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Peanut Allergens Attached with *p*-Aminobenzamidine Are More Resistant to Digestion than Native Allergens

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Abstract

Despite being known as resistant proteins, peanut allergens (Ara h 1 and Ara h 2) can be digested and cause allergic reactions. Making the allergens more resistant to digestion may aid in non-absorption and excretion of the allergens. Our objectives were to make Ara h 1 and Ara h 2 more resistant to digestion and test them in a model system using trypsin as the digestive enzyme. The resistant allergens were prepared by covalently attaching *p*-aminobenzamidine (pABA), a protease inhibitor, to peanut allergens in an extract or on a PVDF membrane using glutaraldehyde, and were then tested for resistance to trypsin digestion. SDS-PAGE and Western blot were performed to determine the allergenic capacity of the modified allergens. A control was prepared using glycine instead. Results showed that Ara h 2, when covalently attached with pABA, was more resistant to trypin digestion than the native allergen. Similarly, Ara h 1, prepared on a PVDF membrane and treated with pABA, displayed a resistance to trypsin digestion. Treatment of the allergens with glycine (a control) instead of pABA showed that the modified allergens were as digestible as native allergens. Blot assays showed that the pABA-treated allergens exhibited a lower allergenic capacity than native allergens. It was concluded that pABA, when attached to peanut allergen Ara h 1 or Ara h 2, inhibited digestion of the allergen by trypsin and reduced their allergenic capacity as well.

Keywords

p-Aminobenzamidine, Peanut Allergens, Ara h 1 and Ara h 2, Resistance to Digestion

1. Introduction

Proteins are usually digested into peptide fragments before being absorbed into the bloodstream. Food allergens are proteins, and, therefore, can be digested despite being known as resistant to digestion [1]. Once absorbed, peptide fragments from the digested allergens could bind to immunoglobulin E (IgE) antibodies and cause an allergic reaction in allergic individuals. To reduce food allergy, one approach is to inhibit or limit the digestion of allergens, so they would be excreted rather than absorbed.

The proposed approach is possible. This is because there have been studies showing that when proteins are not digested or absorbed due to being not accessible to digestive enzymes or interference by polysaccharides, an increase in fecal nitrogen excretion (a measure of protein excretion) is observed [2] [3]. In addition, our concept is also based on the successful development of several calorie-reducing commercial products which contain fats or starches that cannot be digested by enzymes such as lipase or carbohydrase. As a result of indigestion, the products reduce calorie or weigh gain after consumption. The followings are two examples:

1) *Olestra.* Olestra is a non-calorie and non-digestible fat substitute made by esterifying sucrose with fatty acids from edible oil [4] [5]. Olestra is also called Olean, a product produced by Procter & Gamble. Olestra has been clinically shown to reduce calories [6] and was approved by US Food & Drug Administration (FDA) for food use (e.g., in potato chip). Like fiber and cellulose, olestra is not absorbable because it is not digested by pancreatic lipase, and so olestra has no calorie value. With olestra, one gets the taste of the fat without any of the calories from the fat.

2) *Phase* 2. Phase 2, a carbohydrate blocker produced by Pharmachem Labs, is a white kidney bean extract with amylase inhibitors. Phase 2 has been clinically shown to reduce digestion and absorption of starch and carbohydrates by up to 66% [7]. For this reason, Phase 2 helps to control weigh gain by reducing the caloric impact and lowering the glycemic index of carbohydrates consumed. One study [8] has shown that after eight weeks of consuming Phase 2, a group lost an average of 3.79 pounds, compared with the placebo group which lost only an average of 1.65. Another study [9] has also shown a weight loss in 30 days after Phase 2 consumption, as compared to the placebo. On this basis, Phase 2 has recently received a GRAS (Generally Recognized As Safe) status from FDA.

Additionally, it has been reported that tannic acid, a polyphenol, can inhibit carbohydrate digestion and is more effective in inhibiting α-amylase than Phase 2 or other polyphenols from green tea [10]. Tannic acid may thus represent a potential method for controlling diabetes mellitus or obesity. Also, Stojadinovic *et al.* [11] has reported that digestion of β-lactoglobulin by pepsin and pancreatin is reduced when β-lactoglobulin forms complexes with polyphenols, and, thereby, hinders the release of bioactivity peptides. More recently, Sudhair *et al.* [12] has shown that when resistant starch is increased in rice, less of the rice is digested and fewer calories are absorbed.

All of the above indicates that partial inhibition of digestion of certain food components may improve or benefit health. We wanted to apply the same approach to peanut

allergens to prevent absorption and allergic reactions in allergic individuals. Peanut allergy is a health problem and on the rise. Proteins in the peanuts are the culprits, thirteen of which have been identified as allergens [13]. The major peanut allergens are Ara h 1, Ara h 2, and Ara h 3. Methods for reducing the allergenic properties of these allergens have previously been reported [14]. Recent studies have also focused on the use of pulsed ultraviolet light, D-amino acids, and oleic acid to reduce peanut allergens [15] [16] [17].

In this study, we hypothesized that peanut allergens would be more resistant to digestion if they are attached to a protease inhibitor such as *para*-aminobenzamidine (pABA) [18]. A derivative of benzamidine, pABA has been shown to be highly fluorescent when bound to trypsin or thrombin [19]. The compound has an excitation and emission maxima at 293 and 376 nm, respectively. Binding of pABA to trypsin shifts the emission peak to 362 nm with a 50-fold increase in fluorescence while binding to thrombin leads to a shift at 368 nm and a 230-fold increase in fluorescence. In the model system, we used trypsin as the protease. Our objectives were to prepare peanut allergen-pABA conjugates and determine if the conjugates would be more resistant to trypsin digestion than native peanut allergens. The model system is a step towards developing more complex systems that may further support the above concept experimentally and clinically.

2. Materials and Methods

2.1. Materials

Para-aminobenzamidine hydrochloride (pABA), glycine sodium salt, 50% glutaraldehyde, trypsin, sodium borate decahydrate, Tris buffered saline (TBS), and Tween 20 (Tw 20) were purchased from Sigma Co. (St. Louis, MO). Tris-glycine pre-cast gels (4% - 20%), Novex mini-cell apparatus for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), polyvinylidene difluoride (PVDF) membrane kit, Ponceau S solution, and bicinchoninic acid (BCA) protein assay kit were purchased from Thermo Fisher Scientific (Waltham, MA). Aqua Stain was obtained from Bulldog Bio, Inc. (Portsmouth, NH). Monoclonal anti-human IgE-peroxidase was purchased from Southern Biotechnology (Birmingham, AL). Human plasmas from five individuals with peanut allergy (IgE levels determined by CAP-FEIA = 70 - 100 kU/l) were obtained from PlasmaLab International (Everett, WA). Raw jumbo peanuts were purchased from a local grocery store. Amicon Ultra Centrifugal filters (0.5 mL, 10 K) were purchased from Millipore (Billerica, MA).

2.2. Preparation of Peanut Extract

Peanut extract was prepared according to the method of Chung & Reed [16]. Briefly, peanuts were ground and defatted with ether into defatted peanut meals. The resultant peanut meals (0.1 g) were stirred in a 50 mM sodium borate buffer, pH 8.5 (1 mL) for 45 min at room temperature, and then centrifuged at 8000 g for 5 min. The supernatant was collected and centrifuged again. The final extract was then analyzed for protein concentration using the BCA assay kit.

2.3. Preparation of pABA- and Glycine-Allergen Conjugates

The pABA conjugate was prepared using glutaraldehyde as the cross-linking agent [18]. The pABA compound contains amino groups which can be linked to the allergens through reaction with glutaraldehdye. To mimic the reaction, glycine was used as a control because it also has an amino group and is a small molecule like pABA. Briefly, pABA at 20 mM in 50 mM sodium borate buffer, pH 8.5 was mixed in equal volume with a peanut extract (1 mg/mL). Glutaraldehyde (a 50% solution) was added at a final concentration of 1%. The mixture solution was then rocked for overnight and centrifuged at 14,000 *g* for 10 min using centrifugal filters (10 K M.W. cut-off; 500 µL each). The filters were then filled with borate buffer and re-centrifuged. This procedure was repeated 6 times before the final sample (*i.e.*, pABA-allergen conjugate) was collected by centrifuging at 1000 *g* for 2 min. Protein concentration in the conjugate was determined using the BCA assay. A control was prepared in the same manner except that glycine was used instead of pABA.

2.4. Test for Allergen-pABA Conjugate's Resistance to Trypsin Digestion

The allergen-pABA conjugate (5 µL, 0.4 mg/mL) which contained Ara h 2 (18 - 20 kDa) but lacked Ara h 1 (63 kDa) was incubated with or without trypsin (5 µL, 8 µg/mL) in 5 µL of 50 mM borate buffer, pH 8.5 for 15 min at 37˚C. A control conjugate (*i.e.*, allergen-glycine conjugate) or the original peanut extract containing native peanut allergens was also treated with trypsin in the same way. The samples were then applied to a 4% - 20% Tris-glycine gel for SDS-PAGE (non-reduced) analysis. After SDS-PAGE, the gel was stained with Aqua Stain and destained with water. The allergen-pABA conjugate is said to be resistant to trypsin digestion when Ara h 2 in the conjugate remains undigested after trypsin treatment and appear in the SDS-PAGE profile. The digestion profile was also compared to that of the control glycine conjugate or the original peanut extract.

2.5. Determination of pABA Level on pABA-Allergen Conjugate

A pABA standard curve was first prepared by incubating the pABA compound (not conjugate) at various concentrations (0.1 - 0.3 mg/mL; 15 µL each) with trypsin (8 µg/mL, 15 µL) and a substrate solution containing succinylated casein (2 mg/mL, 40 µL in 50 mM borate buffer, pH 8.5) for 20 min, 37˚C. TNBS (0.67%, 25 µL) was then added, and the mixture was again incubated for 20 min, 37˚C, according to the manual from Thermo Scientific, Inc. After the TNBS reaction, the absorbance was measured at 450 nm, and a pABA standard curve was plotted. To determine the level of pABA on the surface of the pABA-allergen conjugate, the conjugate was incubated with trypsin, substrate and TNBS as described above. The level of pABA on the conjugate was determined in duplicates based on the standard curve.

2.6. Conjugation of pABA to Ara h 1 on PVDF Membrane and Test for Resistance to Trypsin Digestion

A membrane strip containing the major peanut allergen Ara h 1 (63 kDa) was prepared

by applying a peanut extract (1 mg/mL, 8 μL) to SDS-PAGE, transferring the proteins to a PVDF membrane in an iBlot apparatus, and cutting out the membrane containing the Ara h 1 band only. The membrane strip was then treated with 0.5% glutaraldehyde (1 mL) in 50 mM sodium borate, pH 8.5 for 2 hr, washed, and incubated with the pABA compound (10 mM) for 3 hr. The resultant membrane containing the pABA-Ara h 1 conjugate was washed and then subjected to treatment with trypsin described below. A control membrane strip was prepared using glycine instead of pABA.

To determine the resistance of Ara h 1-pABA conjugate to trypsin digestion, the membrane strip containing the Ara h 1 conjugate or Ara h 1-glycine conjugate (control) was treated with trypsin (5 μg/mL, 300 μL) for 30 min at 37˚C with some modification of the methods of Fernandez *et al*. [20] and Vestling & Fenselau [21]. After treatment, the membrane was washed, stained with a Ponceau S solution overnight, and then destained with deionized water. The Ara h 1-pABA conjugate is said to be resistant to trypsin if the Ara h 1 band remains undigested and appear on the membrane.

2.7. Determination of IgE Binding to Ara h 1- and Ara h 2-pABA Conjugates

IgE binding was determined in Western blot as previously described [17]. PVDF membrane strip containing Ara h 1-pABA conjugate, Ara h 2-pABA conjugate, or native allergens (Ara h 1 & Ara h 2) from the original peanut extract was incubated with a pooled plasma containing IgE antibodies against peanut allergens (1:10) for 30 min. IgE binding was detected using a mouse anti-human IgE-peroxidase (1:500) and a substrate solution of 4-chloro-1-naphthol (0.5 mg/mL) in triethanolamine buffered saline containing 0.03% hydrogen peroxide.

3. Results

3.1. Profiles of pABA- and Glycine-Allergen Conjugates in SDS-PAGE

Peanut allergens and pABA were covalently linked and became conjugates because of the reaction between the aldehyde groups of glutaraldehyde (GA) and the amino groups of pABA and peanut allergens (**Figure 1(a)**). However, during the reaction with pABA/glutaraldehyde, some proteins including allergen Ara h 1 (63 kDa) were found to form insoluble aggregates. Ultimately, the aggregates were removed through centrifuging and filtering (which also removed excess pABA), thus leaving a conjugate solution that contained mostly the soluble allergen Ara h 2 (18 - 20 kDa) and a smaller protein (15 kDa) seen in SDS-PAGE (**Figure 1(b)**). By contrast, a control conjugate that was prepared using glycine instead of pABA contained mostly soluble protein polymers (>113 kDa) and Ara h 2 (**Figure 1(b)**) (note: lane may not be in alignment with other lanes because it was from another gel). Ara h 3 was not shown in the profile because all SDS-PAGEs in this study were performed under a non-reducing condition. As Ara h 2 was the major protein in the pABA conjugate, we focused on its resistance, if any, to digestion by trypsin in comparison with Ara h 2 in the control glycine conjugate and original peanut extract.

3.2. Resistance of Ara h 2-pABA Conjugate to Trypsin Digestion

Figure 2 (SDS-PAGE) shows the trypsin digestion profiles of Ara h 2 in the pABA and

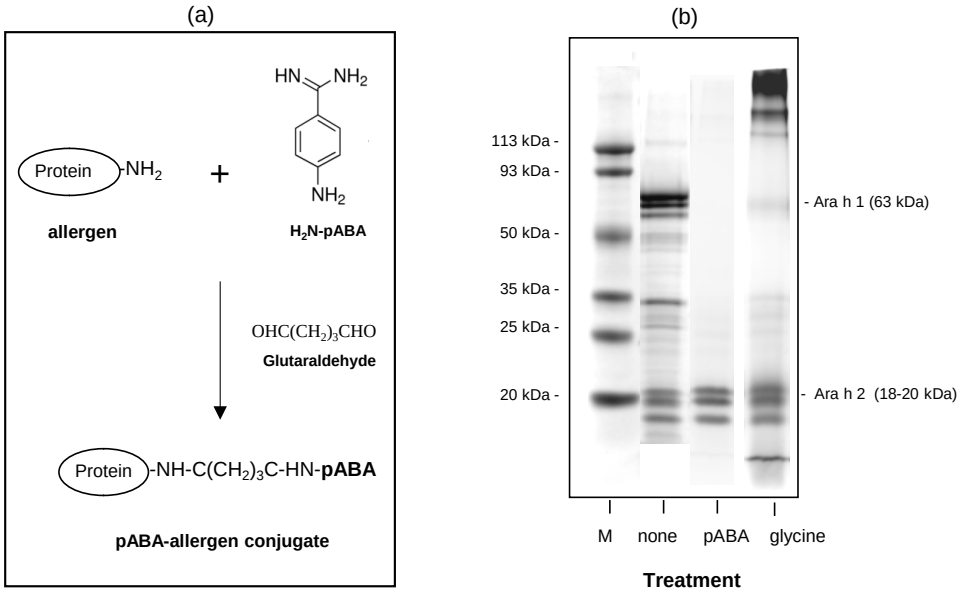


Figure 1. (a) Scheme for preparation of pABA-allergen conjugates and (b) SDS-PAGE of allergen conjugates in peanut extracts treated with pABA or glycine in the presence of glutaraldehyde. After treatment, excess of chemicals were removed by centrifugation and filtration. M = markers.

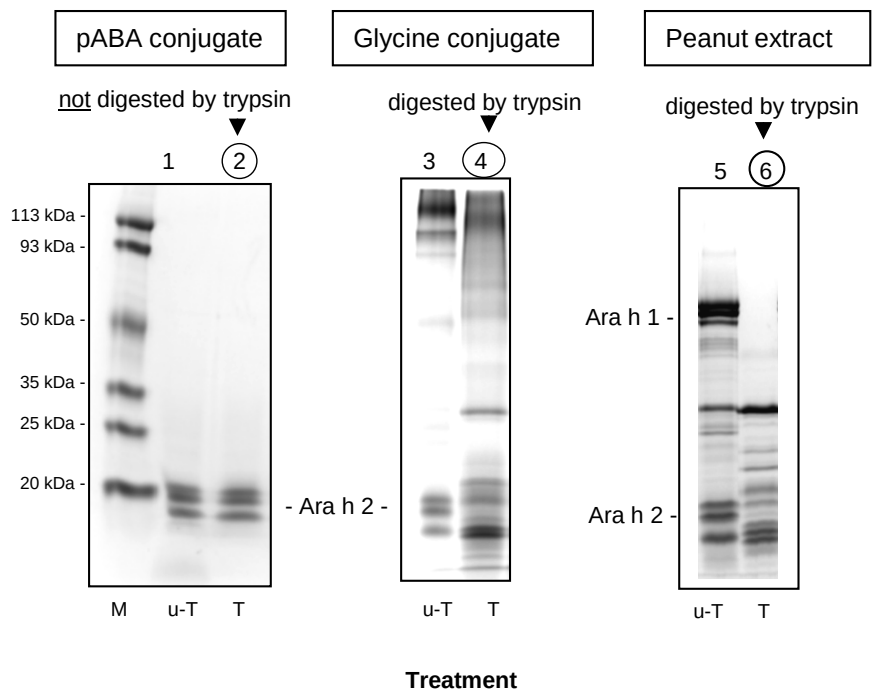


Figure 2. Resistance of pABA-Ara h 2 conjugate to trypsin digestion. Glycine conjugate (control), pABA conjugate, and original peanut extract containing native allergens were each incubated with trypsin and then analyzed by SDS-PAGE. u-T = untreated; T = trypsin-treated; M = markers.

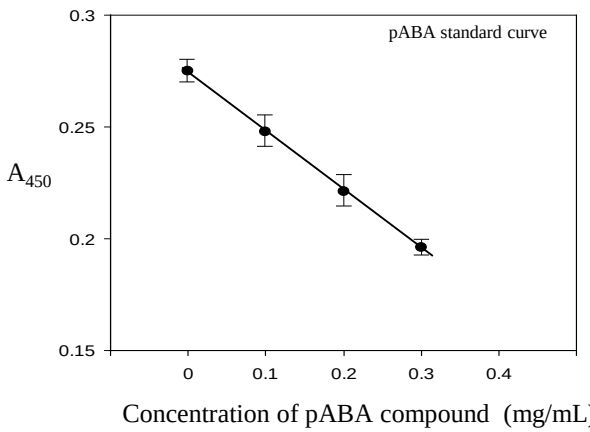
glycine conjugates (a control) and the original peanut extract (alternative control). Data showed that Ara h 2 in the pABA conjugate was not digested by trypsin (Figure 2, lane 2), whereas Ara h 2 in the glycine conjugate or in the original peanut extract was digested by trypsin (Figure 2, lanes 4 and 6). Also, native Ara h 1 in the original extract was digested as well (Figure 2, lane 6). To further prove that an Ara h 1-pABA conjugate, which was not obtained in solution due to insolubility, was more resistant to digestion than native Ara h 1, the Ara h 1 conjugate was prepared on a PVDF membrane rather than in solution (see below). Above all, data on the Ara h 2-pABA conjugate indicates that the Ara h 2-pABA conjugate was more resistant to trypsin digestion than the Ara h 2-glycine conjugate (control) or the native Ara h 2 in the original peanut extract.

3.3. Level of pABA on pABA-Allergen Conjugate

Prior to measuring the level of pABA on the conjugate, a pABA standard curve was prepared, using various concentrations of pABA compound (not the conjugate) to inhibit trypsin digestion. As shown in the pABA standard curve (Figure 3), the degree of digestion (*i.e.*, absorbance value on Y-axis) decreased as the concentration of the pABA compound increased, indicating that digestion was inhibited by increased level of pABA compound. Based on the standard curve, the level of pABA on the allergen-pABA conjugate was determined to be approximately 50 μg/mg protein after subjecting the allergen conjugate to trypsin and TNBS assays (Figure 3).

3.4. Ara h 1-pABA Conjugate on PVDF Membrane and Its Resistance to Trypsin Digestion

Because the major peanut allergen Ara h 1 could not be obtained as a Ara h 1-pABA conjugate in solution due to solubility problem, we prepared the Ara h 1-pABA conjugate on a PVDF membrane strip to further prove that Ara h 1 attached with pABA, like



Level of pABA in allergen conjugate

Level calculated = 50 μg pABA/mg protein

Calculation based on:

total protein (15 μL)	= 15 μg
A-450	= 0.25
pABA based on std curve	= 0.05 mg/mL or 50 ng/uL
total pABA (15 μL)	= 750 ng (=50 x15)
pABA/ protein	= 750 ng/ 15 μg protein
	= 50 μg /mg protein

Figure 3. pABA standard curve and level of pABA in allergen conjugate. For standard curve, pABA compound at the concentration indicated was incubated with trypsin, succinylated casein and then with TNBS. Absorbance was read at 450 nm. For determining pABA in the conjugate, the pABA compound was replaced with the pABA-allergen conjugate. Values are means ± SD of duplicate.

Ara h 2-pABA conjugate described above, is resistant to trypsin digestion. To visualize the result, the membrane strip containing the Ara h 1-pABA conjugate or the control (*i.e.*, Ara h 1-glycine conjugate) was stained with a Ponceau S solution after treatment with trypsin. The band color was pink after staining, but it was presented here as black and white. Results showed that after treatment with trypsin, Ara h 1 in the control glycine conjugate disappeared from the membrane while Ara h 1 in the pABA conjugate remained present on the membrane (**Figure 4(a)**). The finding indicates that Ara h 1-pABA conjugate was more resistant to trypsin digestion than the control glycine conjugate. All these including the data on Ara h 2-pABA conjugate (**Figure 2**) indicate that pABA, when attached to the allergen, can prevent the allergen from being digested by trypsin.

3.5. Allergenic Capacity of Ara h 1- and Ara h 2-pABA Conjugates

The allergenic capacity of Ara h 1 and Ara h 2 may or may not change after conjugation with pABA. To determine if there is a change in allergenic capacity, IgE bindings of Ara

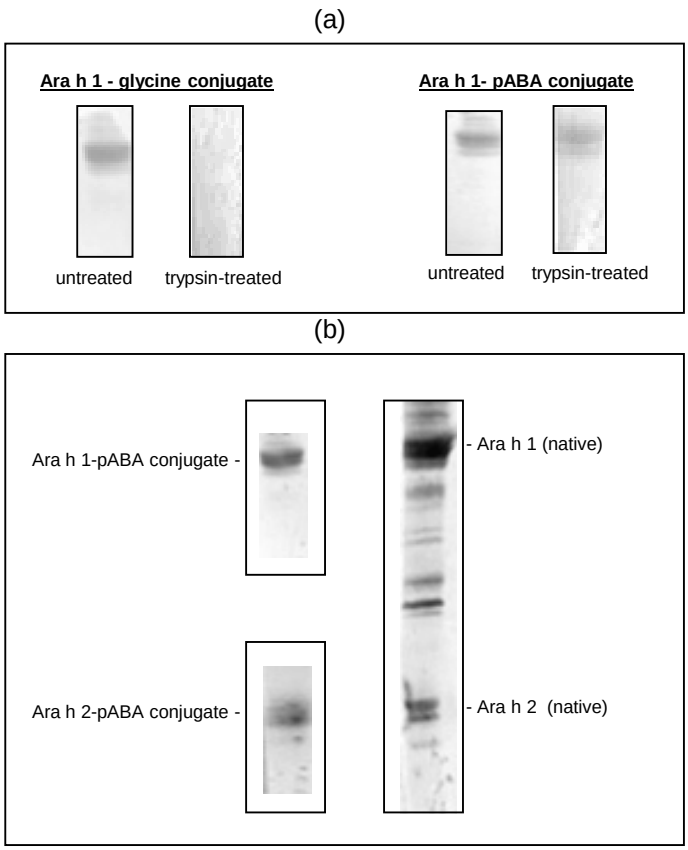


Figure 4. (a) Digestion profiles of Ara h 1-pABA and Ara h 1-glycine conjugates on PVDF membrane and (b) IgE bindings of Ara h 1-pABA, Ara h 2-pABA, and native Ara h 1 & Ara h 2 in Western blots. In (a), membranes were stained with a ponceau S solution after trypsin treatment. In (b), membranes were treated with a pooled plasma (1:10) containing IgE antibodies against peanut allergens, followed by detection with a mouse anti-human IgE peroxidase (1:500) and a colorimetric substrate.

h 1- and Ara h 2-pABA conjugates were determined in Western blot using a pooled human plasma containing IgE antibodies against peanut allergens. Membrane strips with Ara h 1-pABA and Ara h 2-pABA conjugates after plasma treatment are shown in **Figure 4(b)**. It should be noted that the two membrane strips were prepared differently. As described in Methods, Ara h 1-pABA was prepared from native Ara h 1 (from original peanut extract) on a PVDF membrane which was treated with glutaraldehyde/pABA, whereas Ara h 2-pABA was prepared by first treating the original peanut extract with glutaraldehyde/pABA and then transferring to a PVDF membrane. Results showed that both Ara h 1- and Ara h 2-pABA conjugates were recognized by IgE antibodies to some extent, as compared to the native Ara h 1 and Ara h 2 from the original peanut extract (**Figure 4(b)**). Despite the allergenic capacity, the allergen conjugates may not illicit an allergic response based on the assumption that they are indigestible and non-absorbable and could be removed through excretion. This still needs to be further proven clinically.

4. Discussions

This study was conducted based on the concept that peanut allergens that are indigestible may bypass the digestive tract and be excreted without causing an allergic reaction. The concept (*i.e.*, indigestibility) is not new and has been shown to be feasible (*i.e.*, proteins excreted due to indigestibility) [2] [3]. Also the approach has been adopted in the successful development of such commercial products as Phase 2 [7] and Olestra [4] [5] which aim at reducing calorie intake by inhibiting the digestion of starch or fat. Although protease treatment may be another option to reduce peanut allergy, it has been shown that digestion of peanut allergens by proteases does not totally eliminate the allergenic potential or IgE binding of the allergens [22] [23], and besides, high allergenic potency or mediator release from mast cells still exists, suggesting that reduction in IgE binding due to protease treatment does not necessarily translate into reduced allergenic potency [22]. We, therefore, used a different approach—that is, inhibiting rather than enhancing the digestion of peanut allergens to reduce their allergenic potential. Our objective was to demonstrate that peanut allergens can be made more resistant to digestion in a simple model system.

In the model system, we prepared digestion-resistant allergen conjugates by covalently attaching pABA, a protease inhibitor, to peanut allergens in an extract (**Figure 1(a)**). Results (SDS-PAGE) showed that after treatment with pABA, only the allergen Ara h 2 (18 - 20 kDa) and a lower band remained as the pABA conjugates in the extract (**Figure 1(b)**). Other proteins including major peanut allergen Ara h 1 (63 kDa) were not seen in SDS-PAGE because they formed insoluble aggregates during pABA treatment and were removed by centrifuging and filtering. The aggregates were formed mostly because Ara h 1 cross-linked with each other and/or other proteins in the presence of glutaraldehyde. It is not clear why Ara h 2 did not form insoluble aggregates. Insoluble aggregates are known to hardly break down in simulated digestive system, and, thereby, may be removed through excretion [24].

Ara h 2 is reported to contain a high proportion of amino acids such as glutamine and arginine [22]. The high proportion of glutamine and arginine in Ara h 2 suggests that a reasonable amount of pABA would be attached to Ara h 2 after treatment with pABA and glutaraldehyde, and ultimately, an Ara h 2-pABA conjugate with a higher resistance to digestion would be obtained. Indeed, data showed that the Ara h 2 conjugate was not digested by trypsin or was more resistant to digestion than the native Ara h 2 from an original peanut extract or the Ara h 2-glycine conjugate (**Figure 2**). The latter glycine conjugate was prepared in order to confirm that Ara h 2-pABA conjugate became more resistant not because Ara h 2 itself contributed to the resistance [25] but because it was the bound pABA that conferred a higher resistance property to Ara h 2. Consistently, we found that Ara h 1-pABA conjugate, which was prepared on a PVDF membrane, was more resistant to trypsin digestion than native Ara h 1 or Ara h 1-glycine conjugate (**Figure 4(a)**). All these indicate that pABA, when attached to peanut allergens, could prevent the allergens from being digested by trypsin.

Additionally, we did IgE binding because in many cases (after modification), allergens could change in their affinity for IgE, and this additional information (a reduced IgE binding in this case) may be useful to researchers who are interested in studying the change in IgE binding to modified allergens.

5. Conclusion

Peanut allergens Ara h 1 and Ara h 2, when covalently attached with pABA, were shown to be more resistant to trypsin digestion than native allergens. Treatment of the allergens with glycine (control) instead of pABA in the presence of glutaraldehyde resulted in a digestion profile that was comparable to that of native allergens. The model system thus demonstrated that peanut allergens can be made more resistant to digestion by attaching protease inhibitors to the allergens. Further investigations or *in vivo* studies (e.g., animal studies) are still needed to confirm the resistance of modified allergens to digestion.

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