Identification of a major peanut allergen, *Ara h* I, in patients with atopic dermatitis and positive peanut challenges

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Peanuts are among the most common causes of immediate hypersensitivity reactions to foods. Serum from nine patients with atopic dermatitis and a positive double-blind, placebo-controlled, food challenge to peanut were used to begin the process of identification and purification of the major peanut allergens. Identification of a major peanut allergen was accomplished by use of anion-exchange column chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ELISA, thin-layer isoelectric focusing, and IgE-specific immunoblotting. Anion-exchange chromatography revealed several fractions that bound IgE from the serum of the challenge-positive patient pool. By measuring antipeanut-specific IgE in the ELISA and in IgE-specific immunoblotting, we identified an allergenic component with two Coomassie brilliant blue staining bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a mean molecular weight of 63.5 kd. Examination of this fraction by the IgE antipeanut ELISA with individual serum and by the ELISA-inhibition assay with pooled serum, we identified this fraction as a major allergen. Thin-layer isoelectric focusing and immunoblotting of this 63.5 kd fraction revealed it to have an isoelectric point of 4.55. Based on allergen nomenclature of the IUIS Subcommittee for Allergen Nomenclature, this allergen is designated, Ara h *I* (Arachis hypogaea). *(J ALLERGY CLIN IMMUNOL 1991;88:172-9.)*

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

In the past several years, multiple allergens have been identified that stimulate IgE production and cause IgE-mediated disease in man. Identification and purification of allergens is essential for various structural and immunologic studies to investigate why these molecules cause IgE Ab formation.[1] A significant body of work has been done to identify and purify inhaled allergens from a wide variety of sources, including pollens, dust mites, animal danders, insects, and fungi.[1] In comparison, very little work has been completed on food allergens that are known to cause IgE-mediated reactions.[2-4]

Abbreviations used

DBPCFC:	Double-blind, placebo-controlled, food challenge
SDS:	Sodium dodecyl sulfate
PAGE:	Polyacrylamide gel electrophoresis
TLIEF:	Thin-layer isoelectric focusing
AD:	Atopic dermatitis
pI:	Isoelectric point
CRIE:	Crossed radioimmunoelectrophoresis
Ab:	Antibody
IUIS:	International Union of Immunological Societies
FPLC:	Fast protein liquid chromatography
PAS:	Periodic acid–Schiff
MAb:	Monoclonal antibody
IEF:	Isoelectric focusing
Con A:	Concanavalin A
PBS:	Phosphate-buffered saline

During the past 10 years, emphasis has been placed on proper documentation of food-hypersensitivity reactions in children and adults. A large part of this work has been completed in children with AD.[5,6] After

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the identification of patients who are truly allergic to a specific protein, additional work could then be started on the identification of allergens in a particular food.

By use of the sera of patients with AD who had positive DBPCFCs to peanut, we were able to begin the process of identification and purification of the major peanut allergens. A major peanut allergen was identified by use of anion-exchange column chromatography, SDS-PAGE, ELISA, TLIEF, and IgE-specific immunoblotting. This allergen is a glycoprotein from a pure strain of Florunner peanuts that has a mean molecular weight of 63.5 kd and a mean pI of 4.55. After allergen nomenclature based on the IUIS Subcommittee for Allergen Nomenclature, we have designated this allergen, *Ara h* I *(Arachis hypogaea)*.[7]

MATERIAL AND METHODS

Peanut-sensitive patients

Approval for this study was obtained from the Human Research Advisory 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, laryngeal edema, severe wheezing and/or hypotension.) Details of the challenge procedure and interpretation have been discussed previously.[8] Five milliliters of venous blood was obtained from each patient and allowed to clot, and then the serum was collected. An equal volume of serum from each donor was mixed to prepare a nine-person, peanut-specific, IgE Ab pool.

Crude peanut extract

Three commercial lots of southeastern runners *(Arachis hypogaea)* (Florunner), 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 they were 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 according to the recommendations of Yunginger and Jones[9] in 1 mol/L of NaCl to 20 mmol/L of sodium phosphate (pH 7.0), with the addition of 8 mol/L of urea for 4 hours at 4° C. The extract was isolated by centrifugation at 20,000 *g* for 60 minutes at 4° C.

Chromatography

Analytic and preparative anion-exchange chromatography was performed with the FPLC system (Pharmacia, Piscataway, N.J.). Anion-exchange chromatography was used with the Mono Q 5/5 and 10/10 columns (Pharmacia). The crude peanut extract was dialyzed against 20 mmol/L of Tris-*bis*-propane (pH 7.2) and 8 mol/L of urea, and 40 mg was loaded onto the Mono Q 10/10 column. A stepwise salt gradient of 0 to 1.5 mol/L of 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. The collected fractions (200 μl) were blotted with the Mini Blot apparatus (Schleicher & Schuell Inc., Keene, N.H.) onto 0.45 micron nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.). After 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 PBS) for 1 hour. The procedure is then identical to the immunoblotting for IgE.

Electrophoresis and immunoblotting

The electrophoresis procedure[10] was a modification of the method of Sutton et al.[11] SDS-PAGE was performed with a 12.5% polyacrylamide separating gel and a stacking gel of 3%. Twenty microliters of a 1 mg/ml solution of each protein 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 and Zebrowski.[12]

Proteins were electrophorectically 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) (regular size transfer apparatus for crude peanut and minitransfer apparatus for fraction 3). 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 at 4° C. The nitrocellulose blot was then washed three times with PBS (PBS with 0.05% Tween 20) and incubated with the pooled serum (1:20 vol/vol dilution) for 2 hours at 4° C with rocking. After the nitrocellulose blot was again washed 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 an additional wash with PBS three times, the blot was developed with 250 μl of 30 mg of nitro blue tetrazolium in 70% dimethylformamide and 250 μl of 15 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 70% dimethylformamide (Bio-Rad Laboratories) solutions in 25 ml of carbonate buffer (0.2 mol/L, pH 9.8) at room temperature for 15 minutes. The reaction was then stopped by decanting the 30 mg of nitro blue tetrazolium in 70% dimethylformamide/15 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 70% dimethylformamide solution and incubating the nitrocellulose for 10 minutes with distilled water. The blot was then air-dried.

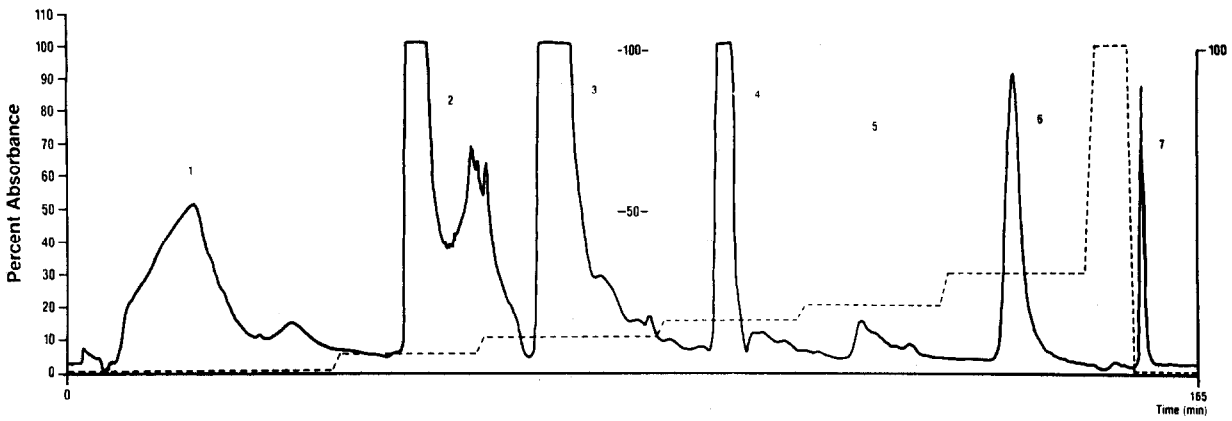

FIG. 1. Anion exchange chromatogram of the defatted crude peanut extract fractionated over the FPLC Mono Q 10/10. The elution pattern of proteins (A_{280}) is illustrated by the *solid line.* A stepwise salt gradient of 0 to 1.5 mol/L of NaCl is illustrated by the *dotted line.* Fractions were pooled as numbered (fraction 2 is divided into 2a and 2b) and applied to SDS-PAGE for analysis.

ELISA for IgE

A biotin-avidin ELISA was developed to quantify IgE antipeanut protein Abs 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 MAbs, 7.12 and 4.15 (kindly provided by Dr. A. Saxon) in coating buffer (0.1 mol/L of sodium carbonate-bicarbonate buffer, pH 9.5). The remainder of the plate was coated with one of the peanut extracts at a concentration of 1 μg/ml in coating buffer. 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 Chemical Co.) immediately and between subsequent incubations. In the upper two rows we used a secondary standard IgE reference to generate a curve for IgE, ranging from 0.05 to 25 ng/ml.

The serum pool and individual 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 a subsequent washing, biotinylated, affinity-purified, goat antihuman IgE (KPL, Gaithersburg, Md.) (1:1000 vol/vol of PBS) was added to all wells. Plates were incubated again for 1 hour at 37° C and washed, and 100 μl of horseradish peroxidase–avidin conjugate (Vector Laboratories, Burlingame, Calif.) was added for 30 minutes. After plates were washed, they were developed by the addition of a buffer containing o-phenylenediamine (Sigma Chemical Co.). The reaction was stopped by the addition of 100 μl of 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 a 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 analysis was done with the FPLC fractions. One hundred microliters of pooled serum (1:20 vol/vol) from the positive-challenge patients was incubated with various concentrations of the FPLC protein fractions (0.00005 to 50 ng/ml) for 18 hours at 4° C. 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.

IEF

The samples were focused with the LKB Multiphor system with LKB PAG plates, pH gradient, 3.5 to 6.85 (LKB, Bromma, Sweden). Five microliters of sample (100 μg of protein) 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). For IgE immunoblotting, the protein was transferred to nitrocellulose by capillary transfer[17] and stained as described in the immunoblotting section above.

RESULTS

Chromatography

Pilot experiments were conducted with the analytical Mono Q 5/5 anion-exchange column to determine the optimal buffer system and salt gradient. Scale up and optimization was completed with the Mono Q 10/10, with a stepwise salt gradient (0 to 1.5 mol/L of NaCl). This procedure separated the crude peanut extract into seven peaks (Fig. 1). Preliminary dot blotting from this separation identified IgE-binding ma-

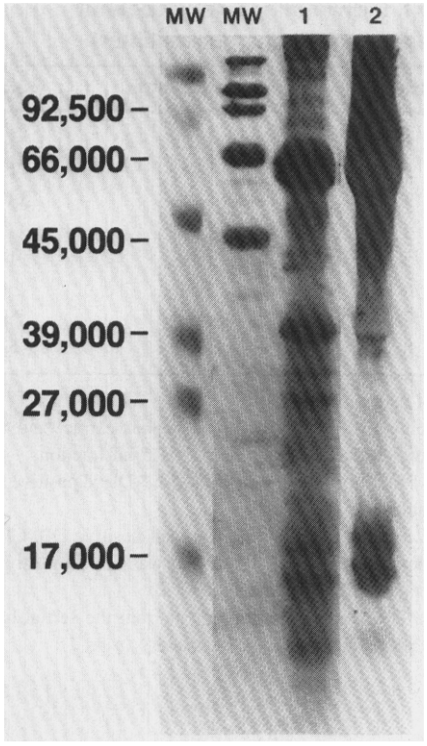

FIG. 2. SDS-PAGE (14 cm by 12 cm) analysis of the defatted crude peanut extract stained with Coomassie brilliant blue *(lane 1)* and immunoblotted for antipeanut-specific IgE *(lane 2)* with the pooled serum from the patients with AD and positive DBPCFCs to peanut; *MW,* molecular weight markers.

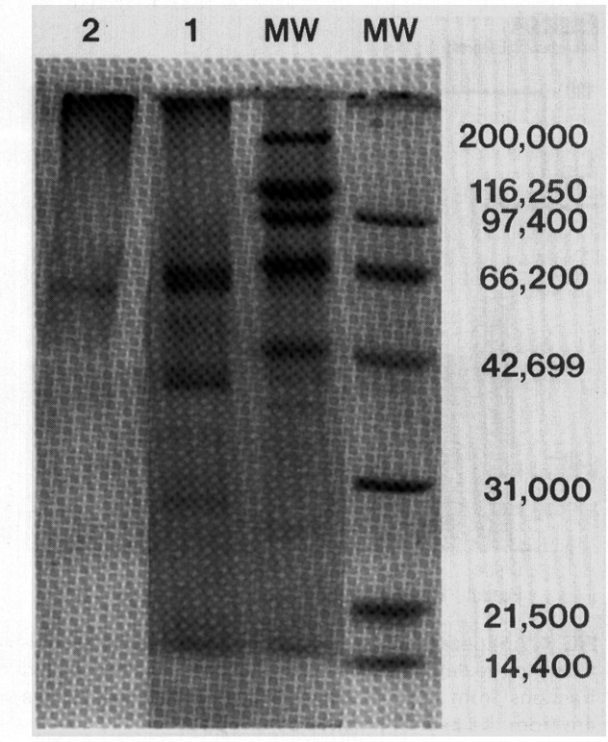

FIG. 3. SDS-PAGE (8 cm by 7.5 cm) analysis of the fraction 3 from the FPLC. The gel stained with Coomassie brilliant blue *(lane 1)* and the IgE-specific immunoblot *(lane 2)* with the pooled serum from the patients with AD and positive DBPCFCs to peanut are illustrated; *MW,* molecular weight markers.

terial in each peak (data not presented). Multiple runs of this fractionation procedure were performed, and each isolated peak was pooled, dialyzed against 100 mmol/L of NH_4HC0_3, and lyophilized. Preliminary separation of crude peanut extract with gel filtration (Superose) did not significantly enrich the purification process.

Electrophoresis and immunoblotting

Initial SDS-PAGE and immunoblotting of the crude peanut extract revealed multiple protein fractions with several IgE-staining bands (Fig. 2). Aliquots of the seven lyophilized fractions from the anion-exchange column were analyzed by SDS-PAGE (data not presented). Immunoblotting for specific IgE with the pooled serum revealed two closely migrating bands that bound significant IgE in Fig. 3. Preliminary blots with normal control serum and with serum from patients with elevated serum IgE values revealed no nonspecific binding to this fraction. The two bands in fraction 3 stained positive for PAS (data not presented). In addition, this fraction did not bind to Con A (after staining with biotinylated Con A and alkaline phosphatase–conjugated antibiotin) (data not presented).

ELISA and ELISA inhibition

ELISA results comparing the crude peanut extract to each isolated fraction are illustrated in Fig. 4. Mono Q 10/10 fractions 2a, 3, and 4 had significant amounts (>50 ng/ml) of IgE binding compared to the crude peanut extract. We additionally tested the serum of six patients (members of the pooled serum) to determine the relative IgE binding to both the crude and the enriched allergen fraction containing the 63.5 kd component (fraction 3). The results are presented in Table I. Each patient's serum had measurable amounts of peanut-specific IgE to both the crude extract and the 63.5 kd fraction. Serum from patients with AD, elevated serum IgE values, and positive DBPCFCs to milk (patient No. 7) and from healthy normal controls

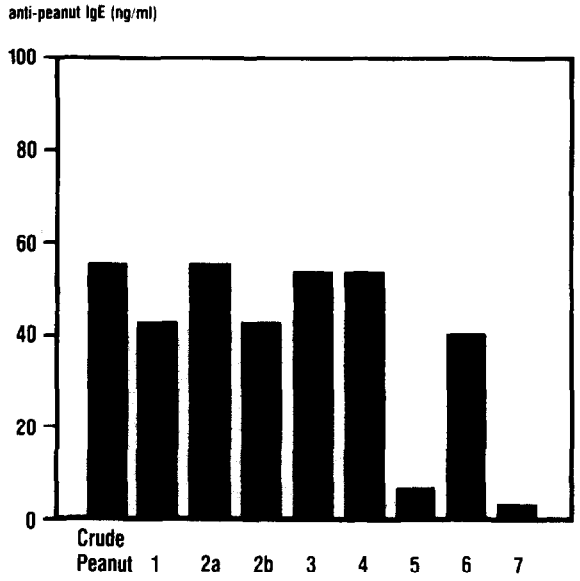

FIG. 4. Antipeanut IgE-specific ELISA (nanograms per milliliter) to the defatted crude peanut extract and the pooled fractions from the anion-exchange column. The results are from the peanut-positive serum pool.

TABLE I. Individual IgE Ab to peanut allergens (nanograms per milliliter)

Patient	Crude peanut	63.5 kd
1	4.21	4.6
2	7.0	13.0
3	285.2	380.0
4	1.0	3.2
5	11.4	17.0
6	5.8	9.8
7	ND	ND
8	ND	ND

ND, Not detectable.
IgE-specific ELISAs to the crude peanut extract and the anion-exchange fraction containing the 63.5 kd fraction.
Patients 1 to 6 are from the 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 was not skin test positive or challenge positive to peanut (n = 2).
Patient 8 is a healthy control patient from the serum bank in the ACH Special Immunology Laboratory (n = 2).

(patient No. 8) did not have detectable peanut-specific IgE to this allergen.

The ELISA-inhibition results are illustrated in Fig. 5. The concentration of the 63.5 kd fraction required to produce 50% inhibition was 5.5 ng/ml compared to 1.4 ng/ml of the crude peanut extract.[18] Control experiments with other food proteins did not demonstrate significant inhibition, demonstrating the specificity of the inhibition assay (data not presented).

TLIEF

Because immunoblotting and ELISAs of the various anion-exchange fractions demonstrated that fraction 3 contained a major allergen, IEF and immunoblotting were done on this fraction. In Fig. 6, the two bands can be observed in this allergen that migrated closely together at 63.5 kd on SDS-PAGE, stained with Coomassie brilliant blue, to have a mean pI of 4.55 (Fig. 6). This protein fraction readily binds IgE from the pooled serum (data not presented).

DISCUSSION

Allergens are usually believed to be proteins, glycoproteins, or carbohydrates of foreign animal or vegetable origin.[19] No unique features have been found to distinguish allergens as a subset of antigenic molecules.[20] Indeed, certain individuals are likely to be genetically more prone to develop significant IgE responses than other individuals and thus have atopic disease. Several factors regulate the development of specific IgE-mediated disease. The patient's genetic and other host-related factors, as well as the route of allergen administration, the dosage, and the frequency of exposure are all important in this process. From clinically related studies in children with challenge-positive food reactions, six foods account for 90% of the positive food challenges in children.[5, 6, 8] Therefore, some characteristics of the antigen/allergen must also be important in food hypersensitivity reactions.

More than 50 allergens have been purified from pollen, mite, animal dander, and other allergen extracts with biochemical techniques (gel filtration, anion-exchange chromatography, and IEF.[1]

Most allergens are low-molecular-weight proteins or glycoproteins (5 to 50 kd). The identification and purification of allergens is essential for developing an understanding of the allergic response. The recent advances in chromatography, including the FPLC (Pharmacia), and the use of MAbs to identify and purify proteins have greatly enhanced this process.

Allergens are a very diverse family of proteins and glycoproteins. Increased knowledge concerning the amino acid sequence of known and purified allergens has not yet revealed any special structural features that would be associated with IgE Ab formation.[1, 20]

Very few studies have been done on the purification of food allergens, compared with the isolation of mite, pollen, animal dander, and venom allergens. Aas[21] purified an allergen from cod (Allergen M, *Gad c* I)

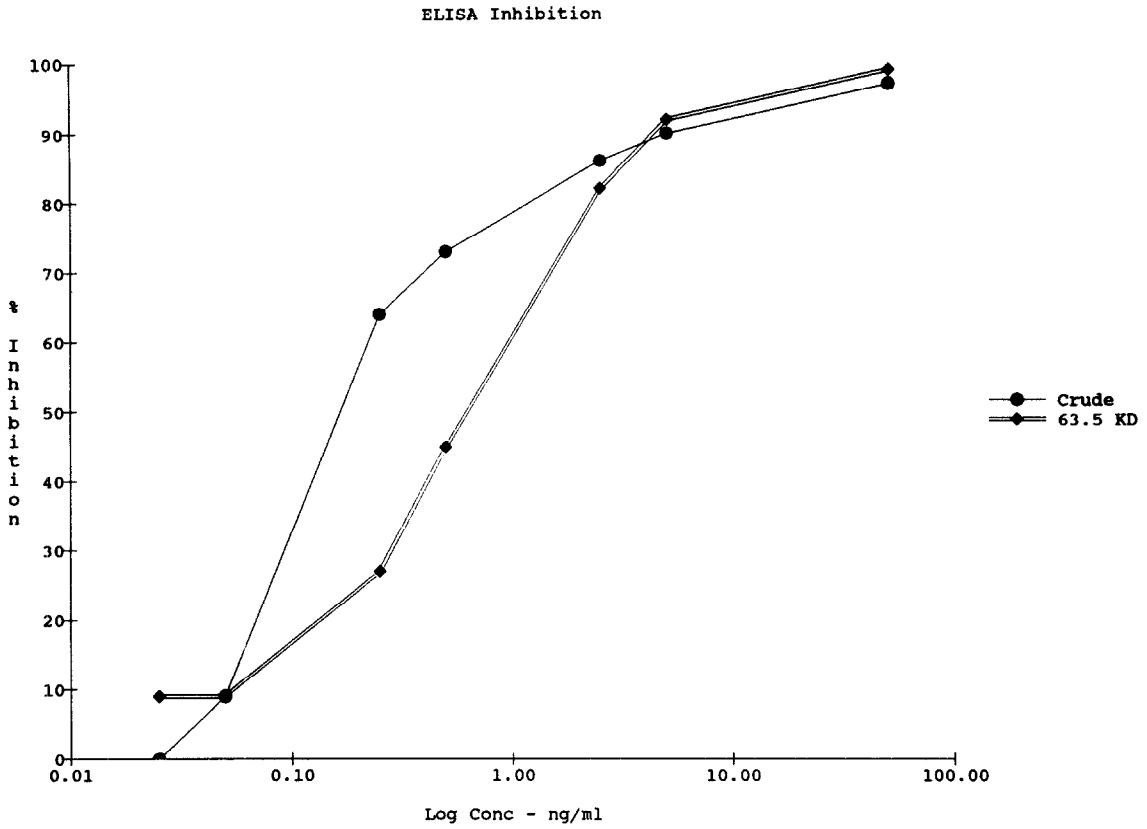

FIG. 5. IgE ELISA-inhibition results of crude peanut extract and anion-exchange fraction 3 (63.5 kd fraction) in ELISA for crude peanut.

and used this purified allergen for studies of patients with cod hypersensitivity as well as for biochemical studies. The immunologic reactivity of one region (amino acids, 41 to 64) of *Gad c* I is reported to be determined by three homologous tetrapeptides, repeated in three sites interspaced by six amino acids, in a segment of 24 residues.[22] *Gad c* I is also said to be relatively resistant to proteinases, heat, and denaturants. Other studies have examined ovomucoid (*Gal d* I), ovalbumin (*Gal d* II), and ovotransferrin (*Gal d* III) from chicken egg white.[23, 24] Hoffman et al.[25] have described two antigens in shrimp, antigen I and II, with molecular weights of 21 and 38 kd, respectively. Recently, Nagpal et al.[26, 27] identified two heat-stable allergens, *Sa* I and *Sa* II from shrimp, with molecular weights of 8.2 and 34 kd, respectively, and a shrimp-derived tRNA allergen.

Several previous studies have examined peanut proteins as potential allergens. Nordlee et al.[28] initially demonstrated peanut allergens in several different peanut products, including peanut butter syrup, peanut butter powder, and peanut flours with RAST inhibition. Heiner and Neucere[29] used direct RAST to demonstrate peanut allergens in different peanut components, including the cotyledons, axial germ tissue, and skin. Various immunologic techniques have been used to identify peanut allergens. Barnett et al.[30] used CRIE to identify 16 IgE-binding allergens in raw peanut. In 2-D electrophoresis, 32 antigens were identified in crude peanut extracts. By RAST and CRIE, sera from several of the 10 patients had IgE binding to arachin and conarachin I but not peanut agglutinin or phospholipase. In another work, Meier-Davis et al.[31] identified 37 antigens in a crude raw peanut extract by 2-D electrophoresis, of which 11 bound IgE. With SDS-PAGE and immunoblotting, they identified three major allergenic proteins that had molecular weights of 15, 20, and 66 kd. In a separate study, an Australian group[32] demonstrated by RAST inhibition that a Con A-reactive glycoprotein bound IgE in >50% of their patients. In CRIE, this glycoprotein migrated with arachin, but it is unknown as yet whether this protein is a subunit of arachin. Another peanut allergen with a high rate of skin test reactivity is a wheat germ lectin-reactive material.[31] By use of RAST inhibition, Taylor et al.[33] demonstrated that both arachin and conarachin had allergenic activity. The significance of the above studies is that there are multiple allergens in peanut extracts that will be identified and possibly purified.

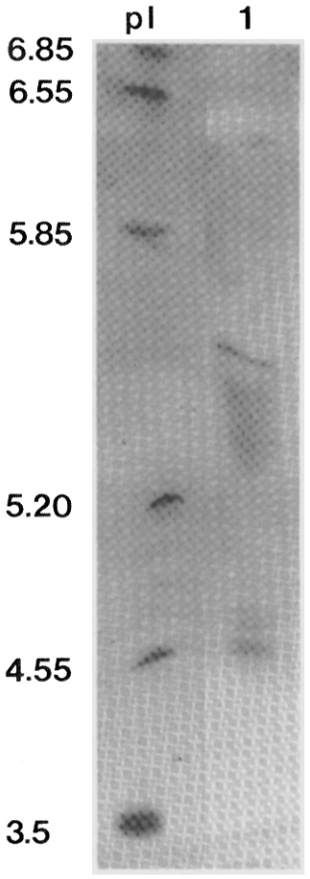

FIG. 6. Coomassie brilliant blue stain of TLIEF gel (pH 3.5 to 6.85) with fraction 3 (anion-exchange column) *(lane 1)* containing the 63.5 kd fraction; *pI*, standards.

There have been previous efforts to purify peanut allergens. Sachs et al.[34] identified a peanut allergen known as Peanut-1. Peanut was extracted in an NH_4HC0_3 buffer and, with anion-exchange chromatography and preparative PAGE, was purified to demonstrate that it contained two major bands with molecular weights of 20 and 30 kd. Peanut-1 contained 8.7% carbohydrate and 11% nitrogen content. On SDS-PAGE and IEF, several protein bands were evident, in addition to the 20 and 30 kd major bands. Peanut-1 was biologically active, as demonstrated by positive skin tests and leukocyte histamine-release assays in patients with peanut allergy. As evidenced by RAST-inhibition assays, this purified fraction did not account for all the allergenic activity in crude peanut extracts.

In our study, by preliminary IgE blotting, there appeared to be several fractions of the crude peanut extract that bound IgE. IgE-specific ELISA and immunoblotting of SDS-PAGE revealed two major allergenic bands migrating with an apparent mean molecular weight of 63.5 kd. This fraction, when it is used in the ELISA inhibition, significantly inhibited IgE binding to the crude peanut extract. By immunoblotting the fraction after IEF, the IgE-binding proteins had approximate pIs of 4.55. These proteins appear to be two distinct bands migrating together with apparent molecular weights of 63.5 kd and having a pI of 4.55. Work is underway to sequence the two distinct bands to determine their similarity. These protein bands are similar to the Con A-reactive glycoprotein in molecular weight described by Barnett et al.,[32] although, after our isolation procedure, this protein does not bind Con A. The protein described by Barnett et al.[32] had a molecular weight of 65 kd and a pI of 4.6 with a carbohydrate content of 2.4%.

Yunginger and Jones,[9] in a review of peanut chemistry and peanut allergens, suggested that, for standardization of peanut extracts, a mixture of peanut varieties, including the Virginia, Spanish, and runner types be used. We chose to use only one variety to begin the allergen identification process, and, in future studies, to incorporate comparisons of these three types of peanuts. With the different polypeptide composition known, it will not be surprising to find some differences in the allergen content.[35]

A recent international committee has been formed for recommendations concerning the proper identification of allergens (IUIS).[1, 7] This committee has put forth guidelines for appropriate nomenclature for identified and purified allergens. This process is important so that different investigators will have comparable protein products for experimental studies. Because these protein bands that bind IgE in pooled and individual sera from patients who have peanut hypersensitivity have been identified from multiple techniques, including SDS-PAGE, ELISA, ELISA inhibition, and TLIEF, we have designated this fraction *Ara h* I. *Ara h* I has a relative molecular weight of 63.5 kd and a pI of 4.55. By PAS staining, these protein bands are glycoproteins.

As evidenced by the multiplicity of previously identified peanut allergens, *Ara h* I is likely to be only the first of several major and minor allergens identified from peanut. The purification process will be greatly enhanced by the production of MAbs to this allergenic fraction. This work is currently underway. Identification and purification of food allergens is vitally important to study comprehensively the complex issues of digestion and absorption, to understand the immune response of the body to food allergens, and to begin to discuss further treatment and prevention of food hypersensitivity.

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