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Effects of Phytic Acid on Peanut Allergens and Allergenic Properties of Extracts

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Phytic acid would form soluble and insoluble complexes with proteins. Our objective was to determine if phytic acid forms insoluble complexes with major peanut allergens, and if such reaction results in a peanut extract with a lower level of soluble allergens and allergenic property. Extracts from raw and roasted peanuts were treated with and without phytic acid at various pH values and then analyzed by SDS-PAGE and a competitive inhibition ELISA (ciELISA). The ciELISA measured IgE binding using a pooled serum from peanut-allergic individuals. Results showed that phytic acid formed complexes with the major peanut allergens (Ara h 1 and Ara h 2), which were insoluble in acidic and neutral conditions. Succinylation of the allergens inhibited complex formation, indicating that lysine residues were involved. A 6-fold reduction in IgE binding or allergenic potency of the extract was observed after treatment with phytic acid. It was concluded that phytic acid formed insoluble complexes with the major peanut allergens, and resulted in a peanut extract with reduced allergenic potency. Application of phytic acid to a peanut butter slurry presented a similar result, indicating that phytic acid may find use in the development of hypoallergenic peanut-based products.

KEYWORDS: Phytic acid; peanut allergens; Ara h 1; Ara h 2; peanut butter; IgE antibodies; ELISA; succinylation

INTRODUCTION

Phytic acid, a *myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($C_6H_{18}O_{24}P_6$), is the chief storage form of phosphate and inositol in mature oilseeds (*1*). On a dry basis, whole oilseeds may contain about 1% phytic acid. For a long time, phytic acid has been related to human health only as an antinutrient because it chelates minerals such as calcium, zinc, iron, magnesium, and potassium and, thereby, reduces their bioavailability (*2*). However, during the last decade, it has been shown that phytic acid is also an antioxidant (*3, 4*), an anticancer agent (*5, 6*) and a useful agent against diabetes (*7, 8*).

Besides minerals, phytic acid binds to and forms soluble and insoluble complexes with proteins (*9, 10*). Insoluble complexes reduce the bioavailability of proteins. The binding between proteins and phytic acid is reported to be electrostatic in nature and involves the anionic phosphate groups of phytic acid and the cationic groups of proteins. However, not all proteins or proteins from the same seed bind to phytic acid. Proteins from corn germ are reported not to bind to phytic acid (*11*).

Peanut allergens are proteins that are associated with peanut allergy. Because the incidence of peanut allergy is on the rise, there have been increased investigations on how to prevent peanut allergy. Changing the allergenic potency or levels of peanut allergens is one approach that may lower the risk of peanut allergy. Previous studies in this laboratory have shown that the allergenic potency of peanut allergens can be reduced, using various enzymes (*12, 13*). To date, several proteins have been identified as peanut allergens (Ara h 1 to Ara h 8), among which Ara h 1 (a vicilin-like protein) and Ara h 2 (a conglutin-like protein) are considered the major allergens because they are recognized by 70 to 90% of sensitized individuals (*14*). Ara h 3, a glycinin protein, is considered a minor peanut allergen because only 45% of patients with allergy show specific immunoglobulin E (IgE) in their sera (*15*). Other minor peanut allergens include Ara h 4 (glycinin), Ara h 5 (profilin), Ara h 6/ Ara h 7 (conglutinin), and Ara h 8 (Bet v 1) (*16*). It is believed that solely by removal of the major peanut allergens, the allergenicity of peanuts is reduced. In this study, we hypothesized that phytic acid may form insoluble complexes with the major peanut allergens, and through this reaction, the allergenic potency of a peanut extract is reduced. Our objective was to determine the validity of this hypothesis. Also, the application of phytic acid to natural peanut butter and related products was discussed.

MATERIALS AND METHODS

Materials. Tris-glycine precast gels (4–20%) were purchased from Invitrogen (Carlsbad, CA). Rabbit antihuman immunoglobulin E (IgE)-peroxidase was purchased from Dako Corporation (Carpinteria, CA). Phytic acid monocalcium salt, succinic anhydride, *o*-phenylenediamine, Tween 20, 96-well microtiter plates, Trizma Base, and phosphate buffer

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saline (PBS) were purchased from Sigma Co. (St. Louis, MO). Superblock blocking buffer, GelCode Blue Stain Reagent, and bicinchoninic acid (BCA)-protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL). Human sera from five patients with peanut allergy (determined by skin prick test and CAP-FEIA assay for IgE) were obtained from the University of Arkansas for Medical Sciences (Little Rock, AR). Raw and roasted high-oleic peanut seeds (SunOleic) were obtained from the University of Florida, Gainesville, FL. Natural peanut butter (creamy and nonhydrogenated) was purchased from a food store.

Treatment of Peanut Extracts and Natural Peanut Butter Slurries with Phytic Acid. Peanut extracts were prepared as previously described (*13*) in 20 mM Tris buffer at pH 3, 7, or 8.5 from defatted meals of raw and roasted peanuts. Extracts (5 mg/mL, 50 μL) were incubated with phytic acid monocalcium salt (5 μL) at a final concentration of 2 mM at room temperature for 10 min and centrifuged (8000 g, 5 min, 4 °C). Protein concentration was determined using the BCA kit assay. The resultant supernatant was subjected to SDS-PAGE (nonreducing), using Tris-glycine precast gels (4–20%) and a Novex Gel Electrophoresis Apparatus. After SDS-PAGE, gels were stained with GelCode Blue Stain and destained with water. In the case of natural peanut butter (nonhydrogenated), slurries were prepared and treated by adding phytic acid (10 mg) to natural peanut butter (0.5 g) in 20 mM Tris buffer, pH 7 (1.5 mL), and stirring the mixture for 60 min at room temperature. The slurries were then centrifuged and the resultant supernatants analyzed by SDS-PAGE and competitive inhibition enzyme-linked immunosorbent assay (ciELISA) (see below).

Determination of Protein Complex Solubility. The precipitates (i.e., protein complexes) were each isolated from the above phytic-treated peanut extracts by centrifuging the extracts and discarding the supernatants. The isolated complexes were then resuspended in 0.02 M Tris buffer (100 μL) at various pH values (3, 7, and 9), vortexed, and centrifuged. The resultant supernatants were then analyzed for their protein concentration using the BCA assay kit. At pH 9, the complex dissolved completely. The solubility of the complex at a pH was calculated as: complex solubility (%) = $(C/C_o) \times 100$, where C = protein concentration at pH 3, 7, or 9, and C_o = protein concentration at pH 9.

Succinylation of Peanut Extracts. Treatment of peanut extracts (raw and roasted) with succinic anhydride was performed according to the method of Lawai and Adebowale (*17*). Briefly, succinic anhydride (25 mg) was added slowly to the peanut extracts in 20 mM Tris buffer, pH 8 (5 mg/mL, 5 mL), and during the reaction, the pH was maintained at pH 8 with 1 N NaOH. The resultant extracts were dialyzed against 0.02 M Tris buffer, pH 7, and then treated with phytic acid as described above. Trinitrobenzenesulfonic acid (TNBS) was used to confirm the reduced availability of free amino groups after succinylation (*18*).

Determination of IgE Binding of Phytic-Treated Peanut Extracts and Natural Peanut Butter Slurries. A ciELISA was carried out (n = 3) as previously described (*13*). Briefly, a peanut extract (50 μL; 0.01–10 μg/mL) treated with and without phytic acid was mixed with a pooled serum (1:20, 50 μL) and then incubated for 45 min in a plate coated with a roasted peanut extract. Immunoglobulin E (IgE) detection was performed using a rabbit antihuman IgE peroxidase conjugate (1:500, 100 μL) and a substrate solution (100 μL) containing *o*-phenylenediamine (0.5 mg/mL) and 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5. The substrate–enzyme reaction was stopped with 4 N sulfuric acid (50 μL), and the absorbance was read at 490 nm with a CERES 900C plate reader (Bio-Tek Instruments, Inc., Winooski, VT). All samples except the substrate were diluted in 1:1 [Superblock]:[PBS/Tween 20]. The absorbance value of a sample containing IgE antibodies and the extract was represented by B, whereas B_o represented the absorbance value of a control containing IgE only. Values are means ± SD (n = 3). Statistical analyses were performed using Student's t-test at a $P < 0.05$ level of significance. Phytic-treated and untreated peanut butter slurries, after being centrifuged, were analyzed in the same way.

Determination of the Effect of Phytic Acid (Bound To Allergens) on IgE Binding. A plate coated with allergens, to which phytic acid was attached, was prepared. Briefly, the plate was first coated with the untreated peanut extract as described above, followed by incubation with the phytic acid monocalcium salt (2 mM) in 0.02 M Tris buffer, pH 7. After 15 min, the resultant phytic-allergen coated plate was washed, incubated with a pooled serum (IgE) (1:20, 100 μL), and detected in the ciELISA as described above. A control plate coated with peanut allergens but without phytic acid attached was also carried out.

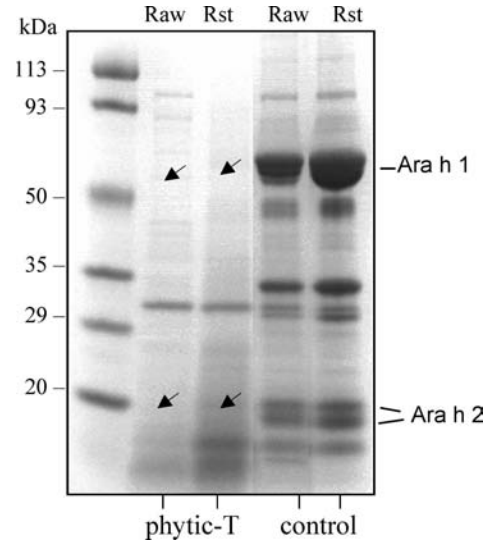

Figure 1. SDS-PAGE of raw and roasted peanut extracts (pH 7) treated and centrifuged with and without phytic acid. Rst = roasted; phytic-T = phytic acid-treated. Arrows indicate absence of peanut allergens.

RESULTS AND DISCUSSIONS

Effect of Phytic Acid on Peanut Allergens. Extracts of raw and roasted peanuts (pH 7) were analyzed by SDS-PAGE, following treatment with phytic acid and centrifugation. The minimal final concentration of phytic acid used to form insoluble complexes with the allergens was 2 mM. **Figure 1** shows the SDS-PAGE profiles of proteins remaining in the extracts after treatment with phytic acid and centrifugation. Compared to the control, a number of protein bands, particularly those corresponding to 63 and 18 kDa, were hardly detected in the treated extracts. These bands were the major peanut allergens, namely, Ara h 1 and Ara h 2 (*13*). A band around 50 kDa was also hardly detected. This band was probably the minor peanut allergen Ara h 3 because it had a molecular weight similar to that of Ara h 3 reported by Rabjohn et al. (*15*) and was weakly recognized by IgE in a Western blot of an untreated peanut extract (data not shown). Another band around 35 kDa was also missing and was not an allergen because it had no reactivity toward IgE in a Western blot of an untreated extract (data not shown). Other protein bands such as those between 20 and 35 kDa and below Ara h 2 were clearly seen. This indicates that phytic acid reacted predominantly with the major peanut allergens from the extracts, and together they formed insoluble complexes in the extracts. Such extracts may experience a change in allergenic properties due to changes in protein or allergen levels in the extracts, and in this case, protein levels changed from 100% (control) to 42% for the roasted extract and 28% for the raw after treatment. Investigation of the solubility of protein complexes (i.e., precipitates isolated from the phytic-treated extracts) indicated that they were not soluble at acidic and neutral pH, but were soluble in alkaline conditions (**Figure 2**).

Raw versus Roasted. Allergens from raw and roasted peanuts have previously been reported to behave differently, in particular, in their reactivity toward enzymes (*12*, *13*), carbohydrates in Maillard reactions (*19*, *20*), and IgE antibodies (*21*, *22*). In

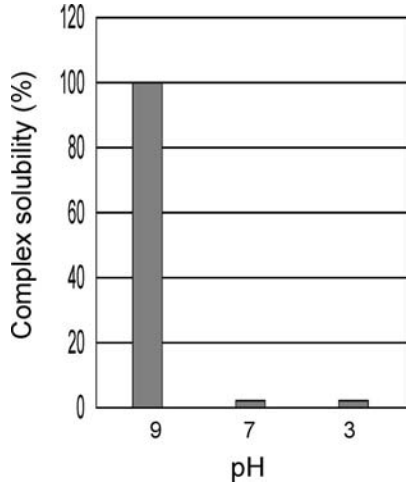

Figure 2. Protein complex solubility (%) at various pH. Complex solubility was determined on the basis of the concentration of protein complex dissolved at the pH indicated, and divided by the concentration at pH 9.

this study, a difference between raw and roasted peanut allergens in their reaction with phytic acid was also noted. Data showed that more insoluble allergen–phytic complexes were formed in the raw, treated extract than in the roasted. As shown in SDS-PAGE (**Figure 1**), in addition to Ara h 1 and Ara h 2, several allergen bands below Ara h 2 were hardly detected in the raw/treated extract, compared to the roasted. Analyses of the protein concentrations in the extracts showed that the raw/treated exhibited a lower protein concentration (28%) than the roasted/treated (42%). All this indicates that raw peanut allergens were more susceptible to phytic treatment than the roasted.

Role of Lysine Residues in Complex Formation. Raw peanut allergens are more reactive with phytic acid, as indicated above, than the roasted probably because raw peanut allergens possess more free amino groups (from lysine residues). Free amino groups are deemed important because they are thought to bind to the phosphate groups of phytic acid and contribute to complex formation. During roasting, these free amino groups may become unavailable because they are involved in a reaction called the Maillard reaction, where they react with the carbohydrates to form protein adducts (*19*, *20*). As a result, roasted peanut allergens contained limited free amino groups and, therefore, were not as reactive with phytic acid as the raw. To verify that free amino groups from lysine residues play a role in the allergen–phytic acid reaction, we subjected allergens from a raw peanut extract to succinic anhydride and then phytic acid treatment. Succinic anhydride was used because it reacts with free amino groups at pH 8 or higher and makes them unavailable. In this case, our hypothesis was that allergens treated with succinic anhydride can not form insoluble complexes with phytic acid. Indeed, data showed that the majority of the major peanut allergens (Ara h 1 and Ara h 2) that were treated with succinic anhydride and then with phytic acid remained in the extract and were detected by SDS-PAGE (**Figure 3**). Very little complex was formed in this case. This indicates that lysine residues are an important factor in the formation of allergen–phytic complexes. In addition to lysine, phytic acid has been shown to bind to arginine and histidine residues of proteins from other sources (*23*). Therefore, these residues may also play a part in the peanut allergen–phytic reaction.

Effect of pH on Phytic–Allergen Interactions. The fact that lysine is involved in the formation of phytic-allergen

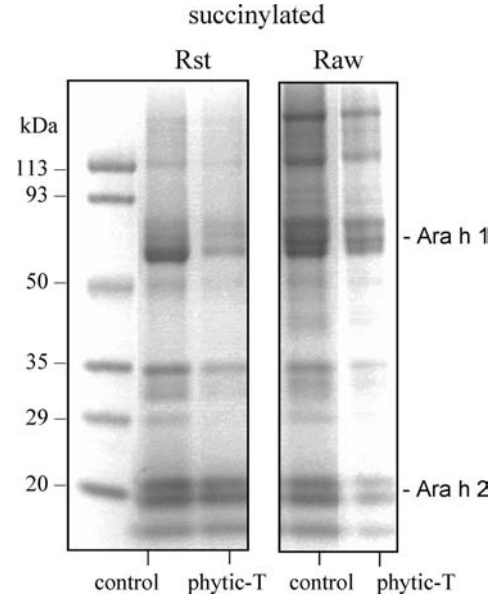

Figure 3. SDS-PAGE of raw and roasted peanut extracts treated with succinic anhydride and then with and without phytic acid. Rst = roasted; phytic-*T* = phytic acid-treated.

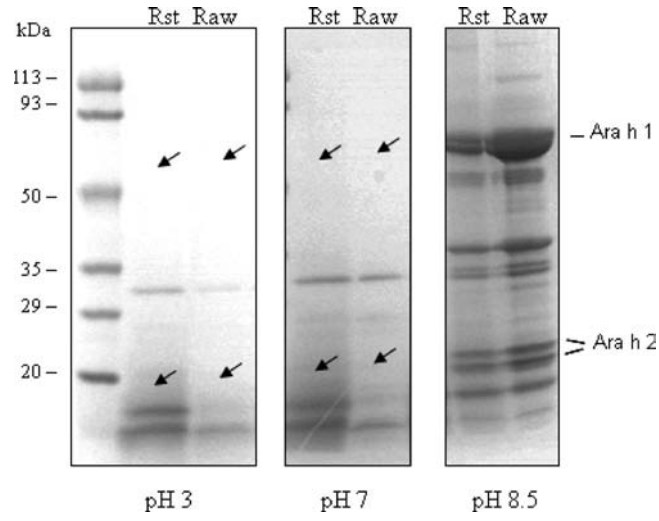

Figure 4. SDS-PAGE of raw and roasted peanut extracts treated at different pH and centrifuged with phytic acid. Arrows indicate absence of peanut allergens.

complexes could also be demonstrated by studying the pH of the reaction. At pH less than 8, lysine in proteins tends to be positively charged due to the free α- and ε-amino groups in the lysine residues (pK_a range of 9–10.5). These positive charges allow the allergens to bind to phytic acid through its phosphate groups, which carry negative charges at these pK_a values of the phosphate esters. However, at pH 8.5 or above, such binding may be difficult because lysine tends to have a zero charge at that pH or above. To verify that pH has an effect on the phytic acid–allergen reaction, we treated the allergens from raw and roasted peanut extracts with phytic acid at three different pH values (3, 7, and 8.5), centrifuged them, and then analyzed them by SDS-PAGE. Results showed that at pH 3 and 7, the majority of major peanut allergens (Ara h 1 and Ara h 2) were precipitated as insoluble complexes by phytic acid, and small amounts of allergens remained in the extract as shown by SDS-PAGE (**Figure 4**). By contrast, at pH 8.5, a significant amount of peanut allergens was detected by SDS-PAGE, indicating that the

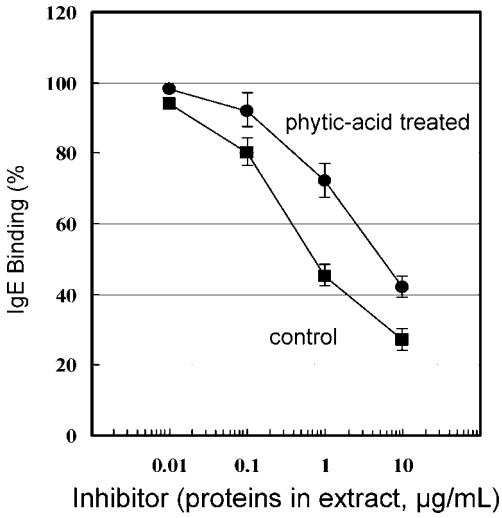

Figure 5. IgE binding of phytic-acid-treated and untreated roasted peanut extracts. A ciELISA was performed in which the extract was diluted at the concentration indicated, mixed with a pooled serum from peanut-allergic individuals and then incubated in an allergen-coated microtiter plate. IgE detection was performed using a rabbit antihuman IgE-peroxidase and a substrate solution of *o*-phenylenediamine and hydrogen peroxide. Upper curve represents a lower IgE binding or inhibitory effect. Values are means ± SD ($n = 3$). Values of the treated samples at 0.1–10 μg/mL are significantly different from those of the control ($P < 0.05$, $n = 3$).

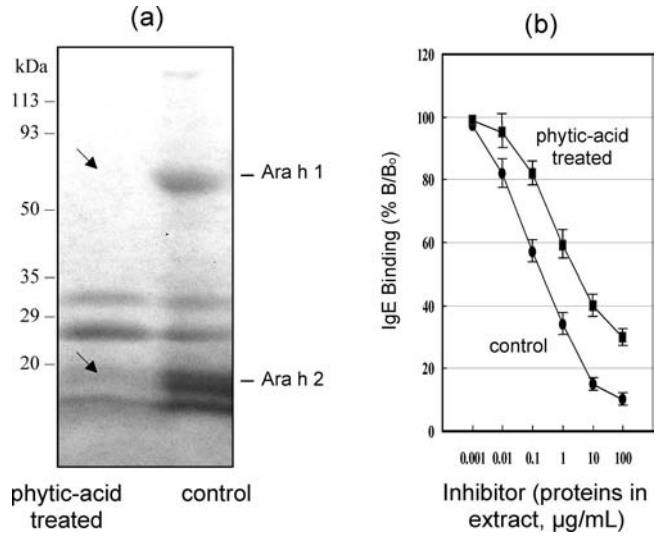

Figure 6. (**a**) SDS-PAGE and (**b**) ciELISA of a natural peanut butter slurry treated and centrifuged with and without phytic acid. Slurry was prepared and treated by stirring natural peanut butter in 0.02 M Tris buffer, pH 7, in the presence of phytic acid, for 60 min. After treatment, the slurry was centrifuged, and the resultant supernatant analyzed by SDS-PAGE and ciELISA as described above. Upper curve represents a lower IgE binding or inhibitory effect. Values are means ± SD ($n = 3$). Values of the treated samples at 0.01–100 μg/mL are significantly different from those of the control ($P < 0.05$, $n = 3$).

majority of the allergens were not affected or precipitated by phytic acid at this pH or higher. However, in the case of proteins from soy, it was reported that the soy proteins form complexes with phytic acid even at pH 9 (*24*).

IgE Binding of Phytic-Treated Peanut Extracts and Phytic-Bound Allergens. Our postulation was that phytic-acid-treated peanut extracts are less allergenic because they contain less soluble allergens after treatment and centrifugation. To support this postulation, we subjected phytic-treated and untreated extracts from roasted peanuts to a ciELISA assay. In the assay, extracts were tested in an allergen-coated microtiter plate for their inhibitory effect on IgE antibodies from a pooled serum of peanut allergic individuals. The higher the inhibitory effect, the more allergenic the extract. Results (**Figure 5**) showed that the phytic-treated extract exhibited a significant, lower inhibitory effect on IgE with a IC_{50} value of 5 μg/mL than the untreated, which had a IC_{50} of 0.8 μg/mL. The IC_{50} is defined as the concentration of proteins (inhibitors) required to inhibit IgE binding by 50%. The significant difference in IC_{50} indicated a 6-fold reduction in the allergenic potency of the treated extract. This suggests that the phytic-treated extract was less allergenic than the untreated.

Additionally, we determined that when phytic acid was attached to peanut allergens, it had an effect on the allergens or their IgE-binding capacity. This was interesting because we previously have demonstrated that the IgE-binding capacity of peanut allergens was altered when the allergens were attached to chemical compounds, such as the Maillard reaction products produced during roasting (*19*, *21*, *22*). We hypothesized that phytic acid may change the IgE-binding capacity of peanut allergens as well when they are linked to phytic acid. To support this hypothesis, a ciELISA was performed. In this case, microtiter plates coated with native or phytic-bound peanut allergens were used and incubated with IgE antibodies. Results showed that there was no significant difference in IgE binding between allergens attached with and without phytic acid (data not shown). This indicates that unlike the Maillard reaction adducts (*19*, *21*), phytic acid (bound to allergens) did not enhance or reduce the IgE-binding capacity of peanut allergens. However, as described above, phytic acid helps to reduce the level of peanut allergens in an extract by forming insoluble allergen complexes and, therefore, reduces the allergenic potency of the peanut extract.

Potential Application. The health benefit of phytic acid (*5*–*8*) and its use as a food additive and antioxidant in cooked meats (*3*, *4*) are known. In this study, phytic acid was shown to form complexes with the major peanut allergens, and they were insoluble in acidic and neutral conditions. Similar insoluble complexes were formed when phytic acid was added to a slurry made from natural peanut butter (nonhydrogenated). As shown in SDS-PAGE (**Figure 6a**), small amounts of allergens (Ara h 1 and Ara h 2) were seen in a butter slurry treated with phytic acid, as compared to the control. Analyses by ciELISA (**Figure 6b**) indicated a significant, lower IgE binding ($IC_{50} = 4$ μg/mL) or an 8-fold reduction in the allergenic potency of the treated slurry, compared to the control ($IC_{50} = 0.5$ μg/mL). As these complexes are insoluble and probably not absorbed by the human body, the research implies that phytic acid may find use in the development of a hypoallergenic peanut butter (slurry) that can be added to a fruit-blended beverage like a smoothie. Currently, only regular peanut butter is used in one of the commercial smoothie beverages called "Peanut Power Plus" (from Smoothie King, a nutrition store selling fruit-blended drinks), but such a beverage is limited to only people who are not allergic to peanuts. Should a hypoallergenic peanut butter (slurry) be developed, it probably would be favored by people or children who are at risk of peanut allergy but who wish to be able to use some peanut product and build up a tolerance for peanuts. The reason behind this is that there has been increasing evidence that exposure to peanuts on a regular basis, rather than avoiding them as

previously proposed, helps develop tolerance to peanuts (*25*). We believe that a hypoallergenic peanut product rather than a regular one may better serve this purpose.

Conclusions. This study demonstrated that phytic acid formed complexes with the major peanut allergens (Ara h 1 and Ara h 2) and that these complexes were insoluble at neutral or acidic pH. Lysine residues were involved as evidenced by the failure of complex formation in a reaction between phytic acid and allergens that had been treated with succinic anhydride. As a result of phytic acid treatment and insoluble complex formation, levels of soluble allergens and IgE binding of a peanut extract were reduced (a 6-fold reduction in allergenic potency). Application of phytic acid to a slurry made from natural peanut butter (nonhydrogenated) demonstrated similar results, indicating that phytic acid may find use in the development of hypoallergenic peanut-based products or beverages such as smoothies.

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