

Comparison of commercial peanut skin test extracts

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Background: Skin prick testing is a major tool for diagnosing food allergy. Food allergen extracts have not been standardized; this may lead to great variability in the predictive accuracy of skin prick tests.

Methods: Six commercial peanut skin test extracts were compared in vitro with RAST inhibition assays, ELISA, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with sera from peanut-allergic adults and in vivo by skin prick testing.

Results: ELISA showed that the content of peanut allergens Ara h I and Ara h II in the extracts ranged from 0.0015 to 0.0236 and 0.0001 to 0.0164 mg Eq/ml, respectively. RAST inhibition studies showed that the extracts produced curves of similar slope, suggesting conservation of allergenic epitopes. SDS-PAGE revealed differences in protein profiles because roasted extracts generally possessed the same number and proportion of major protein bands but raw extracts varied more in both respects. SDS-PAGE and immunoblotting showed that two of the extracts contained major IgE-binding protein bands that did not appear in the others. One roasted extract gave little protein banding and consequently little IgE binding.

Conclusions: Skin testing results showed no differences in the ability of the extracts to provoke a positive skin test response in peanut-sensitive subjects. (*J ALLERGY CLIN IMMUNOL* 1995;95:837-42.)

Key words: Peanut allergy, skin testing, allergenic extracts

Skin prick testing is the most popular diagnostic tool for evaluating food allergy. Food allergen extracts are typically crude preparations.¹ They are usually manufactured from raw food material and are complex mixtures of antigenic, nonantigenic, and allergenic substances (carbohydrates, proteins, lectins, enzymes, histamine, among others).^{2,3} Lack of standardized food allergen extracts may be the basis for the variability in the predictive accuracy of food skin prick tests reported in the literature.

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Abbreviations used

BSA:	Bovine serum albumin
CPE:	Crude peanut extract
PBS:	Phosphate-buffered saline solution
PBS-T:	Phosphate-buffered saline solution with polysorbate 20
SDS-PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

This study investigated peanut skin test extracts from six commercial sources. Comparison was made of their potency by skin prick testing of six peanut-sensitive adults, and the extracts were also compared with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), ELISA for two known peanut allergens (*Ara h I*^{4,5} and *Ara h II*⁶), RAST inhibition, and IgE immunoblotting.

METHODS

Commercial skin test extracts

Six commercial skin test extracts were purchased from their manufacturers: Allergy Laboratories (Oklahoma

City, Okla.), Antigen Laboratories (Liberty, Mo.), Center Laboratories (Hawthorne, N.Y.), Greer Laboratories (Lenoir, N.C.), Hollister-Stier (Spokane, Wash.), and Nelco Laboratories (Deer Park, N.Y.). Two different lots were purchased, 2 months apart. All extracts were glycerinated and were either 1:20 or 1:10 wt/vol, depending on which strength was available for purchase. Manufacturers provided source information.

Protein assay

A tannin precipitation assay method was used to determine the protein concentration of the extracts.⁷

Peanut-sensitive subjects

Serum samples were obtained from patients of the University of Wisconsin Hospital Allergy Clinic in Madison. Patients with histories of allergic reactions to peanuts (ranging from angioedema and urticaria to anaphylaxis) and positive skin test and RAST results to commercial peanut extracts participated. Control sera were obtained from six persons who were not allergic to peanuts and also from three persons with *Alternaria* sensitivity. The control subjects consumed a typical amount of peanuts as part of their diet. All subjects gave written informed consent to the procedures, which had the approval of the University of Wisconsin-Madison Human Subjects Committee.

Skin tests

Positive controls for the tests contained 1 mg/ml histamine in 50% glycerin plus 0.4% phenol. A diluent control was also included. Wheal sizes and flare sizes (sum of length and width values in millimeters) were recorded approximately 10 to 15 minutes after puncture of the skin, according to the method of Turkeltaub et al.⁸

Crude peanut extract

A commercial mix of dry-roasted peanuts (40% Flo-runner, 40% Chinese, and 20% Virginia varieties) was generously donated by Kraft General Foods Ltd. (Canada). The peanuts were vacuum packaged and stored at -20°C . For preparation of crude peanut extract (CPE) the peanuts were blended 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 mol/L NaCl with 20 mmol/L sodium phosphate (pH 7.0; 1:20 wt/vol).⁹ The extract (200 ml) was clarified by centrifugation at 20,000g for 20 minutes at 4°C , and the supernatant solution was dialyzed overnight against 0.01 mol/L phosphate-buffered saline solution (PBS) (pH 7.5; three changes) at 4°C . The CPE was concentrated with stirred cell ultrafiltration (Amicon Corp., Danvers, Mass. 2 kd cutoff for membrane), and the protein content was determined.

SDS-PAGE

Vertical slab SDS-PAGE was carried out by the method of Laemmli¹⁰ (apparatus from Bio-Rad Labora-

tories, Richmond, Calif.). Resolving gels (8×7.3 cm) were 12.5% wt/vol polyacrylamide, with 4% wt/vol stacking gels. Samples were boiled at 95° to 100°C for 5 minutes in sample buffer containing 60 mmol/L dithiothreitol. Approximately 50 μg of sample were loaded per lane. Gels were run at 150 V (constant voltage) for approximately 45 minutes, then stained with FAST stain (Zion Research, Allston, Mass.).

Immunoblotting of extracts with patient sera

Extract proteins were separated by SDS-PAGE as described previously, then were electrophoretically transferred to an Immobilon P membrane (0.45 μm ; Millipore Corp., Bedford, Mass.) according to the method of Towbin et al.,¹¹ except that dithiothreitol was present at a concentration of 1 mmol/L in the blotting buffer. The procedure was performed in a Transblot apparatus (Bio-Rad Laboratories) for 1 hour at 100 V (constant voltage). An india ink stain¹² of a strip of the blotted membrane was done to assure protein transfer.

After removal from the Transblot apparatus, the membrane was blocked with a solution of 0.2% bovine serum albumin (BSA) in 0.01 mol/L PBS containing 0.05% polysorbate 20 (PBS-T) for 1 hour at 25°C . The membrane was washed three times with PBS-T and then cut into strips approximately 0.5 cm wide. The strips were then incubated with sera from peanut-sensitive patients (1:5 dilution in PBS-T containing 3% BSA) for 18 to 24 hour at 25°C . After another wash step the strips were incubated overnight with rabbit anti-human IgE labeled with iodine 125 (40,000 counts/min per strip, Phadebas RAST kit, Pharmacia, Piscataway, N.J.). Autoradiographic detection of IgE binding was done by exposing X-OMAT AR x-ray film (Eastman Kodak, Rochester, N.Y.) to the membrane at -70°C . Exposure times ranged from 1 to 3 days.

RAST inhibition

CPE (3.4 mg/ml) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Biochemicals) (5 to 10 mg peanut protein per milliliter Sepharose 4B), according to the manufacturer's directions. A 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 peanut-allergic subjects or control subjects were incubated in test tubes at room temperature for 18 to 24 hours with rotation, together with various concentrations of the skin test extracts or CPE. The dilution buffer (RAST buffer) consisted of 0.05 mol/L sodium phosphate, 0.43 mol/L sodium chloride, 3% BSA, 0.05% sodium azide, and 0.05% polysorbate 20. After incubation the solid phase was washed three times with 3 ml of RAST buffer and then ^{125}I -labeled rabbit anti-human IgE (25,000 to 35,000 counts/min) (Phadebas RAST test kit, Pharmacia) was added to each tube. The reagents were incubated again as described

previously. The solid phase was again washed and the bound radioactivity measured (5 min/tube) with a gamma scintillation counter (Searle Laboratories, Des Plaines, Ill.).

ELISA

A biotin-avidin ELISA inhibition assay was developed to quantify specific amounts of *Ara h I* and *Ara h II*. A 96-well microtiter plate (Gibco, Santa Clara, Calif.) was coated with 100 μ l each (0.5 μ g/ml) of *Ara h I* in coating buffer (0.1 mol/L sodium carbonate-bicarbonate buffer, pH 9.5). The plate was incubated at 37° C for 1 hour and then washed five times with rinse buffer (0.1 mol/L PBS, pH 7.4, containing 0.05% polysorbate 20, Sigma Chemical Co., St. Louis, Mo.) immediately and between subsequent incubations. In the upper two rows a standard curve ranging from 0.02 to 1,236 ng/ml of *Ara h I* was used to generate the inhibition curve. The standard curve samples and the commercial peanut extract samples were incubated with equal amounts of a biotinylated mouse IgG1 monoclonal antibody (8F10, generated against *Ara h I*, 0.05 μ g/ml). After incubation for 1 hour at 37° C, the plates were washed and 100 μ l horseradish peroxidase-avidin conjugate (1:1000 vol/vol in 0.1 mol/L PBS, Spectrum Laboratories, Burlingame, Calif.) were added and allowed to incubate for 5 minutes at room temperature. After washing again, enzyme binding was detected with the addition of *o*-phenylenediamine (Sigma) substrate. The reaction was stopped by the addition of 100 μ l of 2 N hydrochloric acid to each well, and absorbance was read at 492 nm (microplate reader; Bio-Rad Laboratories). A standard curve was plotted on a log-logit graph by means of simple linear regression analysis.

A similar ELISA inhibition assay was performed for *Ara h II*, in which *Ara h II* was the coating antigen and biotinylated monoclonal antibody (4996C3, generated against *Ara h II*) was used as the detection antibody.

RESULTS

Skin tests

Skin test results are shown in Fig. 1. There was no significant disparity in subject responses to the different extracts, and all produced a positive response far greater than that of the saline solution control. Skin tests in nonallergic persons produced negligible responses (0 to 2 mm).

Protein concentrations

Protein determinations for each of the extracts are indicated in Table I.

Inhibition RAST

Fig. 2 shows the RAST inhibition curves resulting from use of raw extracts as inhibitors in the CPE RAST. Curves from extracts B, C, and D have similar slopes to the reference (CPE) curve, indicating the presence of similar IgE-binding deter-

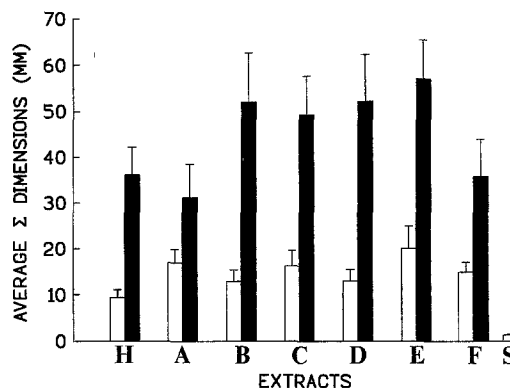


FIG. 1. Skin test results for six commercial peanut extracts in six peanut-sensitive adults. Average sum of orthogonal dimensions (in millimeters) of wheals (white boxes) and flares (black boxes) are shown in y-axis. H, Positive histamine control; S, negative saline solution control.

minants in these extracts.¹³ Extract B was made from Spanish peanuts, and C and D were made from Virginia peanuts; however, the curves are similar, indicating that perhaps there may be some conserved allergenic epitopes in different peanut varieties. Extract E, which was made from a mixture of runner, Spanish, and Virginia varieties, produced an inhibition curve very different from the others tested. However, this extract produced a curve that was similar to one of the roasted extract curves (extract A; Fig. 3).

Fig. 3 shows the RAST inhibition curves resulting from use of the roasted extracts as inhibitors in the CPE RAST. Extract F produced a curve with a similar slope to the CPE curve, but the allergenic content of the extract appears to be much lower. Extract A produced a curve with a dissimilar slope to the CPE curve but very similar to the curve produced by extract E in Fig. 2.

SDS-PAGE

Fig. 4 shows an SDS-PAGE gel of the extract proteins and the CPE. Extract F shows little protein content. Extract C contains a large amount of a protein at 65 kd, which is also seen in the other extracts in varying quantities; this protein has been identified as the major peanut allergen, *Ara h I*. Most of the extracts contain an appreciable amount of protein at 17 kd, which has been identified as *Ara h II*. Extract C also contains larger amounts of protein bands at approximately 34 and 55 kd. Extract E and the CPE contain a major band at 27 to 28 kd, and extracts A, B, and E, and the CPE contain a large band at 25 kd.

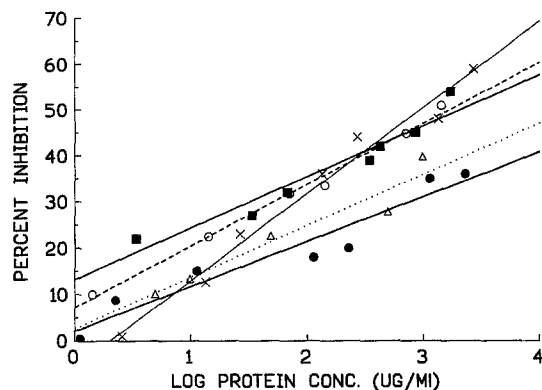


FIG. 2. RAST inhibition by raw commercial peanut extracts (B, Allergy Laboratories, ●; C, Center Laboratories, △; D, Greer Laboratories, ○; E, Hollister-Stier, ×) and CPE (■).

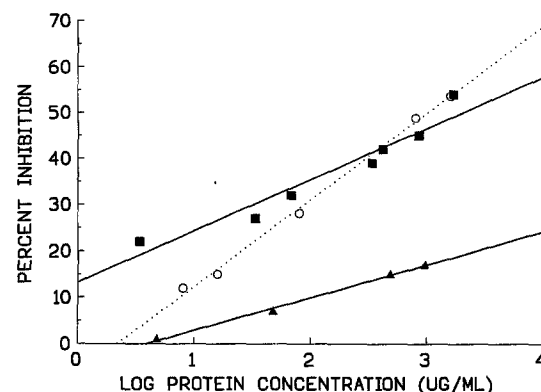


FIG. 3. RAST inhibition by roasted commercial peanut extracts (A, Antigen Laboratories, ○; F, Nelco Laboratories, ▲) and CPE (■).

TABLE I. Analysis of commercial peanut skin test extracts

Manufacturer	Label	Source*	Variety	Dilution (wt/vol)*	Protein (mg/ml)	Mean sum of flare size (mm)	Mean sum of wheal size (mm)
Antigen Labs	A	Roasted	Unknown	1:20	7.4	31 ± 7	16 ± 3
Allergy Labs	B	Raw	Spanish	1:20	20.3	52 ± 10	13 ± 2
Center Labs	C	Raw	Virginia	1:10	3.1	49 ± 8	16 ± 3
Greer Labs	D	Raw	Virginia mixture	1:20	7.5	52 ± 8	13 ± 3
Hollister-Stier	E	Raw	Runner, Virginia, Spanish mixture	1:10	30.5	57 ± 8	20 ± 5
Nelco Labs	F	Roasted	Unknown	1:20	0.29	35 ± 8	15 ± 2
CPE†	CPE	Roasted	Florunner, Chinese, Virginia mixture	1:20	4.2	NT	NT

NT, Not tested.

*Company-supplied information.

†Reference extract.

Immunoblotting of extracts with sera

Fig. 5 shows an autoradiogram of the SDS-PAGE-separated extract proteins immunoblotted with a pooled serum sample from peanut-allergic subjects. Most of the extracts show IgE-binding to proteins in the range of 14 to 25 kd, to *Ara h* I (65 kd), and to *Ara h* II (17 kd). Extracts C and D show heavy IgE binding to bands at approximately 34 and 55 kd. The 28 kd band found in extract E and the CPE do not bind IgE.

ELISA

Table II shows the results of the ELISA for *Ara h* I and *Ara h* II. The interassay variability of the ELISA was 10%, the intraassay variability was 1%, and the correlation coefficient was greater than 0.9.

Extract B contains more *Ara h* I per milliliter than do the other extracts, whereas extract E contains

more *Ara h* II per milliliter than the others. Extracts C and D are made from Virginia peanuts exclusively, and the relative quantities of *Ara h* II per milliliter are similar. The varietal composition of the roasted extracts (extracts A and E) is unknown, so no conclusions can be drawn regarding the effect of variety on *Ara h* I and *Ara h* II content.

DISCUSSION

Commercial skin test extracts vary in protein content, allergenic potency, and allergen composition. Most food extracts are complex mixtures with undefined concentrations of various components including proteins, lipids, carbohydrates, and pigments. Differences in starting material, methods of extraction, and storage conditions can be responsible for differences in protein profile and composition of the extracts.¹⁴

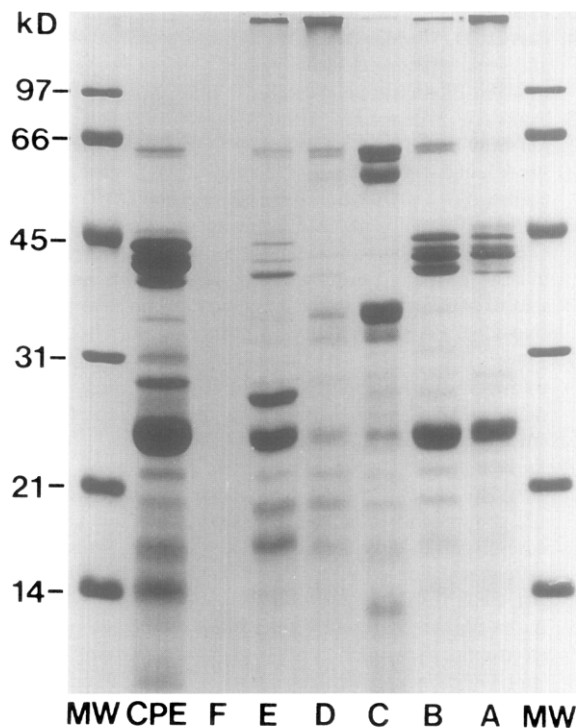


FIG. 4. SDS-PAGE gel of commercial peanut extracts and CPE. Approximately 35 μ g of protein were loaded per lane. Molecular weight markers are located at *far left* and *far right* lanes. Gel was stained with FAST stain.

Peanut-sensitive patients have IgE that binds to components of both raw and roasted peanuts.^{15,16} Most extract manufacturers choose to use raw peanuts to make their extracts because more allergens are present; however, human beings do not generally consume raw peanuts, so roasted peanuts are a logical choice of extract material also.

Most extracts contain numerous IgE-binding proteins in the range of 17 to 25 kD and 65 kD as analyzed by SDS-PAGE. Not all the protein bands in peanuts bind IgE; the 28 kD band found in large quantity in extract E and in the CPE is a good example. Raw Virginia peanut extracts possess a large quantity of 34 kD protein. Although present in small quantities in other extracts, in five of the seven extracts the 34 kD band bound IgE as seen on the immunoblot. This is also true of the 55 kD band. These two bands have not been previously described as major peanut allergens.

There may be some relationship between the 17 to 25 kD IgE-binding proteins; they seem to be conserved in all the extracts, regardless of source variety. Some of the bands could be proteolysis products or subunits of the other, larger-molecular-weight proteins. Because the allergenicity of

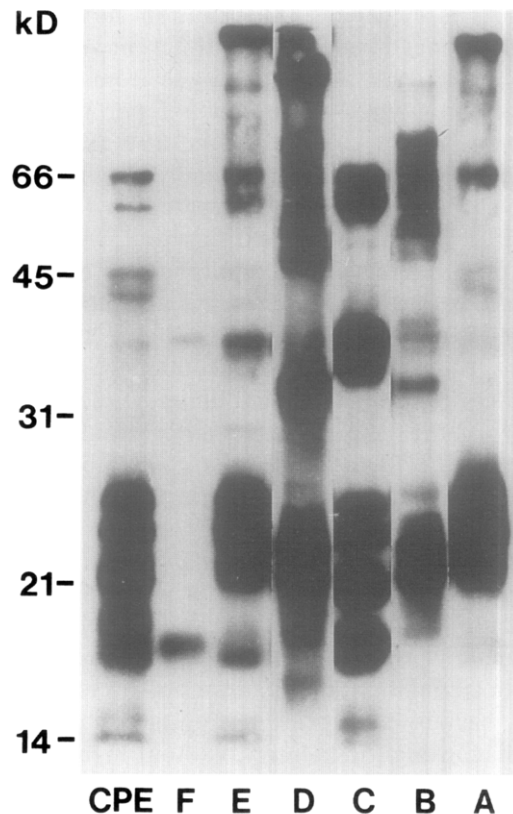


FIG. 5. Autoradiogram of immunoblot of SDS-PAGE-separated extracts with pool of sera from peanut-allergic patients. Bound IgE was detected with rabbit anti-human IgE labeled with ¹²⁵I. Exposure time was 36 hours. Approximately 30 μ g of protein were loaded per lane. Molecular weights (in kilodaltons) are indicated at *left column*.

TABLE II. Analysis for *Ara h I* and *Ara h II* by ELISA

Manufacturer	Label	<i>Ara h I</i> (mg Eq/ml)	<i>Ara h II</i> (mg Eq/ml)
Antigen Labs	A	0.0018	0.0059
Allergy Labs	B	0.0236	0.0081
Center Labs	C	0.0015	0.0042
Greer Labs	D	0.0022	0.0042
Hollister-Stier	E	0.0015	0.0164
Nelco Labs	F	0.0044	0.0001

peanuts is spread throughout the different storage protein fractions,^{17,18} another possibility is that certain peanut proteins that differ in other physicochemical aspects possess similar determinant groups. This is supported by the RAST results, which indicate either conserved or similar allergenic epitopes in separate cultivars of

peanut, as evidenced by inhibition curves with similar slopes resulting from different varieties.

In the ELISA, an extract made from Spanish peanuts contained more *Ara h* I per milliliter than any of the other extracts, and an extract made from a mixture of Runner, Virginia, and Spanish peanuts contained more *Ara h* II per milliliter than the other extracts did. This could be a reflection of varietal differences or, again, of variations in extraction protocol and storage. In comparison, the two separate extracts made from Virginia peanuts contained similar amounts of *Ara h* II per milliliter. Direct correlation of the ELISA results with visual assessment in the SDS-PAGE gels cannot be done, however, because individual proteins have different dye-binding abilities.

One of the extracts contained little protein (extract E) but still elicited a wheal-and-flare response comparable to that caused by the other extracts. This is testimony to the exquisite allergy manifested in our adult patient population; however, because skin prick testing is a major tool for diagnosing food allergy, without high quality extracts, false negatives or false positives could occur. Yunginger and Jones¹⁹ have suggested a method for preparing and testing a standardized peanut extract, but standard, industry-wide procedures to assess quality and potency of peanut extracts are not currently being performed. Numerous problems would have to be overcome to obtain standardized peanut extracts, including identification of adequate source materials, development of reliable assay methods, and preparation of reference standards.¹ Obstacles associated with developing standardized extracts have been solved with for other systems such as the venoms, cat, dust mite, and some of the pollens. In the future, with more insight into the nature of the allergenic proteins of the peanut, standardized peanut extracts will likely be available.

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