Composition and Rheological Properties of Peanut Oil Bodies from Aqueous Enzymatic Extraction

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Abstract: In this study, the relationship between the composition and rheological properties of peanut oil bodies from aqueous enzymatic extraction was evaluated. Aqueous enzymatic extraction using a combination of cellulase and pectinase at a 1:1 ratio effectively destroyed the structure of the cell wall and resulted in the maximum oil body yield of 90.7%. The microstructure and interfacial membrane composition of the peanut oil bodies were observed by confocal laser scanning microscopy. The oil bodies contained three inherent proteins (oleosin, caleosin, and steroleosin) along with two adsorbed foreign proteins (arachin and lipoygenase). Five phospholipids were detected using $^{31}$P nuclear magnetic resonance spectroscopy. Among them, phosphatidylcholine, which plays a major role in the stability of oil bodies, was the most abundant. The measured rheological properties indicated that the oil bodies were a typical elastic system. Elevated temperature and high-speed shear destroyed the binding between proteins and phospholipids, reducing the oil body stability. The findings will facilitate the commercial application of peanut oil bodies by improving the extraction rate of peanut oil bodies and clarifying their stabilization mechanism.

Practical Application: This paper studies the enzymatic extraction, composition and rheological properties of peanut oil bodies. It provides a theoretical basis for the large-scale application of peanut oil bodies in the food and cosmetic industries. It is beneficial to improve the application value of peanut resources.

Key words: peanut oil bodies, rheological properties, protein, phospholipid

1 Introduction

The oil bodies (OBs) inside plant seeds are dispersed subcellular structures and one of the smallest organelles in plant seed cells. Plant lipids are usually stored in the subcellular organelles in the form of triacylglycerols. These subcellular organelles are structurally stable due to the phospholipid monolayer membrane and OB proteins. As a kind of stable natural emulsion, OBs can be used in the food industry without any emulsifier or homogenization process. Peanuts are one of the six major oil crops in the world and constitute an important source of oil and protein in the food industry. Peanut OBs are rich in nutrients such as essential amino acids, unsaturated fatty acids, vitamin E, and phytosterols, making peanut OBs an ideal alternative to emulsified oils.

The methods for extracting peanut OBs are mainly divided into aqueous extraction and aqueous enzymatic extraction. In aqueous extraction, a series of mechanical operations (e.g., filtration and centrifugation) to separate peanut OBs. While aqueous extraction methods are simple, convenient, and do not require a large amount of organic solvent, the purity and extraction rate of the obtained OBs are not high. In aqueous enzymatic extraction, enzyme preparations are applied to mechanically crushed peanuts to increase the degree of damage to the cell wall. Although the reaction time required for aqueous enzymatic extraction is increased compared to aqueous extraction, the peanut OB extraction efficiency is higher. The main enzymes used in aqueous enzymatic extraction are cellulase, pectinase, and other enzymes that can hydrolyze cell walls. Cell wall-degrading enzymes destroy the cell structure of plants and promote the release of oil and protein into the cells.

Al Loman et al. used cellulase to extract soybean OBs and obtained an extraction rate of 87%. Recently, some scholars have studied the compositions and structures of OBs. Zaaboul et al. extracted peanut OBs using aqueous extraction and evaluated their composition. They found that the extraction conditions affected the fatty acid composition of the peanut OBs. Zhou et al. extracted OBs using only Viscozyme L and studied the OB
stability under different salt concentrations and pH conditions.

At present, an efficient extraction method for peanut OBs is lacking. Since OBs are naturally stable emulsions, the rheological properties of OBs are an important indicator of their application value in the food and cosmetic industries. No studies have evaluated the effect of peanut OB composition on the rheological properties of the OBs. In this study, we have compounded cell wall degrading enzymes and improved the extraction process of peanut OBs. Scanning electron microscopy (SEM) was used to verify the hydrolysis effects of different enzymes on the cell walls. Gel electrophoresis and nuclear magnetic resonance (NMR) spectroscopy were used to evaluate the protein and phospholipid compositions of the peanut OBs. For the first time, the rheological properties of peanut OBs were analyzed using a rheometer. Based on these characterizations, the relationship between the structural composition and rheological properties of peanut OBs was assessed. The findings will help to improve the commercial application value of peanut OBs.

2 Materials and Methods

2.1 Materials

Peanuts (YuHua YH-23) were purchased from the Henan Academy of Agricultural Sciences (Zhengzhou, China). Cellulase is from Trichoderma reesei produced by submerged fermentation (optimal pH, 5.5; optimal temperature, 40°C); pectinase is from Aspergillus aculeatus (optimal pH, 5.5; optimal temperature, 45°C); hemicellulase is from Aspergillus niger (optimal pH, 7.0; optimal temperature, 45°C); Viscozyme L (cellulolytic enzyme mixture, optimal pH, 7.0; optimal temperature, 52°C) were procured from Sigma Chemical Company (St. Louis, USA). Other reagents and chemicals of analytical reagent grade or higher were both purchased from Sigma Chemical Company (St. Louis, USA).

2.2 Preparation of OBs

The method to prepare OBs referred to Liu et al. The peeled peanut was crushed with a high-speed universal grinder for 10 s, and the crushed peanuts (20 g) were dispersed in distilled water at 1:4 (w/v). Cell wall-degrading enzyme was added to the mixture followed by incubation for 80 min at its optimum temperature. After enzymolysis, the mixture was boiled for 5 min to inactivate the enzyme. After cooling to room temperature, the solution was centrifuged at 5000 × g for 20 min to obtain the OBs as the upper component. The OB extraction rate was determined as the ratio of the percentage of OB fat to the percentage of total fat in the peanuts.

2.3 SEM analysis

The hydrolysis effects of cell wall-degrading enzymes on the peanut cell walls were evaluated by SEM. After enzymolysis, the peanut emulsion was centrifuged at 4000 × g for 20 min, and the bottom peanut residue was freeze-dried. The freeze-dried peanut powder was smeared on conductive tape, and excess powder was blown off with an ear wash ball. After coating the peanut powder with gold, the morphology of the peanut cell walls was observed by SEM (Quanta Feg 250; Hillsboro, USA).

2.4 Confocal laser scanning microscopy (CLSM)

Sample preparation and dying for CLSM were carried out according to Greenspan et al. The peanut OBs were stained with Nile red (0.1%, w/v) and Nile blue (1%, w/v) dissolved in propanol for 30 min. After staining, 10 μL of emulsion was placed on a glass slide, and the microstructure of the oil droplets was observed by CLSM (Leica DM6000B; Heidelberg, Germany) with an excitation wavelength of 488 nm.

2.5 Gel electrophoresis and two-dimensional electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the composition of interfacial proteins in the OBs. The OBs were defatted and freeze-dried to obtain protein powder. After accurately weighing the powder, it was dissolved in deionized water at a concentration of 4 mg/mL and centrifuged at 8000 × g for 10 min. Subsequently, the supernatant was combined with sample buffer solution in a 1:1 ratio. After boiling for 5 min in a boiling water bath, 10 μL of sample was loaded into each lane of the SDS-PAGE gel. After electrophoresis, the gel was placed in the fixing solution for 1 h and then removed and stained in staining solution for 1.5 h. Finally, the gel was decolored using a decolorizing solution until the protein band was clear. Compared to SDS-PAGE, two-dimensional electrophoresis can provide a more comprehensive analysis of the OB protein composition. Protein (2 mg) was dissolved in hydration loading buffer, swelled with a 7-cm pH 3.0–10.0 immobilized pH gradient (IPG) to 500 μL, and hydrated at room temperature for 16 h. An IPG-phor instrument (Hercules, CA, USA) was used for isopoint focusing and separation, and finally after reaching 40 kVh. Subsequently, the IPG strips were shaken for 15 min in a solution containing 0.13 mol/L dithiothreitol (DTT) and 0.14 mol/L indole-3-acetic acid (IAA). Finally, the gel strip was transferred to SDS-PAGE gel for separation. After electrophoresis, the gel was fixed, stained, and decolorized, and pictures with taken using a gel imager.

2.6 MALDI–TOF/TOF MS analysis

The target bands (dots) on the electrophoresis gel were
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3 Results and Discussion

3.1 Effects of different enzymes on OB yield

The peanut OB yields obtained using different cell wall-degrading enzymes are shown in Fig. 1. Enzymatic extraction produced significantly higher OB yields than aqueous extraction. Among the single enzyme preparations used for extraction, cellulase had the best OB yield (86.7%) followed by Viscozyme L (85.3%) and pectinase (84.8%). This may be because cellulose is the polysaccharide with the highest content in peanut cell walls, whereas the contents of pectin and hemicellulose are low. Combining different cell wall-degrading enzymes may further facilitate the extraction of peanut OBs. In this study, only the combination of cellulase and pectinase resulted in a higher OB yield than cellulase alone. When cellulase and pectinase were compared at a 1:1 ratio, the OB yield reached 90.7% (45°C, add at the same time). In contrast, hemicellulase, either alone or combined with other enzymes, resulted in a lower OB yield. In summary, the combined use of cellulase and pectinase resulted in the highest yield of peanut OBs, and the optimal ratio of cellulase to pectinase was 1:1.

3.2 SEM analysis

In the aqueous enzymatic extraction of peanut OBs, mechanical pulverization is the process that destroys the cell wall into small pieces, eluted with decolorizing solution, and lyophilized. After adding 5 μL trypsin solution to the gel block, it was placed at 4°C for 1 h to allow the block to fully swell. Subsequently, the block was reacted with 200 μL 25 mmol/L NH4HCO3 solution at 37°C for 20 h. After enzymatic hydrolysis, the peptide solution was aspirated and transferred to a new tube. The lyophilized sample was reconstituted with 3 μL 0.1% trifluoroacetic acid solution. Next, 0.7–1 μL of the sample was placed on the sample target and allowed to dry naturally. An equal volume of matrix (α-cyano-4-hydroxycinnamic acid) was then loaded onto the sample target. Finally, 5 μL of 0.1% trifluoroacetate was used for desalting. Detection was carried out using a MALDI-TOF/TOF analyzer (AB Sciex, Framingham, USA). The data were analyzed with Flex Analysis and Biotools software. The NCBI database was used to match peptides and determine protein species.

2.7 31P-NMR analysis

The phospholipids in OBs were extracted according to the method of Yao & Jung. To 20 g of OBs, 200 mL of methanol and 400 mL of chloroform were added. After stirring, the crude oil was obtained by rotary evaporation. Crude oil (10 g) was mixed with 45 mL of n-hexane and 15 mL of ethanol. The ethanol extract was then mixed with 130 mL of chloroform and 111 mL of ethylenediaminetetraacetic acid (0.1 mol/L). The chloroform phase was collected and dried over sodium sulfate, and the solvent was removed. Phospholipids were stored at -26°C until use. OB phospholipids were determined using 31P NMR spectroscopy. Triphenyl phosphate (10 mg) was added to the phospholipid (80–90 mg) followed by dissolution in chloroform, methanol, and ethylenediaminetetraacetic acid to form a mixed solution. After centrifugation at 4000 × g for 10 min, the lower layer was transferred to a 5-mm NMR tube for measurement.

2.8 Rheological properties

The OBs were analyzed within 6 h of emulsion preparation to ensure uniformity. Under a shear speed of 5 s⁻¹ and a heating rate of 5°C/min, the viscosity of the emulsion was measured as a function of temperature using a Hugg rheometer (RS-6000; Waltham, USA). The shear ramp was constructed by measuring the shear rate from 0.01 to 1 s⁻¹. In the linear viscoelastic region, the storage modulus G’ and the loss modulus G″ of the emulsion were measured as functions of the oscillation frequency.

2.9 Statistical analysis

Data are presented as means ± standard deviation (SD). The level of significance was set at p < 0.05 and was determined using Tukey’s test with SPSS software (version 20, SPSS Inc., Chicago, IL, USA). The same letters indicate no significant difference, while different letters indicate significant differences.
For the peanut cell wall to be degraded to the greatest extent possible, the cell wall-degrading enzymes must effectively degrade cellulose, hemicellulose, pectin, and other substances in the cell wall structure. The degree of damage to the peanut cell wall resulting from the cell wall-degrading enzymes was studied by SEM. As shown in Fig. 3A, the peanut cell wall after aqueous extraction appeared smooth and complete, which is not conducive to the release of oil and protein from the peanut cells. This finding agrees with the low OB yield obtained using aqueous extraction. After aqueous enzymatic extraction using different cell wall-degrading enzymes, the peanut cell walls showed different degrees of damage. After hemicellulase treatment, the cell wall did not change greatly compared to after aqueous extraction; the only change observed was the formation of some holes. After cellulase treatment, cellulase + pectinase (1:1), or Viscozyme L, the cell walls showed obvious disintegration and opening because the enzymes hydrolyzed their corresponding substrates, opening the network structure of the cell wall. Cellulase degrades cellulose, which loosens the cellulose microfibril–hemicellulose–pectin structure of the cell wall. Pectinase can hydrolyze the side chain of pectin that binds to other macromolecular polysaccharides, thereby preventing bonding with the polysaccharides between cell walls. Among the treatments in this study, the combination of cellulase and pectinase (1:1) resulted in the highest degree of hydrolysis of the cell wall, the roughest cell wall surface, and the largest hole. Thus, this enzyme combination was used for the subsequent enzymatic extraction of peanut OBs.

### 3.3 OB microstructure

The basic components of the enzymatically extracted peanut OBs were water (mass fraction of 18.2%), fat (79.2%), protein (22.2%), and phospholipid (0.45%). Thus, most of the proteins were separated during the extraction of peanut OBs, resulting in a relatively high purity of peanut OBs. The protein fraction showed red fluorescence after dying with Nile blue, and the OBs showed green fluorescence after dying with Nile red. Figure 4 shows that the proteins...
were located on the outside of the peanut OBs, while the inside of the OBs were filled with oil. These results are in agreement with the structural model of corn OBs proposed by Tzen & Huang. The OBs were composed of a large amount of triglycerides and a small amount of phosphorous esters and proteins. The triglycerides were located in the centers of the OBs, whereas the phospholipids and proteins were located outside the OBs. The proteins and phospholipids form an interfacial membrane that wraps around the internal triglycerides, preventing them from being released and forming a stable emulsion.

### 3.4 OB protein composition

In peanut OBs, protein is the most important emulsifier. Determining the composition of peanut OB proteins is thus of great significance to understanding the mechanism of OB stabilization. As shown in Fig. 5A, the compositions and distributions of peanut OB proteins and peanut raw proteins were significantly different. The peanut globulin in peanut raw protein mainly consists of four hydrophilic acidic subunits (40.5, 37.5, 35.5, and 23.5 kDa) and three hydrophobic basic subunits (18.0, 17.0, and 15.5 kDa). Based on the color shade of the band, the proportions of conglycinin subunits and hydrophilic acidic subunits in OB proteins were significantly less than those in raw proteins.

In addition, the OB proteins had a higher content of hydrophobic basic subunits than the raw proteins. Figure 5B is a two-dimensional electrophoresis image of peanut OB protein. Compared with one-dimensional electrophoresis, two-position electrophoresis has one more pH axis, which can better distinguish the basic and acidic subunits of proteins. Oleosin is the most important interface protein in peanut OB, which is divided into three basic subunits in Fig. 5B. More hydrophobic groups would improve the hydrophobic properties of the interfacial proteins, thereby enhancing the emulsifying activity and stability of the emulsion.

The bands and spots of the protein OBs were excised and identified by mass spectrometry. As shown in Table 1, bands 1 and 6 were matched to arachin 6 and lipoxygenase, respectively, indicating that the foreign protein was mainly composed of these two proteins. Bands 7 and 8 were respectively matched to steroleosin-A and steroleosin-B, the two subtypes of steroleosin (A7LB60 and A7LB59). Band 4 was matched to caleosin, the Ca\(^{2+}\)-binding EF-hand protein. The N-terminus of caleosin contains an EF-chiral region that binds to calcium ions, indicating that calcium is likely present in caleosin in the form of calcium ions. Bands 5, 10, and 11 were matched to oleosin, indicating the presence of the three main subtypes of oleosin in peanut OBs (Q6J118, Q647G3, and Q647G5).

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**Fig. 4** CLSM image of peanut OBs (400×).

**Fig. 5** Electrophoretic analysis of the molecular weights of OB proteins: (A) SDS-PAGE (Line 1, OB proteins; Line 2, peanut raw proteins); and (B) two-dimensional electrophoresis of OB proteins.
In summary, the endogenous interfacial proteins in peanut OBs are primarily oleosin, caleosin, and steroleosin, while the foreign proteins were mainly arachin and lipoxygenase.

3.5 Phospholipid composition of OBs

The phospholipid composition of peanut OBs was determined by $^{31}$P NMR (Fig. 6). The phospholipid with the highest content in peanut OBs was phosphatidylcholine (PC; 50.6%) followed by phosphatidylinositol (PI; 13.6%), phosphatidylethanolamine (PE; 12.7%), phosphatidylserine (PS; 10.8%), and phosphatidic acid (PA; 10%). The results of this study differ from those of Zhou, Chen, Hao, Du, & Liu (2019), who studied the contents of PC, PS, PI, and PE in peanut phospholipids using only the liquid phase. In this study, $^{31}$P NMR was used to detect phospholipids. This method can simultaneously detect multiple phospholipid components with simple sample processing, rapid analysis, and high precision, thereby allowing facile and low-cost quantitative analysis.

Phospholipids account for approximately 80% of the interfacial membrane in peanut OBs, and their composition...
is directly related to the stability of the interfacial membrane. PC, which is the most important phospholipid component, has two hydrophobic acyl groups that are more hydrophobic than in other phospholipid components. The two hydrophobic acyl groups at the Sn1 and Sn2 positions bind to proteins through hydrophobic interaction, which enhances the interfacial expansion ability of the protein on the interfacial membrane and promotes the stability of the emulsion. The presence of PA may affect the quality of peanut oil, and changes in the PA content depend on the peanut storage temperature and processing method. PS improves brain function and Alzheimer’s disease. In summary, phospholipids as an important component of the OB interfacial membrane and play an important role in OB stability.

3.6 Rheological properties

Figure 7A shows the viscosity of peanut OBs as a function of temperature. As the temperature increased, the OB viscosity first decreased and then increased slightly. When the temperature increased from 25°C to 80°C, the viscosity of the emulsion decreased significantly. Increasing temperature speeds the thermal movement of the molecules in the emulsion, destroys the forces between molecules in the oil–water interfacial film, and increases the fluidity of the system, thereby reducing the viscosity. When the temperature exceeded 80°C, the viscosity increased slightly. This may be due to the severe evaporation of water caused by the high temperature, which will cause the emulsion concentration to increase sharply. Proteins and other molecules aggregate to form larger aggregates, resulting in a slight increase in the viscosity of the system.

As shown in Fig. 7B, as the shear rate increased, the viscosity of the emulsion gradually decreased and finally stabilized. This indicates a shear thinning phenomenon characteristic of pseudoplastic fluids and demonstrates that the viscosity of the emulsion is greatly affected by the shearing effect; a small shear rate can destroy the structure of the oil–water interfacial film and reduce the stability of the emulsion. This may be due to the destruction of cross-linking between molecules when the stable emulsification system of amphoteric substances (e.g., proteins and phospholipids) is subjected to shearing.

The viscoelasticity of an emulsion can be characterized by the storage modulus $G'$ and loss modulus $G''$ (Fig. 7C). Within the oscillation frequency studied herein, the $G'$ value of the emulsion was always greater than the $G''$ value, indicating that the emulsion was a typical elastic-based system. The elastic properties of emulsions are mainly determined by the elastic deformation of the macromolecular substances (e.g., proteins and phospholipids) adsorbed in the system. Such a macromolecule-stabilized system is
better able to withstand mechanical shock than a system stabilized by small molecules, resulting in greater emulsion stability. In summary, proteins and phospholipids combine to form an interfacial membrane, which stabilizes the OB. Elevated temperature and high-speed shear will destroy the binding between proteins and phospholipids, thereby reducing the OB stability.

4 Conclusions

We used a combination of cellulase and pectinase to extract peanut OBs and studied their composition and rheological properties. Treatment with cell wall-degrading enzymes destroyed the structure of the peanut cell wall and promoted the release of peanut OBs. Among the enzyme treatments applied in this study, the 1:1 combination of cellulase and pectinase resulted in the highest OB extraction rate (90.7%). An analysis of the OB microstructure showed that triacylglycerol was wrapped in an interfacial membrane formed by proteins and phospholipids. The endogenous proteins of peanut OBs mainly comprised oleosin (18 kDa, three subtypes), caelosin (27 kDa), and stereosin (39 kDa, two subtypes). Two foreign proteins (arachin and lipoxygenase) were adsorbed on the OB surface. The main phospholipid in the OBs was PC, which combined with proteins to stabilize the OBs. The measured rheological properties indicate that the OBs are a stable system dominated by elasticity. High temperature and high-speed shear destroyed the structure of the proteins and phospholipids to destabilize the OBs. The findings provide a theoretical basis for the commercial application of peanut OBs.

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Conflict of Interest

We declare that we have no conflict of interest.

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