Original paper

Effect of Oleosins on the Stability of Oil Bodies in Soymilk

Shiori IDOGAWA^{1,2*}, Naoki ABE², Keietsu ABE² and Tomoyuki FUJII²

¹Taishi Food Inc., 51-1 Shinden, Furukawashimizu, Osaki, Miyagi 989-6228, Japan.

²Graduate School of Agricultural Science, Tohoku University, 468-1 Aramaki Aza Aoba, Aoba-ku, Sendai, Miyagi 980-8572, Japan.

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The aim of this study was to describe the effect of oleosins on the stability of oil bodies in the production of soymilk. We investigated the protein content, average particle diameter and SDS-PAGE analysis of oil bodies at different stages of the soymilk manufacturing process. N-terminal sequencing analysis showed that 11-kDa and 9-kDa fragments, which were not digested by papain, were present at the hydrophilic C-terminal domains of the 24-kDa oleosin and did not undergo decomposition. In terms of colloidal stability, oil bodies from soybeans had the highest floating rate. The binding of oleosin and soymilk proteins was shown to play a role in colloidal stability, especially in solutions with less than 2.5 mg/mL protein. Using transmission electron microscopy, it was found that the surface of the oil bodies was coated in proteins, increasing their stability.

Keywords: oleosin, stability, oil body, pH, protein, soymilk.

Introduction

The production of soymilk involves the formation of a stable colloidal system from soybeans. In soybeans, proteins are mainly located within protein bodies and lipids are present in oil bodies. Soaking crushed soybeans breaks down these intracellular structures. In general, grinding causes the intracellular tissues to break; the protein is then eluted from the protein bodies, but the oil bodies are believed to disperse without breaking (Cambell and Glantz, 2009). The surface of oil bodies binds non-specifically to a wide range of proteins. The oil bodies and proteins in soymilk are then dispersed using heat (Guo *et al.*, 1997). Particles that are not fully dispersed, along with the cell walls, are filtered out as okara (Idogawa *et al.*, 2013); this leaves the oil bodies to be dispersed in the soymilk in a steady colloidal state (Chen and Ono, 2010a; Waschatko *et al.*, 2012). Although the colloidal nature of soymilk is an important issue in food processing, it has not been fully clarified.

In particular, the discussion of protein and lipid interactions is important for the colloidal stability of soymilk. In previous studies, the behavior of proteins and lipids during the heating process of soymilk production has been investigated. The

studies found that proteins on the surface of oil bodies were liberated through heating (Guo *et al.*, 1997; Yan *et al.*, 2016). Additionally, regarding the stability of colloidal particles at different pH values, it was suggested that the lipid content of the soybeans affected the stability of the soymilk (Oizumi *et al.*, 2016). Furthermore, in the colloidal soymilk dispersion system, an interaction between lipids and proteins was identified; the effect of increased concentrations of magnesium chloride on the lipids was also examined (Toda *et al.*, 2008; Idogawa and Fujii, 2015; Fujii, 2017). Based on these findings, it was suggested that the presence of proteins and lipids in soymilk has an important effect on colloidal stability. In fact, it was shown that the state of the emulsion particles influences the elastic behavior of tofu produced from soymilk (Ito and Idogawa, 2013), and that lipids form a part of the protein network making up yuba (Chen and Ono, 2010b). However, the majority of studies on soymilk have focused on proteins, while few have focused on lipids.

Oil bodies have been investigated in a wide range of studies as an energy-storing organelle (Pyc *et al.*, 2017). In particular, in the most complex angiosperms, oil bodies have a particle size of 0.2–2.0 μm , and it is generally assumed that the

*To whom correspondence should be addressed.

E-mail: s-itono@taishi-food.co.jp

neutral lipids are surrounded by a single layer composed of phospholipids bound to a specific protein (Tzen and Huang, 1992; Tzen *et al.*, 1993; Schmidt and Herman, 2008; Maurer *et al.*, 2013). Oil bodies are typically studied following extraction from the plant cell walls and resuspension in an aqueous solution (White *et al.*, 2009). The remarkable stability of oil bodies in an aqueous solution may be due to the presence of phospholipids and proteins on their surface (Slack *et al.*, 1980; Qu *et al.*, 1986). Specifically, oleosin, which is the most common oil body protein, is presumed to play the most important function in stabilizing the oil body through steric and electrostatic effects (Tzen and Huang, 1992; Capuano *et al.*, 2007; Shimada and Nishimura, 2010). Oleosin consists of three structural domains: an N-terminal amphipathic domain, a central hydrophobic domain, and a C-terminal amphipathic domain. The N- and C-terminal domains are not conserved across the various species of oleosins, and oleosin homologs with different lengths have been identified. In contrast, the central anchor domain of oleosins is conserved across a wide variety of species; the central hydrophilic proline knot is known to be especially highly conserved (Huang and Huang, 2015). In a previous study investigating the stability of oil bodies using recombinant oleosins in sesame seeds, artificial oil bodies constructed using truncated oleosins lacking more than half of the original central hydrophobic domain tended to coalesce upon collision or aggregation (Peng *et al.*, 2007). It was suggested that the hydrophobic domain of oleosin contributes to its stability. However, several structural models have been proposed, but not proven, for the central hydrophobic domain of oleosin: insertion of the domain into the triacylglycerol matrix (Huang, 1992; Alexander *et al.*, 2002) or a β -barrel structural model (Tzen, 2012). There are also models for the formation of a complex of oleosins (Ting *et al.*, 1996; Wahlroos *et al.*, 2015).

The aim of this study was to describe the stability of oil bodies and the role of the oleosin throughout the process of soymilk production. First, oil bodies were extracted and purified at various steps of the soymilk production process to examine their properties. Using the proteolytic enzyme papain, the presence of oleosins on the surface layer of the oil bodies was determined and the effect of oleosins on colloidal stability was evaluated.

Materials and Methods

Preparation of oil bodies from soybeans In order to verify the effects of soaking on the properties of oil bodies, oil bodies were prepared after soaking soybeans for 1 h or 20 h. Osuzu soybeans (Aomori, Japan) were stored at 8 °C until use. Soybean samples (10 g) were soaked in de-ionized water for 1 or 20 h at 10 °C and ground with a blender in 100 g 0.1 M Tris-HCl and 3 mM MgCl₂ (pH 8.6) for 1 min. The homogenate was separated into the filtration liquid (supernatant) and okara (pellet) by centrifugation at $1,400 \times g$ for 1 min (H-112,

Kokusan Co. Ltd., Tokyo, Japan). The supernatant was transferred to two clean 50 mL tubes and centrifuged at $22,600 \times g$ for 50 min (CR21N, Hitachi Koki Co. Ltd., Tokyo, Japan). The lighter upper oil body layers were collected and washed in 50 mL 0.1 M Na₂CO₃. The supernatant and pellet were discarded. The mixture was centrifuged, and the upper layer was collected after centrifugation. The upper layer was washed three times in 0.1 M Na₂CO₃, dispersed in 50 mL de-ionized water, centrifuged, and collected as purified oil bodies from soybeans.

Preparation of oil bodies from soymilk In order to verify the effects of heating on the properties of oil bodies, oil bodies were prepared from soymilk and then heated. Soybeans (10 g) were soaked in de-ionized water for 20 h at 10 °C and then ground in 100 g de-ionized water for 1 min. The homogenate was heated in boiling water for 5 min and centrifuged at $1,400 \times g$ for 1 min (H-112, Kokusan Co. Ltd.). The supernatant was then cooled in ice water and centrifuged at $22,600 \times g$ for 50 min (CR21N, Hitachi Koki Co. Ltd.). The upper layers were collected and washed in 50 mL 0.1 M Na₂CO₃. The mixture was centrifuged, and the upper layer was collected after centrifugation. The upper layer was washed three times in 0.1 M Na₂CO₃, dispersed in 50 mL de-ionized water, centrifuged, and collected as purified oil bodies from soymilk.

Preparation of soymilk proteins To examine the effect of pH on the stability of soymilk proteins, soymilk proteins were investigated at different pH values after removing water-soluble components, such as sugars and minerals. Soymilk prepared using the same method described above (50 mL) was centrifuged at $22,600 \times g$ for 50 min (CR21N, Hitachi Koki Co. Ltd.). The upper oil body layer was discarded; the supernatant and pellet were mixed, collected in a clean 50-mL tube, and centrifuged at $22,600 \times g$ for 50 min. In order to completely remove the upper oil body layer from the soymilk protein solution, this step was performed three times. The supernatant and pellet were mixed and adjusted to pH 4.8 using 1 N HCl, centrifuged at $3,000 \times g$ for 15 min (Thanh and Shibasaki, 1976), and the supernatant was discarded. The protein pellet was dissolved in 50 mL phosphate buffer (50 mM, pH 6.0).

Determination of moisture and protein contents The moisture content was determined by placing the samples in aluminum cups and heating them in a dry oven at 105 °C for 24 h. The protein content was measured using the improved Dumas method (Jung *et al.*, 2003) using a nitrogen analyzer (SUMIGRAPH NC-220F, Sumika Chemical Analysis Service, Ltd., Tokyo, Japan) and using a nitrogen to protein conversion factor 5.71.

Particle size analysis Particle size distribution of the oil body emulsions was determined using a Horiba LA-750 Laser Scattering Particle Size Distribution Analyzer (Horiba Instruments, Kyoto, Japan). The instrument reservoir was filled with 50 mM phosphate buffer (pH 7.0) and 1 μ L oil bodies was

Table 1. Protein content and particle distribution of each oil body. Data are means $\pm$ standard deviation of triplicate experiments.

Condition	Oil bodies from soybeans soaked for 1 h	Oil bodies from soybeans soaked for 20 h	Oil bodies from soymilk	Papain-digested oil bodies from soymilk
Protein (% dry weight)	4.21 $\pm$ 0.08	4.47 $\pm$ 0.20	5.48 $\pm$ 0.23	2.29 $\pm$ 0.06
Arithmetic mean particle diameter (nm)	473.5 $\pm$ 2.3	447.0 $\pm$ 1.3	426.1 $\pm$ 1.1	424.5 $\pm$ 1.9

added gradually over the course of the measurements. The average particle size distribution was then calculated.

Digestion of oil bodies with papain Oil bodies (25 mg) were suspended in 500 μ L 20 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/mL papain (Wako Pure Chemical Industries, Ltd., Osaka, Japan or Nagase ChemteX Corp., Osaka, Japan) and incubated at 50 $^{\circ}$ C for 30 min. The proteinase was then inactivated by boiling for 10 min or by adding 10 μ M leupeptin (Peptide Institute, Inc., Osaka, Japan).

Stability test Various oil bodies and soymilk proteins were subjected to a stability test. The soymilk protein and oil body solutions were adjusted to pH 5.6, 5.8, or 6.0 with 1 N HCl and diluted with 50 mM phosphate buffer to a concentration of 0, 1.25, 2.5, 3.75, 5.0, 6.25, 7.5, 8.75, 10.0, or 20.0 mg/mL. The mixture (500 μ L) was dispensed into 1.5 mL tubes and centrifuged at 2,000 $\times$ g (1720, Kubota Corp.) for 1, 2, or 3 min at 25 $^{\circ}$ C. The precipitates from each tube were transferred to aluminum cups, and the dry weights were measured. The sedimentation rates were calculated by observing the slope of the dry weights after 1, 2, and 3 min. The above test was conducted in triplicate and average values were obtained.

SDS-PAGE. SDS-PAGE was conducted according to the method of Laemmli (Laemmli, 1970) at concentrations of 4.5 % and 15 % for the stacking and separating gels, respectively. An SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, and 0.1 % SDS) was used. Oil bodies (25 mg) were suspended in 500 μ L de-ionized water to measure their protein contents. Oil body proteins (20 μ g) were mixed with SDS sample buffer at a final concentration of 1 % SDS, 25 mM Tris-HCl (pH 6.8), 5 mM β -mercaptoethanol, 0.005 % bromophenol blue, and 5 % glycerol. SDS-PAGE was performed at 50 V for 30 min and at 300 V for 40 min. The gel was stained with Coomassie Brilliant Blue G-250.

N-terminal amino acid sequence analysis Papain-hydrolyzed oleosin samples obtained from soymilk were separated by SDS-PAGE with a 20 % acrylamide precast gel (TEFCO, Tokyo, Japan) at 200 V. Proteins were then transferred to a PVDF membrane (ATTO, Tokyo, Japan) with commercially available blotting buffer (ATTO) at 80 mA for 120 min. Proteins were detected with 1 % Ponceau S, and the bands corresponding to 11-kDa and 9-kDa proteins were excised. The 11-kDa amino acid sequence was determined using a gas phase protein sequencer (PPSQ-22A, Shimadzu,

Kyoto, Japan). The 9-kDa amino acid sequence was determined using a gas phase protein sequencer (Procise 492cCL, Applied Biosystems, Foster City, CA, USA).

Electron microscopy The oil bodies were fixed with an equal amount of 4 % paraformaldehyde (PFA) and 4 % glutaraldehyde (GA) in 0.1 M cacodylate buffer at pH 7.4 and 4 $^{\circ}$ C for 1 h. Thereafter, samples were fixed with 2 % GA in 0.1 M cacodylate buffer at pH 7.4 and 4 $^{\circ}$ C overnight. After fixation, the samples were washed three times with 0.1 M cacodylate buffer for 20 min each and postfixed with 2 % osmium tetroxide (OsO₄) in 0.1 M cacodylate buffer at 4 $^{\circ}$ C for 2 h. The samples were dehydrated in graded ethanol solutions (50 %, 70 %, 90 %, and 100 %) as follows: 50 % and 70 % for 20 min each at 4 $^{\circ}$ C, 90 % for 20 min at room temperature, and 100 % repeated four times for 20 min each at room temperature. The samples were infiltrated with propylene oxide (PO) twice for 20 min each, incubated in a 70:30 mixture of PO and resin (Quetol-812, Nissin EM Co., Tokyo, Japan) for 1 h, then the cap of tube was left open to allow the PO to become volatilized overnight. The samples were transferred to fresh 100 % resin and polymerized at 60 $^{\circ}$ C for 48 h. The polymerized resins were sliced into 70-nm ultra-thin sections with a diamond knife using an ultramicrotome (Ultracut UCT, Leica Microsystems, Wetzlar, Germany), and the sections were mounted on copper grids. The sections were stained with 2 % uranyl acetate at room temperature for 15 min, then washed with distilled water, followed by secondary staining with Lead stain solution (Sigma Aldrich, St. Louis, MO, USA) at room temperature for 3 min. The grids were observed through a transmission electron microscope (JEM-1400Plus, JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (EM-14830RUBY2, JEOL Ltd.).

Results and Discussion

Stability of oil bodies and soymilk protein particles Oil bodies in the soymilk samples were collected by centrifugation; an alkaline Na₂CO₃ solution was used to wash the proteins off the surface layer (Fujiki *et al.*, 1982). The results of the component analysis and the average particle diameter of various oil bodies are shown in Table 1. The protein mass of the oil bodies extracted from soybeans after soaking for 1 h and 20 h were lower than those reported previously, where

Fig. 1. The stability of protein particles from soymilk centrifuged at $2,000 \times g$ for 3 min. Solid diamond ($\blacklozenge$), pH 6.0; square ($\square$), pH 5.8; solid triangle ($\blacktriangle$), pH 5.6.

extractions were performed at pH 11.0 (Cao *et al.*, 2015; Zhao *et al.*, 2016). Therefore, compared to the oil bodies extracted from soymilk, it is possible that more protein would be eluted from the oil bodies extracted from soybeans using an alkaline treatment. The protein content was reduced to approximately 40% in papain-digested oil bodies from soymilk. The particle size distributions of all oil bodies showed a single peak. The average particle diameter of oil bodies from soymilk was small compared to that of the oil bodies from soybeans, despite the large mass of the proteins. Based on this result, the oil bodies derived from soybeans were considered to partially aggregate at pH 7.0. As the average particle diameter of papain-digested oil bodies from soymilk was smaller than that of undigested oil bodies derived from soymilk, this likely reflects the presence of proteins on the particle surface.

To validate if soymilk stability is affected by oil bodies, protein and oil body mixtures were prepared from each sample. At a high pH, protein particles and oil bodies were stably dispersed in the soymilk and changes in viscosity were negligible. However, when the pH was lowered, a rapid increase in viscosity was observed (Sato *et al.*, 2017). Therefore, when the oil bodies were centrifuged at $2,000 \times g$, the rate of sedimentation was measured and the stability was evaluated at each pH value.

First, the sedimentation rate was measured in a solution containing only protein particles recovered from soymilk, and compared with that of a system containing both proteins and oil bodies (Fig. 1). Proteins present in the continuous phase tended to settle by aggregation as the pH decreased. At higher protein concentrations, this effect became more pronounced. The sedimentation rates when the oil bodies were dispersed in the protein solutions at a concentration of 20 mg/mL are shown in Fig. 2. A negative sedimentation rate value indicates floating. At pH 6.0, the floating rate tended to slow in the oil

bodies as the protein concentration increased. This was thought to be due to the higher effective density of proteins that adsorbed to the oil bodies. While there was no major difference between oil bodies and papain-digested oil bodies from soymilk, oil bodies from soaked soybeans tended to have a substantially higher floating rate. In addition, when oil bodies from soaked soybeans were digested by papain, their floating rates were the same as those of oil bodies from soymilk digested by papain (data not shown). Therefore, there may have been a difference in the oleosin conformation of the oil bodies from soaked soybeans compared to those from soymilk. In particular, oleosin from soybeans was thought to have retained its native state. As the isoelectric point of soybean oil bodies has been reported to be pH 4–5 (Iwanaga *et al.*, 2007; Maurer *et al.*, 2013), it was thought that their stability decreased as the pH decreased. However, in the oil bodies from soymilk, it is possible that oleosins aggregated and electrostatic interactions were affected by heating. In the stability test, at pH 5.8 and 6.0, the oil bodies from soaked soybeans tended to have a substantially higher floating rate. Oil bodies from soymilk showed an increased floating rate at protein concentrations of less than 2.5 mg/mL. This was because the aggregates were generated using a protein associated with oleosin rather than coalesced oleosin, leading to an increase in the floating rate at a low protein concentration. At a high protein concentration, a strong association was observed between the proteins, which had high stability regardless of the state of oleosin. However, this tendency was not observed in papain-digested oil bodies from soymilk at low protein concentrations. These results suggest that the presence of oleosin had an effect on the interaction of oil bodies with proteins. A previous report showed that isolated soybean and soymilk oil bodies differed in their dispersion stability as a function of pH (Chen *et al.*, 2014). However, at pH 5.6, no differences were observed in the state of oleosin in each oil body when the oil bodies and proteins were together in solution. Since protein aggregation is a common phenomenon, the association and cohesion of proteins has been determined to be an indicator of colloidal stability. Thus, at low protein concentrations, oil bodies that aggregated because of cross-linking were found to float. Oil body floating was suppressed by the binding and polymerization of proteins at high protein concentrations. Therefore, when no proteins were present in the solution, all the oil bodies floated at pH 6.0–5.6. When low protein concentrations were present, oleosin was found to cross-link and aggregate with other proteins. On the other hand, at high protein concentrations, when the pH was low, the adsorption of proteins caused high-density particles to form; it is thought that aggregates of dispersed oil bodies in protein solutions were formed as a result. The association of oleosin with other proteins, especially at low concentrations, was shown to play a strong role in the colloidal stability of soymilk.

N-terminal amino acid sequence analysis of oleosins

Fig. 2. The stability of proteins from soymilk and oil bodies centrifuged at $2,000 \times g$ for 3 min. Solid diamond (◆), oil bodies from soybeans soaked for 1 h; square (□), oil bodies from soymilk; solid triangle (▲), papain-digested oil bodies from soymilk. (a), (b), and (c) represent pH 6.0, 5.8, and 5.6, respectively.

Fig. 3. Protein composition of oil bodies and oil bodies digested with papain. Lane 1, marker; lane 2, oil bodies from soybeans soaked for 1 h; lane 3, oil bodies from soybeans soaked for 20 h; lane 4, oil bodies from soymilk; lanes 5–7, papain-digested oil bodies obtained after soaking soybeans for 1 h or 20 h, and oil bodies from soymilk, respectively.

digested by papain SDS-PAGE analysis of proteins present in the isolated purified oil bodies is shown in Fig. 3. In the oil bodies derived from soybeans soaked for 1 or 20 h and from soymilk, clear bands were observed at the 24-kDa and 18-kDa regions; these were identified as oleosins (Herman, 1987; Zhao *et al.*, 2013) and the electrophoresis patterns of the three oil bodies were highly similar. According to a previous report (Chen and Ono, 2010a), there were no differences in the SDS-PAGE patterns and surface hydrophobicity of unheated and heated oil bodies. In addition, oil bodies from all of the above groups were digested by papain and examined for oleosin peptide fragments by SDS-PAGE. The major protein fragments in all of the samples had a molecular weight of 11-kDa and 9-kDa. Therefore, it was shown that no changes in the primary structure of oleosin occurred during the soymilk production process. Purified oleosins were further digested by papain, and SDS-PAGE was carried out on the papain-digested fragments. No difference was observed before and after purification (data not shown). The 11-kDa protected fragment was purified from


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MTTQVPPHSV QVHTTTTHRY EAGVVPPGAR FETSYEAGVK AASIIHSESG PTTSQVLAVL 60
AGLPVGGILL LLAGLTLAGT LTGLAVATPL FVLFPVLVP ATVAIGLAVA GFLTSGAFGL 120
TALSSFSWIL NYIRETQPAS ENLAA↓AKHH LAEAAEYVGQ KTKEVGQKTK EVGQDIQSKA 180
QDTREAAARD AREAAARDAR EAAARDAKVE ARDVKRTTVT ATTATA

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Fig. 4. The deduced amino acid sequence of a 24-kDa oleosin.

The N-terminal sequence of the 11-kDa papain-protected fragment is shown at the underlined position. The putative cleavage sites are indicated by the arrow. The N-terminal sequence of the 9-kDa papain-protected fragment is shown at the double underlined position.

digested oil bodies from soymilk and subjected to N-terminal sequencing. The first ten residues (AAKHHLAE(E or A)(E or A) and AKHHLAE(A or E)(A or E)(A or E)) were found to correspond to the hydrophilic C-terminal domain of a 24-kDa oleosin (P24 oleosin isoform A and B [*Glycine max*]; P29530 and P29531), confirming the identity of the protein as oleosin (Fig. 4). Because the mixture of two sequences was suggested, we considered the possibility that there were two cleavages sites or two oleosin isoforms. The 9-kDa protected fragment was purified from digested oil bodies and subjected to N-terminal sequencing. The first residues ((A or E)VGQDIQ(E or S)) were found to correspond to part of the 11-kDa fragment. Therefore, of the oleosins present in the surface layer of oil bodies, it was found that the C-terminal region of the 24-kDa oleosin did not undergo decomposition. The central anchor domain of oleosins was not difficult to digest by papain. In a previous study, oleosins were found to dissociate freely from oil bodies after centrifugation at a high pH (Cao *et al.*, 2015). Based on this result, and the results of the papain digestion, it was suggested that oleosins present on the surface of oil bodies were not embedded in lipids; the majority of the structure decomposed due to exposure of the surface to the water phase. It can be proposed that the C-terminal domain of oleosins, especially 24-kDa oleosins, is not digested by papain. It has been speculated that the N- and C-terminal domains of neighboring oleosins could associate with each other to form dimers, trimers, or oligomers (Li *et al.*, 2002; Pons *et al.*, 2005). In safflower, 8-kDa protected fragments formed after exposure to proteinase K represented the hydrophobic domain of the oleosins (Lacey *et al.*, 1998); these results are not consistent with Fig. 4. In this study, oleosin was completely degraded by proteinase K (data not shown). Phylogenetic classification of seed-derived oleosins confirmed the existence of two isoforms (low- and high-molecular weight) that were originally found to have distinct immunological features (Tzen *et al.*, 1990). High-molecular weight oleosins are usually characterized by additional sequences at the C-terminal domain. The proportion of each isoform in the oil body depends on the plant species under investigation. The diverse proteolytic sensitivity of different oleosins in oil body membranes is a potential determinant of oil body longevity during seed germination (Sadeghipour and Bhatla, 2002). The results might also reflect interspecific differences in oleosins.

Examination of oil bodies by transmission electron microscopy In order to determine the presence of phospholipids and proteins on the oil body surface layer, we performed transmission electron microscopy observations of each oil body using chemical fixation (Fig. 5). In all samples, oil bodies with a diameter of less than 1 μm were identified, which matched the size of the oil bodies extracted from soybeans (Krishnan, 2008). In oil bodies from soybeans, mainly spherical oil bodies were observed, while coalescence was seldom observed. In oil bodies from soymilk, some oil bodies containing a small lump were identified (Fig. 5, arrow), suggesting a potential coalescence of oil bodies due to heating during soymilk production. One thin-layer structure was observed on the surface of oil bodies from soybeans and soymilk (Fig. 6). There was a blurred-looking structure on the surface, and the thickness of the layer was approximately 6–10 nm. Since oil bodies may be surrounded by a half-unit phospholipid membrane (Slack *et al.*, 1980), it was suggested that this inconsistency in thickness was caused by the presence of oleosin. Additionally, in both oil bodies, there were very few staining points other than on the membrane structure. On the other hand, in papain-digested oil bodies from soymilk, deformed or coalescing oil bodies were observed. Since they did not show a tendency to coalesce, and because the average particle diameter was not found to be increased in our measurement of particle size distribution of papain-digested oil bodies from soymilk, coalescence seemed to be promoted by pretreatment for electron microscopy. Moreover, some boundaries of the oil bodies were unclear in the micrographs. Therefore, without oleosin, even with a phospholipid coating, the surface layer of the oil body appears to be easily deformed.

It has been shown in previous studies that the loss of some or most oleosins affects the stability of oil bodies (Peng *et al.*, 2007). In general, polymers such as proteins contribute to the stability of oil bodies through electrostatic repulsion forces and steric effects (Dickinson, 1999). In oil bodies digested by papain, it can be suggested that deformation was observed because the majority of the proteins on the surface layer were degraded, and a membrane fluidity-induced Gibbs-Marangoni effect was noticeable. From the above, it can be concluded that the surface of the oil body is dependent on the presence of a protein coating, which greatly increases its stability. In addition, differences in the degenerative state of oleosin are

Fig. 5. Electron micrograph of oil bodies from (a) soybeans soaked for 1 h, (b) from soymilk, and (c) from soymilk and digested with papain. Scale bar = 5 μm .

Fig. 6. Electron micrograph of oil bodies from (a) soybeans soaked for 1 h, (b) from soymilk, and (c) from soymilk and digested with papain. Scale bar = 0.2 μm .

thought to affect the stability of oil bodies derived from soybeans and soymilk. In an oil-in-water emulsion, aggregates of hydrophobic proteins (containing oleosin and similar proteins) have been found to contribute more to stability than other soybean proteins (Gao *et al.*, 2013). Therefore, the difference in the aggregation state of oleosin derived from soybeans and soymilk was thought to directly affect oil body stability.

From the above results, we can conclude that oleosin is

important for the colloidal stability of soymilk. The oil bodies were remarkably stable and did not aggregate or coalesce throughout the soymilk production process. However, the presence of oleosins affected the electrostatic properties of the oil bodies, as well as the stability of the soymilk. In addition, it was suggested that oleosins may be present in the surface layer, but not embedded in lipids. Future studies are required to obtain further knowledge of the requirements for a stable emulsion.

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