

A two-chain aspartic protease present in seeds with high affinity for peanut oil bodies



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ABSTRACT

Peanut seeds are rich in oil, which exists as oil bodies (OBs). By extraction, peanut crude OBs are obtained and can be used as a food ingredient. In a previous study, it was found that the crude OBs contained an unknown protease, which hydrolyzed the oleosins. This would disrupt the integrity of OBs, and therefore, affect their physical and oxidative stability. In this study, the protein composition of crude OBs and some properties of the unknown protease were examined. The results showed that the protease was a two-chain (32 and 9 kDa) aspartic protease, which showed high affinity for OBs. The optimal pH and temperature for oleosin hydrolysis by the protease were pH 4.0 and 60 °C. Interestingly, the aspartic protease not only hydrolyzed OB intrinsic proteins (oleosin, caleosin, and steroleosin), but also extrinsic proteins (especially Ara h 1 allergen and 26–30 kDa arachin).

1. Introduction

Oil bodies (OBs) are lipid-storing organelles in plant seeds, having a neutral lipid (mainly triacylglycerols, TAGs) matrix core, which is coated by the membrane comprised of a monolayer of phospholipids embedded with OB intrinsic proteins (major oleosin and minor caleosin and steroleosin) (Lin, Liao, Yang, & Tzen, 2005; Zhao, Chen, Cao, Kong, & Hua, 2013). In addition, OBs also contain some functional components, such as vitamin E and phytosterol (Chen, Cao, Zhao, Kong, & Hua, 2014; Fisk, White, Carvalho, & Gray, 2006; Gallier, Gordon, & Singh, 2012). In recent years, OBs or OB-rich products have been extracted from oilseeds (such as safflower and soybean) for the practical utilizations in food and personal care (Deckers, Van Rooijen, Boothe, Goll, & Moloney, 2003; Samoto, Kanamori, & Shibata, 2011). Peanut is an important oilseed in China, and more than 14 million tons of peanut are produced per year in recent years. At present, peanut is mainly used for oil production, and also for dry-roasted peanut and peanut butter. It is considered that peanut OBs, which are rich in oleic acid and some other functional components (Arya, Salve, & Chauhan, 2016), may be used as a functional food ingredient.

It was reported that the size of peanut OBs varied in a range of 0.14–5.5 µm, and had an average size of 1.95 µm (Jacks, Yatsu, & Altschul, 1967; Jayaram & Bal, 1991). By weight (dry basis), purified peanut OBs consist of approximately 98% neutral lipids, 1%

proteins, and 1% phospholipids (Jacks et al., 1967; Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). The intrinsic proteins of peanut OBs include 17 and 14 kDa oleosins, 30 kDa caleosin, and 39 kDa steroleosin (Schwager et al., 2015). The intrinsic phospholipids contain 62% phosphatidyl choline, 25% phosphatidyl serine, 8% phosphatidyl inositol, and 5% phosphatidyl ethanolamine (Tzen et al., 1993). It is known that OB intrinsic proteins and phospholipids maintain the integrity of OBs in response to various environmental stresses, such as desiccation and freezing (Leprince, van Aelst, Pritchard, & Murphy, 1998; Shimada, Shimada, Takahashi, Fukao, & Hara-Nishimura, 2008). It was reported that OBs could survive high temperature, 9 M urea, and relatively high shearing and centrifugation speed in the aqueous suspension (Chen, McClements, Gray, & Decker, 2012; Chen & Ono, 2010; Wu et al., 2012). Further, it was clarified that phospholipids were needed to stabilize OBs, but oleosins were mandatory to avoid coalescence (Deleu et al., 2010). These revealed that phospholipids, especially oleosins, were important for keeping the physical stability of OBs.

It was reported that the oleosins of peanut crude OBs were hydrolyzed by an unknown endogenous protease at pH 6.5 and 35 °C (Zhao, Chen, Chen, Kong, & Hua, 2016). The similar phenomenon was also observed in soybean, jicama, sunflower, rapeseed, castor bean, and sesame (Zhao et al., 2016). The effects of the endogenous protease on physical stability of peanut OBs have been examined in our lab, and it was found that the endogenous protease could induce the coalescence

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of peanut OBs and the subsequent release of free oil. However, the information concerning the endogenous protease is few. In this study, the optimal pH and temperature for oleosin hydrolysis, the catalytic residue of the endogenous protease, and the interaction between endogenous protease and OBs were examined. Finally, the endogenous protease was identified by two-dimensional electrophoresis and MALDI-TOF/TOF MS. It is considered that this study is meaningful not only for extraction of peanut OBs, but also for aqueous processing of peanut free oil.

2. Materials and methods

2.1. Materials

Peanut seeds (Haihua 14), harvested in 2016, were purchased from Laiwu, Shandong, China. The seeds were stored at 4 °C until it was used. E-64 (N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine) was purchased from Abcam (Shanghai, China). Phenylmethanesulfonyl fluoride (PMSF), disodium EDTA (EDTA-2Na), pepstatin A, sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue G-250, and β -mercaptoethanol were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Urea, thiourea, CHAPS, dithiothreitol (DTT), ampholyte, and bromophenol blue were purchased from Bio-Rad Laboratories (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of peanut crude OBs

Peanut (20 g) was soaked in deionized (DI) water at 4 °C for 18 h. The soaked seeds were washed three times with fresh DI water, after the soaking water was poured out. Precooled (4 °C) DI water was added to make the final weight of 200 g. The mixture was ground for 90 s by a Waring blender (15,000 rpm; MJ-60BE01B, Midea, Foshan, Guangdong, China), and filtered through four layers of gauze, and the filtrate was centrifuged (5000g for 15 min at 4 °C) into floating, supernatant, and precipitate fractions. The floating fraction (OB pad) was collected as crude OBs, which were used in the following experiments, or dispersed into DI water (crude OBs/DI water = 1/5, wet weight/volume) to obtain crude OB suspension.

2.3. OBs extracted at pH 7.0, 8.0, 9.5, and 11.0

This was conducted using the method of [Chen and Ono \(2010\)](#) with the modification that the temperature was controlled at 4 °C or less than 4 °C. The aqueous extract of peanut was equally divided into four parts, and the pH value of each part was adjusted to 7.0, 8.0, 9.5, or 11.0 with 0.1 and 1 M NaOH solutions. They were centrifuged (5000g for 30 min at 4 °C) by a high-speed refrigerated centrifuge (CR21N, Hitachi, Tokyo, Japan), and the floating fractions (OB pads) were collected and dispersed into DI water (OB pad/DI water = 1/10, wet weight/volume). The pH adjustment and subsequent centrifugation was repeated twice. The OB pads were collected and dispersed into DI water (OB pad/DI water = 1/5, wet weight/volume) in an ice water bath to obtain the suspensions of pH 7.0-OB, pH 8.0-OB, pH 9.5-OB, and pH 11.0-OB.

2.4. Crude OBs washed by 8 M urea solution

The crude OBs were dispersed into 8 M urea solution (crude OBs/urea solution = 1/9, wet weight/volume), and stirred in an ice water bath for 10 min. The suspension was centrifuged (5000g for 10 min at 4 °C), and the floating fraction (OB1) and supernatant (S1) were obtained. The OB1 was treated by urea washing as above for four more times. As a result, OB2–5 and S2–5 were obtained. The obtained OB pads were washed with DI water (4 °C, OB sample/DI water = 1/9, wet weight/volume) for three times. The supernatants (30 ml) were dialyzed (3.5 kDa cutoff) at 4 °C for 18 h (three times, 6 h each time), and

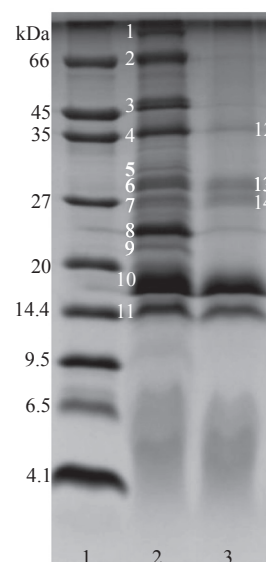


Fig. 1. Tricine-SDS-PAGE protein profiles of peanut crude OBs and pH 7.0-OB. Lane 1, marker; lane 2, crude OBs; lane 3, pH 7.0-OB.

concentrated by ultrafiltration (3 kDa cutoff) to 0.5 ml.

2.5. Effects of pH and temperature on the hydrolysis of proteins in crude OBs

NaN_3 was added into 300 ml of crude OB suspension to a final concentration of 0.02%. The suspension was equally divided to seven parts, and respectively adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. Then they were incubated in a 35 °C water bath. After 0, 1, 3, 6, and 9 h, 1 ml of suspension was collected and cooled in an ice water bath.

Crude OB suspension (300 ml) containing 0.02% NaN_3 was adjusted to pH 4.0, and then equally divided into eight parts, which were respectively incubated in 30, 40, 50, 60, 70, 80, 90, and 100 °C water baths. After 0, 1, 3, 6, and 9 h, 1 ml of suspension was collected and cooled in an ice water bath.

2.6. Assay of inhibition of proteolysis

This was conducted with the method by [Wilson and Tan-Wilson \(2015\)](#) with some modifications. Crude OB suspension was incubated with inhibitor in final concentrations of 1, 2, and 4 mM PMSF, 1, 2, and 4 μM pepstatin A, 16, 160, and 830 μM E-64, or 0.5, 1, and 2 mM EDTA-2Na for 1 h on ice. Then these suspensions were all adjusted to pH 4.0, and incubated in a 60 °C water bath. After 0, 1, 3, 6, and 9 h, 1 ml of suspension was collected and cooled in an ice water bath, and used for Tricine-SDS-PAGE analysis.

2.7. Tricine-SDS-PAGE

The protein concentrations of suspensions of crude OBs, alkali-washed OBs, and urea-washed OBs were determined using the amino acid analysis ([Ying et al., 2015](#)). The suspensions (0.5 ml) were mixed with Tricine-SDS-PAGE sample buffers (with different SDS concentrations) to obtain a protein concentration of 2 mg/ml with the SDS/protein mass ratio of 1.52/1 ([Ying et al., 2015](#)). The protein concentrations of supernatants 1–5 (S1–5) were determined by the bicinchoninic acid method, and respectively mixed with Tricine-SDS-PAGE sample buffer to obtain a protein concentration of 2 mg/ml.

Tricine-SDS-PAGE was conducted according to the method by [Schägger \(2006\)](#). The concentrations of stacking and separating gels were 4% and 16%, respectively. β -mercaptoethanol was added into the

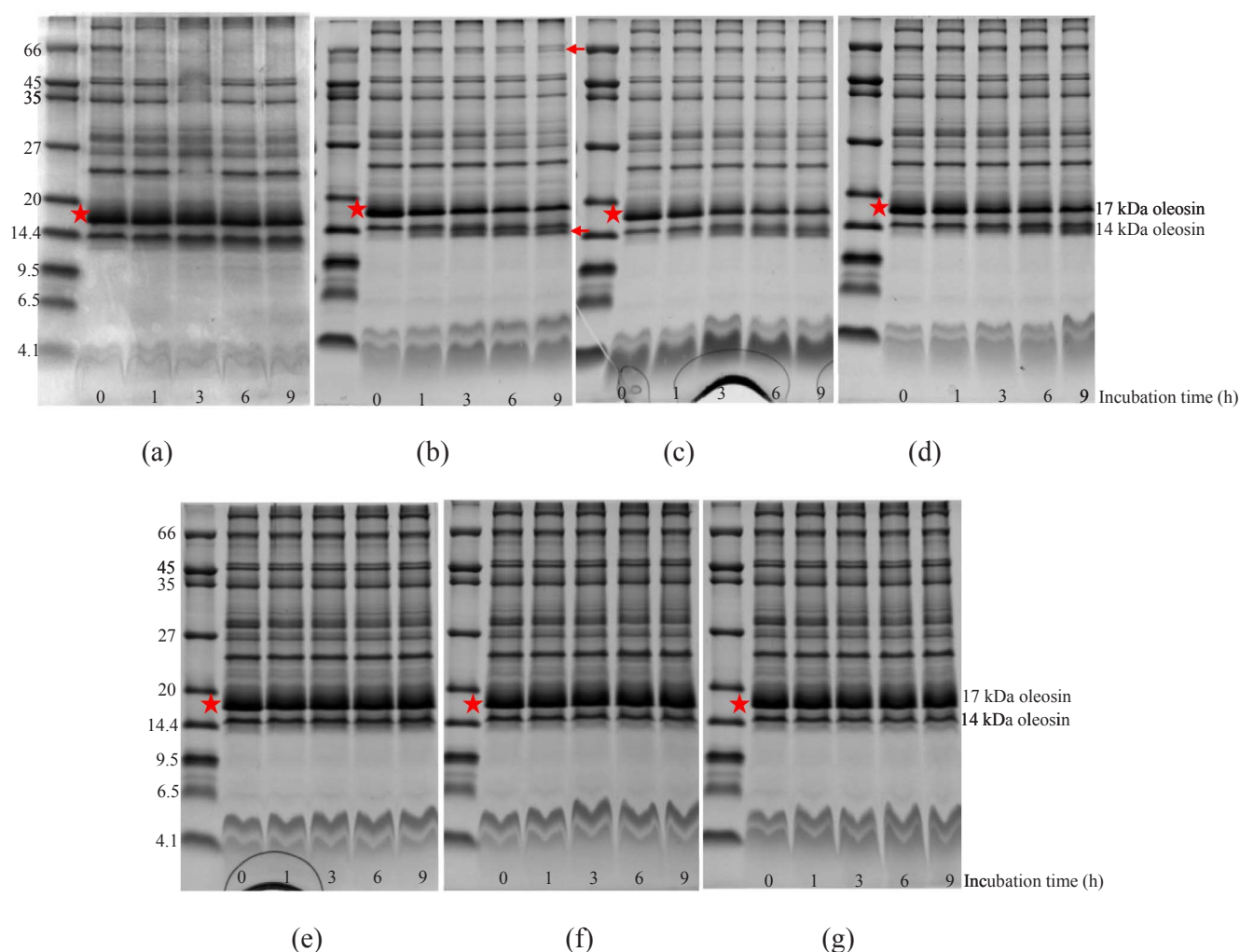


Fig. 2. Effects of pH on the hydrolysis of proteins in crude OBs. The incubation was conducted at 35 °C and pH 3 (a), 4 (b), 5 (c), 6 (d), 7 (e), 8 (f), and 9 (g) for 0, 1, 3, 6, and 9 h.

samples above to the concentration of 2% (v/v), and were heated for 3 min in a boiling water bath. Then 10 μ l of each sample was loaded into a sample well, and the samples were electrophoresed at a constant voltage of 30 mV until all samples entered into the stacking gel and then at a constant voltage of 100 mV until the end. The gel was stained using Coomassie Brilliant Blue G-250, and band intensities were analyzed with Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Two-dimensional (2-D) electrophoresis

pH 7.0-OB was adjusted to pH 9.0, and mixed for 30 min in an ice water bath. By centrifugation (25,000g for 30 min at 4 °C), the supernatant was obtained. Trichloroacetic acid at a final concentration of 10% (w/v) was added into the supernatant above. The mixture was then placed in a –20 °C refrigerator for 5 min, and a 4 °C refrigerator for 15 min. After centrifugation (15,000g for 15 min at 4 °C), the precipitate was dispersed into acetone at 4 °C for 15 min. The precipitate was obtained by centrifugation (15,000g for 15 min at 4 °C), and treated by acetone one more time as above. The precipitate was then placed in a fume hood to evaporate organic solvent and obtain the defatted protein sample. The protein sample (100 μ g) was treated in accordance with the procedures by Cao et al. (2015), with some modifications. The sample preparation solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 200 mM DTT, 0.2% (w/v) ampholyte 3–10, 0.001% bromophenol blue] was used.

2.9. MALDI-TOF/TOF MS

This was conducted according to the exact method of Zhao et al. (2013). Please check the details in this reference.

2.10. Size exclusion chromatography (SEC)-HPLC analysis

Suspensions (0.02% NaN_3) of crude OBs and urea-washed OBs (OB2) were incubated at pH 4.0 in a 60 °C water bath. After 0, 1, 3, 9, and 24 h, 3 ml of suspension was collected and cooled in an ice water bath. These suspensions (2 ml) were separated into floating, supernatant, and precipitate (little) by centrifugation (25,000g for 30 min at 4 °C). These supernatants were collected by syringe, and filtered through a 0.22 μ m membrane.

The HPLC equipment consisted of an Agilent 1100 series chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a column oven (G1311C), a quaternary pump (G1216A), and a UV detector (G1321B). A TSK gel 2000 SWXL (300 mm $\times$ 7.8 mm, Tosho, Tokyo, Japan) column was used for separation. Gradient elution was carried out at a flow rate of 0.5 ml/min using a solvent: acetonitrile/water/trifluoroacetic = 45/55/0.1. The column temperature was kept at 25 °C, and the detection was done at 214 nm. The injection volume was 20 μ l.

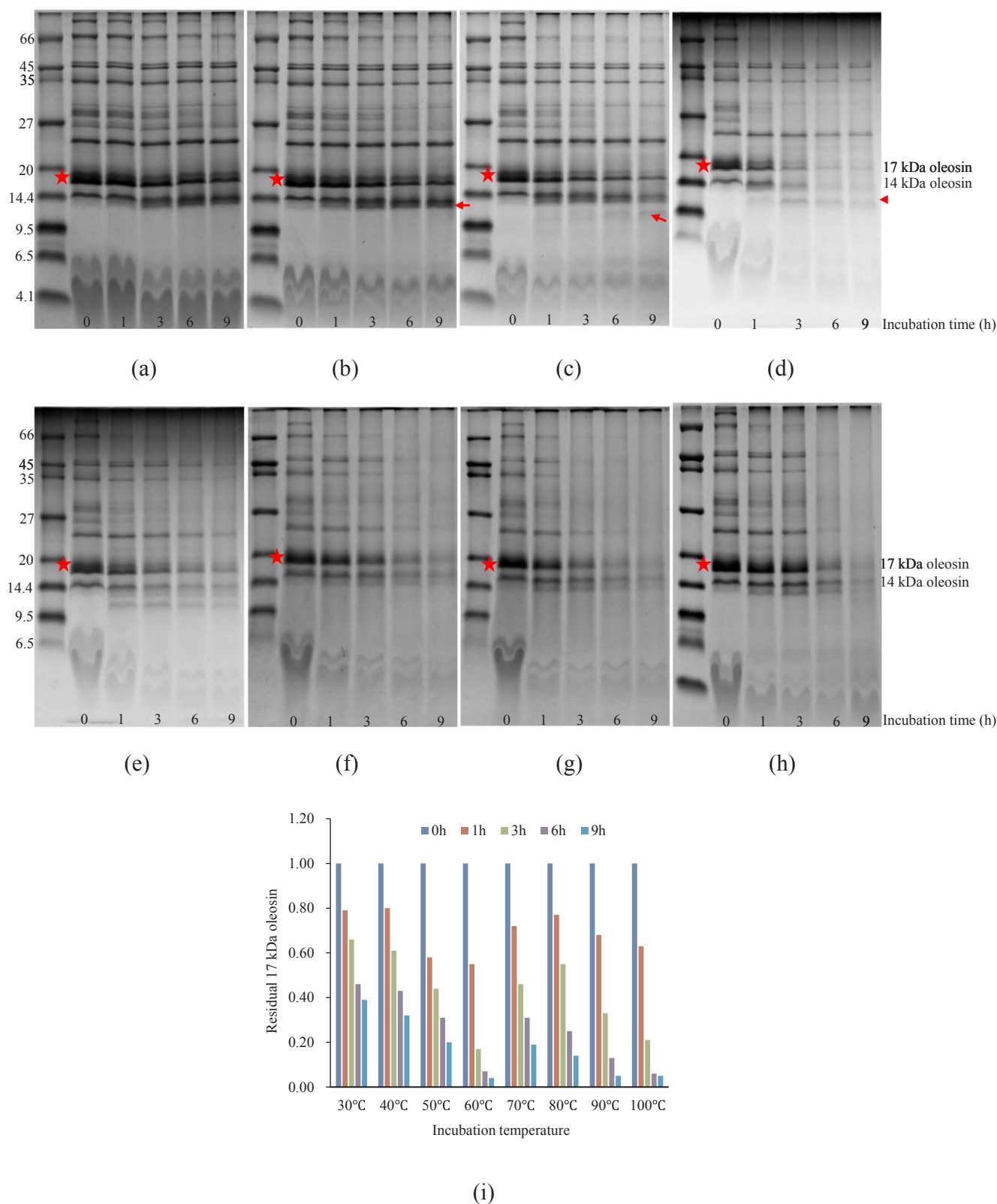


Fig. 3. Effects of temperature on the hydrolysis of proteins in crude OBs. The incubation was conducted at pH 4 and 30 (a), 40 (b), 50 (c), 60 (d), 70 (e), 80 (f), 90 (g), and 100 °C water baths (h) for 0, 1, 3, 6, and 9 h. (i) Effects of temperature on the band intensity of 17 kDa oleosin as function of temperature.

3. Results

3.1. Protein composition of peanut crude OBs and pH 7.0-OB

Fig. 1 shows that crude OBs contained many OB extrinsic proteins,

whereas pH 7.0-OB contained obviously less extrinsic proteins, indicating that extrinsic proteins had weak interaction with OBs.²¹ Bands 1–14 were examined by MALDI-TOF/TOF MS (Table S1, supplementary material). Band 1 was matched to lipoxygenase, band 2 to Ara h 1 allergen, bands 3–8 to arachin, band 9 to conglutin, band 10 to two

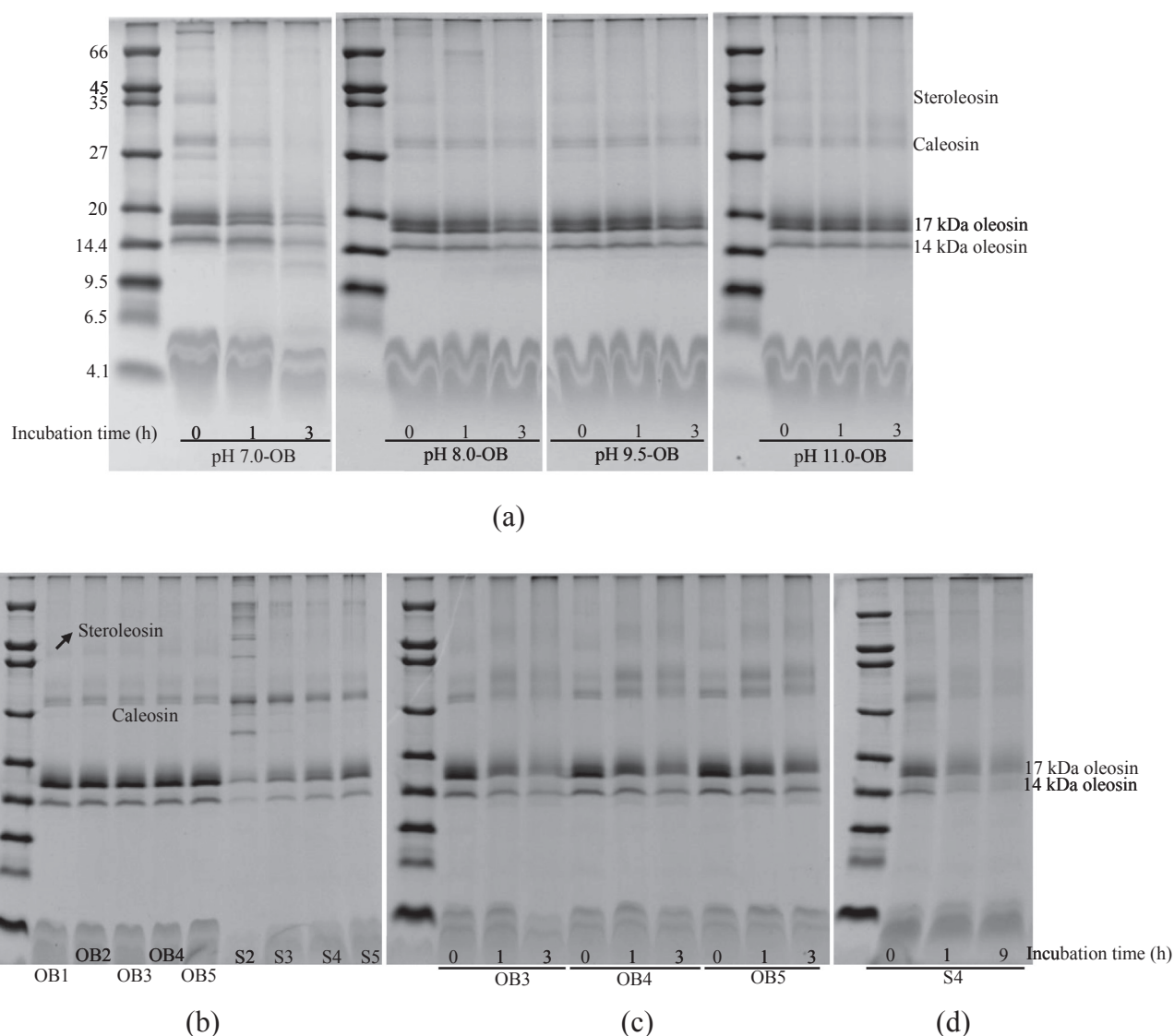


Fig. 4. (a) Tricine-SDS-PAGE protein profiles of pH 7.0-, 8.0-, 9.5-, and 11.0-OB as a function of incubation time. (b) Tricine-SDS-PAGE protein profiles of OB1–5 and S2–5. (c) Tricine-SDS-PAGE protein profiles of OB3–5 as a function of incubation time. (d) Tricine-SDS-PAGE protein profile of S4 as a function of incubation time. The incubation was conducted at pH 4.0 and 60 °C.

17 kDa oleosins, band 11 to two 14 kDa oleosins, band 12 to steroleosin (40 kDa), band 13 to caleosin (also called peroxxygenase) (Hanano et al., 2006; Meesapyodsuk & Qiu, 2011), and band 14 to ferritin. These results showed that most of extrinsic proteins in crude OBs could be removed by pH 7.0 washing except ferritin, and that peanut OBs contained two major oleosins (17 and 14 kDa) as well as caleosin and steroleosin.

3.2. Effects of pH on the endogenous protease-induced hydrolysis of proteins in crude OBs

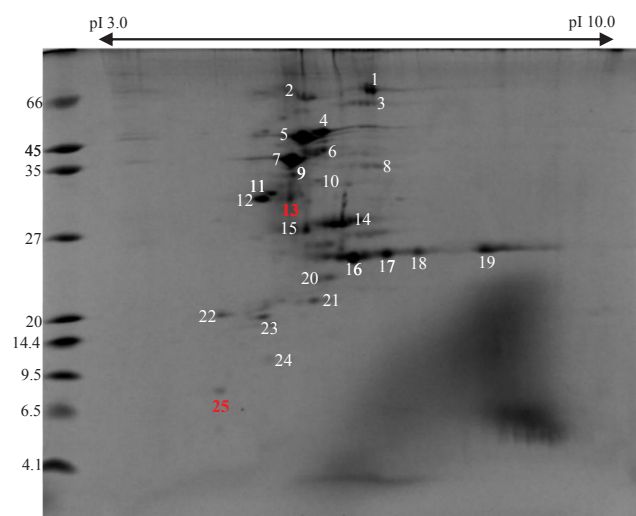
Crude OBs were incubated at 35 °C and pH 3–9 (Fig. 2), and it was found that oleosins, lipoxygenase, arachin (35 and 30 kDa) were the most quickly hydrolyzed at pH 4.0; interestingly, Ara h 1 allergen was the most quickly hydrolyzed at pH 3.0; arachin (46–49, 32, 26, and 22 kDa) and conglutin (21 kDa) were not or slightly hydrolyzed in the pH range of 3.0–9.0 (Fig. S1, supplementary material); at pH 4.0, it was found that just two new bands (66 and 13 kDa, indicated by red arrows) were observed, and this could not well explain the hydrolysis of proteins, which would be further examined in the following Section 3.6.

3.3. Effects of temperature on the endogenous protease-induced hydrolysis of proteins in crude OBs

Crude OBs were incubated at pH 4.0 and 30–100 °C. The band intensity of 17 kDa oleosin as function of incubation time was analyzed (Fig. 3), and it was found that 17 kDa oleosin was the most quickly hydrolyzed at 60 °C. Strangely, the band intensity of 17 kDa oleosin decreased more quickly at 90 and 100 °C than at 80 °C. It was considered that this phenomenon might be induced by the combined effects of high temperature and acidic pH (Saito, Miyakawa, Tamura, Shimamura, & Tomita, 1991).

3.4. Effects of alkaline pH and urea washing on the protein composition of peanut OBs

It is known that pH can obviously affect the protein composition of extracted OBs from various plant seeds (Chen & Ono, 2010; Chen, Zhao, Cao, Kong, & Hua, 2014; Zhao et al., 2016). For example, the extraction at pH 6.5 could obtain soybean OBs that contained many OB extrinsic proteins, whereas that at pH 11.0 could get OBs without extrinsic proteins (Chen & Ono, 2010). Therefore, peanut OBs were extracted at



(a)

1 MGSKANAVVL WLFVSSLLS VVLSAPNDGL RRIGLKKVKP DLNNRLAARL GSNDEALRAS
 61 IRKHHLQNNL GGDDDETIVG LKNYLDAQYY GEIGIGTPPQ KFSVIFTGS ANLWVPSSKC
 121 TFSIACYFHS KYSAKSTTY KNGTAVAIQ YGTGAISGFF SYDNVKGDI VVKNQEFIEA
 181 NREPSVTLFV AKFDGILGLG FQEIAGSAV PVWYNMLDQG LVKEPVFSFW LNRNPEEENG
 241 GELVFGGVDP NHYK GKHTYV PVSRRGYWEF DMGDILIGDQ KTGICADSCS AIADSGTSL
 301 AGPTTVITMI NHAIGAAGVV SQUECKAVVAQ YGQIIDLIA ADAPPKICT QIGLCTFDGT
 361 RGVSGGIESV VDEHQRSSSG GGIACSACEM AVVWMRSQNL QNQTRDRILN YVNQLCDKMP
 421 SPMGESSVDC GDLSKMPTIS FSIGGR TFDL SPDEYILKVG EGVAACQISG FTAIDIPPR
 481 GPLWILGDVF MGRYHTVFDF GQGRVGFAEA A

(b)

pH 7.0, 8.0, 9.5, and 11.0. It was found that Ara h 1 allergen could be removed by pH 9.5 washing, and ferritin was removed by pH 11.0 washing (Fig. 4a). Interestingly, one 32 kDa protein was still contained in pH 11.0-OB. By incubation, it was found that hydrolysis of caleosin and oleosins occurred in pH 7.0-, 8.0-, 9.5-, and 11.0-OB, and pH 7.0-OB had the highest proteolytic activity, followed by pH 8.0-, 9.5-, and 11.0-OB. These results revealed that the endogenous protease was gradually removed from OBs with increasing pH, but it was still contained in pH 11.0-OB, indicating that the protease had high affinity for peanut OBs.

The urea washing was used to confirm the high affinity between the endogenous protease and peanut OBs. Fig. 4b shows the protein profiles of OB1–5 and S2–5, and it was found that OB1–5 had six bands: steroleosin, 32 kDa protein, caleosin, ferritin, and oleosins. S2 had similar protein bands to crude OBs, and interestingly, caleosin and oleosins could be removed from OBs by urea washing. S5 had similar protein bands to OB5. By incubation, it was found that band intensities of oleosin, caleosin, and steroleosin gradually decreased, and one new 13 kDa band appeared in OB3–5 (Fig. 4c). Further, S4 was also incubated, and interestingly, the band intensities of caleosin and oleosins gradually decreased with prolonged incubation time (Fig. 4d). These results revealed that the endogenous protease was contained in OB1–5 and S1–4, and that the protease showed high affinity for peanut OBs.

3.5. Identification of the endogenous protease

Suspension of pH 7.0-OB was adjusted to pH 9.0, and mixed for 30 min in an ice water bath. Then this was separated into floating,

Fig. 5. (a) 2-D electrophoresis protein profile of a supernatant containing endogenous protease. The supernatant was obtained as follows: pH 7.0-OB was adjusted to pH 9.0, and mixed for 30 min in an ice water bath; then the supernatant was obtained by centrifugation (25000g for 30 min at 4 °C). (b) The amino acid sequence of the aspartic protease (*Arachis ipaensis*) identified by MALDI-TOF/TOF MS. The matched peptide is shaded gray for spot 13, and the matched peptides are shaded black for spot 25.

supernatant, and precipitate fractions by centrifugation. The supernatant was collected, and treated by 2-D electrophoresis. Fig. 5a shows that the supernatant contained many protein spots, which were examined by MALDI-TOF/TOF MS (Table S2, supplementary material). It was found that most of protein spots originated from arachin. Interestingly, spots 13 (approximately 32 kDa) and 25 (approximately 9 kDa) were matched to the same protein, an aspartic protease (511 amino acid residues, AARs) (Fig. 5b). Spots 13 and 25 had isoelectric points of 5.6 and 4.7, respectively. According to one review about plant aspartic proteases, the majority of plant aspartic proteases identified so far are synthesized with a prepro-domain and subsequently converted to mature two-chain enzymes (Simoes & Faro, 2004). For example, cardoon procarnosin-A (65 kDa) was converted to mature two-chain (31 and 15 kDa) aspartic protease with the removal of one plant-specific insert (15 kDa) and one prosegment (4 kDa) (Ramalho-Santos et al., 1998). Thus, it was concluded that the mature form of the aspartic protease (511 AARs) was a two-chain (32 and 9 kDa) protease. Assay of inhibition of proteolysis showed that the endogenous protease in crude OBs was an aspartic protease (Fig. S2, supplementary material), which was in good agreement with the results above.

3.6. Release of hydrolyzed products of crude and urea-washed OBs during incubation

The results above showed that the aspartic protease hydrolyzed both OB intrinsic and extrinsic proteins. However, just a few of new bands were observed on Tricine-SDS-PAGE gel. Therefore, two experiments were conducted as below. The suspensions of crude OBs and

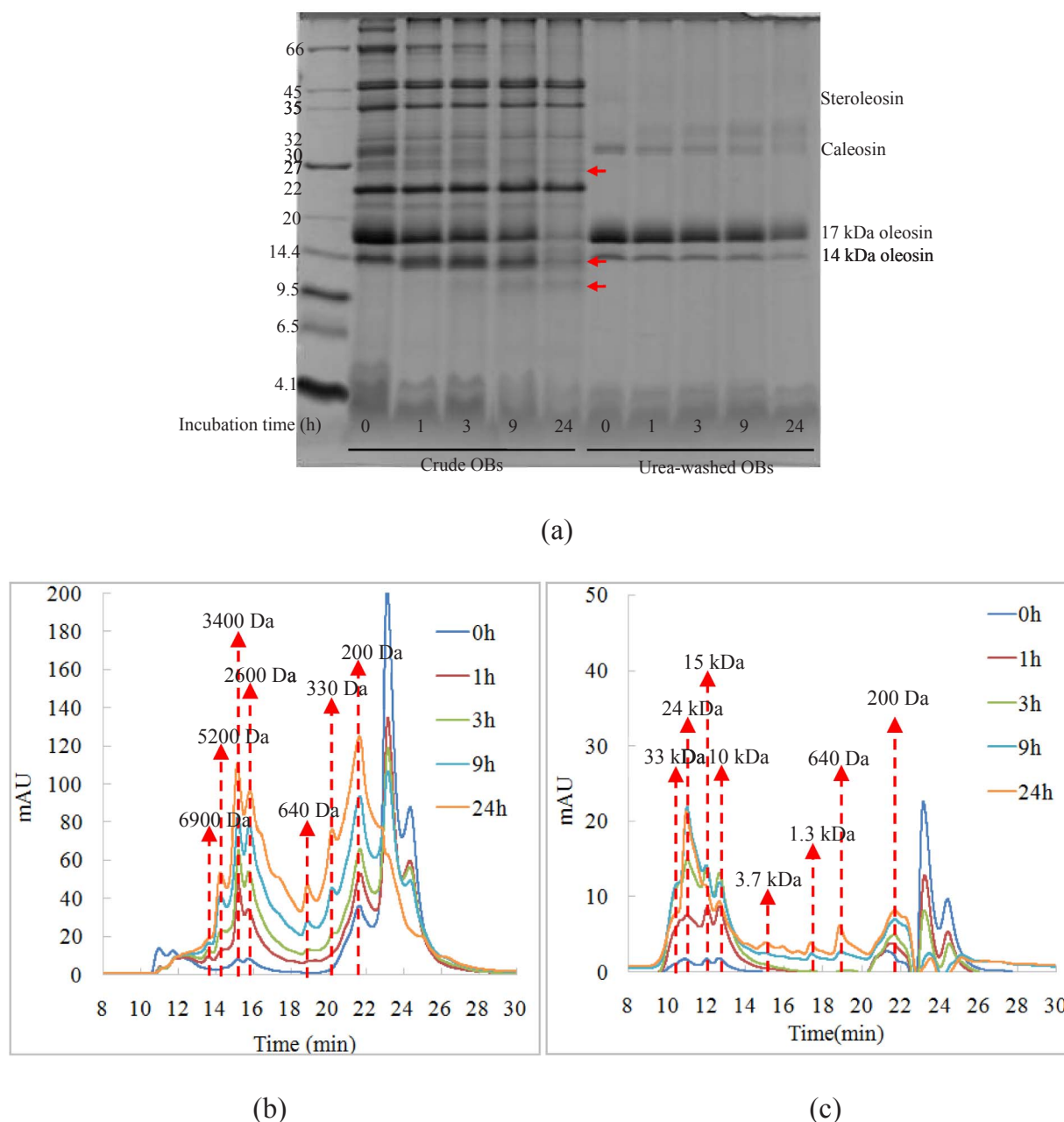


Fig. 6. (a) Tricine-SDS-PAGE protein profiles of crude and urea-washed OBs as a function of incubation time. (b) SEC-HPLC chromatographic profiles of supernatants from incubated crude OBs by centrifugation (25000g for 30 min at 4 °C). (c) SEC-HPLC chromatographic profiles of supernatants from incubated urea-washed OBs by centrifugation (25,000g for 30 min at 4 °C). The incubation was conducted at pH 4.0 and 60 °C for 0, 1, 3, 9, and 24 h.

urea-washed OBs (OB2) were incubated at pH 4.0 and 60 °C. For crude OBs, it was found that 1) in addition to oleosins, the band intensities of lipoxygenase, Ara h 1 allergen, and arachin (30 and 26 kDa) also obviously decreased with prolonged incubation time, 2) those of arachin (46–49, 35, and 22 kDa) and conglutin gradually decreased, 3) that of 32 kDa protein slightly decreased, 4) just a few of new bands (indicated by red arrows) were observed (Fig. 6a). In the case of urea-washed OBs, it was found that the band intensities of steroleosin, caleosin, and oleosins gradually decreased with prolonged incubation time (Fig. 6a).

To confirm whether proteins of crude OBs and urea-washed OBs were hydrolyzed during incubation, the incubated (0, 1, 3, 9, and 24 h) samples were treated by centrifugation, and the supernatants were collected and analyzed by SEC-HPLC. In the case of crude OBs, it was found that peaks mainly appeared in the molecular weight (MW) range of 200–6900 Da and increased with prolonged incubation time

(Fig. 6b), indicating that these polypeptides and oligopeptides were gradually produced on the surface of crude OBs and released into aqueous phase. Differently, four major peaks appeared in the MW range of 10–33 kDa, and several small peaks in the MW range of 200–3700 Da for the urea-washed OBs (Fig. 6c). These results confirmed that the aspartic protease indeed hydrolyzed both OB extrinsic and intrinsic proteins; further, the aspartic protease hydrolyzed oleosins, Ara h 1 allergen, and arachin (30 and 26 kDa) quickly, but arachin (46–49, 35, and 22 kDa) and conglutin (21 kDa) slowly.

4. Discussion

The hydrolysis of oleosins was reported to happen during the plant seed germination and seedling growth, such as sesame (Hsiao & Tzen, 2011), sunflower (Babazadeh, Poursaadat, Sadeghipour, & Colagar,

2012), soybean (Chen et al., 2014), and *Arabidopsis thaliana* (Deruyffelaere et al., 2015), which should be the initial step for the lipase-induced TAGs mobilization. It was reported that oleosins of sesame and *Arabidopsis thaliana* were degraded by the ubiquitin/proteasome system during germination (Deruyffelaere et al., 2015; Hsiao & Tzen, 2011). Differently, sunflower oleosins were reported to be hydrolyzed by a cysteine protease, which was regulated by thioredoxin *h* (Babazadeh et al., 2012). Recently, it was found that the oleosins of crude OBs from aqueous extracts of ungerminated soybean, jicama, sunflower, peanut, rapeseed, castor bean, and sesame were hydrolyzed by incubation (Zhao et al., 2016), indicating that the proteases were already bioactive in the ungerminated plant seeds above. In them, P34 probable thiol protease showed high affinity with ungerminated soybean OBs and might be responsible for the hydrolysis of soybean oleosins (Chen et al., 2014; Kalinski, Melroy, Dwivedi, & Herman, 1992; Kalinski, Weisemann, Matthews, & Herman, 1990). Jicama SPE31 showed high similarity to P34 probable thiol protease (Zhang, Wei, Chang, Teng, & Gong, 2006), so it was considered that SPE31 might be correlated with the hydrolysis of oleosins of ungerminated jicama OBs. Until now, there has been no information about the proteases responsible for the hydrolysis of oleosins of ungerminated sunflower, castor bean, peanut, rapeseed, and sesame OBs.

A recent reference gave some information about an unidentified aspartic protease in peanut. The Ara h 1 allergen and arachin of peanut were subjected to proteolysis two days after seed imbibition, with Ara h 1 allergen and arachin acidic chains being among the storage proteins to be mobilized. And the proteolytic activity was the greatest at pH 2.6–3 (Wilson and Tan-Wilson, 2015). Figs. 2 and S1 show that Ara h 1 allergen and arachin acidic chains (46–49 kDa) in crude OBs were the most quickly hydrolyzed at pH 3, which made us realize that the aspartic protease might be the same protease examined in this study. However, further research is needed to confirm this. Interestingly, the optimal pH for oleosin hydrolysis was 4.0, which was different from that for Ara h 1 allergen and arachin acidic chains (46–49 kDa). This might be due to their different biological functions and structures (Ara h 1 allergen and arachin, storage proteins; oleosins, membrane proteins).

5. Conclusion

A two-chain (32 and 9 kDa) aspartic protease was identified in this study, which showed high affinity for peanut OBs. The optimal pH and temperature for oleosin hydrolysis were pH 4.0 and 60 °C. Interestingly, the aspartic protease hydrolyzed not only OB intrinsic proteins (oleosin, caleosin, and steroleosin), but also OB extrinsic proteins; Ara h 1 allergen and arachin (26–30 kDa) were the most quickly hydrolyzed, followed by arachin (35 kDa), and arachin (22 kDa) was the most slowly hydrolyzed. In all, this study is meaningful not only for extraction and utilization of peanut OBs, but also for aqueous processing of peanut free oil.

Conflict of interest statement

The authors declare no conflicts of interest.

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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.2017.09.020>.

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Table S1. Identification of protein bands in Fig. 1 by MALDI-TOF/TOF MS.

Band	Protein ID	Protein score	MW (Da)	Peptides mached	NBCInr
1	Lipoxygenase 1 [Arachis hypogaea]	334	97954	6	gi 7331095
2	Ara h 1 allergen [Arachis hypogaea]	139	71143	3	gi 312233063
3	Conarachin [Arachis hypogaea]	301	66934	5	gi 46560478
	Chain A, crystal structure of Ara H 1 [Arachis hypogaea]	237	47558	5	gi 347447588
	Glycinin [Arachis hypogaea]	97	61430	2	gi 5712199
	Gly1 [Arachis hypogaea]	97	60754	2	gi 9864777
	Allergen Arah3/Arah4 [Arachis hypogaea]	97	62042	2	gi 21314465
	Storage protein [Arachis hypogaea]	97	61803	2	gi 37789212
	Arachin Ahy-1 [Arachis hypogaea]	97	61810	2	gi 52001219
	Arachin Ahy-2 [Arachis hypogaea]	97	61836	2	gi 52001221
	Arachin Ahy-4 [Arachis hypogaea]	97	61041	2	gi 57669861
	Arachin Arah 3 isoform [Arachis hypogaea]	97	60929	2	gi 199732457
4	Iso-Ara h3 [Arachis hypogaea]	209	58612	1	gi 112380623
	Ara h 3 allergen [Arachis hypogaea]	209	58612	1	gi 312233065
5	Arachin 6 [Arachis hypogaea]	681	60681	6	gi 118776570
	Arachin Ahy-4 [Arachis hypogaea]	171	61041	3	gi 57669861
6	Allergen Arah3/Arah4 [Arachis hypogaea]	200	62042	3	gi 21314465
	Storage protein [Arachis hypogaea]	200	61803	3	gi 37789212
	Arachin Ahy-1 [Arachis hypogaea]	200	61810	3	gi 52001219
	Arachin Ahy-2 [Arachis hypogaea]	200	61836	3	gi 52001221
7	Gly1 [Arachis hypogaea]	185	60754	3	gi 9864777
	Arachin Ahy-4 [Arachis hypogaea]	142	61041	3	gi 57669861
8	Gly1 [Arachis hypogaea]	200	60754	4	gi 9864777
	Glycinin [Arachis hypogaea]	139	61430	3	gi 5712199
9	Conglutin-7 [Arachis hypogaea]	153	20558	5	CONG7_ARAHY
10	Oleolin 3 [Arachis hypogaea]	216	16922	3	gi 52001241
	Oleolin 1 [Arachis hypogaea]	128	17798	2	gi 113200509
11	Oleolin 1 [Arachis hypogaea]	102	14299	2	gi 71040655
	Oleolin 2 [Arachis hypogaea]	102	14345	2	gi 71040657
12	Steroleolin-B [Arachis hypogaea]	239	39857	4	gi 152032028
13	PREDICTED: peroxygenase [Arachis duranensis]	91	27690	4	XP_015940914.1
14	PREDICTED: ferritin-4, chloroplastic-like [Arachis duranensis]	145	29232	1	XP_015954000.1

Protein scores that represent those searches performed with the NCBi database are given. MWs are given as theoretical values. Peptides matched and accession numbers within NCBI are given.

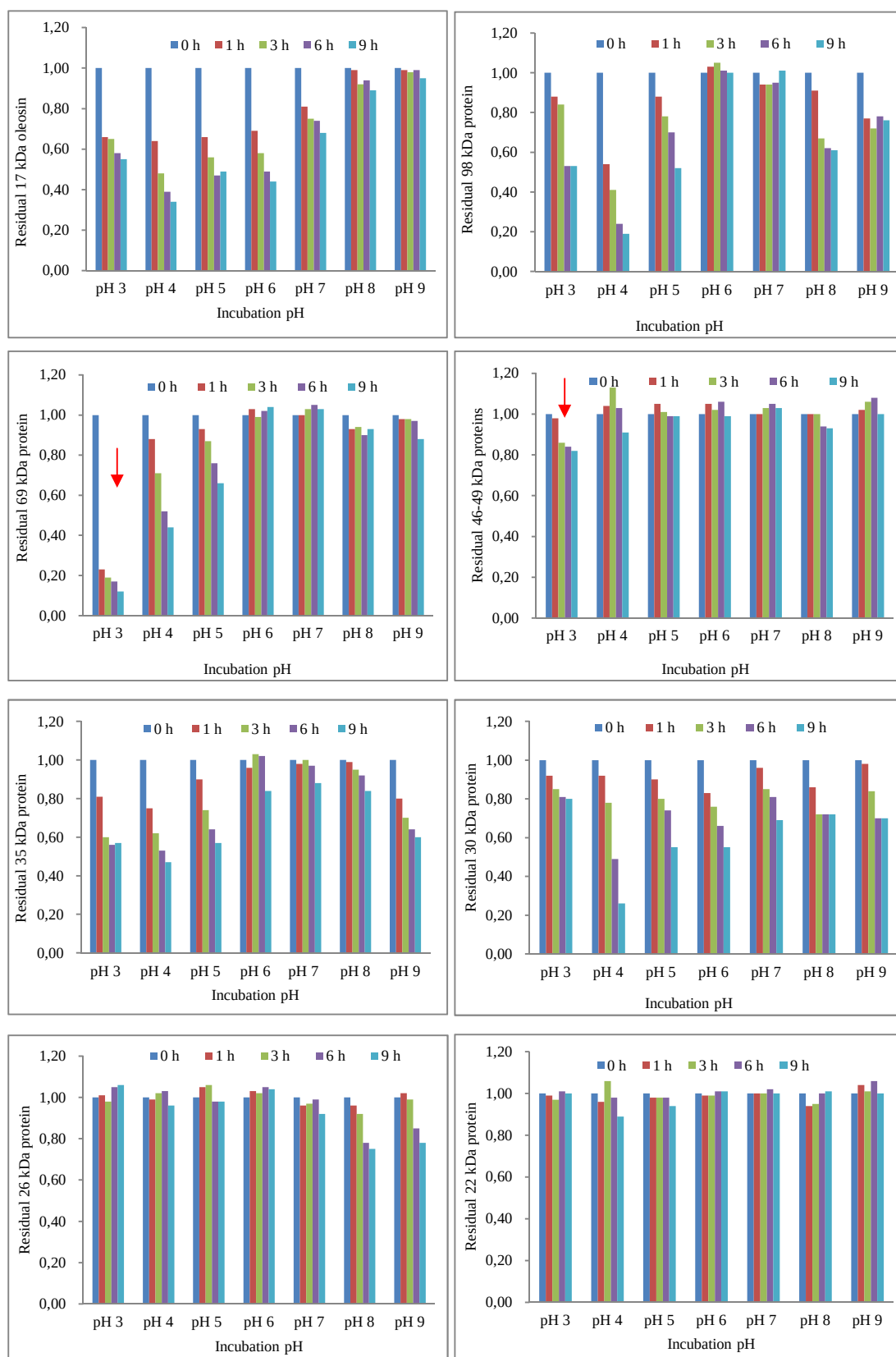


Fig. S1. Endogenous protease-induced hydrolysis of 98, 69, 46-49, 35, 30, 26, and 22 kDa proteins and 17 kDa oleosin as function of incubation time. Suspension of crude OBs was incubated at 35 °C and pH 3–9.

Table S2. Identification of protein spots in Fig. 5a by MALDI-TOF/TOF MS.

Spot	Protein ID	MW	Score	Peptides matched	NCBI Inr
1	RecName: Full=Allergen Ara h 1, clone P41B [Arachis hypogaea]	71701	825	11(7)	gi 1168391
	Ara h 1 allergen [Arachis hypogaea]	71143	735	10(6)	gi 312233063
	RecName: Full=Allergen Ara h 1, clone P17 [Arachis hypogaea]	70639	709	10(6)	gi 1168390
2	PREDICTED: vicilin-like seed storage protein At2g18540 [Arachis ipaensis]	77398	118	1(1)	XP_016201833.1
3	glycinin, partial [Arachis hypogaea]	58368	151	2(1)	gi 47933675
4	Allergen Arah3/Arah4 [Arachis hypogaea]	62042	508	7(5)	gi 21314465
5	Gly1 [Arachis hypogaea]	60754	876	8(7)	gi 9864777
	Arachin Arah3 isoform [Arachis hypogaea]	60929	793	7(6)	gi 199732457
	Glycinin [Arachis hypogaea]	61430	679	7(6)	gi 5712199
	Arachin Ahy-4 [Arachis hypogaea]	61041	679	7(6)	gi 57669861
6	Glycinin [Arachis hypogaea]	61430	113	3(0)	gi 5712199
7	Glycinin, partial [Arachis hypogaea]	58368	117	2(1)	gi 47933675
8	PREDICTED: vicilin-like seed storage protein At2g28490 [Arachis duranensis]	67521	165	2(2)	XP_015952363.1
9	Conarachin [Arachis hypogaea]	76343	113	1(1)	AAU21493.1
10	Glycinin, partial [Arachis hypogaea]	58368	98	2(1)	gi 47933675
11	Arachin Ahy-4 [Arachis hypogaea]	61041	249	3(2)	AAW56067.1
12	PREDICTED: arachin Ahy-3-like [Arachis ipaensis]	60681	808	7(6)	XP_016205798.1
13	PREDICTED: aspartic proteinase [Arachis ipaensis]	55647	94	1(1)	XP_016195356.1
14	glycinin, partial [Arachis hypogaea]	58368	144	1(1)	gi 47933675
15	PREDICTED: vicilin-like seed storage protein At2g28490 [Arachis duranensis]	67521	252	5(3)	XP_015952363.1
	PREDICTED: ferritin-4, chloroplastic-like [Arachis duranensis]	29232	129	2(1)	XP_015954000.1
16	Glycinin, partial [Arachis hypogaea]	58368	279	2(2)	gi 47933675
17	Storage protein [Arachis hypogaea]	61803	361	5(4)	gi 37789212
	Arachin Ahy-1 [Arachis hypogaea]	58543	218	3(2)	gi 3703107
18	Glycinin [Arachis hypogaea]	58543	184	3(2)	gi 3703107
19	Glycinin [Arachis hypogaea]	58543	257	4(2)	gi 3703107
	Arachin Arah3 isoform [Arachis hypogaea]	60929	250	4(2)	gi 199732457
	Arachin 6 [Arachis hypogaea]	60681	246	4(2)	gi 118776570
20	Allergen II [Arachis hypogaea]	18612	98	1(1)	gi 15418705
21	Conglutin [Arachis hypogaea]	17349	144	2(1)	gi 17225991
22	Lipase/lipoxygenase [Arabidopsis thaliana]	20408	63	1(1)	gi 15236014
23	Arachin 6 [Arachis hypogaea]	60681	711	7(5)	gi 118776570
24	Conglutin [Arachis hypogaea]	17479	60	2(1)	CONG_ARAHY
25	PREDICTED: aspartic proteinase [Arachis ipaensis]	55647	158	3(2)	XP_016195356.1

Protein scores that represent those searches performed with the NCBI database are given. MWs are given as theoretical values. Peptides matched and accession numbers within NCBI are given.

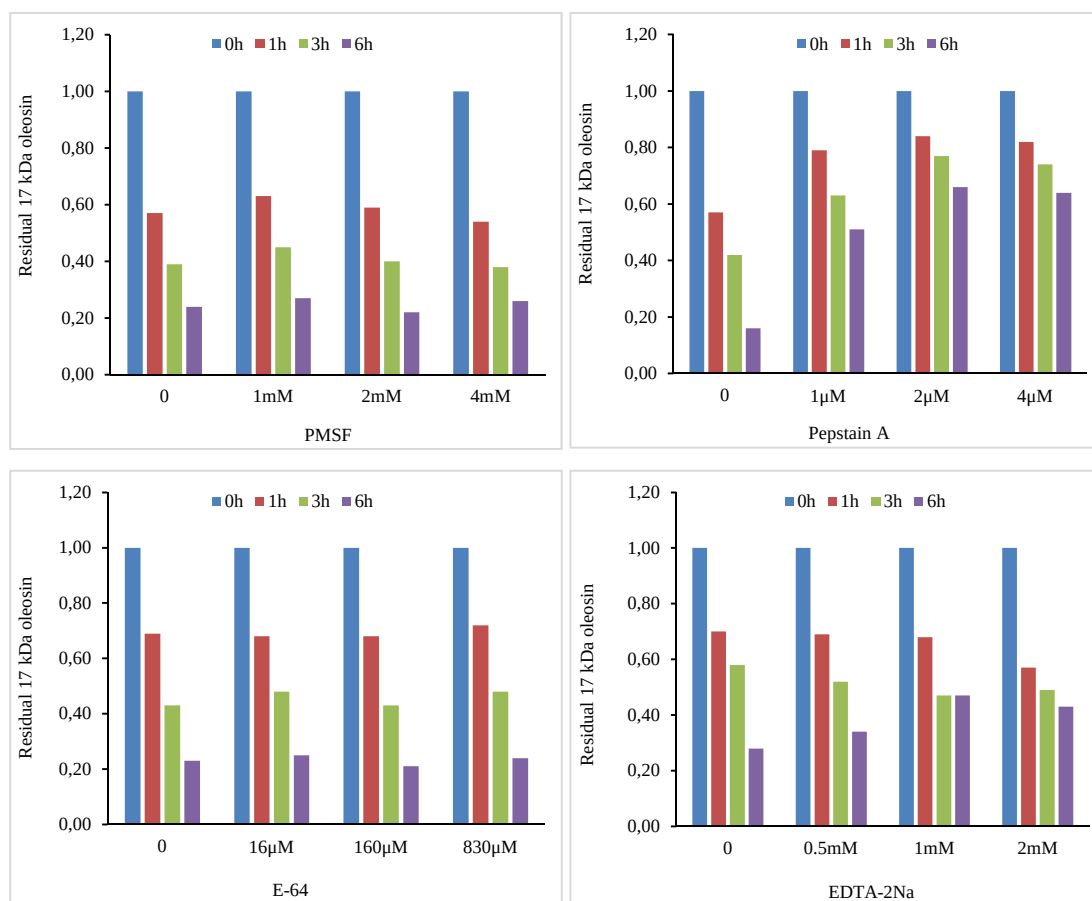


Fig. S2. Effects of protease inhibitors (PMSF, pepstatin A, E-64, and EDTA-2Na) on the proteolytic activity of endogenous protease in crude OBs.

The proteolytic activity of crude OBs at pH 4.0 and 60 °C was examined for sensitivity to PMSF, pepstatin A, E-64, and EDTA-2Na, reagents that respectively inhibit serine, aspartic, cysteine, and metallo- proteases. It was found that the proteolysis was obviously inhibited by pepstatin A, and weakly by EDTA-2Na, indicating that the proteolysis was due to one aspartic protease.