B**) formation of separate protein aggregates; and (**C**) formation of the network of aggregates. The black balls in the graphs represent protein molecules or particles without aggregation, and the gray balls represent protein molecules or particles aggregated (mainly intermolecular disulfide bonds).

(nonfrozen water), possibly due to the changes in protein conformation at higher temperature, which caused the peptide chain to be more accessible to water molecules forming new structures through interactions like hydrogen-bonding and water−ion interactions.

Changes in Microstructure by SEM. The changes in the SEM-determined microstructure of the protein/buffer model system are shown in **Figure 7** before and after the storage at 45 °C. The scans suggest that the model system had a relatively smooth structure initially, and aggregated particles (100−200 nm) formed during 3 months of storage at 45 °C.

Changes in microstructure during whey protein thermal gelation in solution have been widely studied. Under conditions of strong electrostatic repulsions (low salt level), whey protein molecules would form a fine-stranded gel structure. However, under conditions of low electrostatic repulsion, whey protein molecules would form a gel containing large aggregated particles, and the size of these particles ranges from several hundred nanometers to several micrometers (*13, 14, 30*). After 3 months of storage at 45 °C ($a_w \sim 0.98$), the concentrated protein/buffer model system in the present study formed a structure similar to that of thermally denatured particulate gels. In diluted solution, the formation of aggregated particles of whey proteins, in most cases, requires the denaturation of protein molecules to expose their nonpolar amino acids, so the increased hydrophobic interactions can drive protein molecules close to one another. On the other hand, for the protein/buffer model systems in this study, they are very concentrated systems containing only 40% water content so that they would represent a soft textured bar. The protein molecules in these systems are close to one another already and can form intermolecular bonds on their surface even at storage temperatures that are below the protein denaturation temperature. However, it will take much longer time to form this aggregated structure, as compared with the high temperature thermal gelation process, where the heating temperatures (>denaturation temperature) are much higher than the storage temperatures used at the present study.

Hardening of Protein/buffer Systems during Storage. The hardening of the protein/buffer system during storage was also monitored as the function of storage temperature and time (**Figure 8**). When stored at 45 °C, the protein/buffer bar system became significantly harder after 3 days, while when stored at 34 or 23 °C, the time for the model system to get harder was delayed by 30 or 60 days, respectively. In addition, no hardening was observed for the protein/buffer model system during 100 days of storage at 4 °C.

The correlation between the protein aggregation and the hardening of protein/buffer matrix is shown in **Figure 9**. At 45 °C, the texture did not show significant hardening during storage for the first day, although more than 15% protein became insoluble, but when about 25% of total whey proteins became insoluble at the third day, the texture began to change dramatically. At 34 or 23 °C, the critical amount of insoluble protein causing hardening is 15 or 12%, respectively. These results suggested that during storage, whey proteins begin to form segregated protein aggregates (**Figure 10a** going to **Figure 10b**), and the formation of these segregated protein aggregates did not cause the texture to change significantly. When the separate aggregates begin to interact with each other, a more global aggregation network forms (**Figure 10c**) and a significant change in texture (hardening) is observed (**Figure 9**).

In conclusion, moisture-induced whey protein aggregation in protein/buffer model system results in the formation of an aggregated protein network in such a high-protein-containing matrix and further results in a harder texture.

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LITERATURE CITED

(1) Fox, P. F. Milk proteins: General and historical aspects. In *Advanced Dairy Chemistry: Vol. I: Proteins*; Fox, P. F., McSweeney, P. L. H., Eds; Kluwer Academic/Plenum Publishers: New York, 2003; pp 1−48.

(2) De Wit, J. Thermal stability and functionality of whey proteins. *J. Dairy Sci.* **1990**, *73*, 3602–3612.

(3) Ju, Z.; Kilara, A. Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *J. Dairy Sci.* **1998**, *81*, 925–931.

(4) Vardhanabhuti, B.; Foegeding, E. A. Rheological properties and characterization of polymerized whey protein isolates. *J. Agric. Food Chem.* **1999**, *47*, 3649–3655.

(5) Alting, A. C.; Hamer, R. J.; de Kruif, C. G.; Visschers, R. W. Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. *J. Agric. Food Chem.* **2000**, *48*, 5001–5007.

(6) Mleko, S.; Foegeding, E. pH induced aggregation and weak gel formation of whey protein polymers. *J. Food Sci.* **2000**, *65*, 139–143.

(7) Bryant, C. M.; McClements, D. J. Influence of NaCl and $CaCl_2$ on Cold-set gelation of heat-denatured whey protein. *J. Food Sci.* **2000**, *65*, 801–804.

(8) Havea, P.; Singh, H.; Creamer, L. K. Heat-induced aggregation of whey proteins: comparison of cheese WPC with acid WPC and relevance of mineral composition. *J. Agric. Food Chem.* **2002**, *50*, 4674–4681.

(9) Patel, H. A.; Singh, H.; Havea, P.; Considine, T.; Creamer, L. K. Pressure-induced unfolding and aggregation of the proteins in whey protein concentrate solutions. *J. Agric. Food Chem.* **2005**, *53*, 9590–9601.

(10) Liu, X. M.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. *Innovat. Food Sci. Emerg. Technol.* **2005a**, *6*, 310–317.

(11) Liu, X. M.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. *J. Food Sci.* **2005b**, *70*, C581−C585.

(12) Boye, J. I.; Alli, I.; Ismail, A. A.; Gibbs, B. F.; Konishi, Y. Factors affecting molecular characteristics of whey protein gelation. *Int. Dairy J.* **1995**, *5*, 337–353.

(13) Bryant, C. M.; McClements, D. J. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci. Technol.* **1998**, *9*, 143–151.

(14) Foegeding, E. A. Food biophysics of protein gels: A challenge of nano and macroscopic proportions. *Food Biophys.* **2006**, *1*, 41–50.

(15) Liu, W. R.; Langer, R.; Klibanov, A. M. Moisture-induced aggregation of lyophilized proteins in the solid state. *Biotechnol. Bioeng.* **1991**, *37*, 177–184.

(16) Costantino, H. R.; Langer, R.; Klibanov, A. M. Solid-phase aggregation of proteins under pharmaceutically relevant conditions. *J. Pharm. Sci.* **1994**, *83*, 1662–1669.

(17) Stote, R. M.; Feldman, F. Intranasal insulin−A potential new treatment for diabetes mellitus. *Drug Delivery Technol.* **2007**, *7*, 86–92.

(18) Zhou, P.; Labuza, T. P. Effect of water content on glass transition and protein aggregation of whey protein powders during short-term storage. *Food Biophys.* **2007**, *2*, 108–116.

(19) Zhou, P.; Liu, X. M.; Labuza, T. P. Moisture-induced aggregation of whey proteins in a protein/buffer model system. *J. Agric. Food Chem.* **2008**, *56*, 2048–2054.
(20) Ahmad, M. B.; Huglin, M. B. DSC studies on states of water in crosslinked poly(methyl methacrylate-co-*N*-vinyl-2-pyrrolidone) hydrogels. *Polym. Int.* **1994**, *33*, 273–277.
(21) Rüegg, M.; Moor, U.; Blanc, B. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *J. Dairy Res.* **1977**, *44*, 509–520.
(22) Boye, J. I.; Alli, I. Thermal denaturation of mixture of α-lactalbumin and *β*-lactoglobulin: A differential scanning calorimetric study. *Food Res. Int.* **2000**, *33*, 673–682.
(23) Boye, J. I.; Alli, I.; Ismail, A. A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. *J. Agric. Food Chem.* **1997**, *45*, 1116–1125.
(24) Boye, J. I.; Alli, I.; Ismail, A. A. Molecular and microstructural studies of thermal denaturation and gelatin of *β*-lactoglobulin A and B. *J. Agric. Food Chem.* **1997**, *45*, 1608–1618.
(25) Quinn, F. X.; Kampff, E.; Smyth, G.; McBrierty, V. J. Water in hydrogels. 1. A study of water in poly(*N*-vinyl-2-pyrrolidone/methyl methaccrylate) copolymer. *Macromolecules* **1988**, *21*, 3191–3198.
(26) Liu, W. G.; Li, F.; Yao, K. D. Thermal and NMR investigation of the change in the states of water caused by volume phase transition of gelatin gel. *Polym. Int.* **2000**, *49*, 1624–1628.
(27) Frommer, M. A.; Lancet, D. Freezing and nonfreezing water in cellulose-acetate membranes. *J. Appl. Polym. Sci.* **1971**, *16*, 1295–1303.
(28) Kuntz, I. D.; Kauzmann, W. Hydration of proteins and polypeptides. *Adv. Protein Chem.* **1974**, *28*, 239–345.
(29) Zhang, J.; Teng, H.; Zhou, X.; Shen, D. Frozen bound water melting induced cooperative hydration of poly(vinyl methyl ether) in aqueous solution. *Polym. Bull.* **2002**, *48*, 277–282.
(30) Verheul, M.; Roefs, S. P. F. M. Structure of whey proteins gels, studied by permeability, scanning electron microscopy and rheology. *Food Hydrocolloids* **1998**, *12*, 17–24.

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