Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen

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Shrimp is a common cause of seafood hypersensitivity. To study the mechanism of seafood hypersensitivity at the molecular level, we have determined the primary structure of the major heat-stable allergen of shrimp by cloning, expression, nucleotide sequencing, and amino acid sequence determination of an IgE-reactive cDNA clone, Met e *I, isolated from a* Metapenaeus ensis *expression library in λgt 11. We first constructed a cDNA library from the shrimp* M. ensis *in λgt 11. We then screened the library with sera from patients with hypersensitivity reactions to shrimp and identified a positive IgE-reactive clone, designated as* Met e *I. This cDNA was purified to homogeneity and subsequently expressed in the plasmid pGEX. Serum antibodies from patients with shrimp allergy demonstrated positive IgE reactivity by immunoblotting to a protein encoded by the clone* Met e *I; sera from nonallergic control subjects were not reactive. The nucleotide sequence of this cDNA clone revealed an open reading frame of 281 amino acid residues, coding for a protein of 34 kd. Comparison of the* Met e *I amino acid sequence with the Genbank database showed that* Met e *I is highly homologous to multiple isoforms of tropomyosin. (J ALLERGY CLIN IMMUNOL 1994;94:882-90.)*

Key words: *Shrimp allergy,* Metapenaeus ensis, *IgE reactivity, cDNA clone, tropomyosin, recombinant protein, epitope*

It is well established that shellfish-sensitive individuals who ingest crustaceans can experience urticaria, angioedema, laryngospasm, wheezing, and severe anaphylaxis.[1, 2] Although crustaceans have been reported to be one of the more common causes of hypersensitivity reactions to foods, most clinical studies of crustacean allergy have been primarily based on skin testing and RASTs.[3, 4] In both skin testing and RAST, the results are evaluated with a crude crustacean extract. In general, subjects with a history of shrimp sensitivity have a positive skin test response to a low concentration of crustacean extract and positive RAST results in response to a crustacean extract.[1] Daul et al.[5] reported serum IgG, IgM, and IgE levels against shrimp allergens from 11 individuals with shrimp hypersensitivity during a 24-month period. Shrimp antigen--specific IgE and IgG levels, but not IgM and IgA levels, were significantly higher in the

Abbreviations used

LB:	Luria broth
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate–polyacrylamide gel electrophoresis

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group with a history of hypersensitivity compared with control subjects. However, there is also a population of nonatopic individuals with histories of shrimp hypersensitivity who have negative skin test or negative RAST results.[1, 2, 6]

In light of the successful application of molecular cloning in the identification of allergens, we have cloned and expressed an IgE-reactive cDNA clone, obtained by screening a *Metapenaeus ensis* cDNA library with sera from subjects with hypersensitivity to shrimp. The clone is termed *Met e* I according to the nomenclature system proposed by Marsh et al.[7] The nucleotide sequence of *Met e* I and the deduced amino acid sequence of *Met e* I showed significant homology to that of *Drosophila melanogaster* tropomyosin II. Recently, Shanti et al.[8] reported the identification of Sa-II, the major heat-stable shrimp allergen, as tropomyosin by comparison of tryptic digest peptides of Sa-II with the amino acid sequence of *D. melanogaster* protein; our data are consistent with this amino acid analysis. The cloning and expression of shrimp allergen will provide a valuable source of pure allergens for further characterization and subsequent focus in defining the physiologic basis of shrimp sensitivity.

METHODS

Construction of cDNA library

Messenger RNA was isolated from tissues of the live shrimp, *M. ensis* by the guanidium isothiocyanate method.[9] Poly A+ RNA was subsequently purified by oligo-dT cellulose (Collaborative Research, Lexington, Mass.), and a cDNA library was constructed in bacteriophage λgt 11 as described by Young and Davis.[10]

Screening of cDNA library

Serum samples from patients with a known history of shrimp anaphylaxis were obtained from the sera bank at the Division of Rheumatology/Allergy and Clinical Immunology at University of California, Davis. All patients had a documented history of an immune hypersensitivity reaction, including bronchospasm or urticaria after ingestion of shrimp. Other clinical data on these patients are not available. With these sera as probes, the *M. ensis* cDNA library was plated on Luria broth (LB) agar by infecting *Escherichia coli* Y1090 at a density of 20,000 to 30,000 plaques per plate. Plaques were transferred onto multiple nitrocellulose filters. Thereafter, the filters were blocked with Blotto (3% milk in phosphate-buffered saline [PBS]) and incubated individually with sera from patients with shrimp allergy (1:10 dilution) at 4° C for 8 to 10 hours. The filters were then washed with PBS containing 0.05% Tween-20 three times and then incubated with iodine 125–labeled anti-human IgE (Sanofi-Pasteur Diagnostics, Ohaska, Minn.) at 4° C for 8 to 10 hours. The filters were washed as before and exposed to x-ray film. Those plaques that produced positive signals with sera from eight patients with shrimp allergy, but not the 10 nonallergic control subjects, were purified to homogeneity.

Nucleotide sequence determination of cDNA

Phage DNA was isolated from the purified IgE-reactive λgtll clone as previously described,[11] and the cDNA insert was excised by EcoRI digestion. The cDNA was then gel-purified on low melting temperature agarose and ligated into the EcoRI site of plasmid vector pSK (Stratagene, La Jolla, Calif.). After transformation, miniprep plasmid DNA was prepared from the transformants. The nucleotide sequence of the cDNA was determined by dideoxy sequencing[12] with Sequenase (United States Biochemical Corp., Cleveland, Ohio) from double-stranded DNA by using dye, labeled primers and terminators. Gels were run on an Applied Biosystems 373A sequencer (Applied Biosystems, Inc., Foster City, Calif.).

Expression of cDNA

The cDNA encoding the IgE-reactive protein was cloned into the EcoRI site of the plasmid expression vector pGEX[13] (Pharmacia Biotech, Alameda, Calif.). Transformants were selected, and a colony immunoassay was performed with sera from patients with shrimp allergy ($n = 8$). Briefly, transformants were transferred onto nitrocellulose filters, and the filters were incubated on LB agar plates containing 25 μg/ml ampicillin for 10 hours at 37° C and then induced by transferring the filter to LB agar plates containing 25 μg/ml ampicillin and 1 mmol/L isopropyl thiogalactose. After 4 hours of incubation at 37° C, cells were lysed by chloroform vapor, and the filters were incubated with Blotto for blocking. After blocking, the filters were incubated with sera from patients with shrimp allergy (1:10 dilution) at 4° C for 8 to 10 hours. The filters were then washed with PBS containing 0.05% Tween-20 three times and then incubated with I^{125}-labeled anti-human IgE (Sanofi-Pasteur Diagnostics) at 4° C for 8 to 10 hours. The filters were washed as before and exposed to x-ray film. Colonies corresponding to positive signals on the x-ray film were chosen for immunoblotting. Sera from 10 nonallergic volunteers were run in parallel as controls.

Determination of molecular weight of expressed IgE-reactive shrimp protein by immunoblotting

Positive expression clones were grown in LB agar containing 25 μg/ml ampicillin at 37° C overnight and then induced by 1 mmol/L isopropyl thiogalactose for 4 hours at 37° C. The cell lysate was resolved on

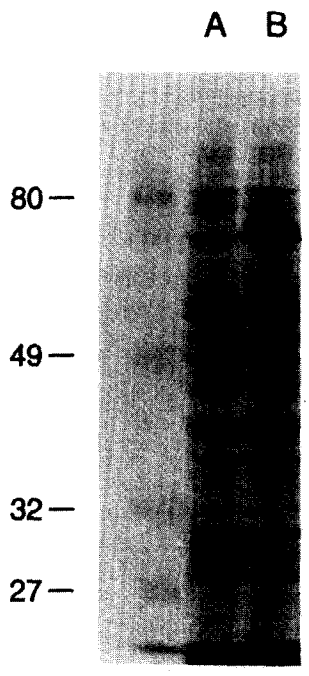

FIG. 1. SDS-PAGE of *E. coli* lysate of pGEX1 containing recombinant *Met e* I fused to glutathionine-S-transferase. Note the presence of a 60 kd recombinant fusion band in *lane B* but not in control *lane A*. The 60 kd band is composed of a 26 kd glutathione-S-transferase fused to a 34 kd protein.

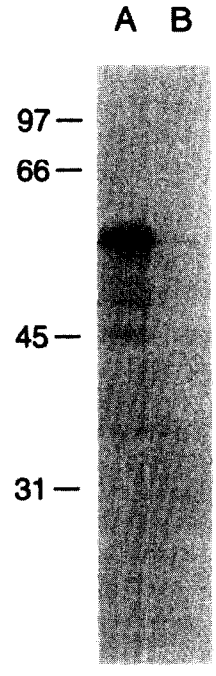

FIG. 2. IgE immunoblot of pGEX *Met e* I with sera from patients with shrimp allergy. Note the presence of a 60 kd reactive band in *lane A* but not in control *lane B*. Normal sera are nonreactive.

10% sodium dodecylsulfate–polyacrylamide gel (SDS-PAGE), which was then transferred onto nitrocellulose filter as previously described.[14] Sera from patients with shrimp allergy (1:10 dilution) were then used to probe the filters, and IgE reactivity was again deduced by using I^{125}-labeled anti-human IgE. After washing and exposure to x-ray film, the position of the positive band was located, and the approximate molecular weight was determined with standard molecular weight markers.

Purification of IgE-reactive *M. ensis* protein, *Met e* I

A 500 ml overnight culture of a pGEX-expressing clone of the IgE-reactive *M. ensis* protein was induced with 1 mmol/L isopropyl thiogalactose, and the cells were harvested. The cells were suspended in 5 ml of lysis buffer (1% Triton X-100, 1% Tween 20, 10 mmol/L dithiothreitol) and sonicated. The cell lysate was then centrifuged to pellet the debris, and the supernatant was added to glutathionine agarose. After incubating for 2 hours on a shaker, the glutathionine agarose was washed three times with PBS containing 1% Triton X-100 (pH 7.3), and the bound protein was eluted with 2 ml of 5 mmol/L reduced glutathione in 50 mmol/L Tris-HCl (pH 8.0). The purified protein was quantitated, and the purity was determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described above.

RESULTS

Identification of an IgE-reactive clone from *M. ensis* cDNA library

The unamplified cDNA library from *M. ensis* muscle had 9.8×10^6/ml total plaque-forming unit primary recombinant clones; this corresponds to 82% of the library. Five IgE-reactive clones were identified by screening the cDNA library with sera from patients with shrimp allergy. The five clones cross-hybridized with each other. One of them was purified and designated *Met e* I according to the nomenclature proposed by Marsh et al.[7] Protein encoded by *Met e* I reacted by Western blot to all eight sera from patients with histories of shrimp anaphylaxis but not to any of the control sera. Digestion of purified phage DNA with EcoR I produced a 2 kb cDNA fragment on agarose gel (data not shown).

Expression of *Met e* I

The *Met e* I cDNA was subcloned into the frame-shifted plasmid expression vectors pGEX, and the protein was expressed in pGEX 1. Com-

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1                                                  10                                                  20
E  F  C  G  R  Q  A  M  K  L  E  K  D  N  A  M  D  R  A  D

21                                                 30                                                  40
T  L  E  Q  Q  N  K  E  A  N  N  R  A  E  K  S  E  E  E  V

41                                                 50                                                  60
E  N  L  Q  K  R  M  Q  Q  L  E  N  D  L  D  Q  V  Q  E  S

61                                                 70                                                  80
L  L  K  A  N  N  Q  L  V  E  K  D  K  A  L  S  N  A  E  G

81                                                 90                                                  100
E  V  A  A  L  N  R  R  I  Q  L  L  E  E  D  L  E  R  S  E

101                                                110                                                 120
E  R  L  N  T  A  T  T  K  L  A  E  A  S  Q  A  A  D  E  S

121                                                130                                                 140
E  R  M  R  K  V  L  E  N  R  S  L  S  D  E  E  R  M  D  A

141                                                150                                                 160
L  E  N  Q  L  K  E  A  R  F  L  A  E  E  A  D  R  K  Y  D

161                                                170                                                 180
E  V  A  R  K  L  A  M  V  E  A  D  L  E  R  A  E  E  R  A

181                                                190                                                 200
E  T  G  E  S  K  I  V  E  L  E  E  E  L  R  V  V  G  N  N

201                                                210                                                 220
L  K  S  L  E  V  S  E  E  K  A  N  Q  R  E  E  A  Y  K  E

221                                                230                                                 240
Q  I  K  T  L  T  N  K  L  K  A  A  E  A  R  A  E  F  A  E

241                                                250                                                 260
R  S  V  Q  K  L  Q  K  E  V  D  R  L  E  D  E  L  V  N  E

261                                                270                                                 280
K  E  K  Y  K  S  I  T  D  E  L  D  Q  T  F  S  E  L  S  G

231
Y
```

FIG. 3. Deduced amino acid sequence of *Met e* I. Amino acid sequence is shown by one-letter amino acid code. The cDNA has an open reading frame that codes for a protein of 281 amino acids. The first five amino acids are believed to be derived from the linker; see Fig. 4. Genebank accession number U08008.

parison of cell lysates of *Met e* I in pGEX 1 and the pGEX 1 wild-type control by SDS-PAGE demonstrated the presence of a 60 kd fusion protein band in pGEX 1 *Met e* I (Fig. 1). Further, immunoblotting of the cell lysate demonstrated a 60 kd band reactive with IgE from patients with shrimp allergy (Fig. 2). The fusion protein was further purified by glutathionine agarose; the purified fusion protein showed a protein band at 60 kd, which still retained its IgE reactivity (data not shown). Because the gluthionine S-transferase has a molecular weight of 26 kd, the deduced molecular weight of *Met e* I was estimated to be 34 kd by SDS-PAGE.

Amino acid determination of *Met e* I

The deduced amino acid sequence of *Met e* I are shown in Fig. 3. Several points should be

TABLE I. Amino acid composition comparison of *Met E* I with other heat-stable shrimp allergens

Amino acid	*Met e* I	Antigen II*	Sa-IIt	*Pen a* I‡
Alanine	31	31	21	33
Cysteine	1	2	3	ND
Aspartic acid	17	58	39	40
Glutamic acid	55	61	75	80
Phenylalanine	4	9	6	4
Glycine	5	20	6	10
Histidine	1	4	3	1
Lysine	25	27	27	26
Leucine	33	30	30	35
Methionine	6	9	6	8
Asparagine	18	ND	ND	ND
Proline	0	6	3	2
Glutamine	17	ND	ND	ND
Arginine	22	19	30	26
Serine	15	15	12	14
Threonine	9	12	9	12
Valine	14	19	15	13
Tryptophan	0	ND	4	ND
Tyrosine	4	7	6	4
Isoleucine	4	12	6	6
Total	281	341	301	312

Amino acid compositions are derived from deduced amino acid sequence from cDNA sequences of clone pSKWK1 from *M. ensis*.
ND, Not measured.
*Major heat-stable shrimp allergen.[28]
†Heat stable allergen of *Penaeus indicus*.[27]
‡From Musmand et al.[26]

TABLE II. Amino acid sequence comparison of putative IgE-reactive epitopes of shrimp tropomyosin and related proteins

Organism	Peptide 1	Peptide 2	Reference
Shrimp			
M. ensis	MQQLENDLDQVQESLLK	FLVEEARDK	This article
P. indicus	MQQLENDLDQVQESLLK	FLAEEADRK	8
Fruit fly			
D. melanogaster	IQTVENELDQTQEALTL	FLAEEADKK	29
Snail			
Biomphalaria glabrata	FSNLQNDFDTANEGLTE	YIAEDAERK	Genbank
Roundworm			
Schistosoma mansoni	LKQAIDCDEAETLQEQM	YIAEDADRK	50
Chicken			
Gallus gallus	LKGTEDEVEKYSESVKE	HIAEEADRK	32
	LKGTEDELDKYSESLKD	HIAEEADRK	31
Quail			
Coturnix coturnix	LKGTEDELDKYSESLKD	HIAEEADRK	51
Fish			
Zebra fish	LKATEDELDKYSEALKD	HIAEEADRK	52
Frog			
Xenopus laevis	LKGTEDELDKYSEALKD	HIAEEADRK	53
Pig			
Sus scrofa	LKATEDELDKYSEALKD	HIAEDADRK	Genbank
Rat			
Rattus norvegicus	IRSLQEQADAAEERAGS	HIAEDADRK	34

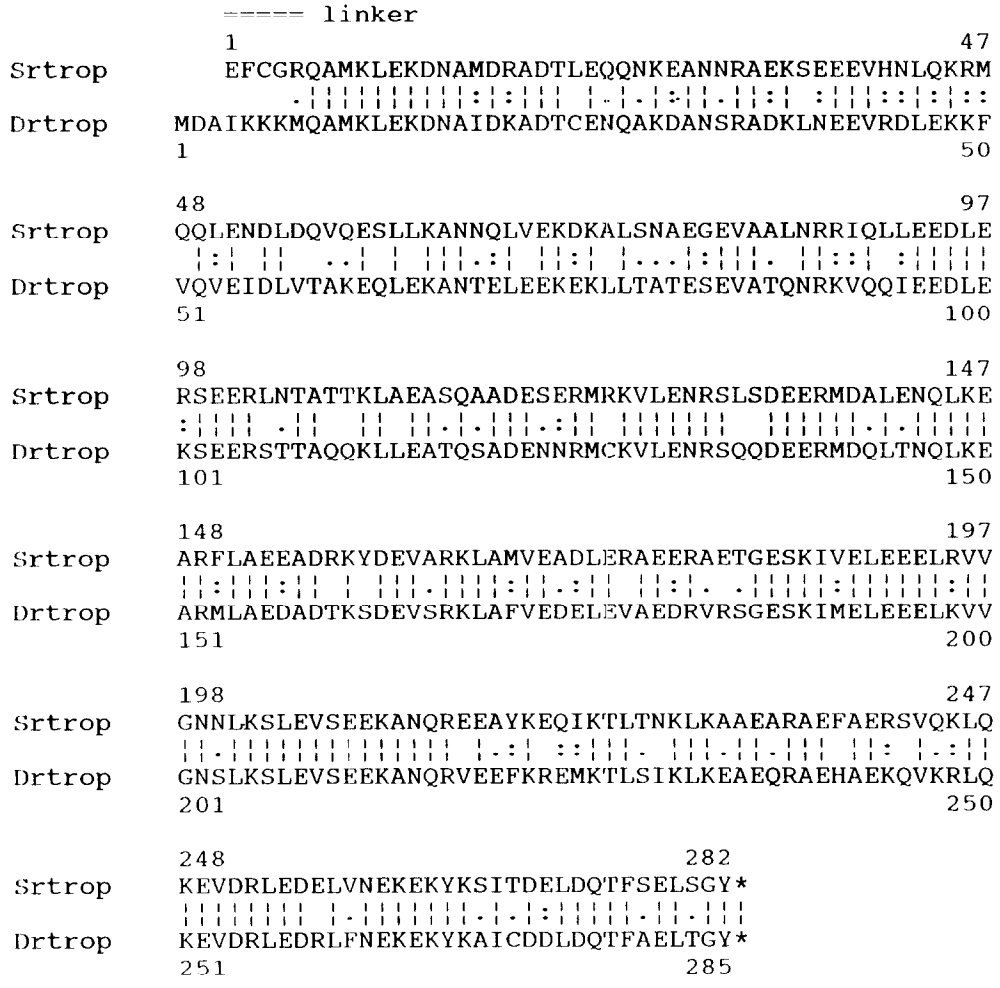

FIG. 4. Alignment of the cloned shrimp cDNA *(Srtrop)* with tropomyosin isoform from *Drosophila* (*Drtrop,* Genbank accession Nb K93277). The percent similarity is 76.596; percent identity is 65.603. *Drtrop* represents the coding sequence of the isoform T of the adult thoracic tropomyosin of *Drosophila.* This coding sequence is encompassed in exons 2, 3, and 4 of the *Drosophila* tropomyosin gene. Exon 1 has been shown to remain untranslated.

made. First, the nucleotide sequence analysis of *Met e* I cDNA shows an open reading frame. The protein is rich in alanine, aspartic acid, lysine, leucine, glutamic acid, and arginine (Table I). A Genbank data search of *Met e* I reveals that the protein is very similar, at the amino acid level, to the previously described sequence of *D. melanogaster* tropomyosin II (Fig. 4).

Finally, two oligopeptides (MQQLENDLDQVQESLLK and FLAEEADRK) of *Pen a* I have been reported to contain IgE-reactive epitopes.[8] We have compared these sequences with tropomyosin from fruit fly, snail, roundworm, chicken, quail, fish, frog, pig, and rat (Table II). In general, a higher degree of identity is found between the shrimp and *Drosophila* tropomyosin than other animal species in the peptide MQQLENDLQVESLLK. On the other hand, the peptide FLAEEADRK is strikingly similar among the animal species, except in the first two amino acid residues. Sequence analysis of the whole protein also revealed other hypervariable regions, which may include other unidentified IgE epitopes. Further work in mapping these unidentified epitopes is in progress.

DISCUSSION

In the past, identification and characterization of antigens were primarily based on conventional biochemical and immunologic techniques, including subfractionation, chromatography, gel electrophoresis, immunoblotting, and immunoassays.

However, advances in molecular biology have allowed the rapid identification of antigens by molecular cloning of cDNA in an expression library. With this approach, a number of antigens have been cloned, sequenced, and identified (including grass and mite allergens).[15-22] To date, the best known examples are allergens of rye grass pollen,[15, 16] ragweed,[17, 18] the major mite allergen *Der p* II,[19, 20] and white-faced hornet venom allergen, antigen 5.[21, 22]

Crustacea has long been known to be a common cause of food hypersensitivity reactions.[23] Several attempts have been made to understand the mechanism of the pathogenesis of crustacean allergy, including double-blind placebo-controlled food challenges. However, a major difficulty in these studies is that only a small percentage of subjects claiming food allergy actually showed an objective reaction by double-blind placebo-controlled food challenges. In addition, shrimp-sensitive individuals often demonstrate species-specific reactivity to shrimp by skin testing and RASTs.[24, 25] Several studies in the past have characterized major IgE-reactive proteins with respect to their molecular weight, isoelectric point, and amino acid composition.[26-28] However, the identity of shrimp allergens has only recently been identified as tropomyosin.[8]

To assist in the study of the allergen at the molecular level, we have constructed a cDNA library from *M. ensis*. *M. ensis* is a commercially important shrimp in the South China Sea.[29] It is among the major commercial shrimp harvests in the waters of Malaysia, Singapore, and Indonesia.[30] This species is extensively cultured in many areas of Southeast Asia, including Malaysia, Thailand, the Philippines, Taiwan, and Hong Kong. It is exported to the United States. The species was convenient for use in this study because it can be easily obtained from local markets in Hong Kong and Southeast Asia throughout the year.[31] We have cloned a major IgE-reactive protein from an *M. ensis* cDNA library with sera from subjects with hypersensitivity to shrimp. The expressed protein is heat-stable because it retains its IgE reactivity in immunoblots in which the protein is boiled and denatured before resolution on polyacrylamide gel. The nucleotide sequence and amino acid sequence analysis of *Met e* I showed significant homology to fruit fly tropomyosin. Amino acid sequence analysis of a short peptide of a previously reported shrimp allergen *Pen a* I[26] also showed strong homology with the fruit fly muscle protein tropomyosin.[32] *Pen a* I was reported to constitute at least 20% of the shrimp protein and has a molecular weight of 36 kd as determined by SDS-PAGE.[26] Likewise, Hoffman et al.[28] have reported a 38 kd shrimp allergen II with an isoelectric point of 5.4, and Nagpal et al.[27] have reported a 34 kd shrimp protein, Sa-II, both of which were remarkably similar to *Pen a* I in amino acid composition. Nonetheless, the most important contribution was the recent study that identified this protein as tropomyosin.[8] Using our clone, we have analyzed the amino acid sequence of *Met e* I and also found significant identity with that of fruit fly tropomyosin. We have also compared the amino acid composition of our cloned shrimp allergen *Met e* I with that of *Pen a* I, Sa-II, and antigen II and found that they are extremely similar, suggesting that the major shrimp allergen is likely to include tropomyosin isoforms among various shrimp species. Isoforms in tropomyosin have been reported in a variety of species, including fruit flies, chickens, and mammals.[32-37]

Tropomyosins are a diverse group of proteins with distinct isoforms found in muscle (skeletal, cardiac, smooth), brain, and nonmuscle cells. In muscles, tropomyosin interacts with actin and troponin T in regulating muscle contraction.[38, 39] Structurally, tropomyosins are elongated two-stranded proteins with a dimeric α-helical coiled structure along their entire length. The coiled structure is based on a repeated pattern of seven amino acids with hydrophobic residues at its first and fourth positions, which is highly conserved among tropomyosins. Tropomyosins bind to themselves in a head-to-tail manner. Although tropomyosins are highly homologous, structural differences do exist among isoforms, which appear to correspond to functional domains of the proteins, such as actin-binding sites, troponin-binding regions, and sequences involved in head-to-tail polymerization.[39-42]

The cross-reactivity of IgE to shrimp with other crustaceans has been well documented.[1-2, 43, 44] This cross-reactivity has also been reported between shrimp and other animals, such as cockroaches, chironomid larvae, caddis flies, and mollusks.[45-49] The expression of recombinant shrimp allergen will permit us to test for cross-reactivity of these allergy sera against the shrimp tropomyosin and assist in the identification of allergens among other arthropods and mollusks. In addition, the availability of this cDNA clone may allow the development of specific and reliable diagnostic and therapeutic approaches to treatment of patients with shrimp allergy.

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