

Identification of the first major allergen of a squid (*Todarodes pacificus*)

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Background: In Japan, squid is an important seafood, and some patients with food allergies are sensitive to squid. There has been no report, however, describing the major allergens of squid.

Objective: To characterize squid allergens, we isolated a major allergen from the Pacific flying squid (*Todarodes pacificus*) and compared it with a major allergen from a shrimp (*Penaeus orientalis*).

Methods: The major squid and shrimp allergens were isolated by column chromatography on diethylaminoethyl-Sepharose (Pharmacia, Uppsala, Sweden), hydroxylapatite, and Sephacryl S-300 (Pharmacia). The IgE reactivity of the isolated allergens was assessed by immunoblotting. The cross-reactivity between the squid and shrimp allergens was examined by use of mouse polyclonal and monoclonal antibodies to the major allergens. Amino acid sequence analyses of the isolated allergens were done.

Results: The isolated squid allergen is a 38 kd, heat-stable protein. IgE antibody binding to the purified squid allergen was demonstrated by immunoblotting. Cross-reactivity between major squid and shrimp allergens was demonstrated with sera from patients allergic to squid or shrimp or with allergen-specific monoclonal antibodies. The amino acid sequence analysis of the major squid allergen showed a marked homology with tropomyosin from blood fluke planorbid (*Biomphalaria glabrata*), which is a common vector snail of *Schistosoma mansoni*.

Conclusion: This 38 kd protein is a major allergen of the squid, *Todarodes pacificus*, and is believed to be squid muscle protein tropomyosin. We named it *Tod p 1* according to International Union of Immunological Societies allergen nomenclature regulation. (*J Allergy Clin Immunol* 1996;98:948-53.)

Key words: Allergen, squid, shrimp, tropomyosin, food allergy

IgE-mediated allergic reaction to shellfish is one of the most frequent food allergies.¹ Symptoms range from mild oral reactions to fatal anaphylactic shock.¹ Crustaceans (such as shrimp, crab, and lobster) are recognized as a common cause of food hypersensitivity reactions.² Allergic reactions to

Abbreviations used

BSA:	Bovine serum albumin
FU:	Fluorescence unit
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis

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Received for publication Jan. 18, 1996; revised Apr. 11, 1996; accepted for publication Apr. 17, 1996.

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0091-6749/96 \$5.00 + 0 1/1/74342

mollusks (such as squid, clams, and oysters) have not been as well studied.²

In Japan, squid is an important seafood. In 1992, the Japanese bought 5.3 kg of squid per household (1.7 kg per person).³ This figure does not include the squid processed and served in restaurants. In the few reports on squid allergy,^{4,5} patients generally had immediate hypersensitivity reactions after eating squid or inhaling vapors while cooking squid.

Shrimp allergens have been well studied among shellfishes. The major shrimp allergens have been reported to be Antigen II, Sa II, and Pen a 1.⁶⁻⁸ Amino acid sequence homology studies of these major allergens have shown that the protein is shrimp tropomyosin.⁹⁻¹⁰ The complete primary structure of the major shrimp allergen (Met e 1) from *Metapenaeus ensis* is homologous to multiple isoforms of tropomyosin.¹¹ No report, however, has been made on the major allergen of squid.

To characterize this allergen, we isolated a major allergen from the Pacific flying squid (*Todarodes pacificus*) and compared it with the major shrimp allergens.

METHODS

Patients

Serum was obtained from four patients who showed immediate hypersensitivity reactions after ingesting squid. Specific IgE antibody reactivity to squid and shrimp allergens was demonstrated in sera of all the patients by use of the CAP system (Pharmacia). Also, serum samples were collected from seven American patients sensitive to shrimp who had little or no exposure to squid as a food.

Purification of the major allergens from squid and shrimp

The extraction and purification procedures for the major allergens from squid (*Todarodes pacificus*) and shrimp (*Penaeus orientalis*) meat was as follows: fresh squid or shrimp (100 gm) that had been cut into small pieces with scissors was boiled in distilled water (300 ml) at 100° C for 10 minutes, then centrifuged at 20,000 g for 20 minutes. The supernatant was used as the crude extract. Ammonium sulfate was added with stirring to 50% saturation. After centrifugation at 20,000 g for 20 minutes, the resulting precipitate was collected and dissolved in 0.05 mol/L Tris-HCl buffer, pH 8.0, and then dialyzed against the same buffer. The sample was applied to a DEAE-Sepharose column (Pharmacia). Fractions were collected with a linear gradient of 0.18 to 0.6 mol/L NaCl in Tris buffer. Those with allergenic activity were pooled and dialyzed overnight in 0.01 mol/L phosphate buffer (pH 6.8). The sample obtained was applied to a hydroxylapatite column (Bio-Rad Laboratories, Hercules, Calif.). Fractions were collected with a 0.01 to 0.5 mol/L NaCl gradient in the same buffer, and those with allergenic activity were pooled and dialyzed against phosphate-buffered saline (PBS). The sample obtained was applied to a Sephacryl S-300 column (Pharmacia) equilibrated with PBS. The allergenic activity of the fraction in the peak of the gel filtration was verified by a fluorometric IgE ELISA and immunoblotting as described below.

Fluorometric indirect IgE ELISA

Specific IgE antibodies to the isolated major allergens were assayed by a fluorometric ELISA as described elsewhere.^{12, 13} Briefly, microplates were coated with the purified allergen protein (1 µg/ml). After incubation of the pooled serum with the immobilized antigens, anti-human IgE antibody conjugated with β-D-galactosidase (Pharmacia) diluted 1:30 was added to each well. As the enzyme reaction substrate, 0.1 mmol/L 4-methylumbelliferyl-β-D-galactoside (Sigma Chemicals, St. Louis, Mo.) was added, and the plates were incubated at 37° C for 2 hours. Then, 0.1 mol/L glycine-NaOH (pH 10.3) was added to stop the reaction. Fluorescence unit was measured on a fluorometric microplate reader (Fluoroskan; Flow Laboratories, McLean, Va.).

The allergenic activity of each fraction obtained by the chromatography was determined as follows: 100 µl per fraction, diluted 1:1,000 in 0.05 mol/L carbonate-bicarbonate buffer, pH 9.6, was added to the wells of microplates. The loaded plates were incubated at 4° C overnight to adsorb the proteins in the fraction. After being washed, the wells of plates were incubated with the patients' pooled serum diluted 1:20. The following procedures were the same as for the specific IgE assay.

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

SDS-PAGE was performed according to the procedure of Laemmli.¹⁴ The sample was applied to a 5% to 15% gradient SDS-PAGE gel under reducing conditions. The gel then was stained with Coomassie Blue (Bio-Rad Laboratories) or the proteins in the gel were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was rinsed in PBS, and blocked with 1% bovine serum albumin (BSA) in PBS. After another rinse with PBS containing 0.02% Tween-20, it was cut into strips, which were incubated overnight with the patients' pooled serum diluted 1:2. After being washed, the strip was incubated with anti-human IgE antibody-conjugated alkaline phosphatase (Quidel, San Diego, Calif.) overnight, and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.¹⁵

Antibodies to major allergens

Mouse polyclonal antibodies to the major squid and shrimp allergens were obtained from BDF1 mice immunized with the purified allergens. These antibodies and mouse monoclonal antibodies to major shrimp allergen (Pen a 1)¹⁶ were used to study the cross-reactivity between the squid and shrimp allergens.

Indirect ELISA for specific IgG from the mouse

Specific IgG antibodies to allergens were assessed by an indirect ELISA as described elsewhere.¹² Briefly, microplates were coated with the purified allergens (1

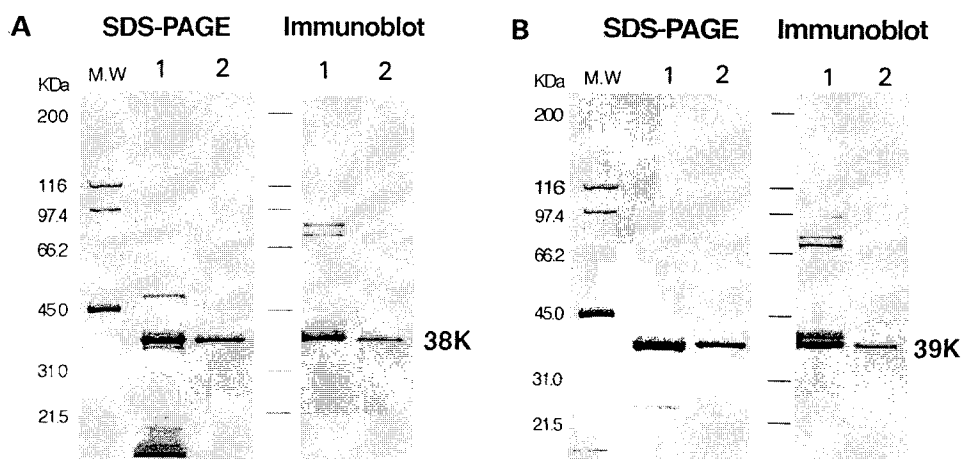


FIG. 1. Analysis of SDS-PAGE and immunoblotting. **A**, Squid allergen. **B**, Shrimp allergen. Lane 1, crude extract; lane 2, purified major allergen.

$\mu\text{g/ml}$). After incubation of mouse polyclonal or monoclonal anti-major allergen IgG antibodies with immobilized antigens, anti-mouse IgG antibody conjugated with peroxidase (Zymed Laboratories, San Francisco, Calif.) was added to each well. Color then was developed with a mixture of hydrogen peroxide and orthophenylenediamine dihydrochloride. After the enzyme reaction was stopped with 4N sulfuric acid, the absorbance at 492 nm was measured with a colorimetric microplate reader (Flow Laboratories).

Amino acid sequence determination

Because the N-amino termini of both the purified squid and shrimp allergens were blocked, the proteins were digested with Acromobacter protease (Wako Pure Chemical Industries, Osaka, Japan). The peptides were separated by reverse-phase high-performance liquid chromatography. Amino acid analysis was done with a Shimadzu gas-phase sequencer (model PSQ-10, Shimadzu, Kyoto, Japan).

RESULTS

Isolation of squid and shrimp allergen

SDS-PAGE showed several protein bands in the crude boiled squid extract (Fig. 1, A, lane 1). A major protein band was present at 38 kd. In immunoblots, IgE antibodies in pooled serum from patients sensitive to squid strongly reacted with this protein (Fig. 1, A, immunoblot lane 1). This 38 kd protein was isolated by column chromatography on DEAE-Sepharose, hydroxylapatite, and Sephacryl S-300 and verified by SDS-PAGE (Fig. 1, A, lane 2). IgE antibodies from the pooled serum of patients with squid allergy reacted with this purified protein in immunoblots (Fig. 1, A, immunoblot lane 2). This band was designated

Tod p 1 according to International Union of Immunological Societies allergen nomenclature. In electrofocusing, Tod p 1 produced multiple bands with an isoelectric point range of 5.1 to 5.9 (data not shown).

Similarly, several protein bands were detected in boiled shrimp crude extract, and one major band was present in the crude extract at 39 kd (Fig. 1, B, lane 1). This 39 kd protein was isolated by the procedure used to purify the major squid allergen. In immunoblotting, IgE antibody from the pooled serum of patients with shrimp allergy reacted with the purified shrimp allergen (Pen o 1) (Fig. 1, B, immunoblot lane 2).

Cross-reactivity of the shrimp and squid allergens

The antigenic cross-reactivity between the major squid (Tod p 1) and shrimp (Pen o 1) allergens was examined by use of mouse polyclonal and monoclonal antibodies to the allergens (Fig. 2). Polyclonal anti-Tod p 1 IgG reacted with both Tod p 1 and Pen o 1, as did polyclonal anti-Pen o 1 IgG. Furthermore, monoclonal anti-Pen a 1 IgG also reacted with both of them. Cross-reactivity between these two allergens was then examined by an ELISA inhibition with IgE in pooled serum from patients with squid allergy. When the serum was preincubated with Pen o 1, the reactivity of the serum with Tod p 1 was markedly abolished (data not shown).

We next evaluated the allergenic cross-reactivity between these two allergens. We examined the reactivity of IgE antibody in the patients' sera with both Tod p 1 and Pen o 1 proteins. IgE antibody in

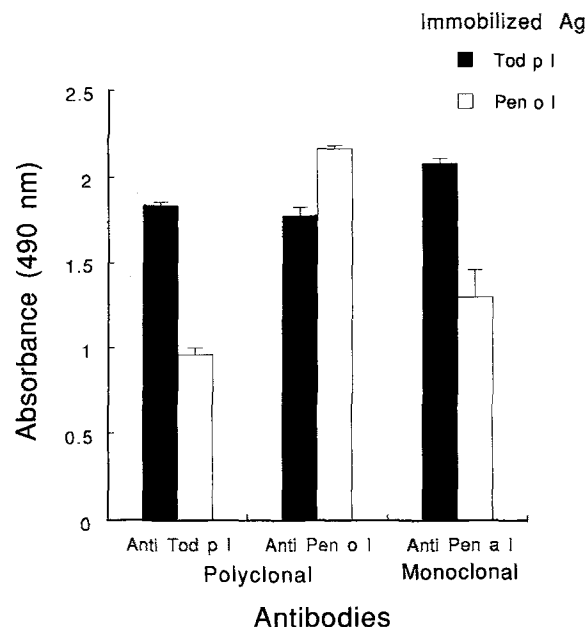


FIG. 2. Cross-reactivity of the major squid and shrimp allergens with polyclonal and monoclonal antibodies. AG, Antigen.

the sera from the American patients sensitive to shrimp, who had never or seldom eaten squid, reacted with Tod p 1 (Fig. 3). Moreover, there was a significant correlation between levels of reactivity of IgE to Pen o 1 and Tod p 1. IgE antibodies in sera of four Japanese patients with squid and shrimp allergies reacted with both the allergens.

Amino acid sequence of peptides derived from the major squid and shrimp allergens

Five peptide fragments of Tod p 1 were isolated by reverse-phase high-performance liquid chromatography (data not shown). These peptides of 12 to 18 amino acid residues were sequenced and then aligned with tropomyosin of the blood fluke planorbis (*Biomphalaria glabrata*)¹⁷ and with Met e 1 (Fig. 4). The homology with the snail protein ranged from 42% to 100% whereas that with Met e 1 was from 40% to 85% (Table I). These findings suggest that Tod p 1 is squid muscle protein tropomyosin.

Three peptides of Pen o 1 were also sequenced and aligned with Met e 1, *B. glabrata* tropomyosin, and Tod p 1. The sequences of peptides I and III were identical with Met e 1 sequences; the peptide II sequence differed in only one amino acid residue. The homologies of Pen o 1 peptides with *B. glabrata* tropomyosin and Tod p 1 ranged from 62% to 85% (Table II).

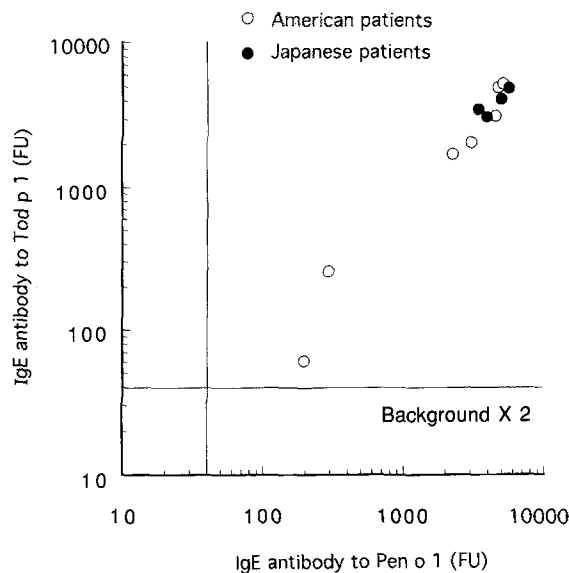


FIG. 3. Correlation of IgE to squid and shrimp major allergens. FU, Fluorescence units.

DISCUSSION

The Japanese eat many kinds of fish and shellfish. In 1992, they consumed about 50 kg per household (about 16 kg per person),³ which does not include the fish and shellfish processed and served in restaurants. They eat various mollusks (e.g., squid, clam, and octopus). More squid is consumed than any other seafood eaten in Japan.³ The most abundant species found in the seas around Japan is the Japanese common squid, *Todarodes pacificus*. The number of persons with fish and shellfish allergies appears to be greater in Japan than in other countries. The prevalence of those allergies in Japan, however, has not yet been established.

We found antigenic cross-reactivity between the major squid and shrimp allergens by use of polyclonal and monoclonal antibodies to the allergens. We also found that the amino acid sequences of two of the peptides of Tod p 1 have strong homology to those of the shrimp allergen. In a previous study, cross-reactivity between squid and shrimp was demonstrated by reverse immunoassay inhibition.⁵ We reconfirmed this finding using anti-purified allergen IgG and amino acid sequence analysis. Cross-reactivity among the Crustacea is well documented.^{18,19} Cross-reactivity also has been reported between shrimp and other animals: oyster, cockroaches, chironomidae larvae and cad-dis flies.²⁰⁻²³ Furthermore, tropomyosin is reported to be a common allergen in the cross-reactivity

	10	20	30	40	50	60
<i>B. glabrata</i>	MDAIKKKMLAMKMEKENAIDRAEQMEQKL RDVEETKNKLEEEFNQNF SNLQNDFDTA					
Tod p 1	NTIEEDLSTLQK YSNLENDFDNA					
Met e 1	EFCGRQAMKLELDNAMDRADTLEQQNKEANNRAEKSEEEVHNLRMQQLLENDLDQV					
	70	80	90	100	110	120
<i>B. glabrata</i>	NEGLTEAQTKEASEKHVAELES DTAGLNRRIQ LLEEDLERSEERLQSATEKLEEASKAA					
Tod p 1	KENLTVANTNLEASEK					
Met e 1	QESLLKANNQLVEKDKALSNAEGEVAALNRRIQ LLEEDLERSEERLNTATTKLAEASQAA					
	130	140	150	160	170	180
<i>B. glabrata</i>	DESERGRKVLESRS LADDERLDGLEAQLKEAKYIAEDAERKYDEAARKLAITEVDLERAE					
Tod p 1	VLENRSQGDEERIDLLEK					
Pen o 1	VLENRSLSDEERMDALENQLK					
Met e 1	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEADRKYDEVARKLAMVEADLERAE					
	190	200	210	220	230	240
<i>B. glabrata</i>	ARLEAAEAKVWELDEELHIVGNNIKTLSIQNDQASQREDSYQETIRDLTQRLKDAENRAT					
Pen o 1	ERAETGESK					
Met e 1	ERAETGEFKIVELEELRVVGNLKSLEVSEKANQREEAYKEQIKTLTNKLKAAEARAE					
	250	260	270	280		
<i>B. glabrata</i>	EAERTVSKLQKEVDRLEDELLAEKERYKSI SDELDSTFAELAGY					
Tod p 1	EVDRLDELLAEK					
Pen o 1	EVDRLDELVNEK					
Met e 1	FAERSVQKLQKEVDRLEDELVNEKEYKSI TDELDQTFSELGY					

FIG. 4. Analysis of amino acid sequences of the major squid and shrimp allergens.

TABLE I. Homology of Tod p 1 peptides with *B. glabrata* tropomyosin and Met e 1

Tod p 1 peptides		No. of amino acid residues identical to Tod p 1 peptides			
		<i>B. glabrata</i> tropomyosin		Met e 1	
No.	Size				
I	12 aa	5 aa	42%	5 aa	42%
II	12 aa	8 aa	67%	5 aa	42%
III	15 aa	11 aa	73%	6 aa	40%
IV	18 aa	11 aa	61%	13 aa	72%
V	13 aa	13 aa	100%	11 aa	85%

TABLE II. Homology of Pen o 1 peptides with Met e 1, *B. glabrata* tropomyosin, and Tod p 1 peptides

Pen o 1 peptides		No. of amino acid residues identical to Pen o 1 peptides			
		Met e 1		<i>B. glabrata</i> tropomyosin	
No.	Size				Tod p 1
I	21 aa	21 aa	100%	15 aa	71%
II	21 aa	20 aa	95%	13 aa	62%
III	13 aa	13 aa	100%	11 aa	85%

*The IV peptide of Tod p 1 has only 18 amino acid residues.

between shrimp and other groups of Arthropoda (i.e., mites and insects).²⁴

Analysis of the amino acid sequences of the peptides of Tod p 1 strongly suggested that Tod p 1 is squid tropomyosin. Some recent studies have shown that the major allergen from shrimp is tropomyosin.⁹⁻¹¹ Their findings indicate that tropo-

myosin may be the common major allergen in shellfish. The amino acid sequence of Tod p 1 shows strong homology to the tropomyosin of *B. glabrata*, the most common vector snail of *Schistosoma mansoni* throughout tropical Central and South America. Both squid and snail are members of the phylum Molluscs. Conversely, the amino

acid sequence of the major shrimp allergen showed strong homology to the tropomyosin of the fruit fly.⁹⁻¹¹ Both the shrimp and the fruit fly are members of the phylum Arthropoda. Thus we found that there is similarity in the amino acid sequences of tropomyosin between Mollusca and Arthropoda phyla.

In squid and shrimp extracts, we found protein bands at 70 to 90 kd visible by immunoblotting, but not by SDS-PAGE (Fig. 1, A and B). Mouse polyclonal anti-Tod p 1 IgG antibody also reacted with these bands in immunoblots (data not shown). We think that these proteins might share some antigenicity with Tod p 1 (squid tropomyosin).

Seafood allergens are a major cause of allergic reactions in adults. We have now isolated and partially characterized the major allergens (Tod p 1 and Pen o 1) of squid and shrimp. The isolation and further characterization of these major allergens will facilitate not only the elucidation of cross-reactions to crustaceans and mollusks but also the advancement of the diagnosis and treatment of seafood allergy.

REFERENCES

1. Sampson HA. Adverse reactions to foods. In: Middleton E Jr, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, Busse WW, editors. Allergy: principles and practice. St. Louis: C.V. Mosby, 1993:1661-86.
2. Daul CB, Morgan JE, Lehrer SB. Hypersensitivity reactions to crustacea and mollusks. Clin Rev Allergy Immunol 1993;11:201-22.
3. Annual report on the family income and expenditure survey. Tokyo: Statistics Bureau, Management and Coordination Agency, Japan. 1993:290-3 (in Japanese).
4. Miyake T, Kawamori J, Yoshida T. Detection of specific IgE antibodies to squid by radioimmunoassay with squid extract for scratch testing: a case of anaphylactic shock induced by squid. Jpn J Pediatr Allergy Clin Immunol 1987;1:14-7 (in Japanese).
5. Carrillo T, Castillo R, Caminero J, et al. Squid hypersensitivity: a clinical and immunologic study. Ann Allergy 1992;68:483-7.
6. Hoffman DR, Day ED, Miller JS. The major heat stable allergen of shrimp. Ann Allergy 1981;47:17-22.
7. Nagpal S, Rajappa L, Metcalfe DD, Subba Rao PV. Isolation and characterization of heat-stable allergens from shrimp (*Penaeus indicus*). J Allergy Clin Immunol 1989;83:26-36.
8. Daul CB, Slattery M, Morgan JE, Lehrer SB. Isolation and characterization of an important 36 kd shrimp allergen [abstract]. J Allergy Clin Immunol 1991;87:192.
9. Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Subba Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. J Immunol 1993;151:5354-63.
10. Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. Int Arch Allergy Appl Immunol 1994;105:49-55.
11. Leung PSC, Chu KH, Chow WK, et al. Cloning, expression, and primary structure of *Metapenaeus ensi* tropomyosin, the major heat-stable shrimp allergen. J Allergy Clin Immunol 1994;94:882-90.
12. Miyazawa H, Inouye S, Sakaguchi M, Koizumi K. A reverse-sandwich ELISA for IgG antibody to a pollen allergen. J Allergy Clin Immunol 1988;82:407-13.
13. Sakaguchi M, Inouye S, Taniai M, Ando S, Usui M, Matuhasi T. Identification of the second major allergen of Japanese cedar pollen. Allergy 1990;45:309-12.
14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
15. Takahashi Y, Nagoya T, Watanabe M, Inouye S, Sakaguchi M, Katagiri S. A new method of counting airborne Japanese cedar (*Cryptomeria japonica*) pollen allergens by immunoblotting. Allergy 1993;48:94-8.
16. Musmand JJ, Daul CB, Lehrer SB. Crustacea allergy. Clin Exp Allergy 1993;23:722-32.
17. Despommier DD, Karapelou JW. Parasite life cycles. New York: Springer-Verlag, 1987:72-3.
18. Halmeppuro L, Salvaggio JE, Lehrer SB. Crawfish and lobster allergens: identification and structural similarities with other crustacea. Int Arch Allergy Appl Immunol 1987;84:165-72.
19. Lehrer SB. The complex nature of food antigens: studies of cross-reacting crustacea allergens. Ann Allergy 1986;57:267-72.
20. O'Neil CE, Stankus RP, Lehrer SB. Antigenic and allergenic cross-reactivity between cockroach and seafood extract [abstract]. Ann Allergy 1985;55:174.
21. Lehrer SB, McCants ML. Reactivity of IgE antibodies with crustacea and oyster allergens: evidence for common antigenic structures. J Allergy Clin Immunol 1987;80:133-9.
22. Eriksson NE, Ryden B, Jonsson P. Hypersensitivity to larvae of chironomids (non-biting midges): cross-sensitization with crustaceans. Allergy 1989;44:305-13.
23. Koshte VL, Kagen SL, Aalberse RC. Cross-reactivity of IgE antibodies to caddis fly with arthropoda and mollusca. J Allergy Clin Immunol 1989;84:174-83.
24. Witteman AM, Akkerdaas JH, van Leeuwen J, van der Zee JS, Aalberse RC. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. Int Arch Allergy Appl Immunol 1994;105:56-61.