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Characterizing the structural and surface properties of proteins isolated before and after enzymatic demulsification of the aqueous extract emulsion of peanut seeds

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ABSTRACT

We isolated and characterized various proteins (adsorbed and unadsorbed) from the aqueous extract emulsion of peanut seeds. The peanut emulsion was further subjected to a freeze-thaw treatment or enzymatic hydrolysis by the alkaline endopeptidase Mifong®2709. The proteins in the residual cream layer and the aqueous phase, obtained from the freeze-thaw treatment as well as the cream layer peptides and aqueous phase peptides obtained at various periods of hydrolysis (namely 5, 10, 30, and 60 min), were isolated and analyzed for structural (molecular weight, disulfide bond content, and surface hydrophobicity) and surface (dynamic interfacial tension, emulsifying activity, and stability indices) properties. The peanut proteins adsorbed at the oil-water interface showed superior emulsification properties and unique structural characteristics consisting of large molecular weight proteins and small molecular weight oleosins, and also possessed a higher disulfide bond content and greater surface hydrophobicity. The proteins in the residual cream layer derived from the freeze-thaw treatment had a higher disulfide bond content, surface hydrophobicity, and emulsification indices than the proteins in the aqueous phase. In contrast, following the hydrolysis of the emulsion, the resulting cream layer peptides and the aqueous phase peptides exhibited a significantly lower disulfide bond content, weaker surface hydrophobicity and lower emulsification indices than the intact proteins. These protein structural changes following hydrolysis contributed to the destabilization of the peanut emulsions. We also observed a positive correlation for the disulfide bond content and the surface hydrophobicity with the emulsifying activity index (EAI), $R^2 = 0.963$ and 0.982, respectively as well as the emulsion stability index (ESI), $R^2 = 0.920$ and 0.906, respectively.

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1. Introduction

Peanuts are one of the most important oilseeds in the world. In China, the production of peanuts exceeds 14 million tons per year. To obtain edible oil, about one-half of this supply is processed by mechanical pressing or hexane extraction. Hexane extraction is currently the most effective oil recovery method. However, the organic solvent is highly flammable and toxic, forming fugitive emissions of volatile organic compounds (VOCs) (Johnson, 2000). Due to the environmental safety and health concerns, and the desire for simultaneous extraction of oil and protein, aqueous extraction processing (AEP) of peanuts (Li, 1984; Rhee, Cater, & Mattil, 1972; Sharma, Khare, & Gupta, 2002; Zhang, Lu, Yang, Li, & Wang, 2011) and other oilseeds (Dominguez, Nunez, & Lema, 1995; Fullbrook, 1983; Liang, Zhang, & Cong, 2012; de Moura, Maurer, Jung, & Johnson, 2011; Zhang, Wang, & Xu, 2007) has attracted increased interest. The process usually consists of an aqueous (enzymatic) extraction of the comminuted materials, followed by a centrifugal separation of the slurry into oil, emulsion, aqueous, and solid phases. The proteins may be recovered from the aqueous or solid phases, depending on the conditions that are selected. Compared with the traditional technology, this process is mild and safe as organic solvents are completely avoided. Nevertheless, the existence of naturally formed oil-in-water emulsions usually results in a significant decrease in the free oil yield.

Some physical and physicochemical treatments were previously utilized to demulsify the emulsions formed during the AEP of peanuts (Li, 1984; Rhee et al., 1972; Zhang et al., 2011). These

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methods are effective, though they are energy-intensive. The use of proteases in destabilizing the emulsions formed during the AEP of soybeans achieved desirable results (Chabrand & Glatz, 2009; Chabrand, Kim, Zhang, Glatz, & Jung, 2008; de Moura et al., 2008; Wu, Johnson, & Jung, 2009). We also found that the alkaline endopeptidase Mifong®2709 is an effective peanut demulsifier, and the optimal hydrolysis conditions produced a free oil yield of ~94% (Zhang, Liu, Lu, Wang, & Zhao, 2013). Enzymatic treatment is therefore an effective biological approach to destabilize the AEP emulsions. The cleavage of protein molecules at the water–oil interface is now considered a major contributor to demulsification. Chabrand et al. (2008) compared the molecular weight changes of proteins from soybean oil emulsions that are subjected to various treatments. They found larger proteins and subunits dissociated from the interface during heating, and only polypeptides that were smaller than 14 kDa remained after enzymatic treatment. However, further structural and surface information (such as the disulfide bond content, surface hydrophobicity, and interfacial tension) were not reported. In the case of peanut emulsions, though the quantity of such emulsion proteins is small, they may also play a crucial role in the stabilization of the peanut emulsion. To explore the enzymatic demulsification mechanism, the specific structural and interfacial properties of these emulsion proteins before and after enzymatic treatment require further investigation and comparison.

Many researchers have attempted to understand the effects of enzymatic hydrolysis on the emulsification properties of proteins (Avramenko, Low, & Nickerson, 2013; Guan, Yao, Chen, Shan, & Zhang, 2007; Jung, Murphy, & Johnson, 2005; Nishinari, Fang, Guo, & Phillips, 2014; Yust & Pedroche, 2010; Zhao, Liu, Zhao, Ren, & Yang, 2011). These studies were generally carried out by preparing model emulsions using target proteins at various degrees of hydrolysis (DH). The structural properties of the hydrolyzed proteins and their emulsifying actions were then correlated. A few experiments were performed with the reverse approach, i.e., gradually hydrolyzing the naturally occurring emulsions (like AEP emulsion), isolating the peptides with different DH from the destabilized emulsions, and analyzing the structural changes of the hydrolyzed proteins. This seems to be a new method to investigate the relationship between enzymatic hydrolysis of proteins and their surface properties.

In the present study, the structural and the surface properties of the interfacial and the unadsorbed proteins from the peanut emulsion were compared with those of the peanut protein isolate (PPI). The emulsion proteins extracted at various enzymatic demulsification stages were further determined for their molecular weight, disulfide bond content, surface hydrophobicity, dynamic interfacial tension, emulsifying activity, and stability indices. In addition, the relationship between the structural characteristics and the emulsifying abilities of the peanut proteins, were investigated.

2. Materials and methods

2.1. Materials

Undehulled (unshelled) peanut seeds were obtained from a local market. The peanut seeds contained 48.6% oil and 27.3% protein (N × 5.46), on a dry basis. The enzyme used in this study was Mifong®2709 (an alkaline endopeptidase from *Bacillus licheniformis*, optimal pH 9.0, optimal temperature 50 °C, enzyme activity 112,000 IU/g) that was purchased from Donghua-Qiangsheng Biotechnology Co, Ltd, Beijing, China. The peanut protein isolate (PPI) containing 85.4% protein (N × 5.46) was obtained according to the method of Zhao et al. (2011). 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 1,8-anilinonaphthalenesulphonate (ANS) were purchased from Sigma (Sigma–Aldrich Co. LLC., MO, USA). All other reagents were of analytical grade.

2.2. Aqueous extraction and phase separation

Clean undehulled peanut seeds (200 g) were soaked in 500 mL distilled water in a beaker overnight and then reduced in size in a pulverizer (model JJ-2; Jiangsu Jingtan Instrument Co., Ltd, Jingtan, China). The material was then placed in a colloid mill (model JMS-30B; Langfang Langtong Instrument Co., Ltd, Langfang, China) for further wet-grinding for 5 min to obtain a sufficient breakdown of the material. The slurry was then transferred to a 3-L jacketed glass reactor connected to a temperature-controlled water bath. The seed-to-water ratio was fixed at 1:10 (w/v) and the slurry pH was adjusted to 10.5. The diluted slurry was incubated at 50 °C for 1 h at 150 rpm agitation rate. Following incubation, the suspension was centrifuged at 2800 × g for 15 min (model TD5-4B; Era Beili Centrifuge Co., Ltd, Beijing, China). The upper cream layer (emulsion phase) was collected on top of a 200-mesh sieve (micro-pore size: 74 μm) by decanting the supernatant gently through the screen. The obtained emulsion was stored at 8 °C for further use.

2.3. Measurement of the surface protein concentration

The surface protein concentration of the peanut emulsion was measured by the methods of Agboola, Singh, Munro, Dalgleish, and Singh (1998) and Chabrand et al. (2008) with some modifications. After 8 g of the original cream layer was washed by dispersion into 4 parts of distilled water, followed by centrifugation (20,050 × g for 30 min; model GL-20G; Anting Scientific Instrument Factory, Shanghai, China), the resulting bottom aqueous phase was then analyzed for the protein content by the method of Lowry, Rosebrough, Farr, and Randall (1951). The difference between the concentration of the total original cream layer proteins and the aqueous phase proteins provided the concentration of the surface proteins. This value was then related to the amount of total cream oil to calculate the milligrams of protein per gram of oil. The surface protein concentration, Γ, was calculated using Eq. (1).

$$\Gamma = \frac{M_{P/O}}{\mathrm{SSA}} \tag{1}$$

where $M_{P/O}$, the mass ratio of the protein to oil, was from the cream layer and SSA, the specific surface area of the oil droplets, was calculated using Eq. (2).

$$\mathrm{SSA} = \left(\frac{6}{D_{3,2}}\right) \times \left(\frac{1}{\rho_{oil}}\right) \tag{2}$$

where $D_{3,2}$, the average particle size, was 4.85 μm according to our previous study (Zhang et al., 2013) and the peanut oil density was 0.91 g/cm^3.

2.4. Extraction of the adsorbed and the unadsorbed proteins

The adsorbed and the unadsorbed proteins were extracted from the peanut emulsion according to the procedure shown in Fig. 1. Specifically, the peanut emulsion (120 g) was washed 4 times with distilled water and then recovered by centrifugation (20,050 × g for 30 min). Both the upper washed cream layer and the bottom aqueous phase were stored at 4 °C for later use. The proteins adsorbed on the emulsion droplet surface in the upper cream layer were extracted as follows: The washed cream layer was mixed with an equal volume of distilled water containing 1.5% (v/v) Tween 20 (used to replace the adsorbed proteins) and agitated for 60 min at

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