Identification and characterization of a second major peanut allergen, *Ara h* II, with use of the sera of patients with atopic dermatitis and positive peanut challenge

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Peanuts are frequently a cause of food hypersensitivity reactions in children. Serum from nine patients with atopic dermatitis and a positive double-blind, placebo-controlled, food challenge to peanut were used in the process of identification and purification of the peanut allergens. Identification of a second major peanut allergen was accomplished with use of various biochemical and molecular techniques. Anion exchange chromatography of the crude peanut extract produced several fractions that bound IgE from the serum of the patient pool with positive challenges. By measuring antipeanut specific IgE and by IgE-specific immunoblotting we have identified an allergic component that has two closely migrating bands with a mean molecular weight of 17 kd. Two-dimensional gel electrophoresis of this fraction revealed it to have a mean isoelectric point of 5.2. According to allergen nomenclature of the IUIS Subcommittee for Allergen Nomenclature this allergen is designated, Ara h *II* (Arachis hypogaea). *(J ALLERGY CLIN IMMUNOL 1992;90:962-9.)*

Key words: *Food hypersensitivity, peanut, allergens, atopic dermatitis*

Identification and purification of allergens is crucial to the understanding of IgE-mediated disease. Immunologic and structural studies with these purified allergens are the next steps in unraveling this process. Several allergens have been identified that stimulate IgE production and cause IgE-mediated disease in humans. In comparison with the body of work done to identify and purify inhaled allergens, significantly less work has been done on the food allergens.[1-3] Because peanuts are a relatively common and often fatal cause of food hypersensitivity reactions we chose to use this model to study IgE-mediated reactions.[4]

Abbreviations used

DBPCFC:	Double-blind, placebo-controlled, food challenge
SDS:	Sodium dodecyl sulfate
PAGE:	Polyacrylamide gel electrophoresis
NBT:	30 mg nitro blue tetrazolium in 70% dimethylformamide
BCIP:	15 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 70% dimethylformamide
TLIEF:	Thin-layer isoelectric focusing
ELISA:	Enzyme-linked immunosorbent assay
AD:	Atopic dermatitis
pI:	Isoelectric point
PBS:	Phosphate-buffered saline

Approximately 60% of children with severe atopic dermatitis (AD) have food hypersensitivity reactions.[5,6] The ability to document food hypersensitivity reactions by double-blind, placebo-controlled, food challenges (DBPCFCs) in this group has allowed appropriate scientific work to be done on the identification of the allergens causing disease.

With the sera of patients who had positive DBPCFCs to peanut, we were able to begin the process

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Received for publication July 16, 1991.
Revised July 17, 1992.
Accepted for publication July 28, 1992.
Supported by National Institute of Health grants R29AI26629-02 and RO1CA40406. Partial support provided by a grant from the Arkansas Science and Technology Authority and Ross Laboratories. Supported in part by the USDA/DOE/NSF Plant Science Centers program (DE-FG09-87-ER13810).
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1/1/41440

of identification and purification of the major peanut allergens. In our previous study we identified and partially purified *Ara h* I, a protein with a mean molecular weight of 63.5 kd and a mean isoelectric point (pI) of 4.55.[7] Identification of a second major peanut allergen, *Ara h* II, was accomplished by use of anion exchange column chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assays (ELISAs), thin-layer isoelectric focusing (TLIEF), two-dimensional gel immunoelectrophoresis, IgE-specific immunoblotting, carbohydrate analysis, amino acid analysis, and sequencing. This protein has a mean molecular weight of 17 kd and a mean pI of 5.2.

MATERIAL AND METHODS

Patients sensitive to peanuts

Approval for this study was obtained from the Human Use Committee at the University of Arkansas for Medical Sciences. Nine patients (mean age, 4.2 years) with AD and a positive immediate prick skin test to peanut had either a positive DBPCFC or a convincing history of peanut anaphylaxis (the allergic reaction was potentially life threatening, that is with laryngeal edema, severe wheezing, and/or hypotension) (7 patients had positive DBPCFCs). Details of the challenge procedure and interpretation have been previously discussed.[8] Five milliliters of venous blood were drawn from each patient, allowed to clot, and the serum was collected. An equal volume of serum from each donor was mixed to prepare a nine-person peanut-specific IgE antibody pool.

Crude peanut extract

Three commercial lots of southeastern runners *(Arachis hypogaea)*, medium grade, from the 1979 crop (North Carolina State University) were used in this study. The peanuts were stored in the freezer at −18° C until roasted. The three lots were combined in equal proportions and blended before defatting. The defatting process (defatted with hexane after roasting for 13 to 16 minutes at 163° to 177° C) was done in the laboratory of Dr. Clyde Young (North Carolina State University). The powdered crude peanut was extracted per the recommendations of Yunginger and Jones[9] in 1 mol/L NaCl to 20 mmol/L sodium phosphate (pH 7.0) with the addition of 8 mol/L urea for 4 hours at 4° C. The extract was isolated by centrifugation at 20,000 *g* for 60 minutes at 4° C. The total protein determination was done by the (BCA) method (Bio-Rad Laboratories, Richmond, Calif.).

Chromatography

Analytic and preparative anion-exchange chromatography was performed with the FPLC system (Pharmacia, Piscataway, N.J.). Anion-exchange chromatography used the PL-SAX column (anion exchange column, Polymer Laboratories, Amherst, Mass.). The crude peanut extract was dialyzed against 20 mmol/L Tris-*bis*-propane (pH 7.2) without urea and 40 mg loaded on the PL-SAX column. A stepwise salt gradient of 0 to 1.5 mol/L NaCl was applied. All fractions of each resolved peak were pooled, dialyzed, and lyophilized.

Dot blotting was done to determine which fractions from the anion exchange column chromatogram contained IgE-binding material. Two hundred microliters of each fraction were blotted with the Mini Blot apparatus (Schleicher and Schuell, Keene, N.H.) onto 0.45 μm nitrocellulose membranes (Bio-Rad). After the membranes were dried, the remaining active sites were blocked with 20 ml of blocking solution (0.5% gelatin with 0.001% thimerosal in 500 ml of phosphate-buffered saline [PBS]) for 1 hour. The procedure is then identical to the immunoblotting for IgE.

Electrophoresis and immunoblotting

The electrophoresis procedure is a modification of Sutton et al.[10, 11] SDS-PAGE was carried out with a 12.5% polyacrylamide separating gel and a stacking gel of 3%. Twenty microliters of a 1 mg/ml solution of each fraction was applied to each well. Replicate samples were applied for independent analysis. Electrophoresis was performed for 4 hours at 0.030 A per gel (E-C Apparatus Corp., St. Petersburg, Fla.) for the 14 cm by 12 cm gels, and for 1 hour at 175 V per gel for the 8 cm by 7.5 cm gels (Mini-Protean II system, Bio-Rad Laboratories). To assure proper protein separation and visualization, Coomassie brilliant blue (Sigma Chemical Co., St. Louis, Mo.) stains were done on gels. For detection of carbohydrate staining material, gels were stained with the modified PAS stain according to the method of Kapitany et al.[12]

Proteins were transferred from the separating gel to a nitrocellulose membrane in a transfer buffer (tris-glycine) with 10% SDS and 40% methanol.[13] The procedure was done in a transblot apparatus (Bio-Rad Laboratories) for 2 hours (0.150 A). An amido black stain (Bio-Rad Laboratories) was done to assure transfer of the protein.

After removal from the transblot apparatus, the nitrocellulose was placed in blocking solution overnight. The nitrocellulose blot was then washed three times with PBS (PBS with 0.05% Tween 20) and incubated with the pooled peanut-sensitive IgE serum (1:20 dilution) for 2 hours at 4° C with rocking. After washing again with PBS three times, alkaline phosphatase–conjugated goat antihuman IgE (1:1000 vol/vol of PBS, Bio-Rad Laboratories) was added and incubated at room temperature with rocking for 2 hours. After again washing with PBS three times, the blot was developed with 250 μl of 30 mg nitro blue tetrazolium in 70% dimethylformamide (NBT) (Bio-Rad Laboratories) and 250 μl of 15 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 70% dimethylformamide (BCIP) (Bio-Rad Laboratories) solutions in 25 ml carbonate buffer (0.2 mol/L, pH 9.8) at room temperature for 15 minutes. The reaction was then stopped by decanting the NBT/BCIP solution and incubating the nitrocellulose for 10 minutes with distilled water. The blot was air-dried for visual analysis.

ELISA for IgE

A biotin-avidin ELISA was developed to quantify IgE antipeanut protein antibodies with modifications from an assay previously published.[14] The upper two rows of a 96-

well microtiter plate (Gibco, Santa Clara, Calif.) were coated with 100 μl each of equal amounts (1 μg/ml) of antihuman IgE monoclonal antibodies, 7.12 and 4.15 (kindly provided by Dr. A. Saxon). The remainder of the plate was coated with one of the peanut products at a concentration of 1 μg/ml 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 was washed five times with rinse buffer (PBS, pH 7.4, containing 0.05% Tween 20; Sigma Chemicals Co.) immediately and between subsequent incubations. The upper two rows used a secondary standard reference to generate a curve for IgE, ranging from 0.05 to 25 ng/ml.

The serum pool and patient serum samples were diluted (1:20 vol/vol) and dispensed in duplicate in the lower portion of the plate. After incubation for 1 hour at 37° C and washing, biotinylated, affinity-purified goat antihuman IgE (KPL, Gaithersburg, Md.) (1:1000 vol/vol PBS) was added to all wells. Plates were incubated for 1 hour at 37° C, washed, and 100 μl horseradish peroxidase–avidin conjugate (Vector Laboratories, Burlingame, Calif.) added for 30 minutes. After washing, the plates were developed by the addition of a buffer containing O-phenylenediamine (Sigma Chemical Co.). The reaction was stopped by the addition of 100 μl 2-*N*-hydrochloric acid to each well, and absorbance was read at 492 nm (Titertek Multiscan, Flow Laboratories, McLean, Va.). The standard curve was plotted on log-logit scale by means of simple linear regression, and values for the pool and individual patient samples were read from the curve as "nanogram-equivalent units" per milliliter (nanogram per milliliter).[15, 16]

ELISA inhibition

A competitive ELISA inhibition was done with the FPLC fractions. One hundred microliters of pooled serum (1:20) from the patients with positive challenges was incubated with various concentrations of the FPLC protein fractions (0.00005 to 50 ng/ml) for 18 hours. The inhibited pooled serum was then used in the ELISA described above. The percent inhibition was calculated by taking the food-specific IgE value minus the incubated food-specific IgE value divided by the food-specific IgE value. This number is multiplied by 100 to get the percentage of inhibition.

Isoelectric focusing

The samples were focused with the LKB Multiphor system using LKB PAG plates, pH gradient 3.5 to 9.5 (LKB, Bromma, Sweden). Five microliters of sample (1 mg/ml) was applied, and an electric current of 200 V was applied for 30 minutes and then increased to 900 to 1200 V for 30 minutes. The gel was fixed and stained with Coomassie brilliant blue following the standard protocol (LKB).

Two-dimensional gel electrophoresis

The samples were run according to the method of O'Farrell et al.[17] The first dimension is an isoelectric focusing gel in glass tubing. After making the gel mixture the samples are loaded with overlay solution and 0.02 mol/L NaOH. The samples are run at 400 V for 12 hours and at 800 V for 1 hour. After removing the gel from the tube, the isoelectric focusing gel is equilibrated for 2 hours in SDS sample buffer. The second dimension is 14 cm by 12 cm, 12.5% polyacrylamide gel described in the electrophoresis section. The gels were stained with the pooled peanut-positive serum for IgE-specific bands as above.

Amino acid analysis, amino acid sequencing, and carbohydrate analysis

The 17 kd fraction was run on a 10% mini-gel (Bio-Rad Laboratories) in triglycine buffer and stained with Rapid Reversible Stain (Diversified Biotech, Newton Centre, Mass.). The two bands were cut separately from the gel and electroluted in tris-glycine SDS buffer. After lyophilization the bands were sequenced individually. Automated gas-phase sequencing was performed on an Applied Biosystems model 475A sequencing system (Dr. Bill Lewis, University of Wyoming, Laramie, Wyo.). Amino acid analysis was done with a Hitachi (Hitachi Instruments, Inc., Danbury, Conn.) HPLC L5000 LC controller with a C18 reverse-phase column.

The electroluted 17 kd fraction was analyzed for carbohydrate analysis (Dr. Russell Carlson, Complex Carbohydrate Research Center, University of Georgia, Athens, Ga.). Glycosyl composition analysis on these samples was performed by the preparation and analysis of trimethylsiyl methylglycosides.

RESULTS

Chromatography

Pilot experiments were conducted with the analytical Mono Q 5/5 (Pharmacia) anion exchange column to determine the optimal buffer system and salt gradient. Screening for IgE-specific peanut binding components was done by dot blotting of these fractions. Scale up and optimization was completed with the PL-SAX column (anion exchange), with a stepwise salt gradient (0 to 1.5 mol/L NaCl). This procedure separated the crude peanut extract into seven major peaks (Fig. 1). Preliminary dot blotting from this separation identified IgE-binding material in each peak (picture not shown). Multiple runs of this fractionation procedure were performed, and each isolated peak was pooled, dialyzed against 100 mmol/L NH_4HCO_3, and lyophilized.

Electrophoresis and immunoblotting

Initial SDS-PAGE and immunoblotting of the crude peanut extract revealed multiple fractions with several IgE-staining bands.[7] Aliquots of the seven lyophilized fractions from the anion exchange column were analyzed by SDS-PAGE (data not shown). Each fraction showed 2 to 5 Coomassie brilliant blue staining protein

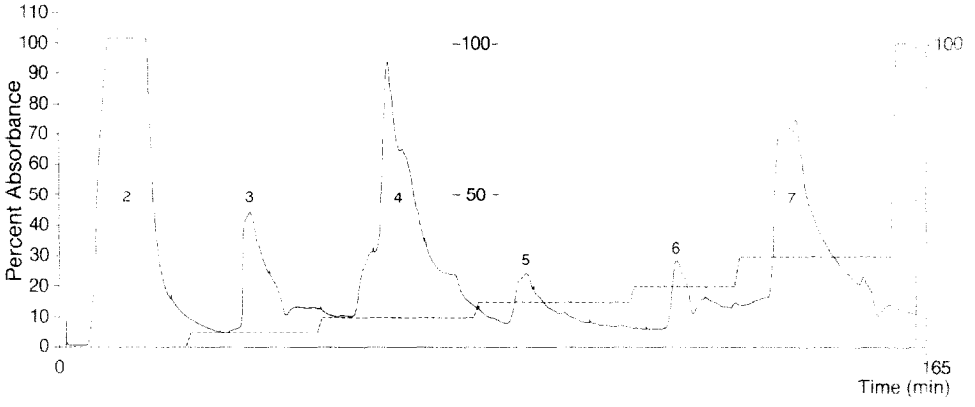

FIG. 1. Anion exchange chromatogram of the defatted crude peanut extract fractionated over the FPLC PL-SAX column. Elution pattern of proteins (A_{280}) shown by the *solid line.* Stepwise salt gradient of 0 to 1.5 mol/L NaCl shown by the *dotted line.* Fractions were pooled as numbered and applied to SDS-PAGE for analysis.

bands. Immunoblotting for specific IgE with the pooled serum revealed IgE-staining bands in each fraction. Fraction 4 showed two large, closely migrating, IgE-specific bands with a mean molecular weight of 17 kd (Fig. 2) (6% by weight of crude peanut extract).

ELISA and ELISA inhibition

ELISA results comparing the crude peanut extract with each isolated fraction are shown in Fig. 3. Fractions 1 through 7 all had IgE-binding from the peanut-positive serum pool. We tested individually the serum of six patients (members of pooled serum) to determine the relative IgE-binding material to both the crude peanut, fraction 4 (which contained the 17 kd component), and *Ara h* I (major component, 63.5 kd fraction). Each patient's serum had measurable amounts of peanut-specific IgE to each. Three of the patients had more peanut-specific IgE (nanogram/milliliter) to the 17 kd fraction than to the 63.5 kd fraction (Table I).

The ELISA inhibition results are shown in Fig. 4. The concentration of the 17 kd fraction required to produce 50% inhibition was 0.4 ng/ml compared with 0.1 ng/ml of the crude peanut extract.[18]

Two-dimensional gel electrophoresis

Because immunoblotting and ELISAs of the various anion exchange fractions suggested that fraction 4 appeared to contain a major allergen, isoelectric focusing was done on this fraction. The two bands in this allergen, which migrated closely together at a mean molecular weight of 17 kd on SDS-PAGE stained with Coomassie brilliant blue, had a pI of 5.2 (gel not shown). Fig. 5 shows the Coomassie-stained gel of the 17 kd fraction. One can see the protein divided

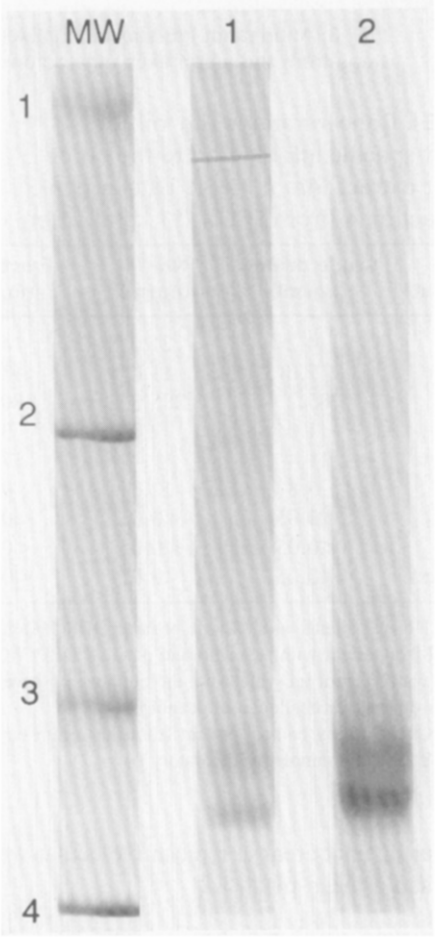

FIG. 2. SDS-PAGE (14 cm × 12 cm) analysis of the defatted crude peanut extract stained with Coomassie brilliant blue (lane 1) and immunoblotted for antipeanut specific IgE (lane 2) with pooled serum from patients with AD and positive DBPCFCs to peanut. Molecular weights: 1, 50,000; 2, 39,000; 3, 27,500; 4, 14,500.

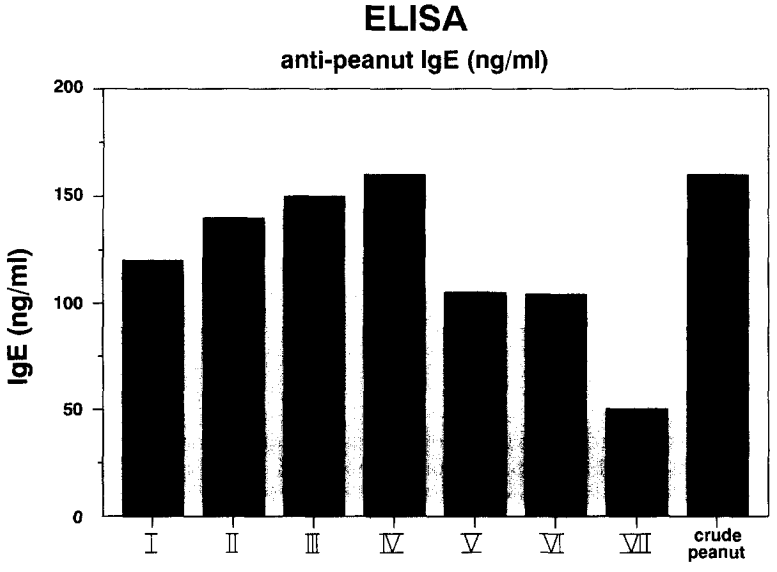

FIG. 3. Antipeanut IgE-specific ELISA (ng/ml) to defatted crude peanut extract and pooled fractions from the anion exchange column. Results are from the peanut-positive serum pool.

TABLE I. Concentrations (ng/ml) of peanut-specific IgE binding to the crude peanut extract, *Ara h* I (63.5 kd allergen), and fraction 4 (from FPLC) (17 kd allergen)

Patient	Crude peanut (ng/ml)	*Ara h* I (ng/ml)	Fraction 4 (ng/ml)
1	4.2	21.0	14.6
2	7.0	11.4	13.0
3	285.2	285.8	380.0
4	1.0	2.0	3.2
5	11.4	19.4	17.0
6	5.8	12.0	9.8
7	<0.05	<0.05	<0.05
8	<0.05	<0.05	<0.05
Normals	<0.05	<0.05	<0.05

Patients 1-6 are patients with AD and positive DBPCFCs to peanut.
Patient 7 is a patient with AD who had positive DBPCFC to milk and elevated serum IgE values but did not have positive skin test results or positive challenge to peanut (n = 2).
Patient 8 is a healthy control patient from the serum bank in the ACH Special Immunology Laboratory (n = 2).

into four distinct areas at a mean molecular weight of 17 kd and a mean pI of 5.2.

Amino acid analysis, amino acid sequencing, and carbohydrate analysis

Table II shows the complete amino acid analysis of the purified peanut fraction. The fraction was particularly rich in glutamic acid, aspartic acid, glycine, and arginine.

The amino acid sequence for both 17 kd bands is shown in Table III. The sequence for the second 17 kd band was essentially identical. Molecular weight discrepancies may be a result of carbohydrate composition in the two isoallergens. There were no known similar N-terminal sequences found in PIR, GENBANK, or SWISS-PROTEIN.

The 17 kd fraction was found to be 20% carbohydrate with significant levels of galacuronic acid, arabinose, and xylose (Table IV).

DISCUSSION

Individuals are genetically prone to produce IgE to specific antigens and to have allergic disease. No known features have been found to distinguish allergens as unique antigens.[19] The route of allergen administration, the dosage, the frequency of exposure, and genetic factors all determine the type and severity of the individual's allergic response. Three seemingly distinct foods account for approximately 80% of positive food challenges in children.[5-7] It is apparent that in addition to the fact that these foods are consumed frequently in the diet of children, other factors in either the allergen (food) itself or in the processing of these allergens cause these foods to be responsible for most food hypersensitivity reactions.

Most allergens are low molecular weight proteins or glycoproteins (5 to 50 kd).[20] Recent knowledge about the amino acid sequence of known allergens has not revealed any special features that would be associated with IgE antibody formation.[19] To first identify and then purify the allergens is crucial to a better

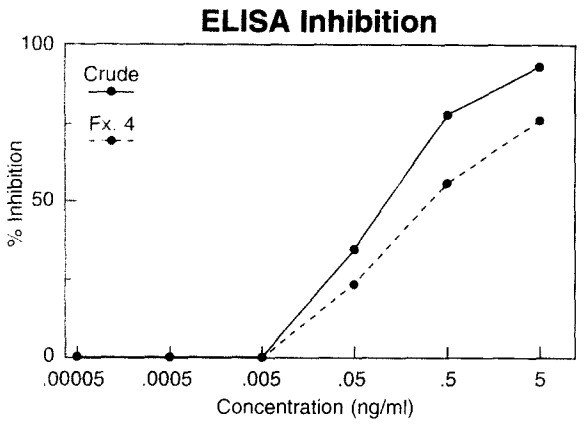

FIG. 4. IgE ELISA inhibition results of crude peanut extract and anion exchange fraction 4 in the ELISA for crude peanut.

understanding of the allergic response. Various biochemical techniques have been used to purify allergens from pollen, mite, and animal dander. These techniques include gel filtration, anion exchange chromatography, and isoelectric focusing.[19] Recent advances in chromatography, including the FPLC (Pharmacia) and immunoaffinity columns with monoclonal antibodies specific for the purified allergens, has allowed easier and faster identification and purification of allergens.

House dust mite, ragweed, and venom allergens are among those allergens that have been isolated and well characterized. Food allergens have been less studied. The food allergens that have been identified and characterized include *Gad c* I (cod), *Gal d* II (ovalbumin), and antigen I (shrimp).[1, 2]

Because of the prevalence and severity of adverse reactions to peanuts, several previous studies have examined the possibly relevant peanut allergens.[21-27] Multiple molecular weight peanut proteins have been identified from these various studies. Meier-Davis et al.[24] identified three major allergenic fractions, one of which had a molecular weight of 15 kd, which is close to the molecular weight of the allergen we have identified. No further identification or characterization of this protein is available.

In a previous study we identified *Ara h* I, a 63.5 kd allergen from peanuts with a pI of 4.55.[7] This allergen was similarly purified and identified with a combination of anion exchange chromatography, SDS-PAGE, ELISA, TLIEF, and ELISA inhibition. The allergen described in this report has two major bands, with an apparent mean molecular weight of 17 kd on SDS-PAGE and a mean pI of 5.2. This fraction bound specific antipeanut IgE from the peanut-positive pool in the ELISA and in the immunoblotting experiments. When used in the ELISA-inhibition studies, the 17 kd fraction significantly inhibited the IgE binding from the peanut-positive pool. In preliminary studies we have used the 17 kd allergen to inhibit binding from the pooled peanut-positive IgE serum to our previously described *Ara h* I. There does appear

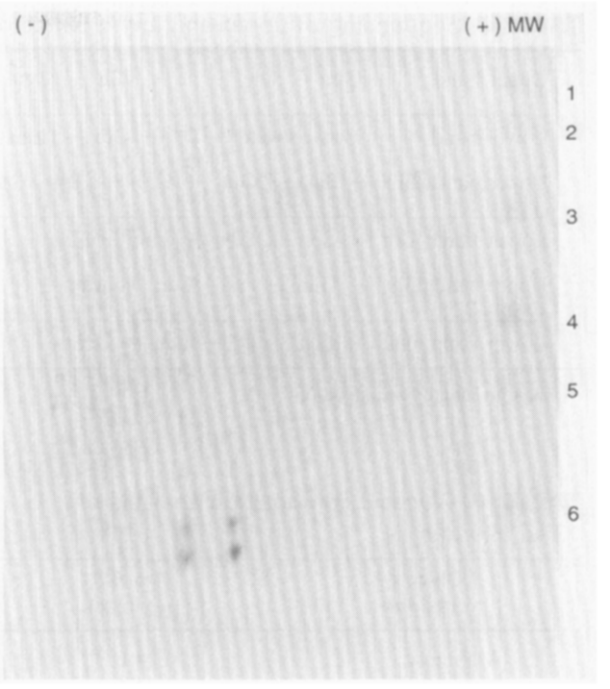

FIG. 5. Coomassie brilliant blue stain of two-dimensional gel with fraction 4 (anion exchange column) containing the 17 kd allergen. Molecular weights: 1, 112,000; 2, 75,000; 3, 50,000; 4, 39,000; 5, 27,500; 6, 17,000.

TABLE II. Amino acid analysis of *Ara h* II

Amino acid	Residues/molecule
Asp	12.2
Glu	24.8
Ser	9.8
His	1.3
Gly	11.3
Thr	2.2
Arg	10.8
Ala	5.4
Tyr	3.9
Met	2.7
Val	2.4
Phe	2.4
Ile	2.9
Leu	7.9

TABLE III. Sequencing of upper and lower bands of electroluted 17 kd peanut allergen

Upper band	* – Gln – Gln – * – Glu – Leu – Gln – * – Asp – * – * – *
Lower band	* – Gln – Gln – * – Glu – Leu – Gln – *Asp*– * – *Glu*– * – *
Upper band	Gln – Ser – Gln – Glu – Arg – Ala – Asp – Leu – Arg – Pro – (Gly)
Lower band	Gln – Ser – Gln – Leu – *Asp* – Ala – *Asn* – Leu – Arg – Pro – *Arg*
Upper band	Glu – Gln – * – Leu – Met – * – Lys – Ile
Lower band	Glu – Gln – * – Leu – Met – * – Lys – Ile

*Unable to identify amino acid.

TABLE IV. Glycosyl composition analysis of 17 kd allergen

Glycosyl residue	*Ara h* II (μg/total)
Arabinose	14.0
Rhamose	2.8
Fucose	0.58
Xylose	9.3
Mannose	2.5
Galactose	4.4
Glucose	5.0
Galacuronic acid	41.0
Galactosamine	ND

ND, None detected.

to be a moderate amount of inhibition of IgE binding to *Ara h* I produced by the 17 kd allergen. Amino acid sequencing of *Ara h* I will help to resolve the identity of similar epitopes for IgE between the unique allergens (investigation in progress).

According to recent recommendations by a recent international committee (IUIS) for proper identification of allergens we have designated this fraction *Ara h* II.[20] This fraction has been purified from a crude peanut extract from Florunner peanuts *(Arachis hypogaea)* by anion exchange chromatography. The fraction was identified as a major allergen by SDS-PAGE, ELISA, ELISA inhibition, TLIEF, amino acid analysis, and sequencing, carbohydrate analysis, and two-dimensional gels.

As we have previously speculated, *Ara h* II is likely to be the second of several major and minor allergens isolated from peanuts. The identification of the allergenic components in foods will allow new studies to elucidate more comprehensively the body's immune response to these allergens. Future work in this area will be directed toward molecular identification and characterization of both B- and T-cell epitopes.

We thank Dr. Jim Brooks of Ross Laboratories for helpful discussions concerning the protein purification schemes for peanut. We also thank Dr. Clyde Young of North State University (Department of Food Science) for suggestions and preparations of the crude peanut powder.

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