Biochemical and Structural Analysis of the IgE Binding Sites on Ara h1, an Abundant and Highly Allergenic Peanut Protein*

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Allergy to peanuts is a significant IgE-mediated health problem because of the high prevalence, potential severity, and chronicity of the reaction. Ara h1, an abundant peanut protein, is recognized by serum IgE from >90% of peanut-sensitive individuals. It has been shown to belong to the vicilin family of seed storage proteins and to contain 23 linear IgE binding epitopes. In this communication, we have determined the critical amino acids within each of the IgE binding epitopes of Ara h1 that are important for immunoglobulin binding. Surprisingly, substitution of a single amino acid within each of the epitopes led to loss of IgE binding. In addition, hydrophobic residues appeared to be most critical for IgE binding. The position of each of the IgE binding epitopes on a homology-based molecular model of Ara h1 showed that they were clustered into two main regions, despite their more even distribution in the primary sequence. Finally, we have shown that Ara h1 forms a stable trimer by the use of a reproducible fluorescence assay. This information will be important in studies designed to reduce the risk of peanut-induced anaphylaxis by lowering the IgE binding capacity of the allergen.

It is estimated that up to 8% of children and 2% of adults have allergic reactions to foods (1–3). Peanut allergy is one of the most common and serious of the immediate hypersensitivity reactions to foods in terms of persistence and severity of reaction. Unlike the clinical symptoms of other food allergies, the reactions to peanuts are rarely outgrown; therefore, most diagnosed children will have the disease for a lifetime (4, 5). In a sensitized individual, ingestion of peanuts results in mast cell-bound IgE binding to a specific allergen. The IgE-allergen complex causes mast cell receptors to cross-link, inducing a signal transduction cascade that ends in degranulation and release of a variety of mediators that give rise to the clinical symptoms of peanut hypersensitivity (6, 7). The majority of cases of fatal food-induced anaphylaxis involve ingestion of peanuts (8, 9). Currently, the only effective treatment for food allergy is avoidance of the food. For peanut-allergic individuals, total avoidance is difficult since peanuts are increasingly being used in the diet as an economical protein source in processed foods.

Because of the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed foods, there is increasing interest in defining the allergenic proteins and exploring ways to decrease the risk to the peanut-sensitive individual. Various studies over the last several years have identified the major allergens in peanuts as belonging to different families of seed storage proteins (10, 11). For example, two of the major peanut allergens Ara h1 and Ara h2 belong to the vicilin and conglutin families of seed storage proteins, respectively. The vicilins represent one of the most abundant proteins found in legumes used for human consumption. This class of proteins does not have any known enzymatic activity but is thought to interact with each other to form unique higher order oligomeric structures that may help in packaging these proteins into seeds (12). Because the vicilins represent such a large percentage of the total protein in a seed, any approach designed to alter the IgE binding capacity of this protein would require that the genetically engineered gene product retain its native function, properties, and three-dimensional structure.

Genetically modified plants are being used more frequently as food sources for human consumption. The major emphasis has been on the introduction of genes whose products would enhance the nutritional value or disease resistance of the transgenic plant. One of the major concerns of this approach is that a gene will be introduced that encodes an unwanted or unknown allergen that would put allergic individuals at risk. Indeed, the introduction of a gene encoding a major Brazil nut allergen into soybeans, ostensibly to increase the nutritional value of soybeans, is a prime example (13). In cases where allergens are transferred into plants, consumers must be informed of the existence of the allergen by labeling as suggested by the United States Food and Drug Administration. In addition, a range of tests that compare the physicochemical properties of known allergens with expressed transgenic products has been proposed for those gene products of unknown allergenicity (14–16). Currently, there is little known about the physicochemical properties of many of the plant allergens, and there have been few investigations aimed at modifying allergenic proteins.

Previous work on the allergenic aspects of the Ara h1 protein has shown that it is recognized by serum IgE from >90% of the peanut-sensitive individuals, indicating that it is a major allergen involved in the clinical etiology of this disease (17). Recently, using pooled serum IgE from a population of peanut-hypersensitive individuals, 23 linear IgE binding epitopes of this allergen have been mapped (10). There was no obvious

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IgE binding to this epitope. The type of each amino acid within the Ara h1 epitopes was assessed relative to its importance to IgE binding. The closed boxes represent the total number of a particular type of amino acid residue found in all of the Ara h1 epitopes, whereas the open boxes represent the number of that type of residue which, when replaced, was found to result in the loss of IgE binding.

**TABLE I**

**Amino acids critical to IgE binding**

The Ara h1 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h1 protein coding sequence is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. Epitopes 16 and 23 were not included in this study because they were recognized by a single patient who was no longer available to the study.

| Peptide | Amino acid sequence | Position |
|---------|---------------------|----------|
| 1       | ARSPYCKKT           | 25–34    |
| 2       | QERDDLQKA           | 48–57    |
| 3       | LEYDRIVYD           | 65–74    |
| 4       | GERGRIQPG           | 89–98    |
| 5       | PGDYDQRQ            | 97–106   |
| 6       | PRREDGRNG           | 107–116  |
| 7       | RREEEDOGP           | 123–132  |
| 8       | EDRERRSHQ           | 134–143  |
| 9       | OFPEOFQF            | 143–152  |
| 10      | HPGRHPFF            | 294–303  |
| 11      | SLYERFSRT           | 311–320  |
| 12      | FMAFENIRR           | 325–334  |
| 13      | CCCAE               | 341–345  |
| 14      | EEREGERQW           | 343–353  |
| 15      | DINGNLKE            | 383–402  |
| 16      | NNFLPEVK            | 409–418  |
| 17      | RRTARLKEG           | 498–507  |
| 18      | EIRLIGFCIN          | 525–534  |
| 19      | HRFILAGED           | 539–548  |
| 20      | IDIEKAPF            | 551–560  |
| 21      | KDLAPPSGE           | 559–568  |
| 22      | KESHFFVSRP          | 578–587  |

sequence motif shared by the epitopes. In this communication, we have determined the critical amino acids within each of the IgE binding epitopes of Ara h1 that are important to immunoglobulin binding. Surprisingly, substitution of a single amino acid within each of the epitopes led to loss of IgE binding. In addition, the hydrophobic residues located in the center of the epitope appeared to be most critical to IgE binding. The position of each of the IgE binding epitopes on a homology-based tertiary structure model of Ara h1 showed that they were clustered into two main regions. This was in contrast to previous observations that showed the IgE binding epitopes distributed evenly along the linear sequence of the molecule. Finally, we have shown that, like other vicilins, Ara h1 forms a stable tertiary structure. This information will be important in studies designed to reduce the risk of peanut-induced anaphylaxis by lowering the IgE binding capacity of the allergen.

**MATERIALS AND METHODS**

**Serum IgE**—Serum from 15 patients with documented peanut hypersensitivity reactions (mean age, 25 years) was used to determine relative binding affinities between wild type and mutant Ara h1 synthesized epitopes. The patients had either a positive double-blind, placebo-controlled food challenge (DBPCFC) or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension; Ref. 18). At least 5 ml of venous blood was drawn from each patient and allowed to clot, and serum was collected. A serum pool from 12 to 15 patients was made by mixing equal aliquots of serum IgE for our experiments. The pools were then used in immunoblot analysis. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences.

**Peptide Synthesis**—Individual peptides were synthesized on a derivatized cellulose membrane using N-(9-fluorenylmethoxycarbonyl (Fmoc) amino acid active esters according to the manufacturer instructions (Genosys Biotechnologies, Woodlands, Texas; Ref. 19). Peptide synthesis reactions were monitored by bromphenol blue color reactions during certain steps of synthesis. Cellulose-derivitized membranes and Fmoc amino acids were supplied by Genosys Biotechnologies. All other chemicals were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) or Fluka (Buchs, Switzerland). Membranes were either probed immediately or stored at −20 °C until needed.

**IgE Binding Assays**—Cellulose membranes containing synthesized peptides were washed 3 times in Tris-buffered saline (TBS; 136 mM NaCl, 2.7 mM KCl, and 50 mM Trizma (Tris base, pH 8.0) for 10 min at room temperature and then incubated overnight in blocking buffer (TBS, 0.05% Tween 20; concentrated membrane-blocking buffer supplied by Genosys; and sucrose (9.0:1.0:0.5)). The membrane was then incubated in pooled sera diluted 1:5 in 20 mM Tris–Cl, pH 7.5, 150 mM NaCl, and 1% bovine serum albumin overnight at 4 °C. Primary antibody was detected with 125I-labeled equine anti-human IgE (Kallestad, Chaska, MN) followed by autoradiography.

**Quantitation of IgE Binding**—Relative amounts of IgE binding to individual peptides were determined by scanning autoradiographs using a Bio-Rad (Hercules, CA) model GS-700 imaging laser densitometer and quantitated with Bio-Rad molecular analyst software. A back-

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1 The abbreviations used are: DBPCFC, double-blind, placebo-controlled food challenge; Fmoc, F-(9-fluorenylmethoxycarbonyl; TBS, Tris-buffered saline; DSP, dithiodisuccinimidyl propionate).
ground area was scanned and subtracted from the obtained values. Following quantitation, wild type intensities were normalized to a value of one, and the mutants were calculated as percentages relative to the wild type.

**Homology-based Model of Ara h1**—Molecular modeling and computations were performed on Silicon Graphics workstations running IRIX 6.2. The Wisconsin Genetic Computer Group (GCG) software package (20) was also used on a digital ALPHA workstation using OpenVMS Version 6.1.

The x-ray crystal structure of the phaseolin A chain (2PHL A, 2.2 Å resolution) from Phaseolus vulgaris was used as the template for homology-based modeling (12, 21, 22). Ara h1 was modeled as a monomer using the COMPOSER module of SYBYL Version 6.3 from Tripos Inc. (St. Louis, MO). Phaseolin is a smaller protein than Ara h1, and it only allowed for the modeling of the region between residues 172–586. Residues Ser211-Asp219 and Asn281-Lys282 on the structure of phaseolin were not solved because of low electron density (12). Before attempting to use the structure for modeling, the regions were constructed using the protein loop search option in SYBYL and minimized using local annealing and the Powell algorithm.

**Fluoresence Polarization of Ara h1 Higher Order Structure**—Ara h1 was purified to >95% homogeneity from crude peanut extract and the atomic coordinates for the crystal structure for this protein can be accessed through the Protein Data Bank, Brookhaven National Laboratory, under code 2PHL (12).
Biochemical and Structural Analysis of IgE Binding to Ara h1

RESULTS

IgE Binding Characteristics of the Ara h1 Epitopes—The amino acids essential to IgE binding in the Ara h1 epitopes were determined by synthesizing duplicate peptides with single amino acid changes at each position. These peptides were then probed with pooled serum IgE from 15 patients with peanut hypersensitivity to determine if the changes affected peanut-specific IgE binding. An immunoblot strip containing the wild type and mutated peptides of epitope 9 is shown in Fig. 1. Binding of pooled serum IgE to these individual peptides was dramatically reduced when either alanine or methionine was substituted for the wild type amino acid, resulting in less than 1% of peanut-specific IgE binding to these peptides. In contrast, the substitution of an alanine for arginine at position 152 resulted in increased IgE binding. The remaining Ara h1 epitopes were tested in the same manner, and the intensity of IgE binding to each spot was determined as a percentage of IgE binding to the wild type peptide (Table I). Each epitope could be mutated to a non-IgE binding peptide by the substitution of a single alanine or methionine residue.

The amino acids within each epitope were classified according to whether they were hydrophobic (apolar), polar, or charged residues (Fig. 2). There were a total of 196 amino acids present in the 21 epitopes of Ara h1 that were studied. Charged residues occurred most frequently (89/196), with hydrophobic residues (71/196) being the next frequent type of amino acid in the epitopes and polar residues representing the least frequent amino acid group (36/196). Thirty-five percent of the mutated hydrophobic residues resulted in loss of IgE binding (<1% IgE binding), whereas only 25 and 17% of mutated polar and charged residues, respectively, had a similar effect. These results indicated that the hydrophobic amino acid residues within these IgE binding epitopes were the most sensitive to changes. In addition, results from this analysis indicated that the amino acids located near the center of the epitope were more critical for IgE binding.

Location of the IgE Binding Epitopes on the Three-dimensional Structure of Ara h1—A homology-based model of Ara h1 tertiary structure was generated to determine the location of the epitopes on this relatively large allergenic molecule. To construct this model, the primary amino acid sequence of Ara h1 was aligned to the highly homologous protein phaseolin, for which x-ray crystal structure data was available (Fig. 3). The quality of the Ara h1 model was assessed using the protein health module of QUANTA and PROCHECK Version 2.1.4 (28) from Oxford Molecular Inc. (Palo Alto, CA) and compared with the quality of the structures of phaseolin and canavalin4 (21, 22, 29). Most of the backbone torsion angles for non-glycine residues lie within the allowed regions of the Ramachandran plot (Fig. 4). Only 1.4% of the amino acids in the Ara h1 model have torsion angles that are disallowed as compared with 0.3 and 0.6% of amino acids in phaseolin and canavalin, respectively (Table II). In addition, the number of buried polar atoms, buried hydrophilic residues, and exposed hydrophobic residues in the Ara h1 model are comparable with those found in the structures of phaseolin and canavalin (Table II). Taken together, these data indicate that the homology-based model of Ara h1 tertiary structure is reasonable and similar to the structures of other homologous proteins that have been solved.

### TABLE II

|                | Ara h1 | Phaseolin | Canavalin |
|----------------|--------|-----------|-----------|
| Buried polar atoms | 52     | 42        | 67        |
| Buried hydrophilic | 18     | 7         | 10        |
| Exposed hydrophobic | 2      | 2         | 3         |
| Ramachandran highly favored* | 308 | 280       | 250       |
| Ramachandran allowed* | 56     | 40        | 71        |
| Ramachandran disallowed* | 5      | 1         | 2         |

* Terminal amino acid residues, glycines, or prolines are not included in these categories.

\[3\] S. J. Maleki, R. Kopper, D. Shin, H. Sampson, A. W. Burks, and G. A. Bannon, manuscript in preparation.

\[4\] The atomic coordinates for the crystal structure for this protein can be accessed through the Protein Data Bank, Brookhaven National Laboratory, under code 1CAU (29).
the molecule consists of two sets of opposing anti-parallel β-sheets in Swiss roll topology joined by an interdomain linker. The terminal regions of the molecule consist of α-helical bundles containing three helices each. Epitope 12 resides on an N-terminal α-helix while epitopes 20 and 21 are located on C-terminal α-helices. Epitopes 14, 15, and 18 are primarily β-strands on the inner faces of the domain, and epitopes 16, 17, 19, and 22 are β-strands on the outer surface of the domain.

The remainder of the epitopes are without a predominant type of higher secondary structure. A space-filled model depicting the surface accessibility of the epitopes and critical amino acids is shown in Fig. 5B. Of the 35 residues that affected IgE binding, 10 were buried beneath the surface of the molecule, and 25 were exposed on the surface.

_Ara h1 Interacts with Itself to Form a Stable Trimeric Structure_—A rapid, reproducible fluorescence assay was developed
in order to determine if the peanut allergen formed higher order structures similar to those observed for soybean vicilins. Purified, fluorescein-labeled Ara h1, 10 nM, was mixed with various concentrations of unlabeled Ara h1. The fluorescence polarization observed at each concentration was then determined and plotted as milli-polarization units (mP) in arbitrary units versus the concentration of Ara h1 (Fig. 6). Measurement of fluorescence reveals the average angular displacement of the fluorophor, which is dependent on the rate and extent of rotational diffusion. An increase in the size of the macromolecule through complex formation results in decreased rotational diffusion of the labeled species, which in turn results in an increase in polarization. The plateaus observed at protein concentrations between 0 and 20 nM and between 200 nM and 2 μM indicate the presence of a homogeneous species at these concentrations. The sharp increase in polarization observed at concentrations of Ara h1 above 50 nM indicates that a highly cooperative interaction between Ara h1 monomers had occurred that results in the formation of a stable homo-oligomeric structure. In order to determine the stoichiometry of this interaction, cross-linking experiments were performed following by SDS-polyacrylamide gel electrophoresis analysis of the cross-linked products (Fig. 6, inset). Ara h1 oligomers representing samples taken at the 200 nM concentration were subjected to limited chemical cross-linking with DSP. Cross-linked and noncross-linked samples were resolved by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie staining. Lower arrow indicates the Ara h1 monomer (~60 kDa), and the upper band represents the Ara h1 trimer (~180 kDa). The Ara h1 allergen forms a stable trimeric structure. Trace fluorescein-labeled Ara h1 was mixed with unlabeled Ara h1, and fluorescence polarization measurements (mP) were made at each concentration. Each point represents the average of three different experiments. Samples from the 200 nM concentration were then subjected to cross-linking with constant amounts of DSP for varying lengths of time, and the products were electrophoresed on SDS-polyacrylamide gels. Protein bands were visualized by Coomassie staining. Lower arrow indicates the Ara h1 monomer (~60 kDa), and the upper band represents the Ara h1 trimer (~180 kDa).

DISCUSSION

Food allergies are mediated through the interaction of IgE to specific proteins contained within the food. While the IgE binding epitopes from the major allergens of cow milk (30), codfish (31), hazel (32), soy (33), and shrimp (34) have all been elucidated there have been few, if any, characteristics found in common with these binding sites. Our work on the IgE binding epitopes of Ara h1 also indicates that there is no common amino acid sequence motif found in all epitopes (10). However, we have determined that, once an IgE binding site has been identified, it is the hydrophobic amino acid residues that appear to play a critical role in immunoglobulin binding. The observation that alteration of a single amino acid leads to the loss of IgE binding in a population of peanut-sensitive individuals is significant because it suggests that, while each patient may display a polyclonal IgE reaction to a particular allergen (10, 11), IgE from different patients that recognize the same epitope must interact with that epitope in a similar fashion. Besides finding that many epitopes contained more than one residue critical for IgE binding, it was also determined that more than one residue type (Ala or Met) could be substituted at certain positions in an epitope with similar results. This may allow for the design of a hypoallergenic protein that would be effective at blunting allergic reactions for a population of peanut-sensitive individuals. Furthermore, a peanut where the IgE binding epitopes of the major allergens have been removed may prevent the development of peanut hypersensitivity in individuals genetically predisposed to this food allergy.

The characteristics that have been attributed to allergenic proteins include their abundance in the food source, their resistance to food processing, and their stability to digestion by the gastrointestinal tract (14, 15). The major peanut allergen, Ara h1, has been shown to be an abundant protein (35) that survives intact in most food processing methods (36) and is stable to digestion in in vitro systems designed to mimic the gastrointestinal tract (37). However, the physical characteristics that allow this protein to exhibit these properties have not previously been examined. Our observations on the tertiary structure of the Ara h1 monomer and the determination that this protein readily forms a trimeric complex may help to determine why this protein is allergenic. For example, we have described the tertiary structure of the Ara h1 protein as consisting of two sets of opposing antiparallel β-sheets in Swiss roll topology with the terminal regions of the molecule consisting of α-helical bundles containing three helices apiece. While there are numerous protease digestion sites throughout the length of this protein, the structure may be so compact that potential cleavage sites are inaccessible until the protein is denatured. In addition, the formation of a trimeric complex and further higher order aggregation may also afford the molecule some protection from protease digestion and denaturation and allow passage of Ara h1 across the small intestine. It has been shown that some atopic individuals transfer more antigen
across the small intestine in both the intact and partially degraded state (38). These physical attributes of the Ara h1 molecule may help to explain the extreme allergenicity exhibited by this protein.

The only therapeutic option currently available for the prevention of a peanut hypersensitivity reaction is food avoidance. Unfortunately, for a ubiquitous food such as the peanut, the possibility of an inadvertent ingestion is great. This is complicated by the fact that all of the peanut allergens identified to date have sequence homology with proteins in other plants. This may explain the cross-reacting IgE antibodies to other legumes that are found in the sera of patients that manifest clinical symptoms to only one member of the legume family (39). The elucidation of the position of the Ara h1 IgE binding epitopes clustered on the surface of the molecule may enable us to better understand why these regions elicit the clinical symptoms associated with peanut hypersensitivity. Perhaps the presentation of multiple, clustered epitopes to mast cells results in a more efficient and dramatic release of mediators that result in the more severe clinical symptoms observed in patients with peanut hypersensitivity. We are currently exploring this possibility by comparing the IgE binding epitopes and tertiary structures of other legume allergens.

Finally, it has been suggested that an altered Ara h1 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 (10). Since the Ara h1 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 development of a hypoallergenic vicilin may be feasible. However, the effect that altering critical amino acids within each of the IgE binding epitopes has on the properties of this seed storage protein is currently unknown. Given the widespread use of peanuts in consumer foods and the potential risk this poses to individuals genetically pre-disposed to developing peanut allergy and to the health of individuals already peanut-sensitive, this approach is currently being explored in our laboratories.

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