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

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

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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.
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
20 mg 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 x g for 30 min at 4°C, and subjected to ammonium sulfate precipitation (15). Ammonium sulfate was added to 100% 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, Piscataway, NJ) and loaded onto a High Prep S. cation exchange resin column (2.5 x 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 trypsin 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 were 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, diethylbis(succinimidylpropionate) (Pierce, Rockford, IL) was used. Dithiobis(succinimidylpropionate) was dissolved in N,N-dimethylformamide as a 74 mM stock solution. 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 isotherms and midpoints between top and bottom plateaus allowed the calculation of the cooperativity values (p) and apparent dissociation constants (Kd) for Ara h 1 oligomer formation (18). In the equation used by Origin program for fitting a sigmoidal curve, the Kd is defined as the midpoint between the lower and upper plateaus and the slope of the curve (p value) defines the cooperativity of the interaction. This equation is described in detail by Czernecki 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–Val190, Pro226–Phc240, Asp249–Asp259, and Arg300–Arg327 were omitted during trimer construction because of structural uncertainty. The coordinates of the Cu atoms, of Asn48, Ile117, Phe174, Glu202, Ala155, and Gly252 from the x-ray crystal structure of phaseolin (4) were used as reference points to fit the Ara h 1 crystals. The generated model was subjected to energy minimization (500 iterations using the Charmm Minimization program resident in QUANTA (version 9.6, 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 done on a Silicon Graphics workstation running IRIX 6.4.

4The atomic coordinates for the crystal structure of phaseolin can be accessed through the Brookhaven Protein Data Bank under PDB 2PHL.
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 NaCl. Fluorescence measurements are expressed as arbitrary millianisotropy values.

Minimal transition is seen in the anisotropy 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 $p$ 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 (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 Ramanchandran 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, $\chi$-1 and $\chi$-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 1](http://www.jimmunol.org/figview/5846/4)

**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.

![FIGURE 2](http://www.jimmunol.org/figview/5846/5)

**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.

<table>
<thead>
<tr>
<th>NaCl Concentration (mM)</th>
<th>$K_{app}$ (μM)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
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<td>900</td>
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<td>2.20</td>
</tr>
<tr>
<td>1400</td>
<td>0.170</td>
<td>2.10</td>
</tr>
<tr>
<td>1800</td>
<td>0.170</td>
<td>2.10</td>
</tr>
</tbody>
</table>

* Values were determined as described in Materials and Methods.
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, $\zeta$ 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 $\alpha$ 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 $\alpha$ helical bundle of one monomer and epitopes 20 and 21 on the $\alpha$ 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, $\sim40\%$ 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 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 covalently stabilized 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 $300 \mu M$, 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...
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-binding epitopes 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 supports 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 has 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.

### Table II. Protease-resistant Ara h1 peptides

<table>
<thead>
<tr>
<th>Digestion time (min)</th>
<th>MW (kDa)</th>
<th>IgE binding</th>
<th>Digestion time (min)</th>
<th>MW (kDa)</th>
<th>IgE binding</th>
<th>Digestion time (min)</th>
<th>MW (kDa)</th>
<th>IgE binding</th>
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<td>0.5–15</td>
<td>60</td>
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<td>8–15</td>
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<td>0.5–180</td>
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<td>1–180</td>
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* MWs of peptides were established from MW standards on SDS-PAGE. ***, IgE binding fragments.

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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 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