
Identification of the basic subunit of Ara h 3 as the major allergen in a group of children allergic to peanuts

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Background: Several proteins have been identified as peanut allergens; among them, Ara h 1 (7S globulin) and Ara h 2 (2S globulin) are usually considered the major allergens.

Objective: To identify the major allergens in a group of children selected for their specific pattern of immunoreactivity.

Methods: We identified the dominant allergen by using (1) amino acid sequencing of the bands that show the strongest IgE immunoreactivity in 1-dimensional electrophoresis and immunoblotting and (2) specific animal IgGs raised against the dominant immunoreactive band to pinpoint the allergen(s) in peanut proteins separated by 2-dimensional electrophoresis and immunoblotting. To confirm these data, we further examined the peanut proteome using serum samples from the children with the unusual immunoreactivity.

Results: We found a group of children with marked peanut allergy who are specifically sensitized to the basic subunit of Ara h 3 (11S globulin family).

Conclusion: That the dominant immunoreactivity in these patients is in a basic subunit of Ara h 3 was unexpected, because previous studies had indicated that Ara h 3 was only a minor peanut allergen and that the identified allergenic epitopes occurred mainly in the acidic Ara h 3 subunit.

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INTRODUCTION

Peanut allergy is one of the most dangerous food hypersensitivities because of its severity, prevalence, and lifetime duration. Epidemiologic studies^{1–4} indicate a prevalence of 0.6% to 1% in the general population in the United States and the European Union. Although there are no reliable data on the prevalence and incidence of fatal and near-fatal anaphylactic reactions, peanut is considered to account for 10% to 47% (depending on geographic area) of immediate reactions.^{5–7} Several proteins have been identified as peanut allergens (Ara h 1 to Ara h 8); among them, Ara h 1 (7S globulin) and Ara h 2 (2S globulin) are usually considered the major allergens because they are recognized by 70% to 90% of sensitized individuals.^{8,9} Ara h 3 (11S globulin family), a glycinin protein, is considered a minor peanut allergen because only 45% of patients with allergy show specific IgE in their sera.¹⁰ Because the sera of patients with allergy generally contain IgEs specific to more than 1 peanut protein, correlation of a specific allergen with clinical symptoms is difficult.

The aim of this research was to identify the major allergens in a group of children with immediate allergic reactions to peanut matter who presented to the Macedonio Melloni Hospital emergency department between January 1, 2001, and December 31, 2002. Serologic characterization of these children's sera provided information about the specificity and sensitivity of the in vitro diagnostic tests and might allow correlations between allergens and clinical symptoms; the identification of specific epitopic sequences could provide the basis of analytical methods to detect "hidden" allergens in commercial foods.

MATERIALS AND METHODS

Allergens and Antibodies

Raw and roasted peanuts (*Arachis hypogaea* L) were of the variety Israel and were kindly supplied by Vincenzo Brandolini, University of Ferrara, Ferrara, Italy. The band corresponding to Ara h 3 basic subunit, with a molecular weight (MW) of approximately 23 kDa, was excised with a scalpel from 1-dimensional gels stained with Coomassie blue. The preparation was divided into 2 parts: 1 part underwent N-terminal amino acid sequencing (see the "Image Analysis and Automatic Determination of the N-terminal Amino Acid Sequences" subsection), and the other part was used for antibody production. Rabbit antiserum to Ara h 3 basic subunit was prepared using conventional immunization procedures. The immunogen (the excised Ara h 3 band) was combined with the complete Freund adjuvant and injected subcutane-

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ously into 2 New Zealand rabbits; after 4 further boosters and a screening of sensitization by enzyme-linked immunosorbent assay, the total bleeding of the animal allowed recovery of approximately 40 mL of blood per animal, used to prepare the antibody solution.

Children With Allergy

Serum samples were examined from 16 children who arrived at the emergency department between January 1, 2001, and December 31, 2002, with acute allergic symptoms after ingestion of peanuts or peanut derivatives (mainly cookies). All the children had positive skin prick test results, performed using a panel of commercial reagents supplied by Stallergenes (Paris, France). Wheal diameters were read through a plastic caliper disk scaled in tenths of millimeters under ($\times 4$) magnification; a wheal with a diameter at least 3 mm larger than that of the positive control (histamine) was considered a positive response.

Specific circulating IgE determinations were performed using the Pharmacia CAP System (Pharmacia Diagnostics AB, Uppsala, Sweden); allergen specific IgE titers of 0.35 IU/mL or greater was considered positive. The children were not given an oral challenge because of the high risk of anaphylaxis. The 5 children (3 boys and 2 girls aged 25 months to 13 years 8 months) selected for this study had a specific pattern of immunoreactivity. Informed consent was obtained from the parents of all children participating in this study.

1-Dimensional Electrophoresis (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis)

Raw and roasted peanuts were ground to a meal. The resultant flour was suspended in diethyl ether to remove the lipid fraction, and after 2 hours at 4°C the supernatant was eliminated. This treatment was repeated 3 times. The defatted peanut flours were suspended in sample buffer-water (1:1 vol/vol) at a final concentration of 10 mg/mL, and 15 μ L of each sample was loaded onto the gel. The sample buffer contained 0.25M Tris hydrochloride buffer (pH 6.8), 7.5% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% β -mercaptoethanol. All chemicals and instruments used in the electrophoretic analysis were purchased from Bio-Rad Laboratories (Segrate, Italy).

The running gel was composed of 15% acrylamide, 0.14% bis-acrylamide, 0.36M Tris hydrochloride buffer (pH 8.8), 35% glycerol, 0.1% SDS, 0.02% ammonium persulfate, and 0.15% TEMED (N,N,N',N'-tetramethylethylenediamine). Bromophenol blue was added to the gel as a run marker. Stacking gel was composed of 3.5% acrylamide, 0.09% bis-acrylamide, 0.125M Tris hydrochloride buffer (pH 6.8), 0.1% SDS, 0.02% ammonium persulfate, and 0.15% TEMED. The running buffer was composed of 25mM Tris, 0.19M glycine, and 0.1% SDS (weight per volume) (pH 8.8).

After the electrophoretic run (90 V at room temperature for approximately 6 hours), gels were dyed with Coomassie brilliant blue G-250.¹¹ Prestained molecular weight marker

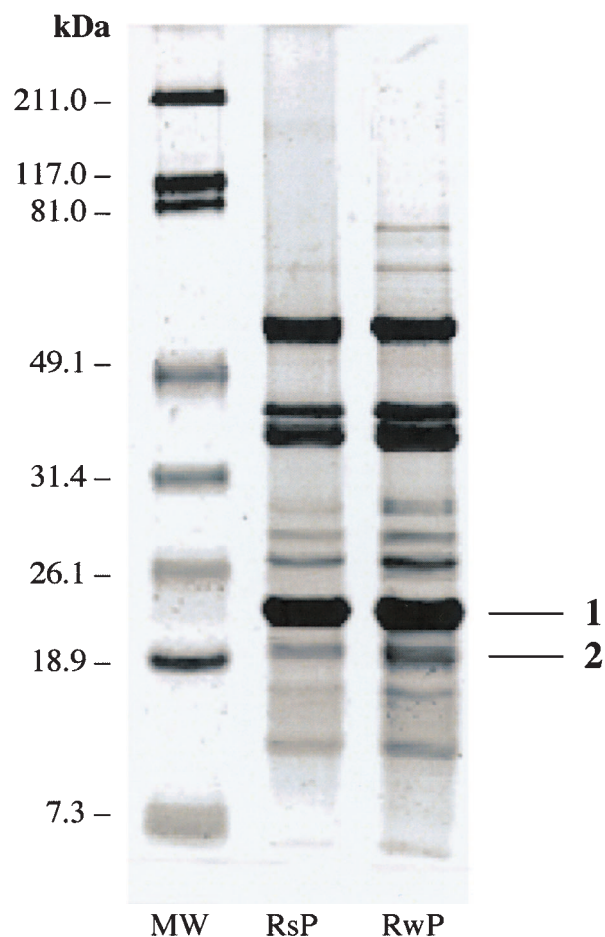


Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of raw (RwP) and roasted (RsP) peanuts. The numbers on the right indicate the bands analyzed for their N-terminal amino acidic sequences and discussed in this article (Table 1). MW indicates prestained molecular weight markers

solution (broad range, Bio-Rad) contained myosin (211 kDa), β -galactosidase (117 kDa), bovine serum albumin (81.0 kDa), ovalbumin (49.1 kDa), carbonic anhydrase (31.4 kDa), soybean trypsin inhibitor (26.1 kDa), lysozyme (18.9 kDa), and aprotinin (7.3 kDa).

2-Dimensional Electrophoresis

The defatted peanut flour was extracted using 25mM Tris hydrochloride buffer (pH 8.2) in a ratio of 1:30 wt/vol, with stirring for 2 hours at 4°C. The pH was monitored regularly and was adjusted to the original value as needed. The alkaline pH of the extraction buffer guaranteed the complete solubilization of the acidic storage globulins, as confirmed by the 2-dimensional electrophoretic pattern. The slurry was centrifuged at 10,000g for 30 minutes, and the pellet was extracted again after the same procedure but with a ratio of 1:5 wt/vol. The 2 supernatants were combined before analysis. The extracted proteins were analyzed immediately or were kept frozen at -80°C until used.

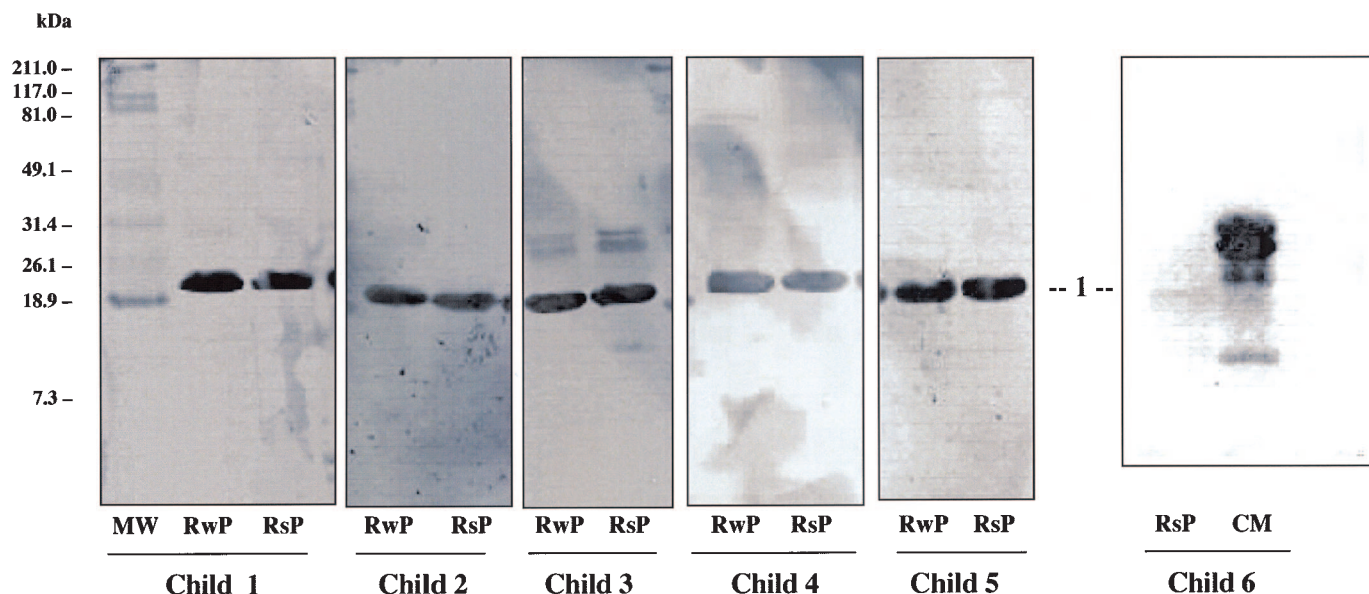


Figure 2. Immunoblottings obtained after the transfer of samples shown in Figure 1 and the incubation of membranes in the serum of the 5 children allergic to peanuts included in this study. Child 6, used as negative control, is allergic to cow's milk (CM). MW indicates prestained molecular weight markers; RwP, raw peanuts; RsP, roasted peanuts.

Isoelectric focusing of peanut samples was performed using 7-cm (pH 3–10) nonlinear immobilized pH gradient strips (Amersham Biosciences, Cologno Monzese, Italy). The strips were rehydrated overnight in a solution consisting of 7M urea, 2M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 65mM 1,4-dithiothreitol, and 0.5% Pharmalytes 3 to 10 (Amersham Biosciences) containing 70 μ g of the protein sample. Strips were focused at 20°C, 6,500 V/h at a maximum of 3,000 V using the Multiphor II electrophoresis unit (Amersham Pharmacia Biotech). Before the second dimension, strips were incubated for 15 minutes in equilibration buffer (6M urea, 2% SDS, 20% glycerol, and 375mM Tris hydrochloride [pH 8.8]) with 65mM dithiothreitol followed by 243mM iodoacetamide. The separation was performed in 12% SDS–polyacrylamide gel electrophoresis gels, according to the method of Laemmli,¹² using a Mini-PROTEAN 3 cell (Bio-Rad Laboratories). The gels were stained with Coomassie blue.¹¹

Immunoblotting

One- and 2-dimensional gels were transferred to polyvinylidene fluoride membranes (Millipore, Vimodrone, Italy) by Western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad Laboratories). The membranes were blocked with 1% gelatin and were washed 3 times with 0.25% gelatin solution (in 150mM sodium chloride, 5mM EDTA, 50mM Tris, and 0.05% Triton-X) to prevent nonspecific adsorption of the immunologic reagents. Membrane was then immersed in 10 mL of 0.25% gelatin solution containing 10 μ L of rabbit anti-Ara h 3 polyclonal antibodies or 300 μ L of serum from allergic children.

Antigen-IgG or antigen-IgE complexes were detected using 10 μ L of mouse anti-rabbit IgG monoclonal antibodies or

20 μ L of goat anti-IgE polyclonal antibodies labeled with alkaline phosphatase (Sigma-Aldrich Corp, St Louis, MO). Finally, after incubation in the bromochloroindolyl phosphate–nitroblue tetrazolium solution, an intense black-purple precipitate developed at the site of enzyme binding. The developing solution contained 15% bromochloroindolyl phosphate and 30% nitroblue tetrazolium in alkaline phosphatase buffer (100mM Tris, 100mM sodium chloride, and 5mM magnesium chloride [pH 9.5]).

Image Analysis and Automatic Determination of the N-terminal Amino Acid Sequences

Gels and membranes were analyzed using a gel scanner and 1- and 2-dimensional image software (Image Master; Amersham Pharmacia). Gels prepared for sequencing were stained as previously described, and protein bands were cut using a surgical scalpel. Amino acid sequences were determined using a Perkin-Elmer Applied Biosystems 492 pulse liquid-phase sequencer. Phenylthiohydantoin-derivative amino acids were identified using reverse-phase high-performance liquid chromatography (Perkin-Elmer Corp, Wellesley, MA).

RESULTS

Figure 1 illustrates the electrophoretic profile of raw and toasted peanuts coming from the same batch of the variety Israel, and Figure 2 shows the corresponding results of immunoblotting obtained using serum samples from 5 children allergic to peanut. In Figure 1, raw and toasted peanuts showed similar protein patterns, with some difference in high-MW proteins; raw peanuts yield 2 bands, absent from toasted peanuts, with electrophoretic runs between those of the MW standards of 81.0 and 49.1 kDa. Both separations, as

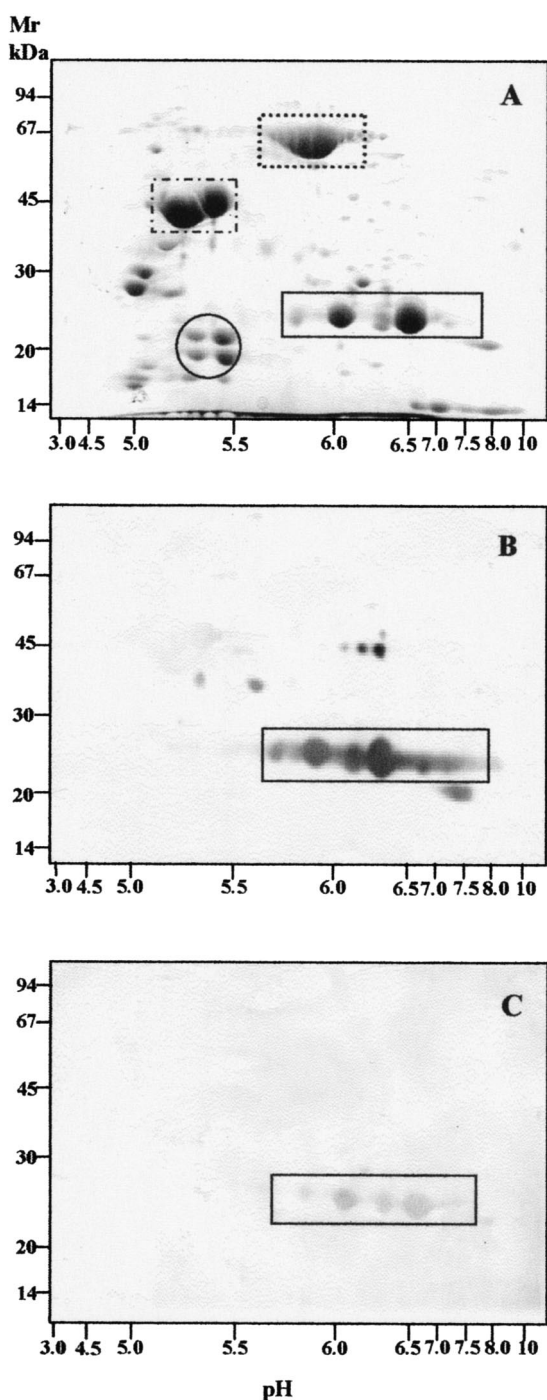


Figure 3. Two-dimensional electrophoretic maps of peanut protein extracts, approximately 70 μ g (A), and immunoblotting analyses with anti-Ara h 3 basic subunit animal antibodies (B) and a patient's serum (C). The solid rectangles enclose the basic subunits of Ara h 3; the dash-dotted rectangle, the acidic subunits of Ara h 3; the dotted rectangle, Ara h 1 polypeptides, according to Burks et al.¹⁵; and the solid circle, Ara h 2 polypeptides, according to Koppelman et al.¹³ MR, relative molecular mass.

Table 1. N-terminal Amino Acid Sequencing and Bioinformatic Identification of Polypeptide Bands 1 and 2 in Figure 1

Band No.	N-terminal amino acid sequence	Accession No. (NCBI)	Protein type
1	GIEETIXTASVKKN	AAG01363	Gly 1
		AAD47382	Glycinin
		AAC63045	Glycinin
		AAM46958	Allergen Ara h 3/Ara h 4
		AAR02860	Storage protein
2	REQXELEGGDRRX	AA061750	Seed storage protein
		AAN77576	Allergen Ara h 2.02
		AAK96887	Allergen II
		AAM78596	Allergen Ara h 2 isoform

Abbreviation: NCBI, National Center for Biotechnology Information.

well as the 2-dimensional one shown in Figure 3, are representative of peanut protein patterns, where the major storage globulins and allergens, including Ara h 1, Ara h 2, and Ara h 3, are clearly identified on the basis of their size, isoelectric points (pI), and sequencing. The same peanut samples were incubated with the 16 serum samples from allergic individuals (data not shown); most of them (15/16, 95%) presented IgEs specific for band 1 (Fig 1), and in 5 cases (Fig 2), this polypeptide was the only significant allergen detected in immunoblotting. There was no difference in immunologic responses between raw and roasted peanuts, and the high-MW bands present only in raw peanuts did not react with the serum of sensitized individuals. To verify the specificity of immunoreactivity to band 1, immunoblotting was performed under identical conditions using a serum sample from a child highly allergic to milk (Fig 2, child 6); no positive reaction was shown with any bands in the peanut electrophoretic profile.

Because band 1 seemed to be the dominant, if not the only, allergen in 5 of the children, we studied this band in greater detail. We sequenced the amino acids in the major bands in the area showing the strongest IgE reactivity, and we screened the peanut proteome using specific rabbit IgGs raised against band 1. The N-terminal amino acid sequences of bands 1 and 2 in Figure 1 are reported in Table 1. Several matches for both sequences were found in the National Center for Biotechnology Information database for *Arachis hypogaea* proteins. Band 1 corresponded to variants of glycinin, the 11S globulin family in peanuts to which Ara h 3 belongs. Band 2 corresponded to 4 isoforms of Ara h 2 in the 2S globulins of peanuts.

To confirm our finding and identify the 11S globulin subunit responsible for the reactivity, we investigated further the spots reacting with specific animal IgGs raised to band 1 of Figure 1, using 2-dimensional electrophoresis. As Figure 3B shows, the animal IgG raised against band 1 reacted strongly with a series of spots having a MW of approximately 23 kDa and pI ranging from 5.8 to 7.2. From the size and pI of these bands and comparison with a Coomassie blue-stained 2-dimensional electrophoretic map of peanut proteins

(Fig 3A), the reactive spots were unequivocally identified as the basic subunits of Ara h 3. These were also the sole spots reacting with the patients' circulating IgEs (Fig 3C). The IgG to band 1 also reacted slightly with Ara h 3 acidic subunits, whereas the serum of the individual in Figure 3C reacted neither to these spots nor to Ara h 1 or Ara h 2. Similar results were obtained using serum samples from the other 4 children allergic to peanuts.

DISCUSSION

Our results showed that the major (15 patients) or unique (5 of these patients) peanut allergen for the 16 children included in this study was the basic subunit of Ara h 3. This finding was unexpected because only Ara h 1 and Ara h 2 are usually considered major allergens; furthermore, studies on the epitopic sequences responsible for allergic reactions to peanut identified epitopes mainly in the acidic Ara h 3 subunits.¹⁰ Nevertheless, more recent studies^{13,14} reconsidering the relative importance of individual peanut allergens have shown that Ara h 3 basic subunits more than the acidic ones may act as allergenic peptides.

Considering all children allergic to peanut included in this study ($n = 16$), Ara h 3 resulted in a peanut major allergen (95% reactivities); it was not possible to find a specific clinical pattern for the 5 children who reacted only to Ara h 3 compared with those who also reacted to Ara h 1 or Ara h 2. On the other hand, some differences can be found in their pattern of sensitization: (1) they were not allergic to milk and eggs, whereas in the group of 11 children who reacted to Ara h 3 and other allergens (Ara h 1, Ara h 2, or both), 7 and 6 were allergic to milk and eggs, respectively; and (2) all were sensitized to nuts (mainly hazelnut).

The different roles of each specific peanut allergen are likely to arise from geographic distribution of the populations, especially when European and North American populations are compared. Although the dominance of one allergen cannot be established on the basis of a small study population, the present results address the epitopic analysis of peanut and other legume allergens to previously neglected target molecules.

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