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

Yeming Chen[a,b,*], Yajing Chen[a,b], Luping Zhao[a,b], Xiangzhen Kong[a,b], Zhaoqi Yang[c], Yufei Hua[a,b]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, China
[b] School of Food Science and Technology, Jiangnan University, China
[c] School of Pharmaceutical Sciences, Jiangnan University, China

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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

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, China.
E-mail address: chenyeming@jiangnan.edu.cn (Y. Chen).

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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 (5000*g* 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 (5000*g* 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 (5000*g* 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 concentrated by ultrafiltration (3 kDa cutoff) to 0.5 ml.

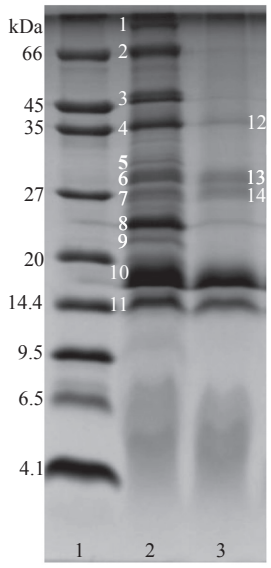

Fig. 1. Tricine-SDS-PAGE protein profiles of peanut crude OBs and pH 7.0-OB. Lane 1, marker; lane 2, crude OBs; lane 3, pH 7.0-OB.

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