

Original article

Enzymatic treatment of peanut butter to reduce the concentration of major peanut allergens

Jianmei Yu,¹ Ipek Goktepe² & Mohamed Ahmedna^{3*†}

1 Department of Family and Consumer Sciences, North Carolina A&T State University, 1601 East Market Street, Greensboro, NC 27411, USA

2 College of Arts and Sciences, Qatar University, P.O. Box 2713, Doha, Qatar

3 Centre for Excellence in Post-Harvest Technologies, North Carolina Research Campus, 500 Laureate Way, Suite 4222, Kannapolis, NC 28081, USA

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Summary This study investigated the effects of enzymatic treatment of peanut butter on two-major peanut allergens (Ara h 1 and Ara h 2). Home-made and commercial peanut butter samples were treated with alpha-chymotrypsin, trypsin or the combination of these enzymes and incubated at room temperature for 24 h or at 37 °C for 3 h. Treated peanut butter samples were sampled weekly for evaluation of total soluble proteins and extractable Ara h 1/Ara h 2. Data show that 1:1 alpha-chymotrypsin: trypsin at 0.04% of enzyme-to-peanut butter ratio resulted in near complete reduction of extractable Ara h 1 and Ara h 2 respectively. Treatment of peanut butter with a combination of trypsin and alpha-chymotrypsin resulted in a decrease in IgE-binding, suggesting that enzymatic treatment has the potential to reduce the allergenicity. However, clinical tests are needed to confirm any reduction in allergenic potential. The amount of water used to disperse enzyme did not have significant effect on allergen reduction but affected the consistency and colour of treated products, especially when the amount of water added was above 5% of peanut butter weight.

Keywords Enzymatic treatment, peanut butter, peanut allergen reduction, α -chymotrypsin and trypsin.

Introduction

Peanut butter is one of the major food products made from peanuts and is a nutrient and energy-dense food. It is rich in unsaturated lipid, protein, fibre and many micronutrients, such as vitamin E, folate, potassium, magnesium and zinc (Özcan & Seven, 2003). Although peanuts and peanut butter have relatively high total fat contents, their fats consist predominantly of unsaturated fatty acids with a proportionately higher mono-unsaturated fatty acid (MUFA) than polyunsaturated fatty acid (PUFA) (Sanders, 1980). High MUFA diets effectively lower LDL-cholesterol without raising triacylglycerol or decreasing HDL concentrations (Kris-Etherton *et al.*, 1999; Sales *et al.*, 2008; Chiavaroli, 2010). Peanuts also contain several bioactive compounds including phytosterols, resveratrol, flavonoids and antioxidants. The health benefits of these phytochemicals are well documented and continue to be

studied and discovered. Furthermore, like all plant foods, peanuts and peanut butter are naturally cholesterol-free. Hence, consumption of peanuts and peanut butter has been associated with reduced heart diseases (O'Byrne *et al.*, 1997; Alper & Mattes, 2003; Kris-Etherton *et al.*, 2008) and cancer (Awad *et al.*, 2000; Yeh *et al.*, 2006).

However, not all consumers can enjoy the nutritious and flavourful peanut butter because of allergy to peanuts. In 2002, about 0.8% of young children and 0.6% of adults were reported to be allergic to peanuts in the United States (Sampson, 2004). The rate of allergy to peanut among US children nearly doubled between 2002 and 2008, reaching 1.4% in 2008 (Sicherer & Sampson, 2010). In Canada, the percentage of children allergic to peanuts also increased from 1.3% in 2000–2002 to 1.6% in 2005–2007 (Ben-Shoshan *et al.*, 2009). The health risk associated with peanut allergy is such that accidental ingestion of a small amount of any peanut product can produce lethal allergic reactions among hypersensitive individuals. For children under 5 years old, 10 mg of defatted peanuts caused subjective symptoms and 100 mg to 3 g caused

*Correspondent: E-mail: ahmedna@qu.edu.qa

†Current address: Mohamed Ahmedna, College of Arts and Sciences, Qatar University, PO Box 2713, Doha, Qatar

objective symptom. For the adults, the doses of defatted, roasted peanut flour that trigger subjective and objective symptoms were found to be 10 mg and 300 mg–3 g respectively (Sicherer & Sampson, 2007).

Research has demonstrated that peanut allergy was caused by the ingestion of allergenic peanut proteins. Among eleven allergenic proteins identified so far (ANSCUIS, 2009), Ara h 1 and Ara h 2 are major allergens that account for 12–16% and 5.9–9.3% of total peanut protein content, respectively, as quantified by Koppelman *et al.* (2001) using SDS-PAGE and densitometry methods. Furthermore, over 95% of peanut allergic individuals were found to have specific IgE-binding to Ara h 1 and Ara h 2 (Scurlock & Burks, 2004). Therefore, Ara h 1 and Ara h 2 represent good indicator of allergens for assessing the impact of processing and their elimination could significantly reduce the allergenic potential of peanuts and peanut products, thus increasing the amount (threshold) of peanut that could trigger serious allergic reactions among peanut allergic individuals. Peanut or peanut butter with reduced allergens can serve as a potential ingredient in functional foods because of its improved safety and high nutritional quality (Arvanityannis & Van Houwelingen-Koukaliaroglou, 2005).

Our previous study (Yu *et al.*, 2011) showed that treatment of roasted peanut kernels with food-grade endopeptidase significantly increased the solubility of peanut protein and reduced Ara h 1 and Ara h 2 in both soluble and non-soluble portions of peanuts. Peanut butter, although made from peanut, has more complicated composition than peanut kernel because of the inclusion of additives such as salt, sugar, molasses, soy protein and hydrogenated vegetable oil. Some of these ingredients may affect the proteolytic activity of α -chymotrypsin and trypsin. Therefore, this study evaluated the effectiveness of endopeptidase treatment in reducing allergenic proteins in peanut butter without additives and in some commercial peanut butter brands. The effects of amount of water, added to assist the enzyme dispersion, on the reduction of Ara h 1/Ara h 2 and the sensory quality (texture and colour) of peanut butter were also evaluated.

Materials and methods

Materials

Light roasted peanut kernels were purchased from Good Earth Peanut Company (Skippers, VA, USA). According to company's information, the peanuts were oil roasted. Commercial peanut butter samples were purchased from a local grocery store (Greensboro, NC, USA). Enzymes used for peanut butter treatment were α -chymotrypsin (EC3.4.21.1, ≥ 40 units mg^{-1} protein, from bovine pancreas) and trypsin (EC 3.4.21.4,

12,100 units mg^{-1} protein, from bovine pancreas). ELISA kits for Ara h 1 and Ara h 2 quantification were purchased from Indoor Biotechnologies (Charlottesville, VA, USA). Other reagents were peroxidase conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Streptavidin-Peroxidase and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) in 70 mM citrate buffer (pH 4.2) (Sigma-Aldrich, St. Louis, MO, USA). All these reagents were prepared according to the instruction of Indoor Biotechnologies. Tris Buffer solution (2 M) was purchased from Fisher Scientific (Atlanta, GA, USA), diluted to 20 mM and adjusted to pH 8.3 before use.

Enzymatic treatment of home-made peanut butter

In this study, peanut butter was made from roasted peanuts and vegetable oil without addition of sugar, salt or any other additives. Fifty gram of roasted peanuts were ground for 30 s using Miracle Mincer TR30 (Miracle Exclusives, Springfield, MO, USA), a small peanut butter maker, followed by addition of 5 mL of vegetable oil. The mixture was then ground for another 20 s to form a paste. An enzyme suspension (in 2.5 mL of neutralised water) was added and the mixture was then ground for about 20–30 s. The amount of enzyme added was pre-calculated to yield enzyme-to-peanut butter ratios of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06% (w/w), which correspond to 4, 8, 12, 16, 20 and 24 U of α -chymotrypsin activity and 1210, 2460, 3630, 4840, 6050 and 7260 U of trypsin activity respectively. In control samples, the enzyme suspensions were replaced with equal volume of neutralised water (DI water adjusted to $\text{pH} 7.0 \pm 0.1$). Duplicate experiments were run for each treatment. Treated peanut butter samples were transferred to pre-sterile plastic containers, capped and kept/incubated at room temperature for 24 h. One gram sample was taken from each batch of treated and control peanut butter for determination of soluble proteins and allergens (Ara h 1 and Ara h 2) levels. The remaining peanut butter was refrigerated and tested weekly for soluble proteins and allergen concentrations. Each analysis, except SDS-PAGE, was replicated three times.

Preliminary trials were conducted to determine the adequate volume of aqueous solution sufficient for delivery of enzymes without negatively affecting the consistency/quality of peanut butter. Volumes of 1.25, 2.5 or 5.0 mL of enzyme suspensions containing 20 mg of dry enzyme were mixed with 50 g of peanut butter to yield an enzyme-to-peanut ratio of 0.04%.

Enzymatic treatment of commercial peanut butter

Five creamy peanut butter samples from three commercial brands were treated with the enzyme suspension,

Table 1 Nutritional compositions of commercial peanut butter samples used in the study (data obtained from the product labels and converted into g 100 g⁻¹ peanut butter from per serving)

Components	Brand 1		Brand 2		Brand 3
	Low Fat	Brand 1 Regular	Low Fat	Brand 2 Regular	
Total fat (g)	33	50	33	50	50
Saturated fat (g)	8	9	7	9	9
Total Carbohydrate (g)	42	22	42	22	19
Sugar (g)	11	6	14	9	9
Dietary fibre (g)	3	6	6	6	6
Protein (g)	22	25	19	22	22
Sodium (mg)	694	469	528	469	438

as described above, to reduce their inherent allergens. The specific commercial products were Brand 1-low fat (Jif Reduced Fat), Brand 1-regular (Jif Regular), Brand 2-low fat (Sippy Reduced Fat), Brand 2-regular (Sippy Regular) and Brand 3-regular (Market Pantry regular). The macronutrient composition of these commercial peanut butter samples is shown in Table 1. A 1:1 α -chymotrypsin: trypsin mixture was used for treatment of commercial peanut butter samples at an enzyme-to-peanut butter ratio of 0.04% (w/w, equivalent to 40 mg/100 g butter). Samples were capped and incubated at room temperature (22 °C) for 24 h or at 37 °C for 3 h. After initial sampling, treated peanut butter samples were stored at 4 °C for 2 weeks during which they were sampled and analysed for total soluble proteins and Ara h 1 and Ara h 2 allergen concentrations. Duplicated samples were run for each treatment. Each analysis, except SDS-PAGE, was replicated three times.

Evaluation of colour change in enzymatically treated commercial peanut butter

The colour of commercial peanut butter samples treated with different enzyme concentrations and water-to-enzyme ratios were determined using a Minolta CM-3500d Spectrophotometer (Konica Minolta, Ramsey, NJ, USA). Briefly, peanut butter was evenly spread at the bottom of a Petri Dish which was then placed on the surface of the spectrophotometer to measure Hunter's L- (white and black), a- (red and green) and b- (yellow and blue) values. Untreated peanut butter samples from the same brand were used as controls (standard samples).

Extraction of peanut proteins

One gram (1.00 ± 0.01) of each peanut butter sample 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 3000 g for 25 min. Supernatant of each sample was poured into a clean tube, whereas the precipitate was extracted one more time with 20 mL of Tris buffer. The combined supernatants were centrifuged for 15 min at 3000 g. The volume of the clear portion was recorded. The lipid layer on the top of the supernatant was subsequently removed using transfer pipettes and the supernatant was stored at -20 °C until further analysis.

Determination of soluble proteins in extracts

The concentration of soluble proteins in each extract was determined by BCA method using bovine serum albumin (BSA) as a standard. BCA kit was purchased from PIERCE (Rockford, IL, USA). Peanut protein extracts were diluted 5–10 times with deionised water to make the protein concentration in test samples within the linear range of the calibration curve (0–1.0 mg mL⁻¹). Protein concentration in peanut extract was expressed as mg protein per mL extract and the total soluble protein was expressed as mg protein per g peanut butter.

Determination of Ara h 1 and Ara h 2 in peanut butter samples

Allergenic proteins, Ara h 1 and Ara h 2, in peanut butter samples were determined by sandwich ELISA using EL-AH1 and EL-AH2 kits from Indoor Biotechnology (Indoor Biotechnology, Charlottesville, VA, USA), respectively, according to the protocols supplied by the kit's manufacturer. Purified Ara h 1 and Ara h 2 were used as positive controls, and untreated samples were used as sample controls. The same protocols were used to construct standard curves for Ara h 1 and Ara h 2 using purified Ara h 1 and Ara h 2 (standards included in the commercial kits). Peanut protein extracts were properly diluted in PBS (pH 7.4) prior to the ELISA assays to keep protein concentration in the linear ranges of the standard curves. The Ara h 1 and Ara h 2 concentrations in diluted extracts (ng mL⁻¹) were obtained from standard curves based on their absorbance at 405 nm. Final Ara h 1 and Ara h 2 concentrations were expressed as a function of the weight of peanut butter and expressed as μ g Ara h 1 or (h2) g⁻¹ peanut butter. The average of four replications for each sample was reported in the results. The percentage reduction of Ara h 1 and h2 was calculated as follows:

$$\% \text{Reduction} = (C_0 - C_t) * 100 / C_0$$

Where C_0 is the concentration of Ara h 1 or Ara h 2 in untreated peanut butter sample, C_t is the concentration of Ara h 1 or Ara h 2 in treated peanut butter sample.

Preparation of SDS-PAGE for peanut butter proteins

Soluble and insoluble peanut butter protein fractions were extracted according to the procedure of Schmitt *et al.* (2010) with a slight modification. Briefly, 100 mg of peanut butter was weighed directly into a 1.5 mL microcentrifuge tube and 1 mL of tris-HCl buffer (pH 8.3) was added in the tube. The mixture was then sonicated and centrifuged at 10 000 rpm for 20 min. The supernatant was poured into another tube and centrifuged again to remove the fat layer. The supernatant was then diluted with standard sample treatment buffer (containing glycerol, SDS and dithiothreitol) at 1:5 ratio, boiled for 5 min, aliquoted in tubes and stored at -20°C until use in gel separation. Pellet was solubilised with 1 mL of standard sample treatment buffer and boiled for 5 min. After centrifugation, the pellet extract was aliquoted and stored at -20°C until use in gel separation. Pellet extracts were diluted with sample treatment buffer (ratio 1:5) before loading onto the gel. Purified Ara h 1 and Ara h 2 were used as references. For all samples, the loading volume was 5 μL per well at a protein concentration of 2 mg mL^{-1} . Protein per peptide profiles of supernatant and pellet fractions from peanut butter, with and without enzyme treatment, were resolved by SDS-PAGE in polyacrylamide gel (12.5%) using a Bio-Rad Mini Protein III system (Bio-Rad, Hercules, CA, USA). 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). 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 used as references.

IgE-binding test

A competitive Inhibition ELISA was carried out in triplicate ($n = 3$) as previously described by Chung *et al.* (2005). Briefly, a microplate was coated with untreated peanut butter extract (1:20 diluted with PBS/Tween 20) and incubated at 37°C for 2 h. After washing four times with PBST, 50 μL treated/untreated peanut samples (0.01–100 $\mu\text{g mL}^{-1}$) mixed with 50 μL of pooled human plasma containing IgE antibodies (1:30 diluted with Superblock) were added and incubated for 45 min at room temperature. Detection of IgE antibodies binding to the plate was performed, using a goat anti-human IgE peroxidase conjugate (1:1000 diluted with Superblock) and a substrate solution (100 μL) containing o-phenylenediamine (0.5 mg mL^{-1}) and 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5. After stopping the enzyme reaction with 2 N sulphuric acid (50 μL), the absorbance was read at 490 nm with an ELx Synergy Plate Reader (Bio-Tek Instruments, Inc., Winooski,

VT, USA). The absorbance value of a sample containing IgE antibodies and the peanut sample was represented by B, whereas B_0 represented the absorbance value of a control containing IgE only. The % IgE-binding inhibition was calculated and graphed as a function of peanut protein concentration.

Statistical analysis

SAS software (SAS Institute Inc., Cary, NC, USA) was used to generate summary statistics expressing the results from various treatments as means $\pm$ standard deviations. The effects of treatments (Type of enzyme, enzyme concentration, enzyme-to-peanut butter ratio and water-to-peanut butter ratio) on concentration of allergens (Ara h1 and Ara h2), protein solubility and product colour were evaluated using Analysis of Variance (ANOVA). Dunnett's post-ANOVA test was used to evaluate significance of differences between treatment means and untreated control samples. Difference in treatment means was judged as significant at the 5% significance level.

Results and discussions

Effect of enzyme concentration on protein solubility and allergen concentration in Home-made peanut butter

α -chymotrypsin and trypsin are endopeptidases that catalyse the degradation of proteins and peptides. Sufficient amount of enzyme is needed to achieve rapid and complete hydrolysis of certain amount of target substrates. However, adding too much enzyme in the substrate will result in wastage of enzyme and higher production cost. Therefore, it is important to determine the optimal enzyme-to-peanut butter ratio.

Figure 1 shows the changes of total soluble proteins in peanut butter samples following treatment with different peptidases at various concentrations. Treatment of peanut butter with enzyme significantly increased total soluble protein, even at enzyme concentration as low as 0.01% (10 $\text{mg } 100 \text{ g}^{-1}$ butter). The increase in soluble protein levelled off as enzyme concentration increased. α -chymotrypsin (E1) treatment increased detectable soluble protein more than trypsin (E2) treatment. The lowest level of detectable soluble proteins for E2 seems to occur at 0.03% instead of higher E2 concentrations. Hence, this point could be an "outlier" due to analytical error. At the same total enzyme concentration, treatment of peanut butter with a mixture of E1 and E2 (1:1) yielded lower detectable soluble protein content in home-made peanut butter samples. These variations may be explained by the different cleaving sites of the enzymes used. Chymotrypsin preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond is a tyrosine,

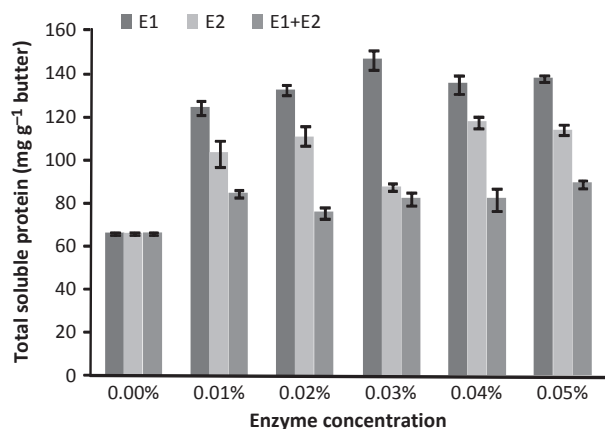


Figure 1 Effect of enzyme-to-peanut butter ratio on the detectable peanut protein in Home-made peanut butter samples. (E1 = α -chymotrypsin, E2 = trypsin and E1 + E2 = 1:1 mixture of α -chymotrypsin and trypsin at equal amount. All peanut butter samples were treated with enzyme at room temperature for 24 h).

tryptophan or phenylalanine. Trypsin predominantly cleaves proteins at the carboxyl side of the amino acids lysine and arginine, except when either is bound to a C-terminal proline (Whitaker, 2003). Therefore, the treatment by the combination of two enzymes may have resulted in very small peptides or even some

amino acids leading to a lower protein detection as BCA method used in this study is based on the binding between Cu^{2+} and protein side-chains, and protein backbone and it does not detect dipeptides (Owusu-Apenten, 2002).

Table 2 shows the reduction of extractable Ara h 1 and Ara h 2 in home-made peanut butter treated with E1, E2 or E1 + E2 at different enzyme-to-peanut ratios. The extractable Ara h 1 and Ara h 2 contents in untreated peanut butter were 1102.27 ± 200.50 and $1015.49 \pm 204.74 \text{ mg g}^{-1}$ butter respectively. After 24 h of enzymatic treatment at room temperature (22°C), the detectable Ara h 2 in home-made peanut butter samples varied significantly ($P < 0.05$). At the same total enzyme concentration, treatment with E1 resulted in smaller reduction of detectable Ara h 1 and Ara h 2, whereas E2 and the mixture of E1 and E2 showed higher efficiency in reducing detectable Ara h 1 and Ara h 2. In all cases, enzyme concentrations above 0.03% did not further reduce Ara h 1 level, but the lowest detectable Ara h 2 was detected at enzymes concentrations of 0.03% or higher, especially in samples treated with the enzyme mixture. Accordingly, the combination of E1 and E2 (1:1) at 0.03–0.04% of total enzyme-to-peanut butter ratio was the most effective in reducing the detectable peanut allergens, especially Ara h2.

Table 2 Effect of enzyme concentration and post-processing storage at 4°C on the reduction of extractable Ara h 1 and Ara h 2 in home-made peanut butter at water-to-peanut ratio of 5% (v/w)

Type of Enzyme	% Enzyme	Treated with enzyme at 22°C for 24 h		Stored at 4°C for 1 week after enzyme treatment		Stored at 4°C for 2 week after enzyme treatment	
		%Ara h1 Reduction	%Ara h2 Reduction	%Ara h1 Reduction	%Ara h2 Reduction	%Ara h1 Reduction	%Ara h2 Reduction
E1	0.01	77.38 ± 2.47	59.41 ± 1.71	78.83 ± 1.43	55.91 ± 2.30	66.91 ± 1.33	49.00 ± 2.27
	0.02	87.03 ± 2.40	76.91 ± 4.78	88.66 ± 0.87	75.97 ± 0.77	85.59 ± 0.69	73.47 ± 2.98
	0.03	91.26 ± 1.11	83.20 ± 2.25	93.65 ± 1.07	83.62 ± 4.98	89.68 ± 0.36	82.80 ± 2.88
	0.04	85.07 ± 0.86	71.98 ± 3.48	95.4 ± 1.48	90.6 ± 2.86	96.8 ± 3.22	87.3 ± 2.65
	0.05	86.83 ± 0.91	81.98 ± 0.49	–	–	–	–
	0.06	88.32 ± 0.66	84.43 ± 1.51	–	–	–	–
E2	0.01	88.61 ± 2.05	73.19 ± 1.29	92.77 ± 1.47	78.78 ± 1.71	87.05 ± 0.96	69.02 ± 0.99
	0.02	97.86 ± 0.77	87.49 ± 1.01	100.00	89.34 ± 2.12	98.05 ± 1.17	91.16 ± 1.77
	0.03	98.49 ± 0.46	88.86 ± 2.56	100.00	94.72 ± 0.84	99.79 ± 0.35	93.58 ± 1.64
	0.04	98.63 ± 0.51	89.14 ± 1.43	99.7 ± 0.44	94.8 ± 0.97	100.00	96.1 ± 1.32
	0.05	98.31 ± 0.39	91.34 ± 1.25	–	–	–	–
	0.06	99.40 ± 0.14	93.96 ± 1.52	–	–	–	–
E1 + E2	0.01	90.72 ± 2.95	78.03 ± 0.76	95.13 ± 0.54	81.29 ± 5.45	90.59 ± 0.99	77.34 ± 3.02
	0.02	97.25 ± 0.69	90.07 ± 1.26	100.00	94.23 ± 2.01	98.77 ± 0.26	93.31 ± 0.85
	0.03	99.82 ± 0.21	91.94 ± 1.43	100.00	95.32 ± 2.29	100.00	97.55 ± 2.17
	0.04	100	91.71 ± 2.50	100.00	95.6 ± 2.73	100.00	98.00 ± 1.96
	0.05	100.00	94.89 ± 1.32	–	–	–	–
	0.06	100.00	95.11 ± 1.39	–	–	–	–

E1, α -chymotrypsin, E2, Trypsin and E1 + E2, combination of α -chymotrypsin and trypsin at a ratio of 1:1.

The Ara h 1 and Ara h 2 contents in untreated home-made peanut butter were 1102.27 ± 200.50 and $1015.49 \pm 204.74 \text{ } \mu\text{g g}^{-1}$ peanut butter respectively.

Table 2 also shows that, at the optimum enzymatic concentration (0.04%), further reduction in detectable Ara h 1 and h 2 were observed during the 2 weeks of refrigerated storage, which can be explained by the continuation of slow enzymatic hydrolysis of peanut protein at low temperature by residual enzyme activity. Ara h 1 reduction increased from 85% at day 1 to 96% after 2 weeks of storage for samples treated with 0.04% E1 and from 98% to 100% for samples treated with 0.04% E2 in the same period. The combination of E1 and E2 at 0.04% led to 100% reduction in Ara h 1, with no significant change during storage. The additional decrease in Ara h 2 (the more potent allergen) during storage is particularly encouraging, given the reported resistance of Ara h 2 to enzymatic treatment. After 2 weeks of refrigerated storage, Ara h 2 reduction increased from 71% to 87%, 89% to 96% and 91% to 98% in samples treated with 0.04% E1, E2 and E1 + E2 respectively. As the combination of E1 and E2 at a total concentration of 0.04% was most effective in reducing the levels of Ara h 1 (100%) and Ara h 2 (98%), it was selected for treatment of commercial peanut butter samples.

Effect of water in enzyme suspension on the quality and allergen levels of home-made peanut butter

Trypsin and α -chymotrypsin are hydrolytic enzymes. Hydrolysis of proteins by these enzymes requires the presence of water. In addition, using enzyme suspension instead of dry enzyme powder ensures homogeneous distribution of enzyme into the peanut butter matrix during processing. However, adding too much water may cause loss of consistency and syneresis in peanut butter. In this study, the optimal enzyme-to-peanut ratio (0.04%) was used to investigate the

effects of water, used for preparation of enzyme suspensions, on the reduction of allergens and on consistency of peanut butter. Data in Table 3 show that, at constant enzyme concentration of 0.04%, the volume of water used to hydrate enzyme in the range 1.25–2.5 mL 50 g⁻¹ peanut butter (or 2.5–5%, w/w) did not impact allergen reduction and caused no undesirable colour change in treated samples. The consistency of these home-made peanut butter samples remained similar to that of the control peanut butter (untreated). However, at the highest level of hydration (above 5 mL 50 g⁻¹ butter or 10% v/w), peanut butter became coarser than the control. Therefore, enzyme suspensions should not exceed 5% water-to-peanut butter ratio to maintain a desirable peanut butter consistency without compromising the level of allergen reduction.

Effect of enzyme treatment on the concentrations of allergens in commercial peanut butter samples

The composition of commercial peanut butter is more complex than that of home-made peanut butter. Based on product labels, commercial butter samples contain sugar, corn syrup/molasses, fully hydrogenated vegetable oil, salt and mono- and diglyceride in addition of peanuts. Soy protein was also present in two low fat peanut butters used in the study. Some of these ingredients may affect the proteolytic activity of α -chymotrypsin and trypsin. For instance, soy protein may compete with peanut protein for enzymes. Furthermore, Table 1 shows that the macronutrient contents differed among the five commercial products tested. For example, low fat peanut butter contained significantly higher levels of carbohydrate whereas total protein contents varied from 19–25% among the products tested in

Table 3 Effect of water volume used in enzyme suspension on the reduction of extractable Ara h 1 and Ara h 2 in home-made peanut butter at total enzyme concentration of 0.04%

Type of Enzyme	% water	Treated with enzyme at 22 °C for 24 h		Stored at 4 °C for 1 week after enzyme treatment		Stored at 4 °C for 2 week after enzyme treatment	
		% Ara h1 Reduction	% Ara h2 Reduction	% Ara h1 Reduction	% Ara h2 Reduction	% Ara h1 Reduction	% Ara h2 Reduction
E1	2.5	93.91 ± 1.31	77.57 ± 0.68	91.85 ± 0.67	76.56 ± 3.49	95.06 ± 0.21	82.17 ± 2.32
	5.0	92.74 ± 1.37	82.79 ± 2.53	92.21 ± 0.73	80.99 ± 1.27	95.73 ± 0.13	85.10 ± 2.55
	10.0	94.91 ± 1.25	85.82 ± 2.97	93.18 ± 0.65	80.49 ± 2.49	96.63 ± 0.10	85.67 ± 2.84
E2	2.5	100	94.70 ± 2.26	98.73 ± 0.65	92.36 ± 2.70	100	94.38 ± 1.41
	5.0	100	94.85 ± 4.53	99.51 ± 0.01	90.75 ± 1.76	100	94.87 ± 2.40
	10.0	100	94.78 ± 4.11	99.26 ± 0.60	90.17 ± 2.08	100	96.63 ± 0.67
E1 + E2	2.5	100	94.58 ± 3.04	100	93.03 ± 2.53	100	97.05 ± 0.90
	5.0	100	93.96 ± 4.55	100	93.66 ± 1.42	100	95.53 ± 1.13
	10.0	100	93.54 ± 5.18	100	94.07 ± 1.32	100	96.35 ± 1.55

E1, α -chymotrypsin, E2, Trypsin, E1 + E2, combination of α -chymotrypsin and trypsin at ratio of 1:1.

The Ara h 1 and Ara h 2 contents in untreated home-made peanut butter were 1102.27 ± 200.50 and 1015.49 ± 204.74 respectively.

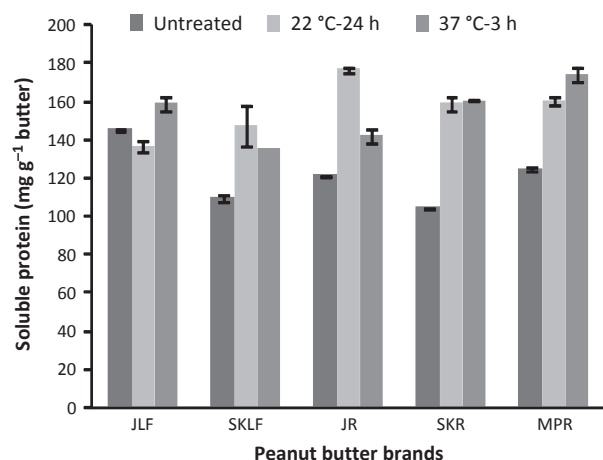


Figure 2 Effect of enzyme treatment on the detectable peanut protein in commercial peanut butter (at enzyme-to-peanut butter ratio of 0.04% w/w with 1:1 α -chymotrypsin and trypsin suspended in neutralised water and a water-to-peanut butter ratio of 5%). JLF- Brand 1-low fat, SKLF- Brand 2-low fat, JR- Brand 1-regular, SKP- Brand 2-regular and MPR- Brand 3-regular.

this study. Consequently, brand and products within brand showed different percentages of soluble proteins (Fig. 2). Figure 1 and Table 2 show that, at 0.04%, the treatment with E1 resulted in higher protein solubility but lower allergen reduction, whereas E2 led to lower protein solubility but higher allergen reduction.

A synergistic effect between E1 and E2 was observed in the reduction of Ara h2, the more resistant and potent allergen (Yu *et al.*, 2011). Therefore, to take advantages of E1 and E2 synergy, the combination of E1 and E2 was used in the treatment of commercial peanut butter. This combination appears to provide the best activity of each enzyme.

Following treatment with 0.04% α -chymotrypsin and trypsin mixture at 22 °C for 24 h or at 37 °C for 3 h, the level of soluble proteins in peanut butter increased significantly, with the exception of Brand 1-low fat peanut butter (Fig. 2). This increase in protein solubility was concurrent with a sharp decline in the level of detectable Ara h 1 and Ara h 2 in all treated samples (Table 4). Enzymatic treatment of peanut butter at 37 °C for 3 h lowered the detectable Ara h 1 in all samples to the levels below the detection limit of the commercial Sandwich ELISA kit ($0.16 \mu\text{g mL}^{-1}$). The Sandwich ELISA is considered the most sensitive ELISA. Ara h 2 exhibited higher resistance to enzymatic hydrolysis as was observed in the case of home-made peanut butter (Tables 2 and 3). Although Ara h 1 could not be detected after 24 h of enzymatic reaction at room temperature (22 °C), the concentration of Ara h 2 in peanut butter samples decreased significantly but remained detectable. This further confirms

that Ara h 2 is more resistant to enzymatic digestion than Ara h1. The results are consistent with the findings recently reported in peanut kernels (Yu *et al.*, 2011). This resistance can be partially explained by findings of Maleki *et al.* (2003) who reported that native Ara h 2 functioned as a trypsin inhibitor. Resistance to denaturation and digestion was thought to be an important characteristic of food allergens (Bannon, 2004). However, roasting might have altered (reduced) the trypsin inhibitory activity of Ara h2 since our data show that over 90% of extractable Ara h 2 in peanut butter made from roasted peanut was hydrolysed by trypsin and chymotrypsin (Table 4). Similar data were also reported in our previous study on enzymatic treatment of peanut kernels (Yu *et al.*, 2011). Table 4 also shows that enzymatic treatment at 37 °C for 3 h was as effective as at room temperature for 24 h. Hence, considering the practicality and efficiency, enzymatic incubation at 37 °C for 3 h or less would be recommended as a preferred process for potential commercial applications.

The extractable Ara h 1 and Ara h 2 contents in untreated home-made peanut butter was lower than that of commercial peanut butter. This difference could be attributed to one or more of the following: (i) the variety of peanuts might be different from those used to produce commercial peanut butter, (ii) peanuts used for home-made peanut butter were oil roasted (according the information provided by the vendor), whereas commercial peanut butter is usually made from dry roasted peanuts and (iii) the relatively large particle size of home-made peanut butter which was not as fine as commercial samples because of the limitations of the lab-scale grinder used to prepare home-made peanut butter. This likely affected the dissolution of peanut protein, and ultimately the extractability of allergens, leading to lower extractable Ara h1 allergens, especially given its larger large molecular weight.

SDS-PAGE for protein in peanut butter supernatants and pellets

Figure 3 provides a visual illustration of the protein profile of treated and untreated peanut butter samples. The Ara h 1 band appears in untreated supernatants (although the intensity of the band is low due to the reasons mentioned above) and pellets (Fig. 3a-lanes 3 and 6) but the band was not apparent in extracts from treated peanut butter (Fig., 3a-lanes 4, 5, 7 and 8), suggesting complete degradation of Ara h1 following enzymatic treatment. In the same gel, Ara h 2 band significantly faded in the supernatant and pellets of treated home-made peanut butter (Fig. 3a lanes 4, 5 and 7, 8) compared with untreated control samples (Fig. 3a-Lanes 3 and 6). Similar pattern was observed in the gel for treated and untreated commercial peanut

Table 4 Effect of enzyme treatment* on the levels of Ara h 1 and Ara h 2 in commercial peanut butter samples

Treatment	Soluble Protein (mg g ⁻¹ butter)	Ara h1 (μg g ⁻¹ butter)	Ara h2 (μg g ⁻¹ butter)	% Ara h1 Reduction	% Ara h2 Reduction
Untreated					
JLF	144.78 ± 0.93	2528.07 ± 765.92	3902.62 ± 173.23	N/A	N/A
SKLF	109.49 ± 1.66	1504.75 ± 88.16	1380.50 ± 19.25	N/A	N/A
JR	120.99 ± 0.13	5833.84 ± 775.44	5736.22 ± 329.68	N/A	N/A
SKR	104.26 ± 0.38	6427.2 ± 855.54	6338.90 ± 116.16	N/A	N/A
MPR	124.38 ± 0.89	9864.8 ± 0.00	6108.14 ± 348.84	N/A	N/A
Treated at 22 °C for 24 h					
JLF	136.32 ± 3.36	nd	157.87 ± 6.17	100.00	96.05
SKLF	147.10 ± 10.91	nd	371.39 ± 1.81	100.00	90.72
JR	176.57 ± 1.79	nd	236.94 ± 4.74	100.00	95.38
SKR	158.98 ± 3.32	nd	400.71 ± 25.00	100.00	93.79
MPR	160.16 ± 1.84	nd	169.32 ± 6.41	100.00	97.40
Treated at 22 °C for 24 h Stored at 4 °C for 1 week					
JLF	148.46 ± 0.99	nd	206.08 ± 9.23	100.00	94.85
SKLF	157.77 ± 0.81	nd	435.51 ± 35.73	100.00	92.72
JR	116.14 ± 3.19	nd	231.24 ± 8.90	100.00	95.49
SKR	196.07 ± 0.23	nd	434.31 ± 17.40	100.00	93.27
MPR	169.8 ± 3.06	nd	343.58 ± 0.21	100.00	94.72
Treated at 37 °C for 3 h					
JLF	158.77 ± 3.49	nd	182.74 ± 6.34	100.00	95.43
SKLF	135.09 ± 0.10	nd	217.49 ± 14.24	100.00	96.37
JR	142.12 ± 3.55	nd	419.93 ± 25.46	100.00	91.82
SKR	160.33 ± 0.51	nd	448.99 ± 34.19	100.00	93.04
MPR	174.45 ± 3.92	nd	408.89 ± 41.66	100.00	93.71
Treated at 37 °C for 3 h Stored at 4 °C for 1 week					
JLF	152.06 ± 7.12	nd	297.04 ± 19.97	100.00	92.58
SKLF	140.85 ± 0.63	nd	503.97 ± 27.85	100.00	91.58
JR	162.45 ± 2.17	nd	247.72 ± 7.90	100.00	95.17
SKR	174.27 ± 0.26	nd	449.25 ± 1.26	100.00	93.04
MPR	202.51 ± 3.70	nd	374.63 ± 62.30	100.00	94.24

*Enzyme treatment condition: 1:1 mixture of α -chymotrypsin and trypsin at total enzyme-to-peanut butter ratio of 0.04% (w/w). Amount of water used to suspend enzyme was 5% of peanut butter (v/w). JLF = Brand 1-low fat, SKLF = Brand 2-low fat, JR = Brand 1-regular, SKP = Brand 2-regular and MPR = Brand 3-regular.

butter samples (JIF Regular) (Fig. 3b) where Ara h 1 bands appeared denser in untreated supernatant and pellet lanes (Fig. 3b-lanes 3 and 6), but the band for Ara h 1 was clearly absent in all supernatants and pellets of enzymatically treated peanut butter (Fig. 3b-Lane 4, 5, 7 and 8). Ara h 2 band was also less visible in all enzymatically treated peanut butter samples (Fig. 3b-lanes 4, 5, 7 and 8).

After enzyme treatment, the Ara h 1 band was not apparent in the SDS-PAGE and Ara h 2 was partially degraded because co-migrating bands faintly appeared in SDS-PAGE of treated samples. This suggests that the individual enzyme treatment protocol might be less effective in degrading Ara h 2 than Ara h 1. Overall, the above discussed ELISA data and SDS-PAGE seem to converge in suggesting that the hydrolytic activity by the enzymes used to treat peanut butter samples significantly decreased the detectable levels of Ara h1 and Ara h2 through breakdown of these two allergenic proteins into lower molecular weight peptides. The

allergenicity and specific molecular weight of such peptides were not, however, evaluated in this study.

In vitro IgE-binding tests with competitive indirect ELISA (ciELISA)

The results of IgE-binding tests are presented in Figure 4. Samples treated with E1 + E2 at 22 °C for 24 h showed higher inhibition of IgE-binding (less allergens available for competitive binding) at all the peanut protein concentrations tested. The largest IgE-binding inhibition was observed at protein concentration of 0.1 and 1.0 μg mL⁻¹. The increased IgE-binding inhibition suggests that enzymatic treatment may have reduced the allergenic potential of treated peanut butter samples. However, in the absence of clinical allergy testing, the degree of inhibition achieved under the experimental condition used in this study may not be high enough to consider the treated peanut butter as hypoallergenic. This residual IgE-binding may be explained by incomplete cleavage of binding sites on

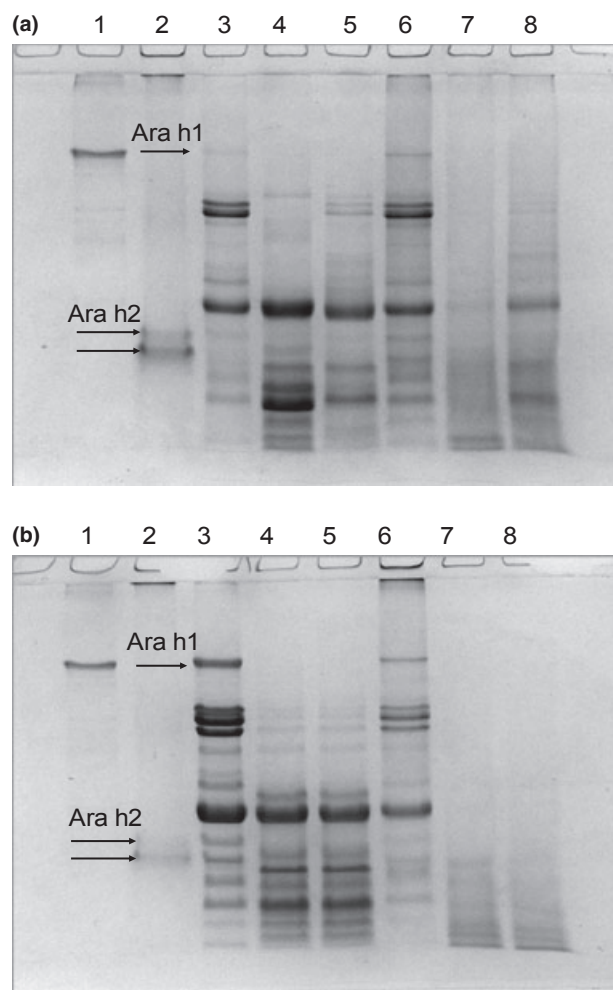


Figure 3 SDS-PAGE of supernatants and pellets of (a) home-made peanut butter (b) commercial peanut butter (JIF regular) used for illustration. Gel (a): Lanes 1-Ara h 1, 2-Ara h 2, 3-untreated supernatant, 4- supernatant treated with E1 at 22 °C for 24 h, 5- supernatant treated with E2 at 22 °C for 24 h, 6-untreated pellet, 7-treated pellet-E1 and 8-treated pellet-E2. Gel (b): Lanes 1-Ara h 1, 2-Ara h 2, 3-untreated supernatant, 4- supernatants treated at 22 °C for 24 h, 5-treated supernatant at 37 °C-3 h, 6-untreated pellet, 7-treated pellet (22 °C-24 h) and 8-treated pellet (37 °C-3 h).

the peptides (Bannon *et al.*, 2003). Further optimisation of the enzymatic process is needed to achieve higher inhibition of IgE-binding.

Effects of enzymatic treatment on the colour of peanut butter

Table 5 shows the changes in peanut butter colour expressed as Hunter's L, a and b-values (L- white and black, a- red and green and b- yellow and blue) as affected by the enzyme-to-peanut butter ratio and the

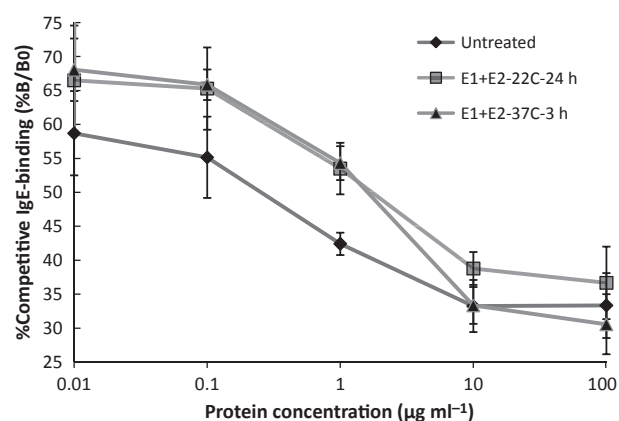


Figure 4 Competitive IgE-binding of extracts from treated and untreated commercial peanut butter samples (Jif regular)*. *Higher % IgE-binding on ELISA plates indicates less allergens in peanut butter extract for competitive IgE-binding.

Table 5 Effects of water in enzyme suspension on the colour parameters of peanut butter samples incubated at room temperature

%Water	% Enzyme	L	a	b
Control	0	53.40 ± 0.38 ^a	8.46 ± 0.10 ^a	25.78 ± 0.37 ^a
5	0.05	51.36 ± 0.09 ^b	8.35 ± 0.08 ^a	21.89 ± 0.11 ^b
	0.10	52.52 ± 0.08 ^b	8.32 ± 0.07 ^a	22.20 ± 0.09 ^b
	0.15	50.49 ± 0.19 ^b	8.01 ± 0.09 ^a	21.35 ± 0.36 ^b
7.5	0.05	46.74 ± 0.17 ^c	8.49 ± 0.10 ^a	19.40 ± 0.16 ^c
	0.10	47.43 ± 0.25 ^c	8.23 ± 0.08 ^a	19.71 ± 0.15 ^c
	0.15	49.62 ± 0.20 ^c	8.01 ± 0.14 ^a	20.37 ± 0.07 ^c
10	0.05	37.17 ± 0.18 ^d	11.47 ± 0.14 ^b	31.32 ± 0.18 ^d
	0.10	36.76 ± 0.47 ^d	11.33 ± 0.28 ^b	30.68 ± 0.93 ^d
	0.15	37.10 ± 0.22 ^d	11.72 ± 0.10 ^b	31.65 ± 0.31 ^d

Enzyme suspension used = 1:1 mixture of α -chymotrypsin and trypsin. Column values with same superscripts are not significantly different at 5% significance level.

Peanut butter used here is Brand 1-Regular creamy peanut butter.

amount of water used in enzyme suspension (Table 3). At the same water-to-peanut butter ratio, the enzyme-to-peanut butter ratio did not have significant effect on the colour of peanut butter as judged by L-, a- and b-values. However, when the same enzyme-to-peanut butter ratio was used at different hydration levels, the added water significantly impacted the colour parameters of peanut butter ($P < 0.05$). For instance, a decrease in whiteness (lower L-values) was proportional to the amount of added water in enzyme suspensions. At 5% of water, the colour change in commercial peanut butter was not noticeable. At 10% water, the L-value decreased from 53 (control) to 37 in treated samples resulting in an unacceptable dark brown colour. This darkening occurred concurrently with increases in a-values. The change in b-values was

less consistent. Based on data in Table 3, the enzyme suspensions should not exceed 5% water- to- peanut butter ratio.

The darkening of peanut butter because of enzymatic treatment in samples receiving more added water can be interpreted by Maillard reaction between amino acids and reducing sugar from corn syrup and/or molasses typically added to commercial peanut butter. Maillard reaction takes place even at room temperature (Ramírez-Jiménez *et al.*, 2003). Protein hydrolysis occurred immediately to produce short peptides and even amino acids when peanut butter was mixed with enzyme solution. The short peptides and amino acids are suspected to react with reducing sugars in commercial peanut butter to produce dark brown pigments. Higher water content in peanut butter could result in increased molecule mobility which might accelerate browning, as the maximum non-enzymatic reaction rate occurs at 14% moisture content for L-cysteine/D-glucose model system (Yeo & Shibamoto, 1991).

Conclusion

This study demonstrated that the contents of the main allergenic proteins (Ara h 1 and Ara h 2) in peanut butter can be significantly reduced by enzymatic treatment of peanut butter with endopeptidases, such as α -chymotrypsin and trypsin, under very mild conditions. Degradation of extractable Ara h 1 and Ara h 2 was basically completed within 24 h of incubation at room temperature or 3 h incubation at 37 °C following the addition of enzymes into the peanut butter matrix. Treated peanut butter can be pasteurised to inactivate the residual enzyme activity without major changes in its quality and allergen content. Enzymatically treated peanut butter retained its original aroma, colour and consistency when the volume of enzyme suspension kept at or below 5% of peanut butter weight. In the presence of reducing sugar (from corn syrup, molasses or sugar), treatment of peanut butter with α -chymotrypsin and trypsin caused darkening of the products at higher water levels used to hydrate enzymes. In addition, SDS-PAGE of soluble fraction and insoluble fraction (pellet) of peanut butter can qualitatively ascertain that Ara h 1 and Ara h 2 are broken down after enzyme treatment. IgE-binding test results indicate that the treatment of peanut butter with alpha-chymotrypsin and trypsin has the potential to reduce the allergenicity of peanut butter. Further study is needed to find the optimal E1/E2 ratio, treatment time and temperature that could result in higher IgE-binding inhibition. Clinical tests are also needed to ascertain the absence of allergic reactions in human subjects exposed to treated peanut butter.

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