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Identification of the Major Brown Shrimp (*Penaeus aztecus*) Allergen as the Muscle Protein Tropomyosin

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Abstract
Shrimp, a major seafood allergen, was investigated as a model food allergen. Extracts from both shrimp (*Penaeus aztecus*) meat and cooking fluid contain a substantial and similar amount of allergenic activity. A 36-kD allergen, demonstrated in both extracts by SDS-PAGE/Western blot analysis, reacted with 28/34 (82%) sera from shrimp-sensitive, skin test and RAST-positive, individuals. This allergen, named *Pen a* I, was isolated by SDS-PAGE; its amino acid composition was rich in aspartic and glutamic acids. A 21-residue peptide, obtained from endoproteinase Lys-C digested *Pen a* I by high-performance liquid chromatography, demonstrated significant homology (60–87%) with the muscle protein tropomyosin from various species and origins. The greatest homology (87%) was noted with tropomyosin of the fruit fly (*Drosophila melanogaster*) reflecting the phylogenic relationship between these two arthropods. These studies demonstrate that tropomyosin is the major shrimp allergen. Although the amino acid sequence of this shrimp muscle protein shares considerable homology with tropomyosins of other species including man, significant differences remain in allergenic activity.

Introduction

Hypersensitivity reactions to ingested seafood are one of the most frequently reported food allergies [1–3]. Symptoms can range from mild oral allergy syndrome to fatal anaphylactic shock. Although no precise information on the prevalence of seafood allergy exists, tentative estimates suggest that approximately up to 250,000 Americans are at risk of serious allergic reactions to seafood products [4]. During the last decade, seafood has become more widely consumed in many parts of the world. In 1990, consumption of seafood reached 15 pounds per capita in the United States [5, 6]. Total seafood consumption of United States commercial landings and imports equaled 65 million pounds for 1990. Of this amount, approximately 6 million pounds were *Crustacea*, a major class of seafood that includes shrimp, crab, lobster and crawfish [6]. As consumption continues to rise, the number of individuals who develop hypersensitivity reactions to *Crustacea* can be expected to expand.

The three major phyla that make up edible seafood are *Chordata* (subphylum *Vertebrata* – including the class *Osteichthyes* = bony fish); *Mollusca* (class *Bivalvia* – containing mussels or clams) and *Arthropoda* (class *Crustacea*, composed of shrimp, crab, lobster and crawfish). To date, *Gad c* I, the major codfish allergen [7–10], is the best studied seafood allergen. Its amino acid sequence has been determined and its allergenic epitopes have been identified. Other seafood allergens have not been well characterized.

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Shrimp is a major cause of seafood allergy and we frequently see subjects allergic to this seafood in our clinical practice [3, 4, 11]. This report details the demonstration and isolation of the major shrimp allergen, a 36-kD protein named *Pen a* I. The amino acid composition suggests that *Pen a* I is similar to, if not identical with the shrimp allergens Antigen II (38 kD) and Sa II (34 kD) previously described by Hoffman et al. [12] and Nagpal et al. [13]. Amino acid sequence homology studies of *Pen a* I demonstrated that this protein is shrimp tropomyosin.

Materials and Methods

Crustacea Allergen Extracts

Raw brown shrimp (*Penaeus aztecus*), obtained from a local seafood market, were boiled for 15 min in ion-depleted water. The meat was removed, deveined, and homogenized in a Waring® blender (Waring Products Division, New Hartford, Conn., USA) in phosphate-buffered saline (PBS) pH 7.2. The homogenate was centrifuged (44,000 *g*) and the supernatant was concentrated by Amicon-YM2 ultrafiltration (Amicon, Danvers, Mass., USA; molecular weight cutoff <1,000 D) at 4°C. The concentrate was recentrifuged (78,000 *g*) and the supernatant was aliquoted and stored at –20°C [13]. Since our previous studies indicated that shrimp cooking fluid contains substantial levels of shrimp allergen [14], this fluid was filtered (Whatman No. 1), concentrated (Amicon-YM1-MWCO >1,000 D) dialyzed against ion-depleted water (MWCO >3,500 D), lyophilized and stored at –20°C [14]. Inhibition of either shrimp meat or shrimp water RAST with shrimp meat or shrimp water extract demonstrated identical allergenic activity [14]. The meat of boiled blue crab (*Callinectes sapidus*), spiny lobster (*Panulirus argus*) and crawfish (*Procambarus clarkii*) were extracted as described for shrimp meat [11].

Patients' Sera

Sera were collected from 34 atopic shrimp-sensitive individuals, all with a history of respiratory (wheezing or shortness of breath), dermatologic (urticaria), or gastrointestinal (nausea, vomiting, and/or diarrhea) symptoms occurring within 1 h following ingestion of shrimp. All individuals were skin test positive (wheal ≥3 mm) with shrimp extract and demonstrated elevated levels of IgE antibodies (RAST binding ≥3%) to shrimp extract. Sera from 4 atopic shrimp-tolerant (no history of shrimp sensitivity) subjects who were skin test and RAST negative to shrimp, were used as controls. These sera contained total IgE levels similar to those of shrimp-sensitive subjects.

SDS-PAGE/Immunoblot Reactions

Proteins from shrimp meat and water extracts were separated by discontinous SDS-PAGE using Laemmli's buffer system (0.025 *M* Tris, 0.192 *M* glycine, pH 8.3, 0.1% SDS). A 4%T 2.7%C stacking gel was poured on top of 15%T 2.7%C separating gel in a minigel format (Hoefer SE200). Samples were denatured and reduced by boiling the sample in a SDS (2%) and 2-ME (1%) containing sample buffer. The samples and molecular weight markers (14–67 kD; BioRad, Hercules, Calif., USA) were applied to the gel and the electrophoresis was performed at 20 mA/gel (V_{max} = 200 V) until the bromphenol blue reached the bottom of the gel. Gels were either stained with Coomassie Blue or the proteins were transferred to nitrocellulose membrane (0.20 µm pore size, BA-83; Schleicher & Schüll, Dassel, Germany) at 100 V/350 mA for 75 min using a Mini Transblot tank (BioRad).

The blots were rinsed in PBS, blocked with PBS containing 10% fetal calf serum + 1% bovine serum albumin (BSA) (or 0.5% nonfat dry milk + 0.2% BSA), rinsed again with PBS-0.02% Tween 20, and cut into strips. These strips were incubated overnight with subjects' sera diluted 1:12 with PBS, and after washing 3 times with PBS-Tween incubated with 25,000 cpm/strip in ^{125}I goat-anti-human IgE (New England Nuclear Research Products, Boston, Mass., USA) for 16–20 h. The strips were washed in PBS (no Tween) and then dried and exposed to autoradiography film (Kodak X-OMAT AR; Kodak, Rochester, N.Y., USA) for 24–96 h at –70°C.

Isolation of the 36-kD Shrimp Allergen by Electroelution

Following SDS-PAGE, the gel was immersed in double distilled water, gently agitated for 10 s, and subsequently stained with Rapid Reversible Stain (Diversified Biotech, Newton Centre, Mass., USA) at RT for 5–10 min. Protein bands were visualized as unstained, clear bands on a uniformly stained semiopaque bluish-green background. The gel was washed twice in distilled water (5 min) and a gel strip containing the 36-kD band was cut with a razor blade, minced, placed in an elution tube, and eluted into the aqueous phase of 25 m*M* Tris, 0.192 *M* glycine, pH 8.3 with a BioRad model 422 electroeluter at 10 mA/tube over 4 h with stirring in the buffer chamber at RT. The aqueous phase was recovered (0.6 ml of eluted protein) and dialyzed against 25 m*M* Tris-HCl, pH 7.0. Eluted samples were analyzed for the 36-kD allergen by immunoblotting with pooled patients' sera. Samples containing the 36-kD allergen were pooled and concentrated on a speed VAC to dryness 4–6 h at RT. The residue was resuspended in ion-depleted water.

Amino Acid Composition and Amino Acid Sequence Determination

The amino acid composition of the 36-kD shrimp allergen was determined by W.M. Keck Foundation, Biotechnology Resource Lab., New Haven, Conn., USA. Since the N-amino terminus of the protein was blocked, the 36-kD allergen was digested with endoproteinase Lys-C. The amino acid sequence of a 21-residue peptide, isolated by HPLC, was determined and compared to 2,649,600 proteins in the Swiss protein data base.

RAST Inhibition Assay

Cyanogen bromide-activated discs were coupled with shrimp extract (2.5 µg/disc) or purified *Pen a* I (0.5 µg/disc) according to established procedure [11]. The discs were incubated with a diluted (1:10 or 1:12) serum pool (RAST-positive shrimp-sensitive patients) in presence of *Pen a* I (5, 0.5, 0.05, or 0.005 µg), or shrimp meat, crab or crawfish extract (25, 2.5, 0.25 or 0.025 µg) in triplicate. After washing 3 times, discs were incubated with ^{125}I-labeled goat anti-human IgE (15,000 cpm/disc) and measured with a gamma counter.

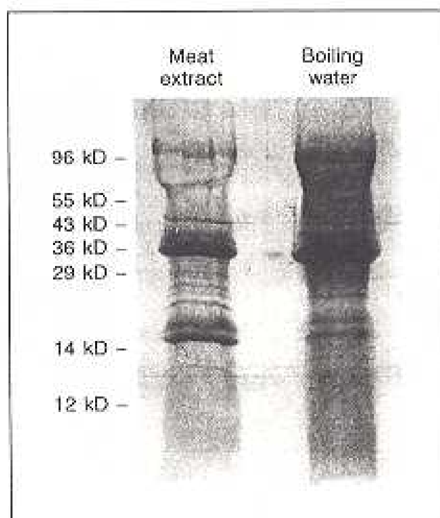

Fig. 1. Protein banding patterns of shrimp (*P. aztecus*) meat and boiling water extracts. 50 μg of brown shrimp meat or water extract were applied to the gel and SDS-PAGE performed. Protein bands were identified by Coomassie Blue staining.

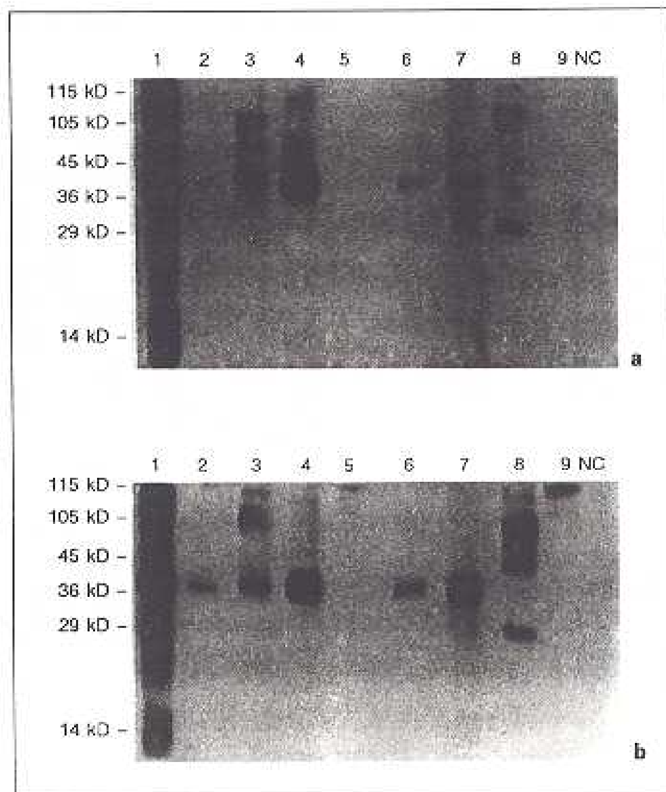

Fig. 2. IgE antibody reactivity to shrimp allergens present in meat (**a**) or water extracts (**b**) by SDS-PAGE and immunoblotting. Representative sera from the 34 shrimp-sensitive subjects were used to demonstrate that a number of proteins primarily in the molecular weight range of 20–115 kD bound IgE antibodies. The most frequent reaction was to a 36-kD protein band. 1–9 = IgE reactivities; NC = negative control.

Results

Shrimp Allergen Reactivity

Both shrimp meat and shrimp water extracts had similar protein banding patterns by Coomassie Blue staining (fig. 1). A major protein band was present in both extracts at 36 kD. The shrimp water extract had noticeably more small molecular weight proteins which did not band discretely. Representative IgE antibody reactivity of 10 of the 34 sera from shrimp-sensitive subjects to shrimp allergens by SDS-PAGE/immunoblot reaction is shown in figure 2. Most sera reacted similarly to both shrimp meat and shrimp water extracts. None of the control sera demonstrated any reactivity to shrimp proteins. The IgE antibody reactivities to the different shrimp allergens of all 34 sera tested are summarized in figure 3. IgE antibodies from the 34 shrimp-sensitive patients reacted to 13 protein bands in the shrimp meat extract ranging from <16 to 166 kD (fig. 3). The most reactive band to which 82% of the sera bound was the 36-kD protein band (fig. 3). This band was called *Pen a* I according to the IUIS allergen nomenclature [15].

Properties of Pen a *I*

Pen a I was purified from crude shrimp meat extract by SDS-PAGE. A comparison of purified *Pen a* I to crude shrimp extract by SDS-PAGE is shown in figure 4. Substantial purification of *Pen a* I was achieved by electroelution, although several bands corresponding to large molecular weight proteins (probably representing aggregates of *Pen a* I) remained. *Pen a* I has an isoelectric point of 5.2 by isoelectrofocusing and a carbohydrate content of 2.9% (analyzed by Complex Carbohydrate Research Center, University of Georgia, Athens, Ga., USA). Amino acid composition of *Pen a* I (table 1) indicates that it is rich in the acidic amino acids aspartic and glutamic acid. Comparison of *Pen a* I amino acid composition with the previously studied shrimp allergens – Antigen II [12] and Sa II [13] – indicates some degree of similarity (table 1).

Amino Acid Sequence of the 21-Amino Acid Residue from Pen a *I*

The amino acid sequence of the 21-residue peptide from *Pen a* I is shown in figure 5. The most remarkable aspect of this sequence is its substantial homology with tropomyosin

Fig. 3. Individual IgE reactivities of 34 shrimp-allergic subjects by SDS-PAGE and immunoblotting to shrimp meat extract. The most reactive band is the 36-kD protein to which 28/34 (82%) of the sera reacted. This major allergen was designated *Pen a* I. The second most reactive band was the 105-kD protein (8/34, 24%).

Fig. 4. Comparison of purified *Pen a* I to crude shrimp extract (meat) by SDS-PAGE. 8 μg of *Pen a* I and 30 μg of shrimp extract were loaded on the gel and the proteins separated by SDS-PAGE followed by Coomassie Blue staining. The results demonstrate substantial purification on *Pen a* I.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1.	Val	Leu	Glu	Asn	Arg	Ser	Leu	Ser	Asp	Glu	Glu	Arg	Met	Asp	Ala	Leu	Glu	Asn	Gln	Leu	Lys
2.	Ile	Leu	Glu	Asn	Arg	Ala	Leu	Ala	Asp	Glu	Glu	Arg	Met	Asp	Ala	Leu	Glu	Asn	Gln	Leu	Lys
3.	Val	Leu	Glu	Asn	Arg	Ser	Gln	Gln	Asp	Glu	Glu	Arg	Met	Asp	Gln	Leu	Thr	Asn	Gln	Leu	Lys
4.	Val	Leu	Glu	Asn	Arg	Ala	Leu	Lys	Asp	Glu	Glu	Lys	Met	Glu	Leu	Gln	Glu	Ile	Gln	Leu	Lys
5.	Val	Leu	Glu	Asn	Arg	Ala	Met	Lys	Asp	Glu	Glu	Lys	Met	Glu	Leu	Gln	Glu	Met	Gln	Leu	Lys

1. *Pen a I* (major shrimp allergen)
2. fruit fly tropomyosins; homology 87%
3. fruit fly tropomyosins; homology 72%
4. human tropomyosins; homology 62%
5. chicken, mouse, rat, human tropomyosins; homology 60%

Fig. 5. The amino acid sequence of the 21-residue peptide from *Pen a* I. The most remarkable aspect of the sequence of this peptide is the substantial homology (60–87%) with the muscle protein tropomyosin from various species.

of other species. The most significant homology (72–87%) was observed with fruit fly (*Drosophila melanogaster*) tropomyosin amino acid residues 129–149 and tropomyosins from mammalian species (60–62%). The homology of *Pen a* I exclusively to tropomyosins from sources as diverse as fruit fly and man, identifies the major shrimp allergen *Pen a* I as shrimp tropomyosin.

Pen a *I-Like Molecules in Other* Crustacea

Immunoblot analysis of IgE reactivities of shrimp-sensitive subjects to crab, lobster, and crawfish (fig. 6) demonstrate a 36-kD allergen in these species. RAST IgE reactivity to *Pen a* I can be inhibited in a dose-dependent manner (fig. 7) by crawfish, lobster, and crab extracts to the same degree as by shrimp extract. These results indicate that *Pen a* I and *Pen a* I-like proteins (= tropomyosins) are major *Crustacea* allergens.

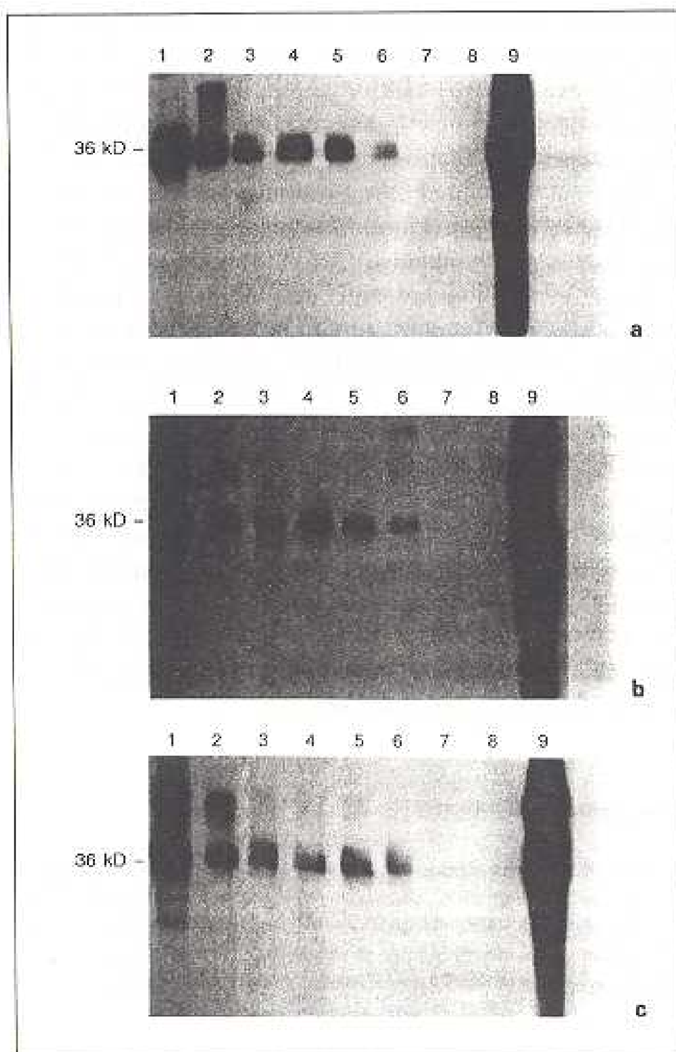

Fig. 6. Immunoblot reactivity of IgE antibodies in sera of shrimp-allergic subjects to proteins present in lobster (**a**), crawfish (**b**) and crab (**c**). The reactions of these sera demonstrate that *Pen a* I-like 36-kD allergens are present in each species.

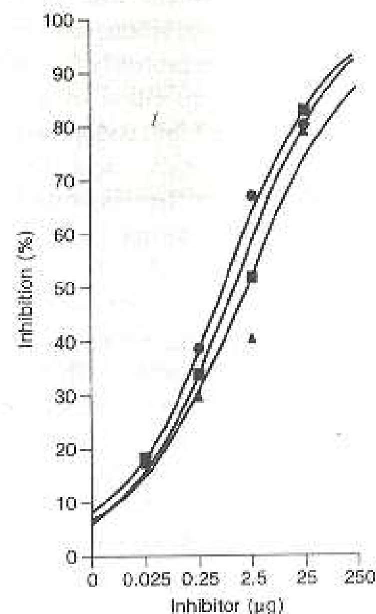

Fig. 7. Inhibition of *Pen a* I RAST by shrimp (■), crawfish (●), and crab (▲) proteins.

Table 1. Amino acid composition of shrimp allergens *Pen a* I, Antigen II and Sa II

Amino acid	*Pen A* I	Antigen II [12]	Sa II [13]
Aspartic acid	40	58	39
Threonine	12	12	9
Serine	14	15	12
Glumatic acid	81	61	75
Proline	2	6	3
Glycine	10	20	6
Alanine	33	31	21
Cysteine	ND	2	3
Valine	13	19	15
Methionine	8	9	6
Isoleucine	35	30	30
Leucine	6	12	6
Tyrosine	4	7	6
Phenylalanine	4	9	6
Histidine	1	4	3
Lysine	26	27	27
Arginine	26	19	30
Tryptophan	ND	ND	4
Total	312	341	301
Calculated MW, kD	36.2	37.0	36.1
MW by SDS-PAGE, kD	36.0	38.8	34.0

Discussion

Our studies have demonstrated that more than 80% of the shrimp-sensitive subjects tested had substantial IgE antibody reactivity to the shrimp allergen *Pen a* I, whereas all of the other shrimp allergens were detected by less than 50% of the subjects tested. *Pen a* I comprises approximately 20% of the total shrimp meat is a water-soluble protein [14] and inhibits 75% RAST reactivity of pooled patients' IgE antibody to whole shrimp meat extract. Based on these observations, we conclude that *Pen a* I is the only major shrimp al-

lergen. Shrimp allergens with similar molecular weights and amino acid compositions have been identified by other investigators and are probably the same molecule [12, 13].

Amino acid composition of a 21-residue peptide obtained from *Pen a* I by endoproteinase Lys-C digestion strongly suggests that the major shrimp allergen, *Pen a* I, is shrimp tropomyosin. Tropomyosin is a major muscle protein present in crude shrimp extracts. Recent evidence from the laboratory of Nagpal et al. [13] also demonstrates that a *Pen a* I-like molecule from another shrimp species (*Penaeus indicus*) is shrimp tropomyosin based on the amino acid sequences of peptides, which confirms our observations [17]. Furthermore, immunoblot and RAST inhibition studies utilizing sera from shrimp-sensitive subjects [18, 19] and *Pen a* I-specific monoclonal antibodies [19, 20] demonstrate the presence of 36-kD allergens in other *Crustacea* (lobster, crawfish and crab). Probably, tropomyosin is a major *Crustacea* allergen present in crab, shrimp and crawfish. These results confirm our earlier clinical [11] and experimental [18] findings that there is substantial cross-reactivity among *Crustacea* species.

In contrast to inhalant allergens [19–23], the molecular basis of the interaction between food allergens and the immune system has not been well studied. *Pen a* I and other tropomyosins provide the unique opportunity to study a group of closely related proteins that are both allergenic (shrimp-derived) and nonallergenic (human-derived) and to learn more about those pathophysiological processes that result either in tolerance or allergic disease to orally ingested proteins. The characterization of B- and T-cell epitopes will contribute to better diagnoses and treatment of food allergy, for example by blocking of the allergic reaction with haptenic peptides or by inducing protective antibodies or allergen-specific T-cell anergy.

The main function of the gastrointestinal tract is to process and absorb ingested food. Gut-associated lymphoid tissue is the largest lymphoid organ in the body and is exposed to the greatest antigenic load confronting any of the host-defense systems. It must remain unresponsive to a wide range of nutrient materials and yet stand ready to mount a rapid and potent response against pathogenic viruses, bacteria, parasites and other foreign substances. In spite of its substantial responsibilities, we know far less about the role of gut lymphoid tissue in the immune response compared to other better studied organs as the spleen, thymus or even the lungs. Through utilization of a well-characterized food antigen such as *Pen a* I, further studies can address important gut immune processes such as T-cell tolerance induction, mucosal membrane immunization, as well as those events that result in food allergy sensitization.

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