



Effectiveness of different proteases in reducing allergen content and IgE-binding of raw peanuts

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ABSTRACT

The effectiveness of some non-specific proteases in reducing raw peanut allergenicity was investigated. Peanut kernels were treated by Alcalase, papain, Neutrase and bromelain, respectively. The residues of major peanut allergens Ara h 1, Ara h 2 and Ara h 6 were determined by sandwich ELISA and SDS-PAGE, and the allergenicities of treated peanuts were compared to that of untreated peanuts by western blot. All tested proteases were effective in reducing Ara h 1, but their effectiveness in hydrolyzing Ara h 2 and Ara h 6 varied greatly. The maximal reductions of extractable Ara h 1, Ara h 2 and Ara h 6 were 100%, 100% and 99.8%, respectively, achieved by Alcalase hydrolysis. Alcalase was more effective in overall allergenicity reduction; bromelain and Neutrase were the least effective in reducing Ara h 2 and Ara h 6, respectively. The hydrolysis of original allergens also produced some smaller peptides with strong IgE-binding.

1. Introduction

Peanuts are an inexpensive but nutritious food crop, rich in protein, healthy fat, dietary fibre, vitamins and minerals. They are consumed worldwide as snacks, peanut butter, peanut oil and food ingredients. However, peanut is a frequent cause of food allergy and the most common cause of fatal food-induced anaphylaxis in the United States and other developed countries (Finkelman, 2010). Accidental ingestion of a small amount of any peanut product can produce lethal allergic reactions among individuals with severe peanut allergy. About 2.2% of children and 1.8% adults are allergic to peanuts in the U.S. (Jiang et al., 2018; Gupta et al., 2019). These percentages have increased significantly from what were reported earlier (Sicherer, Munoz-Furlong, Godbold, & Sampson, 2010; Gupta et al., 2011). Allergy to peanut is persistent and often continues into adulthood and only about 20% of children outgrow their peanut allergy (Finkelman, 2010). Some people develop allergy to peanuts in their adulthood (Gupta et al., 2019). Peanut allergy greatly reduces the life quality of patients and increased cost to the food industry, due to product labelling and recall. It is necessary to explore strategies to decrease the allergenicity of peanuts to protect consumers with peanut allergy.

The threshold dose inducing allergic symptoms varies greatly from individual to individual, and from study to study (Sicherer & Sampson, 2007; Eller, Hansen, & Bindslev-Jensen, 2012; Klemans et al., 2015;

Zhu, Pouillot, Kwegyir-Afful, Luccioli, & Gendel, 2015; Hourihane et al., 2017). The lowest reported eliciting dose of peanut protein/flour was 1.5 mg (Klemans et al., 2015) and the highest was 3 g (Sicherer & Sampson, 2007). The determination of eliciting dose can help industry choose tests with a level of sensitivity capable of detecting food allergens hidden in industrial products and to specify protective measures for highly allergic individuals in order to prevent recurrent severe anaphylaxis (Moneret-Vautrin & Kanny, 2004). The eliciting dose can also serve as a reference/goal for researchers aiming to reduce peanut allergenicity.

Peanut allergy is a typical IgE-mediate type I hypersensitivity (Al-Muhsen, Clarke, & Kagan, 2003) triggered by allergenic proteins and peptides in peanuts. Among 17 allergenic proteins identified so far, Ara h 2 and Ara h 6 were recognized as the most potent (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004; Chen, Wang, El-Mezayen, Zhuang, & Dreskin, 2012; Kulis et al., 2012; Koid et al., 2014) although Ara h 1 and Ara h 3 were the most abundant proteins in peanuts (Johnson et al., 2016). Each peanut allergen contains many IgE-binding epitopes and so far the epitopes of Ara h 1, Ara h 2, Ara h 3, Ara h 6 have been identified by different researchers (Matsuo, Yokooji, & Taogoshim, 2015). To reduce the allergenicity of peanuts, the epitopes in the peanut protein molecules have to be modified or destroyed. Proteolytic hydrolysis is a way to destroy epitopes of allergenic protein.

Our previous studies elucidated that proteases such as trypsin,

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alpha-chymotrypsin and Alcalase reduced the Ara h 1 and Ara h 2 in roasted and raw peanut kernels to various degrees with Alcalase being the most effective among the proteases tested (Yu, Ahmedna, Goktepe, Maleki, & Cheng, 2011; Yu et al., 2015; Mikiashvili & Yu, 2018). The specificity of a protease determines whether an allergenic protein can be completely hydrolyzed. Some proteases are highly specific, while some are less specific. For example, trypsin acts on lysine and arginine residues, while chymotrypsin acts on large hydrophobic residues, such as tryptophan, tyrosine and phenylalanine (Whitaker, 2003). Pepsin, an aspartic endopeptidase, is much less specific than serine endopeptidase trypsin and chymotrypsin (Ahn, Cao, Yu, John, & Engen, 2013). The non-specific or less specific proteases are anticipated to be more effective in reducing allergenicity of a protein molecule because they can cleave the larger peptide at many more positions than can specific proteases, which should destroy more epitopes. However, no study about the effectiveness of different non-selective proteases in hydrolyzing specific peanut allergen and overall peanut allergenicity has been reported.

In this study, the effectiveness of four non-specific proteases, including Alcalase (a protease preparation derived from *Bacillus licheniformis*), papain, Neutrase and bromelain, in reducing major potent allergens Ara h 1, Ara h 2 and Ara h 6 in raw peanuts (or the overall allergenicity of the peanut extracts and precipitates) was evaluated. These proteases are on the GRAS (Generally Recognized As Safe) list except Neutrase (FDA, 2018). Although these proteases are considered as “non-specific”, they have different preferred cleave sites, optimal pHs and temperatures.

2. Materials and methods

2.1. Materials

Raw Virginia peanuts were purchased from a North Carolina peanut producer, and stored at -20°C in Ziploc bags until used. Protease preparations used in this study included Alcalase (consisting primarily of subtilisin A, EC3.4.21.62, a serine endopeptidase from *Bacillus licheniformis*, with activity of 2.59 AU/ml), papain (a cysteine protease from *Carica papaya* latex, EC 3.4.22.2, with activity of 52431 USP-U/mg powder.), bromelain (a sulfhydryl protease from pineapple stem, EC 3.4.22.32, with activity of 1.2 GDU/mg powder) and Neutrase (a zinc metallo endo-protease from *B. amyloliquefaciens*, E.C.3.4.24, with activity of 0.8 U/g product). These were purchased from Fisher Scientific (Suwanee, GA, USA).

2.2. Enzymatic treatments of raw peanuts by single protease

Raw peanut kernels were treated by four single proteases (Alcalase, bromelain, papain, Neutrase) at the optimal pH and temperature of each enzyme in the phosphate buffer (PB, 20 mM). The optimal pHs of Alcalase, papain, Neutrase, and bromelain are 7.5, 6.0, 7.0, and 7.0, respectively; and the optimal temperature of these proteases are 50, 48, 50 and 45°C , respectively. The concentrations of proteases (enzyme to peanut ratio) varied with enzymes and were 1–3.5% (v/w) for Alcalase, 0.1–0.5% (w/w) for papain, 0.2–1.6% for Neutrase and 0.02–0.2% for bromelain. Treatment time was 2.0 h for all proteases. Sample size was 100 g of raw peanuts. After treatment, samples were drained and vacuum-dried at 75°C overnight (18 h), then stored at 4°C for allergen residual measurement, SDS-PAGE and IgE-binding test. The enzyme-buffer solutions after treatment were collected and their volumes were recorded. The Neutrase used in this study was in liquid form, produced by Novozymes, and it has activity of 0.8 AU/g of product in the pH range 5.5–7.5 and temperature range 30 – 55°C . To check the effects of protease treatment on the pH of peanuts, 10 g of each sample were ground into butter which was suspended in 50 ml of deionized water, and the pH of the suspension was measured.

2.3. Soluble protein extraction and determination

Peanut samples were ground into peanut butters and stored at -20°C . The soluble protein of each peanut butter sample was extracted using phosphate buffer (pH 8.0, 20 mM). The extraction of each sample was conducted in duplicate at a peanut to buffer ratio of 1:20 (w/v) at room temperature under constant stirring (60 min), followed by centrifugation at 3000 g for 20 min. The precipitate was extracted for a second time using the same buffer for 30 min. The combined supernatants from the two-step extraction were centrifuged again and the fat layer on the top was removed. The protein concentrations of extracts were determined by the Bicinchoninic acid (BCA) method, using a Pierce BCA Protein Assay Kit (Rockford, IL), and expressed as mg of protein per gramme of peanuts (Mikiashvili & Yu, 2018).

2.4. Determination of allergen residues in the soluble portions of peanut samples

The effectiveness of enzyme treatment was evaluated by quantifying the residues of three major peanut allergens, Ara h 1, Ara h 2 and Ara h 6, in the peanuts treated with different proteases using untreated raw peanuts as control. The Ara h 1, Ara h 2 and Ara h 6 concentrations in peanut extracts were determined using sandwich ELISA kits (Indoor Biotechnologies, Charlottesville, VA, USA) according to manufacturer's instructions. Each kit includes allergen-specific primary antibody, secondary antibody and allergen standard solution. The extracts were diluted to various degrees with phosphate buffered saline (PBS) to ensure that the allergen concentrations were in the detection ranges of the kits. Each allergen analysis was conducted in triplicate for the same sample. Results were expressed as μg of allergen per gramme of peanuts according to the sample weight and the volume of extract.

2.5. Detection of allergen residue by SDS-PAGE

The allergens in the peanut extracts and precipitates (insoluble portions) were also elucidated by SDS-PAGE. The extracts (soluble portions) were diluted with PBS to a protein concentration of 1 mg/ml and 25 μl of each diluted sample was loaded onto the gel (25 μg /well) to ensure that all allergens in the extract could be detected. Molecular weight ladder (Bio-Rad, Hercules, CA, USA) and purified Ara h 1, Ara h 2 and Ara h 6 (Indoor Biotechnologies, Charlottesville, VA, USA) were used to identify the allergen bands in the samples. The allergens in the precipitates of peanuts were extracted using sample treatment buffer for electrophoresis (pH 6.8, Tris buffer containing glycerol, SDS and dithiothreitol), and qualitatively detected by SDS-PAGE, using 4/12% acrylamide gel (4% acrylamide in stacking gel and 12% acrylamide in resolving gel) as described by Mikiashvili and Yu (2018). For each sample, two gels with the same protein load in each well were prepared. One was stained with Coomassie brilliant blue R-250 staining solution (Bio Rad, Hercules, CA, USA); the unstained gel was used for western blot. The stained gel was destained by two-step destaining procedure, and the gel image was obtained by scanning the gel using an EPSON Scanner.

2.6. Western blot of untreated and treated samples

The IgE-bindings of peanut extracts and their corresponding precipitates were tested by western blot as described by Mikiashvili and Yu (2018). The purified Ara h 1, Ara h 2 and Ara h 6 were used as references. The proteins were transferred from the unstained SDS-PAGE gel to a nitrocellulose membrane, using the Trans-Blot® Turbo Blotting System (Bio-Rad, Hercules, CA, USA). After blocking with 3% BSA TBST (Tween 20 in TBS) for 1 h, the membrane was incubated with the pooled plasma of 7 peanut allergic patients (PlasmaLab International, Everett, WA, USA) (1:60 dilution) for 1 h with gentle agitation at room temperature. The membrane was then washed and incubated with goat

Table 1
Allergen contents of raw Virginia peanuts treated with different proteases.

Type of Enzyme	Enzyme % (v/w)	pH	Ara h 1 (μg/g peanut)	Reduction (%)	Ara h 2 (μg/g peanut)	Reduction (%)	Ara h 6 (μg/g peanut)	Reduction (%)
Alcalase	0.0	6.77	5283 ± 84.3	–	4492 ± 422	–	3207 ± 46.0	–
	1.0	6.55	ND	100	3.09 ± 0.51	99.9	1066 ± 83.4	66.8
	2.0	6.58	ND	100	ND	100	131 ± 5.28	95.9
	3.0	6.55	ND	100	ND	100	110 ± 11.8	96.6
	3.5	6.52	ND	100	ND	100	6.86 ± 0.19	99.8
Bromelain	0	6.77	5283 ± 84.3	–	4492 ± 422	–	3207 ± 46.0	–
	0.02	6.57	360 ± 5.92	93.2	2098 ± 17.1	53.3	1985 ± 198	38.1
	0.04	6.65	12.3 ± 1.48	99.8	979 ± 30.4	78.2	1847 ± 89.1	42.4
	0.08	6.65	ND	100	984 ± 17.0	78.1	1461 ± 63.0	54.4
	0.16	6.57	ND	100	791 ± 84.8	82.4	906 ± 22.8	71.8
	0.2	6.59	NN	100	791 ± 14.1	82.4	653 ± 20.2	79.7
Neutrase	0	6.77	5283 ± 84.3	–	4492 ± 422	–	3207 ± 46.0	–
	0.2	6.62	28.9 ± 1.66	99.5	23.3 ± 1.93	99.5	2657 ± 21.2	17.2
	0.4	6.62	25.0 ± 1.60	99.5	13.7 ± 0.42	99.7	1625 ± 55.8	40.3
	0.8	6.60	14.8 ± 0.80	99.7	8.37 ± 0.65	99.8	1313 ± 24.2	59.1
	1.2	6.59	15.2 ± 0.67	99.7	8.28 ± 0.30	99.8	1263 ± 35.7	60.6
	1.6	6.59	17.0 ± 1.17	99.7	7.21 ± 0.33	99.8	1767 ± 39.4	44.9
Papain	0	6.77	5283 ± 84.3	–	4492 ± 422	–	3207 ± 46.0	–
	0.1	6.60	159 ± 10.3	97.0	110 ± 5.01	97.6	2674 ± 161	16.6
	0.2	6.60	32.7 ± 2.59	99.4	58.5 ± 1.31	98.7	3126 ± 189	2.51
	0.3	6.60	11.3 ± 0.61	99.8	26.9 ± 0.70	99.4	721 ± 31.5	77.5
	0.4	6.71	8.12 ± 0.62	99.9	27.4 ± 1.77	99.4	699 ± 19.8	78.2
	0.5	6.67	5.43 ± 0.98	99.9	17.1 ± 1.99	99.6	703 ± 59.0	78.1

ND: non-detectable.

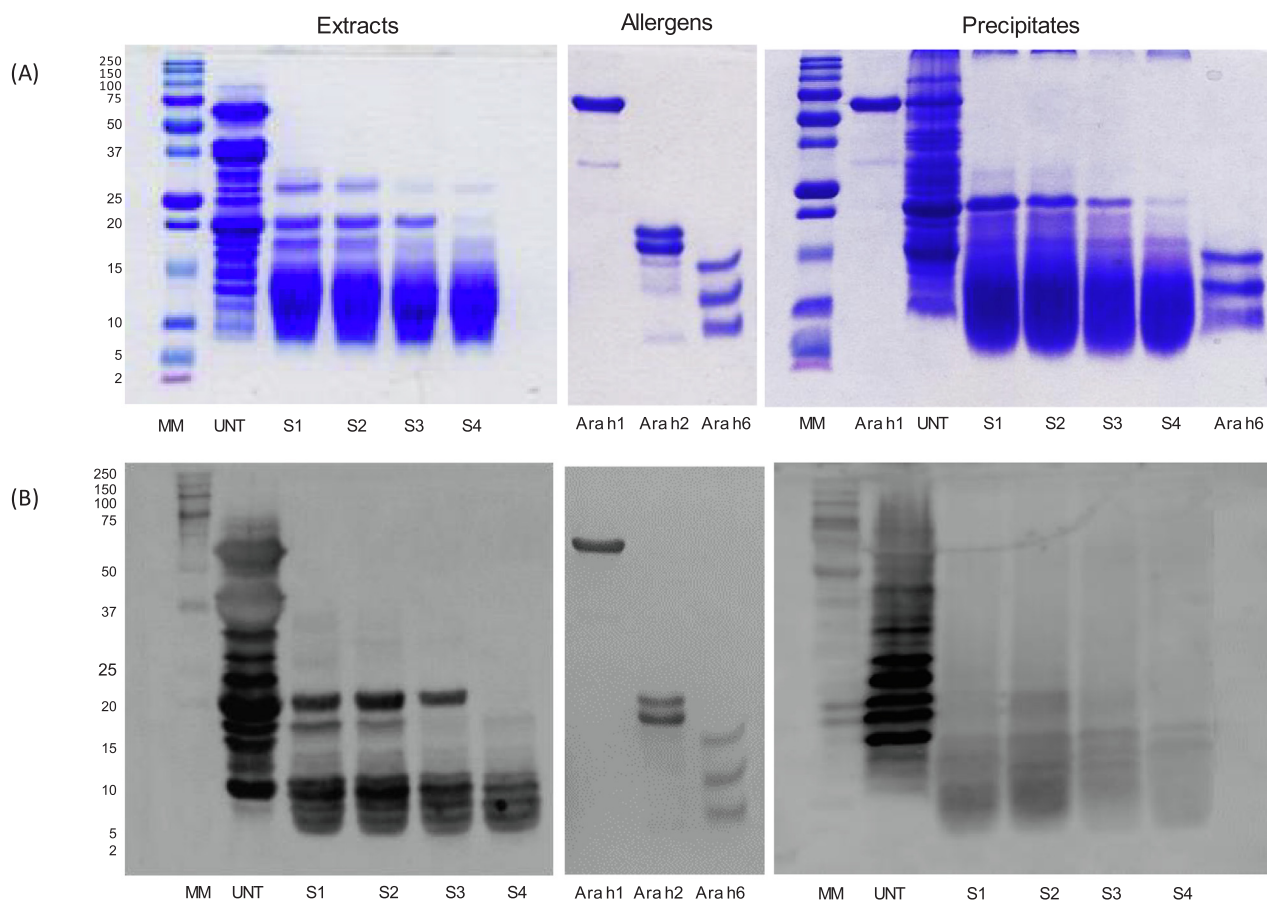


Fig. 1. SDS-PAGE (A) and western blot (B) of Alcalase-treated raw peanut samples: MM-molecular marker, UNT- untreated sample, S1-S4 samples treated with Alcalase at different concentrations: S1 –1%, S2 – 2%, S3 – 3%, S4 – 3.5%, Protein loaded: 25 μg/well (10 μg/well for MM and purified allergens).

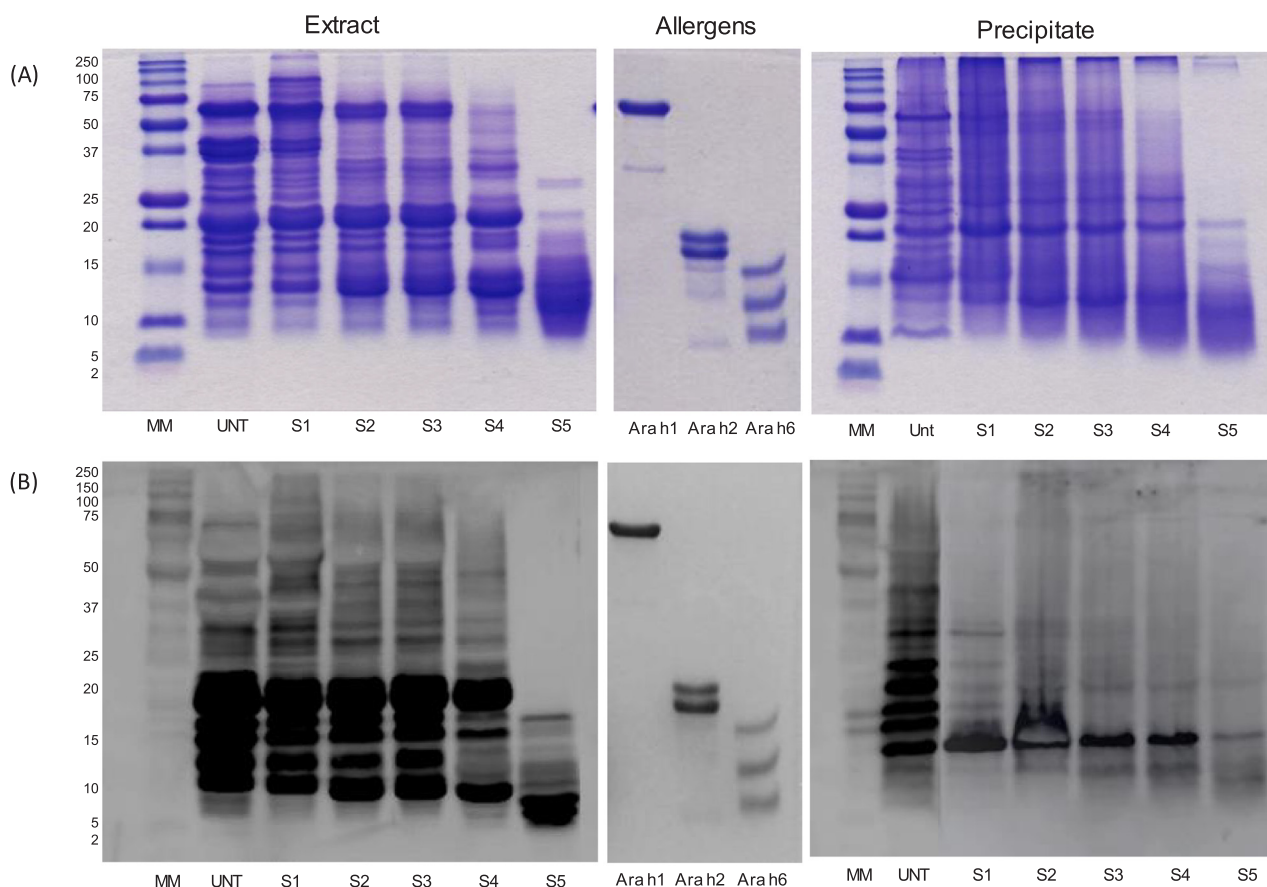


Fig. 2. SDS-PAGE (A) and western blot (B) of bromelain-treated raw peanut samples. MM-molecular marker, UNT- untreated sample, S1-S5 samples treated with bromelain at different concentrations: S1 – 0.02%, S2 – 0.04%, S3 – 0.08%, S4 – 0.16%, S5 – 0.2%. Protein loaded: 25 µg/well (10 µg/well for MM and purified allergens).

anti-human IgE peroxidase-conjugated antibody (Sigma-Aldrich, St. Louis, MO USA) (1:5000 dilution in 3% BSA-TBST) for 1 h. The membrane was washed 3 times with TBST and then the blot was developed with ECL substrate (Pierce, Rockford, IL USA) according to the manufacturer's instructions (Thermo scientific, Rockford, IL). The image of blot was visualized using a Amersham Imager 600 (GE Healthcare, South Plainfield, NJ, USA).

2.7. Data analysis

The allergen content in untreated and treated raw peanuts was expressed as mean $\pm$ standard deviation ($n = 3$). The effectiveness of enzyme treatment in reducing a specific allergen was calculated as the percentage reduction of the allergen according to the allergen contents in treated and untreated peanuts (control).

3. Results and discussion

3.1. Effects of different enzymes on the allergen contents of peanuts

Table 1 shows the pH, Ara h 1, Ara h 2 and Ara h 6 contents of untreated and protease-treated raw Virginia peanuts. Protease hydrolysis lowered the pH of peanuts slightly from 6.77 to 6.52, depending on the type of protease, which might be the consequence of amino acid release during hydrolysis. The extractable Ara h 1, Ara h 2 and Ara h 6 contents of untreated peanut were 5283, 4492 and 3207 µg/g, respectively. Alcalase effectively hydrolyzed Ara h 1 and Ara h 2. The treatment at 1% of enzyme to peanut ratio (E/S, v/w) resulted in near

complete degradation of Ara h 1 and Ara h 2 but not Ara h 6. This is consistent with the finding of a previous study (Mikiashvili & Yu, 2018). The Ara h 6 was more resistant to Alcalase hydrolysis, and it was still detectable at E/S of 3% for 2 h. The maximum Ara 1, Ara h 2 and Ara h 6 reductions achieved by Alcalase treatment were 100%, 99.99% and 96.57%, respectively, at Alcalase concentration of 3% (pH 7.5, 50 °C). Ara h 2 and Ara h 6 have 59% amino acid homology, especially in the middle part and at the C-terminal part of the protein, and at least part of the epitopes of Ara h 6 are cross-reactive with epitopes on Ara h 2 (Koppelman et al., 2005). The protease that can completely hydrolyze Ara h 2 may not completely hydrolyze Ara h 6, due to the 41% difference in amino acid heterology. It was also observed that incubating peanuts in the phosphate buffer without protease for 2 h resulted in various degrees of reduction in Ara h 1, Ara h 2 and Ara h 6. This most likely was caused by the dissolution of these proteins at pH 7.5 which is higher than their isoelectric points (Wen, Borejsza-Wysocki, DeCory, & Durst, 2007). Alcalase used in this study is a liquid enzyme preparation from *B. licheniformis*, which is dominated by serine endopeptidase subtilisin. Although subtilisin is considered as a non-specific enzyme and can hydrolyze most peptide bonds with high specificity for aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) amino acid residues (Doucet, Otter, Gauthier, & Foegeding, 2003), a small number of the peptide bonds crucial for maintaining IgE-epitopes may not be cleaved by Alcalase.

Bromelain effectively hydrolyzed Ara h 1 at a concentration of 0.08%, but could not completely hydrolyze Ara h 2 and Ara h 6 at the highest concentration (0.2%) used in this study. Increasing bromelain

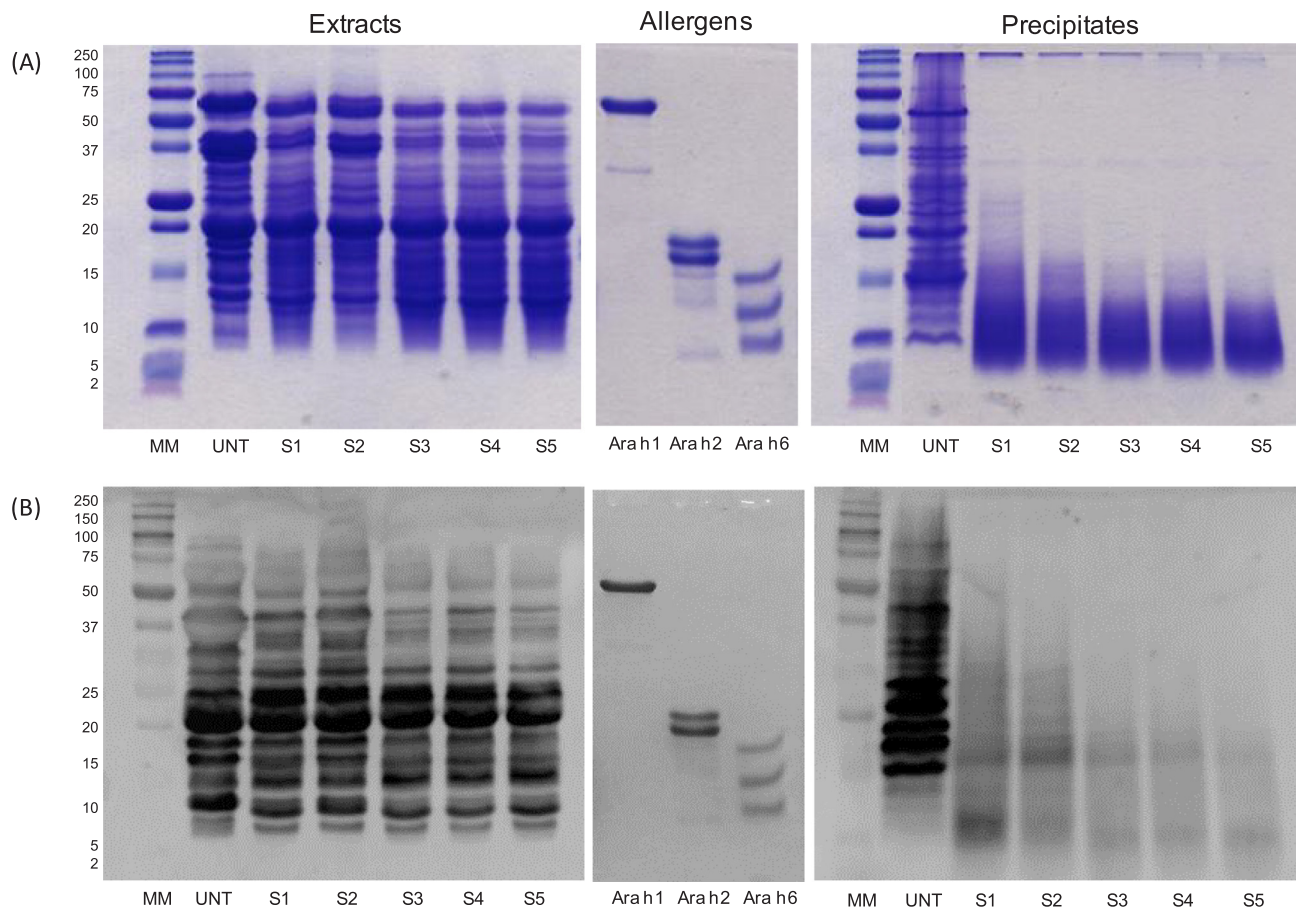


Fig. 3. SDS-PAGE and western blot of Neutrase-treated raw peanuts: MM-molecular marker, UNT- untreated sample, S1-S5 Neutrase-treated samples with concentrations: S1 – 0.2%, S2 – 0.4%, S3 – 0.8%, S4 – 1.2%, S5 – 1.6%. Protein loaded: 25 µg/well (10 µg/well for MM and purified allergens).

concentration from 0.16% to 0.2% did not lead to further reduction in Ara h 2. The highest reductions of Ara h 2 and Ara h 6 achieved by bromelain hydrolysis were 82.4% and 79.7%, respectively (Table 1). The bromelain used in this study is a cysteine endopeptidase from pineapple stem in powder form with low solubility in phosphate buffer (about 0.2%). It hydrolyzes proteins, peptides, amides and esters of amino acids/peptides; preferential cleavage site is the carbonyl end of lysine, alanine, tyrosine and glycine, but there is strong preference for Z-Arg-ArgNHMe amongst small molecule substrates (Rowan, Buttle, & Barrett, 1990). It can be seen that bromelain has fewer preferred cleavage sites than has Alcalase, and thus lower hydrolytic power.

Similar to Alcalase, Neutrase was very effective in reducing Ara h 1 and Ara h 2, but less effective in reducing Ara h 6 (Table 1). At the enzyme to peanut ratio of 0.8% (v/w), Neutrase led to 99.7 and 99.8% reductions of Ara h 1 and Ara h 2, but only 59.1% reduction of Ara h 6. Increasing Neutrase concentration from 0.8% to 1.6% did not further reduce Ara h 1, Ara h 2 and Ara h 6. The highest Ara h 6 reduction (60.6%) was observed at 1.2% Neutrase, and further increase of Neutrase concentration resulted in a higher Ara 6 content in the extract. Neutrase (EC 3.4.24.28) is a neutral, zinc metallo-*endo*-protease from *B. amyloliquefaciens*. This enzyme not only randomly hydrolyzes internal peptide bonds, but also facilitates enzymatic synthesis of oligopeptides by the reverse proteolysis reaction with zinc metal as a co-catalyst (Novozymes, 2016), while peanuts contain a moderate amount of zinc (6 mg/100 g) (Ahmed & Young, 1982). Therefore, it is possible that higher Neutrase concentration could catalyze the formation of Ara h 6 from the amino acids and peptides produced during proteolytic hydrolysis.

Papain used in this study was dry powder with activity of 52431 USP/NE U per mg. Table 1 shows that the papain was effective in

reducing Ara h 1 but less effective in reducing Ara h 2 and Ara h 6 compared with Alcalase. The Ara h 2 was still detectable at the highest papain concentration (0.5%) used in the study. The obvious reduction of Ara h 6 (77.5%) was observed at a papain to peanut ration 0.3%, but increasing papain concentration from 0.3% to 0.5% did not enhance Ara h 6 hydrolysis. Papain is a typical cysteine protease and has seven subsites for binding; the S1 subsite is relatively nonselective, but the S2 subsite has strong preference for the hydrophobic amino acid side chains of Phe, Tyr, Val and Leu of substrates (Whitaker, 2003). Although papain has fairly broad specificity, it has endopeptidase, amidase, and esterase activities, but it is not as effective as Alcalase in hydrolyzing peanut allergens, particularly, Ara h 6. The structural difference between Ara h 2 and Ara h 6 might contribute to the difference of their resistance to papain.

3.2. SDS-PAGE and IgE-binding of Alcalase-treated raw peanuts

The SDS-PAGE of Alcalase-treated peanuts (Fig. 1A) shows that Ara h 1 (64.5 kDa) and Ara h 3 (61, 45 and 37 kDa) were present in the untreated raw peanuts in highest quantities (Koppelman et al., 2004), followed by Ara h 2 (17–19 kDa). Alcalase treatment of raw peanuts, at concentrations 1% and 2%, completely hydrolyzed Ara h 1 and Ara h 3 but the majority of proteins smaller than 20 kDa remained; as Alcalase concentration was increased to 3.0%, proteins of 15–20 kDa including Ara h 2, Ara h 8 (17 kDa), Ara h 10 (16 kDa), Ara h 14 (17.5 kDa) and Ara h 15 (17.5 kDa) (WHO/IUIS, 2018) were mostly degraded in both extracts and precipitates of peanuts. However, as larger protein molecules were broken down, smaller peptides (≤ 10 kDa) formed which were not further hydrolyzed by increasing Alcalase concentration (3.5%). Some of these peptides may have overlapped with Ara h 9

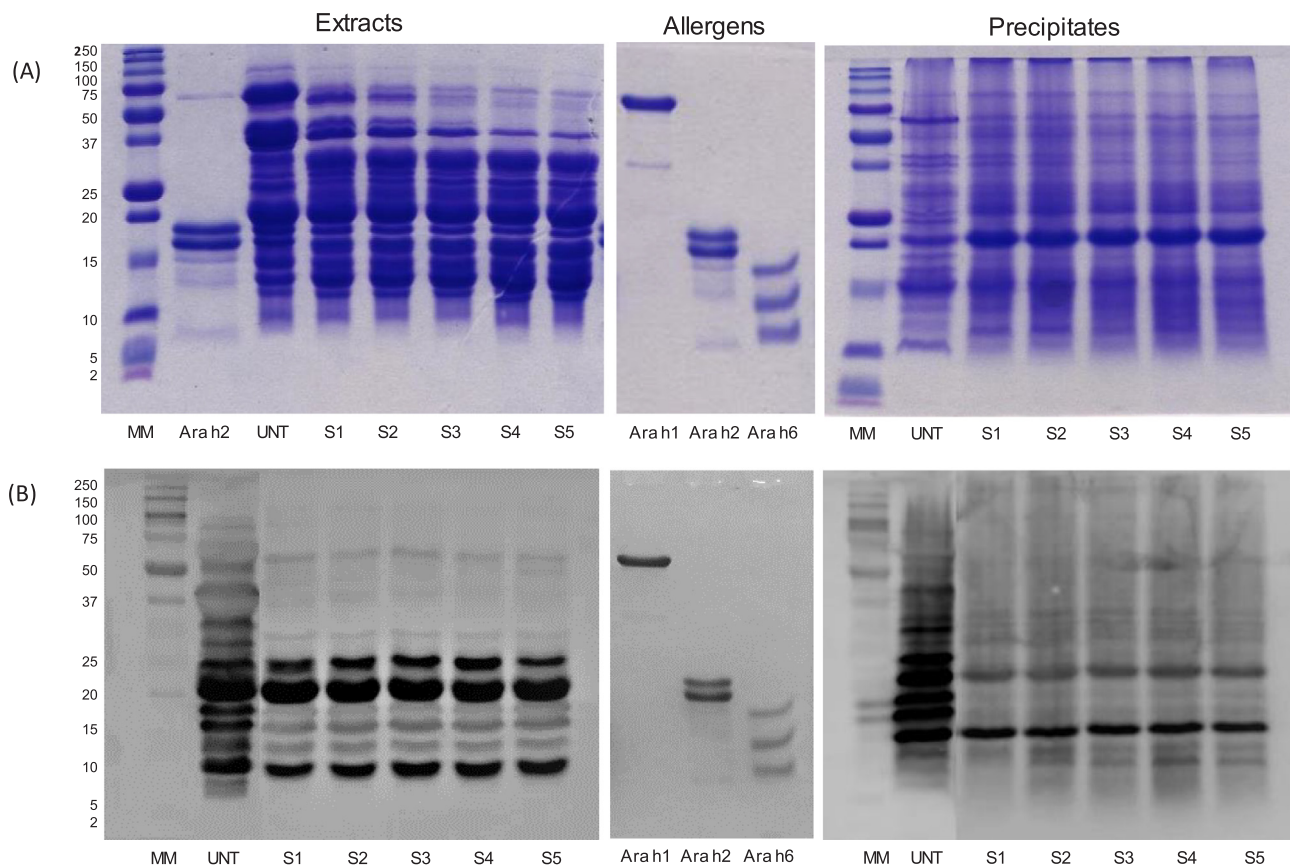


Fig. 4. SDS-PAGE and western blot of papain-treated raw peanuts. MM-molecular marker, UNT- untreated sample, S1-S5 samples treated with papain at different concentrations: S1 – 0.1%, S2 – 0.2%, S3 – 0.3%, S4 – 0.4%, S5 – 0.5%. Protein loaded: 25 µg/well (10 µg/well for MM and purified allergens).

(9.8 kDa), Ara h 16 (8.5 kDa), Ara h 17 (11 kDa), and the peptides around 5 kDa might overlap with fragments of Ara h 12 (5.2 kDa) and Ara h 13 (5.5 kDa) (WHO/IUIS, 2018). The peptides smaller than 5 kDa did not appear clearly on the SDS-PAGE and higher concentrations acrylamide gel or Tris-Tricine gel may be needed to elucidate these smaller peptides.

Although Ara h 1 and Ara h 3 were present in the untreated raw peanuts in highest quantities, their IgE-binding was weak, while the IgE-binding of proteins smaller than 37 kDa was stronger, particularly Ara h 2, Ara h 6 and the proteins with molecular weight about 10 kDa which could be Ara h 9 and Ara h 17 (Fig. 1B, lane UNT). Ara h 9 is a non-specific lipid-transfer protein in peanuts, which was reported to be a major allergen of peanut-allergic patients in the Mediterranean area (Lauer et al., 2009; Krause et al., 2009). The results of this study indicate that Ara h 9 might be more resistant to Alcalase hydrolysis than Ara h 17. Another protein with molecular weight about 25 kDa, which is a fragment of Ara h 3 (Koppelman et al., 2003), also exhibited strong IgE-binding but this protein was sensitive to Alcalase hydrolysis (Fig. 1A). Treatment of peanut kernels with 1–2% of Alcalase eliminated the allergenicity of Ara h 1 and Ara h 3, and 3.5% Alcalase significantly reduced the IgE-binding of proteins around 17 kDa, including Ara h 2. The allergenicity of all proteins larger than 10 kDa was greatly reduced at 3.5% Alcalase, but significant allergenicity of peptides of 5–10 kDa remained (Fig. 1B, lane S4). Higher Alcalase concentration or different protease may be needed to break down the epitopes of these peptides.

3.3. Allergen residues and allergenicity of bromelain-treated raw peanuts

For bromelain treatment, the maximal enzyme to peanut ratio used was 0.2%, due to the low solubility of bromelain in the phosphate

buffer. Fig. 2A shows that the treatment of raw peanuts with bromelain at lower concentrations (0.02–0.08%) obviously reduced Ara h 1 and Ara h 3 but not other allergens; Ara h 1 was completely hydrolyzed and Ara h 2 was mostly hydrolyzed at 0.2% of bromelain, but the Ara h 6 and Ara h 9 as well as proteins with similar sizes were still obvious. Therefore, Ara h 6 and Ara h 9 were resistant to bromelain hydrolysis. The change of Ara h 6 due to bromelain treatment is consistent with the quantitative results obtained by sandwich ELISA (Table 1). Increasing bromelain concentration resulted in the formation of peptides of 5–15 kDa in both extract and precipitate (Fig. 2A, lane S2-S4 and S5). Obvious reduction of IgE-binding was observed for both extracts and precipitate at a bromelain concentration of 0.16% (Fig. 2B, S4). At 0.2% bromelain, the IgE-binding of proteins larger than 17 kDa was eliminated and great reduction in IgE-binding of 10–17 kDa proteins was observed. However, the small peptides (5–10 kDa) formed during hydrolysis showed strong IgE-binding (Fig. 2A and 2B, S5) at 0.2% bromelain, which suggests that the hydrolysis of larger allergens by bromelain resulted in the formation of some smaller allergenic peptides.

3.4. Allergen residues and allergenicity of Neutrase-treated raw peanuts

Fig. 3A shows that Neutrase was effective in reducing Ara h 1 and Ara h 3 at higher enzyme concentrations (0.8–1.6%), but only slightly reduced Ara h 2 (17–19 kDa) and Ara h 6 (14.5 kDa). All major allergens were still detectable by SDS-PAGE after 2 h of hydrolysis at 1.6% Neutrase (Fig. 3A, S5). This is somehow different from ELISA results which show great reduction of Ara h 1 and Ara h 2 even at low Neutrase concentration (Table 1). Accordingly, the IgE-binding/allergenicity of Neutrase-treated peanut extract was similar to that of untreated sample with the exception of proteins of about 30 kDa (Fig. 3B). The IgE-binding of the 30 kDa protein(s) increased for all Neutrase-treated

sample extracts. This might be caused by the formation of advanced glycation end-products during drying of Neutrase-treated peanuts because it was reported that roasting resulted in the formation of high MW, IgE-reactive complexes containing peanut allergens (Hebling, McFarland, Callahan, & Ross, 2013). Based on these results, higher Neutrase concentration or longer treatment time may be needed to achieve higher allergenicity reduction. Interestingly, Neutrase treatment of raw peanuts did not increase the peptides with molecular weights 5–10 kDa in the extracts, but increased these peptides in the precipitates. However, the overall IgE-binding of precipitates of Neutrase-treated peanuts was obviously lower than that of untreated peanut precipitates.

3.5. Allergen residues and allergenicity of papain-treated raw peanuts

Similar to Neutrase, the treatment of raw peanut kernels with papain reduced the Ara h 1 and Ara h 3 in an enzyme dose-dependent manner and did not produce molecules of 5–10 kDa, but increased the band intensity of molecules around 20 kDa in the precipitates and 10–15 kDa fractions in the extracts (Fig. 4A). The western blot shows that the IgE-bindings of proteins larger than 25 kDa (including Ara h 1 and Ara h 3) and from 10 to 20 kDa (including Ara h 2 and Ara h 6) were significantly reduced by papain treatment, even at low papain concentration, but the IgE-bindings of proteins around 25, 20 and 10 kDa were basically unchanged for both extracts and precipitates (Fig. 4B). The SDS-PAGE and western blot results are consistent with the sandwich ELISA results (Table 1).

4. Conclusion

All proteases tested in this study could hydrolyze major allergens in the raw peanuts and reduce the IgE-binding/allergenicity of both soluble and insoluble portions of the peanuts to varying degrees, but their effectiveness in hydrolyzing specific allergens varied greatly. Regardless of type of protease, the concentration of protease also plays an important role in peanut allergen hydrolysis. Overall, Alcalase was the most effective enzyme for reducing raw peanut allergens and allergenicity under the experimental condition of this study. These results indicate that the allergenicity of raw peanuts can be dramatically reduced by hydrolyzing allergenic proteins within the peanut kernel using non-specific proteases. This approach is applicable in peanut processing companies and other food companies using peanut as an ingredient. However, the hydrolysis of raw peanuts by proteases also resulted in some small peptides with strong IgE-binding. Hence, further research is needed to characterize these peptides and to inactivate their immunoreactivity. In addition, the effects of protease hydrolysis on sensory properties and storage stability of peanuts, as well as the cost of enzymatic processing and consumer affordability of allergen-reduced peanuts also need to be evaluated.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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