

MOLECULAR AND IMMUNOLOGICAL CHARACTERIZATION OF SHELLFISH ALLERGENS

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1. ABSTRACT

Shellfish (crustaceans and mollusks) have long been known as a common cause of allergic reactions to food. Like other food allergies, the allergic reactions to shellfish involve IgE-mediated Type I hypersensitivity. Biochemical and molecular studies have documented the major shrimp allergen is the muscle protein tropomyosin. Subsequent molecular cloning studies on lobsters and crabs have characterized this protein as the common allergen in crustaceans. There has also been strong immunological evidence that tropomyosin is a cross-reactive allergen among crustaceans and mollusks. This is further confirmed by recent studies on the identification of allergens in squid and abalone. The advances in the characterization of shellfish allergens will not only enhance our understanding on the physiological basis of shellfish allergy but also lay the groundwork for the development of diagnostic and therapeutic design in food allergies.

2. INTRODUCTION

Seafood constitutes an important food sources for human, particularly as a major source of animal protein. Global fishery production now exceeds 100 million tonnes per year, and about 70% is available for direct human consumption. The current global seafood consumption is 14 kg/capita/year. Due to the high nutritive value of seafood and the promotion of a healthy diet, the demand for seafood products will continue to increase. Yet seafood, particularly shellfish, has long been known as a common cause of allergic reactions to food (1-2). It was estimated more than 250,000 people in the United States alone have a risk of developing hypersensitive reactions to seafood (3-4). The risk will be enhanced with the increasing use of fishery products in processed food. Despite extensive studies on various inhalant and contact allergens, until the past decade there have been few studies defining food allergens at the molecular level. In this review the advances in the identification and characterization of shellfish allergens will be discussed with a view to stimulate further research to explore the immunological and molecular basis of shellfish allergy. The ultimate goal is to develop clinical reagents for diagnosis and immunotherapy tactics.

3. ALLERGIC REACTIONS TO SHELLFISH

Allergic reactions to shellfish is highly exemplified by the hypersensitive reactions to ingestion of

crustaceans. The reaction is a IgE mediated type I hypersensitive reaction, manifested by flushing, urticaria, angioedema. Other reactions may include gastrointestinal (nausea, diarrhea, abdominal cramping), respiratory (laryngospasm, wheezing) and ocular (conjunctivitis). The initial development of a specific IgE response is mediated through the presentation of the allergen by antigen presenting cells in the context of MHC class II to T lymphocytes which in turn stimulate B lymphocytes to mature to plasma cells that secrete the specific IgE antibodies. Upon reexposure to the allergen, the sensitized subject will develop an allergic reaction through interaction of IgE bound on IgE receptors on surfaces of mast cells and basophils. The recognition of IgE epitopes of these allergens will trigger the release of chemical mediators such as histamines, leukotrienes, slow reacting substance of anaphylaxis (SRA-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), resulting in increase in vascular permeability, constriction of bronchial smooth muscles, mucus secretion and migration of inflammatory cells to the reaction site (figure 1). The reactions are usually immediate, beginning minutes to an hour after the food is taken in. Life threatening reactions of systemic anaphylaxis may occur after ingestion of shellfish among sensitized individuals (5). Such symptoms include urticaria, angioedema, generalized pruritus, palate itching, difficulty in swallowing, upper airway obstruction, hypotension with a sensation of faintness and loss of consciousness in serious cases.

In addition to hypersensitivity reaction to shellfish by ingestion, occupational asthma to shellfish have been reported in fisherman, processing workers, shell grinders, cooks as well as restaurant workers (6-9). Lobster, shrimp and crab have been documented to cause occupational dermatitis, rhinitis and conjunctivitis (10-13). Thus allergic reactions to shellfish is a significant concern in shellfish industry and environmental health.

4. CHARACTERIZATION AND IDENTIFICATION OF THE MAJOR SHRIMP ALLERGEN.

Although hypersensitive reactions caused by shellfish has long been known, biochemical and immunological studies on shellfish allergies had only begun in the 1980s. Using crossed immunoradioelectrophoresis, Lehrer *et al.* (14) demonstrated the presence of at least

Table 1. Taxonomic Classification of Commonly Consumed Shellfish.

PHYLUM ARTHROPODA	
Class Crustacean	Shrimp, Lobster, Crab, Crayfish
PHYLUM MOLLUSCA	
Class Gastropoda	Abalone, Whelk, Snail, Limpet
Class Bivalvia	Mussel, Scallop, Oyster, Clam
Class Cephalopoda	
	Cuttlefish, Squid, Octopus

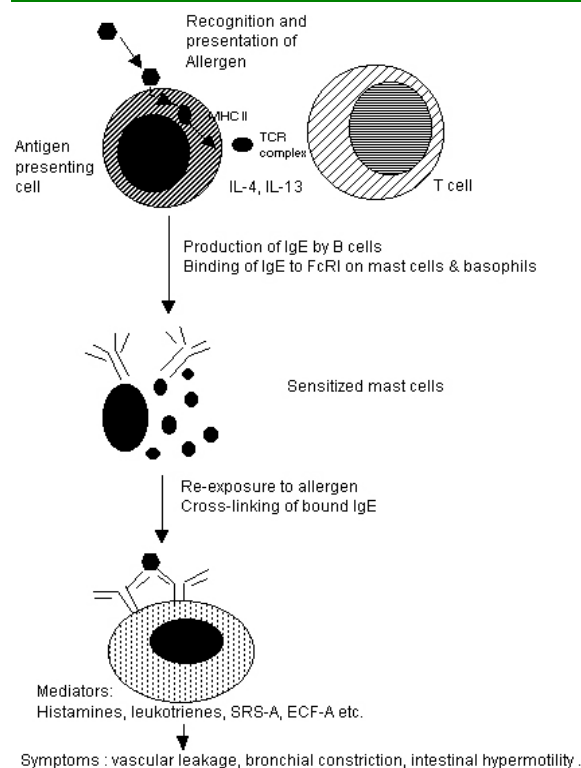


Figure 1. Cascade of hypersensitive reactions upon sensitization by an allergen. Firstly, the allergen is presented by antigen presenting cells in the presence of MHC to T cells which in turn stimulate B cells to produce allergen specific IgE. These IgE are bound on surfaces of mast cells and basophils. Upon re-exposure to the same allergen and cross-linking of bound IgE to the allergens, the mast cells and basophils release a variety of mediators resulting in increase in vascular permeability, constriction of bronchial smooth muscles, mucus secretion and migration of inflammatory cells to the reaction site.

seven allergens in Shrimp. Hoffman *et al.* (15) reported the purification of two allergens, termed antigen I and antigen II from raw and cooked shrimp respectively. Antigen II is a heat stable IgE reactive protein of 38 kD and 341 amino acid residues with an estimated pI of 5.4-5.8. By SDS-PAGE, Daul *et al.* (16) showed that a 36kD protein from *Panaeus aztecus* was able to inhibit 75% of the RAST activity to the whole shrimp. This protein, *Pen a I* is rich in aspartic acid and glutamic acid and is similar to antigen II described by Hoffman *et al.* (15). A similar protein from *Penaeus indicus* designated as Sa-II was described (17). In 1993 and 1994, three groups independently reported the molecular identification of the major shrimp allergen as tropomyosin (18-20).

To characterize the shrimp allergen at the molecular level, we isolated an IgE reactive clone, coined

Met e I from a cDNA library from *Metapenaeus ensis*. *M. ensis* (family Penaeidae) is a commercially important shrimp in South China Sea and is extensively cultured in Southeast Asia. Serum antibodies from patients with shrimp allergy demonstrated specific positive IgE reactivity to recombinant *Met e I* by immunoblotting whereas sera from non-allergic control do not react (figure 2). *Met e I* has an open reading frame of 281 amino acids, coding for a protein of 34kD (19). Comparison of amino acid composition of *Met e I* with that of other shrimp allergens such as *Pen a I*, Sa-II and antigen II showed that they are highly similar, suggesting that the major shrimp allergen is likely to include multiple isoforms of shrimp tropomyosin. (table 2). Furthermore, the deduced amino acid sequence of *Me t e I* is highly homologous to that of *Drosophila melanogaster*. Thus now, it is well established that the shrimp tropomyosin is the major heat stable shrimp allergen.

In addition to tropomyosin, Nagpal *et al.* (17) also reported that shrimp tRNA as a shrimp allergen. However, the data was obtained only with sera from two patients and the nucleic acid preparation was not totally protein free. Since then, this data has not been reproduced.

5. IDENTIFICATION OF ALLERGENS IN OTHER CRUSTACEANS

Hefle *et al.* (21) reported the presence of multiple IgE reactive proteins of 25-45 kD and 5 IgE proteins of 14 kD or less in snow crab meat and cooking water extracts. Moreover, they reported that 5/18 serum samples studied showed IgE binding at 38-41kD which may represent the snow crab tropomyosin. When 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 have been previously shown to react to recombinant shrimp tropomyosin (19) reacted to muscle proteins at approximately 38kD in the crustacean samples (table 3) (22). Sera preabsorbed with recombinant shrimp tropomyosin lost their IgE reactivity totally 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.

To further investigate the actual molecular identity of the lobster allergen, we have constructed a λ gt11 expression cDNA library from the spiny lobster *Panulirus stimpsoni* (Family Palinuridae). This library was screened for IgE reactive clones using sera from subjects with crustacean allergy. A 2 kb cDNA, coined *Pan s I* was identified. Expression of *Pan s I* in plasmid vector pGEX produced a 60 kD recombinant fusion protein reactive to the IgE antibodies from crustacean allergies (figure 2). This protein consists of a 26 kD glutathione transferase fused to protein with an opening reading frame of 274 amino acids. *Pan s I* is the first identified major allergen of the spiny lobster and showed significant homology to tropomyosin of shrimp and fruitfly *Drosophila melanogaster* (23-24). In addition, we have also expressed recombinant fast muscle tropomyosin from the American lobster, *Homarus americanus* (family Nephropidae) (25) and tested for the IgE recognition of this protein, coined *Hom a I* by immunoblotting. *Hom a I* was also recognized by IgE from patients with crustacean allergies (figure 2). Antibodies from subjects with crustacean allergies, when preabsorbed with recombinant proteins *Pan s I* or *Hom a I* lost their IgE reactivity to muscle extract of *P. homarus* and *H. americanus*. Likewise, preincubation of crustaceanallergy sera with the shrimp recombinant tropomyosin *Met e I*, removed their IgE reactivity to *Homarus americanus*

Table 2. Amino acid composition of tropomyosin from shrimp, lobster, fruitfly and chicken

	SHRIMP				LOBSTER			OTHERS	
Amino Acid	Met e I [#]	Pen a I ⁺	Sa-II [^]	Antigen II [*]	Pan s I ^ψ	Hom a I ^ψ	Ha sTm	Fruitfly ^τ	Chicken ^π
Alanine	31	33	21	31	31	33	33	25	34
Cysteine	1	ND	3	2	0	0	0	3	1
Aspartic Acid	17	40	39	58	17	18	16	22	25
Glutamic Acid	55	80	75	61	55	55	57	51	54
Phenylalanine	4	4	6	9	4	3	3	5	1
Glycine	5	10	6	20	4	4	4	3	3
Histidine	1	1	3	4	1	1	1	1	1
Isoleucine	4	6	6	12	4	6	8	7	9
Lysine	25	26	27	27	25	28	29	35	40
Leucine	33	35	30	30	33	33	31	28	38
Methionine	6	8	6	9	6	8	8	8	4
Asparagine	18	ND	ND	ND	16	16	15	13	6
Proline	0	2	3	6	0	0	0	0	0
Glutamine	17	ND	ND	ND	16	17	17	19	12
Arginine	22	26	30	19	21	21	21	17	13
Serine	15	14	12	15	14	15	13	14	17
Threonine	9	12	9	12	10	9	11	14	10
Valine	14	13	15	19	13	13	13	17	9
Tryptophan	0	ND	4	ND	0	0	0	0	0
Tyrosine	4	4	6	7	4	4	4	2	7
Total	281	312	301	341	274	284	284	284	284

Leung *et al.* ¹⁹, +Musmand *et al.* ³⁴, ^Nagpal *et al.* ¹⁷, *Hoffman *et al.* ¹⁵, ψChu *et al.* ²³, τ Basi and Storti. ²⁴, Goodling, *et al.* ³⁸ Ha sTm : Homarus americanus slow muscle tropomyosin ²⁵. ND: not detected.

and another spiny lobster Panulirus homarus (figure 3). These data demonstrate that subjects with crustacean allergies have common IgE epitopes in shrimp and lobster and lobster tropomyosin is a major lobster allergen. Similarly, using a cDNA library from Charybdis feriatus (family Portunidae) a common crab species consumed as seafood throughout its geographical range extending from Japan to the east coast of Africa, we have obtained an IgE reactive clone coined Cha f I and demonstrated that the major allergen in crab is also tropomyosin 923).

6. CROSS-REACTIVITY BETWEEN CRUSTACEAN AND MOLLUSK ALLERGENS

Although crustaceans are well known allergens in susceptible individuals, similar adverse reactions have been described in patients consuming mollusks such as cuttlefish (26), abalone (27), limpet (27-29) and squid (30). Significant RAST reactivity to oyster extracts has been found in sera from crustacean-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 (31). 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 (31). Thus, the molecular basis of cross-reactivity between allergy to crustaceans and mollusks is of particular interest.

Recently, we have 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 edible mollusks including class Gastropoda, class Bivalvia and class Cephalopoda. IgE reactivity of the nine sera obtained from patients with a documented clinical history of shrimp hypersensitivity was tested against ten common mollusks in three different classes (table 3) (22). All nine sera demonstrated IgE reactivity to the 38 kD protein present in the boiled muscle extracts of all mollusks. Representative immunoblots of five out of the nine sera against the mollusk muscle extracts are shown in

Figures 4-6. 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 out of the nine sera tested in whelk; in seven out of the nine sera tested in squid; in four out of the nine sera tested in fan shell; and in two out of the nine sera tested in oyster (Table 3). It was also found that serum from subject # 3 detected other antigens in some mollusk samples tested (figures 4-6). The IgE of this serum reacted with the allergen of 68 kD in oyster and fan shell extracts; and 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 (figure 6). Furthermore, 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 figure 7. Moreover, preabsorption with the shrimp allergen (Met e I) 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 GST fusion protein of human PDC-E2 did not lose their reactivities against the 38 kD band (data not shown). 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 (14, 32-34) as well as a major cross-reactive 35-39 kD allergen in shrimp and scallop (35).

Our data showed that sera from shrimp allergy subjects but not normal controls react with a protein band of approximately 38kD in both crustaceans and mollusks. Furthermore, the reactivity to this 38kD protein in mollusks can be totally absorbed by preincubating the sera with recombinant shrimp tropomyosin. In accordance with our findings, a 38 kD squid (Todarodes pacificus) protein, termed Tod p I which is reactive to sera from patients with shellfish allergy as well as shrimp and squid

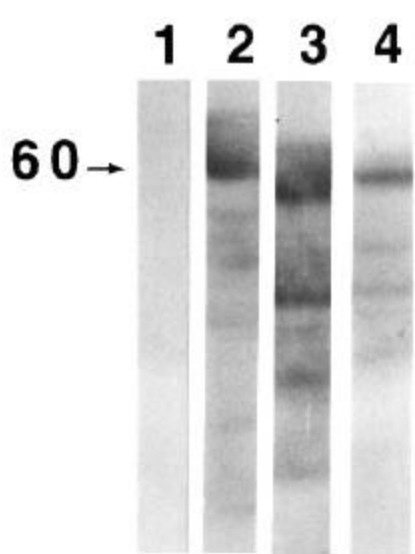


Figure 2. IgE reactivity of sera from subject with shellfish allergy with recombinant Met e I (lane 2), Pan s I (lane 3), Hom a I (lane 4) by immunoblotting. Purified recombinant proteins of Met e I, Pan s I and Hom a I were resolved on SDS-PAGE and analysed for IgE reactivity with sera from subjects with shellfish allergy. Please note that the sera reacted to recombinant fusion proteins of 60kD in each of the recombinant proteins (lanes 2-4) but not to the control (lane 1). Sera from normal controls did not react to the recombinant proteins. The minor bands below 60kD are probably due to breakdown products of the recombinant proteins.

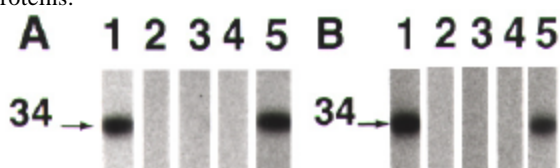


Figure 3. Specific inhibition of IgE reactivity against (A) Panulirus homarus and (B) Homarus americanus proteins by Pan s I, Hom a I and Met e I. Muscle extracts of Panulirus homarus and Homarus americanus were resolved on SDS-PAGE, transferred to nitrocellulose filters and probed with sera. Note the presence of a reactive band with unabsorbed sera at 34 kD (lane 1) and the loss of reactivity when the sera were absorbed by recombinant proteins of Pan s I (lane 2), Hom a I (lane 3) and Met e I (lane 4). Sera absorbed by an irrelevant control recombinant protein PDC-E2 did not inhibit the reactivity (lane 5).

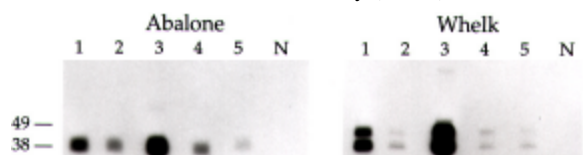


Figure 4. Immunoblotting of sera from shrimp-sensitive subjects against the muscle extracts from abalone and whelk (class Gastropoda). Muscle proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. 3mm strips from each blot were tested for their reactivities to sera IgE from shrimp allergy subjects and normal controls. Lanes 1-5 represent five shrimp-sensitive sera respectively. Lane N represents a negative control of normal sera. The molecular weight of the reactive bands are indicated in kD.

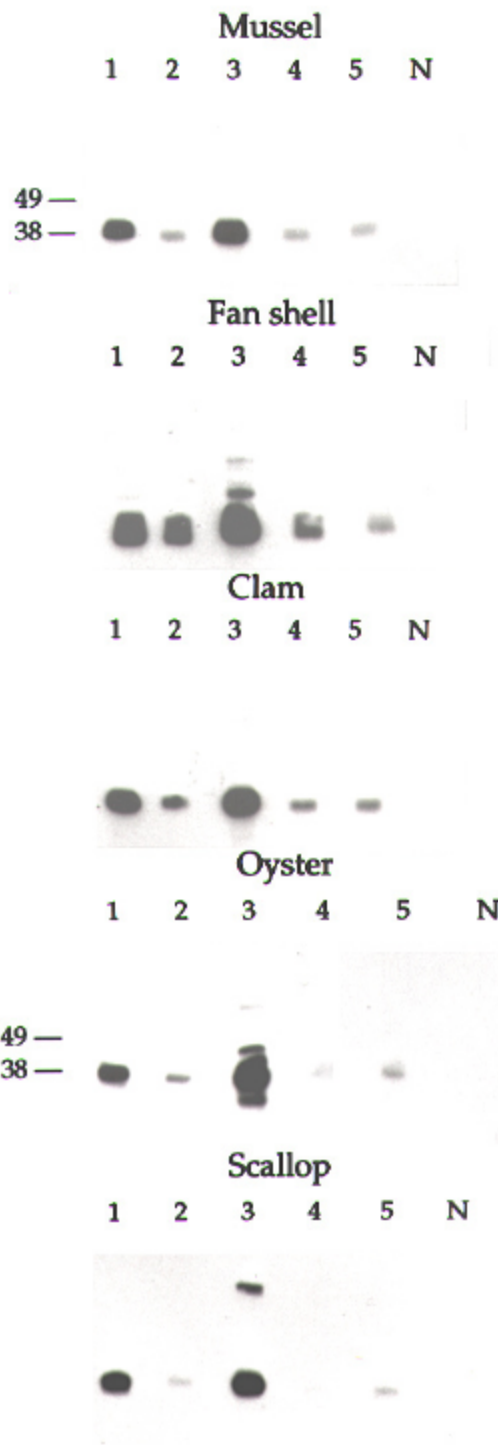


Figure 5. Immunoblotting of sera from shrimp-sensitive subjects against the muscle extracts from mussel, fan shell, clam, oyster and scallop (Class Bivalvia). Muscle proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. 3mm strips from each blot were tested for their reactivities to sera IgE from shrimp allergy subjects and normal controls. Lanes 1-5 represent five shrimp-sensitive sera respectively. Lane N represents a negative control of normal sera. The molecular weight of the reactive bands are indicated in kD.

Table 3. IgE reactivities of shellfish allergic 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>)		
Cockroach (<i>Periplaneta americana</i>)		
Fruitfly (<i>Drosophila melanogaster</i>)	38	9/9
	38	9/9
	38	9/9
PHYLUM MOLLUSCA		
Class Gastropoda		
Abalone (<i>Haliotis diversicolor</i>)	38	9/9
Whelk (<i>Hemifusus ternatana</i>)	38	9/9
	49	8/9
Class Bivalvia		
Mussel (<i>Perna viridis</i>)	38	9/9
Fan shell (<i>Pinna 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.

allergen specific monoclonal antibodies has been reported. Amino acid sequence analysis of Tod p I showed that it has marked homology with the snail tropomyosin (36). Similarly, Lopata *et al.* has reported the presence of a 38kD and a 49kD allergenic protein in abalone (*Haliotis midae*). It is likely that the 38kD protein is also tropomyosin. However whether this 49 kD abalone allergen is similar to that reported earlier from whelk, fan shell, squid and oyster (22), and their biochemical identities are still unknown (37). The presence of a common allergen tropomyosin accounts for the cross-reactivity between crustaceans and mollusks and enhances our understanding of the molecular basis of the hypersensitive response against a highly conserved molecule.

7. PERSPECTIVE

With the application of molecular and immunological techniques in the identification of shellfish allergens, our understanding on the biochemical nature and cross-reactivity of the allergens has significantly increased in the last five years. Although other shellfish allergens remain to be characterized, it is now well established that tropomyosin is the major common shellfish allergen. The

availability of recombinant shellfish tropomyosin now enables us to tackle questions which we could not address before. These include: what are number and identity of the epitopes that initiate the cascade of immunological responses, what is the nature of cross-sensitivity at the cellular level, and why some individuals become sensitized and develop allergies to shellfish. Answers to these questions would provide insights to the cellular signals involved in the allergic responses and thus initiate the development of novel diagnostic and therapeutic approaches to shellfish allergy. Furthermore, studies on shellfish allergy can serve as model in the understanding of the physiological basis of food allergy as well as food tolerance in general.

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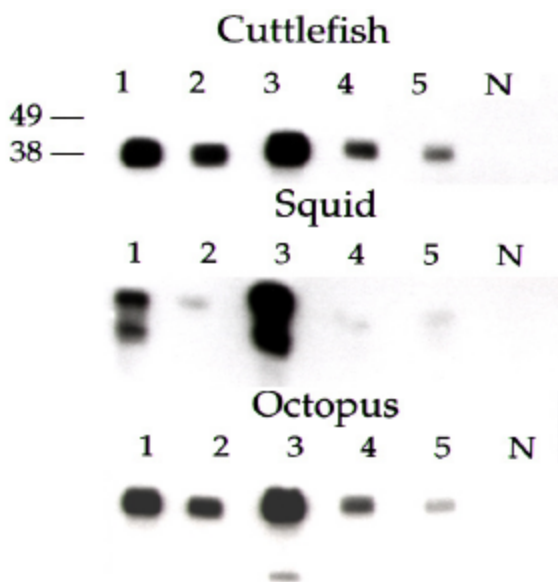


Figure 6. Immunoblotting of sera from shrimp-sensitive subjects against the muscle extracts from cuttlefish, squid and octopus (Class Cephalopoda). Muscle proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. 3mm strips from each blot were tested for their reactivities to sera IgE from shrimp allergy subjects and normal controls. Lanes 1-5 represent five shrimp-sensitive sera respectively. Lane N represents a negative control of normal sera. The molecular weight of the reactive bands are indicated in kD.

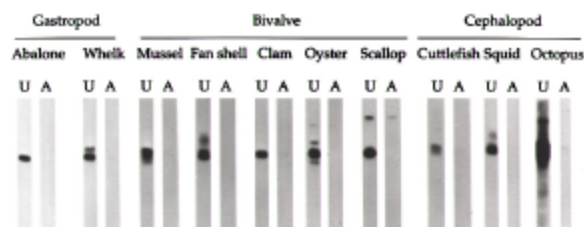


Figure 7. Representative inhibition immunoblot of the IgE reactivity against mollusk muscle extracts. Briefly, 1ml of a 1:10 diluted sera were incubated with 50µg of purified recombinant shrimp tropomyosin at 4°C overnight. The preabsorbed sera were tested for their IgE reactivities against mollusk extracts by immunoblotting. Note the presence of reactive band in the unabsorbed lanes (U) and the loss of reactive bands in the 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 the mollusk preparations.

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