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Effect of concentration, ionic strength and freeze-drying on the heat-induced aggregation of soy proteins

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Abstract

The effects of protein concentration, ionic strength, and lyophilization on heat-induced aggregation of soy proteins were analyzed by SDS-PAGE, SEC-HPLC and laser light scattering (LLS). SDS-PAGE profile suggested that the aggregates were formed via non-covalent forces and/or disulfide bonds. At ionic strength of zero, SEC-HPLC revealed that the samples were composed of three major fractions: aggregates, intermediate fractions and non-aggregated molecules. Furthermore, the relative proportion of the aggregate fraction increased as protein concentration increased. Similarly, LLS indicated that the average hydrodynamic radius (R_h) increased at higher protein concentration. In sample with an ionic strength of zero, the intermediate fraction decreased after freeze-drying with a concomitant increase of the aggregate fraction. When the sample was heated at elevated ionic strength, the SEC-HPLC and LLS profiles changed substantially, the intermediate fractions decreased, and lyophilization had effect on the fraction of aggregates. These experiments suggest novel strategies for producing soy protein aggregates with controlled properties.

Keywords: Soy protein; Aggregate; Intermediate fractions

1. Introduction

The ability of globular proteins to form heat-induced gels has long been exploited to produce foods with different structural and textural characteristics (Clark, 1998). For many years, researches on a number of different proteins, including soy proteins, have proposed a multi-stage mechanism for globular protein gelation: thermally induced unfolding is the first step, followed by aggregation and cross-linking. More recently, the influence of phase separation on gel structure has received more attention (Hua, Cui, & Wang, 2003). It is found that phase separation introduces a fourth stage in the protein gelling mechanism (Clark, Kavanagh, & Ross-Murphy, 2001). The gel structure is determined by the relative kinetics of cross-linking and phase separation. It is thus possible that microstructure can be manipulated by controlling molecular ordering, aggregation, and phase separation. Vardhanabhuti, Foegeding, McGuffey, Daubert, and Swaisgood (2001) found that replacing native whey protein isolate with whey protein aggregates increased fracture stress, fracture modulus, water holding, and the translucency of whey protein gels. Protein aggregation also plays an important role in many protein and polysaccharide mixtures, with consequences for the rheology and microstructure of food systems (Durand, Gimel, & Nicolai, 2002). It is reported that exocellular polysaccharide (EPS)-aggregated whey protein mixture exhibited phase separation, but mixing native whey protein with EPS did not show demixing (Tuinier, Dhont, & De Kruif, 1999). Indeed, phase separation threshold depends mainly on the excluded volume of the biopolymer and it

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has been noted that the increase in particle size as a consequence of heat-induced aggregation will enhance demixing.

Both soy 7S and 11S globulin in their isolated form can aggregate on heating, the nature of which depends upon the precise conditions of protein concentration, pH and ionic strength. Several models were proposed to describe the mechanism underlying soy protein aggregate formation as well as the role that such aggregates may play in gelation (Yamauchi, Yamagishi, & Iwabuchi, 1991). Hydrogen bonds, hydrophobic interactions and disulfide bonds were all found to play important roles in aggregate formation. When 7S and 11S globulins are mixed, as in the case of conventional soy protein products, protein aggregation may involve the interaction between the two types of soy globulin. Previous work found that the basic polypeptide of 11S interacted preferentially with the β-subunits of 7S, and the resulting aggregates were precipitates; however, the α and α′ subunits and the acidic polypeptide interacted through disulfide bonds to form soluble aggregates (Yamagishi, Miyakawa, Noda, & Yamauchi, 1983). These studies have focused on the structure of protein aggregates at the molecular level (the protein secondary or tertiary structure), yet mesoscale structural parameters, such as molecular weight distribution and particle size, have been largely ignored. However, mesoscale parameters are important for understanding phase separation. It is the object of this paper to understand the mesoscale structure of the thermally induced aggregates. To avoid the oxidized lipid induced protein aggregates, an alcohol washing method was used in preparing soy protein. The heat-induced aggregates were obtained by heating the soy protein solution under controlled conditions. SEC-HPLC and LLS were used to determine the molecular weight distribution and particle size. This study would contribute to our further study on the relationship of aggregation and phase separation properties of soy protein–polysaccharide mixtures.

2. Materials and methods

2.1. Materials

Low-denatured, defatted soy flake was provided by Shandong Gushen Industrial & Commercial Co., Ltd. The flake had a protein content of 55.0% ($N \times 6.25$, dry base) and a nitrogen solubility index of 87% (AOAC, 1970). All other reagents and chemicals were of analytical grade.

2.2. Aqueous alcohol washing of defatted soy flake

Defatted soy flake, which had been ground to pass 80 meshes, was extracted with 85% aqueous alcohol at 25 °C for 1 h with a ratio of 1:5 of flake to solvent. The slurry was vacuum filtered and the filter cake was extracted with 95% aqueous alcohol again at 25 °C for 1 h with a ratio of 1:2 of flake to solvent. The slurry was also vacuum filtered and the filter cake was air-dried at room temperature. The dried material was ground to pass 80 meshes and stored at 4 °C.

2.3. Preparation of soy protein devoid of oxidized lipid induced protein aggregation

Aqueous alcohol washed soy flake precipitate were suspended in distilled water and pH was adjusted to 7.0 with 2 N NaOH. After stirring for 1 h at room temperature, the suspension was centrifuged at 10,000*g* for 30 min at 4 °C to recover the supernatant. Soy protein was precipitated by adjusting pH to 4.5 with 2 N HCl and centrifuged at 10,000*g* for 30 min at 4 °C. After washing the curd with distilled water, the protein precipitate was re-suspended in distilled water and neutralized to pH 7.0 with 2 N NaOH and kept at 4 °C overnight. After centrifuging at 10,000*g* for 30 min at 4 °C to remove small quantity of insoluble substances, the supernatant was dialyzed at 4 °C for 24 h, freeze dried, and then stored at 4 °C.

2.4. Turbidity measurement of soy protein dispersions

Soy protein dispersions were prepared in distilled water to desired concentration, stirred magnetically at room temperature for 1 h. Absorbance of the dispersions which was determined at 600 nm on an UV–Vis spectrophotometer (Unico Model 2100, The Biotechnology Education Company, USA) was used as an indicator of turbidity. All samples were measured in triplicate and the means reported.

2.5. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) in a Bio-Rad mini-Protein electrophoresis cell at a constant voltage (200 V) with a gel concentration of 10%. A continuous and dissociating buffer system, containing 0.375 mol/L Tris–HCl, pH 8.8, and 0.1% SDS for the separating gel and 0.025 mol/L Tris–HCl, 0.192 mol/L Gly, and 0.1% SDS, pH 8.3, for the running buffer, was used. Soy protein samples were mixed with reductive sample buffer (10% SDS, 2.5% β-mercaptoethanol) to give a concentration of 2 mg/mL and the solutions were heated at 95 °C for 5 min. To each well, 20 μL of sample solution was loaded. Gel slabs were fixed and stained simultaneously using the Bio-Rad Coomassie blue R-250 stain solution (40% methanol, 10% acetic acid, 0.1% Coomassie blue R-250) for 1 h and destained by Bio-Rad Coomassie blue R-250 destain solution for 5 h with 2–3 changes of destain solution. Molecular weight of each band was determined using Sigmamarker (aprotinin, 6500, a-lactalbumin, 14,200, trypsin inhibitor, 20,000, trypsinogen, 24,000, carbonic anhydrase, 29,000, glyceraldehydes-3-3phosphate dehydrogenase, 36,000, ovalbumin, 45,000, glutamic dehydrogenase, 55,000, albumin, 66,000, fructose-6-6phosphate kinase, 84,000, phosphorylase *b*, 97,000, galactosidase,

116,000, myosin, 205,000) as molecular weight standards. Triplicate runs were carried out.

2.6. Preparation of heat-induced soy protein aggregates

Soy protein powders were suspended in distilled water at 0.5, 1, 2, 3, 4 and 5 (wt%) and magnetic stirred thoroughly. The suspensions were centrifuged at 10,000*g* for 15 min and the supernatants were filtered through cellulose acetate membranes with pore size of 0.45 μm (Merck, Germany) to remove any insoluble particles. The obtained soy protein solutions were injected into screw caped test tubes and heated in a water bath at 100 °C for 15 min and cooled to room temperature in an ice bath for 5 min. In order to study the effect of ionic strength on the heat-induced aggregation, soy protein dispersions with ionic strength of 0.1, 0.2 and 0.4 were prepared by adding NaCl to protein solutions with protein concentration of 1%. Parts of above dispersions were freeze-dried under the pressure of 13.5 Pa and plate temperature of −25 °C. According to Croguennoc, Nicolai, and Durand (2001), the aggregates were stable to dilution and could therefore be investigated by various techniques, so all the aggregate samples were adjusted the concentration to 1% for SEC-HPLC and LLS measurements.

2.7. Molecular weight distribution by SEC-HPLC

The molecular weight distribution was determined by SEC-HPLC. An Agilent liquid chromatography system equipped with a Shodex protein KW-804 Column (Shodex Separation and HPLC Group, Tokyo, Japan) and an Agilent ultraviolet detector was used. The column had an exclusion limit of 1,000,000 Da and separation efficiency larger than 20,000 Da. The flow rate was 1 mL/min with 50 mmol/L phosphate buffer (pH 7.0) and the eluent was monitored at 280 nm. All samples were measured in triplicate and the representative examples were selected for discussion. An Agilent Chemistry Station was used to analyze the peak area by integral and transmit to percentage.

2.8. Particle size distribution determined by laser light scattering

An ALV-5000/E Laser light scattering analyzer (ALV-Laser Vertriebsgesellschaft GmbH, Germany) was used in our experiment. Dynamic light scattering works by measuring the intensity of light scattered by the molecules in the sample as a function of time. If the molecule was stationary then the amount of light scattered would be a constant; however, as all molecules in solution diffuse with Brownian motion in relation to the detector, there will be interference (positive or negative) which causes a change in this intensity. If the temperature and solvent are constant and known, the variation in the intensity of the scattered light is directly related to the hydrodynamic radius, R_h of the particles through a correlation function that is interpreted in terms of the particle translational diffusion coefficient and the diameter is calculated using Stokes–Einstein equation. The method has been described in detail by Lyn et al. (1990). Generally, the mean particle diameter determined by LLS increases with the decrease in the angle of measurement. The lower apparent diameters at the larger angles of measurement were explained by the Mie scattering theory (McConnell, 1981), where the light scattering factor (q) times the radius of gyration was greater than 1 ($qR_g > 1$), and the theory predicted that the radius would be underestimated. Besides, the light intensity at a given angle increases with the particle diameter (sixth power) and that the maximum of the scattered light intensity distribution in space shifts to smaller angles. For a polydispersed protein dispersion, the measured diameter at the small angles represents the mean particle diameter somewhat weighed in favor of large diameters. The particle size value reported is also depended on data analysis model. In this study, the size distribution as revealed by multimodal analysis, using the middle group and giving slightly higher diameters than the unimodal diameter (by Gaussian analysis), was retained in this work because it gave more consistent and reproducible results. Similarly, polydispersity indexes were obtained to characterize the uniformity of the dispersions. Generally, a lower value of the polydispersity index indicates a more uniform distribution. The instrument was operated at room temperature and the wavelength and scattering angle were fixed at 632 nm and 90°, respectively. Although the precise average particle size distribution cannot be determined with certainty, the mean diameters determined at the fixed 90° were significant in evaluating the changes in average particle size. All samples were measured in triplicate and the representative examples were selected for discussion.

2.9. Statistical analysis

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

3. Results and discussion

3.1. Soy protein preparation

Soy protein prepared from alcohol washed soy flakes (ASP) contains 98.09% of protein, substantially higher than that of soy protein directly from defatted soy flakes (conventional soy protein, CSP). Furthermore, the turbidity of the former was substantially lower than the latter (Fig. 1). Huang, Hua, and Qiu (2006) studied the effect of linoleic acid oxidation catalyzed by lipoxygenase on the physicochemical properties of soy protein and their experiments indicated that non-heat-induced aggregates with high molecular weight and large particle size were formed mainly via non-covalent interactions. Alcohol washing of defatted soy flakes undertaken in our experi-

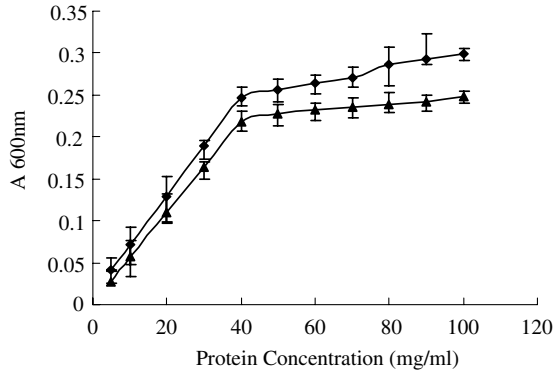

Fig. 1. Turbidity of soy protein samples as the function of protein concentration. ASP (soy protein prepared from alcohol washed soy flakes, ▲) and CSP (soy protein directly from defatted soy flakes, i.e. conventional soy protein, ♦) were dispersed in distilled water, respectively, to desired concentration and the absorption of the dispersions was determined at 600 nm.

ment could inhibited lipoxygenase-catalyzed linoleic acid oxidation induced protein aggregation via removing residual lipids in soybean and thus was proved to modify soy protein functional properties considerably (Hua, Huang, Qiu, & Liu, 2005). In the following experiments, soy proteins prepared from alcohol washed soy flakes were used.

3.2. Electrophoresis

Comparing to unheated sample (lane 1), which presented the characteristic bands for 7S's subunits and 11S's polypeptides, heat-induced soy protein aggregates at 1% and 5% protein concentration respectively (lane 2 and 3) displayed essentially the same electrophoretic profile with regarding to the subunit and polypeptide compositions (Fig. 2). Yet some minor changes were observed for aggregates in 7S's α and α′ subunits, where α and α′ bands seemed to be diffused. The result suggested that the aggregates were formed via non-covalent forces and/or disulfide bonds, since these interactions could be disrupted in the solution containing SDS and β-mercaptoethanol. Thermal aggregation of soy protein has been studied by several groups (German, Damodaran, & Kinsella, 1982; Utsumi, Damodaran, & Kinsella, 1984), but no research indicated the changes in the major 7S's subunit and 11S's polypeptide compositions. Therefore, our result was consistent with other researches.

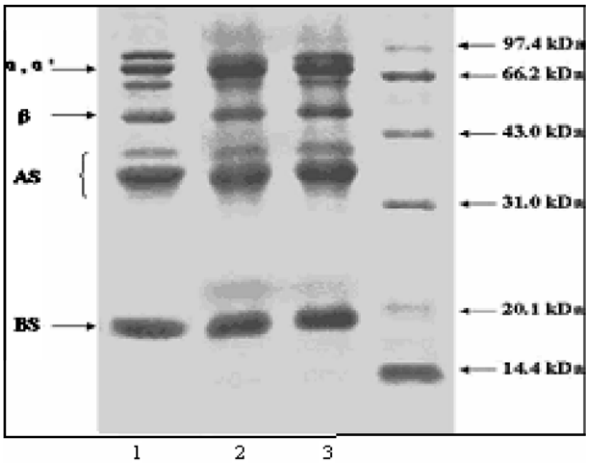

Fig. 2. SDS-PAGE analysis of samples of 1 (soy protein prepared from alcohol washed soy flakes), 2 and 3 (heat-induced soy protein aggregates at 1% and 5% protein concentration respectively) and the standard low molecular weight markers. The protein samples were resolved on a 10% SDS polyacrylamide gel and stained with Coomassie brilliant blue at the room temperature. α, α′ and β indicate subunits of 7S. AS and BS indicate acidic and basic polypeptides of 11S, respectively.

3.3. Aggregation of soy protein at ionic strength of zero

SEC-HPLC measurements were performed at pH 7.0 on heat-induced soy protein aggregates formed at different concentrations at ionic strength of zero (Fig. 3). The elution pattern corresponding to the soy protein before heat treatment displayed two major elution peaks at 280 nm. The first peak with relative intensity of above 70% was characterized by retention time around 9 min, correspond-

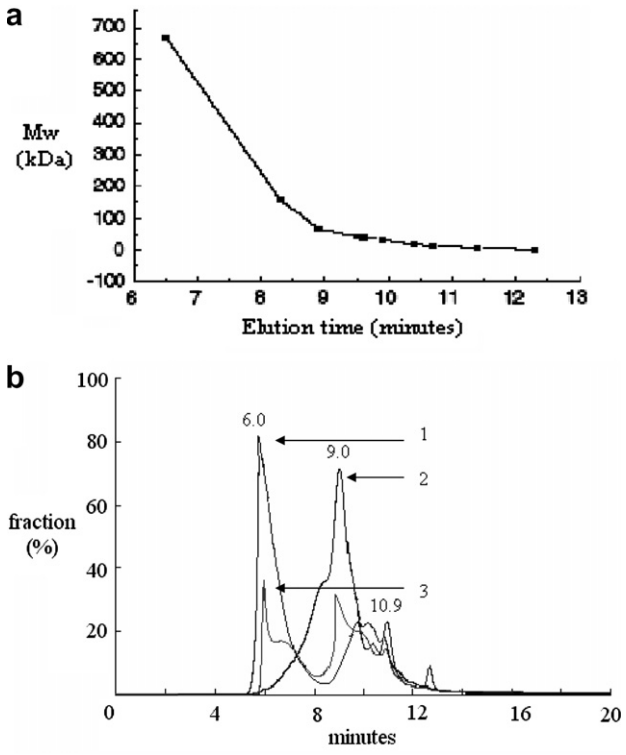

Fig. 3. High performance size exclusion chromatography of soy proteins. (a) Calibration curve of standard proteins on Shodex protein KW-804. Ten standard proteins were thyroglobulin (MW: 669,000), aldolase (MW: 158,000), BSA (MW: 67,000), ovalbumin (MW: 43,000), peroxidase (MW: 40,200), adenylate kinase (MW: 32,000), myoglobin (MW: 17,000), ribonuclease A (MW: 13,700), aprotinin (MW: 6500) and vitamin B12 (MW: 1350), respectively. (b) Native soy protein (line 2), 1% soy protein aggregate (line 3), and 5% soy protein aggregate (line 1). The column was operated at a flow rate of 1 mL min^{-1} with 50 mM sodium phosphate buffer (pH 7.0). Absorbance of the eluent was monitored at 280 nm.

ing to MW of 200,000 Da. A small second peak was also detected around the retention time of 10.9 min. It corresponded to MW of 60,000 Da with relative intensity of 20%. These two peaks (9 min and 10.9 min) corresponded to the intact soy globular protein molecule and subunit, respectively (Koshiyama, 1968). The chromatography of heated soy proteins at ionic strength of zero showed a peak eluting at the void volume with retention time of 6 min corresponding to the aggregated proteins. Since the exclusion limit of the column as provided by the supplier was 10^6 Da, the aggregates were thus estimated to have molecular weight larger than 10^6 Da. There were some peaks eluted around 9–10 min which could probably be contributed by non-aggregated molecules and thermally dissociated subunits. Between the aggregate peak and molecules/subunit peaks, intermediate fractions with molecular weight between 2×10^5 and 10^6 Da were observed, indicating the presence of aggregate with lower degree of aggregation. Furthermore, for the 5% soy protein aggregate, there were higher aggregate fractions (above 70%) and lower intermediate fractions than that of the 1% soy protein aggregate.

Particle size distributions of soy protein dispersions at ionic strength of zero as determined by LLS were shown in Fig. 4. In the unheated sample (native soy protein), there was a large relative contribution to decay in autocorrelation curve of particles with hydrodynamic radius centered at about 10 nm, which could be attributed to be native soy protein molecular as hydrodynamic radius was in consistent with reported dimension for 7S (12.5 nm × 12.5 nm × 3.75 nm) and 11S (12.6 nm × 12.6 nm × 7.5 nm) globulin. In addition, there was also a large contribution of particles with hydrodynamic radius centered at about 100 nm. Presumably, these particles were aggregates formed during soy protein sample preparation or small dust particles. Because large particles scatter much more light than smaller particles, this peak was relatively large, although the quantity was quite small.

After heating, contribution of native-like soy protein molecules decreased, whereas that of large particles increased. The size of the heat-induced aggregates was almost the same as that of particles found in unheated sample but the distribution became narrower, probably because of the decreased relative contribution of the large impurities present in the unheated solution (i.e. relative as compared with newly formed aggregates with slightly smaller size) or possibly because the impurities had precipitated. A shoulder peak with radius around 20–50 nm was observed, indicating the presence of the oligomer which was compatible with the intermediate fractions found in SEC-HPLC analysis. Petruccelli and Anon (1995) studied the heat-induced aggregates by both non-reducing and reducing SDS-PAGE. They also found that intermediate aggregates with molecular weight of 180,000–190,000, formed by α and α′ subunits of 7S as well as intermediate aggregates with molecular weight of 115,000–120,000 formed by α′, α subunits of 7S and a polypeptides of 11S. For the 5% soy protein aggregate, the radius became larger.

In light scattering analysis, particle diameter was measured through the diffusive transport coefficient, but non-elastic particle–particle collisions could reduce the diffusion coefficient and gave larger apparent diameter at high protein concentrations. However, this effect was expected to be relatively small with Newtonian dispersions as the case in this study. Thus protein aggregation behavior would be probably the most important factors determining the diameter changes.

A three-step mechanism for the aggregation of β-lactoglobulin had been proposed which included monomer activation (denaturation), formation of oligomer via disulfide bond and finally, the aggregate formation by non-covalent forces. Since globular proteins formed aggregates and gels in a similar way (Kavanagh, Clark, & Ross-Murphy, 2000), presumably, soy proteins formed aggregates by a similar progressive mechanism. Formation of thermally induced aggregates of the 7S soy globulin was studied using atomic force microscopy (Mills, Huang, Noel, Gunning, & Morris, 2001). The results showed that the small linear aggregates formed at lower protein concentrations, but at higher protein concentrations these small linear aggregates rapidly transformed into large macro-aggregates through interacting with each other, which could be the precursors of protein gel formation. Marangoni, Barbut, McGauley, Marcone, and Narine (2000) found that the aggregation process of whey protein was protein concentration dependent, the higher the initial WPI protein concentration, the greater the SEC-HPLC excluded volume peak. Le Bon, Nicolai, and Durand (1999) showed that the protein concentration had a marked effect on the thermal denaturation and aggregation of β-lactoglobulin. Hoffmann, Sala, Olieman, and De Kruif (1997) evaluated the effect of protein concentration in the range 0.1–1% on β-lactoglobulin using SEC-MALLS and showed that, with increasing concentration, the aggregate peak shifted towards lower elution volumes, demonstrating that the average size of the aggregates increased with increasing concentration. This shift towards higher molecular masses was much stronger for protein concentration than that for heating time.

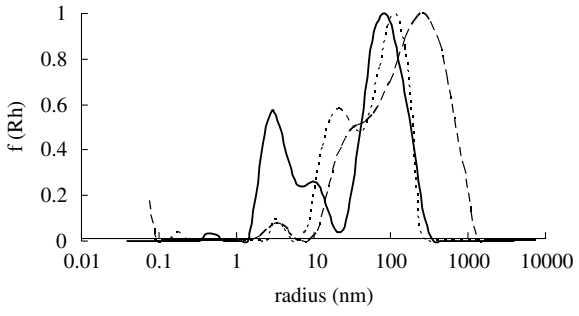

Fig. 4. Particle size distributions of soy proteins suspensions determined by laser light scattering (ALV-5000/E) at 25 °C with the wavelength of 632 nm and scattering angle of 90°. Native soy protein (solid line), 1% soy protein aggregate (dotted line), and 5% soy protein aggregate (dashed line).

3.4. Aggregation of soy protein at higher ionic strength

Fig. 5 showed the size exclusion chromatography of soy protein aggregates with protein concentration of 1% at ionic strength of 0.1, 0.2 and 0.4, respectively. The results showed that, with the increase of ionic strength, the fractions of aggregate peak which were eluted around 6 min increased dramatically with the concomitant decreasing of small fractions. Another common feature of chromatography as comparing with those obtained at ionic strength of 0 (Fig. 3) was that a narrow and sharp aggregate peak distinctively separated from other peaks, indicating a relatively more uniform distribution of aggregate particle weight. Moreover, there were only small part of intermediate fractions between the aggregate peak and molecular or subunit peaks.

Particle size (R_h) as determined by LLS of heat treated soy protein solution at elevated ionic strength (shown in Table 1) also revealed a more homogenous distributions comparing with those obtained at ionic strength of 0. Because as the ionic strength increased from 0.1 to 0.4, the average R_h value increased from 49.12 nm to 102.30 nm, and the polydispersity index decreased, which showed the particles had a more uniform distribution. The impact of ionic strength on the aggregation behavior of soy protein had been noticed by several researchers but the quantitative relationship was lack. Iwabuchi, Watanabe, and Yamauchi (1991) found that heat denatured β-conglycinin formed aggregates above the ionic strength of 0.02. Mills et al. (2001) also found a dramatic change in AFM image of the thermally induced soy 7S globular aggregates structure when the ionic strength increased from 0.01 to 0.2. The thermal aggregation behavior of soy 11S globulin under ionic strength of 0.5 was studied by Mori, Nakamura, and Utsumi (1982), and soluble aggregates with molecular weight of 8×10^6 were formed irrespective as to whether the protein concentration being of 0.5% or 5%.

Ionic strength reduces the intermolecular repulsion between molecules because of the screening of charged groups, thereby increasing the aggregation rate. Moreover, because of the charge distribution on the molecules at low ionic strength, the molecules can aggregate only when they are in a specific orientation; however, at higher ionic strength aggregation may occur more randomly (Schokker, Singh, Pinder, & Creamer, 2000). Some recent study showed that, in heated β-lactoglobulin solutions, very elongated aggregates with low fractal dimensionality are formed at low ionic strength and more globular, branched aggregates with higher fractal dimensionality are formed at higher ionic strength (Aymard, Durand, & Nicolai, 1996). On the other hand, an increase in ionic strength increases the denaturation temperature of the protein, because screening of charged groups reduces intra-molecular repulsion, thereby increasing conformational stability. Because the unfolding of the protein molecule is the initial reaction leading to aggregation, a decrease in the rate of unfolding would lead to a decreased aggregation rate. This effect would be especially noticeable at around the denaturation temperature because the unfolding reaction may become rate limiting (Clark et al., 2001). In our experiments, soy protein were heated at 100 °C, which was far away the denaturation temperature of soy protein (80 °C); and in previous works we had obtained that soy protein formed gel below 100 °C at the ionic strength of 0.5. So considering the temperature and salt concentrations used in our experiment, the first effect clearly outweighed the second effect.

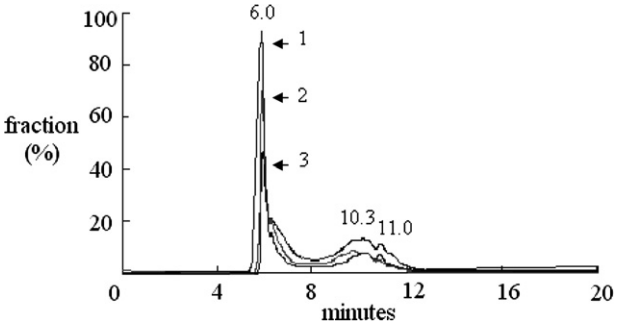

Fig. 5. SEC-HPLC of heat-induced aggregates of 1% soy protein with different ionic strength (I). $I = 0.1$ (line 3), $I = 00.2$ (line 2) and $I = 0.4$ (line 1).

Table 1
Average hydrodynamic radius (R_h, nm) and polydispersity index (Poly. index) of thermal induced aggregates of 1% soy protein

Samples	R_h (nm)	Poly. index
NF, $I = 0$[a]	37.02 ± 0.3A[c]	0.685 ± 0.04A
F, $I = 0$[b]	44.99 ± 0.02B	0.554 ± 0.04B
NF, $I = 0.1$	49.12 ± 0.1C	0.361 ± 0.03C
NF, $I = 0.2$	54.50 ± 0.8D	0.342 ± 0.03D
NF, $I = 0.4$	102.30 ± 0. 7E	0.256 ± 0.03E
F, $I = 0.2$	108.21 ± 0.02F	0.149 ± 0.02F

[a] NF, non-freeze-dried.
[b] F, freeze-dried.
[c] Least significant difference values were computed at the 5% level. The different letter in columns indicates significant difference at $P < 0.05$.

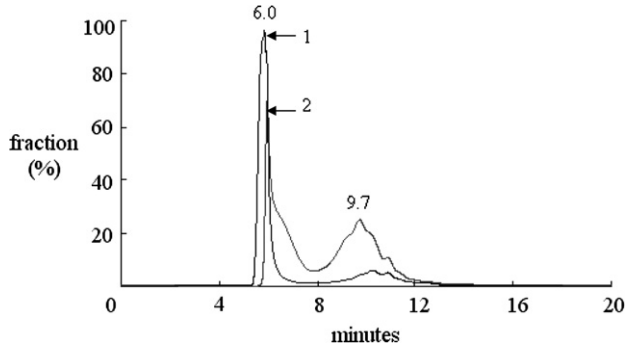

Fig. 6. Molecular distribution of freeze-dried heat-induced soy protein aggregates of 1%. $I = 0$ (line 2), $I = 0.2$ (line 1).

Table 2
Effect of freeze-drying on the fraction of aggregate under different soy protein concentration ($P < 0.05$)

Protein concentration (%)	Fraction of aggregate (%)					
	0.5	1	2	3	4	5
Non-freeze dried sample	14.7 ± 0.1	28.8 ± 0.6	32.6 ± 0. 3	40.0 ± 0.04	49.4 ± 0.2	71.1 ± 0.1
Freeze dried sample	47.5 ± 0.07	52.8 ± 0.4	54.3 ± 0.05	60.7 ± 0.5	65.7 ± 0.01	79.9 ± 0.03

Least significant difference value was computed at the 5% level. $P < 0.05$ indicates that all the fraction of aggregate of freeze dried and non-freeze dried samples are significant different.

3.5. Effect of freeze-drying on soy protein aggregates

The molecular weight distribution of freeze-dried heat-induced soy protein aggregates at the ionic strength of zero and protein concentration of 1% as determined by SEC-HPLC was shown in Fig. 6. Remarkable changes in elution profile were noted comparing with that of non-freeze dried sample (Fig. 3): firstly, the aggregate peak became narrower and sharper, and secondly, the intermediate fractions between the aggregate peak and non-aggregated molecule peaks decreased largely. R_h analyzed by LLS was also affected remarkably by freeze drying. As could be found in Table 1, R_h value became higher from 37.02 nm to 44.99 nm with the polydispersity index decreased.

We also compared the non-freeze dried soy protein aggregates with freeze dried soy protein aggregates at ionic strength of zero as shown in Table 2. With HPLC data we calculated the proportion of aggregate peak (i.e. eluted at 6.0 min) under different concentration. The former increased and the intermediate fractions decreased as the concentration increased. For the freeze-dried sample, the intermediate fractions decreased to a lower proportion at the lower concentration, concomitantly the fractions of aggregate increased.

At higher ionic strength ($I = 0.2$) the molecular weight distribution of freeze-dried heat-induced soy protein aggregates of protein concentration of 1% as determined by SEC-HPLC was also shown in Fig. 6. The profile was similar comparing with that of non-freeze dried sample (Fig. 5), but the fraction of aggregate increased from 72.5% to around 95%. Particle size analyzed by LLS could be found in Table 1, the size of the major aggregates increased to 108.21 nm. Comparing to the sample without freeze-drying, the former formed more uniform structure with larger aggregates and lower polydispersity index.

The amount of water covering the surface of a protein in a fully hydrated state was around 0.3 g/g protein (Kuhlman, Yang, Boice, Fairman, & Raleigh, 1997), while the water content of a dried protein product was usually less than 0.1 g/g. Therefore, the drying process removed part of the hydration layer, which disrupted the protein structure and caused aggregation.

Now the research of the effect of freeze-drying on the aggregation behavior of soy protein was scarce. There was a small but significant body of literature supporting the idea that loss of protein integrity during freezing and freeze-drying is, at least in part, an interfacial phenomenon involving partial denaturation of protein at the ice/freeze-concentrate interface. Strambini and Gabellieri (1996) studied fluorescence lifetimes of tryptophan residues in several proteins and demonstrated that freezing of aqueous solutions of these proteins is accompanied by loosening of the native fold and considerable loss of secondary and tertiary structure. More researches are needed to study the mechanism of the effect of freeze-drying on the protein aggregates.

4. Conclusions

The result of SDS-PAGE suggested that the aggregates were formed via non-covalent forces and/or disulfide bonds, which was consistent with other researches. Molecular weight distribution and particle size of aggregate were affected by protein concentration, ionic strength. At ionic strength of zero, both SEC-HPLC and LLS analysis suggested the presence of intermediate aggregates; however at elevated ionic strength, the intermediate fractions decreased markedly. The polydispersity indexes of protein aggregates indicated that after freeze-drying the systems formed more uniform distributions. Further studies are needed to focus on the phase separation of these aggregates and polysaccharides, and the influences of their excluded volume on the phase separation threshold.

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