

Identification and Mutational Analysis of the Immunodominant IgE Binding Epitopes of the Major Peanut Allergen *Ara h 2*

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A major peanut allergen, *Ara h 2*, is recognized by serum IgE from >90% of patients with peanut hypersensitivity. Biochemical characterization of this allergen indicates that it is a glycoprotein of ~17.5 kDa. Using N-terminal amino acid sequence data from purified *Ara h 2*, oligonucleotide primers were synthesized and used to identify a clone (741 bp) from a peanut cDNA library. This clone was capable of encoding a 17.5-kDa protein with homology to the conglutin family of seed storage proteins. The major linear immunoglobulin E (IgE)-binding epitopes of this allergen were mapped using overlapping peptides synthesized on an activated cellulose membrane and pooled serum IgE from 15 peanut-sensitive patients. Ten IgE-binding epitopes were identified, distributed throughout the length of the *Ara h 2* protein. Sixty-three percent of the amino acids represented in the epitopes were either polar uncharged or apolar residues. In an effort to determine which, if any, of the 10 epitopes were recognized by the majority of patients with peanut hypersensitivity, each set of 10 peptides was probed individually with serum IgE from 10 different patients. All of the patient sera tested recognized multiple epitopes. Three epitopes (aa27-36, aa57-66, and aa65-74) were recognized by all patients tested. In addition, these three peptides bound more IgE than all the other epitopes combined, indicating that they are the immunodominant epitopes of the *Ara h 2* protein. Mutational analysis of the *Ara h 2* epitopes indicate that single amino acid changes result in loss of IgE binding. Two epitopes in region aa57-74 contained the amino acid sequence DPYSP that appears to be necessary for IgE binding. These results may allow for the design of im-

proved diagnostic and therapeutic approaches to peanut hypersensitivity. © 1997 Academic Press

Immediate hypersensitivity reactions to foods occur in 6–8% of children and about 1% of adults (1, 2) and are mediated by the production of immunoglobulin E (IgE)² antibodies to glycoproteins present in the food (3). Peanuts are a major cause of serious allergic reactions in both children and adults. The hypersensitivity to peanuts often starts in childhood and continues throughout life. This is in contrast to other childhood food allergies such as to milk and eggs which generally resolve spontaneously with age (4). In addition, peanut allergy is more likely to cause fatal anaphylaxis than any other food allergy (5–7). Currently, avoidance is the only effective means of dealing with food allergy, but the use of peanuts and peanut by-products as supplements in many different foods makes accidental consumption almost inevitable. Thus, the prevalence and chronic nature of peanut allergy, the potential severity of the allergic reaction, and the widespread use of peanuts in consumer foods necessitates improved methods for managing peanut hypersensitivity.

Food hypersensitivity reactions occur shortly after contact of a specific allergen with its corresponding IgE antibodies which are bound to mast cells. Cross-linking of the allergen-specific IgE by the respective allergen activates the mast cells to release histamine, heparin, and other mediators responsible for the clinical symptoms observed. Thus, the IgE-binding epitopes of the allergens play an important role in the disease process.

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² Abbreviations used: IgE, immunoglobulin E; DMF, *N,N*-dimethylformamide; TBS, Tris-buffered saline.

If improved diagnostic and therapeutic capabilities are to be developed, it is important to determine the primary structure of the major allergens, the IgE-binding sites of these allergens, and the frequency of recognition of any IgE-binding epitopes. Characterization of allergens will provide a better understanding of the human immune response involved in food hypersensitivity reactions.

Several studies have shown that the most allergenic portion of the peanut is the protein fraction of the cotyledon (8–10). Two highly abundant glycoproteins found in the cotyledon are the peanut allergens, *Ara h 1* and *Ara h 2*. These proteins are recognized by serum IgE from >90% of peanut-sensitive patients, thus establishing them as important allergens (11, 12). The majority of serum IgE recognition of the *Ara h 1* and *Ara h 2* allergens appear to be due to epitopes within these proteins that are linear amino acid sequences that do not contain significant amounts of carbohydrate (13). We have recently described the cloning of the gene encoding the *Ara h 1* allergen and identified it as a seed storage protein belonging to the vicilin family of legume storage proteins (13).

In this paper we report the cloning and nucleotide sequence of the second major peanut allergen, *Ara h 2*. The derived amino acid sequence has been used to construct synthetic peptides and perform a detailed examination of the linear IgE binding epitopes of this protein. These results may allow for the design of improved diagnostic and therapeutic approaches to peanut hypersensitivity.

MATERIALS AND METHODS

Serum IgE. Serum from 15 patients with documented peanut hypersensitivity (mean age, 25 years) was used to identify peanut allergens. Each of these individuals had a positive immediate skin prick test to peanut and either a positive double-blind, placebo-controlled, food challenge or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension). Details of the challenge procedure and interpretation have been discussed previously (11). Representative individuals with elevated serum IgE levels (who did not have peanut specific IgE or peanut hypersensitivity) were used as controls in these studies. At least 5 ml of venous blood was drawn from each patient and allowed to clot, and the serum was collected. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences.

Isolation and amino acid sequence analysis of peanut allergen *Ara h 2*. *Ara h 2* was purified to near homogeneity from whole peanut extracts according to the methods of Burks *et al.* (12). Purified *Ara h 2* was electrophoresed on 12.5% acrylamide mini-gels (Bio-Rad Laboratories, Hercules, CA) in Tris/SDS/glycine buffer. The gels were stained with 0.1% Coomassie blue in 10% acetic acid and 50% methanol and destained in 40% methanol for 3 h with continuous shaking. Gel slices containing *Ara h 2* were sent to the W. M. Keck Foundation (Biotechnology Resource Laboratory, Yale University, New Haven, CT) for amino acid sequencing. Amino acid sequencing of intact *Ara h 2* and tryptic peptides of this protein was performed on an Applied Biosystems sequencer with an on-line HPLC column that was eluted with increasing concentrations of acetonitrile.

Peanut RNA isolation and Northern (RNA) gels. Three commercial lots from the 1979 crop of medium grade peanut species, *Arachis hypogaea* (Florunner), were obtained from North Carolina State University for this study. Total RNA was isolated from 1 g of this material according to procedures described by Larsen (14). Poly(A)⁺ RNA was isolated using a purification kit (Collaborative Research, Bedford, MA) according to manufacturer's instructions. Poly(A)⁺ RNA was subjected to electrophoresis in 1.2% formaldehyde agarose gels, transferred to nitrocellulose, and hybridized with ³²P-labeled probes according to the methods of Bannon *et al.* (15).

Computer analysis of *Ara h 2* sequence. Sequence analysis of the *Ara h 2* gene was done on the University of Arkansas for Medical Science's Vax computer using the Wisconsin DNA analysis software package. The algorithm of Needleman and Wunsch was used to align the complete amino acid sequence of *Ara h 2* with homologous proteins before determining the percent identity.

cDNA expression library construction and screening. Peanut poly(A)⁺ RNA was used to synthesize double-stranded cDNA according to the methods of Watson and Jackson (17) and Huynh *et al.* (18). The cDNA was treated with *EcoRI* methylase and then ligated with *EcoRI* and *XhoI* linkers. The DNA was then ligated with *EcoRI*–*XhoI* cut, phosphatase treated λ-ZAP XR phage arms (Stratagene, LaJolla, CA), and *in vitro* packaged. The library was 95% recombinants carrying insert sizes >400 bp. The library was screened using an IgE antibody pool consisting of an equal volume of serum from each patient with peanut hypersensitivity. Detection of primary antibody was with I¹²⁵-labeled anti-IgE antibody performed according to the manufacturer's instructions (Sanofi, Chaska, MN).

PCR amplification of the *Ara h 2* mRNA sequence. Using the oligonucleotide CA(AG)CA(AG)TGGGA(AG)TT(AG)CA(AG)GG(N)-GA(TC)AG derived from amino acid sequence analysis of the *Ara h 2* peanut allergen as one primer and a 23-nt primer which hybridizes to the Bluescript vector, the cDNA that encodes *Ara h 2* was amplified from the IgE-positive clones. Reactions were carried out in a buffer containing 3 mM MgCl₂, 500 mM KCl, and 100 mM Tris–HCl, pH 9.0. Each cycle of the polymerase chain reaction consisted of 30 s at 95°C, followed by 1 min at 56°C, and 2 min at 72°C. Thirty cycles were performed with both primers present in all cycles. From this reaction, a clone carrying an approximately 700-bp insert was identified.

DNA sequencing and analysis. DNA sequencing was done according to the methods of Sanger *et al.* (19) using either ³²P-end labeled oligonucleotide primers or a automated ABI model 377 DNA sequencer using fluorescently tagged nucleotides. Most areas of the clone were sequenced at least twice and in some cases in both directions to ensure an accurate nucleotide sequence for the *Ara h 2* gene.

Peptide synthesis. Individual peptides were synthesized on a derivatised cellulose membrane using Fmoc amino acid active esters according to the manufacturer's instructions (Genosys Biotechnologies, Woodlands, TX). Fmoc–amino acid derivatives were dissolved in 1-methyl-2-pyrrolidone and loaded on marked spots on the membrane. Coupling reactions were followed by acetylation with a solution of 4% (v/v) acetic anhydride in *N,N*-dimethylformamide (DMF). After acetylation, Fmoc groups were removed by incubation of the membrane in 20% (v/v) piperidine in DMF. The membrane was then stained with bromophenol blue to identify the location of the free amino groups. Cycles of coupling, blocking, and deprotection were repeated until the peptides of the desired length were synthesized. After addition of the last amino acid in the peptide, the amino acid side chains were deprotected using a solution of dichloromethane/trifluoroacetic acid/triisobutylsilane (1/1/0.05). Membranes were either probed immediately or stored at –20°C until needed.

IgE binding assay. Cellulose membranes containing synthesized peptides were washed with Tris-buffered saline (TBS) and then incubated with blocking solution overnight at room temperature. After blocking, the membranes were incubated with serum from patients with peanut hypersensitivity diluted (1:5) in a solution containing TBS and 1% bovine serum albumin for at least 12 h at 4°C or 2 h at

TABLE II
Ara h 2 Sequence Similarities

Protein	Source	% Similar
Conglutin- δ	Lupin	39
Mabinlin I (chain B)	Caper	32-35
2S albumin	Sunflower	34
	Castor bean	30
α -Amylase inhibitor	Wheat	29
CM3 protein	Wheat	27

Note. The *Ara h 2* nucleotide and derived amino acid sequences were used to search the GenBank, Swiss-Prot, and EMBL databases for any homologous proteins. The table lists the proteins that had the highest similarity to the *Ara h 2* sequence, the plant source of those proteins, and the percentage similarity between that protein and *Ara h 2*.

and ending with a TAA stop codon at nucleotide position 474 (Fig. 1). The calculated size of the protein encoded by this open reading frame was ~ 17.5 kDa, which is in good agreement with the molecular weight of *Ara h 2* that has been determined experimentally

(12). With the exception of a single cysteine residue at position 30, the amino acid sequence that was determined from the purified *Ara h 2* protein (Table I) was found in this clone. The additional coding region on the amino terminal end of this clone probably represents a signal peptide which would be cleaved from the mature *Ara h 2* allergen.

To determine what size mRNA this clone identified, a ^{32}P -labeled insert was used as a hybridization probe of a Northern blot containing peanut poly(A) $^+$ RNA (data not shown). This insert hybridized to an ~ 0.7 -kb mRNA. The size of the cloned insert and the size of the mRNA were in good agreement. In addition, there was good agreement between the calculated and determined size of the *Ara h 2* protein. Furthermore, the identity of the determined amino acid sequence from the *Ara h 2* peptides agreed with that which was determined from the clone. From these data we concluded that an *Ara h 2* specific clone had been isolated.

Peanut allergen Ara h 2 is a conglutin-like seed storage protein. A search of the GenBank, Swiss-Prot, and EMBL databases revealed significant amino acid sequence homology between the *Ara h 2* protein and

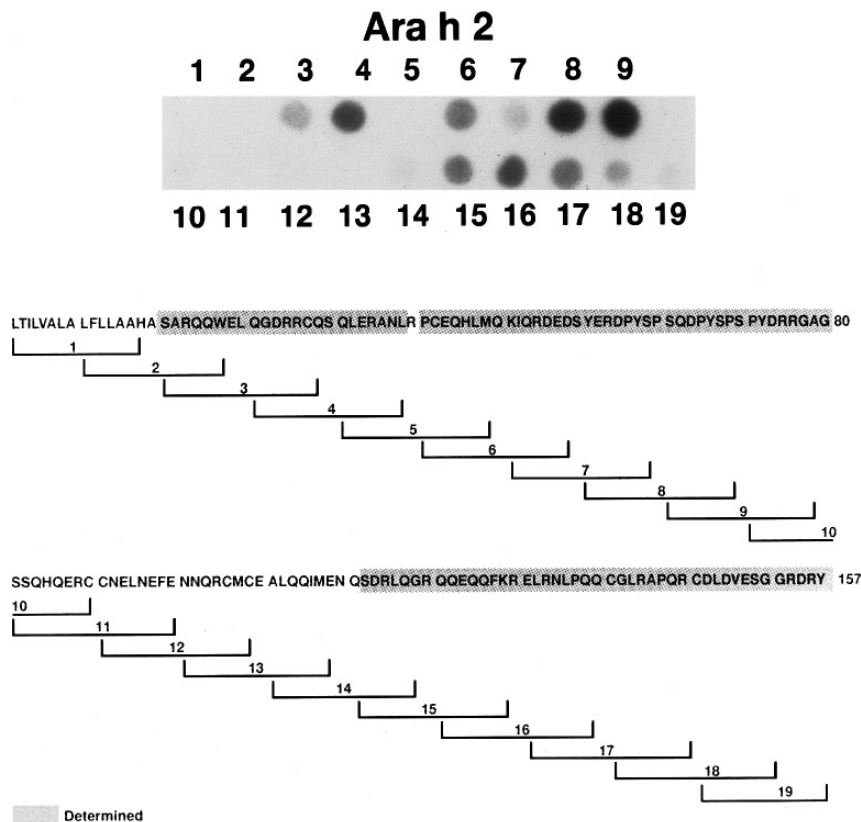


FIG. 2. Multiple IgE-binding sites identified in the *Ara h 2* allergen. Epitope analysis was performed on the *Ara h 2* allergen by synthesizing 15 amino acid long peptides, offset from each other by 8 amino acids for the entire protein molecule. These peptides, represented as spots 1-19, were then probed with a serum pool consisting of 15 patients with documented peanut hypersensitivity. The *Ara h 2* amino acid sequence is shown as the one-letter amino acid code in the bottom half of this figure. The shaded boxes (D1-D3) correspond to the determined IgE binding regions.

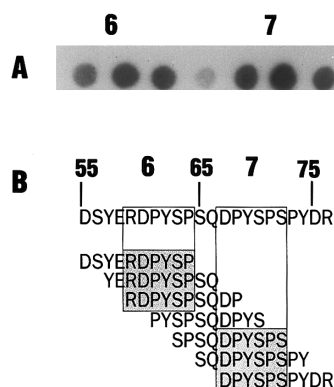


FIG. 3. Core IgE-binding epitopes identified on the *Ara h 2* allergen. Epitope analysis was performed on the IgE-binding sites identified in Fig. 2 by synthesizing 10-amino-acid-long peptides offset by two amino acids. These peptides were then probed with the 15-patient serum pool. (A) The peptide analysis of *Ara h 2* amino acid residues 55–76. This region contains peptides 6 and 7 identified in Table III. (B) The amino acid sequence of this region.

seed storage proteins from a variety of different plants (Table II). The highest percent identity (40%) was observed between the *Ara h 2* protein and conglutin- δ , a sulfur-rich protein from the lupin seed (20). 2S albumins and mabinlins also had a high degree of homology (30–35%) with the *Ara h 2* protein sequence (21). Interestingly, the *Ara h 2* protein had some similarity (26–29%) with α -amylase inhibitors from wheat (22, 23), which are the major allergens in baker's asthma (24, 25) and are important allergens in patients experiencing hypersensitivity reactions following the ingestion of wheat protein (26).

Multiple IgE binding epitopes on the *Ara h 2* protein. Nineteen overlapping peptides representing the derived amino acid sequence of the *Ara h 2* protein were synthesized to determine which regions were recognized by serum IgE. Each peptide was 15 amino acids long and was offset from the previous peptide by 8 amino acids. In this manner, the entire length of the *Ara h 2* protein could be studied in large overlapping fragments. These peptides were then probed with a pool of serum from 15 patients with documented peanut hypersensitivity or serum from a representative control patient with no peanut hypersensitivity. Serum IgE from the control patient did not recognize any of the synthesized peptides (data not shown). In contrast, Fig. 2 shows that there are three IgE binding regions along the entire length of the *Ara h 2* protein that are recognized by this population of patients with peanut hypersensitivity. These IgE-binding regions represent amino acid residues 17–39, 41–80, and 114–157.

In order to determine the exact amino acid sequence of the IgE binding regions, small peptides (10 amino acids long offset by two amino acids) representing the larger IgE-binding regions were synthesized. In this manner it was possible to identify individual IgE-binding

epitopes within the larger IgE-binding regions of the *Ara h 2* molecule (Fig. 3). The 10 IgE-binding epitopes that were identified in this manner are shown in Table III. The size of the epitopes ranged from 6 to 10 amino acids in length. Three epitopes (aa15–24, aa21–30, and aa27–36), which partially overlapped with each other, were found in the region of amino acid residues 17–39. Four epitopes (aa39–48, aa49–58, aa57–66, and aa65–74) were found in region 41–80. Finally, three epitopes (aa115–124, aa127–136, and aa143–152) were found in the region represented by amino acid residues 114–157. Sixty-three percent of the amino acids represented in the epitopes were either polar uncharged or apolar residues. There was no obvious amino acid sequence motif that was shared by all the epitopes, with the exception of epitopes 6 and 7, which contained the sequence DPYSP.

Identification of the immunodominant *Ara h 2* epitopes. In an effort to determine which, if any, of the 10 epitopes was immunodominant, each set of 10 peptides was probed individually with serum IgE from 10 different patients. Five patients were randomly selected from the pool of 15 patients used to identify the common epitopes, and 5 patients were selected from outside this pool. Figure 4A shows an immunoblot strip containing these peptides that has been incubated with an individual patient's serum. This patient's serum IgE recognized peptides 1, 3, 4, 6, and 7. The remaining patients serum IgE were tested in the same manner and the intensity of IgE binding to each spot was determined as a function of that patient's total IgE binding to these 10 epitopes (Fig. 4B) All of the patient sera tested (10/10) recognized multiple peptides. Epitopes 3, 6, and 7 were recognized by serum IgE of all patients tested (10/10). In addition, serum IgE that recognizes these peptides represent the majority of *Ara h 2*-specific IgE found in these patients. These results indicate

TABLE III
Ara h 2 IgE Binding Epitopes

Peptide	AA Sequence	<i>Ara h 2</i> position
1	HASARQWEL	15–24
2	QWELQDRR	21–30
3	DRRCQSQLER	27–36
4	LRPCEQHLMQ	39–48
5	KIQRDEDSYE	49–58
6	YERDPYSPSQ	57–66
7	SQDPYSPSPY	65–74
8	DRLQGRQQEQ	115–124
9	KRELRLNPQQ	127–136
10	QRCDLDVESG	143–152

Note. The *Ara h 2* IgE-binding epitopes (1–10) are indicated as the bold, underlined, single-letter amino acid code. The position of each peptide with respect to the *Ara h 2* protein is indicated in the right-hand column.

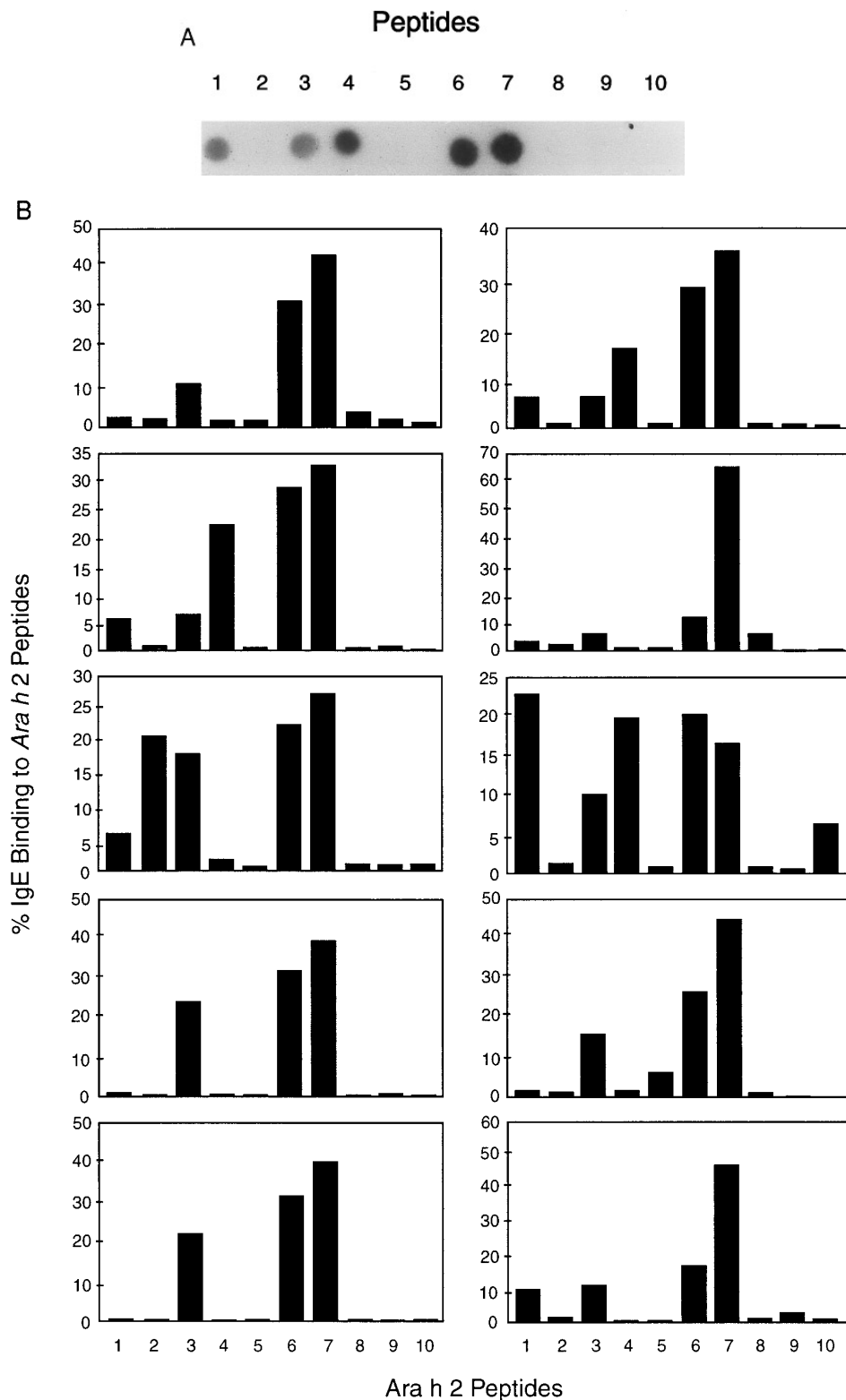


FIG. 4. Identification of the immunodominant *Ara h 2* epitopes. IgE-binding peptides (1–10) were synthesized as 10 amino acid peptides and probed with serum IgE from 10 patients with documented peanut hypersensitivity. IgE binding to individual peptides was detected by ^{125}I -labeled anti-human IgE. (A) A representative SPOTS membrane containing *Ara h 2* epitopes 1–10 and probed with serum IgE from a single patient. (B) The relative intensity of IgE binding to each peptide by individual patients was then determined by densitometry and expressed as a percentage of that patient's total IgE binding to all of the *Ara h 2* peptides. Five patients were selected at random from the 15-patient serum pool and 5 were patients with peanut hypersensitivity that were not included in the pool.

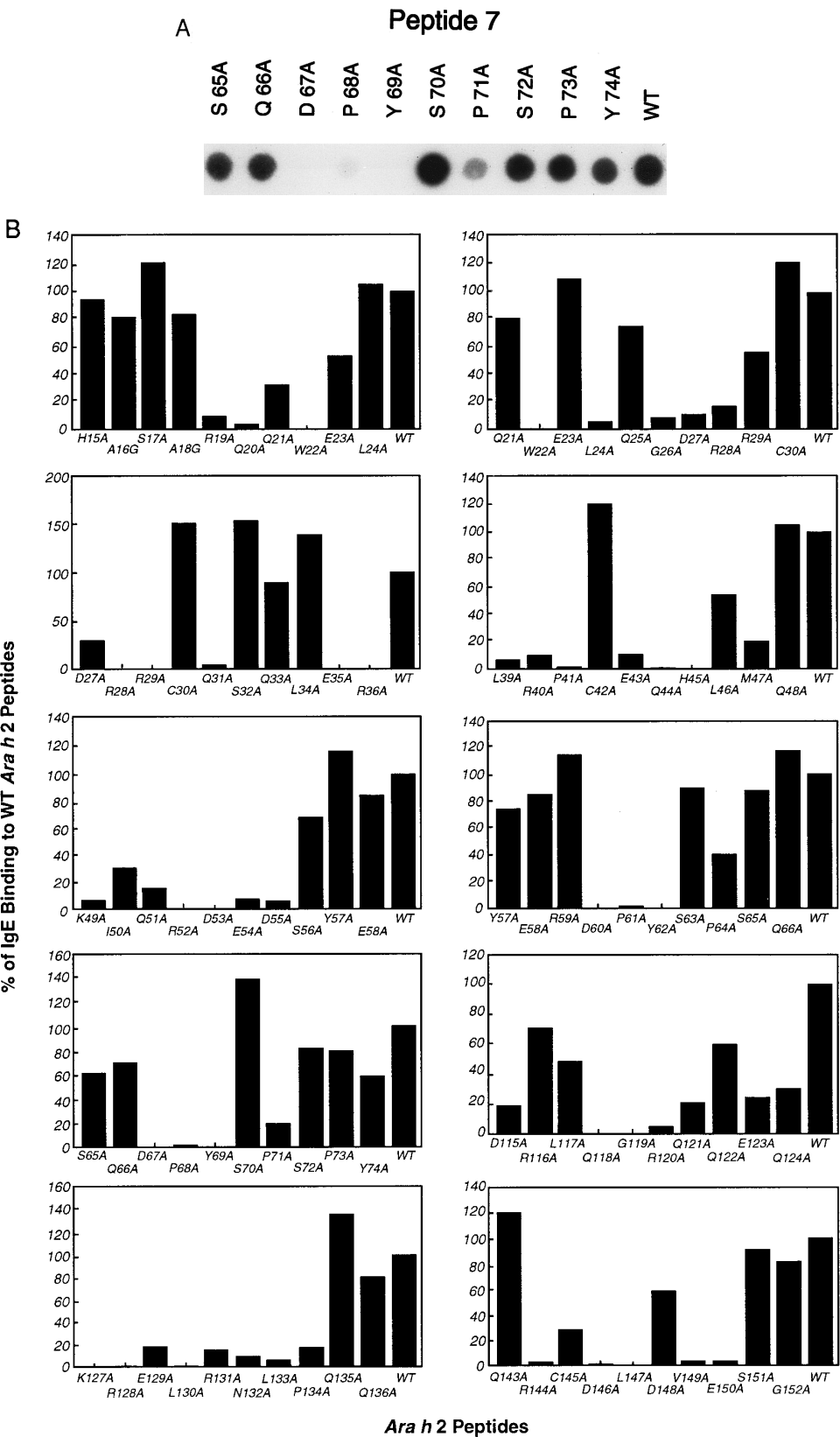


FIG. 5. All of the *Ara h 2* peptides can be mutated to non-IgE-binding epitopes by single amino acid changes. All of the *Ara h 2* epitopes (peptides 1–10) were synthesized with an alanine residue substituted for one of the amino acids and then probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. IgE binding to individual peptides was detected by ¹²⁵I-labeled anti-human IgE.

that peptides 3, 6, and 7 contain the immunodominant epitopes of the *Ara h 2* protein.

The Ara h 2 peptides can be mutated to non-IgE-binding epitopes by single amino acid changes. To assess the importance of individual amino acids in each of the *Ara h 2* epitopes they were synthesized as 10 amino acid residue peptides with alanine residues being substituted one at a time for each of the amino acids in the epitope. These peptides were then probed with pooled serum IgE from 15 patients with documented peanut hypersensitivity. Figure 5A shows an immunoblot strip containing the wild-type and mutated peptides of epitope 7. The pooled serum IgE did not recognize this peptide or binding was drastically reduced when alanine was substituted for amino acids at position 67, 68, or 69. In contrast, the substitution of an alanine for a serine residue at position 70 resulted in increased IgE binding. The remaining *Ara h 2* 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 (Fig. 5B). In general, each epitope could be mutated to a non-IgE-binding peptide by the substitution of an alanine for a single amino acid residue. There was no obvious position within each peptide that, when mutated, would result in loss of IgE binding. Furthermore, there was no consensus in the type of amino acid that, when changed to alanine, would lead to loss of IgE binding.

DISCUSSION

Peanuts are one of the most common food allergens in both children and adults. In addition, peanut hypersensitivity is less likely to resolve spontaneously and more likely to result in fatal anaphylaxis. Because of the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed food, the risk to the peanut-sensitive individual is increasing.

Various studies over the last several years have examined the nature and location of the multiple allergens in peanuts (8–10). Taylor *et al.* (9) demonstrated that the allergenic portion of peanuts was in the protein portion of the cotyledon. Our laboratory recently identified two major allergens from peanut extracts, designated *Ara h 1* (11) and *Ara h 2* (12). Greater than 90% of our patients who were challenge positive to peanut had specific IgE to these proteins. The *Ara h 1* allergen has been identified as a seed storage protein

with sequence homology to the vicilins (13), a family of proteins commonly found in many higher plants (27, 28). The *Ara h 2* nucleotide sequence identified in this report has sequence homology with another class of seed storage proteins called conglutins (20). It is interesting to note that two of the major peanut allergens thus far identified are seed storage proteins that have sequence homology with proteins in other plants. This may explain the cross-reacting antibodies to other legumes that are found in the sera of patients that manifest clinical symptoms to only one member of the legume family (29).

There are at least 10 IgE recognition sites distributed throughout the major peanut allergen *Ara h 2*. The identification of multiple epitopes on a single allergen is not novel, there being reports of multiple IgE binding epitopes on allergens from many foods that cause immediate hypersensitivity reactions (30–36). The observation that most of these proteins have multiple IgE-binding sites probably reflects the polyclonal nature of the immune response to them and may be a necessary step in establishing a protein as an allergen.

Recent evidence suggests that there is a preferential variable heavy chain usage in IgE synthesis and a direct switching from IgM production to IgE synthesis (37). This would suggest that epitopes responsible for antigen-specific IgE antibody production may differ from those promoting antigen-specific IgG antibodies and that there may be some structural similarity between peptides that elicit IgE antibody production. However, there was no obvious sequence motif that was shared by the 23 different IgE-binding epitopes of the peanut allergen *Ara h 1* (13). In the present study, two epitopes in *Ara h 2* share a hexameric peptide (DPYSPS). It is significant to note that these peptides are recognized by serum IgE from all the peanut hypersensitive patients tested in this study. In addition, serum IgE that recognize these peptides represent the majority of *Ara h 2*-specific IgE found in these patients. Whether there is any further structural similarity between the IgE binding epitopes of *Ara h 2* remains to be determined.

The elucidation of the major IgE binding epitopes on *Ara h 2* may enable us to design better therapeutic options for the prevention of anaphylaxis as a result of peanut hypersensitivity. 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 an

(A) A representative SPOTS membrane containing the wild-type (WT) peptide 7 and the mutated forms in which the amino acid at each position was substituted with alanine. (B) The relative intensity of IgE binding to the mutated forms of each peptide was then determined by densitometry and expressed as a percentage of IgE binding to the wild-type peptide. The letters and numbers across the top of (A) and across the bottom of each panel in (B) indicate the one-letter amino acid code for the residue normally at that position, the position of each residue in the *Ara h 2* protein, and the amino acid that was substituted at that position. WT, indicates the wild-type peptide (no amino acid substitutions).

inadvertent ingestion is great. One therapeutic option used extensively for patients with allergic reactions to various aeroallergens and insect sting venoms is allergen desensitization immunotherapy. Allergen immunotherapy consists of injections of increasing amounts of allergens to which a patient has Type I immediate hypersensitivity (38, 39). While the absolute mechanism of immunotherapy is unknown, an increase in IgG or IgG₄ antibody activity, a decrease in allergen-specific IgE levels, and a decrease in basophil activity have all been implicated in mediating this response (40–43). Because allergen immunotherapy has been proven efficacious for treatment of some allergies, treatment with peanut immunotherapy is now being studied as a possible option (44). Our work showing that the IgE-binding epitopes of a major peanut allergen may allow for the use of immunodominant epitopes in this approach.

Finally, our data show that it may be possible to mutate the *Ara h 2* allergen to a protein that no longer binds IgE. This raises the possibility that an altered *Ara h 2* gene could be used to replace its allergenic homologue in the peanut genome. In this manner, a hypoallergenic peanut could be developed that may blunt allergic reactions in sensitive individuals who inadvertently ingest this food. Such an approach would require that the altered *Ara h 2* gene product retain its native function and properties. Given the potential severity of peanut allergic reactions and the widespread use of peanuts in consumer foods, this possibility is actively being pursued in our laboratories.

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