A Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for the Quantitation of Selected Peanut Proteins in Foods

SUSAN L. HEFLE1,2, ROBERT K. BUSH1,2, JOHN W. YUNGINGER3 and FUN SUN CHU3

1Department of Medicine, University of Wisconsin, Madison, Wisconsin and William S. Middleton Memorial Veterans Hospital, 2500 Overlook Terrace, Madison, Wisconsin 53705; 2Food Research Institute, University of Wisconsin, 1925 Willow Drive, Madison, Wisconsin 53706; and 3Departments of Pediatrics and Internal Medicine, Rm. 406 Guggenheim Bldg., Allergic Disease Research Laboratory, Mayo Medical School, Rochester, Minnesota 55905

(Received September 7, 1993/Accepted January 10, 1994)

ABSTRACT

A sandwich-type, enzyme-linked immunosorbent assay (ELISA) was developed for the detection of selected peanut proteins in foods. Monoclonal antibodies against a series of allergenic peanut proteins were used as the capture antibody. Food sample extracts were then added, and polyclonal rabbit antibodies directed against roasted peanut proteins were employed as secondary antibodies. The amount of allergen bound to the solid-phase was determined by a biotin and streptavidin-peroxidase system. Radioallergosorbent assay (RAST) inhibition studies of the food extracts were done as a comparison. The coefficient of determination for the ELISA and RAST assays was 0.85. Selected food samples were tested by RAST inhibition at another laboratory for comparison. Skin tests were done with selected samples in peanut-allergic adults, and the results correlated to the ELISA and RAST inhibition results.

In other studies, defatted peanut protein (0.01 to 5.0%) were added to vanilla ice cream, then extracted and analyzed using ELISA and skin tests. The sensitivity of the ELISA in ice cream was approximately 40 µg/ml. In six of seven peanut-sensitive adults tested, the lowest level of added peanut protein (0.01%, 21 µg/ml) still caused a positive skin test reaction.

Key Words: Allergens, peanut, ELISA, food allergy, monoclonal antibody

Peanuts are one of the most allergenic foods known, and in sensitive individuals, can cause adverse reactions ranging from mild urticaria to life-threatening anaphylactic shock (3-6). For the peanut-sensitive individual, the identification of peanut allergens in foods is important, since the ingestion of minute amounts may trigger anaphylaxis (14). Peanut-sensitive individuals can find it difficult to identify and avoid peanuts, due to the increased use of peanut products in formulated foods to enhance flavor and nutritional values. An interesting example of a new peanut product is deflavored peanuts, which are peanuts that are pressed and deflavored, then refavored and sold as other types of nuts, such as almonds (10). Nordlee et al. (12) using RAST inhibition tests, found that these deflavored peanuts retained their allergenic qualities.

Sensitive individuals may also be inadvertently exposed to nonpeanut-containing foods contaminated with peanut proteins. This can occur during processing, from carryover due to inadequate cleaning of processing equipment used for nonpeanut and peanut products, or in reuse of peanut-containing products. Yunginger et al. (19) found peanut butter cross-contaminating sunflower butter due to inadequate cleaning of common equipment; the contaminated sunflower butter elicited an allergic reaction in a peanut-sensitive patient.

Currently, the only available method for quantitation of peanut allergens in foods is the RAST assay, a radioimmunoassay which requires peanut-allergic human sera and radiolabelled iodine (10). While the assay is sensitive and specific, the presence of these potentially hazardous substances precludes the use of this assay in a food-processing setting. This study describes a sandwich-type, ELISA system for the determination of peanut proteins/allergens in foods. The assay employs monoclonal antibodies raised against a series of allergenic peanut proteins and rabbit polyclonal antibodies. This type of immunoassay, like the radioimmunoassay, is specific and sensitive, but avoids the hazards associated with handling human sera and radioisotopes. Our efforts to use this assay to detect peanut proteins in foods are described.

MATERIALS AND METHODS

Food extractions

A commercial mix of dry-roasted peanuts (40% U.S. split Florunner, 40% Chinese and 20% Virginia varieties) was generously donated by Kraft General Foods, Limited (Canada). The peanuts were vacuum-packaged and stored at -20°C. For preparation of crude peanut extract (CPE), the peanuts were ground into a paste, then defatted with alternate washes of acetone and petroleum ether. After air-drying overnight, the residue was ground to a fine powder, then extracted in 1 M NaCl-20 mM sodium phosphate (pH 7.0) (1:20 wt/vol) (2). The extract (200 ml) was clarified by centrifugation at 20,000 x g for 20 min at 4°C, and the supernatant solution was dialyzed overnight against 0.01 M phosphate-buffered saline (pH 7.5, PBS) (3 changes) at 4°C. The CPE was concentrated using stirred cell ultrafiltration (Amicon Corp., Danvers, MA: membrane 2 kD cutoff), and the protein content determined using the biocinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).
Food samples

Food samples were obtained from grocery stores in Madison, WI, and were ground to a crude paste in a mortar and pestle, then defatted, extracted and dialyzed as described above for the CPE. The mortars and pestles were washed extensively after each extraction to avoid possible cross-contamination. The protein content was determined using the bicinchoninic acid protein assay (BCA) (Pierce Chemical, Rockford, IL). If the protein content was below 0.5 mg/ml, the extract was concentrated by placing it in a dialysis bag, then placing the bag in crystalline polyethylene glycol (M.W. 20,000, J. T. Baker, Phillipsburg, NJ) until the volume was sufficiently reduced. The extracts were stored at -20°C.

Vanilla ice cream was obtained from the Babcock Dairy Store, Department of Food Science, University of Wisconsin-Madison, Madison, WI. Defatted peanut protein in the range of levels of 0 to 5% (wt/wt) was added to samples of melted ice cream (20 g per sample) and mixed for 2 min using a Polytron (Brinkman, Westbury, NY). The samples were stored overnight at -20°C. The samples were then melted, defatted and extracted, as described above for the CPE. The extracts were stored at -20°C.

Peanut-allergic subjects

Sera were obtained from patients of the University of Wisconsin Hospital Allergy Clinic (Madison, WI). Seven adult patients with convincing histories of peanut allergy, and positive skin test (greater than the histamine control) and RAST (greater than two-times control binding) results to commercial peanut extracts were used. Control serum was obtained from six individuals who were not allergic to peanuts, and were peanut skin test and RAST-negative. All subjects gave informed consent and the protocol had the approval of the Human Subjects Committee, University of Wisconsin-Madison.

Skin testing

Individuals were skin-tested at the University of Wisconsin Hospital Allergy Clinic. Serial tenfold dilutions of each extract were prepared by diluting the stock extracts with saline. These were used to prick skin test the subjects. Positive controls for the tests contained 1 mg histamine/ml in 50% glycerin plus 0.4% phenol. The saline diluent was used as a negative control. Wheal size (average of length and width values, in mm) was recorded approximately 10 to 15 min after puncture of the skin.

Enzyme-linked immunosorbent assay (ELISA)

Monoclonal antibodies (IgM class), which recognize peanut proteins with molecular weights of 14, 25, 38, 40 and 44 kD in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (the 38 and 44 kD bands possess allergenic properties in that they bind IgE) (9), were purified from ascites fluid using a commercially available mannan-binding protein column (Pierce), according to manufacturer's directions. Polyclonal antibodies were obtained by immunization of a rabbit with CPE using standard procedures (8), and partial purification using 50% saturated ammonium sulfate. The anti-CPE antibodies used in this series of experiments had a titer of approximately 1:100,000 by indirect ELISA.

Microtiter plates (Nunc, Denmark) were coated with monoclonal antibody in PBS at a concentration of 10 μg IgM/ml (100 μl/well) at 4°C overnight. The unoccupied sites on the plate were blocked with 1% bovine serum albumin in PBS for 1 h at 37°C before further steps. Between each step in the procedure, the plates were washed four times with PBS containing 0.05% Tween 20. Food extracts or CPE, at various concentrations, were added, and incubated for 1 h at 37°C. Anti-CPE polyclonal rabbit antiserum at a concentration of 1 μg IgG/ml (100 μl/well) was added, and allowed to incubate in the plate for 1 h at 37°C. Goat anti-rabbit-IgG labelled with biotin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at a 1/20,000 dilution (.042 μg IgG/ml) was then added to the plate, and incubated for 1 h at 37°C. Bound secondary antibody was detected by addition of streptavidin-peroxidase (Boehringer-Mannheim Biochemicals, 1/10,000 dilution, 50 mU conjugate/ml) for 30 min at 37°C, then o-phenylenediamine; the enzyme reaction was stopped by addition of 1 N HCl. The absorbance at 490 nm was measured in a microtiter plate reader (ThermoMax Kinetic; Molecular Devices, Menlo Park, CA).

Radioallergosorbent (RAST) inhibition assays

Crude peanut extract (3.4 mg/ml) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Biochemicals, Piscataway, NJ) (5-10 mg peanut protein per ml Sepharose 4B), according to manufacturer's directions. One-half milliliter of a 3% wt/vol solution of coupled Sepharose-CPE and 0.1 ml of diluted serum (1.5 dilution in RAST buffer) from a pool of peanut-allergic subjects or controls were incubated in test tubes at room temperature for 18 to 24 h with rotation, along with 0.1 ml of various concentrations of food extracts or CPE. The dilution buffer (RAST buffer) consisted of 0.05 M sodium phosphate, 0.43 M sodium chloride, 3% bovine serum albumin, 0.05% sodium azide and 0.05% Tween 20. After incubation, the solid phase was washed three times with 3 ml of RAST buffer, and then 125I-labelled rabbit anti-human IgE (25,000 to 35,000 cpm) (Phadebas RAST test kit, Pharmacia Biochemicals) was added to each tube. The reagents were incubated again as described above. The solid phase was again washed, and the bound radioactivity measured (5 min/tube) using a gamma scintillation counter (Searle Laboratories, Des Plaines, IL).

Additional RAST inhibition analyses for selected food extracts were performed at the Allergic Diseases Research Laboratory, Mayo Clinic and Foundation (Rochester, MN) by methods previously described (10).

RESULTS

Enzyme-linked immunosorbent assay (ELISA) results

The standard curve for the reference peanut extract (CPE) in the sandwich ELISA is shown in Fig. 1. This curve was used to calculate the relative amounts of peanut protein in each of the food samples, expressed as a percentage of the absorbance given by the CPE, shown in Table I.

![Figure 1. Standard curve for crude peanut extract in the sandwich ELISA system. Inset shows the resulting linear regression curve when the y axis depicts percentage of maximum absorbance. The points are the means of nine trials; standard deviations for all values were below 4%.](image-url)
A relatively high extract protein concentration of 500 µg/ml was chosen for analysis because of the unknown quantity of peanut protein in these samples.

The protein concentrations of the defatted and dialyzed food extracts were determined using the BCA assay and ranged from 0.35 mg/ml (0.7% protein) for the fortune cookies to 3.74 mg/ml (7.5% protein) for trail mix style B (Table 1). All foods except the fortune cookies and cashews contained peanuts. Trail mix style B was additionally assayed with the peanut pieces hand-removed, to determine the amount of residual allergic activity.

Radio allergosorbent (RAST) inhibition results

The total allergenic activity of the food extracts was measured in a RAST inhibition assay in which the food extracts were tested for their ability to inhibit binding of peanut-specific IgE to solid-phase CPE. Food extracts were tested at a protein concentration of 500 µg/ml, and the inhibition was expressed relative to the CPE (Table 1). The linear regression curve between the ELISA and RAST data had a correlation coefficient \( r \) of 0.92, with \( p < 0.0001 \) (Fig. 2).

Four selected food extracts were additionally assayed by an independent RAST inhibition system utilizing a different standard peanut reference extract for verification of the RAST analyses. The two RAST inhibition assay results, although different, show an identical rank order in the amount of peanut present in the food extracts.

The two foods that did not contain peanuts, cashews and fortune cookies, gave negative results in both the RAST and the ELISA systems. The expected differences in the two RAST inhibition analyses were probably due to the different methods of preparation of the standard reference peanut extracts, the different peanut-sensitive serum pools used to do the testing, different solid-phase supports and radiolabeled anti-human IgE. The RAST inhibition assay measures the total allergenic activity, based on IgE-binding; the ELISA only measures the amount of specific peanut protein that the monoclonal antibody recognizes. This explains the higher results obtained from the RAST inhibition studies. However, the resulting coefficient of determination \( r^2 = 0.85 \) between the RAST and ELISA indicates that the ELISA results are proportional to the results obtained from the RAST.

The allergenicity of trail mix style B was not eliminated by hand-removal of the peanuts, therefore, peanut-containing foods cannot be considered safe for sensitive individuals even if all the peanut pieces have been removed.

### Table 1. Protein concentrations and amount of peanut protein present in food extracts.

<table>
<thead>
<tr>
<th>Product</th>
<th>Protein Conc. (mg/ml)</th>
<th>ELISA</th>
<th>RAST(^a)</th>
<th>MAYO(^b)</th>
<th>SPTd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPE - WI</td>
<td>4.2</td>
<td>100.0 ± 1.1</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CPE - Mayo</td>
<td>9.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cashews</td>
<td>2.87</td>
<td>0.1 ± 0.5</td>
<td>0</td>
<td>0</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Cheesecake mix</td>
<td>2.47</td>
<td>74.6 ± 7.6</td>
<td>92</td>
<td>95</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>Chocolate-peanut cookies</td>
<td>0.96</td>
<td>40.0 ± 6.4</td>
<td>74</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Chocolate-nut granola bars A</td>
<td>2.11</td>
<td>33.1 ± 2.9</td>
<td>65</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Chocolate-nut granola bars B</td>
<td>1.64</td>
<td>10.3 ± 4.7</td>
<td>15</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Confection - peanut brittle</td>
<td>1.36</td>
<td>36.8 ± 8.3</td>
<td>76</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Confection - bridge mix</td>
<td>1.75</td>
<td>26.2 ± 3.8</td>
<td>52</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Confection - chocolate candy</td>
<td>1.62</td>
<td>4.2 ± 2.5</td>
<td>4</td>
<td>0.5</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Doughnuts - peanut-coated</td>
<td>1.01</td>
<td>32.7 ± 5.7</td>
<td>84</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Fortune cookies</td>
<td>0.35</td>
<td>0.2 ± 0.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Peanut butter - chunky</td>
<td>2.85</td>
<td>75.8 ± 8.6</td>
<td>97</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Peanut butter - creamy A</td>
<td>1.84</td>
<td>56.7 ± 8.3</td>
<td>81</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Peanut butter - creamy B</td>
<td>2.03</td>
<td>53.4 ± 3.2</td>
<td>68</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Snack mix style A</td>
<td>0.95</td>
<td>12.3 ± 5.2</td>
<td>14</td>
<td>11</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>Snack mix style B</td>
<td>1.65</td>
<td>17.3 ± 5.3</td>
<td>35</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Trail mix style A</td>
<td>1.66</td>
<td>46.5 ± 7.7</td>
<td>63</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Trail mix style B</td>
<td>3.74</td>
<td>57.7 ± 6.3</td>
<td>84</td>
<td>n.d.(^c)</td>
<td></td>
</tr>
<tr>
<td>Trail mix style B with</td>
<td>1.86</td>
<td>10.3 ± 4.9</td>
<td>23</td>
<td>n.d.(^c)</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>peanuts removed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The relative amount of peanut protein is that percentage of the total which is detected by the primary antibodies in the respective assay. Results are the average of five trials and foods were tested at a concentration of 500 µg protein/ml. Samples were measured relative to the CPE. Standard deviations for the RAST assay were all below 5%.

\(^b\) RAST inhibition performed using CPE as the reference extract.

\(^c\) RAST inhibition performed using Mayo CPE as the reference extract.

\(^d\) Skin prick test results; values are the average sum of the orthogonal dimensions of the flare.

\(^e\) Not determined.
Skin tests of food extracts

Four selected food extracts were skin-tested in peanut-sensitive adults; results showed that the sum of the orthogonal dimensions of the flare in positive tests could be correlated to the amount of peanut protein in the extract, as determined by ELISA and RAST inhibition (Table 1). None of the patients demonstrated a skin test greater than the histamine control to the cashew extract (negative control).

Ice cream samples

Vanilla ice cream was spiked with various amounts of defatted peanut protein, then extracted and analyzed using the ELISA and skin tests. Ice cream was used as a vehicle because it has been previously involved in recall incidents due to peanut contamination (1). Peanut protein was added to the ice cream at levels ranging from 0.01 to 5% by weight. The sandwich ELISA sensitivity in ice cream was approximately 40 μg peanut protein/ml. In most (6/7) of the peanut-sensitive patients tested, the lowest level of added peanut (0.01%; 21 μg/ml) still caused a positive skin test reaction, therefore, the skin test is more sensitive in this food matrix than in the present ELISA system.

DISCUSSION

Despite the valuable nutritional and functional aspects of the peanut, it is one of the most allergenic foods known. Allergic reactions to peanuts are often acute and severe. Life-threatening reactions to peanuts are more common than to other foods (14). The prevalence of peanut allergy is not known, but is estimated to be 10% in individuals who are atopic (13). Approximately one-third of peanut-sensitive people experience severe, anaphylactic reactions (17).

Peanuts are the most common cause of food anaphylaxis in the United States (20) and, unfortunately, peanut allergy is rarely outgrown (3). Some individuals are so sensitive that contact of peanut proteins with unbroken skin can produce urticarial and/or gastrointestinal reactions (11,14). The amount required to produce a reaction is unknown, although estimates are in the milligram amount (17,20). It is of note that in this study, hand-picking the peanuts out of the trail mix did not result in allergenicity being reduced to background; therefore, peanut-containing foods cannot be considered safe for sensitive individuals even if the peanuts are not readily discernible in the food.

Strict avoidance is difficult and often unsuccessful. Despite education and availability of self-medication, the frequency of fatal and near-fatal food-induced anaphylactic reactions has appeared to rise in recent years (16). For sensitive individuals, the threat of hidden peanut proteins in foods is constant. Peanut allergens have been found in nonpeanut foodstuffs prepared on common processing equipment (10,19), and sensitive individuals can be inadvertently exposed in this way, or from rework of products containing peanut. It is the opinion of researchers in the field, that the prevalence of peanut allergy has risen in the past decade (15), and unknown incidents of carryover contamination may contribute to increased risk.

Imunoassays offer a specific, sensitive and rapid method to detect and quantitate trace amounts of allergens in food systems. The availability of immunoassays for food allergens would allow manufacturers who use common equipment producing a variety of potentially allergenic foods, to monitor their food products for possible equipment malfunction or inadequate cleaning (10).

The coefficient of determination (r² = 0.85) between the RAST and ELISA indicates that the ELISA results are proportional to the results obtained from the RAST. Therefore, this ELISA could be used as an indicator assay for peanut contamination in food products. The useful range of detection for the foods analyzed in this study was approximately 40 μg peanut protein/ml to 2.0 mg/ml. The ice cream spiking studies indicate that the peanut protein detection limit of the ELISA in ice cream is less than that of skin tests. Given the exquisite sensitivity displayed by most peanut-allergic individuals (6,14,18), this is not surprising. Use of amplification systems, improved monoclonal and polyclonal antibodies, and assessment of food matrix interference problems will result in increased sensitivity for peanut allergen immunoassay systems.

ACKNOWLEDGMENTS

The authors would like to thank Kraft General Foods, Ltd., Canada for their donation of commercial peanut mixture, and Richard Jones of the Allergic Diseases Research Laboratory at the Mayo Clinic for his RAST inhibition analyses.

This work was supported by a Hatch grant from the College of Agricultural and Life Sciences of the University of Wisconsin-Madison, by gift funds from various food companies to the Food Research Institute, by the Department of Veterans Affairs, and by the Mayo Foundation.

REFERENCES