

IgE reactivity against a cross-reactive allergen in crustacea and mollusca: Evidence for tropomyosin as the common allergen

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Background: Although cross-reactivity between mollusks and other crustaceans in shrimp-sensitive subjects has been reported, the mechanism of this allergenic cross-reactivity has not been studied in detail.

Objective: To investigate this cross-reactivity in vitro, we have taken advantage of a complementary DNA that expresses tropomyosin, the immunodominant shrimp allergen.

Methods: Serum IgE from nine patients with known anaphylaxis to shrimp and five normal volunteers were analyzed by immunoblotting against 13 distinct crustaceans and mollusks. As additional antigens, muscle preparations were isolated from grasshopper, cockroach, fruit fly, chicken, and mouse.

Results: Sera from all patients, but not control subjects, reacted specifically with a 38 kd protein in all crustaceans and mollusks studied. In addition, 8 of 9 sera from patients, but from none of the normal control subjects, recognized proteins of various other molecular weights among the mollusk extracts studied. The 38 kd protein was identified as tropomyosin and was shown to share immunodominant epitopes among all species of crustaceans and mollusks tested by specific absorption studies. Moreover, sera from all nine subjects with shrimp allergy demonstrated IgE reactivities against grasshopper, cockroach, and fruit fly but not chicken or murine muscle.

Conclusion: The allergic epitopes on tropomyosin are conserved among invertebrates including not only shellfish but also insects. This latter observation suggests that persons sensitive to shrimp should undergo further study for potential cross-reactive inhalant or ingested insect sensitivity. (*J Allergy Clin Immunol* 1996;98:954-61.)

Key words: IgE, cross-reactivity, tropomyosin, crustaceans, mollusks, insects

Crustaceans are well-known allergens in susceptible persons.¹ Hypersensitive reactions include oropharyngeal pruritus, urticaria, angioedema, gastrointestinal distress, and anaphylaxis.^{2,3} Similar adverse reactions have been described in patients consuming mollusks such as cuttlefish,⁴ abalone,⁵ limpet,⁵⁻⁷ and squid.⁸

Abbreviations used

PBS: Phosphate-buffered saline
SDS-PAGE: Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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alone,⁵ limpet,⁵⁻⁷ and squid.⁸ Allergy to mollusks is of particular interest. Significant RAST reactivity to oyster extracts has been found in sera from crustacea-sensitive subjects, and a number of patients having sensitivity reactions to both crustaceans and oysters have been reported to have elevated IgE antibodies to extracts of both raw and boiled oysters.⁹ Moreover, inhibition experiments also demonstrated that there is significant correlation of IgE reactivity to oyster with that to crustacean allergens, suggesting that oyster and crustacean may share common antigenic epitopes.⁹

Although cross-reactivity has been reported between crustaceans and mollusks,^{7, 9, 10} between crustaceans and insects,¹¹⁻¹³ and between mollusks and insects,¹⁴ the biochemical and molecular identity of the cross-reactive allergens between arthropods and mollusks has not been reported.

The molecular analysis of seafood allergens has been greatly advanced by the application of recombinant DNA technology. Recently, the major shrimp allergen has been identified as the muscle protein tropomyosin.¹⁵⁻¹⁷ Because tropomyosin is a highly conserved protein among all animal species, it is likely that similar, if not identical, epitopes may be present in tropomyosin of common edible shellfish. We analyzed the pattern of IgE reactivities of a panel of sera from subjects with a clinical history of hypersensitive reactions to shrimp using a variety of common edible mollusks (including Class Gastropoda, Class Bivalvia, and Class Cephalopoda) and animals other than Crustacea and Mollusca (insects and vertebrates) by immunoblotting. Our data demonstrate that sera from subjects with shrimp allergy but not from normal control subjects react with a protein band of approximately 38 kd in both crustaceans and mollusks. Furthermore, the reactivity to this 38 kd protein in mollusks can be totally absorbed by preincubating the sera with recombinant shrimp tropomyosin. The presence of this common allergen explains the cross-reactivity between crustaceans and mollusks and enhances our understanding of the molecular basis of the hypersensitive response against a highly conserved molecule.

METHODS

Serum samples

Sera from nine subjects with a known clinical history of hypersensitivity to shrimp were obtained from the sera bank at Division of Allergy and Clinical Immunology, University of California at Davis. All patients had a documented clinical history of anaphylaxis after ingestion of shrimp. These patients avoided other crustaceans, and it was not known whether they would clinically react to other seafood products. Sera from five volunteers who did not have a clinical history of crustacean or other seafood allergy were used as controls.

Animal muscle extracts

Live samples of abalone (*Haliotis diversicolor*), whelk (*Hemifusus ternatana*), mussel (*Perna viridis*), pen shell (*Pinna atropurpurea*), clam (*Lutraria philipinarum*), cuttlefish (*Sepia madokai*), squid (*Loligo edulis*), octopus (*Octopus luteus*), shrimp (*Metapenaeus ensis*), mud crab (*Scylla serrata*), and spiny lobster (*Panulirus homarus*)

were purchased from a seafood market in Hong Kong. Grasshoppers (*Mecopoda elongata*) were obtained from a local pet shop in Hong Kong. Frozen samples of oyster (*Crassostrea gigas*) and adductor muscle of "Alaskan scallop" (unidentified species of the family Pectinidae) were purchased from a local supermarket in Davis, California. Cockroach (*Periplaneta americana*) and fruit fly (*Drosophila melanogaster*) were obtained from the Department of Entomology, University of California, Davis. Mouse and chicken muscle were obtained locally.

Muscles of crustaceans and mollusks were sampled from the most commonly edible parts. For mollusk samples, the foot muscles were obtained from abalone and whelk; the adductor muscles were obtained from mussel, pen shell, scallop, oyster, and clam; and the muscular mantle tissues were collected from cuttlefish, squid, and octopus. For crustacean samples, abdominal muscles (tail) were collected from shrimp and lobster, whereas cheliped muscles (claw) were obtained from crab. Grasshopper muscle was obtained from the femur and tibia of the third pair of legs; muscles were collected from the femur of mouse and chicken. About 2 gm of muscle proteins were obtained from each animal and were boiled in deionized distilled water for 10 minutes. The boiled muscle was homogenized with lysis buffer (100 mmol/L Tris, 10 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L β -mercaptoethanol) or phosphate-buffered saline (PBS) in a Polytron homogenizer (Brinkman) for 30 seconds and centrifuged at 14,000 rpm for 10 minutes. The supernatant was aliquoted and stored in -70°C .

Whole body extracts from cockroaches (*Periplaneta americana*) and fruit flies (*Drosophila melanogaster*) were prepared by homogenizing the whole insect (cuticle removed from cockroaches) in PBS at 4°C in a Polytron homogenizer. The homogenates were then centrifuged twice at 40,000 rpm for 15 minutes to precipitate the debris, and the supernatant was stored at -20°C until use. The amount of total protein in each soluble muscle extract was determined by using a protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as the standard.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Each muscle protein extract was resolved by SDS-PAGE. Briefly, 100 to 150 μg extract was denatured and boiled in 2X sample loading buffer (0.125 mol/L Tris, pH 6.8, 4% SDS, 10% β -mercaptoethanol, and 0.002% bromophenol blue) for 10 minutes. Each sample was resolved in a 10% separating gel with a 5% stacking gel by using a mini-gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) containing the electrode buffer (0.025 mol/L Tris, pH 8.3, 0.192 mol/L glycine, and 0.1% SDS) at 20 mA for 1 hour. Kaleidoscope prestained molecular weight standards (Bio-Rad) consisting of myosin (207 kd), β -galactosidase (139 kd), bovine serum albumin (84 kd), carbonic anhy-

TABLE I. IgE reactivities of shrimp-sensitive sera against animal muscle extracts

Organisms	Reactive band (kd)	No. reactive sera/total sera tested*
Phylum Arthropoda		
Class Crustacea		
Shrimp (<i>Metapenaeus ensis</i>)	38	9/9
Spiny lobster (<i>Panulirus homarus</i>)	38	9/9
Mud crab (<i>Scylla serrata</i>)	38	9/9
Class Insecta		
Grasshopper (<i>Mecopoda elongata</i>)	38	9/9
Cockroach (<i>Periplaneta americana</i>)	38	9/9
Fruitfly (<i>Drosophila melanogaster</i>)	38	9/9
Phylum Mollusca		
Class Gastropoda		
Abalone (<i>Haliotis diversicolor</i>)	38	9/9
Whelk (<i>Hemifusus tematana</i>)	38	9/9
	49	8/9
Class Bivalvia		
Mussel (<i>Perna viridis</i>)	38	9/9
Pen shell (<i>Pincta atropurpurea</i>)	38	9/9
	49	4/9
	68	1/9
Scallop	38	9/9
	72	1/9
Oyster (<i>Crassostrea gigas</i>)	38	9/9
	49	2/9
	69	1/9
Clam (<i>Lutraria philipinarum</i>)	38	9/9
Class Cephalopoda		
Cuttlefish (<i>Sepia madokai</i>)	38	9/9
Squid (<i>Loligo edulis</i>)	38	9/9
	49	7/9
	72	1/9
Octopus (<i>Octopus luteus</i>)	28	1/9
	38	9/9
	72	1/5
Phylum Chordata		
Chicken	—	—
Mouse	—	—

*Sera were tested at 1:10 dilution for their IgE reactivity against muscle proteins from various organisms by immunoblotting. Normal sera did not react to any of the preparations in this study.

drase (41.7 kd), soybean trypsin inhibitor (32 kd), lysozyme (17.9 kd), and aprotinin (8.6 kd) were run in parallel for each gel.

The resolved proteins in the gel were electrophoretically transferred onto a nitrocellulose filter in a semi-dry transfer cell (Bio-Rad) at 15 V for 1 hour. Each blot was then stained with Ponceau S stain (Sigma, St. Louis, Mo.) to verify the transfer of separated proteins. Strips of 3 mm in width were cut from the blot and were blocked with 3% (wt/vol) nonfat dried milk in PBS for 30 minutes. The strips were then incubated in the test serum (1:10 dilution) in PBS with 3% milk at 4° C overnight with shaking. After incubation of primary serum, the strip was washed in PBS-Tween (0.05% Tween-20 in PBS) at room temperature three times for 20 minutes each. The bound IgE on the strip was detected by incubation with 0.4 μ Ci/ml of iodine 125-labeled anti-human IgE (Sanofi-Pasteur Diagnostics, Ohaska, Minn.) in PBS buffer with 3% milk at 4° C overnight. Nonspecific binding was removed by washing the strip with PBS-Tween at room temperature three times for 20 minutes. The strips were exposed to x-ray film with an intensifying screen from 24 hours to 1 week at -70° C. The approximate molecular weight of the allergens identified on x-ray film was estimated with the standard molecular weight markers.

Preparation of recombinant shrimp allergen

An overnight culture of an expression clone coding for recombinant shrimp tropomyosin, Met e 1 in *Escherichia coli* was grown in Luria-Bertani medium containing 25 μ g/ml ampicillin at 37° C and induced with isopropyl thiogalactose at a final concentration of 1 mmol/L.¹⁵ The cells were harvested by centrifuging at 6500 rpm for 20 minutes at 4° C. Recombinant fusion protein was purified as previously described.¹⁸ Briefly, frozen cells were subjected to three cycles of freezing and thawing and subsequently lysed in lysis buffer containing 1% Triton X-100, 1% Tween 20, and 10 mmol/L dithiothreitol and sonicated for 30 seconds three times. After centrifugation, the supernatant was collected and added to pre-swelled glutathione agarose (Sigma) and rocked overnight at 4° C. The agarose was washed twice with MTPBS (150 mmol/L NaCl, 16 mmol/L Na₂HPO₄, 4 mmol/L NaH₂PO₄), and the fusion proteins were eluted with elution buffer containing 50 mmol/L Tris, pH 8.0, and 5 mmol/L reduced glutathione. The concentration of the recombinant fusion protein was determined as described above.

Serum absorption

Sera from nine subjects with shrimp allergy were diluted 1:10 in PBS containing 3% milk and incubated with 50 μ g of recombinant shrimp tropomyosin at 4° C overnight. These preabsorbed sera were then tested for their IgE reactivities against muscle extracts by immunoblotting. Unabsorbed sera, sera absorbed with an irrelevant protein PDC-E2 (the human dihydrolipoam-

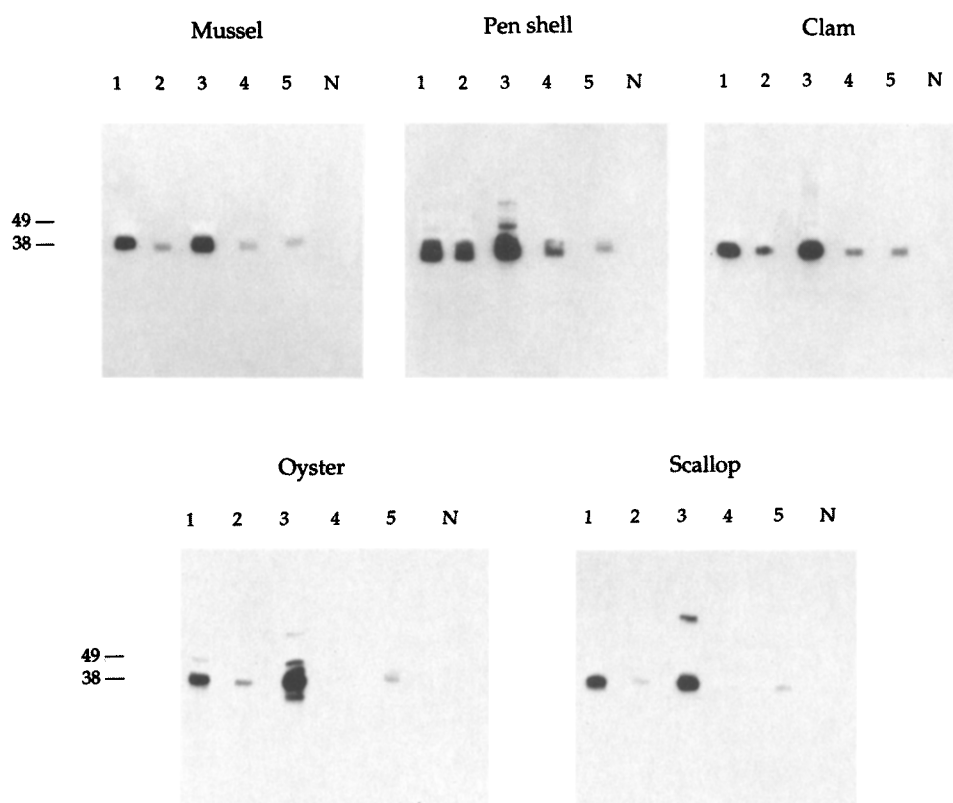


FIG. 1. Immunoblotting of sera from subjects sensitive to shrimp against muscle extracts from Class Bivalvia, including mussel, pen shell, clam, oyster, and scallop. Muscle proteins were resolved by means of SDS-PAGE and transferred to nitrocellulose filters. Three millimeter strips from each blot were tested for their reactivities to serum IgE from subjects allergic to shrimp and from normal control subjects. Lanes 1 through 5 represent five shrimp-sensitive sera, respectively. Lane N represents a negative control of normal sera. Molecular weights of reactive bands are indicated in kilodaltons. Similar data were obtained in immunoblots of subjects sensitive to shrimp against the muscle extracts from Class Gastropoda, including abalone and whelk.

ide acetyltransferase),¹⁹ and sera from normal volunteers were run in parallel as controls.

RESULTS

Reactivity of shrimp-sensitive sera against crustacean muscle extracts

The boiled muscle extracts from shrimp, spiny lobster, and mud crab were subjected to immunoblotting against a panel of sera from subjects with a clinical history of shrimp allergy. Nine sera, which had been previously shown to react to recombinant shrimp tropomyosin,¹⁵ reacted to muscle proteins at approximately 38 kd in the crustacean samples (Table I).

Reactivity of shrimp-sensitive sera against mollusk muscle extracts

IgE reactivity of the nine sera obtained from patients with a documented clinical history of

shrimp hypersensitivity was tested against 10 common edible mollusks (Table I). All nine sera demonstrated IgE reactivity to the 38 kd protein present in the boiled muscle extracts of all mollusks, which included members from Class Gastropoda (abalone and whelk), Class Bivalvia (mussel, pen shell, scallop, oyster, and clam), and Class Cephalopoda (cuttlefish, squid, and octopus). Representative immunoblots of five of the nine sera against the mollusk muscle extracts are shown in Figs. 1 and 2. Normal sera did not react with any of the muscle samples tested.

In addition to the major cross-reactive 38 kd allergen, other minor allergens were found in the immunoblotting experiments. In particular, a 49 kd antigen was detected in eight of the nine sera tested in whelk; in seven of the nine sera tested in squid; in four of the nine sera tested in pen shell; and in two of the nine sera tested in oyster (Table

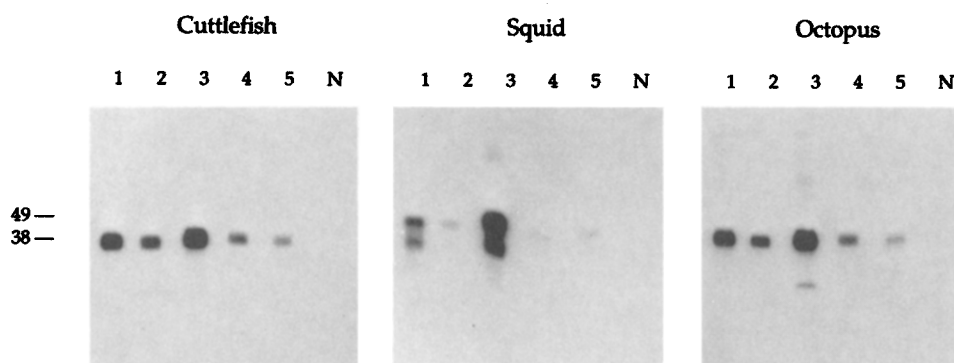


FIG. 2. Immunoblotting of sera from subjects sensitive to shrimp against muscle extracts from Class Cephalopoda, including cuttlefish, squid, and octopus. Muscle proteins were resolved by means of SDS-PAGE and transferred to nitrocellulose filters. Three millimeter strips from each blot were tested for reactivities to serum IgE from subjects allergic to shrimp and from normal control subjects. Lanes 1 through 5 represent five shrimp-sensitive sera, respectively. Lane N represents a negative control of normal sera. Molecular weights of reactive bands are indicated in kilodaltons.

I). It was also found that serum from subject 3 detected other antigens in some mollusk samples tested (Figs. 1 and 2). The IgE of this serum reacted with the allergen of 68 kd in oyster and pen shell extracts and with an allergen of 72 kd in scallop, octopus, and squid extracts. In addition to these antigens, a 28 kd antigen in octopus was recognized by the serum from subject 3 (Fig. 2).

Inhibition of IgE reactivity of shrimp-sensitive sera to crustacean allergen by recombinant shrimp allergen

Sera preabsorbed with recombinant shrimp tropomyosin were tested for their IgE reactivities against shrimp, spiny lobster, and mud crab by means of immunoblotting. Sera preabsorbed with recombinant shrimp tropomyosin lost all of their IgE binding reactivity against the 38 kd protein band in shrimp, spiny lobster, and mud crab, suggesting the presence of similar, if not identical, IgE epitopes among these crustaceans. Unabsorbed sera and sera absorbed with an irrelevant clone, a glutathione-S-transferase fusion protein of human PDC-E2,²⁰ did not lose their reactivity against the 38 kd band (data not shown).

Inhibition of reactivity of shrimp-sensitive sera to mollusk allergen by recombinant shrimp allergen

Preabsorption of the tested sera with recombinant shrimp tropomyosin resulted in complete inhibition of their IgE reactivity to the 38 kd protein present in all the tested mollusks. A representative inhibition immunoblot is shown in Fig. 3. Moreover, preabsorption with the shrimp aller-

gen also completely blocked their IgE reactivity to the other allergens detected by the tested sera. Unabsorbed sera and sera absorbed with an irrelevant recombinant control, a glutathione-S-transferase fusion protein of human PDC-E2,²⁰ did not lose their reactivities against the 38 kd band (data not shown).

IgE reactivity against insect and vertebrate muscle extracts

The IgE reactivities of shrimp-sensitive sera to the muscle extracts from grasshopper, cockroach, fruit fly, chicken, and mouse were tested by means of immunoblotting (Table I). All the tested (9 of 9) shrimp-sensitive sera reacted to a 38 to 40 kd protein band in grasshopper, cockroach, and fruit fly preparations. This IgE reactivity was not detected in muscle proteins from either chicken or mouse. Normal sera did not react with muscle proteins from these insects and vertebrates.

Inhibition immunoblotting demonstrated complete inhibition by the recombinant shrimp allergen of IgE reactivity of the shrimp-sensitive sera to the 38 kd antigen in grasshopper, cockroach, and fruit fly muscle extracts (data not shown).

DISCUSSION

Mollusks—including gastropods such as abalone, bivalves such as oysters, and cephalopods such as squids—are important food sources in many parts of the world. The abalone, whelk, mussel, pen shell, scallop, oyster, clam, cuttlefish, squid, and octopus species chosen for this study encompass the wide range of popular mollusk food items served in different communities.

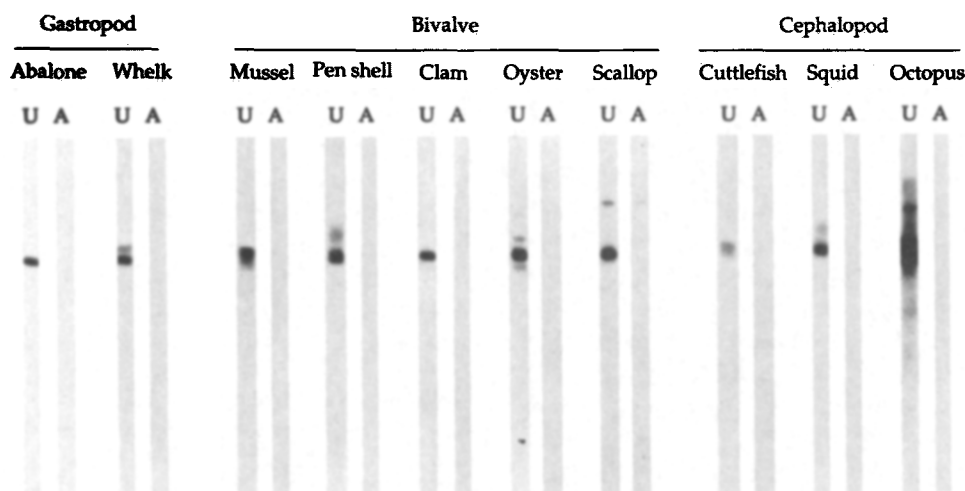


FIG. 3. Representative inhibition immunoblot of IgE reactivity against mollusk muscle extracts. Briefly, 1 ml of 1:10 diluted sera was incubated with 50 mg of purified recombinant shrimp tropomyosin at 4° C overnight. Preabsorbed sera were tested for IgE reactivities against mollusk extracts by means of immunoblotting. Note the presence of reactive bands in unabsorbed lanes (U) and the loss of reactive bands in absorbed lanes (A). Unabsorbed sera and sera absorbed with PDC-E2 were run in parallel as controls. Sera absorbed with PDC-E2 did not absorb out IgE reactivity against mollusk preparations.

In this study the IgE reactivity of the sera from subjects sensitive to shrimp was found to be reactive with a 38 kd muscle protein in crustaceans (shrimp, lobster, and crab), gastropods (abalone and whelk), bivalves (mussel, pen shell, scallop, oyster, and clam), and cephalopods (cuttlefish, squid, and octopus). The results suggest that the 38 kd heat-stable muscle protein is the major cross-reactive allergen present in crustaceans and mollusks. This finding is in accordance with previous reports identifying a 38 kd muscle protein as the common crustacean allergen,^{2, 21-23} as well as a major cross-reactive 35 to 39 kd allergen in shrimp and scallop.¹⁰ Carrillo et al.⁶ have recently reported type I hypersensitivity to cooked limpet extract. This gastropod (*Patella* sp.) is a favorite seafood served in warm maritime regions in Europe. It was also found that extracts from snail, oyster, shrimp, lobster, crawfish, and house dust mite were able to inhibit RAST assays in sera from subjects sensitive to limpet, suggesting the presence of cross-reactive molecules between these organisms. It should be noted that substantial IgE cross-reactivity between the crustacean and mollusk tropomyosin were detected from the nine subjects with shrimp allergy who have avoided other crustaceans. It was not known whether they would clinically react to other seafood products or insects, but the data herein suggest that they are at risk. It has been noted that many patients who are

allergic to shrimp do not have clinical hypersensitivity reactions to mollusks or other shellfish. It is possible that serologic IgE reactivity does not always result in clinical hypersensitivity reactions. The availability of a reliable recombinant allergen should allow these questions to be studied.

A significant degree of amino acid homology has been found between the major shrimp allergen and *Drosophila melanogaster* (fruit fly) tropomyosin.¹⁵⁻¹⁷ Tropomyosins are a group of highly conserved regulatory proteins with multiple isoforms found in both muscle and nonmuscle cells.²⁴ Thus similar, if not identical, allergenic epitopes may be present on tropomyosin of crustaceans and mollusks. In the past the studies on cross-reactivity among invertebrates were based on skin prick test, RAST inhibition, cross-immunoelectrophoresis, ELISA, and inhibition immunoblotting with crude extracts of allergens.^{10-12, 21-23, 25} However, the nature and identity of the cross-reactive molecule were unknown. Furthermore, the analysis of cross-immunoelectrophoresis was primarily based on formed precipitin bands, which are often poorly resolved and do not reveal their biochemical identity. Thus data from these studies were inconclusive and were not able to identify the IgE cross-reactive molecule among crustaceans and mollusks at the molecular level. In contrast, our data clearly reflect the mechanism of allergenic cross-reactivity between crustaceans and mollusks.

We were able to detect significant IgE reactivities to tropomyosin of grasshopper, cockroach, and fruit fly by using sera from patients with shrimp allergy. This observation may be explained by the close phylogenetic relationship between crustaceans and insects (Phylum Arthropoda) and may suggest the presence of highly conserved allergenic epitopes on tropomyosins. Significant amino acid sequence identity between *Drosophila* and shrimp tropomyosin has been reported.¹⁵⁻¹⁷ Therefore it is not surprising that the antibodies are cross-reactive. Similar observations have been reported by Daul et al.,²⁶ in which cross-reactivity between shrimp tropomyosin and fruit fly extract was detected. Likewise, it is highly possible that the tropomyosin of grasshopper and cockroach has similar epitopes that are recognized by serum IgE antibodies from patients with shrimp allergy. A monoclonal antibody, 1A16, raised against house dust mites (*Dermatophagoides pteronyssinus*) was found to be reactive not only to house dust mites, but also to extracts of insects including cockroaches, mosquitoes, chironomids, and other invertebrates such as shrimp, mussels and oysters by RAST. Furthermore, by means of indirect RAST assay and mite RAST inhibition assay, it was shown that the monoclonal antibody 1A16 is likely to react with tropomyosin.²⁵ In fact, the allergenic epitopes on tropomyosin might be very well conserved in many invertebrates. This may explain why cross-reactivity has been reported between insects, crustaceans, arachnids (house dust mites), and mollusks.^{11, 12, 14, 25, 27} Insects have long been and still are a common diet in many parts of the world.^{28, 29} Owing to the presence of possible highly conserved IgE-reactive epitopes between crustaceans and insects, the potential cross-reactive inhalant or ingested insect sensitivity in shrimp-sensitive subjects should be studied further. However, it is of interest that IgE directed to many invertebrate tropomyosins does not recognize vertebrate muscle proteins, suggesting the presence of unique IgE-reactive epitopes on invertebrate tropomyosins.

Our study also demonstrated the presence of other antigens, possibly minor allergens, in some mollusk samples that reacted with sera from subjects sensitive to shrimp. In particular, a 49 kd allergen in whelk, pen shell, oyster, and squid was recognized by some sera from subjects sensitive to shrimp. Peabsorption with recombinant shrimp tropomyosin (Met e 1) removed IgE reactivity against other IgE reactive bands in addition to the 38 kd band as in whelk, pen shell, oyster, squid, and

octopus (Fig. 3). This is probably due to the specific inhibitory effect of other tropomyosin isoforms or other reactive proteins that share a common reactive IgE reactive motif with tropomyosin. However, it should be noted that these minor allergens could not be detected in the crustacean extracts by the shrimp-sensitive sera. Further study in identifying these reactive bands will be necessary to determine whether a common IgE-reactive motif is present between these proteins and tropomyosin and to clarify their clinical importance.

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