



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– α 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 3000g 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 3000g 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 μ l/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 μ l/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 μ l/well) for 30 min at room temperature. After washing 3 times with PBS-T, 100 μ l/well of diluted standard/sample solutions

were added, and incubated for 1 h at 37 °C. Plates were washed again and 100 µl properly diluted secondary antibodies (biotinylated anti-Ara h 1 for Ara h 1 or anti-Ara h 2 for Ara h 2) were added. The plates were incubated for 1 h at room temperature, washed, and 100 µl of diluted Streptavidin-Peroxidase (for Ara h 1) or Peroxidase conjugated Goat anti-Rabbit IgG (for Ara h 2) were added. Plates were incubated for 30 min at room temperature, and then washed. An enzyme substrate (ABTS) was added to the plate (100 µl/well). After 30 min colour development, the absorbance was read at 405 nm using an ELx 808IU Ultra Microplate Reader (Bio-Tek, Winooski, VT).

The concentrations of Ara h 1 and Ara h 2 of each sample was calculated using standard curves developed with purified Ara h 1 and Ara h 2 using the ELISA procedure described above. The final results were calculated as mg Ara h 1 or Ara h 2/g peanuts, and were expressed as mean $\pm$ standard deviation of three replications.

2.5. Determination of IgE binding of treated peanut extracts with human plasma

A competitive inhibition enzyme-linked immunosorbent assay (ciELISA) was carried out as previously described (Chung, Yang, & Krishnamurthy, 2008). Briefly, a microplate was coated with untreated peanut extract (1:20 diluted with PBST) and incubated at 4 °C for overnight. After washing wells 3x with PBST, pH 7.4 (PBS-T), the plate was blocked 3 times (5 min each time) with 200 µl 1% BSA-PBST at room temperature. After washing, 50 µl of a peanut sample (diluted with 1% BSA-PBST) at 0, 0.01, 0.1, 1.0, 10, 100, 1000 µg/ml was mixed with 50 µl of 1:30 diluted pooled human plasma obtained from 10 patients with peanut allergy (Lab-Plasma Inc., Everett WA), and added to each well. The plate was then slowly shaken for 45 min at room temperature, and then washed 3 times. The immunoglobulin E (IgE) antibodies, which were attached to the plate, were then detected using a goat anti-human IgE peroxidase conjugate (1:1000, 100 µl) and a substrate solution (100 µl) containing *o*-phenylenediamine (0.5 mg/ml) and 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5. The enzyme reaction was stopped with 2 N sulfuric acid (50 µl), and the absorbance was read at 490 nm with an ELx Synergy Microplate Reader. The absorbance sample containing IgE and the peanut sample was represented by *B*, while *B*₀ represented the absorbance of a control containing IgE only. The higher the allergen concentration was in the sample, the lower the absorbance, and vice versa (Chung et al., 2008). The IgE binding inhibition of a sample was expressed as $B \times 100 / B_0$.

2.6. Experiment design and statistical analysis

A 3-factor, five-level orthogonal experimental designs was used for each enzyme treatment. The factors were ultrasonic time (0, 0.5, 1.0, 1.5 and 2.0 h), enzyme concentration (0.1%, 0.15%, 0.2%, 0.25% and 0.3%), and enzyme treatment time (1, 3, 5, 7 and 9 h for trypsin; 1, 2, 3, 4 and 5 h for α -chymotrypsin). The data was analysed with a software program for orthogonal test design (Orthogonality Experiment Assistant II, v 3.1.1, China) to obtain optimal treatment parameters. Mean differences were judged at the 0.05 significance level. The highly significant parameter was indicated by two stars and significant parameter was indicated by one star. IgE binding data were means of triplicate measurements ($n = 3$) and its statistical analyses were performed using a Student's *t*-test at a $P < 0.05$ level of significance. IC₅₀ values were calculated by GraphPad Prism Software 5 (GraphPad Software Inc., San Diego, USA).

3. Results and discussions

3.1. Effects of ultrasonic and trypsin treatments on total soluble protein, Ara h 1 and Ara h 2 concentrations in roasted peanut kernel

Table 1 summarizes the results of roasted peanut kernels treated with ultrasound, trypsin and ultrasound followed by trypsin. Table 2 shows the results of significant analysis of data in Table 1. ANOVA was conducted separately for total soluble protein, Ara h 1 and Ara h 2. Results showed that ultrasonic pre-treatment alone increased the solubility of peanut protein, significantly reduced Ara h 1 but not Ara h 2. Ultrasonic treatment may not only loosen the structure of peanut kernel, which makes enzyme easy to diffuse/penetrate into the kernel and contact the protein to be cleaved (Yu et al., 2012), but also cleave peptide bonds due to the shear force. Trypsin concentration had significant effect on Ara h 1 level, but not Ara h 2. Under the experimental conditions in Table 1, enzyme reaction time did not show significant effects on soluble protein Ara h 1 and Ara h 2.

Overall, the reduction of Ara h 1 due to trypsin treatment was similar to the previously reported findings, but the reduction of Ara h 2 due to trypsin treatment was significantly lower (Yu et al., 2011). One explanation is that the Ara h 1 and Ara h 2 were determined by an indirect ELISA which has lower sensitivity than sandwich ELISA. The lower Ara h 2 reduction further confirmed the high resistance of Ara h 2 to trypsin digestion (Bannon et al., 2003; Koppelman, Hefle, Taylor, & de Jong, 2010). According to Table 1, the optimum treatment condition for ultrasound–trypsin combination was 1 h of ultrasonic time, 0.25% of enzyme to peanut ratio, and 1 h reaction time. Under such conditions, highest reductions in soluble allergen levels were obtained. Longer sonication time at higher enzyme concentration did not show further improved protein solubility and allergen reduction. This might be due to the temperature increase during sonication, which resulted in lower cavitation effects (Iida, Tuziuti, Yasui, Towata, & Kozuka, 2008).

3.2. Effects of ultrasonic and α -chymotrypsin treatments on total soluble protein, Ara h 1 and Ara h 2 concentrations in roasted peanut kernel

Table 3 summarizes the results of the three-factor five-level orthogonal experiment with ultrasound–chymotrypsin treatment of roasted peanut kernels. Similar to the treatment with trypsin, α -chymotrypsin treatment significantly increased the soluble protein and reduced Ara h 1 of roasted peanut. In addition, Ara h 2 was reduced by 86.7–90.7% without ultrasound. Ultrasound treatment for 0.5–1.0 h followed by α -chymotrypsin treatment, enhanced the protein solubility but did not significantly increase the reduction of Ara h 2 at all enzyme concentrations and digestion time. When the sonication time increased to 1.5 h, significant decrease in Ara h 2 was observed compared to the treatment without ultrasound treatment. According to Table 3, the optimum treatment conditions for Ara h 2 reduction in the ultrasound–chymotrypsin combination was 2 h of ultrasound treatment, 0.3% of α -chymotrypsin and 4 h of enzyme treatment time. The highest Ara h 2 reduction (97.82%) was achieved under these conditions.

ANOVA of data in Table 4 shows that the total soluble protein content was significantly affected by ultrasonic time and enzyme concentration with *F* values 3.98 and 5.79, respectively; The reduction of Ara h 2 was significantly affected by α -chymotrypsin concentration ($F = 5.26 > F_{(4,12)} = 3.26$); within the range of the enzyme concentrations used in this set of the experiment, enzyme concentration did not show significant impact on the protein solubility and Ara h 1 level.

Table 1

Effects of ultrasonic treatment, trypsin concentration, and treatment time on protein solubility and extractable Ara h 1/h 2 concentration in roasted peanut kernels.

No.	Reaction conditions			Total soluble proteins			Total Ara h 1/h 2 in soluble fractions			
	Ultrasonic time (h)	Enzyme concentration (%)	Reaction time (h)	Soaking solution (mg/g peanut)	Extracts (mg/g peanut)	Total soluble proteins (mg/g peanut)	Ara h 1* (μg/g peanut)	Ara h 1 change %	Ara h 2* (μg/g peanut)	Ara h 2 change %
CTL	0	0	0	0	47.3	47.3	4747 ± 15.8	N/A	463 ± 15.1	N/A
C1	0.5	0	0	4.83	44.5	49.3	585 ± 17.3	−87.7	382 ± 4.07	−17.5
C2	1.0	0	0	7.02	55.6	62.6	744 ± 14.7	−84.3	399 ± 6.50	−13.9
C3	1.5	0	0	8.91	47.7	56.6	732 ± 27.7	−84.6	407 ± 11.1	−12.0
C4	2.0	0	0	9.65	38.4	48.0	721 ± 16.7	−84.8	439 ± 16.0	−4.88
1	0	0.10	1	3.89	98.0	102	18.7 ± 0.13	−99.6	429 ± 6.22	−7.35
2	0	0.15	3	4.67	88.2	92.9	10.5 ± 0.06	−99.8	359 ± 4.64	−22.4
3	0	0.20	5	6.19	99.4	106	6.78 ± 0.39	−99.9	330 ± 14.6	−28.6
4	0	0.25	7	6.28	99.7	106	5.98 ± 0.42	−99.9	308 ± 19.2	−33.5
5	0	0.30	9	9.92	99.0	109	6.38 ± 0.49	−99.9	137 ± 9.88	−70.4
6	0.5	0.10	3	4.59	91.9	96.5	11.3 ± 0.11	−99.8	315 ± 8.02	−31.8
7	0.5	0.15	5	6.32	87.8	94.2	6.62 ± 0.03	−99.9	202 ± 8.52	−56.3
8	0.5	0.20	7	6.78	85.4	92.1	5.43 ± 0.06	−99.9	313 ± 8.08	−32.4
9	0.5	0.25	9	12.1	106	119	8.68 ± 0.23	−99.8	310 ± 5.44	−33.0
10	0.5	0.30	1	4.53	104	109	7.87 ± 0.05	−99.8	273 ± 6.04	−40.9
11	1.0	0.10	5	6.83	101	108	10.4 ± 0.15	−99.8	343 ± 7.38	−25.9
12	1.0	0.15	7	7.40	95.4	103	11.7 ± 0.11	−99.8	387 ± 8.01	−16.3
13	1.0	0.20	9	10.0	103	113	8.72 ± 0.03	−99.8	179 ± 10.6	−61.4
14	1.0	0.25	1	4.55	125	130	6.91 ± 0.34	−99.9	122 ± 2.07	−73.7
15	1.0	0.30	3	5.52	108	113	8.73 ± 0.05	−99.8	361 ± 17.2	−22.0
16	1.5	0.10	7	6.71	78.9	85.6	8.96 ± 0.68	−99.8	351 ± 13.7	−24.1
17	1.5	0.15	9	10.5	87.7	98.1	5.73 ± 0.06	−99.9	230 ± 4.63	−50.2
18	1.5	0.20	1	4.72	101	106	7.24 ± 0.03	−99.9	448 ± 2.05	−3.09
19	1.5	0.25	3	5.50	94.7	100	5.70 ± 0.45	−99.9	169 ± 1.67	−63.5
20	1.5	0.30	5	7.43	97.2	105	7.32 ± 0.17	−99.9	298 ± 1.92	−35.6
21	2.0	0.10	9	9.84	76.8	86.7	12.6 ± 0.06	−99.7	306 ± 1.01	−33.9
22	2.0	0.15	1	4.44	100	105	12.4 ± 0.03	−99.7	386 ± 3.68	−16.5
23	2.0	0.20	3	5.92	67.9	73.8	7.74 ± 0.08	−99.8	274 ± 5.94	−40.8
24	2.0	0.25	5	7.59	83.4	91.0	6.71 ± 0.02	−99.9	288 ± 22.9	−37.7
25	2.0	0.30	7	8.45	90.2	98.7	11.8 ± 0.79	−99.8	378 ± 27.9	−18.3

* Ara h 1 and Ara h 2 concentrations are expressed as mean ± standard deviation.

Table 2Orthogonal test analysis of variance table of Ara h 1/h 2 for peanut treated with chymotrypsin.^a

Variance sources (independent variables)	Dependent variables	S_j	f_e	V_e	F	F_x	S
Ultrasonic time (h)	Total soluble protein	1299	4	325	8.41	$F_{0.01}(4,12) = 5.412$	**
Enzyme concentration (%)		701	4	175	4.54	$F_{0.05}(4,12) = 3.259$	*
Reaction time(hrs)		734	4	183	4.75	$F_{0.05}(4,12) = 3.259$	*
Ultrasonic time (hrs)	Ara h 1	35.3	4	8.82	2.28	$F_{0.05}(4,12) = 3.259$	
Enzyme concentration (%)		99.5	4	24.9	6.43	$F_{0.05}(4,12) = 3.259$	*
Reaction time(hrs)		25.0	4	6.26	1.62	$F_{0.05}(4,12) = 3.259$	
Ultrasonic time (h)	Ara h 2	8112	4	2028	0.245	$F_{0.05}(4,12) = 3.259$	
Enzyme concentration (%)		32132	4	8033	0.972	$F_{0.05}(4,12) = 3.259$	
Reaction time (h)		39498	4	9874	1.20	$F_{0.05}(4,12) = 3.259$	

^a S_j : Sum of squared deviations; f_e : Degrees of freedom; Variance $V_e = S_j/f_e$; Value F ; S : Significance.

** Highly significant.

* Significant.

According to data in Tables 1–4, the Ara h 1 was easily removed by 0.1% trypsin or α -chymotrypsin. The maximum Ara h 2 removal achieved with trypsin and chymotrypsin treatment was 73.7% and 97.8%, respectively. It seems that higher chymotrypsin concentration is required to completely remove Ara h 2 regardless the effect of ultrasound pretreatment.

3.3. Effects of ultrasound and sequential enzymatic treatments on allergen reduction in roasted peanut kernel

Roasted peanut kernels were first treated using trypsin under the optimum conditions obtained from Table 1, then continually hydrolyzed by α -chymotrypsin at different concentrations (0–0.25%) for 1–4 h using a two-factor factorial design. The purpose

of using this sequential enzymatic hydrolysis, is to test the additive effect of two enzymes on Ara h 2 reduction. Results are summarized in Tables 5 and 6. The results showed that a two-step enzyme hydrolysis, slightly increased the amount of soluble protein of peanuts than single enzyme hydrolysis. With pre-hydrolysis of trypsin, 95% reduction in Ara h 2 was achieved at 0.15% α -chymotrypsin for 3 h, compared to 0.3% chymotrypsin without trypsin treatment. When the α -chymotrypsin concentration increased to 0.25% 98% Ara h 2 reduction was achieved. Higher reduction of Ara h 2 is important, because Ara h 2 is the most potent allergen (Palmer et al., 2005). The reduced α -chymotrypsin dosage has economic importance, because α -chymotrypsin is more expensive than trypsin. Under the same ultrasonication and trypsin treatment conditions, the concentration of α -chymotrypsin was highly significant

Table 3
Effects of ultrasonic treatment, chymotrypsin concentration, and treatment time on protein solubility and extractable Ara h 1/h 2 concentration in roasted peanut kernels.

No.	Reaction Conditions			Total soluble proteins			Total Ara h 1/h 2 in soluble proteins			
	Ultrasonic time (h)	Enzyme Concentration (%)	Reaction Time (h)	Soaking solution (mg/g peanut)	Extracts (mg/g peanut)	Total soluble proteins (mg/g peanut)	Ara h 1* (μg/g peanut)	Ara h 1 change %	Ara h 2 (μg/g peanut)	Ara h 2* change %
CTL	0	0	0	0	47.3	47.3	4747 ± 15.8	N/A	463 ± 15.1	N/A
C1	0.5	0	0	4.83	44.5	49.3	585 ± 17.3	−87.7	382 ± 4.07	−17.5
C2	1.0	0	0	7.02	55.6	62.6	744 ± 14.7	−84.3	399 ± 6.50	−13.9
C3	1.5	0	0	8.91	47.7	56.6	732 ± 27.7	−84.6	407 ± 11.1	−12.0
C4	2.0	0	0	9.65	38.4	48.0	721 ± 16.7	−84.8	440 ± 16.0	−4.88
1	0	0.10	1	3.41	98.1	101	21.7 ± 0.86	−99.5	61.6 ± 0.37	−86.7
2	0	0.15	2	5.53	110	116	16.6 ± 0.91	−99.7	43.0 ± 0.15	−90.7
3	0	0.20	3	6.65	106	113	10.2 ± 0.63	−99.8	43.0 ± 0.70	−90.7
4	0	0.25	4	7.50	105	112	9.77 ± 0.48	−99.8	47.8 ± 0.15	−89.7
5	0	0.30	5	9.08	120	129	11.7 ± 0.44	−99.8	47.5 ± 0.35	−89.7
6	0.5	0.10	2	4.85	97.5	102	11.0 ± 0.37	−99.8	70.9 ± 0.95	−84.7
7	0.5	0.15	3	5.93	103	109	10.2 ± 0.92	−99.8	64.1 ± 0.94	−86.1
8	0.5	0.20	4	7.06	117	124	10.2 ± 0.07	−99.8	54.9 ± 0.56	−88.1
9	0.5	0.25	5	8.31	114	122	10.5 ± 0.53	−99.8	40.9 ± 0.96	−91.2
10	0.5	0.30	1	7.14	115	122	11.7 ± 0.64	−99.8	59.3 ± 0.64	−87.2
11	1.0	0.10	3	6.17	112	118	10.4 ± 0.37	−99.8	57.5 ± 0.81	−87.6
12	1.0	0.15	4	6.80	115	121	11.5 ± 0.94	−99.8	44.4 ± 0.94	−90.4
13	1.0	0.20	5	8.34	119	127	20.6 ± 0.97	−99.6	38.0 ± 0.91	−91.8
14	1.0	0.25	1	8.32	116	124	19.0 ± 0.62	−99.6	38.1 ± 0.84	−91.8
15	1.0	0.30	2	7.99	121	129	37.4 ± 0.32	−99.2	37.4 ± 0.67	−91.9
16	1.5	0.10	4	6.94	113	120	10.6 ± 0.35	−99.8	54.3 ± 0.67	−88.3
17	1.5	0.15	5	8.26	105	114	14.92 ± 0.97	−99.7	39.6 ± 0.66	−91.5
18	1.5	0.20	1	6.92	119	126	12.5 ± 0.09	−99.7	45.8 ± 0.59	−90.1
19	1.5	0.25	2	6.93	116	123	13.0 ± 0.87	−99.7	41.5 ± 0.83	−91.0
20	1.5	0.30	3	9.14	119	129	16.5 ± 0.48	−99.7	20.6 ± 0.87	−95.6
21	2.0	0.10	5	7.75	97.5	105	15.3 ± 0.62	−99.7	79.3 ± 0.30	−82.9
22	2.0	0.15	1	6.95	104	111	14.2 ± 0.58	−99.7	59.3 ± 0.22	−87.2
23	2.0	0.20	2	8.34	115	123	11.4 ± 0.99	−99.8	43.1 ± 0.83	−90.7
24	2.0	0.25	3	9.04	104	113	11.9 ± 0.02	−99.8	36.9 ± 0.36	−92.0
25	2.0	0.30	4	9.85	101	110	9.09 ± 0.93	−99.8	10.1 ± 0.47	−97.8

* Ara h 1 and Ara h 2 concentrations are expressed as mean ± standard deviation.

Table 4
Orthogonal test analysis of the variance table of Ara h 1/h 2 for peanut treated with chymotrypsin.^a

Variance sources (independent variables)	Dependent variables	S_j	f_e	V_e	F	F_x	S
Ultrasonic time (h)	Total Soluble Protein	505	4	126	3.98	$F_{0.05}(4,12) = 3.259$	*
Enzyme concentration (%)		734	4	183	5.79	$F_{0.05}(4,12) = 3.259$	*
Reaction time (hours)		33.0	4	8.25	0.26	$F_{0.05}(4,12) = 3.259$	
Ultrasonic time (h)	Ara h 1	235	4	58.8	1.94	$F_{0.05}(4,12) = 3.259$	
Enzyme concentration (%)		67.1	4	16.8	0.553	$F_{0.05}(4,12) = 3.259$	
Reaction time (h)		188	4	47.1	1.55	$F_{0.05}(4,12) = 3.259$	
Ultrasonic time (h)	Ara h 2	925	4	231	1.92	$F_{0.05}(4,12) = 3.259$	
Enzyme concentration (%)		2537	4	634	5.26	$F_{0.05}(4,12) = 3.259$	**
Reaction time (h)		334	4	83.5	0.692	$F_{0.05}(4,12) = 3.259$	

^a S_j : Sum of squared deviations; f_e : Degrees of freedom; Variance $V_e = S_j/f_e$; Value F ; S : Significance.

** Highly significant.

* Significant.

in increasing total soluble protein and reducing Ara h 2 (Table 6). Although a 98% reduction of Ara h 2 was achieved by blanching assisted enzyme treatment in previous study (Yu et al., 2011), the 98% reduction in this current study, is based on the quantification of Ara h 2 by Sandwich ELISA which is much more sensitive than the indirect ELISA used in the previous study.

3.4. SDS-PAGE of proteins in soluble and insoluble fractions of peanuts

The effectiveness of ultrasonic and α -chymotrypsin and trypsin in reducing allergy of Ara h 1 and Ara h 2 was further confirmed by SDS-PAGE (Fig. 1). Both Ara h 1 and Ara h 2 were still present in both extract and pellet of sonicated peanut samples (lane4). Tryp-

sin treatment alone removed Ara h 1 but not Ara h 2 (lane 5); treatment with 0.30% α -chymotrypsin completely removed the Ara h 1 band in both extract and pellet but the Ara h 2 band in the pellet was still visible although it significantly faded (lane 7, Fig. 1A and B); sonication followed by trypsin or α -chymotrypsin or trypsin and α -chymotrypsin treatment completely removed the Ara h 1 and Ara h 2 bands in the soluble fraction (lane 6, 8 and 9, Fig. 1A), but not in pellet (lane 6, 8 and 9, Fig. 1B). SDS-PAGE also shows that ultrasound treatment, increased the solubility of some proteins with molecular weight between 17 and 63 kDa in PBS (Fig. 1A) and in the standard electrophoresis buffer (Fig. 1B). The results shown in Fig 1A are very consistent with those in Tables 1, 3 and 5.

Table 5

Effects of ultrasonic treatment, trypsin and chymotrypsin concentration, and treatment time on protein solubility and extractable Ara h 1/h 2 concentrations in roasted peanut kernels.

Time (h)	Trypsin		α -Chymotrypsin		Total soluble proteins (mg/g peanut)			Total extractable Ara h 1 and Ara h 2			
	Conc. (%)	Time (h)	Conc. (%)	Time (h)	Soaking solution	Extracts	Total	Ara h 1 (μ g/g peanut)*	%Ara h 1 change	Ara h 2 (μ g/g peanut)*	%Ara h 2 change
0	0	0	0	0	0	47.3	47.3	4747 $\pm$ 15.8	N/A	463 $\pm$ 15.1	N/A
1	0.25	1	0	1	4.93	72.9	77.8	72.9 $\pm$ 4.70	98.5	212 $\pm$ 9.54	54.2
1	0.25	1	0.1	1	5.83	120	126	5.43 $\pm$ 0.33	99.9	51.8 $\pm$ 0.58	88.8
1	0.25	1	0.15	1	6.25	117	123	5.17 $\pm$ 0.34	99.9	50.3 $\pm$ 0.54	89.1
1	0.25	1	0.2	1	7.81	132	140	6.21 $\pm$ 0.57	99.9	43.0 $\pm$ 0.94	90.7
1	0.25	1	0.25	1	2.43	137	139	5.33 $\pm$ 0.90	99.9	46.0 $\pm$ 0.01	90.1
1	0.25	1	0	2	5.54	76.1	81.6	17.5 $\pm$ 1.78	99.6	146 $\pm$ 4.57	68.4
1	0.25	1	0.1	2	5.72	117	123	5.12 $\pm$ 0.30	99.9	49.2 $\pm$ 0.81	89.4
1	0.25	1	0.15	2	6.85	125	131	5.40 $\pm$ 0.60	99.9	47.7 $\pm$ 2.67	89.7
1	0.25	1	0.2	2	7.46	142	149	5.86 $\pm$ 1.03	99.9	30.9 $\pm$ 1.37	93.3
1	0.25	1	0.25	2	3.23	134	137	4.98 $\pm$ 0.51	99.9	47.0 $\pm$ 5.67	89.8
1	0.25	1	0	3	5.84	90.4	96.2	16.8 $\pm$ 1.40	99.7	117 $\pm$ 1.66	74.8
1	0.25	1	0.1	3	6.39	129	135	6.22 $\pm$ 0.58	99.9	35.2 $\pm$ 0.34	92.4
1	0.25	1	0.15	3	6.69	123	130	7.40 $\pm$ 0.60	99.8	23.0 $\pm$ 0.18	95.0
1	0.25	1	0.2	3	7.4	138	146	4.98 $\pm$ 0.64	99.9	15.1 $\pm$ 1.82	96.7
1	0.25	1	0.25	3	3.3	121	125	4.98 $\pm$ 0.71	99.9	13.3 $\pm$ 1.53	97.1
1	0.25	1	0	4	6.22	88.7	94.9	10.9 $\pm$ 0.87	99.8	62.0 $\pm$ 1.36	86.6
1	0.25	1	0.1	4	6.87	138	145	5.41 $\pm$ 0.57	99.9	35.4 $\pm$ 1.03	92.3
1	0.25	1	0.15	4	7.15	120	127	5.74 $\pm$ 0.33	99.9	31.4 $\pm$ 1.55	93.2
1	0.25	1	0.2	4	9.06	129	138	4.98 $\pm$ 0.02	99.9	18.7 $\pm$ 1.25	96.0
1	0.25	1	0.25	4	4.93	129	133	5.17 $\pm$ 0.34	99.9	8.29 $\pm$ 1.21	98.2

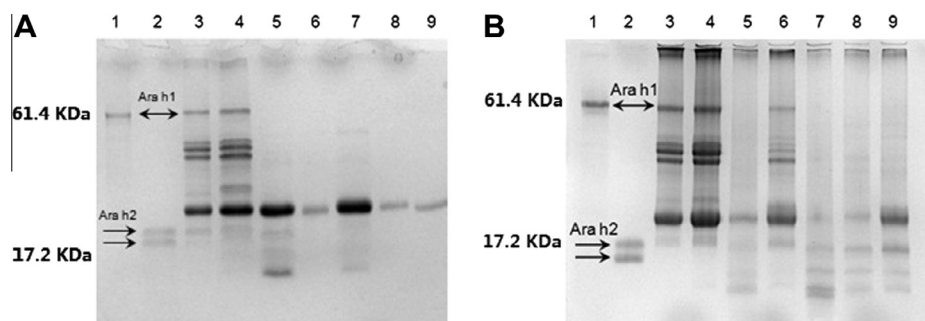
* Ara h 1 and Ara h 2 concentrations are expressed as mean $\pm$ standard deviation.**Table 6**Analysis of the variance table of Ara h 1/h 2 for peanut treated with chymotrypsin.^a

Variance sources (independent variables)	Dependent variables	S_j	f_e	V_e	F	F_x	S
Enzyme concentration (%)	Total soluble protein	7434	4	1859	32.2	$F_{0.01}(4,12) = 5.412$	**
Reaction time (h)		117	3	39.0	0.675	$F_{0.05}(4,12) = 3.259$	
Enzyme concentration (%)	Ara h 1	1846	4	461	2.73	$F_{0.05}(4,12) = 3.259$	
Reaction time (h)		511	3	170	1.01	$F_{0.05}(4,12) = 3.259$	
Enzyme concentration (%)	Ara h 2	32721	4	8180	14.8	$F_{0.05}(4,12) = 3.259$	**
Reaction time (h)		7550	3	2517	4.54	$F_{0.05}(4,12) = 3.259$	*

^a S_j : Sum of squared deviations; f_e : Degrees of freedom; Variance $V_e = S_j/f_e$; Value F ; S : Significance.

** Highly significant.

* Significant.

**Fig. 1.** SDS-PAGE of roasted peanut sample extracts (A) and precipitates (B) from different treatments. Lane 1 – purified Ara h 1, lane 2 – purified Ara h 2, lane 3 – untreated sample, lane 4 – sample sonicated for 1 h, lane 5 – sample treated with 0.25% of trypsin, lane 6 – sonication 1 h + trypsin treatment (0.25%), lane 7 – α -chymotrypsin treatment only; lane 8 – sonication (1 h) + α -chymotrypsin, lane 9 – sonication (1 h) + trypsin treatment (0.25%, 1 h) + α -chymotrypsin.

3.5. Inhibition of peanut protein extracts on IgE binding

It has been reported that fragments of Ara h 1 can still bind IgE even after prolonged trypsin digestion (Van Bostel, 2007). Therefore, a competitive inhibition ELISA (ciELISA) was performed using pooled plasma from 10 patients with clinically confirmed peanut allergy, to test the IgE-binding inhibition of peanut protein extracts

obtained from different treatments. The samples with Ara h 2 removal efficiency of 93% or higher were selected for this test. The untreated peanut extract was used as the control. Fig. 2 shows that ultrasound treatment alone reduced IgE-binding of the extractable protein moderately (by about 10%). The sequential treatment of ultrasound–trypsin–chymotrypsin was most effective in reducing IgE-binding of the extractable protein, followed by ultrasound– α -

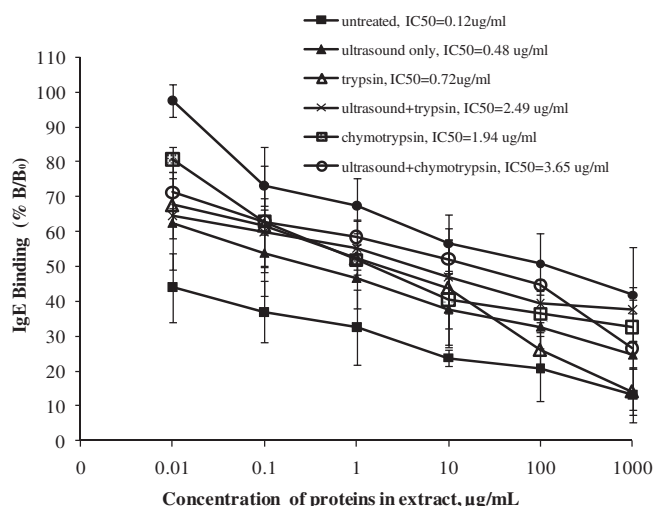


Fig. 2. IgE binding of peanut extracts and their IC_{50} . (Legend: untreated: sample control; ultrasound only: peanut sample sonicated for 1 h; trypsin: peanut sample treated with 0.25% trypsin for 1 h; ultrasound + trypsin: peanut sample sonicated for 1 h and then treated with 0.25% trypsin; chymotrypsin: peanut sample treated with 0.25% chymotrypsin for 1 h; ultrasound + chymotrypsin: peanut sample sonicated for 1 h and then treated with 0.25% chymotrypsin; ultrasound + trypsin + chymotrypsin: peanut sample sonicated for 1 h, then treated with 0.25% trypsin followed by treatment with 0.25% chymotrypsin).

chymotrypsin treatment within the protein concentrations 0.01–1000 $\mu\text{g/mL}$. Fig. 2 also shows that the peanut samples produced by sequential treatment of ultrasonication, trypsin digestion and alpha chymotrypsin digestion had the highest IC_{50} (31.95 $\mu\text{g/mL}$). The IC_{50} , is defined as the concentration of proteins (inhibitors) required to inhibit IgE binding by 50%. The higher IC_{50} value, indicates lower IgE binding. Comparing to the results of Chung et al. (2008), the ultrasound assisted sequential enzymatic treatment is more effective than the pulsed-UV treatment in reducing the IgE binding of the peanut protein extract.

The results in Fig. 2 are in good agreement with those in Fig. 1. Because Lys and Arg are the cleavage sites of trypsin, while Phe, Trp and Tyr are the cleavage sites of α -chymotrypsin, the allergenicities of peptides produced by trypsin and α -chymotrypsin hydrolysis, should be different because they have different amino acid sequences and structure, which play a critical role in allergen stability and IgE binding (Sen et al., 2002). The results indicate that ultrasound treatment might cleave the protein chains at some epitopes, and the sequential hydrolysis of the roasted peanut protein with two enzymes should cleave more peptide bonds than the single enzyme and produce peptides with fewer epitopes and thus reduce allergenicity.

4. Conclusion

This study demonstrated that ultrasound treatment has significantly enhanced the efficiency of enzyme treatment, to reduce the allergens in roasted peanuts. Ultrasonic treatments not only increased the amount of soluble proteins, but also reduced two major peanut allergens Ara h 1 and Ara h 2 levels in peanuts and resulted in a certain inhibition on IgE-binding. Both blanching and ultrasonic pre-treatment showed some enhancement on Ara h 1 and Ara h 2 reduction, but through different mechanisms. Blanching may dissolve small molecules such as Ara h 2 and ultrasonication may reduce the original allergens by molecular destruction due to shear force. The combination of ultrasound treatment and sequential enzymatic treatment of roasted peanut kernels, almost completely removed Ara h 1 and Ara h 2, and significantly reduced IgE binding of the peanut extracts. The ultrasound–enzyme

treatment may preserve more flavouring compounds than the blanching–enzyme treatment due to a lower temperature. The current study provides a post-harvest approach to reduce allergens in roasted peanuts. However, the peptide composition of hydrolysates needs to be studied and the allergenicity of treated peanuts needs to be tested with peanut allergic human individuals.

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