Food Chemistry 177 (2015) 288–294

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

The characterization of soybean oil body integral oleosin isoforms and the effects of alkaline pH on them

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

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, PR China

ARTICLE INFO

Article history:
Received 21 April 2013
Received in revised form 6 January 2015
Accepted 8 January 2015
Available online 14 January 2015

Keywords:
Soybean oil body
Oleosin isoforms
Alkaline pH
Glycoprotein
Thiol group

ABSTRACT

Oil body, an organelle in seed cell (naturally pre-emulsified oil), has great potentials to be used in food, cosmetics, pharmaceutical and other applications requiring stable oil-in-water emulsions. Researchers have tried to extract oil body by alkaline buffers, which are beneficial for removing contaminated proteins. But it is not clear whether alkaline buffers could remove oil body integral proteins (mainly oleosins), which could keep oil body integrity and stability. In this study, seven oleosin isoforms were identified for soybean oil body (three isoforms, 24 kDa; three isoforms, 18 kDa; one isoform, 16 kDa). Oleosins were not glycoproteins and 24 kDa oleosin isoforms possessed less thiol groups than 18 kDa ones. It was found that alkaline pH not only removed contaminated proteins but also oleosins, and more and more oleosins were removed with increasing alkaline pH.

1. Introduction

Oil body (also named as oleosome, lipid body, and spherosome) is discrete intracellular organelle storing triglycerides (TAGs), which is energy resource for seed germination and seedling growth. It is composed of TAGs matrix shielded by a monolayer of phospholipids embedded by its integral proteins (Huang, 1992). Lin, Liao, Yang, and Tzen (2005) reported that there were three identified classes of integral proteins for sesame oil body, named as oleosin, caleosin, and steroleosin, which respectively had isoforms. Oleosin is the major protein (80–90%) of oil body integral proteins (Huang, 1992). Generally, oleosin molecule has a structure composed of N-terminus, hydrophobic central domain, and C-terminus with hydrophobic central domain embedded into TAGs matrix and N-, C-terminus exposed to exterior (Huang, 1992). According to Huang's oil body model, oleosin almost constitutes the whole oil body surface and plays key roles on oil body integrity and stability by supplying electrostatic repulsion and steric hindrance. Therefore, oil body is a kind of naturally stable pre-emulsified oil, which could be used in food industry and personal care products (Deckers, Rooijen, Boothe, Goll, & Moloney, 2001). As a result, researchers have tried to extract oil bodies from diverse plant seeds, including soybean, maize, rapeseed, sunflower and so on (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). And alkaline extraction buffers were well used in the research of food science (Chen & Ono, 2010; Iwanaga et al., 2007; Kapchie, Wei, Hauck, & Murphy, 2008; Karkani, Nenadis, Nikiforidis, & Kiosseoglou, 2013; Matsakidoua, Biliaderisb, & Kiosseogloua, 2013; Nikiforidis, Biliaderis, & Kiosseoglou, 2012; Nikiforidis, Kiosseoglouc, & Scholtena, 2013; Wu et al., 2012), which were beneficial for removing contaminated proteins.

It is known that the pH of extraction buffer has great effects on the protein composition of extracted soybean oil body, which has great effects on oil body properties and subsequently affects the oil body utilization. By neutral pH extraction, lipoxygenase, β-conglycinin, γ-conglycinin, β-amylase, Gly m Bd 30K, and some not well-known proteins are remained on oil body surface in addition to oil body integral proteins; by pH 8–10 extractions, lipoxygenase, β-conglycinin, γ-conglycinin, β-amylase, and glycinin are almost removed from oil body surface; by pH 11 extraction, just oil body integral oleosins are remained on oil body surface (Chen & Ono, 2010; Wu et al., 2012). However, it is not clear whether oleosin could also be removed by the alkaline pH.

Soybean oil body oleosin isoforms with molecular weights (MWs) of 24, 18, and 17 kDa were reported (Herman, 1987; Kalinski, Melroy, Dwivedi, & Herman, 1992). Schmidt and Herman (2008) reported that soybean oil body caleosin was appeared as one thin band (30 kDa) on SDS–PAGE gel, revealing that caleosin was one minor integral protein. Until now, no researches have ever reported soybean oil body steroleosin. Therefore, this study would focus on the major integral oleosin of

* Corresponding author. Tel./fax: +86 510 85329091.
E-mail address: chenyeming19821213@gmail.com (Y. Chen).

http://dx.doi.org/10.1016/j.foodchem.2015.01.052
0308-8146/

soybean oil body. 24 kDa oleosin was confirmed to have at least two isoforms A and B, whose amino acid sequences had been predicted through their cDNA clones (Kalinski, Loer, Weisemann, Matthews, & Herman, 1991; Rowley & Herman, 1997). There were no detailed researches about 18 and 17 kDa oleosins, and whether they had isoforms was not clear. In addition, some fundamental properties of oleosin (pI, glycoprotein or not, containing cysteine residue or not) were not clear as well. Therefore, this study would be divided into two parts: (1) oleosin fundamental properties (isoforms, isoelectric points (pIs), glycoprotein or not, containing cysteine or not); (2) the effects of alkaline pH on the oleosin release from oil body.

2. Materials and methods

2.1. Materials

Soybean (Nannong 88-31), cultivated in 2012, was kindly supplied by Soybean Research Institute, Nanjing Agricultural University (Nanjing, China), and stored at 4 °C until use. Urea, thiourea, CHAPS, dithiothreitol (DTT), and low melting agarose were purchased from Bio-Rad Laboratories (CA, USA). Methylene diacrylamide, acrylamide, sodium dodecyl sulfate (SDS), monobromobimane (mBBr), iodoacetamide, and β-mercaptoethanol were purchased from Sigma–Aldrich (Shanghai, China).

2.2. Preparation of oil body suspension

Twenty grams of soybean was soaked in de-ionized (DI) water for 18 h at 4 °C, ground in fresh DI water (1 g of dry soybean per 9 mL of DI water) with a blender (Midea BE601AB, China) for 2 min. The homogenate was filtered through four layers of cheese cloth to get raw soymilk. Eight grams of sucrose and 32 g of raw soymilk were added into four beakers, respectively, with a final sucrose concentration of 20% (w/w). pH values of the four raw soymilks were respectively adjusted to 6.8, 8.0, 9.5, and 11.0 using 1 M NaOH. They were centrifuged at 25,000*g* for 40 min (Hitachi CR21G, Japan). Oil body pads were collected, dispersed into 39 g of 20% sucrose solution, and correspondingly adjusted to pH 6.8, 8.0, 9.5, and 11.0. They were centrifuged, and oil body pads were collected. Then this was repeated one more time. These four oil body pads were collected, and dispersed into 10 mL of DI water, respectively.

2.3. Protein contents of extracted oil bodies by gravimetric analysis

The suspensions of extracted oil bodies above were dialyzed in 3000 mL of DI water at 4 °C three times, 6 h one time. The dialyzed samples were freeze-dried, and weighed. They were defatted with chloroform/methanol (v/v, 2/1; removing the neutral lipids and polar lipids) three times. The ratio of freeze-dried sample to chloroform/methanol was 1/50 (w/v). The residual matters were air-dried in hood, and weighed. The protein contents in 100 g of extracted oil bodies (dry basis) could be calculated.

2.4. Oil body protein preparation for electrophoresis

The oil body suspensions above were dialyzed against DI water and lyophilized as above. The lyophilized oil bodies were extracted with acetone at 4 °C to remove neutral lipids. After stirred for 30 min, the homogenate was filtered through a layer of filter paper, and the residual was recovered. This was repeated two more times. Then the residual was further defatted by chloroform/methanol (2/1, v/v) for three times. The oil body protein obtained was placed in hood to allow the organic solvent evaporated.

2.5. SDS–PAGE and Tricine–SDS–PAGE

Samples used for SDS–PAGE/Tricine–SDS–PAGE were prepared as below: oil body protein obtained in Section 2.4 was dissolved into 1 mL of SDS–PAGE/Tricine–SDS–PAGE sample buffer to the concentration of 2 mg/mL, and 20 μL of β-mercaptoethanol was added and heated in boiling water bath for 3 min. Ten microliters of each sample was loaded into sample well.

SDS–PAGE was performed according to the method by Chen and Ono (2010), with the concentrations of stacking gel and separating gel being 5% and 12.5%, respectively. After electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250.

Tricine–SDS–PAGE was carried out according to the method by Schägger (2006), using 16% separating gel and 4% stacking gel. After electrophoresis, the gel was also stained with Coomassie Brilliant Blue G-250.

2.6. Two-dimensional electrophoresis (2-D electrophoresis)

Protein of oil body extracted by pH 8.0 obtained in the Section 2.4 was used. The 2-D electrophoresis was performed as the Bio-Rad manufactures instructions. One hundred and fifty micrograms of protein, dissolved in 125 μL of 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% (w/v) bromophenol blue, 0.05% (w/v) SDS), was loaded onto a linear IPG gel strip (pH 3–10, 7 cm, Bio-Rad Laboratories, CA). The first dimensional isoelectric focusing (IEF) was conducted at 40 kVh using Protean IEF Cell (Bio-Rad Laboratories, USA). After IEF, the IPG strip was reduced in equilibration buffer A (6 M urea, 2% (w/v) SDS, 0.375 M Tris–HCl, pH 8.8 and 20% (w/v) glycerol, 0.2% (w/v) DTT) and alkylated in equilibration buffer B (6 M urea, 2% (w/v) SDS, 0.375 M Tris–HCl, pH 8.8 and 20% (w/v) glycerol, 0.25% (w/v) iodoacetamide) for 15 min. After rinsing by SDS–PAGE electrode buffer, the strip was transferred onto 12.5% (w/v) polyacrylamide gel, covered with low melting point agarose, and subjected to the second dimensional SDS–PAGE. The gel was stained with Coomassie Brilliant Blue G-250, and pIs of the interested spots were estimated.

2.7. Protein identification by MALDI-TOF/TOF mass spectrometry (MALDI-TOF/TOF MS)

Interested protein spots from 2-D gel or bands from SDS–PAGE/Tricine–SDS–PAGE gels were treated with trypsin (Promega, China) according to a standard protocol (Shevchenko, Tomas, Havlis, Jesper, & Mann, 2007). After digestion at 37 °C for 20 h, the peptides were extracted, dried in a SpeedVac vacuum centrifuge, and redissolved in 3 μL of 0.1% (w/v) trifluoroacetic acid solution. The sample for detection was prepared by mixing 0.7 μL of the above sample and 0.7 μL of 4-hydroxy-α-cyanocinnamic acid (matrix) on a MALDI target and air-dried. All the peptide mass fingerprint data were obtained by using an ultrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Germany) with the flexAnalysis software. Searches of the peptide mass fingerprint data against NCBI database were performed with the Mascot search engine (http://www.matrixscience.com).

2.8. Whether the oleosins are glycoprotein or not

Oil body proteins were separated by SDS–PAGE. The gel was fixed with 10% TCA, stained by a combination of (i) initial periodic acid oxidation/Alcian blue staining and (ii) subsequent silver nitrate staining. The glycoproteins could be stained as brown bands while the non-glycoproteins as blue bands (Moller & Poulsen, 1995).

2.9. Monobromobimane (mBBr) labeling of proteins

Thiol groups of protein could be visualized after they were labeled by fluorescent reagent of mBBr. Labeling procedure was done according to the method by Buchanan et al. (1997). Ten microliters of 30 mM DTT was added into 50 μL of oil body protein suspension (2–4 μg protein/μL) in water bath at 37 °C for 1 h, allowing disulfide bonds to be reduced. Ten microliters of 50 mM mBBr solution was added. This operation was done under dim light since mBBr was light sensitive. After 20 min of incubation in dark, the sample was exposed to light, and 3 μL of β-mercaptoethanol and 40 μL of SDS–PAGE sample buffer were added. Subsequently, mBBr-labeled samples were resolved by SDS–PAGE. The gel was fixed in fixing solution (5% trifluoroacetic, 33% (v/v) ethanol) overnight, and the fixing solution was changed three times. The fluorescent bands were appeared with UV light excitation on a computing densitometer (Molecular Imager ChemiDoc XRS+, Bio-Rad, USA). The fluorescent bands were considered to be proteins with thiol groups. Afterwards, the gel was stained with Coomassie Blue G-250.

2.10. The effect of alkaline pH on oleosin

Oil bodies extracted from 40 g of raw soymilk (pH 6.8, 20% sucrose) by centrifugation (25,000g, 40 min) was redispersed into DI water, with a total weight of 40 g (20% sucrose, 0.4% (w/w) oil bodies). The oil body suspension was uniformly divided into three beakers, respectively adjusted at pHs 8.0, 9.5 and 11.0 with 0.1 M NaOH and treated by using BECKMAN Optima™ L-XP ultracentrifuge (197,000g, 1 h). The supernatants were collected, and their protein contents were determined as 1.12, 2.35 and 3.11 μg/μL by BCA method (bovine serum albumin as standard). The supernatant without dilution (0.5 mL) was mixed with 0.5 mL of SDS–PAGE sample buffer, and treated by SDS–PAGE. Interested protein bands on gel were identified by MALDI-TOF/TOF MS above.

3. Results and discussion

3.1. The oleosin isoforms are resolved by SDS–PAGE and Tricine–SDS–PAGE

Generally, the oleosin isoforms of plant seed oil body have molecular weights (MWs) in a range of 16–24 kDa (Tzen, Lai, Chan, & Huang, 1990), and are always examined by SDS–PAGE (Chen & Ono, 2010; Herman, 1987; Kalinski et al., 1992; Schmidt & Herman, 2008; Wu et al., 2012). Schägger (2006) reported that Tricine–SDS–PAGE was the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa. Therefore, soybean oil body oleosin isoforms were respectively resolved by SDS–PAGE and Tricine–SDS–PAGE and compared. It is found that four bands are resolved by SDS–PAGE (Fig. 1a), while nine bands are resolved by Tricine–SDS–PAGE (Fig. 1b) in the range of 16–24 kDa. The bands (1–9) on Tricine–SDS–PAGE gel are examined by MALDI-TOF/TOF MS, and the results are shown in Table 1.

Band 1 was matched to 24 kDa oleosin isoform A (I1N747) and band 2 was matched to 24 kDa oleosin isoform B (P29531), in good agreement with the results by Kalinski et al. (1991). Bands 3–5 were matched to the subunits of glycinin (data not shown). Band 6 was matched to 18 kDa oleosin isoforms (I1K1K4 and gi|356515553). Band 7 was matched to 18 kDa oleosin isoform (C3VHQ8). Surprisingly, band 8 was matched to 24 kDa oleosin isoforms (I1N747 and P29531), 18 kDa oleosin isoform (C3VHQ8), and 16 kDa oleosin isoform (C6SZ13). Because the hydrolysis phenomenon is frequently happened in biological system, it is highly possible that the former three ones are hydrolyzed by soybean endoproteases to form polypeptides which have similar MWs to the 16 kDa oleosin isoform (C6SZ13). Unfortunately, band 9 was not matched to any protein in this study. These results confirmed that 24 kDa oleosins (I1N747 and P29531) and 18 kDa oleosin (C3VHQ8) were major isoforms, and 18 kDa oleosins (I1K1K4 and gi|356515553) and 16 kDa oleosin (C6SZ13) were minor isoforms.

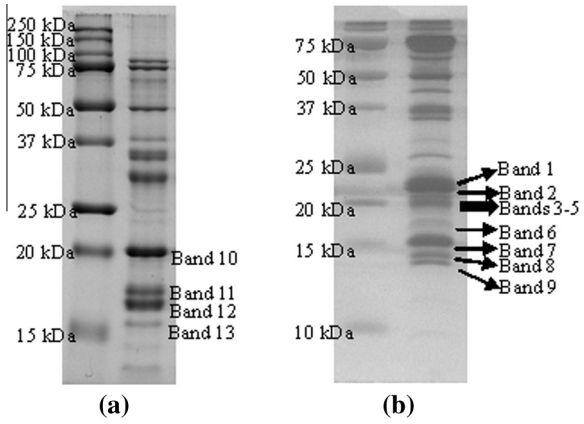


Fig. 1. The protein profiles of pH 6.8 extracted oil bodies resolved by SDS–PAGE (a) and Tricine–SDS–PAGE (b). Twenty micrograms of protein was loaded for SDS–PAGE and Tricine–SDS–PAGE analysis, and the gels were stained by Coomassie Blue G-250.

The four bands (10–13) on SDS–PAGE gel were also examined by MALDI-TOF/TOF MS. Band 10 was matched to 24 kDa oleosin isoforms (I1N747 and P29531). Band 11 was matched to the subunits of glycinin. Interestingly, band 12 was matched to 18 kDa oleosin isoforms (gi|356515553 and C3VHQ8) and 16 kDa oleosin isoform (C6SZ13). Band 13 was matched to 24 kDa oleosin isoforms (I1N747 and P29531), which further showed that some oleosins were hydrolyzed in the extracted oil bodies. Compared with SDS–PAGE, Tricine–SDS–PAGE is better at resolving soybean oleosin isoforms.

3.2. The oleosin isoforms are resolved by two-dimensional electrophoresis

In order to examine the pIs of oleosin isoforms, two-dimensional electrophoresis was performed. Fig. 2 shows that there are more than 40 separated protein spots on gel and there are 14 spots in the range of 16–24 kDa, which are excised from the gel and subjected to MALDI-TOF/TOF MS (Table 1). Spot 1 was matched to 24 kDa oleosin isoform (I1N747), and spot 2 was matched to 24 kDa oleosin isoform (P29530). Spots 3 and 4 were matched to the subunits of glycinin. Spot 5 was matched to 18 kDa oleosin isoform (gi|356515553), and spot 6 was matched to 18 kDa oleosin isoform (C3VHQ8). Spot 7 was matched to one fragment originated from 24 kDa oleosin isoform (P29530). Unfortunately, the spots indicated by blue arrows were not matched to any proteins. These results confirmed one more 24 kDa oleosin isoform (P29530), while three confirmed oleosin isoforms above (P29531, I1K1K4, and C6SZ13) were not identified on the 2-D gel. 24 kDa oleosin isoforms (I1N747 and P29530) showed similar pIs to their theoretical pIs, while 18 kDa oleosin isoforms (gi|356515553 and C3VHQ8) showed lower pIs than their theoretical pIs, which might be induced by the small amount of SDS used in the 2-D electrophoresis. In this study, the 2-D electrophoresis was conducted several times, it was found that oleosin isoforms could not be resolved without SDS. Therefore, it was considered that 2-D electrophoresis was difficult to be used for hydrophobic proteins as oleosins.

Seven oleosin isoforms were unambiguously identified in the results above. 24 kDa oleosin isoforms (P29530, P29531, and

Table 1
Protein bands (pH 6.8 extracted oil bodies) on Tricine–SDS–PAGE gels (Fig. 1) and protein spots (pH 8.0 extracted oil bodies) on 2-D gel (Fig. 2) identified by MALDI-TOF/TOF MS.

Protein ID	Identified in band*/spot#					Length[a]	MW[b]	pI E/T[c]	NCBInr acc.#	UniProtKB acc.#
PREDICTED[d]: P24 oleosin isoform A	1*	8*	10*	13*	1#	226	23,575	9.09/8.89	gi|356571311	I1N747
RecName: P24 oleosin isoform A					2#	226	23,487	8.19/8.01	gi|1709459	P29530
P24 oleosin isoform B	2*	8*	10*	13*		223	23,378		gi|351722277	P29531
PREDICTED: oleosin 5-like	6*					181	19,150		gi|356511688	I1K1K4
PREDICTED: oleosin 16 kDa-like	6*		12*		5#	166	17,534	6.60/7.77	gi|356515553	
16.5 kDa oleosin	7*	8*	12*		6#	165	17,463	6.19/7.74	gi|351726299	C3VHQ8
Uncharacterized protein LOC100306353		8*	12*			147	15,767		gi|351721929	C6SZ13
24 kDa oleosin isoform[e]					7#	152	15,801	8.47/8.23	gi|18720	

* Band identification numbers correspond to those proteins labeled in Fig. 1.
\# Spot identification numbers correspond to those proteins labeled in Fig. 2.
[a] The number of amino acid residues for each protein.
[b] Molecular weight (MW) are given as theoretical values (Da).
[c] Isoelectric point (pI) values of proteins from 2-D gel are given as experimental/theoretical values.
[d] PREDICTED: This record is predicted by automated computational analysis. This record is derived from a genomic sequence annotated using gene prediction method: GNOMON, supported by mRNA and EST evidence.
[e] 24 kDa oleosin isoform is one fragment from 24 kDa oleosin isoform (P29530). The accession numbers (acc.#) are according to the genome sequence of soybean reported by Schmutz et al. (2010).

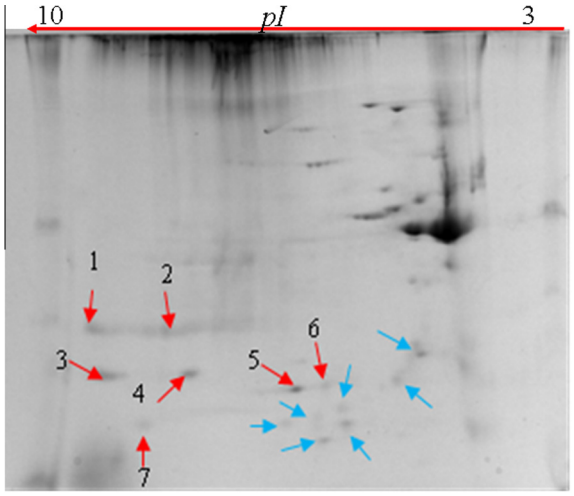


Fig. 2. 2-D gel pattern of protein from oil bodies obtained by pH 8.0 extraction. Proteins were separated by isoelectric focusing on IPG strips (7 cm, pH 3–10), then by SDS–PAGE on a 12.5% gel. The spots indicated by arrows were examined by MALDI-TOF/TOF MS. The gel was stained by Coomassie Blue G-250.

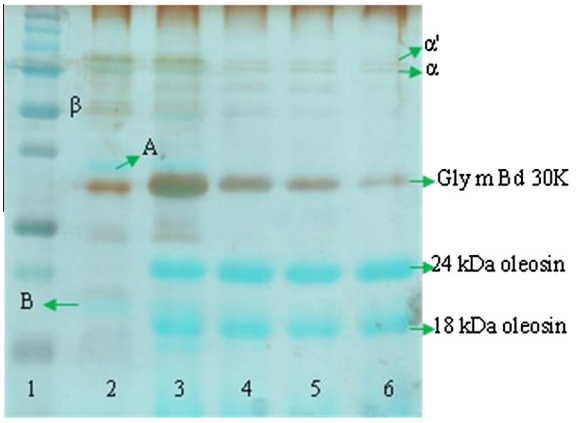


Fig. 3. SDS–PAGE pattern of proteins from pH 6.8, 8.0, 9.5, and 11.0 extracted oil bodies. The gel was stained by a method which could distinguish glycoprotein and non-glycoprotein. Lane 1, marker; Lane 2, supernatant obtained from raw soymilk by centrifugation (25,000*g*, 40 min); Lanes 3–6, protein of oil bodies obtained by pH 6.8, 8.0, 9.5, and 11.0 extractions. α′, α, and β subunits of β-conglycinin and Gly m Bd 30K, which are glycoproteins, are stained as brown color, while acidic (A) and basic (B) polypeptides of glycinin, which are not glycoproteins, are stained as blue color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

I1N747) had been well examined (Kalinski et al., 1991; Rowley & Herman, 1997). The other four isoforms were the first time to be identified by SDS–PAGE, Tricine–SDS–PAGE, and 2-D electrophoresis combining with MALDI-TOF/TOF MS. To our best knowledge, this study should be the first time to confirm that soybean oleosin isoforms were alkaline proteins by 2-D electrophoresis although it could not resolve oleosin isoforms very well.

3.3. 24 and 18 kDa oleosins are not glycoproteins

Fig. 1a shows that oleosin isoforms are mainly appeared as 24 kDa (P29530, P29531, and I1N747) and 18 kDa (gi|356515553, C3VHQ8, and C6SZ13) bands, and 16 kDa band is the products of endoprotease-hydrolyzed 24 kDa oleosin isoforms on the SDS–PAGE gel. Therefore, oleosin isoforms were deemed as 24 and 18 kDa oleosins in the following study.

It was considered that oleosin, one kind of membrane protein, might be glycoprotein. To our best knowledge, there were no researches examining whether oleosin was glycoprotein or not. Fig. 3 clearly shows that Gly m Bd 30K, α′, α, and β subunits of β-conglycinin, which are glycoproteins (Maruyama et al., 1998; Ogawa et al., 1993), are stained as brown color, while acidic (A) and basic (B) polypeptides of glycinin, which are not glycoproteins (Utsumi, Kanamori, Kim, Sato, & Kito, 1991), are stained as blue color. And 24 and 18 kDa oleosins are stained as blue color, which clearly reveals that oleosin isoforms are not glycoproteins. It was reported (Chabrand, Kim, Zhang, Glatz, & Jung, 2008) that the carbohydrate contributed about 2% (dry basis) to the pH 8.0 extracted soybean oil bodies (without washing). It was considered that the carbohydrate might include three parts: contaminated carbohydrate (such as soybean oligosaccharides), contaminated glycoproteins (such as Gly m Bd 30K), and oil body membrane intrinsic glycolipids.

3.4. 24 and 18 kDa oleosins contain different amounts of cysteine residues

Cysteine residues of protein molecule are very reactive owing to their thiol groups. According to the NCBI database, the three identified 24 kDa oleosin isoforms in this study do not contain any cysteine residues, while the three identified 18 kDa oleosin isoforms contain cysteine residues. In order to confirm this, the protein of

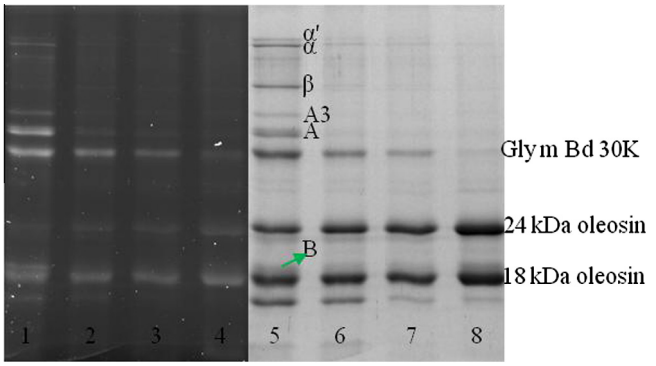
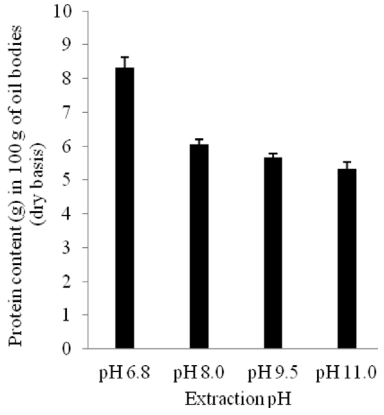


Fig. 4. SDS–PAGE pattern of mBBr-labeled protein of pH 6.8, 8.0, 9.5 and 11.0 extracted oil bodies. The picture with black background and the one with gray background are from the same gel. The former one was visualized under UV light, and the latter one was taken after staining the gel by Coomassie Blue G-250. Protein of oil bodies obtained by pH 6.8 (Lanes 1 and 5), 8.0 (Lanes 3 and 7), 9.5 (Lanes 4 and 8), and 11.0 (Lanes 2 and 6) extractions.

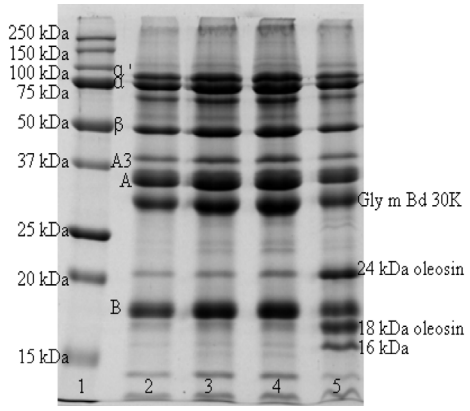
oil body was reduced by DTT, labeled by mBBr and treated by SDS–PAGE. Fig. 4 shows that Gly m Bd 30K, α′, α, acidic and basic polypeptides (thiol-containing proteins) can be clearly visualized, while β (not thiol-containing protein) (Maruyama et al., 1998) cannot be visualized with UV light excitation. Both of 24 and 18 kDa oleosins can be visualized, and the intensity of the former one is obviously weaker than that of the latter one, revealing that 24 kDa oleosin contains less thiol groups than 18 kDa oleosin. It was surprising that thiol groups were detected for 24 kDa oleosin although NCBI database showed that the three confirmed 24 kDa oleosins (P29530, P29531, and I1N747) in this study did not contain any cysteine residues. This similar phenomenon was also found in sunflower oil body oleosin by Babazadeh, Poursaadat, Sadeghipour, and Colagar (2012). In addition, another research about the interaction between soybean oil bodies and Gly m Bd 30K in our lab showed that Gly m Bd 30K could strongly be bound to soybean oil bodies through disulfide bond with 24 kDa oleosin. It was considered that 24 kDa oleosin might have some unknown cysteine-containing isoforms, which needed further researches.

3.5. The effects of alkaline pH on oleosin

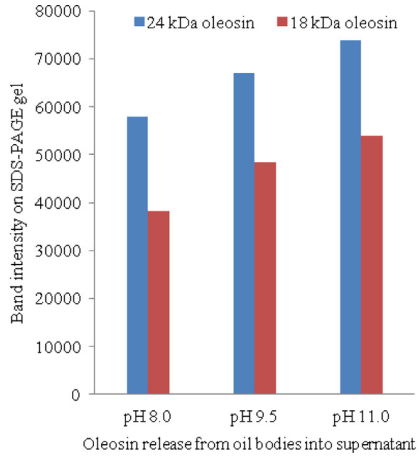
The protein contents of oil bodies extracted by pH 6.8, 8.0, 9.5, and 11.0 were examined by gravimetric analysis. Fig. 5a shows that pH 6.8 extracted oil bodies contain the most protein (8.33 ± 0.26 g/100 g of oil bodies, dry basis), followed by pH 8.0 (6.05 ± 0.15 g/



(a)



(b)



(c)

Fig. 5. (a) The protein contents (dry basis) of pH 6.8, 8.0, 9.5, and 11.0 extracted oil bodies by gravimetric analysis. (b) The SDS–PAGE pattern of protein in the supernatants. Lane 1, marker; Lanes 2–4, the supernatants obtained by ultracentrifuging (197,000*g*, 1 h) pH 6.8 extracted oil body suspensions whose pHs have been adjusted to 8.0, 9.5, and 11.0; Lane 5, protein of pH 6.8 extracted oil bodies. (c) The band intensities of 24 and 18 kDa oleosins on Lanes 2–4 by software Quantity One (version 4.6.2, Bio-Rad, USA).

100 g of oil bodies), 9.5 (5.67 ± 0.13 g/100 g of oil bodies), and 11.0 (5.33 ± 0.20 g/100 g of oil bodies) extracted oil bodies. Tzen et al. (1993) reported that the protein contents of oil bodies from rapeseed, mustard, cotton, flax, maize, peanut, and sesame were in a range of 0.59–3.46% (dry basis) by gravimetric analysis, and were negatively correlated with the particle size of oil bodies (0.65–2.00 μm). Therefore, it was reasonable that soybean oil bodies (400 nm, Chen & Ono, 2010) contained more protein than the oil bodies above. It was reported that the protein content of soybean oil bodies was about 10–15% (dry basis) by using Kjeldahl method (Iwanaga et al., 2007; Wu et al., 2012), the larger value should result from the phospholipids, another important components of oil bodies (Huang, 1992). This was because phospholipids included some nitrogen-containing phospholipids: phosphatidylserines (PS), phosphatidyl cholines (PC), and phosphatidyl ethanolamines (PE). In addition, oleosin was alkaline protein, so a proper conversion factor was needed. Therefore, it was concluded that Kjeldahl method was not good at determining the protein content of oil bodies, and the gravimetric analysis should be the convincing method.

In order to clarify whether alkaline pH could remove oleosin, pH 6.8 extracted oil bodies were dispersed into 20% sucrose solution (w/w), divided into three beakers and adjusted to pH 8.0, 9.5, and 11.0, respectively. They were treated by ultracentrifugation (197,000*g*, 1 h), which could float all of the oil bodies in the suspensions. The protein concentrations of the supernatants were determined as 1.12, 2.35, and 3.11 μg/μL, which clearly showed more and more protein released from oil body with increasing pH. The supernatant without dilution (0.5 mL) was mixed with 0.5 mL of SDS–PAGE sample buffer, and 20 μL of β-mercaptoethanol was added. It was treated by SDS–PAGE. Fig. 5 shows that all the protein bands become denser with increasing pH. The band intensities of 24 and 18 kDa oleosins (lanes 2–4) are analyzed by software Quantity One (Version 4.6.2, Bio-Rad, USA) and shown in Fig. 5c, and it is found that their band intensities are gradually increased with increasing pH, and more amounts of 24 kDa oleosin is released from oil body than 18 kDa oleosin. This clearly revealed that high alkaline pH was beneficial for oleosin releasing from oil body. According to Huang's oil body model (1992), oleosin was composed of three parts: N-terminus, hydrophobic central domain, and C-terminus. The positively charged amino acid residues of N- and C-terminus were bound to negatively charged phospholipids by salt bridges, and hydrophobic central domain was embedded into TAGs matrix, which should make oleosin release from oil body difficult. It was reported that some surface active agents, such as Tween and SDS, could induce oleosin release from oil body, and it was explained that oleosin was replaced by these surface active agents (Nikiforidis & Kiosseoglou, 2011; Tzen, Peng, Cheng, Chen, & Chiu, 1997). The oleosin release induced by alkaline pH was not well examined until now. The reason might be that the positively charged amino acid residues of N- and C-terminus were deprotonated by alkaline pH, which decreased the numbers of salt bridges between oleosins and phospholipids. As a result, oleosin could not anchor on oil body as strong as before. These results showed that alkaline pH extraction not only removed contaminated proteins but also oleosins.

4. Conclusions

Seven oleosin isoforms were unambiguously identified in this study (Table 1). Three oleosin isoforms (P29530, P29531, and I1N747) had MWs around 24 kDa, three oleosin isoforms (I1K1K4, gi|356515553, and C3VHQ8) held MWs around 18 kDa, and one oleosin isoform (C6SZ13) had MW around 16 kDa. It was found that all identified oleosin isoforms were not glycoproteins, and 24 kDa oleosin isoforms possessed less thiol groups than 18 kDa ones, and alkaline pH extraction not only removed contaminated proteins but also oleosins, which was enhanced by high alkaline pH. In all, the study was not only meaningful for oil body utilization, but also meaningful for the enzyme-assisted extraction processing of soybean oil, in which oleosin played a role in inhibiting oil release from oil body. This study might give some hints to modify this processing to increase the oil yield.

Acknowledgments

We gratefully acknowledge the financial support received from Natural Science Foundation of China (31301496), Fundamental Research Fund of Ministry of Education (2050205), Natural Science Foundation of China (31201380), Natural Science Foundation of Jiangsu Province, PR China (BK2011151), and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.01.052.

References

Buchanan, B. B., Adamidi, C., Lozano, R. M., Yee, B. C., Momma, M., Obrehel, K., et al. (1997). Thioredoxin-linked mitigation of allergic responses to wheat. *Proceedings of the National Academy of Sciences, 94*(10), 5372–5377.

Babazadeh, N., Poursaadat, M., Sadeghipour, H. R., & Colagar, A. H. Z. (2012). Oil body mobilization in sunflower seedlings is potentially regulated by thioredoxin h. *Plant Physiology and Biochemistry, 57*, 134–142.

Chabrand, R. M., Kim, H., Zhang, C., Glatz, C. E., & Jung, S. (2008). Destabilization of the emulsion formed during aqueous extraction of soybean oil. *Journal of the American Oil Chemists' Society, 85*(4), 383–390.

Chen, Y., & Ono, T. (2010). Simple extraction method of non-allergenic intact soybean oil bodies that are thermally stable in an aqueous medium. *Journal of Agricultural and Food Chemistry, 58*(12), 7402–7407.

Deckers, H. M., Rooijen, G. V., Boothe, J., Goll, J., & Moloney, M. M. (2001). *Oil body-based personal care products*. U.S. patent, 6183762.

Herman, E. M. (1987). Immunogold-localization and synthesis of an oil-body protein membrane protein in developing soybean seeds. *Planta, 172*(3), 336–345.

Huang, A. H. C. (1992). Oil bodies and oleosins in seeds. *Annual Review of Plant Physiology and Plant Molecular Biology, 43*, 177–200.

Iwanaga, D., Gray, D. A., Fisk, I., Decker, E. A., Weiss, J., & McClements, D. J. (2007). Extraction and characterization of oil bodies from soy beans: A natural source of pre-emulsified soybean oil. *Journal of Agricultural and Food Chemistry, 55*(21), 8711–8716.

Kalinski, A., Loer, D. S., Weisemann, J. M., Matthews, B. F., & Herman, E. M. (1991). Isoforms of soybean seed oil body membrane protein 24 kDa oleosin are encoded by closely related cDNAs. *Plant Molecular Biology, 17*(5), 1095–1098.

Kalinski, A., Melroy, D. L., Dwivedi, R. S., & Herman, E. M. (1992). A soybean vacuolar protein (P34) related to thiol proteases is synthesized as a glycoprotein precursor during seed maturation. *Journal of Biological Chemistry, 267*, 12068–12076.

Kapchie, V. N., Wei, D., Hauck, C., & Murphy, P. A. (2008). Enzyme-assisted aqueous extraction of oleosomes from soybeans (*Glycine max*). *Journal of Agricultural and Food Chemistry, 56*(5), 1766–1771.

Karkani, O. A., Nenadis, N., Nikiforidis, C. V., & Kiosseoglou, V. (2013). Effect of recovery methods on the oxidative and physical stability of oil body emulsions. *Food Chemistry, 139*(14), 640–648.

Lin, L. J., Liao, P. C., Yang, H. H., & Tzen, J. T. C. (2005). Determination and analyses of the N-termini of oil-body proteins, steroleosin, caleosin and oleosin. *Plant Physiology and Biochemistry, 43*(8), 770–776.

Maruyama, N., Katsube, T., Wada, Y., Oh, M. H., Barba de la rosa, A. P., Okuda, E., et al. (1998). The roles of the N-linked glycans and extension regions of soybean β-conglycinin in folding, assembly and structural features. *European Journal of Biochemistry, 258*(2), 854–862.

Matsakidoua, A., Biliaderisb, C. G., & Kiosseogloua, V. (2013). Preparation and characterization of composite sodium caseinate edible films incorporating naturally emulsified oil bodies. *Food Hydrocolloids, 30*(1), 232–240.

Moller, H. J., & Poulsen, J. H. (1995). Improved method for silver staining of glycoproteins in thin sodium dodecyl sulfate polyacrylamide gels. *Analytical Biochemistry, 226*(2), 371–374.

Nikiforidis, C. V., & Kiosseoglou, V. (2011). Competitive displacement of oil body surface proteins by Tween-80 effect on physical stability. *Food Hydrocolloids, 25*(5), 1063–1068.

Nikiforidis, C. V., Biliaderis, C. G., & Kiosseoglou, V. (2012). Rheological characteristics and physicochemical stability of dressing-type emulsions made of oil bodies–egg yolk blends. *Food Chemistry, 134*(1), 64–73.

Nikiforidis, C. V., Kiosseoglouc, V., & Scholtena, E. (2013). Oil bodies: An insight on their microstructure—Maize germ vs sunflower seed. *Food Research International, 52*(1), 136–141.

Ogawa, T., Tsuji, H., Bando, N., Kitamura, K., Zhu, Y. L., Hirano, H., et al. (1993). Identification of the soybean allergenic protein, Gly m Bd 30K, with the soybean seed 34-kDa oil-body-associated protein. *Bioscience, Biotechnology, and Biochemistry, 57*(6), 1030–1033.

Rowley, D. L., & Herman, E. M. (1997). The upstream domain of soybean oleosin genes contains regulatory elements similar to those of legume storage proteins. *Biochimica et Biophysica Acta, 1345*(1), 1–4.

Schmidt, M. A., & Herman, E. M. (2008). Suppression of soybean oleosin produces micro-oil bodies that aggregate into oil body/ER complexes. *Molecular Plant, 1*, 910–924.

Schägger, H. (2006). Tricine–SDS–PAGE. *Nature Protocols, 1*(1), 16–22.

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., et al. (2010). Genome sequence of the palaeopolyploid soybean. *Nature, 463*, 178–183.

Shevchenko, A., Tomas, H., Havlis, J., Jesper, V. O., & Mann, M. (2007). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols, 1*(6), 2856–2860.

Tzen, J. T. C., Cao, Y. Z., Laurent, P., Ratnayake, C., & Huang, A. H. C. (1993). Lipids, proteins and structure of seed oil bodies from diverse species. *Plant Physiology, 101*(1), 267–276.

Tzen, J. T. C., Lai, Y. K., Chan, K. L., & Huang, A. H. C. (1990). Oleosin isoforms of high and low molecular weights are present in the oil bodies of diverse seed species. *Plant Physiology, 94*(3), 1282–1289.

Tzen, J. T. C., Peng, C. C., Cheng, D. J., Chen, E. C. F., & Chiu, J. M. H. (1997). A new method for seed oil body purification and examination of oil body integrity following germination. *Journal of Biochemistry, 121*(4), 762–768.

Utsumi, S., Kanamori, J., Kim, C. S., Sato, T., & Kito, M. (1991). Properties and distribution of soybean proglycinin expressed in *Saccharomyces cerevisiae*. *Journal of Agricultural and Food Chemistry, 39*(6), 1179–1186.

Wu, N. N., Huang, X., Yang, X. Q., Guo, J., Yin, S. W., He, X. T., et al. (2012). In vitro assessment of the bioaccessibility of fatty acids and tocopherol from soybean oil body emulsions stabilized with ι-carrageenan. *Journal of Agricultural and Food Chemistry, 60*(6), 1567–1575.