Allergenicity of Maillard Reaction Products from Peanut Proteins

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It is known that peanut allergy is caused by peanut proteins. However, little is known about the impact of roasting on the allergenicity of peanuts. During roasting, proteins react with sugars to form Maillard reaction products, which could affect allergenicity. To determine if the Maillard reaction could convert a nonallergenic peanut protein into a potentially allergenic product, nonallergenic lectin was reacted with glucose or fructose at 50 °C for 28 days. Browning products from heat-treated peanuts were also examined. The products were analyzed in immunoblot and competitive assays, using a pooled serum (i.e., IgE antibodies) from patients with peanut anaphylaxis. Results showed that the products were recognized by IgE and had an inhibitory effect on IgE binding to a peanut allergen. Thus, the findings suggest that these Maillard reaction products are potentially allergenic and indicate the need to verify whether the Maillard reaction products formed in peanuts during roasting increase their allergenicity.

INTRODUCTION

The Maillard reaction, which occurs in foods during thermal processing and home cooking, is a nonenzymatic browning reaction between a protein and a reducing sugar (Nursten, 1981; Namiki, 1988). In the Maillard reaction, the amino groups of proteins undergo nonenzymatic glycosylation to form Amadori products on the proteins. Through a series of rearrangements, cyclizations, and dehydrations, the Amadori products form structurally diverse compounds known as advanced glycation end (AGE) products (Kato et al., 1994; Kawakishi et al., 1996; Booth et al., 1997). Specific cell receptors for AGE products have been identified, and binding of these products to the receptors has been shown to initiate the release of cytokines, which are important in antibody elicitation (Vlassara et al., 1988; Kirstein et al., 1990). Additionally, many of the protein-bound AGE products are known to be capable of eliciting IgG immune responses (Witztum et al., 1983; Matsuda et al., 1985; Nakayama et al., 1985; Hayase et al., 1989; Kato et al., 1994; Matsuda and Kato, 1996) and have allergenic potential because people expressing high IgG responses to food allergens have been shown to be more likely to develop IgE antibodies to the allergens later in life (Calkhoven et al., 1991; Okuma, 1992). Several studies have found significant correlation between IgE and IgG antibody titers (Campbell et al., 1987; Berrens and Homedes, 1991; Heddleson et al., 1997; Duchateau et al., 1998).

A number of studies have demonstrated the association of Maillard reaction products with food allergy, especially milk allergy. Bleumink and Young (1968) showed that coupling of lactose to the lysine residue of β-lactoglobulin increased skin reaction of patients with cow's milk allergy. Mukoyama et al. (1977) showed that in comparison with the native protein, the specific activity of the lactose–β-lactoglobulin conjugate increased 10–100-fold in direct intradermal tests. Kaminoagawa et al. (1984) showed that the sugar–protein complex from a reagent grade lactose was responsible for the high positive skin test of patients with cow's milk allergy. By contrast, Babiker et al. (1998) showed that conjugation of galactomannan to soy proteins reduced the allergenicity of soy proteins.

In peanut seeds, significant levels of reducing sugars have been detected (Vercellotti et al., 1995). Presumably, Maillard reaction or browning products may occur. However, very little is known about the nature or allergenicity of these products from peanut seeds. Because isolation of these products is difficult and time-consuming, we prepared in this study a model system in which Maillard reaction products were produced from a reaction between a nonallergenic protein (i.e., lectin) and glucose or fructose. Also, Maillard reaction products were produced by heat treatment and extraction of defatted peanut meals. Our objective was to determine if Maillard reaction products from these preparations were potentially allergenic.

MATERIALS AND METHODS

Apparatus. Xcell II mini-cell and blot module were purchased from Novex (San Diego, CA). CERES 900C microtiter plate reader was purchased from Bio-Tek Instruments, Inc. (Winooski, VT).

Reagents and Materials. Precast 4–20% Tris–glycine gels and SDS running buffer were purchased from Novex. Prestained low and high weight molecular standards, Coomassie Brilliant Blue R-250, and alkaline phosphatase conjugate substrate kit were purchased from Bio-Rad Laboratories (Hercules, CA). Immobilon P transfer membrane was purchased from Millipore Corp. (Bedford, MA). Peanut lectin, glucose, fructose, monoclonal anti-human IgE alkaline phosphatase conjugate, bovine serum albumin (BSA), Tween 20,

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phosphate-buffered saline (PBS), Tris-buffered saline (TBS), and nitroblue tetrazolium (NBT) tablets were purchased from Sigma Chemical Co. (St. Louis, MO). Normal human serum and serum from a pool of five patients with peanut anaphylaxis were obtained from the University of Arkansas, Children's Hospital (Little Rock, AR). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL). Mature and immature (classified as black and yellow) peanut seeds (*Arachis hypogaea* L. var. Florunner) were obtained from the USDA–ARS National Peanut Research Laboratory (Dawson, GA) (Chung et al., 1998).

Preparation of a Maillard or Browning Reaction Model System. The model system was prepared with some modification of the method of Kato and Matsuda (1996). The system consisted of a peanut lectin and a reducing sugar (glucose or fructose) in 0.3 M sodium phosphate, pH 8, containing 0.04% sodium azide. The control had no sugar. Maillard or browning reaction was carried out by incubating lectin (5 mg/mL, final) at 50 °C with glucose, fructose (150 mM, final), or no sugar for various times (0 and 1–5 weeks). Aliquots containing the browning products were withdrawn at different times and analyzed in immunoblots for their allergenicity.

Preparation and Measurement of Browning Products from Defatted, Heat-Treated Peanuts. To eliminate the effect of fats on the proteins during heating [note: fats also contribute to browning (Nakamura et al., 1998; Hidalgo et al., 1999)], defatted peanut meals were used. The defatted meals were prepared by grinding mature and immature seeds in acetone and then in hexane (both in dry ice). The meals were heated in a convection oven at 160 °C for 30 min (simulating roasting), extracted (0.1 g) with 0.7 mL of 0.1 M sodium bicarbonate, pH 10.4, and centrifuged at 8500*g* for 10 min. The resultant supernatants or extracts were analyzed in duplicates for browning products, using the NBT colorimetric assay (Ghiggeri and Candiano, 1988). Briefly, the extract (50 μL) was reacted with 0.3 M NBT (300 μL) at 40 °C for 5 min, and absorbance at 562 nm (A_{562}) was read in a microtiter plate using a CERES 900C plate reader (Bio-Tek Instruments, Inc). The reactivity of the browning products was expressed as A_{562} min^{-1} (protein concentration)$^{-1}$. Protein concentration in the extract was determined using the BCA protein assay kit. The extracts were further analyzed in immunoblots (see below) for their ability to inhibit IgE binding to a peanut allergen.

Determination of Allergenicity of Browning Reaction Products by SDS–PAGE and Immunoblotting. SDS–polyacrylamide gel electrophoresis (PAGE) (4–20% gel in Tris–glycine) and blotting were respectively performed with some modification of the method of Chung et al. (1998). Briefly, aliquots of the lectin–sugar reaction mixture and the control (5 μL each; 5 mg/mL) were withdrawn at different times (0 and 1–5 weeks), subjected to SDS–PAGE in the Xcell II mini-cell, and transferred to the Immobilon P membrane in the blot module. After blocking with 1% BSA–PBS–Tween 20 (0.05%), the membrane was incubated for 30 min with a normal human serum or pooled serum (diluted 1:30 in BSA–PBS–Tween) from patients allergic to peanuts, washed three times with PBS–Tween buffer, and then incubated for another 30 min with a monoclonal anti-human IgE alkaline phosphatase conjugate (1:1000, in BSA–PBS–Tween). The membrane was again washed three times. A substrate (substrate kit from Bio-Rad Laboratories) containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and NBT was added. Allergenicity of the products was determined on the basis of development of colored band(s) visualized on the membrane versus the control. Products were considered as potentially allergenic if the bands were significantly darker than the control band (i.e., lectin without sugar) and also if normal human serum gave bands (if any) lighter than the patient serum.

Determination of the Inhibitory Effect of Browning Products on IgE Binding to an Allergen. A peanut allergen, shown in this laboratory to be strongly recognized by IgE from allergic patients (Chung and Champagne, 1999), was prepared in a membrane-bound form by SDS–PAGE on a crude peanut extract, transfer of the proteins to an Immo-

bilon P membrane, and excision of a portion of the membrane containing the allergen only. To determine the inhibitory effect of browning products on IgE binding to the membrane-bound allergen, an aliquot (100 μL) from a lectin–sugar reaction mixture (week 4 at 50 °C) was preincubated for 15 min with a pooled serum (100 μL; 1:20) from allergic patients. The resultant mixture was then incubated with the allergen-bound membrane for 30 min. The membrane was washed three times with PBS–Tween buffer. A monoclonal anti-human IgE–alkaline phosphatase conjugate (1:1000) and substrate were added, respectively, as described above. A control (lectin without sugar at week 4) was performed in the same way. Protein concentration was determined using the BCA–protein assay kit. In this case, the control gave a dark band (allergen) on the membrane, whereas the sample with an inhibitory effect gave a light band or no band.

Additionally, the inhibitory effects of browning products from defatted, oven-heat-treated mature or immature peanuts were evaluated. A diluted extract from nonheated or raw peanuts was used as the control. The control was diluted to a protein concentration of 0.2 mg/mL so that IgE binding to the allergen on the membrane was partially inhibited. Briefly, a mixture of the diluted control (100 μL) and pooled serum (100 μL; 1:20) was incubated with the allergen-bound membrane as described above. Extracts from heat-treated peanuts were also diluted to the same protein concentration prior to the assays. A sample containing buffer only was also analyzed for comparison.

RESULTS AND DISCUSSION

In this study, the Maillard or browning reaction was carried out between a peanut lectin and glucose or fructose at 50 °C for various times (0 and 1–5 weeks). Glycation by glucose is usually a slower reaction. McPherson et al. (1988) showed that fructose reacted with amino groups ~10-fold more efficiently than glucose. Our study showed that browning occurred more rapidly with fructose than with glucose. Lectin was chosen as the reacting protein because it appeared to have little reactivity with IgE antibodies in immunoblot assays, thereby eliminating the chance that the native lectin competes with the products for IgE binding. Also, 50 °C was chosen because browning occurred more quickly at this temperature than at 37 °C and degradation of the products was slower at this temperature than at a higher temperature.

Allergenic Potentials of Maillard Reaction or Browning Products. Prior to determination of allergenicity, products from reactions between lectin and glucose or fructose at various times (0 and 1–5 weeks) were analyzed by SDS–PAGE and Coomassie Blue staining. In each case (i.e., lectin–glucose or lectin–fructose reaction), levels of products were found to increase for weeks (4 weeks for fructose; >5 weeks for glucose) and then decrease (data not shown). For allergenicity characterization, products at week 4 were chosen and subjected to immunoblot assays, using a pooled serum (IgE antibodies) from patients with peanut anaphylaxis. A pooled serum was used because the IgE level and response to the allergen vary with each individual patient. Figure 1 shows the profile of products and the control (i.e., lectin with no sugar) in immunoblots using the pooled serum. Peanut lectin (32 kDa) yielded no products in the absence of sugars and reacted minimally with IgE antibodies (i.e., the lectin band was hardly seen) (Figure 1c). When lectin reacted with the sugar (glucose or fructose), three to four products or protein bands (between 48 and 77 kDa; 32–36 kDa; and 26 kDa) (Figure 1a,b) were recognized by IgE antibodies
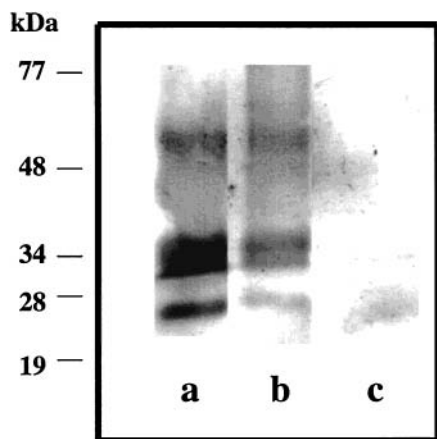
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Figure 1. SDS–PAGE and immunoblot analyses of Maillard reaction products from lectin–sugar reactions at week 4: (a) lectin–fructose reaction; (b) lectin–glucose reaction; (c) control (lectin with no sugar). Products were detected using pooled serum (IgE antibodies) from patients with peanut anaphylaxis and a monoclonal anti-human IgE–alkaline phosphatase conjugate.

Figure 2. Inhibitory effects of lectin–sugar solutions on IgE binding in competitive immunoblots: (a) control (lectin with no sugar); (b) lectin–glucose solution; (c) lectin–fructose solution. Competitive inhibition was carried out by incubating an allergen-bound membrane with a mixture of pooled patient serum (IgE antibodies) and a lectin–sugar solution (a, b, or c). Detection of the allergen on the membrane was carried out using a monoclonal anti-human IgE–alkaline phosphatase conjugate.

Figure 3. NBT assay of browning products in extracts of heat-treated peanuts: (A) control (raw or untreated); (B) heat-treated immature peanuts; (C) heat-treated mature peanuts. Reactivity of browning products with NBT was expressed as U or A_{562} min^{-1} (protein concentration)$^{-1}$ (mean of duplicates).

from patients' serum. Nonspecific binding to these products (e.g., from fructose–lectin reaction) was little when tested with normal human serum (not shown). Fructose gave darker bands than glucose (Figure 1) because browning occurred more rapidly with fructose than with glucose and therefore gave a higher yield of Maillard products than did glucose. This means that allergenicity increases with the levels of products. Early observations (not shown) also indicated that allergenicity increased with incubation times (i.e., 1–4 weeks) due to an increasing level of products produced during those periods. However, after 4 weeks of incubation, allergenicity decreased, probably due to a breakdown of products or a decrease in product levels.

During the browning reaction, AGE products such as carboxylmethyllysine, 3-deoxyglucosone, and pyrraline [ϵ-2-(formyl-5-hydroxymethylpyrrol-1-yl)-L-norleucine] are known to occur. They modify proteins and ultimately result in covalent cross-linking of the proteins and generation of AGE-bound proteins (Kato et al., 1987; Shin et al., 1988; Konishi et al., 1994; Nagarai and Sady, 1996; Nagaraj et al., 1996; Shipanova et al., 1997). In this case, lectin was modified probably by the AGE products and, as a result, yielded several polymers or protein bands (48–77, 32–36, and 26 kDa) (Figure 1). The 48–77 kDa band was formed probably as a result of protein cross-linking, whereas the 32–36 kDa band could be a lectin–AGE conjugate, which, because of the potential attachment of AGE to lectin (32 kDa), shifted slightly in mobility and was located above the lectin band in SDS–PAGE. The 26 kDa could be a breakdown product from lectin or the above bands. Because all of the lectin products except lectin itself bound to IgE antibodies, it can be concluded that products from a Maillard reaction are potentially allergenic.

Inhibition of IgE Binding by Browning Products. To further determine the allergenic potentials of the browning reaction products from the model system, a competitive immunoblot assay was performed. In the assay, pooled serum (IgE antibodies) from allergic patients was preincubated with the glucose– or fructose–lectin solution or the control (i.e., lectin without sugar) (all at week 4). The mixture was then incubated with a membrane conta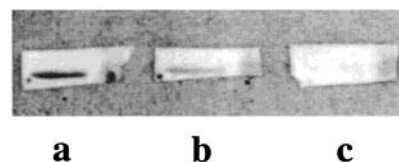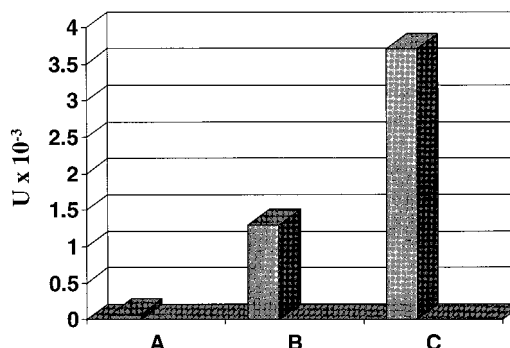ining a peanut allergen (see Materials and Methods for details). In this case, the membrane-bound allergen was competing for IgE binding with the browning products in the lectin–sugar solution. Figure 2 shows the inhibitory effect of the browning products on IgE binding. The control (i.e., lectin with no sugar) (Figure 2a) was shown to give an intense band (i.e., allergen) on the membrane, indicating that IgE binding to the allergen was not affected by lectin. By contrast, treatment of the membrane with glucose– and fructose–lectin solutions resulted in the fading out and disappearance of the band, respectively (Figure 2b,c). This suggests that there was an inhibition of IgE binding and that the extent of inhibition was dependent on the type of sugar involved. In this case, fructose had the most inhibitory effect, probably due to its possessing a higher level of browning products than glucose. This again suggests that Maillard reaction products of peanut proteins are potentially allergenic.

Inhibitory Effects of Browning Products from Heat-Treated Peanuts. Previously we showed that carbohydrate and protein levels increase during peanut maturation (Vercellotti et al., 1995; Chung et al., 1997, 1998). This suggests that levels of browning products may vary between peanuts of different maturity and that mature peanuts may have a higher level of browning products than the immature peanuts. To verify this, levels of browning products from extracts of heat-treated peanuts were determined, using an NBT assay. The assay measured the color change (absorbance at 562 nm) caused by the reduction of NBT to monoformazan by the browning products (Ghigger and Candiano, 1988; Mashiba et al., 1992). Figure 3 shows the difference in the levels of browning products between mature and immature heat-treated peanut extracts. As predicted, mature heat-treated peanuts were shown to have a
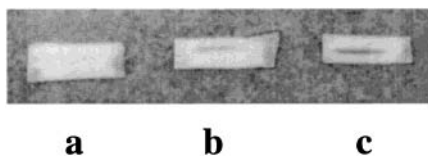
Figure 4. Inhibitory effect of a browning extract from heat-treated mature peanuts on IgE binding in a competitive immunoblot assay: (a) extract of heat-treated mature peanuts; (b) untreated/raw peanuts (a control); (c) PBS buffer only. Competitive inhibition was carried out by incubating an allergen-bound membrane with a mixture of pooled serum (IgE antibodies) and the sample (a, b, or c). The control was diluted so that IgE binding was partially inhibited. Samples were diluted to the same protein concentration. Detection of the allergen on the membrane was carried out by using a monoclonal anti-human IgE−alkaline phosphatase conjugate.

higher level of browning products than the immature peanuts. By contrast, little was seen in the control (i.e., untreated).

The inhibitory effect of browning products from extracts of heat-treated peanuts on IgE binding was evaluated in competitive immunoblot assays as described above. A diluted extract from untreated or raw peanuts was used as the control. The control itself was inhibitory and, therefore, was diluted so that IgE binding to the allergen on the membrane was partially inhibited. Samples were also diluted to the same protein concentration prior to assays. In this case, a difference in the intensity of the band between the heat-treated mature peanuts and the control (untreated) was observed (Figure 4a,b). The former gave a band less intense than the control, suggesting that heat-treated peanuts had a greater inhibitory effect than the control and that browning products probably were responsible for the inhibition (in other words, they were potentially allergenic). On the other hand, immature heat-treated peanuts exhibited no difference from the control (not shown). This probably was due to the level of products being too low to have an inhibitory effect. In comparison, a band with the highest intensity was seen (Figure 4c) when there was no inhibitory effect (i.e., no heat-treated or untreated samples were applied).

Conclusions. Maillard reaction products generated by a model system consisting of a nonallergenic peanut protein (i.e., lectin) and glucose or fructose (incubated at 50 °C, 4 weeks) were recognized in immunoblots by IgE antibodies from a pooled serum of patients with peanut anaphylaxis. The products were shown to have an inhibitory effect on IgE binding to a peanut allergen in competitive immunoblot assays. Maillard reaction products, measured by the NBT assay, from extracts of heat-treated peanuts also showed an inhibitory effect on IgE binding in competitive immunoblots. These findings suggest that these Maillard reaction products are potentially allergenic. Our next goal will be to verify whether the Maillard reaction products formed in peanuts during roasting increase their allergenicity.

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