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Identification of Peanut Agglutinin and Soybean Trypsin Inhibitor as Minor Legume Allergens

Key Words

Food hypersensitivity
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Peanuts
Soybeans

Abstract

Peanuts and soybeans are frequent causes of food hypersensitivity reactions in children. Sera from 12 patients with atopic dermatitis and a positive double-blind placebo-controlled food challenge to peanut and sera from 5 patients with atopic dermatitis and a positive double-blind placebo-controlled food challenge to soybean were used to identify and characterize specific legume allergens. Identification of a minor allergen from peanut and a minor allergen from soybean was accomplished using various physicochemical techniques. The peanut fraction, peanut agglutinin, isolated by anion-exchange chromatography and electroelution and confirmed by amino acid sequencing, bound IgE in only 50% of the peanut challenge positive patients. The soybean fraction, soybean trypsin inhibitor, identified by gel filtration and electroelution and confirmed by amino acid sequencing, bound IgE in only 20% of the soy challenge positive patients. The identification of these two known legume proteins as minor allergens should allow further immunologic and structural investigations to compare the major and minor legume allergens.

Introduction

The identification and characterization of allergens is critical to our understanding of IgE-mediated disease. With the understanding of the major and minor allergens within a known allergen extract, we can better define the immunologic response to that allergen. Peanuts and soybeans are two members of the legume family which are among the five major foods that cause food hypersensitivity reactions in children [1]. Allergic reactions to peanuts, if documented early in life, tend to persist for a lifetime [2]. In comparison, allergic reactions to soybeans are often lost within the first

few years after the initial positive food challenge [3]. Several major allergens have been described in peanuts [4–6], while relatively little has been written about the soybean allergens [7–9]. Both peanuts and soybeans are being increasingly used as food extenders in various food products.

Approximately 60% of the children with severe atopic dermatitis have food hypersensitivity reactions [1, 10]. The ability to document food hypersensitivity reactions by double-blind placebo-controlled food challenges (DBPCFCs) in this group of patients has allowed appropriate scientific work to be done on the identification of the allergens causing disease.

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With the sera of patients who had positive DBPCFCs to both peanuts and soybeans, we were able to begin the process of identification and purification of the major and minor peanut and soybean allergens.

Materials and Methods

Patients

Approval for this study was obtained from the Human Use Advisory Committee at the University of Arkansas for Medical Sciences. Twelve patients with atopic dermatitis 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, i.e., with laryngeal edema, severe wheezing, and/or hypotension. Five patients with atopic dermatitis and a positive immediate prick skin test to soybean had a positive DBPCFC to soybean. Details of the challenge procedure and interpretation have been previously discussed [11]. Venous blood (5 ml) was drawn from each patient, allowed to clot, and the serum collected. An equal volume of serum from each donor was mixed to prepare either a peanut-specific IgE antibody pool or a soy-specific IgE antibody pool.

Crude Peanut Extract and Crude Soybean Extract

Three commercial lots of southeastern runners, peanuts (*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 (with hexane after roasting for 13–16 min at $163\text{--}177^{\circ}\text{C}$) 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 [12] in 1 mol/l NaCl to 20 mmol/l sodium phosphate (pH 7.0) with the addition of 8 mol/l urea for 4 h at 4°C . The extract was clarified by centrifugation at 20,000 g for 60 min at 4°C . The powdered crude soybean isolate (obtained from Dr. Jim Brooks at Ross Laboratories) was extracted in the same manner as peanut without the addition of urea. The total protein determination was done by the BCA method (Pierce Laboratories, Rockville, Md., USA).

Anion-Exchange and Gel Filtration Chromatography

Anion-exchange (Mono Q column) and gel filtration chromatography (Superdex 75 column) were performed with the FPLC system (Pharmacia, Piscataway, N.J., USA). The crude peanut extract was dialyzed against 20 mmol/l Tris-*bis*-propane (pH 7.2) without urea (to enhance for the specific peanut fraction) and 40 mg loaded on the Mono Q column. A stepwise NaCl gradient of 0–1.5 mol/l was applied. The crude soybean extract (40 mg) was loaded on the Superdex 75 column. All fractions of each resolved peak were pooled, dialyzed, and lyophilized.

Electroelution

The peanut protein peak from the FPLC which eluted at 15% NaCl was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mini gel (Bio-Rad) in Tris-glycine buffer and stained with Rapid Reversible stain (Diversified Biotech, Newton, Mass., USA). The soybean protein peak from the Superdex 75 which resolved as peak 2 was similarly treated. A 31-kD peanut

band and a 20-kD soybean band were excised from the gel and electroeluted in Tris-glycine SDS buffer. After dialyzing and lyophilizing, the protein fractions were analyzed for their amino acid sequence. For the peanut fraction, automated gas-phase sequencing was performed using an Applied Biosystems model 475A sequencing system (Dr. Bill Lewis, University of Wyoming, Laramie, Wyo., USA). For the soybean fraction, high-performance liquid chromatography identification of the resulting PTH amino acids was carried out on an Applied Biosystems sequencer equipped with on-line high-performance liquid chromatography systems (W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, Conn., USA).

Enzyme-Linked Immunosorbent Assay (ELISA) for IgE

A biotin-avidin ELISA was developed to quantify IgE antipeanut protein antibodies and antisoymbean antibodies with modifications of an assay previously described [13]. The upper two rows of a 96-well microtiter plate (Gibco, Santa Clara, Calif., USA) were coated with 100 μl each of equal amounts (1 $\mu\text{g}/\text{ml}$) of antihuman IgE monoclonal antibodies, 7.12 and 4.15 (kindly provided by Dr. A. Saxon). The remainder of the plate was coated with either the peanut protein or the soybean protein at a concentration of 1 $\mu\text{g}/\text{ml}$ in coating buffer (0.1 mol/l sodium carbonate-bicarbonate buffer, pH 9.6). The plate was incubated at 37°C for 1 h and then washed five times with rinse buffer (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20; Sigma Chemicals, St. Louis, Mo., USA) immediately and between subsequent incubations. A secondary IgE reference standard was added to the upper two rows 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 into individual wells in the lower portion of the plate. After incubation for 1 h at 37°C and washing, biotinylated, affinity-purified goat antihuman IgE (KPL, Gaithersburg, Md., USA; 1:1,000 vol/vol bovine serum albumin) was added to all wells. The plates were incubated for 1 h at 37°C , washed and 100 μl horseradish peroxidase-avidin conjugate (Vector Laboratories, Burlingame, Calif., USA) added for 5 min. After washing, the plates were developed by the addition of a citrate buffer containing *o*-phenylenediamine (Sigma). The reaction was stopped by addition of 100 μl 2 *N* hydrochloric acid to each well and the absorbance read at 490 nm (Bio-Rad microplate reader model 450). The standard curve was plotted on a log-logit scale by means of simple linear regression analysis, and values for the pool and individual samples were read from the curve as 'nanogram equivalent units' per milliliter (ng/ml) [5, 7].

ELISA Inhibition

A competitive ELISA inhibition was done with the electroeluted fraction. One hundred microliters of pooled serum (1:10) from the patients with positive challenges was incubated with the electroeluted fraction (0.0005–50 ng/ml) for 18 h. 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.

Electrophoresis and Immunoblotting

The electrophoresis procedure is a modification of the method of Sutton et al. [14]. SDS-PAGE was carried out with a 10% 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

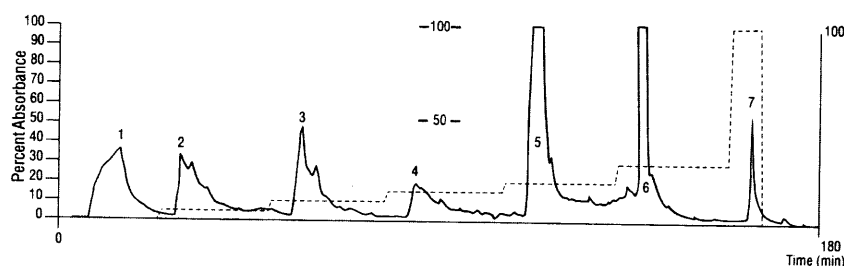


Fig. 1. Anion-exchange chromatography of crude peanut extract. The protein peak at 15% (peak 4) NaCl demonstrated intense IgE binding on SDS-PAGE immunoblot.

performed for 4 h at 0.03 A per gel (Hoefer Scientific Instruments, San Francisco, Calif., USA) for the 14×12 cm gels. To assure proper protein separation and visualization, Coomassie brilliant blue (Sigma) stains were done on the gels.

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

After removal from the transblot apparatus, the nitrocellulose was placed in blocking solution (5% milk fat) for 2 h. The nitrocellulose was then washed three times (phosphate-buffered saline with 0.05% Tween 20) and incubated with the pooled peanut-sensitive IgE serum or the pooled soybean-sensitive IgE serum (1:20 vol/vol) overnight with rocking. After washing again with phosphate-buffered saline three times, alkaline-phosphatase-conjugated goat antihuman IgE (1:1,000 vol/vol of gelatin diluent, KPL) was added and incubated at room temperature with rocking for 2 h. After again washing with phosphate-buffered saline three times, the blot was developed with 250 µl of 30 mg nitroblue tetrazolium in 70% dimethylformamide (NBT) and 250 µl of 15 mg 5-bromo-4-chloro-3-indolyl-phosphate in 70% dimethylformamide (BCIP; Bio-Rad) solutions in 25 ml carbonate buffer (0.2 mol/l, pH 9.8) at room temperature for 15 min. The reaction was then stopped by decanting the NBT/BCIP solution and incubating the nitrocellulose for 10 min with distilled water. The blot was air-dried for visual analysis.

Results

Peanut Allergen

Chromatography. Scale-up and optimization for the separation of the crude peanut material were completed with the Mono Q anion-exchange column with a stepwise salt gradient (fig. 1). Multiple runs of this fractionation procedure were performed, and each isolated peak was pooled and dialyzed against 100 mmol/l NH_4HCO_3 . A protein peak (OD_{280}) which eluted at 15% NaCl demonstrated intense IgE binding on SDS-PAGE immunoblot analysis.

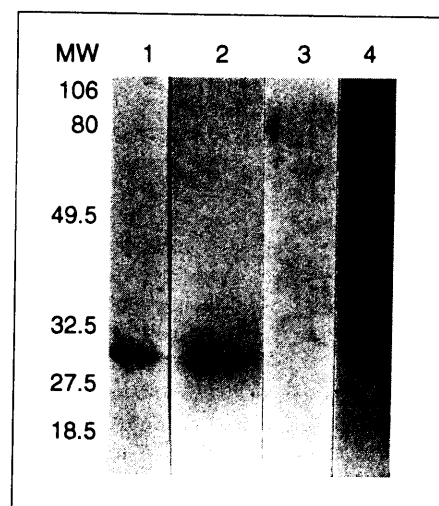


Fig. 2. SDS-PAGE (molecular weight standards; lane 1 = 31-kD peanut protein) and IgE immunoblot (lane 2) with peanut-specific serum. The controls with serum from a patient with elevated serum IgE (lane 3) and a patient without peanut hypersensitivity and a normal serum IgE level (lane 4) are shown.

Electrophoresis and Immunoblotting. Aliquots of the lyophilized fractions from the anion-exchange column were analyzed by SDS-PAGE (data shown). The protein peak which eluted at 15% NaCl had one major and several minor protein bands. The protein peak at 31 kD was then excised from the gel (fig. 2) and electroeluted for IgE immunoblotting. Immunoblotting for specific IgE with the pooled peanut-specific serum revealed a strong IgE-staining band, while pooled serum from patients with elevated serum IgE levels and pooled serum from normal laboratory volunteers revealed no IgE-staining band (fig. 2).

Table 1. Peanut-specific IgE binding to the 31-kD peanut fraction

Patient	31-kD peanut fraction ng/ml
1	0.48
2	32.07
3	0.78
4	<0.05
5	3.02
6	<0.05
7	<0.05
8	1.2
9	<0.05
10	<0.5
11	13.85
12	<0.05
13 ^a	<0.05
14 ^b	<0.05
Pooled peanut challenge positive serum	3.49

^a Patients (n = 2) with elevated serum IgE but not peanut challenge positive.
^b Patients (n = 2) with normal serum IgE and not peanut challenge positive.

ELISA and ELISA Inhibition. We tested individually the sera of all 12 patients to determine the amount of IgE binding to the electroeluted 31-kD peanut fraction. Six of the patient's (50% of the peanut-positive patients) had measurable amounts (0.4–32.07 ng/ml) of peanut-specific IgE to this protein (table 1; 95% confidence interval 0.211–0.789). The ELISA inhibition results comparing the IgE-inhibition of the 31-kD fraction and *Ara h* II (major peanut allergen, 17 kD) against crude peanut are shown in figure 3. As can be seen, a 1,000-fold greater concentration of this 31-kD peanut fraction could not produce 50% inhibition.

Amino Acid Sequencing. The amino acid sequence of the 31-kD electroeluted peanut product revealed 100% homology with the first 32 amino acids at the amino terminal with peanut agglutinin [15]. To further ensure that the electroeluted product was peanut agglutinin, we obtained commercially available peanut agglutinin (Sigma). In the ELISA assay we coated in separate wells the electroeluted fraction and the commercially available peanut agglutinin at concentrations ranging from 0.01 to 10 ng/ml. The curves of IgE binding from the pooled peanut-specific IgE antibody pool were identical (fig. 4).

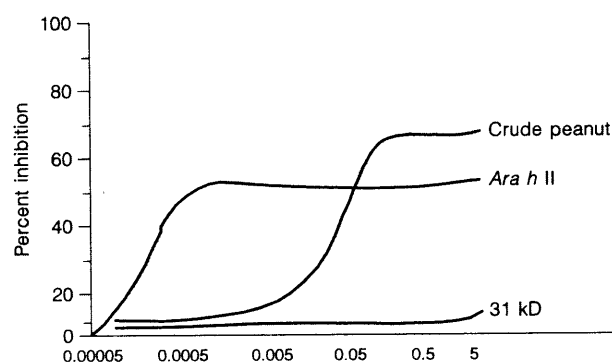


Fig. 3. ELISA inhibition to crude peanut comparing the 31-kD peanut fraction, *Ara h* II, and crude peanut.

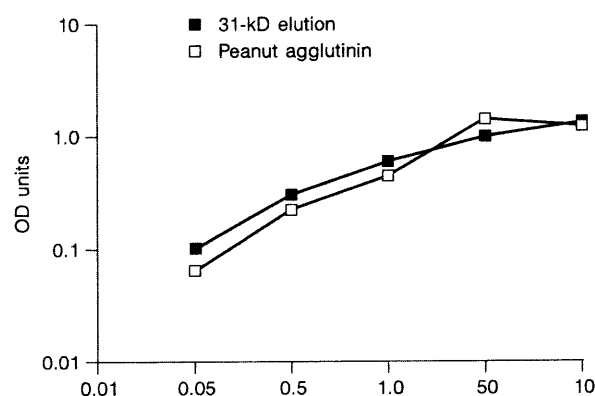


Fig. 4. IgE-specific ELISA comparing commercially available peanut agglutinin and the electroeluted 31-kD peanut fraction.

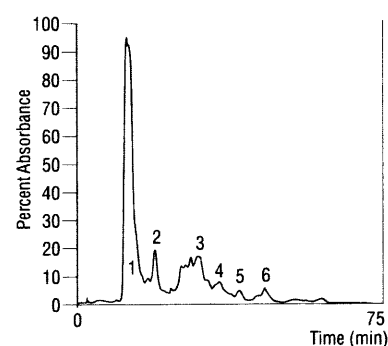


Fig. 5. Gel filtration chromatography of crude soybean extract. Peak 2 contained the 20-kD soybean protein.

Soybean Allergen

Chromatography. Scaleup and optimization for the separation of the crude soybean material were completed with the Superdex 75 column. Multiple runs of this fractionation procedure were performed, and each isolated peak was pooled, dialyzed against 100 mmol/l NH_4HCO_3 and lyophilized (fig. 5).

Electrophoresis and Immunoblotting. Aliquots of the fractions from the Superdex 75 column were run on preliminary 10% SDS-PAGE gels. The fraction which eluted in peak 2 had one major and several minor proteins bands. The protein migrating at 20 kD was excised from the gel and electroeluted and then electrophoresed for immunoblotting. Immunoblotting for specific IgE was done with the soybean-positive serum pool which revealed specific IgE binding to the 20-kD protein (fig. 6). Serum pools from patients with elevated serum IgE levels and from normal controls did not bind to this protein band (fig. 6).

ELISA and ELISA Inhibition. Five patients with positive DBPCFCs to soybean were tested individually to determine the amount of IgE binding to the electroeluted 20 kD soybean fraction. Only 1 of the 5 patients and the soybean-specific IgE serum pool had a measurable amount (0.3 ng/ml) of soybean-specific IgE to this fraction (table 2; 95% confidence interval 0.005–0.716). The ELISA inhibition with the 20-kD soybean fraction inhibiting IgE binding to the crude soybean extract did not rise above 10% inhibition, despite using 1,000-fold greater concentrations of the 20-kD soybean fraction (data not shown).

Amino Acid Sequencing. The amino acid sequence of the first 30 amino acids of the 20-kD soybean fraction revealed 100% homology with soybean trypsin inhibitors A and C (Yale University, W.M. Keck Foundation Biotechnology Resource Laboratory).

Discussion

Individuals are genetically prone to produce IgE to specific allergens and to have allergic disease. To date no distinguishing features have been found to delineate allergens as unique antigens [16]. The route of allergen administration, the dosage, the frequency of exposure, and genetic factors all determine type and severity of the individual's allergic response. Three foods, milk, eggs, and peanuts, account for approximately 80% of positive food challenges [10]. If soybeans and wheat are added, this list accounts for approximately 90% of the positive food challenges [3, 10]. Most allergens are low molecular weight proteins or glycoproteins (5–50 kD) [17]. Recent knowledge about the

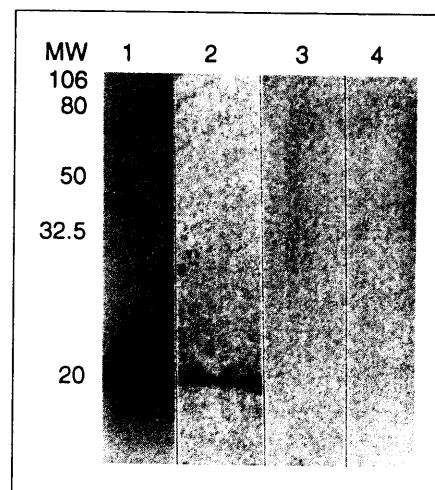


Fig. 6. SDS-PAGE (molecular weight standards; lane 1 = 20-kD soybean protein) and IgE-specific immunoblot (lane 2) with soybean-specific serum. The controls with a patient with elevated serum IgE (lane 3) and a patient without soybean hypersensitivity and a normal serum IgE level (lane 4) are shown.

Table 2. Soybean-specific IgE binding to the 20-kD soybean fraction

Patient	20-kD soybean fraction ng/ml
1	<0.05
2	<0.05
3	0.15
4	<0.05
5	<0.05
6 ^a	<0.05
7 ^b	<0.05

^a Patients (n = 2) with elevated serum IgE but not soybean challenge positive.

^b Patients (n = 2) with normal serum IgE and not soybean challenge positive.

amino acid sequence of known allergens has not revealed any special features that would be associated with IgE antibody formation [16].

Allergen purification is essential to the better understanding of the allergic response. Techniques utilized to purify allergens include gel filtration, anion-exchange chromatography, isoelectric focusing, and immunoaffinity co-

lums [16]. Recent work with recombinant allergens has allowed further characterization of the immune response to certain allergens [18]. House dust mite, ragweed, and venom allergens are among those which have been extensively characterized. Food allergens have been less studied. One of the first food allergens described and still one of the best characterized is *Gad c I* from codfish [19].

Because of the prevalence and severity of reactions to peanuts and the prevalence of reactions to soybeans, several studies have examined the possible major and minor allergens in each of these legumes [4, 20, 21]. A concanavalin A reactive glycoprotein was described as one of the major allergens in peanuts with a molecular weight of approximately 65 kD [22]. Our group has described two major peanut allergens with each of our patients who had positive DBPCFCs to peanut having specific IgE to these purified proteins. The molecular weight of the first allergen, *Ara h I*, is 63.5 kD [5], and the molecular weight of the second allergen, *Ara h II*, is 17 kD [6]. Both of these proteins have acidic pI values. Commercially available peanut agglutinin was utilized in one study to compare the IgE-binding capability of it to other peanut protein fractions, including the concanavalin A reactive glycoprotein [4]. These authors only found 2 patients from among their group of 10 patients who had specific IgE to this fraction. Despite this finding, there has continued to be speculation that patients will have nonspecific IgE binding to peanut agglutinin. Our data presented clearly show that there is not general nonspecific binding of IgE to the peanut agglutinin.

The comparative work in identifying the soybean allergens has been much less. In 2 patients with severe reactions to soybeans, soybean trypsin inhibitor was identified as the protein to which these patients made specific IgE [23]. Many of the purification schemes to separate soybeans into their component proteins have utilized sedimentation coefficients to identify the soybean fractions. Shibaski et al. [7] found the 2S fraction to bind IgE in the greatest number of patients. They did find IgE binding to the 7S, 11S, and 15S soybean fractions. In utilizing our patients with atopic dermatitis and positive DBPCFCs to soybean, in a previous study [8] we found no significant differences in the amount of specific IgE to either the 2S, 7S, 11S, or 15S soybean fractions. Recent work in patients with exacerbations of asthma secondary to exposure to soybean allergens has identified the first major allergen, *Gly m I*, a 14-kD protein [9].

Our findings in this study that only a minority of our challenge-positive patients have specific IgE to peanut agglutinin and soybean trypsin inhibitor indicate that these allergens are minor allergens as compared with the known major peanut and soybean allergens. With the sequence information on these two proteins and purified material available, future immunologic and structural studies will be able to compare the major and minor allergens from each legume. Since both allergens are from the same botanical family, and because the natural history of the food sensitivity for each legume is significantly different, these studies may shed important information on either structure or sequence necessary to bind IgE. Future work with these legumes will be directed toward the characterization of the B and T cell epitopes on these allergens.

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