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Simple Extraction Method of Non-allergenic Intact Soybean Oil Bodies That Are Thermally Stable in an Aqueous Medium

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This study supplied a simple extraction method for intact soybean oil body (ISOB) and examined the heating effect on ISOB. ISOB, which just contained its intrinsic protein (oleosin), could be obtained by pH 11 extraction (50000*g*, 45 min). ISOB suspension was dialyzed to deionized water (1:3600) and named DISOB. DISOB aggregated at pH 5.7, but NaCl pre-addition (5−500 mM) made ISOB disperse well at pH 5.7. The heating (30, 40, 50, 60, 70, 80, and 90 °C and boiling water baths, 30 min) did not affect the particle size distributions of ISOB. The pH and $CaCl_2$ effects on DISOB and its surface hydrophobicity were also not affected by heating (>95 °C, 5 min). Both unheated and heated ISOB were bound to native soybean protein but were not bound to the heat-denatured one. Thus, it was suggested that ISOB changed little by heating. This study was meaningful in two aspects: (1) pH 11 extraction removed β-conglycinin, glycinin, and allergenic proteins (such as *Gly* m Bd 30K), and the obtained ISOB had good stability in an aqueous medium. (2) Heating could denature the contamination allergenic proteins.

KEYWORDS: Soybean; raw soymilk; pH 11 extraction; intact soybean oil body (ISOB); hydrophilic surface; *Gly* m Bd 30K; heating

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] seeds store triacylglycerols (TAGs) in organelles called oil bodies. Oil bodies and protein storage vacuoles (PSVs) are filled in dried soybean seed cells, but they do not coalesce (*1*). Shimada et al. reported (*2*) that oil bodies in *Arabidopsis thaliana* seeds had a good freezing tolerance. These show that the oil body can tolerate some environmental stress.

According to the famous oil body model by Huang (*3*, *4*), the oil body has a TAG matrix core, which is covered by a layer of phospholipids and embedded by oil body intrinsic oleosin. Other than oleosin, some surface phospholipids (hydrophilic parts) are also exposed to the exterior. Thus, it was considered that oil body properties were determined by oleosin and the exposed phospholipids. It was known that the soybean oil body had three intrinsic oleosins: 24, 18, and 17 kDa (*5*, *6*). In addition, there was one more protein, known as P34, *Gly* m Bd 30K, or *Gly* m Bd 1 (*7*). Initially, it was considered oil body oleosin because it could bind to the oil body strongly, but in the natural seed, it was present in PSVs and was an allergenic protein to humans (*6*).

Generally, soybean oil is effectively extracted by hexane and is used as a food oil. However, hexane extraction has some bad points: solvent residue, explosive, and not good for the environment. Therefore, aqueous extraction processing (AEP) was tried to extract the oil body (*8*). This was because the oil body had potential to be used in food processing and cosmetics (*9*, *10*). Kapchie et al. reported (*8*) that a high soybean oil body yield (84.65%) could be obtained by enzyme-assisted aqueous extraction. Iwanaga et al. (*11*, *12*) examined the properties of the soybean oil body that was extracted by a pH 8.6 buffer. The results showed that the oil body was unstable in an aqueous medium. Therefore, they stabilized the oil body using pectin.

Huang reported (*4*) that the oil body had a hydrophilic and negatively charged surface at neutral pH. It was considered that the unstable oil body, which was extracted by the pH 8.6 buffer, might be contaminated by some seed proteins, such as the well-known *Gly* m Bd 30K. Thus, this study aimed to supply an extraction method, which could obtain the intact soybean oil body (ISOB). In addition, the heating effect on ISOB was also examined. This was because heating was important in food processing, which could denature the allergenic proteins, and was used for pasteurization.

MATERIALS AND METHODS

Materials. Soybean Suzuyutaka (2007) was used. It was a typical soybean variety in Iwate, Japan. It was stored at 4 °C until use.

Raw Soymilk and Soymilk Preparations. Soybean (20 g) was soaked in deionized (DI) water for 18 h at 4 °C. The total weight of soybean and DI water was made 200 g and ground with an Oster blender (13 900 rpm, 90 s; Oster, Milwaukee, WI). The homogenate was filtrated through two layers of Kimwipe sheets (Nippon Paper Crecia Co., Ltd., Tokyo, Japan). The filtrate was designated as raw soymilk. The raw soymilk was heated at > 95 °C for 5 min and designated as soymilk.

Oil Body Preparations by pH 6.5−11 Extractions. Raw soymilk (20 g) and sucrose (5 g) were added into five 50 mL beakers, respectively. They were mixed, and their pH values were adjusted to 6.5 (raw soymilk itself), 8, 9, 10, and 11 with 0.1 and 1 M NaOH solutions. They were treated

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by centrifugation (50000*g*, 45 min). The five floating fractions (oil body) were collected and washed twice [50000*g*, 45 min; 20% (w/w) sucrose and DI water; pH 6.5, 8, 9, 10, and 11]. Then, the floating fractions were carefully collected and dispersed into 10 mL of DI water, respectively.

ISOB Preparation. Raw soymilk (120 g) and sucrose (30 g) were added to a 200 mL beaker and mixed. pH was adjusted to 11 by 1 M NaOH. Then, the method was the same as above. The floating fraction was dispersed into 50 mL of DI water and deemed as an ISOB suspension. Then, it was dialyzed in 3000 mL of DI water twice and named as a dialyzed intact soybean oil body (DISOB) suspension, which had a lipid concentration of about 3%. In addition, the oil body was extracted with the same method using pH 9 and named as a dialyzed pH 9 oil body (DpH9OB) suspension, which was used as a control.

pH and NaCl Effects on DISOB. The DISOB (10 mL) suspension was diluted 10 times with DI water. pH was adjusted to 6.0 with 0.01 M HCl. About 5 mL was moved into the 1st tube. Then, pH was adjusted to 5.7. Also, about 5 mL was move into the 2nd tube. Then, pH 5.4−3.3 suspensions were moved into the 3rd−10th tubes, respectively.

DISOB (5 mL) suspension was diluted 10 times with DI water and adjusted to pH 5.7. A total of 5 mL was added into seven test tubes, respectively. A total of 0, 0.05, 0.1, 0.5, 1, 2.5, and 5 mL of 1 M NaCl were added. Then, a total of 5, 4.95, 4.9, 4.5, 4, 2.5, and 0 mL of DI water were added with a pipet. As a result, DISOB suspensions, which contained 0, 5, 10, 50, 100, 250, and 500 mM NaCl, were obtained. The pictures were taken by a digital camera (FinePix F50fd, Fujifilm Holdings, Tokyo, Japan).

Heating Effect on ISOB. ISOB (2.5 mL) suspensions were added to eight test tubes and put into 30, 40, 50, 60, 70, 80, and 90 °C and boiling water baths for 30 min, respectively. Then, they were cooled with tap water before particle size distribution determination.

Particle Size Distribution Determination. The particle size distribution was measured using a laser-light-scattering instrument (Coulter LS230, Beckman Coulter, Fullerton, CA). A prefiltrated (0.2 μm membrane filter, Advantec Toyo Co. Ltd., Tokyo, Japan) 30 mM Tris-HCl buffer (pH 9) was transferred into the instrument and circulated at 50 L/min. Glass was selected for the optical model of light scattering. The refractive index (1.332) was used. After the background light scattering was determined, the oil body sample was dropped into the instrument. The particle size distribution was measured and displayed after about 5 min. The particle volume ratio (%) of the sample was shown against the particle diameters.

pH and $CaCl_2$ Effects on Unheated and Heated DISOB. DISOB suspension (10 mL) was diluted 10 times with DI water, heated at > 95 °C for 5 min, and cooled with tap water. Then, 5 mL of pH 6.0−3.3 suspensions was prepared by the method above.

DISOB suspension (1 mL, heated or not) was added to six test tubes. A total of 0, 0.5, 1, 1.5, 2.0, and 2.5 mL of 100 mM $CaCl_2$ were added, and then a total of 9, 8.5, 8, 7.5, 7.0, and 6.5 mL of DI water were added with a pipet. As a result, DISOB suspensions, which contained 0, 5, 10, 15, 20, and 25 mM $CaCl_2$, were obtained. They were centrifuged at 1600*g* for 20 min. The pictures were taken by the digital camera above.

Protein Fraction Preparation of Raw Soymilk and Soymilk. Sucrose was added to raw soymilk and soymilk to concentrations of 20% (w/w). They were stirred and centrifuged (50000*g*, 45 min). Both raw soymilk and soymilk were separated to three fractions: floating (oil body), supernatant, and precipitate. The supernatant fractions were designated as the protein fractions of raw soymilk (native protein, NP) and soymilk (heat-denatured protein, DP).

Oil Bodies of Four Mixtures. Four mixtures were prepared from 5 mL of the ISOB suspension and 20 mL of the protein fraction of raw soymilk (NP), 5 mL of the heated ISOB suspension (> 95 °C for 5 min) and 20 mL of NP, 5 mL of ISOB and 20 mL of the protein fraction of soymilk (DP), and 5 mL of heated ISOB and 20 mL of DP. They were stirred for 20 min and centrifuged (50000*g*, 45 min). Their floating fractions were obtained and washed twice (50000*g*, 45 min; DI water and 20% sucrose). The floating fractions were dispersed into 5 mL of DI water and termed as oil bodies of four mixtures.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was conducted with the method by Laemmli (*13*), with the concentrations of the stacking and running gels being 5 and 12.5%, respectively. The buffer in the reservoir contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS (w/w), while the buffers in the stacking and running gels were 0.125 M Tris-HCl (pH 6.8) and 0.38 M Tris-HCl (pH 8.8), respectively. Samples [0.01% (w/w)] contained 0.25 M Tris-HCl (pH 6.8), 1% SDS, 2% 2-mercaptoethanol, glycerol, and bromophenol blue. This stood overnight at room temperature. Each sample (about 15 μg) was added into a sample well in the stacking gel and electrophoresed.

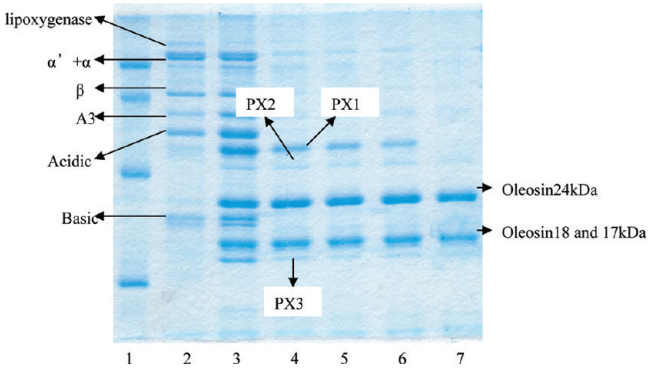


Figure 1. SDS−PAGE patterns of the protein compositions of soybean oil bodies obtained by pH 6.5, 8, 9, 10, and 11 extractions. Lane 1, marker; lane 2, raw soymilk; and lanes 3, 4, 5, 6, and 7, soybean oil bodies obtained by pH 6.5, 8, 9, 10, and 11 extractions, respectively. Markers: bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and lysozyme (14 kDa).

Coomassie Brilliant Blue G-250 was used for staining protein in gel by the method of Blakesley and Boezi (*14*). Gel was destained using tap water and dried on a filter paper (No. 2, Advantec Toyo Co., Tokyo, Japan) in decompression conditions at 75 °C.

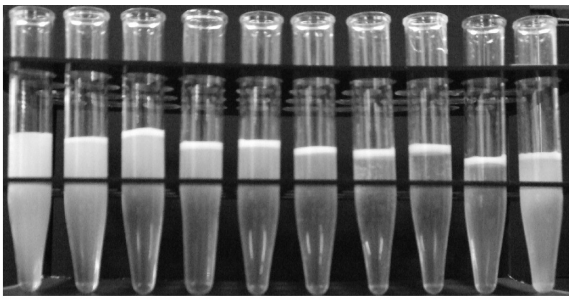
Surface Hydrophobicities of DISOB and Soybean Protein. DISOB suspension (1 mL) was diluted 40 times with 0.1 M phosphate buffer (pH 6.8). A total of 20 mL of this solution was heated at > 95 °C for 5 min. Six samples of 2 mL of diluted heated DISOB suspensions were adjusted to 0, 2, 4, 6, 8, and 10×10^{-5} M 1-anilino-8-naphtalene sulfonate (ANS), with a final volume of 10 mL using the buffer above. The six samples of unheated diluted DISOB suspensions were prepared in the same way. The fluorescence intensity was determined after 2 h by a fluorescence spectrophotometer (RF-5300PC, Shimadzu Co., Tokyo, Japan). The excitation wavelength was 375 nm, and the fluorescence wavelength was 475 nm.

The NP (1 mL) was diluted 40 times with 0.1 M phosphate buffer (pH 6.8). Then, the method for measurement was the same as the unheated and heated DISOB suspensions above.

RESULTS

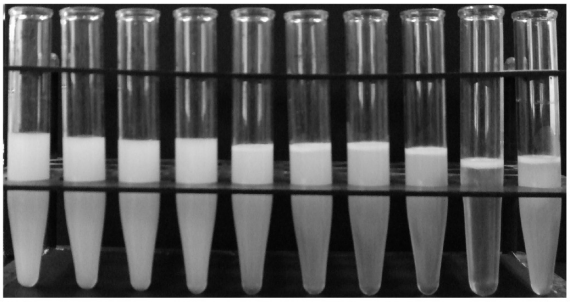
Protein Compositions of Oil Body by pH 6.5−11 Extractions. Guo et al. (*15*) reported that there were many protein molecules binding to the oil body in raw soymilk. Therefore, this section examined the pH effect on oil body surface proteins. Lane 2 (**Figure 1**) showed protein compositions of raw soymilk, which contained β-conglycinin (α', α, and β subunits), glycinin (acidic and basic peptides), and lipoxygenase. Lane 3 showed that the oil body by pH 6.5 extraction contained not only the oil body oleosins (24, 18, and 17 kDa) but also β-conglycinin, glycinin, and some not well-known proteins (PX1, PX2, and PX3). Lanes 4, 5, and 6 showed that oil bodies by pH 8, 9, and 10 extractions had the same protein compositions: oleosins (24, 18, and 17 kDa) and PX1, PX2, and PX3. Lane 7 showed that oil body by pH 11 extraction just contained oleosins (24, 18, and 17 kDa). Thus, it was concluded that (1) PX1, PX2, and PX3 bound more strongly to the oil body than soybean storage proteins (glycinin and β-conglycinin) and (2) ISOB could be obtained by pH 11 extraction.

pH and NaCl Effects on DISOB. As stated above, ISOB could be obtained by pH 11 extraction, and then, DISOB could be obtained by dialysis. In this section, its surface charge property



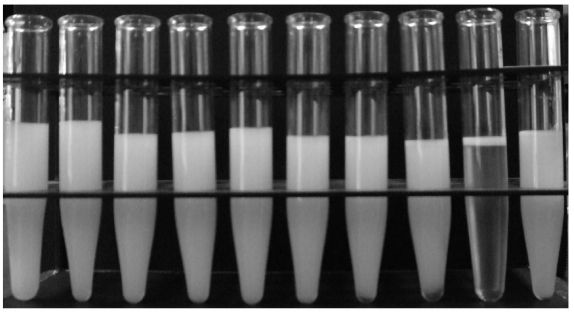
pH 3.3 3.6 3.9 4.2 4.5 4.8 5.1 5.4 5.7 6.0

(a)



pH 3.3 3.6 3.9 4.2 4.5 4.8 5.1 5.4 5.7 6.0

(b)



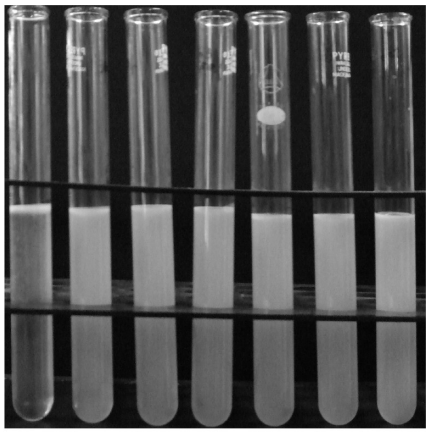
pH 3.3 3.6 3.9 4.2 4.5 4.8 5.1 5.4 5.7 6.0

(c)

Figure 2. pH effect on the soybean oil body. (a) DpH9OB suspension (control), picture taken after 2 h. (b) DISOB suspension, picture taken overnight. (c) Heated (>95 °C, 5 min) DISOB suspension, picture taken overnight.

was examined. **Figure 2a** showed that the DpH9OB (control) suspension, which was obtained by pH 9 extraction and dialysis, aggregated heavily in a larger pH range (3.3−6.0) just after 2 h at room temperature. This trend was in agreement with the results by Iwanaga et al. (*11*), who extracted soybean oil body by a pH 8.6 buffer. In addition, they also showed that soybean oil body would aggregate by NaCl pre-addition. Thus, we considered that the bad stability was caused by PX1, PX2, and PX3 (**Figure 1**). **Figure 2b** showed that DISOB did not aggregate at pH 3.3−4.2 and gradually aggregated from 4.5 to 5.7 and, then, aggregation weakened at pH 6.0 overnight. However, the aggregation was slight, except at pH 5.7. This showed that DISOB had good stability in an aqueous medium, which was in agreement with the report (*4*) that the oil body had a hydrophilic and negatively charged surface at neutral pH.

The results above showed that the DISOB suspension had good stability and just aggregated heavily at pH 5.7. **Figure 3** showed that DISOB suspensions (5−500 mM NaCl) were still



NaCl (mM) 0 5 10 25 100 250 500

Figure 3. NaCl (pre-addition, 0−500 mM) effect on the pH 5.7 DISOB suspension.

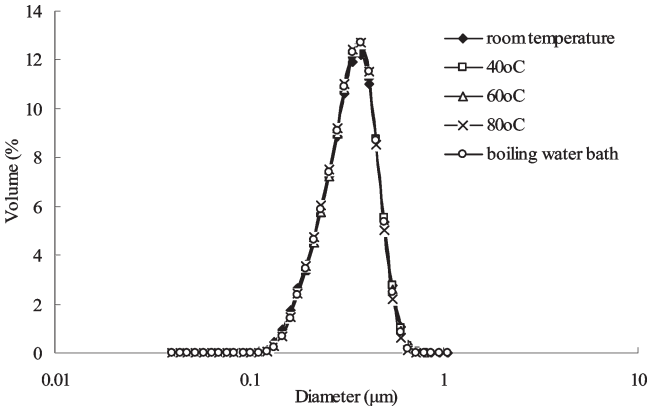


Figure 4. Heating effect (30, 40, 50, 60, 70, 80, and 90 °C and boiling water baths, 30 min) on the particle size distributions of ISOB. The particle size distributions of heated (30, 50, 70, and 90 °C) ISOB suspensions were not shown because they were the same as those (40, 60, and 80 °C and boiling water bath). ISOB (room temperature) was used as a control.

stable overnight, except the one (0 mM NaCl). This revealed that NaCl could increase oil body stability in an aqueous medium.

Heating Effect on ISOB Size. It was considered that there remained little contamination proteins, especially PX1, PX2, and PX3, which might be allergenic proteins to humans, in the ISOB suspension. Thus, the heating effect on ISOB was examined because of its functions: (1) denaturing allergenic protein and (2) pasteurization. At first, the heating (30, 40, 50, 60, 70, 80, and 90 °C and boiling water baths, 30 min; unheated ISOB was used as a control) effect on ISOB size was examined. **Figure 4** showed that heated ISOB had the same particle size distributions. This revealed that ISOB could tolerate high temperatures.

Heating Effect on DISOB Surface Properties. Figure 4 showed the heating effect on ISOB size. In this section, the heating effects on DISOB surface charge and hydrophobicity were examined. **Figure 2c** showed that heated (>95 °C, 5 min) DISOB just aggregated heavily at pH 5.7 overnight, which showed a similar trend with DISOB (**Figure 2b**).

Calcium ion (Ca^{2+}) salt was a kind of coagulant for tofu processing. **Figure 5** revealed that the Ca^{2+} effect on DISOB was not affected by heating. DISOB did not aggregate (1600*g*, 20 min) when $CaCl_2$ was 0 mM, but DISOB aggregated when $CaCl_2$ was 5 mM. Then, the aggregation gradually weakened from 10 mM. As stated above, DISOB aggregated at pH 5.7; therefore, it should be

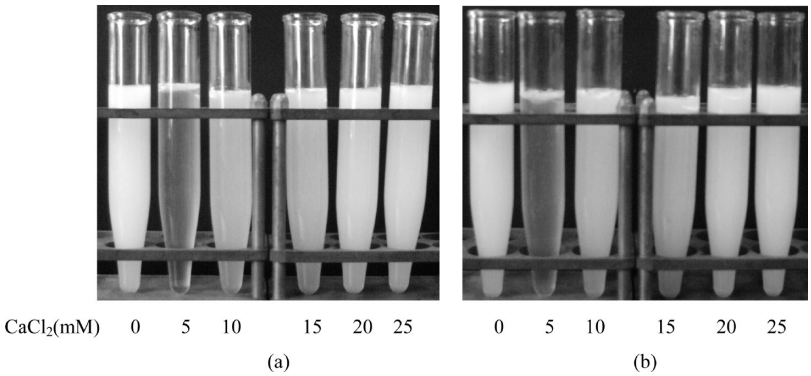


Figure 5. $CaCl_2$ (0−25 mM) effect on the (a) unheated and (b) heated (>95 °C, 5 min) DISOB. The pictures were taken after centrifugation (1600*g*, 20 min).

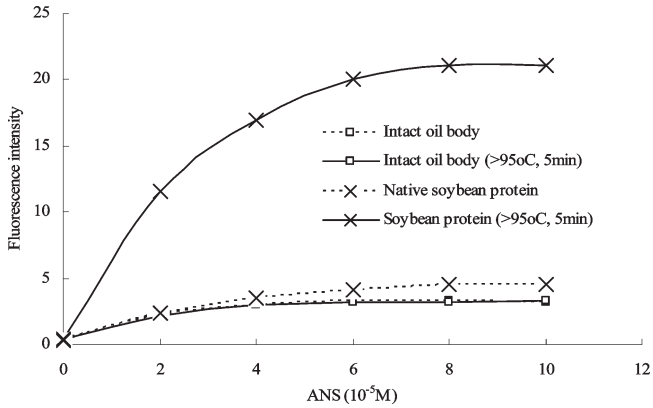


Figure 6. Heating (>95 °C, 5 min) effect on the surface hydrophobicities of DISOB and native soybean protein (NP).

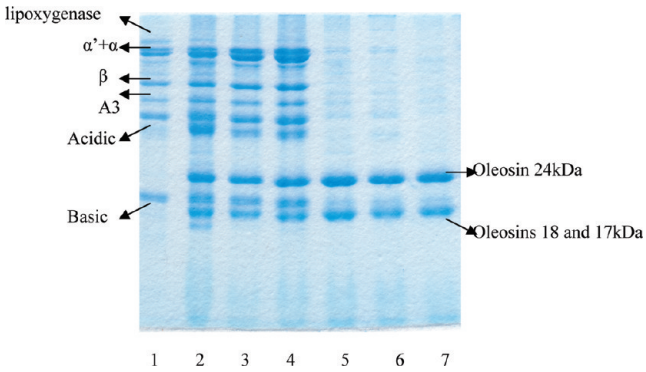


Figure 7. Protein compositions of oil bodies of four mixtures. Lane 1, raw soymilk; lane 2, raw soymilk oil body (by pH 6.5 extraction); lane 3, oil body from the mixture of ISOB and NP; lane 4, oil body from the mixture of heated ISOB and NP; lane 5, oil body from the mixture of unheated ISOB and DP; lane 6, oil body from the mixture of heated ISOB and DP; and lane 7, ISOB.

negatively charged in DI water (neutral pH). Therefore, DISOB (negative charge) dispersed well when Ca^{2+} was 0 mM. It was considered that Ca^{2+} played a role of Ca bridge when its concentration was low, but Ca^{2+} bound to DISOB to make it hold a positive charge when its concentration was high, which made DISOB repel each other again.

The oil body had a phospholipid and oleosin (protein) surface. It was well-known that surface hydrophobicities of many proteins would increase, owing to the exposure of hydrophobic amino acid residues by heating (*16*). Thus, the heating effect on surface hydrophobicity of DISOB was examined. **Figure 6** showed that surface hydrophobicity of DISOB did not change by heating, while that of native soybean protein (NP) was increased by heating. In all, it was suggested that DISOB was not affected by heating.

Soybean Protein-Binding Properties of Unheated and Heated ISOB. The results above showed that size, surface charge, and hydrophobility of ISOB were not affected by heating. Thus, it was considered that heating changed ISOB little. To validate it, soybean protein-binding properties of unheated and heated ISOB were examined. Four mixtures from NP and ISOB, NP and heated ISOB, DP and ISOB, and DP and heated ISOB were prepared. It was found that heated ISOB (lanes 4 and 6 in **Figure 7**) showed the same soybean protein-binding property as ISOB (lanes 3 and 5); both of them could be bound to NP (lanes 3 and 4) but could not be bound to DP (lanes 5 and 6). Lanes 1 and 2 showed the protein compositions of raw soymilk and raw soymilk oil body (pH 6.5 extraction). The raw soymilk oil body did not only contain oleosins (24, 18, and 17 kDa) but also many other proteins (such as acidic and basic peptides, α′, α, and β subunits). Lane 7 showed ISOB just contained oleosins (24, 18, and 17 kDa).

DISCUSSION

ISOB. Other than oleosins (24, 18, and 17 kDa), the raw soymilk oil body was bound weakly to storage proteins (β-conglycinin and glycinin) and enzyme protein (lipoxygenase) but strongly to PX1, PX2, and PX3 (**Figure 1**).

It was well-known that there was lectin, Kunitz trysin inhibitor, Bowman−Birk inhibitor, and three main allergenic proteins (α subunit of β-conglycinin, *Gly* m Bd 30K, and *Gly* m Bd 28K) in soybean seeds. Initially, the *Gly* m Bd 30K was considered oil body olesoin (*5*), because it was bound to the oil body strongly. Kalinski et al. (*6*) reported that *Gly* m Bd 30K, unlike oleosin, was located in the protein storage vacuole. It was reported (*17*−*19*) that *Gly* m Bd 30K on the SDS−PAGE gel was under the acidic peptide band and showed a molecular weight of about 30 kDa. Takahashi et al. reported (*20*) that Suzuyutaka contained *Gly* m Bd 28K, and it was reported (*21*, *22*) that *Gly* m Bd 28K had a molecular weight of about 26 kDa on SDS−PAGE gel. Thus, it was suggested that PX1 and PX2 were *Gly* m Bd 30K and *Gly* m Bd 28K because they showed molecular weights of about 30 and 26 kDa, respectively (**Figure 1**). PX3 was considered as the Kunitz trypsin inhibitor (*23*). In this study, it was clarified that PX1, PX2, and PX3, which strongly bound to the oil body, could be removed by pH 11 extraction and ISOB could be obtained.

The pH effect showed that DISOB should have an isoelectric point near pH 5.7 (**Figure 2b**). Interestingly, NaCl (5−500 mM) pre-addition could inhibit the DISOB aggregate at pH 5.7 (**Figure 3**). Initially, DISOB suspension had an extremely low ionic strength (dialyzed to DI water with 1:3600) before NaCl addition. Therefore, DISOB was dispersed in DI water with its intrinsic negative charge. When pH was adjusted to 5.7, its charge was decreased and aggregation happened (**Figure 2b**). By NaCl pre-addition, the electric double layer was formed, which inhibits the collision among DISOBs (**Figure 3**). With the increase of the ionic strength, the electric double layer would gradually become thin. As a result, colloidal particles would move near each other to cause aggregation. However, **Figure 3** showed that 500 mM NaCl DISOB suspension did not aggregate. This meant that ISOB had a hydrophilic surface or the ionic strength was still not high enough.

Heating Effect on ISOB. pH 11 extraction could remove the allergenic proteins (*Gly* m Bd 30K and *Gly* m Bd 28K) and Kunitz trypsin inhibitor in the laboratory processing method. However, it was possible that ISOB was contaminated by these proteins and some other factors in the industry production. Thus, the heating effect on ISOB was examined, which could denature the allergenic proteins and was used for pasteurization. The particle size distribution of ISOB was not affected by heating (30−90 °C and boiling water baths, 30 min). The pH and $CaCl_2$ effects and surface hydrophobicity were also not affected by heating. In addition, heating did not affect soybean protein-binding property; both unheated and heated ISOB could be bound to native soybean protein but could not be bound to the heat-denatured one. It was considered that these were determined by the phospholipid and oleosin surface of ISOB.

The phospholipid has a simple structure: a hydrophilic part (no charge or negative charge at neutral pH) and a hydrophobic part. The hydrophobic part is embedded into the TAG matrix, and the hydrophilic part is exposed to the exterior. Generally, the oleosin molecule has three parts: N terminus, central domain, and C terminus. The central domain is hydrophobic and embedded into the TAG matrix, while N and C terminuses are located on the ISOB surface. It was suggested (*24*) that the N terminus was a random structure and a small part (4−6 kDa) of the C terminus might be α helix; however, most of it was random structure. The amino acid compositions of oleosin N and C terminuses were characteristic of more alkaline amino acid residues than acidic ones. Huang (*4*) reported that the alkaline ones bound to negatively charged phopholipids by salt bridges, which made N and C terminus bind to the oil body closely; the acidic and hydrophilic ones were exposed to the exterior. Therefore, the surface phospholipid and oleosin made ISOB have a hydrophilic and negatively charged surface at neutral pH. It was considered that heating would not change the hydrophilic parts of phospholipids. However, heating could weaken the salt bridge; thus, N and C terminuses released from oil body surface. However, they might return to their intrinsic locations after cooling, owing to the salt bridges. These should be the reasons why heating did not change the surface charge and hydrophobicity of ISOB. In addition, it was considered that the steric hindrance of phospholipid and oleosin (*4*) and hydrophilic and negatively charged surface of ISOB were the reasons why the particle size distributions of heated ISOB (**Figure 4**) were the same.

In all, it was considered that non-allergenic ISOB could be obtained by pH 11 extraction and heating. The ISOB had good stability in an aqueous medium, especially with NaCl pre-addition. Thus, this study was meaningful for soybean oil body use in food processing and cosmetics.

ABBREVIATIONS USED

TAG, triacylglycerol; ISOB, intact soybean oil body; DISOB, dialyzed intact soybean oil body; NP, protein fraction of raw soymilk; DP, protein fraction of soymilk.

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