Molecular Basis of Arthropod Cross-Reactivity: IgE-Binding Cross-Reactive Epitopes of Shrimp, House Dust Mite and Cockroach Tropomyosins

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Key Words
Shrimp · Dust mite · Cockroach · Allergens · Cross-reactivity

Abstract
Background: Shrimp may cross-react with other crustaceans and mollusks and nonedible arthropods such as insects (cockroach and chironomids), arachnids (house dust mites) and even nematodes. Since the muscle protein tropomyosin has been implicated as a possible cross-reacting allergen, this study characterized the IgE-binding epitopes in shrimp tropomyosin, Pen a 1, that cross-react with other allergenic invertebrate tropomyosins in house dust mites (Der p 10, Der f 10) and cockroaches (Per a 7). Pen a 1-reactive sera from shrimp-allergic subjects were used to evaluate the effect on IgE binding of different amino acid substitutions in Pen a 1 epitopes based on homologous sequences in Per a 7 and Der p 10/Der f 10. Results: 7/8 individually recognized Pen a 1 epitopes (2, 3a, 3b, 4, 5a, 4 and 5a) with Per a 7. In addition, even homologous regions of other arthropod tropomyosins that differ in one or more amino acids from the sequences of Pen a 1 epitopes are still recognized by shrimp-allergic IgE antibodies. In total, shrimp-allergic sera recognize 6/8 peptides homologous to Pen a 1 epitopes in Per a 7, 7/8 in Der p 10/Der f 10, and 7/8 epitopes in Hom a 1. Conclusions: The IgE recognition by shrimp-allergic individuals of identified and/or similar amino acid sequences homologous to Pen a 1 epitopes in mite, cockroach and lobster tropomyosins are the basis of the in vitro cross-reactivity among invertebrate species. Based on amino acid sequence similarity and epitope reactivity, lobster tropomyosin has the strongest and cockroach the least cross-reactivity with shrimp. The clinical relevance of these cross-reactivities in developing allergic reactions to different arthropods needs to be determined.

Introduction
Shellfish are a frequent cause of food-induced allergic reactions, which range from mild oral allergy syndrome to urticaria, angioedema or even systemic anaphylaxis [1, 2].
Most shellfish species elicitting allergic food reactions belong to the class crustacea; shrimp species Penaeus aztecus, P. indicus and Metapenaeus ensis have been the most thoroughly studied. The muscle protein tropomyosin has been identified as the only major shrimp allergen in all three shrimp species studied [3-5]. In addition, tropomyosins from several other invertebrate species have also been shown to be allergenic; tropomyosin is an important allergen in other crustaceans such as spiny lobster Panulirus stimpsoni (Pan p 1), American lobster Homarus americanus (Hoa m 1) [6,7] and crab Charybdis feriata (Cha f 1) [8], mollusks like the snail Turbo cornutus (Tur c 1) [9], oyster Crassostrea gigas (Cro g 1) [10], squid Todarodes pacificus (Tod p 1) [11], abalone Haliotis diversicolor, scallop Chlamys nobilis and mussel Perna viridis [12]. Allergenic tropomyosins are also present in other arthropods such as the cockroach Periplaneta americana (Per a 7) [13,14], and the house dust mites Dermatophagoides farinae (Der f 10) [15] and Dermatophagoides pteronyssinus (Der p 10) [16]. Shrimp-allergic subjects may also react to other crustaceans and mollusks [17-21], and some in vitro evidence suggests that shrimp may cross-react with nonedible arthropods such as the arachnids (mites) [22] and insects such as chironomids [23] and cockroaches [22,24-26]. In this context, tropomyosin has been implicated as the allergen responsible for invertebrate cross-reactivity [27].

Tropomyosin belongs to a family of phylogenetically conserved proteins with multiple isoforms present in both muscle and nonmuscle cells of vertebrates and invertebrates [28]. Previous studies have identified five main IgE-binding regions in the shrimp tropomyosin, Pen a 1, using synthetic overlapping peptides and sera of 18 shrimp-allergic individuals [29,30]. Eight IgE-binding epitopes (minimum peptides with the strongest IgE-binding capacity) have been identified with individual sera of shrimp-allergic subjects reactive to each of the 5 main regions; in region 1: the first epitope (43-55: VHNQKRMQQLEN); in region 2: the second epitope (87-101: ALNRRIQLLEEDLR), within region 3: two epitopes identified centered around the cores 3a (137-141: DEERM) and 3b (144-151: LENOQLKEA); in region 4: the fifth epitope (187-197: ESKVLEELF); in region 5, three epitopes identified: 5a (249-259: LQKEVDRLEDEL), 5b (266-275: KYKSIITDE) and 5c (273-281: ELDQTFSEL) [31].

Given the high degree of tropomyosin amino acid sequence similarity (81% between shrimp and mites and 82% between shrimp and cockroach) and the suggested clinical cross-reactivity among tropomyosins, it could be expected that IgE-binding epitopes in Der p 10 and Pera 7 may be similar to those observed in Pen a 1. However, amino acid differences between Pen a 1 and homologous sequences in other invertebrate tropomyosins could alter IgE binding. The aim of this study was to identify and characterize the cross-reacting epitopes among Pen a 1 and other invertebrate tropomyosins such as those from crustaceans (American lobster, H. americanus; spiny lobster, P. stimpsoni; greasy-back shrimp, M. ensis), arachnids (the house dust mites, D. farinae and D. pteronyssinus) and insects (cockroach, P. americana) using sera from shrimp-allergic individuals and overlapping synthetic peptides of invertebrate tropomyosins. In addition, the effect of amino acid substitutions based on amino acid sequence differences with other invertebrate tropomyosins on the IgE-binding capacity of Pen a 1 epitopes by shrimp-allergic sera is assessed.

Material and Methods

Sera
Sera from 3 adult (18 years or older) shrimp-allergic subjects, each of whom gave informed consent, were selected based on the following criteria: (1) history of respiratory (wheezing or shortness of breath, dermatologic (urticaria or angioedema) or gastrointestinal (nausea, vomiting and/or diarrhea) symptoms occurring within 1 h following ingestion of shrimp; (2) positive immediate skin prick test (wheat >3 mm) to brown shrimp P. aztecus; (3) elevated serumspecific IgE to shrimp by radioallergosorbent test (RAST binding >3%); (4) strong IgE reactivity to purified shrimp tropomyosin by immunoblot analysis, and (5) IgE reactivity to one or more of the five major Pen a 1 IgE-binding regions identified with synthetic overlapping peptides -15 amino acids in length, 9-amino acid overlap [30].

Synthesis of Peptides and IgE-Binding Assay

The procedure for synthesis of overlapping peptides for Pen a 1, Hoa m 1, Per a 7 and Der p 10 was performed according to the method used by Ikeda et al. [32] with some modifications. Briefly, each cycle began by excisionation of an Fmoc amino acid into the SPOTs cellulose membrane (Genosys Biotechnologies, The Woodlands, Tex., USA). After acetylation and biocleavage of uncoupled amino groups, protective Fmoc groups were cleaved by immersion in 20% piperidine (Aldrich Chemicals, Milwaukee, Wis., USA) in DMF (N,N-dimethylformamide, Aldrich) to render nascent peptides reactive. Each additional Fmoc-amino acid is esterified to the previous one by the same process until the desired peptide is generated. After addition of the last amino acid, the protecting groups on the side chains of the amino acids were removed using a 1:1:10:0.05 mixture of dichloromethane/Ac2O/H2O/N-hydroxybenzotriazole (TFA/HOBt) triisopropylsilane (Aldrich), followed by washing in methanol; membranes were either stored immediately or dried and stored at -20°C until needed.

For the IgE-binding assay, the membranes were incubated in blocking solution (Genosys) dissolved 1:10 in TBS for 2 h and then overnight with the epitope-reactive patient’s serum diluted 1:5 in

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blocking buffer. After washing in TBS-Tween (TBS, 0.05% Tween; pH 7.5), IgE reactivities were detected using a 1.3 μCi per membrane of 125I-labeled horse anti-human IgE (Endo Diagnostics Pasteur, Chaska, Minn., USA) diluted 1:10 in Genous blocking solution. The next day, the membranes were washed in TBS-Tween and placed between plastic sheets and exposed to X-ray film for 12 h. The intensity of the IgE reactivities was determined visually (negative, weak, medium, strong, very strong) by agreement of four different investigators, who graded independently the intensities assigning the above scores. IgE reactivities were color-coded based on their intensity as follows: negative (white), weak (yellow), medium (green), strong (red), and very strong (purple).

**Results**

**Sequence Comparison of Pen a 1 IgE-Binding Epitopes with Homologous Sequences in Invertebrate Tropomyosins**

Comparison of the amino acid sequences of eight Pen a 1 IgE-binding epitopes with the homologous sequences of allergenic tropomyosins from other species are shown in figure 1. Lobster tropomyosin, Hom a 1, is composed of both fast (Hom a TM1) and slow (Hom a TM2) tropomyosin isoforms. Generally, Met e 1, Hom a TM1 and Pan s had amino acid sequences most similar to Pen a 1 (substitutions ranged from one to five amino acids). The shaded areas indicate the amino acid sequence homologies in the eight IgE-binding epitopes of Pen a 1. Epitope homologous sequences contained the greatest number of amino acid sequence substitutions, while epitopes 2, 4, and 6 showed no or minimal substitutions.

The amino acid sequence identity (% of amino acids identical) and similarity (% of amino acids within the same amino acid group) of the different Pen a 1 IgE-binding epitopes with corresponding sequences in other invertebrate tropomyosins are shown in table 1. Total identity (100%) was detected between Pen a 1 epitopes and the homologous amino acid sequences in the shrimp tropomyosin (Met e 1), and the fast muscle isoform of Hom a 1 (Hom a TM1) for all eight epitopes. Interestingly, Pen a 1 epitope 1 differed from the slow tropomyosin isoform of D. pulex by seven amino acid residues, while the sequences of the remaining seven epitopes were identical in both tropomyosins. Homology for most Pen a 1 epitopes with homologous sequences from cockroach (Pen a 7) and mite (Der p 10) tropomyosins was very high (up to 100%). Pen a 1 epitopes 3a core, 3b core, 4 and 5a had 100% identity with homologous sequences in house dust mite tropomyosins Der f 10 and Der p 10. Pen a 1 epitopes 2, 3a core, 3b core, 4 and 2a were also identical to the corresponding Pen a 7 sequences. In contrast, epitopes 1, 2, 5b and 5c of Pen a 1 differed in one to six amino acid residues from Der p 10, Der f 10, Per a 7 and Hom a 1. The effect of individual amino acid substitutions transforming Pen a 1 epitopes into

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Fig. 1. Comparison of identified Pen a 1 IgE-binding epitopes with homologous sequences in other allergen tropomyosins: Met e 1 from the shrimp *M. ensis*, Hom e 1 TM from the lobster *H. americanus* (last isoform), Hom a TM from *H. americanus* (slow isoform), Pen a 1 from the spiny lobster *P. setiferus*, Pen a 7 from the cockroach *P. americana*, Der p 10 from the house dust mite *D. pteronyssinus* and Der f 10 from *D. farinae*. Homologous sequences are highlighted. The epitopes are colored in grey. The overlapping areas between two epitopes are dark grey. Since the IgE-binding epitopes 2, 3a, 3b, 4 and 5a vary from patient to patient, the parts that are common (cores) are marked.

Table 1. Identity (Ide) and similarity (Sim) of Pen a 1 IgE-binding epitopes and homologous sequences in other tropomyosins (%)

<table>
<thead>
<tr>
<th></th>
<th>Epitope 1</th>
<th>Epitope 2</th>
<th>Epitope 3a</th>
<th>Epitope 3b</th>
<th>Epitope 4</th>
<th>Epitope 5a</th>
<th>Epitope 5b</th>
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<td>Sim</td>
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<td>Sim</td>
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<td>Pan e 1</td>
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<td>Pen a 7</td>
<td>54</td>
<td>92</td>
<td>100</td>
<td>100</td>
<td>64-100</td>
<td>75-100</td>
<td>100</td>
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<td>Der f 10</td>
<td>62</td>
<td>85</td>
<td>91-93</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>41-100</td>
<td>93-1-100</td>
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<tr>
<td>Der p 10</td>
<td>62</td>
<td>85</td>
<td>93</td>
<td>100</td>
<td>66-100</td>
<td>100</td>
<td>80-100</td>
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IgE-Binding Cross-Reactive Epitopes of
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Fig. 2. Autoradiography with results from epitopes 5b and 5c with serum 4. Pen a 1 epitope 5b: KYKTIDTDE (266–273) (spots 1 and 10); spot 2 substitution F for S at position 269; spot 3 substitution C for T at position 271; spot 4 substitution D for E at position 273; spot 5 substitution F for S and C for T; spot 6 substitution F for S, D for E at position 7; substitution C for T and D for E; spot 8 substitution F for S, C for T, and D for E; and spot 9 substitution S for T at position 271. Pen a 1 epitope 5c: ELDGTFSEL (273–281) (spots 1 and 10); spot 2 substitution D for E at position 273; spot 3 substitution M for Q at position 276; spot 4 substitution T for S at position 279; spot 5 substitution D for E and M for Q; spot 6 substitution D for E and T for S; spot 7 substitution M for Q and T for S; spot 8 substitution D for E, M for Q and T for S; and spot 9 substitution A for S at position 279.

Sequence identity of epitopes 5a and 5b with lobster tropomyosin Hom A TMF was 100% for all epitope varieties identified. Four subjects (No. 4, 6, 10 and 18) showed IgE binding to a mutated Pen a 1 peptide identical to the homologous Per a 7 sequence; one of the 4 sera (number 6) also recognized a peptide with 100% sequence identity with the homologous Der p 10/ Der f 10 peptides. All other sera tested did not recognize the homologous sequences in Der p 10/ Der f 10 and Per a 7 (data not shown). In epitope 5, no combinatorial substitutions were performed. However, Der p 7, Der p 10/ Der f 10 and crustacean tropomyosins present 100% identity with Pen a 1 epitope 4 (except Pan s 1, which differs in one amino acid). Epitope 5a presents 100% identity with homologous sequences in the other arthropod tropomyosins, and thus would also be recognized by the patients’ shrimp-specific IgE. Pen a 1 epitopes 5b and 5c have 100% sequence identity with homologous peptides in other crustacean tropomyosins. Although no IgE binding was detected to homologous peptides of Pen a 1 epitope 5b in Per a 7, significant IgE reactivity was detected to homologous sequence in Der p 10/ Der f 10 with sera tested from all 4 subjects. Only weak IgE binding was detected to Pen a 1 epitope 5c homologous peptide in Per a 7; however, important IgE reactivity was observed to homologous sequence in Der p 10/ Der f 10 (see fig. 4).

A comparison of IgE reactivities of shrimp-allergic sera to Pen a 1 epitopes and homologous sequences in lobster, cockroach, and mite tropomyosins is shown in table 2. Significant IgE antibody reactivity of shrimp allergic sera
Fig. 3. Combinatorial substitutions of epitope 1 and individual IgE antibody reactivities. The IgE reactivities shown on the autoradiography of the SPOF’s membranes were scored according to their intensity and color-coded.
Fig. 4. Combinatorial substitutions of epitopes \(5a, 5b, 5c\) and individual IgE antibody reactivities. The IgE reactivities shown on the autoradiography of the SPOTs membranes were scored according to their intensity and color-coded.

Table 2. IgE reactivity to Pen a 1 epitopes and homologous sequences in other invertebrate tropomyosins (Hom a 1, Per a 7, Der p 10/Der f 110)

<table>
<thead>
<tr>
<th>Epitope 1</th>
<th>Epitope 2</th>
<th>Epitope 3a</th>
<th>Epitope 3b</th>
<th>Epitope 4</th>
<th>Epitope 5a</th>
<th>Epitope 5b</th>
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<td>Pen a 1</td>
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** = IgE reactivity similar to that of Pen a 1 epitopes; *= IgE reactivity weaker in the subjects tested; - = IgE reactivity not detected.
was detected to all three tropomyosins. IgE antibody reactivity to Hom a TM was most similar to Pen a 1, recognizing 8/8 epitope homologous sequences. Reactivity to Der p 10 and Pen a 7 was less similar to Pen a 1, with only 4-5/8 homologous sequences recognized by shrimp IgE antibodies.

Discussion

The fact that tropomyosins are important allergens in many invertebrates, and that they are highly conserved proteins with a high degree of sequence identity suggests that IgE-binding epitopes similar to those identified in Pen a 1 exist in other invertebrate tropomyosins and are responsible for the observed laboratory and clinical cross-reactivity among invertebrate species. This is supported by our findings. In a previous study by our group, five major IgE-binding regions which include eight IgE-binding epitopes were identified in Pen a 1 [30, 31]. Although tropomyosin sequence homology is generally high, homology of Pen a 1 epitopes with homologous regions in crustaceans, mite and cockroach tropomyosins is even higher (>90%). Furthermore, even when the homologous sequences of Pen a 1 epitopes in other arthropod tropomyosins differ from Pen a 1 in one or more amino acids, shrimp-allergic sera may still bind these epitopes, although perhaps with lower affinity or intensity. In addition, comparison of Pen a 1 IgE-binding regions with other allergenic tropomyosins whose B cell epitopes have been partially characterized (such as Pen i 1 from the shrimp P. indicus [4], Tur c 1 from the snail T. cornutus [10], and Cra g 1 from the oyster C. gigas [9]) as well as previously identified Pen a 1 epitopes using a recombinant library [33] show substantial similarity in the location of the IgE-binding epitopes in all tropomyosins. Most of the five IgE-binding regions in Pen a 1 partially or completely overlap with those observed in other allergenic tropomyosins. Of particular importance are regions 2 (Pen a 1: 85-105) and 5 (Pen a 1: 247-284), since homologous sequences in oyster and in the snail tropomyosins (Cra g 1 and Tur c 1, respectively) are recognized by mollusk-allergic sera, suggesting that shared tropomyosin epitopes may be responsible for the cross-reactivity between crustaceans and mollusks [27].

Cross-reactivity among a number of foods of similar types as well as between foods and seemingly unrelated substances has been demonstrated. For example, different members of the legume family such as soybeans, peanuts and peas [34-36], grains [37], fish [38, 39] and crustaceans are recognized in vitro by IgE from food-allergic subjects to that particular group. However, these cross-reactivities may have very different endpoints. Cross-reactivity among legumes or among grains by in vitro tests is not necessarily considered to be clinically significant [36, 37]; provocative challenges only elicited symptoms in one or two members within a vegetable group in less than 5% of subjects tested [36]. In contrast, cross-reactivity among fish exists and is of clinical relevance, although the degree of cross-reactivity between different fish species varies widely among allergic individuals [38, 39]. Cross-reactivity can also occur between foods and apparently unrelated substances such as between pollens (particularly birch pollen) and a variety of fruits and vegetables [40-45]. More recently, cross-reactivity has been shown between latex proteins and those present in fruits and vegetables [46]. Indeed, these cross-reactivities of fruits or foods and vegetables with inhaled allergens may have clinical significance in that some patients develop food allergies subsequent to inhalant pollen exposure.

Since cross-reactivity among crustaceans can be clinically significant, allergy to one crustacean suggests potential reactivity to other crustaceans [17, 18] as well. Although this is not absolute. However, whether or not shrimp-allergic subjects can develop allergy to other invertebrate foods, like mollusks, or inhalants such as cockroach or house dust mites, due to cross-reacting tropomyosin molecules, has not been established. There are reports that shrimp-allergic individuals may also be allergic to mollusks [17, 19-21], and that sensitization to crustaceans can result in development of allergy to mites and cockroaches [47]. Alternatively, it has been reported that exposure of some patients to mite antigens (through mite immunotherapy) may cause sensitization to foods such as shrimp [48], limpet [49], snail [50] and squid [51]. More recently, our laboratory has observed IgE reactivity to shrimp Pen a 1 in mite and cockroach-allergic Orthodon Jews, whose religious beliefs prohibit eating crustaceans or their derivatives [52]. All of these studies suggest that a clinically significant cross-reactivity among arthropods exists.

Our results demonstrate that shrimp-allergic subjects recognize IgE-binding epitopes which are similar if not identical to those in Pen a 1 in other invertebrate tropomyosins. The recognition of epitopes in other invertebrate tropomyosins by shrimp-allergic sera occurs to identical sequences, or even homologous invertebrate tropomyosin sequences that differ from Pen a 1 epitopes by several amino acids. The fact that shared sequences in different tropomyosins are recognized by serum IgE of shrimp-
allergic individuals illustrates the basis for cross-reactivity among invertebrates and the basis for development of allergy to other invertebrates in shrimp-allergic subjects. Whether this is true for individuals allergic to other invertebrate allergens such as cockroach and dust mite, and whether these subjects recognize the same IgE-binding epitopes in Pen a 7 and Der p 10 as those identified in Pen a 1 by shrimp-allergic subjects needs to be determined. The allergen which initiates sensitization, although generally difficult to identify because of multiple allergen exposures, probably depends on the individual immune system, the route of sensitization and frequency and dose of exposure. Further studies performing double-blind placebo-controlled food challenges with crustaceans and provocation tests with cockroach and/or house dust mite extracts will assess the clinical relevance of in vitro cross-reactivity and the risk for a subject to develop multiple allergies to arthropods.

Several important patterns were observed regarding the effect of amino acid substitution on the binding of Pen a 1 peptides to IgE. First, single amino acid substitutions may not modify, decrease or even abolish IgE binding to the mutated peptide, supporting the results obtained in a previous study by Reese et al. [31]. Particular amino acid substitutions were significant in decreasing or abolishing the IgE binding to an epitope in most subjects tested: in epitope 1, 3 individual substitutions exist that abolish IgE binding to the modified Pen a 1 peptide in epitope 2, 3 substitutions: in epitope 3a and 3b, 3 substitutions, and in epitope 5b, 1 substitution. For example, substitution of asparagine (Q) with histidine (H) in position 47 of epitope 1; (43–55) or of serine (S) with phenylalanine (F) in position 269 of epitope 5b; (266–273) completely abolishes IgE binding to the mutated peptide in all subjects tested (see fig. 2). Second, IgE reactivity to the modified peptides decreased with the number of substitutions included demonstrating that in general peptides with two or more amino acid substitutions show little or no IgE recognition from shrimp-allergic sera. Third, most of the critical amino acid substitutions that led to a significant decrease or total loss of IgE binding to the modified epitopes were located at positions in the center rather than in the periphery of the epitope. Fourth, a majority of the critical amino acid substitutions that completely abolish IgE binding to the epitope were nonconservative substitutions (within different amino acid groups) rather than conservative ones (within the same amino acid group).

Identifying the amino acid substitutions that reduce or abolish IgE binding to particular epitopes is important because such information is necessary to produce altered allergens as safer reagents for the treatment of food hypersensitivity. The ideal modified allergen for allergen-specific immunotherapy should have reduced or abolished IgE-binding capacity reducing the risk of anaphylactic reactions while T cell epitopes remain intact in order to induce T cell tolerance/nergy [53]. Hypoallergenic variants of ryegrass pollen allergen Lol p 5 generated by site-directed mutagenesis contain point mutations in highly conserved protein domains outside the identified T cell epitopes [54]. Most of the variants showed significant reduction in IgE reactivity by immunoblotting and ELISA inhibition experiments. Mutational analysis of the epitopes of the major peanut allergens Ara h 1, Ara h 2 and Ara h 3 have identified the amino acids critical for IgE binding. In general, each epitope could be mutated to a non-IgE-binding peptide by substitution of an alanine for a single amino acid residue [32, 55–57]. The studies suggest that altered Ara h 1, Ara h 2 or Ara h 3 genes could be used to replace its allergic homologies in the peanut genome. However, the role of conformational epitopes in allergenicity, and the effect of amino acid substitutions on protein functionality remains to be elucidated. Our group has identified the IgE-binding epitopes in Pen a 1 and the amino acid substitutions that abolish IgE binding to Pen a 1 epitopes. These substitutions are important since they provide information to alter tropomyosin cDNA nucleotide sequence for the production of a tropomyosin variant in hypoallergenic shrimp which could be safely ingested by shrimp-allergic subjects, and produce recombinant nonallergenic tropomyosin, a promising safer therapeutic option for food-allergic individuals.

Acknowledgments

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