



Tropomyosins in gastropods and bivalves: Identification as major allergens and amino acid sequence features

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ABSTRACT

Tropomyosin appears to be a major cross-reactive allergen of crustaceans and molluscs. In this study, four species of gastropods (disc abalone, turban shell, whelk and Middendorff's buccinum) and seven species of bivalves (bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam and short-neck clam) were confirmed to be allergenic by ELISA and their major allergen identified as tropomyosin by immunoblotting. Inhibition immunoblotting data showed the cross-reactivity of gastropod and bivalve tropomyosins with one another and also with cephalopod and crustacean tropomyosins. Then, amino acid sequences of tropomyosins from 10 species except for Middendorff's buccinum were elucidated by cDNA cloning. The known amino acid sequence data including our results reveal that molluscan tropomyosins share low sequence identities (about 60%) with crustacean tropomyosins and that they are highly homologous with one another within the same group (same family or same order) but not among the groups.

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1. Introduction

Food allergy mediated by immunoglobulin E (IgE) antibodies is a public concern in industrialised countries. Sensitised subjects with high levels of IgE to a specific food can develop hypersensitive reactions, such as urticaria, asthma, diarrhoea and even anaphylaxis, immediately after ingestion of the food. In coastal countries, shellfish including crustaceans and molluscs are obviously among the most common causes of food allergy. The major allergen of crustaceans is tropomyosin, a 35–38 kDa myofibrillar protein involved in the muscle contraction, as demonstrated with the following various species: shrimps (Daul, Slattey, Reese, & Lehrer, 1994; Leung et al., 1994; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Shanti, Martin, Nagpal, Metcalfe, & Subba Rao, 1993), crayfishes (Leung et al., 1998a), lobsters (Leung et al., 1998a), crabs (Leung et al., 1998b; Motoyama et al., 2007), krills (Motoyama et al., in press; Nakano, Yoshimura, & Yamada, 2008), mantis shrimps (Motoyama et al., in press) and barnacles (Suma et al., 2007). Moreover, tropomyosin is considered to be a major allergen in molluscs primarily composed of cephalopods, gastropods and bivalves. Molecular evidence for this has been obtained with as many as nine species of cephalopods (cuttlefish, squid and octopus) (Ishikawa, Suzuki, Ishida, Nagashima, & Shiomi, 2001; Miyazawa et al., 1996; Motoyama, Ishizaki, Nagashima, & Shiomi, 2006).

However, despite that gastropods and bivalves include a variety of groups, molecular studies have been limited to the following species: Japanese abalone *Haliotis diversicolor* (Chuo, Wong, & Leung, 2000), turban shell *Turbo cornutus* (Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998a), green mussel *Perna viridis* (Chuo et al., 2000), noble scallop *Chlamys nobilis* (Chuo et al., 2000), Japanese oyster *Crassostrea gigas* (Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998b; Ishikawa, Shimakura, Nagashima, & Shiomi, 1997) and constricted tagelus *Sinonovacula constricta* (Song, Li, Li, & Ran, in press).

IgE cross-reactivity is clinically and experimentally recognised among crustaceans, among molluscs and even between crustaceans and molluscs (Lehrer & McCants, 1987; Leung et al., 1996; Motoyama et al., 2006; Reese, Ayuso, & Lehrer, 1999). Accumulated data on the primary structures of crustacean tropomyosins prove that they share extremely high sequence identities (mostly more than 90%) with one another (Motoyama et al., 2007), except for barnacle tropomyosin having considerably low identities (about 60%) with other crustacean tropomyosins (Suma et al., 2007). In accordance with this, the eight IgE epitopes proposed for brown shrimp *Penaeus aztecus* tropomyosin (Pen a 1) (Ayuso, Lehrer, & Reese, 2002b; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002a; Reese et al., 2005) are well conserved in other crustacean tropomyosins, being a molecular basis for the cross-reactivity among crustaceans. On the other hand, the cross-reactivity either among molluscs or between crustaceans and molluscs is not fully understood due to the shortage of information about the primary

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structures of molluscan tropomyosins, especially those of gastropod and bivalve tropomyosins. Amino acid sequences of gastropod and bivalve tropomyosins even including those with no evidence for allergenicity are known only for three species of abalones, three species of mussels, three species of scallops and one species of clam (refer to Table 2).

For a better understanding of the cross-reactivity either among molluscs or between crustaceans and molluscs, it is essential to elucidate amino acid sequences of allergenic tropomyosins from various species of gastropods and bivalves. In this study, therefore, four species of gastropods (disc abalone, turban shell, whelk and Middendorff's buccinum) and seven species of bivalves (bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam and short-neck clam), which are situated at various taxonomical positions (refer to Table 1), were selected as samples. We report here the identification of tropomyosins as the major allergens in these 11 species and the amino acid sequences of tropomyosins in 10 species (except for Middendorff's buccinum) determined by a cDNA cloning technique. The sequence features of molluscan tropomyosins are also discussed compared to those of crustacean tropomyosins.

2. Materials and methods

2.1. Animal samples

Live specimens of four species of gastropods (disc abalone *Haliotis discus discus*, turban shell *T. cornutus*, whelk *Neptunea polycostata* and Middendorff's buccinum *Buccinum middendorffi*), seven species of bivalves (bloody cockle *Scapharca broughtonii*, Japanese oyster *C. gigas*, Japanese cockle *Fulvia mutica*, surf clam *Pseudocardium sachalinensis*, horse clam *Tresus keenae*, razor clam *Solen strictus* and short-neck clam *Ruditapes philippinarum*) and American lobster *Homarus americanus* and fresh specimens of Japanese flying squid *Todarodes pacificus* were all purchased at the Tokyo Central Wholesale Market. The taxonomical positions of the gastropods and bivalves are summarised in Table 1. Columellar muscle was obtained from disc abalone, foot muscle from turban shell, whelk, Middendorff's buccinum, bloody cockle, Japanese cockle, surf clam and razor clam, adductor muscle from Japanese oyster, siphon (major edible muscle part) from horse clam, soft tissues from short-neck clam, abdominal muscle from American lobster and mantle muscle from Japanese flying squid. Muscle or soft tissue samples for extraction were stored at -20°C until use and those for molecular cloning experiments were immediately immersed in liquid nitrogen and stored at -80°C until use.

Table 1
Gastropods and bivalves used in this study.

Class	Order	Family	Species (common name)
Gastropoda	Vetigastropoda	Haliotidae	<i>Haliotis discus discus</i> (Japanese abalone)
		Turbinidae	<i>Turbo cornutus</i> (turban shell)
	Neogastropoda	Buccinidae	<i>Neptunea polycostata</i> (whelk)
			<i>Buccinum middendorffi</i> (Middendorff's buccinum)
Bivalvia	Arcoida	Arcidae	<i>Scapharca broughtonii</i> (bloody cockle)
	Ostreoida	Ostreidae	<i>Crassostrea gigas</i> (Japanese oyster)
	Veneroida	Cardiidae	<i>Fulvia mutica</i> (Japanese cockle)
		Mactridae	<i>Pseudocardium sachalinensis</i> (surf clam)
			<i>Tresus keenae</i> (horse clam)
		Solenidae	<i>Solen strictus</i> (razor clam)
		Veneridae	<i>Ruditapes philippinarum</i> (short-neck clam)

2.2. Preparation of heated extracts

Each muscle or soft tissue sample was homogenised with four volumes of 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). In view of the fact that tropomyosin, a target protein in this study, is thermostable, the homogenate was then heated in a boiling water bath for 10 min. After centrifugation at 18,000g for 15 min, the supernatant obtained was used as a heated extract. Protein concentrations of the heated extracts were quantified by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard.

2.3. Purification of tropomyosin

American lobster, Japanese flying squid and turban shell tropomyosins were purified from the abdominal, mantle and foot muscles, respectively, as reported previously (Motoyama et al., 2007). In brief, an acetone powder of myofibrillar proteins prepared from the muscle was extracted with 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM CaCl_2 and 1 mM dithiothreitol. The extract was subjected to salting-out with ammonium sulfate (30–60% saturation), followed by isoelectrical precipitation (pH 4.6). Finally, small amounts of contaminants were removed by reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan). The homogeneity of the final preparation was supported by SDS-PAGE.

2.4. Human sera

Sera were donated from 10 crustacean-allergic patients (patients 1–10) with a history of hypersensitive reactions after ingestion of crustaceans. These patients had all been diagnosed to be allergic to crustaceans by the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) performed at hospitals; the determined CAP-RAST classes were 3–6 against shrimp and/or crab. Patients 5, 6, 8 and 9 had been additionally shown to have CAP-RAST classes of 3–6 against squid and octopus. In this study, sera from eight healthy volunteers without adverse reactions after ingestion of any foods were used as controls.

2.5. Fluorescence ELISA

Reactivity of the patient sera to each heated extract was evaluated by fluorescence ELISA as described elsewhere (Hamada et al., 2004). In brief, a polystyrene microtiter plate with 96 wells (Type H (black); Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of the heated extract (diluted 1:2000) and reacted successively with patient or control serum (diluted 1:500) and β -galactosidase-conjugated goat anti-human IgE antibody (0.25 $\mu\text{g}/\text{ml}$; American Qual-ex, San Clement, CA, USA). Enzyme reaction was carried out using substrate solution (0.1 mg/ml 4-methylumbelliferyl- β -D-galactoside) and stopped by the addition of 100 mM glycine-NaOH buffer (pH 10.3). Fluorescence intensity was measured on a SPECTRAMax GEMINI XS (Molecular Devices, Sunnyvale, CA, USA) with excitation at 367 nm and emission at 453 nm. All ELISAs were performed in triplicate and the data obtained were expressed in mean $\pm$ SD.

2.6. SDS-PAGE

SDS-PAGE was performed on a PhastSystem apparatus (GE-Healthcare, Piscataway, NJ, USA) as described in the manufacturer's manual. Ready-made gels (PhastGel Gradient 8–25) and ready-made buffer strips (PhastGel SDS Buffer Strips) were purchased from GE-Healthcare. Each sample was dissolved in 62.5 mM 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, Hercules, CA, USA) were run as a reference, along with samples. After running, proteins were visualised by staining with Coomassie Brilliant Blue R-250.

2.7. Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as reported previously (Kobayashi et al., 2006). Briefly, the proteins separated by SDS–PAGE were electrotransferred from the gel to a nitrocellulose membrane, which was reacted successively with primary and secondary antibodies. In order to detect tropomyosin, an antiserum (diluted 1:30,000) raised in rabbits against king crab tropomyosin, which was kindly gifted from Dr. H. Ushio in our university, was used as a primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:20,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) as a secondary antibody. In IgE-immunoblotting, patient or control serum (diluted 1:500) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:10,000; Kirkegaard & Perry Laboratories) were used as primary and secondary antibodies, respectively. Blots were visualised using an ECL Plus Western Blotting Detection System (GE-Healthcare) and an ECL Mini Camera (GE-Healthcare), as recommended by the manufacturer. For inhibition IgE-immunoblotting, patient serum (diluted 1:250) was preincubated with an equal volume of American lobster, Japanese flying squid or turban shell tropomyosin solution (10 µg/ml) at 37 °C for 1 h and used as a primary antibody.

2.8. cDNA cloning of tropomyosins

The rapid amplification cDNA ends (RACE) method was employed for cDNA cloning of tropomyosins. Total RNA was extracted from 1.5 g of each muscle sample with 15 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For 3'RACE, first-stranded cDNA was synthesised from 5 µg of total RNA using the 3'RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's manual and used as a template. 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 2 min; and 72 °C for 7 min. In PCR, the following forward primers were used in combination of the abridged universal anchor primer (AUAP): 5'-CAGAGGTGACCTGAACGT-3' (corresponding to the region 172–178 of the amino acid sequence) for disc abalone, 5'-GATGCCATCAAGAAGAAGATG-3' (corresponding to the region 2–8) for turban shell and short-neck clam and 5'-GAGGCTGCCCGTAACT(C/T)GC-3' (corresponding to the region 164–170) for the other species. These primers were designed on the basis of the known cDNAs encoding molluscan tropomyosins (refer to Table 2 for the accession numbers of tropomyosins in the DDBJ/EMBL/GenBank databases). The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany) and at least three clones were analysed for nucleotide sequences using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and a PRISM 310 genetic analyser (Applied Biosystems). Based on the determined partial nucleotide sequence, 5'RACE was carried out using the 5'RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's instructions. For each species, first-stranded cDNA was synthesised from 5 µg of total RNA using the gene-specific reverse primer and subjected as a template to PCR using another gene-specific reverse primer and the abridged anchor primer (AAP). In the case of disc abalone, nested-PCR was further performed. The PCR conditions in 5'RACE were the same as those in 3'RACE. All amplified products were subcloned and sequenced as described above.

3. Results and discussion

3.1. IgE reactivity of heated extracts from gastropods and bivalves

IgE reactivity of the heated extracts from gastropods and bivalves was analysed by fluorescence ELISA using five crustacean-allergic patient sera and a control serum pooled from eight healthy subjects. In comparison of the results with the control serum, each patient serum was judged to react with varied potencies to all of the heated extracts not only from American lobster but also from four species of gastropods and seven species of bivalves (Fig. 1). Prior to this study, the CAP-RAT determinations had demonstrated the occurrence of specific IgE against shrimp and/or crab in the five patient sera used and specific IgE against squid and octopus in the patient 5 serum. In the light of the established cross-reactivity among crustaceans (Motoyama et al., 2007; Reese et al., 1999), the reactivity of the five patient sera to the heated extract from American lobster is easily understood. Importantly, the results with the heated extracts from gastropods and bivalves show that at least the five patient sera recognise molluscs as well as crustaceans. This conforms well to the fact that tropomyosin is a cross-reactive allergen between crustaceans and molluscs as described below.

3.2. Identification of tropomyosins as major allergens in gastropods and bivalves

As analysed by SDS–PAGE, a 37 kDa protein, which is comparable in molecular mass to the turban shell tropomyosin used as a reference, was prominently detected in all of the heated extracts from four species of gastropods and seven species of bivalves (Fig. 2A). Irrespective of the species, the 37 kDa protein was judged to be tropomyosin from the positive reactivity with the antiserum against the king crab tropomyosin (Fig. 2B). In IgG-immunoblotting, minor blots above and/or below the 37 kDa protein (tropomyosin) were also observed in several species such as Japanese oyster and horse clam. It is unclear whether these blots are attributable to tropomyosin isoforms or other cross-reactive proteins. IgE-immunoblotting, using the pooled serum from 10 crustacean-allergic patients and individual six patient sera, showed that tropomyosins in all samples were IgE-reactive (Fig. 2C), although the reactivity of Japanese cockle tropomyosin with some patient sera was particularly low. In some species such as bloody cockle and Japanese oyster, IgE-reactive proteins of 100–150 kDa were also recognised. Nevertheless, IgE-immunoblotting data allowed us to conclude that tropomyosin is almost the sole IgE-reactive protein in all gastropods and bivalves examined. When four patient serum samples (pooled serum and three sera from patients 1–3) were individually preadsorbed with American lobster, Japanese flying squid or turban shell tropomyosin as an inhibitor, they completely lost their reactivity to tropomyosins from four species of gastropods and seven species of bivalves (only the results with the patient 1 serum are shown in Fig. 2D). These results qualitatively demonstrate the cross-reactivity among molluscan tropomyosins and also between crustacean and molluscan tropomyosins, as in previous studies (Lehrer & McCants, 1987; Leung et al., 1996; Motoyama et al., 2006; Reese et al., 1999).

3.3. Nucleotide sequences of cDNAs encoding gastropod and bivalve tropomyosins

In 3'RACE, no amplified products were obtained for Middendorff's buccinum. Attempts were made to amplify the cDNAs encoding the Middendorff's buccinum tropomyosin by RT-PCR using various primer sets but were unsuccessful. However, the

Table 2
Amino acid sequence identities (%) among molluscan and crustacean tropomyosins.

	Gastropods			Bivalves									Cephalopods	Crustaceans
	Order: Vetigastropoda		Order: Neogastropoda	Order: Arcoida	Order: Mytiloida	Order: Ostreoida		Order: Veneroida						
	Family: Haliotidae	Family: Turbinidae	Family: Buccinidae	Family: Arcidae	Family: Mytilidae	Family: Pectinidae	Family: Ostreidae	Family: Cardiidae	Family: Mactridae	Family: Solecurtidae	Family: Solenidae	Family: Veneridae		
	Abalones	Turban shell	Whelk	Bloody cockle	Mussels	Scallops	Japanese oyster	Japanese cockle	Surf clam	Constricted tagelus	Razor clam	Short-neck clam		
Gastropods														
Abalones	96.1–99.6	94.0–97.2	75.4–77.8	77.5–79.6	71.5–75.4	68.3–81.0	76.0–78.5	73.6–75.4	72.5–75.7	70.8–73.2	73.9–76.1	74.6–76.8	77.8–82.7	59.2–63.7
Turban shell		100	77.1	80.3	74.3–74.6	70.8–80.6	77.8	75.4	74.6–76.4	73.2	76	76.7	79.9–81.3	60.9–63.4
Whelk			100	72.2	69.0–70.1	68.7–71.1	77.1	69.7	68.7–70.4	67.6	68.7	70.8	73.2–74.3	57.4–60.9
Bivalves														
Bloody cockle				100	75.4–75.7	70.4–73.2	79.2	72.9	72.9–73.6	70.1	73.2	73.9	71.8–73.2	57.7–60.2
Mussels					94.0–99.6	67.6–70.1	78.9–79.2	66.2–66.9	64.8–66.5	65.5–66.2	66.9–67.3	66.2–66.9	69.4–71.5	54.6–58.1
Scallops						84.4–91.9	72.2–73.9	66.2–70.4	66.5–71.5	65.1–67.6	66.9–68.7	68.0–71.5	69.7–74.6	56.7–60.0
Japanese oyster							100	72.5	72.9–74.3	69.7	72.9	74.3	75.0–76.0	59.9–62.3
Japanese cockle								100	85.9–88.7	83.8	85.6	86.6	70.1–70.8	57.0–58.5
Surf clam									94.4	87.7–88.4	92.3–92.6	92.3	71.8–72.9	55.3–57.4
horse clam														
Constricted tagelus										100	89.1	85.9	70.1–71.5	57.0–58.1
Razor clam											100	90.5	72.5–73.6	57.0–58.1
Short-neck clam												100	72.9–73.6	57.4–59.2
Cephalopods													91.2–99.6	61.3–64.4
Crustaceans														89.1–98.6

Gastropods: four species of abalones, tropical abalone *Haliotis asinina* (AY320360), Japanese abalone *Haliotis diversicolor* (AF216518), California red abalone *Haliotis rufescens* (X75218) and disc abalone *Haliotis discus discus*; turban shell *Turbo cornutus*; whelk *Neptunea polycostata*. Bivalves: bloody cockle *Scapharca broughtonii*; three species of mussels, blue mussel *Mytilus edulis* (U40035), Mediterranean mussel *Mytilus galloprovincialis* (AB000907) and green mussel *Perna viridis* (AF216519); three species of scallops, akazara scallop *Chlamys nipponensis* (AB021681), noble scallop *Chlamys nobilis* (AF216520) and Yesso scallop *Patinopecten yessoensis* (AB004636); Japanese oyster *Crassostrea gigas*; Japanese cockle *Fulvia mutica*; surf clam *Pseudocardium sachalinensis*; horse clam *Tresus keenae*; constricted tagelus *Sinonovacula constricta* (EU082209); razor clam *Solen strictus*; short-neck clam *Ruditapes philippinarum*. Cephalopods: golden cuttlefish *Sepia esculenta* (AB218913), big fin reef squid *Sepioteuthis lessoniana* (AB218914), Japanese flying squid *Todarodes pacificus*, neon flying squid *Ommastrephes bartrami* (AB218916) and common octopus *Octopus vulgaris* (AB218917). Crustaceans: brown shrimp *Penaeus aztecus*, pink shrimp *Pandalus eous* (AB270631), American lobster *Homarus americanus* (fast-type tropomyosin) (AF034954), American lobster (slow-twitch tropomyosin) (AF034953), American lobster (slow-tonic tropomyosin) (AY521627), snow crab *Chionoecetes opilio* (slow-tonic tropomyosin) (AB270634), horsehair crab *Erimacrus isenbekii* (slow-twitch tropomyosin) (AB270635), horsehair crab (slow-tonic tropomyosin) (AB270636), king crab *Paralithodes camtschaticus* (fast tropomyosin) (AB270632) and king crab (slow-tonic tropomyosins) (AB270633). Except for the species shown in Fig. 2, accession numbers (DDBJ/EMBL/GenBank nucleotide sequence database) are indicated in parentheses.

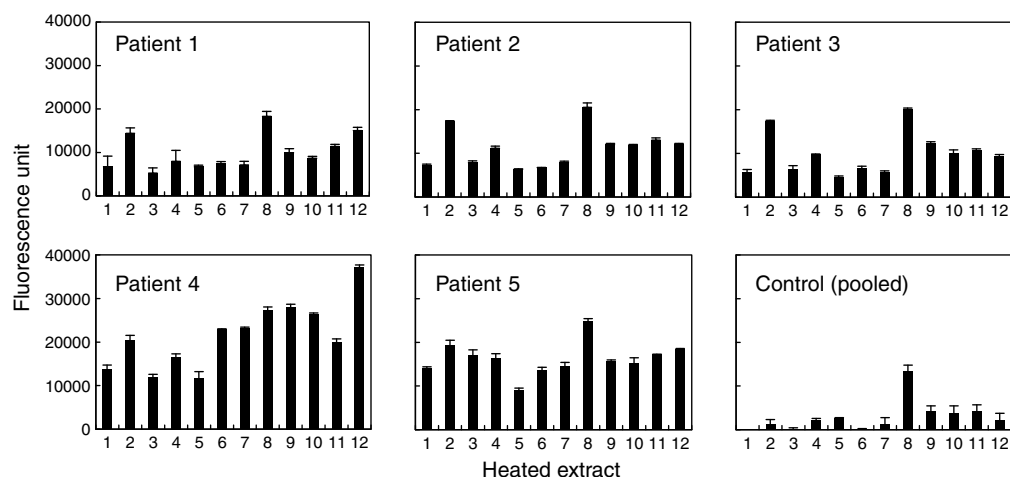


Fig. 1. Analysis by fluorescence ELISA of the IgE reactivity of patient sera to the heated extracts from four species of gastropods, seven species of bivalves and American lobster. Each heated extract (diluted 1:2000) was coated on the ELISA plate and reacted with patient or control serum (diluted 1:500). The control serum was pooled from eight healthy subjects. Heated extracts (protein concentration in mg/ml): 1, disc abalone (0.87); 2, turban shell (2.1); 3, whelk (3.8); 4, Middendorff's buccinum (2.1); 5, bloody cockle (5.5); 6, Japanese oyster (4.2); 7, Japanese cockle (2.8); 8, surf clam (9.3); 9, horse clam (6.2); 10, razor clam (4.6); 11, short-neck clam (7.5); 12, American lobster (3.6).

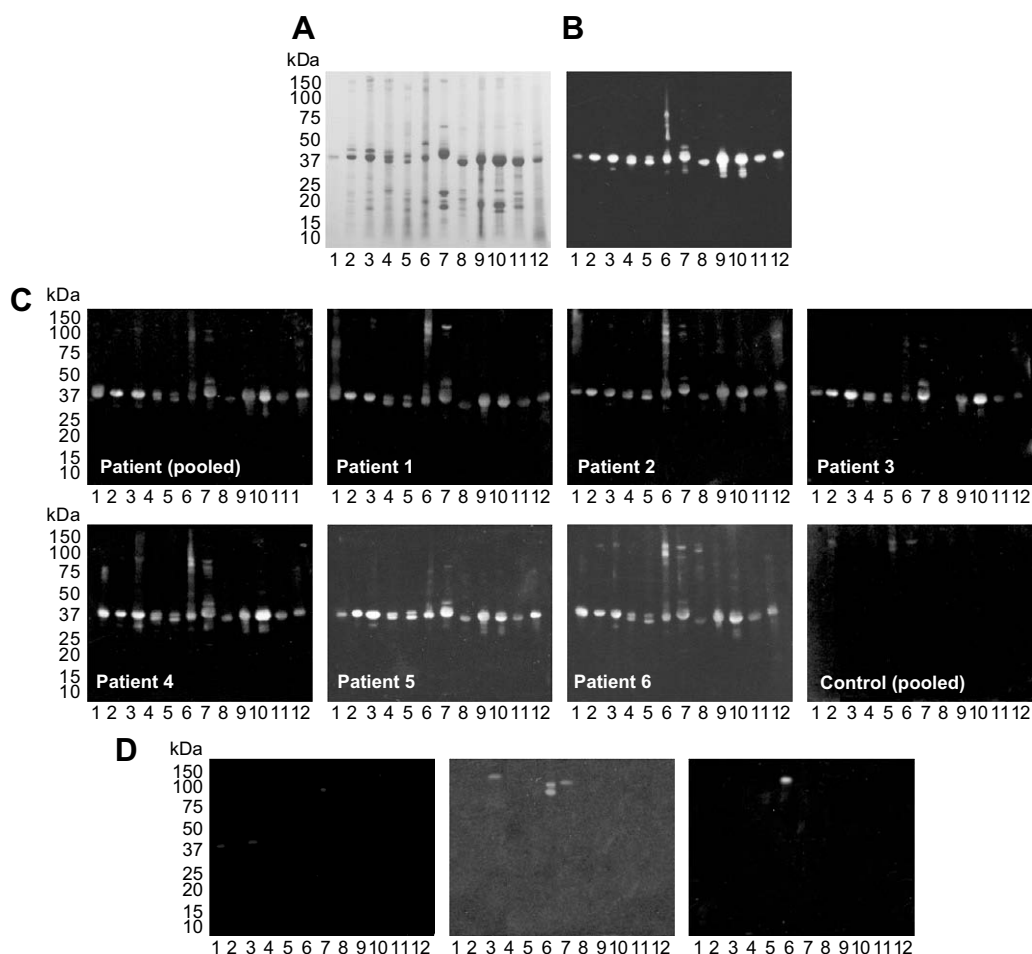


Fig. 2. Analysis of the heated extracts from four species of gastropods and seven species of bivalves by SDS-PAGE (A), IgG-immunoblotting (B), IgE-immunoblotting (C) and inhibition IgE-immunoblotting (D). Lanes: 1, tropomyosin purified from turban shell; 2, extract from disc abalone; 3, extract from turban shell; 4, extract from whelk; 5, extract from Middendorff's buccinum; 6, extract from bloody cockle; 7, extract from Japanese oyster; 8, extract from surf clam; 9, extract from horse clam; 10, extract from razor clam; 11, extract from short-neck clam; 12, extract from short-neck clam. (A) Heated extracts (0.3 μ l each) with the protein concentrations described in the legend for Fig. 1 were subjected to SDS-PAGE. (B) Antiserum against king crab tropomyosin was used to detect tropomyosins. (C) Patient sera (pooled serum from 10 patients and individual six patient sera) were used to detect IgE-binding proteins. (D) The patient 1 serum (diluted 1:250) was preincubated with an equal volume of inhibitor (left: American lobster tropomyosin, middle: Japanese flying squid tropomyosin, right: turban shell tropomyosin) solution (10 μ g/ml) and used as a primary antibody.

full-length cDNAs encoding tropomyosins from the remaining 10 species could be cloned by both 3' and 5'RACE. After subcloning of each PCR product into the pT7Blue-2 T-vector, at least three clones were analysed for nucleotide sequence. For each PCR product, there was no difference in nucleotide sequence among the clones analysed, suggesting that isoforms of the cloned tropomyosin, if present, are trace in the 10 species. The determined nucleotide sequences of the tropomyosin cDNAs, although not shown in this paper to save space, have been deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers: AB444939 for disc abalone (1223 bp), AB444940 for turban shell (1108 bp), AB444941 for whelk (1077 bp), AB444942 for bloody cockle (1079 bp), AB444943 for Japanese oyster (1110 bp), AB444944 for Japanese cockle (1119 bp), AB444945 for surf clam (1020 bp), AB444946 for horse clam (1081 bp), AB444947 for razor clam

clam (1112 bp) and AB444948 for short-neck clam (1198 bp). All cDNAs commonly contain an open reading frame composed of 852 bp coding for 284 amino acid residues.

3.4. Amino acid sequences of gastropod and bivalve tropomyosins

The amino acid sequences of gastropod tropomyosins have so far been elucidated for three species of abalones (ear shell, Japanese abalone and California red abalone) and those of bivalve tropomyosins for three species of mussels (blue mussel, Mediterranean mussel and green mussel), three species of scallops (akazara scallop, noble scallop and Yezo scallop) and one species of clam (constricted tagelus) (refer to Table 2). In this study, the amino acid sequences of tropomyosins from three species of gastropods (disc abalone, turban shell and whelk) and seven species of bivalves

	1	10	20	30	40	50	60	70	80
Disc abalone	MDA	IKKKMLAMKMEKENA	VDRAEQNEQKLRDTEEQKAKI	TEEDLNNLQKKCANLEND	FDNVNEQLQEQAMAKLETSEKRVTE				
Turban shell	MDA	IKKKMLAMKMEKENAL	DRAEQLEQKLRDTEEQKAKI	TEEDLNNLQKKCANLEND	FDNVNEQLQDALSLENSEKRVTE				
Whelk	MDL	IKKKMLSMKMDKENAL	DRADVMEQKFRDAEDQKSKLE	EDDLNNLQKKYSQLENE	FDVRVNEGLDANAKLETQEKRVNE				
Bloody cockle	MDS	IKKKMIAMKMEKENAL	DRSEQLEQKLRDTEEQKAKVEEL	LGAYQKKFSILENDFD	VTNTKWEADSAVKLEEA	EAKLLTE			
Japanese oyster	MDS	IKKKMIAMKMEKENA	QDRAEQLEQKLRDTEEQKAKI	TEEDLTSLQKKHNSLENE	FDQVNEKYQECQTKLEEA	EAKTASE			
Japanese cockle	MEA	IKKKMQSMKNEKENA	IDKAEQLETKLKDTEDSKAKI	TEEDLTSLQKKYTNLENE	FDTVNEKHADSAVKLEEA	EAKRLTE			
Surf clam	MDS	IKKKMQAMKIEKENAL	DKSEQLDQKLDVVEETKAKVEED	LSSSLQKKYTNLENE	FDKVNEQYNEGVVVKLEA	SEKRVTE			
Horse clam	MDS	IKKKMQAMKIEKENAL	DKAEQLDQKLDTEDSKAKI	TEEDLSSSLQKKYTNLENE	FDQVNEKYNDGVVVKLEA	SEKRVTE			
Razor clam	MDS	IKKKMQAMKLEKENAL	DKSEQLDQKLDTEDSKARA	EEDLSSSLQKKFTNLENE	FDKVNEQYQEGVVKLEA	SEKRVTE			
Short-neck clam	MDA	IKKKMQAMKLEKENAL	DKAEQLEQKLRDVEETKAKA	EEDLTLQKKYTNLENE	FDQVNEKYNEGVNKL	LEVSEKRVTE			
Japanese flying squid	MDA	IKKKMLAMKMEKEVAT	DKAEQTEQSLRDLEAAKNIT	EEDLSTLQKKYSNLENE	FDNAKENLT	VANTNLEA	SEKRVNE		
Brown shrimp	MDA	IKKKMQAMKLEKDNAM	DRADTLEQQNKEANNRAEKSE	E	VHNLQKRMQQL	EN	DL	DQVQESLLKANIQL	VEKDKALS
	81	90	100	110	120	130	140	150	160
Disc abalone	MEQ	EVSGTTRKITLL	EEDLERNERLQT	ATERLEEASKA	ADESERGRKVL	ESRSLADDERID	QLEAQLKEAKYIAE	DAER	
Turban shell	MEQ	EVSGTTRKITLL	EEDLERNERLQT	ATERLEEASKA	ANESERGRKVL	ESRSLADDERID	QLEAQLKEAKYIAE	DAER	
Whelk	MEQ	ISGLNRRIQLL	EEDLERSEERLQT	ATEKLEEA	TKADESERARKVL	ESKNQTAEE	SADSLAEQLKESKYIAE	DAER	
Bloody cockle	SEQ	EIASLTKRTTLL	EDDIKNEEKLS	ATQKLEEA	SHADESERGRKVL	ESRSFADDERID	ALEAQLKEAKYIAE	DAER	
Japanese oyster	AEQ	EIQSLNRRIQLL	EEDMERSEERLQT	ATEKLEDA	SKADESERNRKVL	ENLNNA	SEERTDVL	EKLQTEAKLIAE	DAER
Japanese cockle	TEDE	IKGYTRRIQLLE	DDLERTQTKL	DEATGKLEEA	TKADESERGRKVL	ESRSLADDERID	GLKEQVQDAKYVAE	SDR	
Surf clam	CEDE	IKGYTRRIQLLE	DDLERTQVKL	DEALLKLEEA	TKADESERGRKVL	ESRSIADDDRID	GLKEQVQDAKYVAE	ADR	
Horse clam	CEDE	IKGYTRRIQLLE	DDLERTQVKL	DEATLKL	EDATKTADESERGRKVL	ESRSIADDDRID	QLEAQLKEAKYIAE	DAER	
Razor clam	CEDE	IKGYTRRIQLLE	DDLERTQVKL	DEATLKL	EDATKTADESERGRKVL	ESRSIADDDRID	QLEAQLKEAKYIAE	DAER	
Short-neck clam	AED	EIKGYTRRIQLLE	DDLERTQVKL	DEATLKL	EDATKTADESERGRKVL	ESRSIADDDRID	ALEKQVQDAKYVAE	ADR	
Japanese flying squid	CESE	IQGLNRRIQLL	EEDLERSEERL	TSAQSKLEDA	SKADESERGRKVL	ENRSQGD	EERTDLEKQLEAK	KWIAE	ADR
Brown shrimp	AEGE	VALNRRIQLL	EEDLERSEER	LN	TATTKLAEASQA	ADESERMRKVL	ENRSL	SEEDERM	AL
	161	170	180	190	200	210	220	230	240
Disc abalone	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQEASQREDSY	EETIRDLTQRLKDAEN	RAT		
Turban shell	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQEASQREDSY	EETIRDLTQRLKDAEN	RAT		
Whelk	KYDE	AARKLAITEVD	LRAEARLEAAEAKVLE	LEQLHVGNMKSLE	ISEQNDQASQSWDSY	EETIRDLTQRLKDAEN	RAT		
Bloody cockle	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Japanese oyster	KYDE	AARKLAITEVD	LRAEARLEAAEAKVLE	LEQLHVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Japanese cockle	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Surf clam	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Horse clam	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Razor clam	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Short-neck clam	KYDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
Japanese flying squid	KFDE	AARKLAITEVD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQEASQREDSY	EETIRDLTQRLKDAEN	RAT		
Brown shrimp	KYDE	VARKLAMVEAD	LRAEARLEAAEAKI	LEELKLVVGNMKSLE	ISEQNDQASQREDSY	EETIRDLTQRLKDAEN	RAT		
	241	250	260	270	280				
Disc abalone	EAER	TVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Turban shell	EAER	TVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Whelk	EAER	TVTKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Bloody cockle	EAER	TVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Japanese oyster	EAER	TVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Japanese cockle	EAER	VVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Surf clam	EAER	VVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Horse clam	EAER	VVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Razor clam	EAER	VVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Short-neck clam	EAER	VVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Japanese flying squid	EAER	TVSKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		
Brown shrimp	FAER	SVQKLQKEVDRL	EDLLEAEKEKYKAI	SD	ELDQTF	AE	LAGY		

Fig. 3. Amino acid sequences of the gastropod and bivalve tropomyosins determined in this study and Japanese flying and brown shrimp tropomyosins as references. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): disc abalone *Haliotis discus discus*, AB444939; turban shell *Turbo cornutus*, AB444940; whelk *Neptunea polycostata*, AB444941; bloody cockle *Scapharca broughtonii*, AB444942; Japanese oyster *Crassostrea gigas*, AB444943; Japanese cockle *Fulvia mutica*, AB444944; surf clam *Pseudocardium sachalinensis*, AB444945; horse clam *Tresus keenae*, AB444946; razor clam *Solen strictus*, AB444947; short-neck clam *Ruditapes philippinarum*, AB444948; Japanese flying squid *Todarodes pacificus*, AB218915; brown shrimp *Penaeus aztecus*, DQ151457. The residues differing from brown shrimp tropomyosin are shaded. The IgE epitope regions proposed for brown shrimp tropomyosin are boxed.

(bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam and short-neck clam) were newly clarified. It is worth mentioning that, except for disc abalone, the gastropod and bivalve species used in this study are not only classified into diverse families and orders but also taxonomically discriminated from those used in the previous sequence studies. Thus, this study is of particular value in making it possible to understand the overall features of the amino acid sequences of tropomyosins from edible gastropods and bivalves.

The determined amino acid sequences of tropomyosins from the three species of gastropods and seven species of bivalves are aligned in Fig. 3, together with those of Japanese flying squid tropomyosin (a representative of cephalopod tropomyosins) and brown shrimp tropomyosin (a representative of crustacean tropomyosins). It is obvious that the amino acid sequences of gastropod and bivalve tropomyosins are significantly different from that of brown shrimp tropomyosin. As it is not easy to compare the amino acid sequences among molluscs only by the sequence alignment shown in Fig. 3, the sequence identities among molluscan and crustacean tropomyosins are summarised in Table 2, in which gastropods and bivalves are listed according to the family to which they belong. First, it should be noted that gastropod and bivalve tropomyosins share only 60% sequence identities with crustacean tropomyosins and considerably low identities (mostly 70–80%) with cephalopod tropomyosins. Another significant feature of the amino acid sequences of gastropod and bivalve tropomyosins is that they are highly homologous (mostly more than 90% identities) with one another within the same group (same family or same order) but not (mostly 70–80% identities) among the groups. Although further sequence data are needed, the gastropods and bivalves whose tropomyosins have already been sequenced can be divided into the following groups based on the sequence identities: two groups (members of the orders Vetigastropoda and Neogastropoda) for gastropods and five groups (members of the order Arcoida, the order Mytiloida, the families Pectinidae and Ostreidae of the order Ostreoida and the order Veneroida) for bivalves.

As described above, molluscan tropomyosins have low sequence identities with crustacean tropomyosins and are also distinguishable in sequence among the groups. Nevertheless, the cross-reactivity either among molluscan tropomyosins or between molluscan and crustacean tropomyosins was shown in this study as well as in previous studies (Lehrer & McCants, 1987; Leung et al., 1996; Motoyama et al., 2006; Reese et al., 1999). This can be realised to some extent in consideration of the eight IgE epitopes (regions 43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273 and 273–281) proposed for brown shrimp tropomyosin (Pen a 1) (Ayuso et al., 2002a, 2002b; Reese et al., 2005). As discussed in our previous paper (Motoyama et al., 2006), 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, accounting for the cross-reactivity between cephalopod and crustacean tropomyosins. In the case of gastropod and bivalve tropomyosins, the sequence of the region 249–259 is completely conserved as well. This partly supports the cross-reactivity either among molluscan tropomyosins or between molluscan and crustacean tropomyosins. However, gastropod and bivalve tropomyosins have extended replacements in the regions corresponding to the other Pen a 1 epitopes. Elucidation of IgE epitopes of gastropod and bivalve tropomyosins using synthetic peptides is needed to discuss in more detail their cross-reactivity with cephalopod and crustacean tropomyosins.

In conclusion, this study provided experimental evidence that a variety of gastropods and bivalves are allergenic and that their major allergen is tropomyosin cross-reacting with one another and

also with cephalopod and crustacean tropomyosins. Importantly, our sequence data, together with the previous data, revealed that gastropods and bivalves contain tropomyosins with amino acid sequence features associated with their taxonomical positions. This might be a molecular basis for the fact that there are patients sensitive to some species of gastropods or bivalves but not to others. Therefore, future study on IgE epitopes of gastropod and bivalve tropomyosins should be performed in consideration of the taxonomical positions of species. Such study will greatly increase our molecular knowledge on the allergenicity of gastropod and bivalve tropomyosins.

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