

Modification of Peanut Allergen Ara h 3: Effects on IgE Binding and T Cell Stimulation

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Key Words

Immunotherapy · Modified allergen · Peanut allergy

Abstract

Background: Peanut allergy is a major health concern due to the increased prevalence, potential severity, and chronicity of the reaction. The cDNA encoding a third peanut allergen, Ara h 3, has been previously cloned and characterized. Mutational analysis of the Ara h 3 IgE-binding epitopes with synthetic peptides revealed that single amino acid changes at critical residues could diminish IgE binding. **Methods:** Specific oligonucleotides were used in polymerase chain reactions to modify the cDNA encoding Ara h 3 at critical IgE binding sites. Four point mutations were introduced into the Ara h 3 cDNA at codons encoding critical amino acids in epitopes 1, 2, 3 and 4. Recombinant modified proteins were used in SDS-PAGE/Western IgE immunoblot, SDS-PAGE/Western IgE immunoblot inhibition and T cell proliferation assays to determine the effects of these changes on in vitro clinical indicators of peanut hypersensitivity. **Results:** Higher amounts of modified Ara h 3 were required to compete with the wild-type allergen for peanut-specific serum IgE. Immunoblot analysis with

individual serum IgE from Ara-h-3-allergic patients showed that IgE binding to the modified protein decreased ~35–85% in comparison to IgE binding to wild-type Ara h 3. Also, the modified Ara h 3 retained the ability to stimulate T cell activation in PBMCs donated by Ara-h-3-allergic patients. **Conclusions:** The engineered hypoallergenic Ara h 3 variant displays two characteristics essential for recombinant allergen immunotherapy; it has a reduced binding capacity for serum IgE from peanut-hypersensitive patients and it can stimulate T-cell proliferation and activation.

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Introduction

Allergen immunotherapy is defined as controlled exposure to known allergens in an attempt to reduce the severity of the allergic response [1]. Specific allergen immunotherapy is currently indicated for allergic rhinitis and some patients with asthma [2]. Unfortunately, immunotherapy for food allergy is not an option due to the anaphylactic potential associated with the high IgE-binding capacity of purified food allergens. However, genetic engineering of recombinant allergens offers an exciting possi-

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bility for developing new allergen variants for use in allergen immunotherapy. These modified recombinant allergens can be formulated with predetermined protein levels that could replace conventional sources of allergens as a means for treating ongoing IgE-mediated disease as allergen-based vaccines [3].

Protein allergenicity and the allergic response depend upon the stimulation and ultimate interaction of a multivalent allergen with mast-cell- or basophil-bound specific IgE antibodies. A recent approach to allergen immunotherapy has been the production of recombinant protein mutated to reduce the B-cell epitope density but retain the T-cell proliferative response of the allergen. Modifications of recombinant allergens with a reduced capacity to bind cell-bound IgE would ensure a lower risk of IgE-mediated side effects and the retention of their T-cell epitopes within the allergen would allow for a modulation of the immune response [4].

Using this technology, hypoallergens have been developed for group 2 mite allergens, major birch pollen allergens, venom allergens and peanut allergens [5–11]. Engineering of recombinant aeroallergens, in particular, has been successful and represents promising new approaches for immunotherapy of pollen-allergic disease. Most work regarding hypoallergenic forms of food allergens have concentrated on either physical or genetic removal or disruption of specific allergens which could have a deleterious effect on the overall nutritional value of the food [12, 13]. Clones encoding a food allergen mutated to produce a protein with a lower IgE-binding potential would allow higher doses of recombinant protein to be given per injection to induce tolerance in allergic individuals [3]. Previous studies have shown that CD4⁺ T cells from atopic patients produce high levels of IL-4 upon stimulation with low concentrations of allergen. Upon exposure to high concentrations of allergen the amount of IL-4 produced by CD4⁺ T cells drastically decreased, suggesting that allergens should be administered at high doses if the immune response is going to shift from a Th2-allergic response to a Th1-inflammatory response [14].

In a recent publication, the molecular cloning and epitope analysis of a third peanut allergen, Ara h 3, was described [15]. Database searches for sequence similarity revealed that the Ara h 3 cDNA encoded an 11S seed storage protein common to the Leguminosae (legumes) family. These are common storage proteins in legumes that function as a source of nitrogen for the developing plant that are initially synthesized as 60-kD preproglobulins [16]. The amino acid sequence of Ara h 3 showed between 62 and 72% sequence identity to glycinins from *Glycine*

max (soybean) and *Pisum sativum* (pea). In particular, 24 of 26 residues thought to be important for glycinin tertiary structure are present in the Ara h 3 primary sequence, including a conserved cleavage site between Asn-325 and Gly-326 [17]. Cleavage by an asparaginyl endopeptidase at this site separates the 60-kD preproglobulin into an amino-terminal 40-kD acidic subunit and a carboxyterminal 20-kD basic subunit [18].

Immunoblots of expressed protein revealed that approximately 44% (8/18) of a peanut-allergic patient population had serum IgE that recognized recombinant Ara h 3 [15]. Serum IgE from Ara-h-3-allergic patients and synthetic peptides were used to map the linear, B-cell epitopes residing within the primary sequence. An interesting finding was that all four Ara h 3 epitopes reside within the amino-terminal 40-kD acidic subunit. Also, the amino acid alignment showed that none of the critical residues required for IgE binding were structurally conserved residues. Mutational analysis of these epitopes revealed that single amino acid changes at particular residues resulted in a significant decrease in IgE binding.

In this report, the creation of a hypoallergenic variant of the 40-kD subunit of Ara h 3 that exhibits properties favorable for allergen immunotherapy is described. Knowledge of the amino acids that were critical for IgE binding made it possible to genetically engineer a recombinant Ara h 3 cDNA 40-kD clone to encode a protein with a reduced IgE-binding capacity. By altering four critical IgE-binding amino acids within the 40-kD subunit of the Ara h 3 primary sequence, a hypoallergenic variant of recombinant 40-kD Ara h 3 was engineered that activated T cells from Ara-h-3-allergic patients, while still displaying a substantial decrease in IgE-binding capacity.

Materials and Methods

Serum IgE

Serum from patients with documented peanut hypersensitivity was used to probe recombinant proteins to examine IgE-binding reactivity. Each patient had a positive immediate skin prick test to peanut and either a positive, double-blind, placebo-controlled, food challenge (DBPCFC), or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension). Serum IgE from 5 patients previously shown to recognize the Ara h 3 recombinant protein and serum IgE from 3 patients with no clinical reactivity to peanuts served as a control for this study. A serum pool, consisting of equal aliquots of serum IgE from each of the peanut-allergic patients, was used in immunoblot analysis experiments to determine the IgE-binding characteristics of the population. Details outlining the challenge procedure and collection of IgE serum have been discussed previously [19]. The Human Use Advisory Committee at the University of Arkansas for Medical Sciences approved all studies.

Table 1. Amino acids critical to IgE binding

Epitope	AA sequence	Ara h 3 position	Recognition %
1	IETWN <u>P</u> NNQEFECAG	33–47	25
2	GNIFSGFTPE <u>F</u> LEQA	240–254	38
3	VTVRGGLRIL <u>L</u> SPDRK	279–293	100
4	DEDEY <u>E</u> YDEEDRRRG	303–317	38

The Ara h 3 IgE binding epitopes are indicated as the single-letter amino acid code. The position of each peptide with respect to the Ara h 3 primary sequence is indicated in the middle column. The amino acids that, when altered, led to a decrease in IgE binding are shown as the bold, underlined residues. Recognition indicates the number of individuals with serum IgE binding to the epitope.

Site-Directed Mutagenesis of the Ara h 3 Gene by the Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to alter the cDNA encoding Ara h 3 at epitopes 1, 2, 3, and 4. The amino acids that were mutated within each epitope are shown in table 1. The oligonucleotides used for mutagenesis and their corresponding nucleotide sequences are displayed in table 2. Since all four epitopes resided within the 40-kD acidic subunit, the mutagenesis reactions were engineered to produce a truncated cDNA encoding the 325 amino acids prior to the Asn-Gly cleavage site (fig. 1a). Group 1 reactions consisted of PCR amplifications using the designed oligonucleotides and Ara h 3 cDNA templates to produce products 1–5. In each reaction, the primers were engineered with overlapping sequences introducing the alanine substitution at the respective site in the epitope. The template for all group 1 reactions was purified plasmid consisting of the Ara h 3 cDNA derived from the pET 24(b)+ construct wild-type Ara h 3 for cloning purposes. Group 2 reactions utilized the products from group 1 as templates to produce products 6 and 7. Group 3 reactions consisted of a single amplification using VF and 4AS as primers to produce product 8 with all four epitopes modified at a single amino acid site. A fourth amplification was performed using product 8 and product 5 from group 1 with VF and VAS40 primers for this final extension to introduce both restriction sites.

The final PCR product, representing the Ara h 3 mutated cDNA, was obtained and purified. A-overhangs were generated by incubating the purified mutant cDNA with 1 unit of *Taq* polymerase (Promega), 1 mM MgCl₂, 1 × reaction buffer, and 0.2 mM dATP at 70 °C for 30 min. For all cDNA cloning purposes, the resulting product was cloned into the pGEM T-vector as previously described [15], except that a 2 × ligase buffer was substituted, allowing the incubation time to proceed at 25 °C for 1 h followed by 3 h at 4 °C. Following each amplification step, the PCR products were separated on a 1% agarose/TAE/EtBr gel and purified with the Qiagen gel-extraction kit (Qiagen, Valencia, Calif., USA). This truncated, modified cDNA was then cloned into the pET 24(b)+ expression vector under control of

Table 2. Oligonucleotides used to mutate the Ara h 3 cDNA

Name	Sequence
VF	ACATAT <u>GGCTAGCTT</u> CCGGCAG
1AS	CTGGTTGTTGGCGT <u>TCCA</u> AGT
1F	ACTTGGAA <u>CGCCAACA</u> ACCAG
2AS	TTGTTCCAGGG <u>CCTCCG</u> CGT
2F	ACGCCGGAG <u>GCCTGG</u> AACAA
3AS	TGGGCTCAAG <u>GCTCTG</u> AGGCC
3F	GGCCTCAGAG <u>CCTGAG</u> CCCA
4AS	TTCATCGTAG <u>G</u> CATATT <u>CATC</u>
4F	GATGAATATG <u>CCTACG</u> ATGAA
VAS-40	AGCTT <u>GTCTGAC</u> ATTCCCCCTGC

Oligonucleotides containing the desired mutations were annealed to the template and extended using PCR. All oligonucleotides are shown in their 5' to 3' orientation. Boldfaced nucleotides represent mutations that were introduced into the cDNA. Boldfaced nucleotides in VF and VAS-40 represent restriction sites.

the T7 lac promoter with a His tag at the carboxyl terminus (fig. 1b). Expression of this construct in bacteria produced the aminoterminal 40-kD acidic subunit containing the four modified IgE-binding epitopes.

All reaction mixes contained 60 µl dH₂O, 1 × reaction buffer, 1 mM MgCl₂, 0.2 mM dNTP mix, 100 ng of template, 2 pmol of each primer, and 1 unit of cloned *Pfu* polymerase (Stratagene, La Jolla, Calif., USA). The cycle consisted of a 3-min denaturation step at 94 °C, followed by 30 cycles of a 30-second 94 °C denaturation step, a 30-second 56 °C annealing step, and a 1- to 2-min 72 °C elongation step. A final extension for all reactions was performed at 72 °C for 7 min.

Expression and Purification of Recombinant Ara h 3 Proteins

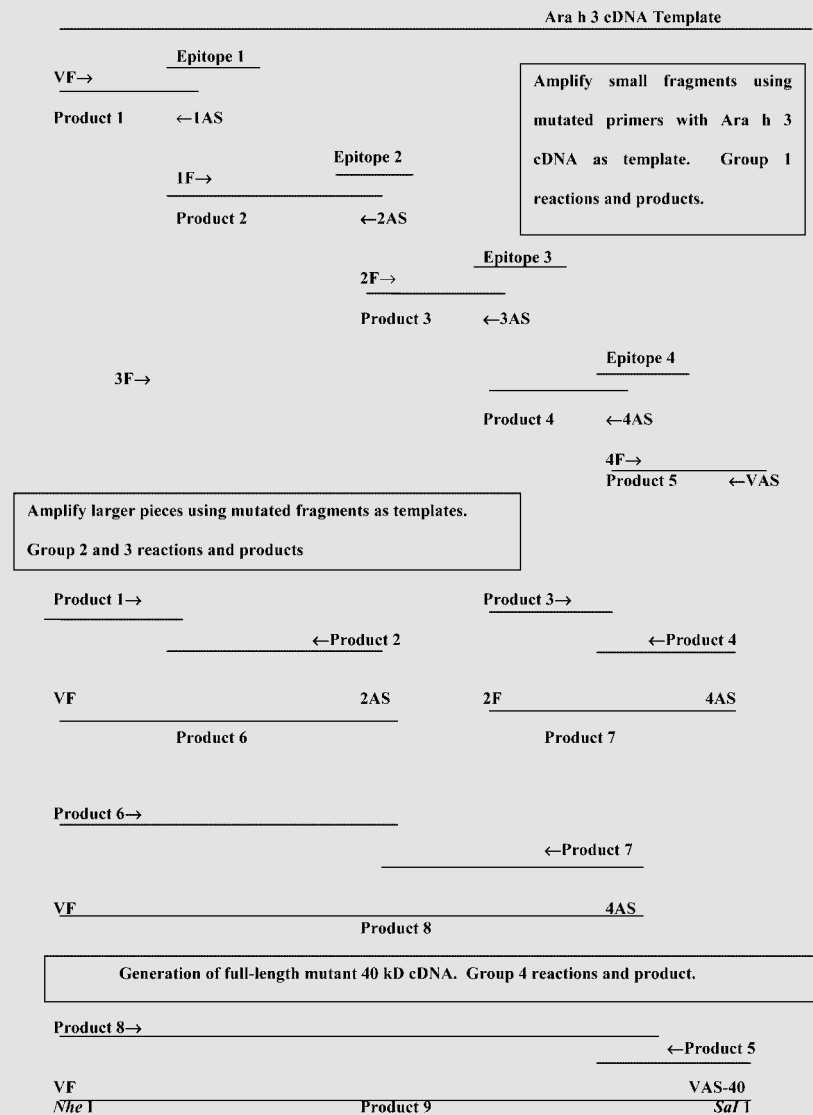
Recombinant proteins were expressed as previously described for wild-type 57-kD Ara h 3 [15]. All recombinant Ara h 3 proteins were expressed with the His tag at the carboxy terminus (40 kD wild type, and 40 kD modified) and purified from bacterial lysates under denaturing conditions using a His-Bind Purification Kit (Novagen, Madison, Wisc., USA). Fractions containing recombinant proteins were subjected to dialysis for the removal of salts followed by lyophilization to concentrate protein. All purified recombinant proteins were stored in PBS, or in a solution of 0.1 M urea in PBS, at 4 °C.

SDS-PAGE, Western Blots, and IgE-Binding Assay

All procedures performed have been described previously [15]. Briefly, purified recombinant proteins and bacterial extracts were analyzed by SDS-PAGE using pre-cast 12% tris-glycine gels (Novex, San Diego, Calif., USA). Proteins were visualized by either Coomassie brilliant blue staining or by using Gelcode Blue Stain Reagent according to the manufacturer's protocol (Pierce, Rockford, Ill., USA). For immunoblot analysis, proteins were electroblotted onto nitrocellulose at 30 V for 90 min. After transfer, blots were blocked using a solution containing Tris/NaCl and 3% bovine serum albu-

a

Mutagenesis Strategy for the 40 kD Ara h 3 cDNA



b

Plasmid Construct for Bacterial Expression of Recombinant Ara h 3

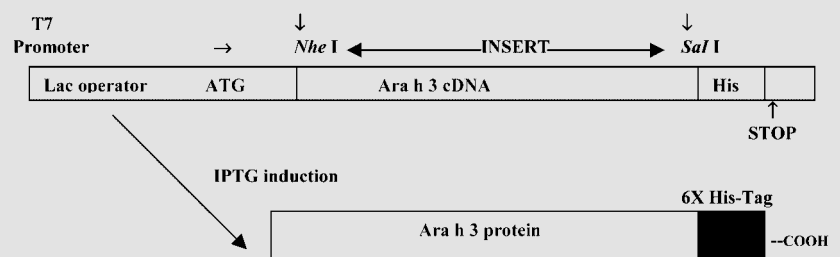


Fig. 1. Diagram of the PCR protocol and the plasmid construct for development of the 40-kD cDNA modified Ara h 3. a Step-by-step outline used to prepare the Ara h 3 modified cDNA product. b Plasmid construct that was used to produce the recombinant 40-kD protein with the His tag.

min. All blots were incubated with a serum pool or individual serum diluted (1:5) in a solution containing Tris/NaCl and 1% bovine serum albumin for 16 h at 4°C. Primary antibody was detected with ¹²⁵I-labeled anti-IgE antibody (Sanofi Pasteur Diagnostics, Paris, France).

Quantitation of IgE Binding

Relative amounts of IgE binding to wild-type and modified proteins were determined by scanning autoradiographs with a Bio-Rad model GS-700 imaging laser densitometer (Bio-Rad, Hercules, Calif., USA). Following scanning, IgE binding was quantitated with Bio-Rad molecular analyst software. Background areas were scanned and subtracted from the values obtained for the scanned images. After quantitation, all wild-type intensities were standardized to a value of one, with intensities from the modified proteins calculated as percentages relative to wild-type binding.

Competitive Inhibition Assays

The competitive inhibition experiments were performed by preincubating the wild-type, modified Ara h 3 or rice protein with serum IgE prior to incubation with blotted proteins. For example, 0.5 µg of SDS-PAGE electrophoresed protein was immobilized onto nitrocellulose. The nitrocellulose blots containing immobilized 40-kD wild-type Ara h 3, modified Ara h 3 and rice were cut into strips. A 1:10 dilution of serum IgE was mixed overnight at 4°C with different concentrations of either the 40-kD modified Ara h 3 protein, the 40-kD wild-type Ara h 3 protein or rice protein. Serum IgE that had been incubated with protein was then used to probe the immunoblots as described in the SDS-PAGE/Western blots and IgE-binding assay section. Autoradiographs of the blots were scanned with a densitometer and IgE binding was quantitated as described in the Quantitation of IgE-binding section. Rice protein was used as nonspecific protein and serum IgE from individuals with no IgE to peanut was used as a control.

T Cell Proliferation Assays

Four to 6 ml of heparinized blood from the 5 peanut-hypersensitive patients and 3 control patients (no IgE to peanut) were added to sterile tubes containing 3 ml of Fico/Lite (Atlanta Biological, Atlanta, Ga., USA). Separation was achieved by spinning the tubes at 1,800 rpm for 30 min in a Sorvall RT6000R centrifuge (Sorvall, Newton, Conn., USA). After separation, the top layer of plasma and the monocyte layer containing the lymphocytes were aspirated and placed into clean, sterile tubes and spun at 1,800 rpm for 10 min. The resulting supernatant was discarded and the cell pellet was lifted with PBS. Following resuspension, the tube was centrifuged at 1,800 rpm for 10 min. Residual red cells present in the cell button were lysed by the addition of 3 ml ammonium chloride, followed by incubation for 5 min in a 37°C water bath with gentle agitation. Tubes were filled with PBS and spun for 5 min at 1,000 rpm. After this final wash, 3.8 ml RPMI 1640 [10% ABS (human AB negative serum); Atlanta Biologicals] was added to each tube as media. Cell viability was determined by adding 20 µl of trypan blue to 20 µl cells and counting the number of dead cells/100 cells. The viable cell number was adjusted to 2×10^6 cells/ml. Protein concentration and incubation time periods were optimized to the following conditions. Proliferation assays were set up in triplicate with 10 µg of protein added to 100 µl of cells in a sterile round-bottom 96-well plate. Wells containing only media served as background controls. Plates were incubated at 37°C for 3 days. On day 3, cells were tagged with 50 µl (1 µCi/well)

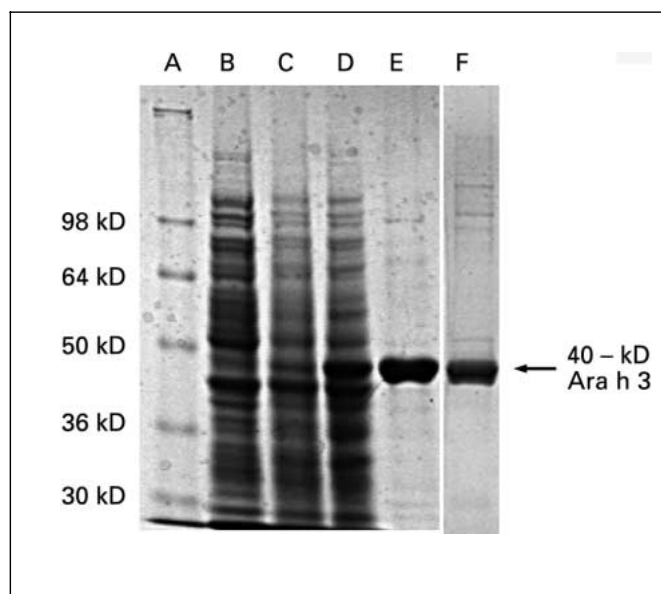


Fig. 2. Bacterial expression and purification of the wild-type and modified Ara h 3 proteins. Recombinant wild-type and modified Ara h 3 proteins were expressed in BL21λ (DE3) cells and purified by chromatography on Ni²⁺ columns. 10-µl samples were separated on a 12% tris-glycine gel and visualized. A = Molecular weight standards; B = induced bacterial product without insert; C = uninduced wild-type bacterial product Ara h 3; D = 4-hour induction of wild-type bacterial product Ara h 3; E = purified wild-type Ara h 3 protein after elution from a Ni²⁺ column; F = purified modified Ara h 3 protein after elution from a Ni²⁺ column.

of [³H]-thymidine (ICN Biomedicals, Costa Mesa, Calif., USA) and incubated for 6 h at 37°C. After incubation, cells were harvested onto glass filter paper (Filtermate 196, Packard, Downer Grove, Ill., USA) and dried overnight. The amount of incorporated label was determined with a β-counter (Matrix 9600 Direct Beta Counter, Packard) according to the manufacturer's protocol. The stimulation index (SI) was calculated on each set by dividing the mean of the triplicate values by the mean of the media.

Results

Expression and Purification of the Wild-Type and Modified Ara h 3 Recombinant Proteins

The wild-type and modified recombinant proteins were expressed in BL21λ(DE3) bacterial cells. Both proteins were optimally expressed following a 4-hour induction by IPTG. A purification protocol employing immobilized divalent cation chromatography was sufficient to obtain pure, recombinant protein. Figure 2 shows a Coomassie-stained gel representing the recombinant wild-

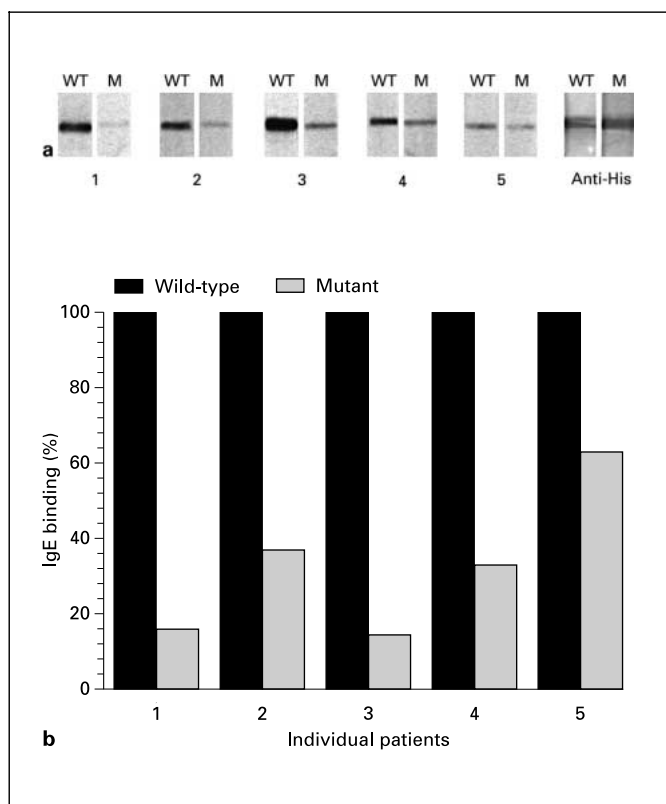


Fig. 3. Purified wild-type and modified Ara h 3 immunoblots probed with individual patient serum. a Equal amounts of purified wild-type and modified Ara h 3 were loaded onto preparative 12% tris-glycine gels, separated by electrophoresis, and blotted to nitrocellulose. 1–5 represents strips of wild-type (WT) and modified (M) protein probed with individual serum from 5 peanut-allergic patients shown previously to recognize the recombinant Ara h 3 protein. The strips labeled anti-His were probed with tetra-His antibody to show that equal amounts of each recombinant protein were loaded on the gel. b The autoradiograph from (a) was scanned with a densitometer and relative values for IgE binding were quantitated. Individual patients are numbered 1–5 on the x-axis. The black bars represent the wild-type protein, while the gray bars represent the modified protein. The amount of IgE binding to the wild-type Ara h 3 was normalized to a value of 100% for each patient. The amount of IgE bound to the modified Ara h 3 is displayed as a percentage of the amount of IgE bound to wild type.

type and modified Ara h 3. Purification of recombinant protein from 250-ml cultures of BL21λ(DE3) cells routinely generated ~ 500–600 µg of wild-type and modified Ara h 3. The recombinant proteins were stable in a solution of 0.1 M urea in PBS.

Individual Patient Serum IgE Binding to the Modified Ara h 3 Protein Is Reduced

The IgE binding activity of the modified Ara h 3 protein containing the four point mutations at critical residues was compared to wild-type Ara h 3 protein by immunoblot analysis. Serum from 5 Ara-h-3-allergic patients was used to probe immunoblots of the purified, recombinant proteins (fig. 3a). Serum IgE from all 5 patients exhibited higher reactivity towards the wild-type protein. Serum IgE from atopic individuals with no peanut allergy failed to bind either the wild-type or modified Ara h 3 protein (data not shown). The blot labeled anti-His denotes recombinant protein probed with anti-tetra-His antibody to serve as a control demonstrating that equal amounts of recombinant proteins were loaded. Binding of serum IgE from each individual patient to wild-type protein was given a value of 100%, with IgE binding to the mutant protein calculated as a percentage of wild-type binding (fig. 3b). Serum from patients 1 and 3 showed the greatest change in IgE binding to the modified Ara h 3 protein, with IgE binding decreasing about 80%. Serum from patients 2, 4, and 5 reacted with the modified protein at levels that were 30–60% of the wild-type allergen. These results indicate that the four single amino acid substitutions introduced into the Ara h 3 recombinant protein decreased serum IgE binding in this peanut-sensitive population.

Higher Amounts of Modified Ara h 3 Protein Is Required to Achieve 50% Inhibition of IgE Binding to the Wild-Type Allergen

The ability of the modified Ara h 3 protein to inhibit IgE binding to the Ara h 3 wild-type protein was examined using a pool of serum IgE from 5 Ara-h-3-allergic patients. Figure 4 is a graph plotting the percent inhibition versus the inhibitor concentration. Pre-incubation of pooled serum IgE with 0.25 µg/ml of Ara h 3 wild-type protein immobilized to the membrane resulted in about 55% inhibition. To achieve the same level of inhibition with the modified Ara h 3 protein, an approximated 28-fold increase in the protein concentration was required. When pooled serum IgE was pre-incubated with 10 µg/ml of Ara h 3 wild-type protein, approximately 80% inhibition was achieved. In comparison, 10 µg/ml of the modified Ara h 3 protein only displayed 64% inhibition of IgE binding to the wild-type protein.

The Modified Ara h 3 Protein Retains the Ability to Activate T Cells

The ability of the modified Ara h 3 protein to interact with T cells from peanut-sensitive individuals was deter-

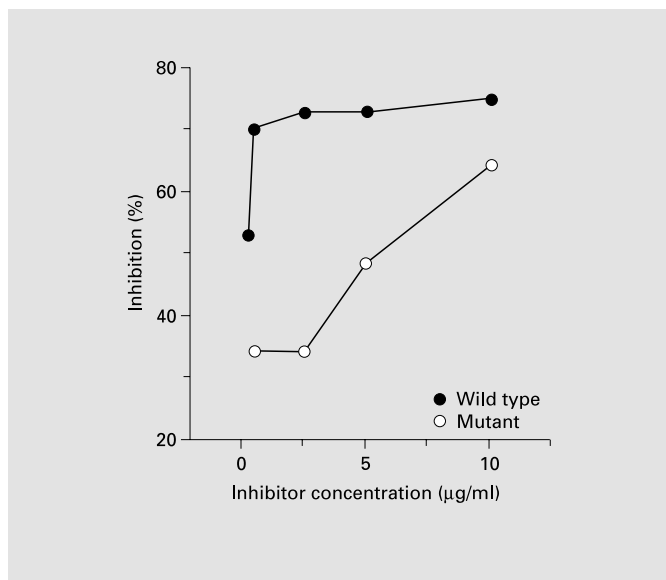


Fig. 4. Inhibition of IgE binding to Ara h 3 wild-type protein using the modified Ara h 3 protein. IgE binding to 0.5 µg/ml Ara h 3 wild-type protein was inhibited by pre-incubation of serum IgE from a pool of 5 Ara-h-3-allergic patients with increasing concentrations of either the Ara h 3 wild-type or the modified Ara h 3 protein.

mined by a T-cell proliferation assay. Peripheral blood mononuclear cells (PBMC) from the 5 Ara-h-3-allergic patients were incubated in the presence of wild-type and modified proteins, and proliferation was assessed by the incorporation of radioactive thymidine into the DNA of dividing cells (table 3). The SI for the patients tested ranged from 2.5 to 17.7 for the wild-type Ara h 3 protein. The SI for these same patients tested with the modified Ara h 3 protein ranged from 1.2 to 16. In general, the modified Ara h 3 protein was capable of stimulating T-cell proliferation to the same extent as the wild-type protein as shown by the ratio of wild-type/modified SI being very close to one (0.85–1.11). However, PBMC from patients 3 and 5 exhibited wild-type/modified SI ratios equal to or greater than 2, indicating that a decrease in T-cell proliferation was observed when the modified Ara h 3 protein was used to stimulate cells.

Discussion

The elucidation of the major IgE binding epitopes on Ara h 3 and the determination of which amino acids within these epitopes are critical for IgE binding provided the necessary information to alter the Ara h 3 cDNA by site-

Table 3. The modified Ara h 3 protein retains the ability to activate T cells

Patient	Ara h 3		Ratio, wild type/modified
	wild type	modified	
1	2.6	2.6	1.00
2	4.7	5.5	0.85
3	2.5	1.2	2.10
4	17.7	16.0	1.11
5	9.1	8.2	1.11

SI of T cells obtained from PBMCs from 5 individual patients in response to wild-type or modified Ara h 3 proteins. All values represent the mean SI from three separate wells.

directed mutagenesis to encode a protein with a reduced binding capacity for serum IgE [15]. As mentioned previously, all four Ara h 3 epitopes reside within the amino-terminal 40-kD acidic subunit. Importantly, there was no overlap between amino acids considered to be important for 11S seed storage protein structure and the critical residues required for IgE binding. Also, previous studies on soybean glycinins had shown that the aminoterminal 40-kD acidic subunit is tolerable to large insertions and deletions [20]. These two findings suggested that the Ara h 3 cDNA representing the aminoterminal 40-kD acidic subunit could be altered to encode a subunit with reduced allergic reactivity that retained its structural properties.

The ability of the modified Ara h 3 protein to inhibit IgE binding to the wild-type Ara h 3 recombinant protein was determined by examining the amount of modified or wild-type protein required to achieve 50% inhibition of IgE binding to immobilized wild-type Ara h 3 recombinant protein. To attain 50% inhibition of IgE binding, an approximate 20- to 30-fold increase in the modified Ara h 3 protein concentration was required as compared to the wild-type protein concentration. Similar experiments have been performed using engineered variants of a variety of aeroallergens. For example, an engineered variant of the house dust mite allergen Der f 2 demonstrated that an 18-fold increase in mutant protein concentration was required to achieve 50% IgE inhibition as compared to the amount of wild-type Der f 2 needed to achieve the same level of IgE inhibition [21]. Another modified aeroallergen, Bet v 1, showed that a 20-fold increase in mutant protein concentration was required to achieve levels of inhibition (50%) comparable to the wild-type protein [4].

All of these results show that relatively small changes in the primary structure of the allergen can significantly disrupt IgE binding and further indicate the potential usefulness of engineered allergen variants in allergen immunotherapy.

The substitution of amino acids critical for IgE binding by alanine into the aminoterminal 40-kD acidic subunit of Ara h 3 significantly reduced the ability of serum IgE from Ara-h-3-allergic patients to recognize modified recombinant protein. Four of the 5 patient sera tested displayed at least a 60% decrease in IgE binding to the modified Ara h 3 protein. Two important conclusions can be drawn from these results. First, small changes (altering 4 out of 325 amino acids) to the aminoterminal 40-kD acidic subunit had a great impact on the ability of the protein to be recognized by serum IgE from Ara-h-3-allergic patients. Secondly, only one hypoallergenic variant had to be created to successfully decrease IgE binding for the Ara-h-3-allergic patient population. Additional modifications may be necessary to further reduce serum IgE binding in these individuals and others. Experiments are underway to modify two or three amino acid sites critical for IgE binding in these epitopes. The caveat being that the more changes are made to the molecule, structural changes may be introduced that will not be properly processed by the plant for production of hypoallergenic peanuts.

Another proposed requisite for recombinant allergens to be successful as immunotherapeutic agents in addition to reduced IgE binding is that they must retain the ability to activate T cells [3]. To determine if the modified Ara h 3 protein met this requirement, its ability to stimulate T cell proliferation was assayed with PBMCs from Ara-h-3-

allergic patients. Of the 5 Ara-h-3-allergic patients tested, the PBMCs of 4 patients exhibited similar wild-type/modified SI ratios indicating that the modified Ara h 3 protein could stimulate T cells to proliferate as well as the wild-type Ara h 3 protein. In the other patient, PBMC showed a decreased T cell activation ability upon exposure to the modified protein. While there was not complete retention of the ability to stimulate T cells isolated from PBMCs donated by these Ara-h-3-allergic patients, their T cells still proliferated upon exposure to the modified Ara h 3 protein. The fact that all of the patients examined retained T cell proliferation when exposed to the modified Ara h 3 protein suggests that the modified protein can still prime T cell activation.

The modified Ara h 3 protein shows promise for being an effective immunotherapeutic agent. Since the Ara h 3 hypoallergenic variant has a decreased IgE binding capacity, an increased amount of protein could be injected during immunotherapy trials. Since the modified Ara h 3 protein displayed a decreased capacity to bind serum IgE and retained the ability to stimulate T-cell proliferation from PBMCs donated by Ara-h-3-allergic patients, the modified protein has the potential to be useful in a clinical setting for allergen-specific immunotherapy aimed at treating peanut-hypersensitive patients.

Acknowledgments

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