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Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds

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ABSTRACT

Phenolic compounds are known to form soluble and insoluble complexes with proteins. The objective of this study was to determine if phenolics such as caffeic, chlorogenic and ferulic acids form insoluble and irreversible complexes with major peanut allergens, and if such complexation reduces immunoglobulin E (IgE) binding. After adding each of the phenolics to peanut extracts and liquid peanut butter, the soluble materials were analysed by SDS–PAGE and inhibition ELISA. Results showed that addition of the phenolics precipitated most of the major peanut allergens, Ara h 1 and Ara h 2, and that complexation was irreversible. IgE binding was reduced approximately 10- to 16-fold. We concluded that reducing IgE binding by phenolics is feasible. The research, if proven by clinical studies, could lead to the development of less allergenic liquid peanut-based products.

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1. Introduction

Phenolic compounds are phytochemicals ubiquitous in fruits, vegetables and plants (Lin & Tang, 2008; Liu, 2007; Naczk & Shahidi, 2006) and have received attention for their biological function as antioxidants (Labuckas, Maestri, Perelló, Martínez, & Lamarque, 2008; Majo, Guardia, Giammanco, Neve, & Giammanco, 2008) and anti-carcinogens (McCann et al., 2007; Ramos, 2008; Yu et al., 2008). They possess a significant binding affinity for proteins, which can lead to the formation of soluble and insoluble protein–phenolic complexes (Labuckas et al., 2008; Naczk, Grant, Zadernowski, & Barre, 2006; Papadopoulou & Frazier, 2004). Insoluble complexes are formed due to the formation of a sufficient hydrophobic coating onto the protein surface by phenolics (Charlton et al., 2002) or a combination of this hydrophobic coating and the cross-linking of different protein molecules with phenols (Baxter, Lilley, Haslam, & Williamson, 1997; Charlton et al., 2002; Papadopoulou & Frazier, 2004). In this case, cross-linking can occur between the free amino/tryptophan groups of proteins and the phenol groups of the phenolic compounds (Labuckas et al., 2008; O'Connell & Fox, 1999; Rawel, Czajka, Rohn, & Kroll, 2002).

Peanut allergens are proteins. Therefore, it is possible that they may form soluble and insoluble complexes with phenolic compounds. In this study, we focused on the insoluble products because we postulated that the complexation is possible and may be irreversible and thus help reducing the allergenic capacity of peanut-based products. To date, at least 8 peanut allergens have been identified (Burks, 2008; Sicherer & Sampso, 2007), of which Ara h 1 and Ara h 2 are considered major peanut allergens because Ara h 1 and Ara h 2 are recognised by 90% of sensitised individuals. We have previously demonstrated that reducing the level of these two major peanut allergens in peanut-based products such as liquid peanut butter may help lower the allergenic capacity of these products (Chung & Champagne, 2007; Chung, Yang, & Krishnamurthy, 2008). According to several studies (Brockow et al., 2008; Taniuchi et al., in press; Yamaoto et al., 2004), foods with reduced allergenicity may be beneficial to people with food allergy because they (people) tend to become tolerant to food allergy after consuming these foods for a period of time. Because peanut allergy is on the rise (Sicherer & Sampso, 2007), studies on peanut tolerance have become an important topic (Burks, 2008).

In this study, we examined three different monomeric phenolic compounds for their ability to irreversibly complex with the major peanut allergens. These compounds were caffeic, ferulic and chlorogenic acids, respectively, commonly found in fruits and vegetables (Naczk & Shahidi, 2006). Our objectives were to determine if these monomeric phenolics form irreversibly insoluble complexes with the major peanut allergens in peanut extracts and liquid

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peanut butter, and if such a complex-formation process reduces the allergenic capacity of the extracts and liquid peanut butter.

2. Materials and methods

2.1. Materials

Phosphate buffer saline (PBS), 96-well microtiter plates, σ-phenylenediamine, and Tween 20 were purchased from Sigma Co. (St. Louis, MO). Tris–glycine pre-cast gels (4–20%), and goat anti-human immunoglobulin E (IgE)-peroxidase were purchased from Invitrogen (Carlsbad, CA). Superblock blocking buffer, GelCode Blue Stain Reagent and bicinchoninic acid (BCA)-protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL). Human plasmas from three individuals with peanut allergy (determined by CAP-FEIA assay for IgE) were obtained from PlasmaLab International (Everett, WA). Roasted high-oleic peanuts were from the University of Florida, Gainesville, FL. Natural peanut butter (creamy and non-hydrogenated) was purchased from Whole Foods Market.

2.2. Preparation of peanut extracts and liquid peanut butter

Peanut extracts in 20 mM phosphate buffer, pH 8 were prepared from defatted roasted peanut meals as previously described (Chung et al., 2008). Liquid peanut butter was prepared by stirring natural peanut butter (non-hydrogenated; oil partially removed) in 20 mM phosphate buffer, pH 8 at a 1:2 ratio (w:v) at room temperature for 60 min (Chung et al., 2008).

2.3. Treatment with phenolic compounds

Roasted peanut extracts and liquid peanut butter at a protein concentration of 5 mg/mL were each treated with a phenolic compound (caffeic, ferulic, and chlorogenic acids; each dissolved in dimethylformamide) at a final concentration of 50–100 mM. The ratios (v:v) of extracts and liquid peanut butter to each of the phenolics were 50:1 and 10:1, respectively. The mixtures were then stirred for 60 min and centrifuged at 8000g for 10 min. The supernatants thus obtained were subjected to SDS–PAGE and ciELISA (competitive inhibition enzyme-linked immunosorbent assay) analyses as described below. Protein concentration was determined, using the BCA kit assay.

2.4. SDS–PAGE of phenolic-treated peanut extracts and liquid peanut butter

SDS–PAGEs were performed under non-reducing condition on the supernatants from the phenolic-treated peanut extracts and liquid peanut butter, using Tris–glycine pre-cast gels (4–20%) and a Novex Gel Electrophoresis Apparatus. After electrophoresis, gels were stained with GelCode Blue Stain, and destained with water.

2.5. Determination of IgE binding of phenolic-treated peanut extracts and liquid peanut butter

ciELISA was carried out in triplicate ($n = 3$) as previously described (Chung et al., 2008). Briefly, a peanut sample (treated and not treated) (50 μL) (0.01–10 μg/mL) was mixed with a pooled human plasma containing IgE antibodies (1:30, 50 μL), followed by incubation for 30 min in a microtiter plate coated with a roasted peanut extract. Detection of IgE antibodies binding to the plate was performed, using a goat anti-human IgE peroxidase conjugate (1:1000) 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. After stopping the enzyme reaction with 4 N sulphuric acid (50 μL), 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 [Superblock]: [PBS/Tween 20] (1:1). The absorbance value of a sample containing IgE antibodies and the peanut sample was represented by B, while Bo represented the absorbance value of a control containing IgE only.

2.6. Statistical analyses

Data presented in Fig. 3 represent mean values of three determinations. Comparisons between data were performed by the Student's *t*-test at a $P < 0.05$ level of significance.

3. Results and discussions

3.1. Effect of phenolics on peanut allergens in extracts and liquid peanut butter

As shown in SDS–PAGE (Fig. 1), after treatment with phenolics (caffeic acid, chlorogenic acid and ferulic acid), some proteins in the peanut extracts and liquid peanut butter were reduced. Among the proteins reduced were the major peanut allergens, Ara h 1 (63 kDa) and Ara h 2 (18–20 kDa). By contrast, proteins between 27 kDa and 35 kDa and below 12 kDa were not reduced but remained soluble. As a result of the phenolic treatment, precipitates or complexes were obtained. Analyses of the precipitates by SDS–PAGE confirmed the presence of the two major peanut allergens in the precipitates (data not shown). According to Spencer et al. (1988) and Baxter et al. (1997), precipitation or complexation of proteins can be irreversible via either multi-site interactions (i.e., several phenolics bound to one protein molecule) or multidentate interactions (one phenolic bound to several protein sites or protein molecules). In this study, we found that the precipitates were insoluble in 1 M NaCl or 2 M urea, suggesting that complexation of peanut allergens with phenolics is irreversible.

Despite the phenolic treatment, it appears that there were still some residual soluble allergens remaining in the sample solution. Therefore, we performed repeated complexation (i.e., treated 2 times) to determine if it helps to precipitate the residual allergens (note: treatment for more than 2 times was not necessary based on the data discussed below). The peanut sample thus treated repeatedly was then analysed by SDS–PAGE against the sample that was treated only once with the phenolic compound. A typical protein profile of the liquid peanut butter samples treated for different times with caffeic acid is illustrated in Fig. 2. A total loss of proteins (including other soluble proteins besides the major peanut allergens) was seen with the repeatedly-treated sample, as compared to the sample that was treated once. This suggests that repeated complexation removed not only the major peanut allergens but also the rest of the soluble proteins. In this case, repeated complexation appeared to produce a peanut product that is no longer a peanut – a quote from Burks (2008) who indicated, in reference to the development of transgenic allergen-free peanut, that altering enough of the peanut allergens to make a modified peanut that is less likely to cause an allergic reaction may result in a plant or product that is no longer a peanut. Because of a lack of proteins, such modified peanut product may have no nutritional value and would exhibit a flavour loss due to the loss of protein-bound Maillard reaction products (Chung & Champagne, 2001; Cämmerer & Kroh, 2009). With a lack of nutritional value and peanut flavours, the modified peanut product is unlikely to be welcome by consumers. On this basis, we did not pursue repeated complexation for the removal of peanut allergens.

Due to the insolubility of the phenolic–allergen complexes, digestion with digestive enzymes was impossible. This suggests

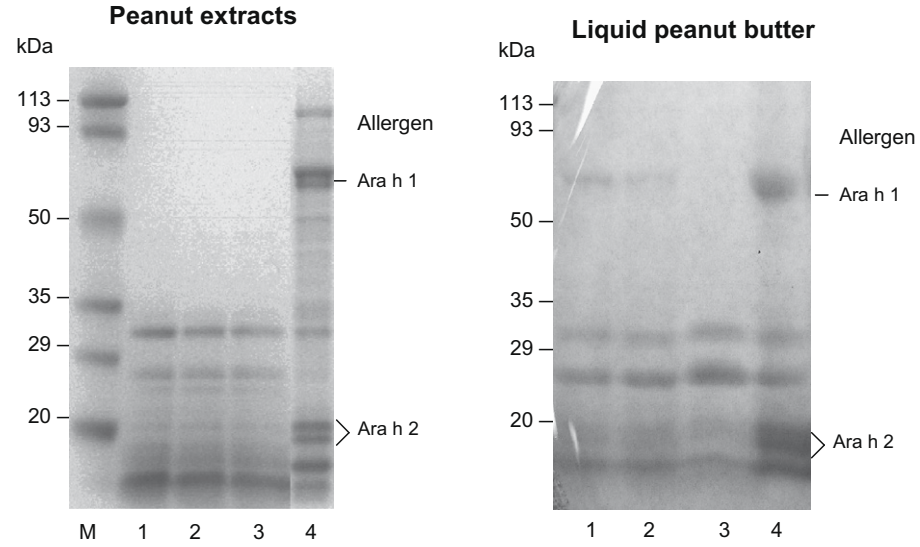

Fig. 1. SDS–PAGE of phenolic-treated and untreated roasted peanut extracts and liquid peanut butter. Phenolics as indicated were each added to the peanut samples, stirred for 60 min and centrifuged. The resultant supernatants were analysed by SDS–PAGE. M = markers; 1 = ferulic-treated; 2 = chlorogenic-treated; 3 = caffeic-treated; 4 = untreated.

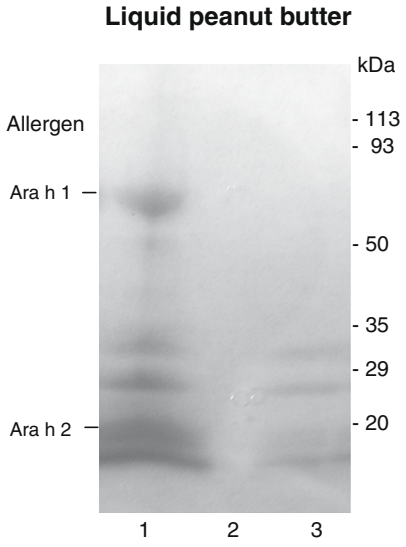

Fig. 2. SDS–PAGE of liquid peanut butter treated 1 and 2 times with caffeic acid. After the first treatment (1st time), the sample was again treated with caffeic acid (i.e., 2nd time) and centrifuged. The resultant supernatants were then subjected to SDS–PAGE. 1 = untreated; 2 = treated 2 times; 3 = treated 1 time.

that the complexes, if ingested, may be resistant to digestion and absorption by the human body, and ultimately are likely to be excreted from the body. This in combination with the reduced amount of soluble peanut allergens resulting from the treatment led us to believe that the phenolic-treated extracts and liquid peanut butter possibly may be less allergenic or have a lower IgE binding (discussed below). Overall, the data demonstrated that the three phenolics tested irreversibly precipitated the major peanut allergens. Of the three phenolics, caffeic acid appeared to be more effective in precipitation of proteins than the others in liquid peanut butter (Fig. 1).

3.2. IgE binding of phenolic-treated extracts and liquid peanut butter

The results above suggested that IgE binding of both phenolic-treated extracts and liquid peanut butter may be reduced because of precipitation of soluble major peanut allergens (Fig. 1). To test our hypothesis, we performed a competitive inhibition ELISA (ciELISA), an *in vitro* immunoassay in which IgE binding was measured as an indicator of the allergenic capacity of peanut samples. In the assay, phenolic-treated and untreated peanut extracts and liquid peanut butter were each tested for their inhibitory effect on IgE antibodies. In this case, the more allergens are in the sample, the more inhibitory and allergenic the sample is. Results (Fig. 3) showed that the inhibitory effect was more pronounced with the untreated (upper curve) than with the phenolic-treated extracts or liquid peanut butter. This means that IgE binding of the untreated was higher than that of the phenolic-treated, and in other words, IgE binding of the phenolic-treated extracts and liquid peanut butter was reduced. There was no significant difference in IgE binding ($P < 0.05$) between the three treatments (i.e., caffeic, chlorogenic and ferulic acids). To determine the extent of reduction in IgE binding, the IC_{50} values of the phenolic-treated and untreated samples were calculated. IC_{50} is defined as the protein concentration required to inhibit IgE binding by 50%. In this case, IC_{50} for the phenolic-treated and untreated peanut extracts were approximately 7 μg/mL and 0.7 μg/mL, respectively (Table 1). For phenolic-treated liquid peanut butter, IC_{50} was 5 μg/mL, compared to 0.3 μg/mL of the untreated. In this case, the phenolic-treated was approximately 10- to 16-fold lower in IgE binding than the untreated. This indicates that IgE binding or the allergenic capacity of the peanut extracts and liquid peanut butter was successfully reduced by the phenolics.

3.3. Possible applications

There have been studies showing that consuming foods with reduced allergenicity may help to induce food tolerance (Brockow et al., 2008; Taniuchi et al., in press; Yamaoto et al., 2004). Among these studies is one supported by the European Union (Brockow et al., 2008), where allergenicity-reduced cow's milk and hen's egg were shown to be partly tolerated by patients with allergy to those foods. Also, the safety and benefits of these foods with reduced allergenicity have been demonstrated (Yamaoto et al., 2004). In that study, hypoallergenic wheat flour and cupcakes have been shown to be safe to children with atopic dermatitis and wheat allergy, based on the finding that 86% of these children

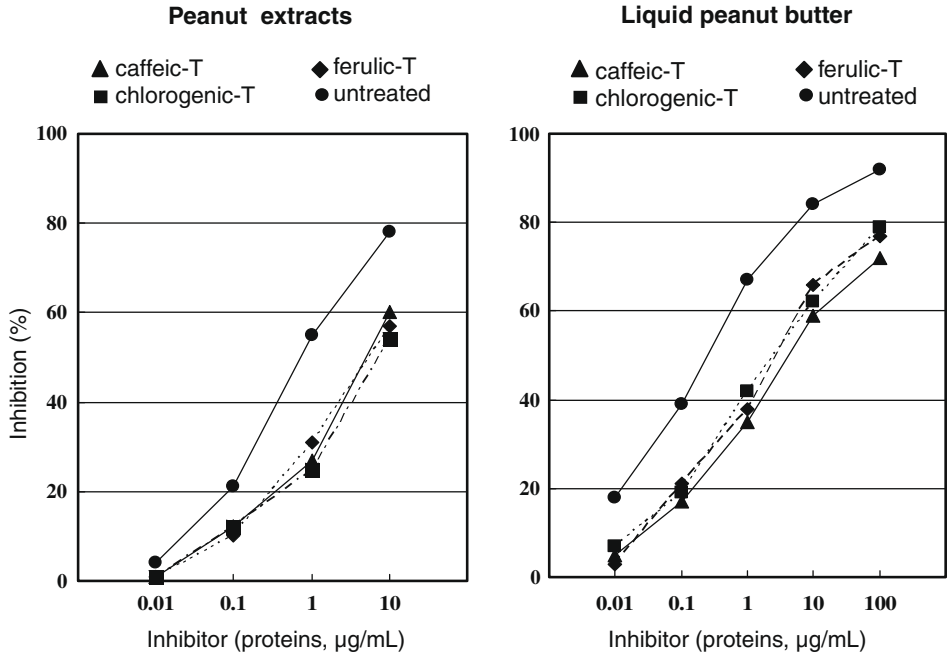

Fig. 3. IgE binding of phenolic-treated and untreated peanut extracts and liquid peanut butter in competitive inhibition ELISA. T = treated. The assay was performed, using a pooled human plasma from peanut-allergic individuals. IgE was detected, using a goat anti-human IgE peroxidase conjugate and *o*-phenylenediamine substrate. Values (on semi-log scale) are mean of three determinations. Values between treatments (caffeic, chlorogenic, and ferulic) are not significantly different from each other but from the untreated ($P < 0.05$).

Table 1
IC_{50}[a] (μg/mL) of phenolic-treated and untreated peanut samples.

Sample	Untreated	Phenolic-treated[b]	Decrease in[c] IgE binding
Peanut extract	0.7	7	10-fold
Liquid peanut butter	0.3	5	16-fold

[a] Defined as the protein concentration required to inhibit IgE binding by 50%.
[b] Caffeic-, chlorogenic-, or ferulic-treated.
[c] Refers to phenolic-treated sample.

showed no adverse response after consuming the hypoallergenic wheat cupcakes. Moreover, by taking the hypoallergenic cupcakes over a long period (more than 6 months), more than half of these children were hyposensitised and able to eat normal wheat products (this means that they became tolerant to wheat). Similar results and tolerance to wheat have been reported in the study of Taniuchi et al. (in press), where three quarter of the children with wheat allergy, who took hypoallergenic wheat flour, achieved the daily intake of normal wheat product.

In this study, we demonstrated that IgE binding or the allergenicity capacity of peanut extracts and liquid peanut butter was reduced, using simple monomeric phenolic compounds. If proven by clinical studies, the research may lead to the development of less allergenic liquid peanut-based products. However, there still remain several major questions and concerns, most prominently regarding whether the hypoallergenic peanut products will be safe and effective in those with the most severe forms of peanut allergy. Therefore, this would be not ready for general use for many years until the allergy problem is better understood. Other strategies with a similar problem are described below:

(1) *Immunotherapy*. Previous attempts to treat peanut allergy have been by immunotherapy with injections of aqueous peanut extract (Nelson, Lahr, Rule, Bock, & Leung, 1997). However, these attempts are always accompanied by significant complications and a high risk of anaphylaxis. Burks et al. (2000) have shown that modifying the allergens and turning them into hypoallergenic proteins provide a better immunotherapeutic agent for treatment of peanut allergy. Whether this is safe remains to be seen.

(2) *Oral tolerance study*. Oral tolerance induction, in which peanuts are ingested in increasingly larger amounts on a regular basis to induce tolerance to peanuts in late life, has been studied and has shown some positive results (e.g., children with peanut allergy can ingest or tolerate more peanuts than before the study is conducted) (Burks, Laubach, & Jones, 2008). However, as indicated by Burks (2008), the potential of anaphylaxis during oral immunotherapy is still present, and therefore, the therapy should not be used in those with peanut allergies. In addition, the treatment is associated with high attendant cost and done only in research settings with intensive care unit support immediately available. Wood (2008) has also indicated that adverse reactions are extremely common in the course of oral immunotherapy, and so the future of oral immunotherapy is far from clear; it is safe to say (quoted) that such treatment will not be ready for general use for many years. Wood (2008) has further indicated that other approaches such as modification of allergens may allow both greater efficacy and improved safety.

(3) *Threshold*. Currently, strict avoidance of peanuts or peanut proteins is the only effective treatment for preventing peanut-induced allergic reaction. This can be challenging because in most cases, peanuts are not labeled or are hidden in trace amounts in foods that are cross-contaminated with peanuts due to processing with the same machinery that processes peanuts. For this reason, accidental ingestion of trace amount of peanut proteins is common. For children with severe peanut allergy, this could be life-threatening. Leung et al. (2003) have shown that treatment with monoclonal anti-IgE antibody markedly increases the threshold

reactivity to peanuts in adults with peanut allergies. Also, Burks (2008) has indicated that the study of oral immunotherapy offers the possibilities of at least raising the threshold of the amount of peanuts. The findings in this study also could have the potential to increase the threshold. To date, the proposed threshold for adventitious food allergens is not to exceed 1–5 mg/kg of protein in industrial food manufacture (Crevel et al., 2008).

(4) *Flavour issue and nutritional value*. Producing an allergen-free peanut or peanut product may seem to be the best approach to treat peanut allergy. However, such an approach may be impractical because as indicated by Burks (2008), altering enough of the peanut allergens to make a modified peanut (or peanut product) that is less likely to cause an allergic reaction may result in a plant or product that is no longer a peanut. In this case, the nutritional value of the modified peanut is reduced and accompanied by a loss of peanut flavours due to the loss of protein-bound Maillard reaction adducts (Chung & Champagne, 2001; Cämmerer, 2009). With a lack of peanut flavour and nutritional value, the modified (allergen-free) peanut or peanut product is unlikely to be welcome by consumers. This may not be good for the food allergy management. As indicated by Leung and Bock (2003), current food allergy management strategies (allergen avoidance and use of epinephrine) are not adequate for dealing with food allergies, and additional strategies are needed. van Putten et al. (2006) have suggested that hypoallergenic foods be part of the additional food allergy management strategies. However, with hypoallergenic peanut products, whether this is possible remains to be seen.

4. Conclusions

Treatment of peanut protein extracts and liquid peanut butter with monomeric phenolic compounds (caffeic, chlorogenic and ferulic acids) resulted in the formation of insoluble complexes and a reduction of the level of soluble major peanut allergens. The complexes were insoluble in 1 M NaCl and 2 M urea, indicating that complexation was irreversible. As a result of the complexation, IgE binding of the extracts and liquid peanut butter was reduced approximately 10- to 16-fold. If proven by clinical studies, the research may lead to the development of less allergenic liquid peanut-based products. However, this would be not ready for general use for many years until the allergy problem is better understood. The mainstay of therapy for IgE-mediated peanut allergy remains avoidance of the offending foods and following the guidelines of food allergy management.

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