

Allergenic Properties of Enzymatically Hydrolyzed Peanut Flour Extracts

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Key Words

Peanut flour · Peanut allergy · Proteolysis · Inhibition ELISA · Western blot · Basophil activation test · IgE cross-linking

Abstract

Background: Peanut flour is a high-protein, low-oil, powdered material prepared from roasted peanut seed. In addition to being a well-established food ingredient, peanut flour is also the active ingredient in peanut oral immunotherapy trials. Enzymatic hydrolysis was evaluated as a processing strategy to generate hydrolysates from peanut flour with reduced allergenicity. **Methods:** Soluble fractions of 10% (w/v) light roasted peanut flour dispersions were hydrolyzed with the following proteases: Alcalase (pH 8.0, 60°C), pepsin (pH 2.0, 37°C) or Flavourzyme (pH 7.0, 50°C) for 60 min. Western blotting, inhibition ELISA and basophil activation tests were used to examine IgE reactivity. **Results:** Western blotting experiments revealed the hydrolysates retained IgE binding reactivity and these IgE-reactive peptides were primarily Ara h 2 fragments regardless of the protease tested. Inhibition ELISA assays demonstrated that each of the hydrolysates had decreased capacity to bind peanut-specific IgE compared with nonhydrolyzed controls. Basophil activation tests revealed that all hydrolysates were comparable ($p > 0.05$) to nonhydrolyzed controls in IgE cross-linking capacity. **Conclusions:** These results indicate that

hydrolysis of peanut flour reduced IgE binding capacity; however, IgE cross-linking capacity during hydrolysis was retained, thus suggesting such hydrolysates are not hypoallergenic.

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Introduction

Peanut allergy is an IgE-mediated adverse immune response to peanut proteins, and is considered one of the most severe food allergies due to its life-threatening nature and persistency [1]. Among all the defined peanut allergens, Ara h 1 and Ara h 2 are considered major allergens as they are recognized by serum IgE from more than 90% of peanut-allergic patients [2, 3]. Ara h 3/4 has been recognized by serum IgE from 44 to 54% of patients with a history of peanut hypersensitivity [4, 5]. Importantly, studies investigating the capacity of peanut proteins to cross-link IgE on effector cells have demonstrated that Ara h 2, together with its homolog Ara h 6, account for more than 80% of effector activity in peanut extracts [6, 7].

Peanut flour generally refers to a high protein, partially defatted powdered material prepared from roasted peanut seed. Peanut flours are well-established as food ingredients, and are also being used as the active ingredient in ongoing peanut oral immunotherapy (OIT) trials

[8, 9]. OIT involves subjects receiving regular oral ingestion of gradually increased doses of an allergen under physician supervision to desensitize an individual with the possibility that, over time, tolerance can be induced [10]. OIT has shown promise in desensitizing peanut allergic subjects [8, 9]; however, there is an inherent risk of adverse immune response during these regimented ingestions of peanut flour [11]. As such, enzymatic hydrolysis represents a potential processing strategy to improve the OIT potential of peanut flour by modulating its immunogenic properties. Peptides derived from enzymatically hydrolyzed flours selected for OIT should ideally contain the epitopes targeting CD4⁺ T cells, but lack the capacity to cross-link IgE on mast cells and basophils. For example, the potential for Ara h 2 peptides to be used in OIT was investigated in model systems [12]. The researchers identified short Ara h 2 peptides containing T cell epitopes that target Ara h 2-specific CD4⁺ T cells but lack the capacity to bind IgE, which suggest these peptides are good immunotherapy candidates [12]. Before enzymatically hydrolyzed flours can be considered for OIT, additional basic characterization data is needed.

Enzymatic proteolysis is a commonly utilized strategy for improving the functionality, digestibility, bioactive and immunogenic properties of food proteins, including peanut [13–15]. Recently, ELISA testing indicated that Ara h 1 and Ara h 2 in peanut kernel extracts were reduced up to 100 and 98%, respectively, after hydrolysis with trypsin/chymotrypsin [16]. When processing food proteins with enzymes, choices are not limited to digestive proteases, and two commonly studied proteases are: (1) Alcalase, which is as an endoprotease with broad specificity, and (2) Flavourzyme, which has both endo- and exoprotease activities, but is primarily used as an exoprotease for debittering of hydrolysates. The effects of these two proteases on the allergenicity of roasted peanut protein extracts were recently examined [13]. Western blotting and ELISA data indicated that IgE binding for the major peanut allergens (Ara h 1, Ara h 2 and Ara h 3) was significantly decreased in the first 30 min of Alcalase hydrolysis, but Ara h 1 and Ara h 3 were more persistent with Flavourzyme hydrolysis.

In the current study, two commercially available enzymes, Alcalase and Flavourzyme, and a digestive enzyme, pepsin, were used individually to hydrolyze the peanut allergens in roasted peanut flour extracts. While it is clear from the literature that enzymatic hydrolysis can substantially reduce IgE binding of peanut protein extracts as measured via Western blotting and ELISA testing, more data is needed evaluating such hydrolysates us-

ing more physiologically relevant assays. Accordingly, Western blotting and inhibition ELISA were used to screen the IgE binding potential of generated hydrolysates, and hydrolysates were then evaluated via basophil activation testing (BAT). BAT is an ex vivo functional assay that measures the capacity of IgE to be cross-linked on the surface of basophils by tracking CD63, a protein associated with granules that is found on the cell surface following degranulation [17]. It was hypothesized that peptides generated via enzymatic hydrolysis showing decreased IgE binding in Western blots and ELISA would in turn have a decreased capacity to cross-link IgE in BAT.

Materials and Methods

Preparation of Peanut Protein Hydrolysates

Light roasted 12% fat peanut flour (Golden Peanut Company, Alpharetta, Ga., USA) dispersions (10% w/v in deionized water) were stirred for 20 min, and the pH was then adjusted to 2.0, 7.0 or 8.0 using 2 N NaOH or 2 N HCl depending on subsequent protease addition. After pH adjustment, dispersions were further stirred for 10 min and then centrifuged at 9,150 g, 10–15°C for 20 min, and the BCA assay (Pierce, Rockford, Ill., USA) was used to determine protein concentration in the supernatants. Supernatants were hydrolyzed with Alcalase from *Bacillus licheniformis* (pH 8.0, 60°C, E/S = 0.048 Anson units/g protein), pepsin from porcine stomach mucosa (pH 2.0, 37°C, E/S = 18,400 units/g protein) or Flavourzyme from *Aspergillus oryzae* (pH 7.0, 50°C, E/S = 10 LAPU/g protein). Proteases were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., USA). Ten-milliliter samples were taken at 0, 5, 30 and 60 min of hydrolysis, and proteases were inactivated by submersing samples at 90°C for 15 min.

Degree of Hydrolysis Assay

The degree of hydrolysis (DH) was determined by the TNBS assay [18]. Briefly, hydrolysates (0.05 ml) were diluted 1:20 with 1% SDS and vortexed. Then, 0.25 ml of each sample was transferred into amber vials containing 2.0 ml 0.2125 M sodium phosphate buffer (pH 8.2) and 2 ml 0.1% trinitrobenzene sulfonic acid (TNBS). Test tubes were equilibrated at 50°C for 60 min in the dark. The TNBS reaction was quenched by adding 4 ml of 0.1 N HCl. Absorbencies were measured at 340 nm using a Monochromator Microplate Reader (Tecan, Grödig, Austria). DH values were calculated using $DH = h/h_{tot} \times 100\%$, where h is the number of peptide bonds cleaved during hydrolysis determined by reference to L-leucine standard, and h_{tot} is the total number of peptide bonds determined by fully hydrolyzing soluble fractions of peanut flour dispersions with 12 N HCl for 24 h at a 1:1 ratio at 90°C.

SDS-PAGE and Western Blotting

NuPAGE® 1.0 mm × 10 well, 4–12% Bis-Tris Gels (Invitrogen, Carlsbad, Calif., USA) were used for SDS-PAGE (10 µg protein/lane) and Western blots (20 µg protein/lane), and were prepared according to the manufacturer's instructions. For Western blots,

proteins were transferred onto Immobilon® transfer membranes and stained by Ponceau S solution. The membranes were blocked using bovine albumin solution Fraction V (Gibco, Life Technologies Corp., Carlsbad, Calif., USA). Blots were incubated overnight in: (1) pooled human sera from peanut-allergic patients (n = 6, containing 0.5, 2.1, 1.3, 2.9, 1.1 and 0.6 ml of serum containing 90.3, 840, 27, 11.3, 42.9 and 159 kU/l, respectively, of peanut-specific IgE; final peanut-specific IgE = 336.6 kU/l as determined by ImmunoCAP method), (2) anti-Ara h 1 rabbit sera or (3) anti-Ara h 2 rabbit sera. Biotin-labeled affinity-purified antibody to human IgE produced in goat (1:4,000) plus NeutrAvidin™ horseradish peroxidase conjugate or goat anti-rabbit IgG- horseradish peroxidase (SouthernBiotech, Birmingham, Ala., USA) were used to bind sera antibodies. The blots were submerged in SuperSignal® West Pico Chemiluminescent Substrate, and a Chemi Doc™ Imaging System was used to visualize the digital signal of the blots.

Inhibition ELISA

Immulon 4 HBX 96-well ELISA plates (Thermo Scientific, Waltham, Mass., USA) were coated with 100 µl per well containing 20 µg/ml of crude peanut extract [19] in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated for 1 h at 37°C. Following incubation, the plates underwent 5 washes with the wash solution (PBS, 0.05% Tween-20) using an automated plate washer. To avoid nonspecific binding, the plates were then blocked with 200 µl of 2% bovine serum albumin (BSA) and incubated for 2 h at 37°C. During the blocking incubation, the samples were prepared as follows. The hydrolysates produced by Alcalase, pepsin or Flavourzyme, and their nonhydrolyzed controls were diluted in 2% BSA to a concentration of 5 mg/ml followed by a 10-fold serial dilution from this concentration. Plasma samples (from the same 4 peanut-allergic subjects used in the BAT assays) were diluted 1:20, also using 2% BSA as a diluent. Equal volumes of plasma and hydrolysates were combined and the mixture was incubated for 1 h at room temperature. Plates were washed as described above and 100 µl of each inhibited plasma sample mixture was added to the plate in duplicates and incubated for 1 h at 37°C. After washing, 100 µl of a 1:4,000 dilution biotinylated goat anti-human IgE (KPL, Gaithersburg, Md., USA) was added to every well and incubated for 1 h at 37°C. The plates were washed and 100 µl of a 1:5,000 dilution of NeutrAvidin™-Horseradish Peroxidase (Thermo Scientific) was added to every well and incubated for 45 min at 37°C. The plates were washed for a final time and 100 µl of TMB substrate (KPL) was added to each well and allowed to develop for 7 min followed by the addition of 100 µl of Stop solution (KPL). The absorbance was read at 450 nm using a microplate reader. Optical Density values were recorded and percent inhibition at each inhibitor concentration was calculated using the following equation: % inhibition = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance without inhibitor (PBS only), while A_1 is the absorbance at each inhibitor concentration. Calculations of IC₅₀ values and statistical analyses were performed using GraphPad version 5.0.

Basophil Activation Test

BAT was performed essentially as described [19]. Briefly, whole blood from peanut-allergic subjects was mixed with basophil media containing human IL-3 (Miltenyi Biotec, Bergisch Gladbach, Germany) with or without antigens for 30 min. Antigens used in this study were 1 or 0.1 µg/ml hydrolysates, nonhydrolyzed con-

trols used at the same concentrations, polyclonal anti-IgE antibody (1 µg/ml; as a positive control) or media alone (negative control). After incubation, reactions were stopped by addition of EDTA, and then cells were stained with antibodies. Following staining for 30 min, red blood cells were lysed and leukocytes were fixed. Basophils were identified as CD203c+CD123+ and CD63, the protein marker for activated basophils, assessed by flow cytometry [19]. BAT tests were performed on whole blood from the same 4 individual subjects used in the inhibition ELISA experiments. These subjects have challenge-proven peanut allergy and peanut-specific IgE levels of 923, 35.1, 836 and 316 kU/l. Statistical analyses were performed using a two-tailed, paired t test on GraphPad version 5.0.

Results

Enzymatic Hydrolysis Results in Decreased Size of IgE-Binding Peptides Although Ara h 2 Fragments Remain Intact

Prior to enzyme addition, protein contents of soluble extracts were determined. Soluble fractions at pH 2.0 (pepsin) had the highest protein content with 18.2 mg/ml, followed by soluble fractions at pH 8.0 (Alcalase) with 10.4 mg/ml, and pH 7.0 (Flavourzyme) with 5.9 mg/ml. DH after 60 min of hydrolysis was greatest for Flavourzyme (26.5%), followed by Alcalase (22.2%) and pepsin (14.9%). SDS-PAGE was used to visualize the proteins in hydrolysates and nonhydrolyzed controls (fig. 1a–c). The large molecular weight proteins in the extracts were evidently hydrolyzed into smaller fragments. Of note, a series of small MW peptides (<10 kDa) were produced during hydrolysis with all enzymes.

To qualitatively screen the hydrolysates for remaining IgE-binding peptides, a Western blotting strategy was employed. Using pooled sera from confirmed peanut allergic subjects, Western blotting revealed peanut allergens were hydrolyzed by all enzymes after 60 min of hydrolysis as demonstrated by a reduction in the size of the IgE-binding peptides (fig. 1d). Additionally, Western blots with anti-Ara h 1 (fig. 1e) and anti-Ara h 2 (fig. 1f) rabbit sera revealed that Ara h 2 was more resistant to hydrolysis than Ara h 1 regardless of the protease tested as evidenced by binding patterns of residual peptides.

Hydrolysates Have Decreased Ability to Bind Peanut-Specific Human IgE in Inhibition ELISA Assays

Inhibition ELISA was conducted using plasma from 4 peanut-allergic subjects to quantitatively compare hydrolyzed and nonhydrolyzed peanut protein extracts. As shown in figure 2, each of the hydrolysates was less effective in binding peanut-specific IgE in plasma than

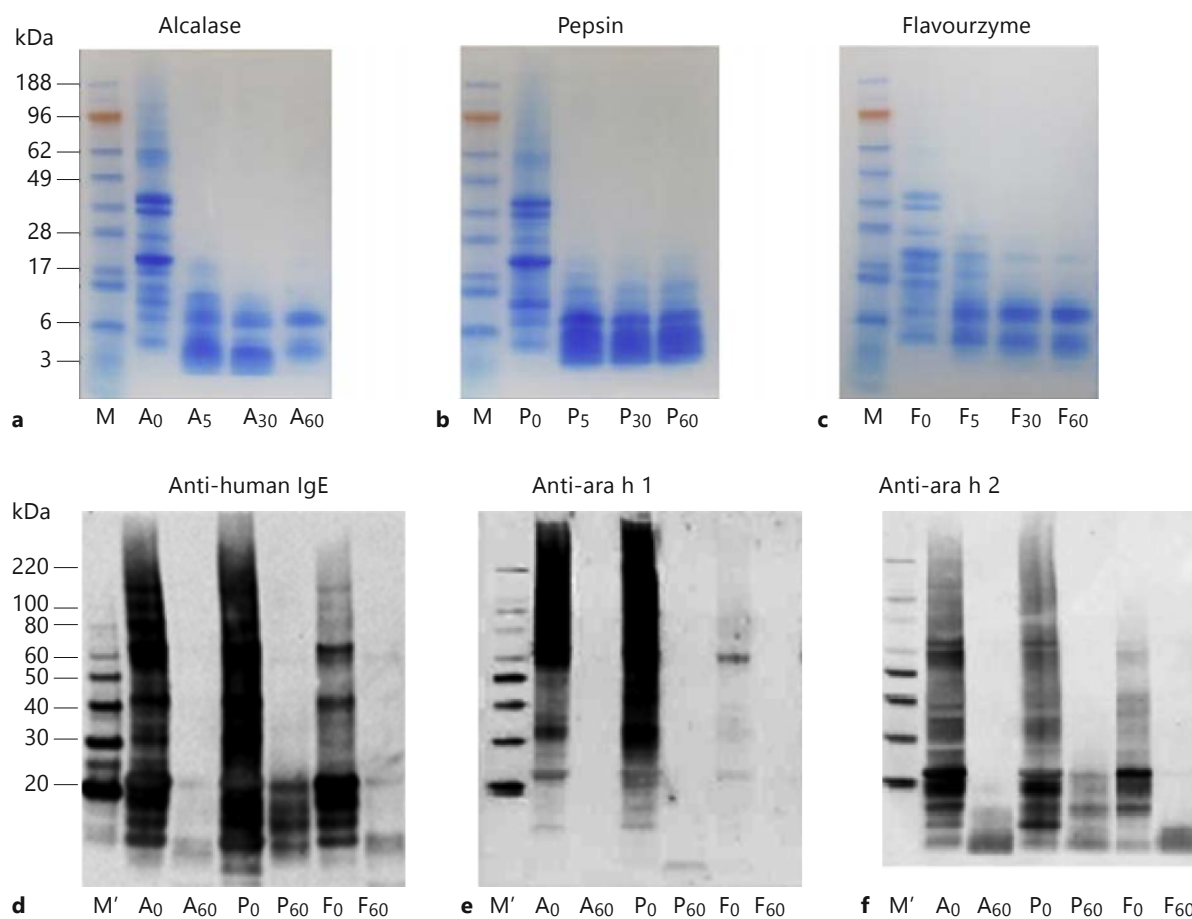


Fig. 1. SDS-PAGE (**a–c**) and Western blots (**d–f**) of soluble peanut flour extracts after various protease treatments. Proteases evaluated after 5, 30 or 60 min of hydrolysis included Alcalase (A), pepsin (P) and Flavourzyme (F). 0, 5, 30 and 60 indicate the hydrolysis time in minutes. Blots were reacted against a human sera pool from

peanut-allergic subjects (**d**), and against rabbit anti-Ara h 1 sera (**e**) and rabbit anti-Ara h 2 sera (**f**). M and M' are MW markers. Exposure times were 208 s (**d**), 51 s (**e**) and 26 s (**f**). Exposure time and contrast were adjusted to optimize visualization of faint IgE binding in hydrolysates.

Table 1. IC₅₀ values of the hydrolysates from inhibition ELISA experiments

Inhibitor	Hydrolysis time, min	IC ₅₀ value, µg/ml	p value
Alcalase	0	2.19	<0.0001
	60	208.60	
Pepsin	0	5.76	0.0982
	60	94.77	
Flavourzyme	0	1.37	0.0045
	60	18.81	
CPE		0.42	

nonhydrolyzed controls. Calculations of IC₅₀ values revealed large differences between the hydrolyzed and nonhydrolyzed samples (table 1). Each hydrolysate had a greater than 10-fold higher IC₅₀ value than its nonhydrolyzed counterpart. Hydrolysates produced by Alcalase and Flavourzyme both proved significantly weaker in terms of inhibition performance than controls, whereas the pepsin-produced hydrolysate did not quite reach statistical significance compared to its control (table 1). These results coincide with the DH data described above which demonstrated that a higher DH was accomplished by Alcalase and Flavourzyme compared to pepsin.

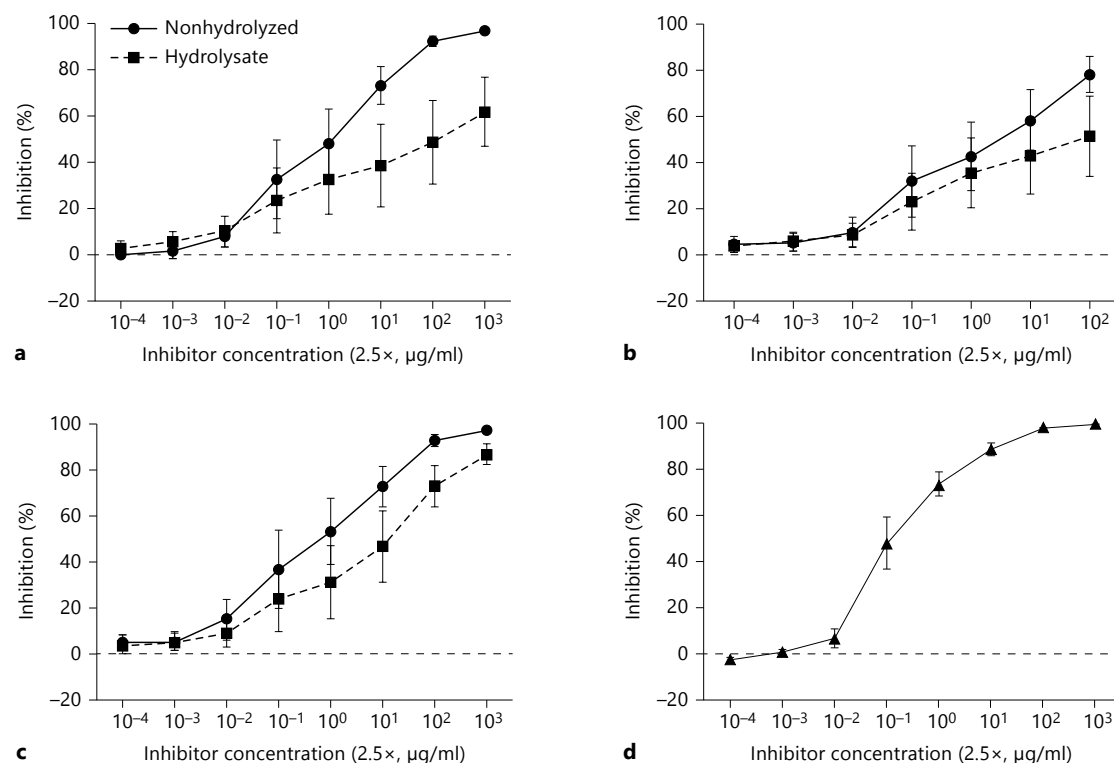


Fig. 2. Inhibition ELISA results comparing hydrolysates and non-hydrolyzed controls. Inhibitions were conducted on individual peanut-allergic subjects' plasma ($n = 4$) with hydrolysates pro-

duced by Alcalase (**a**), pepsin (**b**) or Flavourzyme (**c**). Data shown are means with standard error. The positive control, crude peanut extract (CPE), used on the 4 plasma samples is also shown (**d**).

Hydrolysates Retain the Ability to Activate Human Basophils from Peanut Allergic Subjects

BAT was conducted using whole blood from the same 4 peanut-allergic subjects used in the ELISA experiments. The negative (basophil media alone) and positive controls (anti-IgE and soluble peanut extract) behaved as expected in these 4 subjects, indicating that these subjects' basophils can be activated through the IgE pathway and are reactive to peanut antigens (fig. 3). There were no significant differences measured for any of the hydrolysates compared to their nonhydrolyzed controls in the BAT assay. Comparable results were found for both the concentrations of antigen tested (fig. 3a, b).

Discussion

We hypothesized that enzymatic degradation of peanut proteins would result in decreased size of IgE-binding peptides, decreased capacity to inhibit IgE binding in an

ELISA assay, and ultimately a decreased capacity to activate human basophils. Production of such a hypoallergenic peanut peptide mixture may be of value for peanut allergy immunotherapy applications, such as OIT. Recently, our group demonstrated that hydrolyzed cashew proteins are hypoallergenic and are effective immunotherapy reagents in a mouse model of cashew allergy [20]. In the present study, we assessed the allergenicity of peanut protein hydrolysates using human IgE and basophil assays.

The relative degrading capacities of tested enzymes, based on DH values from the TNBS assay, were: Flavourzyme > Alcalase > pepsin. SDS-PAGE analysis provided visual evidence that peanut proteins were hydrolyzed by each of the enzymes tested. Western blotting suggested that IgE binding of residual peptides was confined to peptides of <20 kDa. Furthermore, Western blotting with anti-Ara h 1 (fig. 1e) and anti-Ara h 2 (fig. 1f) showed that small MW peptides generated by hydrolysis were primarily derived from Ara h 2 regardless of the pro-

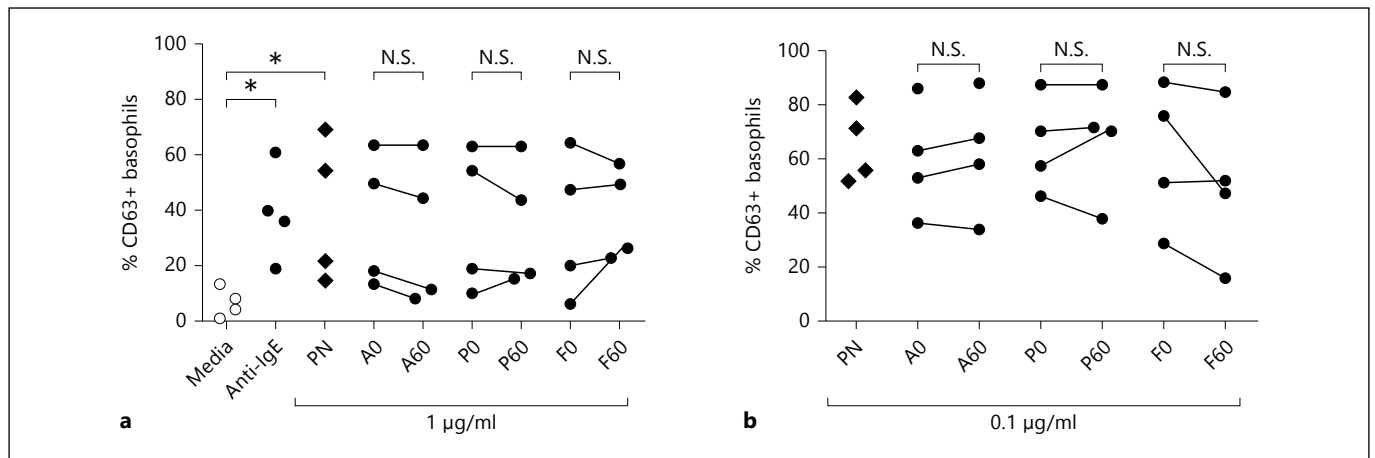


Fig. 3. Summary of BAT conducted on whole blood from peanut-allergic subjects ($n = 4$; note these are the same subjects whose plasma was used in inhibition ELISA assays shown in fig. 2). Individual data points are shown. Negative (media) and positive (anti-IgE) controls are shown along with antigens used

at 1 µg/ml (**a**) and 0.1 µg/ml (**b**). PN is soluble peanut extract from peanut flour as described in [19]; A0, A60, P0, P60, F0 and F60 are 0- and 60-min hydrolysates for Alcalase, pepsin and Flavourzyme, respectively. * $p < 0.05$. N.S. = Not significant (i.e. $p > 0.05$).

tease tested. Ara h 2 belongs to the prolamin superfamily of plant protein allergens which are characterized by a conserved motif of 8 cysteine residues [21, 22]. Structural analysis across this superfamily suggests that these 8 cysteine residues stabilize a core tertiary structure of 3 or 4 α -helix bundles connected by variable loop regions, and its highly structured core, reinforced by disulfide bonds, is resistant to enzymatic hydrolysis [23]. Pepsin resulted in hydrolysates with the darkest bands of peptides in the <20 kDa region, and pepsin resistant Ara h 2 fragments have been well documented [24]. Previously, Western blotting experiments suggested that Ara h 1 and Ara h 3 were more resistant than Ara h 2 to Alcalase and/or Flavourzyme [13]. Discrepancies are attributed to different extraction and hydrolysis protocols between the two studies. Since Western blotting probes IgE-binding proteins/peptides that are reduced and denatured on a membrane, we additionally sought to examine the capacity of the hydrolysates to bind IgE in solution using an inhibition ELISA strategy. The results indicate large differences in the capacity to bind IgE between hydrolysates and nonhydrolyzed controls (fig. 2; table 1). However, it should be noted that the hydrolysates clearly have the capacity to bind a fraction of the peanut-specific IgE, as inhibition is significantly reduced, although not rendered absent.

Despite extensive reduction in the size of IgE-binding peptides as observed by Western blotting experiments (fig. 1d–f), and the substantial decrease in IC_{50} values

from inhibition ELISA assays (table 1), no hydrolysate showed a significant reduction in its capacity to degranulate basophils compared to nonhydrolyzed controls (fig. 3a, b). A previous report found that Ara h 2 treated with trypsin/chymotrypsin displayed minimal reduction in IgE binding capacity, and mediator release from rat basophilic leukemia cells was not reduced by protease treatment, which was attributed to the proteolytic resistant core of Ara h 2 [23]. Additionally, studies have examined the effector activity of the peanut 2S albumins, Ara h 2 and 6, and have demonstrated that these proteins are the most potent peanut allergens in terms of degranulating basophils [7] and inducing anaphylaxis in a mouse model of peanut allergy [25]. Our current data show Ara h 2 fragments are present following any of the enzymatic hydrolyses, and thus may account for the persistent basophil reactivity we measured in response to the hydrolysates. Another consideration is that small peptides with reduced IgE binding capacity in the context of Western blotting or ELISA experiments may be forming larger aggregates in human blood, and these aggregates could in turn cross-link basophils. Importantly, digested Ara h 1 fragments with sizes of ~2.0 kDa were shown to form aggregates with sizes up to 20 kDa, which was hypothesized to explain the retained capacity of Ara h 1 digests to trigger immune response in Brown Norway rats [26].

A consideration for future studies is that, despite these hydrolysates seemingly displaying substantial protein degradation, further protein breakdown is needed to re-

duce IgE cross-linking. Indeed, peptides of only 8 amino acids are known to bind IgE [27] and peptides as small as 30–35 amino acids that contain at least two IgE-binding epitopes are capable of cross-linking IgE molecules on mast cells or basophils [28]. Sequential hydrolysis with Alcalase and Flavourzyme is commonly applied to increase DH, and recent work with peanut protein extracts found that the DH of sequential Alcalase/Flavourzyme hydrolysates was 69% compared to individual DH values of 17 and 29% for Alcalase and Flavourzyme, respectively [13]. Increasing the ratio of enzyme/substrate with increased incubation times may also result in further hydrolysis of the peanut proteins. Such highly hydrolyzed extracts should be evaluated via BAT or similar assays.

In conclusion, despite Western blotting and ELISA data showing significant decreases in IgE binding of various peanut flour hydrolysates, the more robust and phys-

iologically relevant basophil activation test revealed all hydrolysates retained IgE cross-linking capacity. BAT data also suggest that these hydrolysates, as currently prepared, are likely not suitable to be used as an alternative to native peanut flour proteins in OIT. Beyond OIT potential, the unexpected finding that hydrolysates can still cross-link basophils, despite extensive degradation, provides further insight into the allergenic properties of peanut proteins. Further work is needed to characterize structural and allergenic properties of peanut protein hydrolysates to better understand these phenomena.

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