

Structure of the Major Peanut Allergen Ara h 1 May Protect IgE-Binding Epitopes from Degradation¹

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In the past decade, there has been an increase in allergic reactions to peanut proteins, sometimes resulting in fatal anaphylaxis. The development of improved methods for diagnosis and treatment of peanut allergies requires a better understanding of the structure of the allergens. Ara h 1, a major peanut allergen belonging to the vicilin family of seed storage proteins, is recognized by serum IgE from >90% of peanut-allergic patients. In this communication, Ara h 1 was shown to form a highly stable homotrimer. Hydrophobic interactions were determined to be the main molecular force holding monomers together. A molecular model of the Ara h 1 trimer was constructed to view the stabilizing hydrophobic residues in the three dimensional structure. Hydrophobic amino acids that contribute to trimer formation are at the distal ends of the three dimensional structure where monomer-monomer contacts occur. Coincidentally, the majority of the IgE-binding epitopes are also located in this region, suggesting that they may be protected from digestion by the monomer-monomer contacts. On incubation of Ara h 1 with digestive enzymes, various protease-resistant fragments containing IgE-binding sites were identified. The highly stable nature of the Ara h 1 trimer, the presence of digestion resistant fragments, and the strategic location of the IgE-binding epitopes indicate that the quaternary structure of a protein may play a significant role in overall allergenicity. *The Journal of Immunology*, 2000, 164: 5844–5849.

Legume seed storage proteins constitute the third largest source of dietary protein on Earth. They are of particular importance as a nutritional source in developing countries that lack ample supplies of animal protein (1). Peanuts are widely used for the preparation of a variety of foods in the U.S. and are also relied on as a protein extender in developing countries. There has been an increase in the observed incidence of peanut allergies in children over the last 10 years. This is thought to be due to the increased popularity and use of peanut products by the population in the last decade and the introduction of peanut products to children's diets at an early age (2–5). Thus, it is increasingly common for the public to be exposed to an abundantly utilized and often disguised food such as peanuts. This has led to increasing rates of sensitization, accidental ingestion, anaphylaxis, and even death in peanut-allergic patients.

There are a number of characteristics that increases the capacity of a food allergen to provoke a dangerous systemic allergic reaction. These include its ability to stimulate high titers of IgE and to resist gastrointestinal degradation sufficiently to produce fragments

containing multiple IgE binding epitopes. The more degraded an allergen becomes, the more fragments are produced that contain single IgE-binding epitopes. Protein fragments containing single IgE-binding sites are incapable of cross-linking IgE-bound FcεR1 receptors and therefore of causing mast cell degranulation. Thus, the biochemical and structural aspects of allergens play a critical role in the disease process.

The general biochemical characteristics of most food allergens indicate that they are low m.w. glycoproteins (<70 kDa) with acidic isoelectric points that are highly abundant in food. These proteins are usually resistant to proteases, heat, and denaturants, allowing them to resist degradation during food preparation and digestion (6, 7). Several studies have shown that the most allergenic portion of the peanut is the protein fraction of the cotyledon (8–10). The peanut allergen Ara h 1 is a vicilin-like seed storage protein found in the cotyledon. This protein is one of the main storage proteins of the seed and is utilized as a nitrogen and amino acid source during development of a new peanut plant. In addition to its importance to the developing plant, Ara h 1 is recognized by serum IgE from >90% of peanut-sensitive patients, thus establishing it as an important allergen in the etiology of this disease (11, 12). The linear IgE-binding epitopes of this allergen have been mapped and shown to consist of 23 independent binding sites (13). Individual patients with IgE Abs to Ara h 1 have been shown to have IgE that recognizes multiple epitopes on the Ara h 1 protein (13). These sites are evenly distributed along the linear sequence of the molecule. However, a molecular model of the tertiary structure of the Ara h 1 protein shows that the IgE-binding sites were clustered into two main regions. In addition, Ara h 1 forms homotrimers, a physical characteristic that may be important in establishing it as an allergen (14).

Using fluorescence anisotropy, we have shown that the formation of a highly stable Ara h 1 homotrimer is mediated primarily through hydrophobic interactions. A molecular model of the Ara h 1 trimer indicates that hydrophobic residues on α-helical bundles

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Received for publication November 18, 1999. Accepted for publication March 10, 2000.

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¹ This work was supported by National Institutes of Health Grant RO1-AI133596 and a Clarissa Sosin Allergy Research Foundation grant. R.A.K. was supported by a sabbatical leave grant from the Department of Chemistry, Hendrix College, Conway, AR. C.-W.P. was supported by a sabbatical leave grant from the Department of Dermatology, Hallym University, Seoul, Korea.

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located on the ends of each monomer contribute to the stability of the structure. The IgE-binding epitopes are clustered in the regions of monomer-monomer contact. The importance of the trimer structure to the overall allergenicity of the molecule was investigated using assays designed to determine the stability of Ara h 1 to digestion with proteases. In this report, when Ara h 1 was incubated with digestive enzymes, various protease-resistant fragments containing IgE-binding sites were obtained, indicating that the quaternary structure of Ara h 1 may play an important role in protecting these epitopes from digestive enzymes.

Materials and Methods

Purification of peanut allergen Ara h 1

Twenty grams of crude peanut extract were added to 500 ml extraction buffer (50 mM Tris-Cl (pH 8.3), 5 mM DTT, 1 mM EDTA, 1 mM PMSF) containing 200 mM NaCl. In this report, the extraction buffer contains the chemicals indicated within the parentheses, but the salt concentration is varied as indicated for different experiments. The solution was stirred gently at room temperature, cleared by centrifugation at $13,000 \times g$ for 30 min at 4°C, and subjected to ammonium sulfate precipitation (15). Ammonium sulfate was added to 70% saturation. The remaining supernatant was then taken to 100% ammonium sulfate saturation and the Ara h 1 protein collected by centrifugation. The pellet was resolubilized in extraction buffer (pH 8.3) by sonication on ice at 40% power using a Heat Systems Disruptor (Fischer Scientific, Atlanta, GA). After sonication, the solubilized proteins were desalted on disposable PD-10 gel filtration columns (Pharmacia Biotech, Piscataway, NJ) and loaded onto a High Prep S, cation exchange resin column (2.5×12 cm, Bio-Rad Laboratories, Hercules, CA). A linear salt gradient (200–800 mM NaCl) was used to elute Ara h 1 from the column and 2.5-ml fractions were assayed for Ara h 1 content by a 12% SDS-polyacrylamide gel (Novex, San Diego, CA) and Coomassie Brilliant Blue staining. Fractions containing Ara h 1 were pooled and desalted into desired buffers on PD-10 columns just before use in all experiments. The desired buffers used were identical with extraction buffer containing 100 mM NaCl with varying pH values (pH 8.3 for tryptic and chymotryptic digestion reactions and pH 2 for pepsin digestion for the digestion reactions). Protein concentrations were monitored using the Bio-Rad protein assay reagent kit (Bio-Rad Laboratories). At each stage, samples were subject to electrophoresis on 12% SDS-polyacrylamide gels (Novex), and the purity of the Ara h 1 fractions was assessed by Coomassie staining and densitometry of the resulting protein bands. Purified Ara h 1 was stored in aliquots at -80°C .

Serum IgE

Serum from 15 patients with documented peanut hypersensitivity reactions (mean age, 25 yr) was used to identify Ara h 1 during purification. The patients had either a positive double-blind, placebo-controlled food challenge or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension (16). Equal aliquots of IgE-containing serum from 12 to 15 patients was pooled and used for our experiments. Each patient's serum contained IgE that recognized Ara h 1. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences.

Cross-linking reactions

Purified Ara h 1 was desalted into PBS, and two identical samples were diluted to a final concentration of 3 μM . The pH was adjusted with HCl to either pH 2 or pH 7.6, and the solution was allowed to incubate at room temperature for 1 h. After incubation, the Ara h 1 sample at pH 2 was adjusted to pH 7.6, and both samples were then subjected to cross-linking. The process of pH adjustment and cross-linking was minimized (<2 min) to prevent reequilibration of Ara h 1 monomers and trimers before cross-linking. The protein cross-linking reagent, dithiobis(succinimidylpropionate) (Pierce, Rockford, IL) was used. Dithiobis(succinimidylpropionate) was dissolved in *N,N*-dimethylformamide as a 74 mM stock solution. A 1- μl sample of cross-linker stock solution was added to 50 μl of the Ara h 1 samples. The reactions were quenched after 80 s by addition of SDS sample buffer (lacking DTT), heated to 100°C for 5 min, and subjected to SDS-PAGE. Limited cross-linking was performed to minimize the formation of nonspecific complexes.

Protease digestions

Purified Ara h 1 was desalted and diluted to a final concentration of 8 μM in extraction buffer containing 100 mM NaCl adjusted with HCl to pH 8.3

for tryptic and chymotryptic digestions and pH 2.0 for pepsin digestion (all proteases were purchased from Sigma (St. Louis, MO)). The diluted Ara h 1 was incubated in the presence of 0.5 $\mu\text{g}/\text{ml}$ of trypsin, chymotrypsin, or pepsin at 37°C , and aliquots were taken at 0 min, 10 s, and 1, 2, 4, 8, 15, 30, 60, 120, and 180 min. The digestion reaction in each aliquot was quenched by the addition of SDS-sample buffer. Samples were then subjected to SDS-PAGE and either stained or transferred to nitrocellulose for immunoblot analysis using pooled serum from peanut allergic individuals.

Immunoblot analysis

For the detection of Ara h 1 or IgE-binding fragments of Ara h 1, immunoblot analysis was performed using serum IgE from a 15-person pool of peanut-allergic individuals. SDS-PAGE (12%)-resolved proteins were transferred to nitrocellulose membrane (0.45 μm , Schleicher and Schuell, Keene, NH) electrophoretically. The membranes were blocked in Tris-buffered saline with Tween 20 (TBST) plus 1% BSA for 2 h at room temperature. The membrane was then washed in TBST and incubated with a 1:10 dilution of pooled human sera for 1 h. Detection of the bound IgE was accomplished using ^{125}I -labeled anti-human IgE secondary Ab (Sanofi, Chaska, MN) and subsequent exposure to x-ray film.

Fluorescein labeling

Ara h 1 was desalted into NaH_2PO_4 buffer, pH 8, and labeled with FITC according to the methods described by Fernando and Royer (17) for dansyl labeling. Briefly, the FITC was dissolved in *N,N*-dimethylformamide at 250 mg/ml to make the stock solution, and 10 μl of the solution were added to 1 ml of Ara h 1 solution. This mixture was then incubated at room temperature for 10 min. The free FITC was separated from the labeled protein using a desalting column (described above) that was pre-equilibrated in binding buffer (10 mM HEPES/KOH (pH 7.9), 1 mM EDTA, 1 mM DTT, 5% glycerol).

Fluorescence anisotropy measurements of Ara h 1

All fluorescence measurements were made using a Beacon fluorescence polarization spectrometer (Pan Vera, Madison, WI) with fixed excitation (490 nm) and emission (530 nm) wavelengths. Fluorescence measurements were done at room temperature (24°C) in previously described binding buffer containing different salt concentrations from 0 to 1.8 M NaCl, in a final volume of 1.1 ml. Fluorescence anisotropy is described in detail by Fernando and Royer (17). A constant amount of fluorescein-labeled Ara h 1 protein (10 nM) in binding buffer was mixed with serial dilutions (by 0.5 or 0.8 increments) of unlabeled Ara h 1 to analyze oligomer formation at room temperature. Each data point is an average of three independent measurements, and each curve is fitted to a sigmoidal function using Origin (Microcal Software, Northampton, MA). Analysis of the slope of the binding curves and midway point between top and bottom plateaus allowed the calculation of the cooperativity values (ρ) and apparent dissociation constants (K_{app}) for Ara h 1 oligomer formation (18). In the equation used by Origin program for fitting a sigmoidal curve, the K_{app} is defined as the midway point between the lower and upper plateaus and the slope of the curve (ρ value) defines the cooperativity of the interaction. This equation is described in detail by Czernik et al. (18). The intensity of fluorescence remained constant throughout the anisotropy measurements.

Homology-based model of Ara h 1 trimer

A homology-based model of an Ara h 1 monomer (14) was used to construct the trimeric model. Several small areas representing Ara h 1 amino acid residues Leu¹⁶⁶-Val¹⁹³, Pro²²⁶-Phe²⁴⁰, Asp²⁴⁹-Asp²⁵⁹, and Arg³⁰⁰-Arg³²⁷ were omitted during trimer construction because of structural uncertainty. The coordinates of the α atoms, of Asn⁴⁸, Ile¹¹⁷, Phe¹⁷⁴, Glu²⁰², Ala³¹², and Gly³⁶² from the x-ray crystal structure of phaseolin⁴ (Protein Data Bank code 2PHL) were used as reference points to fit the α atoms of Asn³⁷, Ile¹⁰⁰, Phe¹⁵⁸, Glu²⁰⁴, Ala³⁵, and Gly³⁹⁶ from Ara h 1 to form a trimer. The Fit Monomers program from SYBYL (version 6.3, Tripos, St. Louis MO) was used to create the initial framework. The energy of the Ara h 1 trimer was minimized with a harmonic force constraint of 100 using the Charmm force field and the adopted basis Newton-Raphson method for 500 iterations using the Charmm Minimization program resident in QUANTA (version 96, Molecular Simulations, Burlington, MA). The stereochemical quality of the model was assessed by using PROCHECK (version 2.1.4, Oxford Molecular, Palo Alto, CA). The computations were performed on a Silicon Graphics workstation running IRIX 6.4.

⁴ The atomic coordinates for the crystal structure of phaseolin can be accessed through the Brookhaven Protein Data Bank under PDB 2PHL (19).

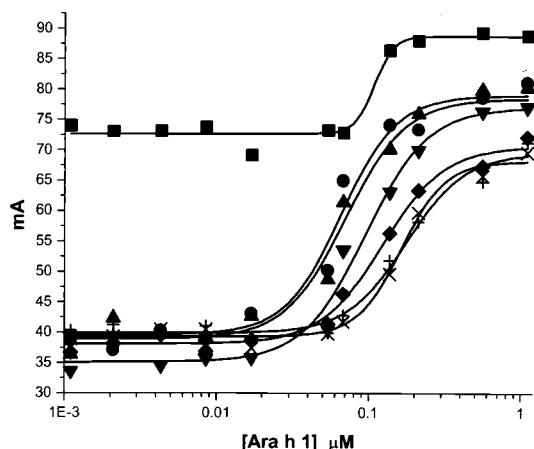


FIGURE 1. The Ara h 1 trimer is a highly stable structure due to the interaction of monomers through hydrophobic bonds. Fluorescence anisotropy was used to follow the formation of Ara h 1 higher order structure. A constant amount of fluorescein-labeled Ara h 1 (10 nM) were mixed with serial dilutions of unlabeled Ara h 1 (x-axis) and different concentrations of NaCl. Fluorescence measurements are expressed as arbitrary millianisotropy units (mA) on the y-axis. Each data point represents the average of three independent measurements. ■, 0 mM NaCl; ●, 100 mM NaCl; ▲, 300 mM NaCl; ▼, 500 mM NaCl; ◆, 900 mM NaCl; +, 1.4 M NaCl; ×, 1.8 M NaCl.

Results

Ara h 1 trimers are stable at high concentrations of NaCl

Fluorescence anisotropy and molecular modeling methods were used to gain a better understanding of the structural properties of Ara h 1 that may contribute to its stability and allergenicity. The Ara h 1 protein is known to form a homotrimer at relatively low concentrations, and this structure has been suggested to be important to the overall allergenicity of the molecule (14). To determine the stability and types of monomer interactions that mediate the formation of a homotrimer, fluorescence anisotropy measurements were performed in the presence of increasing salt concentrations (0–1.8 M NaCl). Purified, fluorescein-labeled Ara h 1 (10 nM) was mixed with various concentrations of unlabeled Ara h 1. The anisotropy of fluorescence observed at each concentration was determined and plotted as milli-anisotropy U vs the concentration of Ara h 1 (Fig. 1). The ability of the trimer to form at NaCl concentrations as high as 1.8 M is indicated by the presence of the plateau observed at Ara h 1 concentrations of 0.1–0.3 μ M. There is an increase in the dissociation constant as indicated by the increase in K_{app} from 0.065 to 0.170 μ M as the NaCl concentration is increased from 0.1 M to 1.8 M. The cooperativity (ρ) of trimer formation also decreased from 2.4 to 2.1 over the same salt concentrations (Table I). Minimal transition is seen in the anisotropy

Table I. Summary of association (K_{app}) and cooperativity (ρ) values for Ara h 1 trimer formation

NaCl Concentration (mM)	K_{app}^a (μ M)	ρ^a
0		
100	0.065	2.40
300	0.070	2.30
500	0.095	2.10
900	0.120	2.10
1400	0.170	2.20
1800	0.170	2.10

^a Values were determined as described in *Materials and Methods*.

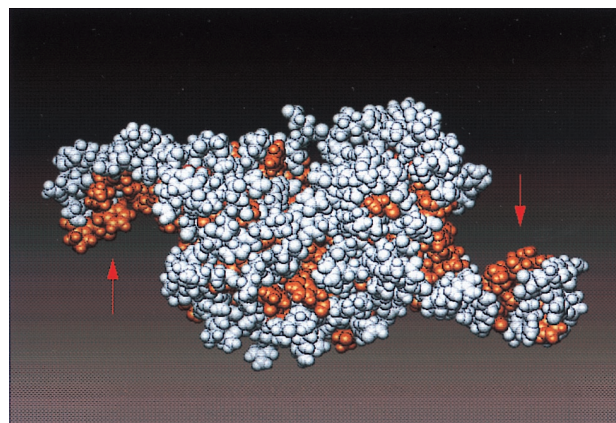


FIGURE 2. The ends of the Ara h 1 molecule contain the majority of the surface accessible hydrophobic amino acid residues. This figure shows a space filled view of the outer surface of an Ara h 1 monomer. The atoms of the hydrophobic amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, valine) are shown in orange, and the remaining atoms are shown in white. Arrows, areas on the Ara h 1 monomer with the highest concentrations of surface-accessible hydrophobic amino acids.

curve on dilution of Ara h 1 in the absence of NaCl. This result is most likely due to accentuated oligomerization of the protein in the absence of counterions in the solution; therefore, only minimal dissociation is seen on dilution of the protein. Thus, due to the minute change in anisotropy the K_{app} and ρ values cannot be obtained with the fitting program used (see *Materials and Methods*). Collectively, these results indicate that although some ionic interactions are involved in the cooperative interaction of monomers, the formation and stability of the Ara h 1 trimer are primarily due to hydrophobic interactions.

Molecular model of the Ara h 1 trimer and location of the IgE-binding epitopes

Because we had demonstrated that hydrophobic interactions were primarily responsible for Ara h 1 trimer formation, we examined the location of any surface-accessible hydrophobic amino acid residues present on the Ara h 1 monomer that may contribute to trimer formation. To accomplish this, a homology-based model of Ara h 1 tertiary structure, representing aa 172–586 (14), was utilized. The tertiary structure of the molecule consists of four domains: an α helical bundle on one end, two sets of opposing anti-parallel β sheets and a α helical bundle on the opposite end. The space-filled model of the Ara h 1 molecule and the position of the hydrophobic amino acid residues (alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, valine) are shown in Fig. 2. The distal regions of the molecule contain the majority of the surface-accessible hydrophobic amino acids.

To construct the model, three Ara h 1 monomers were aligned to the corresponding monomers that form the phaseolin trimer,⁴ a vicilin protein the x-ray crystal structure of which revealed a trimer (19). The quality of the model was assessed using the protein health module of QUANTA and PROCHECK version 2.1.4 and compared with the quality of the phaseolin trimer (19). Most of the backbone torsion angles for nonglycine residues lie within the allowed regions of the Ramachandran plot (data not shown). Only 1.0% of the amino acids in the Ara h 1 trimer have torsion angles that are disallowed as compared with 0.3% of amino acids in phaseolin. Side chain parameters, χ -1 and χ -2 angles, were also tested in the Ara h 1 trimer. The majority of the side chains are within the ideal 2.5 SD range where the percentage of residues

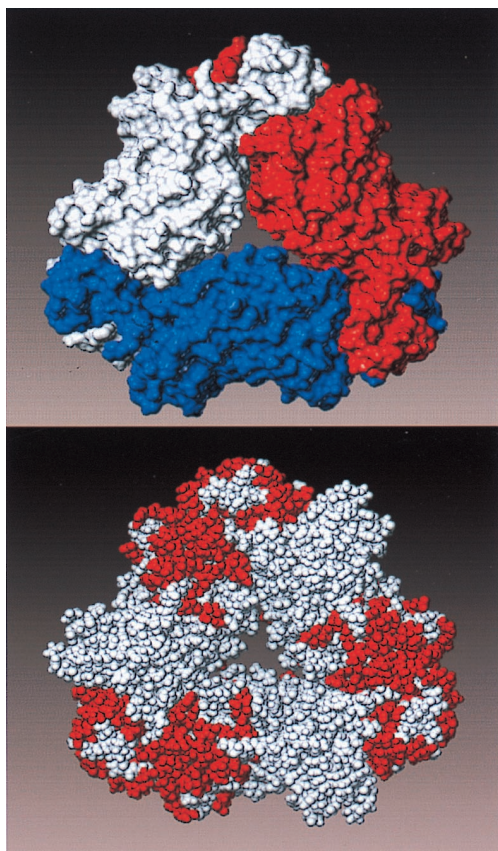


FIGURE 3. Molecular model of the Ara h 1 trimer. *Top*, Solid Connolly surface depiction of the Ara h 1 trimer. The α helical bundles located on the ends of each monomer overlap to form the trimer. *Bottom*, space-filled model of the Ara h 1 trimer with IgE-binding epitopes 10–22 colored in red, with the rest of the atoms in white. The majority of the epitopes lie near the areas of monomer-monomer contact.

outside the criteria is 19.3% for the Ara h 1 trimer and 16.9% for the phaseolin trimer. In addition to these criteria, a variety of main chain parameters (Omega angles, Van der Waals contacts, ζ angles, and H-bond energy) were tested for stereochemical quality and were comparable with that of the phaseolin x-ray crystal structure. Taken together, these data indicate that the homology-based model of the Ara h 1 trimer is reasonable and similar to the phaseolin trimer. The global fold of the Ara h 1 trimer indicates that formation of this structure is due to the α helical bundles on the ends of one Ara h 1 monomer overlapping with those of the adjacent monomer (Fig. 3, *top*). The hydrophobic residues depicted in Fig. 2 are those that form the interface between the monomers, similar to the structure of the phaseolin trimer (19).

The majority of the IgE-binding epitopes (13) are clustered near the regions of Ara h 1 monomer-monomer contact (Fig. 3, *bottom*). Epitopes 11 and 12 on the α helical bundle of one monomer and epitopes 20 and 21 on the α helical bundle of another monomer contact one another when the trimer is formed. Whereas most of the amino acid residues contained within these epitopes are surface accessible in the Ara h 1 monomer, $\sim 40\%$ of the residues within these epitopes lose surface accessibility when the trimer is formed as calculated by the QUANTA molecular simulation program.

Digestion-resistant fragments containing intact IgE epitopes

To determine whether quaternary structure played any role in protecting the Ara h 1 molecule from proteolytic digestion it was essential to determine whether the Ara h 1 trimer would remain

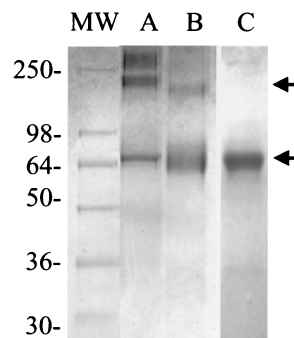


FIGURE 4. The Ara h 1 trimer is stable at pH 2 for 1 h. Ara h 1 trimers were incubated at pH 2 for 1 h and then subjected to a cross-linking reaction. The amount of covalently stabilized Ara h 1 trimer produced under these conditions was assessed by SDS-PAGE analysis. *Lane MW*, MW standards; *Lane A*, Ara h 1 trimers cross-linked after 1 h of incubation at pH 7.6; *Lane B*, Ara h 1 trimers cross-linked after 1 h of incubation at pH 2.0; *C*, Ara h 1 protein without any cross-linking. Arrows, Ara h 1 monomers and trimers.

intact when exposed to the environment of the stomach. Therefore, purified Ara h 1 was exposed to acidic pH. At the end of this incubation period, a cross-linking reaction was performed, and the amount of Ara h 1 trimer formed under these conditions was visualized by SDS-PAGE analysis (Fig. 4). Even though effects of acid hydrolysis can be seen on the integrity of the protein, it is clear that the Ara h 1 oligomer was found to be stable even after incubation at pH 2 and could still bind IgE (data not shown).

According to anisotropy experiments, at concentrations above 300 μM , Ara h 1 is in the form of a highly stable trimer. To determine whether some of the IgE binding epitopes were protected from digestion as predicted by the quaternary structure determinations, Ara h 1 was exposed to trypsin, chymotrypsin, or pepsin; proteases encountered in the gastrointestinal tract. Protease concentrations were such that nonallergenic proteins tested were digested to small (<10 kDa) peptides in a short period of time (<1 h, data not shown). The Ara h 1 peptides produced by these digestions were subject to SDS-PAGE and visualized by Coomassie staining. Peptides ranging in size from 16 kDa to 29 kDa were observed on Coomassie-stained gels up to 3 h after the start of digestion (Table II). A representative digestion of Ara h 1 with chymotrypsin has been shown in Fig. 5 as an example. The data from digestions with pepsin, trypsin, and chymotrypsin have been summarized in Table II. These peptide fragments ranged in size

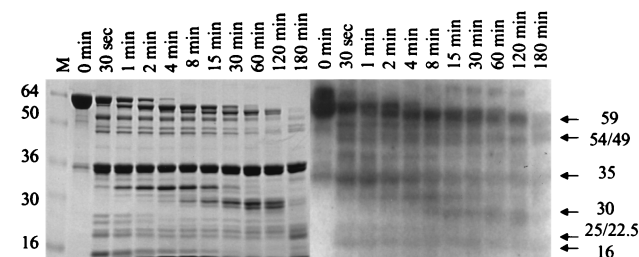


FIGURE 5. Chymotrypsin digestion of Ara h 1. Ara h 1 was incubated with chymotrypsin for the various time points indicated. Samples were run on SDS-PAGE and stained with Coomassie (*left*). The same digestion samples were transferred to nitrocellulose and analyzed by Western blot analysis using serum from allergic individuals. The bound serum IgE was recognized by an ^{125}I -anti-human secondary Ab and subject to autoradiography (*right*). Arrows, location and size of the chymotrypsin-resistant fragments that are recognized by serum IgE.

Table II. *Protease-resistant Ara h 1 peptides*

Pepsin			Trypsin			Chymotrypsin		
Digestion time (min)	MW ^a (kDa)	IgE binding	Digestion time (min)	MW ^a (kDa)	IgE binding	Digestion time (min)	MW ^a (kDa)	IgE binding
0.5–15	62		1–15	61		0.5–30	62	
0.5–15	60	***	8–15	58		2–180	59	***
0.5–30	50	***	0.5–120	50	***	0.5–180	54	***
0.5–30	47.5		0–60	36	***	0.5–180	49	***
0.5–30	45		1–60	35	***	0.5–180	48	
0.5–30	43		2–120	30		0.5–180	35.5	***
2–30	35.5	***	4–180	28	***	0.5–180	34	
2–30	33	***	8–180	26	***	0.5–120	32.5	
0.5–15	31		4–180	23		4–180	30	***
0.5–30	29		4–180	18.5		8–180	29	
0.5–180	27	***	30–180	17		0.5–180	26	
2–180	26.5	***	0.5–8	16		0.5–180	25	***
4–180	23.5	***	4–15	15		1–30	22.5	***
8–180	22.5	***				1–180	16	***

^a MWs of peptides were established from MW standards on SDS-PAGE.

***, IgE binding fragments.

from 16 to 61 kDa during 3 h of digestion. To determine whether the long lived peptides contained IgE binding epitopes, the Ara h 1 digestion products were subjected to immunoblot analysis using serum IgE from a pool of peanut-sensitive patients. In each case, many peptides contained intact binding sites that could be recognized by serum IgE (Table II). Knowing that Ara h 1 contains 23 IgE binding sites that are evenly distributed along the linear sequence of the molecule (13), these results suggest that large proteolytic fragments of Ara h1 contain multiple IgE-binding epitopes and survive digestion by the gastrointestinal enzymes tested.

Discussion

In the past, the study of allergens at a molecular level has been largely limited to biochemical measurements such as size, isoelectric points, glycosylation, and resistance to denaturation and digestion (6, 20–23). Ara h 1 has all of the classic characteristics of a food allergen. It is a 65-kDa glycoprotein with an acidic isoelectric point. It is an abundant protein in the peanut (11) that survives food-processing methods intact (6) and is stable up to 1 h within the in vitro digestion systems designed to mimic the gastrointestinal tract (6). Resistance of allergens to digestive enzymes have been attributed to various factors including protease inhibitors or nonprotein components present in the extracts analyzed (7, 24), direct effects on the secretion of endogenous proteins and/or the structure of the allergen itself (25). To date, there has been limited information regarding the structural basis for the stability and resistance of an allergen to digestion. In addition, the nature of the digestion-resistant fragments, especially the IgE-binding characteristics of these fragments have not been determined for any allergen. The combination of molecular biology, fluorescence anisotropy, and protein computer modeling has allowed us to examine a new set of important allergen characteristics. We have been able to identify IgE-binding sites, the amino acid residues critical for IgE binding (14), monomer tertiary structure, and oligomer formation. In this communication, we have examined the biochemical forces involved in oligomerization, identified the hydrophobic amino acids critical for this interaction, and shown that the locations of these residues coincide with the IgE recognition sites on the tertiary structure. Digestion-resistant fragments containing multiple IgE-binding sites were identified. Together these results show that quaternary structure may play an important role in the allergenic properties of a protein.

The only therapeutic option presently available for the prevention of a food hypersensitivity reaction is food avoidance. Unfortunately, for a ubiquitous food such as peanut, the possibility of inadvertent ingestion is great (26–28). Because of the potential severity of the allergic reaction, it has been suggested that a hypoallergenic Ara h 1 gene could be developed to replace its allergenic homologue in the peanut genome, thus blunting allergic reactions in sensitive individuals who inadvertently ingest this food (14). Because the Ara h 1 gene product is such an abundant and integral seed storage protein, it would be necessary for the altered vicilin to retain as much of its native function, properties, and three-dimensional structure as possible. The data presented here indicate that most of the IgE-binding epitopes are located at the contact points between the monomers in the Ara h 1 homotrimer. Amino acid substitutions designed to reduce the allergenicity of this protein may have deleterious effects on trimer formation and protein function. On the other hand, weakly destabilizing mutations may be desirable in making IgE-binding sites available to digestive enzymes.

It has been shown that Ara h 1 is capable of forming trimeric complexes in vitro similar to that of other vicilins (14). This multimeric form is also observed in the phaseolin x-ray crystal structure (19) and within the initial multiple isomorphous replacement electron density maps of canavalin⁵ (29). For phaseolin, the primary site of monomer contact was overlap of the terminal α helical bundles. A trimeric model of Ara h 1 based on the phaseolin structure revealed that the α helical bundles of the Ara h 1 monomers also appear to be the primary site for monomer contact. In addition, fluorescence anisotropy experiments clearly indicate that trimer formation is primarily mediated through hydrophobic interactions; this is confirmed by the location of hydrophobic residues in the areas of monomer-monomer contact. As previously mentioned, the location of the intramolecular hydrophobic contacts coincides with the location of preponderance of the IgE-binding epitopes. Also, when examining all of the available protease recognition sites located on the Ara h 1 primary sequence, it is clear that several of these sites are protected from digestion. Considering that Ara h 1 contains 23 IgE epitopes that, when the protein is denatured, are somewhat evenly distributed along the primary amino

⁵ The atomic coordinates for the crystal structure of canavalin can be accessed through the Brookhaven Protein Data Bank under PDB 1CAU (29).

acid sequence of the molecule, and the majority of the peanut-allergic individuals are known to recognize a minimum of five epitopes, it becomes obvious that any fragment larger than 10 kDa must contain more than one IgE-binding site. Collectively, these results suggest that the formation of a trimeric complex may afford the molecule some protection from protease digestion and denaturation, allowing passage of large fragments of Ara h 1 containing several intact IgE-binding epitopes across the small intestine, therefore contributing to its allergenicity.

Studies designed to develop hypoallergenic alternatives to replace native allergens in plants must take into account not only those amino acid substitutions that result in IgE binding but also those that will not disrupt the native structure of larger protein complexes. Thus, the development of an assay system that allows us to measure trimer formation and stability will permit us to test the integrity of recombinant proteins before plant transformation. Also, mutations in the recombinant protein that may allow trimer formation while having a destabilizing effect may increase the susceptibility of Ara h 1 to acid hydrolysis and digestion, therefore rendering it less likely to cause sensitization. Given the widespread use of peanuts in consumer foods and the potential risk this poses to individuals genetically predisposed to developing peanut allergy and to the health of individuals already peanut sensitive, these approaches are currently being explored in our laboratories.

References

- Singh, B., and U. Singh. 1991. Peanut as a source of protein for human foods. *Plant Foods Hum. Nutr.* 41:165.
- Hefle, S. L., J. A. Nordlee, and S. L. Taylor. 1996. Allergenic foods. *Crit. Rev. Food Sci. Nutr.* 14:1269.
- Hourihane, J. O., S. A. Roberts, and J. O. Warner. 1998. Resolution of peanut allergy: a case-control study. *Br. Med. J.* 316:1271.
- Bahna, S. L. 1998. Man shall not live by peanut alone. *Pediatrics* 102:148.
- Kmietowicz, Z. 1998. Women warned to avoid peanuts during pregnancy and lactation. *Br. Med. J.* 316:1926.
- Astwood, J. D., J. N. Leach, and R. L. Fuchs. 1996. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* 14:1269.
- Deshpande, S. S., and J. Nielsen. 1987. In vitro digestibility of dry bean (*Phaseolus vulgaris* L.) proteins: the role of heat stable protease inhibitors. *J. Food Sci.* 52:1326.
- Bush, R. K., S. L. Taylor, and J. A. Nordlee. 1989. Peanut sensitivity. *Allergy Proc.* 10:261.
- Gillespie, D. N., S. Nakajima, and G. J. Gleich. 1976. Detection of allergy to nuts by the radioallergosorbent test. *J. Allergy Clin. Immunol.* 57:302.
- Taylor, S. L., W. W. Busse, M. I. Sachs, J. L. Parker, and J. W. Yunginger. 1981. Peanut oil is not allergenic to peanut-sensitive individuals. *J. Allergy Clin. Immunol.* 68:372.
- Burks, A. W., L. W. Williams, R. M. Helm, C. Connaughton, G. Cockrell, and T. O'Brien. 1991. Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* 88:172.
- Burks, A. W., G. Cockrell, J. S. Stanley, R. M. Helm, and G. A. Bannon. 1995. Recombinant peanut allergen Ara h 1 expression and IgE binding in patients with peanut hypersensitivity. *J. Clin. Invest.* 96:1715.
- Burks, A. W., D. Shin, G. Cockrell, J. S. Stanley, R. M. Helm, and G. A. Bannon. 1997. Mapping and mutational analysis of the IgE binding epitopes on Ara h 1, a legume storage protein and a major peanut allergen. *Eur. J. Biochem.* 245:334.
- Shin, D., C. M. Compadre, S. J. Maleki, R. A. Kopper, H. Sampson, S. K. Huang, A. W. Burks, and G. A. Bannon. 1998. Biochemical and structural analysis of the IgE binding sites on Ara h 1, an abundant and highly allergenic peanut protein. *J. Biol. Chem.* 273:13753.
- Green, A. A., and W. L. Hughes. 1955. Protein fractionation on the basis of solubility in aqueous solutions of salt and organic solvents. *Methods Enzymol.* 1:67.
- Burks, A. W., S. B. Mallory, L. W. Williams, and M. A. Shirrell. 1988. Atopic dermatitis: clinical relevance of food hypersensitivity reactions. *J. Pediatr.* 113:447.
- Fernando, T., and C. A. Royer. 1992. Role of protein-protein interactions in the regulation of transcription by trp repressor investigated by fluorescence spectroscopy. *Biochemistry* 31:3429.
- Czernik, P. J., C. A. Peterson, and B. K. Hurlburt. 1996. Preferential binding of MyoD-E12 versus myogenin-E12 to the murine sarcoma virus enhancer in vitro. *J. Biol. Chem.* 271:9141.
- Lawrence, M. C., T. Izard, M. Beuchat, R. J. Blagrove, and P. M. Coleman. 1994. Structure of phaseolin at 2.2 Å resolution: implications for a common vicilin/legumin structure and the genetic engineering of seed storage proteins. *J. Mol. Biol.* 238:748.
- Deshpande, S. S., and J. Nielsen. 1987. In vitro enzymatic hydrolysis of Phaseolin, the major storage protein of *Phaseolus vulgaris* L. *Food Sci.* 52:1326.
- Bush, R. A., and S. L. Hefle. 1996. Food allergens. *Crit. Rev. Food Sci. Nutr.* 36:119.
- Burks, A. W., L. W. Williams, W. Thresher, C. Connaughton, G. Cockrell, and R. M. Helm. 1992. Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *J. Allergy Clin. Immunol.* 90:889.
- Anderson J. A., and D. D. Sogn, eds. Adverse reactions to foods. National Institutes of Health Publication 84-2442, July, 1984, pp. 1–6.
- Becker, W. M. 1997. Characterization of Ara h 1 by two-dimensional electrophoresis immunoblot and recombinant techniques: new digestion experiments with peanuts imitating the gastrointestinal tract. *Int. Arch. Allergy Immunol.* 113:118.
- Baglieri, A., S. Mahe, R. Benamouzig, L. Savoie, and D. Tome. 1995. Digestion patterns of endogenous and different exogenous proteins affect the composition of intestinal effluents in humans. *J. Nutr.* 125:1238.
- Sampson, H. A. 1997. Food allergy. *JAMA* 278:1888.
- Bock, S. A., and S. M. Atkins. 1989. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* 83:900.
- Lemanske R. F., and S. L. Taylor. 1987. Standardized extracts, foods. *Clin. Rev. Allergy* 5:23.
- Ko, T. P., J. D. Ng, and A. McPherson. 1993. The three-dimensional structure of canavalin from jack bean (*Canavalia ensiformis*). *Plant Physiol.* 101:729.