JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Structural Characterization of Heat-Induced Protein Particles in Soy Milk

CHENGANG REN,[†] LU TANG,[†] MIN ZHANG,[‡] AND SHUNTANG GUO*,[†]

College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China, and Food College, Northeast Agricultural University, Harbin 150030, China

This study analyzed the aggregation mode of polypeptides in protein particles of soy milk by using ultracentrifugation, gel filtration, and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The results show that the protein particles in soy milk were mainly formed by various complex protein aggregates. These protein aggregates were mainly composed of the basic and acidic polypeptides of glycinin (11S), which interact with each other via disulfide bonds, and a very small amount of the disulfide-linked α′ and α subunits of β-conglycinin (7S). Moreover, the protein aggregates and a part of monomeric subunits of 7S and 11S form protein particles through non-covalent interactions, especially hydrophobic interactions and hydrogen bonds. It is suggested that the polymerized basic polypeptides should be located inside the protein particles, whereas the acidic polypeptides of 11S, α′ and α subunits are located outside them.

KEYWORDS: Soy milk; protein particles; aggregates; disulfide bond; non-covalent interaction

INTRODUCTION

Soy milk is a traditional protein beverage, and it also forms the base in the production of tofu. Generally, soy milk has 4−6% protein content (*1*, *2*), of which 50% consists of particles of >40 nm in diameter (*3*). It has been found that particulate protein can coagulate at a lower concentration of coagulants in comparison to supernatant protein (*4*). The more protein particles in soy milk results in a harder tofu gel made from the soy milk (*5*, *6*). These results suggest that the composition and quantity of protein particles significantly affect the physical and chemical properties of soy milk.

The protein particles in soy milk are not fragmental protein bodies formed during grinding, but protein aggregates formed by the dissociation and rearrangement of the subunits of soy protein during heating (*3*, *7*, *8*). In soy milk, the basic polypeptides of glycinin (11S) and the β subunit of β-conglycinin (7S) tend to form protein particles, whereas the acidic polypeptides of 11S and the α′ and α subunits of 7S tend to exist in soluble form (*3*, *9*). A higher particle content is the result of a corresponding higher 11S content in soy milk, whereas the increase of the α′ and α subunits of 7S inhibits the formation of protein particles (*10*).

In our previous study, we found that the whey soybean protein contributes to the formation of protein particles and enhances their sizes (*11*). The formation of large particles may be due to S−S bridges between subunits (*3*). Thermal treatment causes the formation of protein aggregates through disulfide bonds and hydrophobic interactions (*12*). Changes in protein surface hydrophobicity have been considered to be related to the precipitate formation of soy milk when heated at two different temperatures (*13*). Tezuka and others (*5*) found that the hydrophobicity of the IIa and IIb subunits of 11S was stronger than the group protein I, and IIa, IIb can form protein particles more easily. Although there are some reports on the ratio of various subunits in protein particles and the kinds of interaction force involved in the formation of protein particles, it is still not very clear about how the subunits interact with each other and form protein particles in soy milk.

In this paper, the interactions between subunits of protein particles in soy milk were analyzed using ultracentrifugation, gel filtration, and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Likewise, the association manner of subunits in protein particles was also discussed.

MATERIALS AND METHODS

Materials. Defatted low-heat soybean meal was provided by Qinhuangdao Jinhai Grain & Oil Industrial Co., Ltd. Sephacryl S-500 Superfine was obtained from GE Healthcare. The chemical reagents used in this study were all of reagent grade.

Preparation of Defatted Soy Milk and Protein Particles. A total of 50 g of defatted low-heat soybean meals was mixed with 450 mL of distilled water. Thereafter, they were ground for 2 min in a homogenate using a blender (JYL-350A, Shandong Joyoung Household Electrical Appliances Co., Ltd., China). To remove the okara, the slurries were centrifuged at 3500*g* for 10 min. The supernatant was heated in a boiling water bath for 10 min, after which it was immediately cooled to room temperature by ice water to obtain the defatted soy milk. In experiments with *N*-ethylmaleimide (NEM), a portion of stock

* Address correspondence to this author at the College of Food Science and Nutritional Engineering, China Agricultural University, No. 17 Qinghua Donglu, Haidian District, Beijing 100083, China (e-mail shuntang@cau.edu.cn; telephone/fax +86-10-62737634).

[†] China Agricultural University.

[‡] Northeast Agricultural University.

10.1021/jf803321n CCC: $40.75
Published on Web 02/12/2009

protein solution was made 0.02 M with NEM by adding the solid reagent before heat treatment.

The defatted soy milk was centrifuged at 156200*g* for 30 min at 20 °C, and the pellet was the protein particles (diameter > 40 nm) (*3, 14*). The supernatant consisted of nonparticle protein.

Analysis of Protein Particle Size Distribution in Defatted Soy Milk. The distribution of the protein particle sizes was measured by laser light scattering using a Series Particle Size analyzer (Beckman-Coulter LS 230) with a Small Volume Module sample platform. The refractive index used for the dispersed phase was 1.471 and the correspondent to water was 1.333. Each sample was measured in triplicate and expressed as a percentage of volumetric particle size distributions using Beckman Coulter LS version 3.29 analytical software.

Dissociation of Protein Particles in Various Solutions. To investigate the type of intermolecular force in protein particles, the pellet (protein particles) of defatted soy milk by ultracentrifugation was mixed with a 30 mM concentration of standard Tris-HCl buffer (TB) (pH 8.0, contain 0.02% NaN_3) at a ratio of 1:1 (w/w). Subsequently, the mixture was homogenized at 10000 rpm for 30 s using a homogenizer (IKA Ultra Turrax T18 basic, equipped with S18N-19G) to obtain the slurry of protein particles. The slurry was mixed at a ratio of 1:5 (v/v) with five types of solvent: (1) 30 mM standard Tris-HCl buffer (pH 8.0, containing 0.02% NaN_3) (TB); (2) 0.6 M NaCl in the TB (TB + NaCl); (3) 0.24 M 2-mercaptoethanol (2-ME) in the TB (TB + 2-ME); (4) 7.2 M urea in the TB (TB + urea); (5) 7.2 M urea and 0.6% SDS in the TB (TB + urea + SDS). The concentrations of NaCl, 2-ME, urea, and SDS in the mixed dispersion reached 0.5 M, 0.2 M, 6 M, and 0.5%, respectively. The dispersions were then incubated at 25 °C for 20 h. After incubation, the samples were centrifuged at 156200*g* for 30 min at 20 °C, and the pellet was dispersed with 0.01 M NaOH and quantified using the Bradford method (*15*).

The content of dissociated protein particles was calculated using the following formula:

$$\%\ \text{dissociated protein particles} = (1 - \text{protein content in the pellet of ultracentrifugation/initial protein content in the dispersions of protein particles}) \times 100\%$$

Sephacryl S-500 Gel Chromatography. Size exclusion chromatography was carried out with a column (1.6 cm × 75 cm) using Sephacryl S-500 (separation range of 4×10^4−2×10^7 Da, dextran standards). After incubation at 25 °C for 20 h, the dispersion of protein particles by TB + urea + SDS was filtered with a 0.45 μm membrane. Aliquots (1 mL) of the filtered dispersion were injected and eluted with 30 mM Tris-HCl buffer (pH 8.0, containing 0.02% NaN_3, 0.15 M NaCl, 6 M urea, and 0.5%SDS) at a flow rate of 0.5 mL/min at room temperature. The eluent was monitored at 280 nm using a UV detector. Lactoferrin (86 kDa) and lysozyme (14 kDa) were used as the control protein of molecular mass. Every eluted fraction was collected, concentrated, and then analyzed by electrophoresis.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE in the presence or absence of 2-ME was carried out on a vertical slab gel of 1 mm thickness (BIO CRAFT model BE-210N, Japan) using an alkaline discontinuous buffer (*16*). The concentrations of the stacking gel and separating gel were 4 and 12.5%, respectively. The sample buffer for nonreducing SDS-PAGE contained 0.125 M Tris-HCl (pH 6.8), 1% SDS, 6 M urea, 20% glycerol, and 1% bromophenol blue, and that of reducing SDS-PAGE contained 0.125 M Tris-HCl (pH 6.8), 1% SDS, 6 M urea, 20% glycerol, 2% 2-mercaptoethanol, and 1% bromophenol blue. The solution was heated at 95 °C for 5 min. Aliquots (5 μL) were then loaded to the gel. A broad range of molecular mass standard proteins was made from lactoferrin (86 kDa), ovalbumin (44 kDa), β-casein (24 kDa), and lysozyme (14 kDa).

After the run, the protein bands were stained with Coomassie brilliant blue G-250 in the gel. Furthermore, they were scanned by an HP scan instrument (HP 1000).

Statistical Analysis. All determinations were carried out at least three times. Data were analyzed by analysis of standard deviations and variance (ANOVA) using EXCEL.

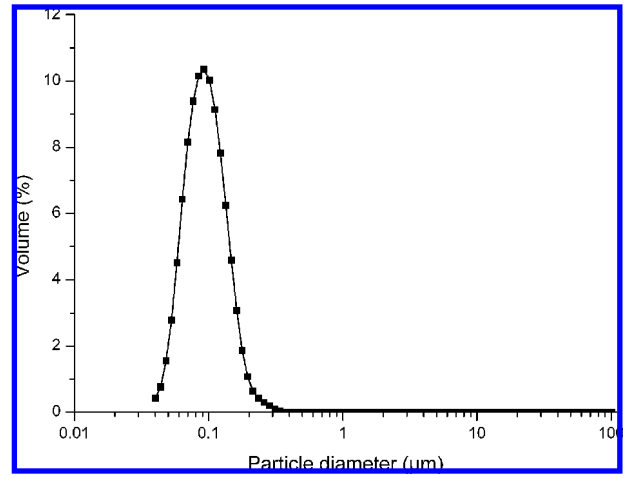

Figure 1. Particle size distribution of defatted soy milk (■).

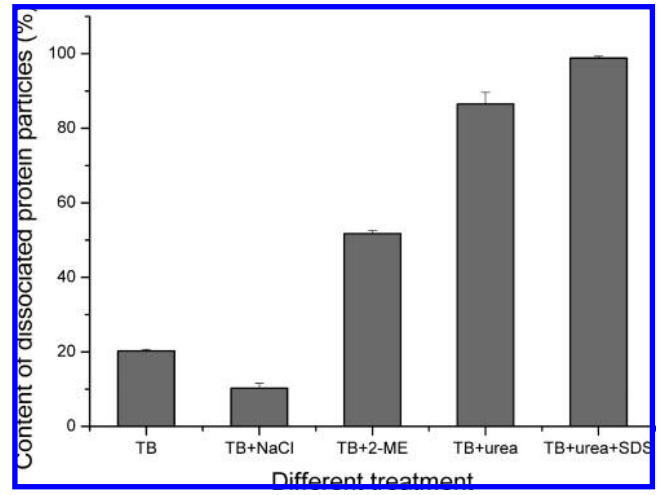

Figure 2. Effects of various reagents on the dissociation of protein particles in defatted soy milk. TB, 30 mM standard Tris-HCl buffer (pH 8.0, contain 0.02% NaN_3); TB + NaCl, 0.5 M NaCl in the TB; TB + 2-ME, 0.2 M 2-mercaptoethanol (2-ME) in the TB; TB + urea, 6 M urea in the TB; TB + urea + SDS, 6 M urea and 0.5% SDS in the TB.

RESULTS AND DISCUSSION

Size Distribution of Protein Particles. The defatted soy milk was prepared from defatted low-heat soybean meal. The size distribution of protein particles in the defatted soy milk was analyzed using the size distribution analyzer. **Figure 1** shows the result. In defatted soy milk, most of the protein particles ranged from 40 to 200 nm in diameter. Only a small amount of particles are larger than 200 nm in diameter. This conformed to the report of others (*2, 3, 8*), in which the size distribution of protein particles in whole soy milk was analyzed by particle size analyzer or ultracentrifugation.

Dissociation of Protein Particles in Buffers of Various Reagents. To investigate the association manner between various polypeptides in protein particles, we obtained the protein particles with diameters of >40 nm by ultracentrifugation at 156200*g* as pellet (*3, 9*) and then determined the quantities of protein dissociated from the protein particles in buffers containing various reagents (**Figure 2**). Less than 20% of the protein in particles was dissociated by the standard Tris-HCl buffer alone. Fewer protein particles (about 10%) were dissociated by TB + NaCl compared to TB, which may be due to the stabilizing effect of NaCl on protein structure (*17*). In comparison to the above-mentioned treatments, the quantity of protein dissociated by TB + 2-ME was >2 times that dissociated

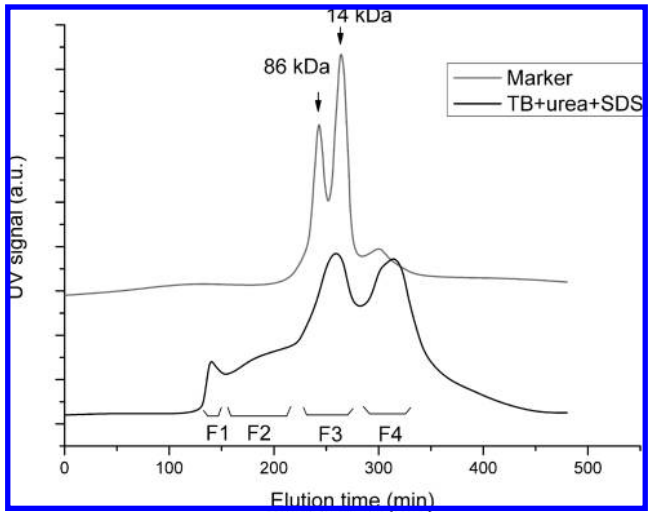

Figure 3. Size exclusion chromatography of dissociated protein particles by TB + urea + SDS. Column, Sephacryl S-500 (1.6 cm × 75 cm); flow rate, 0.5 mL/min at room temperature; eluting buffer, 30 mM Tris-HCl, pH 8.0, containing 0.02% NaN_3, 0.15 M NaCl, 6 M urea, and 0.5% SDS. The eluent was monitored at 280 nm. Lactoferrin (86 kDa) and lysozyme (14 kDa) were used as control proteins of molecular mass.

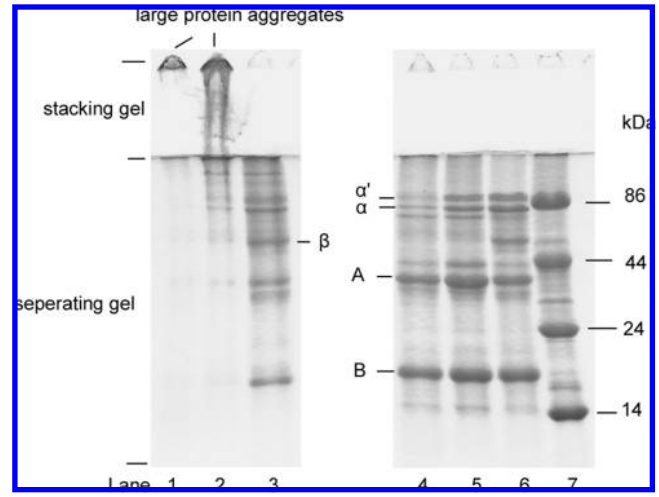

Figure 4. Nonreducing and reducing SDS-PAGE profiles of protein fractions from different eluting fractions of Sephacryl S-500. Lanes 1, 2, and 3 represent F1, F2, and F3 in **Figure 3** (without 2-ME), respectively. Lanes 4, 5, and 6 represent F1, F2, and F3 in **Figure 3** (with 2-ME). Lane 7 shows molecular mass markers (86, 44, 24, and 14 kDa).

by TB alone. 2-ME is a strong reducing agent and has the ability to cleave disulfide bonds that are responsible for holding tertiary and quaternary structure. The observation indicates the presence of disulfide bonds in protein particles. TB + urea caused about 86% dissociation of the protein particles, whereas the protein particles almost disappeared in the Tris-HCl buffer containing urea and SDS. Urea and SDS are agents known to disrupt noncovalent interactions, such as hydrogen bonds and hydrophobic interactions. This result indicates that the structure of the protein particles is stabilized mainly by hydrophobic interactions and hydrogen bonds.

Sephacryl S-500 Analysis of Dissociated Protein Particles by Urea and SDS. Although most of the original protein particles were dissociated after treatment by Tris-HCl buffer containing 0.5% of SDS and 6 M urea, a great amount of the dissociated protein particles could not enter the stacking gel of 4% when analyzed by nonreduced SDS-PAGE (results not shown). This phenomenon indicates that the protein particles were not dissociated into monomeric subunits completely by Tris-HCl buffer containing urea and SDS. The dissociated protein particles were analyzed using Sephacryl S-500 gel chromatography. **Figure 3** shows the result. It can be seen that the dissociated protein particles were separated into four fractions: F1, F2, F3, and F4. According to the retention time of standard protein which contains lactoferrin (86 kDa) and lysozyme (14 kDa) at the same eluting condition, it can be deduced that the average molecular masses of F1 and F2 are >86 kDa. Additionally, the average molecular mass of F3 is between 86 and 14 kDa. The average molecular mass of F4 is <14 kDa. However, the protein was not observed in the F4 fraction by TCA precipitation. Thus, it is considered that F4 should be some small molecular nonprotein components which are strongly absorbing at 280 nm.

Electrophoresis Analysis of Dissociated Protein Particles Eluted by S-500. The eluting fractions (F1, F2, and F3) of S-500 were collected and analyzed by SDS-PAGE in the presence and absence of 2-ME. **Figure 4** shows the protein-banding profiles of each eluting fraction. Electrophoresis of F1 in the absence of 2-ME gave aggregates on top of the stacking gel as major components and aggregates on top of the separating gel as minor components (**Figure 4**, lane 1). F2 gave aggregates not only on top of the stacking gel but also in the stacking gel and on top of the separating gel (**Figure 4**, lane 2). Although most of the material of F3 entered the stacking gel, there is some protein still on top of the separating gel (**Figure 4**, lane 3). F3 also gave monomers of α′, α, and β subunits of 7S and basic and acidic polypeptides of 11S. It is suggested that although the protein particles were dissociated into nonparticles by TB + urea + SDS, most of them were not cleaved to monomeric subunits but exist as aggregates with different degrees of polymerization. On the basis of the areas of every fraction in **Figure 3**, the high-degree polymerized aggregates (F1), medium aggregates (F2), small oligomers and free subunits (F3) account for 9.7 ± 0.6, 39.6 ± 1.8, and 50.7 ± 2.3% of total protein, respectively.

All of the protein aggregates in F1, F2, and F3 were transformed into monomers of α′ and α subunits of 7S and acidic and basic polypeptides of 11S in the presence of 2-ME (**Figure 4**, lanes 4−6). This indicated the presence of disulfide bonds in these aggregates. This result was close to those described by Yamagishi and others (*18*), who reported that the basic and acidic polypeptides of 11S could form high molecular weight aggregates through disulfide bonds during heating and that the molar ratios of basic/acidic polypeptides (B/A) of the aggregates increase with increasing heating time. Meanwhile, the α′ and α subunits of 7S could interact with acidic polypeptides and form soluble aggregates through disulfide bonds (*19*). There is also other research reporting similar results on the sulfhydryl−disulfide exchange reaction between the basic and acidic polypeptides of 11S during thermal denaturation (*20*−*22*). On the other hand, some research has reported that the disulfide bonds were not involved in the gelling of 7S (*23, 24*). This may be because the disulfide-linked α′ and α subunits are too short to be detected in that research. In our experiments, more than half of the 7S components were removed as supernatant when the protein particles were prepared by ultracentrifugation. When the dissociated protein particles were analyzed by S-500, great amounts of the monomeric α′ and α subunits were separated into F3 in **Figure 4** and the disulfide-linked α′ and α subunits were concentrated again. Even so, the amount of the disulfide-linked α′ and α subunits was still very small in F1 and F2. It also can be seen in our result that there

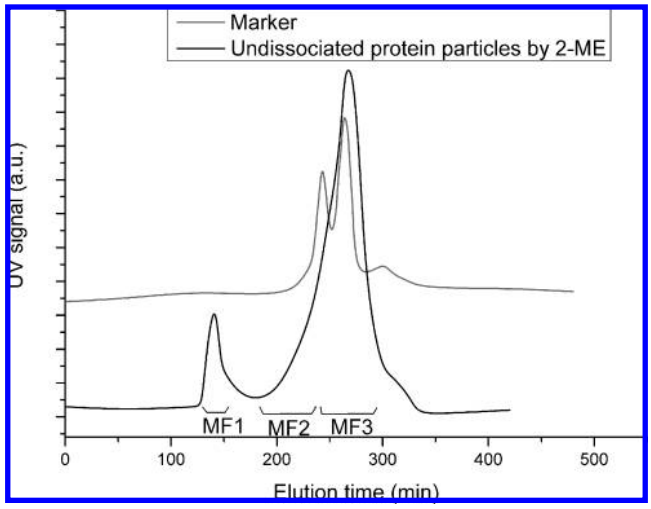

Figure 5. Size exclusion chromatography of undissociated protein particles by TB + 2-ME. Column, Sephacryl S-500 (1.6 cm × 75 cm); flow rate, 0.5 mL/min at room temperature; eluting buffer, 30 mM Tris-HCl, pH 8.0, containing 0.02% NaN_3, 0.15 M NaCl, 6 M urea, and 0.5% SDS. The eluent was monitored at 280 nm. Lactoferrin (86 kDa) and lysozyme (14 kDa) were used as control proteins of molecular mass.

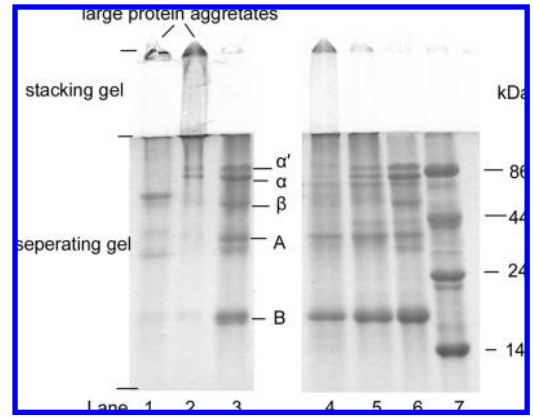

Figure 6. Nonreducing and reducing SDS-PAGE profiles of protein fractions from different eluting fractions of Sephacryl S-500 in **Figure 5**. Lanes 1, 2, and 3 represent MF1, MF2, and MF3 in **Figure 5** (without 2-ME), respectively. Lanes 4, 5, and 6 represent MF1, MF2, MF3 in **Figure 5** (with 2-ME). Lane 7 shows molecular mass markers (86, 44, 24, and 14 kDa).

was no β subunit in F1 or F2 (**Figure 4**, lanes 4 and 5) in the presence of 2-ME. This result suggested that the β subunit was not involved in the formation of protein aggregates via disulfide bonds. This result is in accordance with the report of Utsumi et al. (*25*), who considered that the interaction between the basic subunits of 11S and the β subunit of 7S is predominantly electrostatic in nature. The β subunit has only one cysteinyl residue, whereas the α′ and α subunits have five each. The β subunit has less chance to form disulfide bonds than the α′ and α subunits. It can be concluded that the dissociated aggregates by TB + urea + SDS are mainly disulfide-linked acidic and basic polypeptides of 11S and a very small amount of α′ and α subunits of 7S.

Effect of 2-ME on the Dissociation of Protein Particles. 2-ME can cleave disulfide bonds. However, more than 40% of protein particles were not dissociated by TB + 2-ME (**Figure 2**). To determine whether the disulfide bonds in protein aggregates were cleaved or not, the undissociated protein particles were then treated with TB + urea + SDS and analyzed by S-500 and SDS-PAGE. **Figure 5** shows the eluting profile of the undissociated protein particles. It can be seen that the relative content of protein aggregates decreased evidently compared to that in **Figure 3**. It is indicated that the molecular weight of protein aggregates in particles became smaller after treatment by TB + 2-ME. On the basis of the eluting time, three factions (MF1, MF2, and MF3) (**Figure 5**) were collected and analyzed by nonreducing and reducing SDS-PAGE. **Figure 6** shows the protein-banding profiles of each eluting fraction. Electrophoresis of MF1 in the absence of 2-ME gave aggregates on top of the stacking gel as major components (**Figure 6**, lane 1). MF2 gave aggregates not only on top of the stacking gel but also in the stacking gel and on top of the separating gel (**Figure 6**, lane 2). Although all of the material of MF3 entered the stacking gel, a small amount of protein remained on top of the separating gel (**Figure 6**, lane 3). MF3 also gave monomers of α′, α, and β subunits of 7S and basic and acidic polypeptides of 11S. Most of the protein aggregates in MF1, MF2, and MF3 were transformed into monomers in the presence of 2-ME (**Figure 6**, lanes 4−6). Lanes 4 and 5 mainly gave the band of basic polypeptides of 11S. Compared to the SDS-PAGE profiles of F1 and F2 (**Figure 4**, lanes 4 and 5), the density of the band of acidic polypeptides in **Figure 6** (lanes 4 and 5) decreased a lot. This result suggested that most of the disulfide-linked acidic polypeptides of aggregates in protein particles were cleaved into monomers after treatment by TB + 2-ME. The basic polypeptides have a larger amount of hydrophobic amino acid than the acidic ones (*18*, *26*). The disulfide bonds between basic polypeptides may be buried in the hydrophobic regions and hard for 2-ME to attack in the absence of urea and SDS. Hence, it can be deduced that the disulfide-linked basic polypeptides should be located inside the protein particles, whereas the acidic polypeptides would be located outside them. On the other hand, all of the subunits of 7S have hydrophilic saccharide chains (*27*). Furthermore, both α′ and α subunits have hydrophilic acidic extension regions (*28*). Therefore, the α′ and α subunits should be also located outside the protein particles.

Repression Effect of NEM on the Formation of Protein Aggregates in Protein Particles during Heating. The protein particles of soy milk formed during heating in the presence of NEM were treated by TB + urea + SDS and analyzed by S-500. **Figure 7** shows the result. Compared to the eluting profile of the protein particles formed in the absence of NEM in **Figure 3**, F1 and F2 representing aggregates did not appear in **Figure 7**. The eluting fraction of NF (as shown in **Figure 7**) was also analyzed by SDS-PAGE in the presence and absence of 2-ME, and the result is shown as **Figure 8**. The result showed that there was almost no protein aggregate on top of the stacking and separating gels (**Figure 8**, lane 1). On the other hand, most of the disulfide-linked acidic and basic subunits (AB subunit) of 11S were not broken into basic and acidic polypeptides. This result is in accordance with the report of Mori and others (*29*). The sulfhydryl blocking in the presence of NEM represses the excess release of acidic polypeptides (*30*) and the following disulfide exchange on heating. Therefore, it is suggested that disulfide-linked protein aggregates formed during heating.

On the basis of the above results and discussion, the probable interaction mechanism of polypeptides in heat-induced protein particles of soy milk was proposed. The proteins in soy milk dissociated, rearranged, and aggregated to form protein particles when heated. The protein particles of >40 nm in diameter dissociated into protein aggregates with various molecular masses and monomeric subunits of 7S and 11S protein after treatment by the mixture of 6 M urea and 0.5% SDS. The aggregates were primarily composed of the disulfide-linked basic and acidic polypeptides of 11S, besides a very small amount of

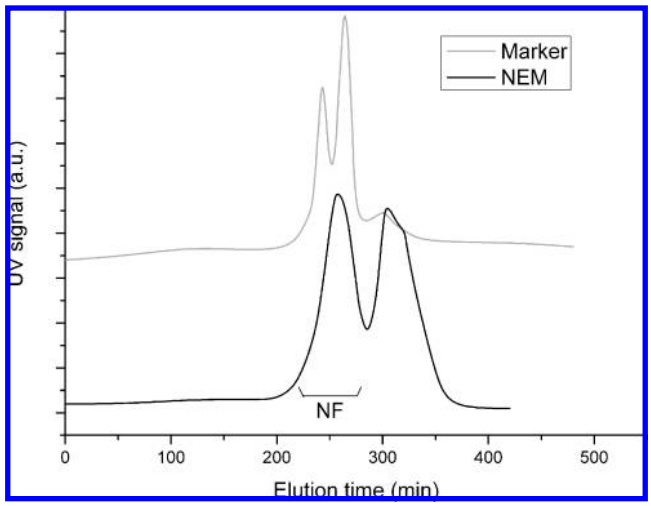

Figure 7. Size exclusion chromatography of protein particles formed in the presence of *N*-ethylmaleimide (NEM). Column, Sephacryl S-500 (1.6 cm × 75 cm); flow rate, 0.5 mL/min at room temperature; eluting buffer, 30 mM Tris-HCl, pH 8.0, containing 0.02% NaN_3, 0.15 M NaCl, 6 M urea, and 0.5% SDS. The eluent was monitored at 280 nm. Lactoferrin (86 kDa) and lysozyme (14 kDa) were used as control proteins of molecular mass.

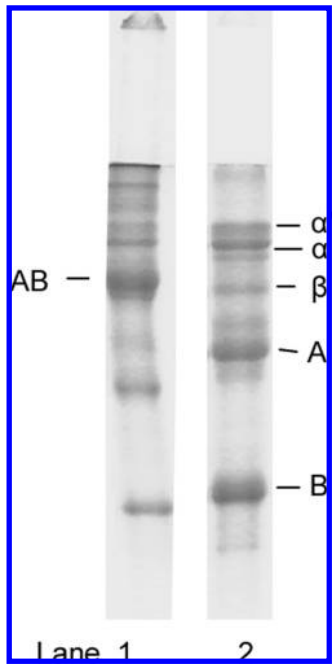

Figure 8. Nonreducing and reducing SDS-PAGE profiles of the eluting fraction (NF) of Sephacryl S-500 in **Figure 7**: lane 1, without 2-ME; lane 2, with 2-ME.

α′ and α subunits of 7S. These aggregates and a part of monomeric subunits of 7S and 11S, as structural units, interact with each other to form protein particles primarily via noncovalent interactions, especially hydrophobic interactions and hydrogen bonding. The disulfide-linked basic polypeptides of the aggregates should be located inside the protein particles, whereas the acidic, α′ and α subunits should be located outside them for their high hydrophilicity.

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Received for review October 23, 2008. Revised manuscript received December 31, 2008. Accepted January 14, 2009. This research was supported by 863 Program (Hi-tech research and development program of China) (Serial No. 2006AA10Z328).

JF803321N