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Allergenic Properties of Roasted Peanut Allergens May Be Reduced by Peroxidase

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Peanut allergy is a public health issue. The culprits are the peanut allergens. Reducing the allergenic properties of these allergens or proteins will be beneficial to allergic individuals. In this study, the objective was to determine if peroxidase (POD), which catalyzes protein cross-linking, reduces the allergenic properties of peanut allergens. In the experiments, protein extracts from raw and roasted defatted peanut meals at pH 8 were incubated with and without POD in the presence of hydrogen peroxide at 37 °C for 60 min. The POD-treated and untreated samples were then analyzed by SDS-PAGE, western blots, and competitive inhibition ELISA. IgE binding or allergenicity was determined in blots and ELISA. Results showed that POD treatment had no effect on raw peanuts with respect to protein cross-linking. However, a significant decrease was seen in the levels of the major allergens, Ara h 1 and Ara h 2, in roasted peanuts after POD treatment. Also, polymers were formed. Despite this, a reduction in IgE binding was observed. It was concluded that POD induced the cross-linking of mainly Ara h 1 and Ara h 2 from roasted peanuts and that, due to POD treatment, IgE binding was reduced. The finding indicates that POD can help reduce the allergenic properties of roasted peanut allergens.

KEYWORDS: Peroxidase; enzymes; raw and roasted peanuts; allergenicity; Ara h 1 and Ara h 2 allergens; cross-linking; IgE antibodies; ELISA; western blots

INTRODUCTION

Peroxidase (POD) is a heme-containing enzyme catalyzing the oxidation of a variety of phenolic compounds such as ferulic acids into *o*-quinones (*1*). The POD-generated *o*-quinones then react with other phenolics, amino, or sulfhydryl compounds to form new cross-linked products (*2*−*4*). For instance, polysaccharides with ferulic acid moieties interact to form cross-links in the presence of peroxidase (*5*, *6*). The end result is a change or an improvement in the functional properties of polysaccharides. In plants, peroxidases are involved in the formation of plant cell walls through cross-linking of the polysaccharides (*2*, *7*). In foods such as wheat dough, peroxidase helps to improve the functional properties by catalyzing the cross-linking of polysaccharides and/or proteins (*6*, *8*). Proteins can become cross-linked with other proteins or polysaccharides in the presence of peroxidase because proteins contain tyrosine residues. Tyrosine carries a phenol group, which can react with the ferulic acid moieties of polysaccharides or the tyrosine residues of another protein to form protein−polysaccharide (*3*, *6*, *8*) or protein−protein cross-links (*9*, *10*). An illustration of a protein−protein cross-link via the tyrosine residues in the presence of peroxidase and hydrogen peroxide is given in **Figure 1**.

Although the use of peroxidase to improve the functional properties of polysaccharides and proteins has been documented, very little is known about whether peroxidase can affect the allergenic properties of proteins. A number of studies have reported the cross-linking of proteins catalyzed by the enzyme transglutaminase (*11*−*15*). Such reaction by transglutaminase is reported to result in a reduction of the immunogenic and allergenic properties of proteins (*15*). This reduction in allergenicity is probably due to the masking of binding sites on the allergenic proteins through cross-linking. Because of the masking of binding sites, immunoglobulin E (IgE) antibodies, which are produced in high levels by peanut allergic persons, are unable to bind to the allergenic proteins. As a result, the allergenic properties of the proteins are reduced. Other methods such as cross-linking with poly(ethylene glycol) have also been used to reduce the allergenicity of proteins (*16*, *17*). On this basis, we postulated in this study that cross-linking induced by peroxidase may affect or reduce the allergenic properties of peanut allergens.

To date, three major peanut proteins or allergens have been identified (*18*). Of these, Ara h 1 and Ara h 2 are the best known. These allergens react with IgE antibodies and, consequently, induce allergic reactions. Reducing the allergenic properties of these proteins will be beneficial to peanut allergic individuals. Previously, we have reported that roasting enhances the immunoglobulin E (IgE) binding or allergenic properties of peanuts

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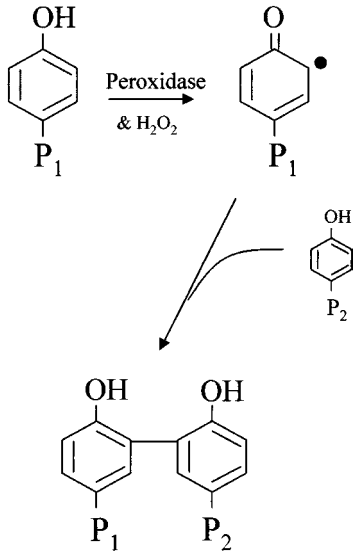

Figure 1. Cross-linking between two proteins (P_1 and P_2) catalyzed by peroxidase in the presence of hydrogen peroxide (*9*). P_1 and P_2 are linked through the phenol group of the tyrosine residue on the protein.

(*19*−*23*). In this study, the objective was to determine if IgE binding or the allergenicity of peanut proteins can be reduced by peroxidase through cross-linking of the peanut proteins.

MATERIALS AND METHODS

Apparatus. Apparatus for gel electrophoresis and western blot analysis was purchased from Invitrogen (Carlsbad, CA). A CERES 900C microtiter plate reader was purchased from Bio-Tek Instruments, Inc. (Winooski, VT).

Reagents and Materials. Bovine serum albumin (BSA), peroxidase (250 units/mg), ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 96-well microtiter plate (Corning), anti-rabbit IgG alkaline phosphatase conjugate, *p*-nitrophenyl phosphate, rabbit anti-human IgE−peroxidase conjugate, *o*-phenylenediamine, Tween 20, and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO); 4−20% Tris−glycine precast gels were purchased from Invitrogen. Immobilon-P membrane was obtained from Millipore Corp. (Bedford, MA). NBT/BCIP and CDP-Star substrates were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Human sera from four patients with peanut allergy were obtained from the University of Arkansas, Children's Hospital (Little Rock, AR). Superblock blocking buffer and bicinchoninic acid (BCA)−protein assay kit were purchased from Pierce Chemical Co. (Rockford, IL). Raw and roasted high-oleic peanut seeds (SunOleic) were obtained from the University of Florida, Gainesville, FL.

Preparation of Peanut Protein Extracts and Purified Allergens. Extracts were prepared as previously described from defatted meals of raw and roasted peanuts (*22*). Briefly, defatted peanut meals (40 mg) were stirred in 0.3 mL of 0.02 M sodium phosphate, pH 8, plus 10 mM EGTA for 20 min at 4 °C, followed by centrifugation at 8500*g* for 10 min. The resultant supernatants (extracts) were then used for peroxidase treatment as described below. Concentration of proteins in the extract was determined using the BCA kit assay. Purification of allergens Ara h 1 and Ara h 2 from roasted peanuts was performed according to the methods of Sen et al. (*24*) and Koppelman et al. (*25*).

Treatment with Peroxidase (POD). Protein extracts from raw and roasted peanuts were diluted to a protein concentration of 5 mg/mL with 20 mM sodium phosphate buffer, pH 8, plus 10 mM EGTA. Hydrogen peroxide (2 μL, 1%) and POD (1 μL, 10 mg/mL) were then added in sequence to the diluted extract (57 μL) and allowed to incubate at 37 °C for 60 min. A control was performed in the same way except that no POD was added. After incubation, the treated and untreated samples were diluted and analyzed by SDS-PAGE, western blots, and competitive inhibition ELISA as described below.

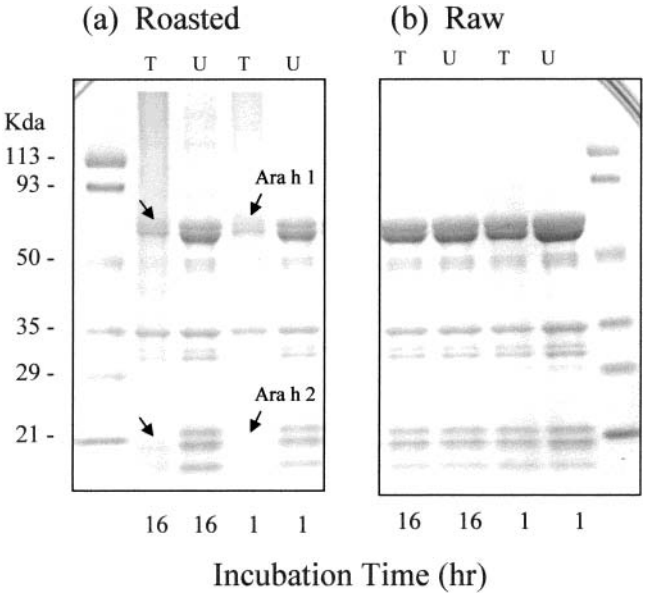

Figure 2. SDS-PAGE of peroxidase-treated (T) and untreated (U) proteins from raw and roasted peanut extracts. Extracts were incubated with and without peroxidase in the presence of hydrogen peroxide at 37 °C for 1 and 16 h. Tris–glycine gels (4–20%) were used in SDS-PAGE and stained with Coomassie Blue. Ara h 1 and Ara h 2 refer to peanut major allergens.

Analyses of Proteins and IgE Binding in SDS-PAGE and Western Blots. SDS-PAGE was performed as previously described using a Novex gel electrophoresis apparatus and 4−20% Tris−glycine precast gels (*22*). Blots were performed using an Immobilon-P membrane, a pooled serum containing IgE antibodies from peanut allergic individuals, an anti-human IgE−alkaline phosphatase secondary antibody, and a chemiluminescent substrate CDP-Star (*20*).

Assay of IgE Binding or Allergenicity in ELISA. A competitive inhibition ELISA was carried out ($n = 3$) as previously described (*22*). All samples were diluted in Superblock/PBS−Tween 20 (1:1). Briefly, a diluted pooled serum (50 μL, 1:20) containing IgE antibodies from peanut allergic individuals was mixed with a peanut extract (50 μL) at various protein concentrations from 0.1 to 1000 μg/mL. The resultant mixture was added to a microtiter plate coated with a roasted peanut extract and allowed to incubate for 45 min at 25 °C. After incubation and washing, a rabbit anti-human IgE peroxidase conjugate (1:500) (100 μL) was added, incubated for 30 min at 25 °C, and washed. A substrate solution (100 μL) of *o*-phenylenediamine (0.5 mg/mL) and 0.03% hydrogen peroxide in 0.1 M citrate buffer, pH 5.5, was added. After incubation for 30 min at 37 °C, the reaction was stopped with 50 μL of 4 N sulfuric acid. The absorbance was read at 490 nm. In **Figure 5**, B represents the absorbance value of a sample containing IgE antibodies and a peanut extract (inhibitor), whereas B_0 represents the absorbance value of a control containing IgE only. Values were means of three determinations. Statistical analyses were performed using Student's t test at 95% confidence.

RESULTS AND DISCUSSION

POD Treatment on Peanut Proteins. Protein extracts from raw and roasted peanuts were incubated, respectively, with peroxidase and hydrogen peroxide at 37 °C for various times (1−16 h). Analyses by SDS-PAGE indicate that treatments between 1 and 16 h were not different in protein profiles. Typical treatment profiles of raw and roasted peanut proteins at 1 and 16 h after POD treatment are shown (**Figure 2**). Significant changes were observed in the protein profiles of roasted peanuts at 1 or 16 h after POD treatment (**Figure 2a**), whereas there was no change in the profiles of raw peanuts (**Figure 2b**). In roasted peanuts, peroxidase appeared to target several protein bands (**Figure 2a**). Two of these bands, which have molecular

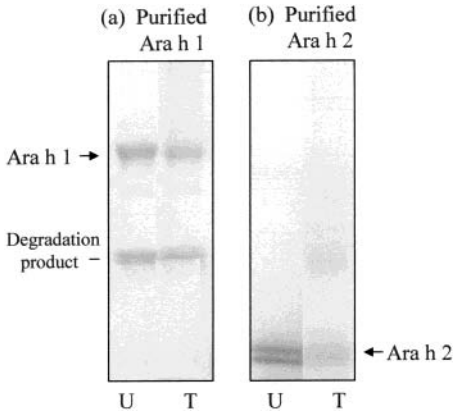

Figure 3. SDS-PAGE of purified peanut allergens (Ara h 1 and Ara h 2 from roasted peanuts) treated with (T) and without (U) peroxidase in the presence of hydrogen peroxide at 37 °C for 1 h. Tris–glycine gels (4–20%) were used and stained with Coomassie Blue.

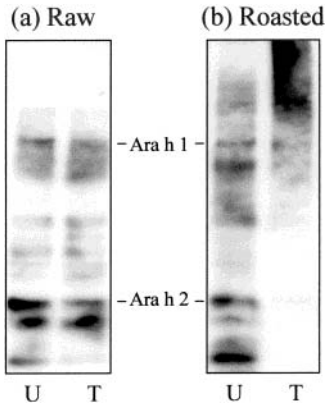

Figure 4. IgE binding of peroxidase-treated (T) and untreated (U) proteins from raw and roasted peanut extracts in western blots. Blots were performed using an Immobilon-P membrane, a pooled serum containing IgE antibodies from peanut allergic individuals, an anti-human IgE–alkaline phosphatase secondary antibody, and a chemiluminescent substrate CDP-Star.

masses of approximately 63 and 18 kDa, respectively, correspond to what we have known as the peanut major allergens, namely, Ara h 1 and Ara h 2 (*18*). The observation that POD targeted a few proteins is not unusual because in one study POD is reported to target only β-lactoglobulin among the whey proteins (*9*).

As shown in SDS-PAGE (**Figure 2a**), bands corresponding to Ara h 1 and Ara h 2 from roasted peanuts exhibited a significant decrease in density or levels after POD treatment. Of the two, Ara h 2 displayed the greatest decrease in density (i.e., the two bands almost missing). High molecular weight protein polymers in the form of dark smears (located above the allergen bands) were also observed. All of this was caused by the cross-linking between the proteins induced by POD. In other words, cross-linking led to not only a decrease in the levels of Ara h 1 and Ara h 2 monomers but also the formation of protein polymers. When individual purified allergens (Ara h 1 and Ara h 2 purified from roasted peanuts) were treated with POD, similar results (i.e., decrease in band density) were obtained (**Figure 3**). Again, purified Ara h 2 exhibited the most distinct reduction (i.e., bands almost missing). High molecular weight protein aggregates in the form of dark smears were also seen above the purified allergens after POD treatment. This finding indicates that the allergen Ara h 2 or Ara h 1 can react with itself in the presence of POD to form oligomers.

The mechanism of why Ara h 1 and Ara h 2 are more susceptible to peroxidase treatment than other proteins in the extract is not clear. Probably Ara h 1 and Ara h 2 may contain a number of tyrosine residues, which may be lacking in other proteins in the extract. Tyrosine residues are important because, as illustrated in **Figure 1**, they are required for the cross-linking of proteins induced by POD. Another explanation is that the tyrosine residues in the allergens may be more accessible to POD than the residues of other proteins in the extract. A good example is the allergens from raw peanuts. These allergens, unlike those from roasted peanuts, appeared to be much less affected by peroxidase treatment (**Figure 2**). The reason for this difference between raw and roasted peanuts is probably because the tyrosine residues are not exposed when the proteins are in the raw or non-denatured state. After roasting, proteins generally are denatured, and this allows the tyrosine residues to be exposed and accessible to peroxidase. Our finding that raw and roasted peanuts behaved differently toward POD supports this assumption.

Another reason for the high susceptibility of Ara h 1 and Ara h 2 to POD treatment is probably because Ara h 1 and Ara h 2 may contain ferulic acids. As previously mentioned, ferulic acids are phenolic compounds commonly found in legumes and plants (*7*). Cross-linking of proteins by peroxidase is mainly dependent on the presence of tyrosine residues and/or ferulic acids. In this study, it was shown that not all proteins (e.g., 35 kDa) in the peanut extracts were cross-linked by POD. Assuming that most proteins contain tyrosine residues, the only reason these proteins were not cross-linked by peroxidase is probably because they do not carry ferulic acids. Ferulic acids may bind to and exist as moieties of polysaccharides and proteins. In the presence of peroxidase, polysaccharides with ferulic acid moieties can cross-link to form new polymers (*2*−*4*). Similarly, proteins or allergens with these moieties may react with either the tyrosyl residues or the ferulic acid moieties of another protein to form new cross-links in the presence of peroxidase (*10*). It is thought that allergens lacking these moieties may be less reactive toward POD than those containing the moieties.

Western Blots and Competitive Inhibition ELISA. In vitro methods such as western blots and ELISA were performed to determine whether the allergenic properties or IgE binding of peanut proteins in the extracts change after POD treatment. In the blot assays, IgE antibodies from a pooled serum of peanut allergic individuals were shown to recognize the major peanut allergens Ara h 1 and Ara h 2 from raw and roasted peanuts (**Figure 4**). Of the two allergens, Ara h 2 exhibited the most distinct IgE binding (i.e., darker bands). Allergens from raw peanuts appeared to exhibit little change in IgE binding after POD treatment (**Figure 4a**). This suggests that the proteins and allergenicity were not affected by peroxidase when peanuts were raw. However, when roasted peanuts were used and treated with POD, changes in IgE-binding profiles were observed. As shown in **Figure 4b**, after POD treatment and blot analysis, protein bands corresponding to Ara h 2 from roasted peanuts were found mostly missing in SDS-PAGE. Also, the band corresponding to Ara h 1 was not distinct. All of this indicates a reduced IgE binding, which is probably due to the decreased levels of Ara h 1 and Ara h 2 monomers after POD treatment. Ara h 1 and Ara h 2 were decreased because they became cross-linked and formed smears after POD treatment, and this may result in the masking of IgE epitopes and, consequently, a reduced IgE binding. It is also possible that Maillard reaction adducts are involved because these adducts, which are bound to the proteins, are reported to be associated with increased IgE binding (*22*). These adducts may decrease with Ara h 1 and Ara h 2 after

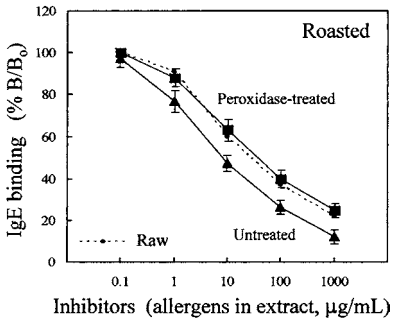

Figure 5. Inhibition of IgE binding by peroxidase-treated and untreated proteins from roasted peanut extracts in competitive inhibition ELISAs. Raw peanut extract (dash line) (treated or untreated) is shown for comparison. Extracts, diluted at the concentration indicated, were each mixed with a pooled serum containing IgE antibodies (1:40$_{final}$) from peanut allergic individuals and then added to a microtiter plate coated with a roasted peanut extract. Detection of IgE antibodies was carried out using a rabbit anti-human IgE–peroxidase conjugate (1:500) and a substrate solution of *o*-phenylenediamine (0.5 mg/mL) and hydrogen peroxide (0.03%). Values are mean ± SD, $n = 3$. Values (below 90% IgE binding) between treated and untreated are significantly different at 95% confidence.

POD treatment and thus contribute to a reduction in IgE binding. Although levels of Ara h 1 and Ara h 2 decreased after POD treatment, smears or high molecular weight protein polymers with IgE-binding properties were seen (**Figure 4b**). These smears are probably the cross-links of Ara h 1 and /or Ara h 2 containing also the Maillard reaction adducts. Because of their IgE-binding properties, these cross-links may compensate for the decrease of Ara h 1 and Ara h 2 observed above. If so, there may be no reduction in IgE binding in the extract overall.

To determine if there is indeed an overall reduction in IgE binding displayed by the POD-treated extract, a competitive inhibition ELISA was performed. In this assay, the degree of IgE binding was measured on the basis of the inhibition of IgE by the total proteins in the extract from roasted or raw peanuts. In this case, the higher the inhibition, the more allergenic the extract is. Results show that after POD treatment, the extract from roasted peanuts displayed a less steep IgE inhibition curve or a lower inhibition than the untreated (**Figure 5**). This means that the POD-treated extract was less allergenic than the untreated and that, overall, there was a reduction in allergenicity or IgE binding after POD treatment. This indicates that IgE binding exhibited by the cross-linked proteins (i.e., smears in **Figure 4**) cannot compensate for the decrease in IgE binding of Ara h 1 and Ara h 2 in the POD-treated extract.

Also, we wanted to determine if the reduction in IgE binding may be due to a reduced solubility of the cross-linked proteins. In the study, protein precipitation during or after POD treatment did not seem to occur. This is based on the following observations: (1) samples remained clear after peroxidase treatment; (2) an alkaline buffer (pH 8), which generally favors the solubility of proteins, was used in the treatment of peanut extracts with POD; (3) supernatants obtained after centrifugation of POD-treated samples gave the same protein profile in SDS-PAGE as the original treated samples; and (4) samples treated with and without 4 M urea were not different in protein profiles. On this basis, it is concluded that the solubility problem did not exist and that the overall reduction in IgE binding observed above was not caused by a reduced protein-solubility problem.

Additionally, we have previously reported that roasted peanuts have a higher IgE binding than raw peanuts (*20*−*23*). In this study, IgE binding of roasted peanuts was reduced by POD treatment. We wanted to determine if roasted peanuts, after POD treatment, continue to exhibit a higher IgE binding than raw peanuts as previously described. As shown in **Figure 5**, after POD treatment, roasted peanuts did not exhibit a higher IgE binding than raw peanuts. Instead, the POD-treated roasted peanuts had an IgE binding very similar to the raw (here, only the untreated raw is shown because treated and untreated raw peanuts were not different in IgE binding, **Figure 1**). The result indicates that after POD treatment, roasted peanuts were no longer more allergenic than the raw as previously found. The reduction in IgE binding achieved in this study is considered to be significant because most of the peanuts consumed in the United States are roasted.

Conclusions. The goal of this study was to determine if peroxidase treatment induces cross-linking of peanut proteins and, consequently, leads to a reduction in the allergenic properties of the proteins. Results showed that POD had no effect on raw peanuts but caused a significant decrease in the levels of the two major peanut allergens, Ara h 1 and Ara h 2, in roasted peanuts. High molecular weight cross-linked proteins were also formed after POD treatment. An overall reduction in IgE binding or allergenicity exhibited by the POD-treated peanut extract was observed. The study demonstrated that POD can help to reduce the allergenicity of roasted peanuts, but not raw peanuts.

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