



Cephalopod tropomyosins: Identification as major allergens and molecular cloning

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Abstract

Heated extracts prepared from the mantle muscles (for decapods) or leg muscles (for octapods) of nine species of cephalopods were shown to be all reactive with serum IgE in crustacean-allergic patients. No marked difference in the reactivity with IgE was recognized among the cephalopods, suggesting that they are almost equally allergenic. Immunoblotting and inhibition immunoblotting data revealed that the major allergen is tropomyosin in common with the nine species of cephalopods and that the cephalopod tropomyosins are cross-reactive with one another and also with crustacean tropomyosins. Molecular cloning experiments first elucidated the primary structures of tropomyosins from five species of cephalopods. The cephalopod tropomyosins show high sequence identity (more than 92% identity) with one another, being the molecular basis for their cross-reactivity. Although the sequence identity between cephalopod and crustacean tropomyosins is only about 63–64%, some of the IgE-binding epitopes proposed for brown shrimp *Penaeus aztecus* tropomyosin (Pen a 1) are well conserved in the cephalopod tropomyosins, supporting the cross-reactivity between cephalopod and crustacean tropomyosins.

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1. Introduction

Shellfish including crustaceans and mollusks are a frequent cause of food hypersensitive reactions mediated by IgE antibodies, such as urticaria, asthma and vomiting, in allergic individuals. Among shellfish, four groups (shrimp, crab, squid and abalone) are assumed to be especially responsible for severe hypersensitive reactions in Japan and hence processed food products containing them are currently recommended to put labels indicating their use on packages or bottles by the Ministry of Health, Labor and Welfare. It is well known that the major allergen of crustaceans is tropomyosin, a myofibrillar protein composed of two identical subunits with a molecular mass of 35–38 kDa, as demonstrated at the molecular level with

three species of shrimp (Shanti et al., 1993; Daul et al., 1994; Leung et al., 1994), two species of lobster (Leung et al., 1998a) and one species of crab (Leung et al., 1998b). Interestingly, allergic cross-reactivity has previously been reported not only among crustaceans but also between crustaceans and mollusks (Lehrer and McCants, 1987). In accordance with this, we have already isolated allergens from oyster *Crassostrea gigas* (Ishikawa et al., 1997, 1998a), turban shell *Turbo cornutus* (Ishikawa et al., 1998b) and common octopus *Octopus vulgaris* (Ishikawa et al., 2001) and identified all of them as tropomyosins based on the determined partial amino acid sequences. Independently, the major allergen in Japanese flying squid *Todarodes pacificus* (Miyazawa et al., 1996), abalone *Haliotis diversicolor* (Chuo et al., 2000), scallop *Chlamys nobilis* (Chuo et al., 2000) and mussel *Perna viridis* (Chuo et al., 2000) has also been identified as tropomyosin. In addition, some inhibition studies have established the cross-reactivity between tropomyosins from crustaceans and mollusks

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(Leung et al., 1996; Miyazawa et al., 1996; Chuo et al., 2000).

In Japan, cephalopods including decapods (cuttlefish and squid) and octapods (octopus) are the most important edible mollusks; approximately 500,000 ton of decapods and 50,000 ton of octapods are caught along the coasts of Japan every year. Thus, it is important to confirm whether the fact that the major allergen of Japanese flying squid (Miyazawa et al., 1996) and common octopus (Ishikawa et al., 2001) is tropomyosin is true for various species of edible cephalopods. Furthermore, it should be noted that the cross-reactivity of cephalopod tropomyosins with those from other shellfish is not fully understood at the molecular level, since no information is available on the primary structures of cephalopod tropomyosins. In this study, therefore, we first showed that tropomyosin is the major allergen in common with nine species of cephalopods. Then, the primary structures of tropomyosins from five species of cephalopods were elucidated by a cDNA cloning technique and compared with those of tropomyosins from other mollusks and crustaceans from the viewpoint of IgE-binding epitopes.

2. Materials and methods

2.1. Cephalopod and crustacean samples

The following cephalopod and crustacean samples were used: one species of cuttlefish (golden cuttlefish *Sepia esculenta*), five species of squid (big fin reef squid *Sepioteuthis lessoniana*, spear squid *Loligo bleekeri*, swordtip squid *Loligo edulis*, Japanese flying squid *T. pacificus* and neon flying squid *Ommastrephes bartramii*), three species of octopus (common octopus *Octopus vulgaris*, ocellated octopus *Octopus ocellatus* and Pacific giant octopus *Paroctopus doylei*) and two species of crustaceans (kuruma prawn *Penaeus japonicus* and Japanese spiny lobster *Panulirus japonicus*). Fresh specimens of each species were purchased at a local retail shop in Tokyo. Mantle muscle (for decapods), leg muscle (for octapods) or tail muscle (for crustaceans) was obtained from each specimen and stored at -20°C until used. For cloning experiments, live specimens of golden cuttlefish, big fin reef squid, Japanese flying squid, neon flying squid and common octopus were purchased at the Tokyo Central Wholesale Market. Mantle muscle or leg muscle obtained from each live specimen was immediately frozen in liquid nitrogen and stored at -80°C until used.

2.2. Preparation of extracts

Each muscle sample was homogenized with four volumes of 0.01 M phosphate buffer (pH 7.0) containing 0.6 M KCl. Since tropomyosin, a target protein in this study, is heat-stable, the homogenate was heated in a boiling water bath for 10 min and centrifuged at 18,000g for 20 min. The supernatant thus obtained was used as heated extract. Protein concentrations of the heated extracts were determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

2.3. Human sera

Sera were obtained from 10 crustacean-allergic patients (patients 1–10) with documented clinical histories of immediate hypersensitive reactions after ingestion of crustaceans. The patients were all diagnosed to be allergic to crustaceans based on the determined CAP-RAST classes of 2–5 against shrimp. Patients 1–8 were also subjected to CAP-RAST tests against crab and shown to have classes of 2–5. However, no CAP-RAST data against mollusks were obtained with any patients. Written informed

consent was obtained from each patient. The patient sera were used as a pooled sample or individually used. In this study, sera from four healthy volunteers without adverse reactions after ingestion of any foods were pooled and used as a control. All sera were stored at -20°C until used.

2.4. ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed using a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan), essentially as reported previously (Ishikawa et al., 1997). Briefly, 50 μl of each heated extract, diluted 1:1000 with 0.05 M carbonate buffer (pH 9.5), was coated on the plate and immunoreacted with 50 μl of patient or control serum, diluted 1:100 with 0.1% BSA in Dulbecco's phosphate-buffered saline (pH 7.4, PBS), followed by 50 μl of peroxidase-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), diluted 1:2500 with 0.1% BSA in PBS. Enzyme reaction was carried out using substrate solution (0.1% *o*-phenylenediamine and 0.03% hydrogen peroxide in 0.05 M phosphate-citrate buffer, pH 5.0) and the developed color was measured at 490 nm on a Kinetic Microplate Reader model 550 (Bio-Rad Laboratories, Hercules, CA, USA). All ELISAs were performed in triplicate and the data were expressed in mean values.

2.5. SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem apparatus (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Ready-made gels (PhastGel Gradient 8–25) and ready-made buffer strips (PhastGel SDS Buffer Strips) were purchased from Amersham Biosciences. Each sample was dissolved in 0.0625 M phosphate buffer (pH 7.5) containing 2.5% SDS and 5% dithiothreitol, heated at 100°C for 10 min and subjected to electrophoresis. Precision plus protein standards (Bio-Rad Laboratories) were run as a reference, along with samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

2.6. Immunoblotting

Following SDS-PAGE, the separated proteins were electrotransferred from the gel to a nitrocellulose membrane using the PhastSystem apparatus equipped with a PhastTransfer (Amersham Biosciences), as described in the manufacturer's manual. The membrane was then washed with 0.05% Tween 20 in PBS (Tween-PBS) and blocked with 5% skimmed milk in Tween-PBS at 4°C overnight. After washing with Tween-PBS, the membrane was reacted successively with patient serum (diluted 1:500 with PBS) at 37°C for 1 h and peroxidase-conjugated goat anti-human IgE antibody (diluted 1:10,000 with PBS) at 37°C for 1 h. Blots were visualized using an ECL Plus western blotting detection system (Amersham Biosciences) and an ECL mini camera (Amersham Biosciences), according to the manufacturer's instructions.

For inhibition immunoblotting, patient serum (diluted 1:250) was mixed with an equal volume of inhibitor solution (20 $\mu\text{g}/\text{ml}$). After incubation at 37°C for 1 h, the mixture was used as a primary antibody. The inhibitors used were tropomyosins from Japanese flying squid and Japanese spiny lobster, gifts from Dr. T. Ojima, Graduate School of Fisheries Sciences, Hokkaido University, who purified by a combination of iso-electrical precipitation and salting-out with ammonium sulfate following the methods of Bailey (1948) and Cummins and Perry (1973). The homogeneity of both tropomyosin preparations was supported by SDS-PAGE.

2.7. Cloning experiments

Total RNA was extracted from 2 g of each muscle sample with the TRIzol reagent (Life Technologies, Rockville, MD, USA) and poly(A)⁺ mRNA was purified using an mRNA purification kit (Amersham Biosciences). A part of the purified mRNA was converted to cDNA, followed

by ligation of AP1 adapters, using a Marathon cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA, USA). The Marathon cDNA library thus constructed was applied to 3' and 5' rapid amplification of cDNA ends (RACE). In combination with the AP1 adaptor primer, a forward primer 5'-GATGCCATCAAGAAGAAGATG-3' (corresponding to ²DAIKKKM⁸ conserved for the known molluscan tropomyosins) and a reverse primer 5'-GCGAGTTTACGGGCAGCCTC-3' (corresponding to ¹⁶⁴EAARKLA¹⁷⁰ conserved for the known molluscan tropomyosins) were used for 3' and 5'RACE, respectively. As compared to the reported nucleotide sequences of the molluscan tropomyosin cDNAs, both primers are completely identical with the cDNAs encoding tropomyosins from two species of abalone, *H. diversicolor* (accession number of the DDBJ/EMBL/GenBank nucleotide sequence databases: AF216518) and *Haliotis rufescens* (X75218), and are different by one to three nucleotides from the cDNAs encoding tropomyosins from three species of mussel, *Mytilus edulis* (U40035), *Mytilus galloprovincialis* (AB000907) and *P. viridis* (AF216519), and three species of scallop, *C. nobilis* (AF216520), *Chlamys nipponensis* (AB021681) and *Patinopecten yessoensis* (AB004636). In 3' and 5'RACE, amplification of cDNA fragments by polymerase chain reaction (PCR) was performed using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 1 min; and 72 °C for 7 min. The PCR products were subcloned into the pT7Blue-2T-vector (Novagen, Darmstadt, Germany) and their nucleotide sequences were determined using a PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and a PRISM 310 genetic analyzer (Applied Biosystems).

3. Results and discussion

3.1. Allergenicity and allergens of cephalopods

IgE-binding ability of each heated extract was evaluated by ELISA using a pooled serum from 10 crustacean-allergic patients and three individual sera. As shown in Fig. 1, all the patient sera reacted to all the heated extracts from nine species of cephalopods, although less potently than to those from two species of crustaceans. No marked difference in the reactivity with IgE was recognized among the cephalopods, suggesting that they are almost equally allergenic. It should be noted that at least the crustacean-allergic patients under investigation are also sensitive to cephalopods. This is understood by the fact that tropomyosin is a cross-reactive allergen between cephalopods and crustaceans, as described below.

On SDS-PAGE, a prominent protein band of 35–38 kDa, which is assignable to tropomyosin from the behaviors of purified tropomyosin preparations from Japanese flying squid and Japanese spiny lobster, was found in each heated extract (Fig. 2A). Immunoblotting experiments revealed that tropomyosin (35–38 kDa protein) is the sole or major IgE-reactive protein in common with the nine species of cephalopods (Fig. 2B). Our results not only reconfirmed the previous finding that the major allergen in Japanese flying squid (Miyazawa et al., 1996) and common octopus (Ishikawa et al., 2001) is tropomyosin but also suggested that the major allergen in the other seven species of cephalopods is tropomyosin as well. Furthermore, preadsorption with tropomyosin purified from either Japanese flying squid or Japanese spiny lobster abolished the IgE-reactivity of patient sera to tropomyosins from the nine species of cephalopods (Fig. 2C). This allowed us to assume that cephalopod tropomyosins are cross-reactive with one another and also with crustacean tropomyosins. Therefore, crustacean-allergic patients are recommended to avoid ingestion of cephalopods as well as crustaceans unless they are diagnosed to be insensitive to cephalopods.

3.2. Nucleotide sequences of cDNAs encoding cephalopod tropomyosins

3'RACE and 5'RACE data established the nucleotide sequences of the full-length cDNAs (1149 bp for golden cuttlefish, 1178 bp for big fin reef squid, 1271 bp for Japanese flying squid, 1293 bp for neon flying squid and 1172 bp for common octopus) encoding tropomyosins from five species of cephalopods. The same nucleotide sequence as the forward primer used for 3'RACE was recognized only in the cDNAs encoding big fin reef squid and neon flying squid tropomyosins and that as the reverse primer used for 5'RACE only in the common octopus tropomyosin cDNA, while one or two nucleotide replacements occurred in the other cases. Nevertheless, cDNA fragments were successfully amplified in all 3' and 5'RACE experiments. In this study, at least five clones were subjected to DNA

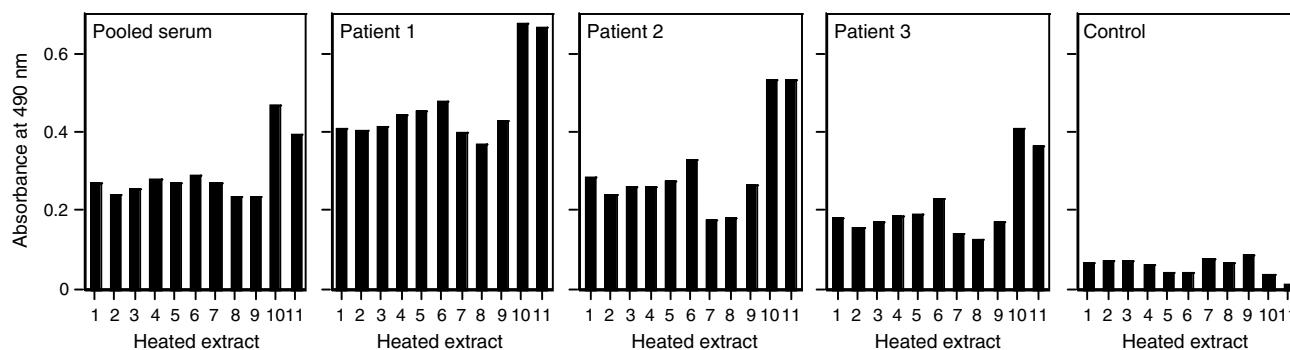


Fig. 1. Analysis by ELISA of the IgE reactivity of patient sera to the heated extracts from nine species of cephalopods and two species of crustaceans. Each heated extract (diluted 1:1000) was coated on the ELISA plate and reacted with patient serum (diluted 1:100). Heated extracts (protein concentration in mg/ml): 1, golden cuttlefish (7.8); 2, big fin reef squid (13.0); 3, spear squid (13.7); 4, swordtip squid (7.7); 5, Japanese flying squid (11.4); 6, neon flying squid (9.2); 7, common octopus (15.1); 8, ocellated octopus (5.2); 9, Pacific giant octopus (3.4); 10, kuruma prawn (7.8); 11, Japanese spiny lobster (7.5).

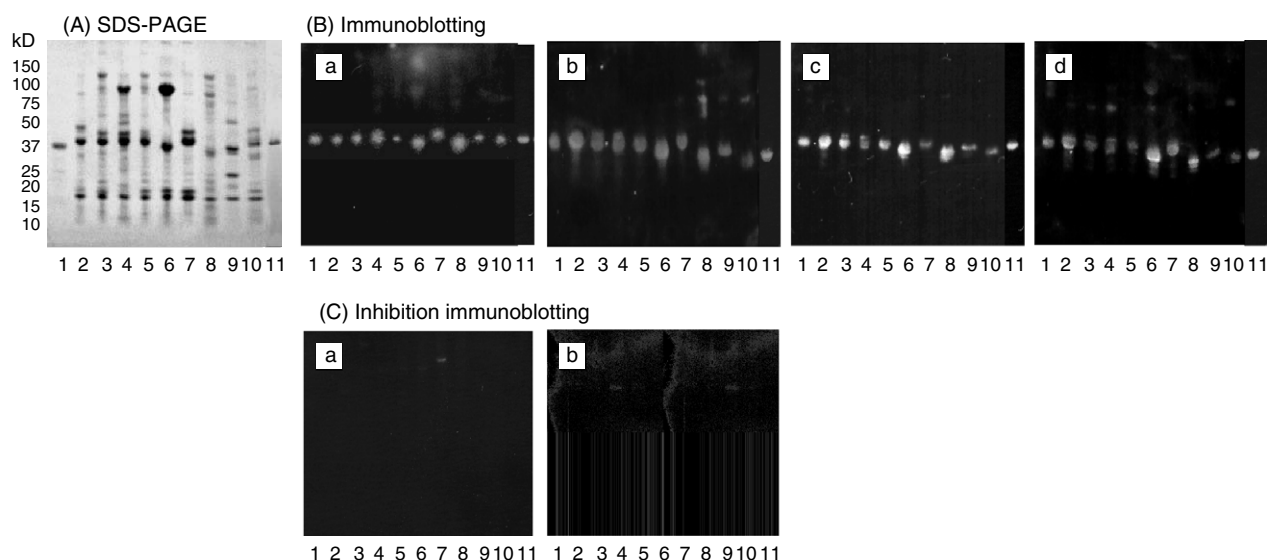


Fig. 2. Analysis of the heated extracts from nine species of cephalopods by SDS-PAGE (A), immunoblotting (B) and inhibition immunoblotting (C). Lanes: 1, tropomyosin purified from Japanese flying squid; 2, golden cuttlefish extract; 3, big fin reef squid extract; 4, spear squid extract; 5, swordtip squid extract; 6, Japanese flying squid extract; 7, neon flying squid extract; 8, common octopus extract; 9, ocellated octopus extract; 10, Pacific giant octopus extract; 11, tropomyosin purified from Japanese spiny lobster. (A) Heated extracts (1 μ l each) with the protein concentrations described in the legend for Fig. 1 were subjected to SDS-PAGE. (B) Sera: a, pooled serum from 10 patients; b, patient 1; c, patient 2; d, patient 3. (C) Inhibitors: a, tropomyosin purified from Japanese flying squid; b, tropomyosin purified from Japanese spiny lobster. Pooled serum (diluted 1:250) from 10 patients was preincubated with an equal volume of inhibitor solution (20 μ g/ml). The same results were also obtained with the sera from patients 1–3.

sequencing following subcloning of each PCR product into the pT7Blue-2T-vector. For each PCR product, no difference in nucleotide sequence was observed among the clones analyzed. The determined nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers of AB218913 (golden cuttlefish), AB218914 (big fin reef squid), AB218915 (Japanese flying squid), AB218916 (neon flying squid) and AB218917 (common octopus), although not shown in this paper to save space.

3.3. Deduced amino acid sequences of cephalopod tropomyosins

This study is the first to report the entire amino acid sequences of cephalopod tropomyosins. An open reading frame of each tropomyosin cDNA coded for 284 amino acid residues. The deduced amino acid sequences of tropomyosins from five species of cephalopods are shown in Fig. 3, together with those from eight species of mollusks (three species of mussel, three species of scallop and two species of abalone), two species of crustaceans (shrimp and American lobster) and human.

Partial amino acid sequences have previously been clarified for tropomyosins from Japanese flying squid (Miyazawa et al., 1996) and common octopus (Ishikawa et al., 2001) by analyzing five peptide fragments produced upon enzymatic digestion. The five enzymatic peptides from Japanese flying squid tropomyosin were completely consistent with the segments 37–42, 50–61, 62–76, 129–146 and 252–264. In the case of common octopus tropomyosin, the five

enzymatic peptides correspond to the segments 50–61, 77–112, 129–146, 234–248 and 269–281. However, three alterations were found in the segment 77–112; Cys-81, Asp-98 and Thr-111 identified in this study were reported to be Glu-81, Asn-98 and Gln-111, respectively, in the previous paper. We revise here the previous results since the amino acid sequence determined in this study is apparently correct as compared to those of tropomyosins from the other four species of cephalopods.

Cephalopod tropomyosins are highly homologous with one another; the sequence identity is more than 96% among the four species of decapods and about 92% between the decapods and the octapod (common octopus). This high sequence identity is the molecular basis for the cross-reactivity among cephalopod tropomyosins. In contrast, cephalopod tropomyosins share only 70–82% sequence identity with other molluscan tropomyosins, 63–64% with crustacean tropomyosins and 51–55% with human α - and β -tropomyosins.

Despite the low sequence identity between cephalopod and crustacean tropomyosins, cephalopod tropomyosins were found to cross-react with crustacean tropomyosins by inhibition immunoblotting. This can be understood at the molecular level to some extent. Eight regions (43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273 and 273–281), each of which has 2–7 alterations compared to the amino acid sequences of human α - and β -tropomyosins, have previously been identified as IgE-binding epitopes of brown shrimp *Penaeus aztecus* tropomyosin (Pen a 1) (Ayuso et al., 2002a,b). These Pen a 1 epitopes are conserved completely in shrimp *Metapenaeus ensis*

	10	20	30	40	50	60	70	80
Golden cuttlefish	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Big fin reef squid	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Japanese flying squid	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Neon flying squid	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Common octopus	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Mussel 1	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Mussel 2	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Mussel 3	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Scallop 1	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Scallop 2	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Scallop 3	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Abalone 1	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Abalone 2	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Brown shrimp	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
American lobster	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Human (α)	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
Human (β)	MDAIKKKMLAMKMEKEVATDKAEQTEQSLRDL	EADAKNKT	EEDLSTLQKKYSNLE	NDFNANEQLTAANTNLE	EASEKRV	AE		
	90	100	110	120	130	140	150	160
Golden cuttlefish	CESEIQGLNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERGRK	VLENRS	QGDDEER
Big fin reef squid	CESEIQGLNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERGRK	VLENRS	QGDDEER
Japanese flying squid	CESEIQGLNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERGRK	VLENRS	QGDDEER
Neon flying squid	CESEIQGLNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERGRK	VLENRS	QGDDEER
Common octopus	CESEIQGLNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERGRK	VLENRS	QGDDEER
Mussel 1	HEQEIQSLTR	KISMLEED	IMKSEERY	TTAASKLE	EASKAADE	SERNRK	VLENLNC	GNDERID
Mussel 2	HEQEIQSLTR	KISMLEED	IMKSEERY	TTAASKLE	EASKAADE	SERNRK	VLENLNC	GNDERID
Mussel 3	HEQEIQSLTR	KISMLEED	IMKSEERY	TTAASKLE	EASKAADE	SERNRK	VLENLNC	GNDERID
Scallop 1	LESQVSLQRR	LLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Scallop 2	LESQVSLQRR	LLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Scallop 3	LESQVSLQRR	LLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Abalone 1	MEQEVSGTTR	KITLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Abalone 2	MEQEVSGTTR	KITLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Brown shrimp	AEGEVALNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
American lobster	AEGEVALNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Human (α)	AEGEVALNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
Human (β)	AEGEVALNRR	IQLLEEDL	ERSEERL	TSAQSKLE	DASKAADE	SERNRK	VLENLNC	GNDERID
	170	180	190	200	210	220	230	240
Golden cuttlefish	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Big fin reef squid	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Japanese flying squid	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Neon flying squid	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Common octopus	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Mussel 1	KYEEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Mussel 2	KYEEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Mussel 3	KYEEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Scallop 1	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Scallop 2	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Scallop 3	KFDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Abalone 1	KYDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Abalone 2	KYDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Brown shrimp	KYDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
American lobster	KYDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Human (α)	KYDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
Human (β)	KYDEAARKLA	ITEVDL	ERAERLE	AAEAKIV	LEELKVV	GNNMKS	LEISEQE	ASQRED
	250	260	270	280				
Golden cuttlefish	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Big fin reef squid	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Japanese flying squid	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Neon flying squid	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Common octopus	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Mussel 1	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Mussel 2	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Mussel 3	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Scallop 1	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Scallop 2	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Scallop 3	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Abalone 1	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Abalone 2	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Brown shrimp	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
American lobster	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Human (α)	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				
Human (β)	EAERTVSKLQ	KEVDRL	EDLLAEK	ERYKSI				

Fig. 3. Alignment of the amino acid sequences of cephalopod tropomyosins with those of mussel, scallop, abalone, brown shrimp, American lobster and human tropomyosins. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): golden cuttlefish, AB218913; big fin reef squid, AB218914; Japanese flying squid, AB218915; neon flying squid, AB218916; common octopus, AB218917; mussel 1 (*Mytilus edulis*), U40035; mussel 2 (*Mytilus galloprovincialis*), AB000907; mussel 3 (*Perna viridis*), AF216519; scallop 1 (*Chlamys nobilis*), AF216520; scallop 2 (*Chlamys nipponensis*), AB021681; scallop 3 (*Patinopecten yessoensis*), AB004636; abalone 1 (*Haliotis diversicolor*), AF216518; abalone 2 (*Haliotis rufescens*), X75218; American lobster (*Homarus americanus*), AF034954; human α , M19713; human β , X06825. The amino acid sequence of brown shrimp (*Penaeus aztecus*) tropomyosin is from the paper of Ayuso et al. (2002a). The residues differing from golden cuttlefish tropomyosin are shaded and the IgE-binding epitopes proposed for brown shrimp tropomyosin are boxed.

(U08008) and American lobster *Homarus americanus* (AF034954; Fig. 3) tropomyosins and almost completely in spiny lobster *Panulirus stimpsoni* (AF030063) and crab *Charybdis feriatus* (AF061783) tropomyosins, suggesting that they are common IgE-binding epitopes of crustacean tropomyosins. Cephalopod tropomyosins have the same sequence in the region 249–259 as Pen a 1 and have only one replacement in the regions 88–101, 137–141, 187–197 and 273–281 compared to Pen a 1. It is thus assumed that these five regions are IgE-binding epitopes for both cephalopod and crustacean tropomyosins, accounting for their cross-reactivity. On the other hand, there are more than two alterations in the regions 43–55, 144–151 and 266–273 between cephalopod and crustacean tropomyosins. Cephalopod tropomyosins also differ from human tropomyosins by more than two amino acid residues in these three regions, leading us to assume that the three regions of cephalopod tropomyosins may be involved in IgE-binding. Future study is needed to clarify whether the three regions are epitopes specific for crustacean tropomyosins or common to cephalopod and crustacean tropomyosins.

Mussel, scallop and abalone tropomyosins obviously have an IgE-binding epitope in the region 249–259 common to cephalopod and crustacean tropomyosins. However, they have extended replacements in the regions corresponding to the other Pen a 1 epitopes and hence their IgE-binding epitopes are not fully discussed. Elucidation of IgE-binding epitopes of molluscan tropomyosins using synthetic peptides is needed to understand in more detail the cross-reactivity among shellfish tropomyosins. The primary structures of cephalopod tropomyosins determined in this study will provide helpful information in designing synthetic peptides to be used in future study on IgE-binding epitopes of shellfish tropomyosins.

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