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Identification of Continuous, Allergenic Regions of the Major Shrimp Allergen Pen a 1 (Tropomyosin)

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Key Words

Tropomyosin · Pen a 1 · Invertebrate allergens · IgE-binding sequences

Abstract

Background: Crustaceans and mollusks are a frequent cause of allergic reactions. The only major allergen identified in shrimp is the muscle protein tropomyosin; at least 80% of shrimp-allergic subjects react to tropomyosin. Furthermore, tropomyosin is an important allergen in other crustaceans such as lobsters, crabs and mollusks, as well as other arthropods such as house dust mites and cockroaches, and has been implied as the cause of clinical cross-sensitivity among invertebrates. In contrast, vertebrate tropomyosins are considered nonallergenic. ***Objective:*** The basis of the allergenicity of proteins has not yet been resolved. Thus, tropomyosin molecules provide an excellent opportunity to study the relationship between protein structure and allergenicity. The aim of the current study was to identify the IgE-binding regions of Pen a 1 and compare these regions with homologous sequences in other allergenic and nonallergenic tropomyosins. ***Methods:*** Forty-six overlapping peptides (length: 15 amino acids; offset: 6 amino acids) spanning the entire Pen a 1 molecule were synthesized and tested for IgE antibody reactivity with sera from 18 shrimp-allergic subjects to identify the IgE-binding regions of shrimp tropomyosin. ***Results:*** Based on the frequency and intensity of the IgE reactivities, five major IgE-binding regions were identified. All five major IgE-binding regions were 15–38 amino acids long. The major IgE-binding regions identified were: region 1: Pen a 1 (43–57); region 2: Pen a 1 (85–105); region 3: Pen a 1 (133–148); region 4: Pen a 1 (187–202), and region 5: Pen a 1 (247–284). In addition, 22 peptides were categorized as minor IgE-binding regions, and 12 peptides did not bind any IgE antibodies. No substantial differences in amino acid group composition in the five IgE-binding regions compared to the whole molecule were detected. Sequence identities and similarities of the Pen a 1 IgE-binding regions with homologous regions of allergenic arthropod tropomyosins were as high as 100%, whereas identities and similarities with homologous vertebrate sequences ranged from 36 to 76% and 53 to 85%, respectively. ***Conclusion:*** Five major IgE-binding regions of the allergenic shrimp tropomyosin, Pen a 1, were identified which are positioned at regular intervals of approximately 42 amino acids (7 heptads), suggesting a relationship with the repetitive coiled-coil structure of the tropomyosin molecule. The high degree of similarity between Pen a 1 IgE-binding regions and homologous sequences in invertebrate tropomyosins and the lower percentage of similarity with homologous regions of vertebrate tropomyosins supports a structural basis for cross-reactivity of allergenic tropomyosins.

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Introduction

Among allergens of animal origin, shellfish (crustaceans and mollusks) are a frequent cause of allergic reactions to foods [1, 2]. Most shellfish species that elicit allergic food reactions belong to the class crustacea and include shrimp, crab, crawfish and lobster; the shrimp genera *Penaeus* and *Metapenaeus* are two of the most frequently studied. The only major allergen identified in shrimp is the muscle protein tropomyosin. At least 80% of shrimp-allergic subjects react to tropomyosin and it binds approximately 85% of the shrimp-specific IgE from shrimp-allergic subjects; all other shrimp allergens bind IgE from less than 25% of the shrimp-allergic subjects [3–5]. Recent studies have demonstrated that tropomyosin is an important allergen in other crustaceans, such as lobsters *Panulirus stimpsoni* and *Homarus americanus* (Pan s 1, Hom a 1) [6, 7], crab *Charybdis feriatus* (Cha f 1) [8], mollusks such as squid *Todarodes pacificus* (Tod p 1) [9], snail *Turbo cornutus* (Tur c 1) [10] and oyster *Crassostrea gigas* (Cra g 1) [11], and in other invertebrates such as house dust mites *Dermatophagoides farinae* (Der f 10) and *D. pteronyssinus* (Der p 10) [12, 13] and cockroach *Periplaneta americana* (Per a 7) [14, 15]. Furthermore, concomitant clinical and in vitro hypersensitivity to crustaceans, insects, arachnids, mollusks and even nematodes has suggested that tropomyosins may be the cause of clinical cross-sensitivity among invertebrates [16–20].

Tropomyosin belongs to a family of proteins present in all eukaryotic cells, where it is associated with the thin filament in muscle and microfilaments in many nonmuscle cells. Together with actin and myosin, tropomyosin plays a role in the contractile activities of these cells, as well as in the regulation of cell morphology and motility [21]. Tropomyosin is present in phylogenetically unrelated vertebrate and invertebrate species, with several tropomyosin isoforms being found in muscle (skeletal, cardiac and smooth), and nonmuscle cells such as those in brain, fibroblasts and platelets. Even though the degree of sequence identity and functional similarity is very high among tropomyosins, vertebrate tropomyosins are considered to be nonallergenic [22, 23]. Nevertheless, the basis for these differences in allergenicity among tropomyosins has not yet been elucidated. Thus, tropomyosin molecules provide an excellent opportunity to study the relationship between protein structure and allergenicity. The aim of the current study was to identify the location of IgE-binding regions of Pen a 1 using overlapping peptides spanning the whole length of the tropomyosin molecule and to compare these regions with homologous sequences in other allergenic and nonallergenic tropomyosins.

Materials and Methods

Sera from Shrimp-Allergic Individuals

Sera collected from 18 adult (18 years or older) atopic shrimp-allergic subjects were used to identify the IgE-binding regions of shrimp tropomyosin. All 18 subjects fulfilled four 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) immediate positive skin prick test (wheal >3 mm) to cooked brown shrimp *(Penaeus aztecus);* (3) elevated shrimp-specific IgE levels demonstrated by radioallergosorbent test (binding >3%) [24], and (4) strong IgE reactivity to purified shrimp tropomyosin by immunoblot analysis. Three shrimp-allergic subjects without IgE reactivity to shrimp tropomyosin by immunoblot but fulfilling the other criteria for shrimp allergy were used as negative controls.

Shrimp Extract and Shrimp Tropomyosin Purification

Shrimp extract from locally purchased raw brown shrimp was prepared as described previously [24]. Pen a 1 was purified from shrimp extract by preparative SDS-PAGE (Model 491 PrepCell, Bio-rad). Briefly, shrimp extract was separated on a 28-mm ID column using a Laemmli discontinuous SDS-PAGE buffer system [25]. A 15-mm-high stacking gel (5% T, 1.5% C) poured on top of the 65-mm-high separation gel (11% T, 1.5% C) was used to separate shrimp proteins, and the fractions containing Pen a 1 were collected and pooled.

IgE Recognition of Pen a 1 by Immunoblot Analysis

Pen a 1 was run by SDS-PAGE [25] and transferred onto a cyanogen-bromide (CNBr)-activated [26] nitrocellulose membrane (0.45 μm; BAS 45, Schneider and Schuell, Germany) at 0.8 mA/cm^2 for 30 min by semidry blotting [27]. The blots were blocked in Tris-buffered saline (TBS)-Tween for 30 min, dried and stored between filter paper until use. Seventy-five microliters of sera from each subject, diluted 1:2 in TBS-Tween (0.1 *M* Tris HCl, pH 7.5, 0.1 *M* NaCl, 2.5 m*M* $MgCl_2$, 0.05% Tween), were incubated for 2 h with the Pen a 1-containing membrane using a Surf-blot apparatus (Idea Scientific, Minneapolis, Minn., USA). The membranes were washed in TBS-Tween and dried. To visualize IgE-binding proteins, the blot was incubated for 2 h with alkaline phosphatase-conjugated, monoclonal mouse anti-human IgE (Southern Biotechnology Associates, Birmingham, Ala., USA) diluted 1:1,000 in TBS-Tween and washed 3 times for 10 min in TBS-Tween. For detection of bound IgE, the membrane was washed for 5 min in 37°C warm TBS-AP (0.1 *M* Tris-HCl, 0.1 *M* NaCl, 5 m*M* $MgCl_2$, pH 9.5), and antibody binding was visualized at 37°C using the substrate-chromogen mixture for alkaline phosphatase containing 450 μ*M* 5-bromo-4-chloro-indolyl-phosphate disodium salt (Sigma) and 400 μ*M* nitroblue tetrazolium chloride (Sigma) solubilized in TBS-AP [28]. The reaction was stopped with deionized water and the blots were dried.

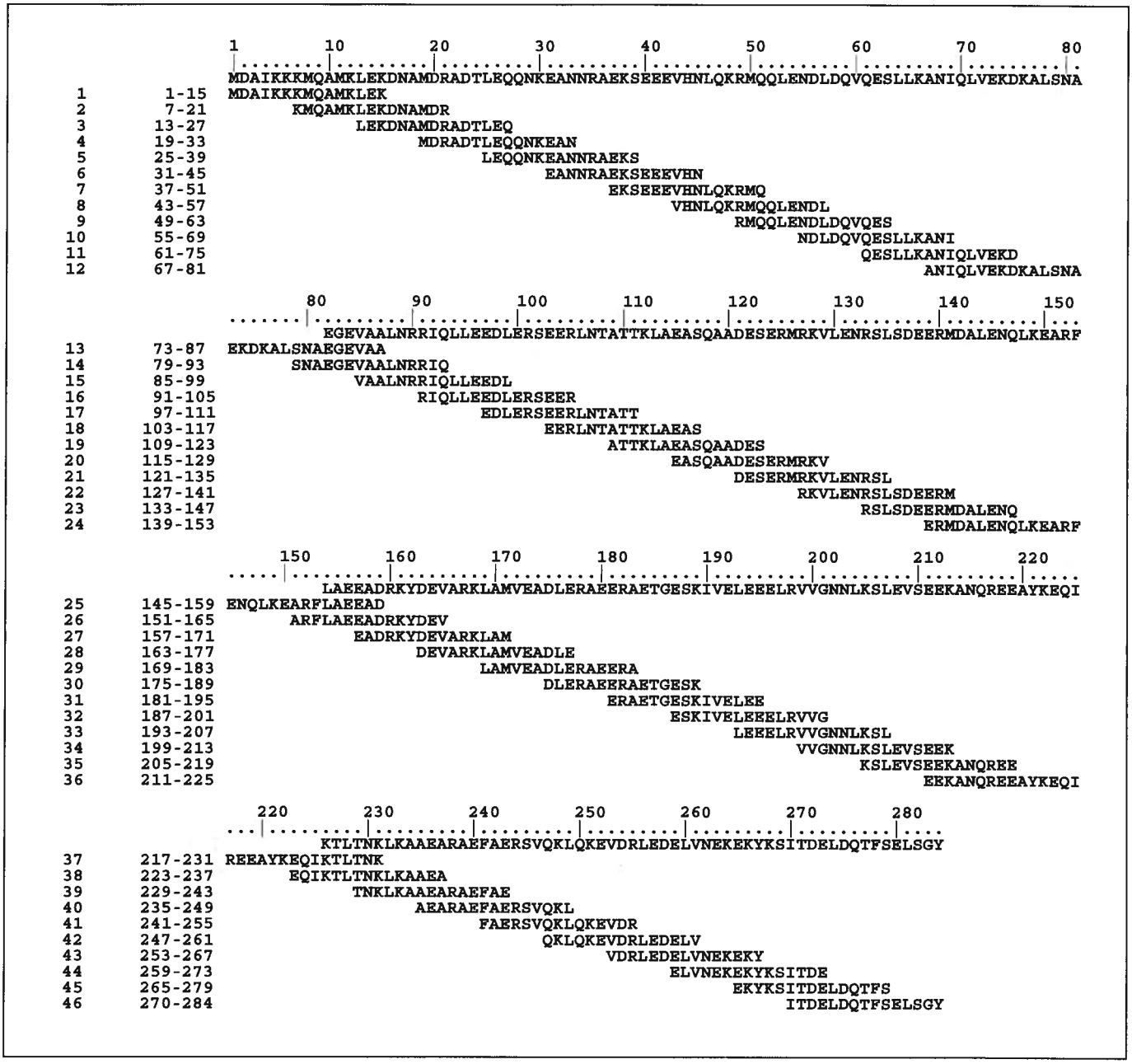

Fig. 1. Forty-six synthetic overlapping peptides spanning the entire sequence of Pen a 1 (length: 15 amino acid residues; offset: 6 amino acids).

IgE Antibody Reactivity to Synthetic Overlapping Pen a 1 Peptides: Peptide Synthesis and IgE-Binding Assay

Forty-six overlapping peptides were synthesized, spanning the entire 284-amino acid residue length of Pen a 1. Each peptide had a length of 15 amino acid residues with an offset of 6 in relation to the previous and following peptides, with the exception of peptide 46, which had an offset of 5 amino acid residues (fig. 1). Overlapping peptides were synthesized with fluorenylmethoxycarboyl (Fmoc) amino acids on cellulose membranes containing free hydroxyl groups according to the instructions of the manufacturer of the SPOTS Epitope Mapping System (Genosys Biotechnologies, The Woodlands, Tex., USA). For the preparation of the Fmoc-amino acid active ester solutions, each amino acid was dissolved in purified 1-methyl-2 pyrolidinone (NMP 99%; Sigma-Aldrich, St. Louis, Mo., USA). Each amino acid solution was aliquoted and stored at −20°C until ready for use. Due to intrinsic instability, arginine was dissolved in NMP immediately prior to each synthesis cycle.

The procedure for peptide synthesis was performed as described previously [29–31]. Briefly, each cycle began with sterification of an Fmoc amino acid to the SPOTs cellulose membrane (Genosys). Following incubation, the membranes were washed in N,N-didethylformamide (DMF; EM Science, Gibbstown, N.J., USA), and 4% acetic anhydride in DMF was added to acetylate to block any uncoupled amino groups to prevent further reaction of these groups and the formation of deletion sequences. After acetylation, protective Fmoc groups were cleaved by incubation in 20% piperidine (Aldrich Chemicals, Milwaukee, Wisc., USA) in DMF, to render nascent peptides reactive. Each additional Fmoc-amino acid was esterified to the previous one by the same process until the desired peptide was generated. After addition of the last amino acid, the protecting groups on the side chains of the amino acids were removed using a 1:1:0.05 mixture of dichloromethane (Aldrich), trifluoroacetic acid (Aldrich) and triisobutylsilane (Aldrich), followed by washing in methanol. Membranes were either probed immediately or dried and stored at −20°C until needed.

For the IgE-binding assay, the membranes were first rinsed in methanol and washed in TBS (pH 7.5) 3 times for 10 min. The membranes were incubated in blocking solution (Genosys) diluted 1:10 in TBS for 2 h and then overnight with the patient's serum diluted 1:5 with blocking buffer. After washing three times for 15 min in TBS-Tween (TBS, 0.5% Tween, pH 7.5), IgE reactivities were detected using 0.8 μCi per membrane of ^{125}I-labeled horse-anti-human IgE (Sanofi Diagnostics Pasteur, Chasca, Minn., USA) diluted 1:10 in Genosys blocking solution. The next day, the membranes were washed 3 times for 15 min in TBS-Tween, placed between plastic sheets and exposed to X-ray film for 72 h.

For interpretation of the results, IgE reactivities were graded according to their intensity into four categories, i.e. negative (0), weak (1), medium (2) and strong (3), and color coded as follows: negative = white; weak intensity = light gray; medium intensity = dark gray; strong intensity = black. The intensity of the IgE reactivities was determined visually by agreement between three different investigators, who graded the reactivities independently, assigning the above scores.

Results

IgE-Binding Regions of Pen a 1

Table 1 shows the frequency and intensity of the IgE recognition of the different peptides by the 18 subjects' sera. A wide range of peptides (from 1 to 16, mean of 8 per subject) were recognized by serum IgE of the shrimp-allergic, Pen a 1-reactive sera. In contrast, none of the three control sera from shrimp-allergic, non-Pen a 1-reactive subjects showed IgE binding to any of the 46 peptides tested (data not shown). As an example, figure 2 shows the peptides recognized by the serum IgE of 6 shrimp-allergic subjects, with each spot representing a different peptide out of all 46 tested. The number of sera which recognized individual peptides varied from 2 (11.1%) for one peptide to 33.3% for ten or more peptides. IgE-binding peptides were detected over most of the tropomyosin molecule. An intensity score (0–3) for each peptide was calculated by adding the individual scores obtained with each individual serum (peptide score). The mean intensity score of all peptides was obtained by adding all the peptide scores and dividing by 46 peptides (mean peptide score). A major IgE-binding region was defined as a region recognized by serum IgE from more than 9 out of 18 subjects (50%) and/or with an intensity score of IgE binding larger than the mean peptide score (5.9 ± 6.1) plus one standard deviation.

Based on the frequency and intensity of the IgE reactivities, five major IgE-binding regions were identified. All five major IgE-binding regions spanned from 1 to 4 peptides, with a length of between 15 and 38 amino acid residues. The major IgE-binding regions identified were: region 1: Pen a 1 (43–57); region 2: Pen a 1 (85–105); region 3: Pen a 1 (133–148); region 4: Pen a 1 (187–202), and region 5: Pen a 1 (247–284). Region 1 was recognized by 10 out of 18 subjects (55.5%), region 2 by 15 out of 18 (83.3%), region 3 by 10 out of 18 (55.5%), region 4 by 5 out of 18 (27.5%) and region 5 by 12 out of 18 (66.6%). The scores for intensity of IgE recognition were 18, 19, 20, 12 and 12.2 for the five regions, respectively.

Amino Acid Composition of the IgE-Binding Regions

To determine if certain groups of amino acids may be important for IgE binding, the relative frequency of different amino acids in the tropomyosin molecule relative to the main IgE-binding regions were analyzed. Five categories of amino acids were considered: nonpolar, aliphatic (alanine A, glycine G, isoleucine I, leucine L, proline P, valine V); polar, uncharged (cysteine C, asparagine N, methionine M, glutamine Q, serine S, threonine T); aromatic (phenylalanine F, tryptophane W, tyrosine Y); positively charged (histidine H, lysine K, arginine R), and negatively charged (aspartic acid D, glutamic acid E). The frequency of the different groups of amino acids in each IgE-binding region was considered in relation to the number of amino acids present in that group, supposing that all amino acids have the same probability of appearing in a protein (probability of 1). For the whole molecule, negatively charged amino acids are 2.5 times more frequent than would be expected by chance. In contrast, aromatic residues are almost absent in the tropomyosin molecule. The other three groups of amino acids are present in the molecule with the frequency expected considering the occurrence of all 20 amino acids to be equally probable. In the five main IgE-binding regions, the frequency of each amino acid group is the same as the frequency observed in the whole molecule. No substantial differences in amino acid group composition in the five IgE-binding regions

Table 1. IgE reactivities of 18 shrimp-allergic subjects to the 46 overlapping peptides of Pen A1

	Peptide No.																																																PR
Subject No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	
3																																																1
9																																																1
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Positive subjects, n	5	6	4	2	3	–	–	10	–	–	1	3	2	–	13	9	1	2	2	1	8	1	10	4	7	–	5	4	2	3	–	5	6	–	–	1	1	1	–	–	–	6	2	5	7	6	
Intensity	7	6	6	4	5	–	–	18	–	–	3	5	3	–	20	18	1	5	4	2	10	1	20	9	9	–	9	8	3	8	–	12	10	–	–	1	3	1	–	–	–	14	4	15	18	10	
≥50% of subjects								+							+	+							+																							
>Mean intensity + 1 SD								+							+	+							+								+												+		+	+		

The color-coded system used is as follows: strong (3): black; medium (2): dark gray; weak (1): light gray; negative (0): white. The five major IgE binding regions include: Pen a 1 region 1 (43–57), region 2 (85–105), region 3 (133–147), region 4 (187–202) and region 5 (247–284).
PR = Positive response.

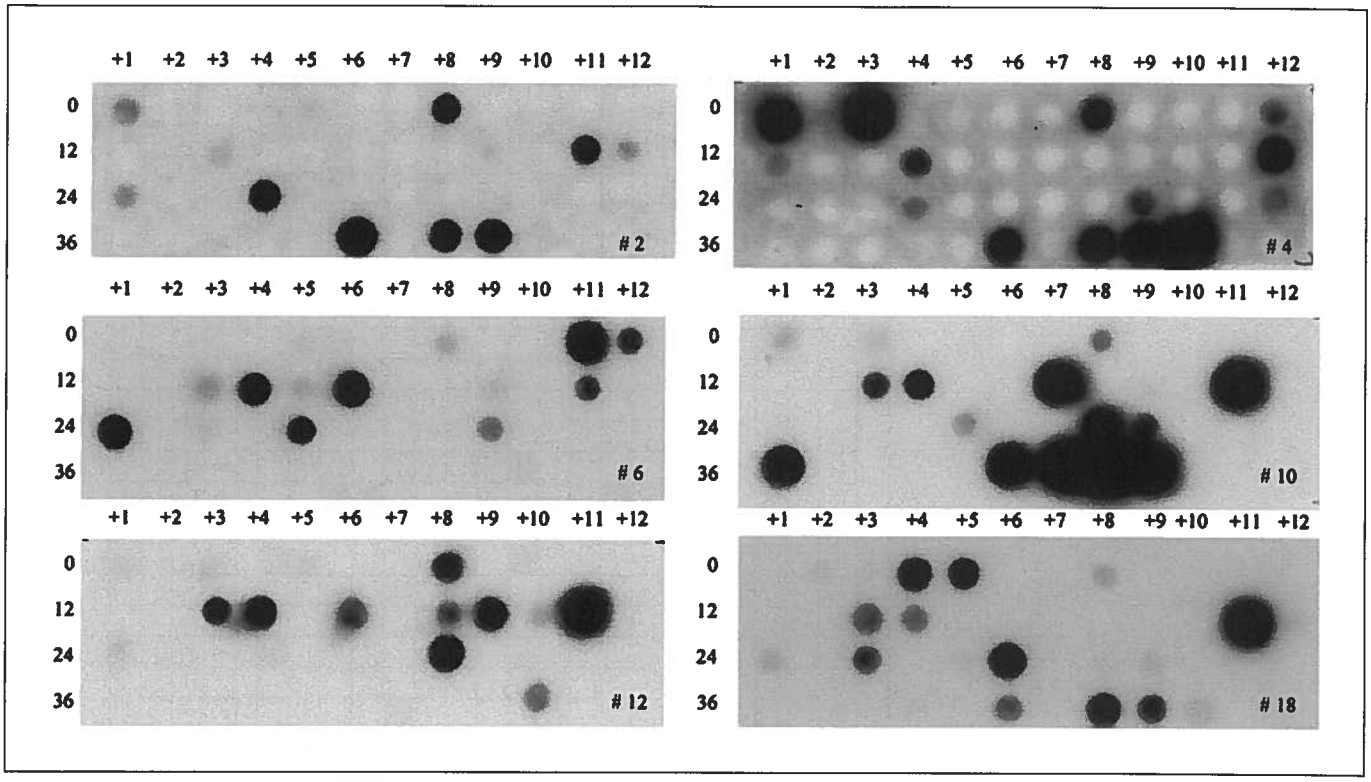

Fig. 2. SPOTs analysis of Pen a 1 peptides with sera from 6 representative shrimp-allergic subjects. For interpretation of the results, see subject 4: spot 1: strong (3); spot 16: medium (2); spot 12: weak (1); spot 37: negative (0).

compared to the whole molecule were detected. When individual amino acids were analyzed, it was noted that several amino acids such as proline, cysteine and tryptophane were absent from the molecule and others such as glycine, isoleucine, tyrosine and histidine were rarely found. In contrast, alanine, leucine, lysine, arginine and glutamic acid were present in the molecule at least 2 times more frequently than expected by chance. Only alanine seems to be less frequent in the IgE-binding regions than in the rest of the molecule. No differences in respect to the distribution of amino acids in the tropomyosin molecule were noticed, since all other amino acids are similarly represented in Pen a 1.

Allergenic Motifs

In previous studies, the presence of tandem amino acid repeats contained within allergens has been reported [32, 33]. In our study, all five major IgE-binding regions identified within Pen a 1 contain the amino acid sequence LEXXL, where L is leucine and X is usually a negatively charged amino acid such as glutamic acid (E) or aspartic acid (D). In regions 1 and 3, X may be an aspartic acid (D), glutamine (Q) or asparagine (N). The tandem motifs occupy positions 53–57 (motif 1), 95–99 (motif 2), 144–148 (motif 3), 193–197 (motif 4) and 256–260 (motif 5). Even though IgE reactivities were detected to Pen a 1 peptides which did not contain a tandem repeat, every tandem amino acid repeat was found to be included within a major Pen a 1 IgE-binding region.

Sequence Identity and Similarity of Pen a 1 and Pen a 1 IgE-Binding Regions with Homologous Regions in Other Allergenic and Nonallergenic Tropomyosins

Sequence identities (percentage of amino acids identical in both compared tropomyosins) and similarities (percentage of amino acids which are identical or belong to the same amino acid group) in the amino acid sequences of different tropomyosins with Pen a 1 IgE-binding regions are represented in table 2. Sequence identity among Pen a 1 and other tropomyosins varied from 56% (rabbit tropomyosin) to 98% (lobster fast tropomyosin); similarity in amino acid composition ranged from 72 to 98%, with the highest similarities (over 80%) being observed

Table 2. Sequence identity and similarity among Pen a 1, Pen a 1 IgE-binding regions and other allergenic and nonallergenic tropomyosins

Tropomyosin	Pen a 1		Region 1		Region 2		Region 3		Region 4		Region 5	
	Ide	Sim	Ide	Sim	Ide	Sim	Ide	Sim	Ide	Sim	Ide	Sim
Hom a TMf	98	98	100	100	100	100	100	100	100	100	100	100
Hom a TMs	93	96	46	80	100	100	100	100	100	100	100	100
Pan s 1	98	98	100	100	100	100	100	100	95	95	100	100
Per a 7	82	90	60	93	100	100	80	87	100	100	81	92
Der f 10	81	89	60	86	95	100	80	100	100	100	89	94
Der p 10	81	89	60	86	95	100	73	100	100	100	89	94
Dro m TM	70	87	46	93	100	100	87	87	95	100	68	81
Myt e TM	57	75	53	73	52	80	40	53	57	80	76	86
Onc v TM	70	83	53	80	80	90	53	73	90	95	81	94
Sch m TM	60	74	26	601	71	76	53	67	90	90	65	86
Gal g TM	58	72	33	53	71	85	47	67	76	85	44	63
Ory c TM	56	72	33	60	71	85	47	67	71	80	44	63

Identity (Ide): percentage of amino acids identical in both tropomyosin sequences compared. Similarity (Sim): percentage of amino acids which are identical or belong to the same amino acid group in both sequences compared. Five different groups were considered: nonpolar aliphatic: glycine, alanine, valine, leucine, isoleucine and proline; polar uncharged: serine, threonine, cysteine, methionine, asparagine and glutamine; aromatic: phenylalanine, tyrosine and tryptophane; positively charged: lysine, arginine and histidine; negatively charged: asparatate and glutamate. Crustacea: *P. aztecus* (brown shrimp, Pen a 1), *H. americanus* (Atlantic lobster, Hom a 1), *H. americanus* slow muscle tropomyosin (Hom a TMs) and *P. stimpsoni* (spiny lobster, Pan s 1). Insects: *P. americana* (American cockroach, Per a 7) and *D. melanogaster* (fruit fly, Dro m TM). Arachnids: *D. pteronyssinus* and *D. farinae* (house dust mites, Der p 10, Der f 10). Mollusks: *Mytilus edulis* (blue mussel, Myt e TM). Nematodes: *Onchocerca volvulus* (Onc v TM). Trematodes: *Schistosoma mansoni* (Sch m TM). Vertebrates: *Gallus gallus* (chicken, Gal g TM) and *Oryctolagus cuniculus* (rabbit, Ory c TM).

within arthropods (insects, arachnids and crustaceans). Remarkably, amino acid sequences of several IgE-binding regions of Pen a 1 showed a significant degree of identity with homologous sequences of other tropomyosins, specially among arthropods such as lobster, house dust mite, cockroach and fruit fly, reaching up to 100% identity in particular sequences (table 2).

Similarities between Pen a 1 regions and tropomyosin of mollusks such as mussels, helminths such as *Onchocerca* and, surprisingly, also vertebrates such as rabbit, although they varied depending on the regions considered, were as high as 86, 94 and 85%, respectively. Region 1 is identical within crustaceans but differs from other invertebrates and vertebrates, with only 26, 60 and 33% identity with *Schistosoma*, insects and vertebrates, respectively. Interestingly, regions 2 and 4 show 100% similarity with homologous regions of arthropod tropomyosins from American cockroach *P. americana* (Per a 7), fruit fly *Drosophila melanogaster* (Dro m TM) and house dust mites *D. pteronyssinus* and *D. farinae* (Der p 10, Der f 10). Furthermore, regions 2 and 4 show significant similarity with corresponding sequences in other tropomyosins, including those from vertebrates (up to 85%). Regions 3 and 5 are identical within crustaceans, and the identity with tropomyosins of other arthropods reaches up to 89%. In contrast, these Pen a 1 regions differ substantially from those of homologous sequences (as low as 40% identity) in helminths and vertebrate tropomyosins.

Comparison of IgE-Binding Regions Identified in Pen a 1 and in Other Allergenic Tropomyosins

Even though many invertebrate tropomyosins have been identified as allergenic, the location of the IgE-binding epitopes has been partially identified for only a small number of them. Figure 3 shows the amino acid sequence comparison of Pen a 1 with other allergenic tropomyosins whose B cell epitopes have been partially characterized, such as Pen i 1 from the shrimp *Penaeus indicus*, Tur c 1 from the snail *T. cornutus* and Cra g 1 from the oyster *C. gigas*. Results from a previous study by our group of IgE-binding epitopes from Pen a 1 using a recombinant peptide library of Pen a 1 are also included (regions E2,

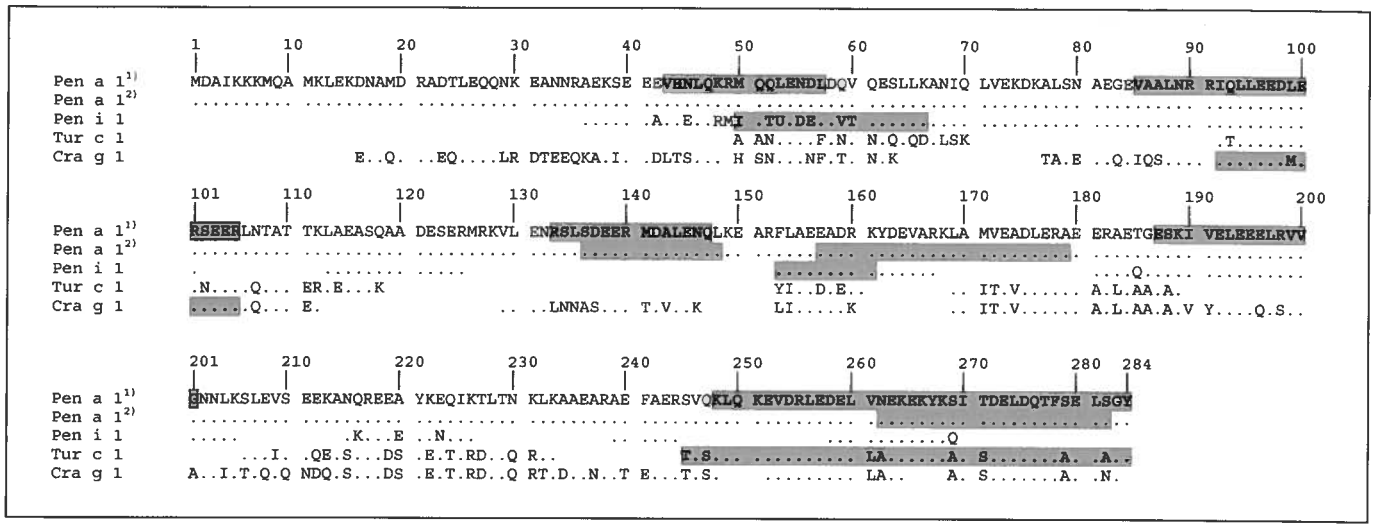

Fig. 3. IgE-binding regions of invertebrate tropomyosins. [1] Pen a 1 *(P. aztecus):* region 1 (43–57)[1], region 2 (85–105)[1], region 3 (133–147)[1], region 4 (187–202)[1] and region 5 (247–284)[1]. [2] Pen a 1 (167–179)[2], Pen a 1 (136–148)[2], Pen a 1 (262–282)[2] and Pen a 1 (157–169)[2] are Pen a 1 IgE-binding regions identified previously as E2, E3, E4 and E6 using a recombinant Pen a 1 peptide library [38]. Pen i 1 *(P. indicus):* Pen i 1 (50–66, 153–161) [4]; Cra g 1 *(C. gigas):* K21d (92–105) [11]; Tur c 1 *(T. cornutus):* MT17 (245–284) [10].

E3, E4 and E6). Most of the five IgE-binding regions identified in Pen a 1 in the current study partially or completely overlap with those observed in other allergenic tropomyosins. Region 5 seems to be of particular importance; it was also identified as an important IgE-binding region in the snail tropomyosin, Tur c 1, since the strongest IgE reactivities of both shrimp-allergic and snail-allergic individuals were directed towards this region.

Discussion

In recent years, the primary structures of a number of allergens have been characterized, and T and B cell epitopes of several allergens have been identified. Knowledge of the B and T cell epitopes in food allergens is important because it can serve as the basis for the development of new, safer recombinant allergens for food immunotherapy and genetically modified hypoallergenic foods; such information would aid in the assessment of the allergenicity of novel proteins in newly developed transgenic foods. Ideally, recombinant allergens developed for immunotherapy would have a reduced capacity to bind serum and mast cell-bound IgE, ensuring a lower risk of IgE-mediated side effects, while retaining their T cell epitopes, allowing for a modulation of the immune response.

In this study, five major Pen a 1 IgE-binding regions have been identified using synthetic overlapping peptides, 15 amino acids long with an offset of 6, spanning the whole length of Pen a 1. The five IgE-binding regions are distributed along the molecule at approximately every 42 amino acid residues. These results suggest a relation with the heptameric repeat pattern characteristic for the α-helical, coiled-coil structure of tropomyosin [21]. The five regions identified contain at least 15 amino acid residues (region 5 spans 37 residues). Since other studies have determined the IgE-binding epitopes of other allergens to consist of approximately 8 amino acids [29–31, 33], the identified IgE-binding regions of Pen a 1 are most probably larger than their epitopes and some regions may contain several epitopes. The smallest IgE-binding sequences within these regions of Pen a 1 remain to be investigated.

Previous studies of the homologous cockroach allergens Per a 1 and Bla g 1 [32] and the latex allergen Hev b 5 [33] have reported the presence of tandem amino acid repeats within allergens. Per a 1 and Bla g 1 sequences contain multiple tandem amino acid repeats of approximately 100 amino acid residues. Hev b 5 presents 9 repeated amino acid sequences of the type XEEX or XEEEX; four peptides of 8 amino acids containing the sequence XEEX were found to be IgE-binding epitopes. In contrast, none of the peptides containing the motif XEEEX bound IgE from latex-allergic subjects. In our

study, all five major IgE-binding regions identified within Pen a 1 contained the amino acid sequence LEXXL. Even though IgE reactivity with other Pen a 1 peptides that do not contain the tandem repeat was detected, every repetition of the tandem motif was found to be included within a major Pen a 1 IgE-binding region.

Tropomyosins are coiled-coil dimers made up of two parallel α-helical tropomyosin molecules that are wound around each other. The tropomyosin monomer contains a heptad repeat, $(abcdefg)_n$, in which generally large hydrophobic nonpolar residues occur at positions a and d, while positions b, c, e, f and g are usually occupied by polar or ionic amino acids [21]. The interaction between two α-helices in a coiled-coil involve these hydrophobic residues in position a and d. Charge-charge interactions between acidic residues found in position e and basic residues in position g also help stabilize the coiled-coil. The outer positions b, c and f must be free to interact with proteins such as actin and troponin (fig. 4). Interestingly, the tandem repeats always occupy positions d, e, f, g and a in the heptameric repeats, which represents the area of interaction between the two α-helices of tropomyosin. Although tropomyosin is a very flexible molecule that may spontaneously unfold and expose those internal sequences, the repeats are probably mostly nonexposed sequences in the native molecule. Since those sequences are usually not exposed in the tropomyosin molecule, they may not be responsible for the positive skin prick test observed in shrimp-allergic subjects, since some prior enzymatic digestion of the molecule would be required for dissociation and epitope exposure. Exposed linear or three-dimensional epitopes could be implicated in the positive skin prick test response to shrimp tropomyosin. In contrast, enzymatic activity in the gastrointestinal tract could theoretically expose protected epitopes that may be responsible for the allergic reactions. However, the importance of exposed versus nonexposed epitopes in relation to clinical symptoms remains to be elucidated.

Shrimp-allergic patients may react to other crustaceans and sometimes to mollusks [24, 34], and substantial in vitro cross-reactivity among crustaceans has been demonstrated. Furthermore, allergenic cross-reactivity between arachnids (house dust mites), crustaceans (shrimps) and mollusks (snails, limpets) has been suggested to be of clinical relevance, especially in subjects receiving mite immunotherapy [17, 19]. Also, it has been reported that some patients may become allergic to mites and/or cockroaches subsequent to their sensitization to crustacean tropomyosin due to unusual ingestion of crabs and shrimp [de Blay, pers. commun.]. Comparison of the amino acid sequence

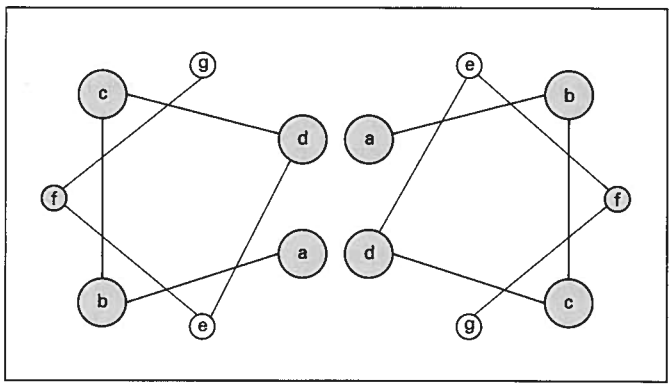

Fig. 4. Schematic end-on view of the coiled-coil structure showing the position of residues in the heptad repeat of tropomyosin.

of the entire Pen a 1 molecule with different tropomyosins showed that the identity ranged from 56% (rabbit tropomyosin) to 98% (lobster fast muscle tropomyosin). Similarities were even higher when conservative substitutions were not considered as different amino acids; the highest similarities were found among arthropods, which reached over 80%. When the amino acid sequences within Pen a 1 IgE-binding regions were compared with corresponding areas in other tropomyosins, the degree of similarity was found to be remarkably high; 100% identity of all five regions with the major lobster allergen Hom a 1 (fast muscle tropomyosin) was detected, explaining the high degree of cross-reactivity among crustaceans. The sequence identity of Pen a 1 IgE-binding regions with Per a 7 and Der p 10 is also very high (60–100%), which might suggest that there are similar IgE-binding epitopes in arthropods. Also consistent with this observation is the identity of Pen a 1 regions 2 and 4 with homologous amino acid sequences of tropomyosin from *D. melanogaster*. Similarities of Pen a 1 regions with those from mollusks and helminths are lower, but still as high as 86 and 94%, respectively. The importance of the cross-reactivity with helminths has been studied far less, but has also been reported [35].

Furthermore, comparison of IgE-binding regions identified in Pen a 1 and in other allergenic tropomyosins supports our results. Figure 3 and table 2 show the amino acid sequence comparison of Pen a 1 with other allergenic tropomyosins whose B cell epitopes have been partially characterized, such as Pen i 1 from the shrimp *P. indicus*, Tur c 1 from the snail *T. cornutus* and Cra g 1 from the oyster *C. gigas*. Results from a previous study by our group of IgE-binding epitopes from Pen a 1 using a recom-

binant library are also included [regions E2 (167–179), E3 (136–148), E4 (262–282) and E6 (157–169)] [36]. Most of the five IgE-binding regions identified in Pen a 1 in the current study partially or completely overlap with those observed in other allergenic tropomyosins. Regions 2 and 5 seem to be of particular importance, since the homologous sequences in oyster (Cra g 1) and snail (Tur c 1) tropomyosins bind IgE antibodies of mollusk-allergic subjects, thus supporting the notion that tropomyosin may be the cause of clinically relevant cross-sensitization between crustaceans and mollusks [24, 37]. Thus, the similarity of homologous regions of anthropod tropomyosins with IgE-binding regions of Pen a 1 supports the reported cross-reactivity of these molecules. Further studies need to identify the IgE-binding epitope(s) in each region and demonstrate inhibition of IgE binding by synthetic peptides representing homologous amino acid sequences of other reported cross-reacting tropomyosins.

In summary, this study has identified multiple IgE-binding epitopes in Pen a 1. As the understanding of the structure of allergens increases, safer and more effective therapeutic reagents may be produced [38]. In addition, the analysis of the structural basis of cross-reactivities of food and aeroallergens will have an impact on new approaches to the diagnosis and treatment of various allergic reactions. In this regard, allergenic and nonallergenic tropomyosins may serve as a model to elucidate the relationship between in vitro cross-reactivity among related proteins and their capacity to induce multiple clinical sensitization. Therefore, investigations of the tropomyosin molecule are not only directed towards a better understanding of seafood allergy but, in addition, will have significance in the broad area of allergy treatment and diagnosis.

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