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Reduction of major peanut allergens Ara h 1 and Ara h 2, in roasted peanuts by ultrasound assisted enzymatic treatment

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ABSTRACT

This study investigated the effects of ultrasound, enzyme concentration and enzyme treatment time on soluble protein and major allergenic proteins (Ara h 1 and Ara h 2) of roasted peanut kernels. A 3-factor, five-level orthogonal experimental design was implemented with various ultrasonication times, concentrations of trypsin or α-chymotrypsin and treatment times. The total soluble proteins were determined by the Bicinchoninic acid (BCA) method, Ara h 1 and Ara h 2 were evaluated by SDS–PAGE and sandwich ELISA. The IgE-binding of peanut extracts was analysed by a competitive inhibition ELISA. Results indicate that ultrasound treatment, followed by protease digestion of peanuts, significantly increased the solubility of peanut protein and decreased the concentrations of Ara h 1 and Ara h 2. The sequential treatment of peanuts by ultrasonication–trypsin–alpha chymotrypsin, resulted in maximum reductions of Ara h 1/Ara h 2, and lowest IgE-binding. This study provides an approach to significantly reduce allergenic proteins in peanut product.

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1. Introduction

Peanut is a nutrient-rich and very popular food, but it also causes a serious allergic reaction to about 1% of the US population. Peanut allergy is one of the most severe food allergies. It is responsible for 59% of deaths caused by food allergy (Bock, Muñoz-Furlong, & Sampson, 2007). Ingestion of extremely small amount of peanuts could lead to highly sensitive patients with allergic reactions, such as gastrointestinal discomfort, allergic dermatitis and other diseases; in some cases severe or even allergic shock and anaphylactic death occurs (Dean, Hugh, & Ronald, 1997; Sicherer & Sampson, 2007). Due to the wide use of peanuts in all kinds of food products, removal of allergenic proteins in peanuts, before it is incorporated into the food products, became imperatively important.

Many methods have been proposed to reduce peanut allergens, but these cannot solve the problem completely. Genetic modification has been utilised to reduce food allergens. The application of RNA interference technology for the silencing of Ara h 2 in peanut, resulted in a significant reduction of Ara h 2 and a subsequent decrease in peanut allergenicity *in vitro* (Dodo, Konan, Chen, Egnin, & Viquez, 2007; Guo, Liang, Chung, Holbrook, & Koppelman, 2008). However, consumer acceptance of genetically modified products is still a big issue, because many people are afraid of the hidden long term harmful effect (Singh & Bhalla, 2008). Application of pulsed-UV to peanuts and peanut butter was reported to effectively reduce IgE-binding of peanut extracts and peanut butter (Chung, Yang, & Krishnamurthy, 2008; Yang, Mwakatage, Goodrich-Schneider, Krishnamurthy, & Rababah, 2011). Gamma irradiation also reduced the IgG binding of whole peanut protein extract and Ara h 6 due to the loss of the α-helix structure (Luo et al., 2013). However, peanuts are rich in fat with very high unsaturated fatty acid content. Exposing peanuts to pulsed-UV or irradiation, may accelerate lipid oxidation which will result in rapid quality deterioration and harmful oxidation products. In addition, the solubility of peanut protein was significantly reduced after pulsed UV treatment (Chung et al., 2008). Therefore, seeking a safe and effective technology/method to remove peanut allergens is demanded by consumers. Our previous study, showed that treatment of roasted peanut kernels with digestive proteases trypsin and chymotrypsin, significantly reduced Ara h 1 and Ara h 2 in peanuts, while increasing the solubility of peanut protein (Yu, Ahmedna, Goktepe, Cheng, & Maleki, 2011). The study also found that Ara h 2 was more resistant to the enzymatic digestion as reported by other researchers (Astwood, Leach, & Fuchs, 1996), but blanching before enzyme treatment enhanced the reduction of Ara h 2, the more potent peanut allergen. It was also reported that digestion of native Ara h 2 by

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trypsin, chymotrypsin or pepsin, produced a 10-kDa protein fragment, which contained intact binding sites that could still be recognised by IgE. This fragment also contains 11 potential chymotrypsin cleavage sites (Bannon, Fu, Kimber, & Hinton, 2003). Therefore, we hypothesise that a pretreatment that can disrupt peanut structure and sequential enzyme treatment may enhance the allergen reduction by digestive enzymes.

Ultrasound technology is a novel technology that recently found a wide application in food processing. High energy (high power, high intensity, low frequency) ultrasonic waves are capable of altering material properties (e.g. physical disruption, acceleration of certain chemical reactions) through generation of immense pressure, shear and temperature gradient in the medium through which they propagate (Dolatowski, Stadnik, & Stasiak, 2007). The toughness of some meats due to the high connective tissue content was reported to be reduced during ultrasound treatment (Jayasooriya, Torley, D'Arcy, & Bhandari, 2007; Smith, Cannon, Novakofski, McKeith, & O'Brien, 1991). Therefore, ultrasound treatment may have potential to loosen the peanut structure or even break peptide chains of peanut proteins.

The objectives of this study were to (1) investigate the impact of ultrasonic treatment on the major peanut allergens; (2) determine the optimum treatment conditions such as ultrasonic time, enzyme concentration and enzyme treatment time, and (3) determine the effectiveness of ultrasound and enzyme treatments on IgE binding of extracts of treated peanuts.

2. Materials and methods

2.1. Materials

The roasted peanut kernels (without skin) were purchased from Good Earth Peanut Company (Skippers, VA). Enzymes used in this study including α-chymotrypsin (EC3.4.21.1, P40 units/mg protein, from bovine pancreas), trypsin (EC 3.4.21.4, P 10,000 units/mg protein, from bovine pancreas), *o*-Phenylenediamine and Streptavidin-Peroxidase were purchased from Sigma–Aldrich (St. Louis, MO). Pierce BCA Protein Assay Kits were purchased from Thermo Scientific (Rockford, IL). Ara h 1 and Ara h 2 ELISA Kits were purchased from Indoor Biotechnologies (Charlottesville, VA). Peroxidase conjugated Goat anti-Rabbit IgG was purchased from Jackson Immuno Research Laboratories (West Grove, PA), and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution was purchased from KPL Inc. (Gaithersburg, MD). Hydrogen peroxide (30%) was purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Ultrasound assisted enzymatic treatment of peanut kernels

Peanut kernels were weighed into a set of beakers (20 g per beaker), and then 40 ml of phosphate buffer (pH 7.5) was added in each beaker. Samples were sonicated in a water bath sonicator (Branson Cleaning Equipment Company, Danbury, CT) for 1–5 h at 50 Hz. Trypsin or α-chymotrypsin was added to the peanut-buffer system to the enzyme/peanut ratio 0%, 0.1%, 0.15%, 0.20%, 0.25% or 0.30% (w/w). Samples were incubated for different time periods at 37 °C. Samples were then drained and soaking solutions were collected separately. Enzyme treated peanuts were dried at 70 °C overnight in a vacuum oven (Fisher Scientific, Pittsburgh, PA) to remove moisture and to inactivate enzymes. The dried peanuts were ground to paste using a high speed blender, followed by mortar and pestle, and then transferred into capped plastic containers and stored at 4 °C until used. The untreated peanut was used as a control.

2.3. Sequential enzyme treatment of roasted peanuts

Peanut kernels sonicated for 1 h were hydrolyzed with trypsin under the optimal condition obtained from Section 2.2, followed by α-chymotrypsin hydrolysis at enzyme to peanut ratios of 0%, 0.1%, 0.15%, 0.20%, 0.25% or 0.30% (w/w) for 1, 2, 3, and 4 h, respectively. Samples were drained, dried and ground into pastes as described in 2.2. They were labelled and stored at 4 °C until used.

2.4. Peanut protein extraction and determination

Two grams of sample from each treatment were mixed with 20 ml of phosphate buffer (pH 7.8) and stirred at room temperature for 1 h. Mixtures were then centrifuged at 3000*g* for 10 min. The supernatants were collected into 50 ml tubes and the precipitates were extracted one more time. Combined supernatants from two extractions were centrifuged at 3000*g* for 10 min. The volumes of supernatants were recorded. The lipid layers on the top of supernatants were removed using transfer pipettes and supernatants were stored at 4 °C. Soluble protein, Ara h 1 and Ara h 2 contents of each sample were determined and statistical analysis of the data was performed to determine the optimal sonication time, enzyme concentration and digestion time. Insoluble protein in the precipitate fractions, were extracted according to the procedure of Schmitt, Nesbit, Hurlburt, Cheng, and Maleki (2010) using standard electrophoresis sample treatment buffer containing glycerol, SDS and dithiothreitol.

2.4.1. Determination of soluble protein by BCA method

Protein concentrations in soaking solutions and extracts were determined by the BCA method using analytical grade BSA as the standard. Samples were diluted 3–5 times with deionised water to bring protein concentration in test sample down to the linear range of BSA calibration curve (0–1.0 mg/ml). Protein concentration in peanut soaking solution/extract was expressed as mg protein/ml. Total soluble protein contents of peanut samples were expressed as mg protein per gram peanuts.

2.4.2. SDS–PAGE of soluble and insoluble protein fractions

The extracts of roasted peanuts, treated and untreated with enzyme, were adjusted to 1 mg/ml with phosphate buffer. They were mixed with the same volume of sample treatment buffer (pH 6.8, Tris buffer containing glycerol, SDS and dithiothreitol), then heated in boiling water bath for 10 min. After cooling to room temperature, samples (20 μl/well) were loaded to 4/12.5% polyacrylamide gel and run using a Bio-Rad Mini Protein III system (Bio-Rad, Hercules, CA). Purified peanut allergens Ara h 1 and Ara h 2 were used as references. The gel was resolved at the power conditions recommended by Bio-Rad (200 V, 120 mA and 45 min) and stained with a Coomassie Brilliant Blue R-250 solution (Bio-Rad, Hercules, CA) for overnight. After destaining, the gels were photographed with a Gel Doc XR imaging system (Bio-Rad, Hercules, CA).

2.4.3. Determination of Ara h 1 and Ara h 2 using sandwich ELISA

Concentrations of Ara h 1 and Ara h 2 in soaking solutions and extracts were determined by sandwich ELISA protocols and kits developed by Indoor Biotechnology (Charlottesville, VA). Anti-Ara h 1/h 2 Costar 96-well polystyrene microtiters were coated with 100 μl/well of properly diluted Anti-Ara h 1/h 2, then incubated overnight at 4 °C followed by 3 washes with pH 7.4 PBS Tween 20 (PBS-T) using a Microplate Washer (Bio-Tek, Winooski,VT). The plate was then blocked with 0.5% non-fat dry milk in PBS-T (100 μl/well) for 30 min at room temperature. After washing 3 times with PBS-T, 100 μl/well of diluted standard/sample solutions