



Enzymatic treatment of peanut kernels to reduce allergen levels

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ABSTRACT

This study investigated the use of enzymatic treatment to reduce peanut allergens in peanut kernels as affected by processing conditions. Two major peanut allergens, Ara h 1 and Ara h 2, were used as indicators of process effectiveness. Enzymatic treatment effectively reduced Ara h 1 and Ara h 2 in roasted peanut kernels by up to 100% under optimal conditions. For instance, treatment of roasted peanut kernels with α -chymotrypsin and trypsin for 1–3 h significantly increased the solubility of peanut protein while reducing Ara h 1 and Ara h 2 in peanut kernel extracts by 100% and 98%, respectively, based on ELISA readings. Ara h 1 and Ara h 2 levels in peanut protein extracts were inversely correlated with protein solubility in roasted peanut. Blanching of kernels enhanced the effectiveness of enzyme treatment in roasted peanuts but not in raw peanuts. The optimal concentration of enzyme was determined by response surface to be in the range of 0.1–0.2%. No consistent results were obtained for raw peanut kernels since Ara h 1 and Ara h 2 increased in peanut protein extracts under some treatment conditions and decreased in others.

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1. Introduction

Peanut is a very nutritious and economically important crop worldwide. It consists mainly of oil (44–56%) and protein (22–30%), and represents a good source of energy and protein, especially in developing countries. Upon roasting, peanuts produce a very distinctive and pleasant flavour. Therefore, roasted peanuts are widely used in many food products to confer the pleasant flavour or for the purpose of protein fortification (Lusas, 1979). However, consumption of peanuts or peanut containing food products can cause severe and even fatal allergenic reactions to many individuals, particularly, children under 5 years old. About 0.8% of children and 0.6% of adults in the United States are allergic to peanuts (Sampson, 2004). The prevalence of peanut allergy is on the rise due to causes that are not well understood (Grundy et al., 2001; Sicherer, Munoz-Furlong, & Sampson, 2003). While avoidance is the best way to guard against manifestation of allergy, the ubiquitous use of peanuts and peanut products by the food industry makes it very hard for allergic individuals to avoid accidental ingestion. Studies suggest that up to 75% of individuals with known peanut allergy experience reactions caused by accidental exposure (Kagan et al., 2003). The latter accounts for about 59%

of the reported allergy related deaths (Sicherer & Sampson, 2007). According to Sicherer and Sampson (2007), objective symptoms for many individuals occur from ingestion of about 1–3 peanut kernels, with the exception of extremely sensitive individuals who showed allergic symptoms for ingesting as little as 0.1 mg peanut protein. Therefore, it would be useful to reduce the level of allergens in peanuts that are mixed with other food ingredients as to protect consumers from potential life threatening allergic reactions among the majority of peanut allergic consumers.

Numerous studies have been conducted to characterise the specific proteins responsible for peanut allergy. To date, 11 such proteins (Ara h 1 through h 11) from peanut have been recognised by the Allergen Nomenclature Sub-committee of the International Union of Immunological Societies (ANSCUIS, 2009). Among these allergens, Ara h 1 (63.5 kDa) and Ara h 2 (16–17 kDa) are major peanut proteins that accounts for 12–16% and 5.9–9.3% of the total peanut protein, respectively. Individual content of these allergens in peanuts of different varieties is similar regardless where such peanuts are grown (Koppelman et al., 2001). Furthermore, more than 95% of peanut allergic individuals in the USA have specific IgE to Ara h 1 and Ara h 2 (Scurlock & Burks, 2004). However, Ara h 2 was found to be a more potent allergen than Ara h 1 (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004; Palmer et al., 2005). The remaining allergens vary in their sensitising rates up to 50% for some (e.g., Ara h 3, and h 4) (Wen, Wysocki, Decory, & Durst, 2007) while others have weak or unknown allergenic potential.

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Various efforts have also targeted reduction of the level of allergens or reduction of the allergenic potential of plant proteins. For instance, researchers are trying to reduce the allergenicity of peanuts through genetic mutations such as gene suppression, gene silencing and site-directed mutagenesis (Bannon et al., 2001; Chu et al., 2008; Herman, Helm, Jung, & Kinney, 2003), conventional breeding (Guo, Liang, Chung, Holbrook, & Maleki, 2008) and processing (Cabanillas et al., 2009; Chung & Champagne, 2009; Mondoulet et al., 2005). A recent study suggests that the majority of proteins in peanut, including the allergens become less soluble with boiling, frying, and roasting, which leads to a reduction in the extractable levels of the allergens (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010).

Enzymatic processing is another approach to reducing/eliminating allergenicity of some foods. A successful example of enzymatic removal of food allergens is the production of hypoallergenic rice by a two-stage enzymatic proteolysis process using actinase that produces hypoallergenic rice grains while retaining an acceptable texture (Watannbe, 1993). Other examples of enzyme treatment to remove allergens include producing hypoallergenic whey protein hydrolysate (Jost, Meister, & Monti, 1991) and psyllium seed husk (Nielsen, 1993) using proteases. Despite these successful examples of processing for hypoallergenicity, many studies have shown that food allergens are commonly resistant to gastric digestion. Peanut allergens, particularly, Ara h 2 and lower molecular weight peanut allergens, had unusually high stability to denaturation and proteolysis (Astwood, Leach, & Fuchs, 1996). Ara h 2 also functions as a trypsin inhibitor and roasting enhanced this function (Maleki et al., 2003). This suggests that processing may be more effective than previously thought if it incorporates a specific sequence of steps that enhance allergen reduction or degradation.

The objectives of this study were to (1) investigate the effectiveness of different endopeptidases under different processing conditions on the reduction/degradation of two major peanut allergens (Ara h 1 and Ara h 2) used as indicators of process effectiveness, and (2) determine the optimum conditions for different enzymatic treatments to achieve maximum reduction in indicator peanut allergens.

2. Materials and methods

2.1. Materials

Both raw and light 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, ≥ 40 units/mg protein, from bovine pancreas) and trypsin (EC 3.4.21.4, $\geq 10,000$ units/mg protein, from bovine pancreas), were purchased from Sigma–Aldrich (St. Louis, MO). Chicken anti-Ara h 1 and anti-Ara h 2 antibodies were provided by Dr. Maleki (Schmitt, Cheng, Maleki, & Burks, 2004; Schmitt et al., 2010), while peroxidase labelled anti-chicken antibody was purchased from Sigma–Aldrich (St. Louis, MO). Enzyme substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic) (ABTS) solution was purchased from KPL Inc. (Gaithersburg, MD).

2.2. Tryptic and alpha-chymotryptic enzyme treatments

Both raw and roasted peanut kernels were used in the study. Batches from each type of peanut kernels were divided into two portions, one of which were blanched for 5 min in boiling water before enzyme treatment, and second were treated with enzyme solution without blanching. The purpose of blanching was to inactivate bacteria and other microorganisms as well as to loosen/soft-

en the kernel structure for enzyme penetration/activity. Blanched kernels were drained and cooled to room temperature, then transferred into plastic containers. Fifty millilitres of enzyme solutions containing 0%, 0.01%, 0.05%, 0.10% or 0.15% (w/w) of α -chymotrypsin or trypsin or 1:1 mixture of the two enzymes were added to individual flasks containing 25 g of peanut kernels (dry weight). The pH of enzyme solutions was adjusted to about the optimal pH for each enzyme, pH 6.8–7.0 for α -chymotrypsin and trypsin. Neutral pH was used because it is close to the optimal pH of these enzymes (pH 7.8) and would be more suitable for processing as it prevents equipment from the corrosive damage associated with low/high pH. The containers were then capped and incubated at 37 °C for 1, 3 and 5 h, respectively. The treated peanut kernels were drained and dried overnight at 65–70 °C in a vacuum oven to remove water and to inactivate enzymes. Dry kernels were ground into fine flour using a high speed blender. Dry flour samples were labelled and stored at 4 °C until used.

2.3. Extraction of peanut protein

One gram of each type of peanut flour was mixed with 20 ml of Tris–HCl buffer (pH 8.3) and stirred at room temperature for 1 h. Mixtures were then centrifuged at 3000g for 20 min. The lipid layer on the top of supernatant was removed using transfer pipettes and supernatant was stored at –20 °C for further analysis. Pellets were mixed with standard electrophoresis sample treatment buffer (pH 6.8, Tris buffer containing glycerol, 1% SDS and 5 mM dithiothreitol) and boiled for 5 min in a water bath to extract proteins that were not soluble in Tris buffer. The extracts were centrifuged at 5500g for 15 min and the supernatants were used in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western Blot analysis.

2.4. Determination of soluble protein in extracts

Soluble protein in each extract (including control: extract from untreated raw or roasted peanuts) was determined by BCA method using analytical grade BSA as standard. BCA kit was purchased from PIERCE (Rockford, IL). Peanut protein extracts were diluted 5–10 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 extract was expressed as mg protein/ml extract. All samples were run with their respective controls for validation.

2.5. ELISA determination of Ara h 1 and Ara h 2

In this study, Ara h 1 and Ara h 2 concentrations in peanut crude protein extracts were quantified by an indirect ELISA. The ELISA protocol used chicken anti-Ara h 1 and anti-Ara h 2 antibodies as primary antibodies and peroxidase labelled anti-chicken antibodies as detection antibodies. Standard curves for Ara h 1 and Ara h 2 were developed using purified Ara h 1 and Ara h 2. Purified peanut protein extracts were serially diluted in PBS (pH 7.4) prior to use in ELISA assays to ensure the absorbance readings for both Ara h 1 and Ara h 2 fall into the linear ranges of the standard curves (0.01–0.25 ppm for both Ara h 1, and Ara h 2). A Costar 96-well polystyrene microplate (Fisher Scientific, Atlanta, GA) was coated with 100 μ l per well of diluted peanut protein extracts or purified Ara h 1 and Ara h 2. Untreated peanut protein extract was used as reference control while known concentrations of purified Ara h 1 and Ara h 2 were used as positive controls for data validation. The plate was incubated at 37 °C for 1 h. The plate was washed four times with PBST using a Microplate Washer (Bio-Tek, Winooski, VT), then blocked with 0.5% non-fat dry milk in PBST for 30 min at 37 °C. After blocking, the plate was washed four times and

1:5000 diluted (in PBS) chicken anti-Ara h 1 or anti-Ara h 2 was added to each well (100 µl/well). The plate was incubated for 1 h at 37 °C. After washing four times with PBST, 100 µl of horseradish peroxidase labelled anti-chicken antibody (1:20,000 diluted in PBS) was added to each well. The plate was then incubated for 1 h at 37 °C and washed four times with PBST. 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 808_{IU} 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 expressed as mg Ara h 1 or Ara h 2/g soluble protein.

Levels of allergen reductions achieved by the most effective treatment were further confirmed with sandwich ELISA using ELISA kits purchased from Indoor Biotechnologies (Charlottesville, VA).

2.6. SDS-PAGE and Western Blot analysis

2.6.1. SDS-PAGE

Protein/peptide profile in soluble fractions of roasted peanuts with and without enzyme treatment was resolved by SDS-PAGE in 4–15% polyacrylamide gel (15 × 10 cm) using a Bio-Rad Mini Protein III system (Bio-Rad, Hercules, CA). Peanut extract was mixed with 2× sample treatment buffer (pH 6.8, Tris buffer containing glycerol, SDS and dithiothreitol) to a total protein concentration of 1 mg/ml and heated at 90 °C for 10 min. After cooling to room temperature, samples were loaded onto the gel at 5 µl/well (5 µg of protein per well). 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). Gels were stained with Coomassie brilliant blue R-250 solution (Bio-Rad) for 4–5 h or overnight. After destaining, the gels were photographed with a Gel Doc XR imaging system (Bio-Rad, Hercules, CA). Protein bands corresponding to Ara h 1 and Ara h 2 were identified based on bands of purified Ara h 1 and Ara h 2.

2.6.2. Western Blot analysis

The proteins in the soluble and insoluble fractions of roasted peanuts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) as described above. For Western Blotting, the proteins were transferred to a PVDF membrane. Membranes were pre-blocked for 1 h at room temperature (RT) in 5% blotto (5% non-fat dry-milk dissolved in PBST). Chicken anti-raw Ara h 1 (1:5000) and chicken anti-raw Ara h 2 (1:8000) were diluted in 5% blotto and incubated with the PVDF membrane for 1 h at RT. Both antibodies have been shown to recognise both raw and thermally processed forms of these proteins. Serum IgE binding was performed in a similar way, with blocking at RT for 30 min in 2% blotto and then incubating with pooled sera from 15 peanut allergic individuals diluted 1:30 and incubated with membranes for 2 h at RT. The horseradish peroxidase-labelled anti-chicken IgY (1:100,000) was diluted in 2% blotto and incubation time was 30 min at RT. Following secondary antibody incubation, membranes were washed extensively with PBS containing 0.05% Tween-20 (PBST) and incubated with ECL substrate according to the manufacturer's instructions. The chemiluminescence was then measured using a FUJIFILM Luminescent Image Analyzer LAS-1000 (Fuji Photo Film Co., Ltd., Duluth, GA).

2.7. Experiment design

A four factor-factorial design was used for the treatment of peanut kernels. The factors were type of proteases (α -chymotrypsin, trypsin, and 1:1 combination of α -chymotrypsin and trypsin), en-

zyme concentration (0.01%, 0.05%, 0.1% and 0.15%), enzyme treatment time (1, 3 and 5 h), and pretreatment of peanut kernels (blanching and non-blanching). Response surface methodology (SAS, 2002) was used to predict regions where experimental conditions yield lowest concentrations of Ara h 1 and Ara h 2. Analysis of variance (SAS, 2002) was used to evaluate the effect of treatment factors on dependent variables such as protein solubility and concentrations of Ara h 1 and Ara h 2. Mean differences were judged at the 5% significance level.

3. Results and discussions

3.1. Effects of α -chymotrypsin on the allergens of raw peanut kernel

Table 1 summarises the effects of α -chymotrypsin concentration, treatment time and blanching, on protein solubility, and extractable Ara h 1 and Ara h 2 concentrations in raw peanut kernels. The protein solubility decreased for both non-blanching and blanching peanuts, most likely due to the heat treatment during blanching and drying at 65 °C to inactivate the enzyme and to remove moisture (Schmitt et al., 2010).

Besides low solubility, treatment of raw peanut kernels with low concentrations (0.01–0.05%) of α -chymotrypsin was found to increase soluble allergens in blanching peanut kernels (Table 1). Both detectable Ara h 1 and Ara h 2 and fragments increased in blanching kernels treated with α -chymotrypsin at 0.05% or lower. However, at 0.1% enzyme or higher, both detectable Ara h 1 and Ara h 2 or fragments in blanching peanut kernels decreased with Ara h 1 showing more resistance to enzyme activity. The increase of detectable Ara h 1 and Ara h 2 at low enzyme concentration might be caused by blanching, which may caused partial unfolding of protein molecules and potentially exposed more binding sites to the anti-Ara h 1/Ara h 2 antibodies used for allergen detection. Furthermore, Ara h 2 in raw peanut may have served as trypsin inhibitor lowering the impact of enzyme treatment (Maleki et al., 2003).

Based on response surface modelling, the optimum enzyme concentration and treatment time for reduction of Ara h 1 in raw peanut kernels were predicted to be 0.12% α -chymotrypsin and 1–3 h of incubation, respectively (Fig. 1a). The optimum conditions for Ara h 2 reductions tended to be in the higher enzyme concentration and longer treatment time region (Fig. 1b). To maximise the reduction of Ara h 1 and Ara h 2 in raw peanut kernels, higher enzyme concentration may be needed without blanching step. The low efficiency of α -chymotrypsin against Ara h 1 and Ara h 2 in raw peanut kernels may be explained by the presence of protease inhibitors as described by Maleki et al. (2003) who found that Ara h 2 purified from raw peanuts functioned as a trypsin inhibitor. Since α -chymotrypsin is structurally very similar to trypsin, the activity of α -chymotrypsin might be also inhibited by Ara h 2.

3.2. Effects of α -chymotrypsin on allergens in roasted peanut kernel

Table 2 summarises the results of treatment of blanching and non-blanching roasted peanut kernels with α -chymotrypsin solution. As expected, the protein solubility of roasted peanut was significantly lower than that of raw peanut (Chiou & Tsai, 1989; Kopper et al., 2005; Schmitt et al., 2010). Upon treatment with α -chymotrypsin solution, the protein solubility increased, but the detectable/extractable levels of Ara h 1 and Ara h 2 decreased in both blanching and non-blanching peanuts. At the same treatment time, higher enzyme concentration resulted in higher reductions in soluble allergen levels. At α -chymotrypsin concentration of 0.01%, Ara h 1 and Ara h 2 reductions were 63–82% and 68–82%,

Table 1
Effects of pretreatment (blanching), α -chymotrypsin concentration, and treatment time on protein solubility and extractable Ara h 1/h2 concentrations in raw peanut kernels.^a

Enzyme concentration	Time (hours)	Non-blanching raw peanuts					Blanched raw peanuts				
		Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change	Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change
Control (untreated)		7.03	3.75	N/A	0.98	N/A	7.03	3.26	N/A	0.98	N/A
0.01%	1	7.02	3.60 ± 0.14	-4.08	0.51 ± 0.04	-47.69	4.48	5.58 ± 0.11	61.78	3.01 ± 0.13	206.83
	3	6.28	4.09 ± 0.18	8.98	0.59 ± 0.06	-39.95	4.75	5.70 ± 0.29	65.23	2.65 ± 0.16	170.89
	5	6.05	3.97 ± 0.16	5.96	0.49 ± 0.07	-49.65	5.08	4.97 ± 0.29	44.08	2.18 ± 0.24	122.46
0.05%	1	6.99	2.96 ± 0.18	-21.14	0.28 ± 0.03	-71.13	5.05	3.97 ± 0.27	15.15	1.75 ± 0.16	78.55
	3	6.80	3.03 ± 0.29	-19.17	0.27 ± 0.04	-72.05	4.92	4.66 ± 0.57	34.75	1.93 ± 0.05	96.95
	5	6.34	3.20 ± 0.21	-14.79	0.25 ± 0.02	-74.87	4.77	4.31 ± 0.20	24.9	2.01 ± 0.06	105.22
0.10%	1	6.82	2.82 ± 0.23	-24.75	0.26 ± 0.06	-72.99	5.25	3.03 ± 0.31	-12.27	1.03 ± 0.02	5.37
	3	6.70	3.03 ± 0.28	-19.18	0.25 ± 0.07	-74.04	5.27	3.29 ± 0.20	-4.7	1.31 ± 0.06	33.86
	5	6.33	3.10 ± 0.18	-17.43	0.23 ± 0.07	-76.91	5.15	2.74 ± 0.14	-20.56	1.20 ± 0.03	22.91
0.15%	1	6.49	2.85 ± 0.27	-24.11	0.23 ± 0.03	-76.58	6.13	1.99 ± 0.14	-42.29	0.51 ± 0.01	-48.16
	3	6.80	2.32 ± 0.18	-38.05	0.17 ± 0.02	-82.82	6.34	2.04 ± 0.16	-40.87	0.62 ± 0.04	-36.71
	5	6.41	3.25 ± 0.18	-13.42	0.23 ± 0.02	-76.06	6.41	2.31 ± 0.13	-32.99	0.60 ± 0.08	-38.77

^a Soluble protein concentration as measured by BCA method, extractable Ara h 1 and Ara h 2 concentration as measured by ELISA.

respectively, in non-blanching peanuts. When the concentration of α -chymotrypsin solution was increased to 0.15%, detectable Ara h 1 and Ara h 2 was decreased by 91–95% and 92–93%, respectively.

Chymotrypsin treatment of blanching, roasted peanuts resulted in reduction of detectable soluble allergens by as much as 100%. In fact, Ara h 1 and Ara h 2 levels in blanching peanut kernels were non-detectable by the indirect ELISA method at enzyme concentration of 0.1–0.15%. The optimum enzyme treatment conditions for roasted peanut kernels consists of 5 min of blanching prior to incubation of peanut kernels in 0.12% enzyme for 3 h as shown by response surface in Fig. 2. Under these conditions, Ara h 1 and Ara h 2 were not detectable in soluble peanut protein extracts (Table 2).

It is possible that Ara h 1 and Ara h 2 in roasted peanuts are more sensitive to α -chymotrypsin than those in raw peanuts and that blanching enhanced the efficiency of enzymatic treatment. Roasting may enhance the digestive efficiency of α -chymotrypsin due to the destruction of enzyme inhibitors (Norioka, Omichi, & Ikenaka, 1982; Perkins & Toledo, 1982) and increased hydrophobicity of peanut protein as evidenced by the decreased concentrations of soluble proteins in untreated roasted peanuts. Because the binding pocket of α -chymotrypsin is hydrophobic (Whitaker, 2003), thermal treatment of peanut protein might expose specific hydrophobic amino acid residues, such as tyrosine, phenylalanine and tryptophane and peptide bonds to the pocket stereospecifically for hydrolysis.

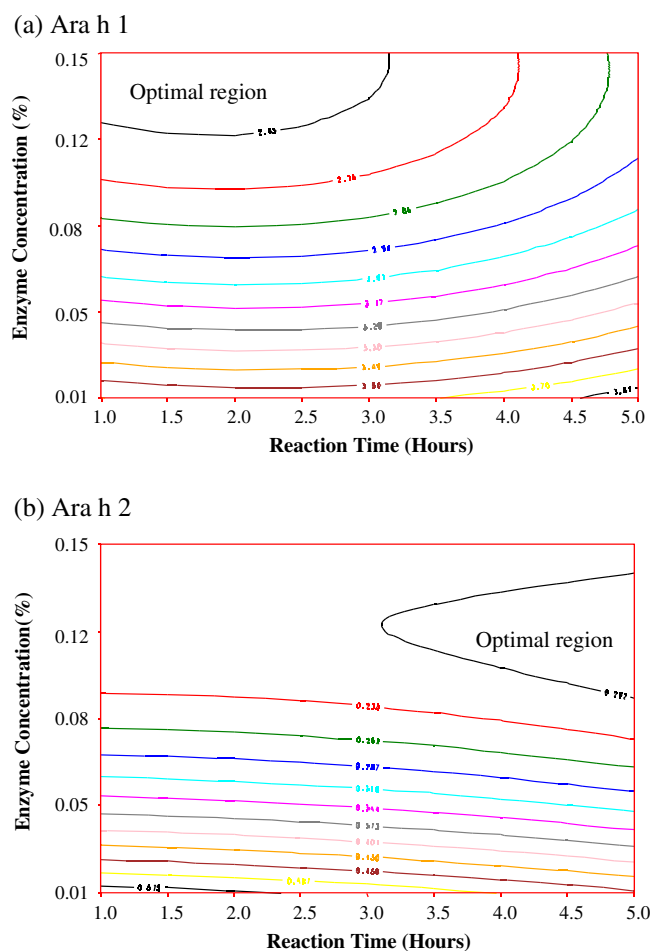


Fig. 1. Response surface showing changes in detectable concentrations of (a) Ara h 1 and (b) Ara h 2 in non-blanching raw peanut kernels as a function of α -chymotrypsin concentration and incubation time.

Table 2
Effects of pretreatment (blanching), α -chymotrypsin concentration, and treatment time on protein solubility and extractable Ara h 1/h 2 concentrations in roasted peanut kernels.^a

Enzyme concentration	Time (hours)	Non-blanch roasted peanuts					Blanched roasted peanuts				
		Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change	Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change
Control (untreated)	0	2.30	3.94 ± 0.47	N/A	1.49 ± 0.06	N/A	2.03	3.94 ± 0.47	N/A	1.49 ± 0.06	N/A
	1	4.1	0.91 ± 0.03	-62.74	0.47 ± 0.03	-68.54	2.58	0.41 ± 0.01	-89.55	0.28 ± 0.09	-81.2
	3	4.05	0.52 ± 0.02	-78.8	0.33 ± 0.02	-77.91	2.79	0.57 ± 0.08	-85.59	0.26 ± 0.05	-82.6
	5	4.21	0.41 ± 0.03	-83.17	0.27 ± 0.01	-81.93	2.74	1.23 ± 0.15	-68.75	0.30 ± 0.06	-79.8
	1	3.49	0.26 ± 0.04	-89.28	0.29 ± 0.07	-80.59	3.96	0.25 ± 0.06	-93.72	0.05 ± 0.04	-97.9
0.05%	3	3.62	0.13 ± 0.01	-94.66	0.23 ± 0.01	-84.61	4.2	0.08 ± 0.06	-98.06	0.03 ± 0.02	-97.9
	5	3.53	0.22 ± 0.02	-90.77	0.19 ± 0.01	-87.28	3.86	0.09 ± 0.04	-97.74	0.01 ± 0.01	-99.6
	1	3.79	0.19 ± 0.02	-92.09	0.11 ± 0.01	-92.64	4.14	0.07 ± 0.04	-98.28	0.00 ± 0.00	-100.0
	3	4.07	0.12 ± 0.03	-95.02	0.12 ± 0.01	-91.90	4.12	0.02 ± 0.03	-99.53	0.00 ± 0.02	-99.96
	5	3.79	0.14 ± 0.01	-94.05	0.09 ± 0.00	-93.98	4.03	0.00 ± 0.00	-100.00	0.00 ± 0.00	-100.0
0.15%	1	3.91	0.14 ± 0.03	-94.15	0.12 ± 0.02	-91.97	4.91	0.00 ± 0.00	-100.00	0.00 ± 0.00	-100.0
	3	3.7	0.12 ± 0.02	-95.13	0.12 ± 0.00	-91.97	4.56	0.00 ± 0.00	-100.00	0.00 ± 0.00	-100.0
	5	4.07	0.23 ± 0.05	-90.57	0.11 ± 0.03	-92.64	4.85	0.00 ± 0.00	-100.00	0.00 ± 0.00	-100.0

^aSoluble protein concentration as measured by BCA method, extractable Ara h 1 and Ara h 2 concentration as measured by ELISA.

3.3. Effects of trypsin treatment on Ara h 1 and Ara h 2 in raw and roasted peanut kernels

Similar to the treatment with α -chymotrypsin, trypsin treatment of raw peanut did not reduce Ara h 1 and Ara h 2 significantly. In both cases it is most likely due to more soluble overall protein and allergen, therefore more enzyme or longer incubation periods are needed to digest these levels of protein to the same level of allergens in roasted peanut extracts. In fact detectable levels of these allergens or fragments increased under all treatment conditions, especially, Ara h 2 in blanched raw peanuts. This is possibly due to digestion of Ara h 2 and enhanced exposure of peptide epitopes to antibody (Table 3). In contrast, trypsin treatment of roasted peanuts significantly reduced Ara h 1 and Ara h 2 while increasing the soluble proteins in both blanched and non-blanch soluble extracts (Table 4). Even at low trypsin concentration, the reduction of peanut allergens was significant and time dependent. However, at enzyme concentration of 0.05% or higher, treatment time had no significant effect, suggesting a fast and complete enzymatic process. The treatment of blanched roasted peanuts at low enzyme concentration (0.01%) led to 67–81% and 52–69% reduction in Ara h 1 and Ara h 2, respectively, or about double the levels observed in the soluble protein fractions obtained from non-blanch peanut samples. At 0.15%, trypsin treatment resulted in near complete digestion of detectable Ara h 1 in the soluble frac-

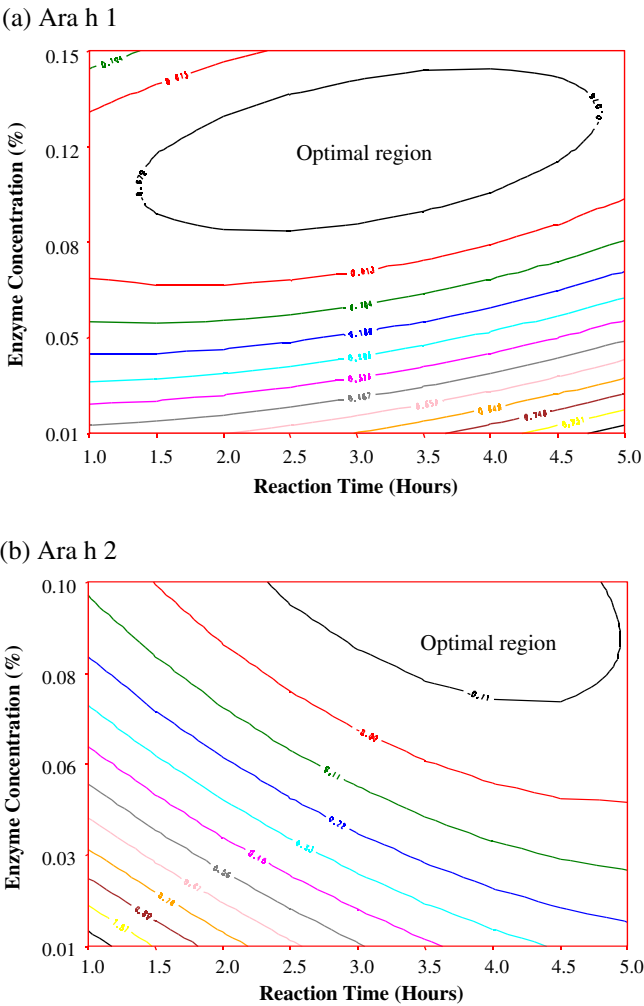


Fig. 2. Response surface showing changes in detectable concentrations of (a) Ara h 1 and (b) Ara h 2 in blanched roasted peanut kernels as a function of α -chymotrypsin concentration and incubation time.

Table 3Effects of pretreatment (blanching), trypsin concentration, and treatment time on protein solubility and extractable Ara h 1/h2 concentration in raw peanut kernels.^a

Enzyme concentration	Time (hours)	Non-blanching raw peanuts					Blanching raw peanuts				
		Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change	Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change
Control (untreated)	0	10.91 ± 0.12	5.19	N/A	0.82	N/A	10.91 ± 0.12	5.19	N/A	0.82	N/A
0.01%	1	10.87 ± 0.31	4.99	−3.33	0.79	−3.72	8.18 ± 0.19	5.66	9.15	1.26	54.06
	3	10.98 ± 0.28	5.15	5.13	0.86	−0.68	8.18 ± 0.04	5.75	10.82	1.31	60.70
	5	11.26 ± 0.28	5.15	−0.63	0.81	−0.68	8.57 ± 0.15	5.28	1.79	1.19	45.67
0.05%	1	10.89 ± 0.03	5.47	5.47	1.41	72.83	8.63 ± 0.21	4.8	−7.44	0.90	10.45
	3	10.611 ± 0.43	5.31	2.37	1.21	48.39	8.36 ± 0.18	5.52	6.37	0.97	18.76
	5	10.243 ± 0.03	5.9	13.69	1.15	40.92	7.96 ± 0.14	4.82	−7.00	1.19	46.30
0.10%	1	10.437 ± 0.55	5.84	12.48	1.35	65.44	8.11 ± 0.05	4.82	−7.02	1.04	27.78
	3	10.827 ± 0.37	5.56	7.23	1.4	72.30	8.23 ± 0.09	4.41	−15.09	1.12	37.74
	5	11.120 ± 0.57	5.14	−0.84	1.16	41.87	8.46 ± 0.01	4.2	−18.98	1.03	26.53
0.15%	1	10.838 ± 0.38	5.83	12.45	1.54	89.10	8.13 ± 0.16	4.28	−17.54	1.07	31.08
	3	10.513 ± 0.50	5.26	1.38	1.1	35.53	8.66 ± 0.01	5.39	3.97	1.32	61.51
	5	9.138 ± 0.06	4.52	−12.86	0.88	7.74	8.74 ± 0.10	5.78	11.38	1.67	104.57

Note: Raw peanuts used in trypsin treatment were different batches from those used in α -chymotrypsin treatment. Therefore, the initial soluble protein, Ara h 1 and Ara h 2 were different from that in Table 1.^a Soluble protein concentration as measured by BCA method, extractable Ara h 1 and Ara h 2 concentration as measured by ELISA.**Table 4**Effects of pretreatment (blanching), trypsin concentration, and treatment time on protein solubility and extractable Ara h 1 and Ara h 2 concentrations in roasted peanut kernels.^a

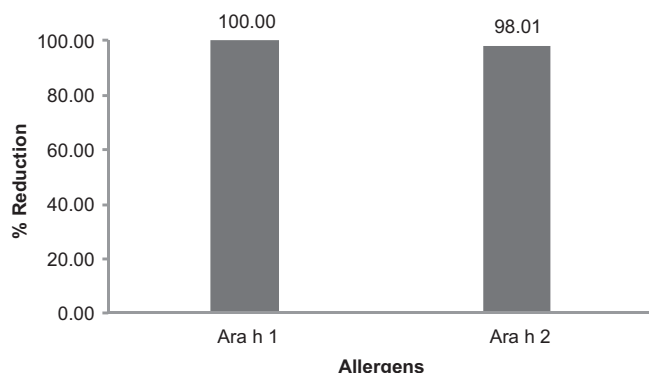
Enzyme concentration	Time (hours)	Non-blanching peanuts					Blanching peanuts				
		Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change	Soluble protein (mg/ml)	Ara h 1 (mg/g protein)	% Ara h 1 change	Ara h 2 (mg/g protein)	% Ara h 2 change
Control (untreated)	0	3.86	3.71		1.21		4.86	3.83		1.18	
0.01%	1	3.94	2.02	−43.92	0.81	−33.69	4.53	1.24	−67.54	0.57	−51.73
	3	3.79	2.23	−39.95	0.73	−40.04	5.37	1.02	−73.45	0.44	−62.98
	5	3.71	1.83	−50.62	0.47	−61.39	4.68	0.74	−80.80	0.36	−69.30
0.05%	1	5.14	0.35	−90.70	0.08	−93.84	6.97	0.03	−99.93	0.10	−91.49
	3	5.32	0.37	−90.04	0.06	−95.33	7.33	0.01	−99.84	0.08	−93.18
	5	5.25	0.36	−90.03	0.08	−93.84	6.88	0.06	−98.54	0.11	−90.88
0.10%	1	5.57	0.29	−92.30	0.06	−94.72	7.86	0.00	−100.00	0.03	−97.93
	3	6.00	0.26	−93.08	0.06	−94.72	6.88	0.00	−100.00	0.04	−96.27
	5	5.79	0.28	−92.54	0.07	−94.05	7.74	0.00	−100.00	0.04	−96.32
0.15%	1	6.28	0.00	−100.00	0.06	−95.22	7.34	0.00	−100.00	0.07	−93.63
	3	6.36	0.00	−100.00	0.04	−96.73	7.65	0.00	−100.00	0.07	−94.44
	5	6.43	0.00	−100.00	0.06	−95.22	7.49	0.00	−100.00	0.08	−93.33

^a Soluble protein concentration as measured by BCA method, extractable Ara h 1 and Ara h 2 concentrations as measured by ELISA.

Table 5Synergistic effect of α -chymotrypsin (E1) and trypsin (E2) on the reduction of Ara h 1 and Ara h 2 in roasted peanuts at low individual enzyme concentration.^a

Treatment type	Soluble protein (mg/ml)	Ara h 1 (μ g/g protein)	% Ara h 1 changes	Ara h 2 (μ g/g protein)	% Ara h 2 change
Untreated	3.441	3.532		1.371	
E1	5.195	0.027	–99.27	0.153	–88.38
E1/E2 = 90/10	5.527	0.015	–99.59	0.135	–89.72
E1/E2 = 75/25	4.742	0.001	–99.97	0.169	–87.17
E1/E2 = 50/50	5.089	0.018	–99.51	0.131	–90.02
E1/E2 = 25/75	5.200	0.001	–99.97	0.154	–88.27
E1 = E2 = 10/90	5.233	0.061	–98.34	0.174	–86.76
E2	5.525	0.17	–95.31	0.26	–80.25

Note: Total enzyme concentration = 0.05%, incubation time = 3 h at 37 °C.

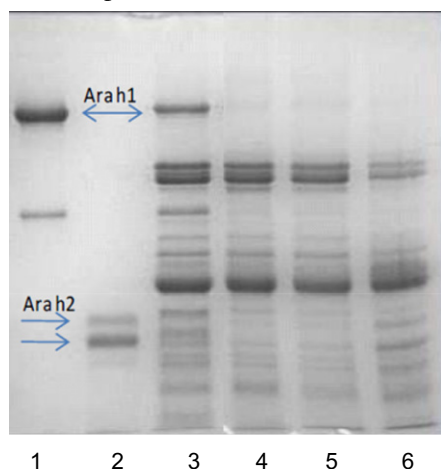
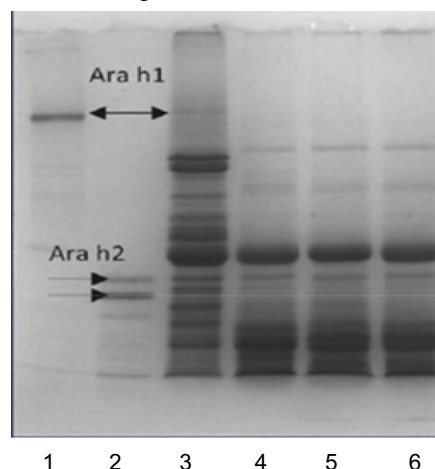
^a Soluble protein concentration as measured by BCA method, extractable Ara h 1 and Ara h 2 concentrations as measured by direct ELISA.**Fig. 3.** Sandwich ELISA results confirming percent reduction of Ara h 1 and Ara h 2 in roasted peanut extracts following enzymatic treatment (chymotrypsin + trypsin).

tion of both blanched and non-blanched peanuts, and over 94% reduction of Ara h 2. As with α -chymotrypsin, it seems that blanching enhanced the efficiency of enzymatic activity on Ara h 1 and Ara h 2 allergens in peanut kernels.

3.4. Combined α -chymotrypsin and trypsin treatment

Although both trypsin and α -chymotrypsin are serine peptidases with the same optimum pH (pH 7.8–8.0), the cleavage sites

of two enzymes slightly differ. While α -chymotrypsin hydrolyses peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, and Met) on the carboxyl end of the bond, trypsin hydrolyses peptide bonds only at lysyl and arginyl residues that furnish the carbonyl moiety of the peptide bond (Whitaker, 2003). Therefore, they may hydrolyse allergenic proteins in a complementary manner. To test this potential synergistic enhancement of digestibility, 0.05% total enzyme solutions with different α -chymotrypsin to trypsin ratio were used to treat peanut kernels. Significant enzyme synergy was observed for Ara h 2 at chymotrypsin/trypsin ratio of 1:1 but not for Ara h 1 (Table 5). However, α -chymotrypsin reduced Ara h 1 more effectively than trypsin and seems to remain as efficient against Ara h 2 at the lower level in the 1:1 ratio. This may be explained by the similarity and difference of mechanisms of trypsin and chymotrypsin, as well as the structure of Ara h 1 (Maleki, Chung, Champagne, & Raufman, 2000a) and Ara h 2. A 10-kDa fragment obtained from gastrointestinal (GI) digestion of native Ara h 2 was reported to contain 11 potential chymotrypsin cleavage sites that are protected from the enzyme action by the compact structure of the protein (Bannon, Fu, Kimber, & Hinton, 2003). The denaturation or partial denaturation of protein caused by roasting and blanching steps may expose some of these sites for α -chymotrypsin cleavage thereby lowering detectable levels of Ara h 2 in kernel extracts as shown in Table 5. Thermal effect on non-protein components of the peanut kernel may also have played a role in facilitating enzymatic activity. To confirm the above findings, extracts of roasted peanuts treated with 1:1 trypsin

(a) Raw peanuts**(b) Roasted peanuts****Fig. 4.** SDS–PAGE of soluble fractions of raw and roasted peanut protein extracts from enzymatically treated and untreated peanut kernels. (a) Raw peanut extracts: lanes 1 and 2 = purified Ara h 1 and Ara h 2; lane 3 = untreated raw peanut; lanes 4–6 = raw peanut treated with 0.15% of E1, E2 and E1 + E2, respectively. (b) Roasted peanut extracts: lanes 1 and 2 = purified Ara h 1 and Ara h 2; lane 3 = untreated roasted peanut supernatant; lane 4–6 = roasted peanut treated with 0.15% of E1, E2 and E3, respectively (E1: α -chymotrypsin and E2: trypsin).

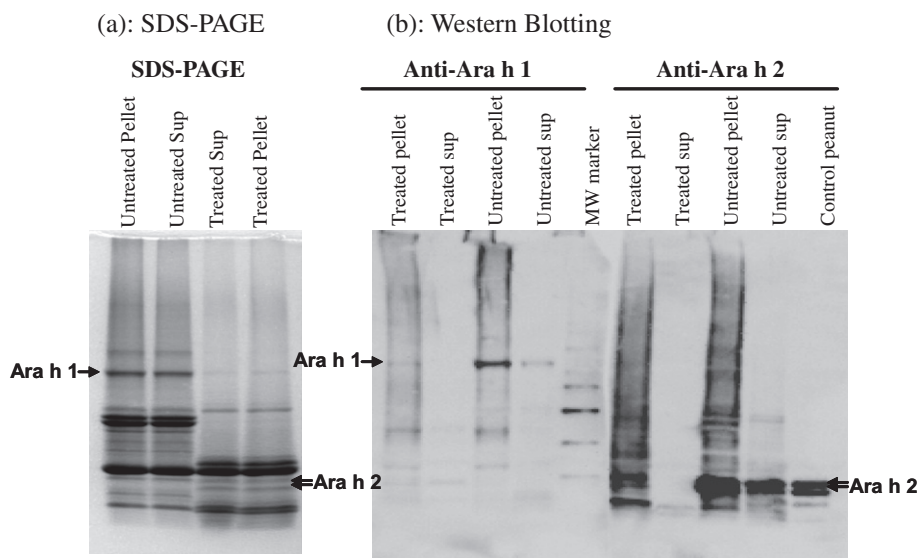


Fig. 5. Images of (a) SDS–PAGE and (b) Western Blot of peanut protein extracts and pellets from enzymatically treated and untreated roasted peanut kernels.

and α -chymotrypsin solution (at total concentration of 0.15%) and untreated peanuts were analysed by Sandwich ELISA which also revealed the absence of Ara h 1 and a 98% reduction of Ara h 2 in the soluble fraction of peanuts (Fig. 3).

3.5. SDA–PAGE of treated and untreated peanut extracts

The effectiveness of α -chymotrypsin and trypsin in hydrolysing Ara h 1 and Ara h 2 was further confirmed by SDS–PAGE and Western Blot of extracts from untreated and enzyme treated peanuts (Fig. 4).

The Ara h 1 band in untreated raw peanut extract (lanes 3, Fig. 4a) appears to be completely degraded while Ara h 2 band significantly faded away in the soluble protein fraction of the kernels treated with 0.15% α -chymotrypsin, trypsin or a mixture of the two enzymes (lanes 4–6, Fig. 4a).

Ara h 1 was significantly reduced in protein extracts from untreated roasted peanuts (lane 3, Fig. 4b) when compared to extract from raw peanut kernel although both samples were loaded on the gel at the same total protein concentration (1.0 mg/ml). In contrast, the two bands corresponding to Ara h 2 migration pattern were more visible on the gel (lane 3). The lower density of Ara h 1 band in untreated roasted peanuts could be the result of oligomerisation of Ara h 1 during roasting (Maleki et al., 2000b; Schmitt et al., 2010) which causes reduced extractability. After enzyme treatment, the Ara h 1 band disappeared from SDS–PAGE and Ara h 1 oligomers also decreased significantly. It appears as if the dual bands of Ara h 2 were only partially degraded because faint co-migrating bands still appeared in SDS–PAGE, although there were significantly reduced (lanes 4–6). This shows that the individual enzyme treatment protocol might be less effective in degrading Ara h 2 than Ara h 1. Fig. 4b also shows that the enzymatic degradation of Ara h 1 and Ara h 2 yielded mostly small protein fragments with MW less than 16 kDa.

3.6. Western Blotting of roasted peanut extracts using antibody binding

Fig. 5 shows the SDS–PAGE and Western Blots using specific anti-Ara h 1 and anti-Ara h 2 antibodies to detect the presence of these allergens in Tris buffer extracts and the pellets (solubilised in SDS-sample buffer) from enzyme treated (with both proteases)

and untreated roasted peanuts. Ara h1 and h2 mostly disappeared in soluble fraction of treated peanut kernels. Higher levels of Ara h 1 and higher molecular weight smears that are recognised by anti-Ara h 1 antibody appeared in both treated and untreated insoluble/pellet fractions. Although residual anti-Ara h 2 antibody binding activity was observed in treated pellet for Ara h 2, it was lower than that of the untreated peanut pellet. Higher molecular weight bands appeared in both pellets, which indicate that Ara h 2 or fragments may be forming oligomeric structures with other fragments or other proteins that are insoluble in aqueous solution. These results are in agreement with data in Tables 2 and 4, and further demonstrate that Ara h 2 is more resistant to protease digestion (Maleki et al., 2003; Sen et al., 2002). The results confirm that our enzyme treatment process degrades the allergens into smaller peptides which may or may not retain their IgE binding and other allergenic properties in the soluble fractions of treated whole roasted peanuts.

4. Conclusion

This study demonstrated that treatment of whole roasted peanuts with proteases such as chymotrypsin and trypsin effectively degraded extractable fraction of the two major peanut major peanut allergens Ara h 1 and Ara h 2. Blanching of roasted peanuts enhanced the efficiency of enzyme treatment. The enzymatic process developed in this study not only degraded extractable Ara h 1 and Ara h 2 but also increased the amount of soluble proteins. Accordingly, it is possible that the thresholds of lowest- and no-observed adverse-effect levels of enzyme-treated roasted peanut kernels might be significantly higher than those of untreated peanuts. Results from this study indicate the potential for producing peanuts with reduced allergenicity using post-harvest processing approaches such as food grade enzymatic treatment. However, the results presented in this paper were from *in vitro* tests only and, therefore, allergenic potential has to be tested *in vivo* to confirm reduced allergenicity in treated peanuts.

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