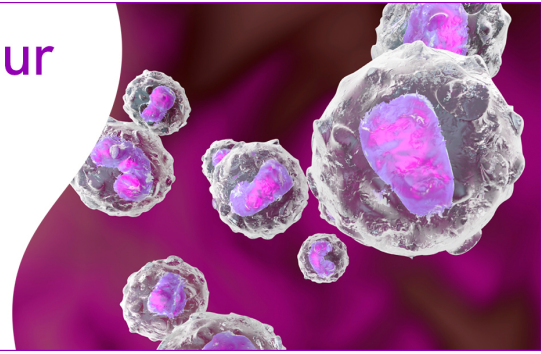


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# Protein Structure Plays a Critical Role in Peanut Allergen Stability and May Determine Immunodominant IgE-Binding Epitopes

Moon Sen,\* Randall Kopper,\* Laurent Pons,\* Edathara C. Abraham,\* A. Wesley Burks,<sup>†</sup> and Gary A. Bannon<sup>1\*</sup>

Hypersensitivity to peanuts is a reaction mediated by IgE Abs in response to several peanut protein allergens. Among these allergenic proteins, Ara h 2 is one of the most commonly recognized allergens. Ara h 2 is a 17-kDa protein that has eight cysteine residues that could form up to four disulfide bonds. Circular dichroism studies showed substantial changes in the secondary and tertiary structures of the reduced Ara h 2 as compared with the native protein. Upon treatment with trypsin, chymotrypsin, or pepsin, a number of relatively large fragments are produced that are resistant to further enzymatic digestion. These resistant Ara h 2 peptide fragments contain intact IgE-binding epitopes and several potential enzyme cut sites that are protected from the enzymes by the compact structure of the protein. The enzyme-treated allergen remains essentially intact despite the action of proteases until the fragments are dissociated when the disulfide linkages are reduced. Amino acid sequence analysis of the resistant protein fragments indicates that they contain most of the immunodominant IgE-binding epitopes. These results provide a link between allergen structure and the immunodominant IgE-binding epitopes within a population of food-allergic individuals. *The Journal of Immunology*, 2002, 169: 882–887.

Food-allergic reactions are causing increasing concern in the U.S., with about one-fourth of American households altering their dietary habits because a member of the family is perceived to suffer from food allergies (1). Prospective studies have indicated that ~5% of children less than 4 years of age experience IgE-mediated food-allergic reactions, with ~1.5% of young children reacting to cow's milk, ~1.3% to hen's egg, and 0.5% to peanut (2). Children with atopic disorders, especially atopic dermatitis, are more often affected by food allergies. About 35% of children with moderate to severe atopic dermatitis have skin symptoms provoked by food hypersensitivity (3). Given the estimated frequency of allergy to a variety of foods, it is likely that ~2% of the adult population, or ~5.5 million Americans are affected by food allergies (2).

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 (4–6). Thus, it is increasingly common for the public to be exposed to an abundantly used and often disguised food such as peanuts. This has led to increasing rates of sensitization, accidental ingestion, anaphylaxis, and even death in peanut-allergic individuals.

Food allergens have several biochemical characteristics in common. These include their glycosylation pattern, abundance in the food, and their resistance to proteases, heat, and denaturants. One of the more significant food allergen characteristics is that they are stable to the proteolytic and acidic conditions of the digestive tract, which imparts an increased probability of reaching the intestinal mucosa, where absorption can occur. A variety of food allergens has been tested in simulated gastric fluid, where they survive for extended periods of time when compared with nonallergenic food proteins (7). Even though allergen stability has been demonstrated for a variety of food allergies, there is little known about why these proteins have the ability to resist degradation.

The Ara h 2 peanut allergen is recognized by serum IgE from >90% of peanut-allergic patients, thus establishing the importance of this protein in the etiology of the disease (8, 9). Ara h 2 has been shown to be resistant to acidic conditions and digestion with gastrointestinal (GI)<sup>2</sup> tract enzymes (7). The linear IgE-binding epitopes of the Ara h 2 allergen have been mapped using overlapping peptides and serum IgE from a population of peanut-sensitive patients. Ara h 2 contains 10 IgE-binding epitopes detected with linear peptides representing the major epitopes recognized by serum from a peanut-sensitive patient population. Immunodominant IgE-binding epitopes were also determined from a population of peanut-sensitive patients for Ara h 2. Ara h 2 contained 3 epitopes (epitopes 3, 6, and 7) that were recognized by serum IgE from the majority of patients tested and represented the majority of allergen-specific IgE found in these patients (10).

There has been very little work performed describing Ara h 2 protein structure. However, there are eight cysteine residues that could form up to four disulfide bonds in Ara h 2, disulfide bonds having been shown in other allergens to contribute to the overall allergenicity of the molecule (11, 12). In this communication, we have used purified allergen to demonstrate that the disulfide bonds

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Received for publication October 5, 2001. Accepted for publication May 10, 2002.

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<sup>2</sup> Abbreviations used in this paper: GI, gastrointestinal; BLG,  $\beta$ -lactoglobulin.

of Ara h 2 contribute significantly to the overall structure and stability of the allergen. The native Ara h 2 protein remains intact even after digestion with GI tract enzymes. Only after reduction of the disulfide bonds does a ~10-kDa protease-resistant fragment become apparent. The protease-resistant Ara h 2 protein fragment was then isolated and subjected to amino acid sequence analysis, in which it was determined that the fragment contained many of the previously determined immunodominant IgE-binding epitopes. These results demonstrate that protein structure plays an important role in the stability of this allergen to resist digestion and may dictate which of the IgE-binding epitopes are immunodominant.

## Materials and Methods

### Patient sera

Pooled serum from 16 patients with documented peanut hypersensitivity reactions was used to identify Ara h 2 epitope-containing fragments produced by digestion with proteolytic enzymes. The patients had either a positive, double blind, placebo-controlled food challenge or a convincing history of peanut anaphylaxis (13). Equal aliquots of IgE-containing serum from each patient were pooled and used for our experiments. Each patient's serum contained IgE that recognized Ara h 2. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences, and informed consent was obtained from each serum donor.

### Purification of the Ara h 2 protein

Peanut seeds (*Arachis hypogaea* L., *Florunner* cultivar) were ground in liquid nitrogen and then defatted three times in a Soxhlet extractor using diethyl ether. The derived peanut flour was dissolved (2:100, w/v) and stirred in TBS buffer (65 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 200 mM NaCl, pH 8.3) for 1 h at room temperature. The extract was cleared by filtration through six layers of cheesecloth and centrifugation at 30,000 × *g* for 30 min at 4°C. The supernatant was fractionated using ammonium sulfate precipitation (14). Ammonium sulfate was added to 40% saturation. The remaining supernatant was then taken to 70% ammonium sulfate saturation and centrifuged at 30,000 × *g* for 30 min at 4°C. The pellet was resuspended in TB buffer and sonicated on ice for 20 s at 50% power using a Sonic Dismembrator Model 300 (Fisher Scientific, Pittsburgh, PA). Undissolved particles were removed by centrifugation (3,000 × *g* for 15 min), and the solution was applied to a MacroPrep High Q anion exchange column (2.5 × 12 cm; Bio-Rad, Hercules, CA). After washing the column with TB containing 40 mM NaCl, bound proteins were eluted using a 400 ml linear salt gradient (40–140 mM NaCl) at 2 ml/min flow rate. Fractions of 2.5 ml were collected and analyzed by SDS-PAGE using 4–20% gradient polyacrylamide gels (Novex, San Diego, CA) and Coomassie brilliant blue R staining. Pooled fractions enriched with Ara h 2 were dialyzed overnight at 4°C against 25 mM Tris-HCl (pH 7.4) and 3 M NaCl and loaded onto a phenyl-Sepharose column (2.5 × 12 cm; Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in the same buffer. Bound proteins were eluted using a 200 ml linear decreasing salt gradient (3 to 0 M NaCl) at 1.5 ml/min flow rate. Collected fractions of 2 ml were analyzed by SDS-PAGE, as described above. Ara h 2-containing fractions were pooled and dialyzed overnight at room temperature against 15 mM ammonium bicarbonate (adjusted to pH 7.0 by bubbling CO<sub>2</sub>), and 2-ml aliquots were lyophilized and stored at –70°C until needed.

### Circular dichroism measurements

To investigate the secondary and tertiary structural differences between native and reduced Ara h 2 protein, their far UV and near UV circular dichroism spectra were recorded at 37°C with a Jasco J710 spectropolarimeter (Easton, MD) using 0.1- and 1.0-cm path-length quartz cuvettes, respectively. Protein concentrations of 0.1 mg/ml for far UV and 1 mg/ml for near UV were used for measurements. Averages of five scans were used for each spectrum with a bandwidth of 2 nm. The scans were then corrected for buffer and smoothed to eliminate background noise. Mean residue ellipticities were referred to a mean residue mass of 115 Da. Secondary structure parameters were calculated using the computer program PROSEC derived from Yang et al. (15).

### Protease digestions of purified proteins

Purified Ara h 2 (50 μM) in 65 mM Tris-HCl, 1 mM EDTA, was adjusted with HCl to pH 8.3 for tryptic and chymotryptic digestions or to pH 2.1 for pepsin digestion. All proteases were obtained from Sigma-Aldrich (St.

Louis, MO). The Ara h 2 protein was incubated at 37°C in the presence of 0.1 μM trypsin, 0.25 μM chymotrypsin, or 10 μM pepsin, and aliquots were taken at timed intervals. The hydrolysis reaction in each aliquot was quenched by the addition of SDS sample buffer. Samples were then subjected to SDS-PAGE and either Coomassie stained or transferred to polyvinylidene difluoride membrane for immunoblot analysis.

### Gel electrophoresis and immunoblotting

For the detection of IgE-binding fragments of Ara h 2, immunoblot analysis was performed using serum IgE from a 16-person pool of peanut-allergic individuals. SDS-PAGE (4–20%)–resolved proteins were transferred to nitrocellulose membrane (0.45 μm; Schleicher & Schuell, Keene, NH) electrophoretically. The membranes were blocked with 2% BSA in TBST 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 <sup>125</sup>I-labeled anti-human IgE secondary Ab (Sanofi, Chaska, MN) and subsequent exposure of x-ray film.

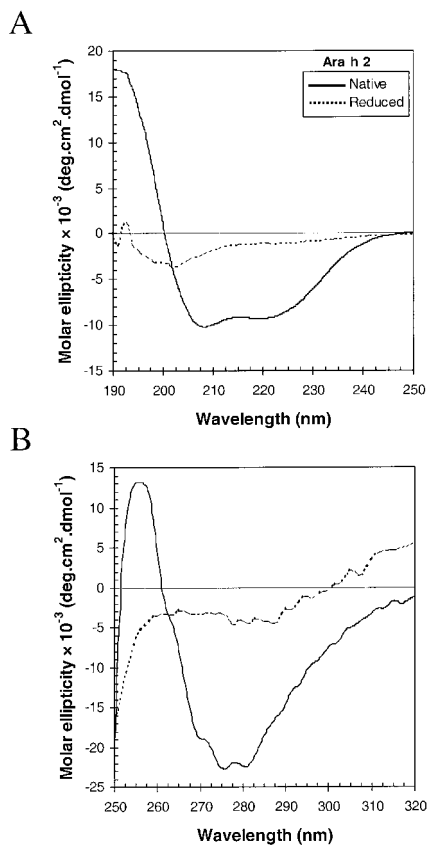
### Amino acid sequence analysis

Purified native Ara h 2 protein was digested with chymotrypsin for varying lengths of time and then electrophoresed on 12% SDS-PAGE gels and blotted to a polyvinylidene difluoride membrane. The blot was stained with Coomassie blue to visualize the 10-kDa peptide, and this area was cut from the blot and sent to the W. M. Keck Molecular Sequencing facility at Yale University (New Haven, CT) for amino-terminal sequencing.

## Results

### Disulfide bonds of the Ara h 2 allergen play a significant role in defining its secondary and tertiary structure

Circular dichroism methods were used to gain a better understanding of the structural properties of Ara h 2 that may contribute to its stability and allergenicity. The Ara h 2 protein does not form any higher order oligomeric structures with itself, but does contain eight cysteine residues that have the potential to form up to four disulfide bonds. To determine whether the disulfide bonds contributed to the secondary or tertiary structure of this protein, circular dichroism measurements were performed in the presence or absence of a reducing agent (DTT). Native or reduced Ara h 2 was monitored either at the far (190–250 nm) or near (250–320 nm) UV ranges, and the molar ellipticity values observed in these UV ranges were plotted (Fig. 1). The best estimates of secondary structure proportions obtained from the far UV data for the native Ara h 2 (Fig. 1A) are 18.2% of the molecule in α-helices, 54% in β-pleated sheet, and 27.7% in a random coil configuration. When the molecule was reduced, there was a significant difference in secondary structure fractions. Reduced Ara h 2 exhibits a secondary structure predominated by β-pleated sheet (82.3%), with the remainder of the molecule mostly in a random coil configuration. Even before estimating the secondary and tertiary structure parameters, it became apparent from the spectrum that in the reduced Ara h 2 the wavelength minima at 207 and 222 nm and the maximum at 191 nm, typical characteristics of α-helical configuration, were absent. The near UV circular dichroism spectra of a protein is generally believed to offer insight into the tertiary structure formed by folding of the secondary structural elements as well as by their subsequent packing to form a compact three-dimensional structure. The phenylalanine, tyrosine, and tryptophan fine structures contribute to the near UV spectra. The intensity differs due to the presence or lack of the rigidity of the protein, with more highly mobile side chains having lower intensities or interactions between aromatic amino acids. The maxima between 250 and 265 nm arise from phenylalanine residues. The remaining transitions between 270 and 290 nm arise from tyrosine and tryptophan residues. Most noteworthy is the observation that all the above transitions were absent after reduction of the Ara h 2, suggesting dramatically different tertiary structure (Fig. 1B).

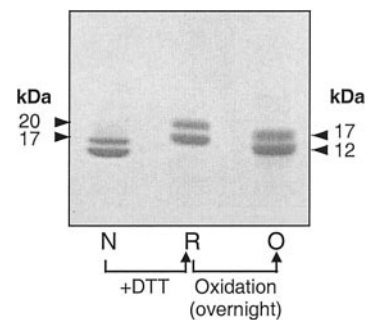


**FIGURE 1.** Disulfide bonds play an important role in defining Ara h 2 protein structure. *A*, Circular dichroism spectrum of Ara h 2 protein structure in the presence or absence of a reducing agent. The molar ellipticity of the native or reduced Ara h 2 protein is shown as a function of wavelength (190–250 nm). *B*, Circular dichroism spectrum of Ara h 2 protein structure in the presence or absence of a reducing agent. The molar ellipticity of the native or reduced Ara h 2 protein is shown as a function of wavelength (250–320 nm).

The importance of the disulfide bonds to Ara h 2 protein structure was further confirmed by denaturing SDS-PAGE electrophoresis of native, reduced, and reoxidized allergen. In this experiment, native Ara h 2 was first reduced with DTT, and then a portion of the sample was allowed to reoxidize by removal of the reducing agent. All samples were electrophoresed on 4–20% SDS-polyacrylamide gels, and then the gel was stained with Coomassie brilliant blue R. Previous work had shown that the reduced Ara h 2 protein migrated as a doublet on SDS-PAGE gels with an average molecular mass of 17 kDa (8). As shown in Fig. 2, the native, nonreduced Ara h 2 protein migrated as a doublet protein with an average molecular mass of 12 kDa. In contrast, the reduced Ara h 2 migrated as a slightly larger doublet with an average molecular mass of 17 kDa. The reduced Ara h 2 protein that was allowed to reoxidize migrated as a mixture of these two forms. These results support the circular dichroism measurements and provide a visual confirmation of the importance disulfide bonds play in defining overall Ara h 2 structure.

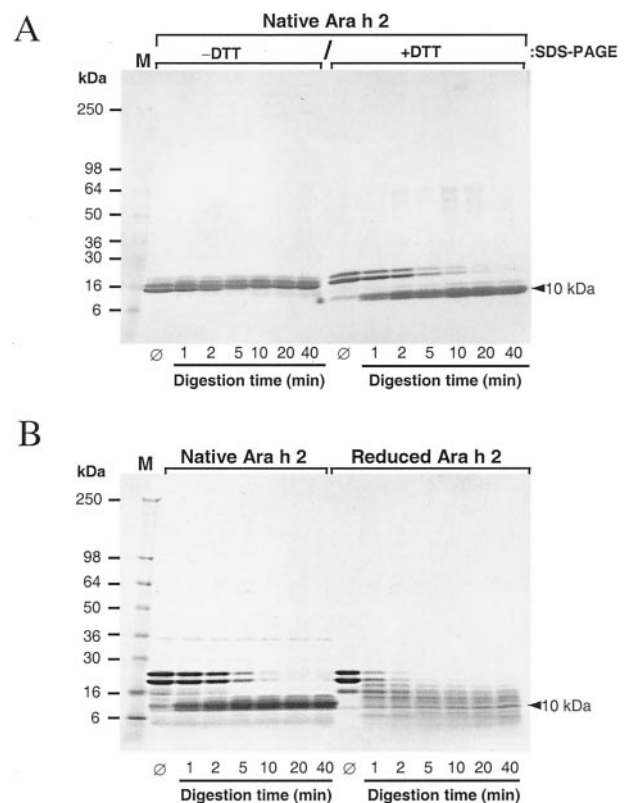
*Reduction of disulfide bonds in the native Ara h 2 protein leads to increased susceptibility to proteolysis*

The Ara h 2 protein was exposed to proteases encountered in the GI tract to determine whether the native protein structure, as mediated by disulfide bonds, played any role in protecting it from degradation. Native Ara h 2 protein was exposed to trypsin, chy-



**FIGURE 2.** SDS-PAGE electrophoresis of native (N), reduced (R), and reoxidized (O) Ara h 2 protein. The native Ara h 2 protein migrates in 4–20% SDS-PAGE gels as a doublet with an average molecular mass of ~12 kDa. When the protein is reduced, it migrates with an average molecular mass of ~17 kDa. When the reduced Ara h 2 protein is allowed to reoxidize, there is a mixture of molecules migrating at 12 and 17 kDa.

motrypsin, or pepsin, and then, after deactivating the protease, half of the sample was reduced with DTT and electrophoresed on 4–20% SDS-PAGE gels. The results of Ara h 2 digestion with trypsin are shown in Fig. 3A. Digestion of Ara h 2 with either

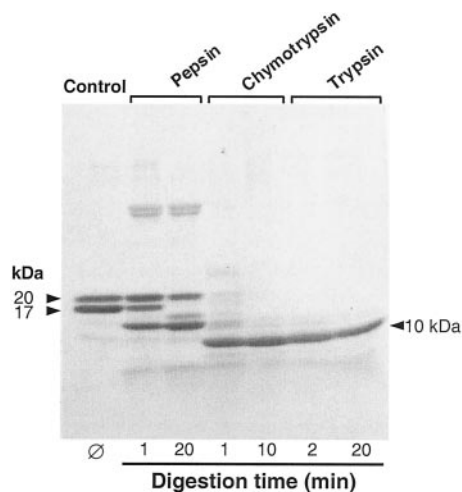


**FIGURE 3.** Digestion of native Ara h 2 with trypsin reveals a 10-kDa protease-resistant fragment. *A*, Native Ara h 2 was digested with trypsin for up to 40 min, and samples taken at different times were then electrophoresed on 4–20% SDS-PAGE gels in the presence or absence of a reducing agent (DTT). The migration of molecular mass standards is indicated along the left side of the gel (kDa). The arrowhead indicates the presence of a 10-kDa protease-resistant Ara h 2 fragment that is in the samples treated with DTT after protease digestion. *B*, Native or reduced Ara h 2 was digested with trypsin for up to 40 min, and samples taken at different times were then electrophoresed on 4–20% SDS-PAGE gels in the presence of a reducing agent. The migration of molecular mass standards is indicated along the left side of the gel (kDa). The arrowhead indicates the presence of a 10-kDa protease-resistant Ara h 2 fragment in the native Ara h 2 samples.

chymotrypsin or pepsin gave essentially similar results (data not shown). The nonreduced Ara h 2 protein showed little change in its migration on polyacrylamide gels even after 40 min of enzyme digestion. However, when the disulfide bonds of the digested protein are reduced, the characteristic protein doublet disappears and a prominent 10-kDa protein fragment is obvious after only a short digestion time. The 10-kDa protein fragment was resistant to digestion for the length of the experiment.

The results described above suggest that the Ara h 2 disulfide bonds may be important in the resistance of this allergen to digestion with proteases commonly encountered in the GI tract. To address this question directly, Ara h 2 was digested with trypsin in the presence or absence of a reducing agent and then electrophoresed on 4–20% SDS-PAGE gels, and the resulting peptides were visualized by staining with Coomassie (Fig. 3B). The native Ara h 2 protein digested with trypsin produced the 10-kDa protein fragment previously observed that was stable for the length of the experiment. In contrast, the Ara h 2 protein that was first reduced and then digested with trypsin did not produce any significant enzyme-resistant protein fragment and appeared to be much more susceptible to the action of the protease when compared with the native protein. Similar results were obtained with chymotrypsin and pepsin digestions (data not shown).

Because most proteins that enter the GI tract encounter not one, but three proteases in succession, the ability of the 10-kDa Ara h 2 fragment to resist proteolysis from all three proteases was tested. In this experiment, purified native Ara h 2 was exposed to pepsin, then chymotrypsin, and finally trypsin at concentrations and for a duration of time that digested reduced Ara h 2 to small nondescript fragments (compare Fig. 3B with Fig. 4). Fig. 4 shows that the 10-kDa Ara h 2 fragment is resistant to proteolysis by the three proteases when they are allowed to digest the protein one after the other.



**FIGURE 4.** Native Ara h 2 is resistant to degradation even after digestion with three proteases. Native Ara h 2 was digested with pepsin for 20 min, chymotrypsin for 10 min, and finally trypsin for 20 min, and samples taken at different times were then electrophoresed on 4–20% SDS-PAGE gels in the presence of a reducing agent (DTT). The migration of undigested native Ara h 2 is indicated along the left side of the gel (arrowheads). The arrowhead on the right side of the gel indicates the presence of a 10-kDa protease-resistant Ara h 2 fragment that is in the samples after digestion with all three proteases.

#### *Urea has no effect on the ability of Ara h 2 to resist degradation by proteases*

The role of Ara h 2 protein structures, mediated by forces other than covalent disulfide bonds, to stabilize this protein against the actions of proteases was also tested. Native Ara h 2 was treated with 1 M urea, a denaturant that disrupts protein structure mediated by hydrogen-bonding interactions, and then exposed to chymotrypsin. The appearance of the 10-kDa resistant peptide fragment was assessed by SDS-PAGE gel electrophoresis and staining with Coomassie. As shown in Fig. 5, 1 M urea had no effect on the stability of the 10-kDa protease-resistant fragment. Only after the disulfide bonds were reduced with DTT did the Ara h 2 protein become susceptible to degradation.

#### *Protease-resistant Ara h 2 fragments contain the immunodominant IgE-binding epitopes*

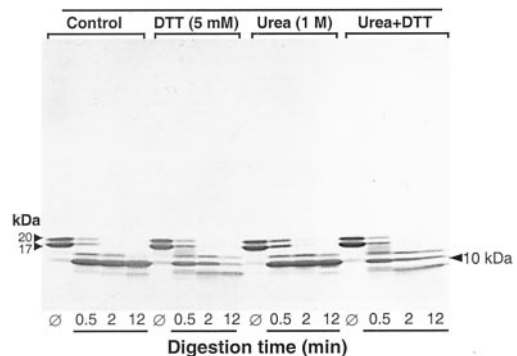
To determine whether the most protease-resistant Ara h 2 fragments contained IgE-binding epitopes, the protein was exposed to chymotrypsin and the reactions were electrophoresed on SDS-PAGE gels, blotted to nitrocellulose, and probed with serum IgE from a pool of peanut-sensitive patients (Fig. 6A). The 10-kDa protease-resistant peptide contained intact binding sites that could be recognized by IgE. Knowing that Ara h 2 contains 10 IgE binding sites that are evenly distributed along the linear sequence of the molecule (10), these results suggest that this fragment of Ara h 2 contains multiple IgE-binding epitopes and survives digestion by the GI enzymes tested.

The 10-kDa protease-resistant fragment was purified, and amino-terminal sequencing was performed to identify what portion of the allergen this peptide represented. The amino acid sequence indicated that the 10-kDa fragment begins at aa position 23 and contains ~90 aa. This portion of the Ara h 2 protein contains IgE-binding epitopes 2–7 and 6 of 8 of the cysteine residues (Fig. 6B). Interestingly, this fragment also contains 11 potential chymotrypsin cleavage sites.

## Discussion

Analysis of a variety of allergenic foods has resulted in the identification of certain biochemical characteristics that are shared by most, but not necessarily all food allergens (16). One characteristic that appears to be shared by most food allergens is that they are extremely stable proteins resistant to denaturation. Resistance to

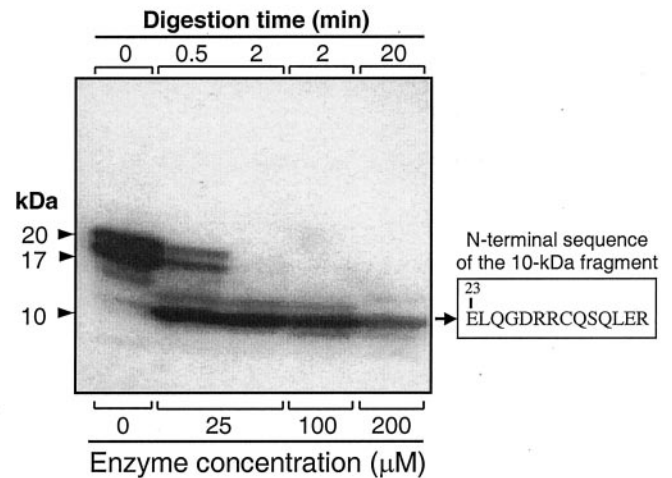
#### *Chymotrypsin digestion conditions of native Ara h 2*



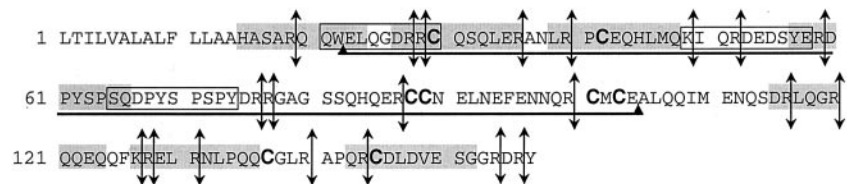
**FIGURE 5.** Hydrogen-bonding interactions do not appear to play a role in allergen stability. Native Ara h 2 was digested with chymotrypsin in the presence of a reducing agent (DTT), a denaturant (Urea), or both (Urea + DTT). The arrowhead indicates the presence of a 10-kDa protease-resistant Ara h 2 fragment present in the control and urea-treated samples.

A

**FIGURE 6.** The 10-kDa Ara h 2 protease-resistant fragment contains the immunodominant IgE-binding epitopes and multiple potential protease cleavage sites. *A*, Native Ara h 2 was digested with chymotrypsin for different lengths of time (digestion time) with different amounts of enzyme (enzyme concentration). In addition to a control sample (0 time), four separate reactions were performed, each with a single chymotrypsin concentration for a single time: 25  $\mu$ M chymotrypsin for 0.5 min, 25  $\mu$ M chymotrypsin for 2 min, 100  $\mu$ M chymotrypsin for 2 min, and 200  $\mu$ M chymotrypsin for 20 min. To each reaction, SDS sample buffer was added to inactivate the enzyme. DTT was then added to reduce the protein; the samples were electrophoresed on 4–20% SDS-PAGE gels, immunoblotted to nitrocellulose membrane, and probed with serum IgE from a pool of peanut-sensitive patients. *B*, Amino acid sequence of Ara h 2 presented as the one-letter amino acid code. Boxes indicate IgE binding sites. Arrows indicate the trypsin digestion sites. Cysteines are bolded, and the 10-kDa protease-resistant fragment is underlined.



B



denaturation of food allergens is thought to be an important characteristic because the longer significant portions of the protein remain intact the more likely it is to trigger an immune response (17). However, this property is not a predominant characteristic of aeroallergens, primarily because their route of sensitization is through the respiratory tract. The observation that many of the food allergens are proteins containing intramolecular disulfide bonds that may be important to their allergenicity (18) has led to the assumption that protein structure may be an important factor in an allergen's ability to resist denaturation. This assumption was tested using an allergen in milk,  $\beta$ -lactoglobulin (BLG). BLG contains two disulfides (19) that, when disrupted by site-directed mutagenesis, changed its structure (20) and the accessibility of IgG- and IgE-binding epitopes (21, 22). The disruption of disulfide bonds in BLG also had an impact on the sensitivity of this protein to digestion with pepsin and its overall allergenicity with respect to skin test responses and GI symptoms in a sensitized dog model of food allergy (12).

We have shown that the overall structure of another food allergen, Ara h 2, also is dramatically changed when disulfide bonds are reduced. Interestingly, the Ara h 2 structure is not completely randomized when the disulfide bonds are reduced, but instead is predominated by a  $\beta$ -pleated sheet and  $\beta$ -turn configuration. These results indicate that the Ara h 2 molecule is a very ordered protein even without its disulfide bonds being intact. In addition, reduced Ara h 2 becomes susceptible to rapid digestion with pepsin, chymotrypsin, or trypsin, indicating a reduction in its overall allergenicity. Models of digestion are commonly used to assess the stability of dietary protein (7, 23, 24). A digestion model using simulated gastric fluid was adapted to evaluate the allergenic potential of dietary proteins (7). In this model, stability to digestion by pepsin has been used as criterion for distinguishing food allergens from safe, nonallergenic dietary proteins. Although these digestibility models are representative of human digestion, they are

not designed to predict the  $t_{1/2}$  of a protein in vivo. Likewise, the in vitro digestion conditions used in this study cannot predict the stability of a protein in vivo; however, they are useful in identifying regions of the protein that are more resistant to protease digestion than other portions of the allergen or other nonallergen proteins. In this manner, the fragments of the allergen most likely to survive the longest in the mammalian GI tract can be identified and studied.

The observation that reduction of disulfide bonds reduces overall allergenicity has led some investigators to propose a molecular genetic approach to the problem of reducing the allergenic potential of some plant proteins. This approach uses a family of 12-kDa proteins called thioredoxins that undergo reversible redox changes through a catalytically active disulfide site (25–29). Thioredoxins have been shown to reduce intramolecular disulfide bonds from a wide variety of proteins, many of which are considered allergens (30–32). Buchanan and colleagues (11, 12) used the biological activity of this ubiquitous protein to determine whether they could reduce the allergenic potential of wheat and milk allergens. Briefly, the authors exposed either the purified allergens or an extract from the food source containing the allergens to thioredoxin purified from *Escherichia coli* and then performed skin tests and monitored GI symptoms in a sensitized dog model. Allergens that had their disulfide bonds reduced by thioredoxin showed greatly reduced skin tests and GI symptoms. These results indicate that it may be possible to approach the problem of food allergens, particularly allergens in cultivated crops, by constructing transgenic cell lines that overproduce thioredoxin. The advantage of using thioredoxin is that it is a general approach that will be useful for reducing the allergenicity of any food crop whose allergens depend on disulfide bonds for their activity. However, the approach may be somewhat limited, especially for those food allergens whose IgE-binding epitopes are not dependent on intact disulfide bonds for them to elicit an allergic response. In fact, when extracts from

peanut seeds were incubated with thioredoxin and then treated with monobromobimane to label sulfhydryl groups, one of the proteins identified was Ara h 2. However, Ara h 1, another major peanut allergen, was not affected by this treatment (33). Previously, we had shown that Ara h 1 can form a stable trimer complex that may afford the molecule some protection from protease digestion and denaturation, allowing passage of Ara h 1 containing several intact IgE-binding epitopes across the small intestine, contributing to its overall allergenicity (34). Collectively, these results provide additional evidence that protein structure, either mediated through disulfide bonds or through higher order protein-protein interactions, plays a critical role in the allergenicity of peanut allergens.

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