JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

The Integral and Extrinsic Bioactive Proteins in the Aqueous Extracted Soybean Oil Bodies

Luping Zhao, Yeming Chen, Yanyun Cao, Xiangzhen Kong, and Yufei Hua*

State Key Laboratory of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu Province 214122, People's Republic of China

ABSTRACT: Soybean oil bodies (OBs), naturally pre-emulsified soybean oil, have been examined by many researchers owing to their great potential utilizations in food, cosmetics, pharmaceutical, and other applications requiring stable oil-in-water emulsions. This study was the first time to confirm that lectin, Gly m Bd 28K (Bd 28K, one soybean allergenic protein), Kunitz trypsin inhibitor (KTI), and Bowman–Birk inhibitor (BBI) were not contained in the extracted soybean OBs even by neutral pH aqueous extraction. It was clarified that the well-known Gly m Bd 30K (Bd 30K), another soybean allergenic protein, was strongly bound to soybean OBs through a disulfide bond with 24 kDa oleosin. One steroleosin isoform (41 kDa) and two caleosin isoforms (27 kDa, 29 kDa), the integral bioactive proteins, were confirmed for the first time in soybean OBs, and a considerable amount of calcium, necessary for the biological activities of caleosin, was strongly bound to OBs. Unexpectedly, it was found that 24 kDa and 18 kDa oleosins could be hydrolyzed by an unknown soybean endoprotease in the extracted soybean OBs, which might give some hints for improving the enzyme-assisted aqueous extraction processing of soybean free oil.

KEYWORDS: *soybean oil bodies, caleosin, calcium, steroleosin, Gly m Bd 30K, disulfide bond, soybean endoprotease*

INTRODUCTION

A soybean seed cell is mainly filled with oil bodies (OBs) and protein storage vacuoles (PSVs), which are the storage organelles for soybean oil and protein, respectively.[1] Soybean OBs, with a particle size range of 200–500 nm,[2] are composed of a core of triglyceride (TAG) matrix covered by one monolayer of phospholipids embedded with OB intrinsic proteins.[3] Generally, TAGs in OBs are extracted by organic solvent and used as vegetable food oil. Recently, aqueous extraction processing (AEP) of soybean oil has greatly advanced owing to its safety and environmental benefits. One strategy of AEP is to directly obtain soybean OBs,[2,4−6] and the other is to de-emulsify OBs to obtain free oil.[7,8]

Soybean OBs, a natural source of pre-emulsified soybean oil,[4] are very stable toward coalescence in the aqueous suspension and could survive high temperature,[2] 9 M urea,[6] and relatively high shearing and centrifugation speeds (50 000*g*),[2] which has great potential to be used in food products in place of emulsified soybean oil, for example, in dressings, sauces, dips, beverages, and desserts,[4] and also could be used to personal care products, pharmaceuticals, and other applications requiring highly stable oil-in-water emulsions.[9,10] Thus, many researchers tried to extract soybean OBs and focused on extraction yield,[5] dispersion stability,[11] oxidative stability[12] and *in vitro* digestibility.[13] In the aqueous extraction of soybean OBs, Gly m Bd 30K (Bd 30K), one soybean allergenic protein[14,15] naturally located in PSVs of soybean seed cell, is released and strongly bound to OBs.[16] Until now, high alkaline pH extraction (pH 11) is the most efficient method for soybean OBs without Bd 30K,[2,17] but this method has negative effects on soybean protein and not good to environment.[18] Therefore, a new and mild method (such as neutral pH or low alkaline pH extraction) for OBs without Bd 30K is hoped, which makes the clarification of the strong interaction between OBs and Bd 30K necessary. Other than Bd 30K, β-conglycinin, glycinin, lipoxygenase, and β-amylase were contained in the neutral pH extracted OBs, which could be removed by ≥pH 8 extraction.[2] However, no research has ever given the information about the interaction between OBs and four bioactive proteins: lectin, Gly m Bd 28K (Bd 28K, another allergenic soybean protein), Kunitz trypsin inhibitor (KTI), and Bowman–Birk inhibitor (BBI)), which might have negative effects on the utilizations of soybean OBs.

In addition to the extrinsic bioactive proteins above, soybean OBs might contain some intrinsic bioactive proteins. Naturally, a plant seed OB surface is composed of phospholipids and OB intrinsic proteins, which are identified to have at least three classes for sesame OBs, oleosin, caleosin, and steroleosin.[19] Oleosin, an abundant OB intrinsic protein (80–90%), has a structure composed of N-terminus, a hydrophobic central domain, and C-terminus; the hydrophobic central domain is embedded in the TAG matrix, and the positively charged amino acid residues of N-terminus and C-terminus are bound to the negatively charged phospholipids of the OBs by salt bridges.[3,19,20] Oleosin is directly involved in regulating the size and stability of the OBs[21] and is thought to play an important role in recruiting lipases and perhaps other enzymes (e.g., phospholipases, proteases) that are involved in OB breakdown.[22] Caleosin has a similar structure to oleosin and contains a single calcium-binding site known as a helix–loop–helix EF-hand motif in the N-terminus.[23] Caleosin can act as a peroxygenase in addition to its structural role as oleosin and plays an important role in producing peroxidized polyunsatu-

Received: July 28, 2013
Revised: September 12, 2013
Accepted: September 12, 2013
Published: September 13, 2013

rated fatty acids,[24,25] which might produce off-flavors for extracted soybean OBs. Different from oleosin and caleosin, steroleosin, also named sterol dehydrogenase, is composed of an N-terminal hydrophobic region (embedded into the TAG matrix) and a C-terminal region, and could dehydrogenate phytosterol into its ketone-containing derivative,[26] one kind of oxidized form of phytosterol.[27] It is reported that almond OBs contain total phytosterol content of 1.91 mg/g lipid.[28] Therefore, it is considered that caleosin and steroleosin might also have negative effects on the utilization of soybean OBs if they were contained in the extracted soybean OBs.

Soybean oleosins could be resolved into three bands with molecular weights (MWs) of 24, 18, and 17 kDa by SDS-PAGE.[1,2] Only one work reported that soybean caleosin appeared as one band on an SDS-PAGE gel with MW of about 30 kDa, and no more information was supplied.[21] Until now, no research has ever reported soybean steroleosin. Therefore, this study aimed to confirm whether caleosin, steroleosin, lectin, Gly m Bd 28K, KTI, and BBI are contained in the extracted soybean OBs and clarify the strong interaction mechanism between soybean OBs and Bd 30K.

MATERIALS AND METHODS

Materials. Soybean Nannong 88-31 (moisture content, 9%), harvested August 2011, was used. It was stored at 4 °C until use. All reagents were purchased from Sigma-Aldrich Trading Co., Ltd., and were analytical reagent grade.

Raw Soymilk Preparation. Twenty grams of soybean was soaked in deionized (DI) water at 4 °C for 18 h. The soaking water was poured off, and soaked soybeans were washed with fresh DI water three times. DI water was added to make the total weight 200 g. The mixture was ground in a Waring blender (18 000 rpm, MJ-60BE01B, Midea) for 90 s. The homogenate was filtered through four layers of gauze. The filtrate was deemed raw soymilk (pH 6.8) and kept in a 250 mL beaker. The procedures after soaking were conducted at room temperature.

Oil Bodies Extracted at pH 6.8, 8.0, and 11.0. If the four bioactive proteins (lectin, Bd 28K, KTI, and BBI) were bound to OBs, then their interaction strength with OBs could be revealed by the effect of pH 6.8−11.0 extractions on the protein compositions of the extracted OBs.[2] Sucrose was added into raw soymilk to make the concentration of 20% (w/w) and mixed well in the 250 mL beaker. Forty grams of raw soymilk (20% sucrose) was added into the first centrifuge tube. The residual raw soymilk in the beaker was adjusted to pH 8.0 by 2 M NaOH, and 40 g was added into the second centrifuge tube. The residual raw soymilk in the beaker was adjusted to pH 11.0, and 40 g was added into the third centrifuge tube. Then they were treated by centrifugation (25 000*g*, 30 min). The floating fractions were collected, dispersed into 39 g of sucrose solution (20%, w/w), and adjusted to the corresponding pH, respectively. They were mixed well and treated by centrifugation (25 000*g*, 30 min). Then the collection of floating fraction, dispersion into sucrose solution (20%, w/w), pH adjustment, and centrifugation as above was repeated twice. The floating fractions were collected and used as themselves, or the floating fractions were collected and dispersed into 10 mL of DI water. The OB suspensions were defatted with the method by Tzen et al.[20] Briefly, 30 mL of diethyl ether was added, and the suspensions were thoroughly mixed for 30 min and treated by centrifugation (15 000*g*, 5 min). The upper diethyl ether was removed, and the procedure was repeated one more time. The residual diethyl ether was allowed to evaporate in the hood, and the residual aqueous solutions were well mixed. The protein concentration of pH 6.8 extracted OBs was determined by the micro-Kjeldahl method, and 0.5 mL of the defatted sample (well mixed) was diluted with SDS-PAGE (or Tricine-SDS-PAGE) sample buffer to make the protein concentration 2 mg/mL. The other two defatted samples (pH 8.0 and pH 11.0), which had lower protein concentrations, were mixed with the same volume of sample buffer above. This method, different from the traditional method, was beneficial for examining the protein release behaviors from OBs.

Calcium Determination of Extracted Oil Bodies. Oil bodies (extracted at pH 6.8, 8.0, and 11.0) without dispersion into DI water were dried in the oven (105 °C) to constant weights. About 1.50 g of dry samples was weighed into crucibles and put into a muffle furnace (550 °C, 5 h). The crucibles were taken out and cooled in a desiccator. Twelve millimolar HNO_3 solution was added to dissolve the ashed samples, and the solution was moved into a 25 mL volumetric flask. The crucible was rinsed with 12 mM HNO_3 three times, and the rinsed solution was added into the volumetric flask. Twelve millimolar HNO_3 was added to make the volume 25 mL, and the solution was mixed well. One milliliter was diluted 50 times with 12 mM HNO_3 with the final La_2O_3 concentration of 0.06 M. The obtained samples were analyzed using atomic absorption spectrometry (SperctrAA220/22Z, Varian, USA). The wavelength is 422.7 nm, spectral width is 0.5 nm, and lamp current is 1 mA.

Oil Bodies Extracted at pH 6.8 at 4 °C. Twenty grams of soybean was soaked in DI water at 4 °C for 18 h. The soaking water was poured off, and the soaked soybeans were washed with precooled fresh DI water (4 °C) three times. Precooled DI water was added to make the total weight of 200 g. The mixture was ground for 90 s. The homogenate was filtered through four layers of gauze, and raw soymilk was obtained. Sucrose was added to make the concentration of 20%, and the suspension was mixed well. Eighty grams was treated by centrifugation (25 000*g*, 30 min; 4 °C). The floating fraction was collected, added into 20 mL of precooled DI water, and mixed well in a 4 °C refrigerator. Then this was kept at room temperature. After 0, 1, 3, 6, and 9 h, 3 mL of suspension was collected and cooled in an ice water bath, respectively. The oil bodies were defatted by the method stated above with some differences: (1) 9 mL of precooled diethyl ether (4 °C) was used; (2) centrifugation was conducted at 15 000*g* for 5 min at 4 °C); (3) thoroughly mixing (30 min) and air-evaporation of diethyl ether combined with fan blowing were conducted in ice water bath. The defatted sample (0.5 mL, well mixed) was diluted with Tricine-SDS-PAGE sample buffer to make the protein concentration 2 mg/mL.

In addition, OBs were obtained as above with extraction conditions of pH 11.0 and 4 °C, defatted by diethyl ether, and used for the analysis of integral proteins of OBs by Tricine-SDS-PAGE.

SDS-PAGE. SDS-PAGE was conducted by the method of Laemmli with the concentrations of the stacking and separating gels being 5% and 12.5%, respectively.[29] 2-Mercaptoethanol (no 2-mercaptoethanol for nonreducing SDS-PAGE) was added into the samples above to the concentration of 2% (v/v), and samples were heated for 3 min in a boiling water bath. Then 10 μL of each sample was loaded into a well. SDS-PAGE was performed at 15 mA for 2 h. The gel was stained using Coomassie Brilliant Blue G-250.

Tricine-SDS-PAGE. Tricine-SDS-PAGE was conducted according to the method of Schagger.[30] The concentrations of stacking and separating gels were 4% and 16%, respectively. 2-Mercaptoethanol (no 2-mercaptoethanol for nonreducing Tricine-SDS-PAGE) was added into the samples above to the concentration of 2% (v/v), and samples were heated for 3 min in a boiling water bath. Then 10 μL of each sample was loaded into a sample well, and the samples were electrophoresed at constant voltage of 30 mV until all of protein sample entered into the stacking gel and then at constant voltage of 100 mV until end. The gel was also stained using Coomassie Brilliant Blue G-250.

Diagonal Electrophoresis. A sample without 2% 2-mercaptoethanol was put into a sample well and electrophoresed. After electrophoresis, the lane was cut from the gel and put into a 25 mL beaker. DI water (24.5 mL) and 0.5 mL of 2-mercaptoethanol were added, and then the sample was put into a boiling water bath for 3 min. The reduced lane was used for the second dimensional electrophoresis of diagonal electrophoresis.

Two-Dimensional Electrophoresis. The pH 6.8 extracted OB suspension (extracted at room temperature) was desalted by dialysis against DI water and lyophilized. The lyophilized OB was extracted

with acetone at 4 °C. After being stirred for 30 min, the homogenate was filtered through a layer of filter paper, and the protein fraction was recovered. This was repeated two more times to remove neutral lipids. Then the sample was further defatted by chloroform/methanol (2/1, v/v) three times. Finally, the protein obtained was placed in the hood to allow the organic solvent to evaporate.

The two-dimensional (2-D) electrophoresis was performed according to the Bio-Rad manufacturer's instructions. Protein (150 μg) obtained above was thoroughly dissolved in 125 μL of 2-D electrophoresis sample preparation solution (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 75 mM DTT, 0.2% (w/v) ampholyte 3−10, 0.001% bromophenol blue), assisted by vortexing and ultrasonic treatment. The sample was loaded onto a linear IPG gel strip (pH 3−10, 7 cm, Bio-Rad Laboratories, CA) and allowed to hydrate for 16 h at 20 °C. The first dimension isoelectric focusing (IEF) was conducted at 40 kVh using a Protean IEF cell (Bio-Rad Laboratories). After IEF, the IPG strip was reduced and alkylated with DTT (20 mg/mL) and iodoacetamide (25 mg/mL) in equilibration buffer (6 M urea, 2% SDS (w/v), 0.375 M Tris-HCl, pH 8.8, and 20% (w/v) glycerol) for 15 min. After the sample was rinsed with SDS-PAGE electrode buffer, it was transferred onto the SDS-PAGE separating gel, covered with low melting point agarose, and subjected to the second dimension SDS-PAGE. The gel was stained with Coomassie Brilliant Blue G-250.

MALDI-TOF/TOF-MS. The protein spots were excised from a Coomassie Brilliant Blue G-250 stained gel, washed in distilled water, and destained completely in a 30% acetonitrile (v/v) containing 100 mM NH_4HCO_3. After a 100% acetonitrile washing, the destained gel piece was placed into reducing solution (10 μL of 100 mM DTT, 90 μL of 100 mM NH_4HCO_3) for 30 min at 56 °C. After a 100% acetonitrile washing, 70 μL of 100 mM NH_4HCO_3 and 30 μL of 200 mM iodoacetic acid were added, and the mixture was incubated for 20 min in dark. After a 100% acetonitrile washing again, 5 μL (10 μg/mL) of trypsin (Promega, V5111) was added, and the mixture was put into a 4 °C refrigerator for 30 min. Then 25 mM NH_4HCO_3 solution was added. The peptides were generated after 20 h incubation at 37 °C. The solution containing peptides was collected and concentrated at 30 °C to get a dry powder. Three microliters of 0.1% trifluoroacetic acid (v/v) was used to dissolve the dry powder. In the final step before MALDI-TOF/TOF analysis, the sample was prepared by mixing 0.7 μL of the sample and 0.7 μL of 4-hydroxy-α-cyanocinnamic acid (used as a matrix) on a MALDI target and air-dried. All mass spectra were obtained with an ultrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Germany). All mass spectra were analyzed by flexAnalysis software provided by Bruker Daltonics Corp. Protein identification was performed by searching plant proteins in the latest version of the NCBInr database.

RESULTS AND DISSCUSSION

Caleosin and Steroleosin of Extracted Soybean Oil Bodies. Chen and Ono reported a simple method for extracting soybean oil bodies (OBs) without contamination from extrinsic proteins by pH 11 washing.[2] It was known that steroleosin and caleosin were minor integral proteins of OBs, so 40 μg of protein of pH 11 extracted OBs (obtained at 4 °C) is treated by Tricine-SDS-PAGE and resolved into 15 bands (lane 2, Figure 1), very different from several other reports.[1,2,13] Unfortunately, the bands above band 1 (Figure 1) were not matched to proteins by MALDI-TOF/TOF-MS. Bands 1−9 are matched to proteins and shown in Table 1. Band 1 was matched to hydroxysteroid 11-β-dehydrogenase 1-like protein (gil356539128), which should be the steroleosin of soybean OBs with MW of 41 kDa.[26] This is the first time to confirm the steroleosin for soybean OBs. Both bands 2 (29 kDa) and 3 (27 kDa) were matched to Ca^{+2}-binding EF hand protein (gil 351725291), which was generally named as caleosin. This was different from the results by Schmidt and Herman,[21] who reported that soybean caleosin just appeared as one band (30 kDa) on SDS-PAGE gel. It was very hard to consider that caleosin could be hydrolyzed in the extraction condition with pH 11.0 and 4 °C, and the NCBI database showed that there were several isoforms for soybean caleosin. Therefore, it was reasonable that two caleosin isoforms were confirmed in this study. Hanano et al. reported that the peroxygenase activity of caleosin was entirely calcium-dependent,[24] so the calcium content of pH 11 extracted soybean OBs was determined as 32.68 mg/100 g of soybean OBs (dry basis) with pH 6.8

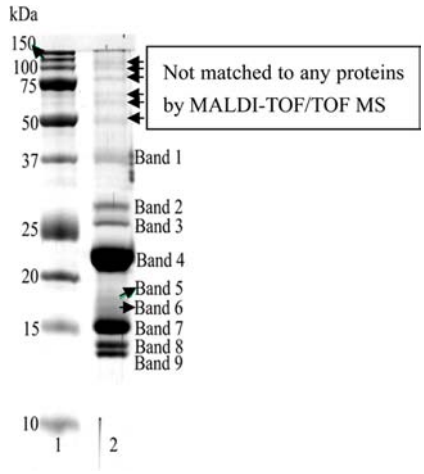

Figure 1. Reducing Tricine-SDS-PAGE pattern of the protein compositions of oil bodies obtained by pH 11.0 extraction at 4 °C. Lane 1, marker; lane 2, protein (40 μg) of oil bodies obtained by pH 11.0 extraction.

Table 1. The Identification of Protein Bands on Lane 2 in Figure 1 by MALDI-TOF/TOF-MS

band	protein ID	protein score[a]	MW[a] (Da)	NBCInr acc no.[a]	Uniprot acc no.[a]
1	PREDICTED:[b] low-quantity quality, hydroxysteroid 11-β-dehydrogenase 1-like protein	84	40972	gil 356539128	none
2	Ca^{+2}-binding EF hand protein	104	27134	gil 351725291	O23959
3	Ca^{+2}-binding EF hand protein	520	27134	gil 351725291	O23959
4	P24 oleosin isoform A	278	23575	gil 356571311	P29530
	P24 oleosin isoform B	334	23378	gil 351722277	P29531
5	PREDICTED:[b] oleosin 5-like	145	19150	gil 356511688	I1MTE2
6	PREDICTED:[b] oleosin 16 kDa-like	91	17534	gil 356515553	I1MUH0
7	16.5 kDa oleosin	199	17463	gil 351726299	C3VHQ8
8	P24 oleosin isoform A	131	23378	gil 356571311	P29530
	uncharacterized protein LOC100306353	140	15767	gil 351721929	C6SZ13
9	oleosin 18.5 kDa-like isoform 1	124	15743	gil 356576403	none

[a]Molecular weights (MW) are given as theoretical values. Protein scores represent those searches performed via Mascot. Accession numbers within each respective database (NBCI and Uniprot) are given. [b]PREDICTED: This record is predicted by automated computational analysis, derived from a genomic sequence annotated using gene prediction method GNOMON, supported by mRNA and EST evidence.

extracted soybean OBs as control (calcium content, 43.94 mg/100 g of soybean OBs, dry basis), revealing that calcium could be closely bound to OBs. These results showed that caleosin and steroleosin, demonstrated as peroxygenase and sterol dehydrogenase,[24,26] were contained in extracted soybean OBs, which might be able to, respectively, catalyze the oxidation of polyunsaturated fatty acids and phytosterol in the OB extraction and subsequent storage.

As expected, band 4 was matched to 24 kDa oleosin isoforms (P29530 and P29531),[25,26] and bands 5−7 were matched to 18 kDa oleosin isoforms (I1MTE2, I1MUH0, and C3VHQ8). Surprisingly, band 8 was not only matched to 16 kDa oleosin (C6SZ13) but also matched to 24 kDa oleosin isoform (P29530). Band 9 was matched to another 16 kDa oleosin isoform (gil356576403). These results showed that 24 kDa oleosin at least had two isoforms (P29530 and P29531),[31,32] The 18 kDa oleosin at least had three isoforms, and 16 kDa oleosin at least had two isoforms. Herman separated the oleosins of soybean OBs into three bands with MWs of 24, 18, and 17 kDa by SDS-PAGE.[1] Compared with our results, it was concluded that each band obtained by Herman should at least have contained two or three kinds of oleosin isoforms, and Tricine-SDS-PAGE was better at resolving oleosins than SDS-PAGE.

Unknown Endoprotease of Extracted Soybean Oil Bodies. The results above showed that 24 kDa oleosin isoform (P29530) might be hydrolyzed by a soybean endoprotease in the extracted OBs. In order to confirm this hypothesis, OBs were obtained at 4 °C by pH 6.8 extraction; then OBs were dispersed well in precooled DI water and kept at room temperature for 0, 1, 3, 6, and 9 h. Figure 2 shows that 24 and

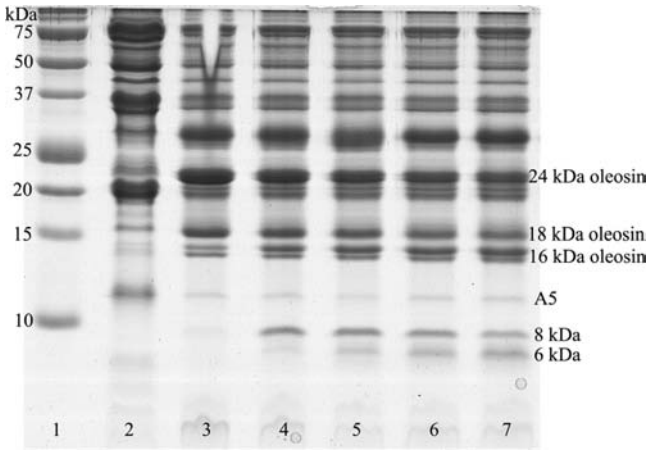

Figure 2. The effect of time on the soybean endoprotease-induced hydrolysis of oleosins. Oil bodies were extracted by pH 6.8 at 4 °C, dispersed well at 4 °C, and kept at room temperature for 0, 1, 3, 6, and 9 h. Lane 1, marker; lane 2, raw soymilk; lanes 3−8, the protein compositions of oil bodies kept at room temperature for 0, 1, 3, 6, and 9 h. A5 is one kind of acidic polypeptide of glycinin.

18 kDa oleosins are obviously hydrolyzed with time, but the other proteins are not; the band below the 18 kDa oleosin gradually becomes dense, and two new bands (apparent molecular weights 8 and 6 kDa) appeared after 1 and 3 h, respectively. As expected, the band below the 18 kDa oleosin on lane 7 is matched to 24 kDa oleosin isoforms (P29530 and P29531), 18 kDa oleosin isoform (C3VHQ8), and 16 kDa olesoin isoform (C6SZ13). Unluckily, the 8 and 6 kDa proteins are not matched to any proteins, but Figure 2 clearly reveals that they should be the endoprotease-hydrolyzed polypeptides of 24 kDa oleosin (P29530 and P29531) or 18 kDa oleosin (C3VHQ8). As stated above, oleosin, a major integral protein of OBs, plays an important role in the stability and integrity of OBs.[21] Therefore, it was considered that the oleosin hydrolysis induced by the endoprotease might have some negative effects on the utilization of soybean OBs, but it might be good news for enzyme-assisted aqueous extraction of soybean free oil, which needs artificially added protease to hydrolyze oleosin to destroy OBs.[8] The unknown endoprotease might be an integral protein of OBs but also might be an extrinsic protein that was bound to OBs in the extraction. It was considered that the identification of this endoprotease should be important for the aqueous extraction processing of soybean free oil and OBs, and we will examine it in our next work.

Extrinsic Bioactive Proteins of Soybean Oil Bodies. Chen and Ono reported that the extracted OBs could contain lipoxygenase, glycinin, β-conglycinin, γ-conglycinin, and Gly m Bd 30K (Bd 30K),[2] but they did not give the information about lectin, Gly m Bd 28K (Bd 28K), Kunitz trypsin inhibitor (KTI), and Bowman−Birk inhibitor (BBI), which might pose hazards to the utilization of OBs. In order to clarify this, SDS-PAGE (target lectin, 30 kDa; Bd 28K, 28 kDa)[33] and Tricine-SDS-PAGE (target KTI, 20 kDa; BBI, 8 kDa) were conducted for the OBs obtained by pH 6.8, 8.0, and 11 extractions at room temperature (Figure 3). Band 10 (30 kDa; lane 2, Figure 3a) was matched to Bd 30K (gil3097321) and P34 probable thiol protease (P34, gil351721011) but not matched to lectin. Generally, Bd 30K and P34 were considered to be similar proteins owing to their identical N-terminal amino acid sequences and amino acid compositions.[15] In order to further confirm whether lectin was contained in extracted OBs, two-dimensional electrophoresis was conducted for the protein of pH 6.8 extracted OBs. Figure 4 clearly shows that 30 kDa proteins are separated into two spots (PX1a and PX1b). Both of them were matched to Bd 30K and P34, which confirmed that lectin was indeed not contained in extracted OBs. In addition, the supernatant separated from pH 6.8 extracted OBs by centrifugation (25 000*g*, 30 min) was treated by SDS-PAGE, and the 30 kDa band was matched to lectin, as well as Bd 30K and P34. Therefore, it was concluded that lectin was not bound to OBs and tended to exist in the supernatant. The reason for lectin not binding to OBs should be correlated with its high solubility in aqueous medium.[34] Bands 11 and 12 (27−29 kDa; lane 2, Figure 3a) were all matched to caleosin, which was in good agreement with the results in Figure 1, but not to Bd 28K (28 kDa).[33] Therefore, it was suggested that Bd 28K was also not contained in extracted OBs.

Bands 13 and 14, around the MW of KTI (lane 6, Figure 3b), were matched to the subunits of glycinin, but not to KTI. Xu et al. reported that almost all KTI remained in the supernatant obtained from raw soymilk by ultracentrifugation (197 000*g*, 1 h),[35] revealing that the result above was reasonable. Figure 3b shows that one 8 kDa protein band (around the MW of BBI) appears for the pH 6.8 and 8.0 extracted OBs but disappears for the pH 11.0 extracted OBs regardless of reducing or nonreducing conditions, revealing that it is strongly bound to OBs by a noncovalent molecular interaction. It was examined by MALDI-TOF/TOF-MS seven times but not matched to any proteins. Lane 3 in Figure 2 shows the protein compositions of OBs obtained by pH 6.8 extraction at 4 °C, and the 8 kDa protein band does not appear, while lane 6 in Figure 3b shows

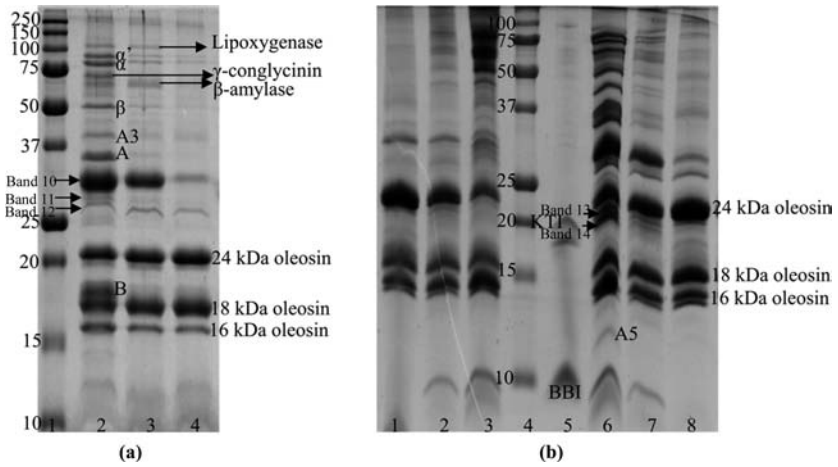

Figure 3. (a) Reducing SDS-PAGE pattern of the protein compositions of oil bodies obtained by 6.8, 8.0, and 11.0 extractions at room temperature. Lane 1, marker; lanes 2–4, oil bodies obtained by pH 6.8, 8.0, and 11.0 extractions. (b) Nonreducing and reducing Tricine-SDS-PAGE patterns of the protein compositions of oil bodies obtained by 6.8, 8.0, and 11.0 extractions. Lanes 1–3 (nonreducing) oil bodies obtained by pH 11.0, 8.0, and 6.8 extractions; lane 4, marker; lane 5, KTI and BBI standards; lanes 6–8 (reducing) oil bodies obtained by pH 6.8, 8.0, and 11.0 extractions. α', α, and β are subunits of β-conglycinin; A3, A, and A5 are acidic polypeptides; B is a basic polypeptide of glycinin.

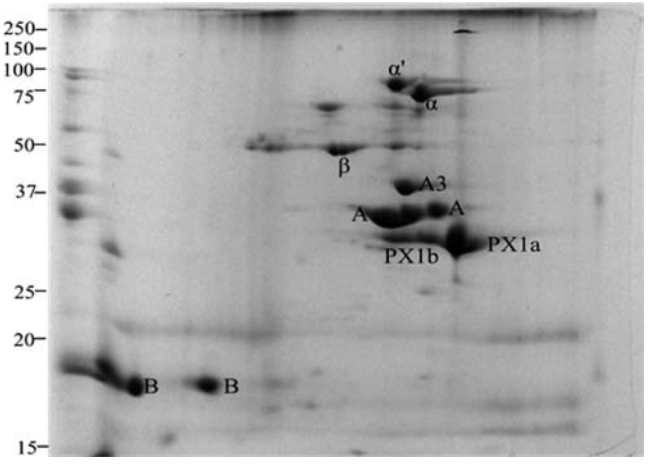

Figure 4. The protein profiles of oil bodies obtained from pH 6.8 extraction (room temperature) by two-dimensional electrophoresis. Proteins with molecular weights of around 30 kDa are separated into two spots, PX1a and PX1b.

the protein compositions of OBs obtained by pH 6.8 extraction at room temperature, and the 8 kDa protein band is clear. These results showed that the 8 kDa protein should be the soybean endoprotease-hydrolyzed polypeptide of oleosin and the hydrolysis was accelerated at room temperature compared with 4 °C. It was very hard to consider that BBI could be strongly bound to OBs by noncovalent molecular interaction owing to its high hydrophicility (composed of about 75% hydrophilic amino acid residues, NCBI database).[35] According to Huang's OB model,[3] oleosin has a structure composed of N-terminus, central hydrophobic domain, and C-terminus. The central hydrophobic domain was embedded into the OB TAG matrix, while the N-terminal and C-terminal regions were exposed to the exterior. The positively charged amino acid residues of the N-terminus and C-terminus were bound to negatively charged phospholipids by salt bridges. According to the Uniprot database, 24 kDa oleosin isoform P29530 was composed of N-terminus (76 amino acid residues), central hydrophobic domain (54 amino acid residues), and C-terminus (96 amino acid residues), while 24 kDa oleosin isoform P29531 was composed of N-terminus (57 amino acid residues), central hydrophobic domain (72 amino acid residues), and C-terminus (94 amino acid residues). Based on the results above, we could not clarify whether the cleavage of the peptide bond occurred on the N-terminus or on the C-terminus, but the salt bridges between the N-terminus/C-terminus and phospholipids should supply the strong interaction between the 8 kDa protein and OBs.

The Strong Interaction between Oil Bodies and Bd 30K. According to Huang's OB model,[3] OBs possessed a hydrophilic and negatively charged surface at neutral pH. And it was reported that even 9 M urea could not remove Bd 30K from OBs.[13] Therefore, it was considered that the strong interaction between OBs and Bd 30K might result from a disulfide bond (SS).

Figure 5a shows that there is just one thin band (band A) at 30 kDa on lane 1 (nonreducing; protein of pH 6.8 extracted OBs). By 2-mercaptoethanol treatment, a thick band is formed at 30 kDa on lane 2. These results mean that 30 kDa proteins are bound to other proteins or themselves by disulfide bonds. Figure 5a also shows that there is a wide zone (zone B), band C and band D on lane 1, which did not exist in lane 2. Therefore, diagonal electrophoresis was conducted. Figure 5b shows that zone B is resolved into α', α, PX2, and PX3, band C is resolved into PX4 and PX5, band D is resolved into PX6 and PX7, and band A is resolved into PX8 and PX9. PX2–9 were examined by MALDI-TOF/TOF-MS; PX4, 5, 8, and 9 were matched to the subunits of glycinin. Both PX2 and PX6 were matched to Bd 30K and P34. It was interesting that both PX3 and PX7 were matched to 24 kDa oleosin isoform P29530 but not to 24 kDa oleosin isoform P29531. It was reported that half of α' and α subunits of β-conglycinin were disulfide (SS) linked, together or with P34, in the soybean seed cotyledon cell: α'/α-SS-α'/α and α'/α-SS-P34 (or Bd 30K).[36] Both α' and α subunits contained only one thiol group (NCBI database). In addition, zone B had a MW around 150 kDa. Therefore, zone B should be the mixture of α'/α-SS-α'/α and α'/α-SS-P34 (or Bd 30K)-SS-24 kDa oleosin isoform P29530. Band D had MW of a little above 50 kDa, which should be P34 (or Bd 30K)-SS-24 kDa oleosin isoform P29530. Interestingly, it was found that P34/Bd 30K could only bind to 24 kDa oleosin isoform P29530

dx.doi.org/10.1021/jf403327e | J. Agric. Food Chem. 2013, 61, 9727–9733

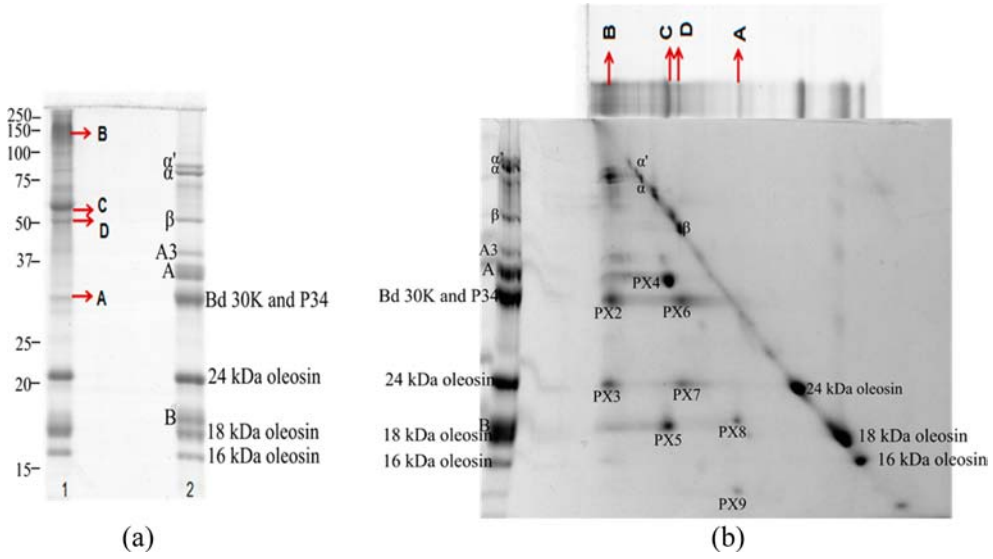

Figure 5. (a) Nonreducing and reducing SDS-PAGE pattern of protein from oil bodies by 6.8 extraction at room temperature. Lane 1, nonreducing protein of oil bodies obtained by pH 6.8 extraction; lane 2, reducing protein of oil bodies obtained by pH 6.8 extraction. (b) The diagonal electrophoresis pattern of protein of oil bodies obtained by pH 6.8 extraction.

although 24 kDa oleosin at least contained two isoforms (P29530 and P29531). It was reported that P34/Bd 30K, originally stored in the PSVs of soybean seed cell,[16] were released into soybean homogenate after the disruption of the soybean and strongly bound to OBs.[1] Generally, the thiol/disulfide exchange reaction widely happened in the biological system. Therefore, it was considered that the thiol/disulfide exchange reaction happened between P34/Bd 30K and 24 kDa oleosin to form disulfide bond.

In all, this study confirmed caleosin (two isoforms) and steroleosin (one isoform), respectively, demonstrated as peroxygenase[24,25] and sterol dehydrogenase,[26] for soybean OBs. Considerable amounts of calcium, necessary for the biological activity of caleosin, were strongly bound to soybean OBs. This study was the first to give information about the interaction between OBs and four bioactive proteins (lectin, Bd 28K, KTI, and BBI), revealing that the four proteins were not contained in the extracted OBs. It was clarified that the extrinsic Bd 30K/P34 was strongly bound to OBs through a disulfide bond with 24 kDa oleosin. A big finding was that 24 and 18 kDa oleosins could be hydrolyzed by unknown soybean endoproteases in the extraction, which might give negative effects on the utilization of soybean OBs but might be good news for enzyme-assisted aqueous extraction processing of soybean free oil.

AUTHOR INFORMATION

Corresponding Author

*Phone: 0510-85917812. Fax: 0510-85329091. E-mail: yfhua@jiangnan.edu.cn.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support received from Fundamental Research Fund of Ministry of Education (Grant 2050205), Natural Science Foundation of China (Grant 21276107), National Great Project of Scientific and Technical Supporting Programs funded by the Ministry of Science and Technology of China during the 12th five-year plan (Grant 2012BAD34B04-1), 863 Program (Hi-tech research and development program of China, Grant 2013AA102204-3), and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

REFERENCES

(1) Herman, E. M. Immunogold-localization and synthesis of an oil body membrane protein in developing soybean seeds. *Planta* **1987**, *172*, 336–345.

(2) Chen, Y.; Ono, T. Simple extraction method of non-allergenic intact soybean oil bodies that are thermally stable in an aqueous medium. *J. Agric. Food Chem.* **2010**, *58*, 7402–7407.

(3) Huang, A. H. C. Oil bodies and oleosins in seeds. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 177–200.

(4) Iwanaga, D.; Gray, D. A.; Fisk, I. D.; Decker, E. A.; Weiss, L.; McClements, D. J. Extraction and characterization of oil bodies from soy beans: A natural source of pre-emulsified soybean oil. *J. Agric. Food Chem.* **2007**, *55*, 8711–8716.

(5) Kapchie, V. N.; Wei, D.; Hauck, C.; Murphy, P. Enzyme-assisted aqueous extraction of oleosomes from soybeans (*Glycine Max*). *J. Agric. Food Chem.* **2008**, *56*, 1766–1771.

(6) Wu, N.; Yang, X.; Teng, Z.; Yin, S.; Zhu, J.; Qi, J. Stabilization of soybean oil body emulsions using κ, ι, λ-carrageenan at different pH values. *Food Res. Int.* **2011**, *44*, 1059–1068.

(7) Lamsal, B. P.; Johnson, L. A. Separating oil from aqueous extraction fractions of soybean. *J. Am. Oil Chem. Soc.* **2007**, *84*, 785–792.

(8) Campbell, K. A.; Glatz, C. E. Mechanisms of aqueous extraction of soybean oil. *J. Agric. Food Chem.* **2009**, *57*, 10904–10912.

(9) Marcoux, D.; Gorkiewicz-Petkow, A.; Hirsch, R.; Korting, H. C. Dry skin improvement by an oleosome emulsion as carrier for sphingolipid. *J. Am. Acad. Dermatol.* **2004**, *293*, P77.

(10) Deckers, H. M.; Van Rooijen, G.; Boothe, J.; Goll, J.; Moloney, M. M. (Sembiosys Genetics Inc.) Products for topical applications comprising oil bodies. U.S. Patent 6,599,513, July 29, 2003.

(11) Iwanaga, D.; Gray, D.; Decker, E. A.; Weiss, J.; McClements, D. J. Stabilization of soybean oil bodies using protective pectin coatings formed by electrostatic deposition. *J. Agric. Food Chem.* **2008**, *56*, 2240–2245.

(12) Chen, B.; McClements, D. J.; Gray, D. A.; Decker, E. A. Physical and oxidative stability of pre-emulsified oil bodies extracted from soybeans. *Food Chem.* **2012**, *132*, 1514–1520.

(13) Wu, N.; Huang, X.; Yang, X.; Guo, J.; Yin, S.; He, X.; Wang, L.; Zhu, J.; Qi, J.; Zheng, E. *In vitro* assessment of the bioaccessibility of fatty acids and tocopherol from soybean oil body emulsions stabilized with ι-carrageenan. *J. Agric. Food Chem.* **2012**, *60*, 1567−1575.
(14) Ogawa, T.; Bando, N.; Tsuji, H.; Okajima, H.; Nishikawa, K.; Sasaoka, K. Investigation of the IgE-binding proteins in soybean by immunoblotting with the sera of the soybean sensitive patients with atopic dermatitis. *J. Nutr. Sci. Vitaminol.* **1991**, *37*, 555−565.
(15) Ogawa, T.; Tsuji, H.; Bando, N.; Kitamura, K.; Zhu, Y.; Hirano, H.; Nishikawa, K. Identification of the soybean allergenic protein, Gly m Bd 30K, with the soybean seed 34-kDa oil-body-associated protein. *Biosci., Biotechnol., Biochem.* **1993**, *57* (6), 1030−1033.
(16) Kalinski, A.; Melroy, D. L.; Dwivedi, R. S.; Herman, E. M. A soybean vacuolar protein (P34) related to thiol proteases is synthesized as a glycoprotein precursor during seed maturation. *J. Biol. Chem.* **1992**, *267* (17), 12068−12076.
(17) Waschatko, G.; Schiedt, B.; Vilgis, T. A.; Junghans, A. Soybean oleosomes behavior at the air-water interface. *J. Phys. Chem. B.* **2012**, *116* (35), 10832−10841.
(18) Friedman, M.; Levin, C. E.; Noma, A. T. Factors governing lysinoalanine formation in soy proteins. *J. Food Sci.* **1984**, *49*, 1282−1288.
(19) Lin, L.; Liao, P.; Yang, H.; Tzen, J. T. C. Determination and analyses of the N-termini of oil body proteins, steroleosin, caleosin and oleosin. *Plant Physiol. Biochem.* **2005**, *43*, 770−776.
(20) Tzen, J. T. C.; Huang, A. H. C. Surface structure and properties of plant seed oil bodies. *J. Cell Biol.* **1992**, *117*, 327−335.
(21) Schmidt, M. A.; Herman, E. M. Suppression of soybean oleosin produces micro-oil bodies that aggregate into oil body/ER complexes. *Mol. Plant* **2008**, *1*, 910−924.
(22) Quettier, A. L.; Eastmond, P. J. Storage oil hydrolysis during early seedling growth. *Plant Physiol. Biochem.* **2009**, *47*, 485−490.
(23) Chen, J. C.; Tsai, C. C.; Tzen, J. T. Cloning and secondary structure analysis of caleosin, a unique calcium-binding protein in oil bodies of plant seeds. *Plant Cell Physiol.* **1999**, *40*, 1079−1086.
(24) Hanano, A.; Burcklen, M.; Flenet, M.; Ivancich, A.; Louwagie, M.; Garin, J.; Blee, E. Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif. *J. Biol. Chem.* **2006**, *281*, 33140−33151.
(25) Meesapyodsuk, D.; Qiu, X. A peroxygenase pathway involved in the biosynthesis of epoxy fatty acids in oat. *Plant Physiol.* **2011**, *157*, 454−463.
(26) Lin, L. J.; Tai, S. S.; Peng, C. C.; Tzen, J. T. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. *Plant Physiol.* **2002**, *128*, 1200−1211.
(27) Garcia-Llatas, G.; Cercaci, L.; Rodriguez-Estrada, M. T.; Lagarda, M. J.; Farre, R.; Lercker, G. Sterol oxidation in ready-to-eat infant foods during storage. *J. Agric. Food Chem.* **2008**, *56*, 469−475.
(28) Gallier, S.; Gordon, K. C.; Singh, H. Chemical and structural characterization of almond oil bodies and bovine milk fat globules. *Food Chem.* **2012**, *132* (4), 1996−2006.
(29) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(30) Schagger, H. Tricine-SDS-PAGE. *Nat. Protoc.* **2006**, *1*, 16−22.
(31) Kalinski, A.; Loer, D. S.; Weisemann, J. M.; Matthews, B. F.; Herman, E. M. Isoforms of soybean seed oil body membrane protein 24 kDa oleosin are encoded by closely related cDNAs. *Plant Mol. Biol.* **1991**, *17*, 1095−1098.
(32) Rowley, D. L.; Herman, E. M. The upstream domain of soybean oleosin genes contains regulatory elements similar to those of legume storage proteins. *Biochem. Biophys. Acta.* **1997**, *1345*, 1−4.
(33) Tsuji, H.; Bando, N.; Hiemori, M.; Yamanishi, R.; Kimoto, M.; Nishikawa, K.; Ogawa, T. Purification and characterization of soybean allergy *Gly* m Bd 28K. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 942−947.
(34) Iwabuchi, S.; Yamauchi, F. Electrophoretic analysis of whey proteins present in soybean globulin fractions. *J. Agric. Food Chem.* **1987**, *35*, 205−209.
(35) Xu, Z.; Chen, Y.; Zhang, C.; Kong, X.; Hua, Y. The heat-induced protein aggregate correlated with trypsin inhibitor inactivation in soymilk processing. *J. Agric. Food Chem.* **2012**, *60*, 8012−8019.
(36) Wadahama, H.; Iwasaki, K.; Matsusaki, M.; Nishizawa, N.; Ishimoto, M.; Arisaka, F.; Takagi, K.; Urade, R. Accumulation of β-conglycinin in soybean cotyledon through the formation of disulfide bonds between α′- and α-subunits. *Plant Physiol.* **2012**, *158*, 1395−1405.