



Paramyosin of the disc abalone *Haliotis discus discus*: Identification as a new allergen and cross-reactivity with tropomyosin

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ABSTRACT

Tropomyosin, a 35–38 kDa myofibrillar protein, represents a major allergen in molluscs, as well as in crustaceans. Besides tropomyosin, a 100 kDa allergen was newly detected in the disc abalone *Haliotis discus discus*. The 100 kDa allergen was purified from the muscle of the disc abalone by salting-out and hydroxyapatite HPLC and identified as paramyosin based on the determined amino acid sequences of the peptide fragments produced by lysylendopeptidase digestion. Based on analysis by fluorescence ELISA, as many as 16 of the 18 patient sera tested, reacted to the disc abalone tropomyosin. The same patient sera also reacted to the disc abalone paramyosin, although rather less potently than to tropomyosin, suggesting that paramyosin is a major allergen. Immunoblotting data showed that IgE-reactive paramyosin is distributed in some species of molluscs other than the disc abalone. Interestingly, cross-reactivity between paramyosin and tropomyosin was demonstrated by inhibition immunoblotting and inhibition ELISA.

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1. Introduction

Food allergy mediated by immunoglobulin E (IgE) is a public concern in industrialised countries. In sensitised subjects with high levels of specific IgE to a certain food, adverse reactions such as urticaria, asthma, diarrhoea and anaphylaxis are induced immediately after ingestion of the food; in severe cases, anaphylactic shock leads to even death. Of a number of allergenic foodstuffs, seafoods (fish, crustaceans and molluscs) are recognised as an important cause of food allergy especially in coastal countries, where their consumption is high. Previous studies showed that parvalbumin, a 12 kDa sarcoplasmic protein, is a major cross-reactive allergen in fish (Lehrer, Ayuso, & Reese, 2003; Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005; Wild & Lehrer, 2005). Besides parvalbumin, collagen has been additionally identified as a fish allergen although not major (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000). As for crustaceans and molluscs, tropomyosin, a 35–38 kDa myofibrillar protein, is a common major allergen (Chuo, Wong, & Leung, 2000; Emoto, Ishizaki, & Shiomi, 2009; Leung et al., 1996; Motoyama, Ishizaki, Nagashima, & Shiomi, 2006; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Reese, Ayuso, & Lehrer, 1999; Wild & Lehrer, 2005). IgE cross-reactivity of tropomyosin can be seen amongst crustaceans, amongst molluscs, between crustaceans and molluscs, and even between crustaceans and terrestrial arthropods, such as cock-

roaches and mites (Arlan, Morgan, Vyszynski-Moher, & Sharra, 2009; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002). Recently, arginine kinase (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007; Yu, Lin, Chiang, & Chow, 2003), a sarcoplasmic calcium-binding protein (Ayuso et al., 2009; Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008) and myosin light chain (Ayuso et al., 2008) have also been confirmed to be new allergens in crustaceans.

To prevent allergic accidents, labelling systems for processed food products containing allergenic food materials have recently been established in some countries. In Japan, 25 kinds of food materials are considered to be especially allergenic, and therefore are obligated or recommended to put labels on packages or bottles of processed food products containing them as raw materials. Abalone, together with other six kinds of seafoods (salmon, mackerel, shrimp, crab, squid and salmon roe), is amongst the 25 kinds of allergenic food materials. Molecular studies with two species of abalones, the Japanese abalone *Haliotis diversicolor* (Chuo et al., 2000), and the disc abalone *Haliotis discus discus* (Emoto et al., 2009), proved that their major allergen is tropomyosin, as in the other molluscs. During our study for allergens in disc abalone by immunoblotting, however, not only tropomyosin but also a 100 kDa allergen was found to be allergenic. This finding prompted us to purify and identify the 100 kDa allergen in the disc abalone. As a result, the 100 kDa allergen was demonstrated to be paramyosin by partial amino acid sequencing of the purified preparation. Subsequent ELISA experiments showed that paramyosin, as well as tropomyosin, is a major allergen in disc abalone. Interestingly,

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the IgE cross-reactivity between paramyosin and tropomyosin was also verified by inhibition experiments. We report here the identification of the 100 kDa allergen in the disc abalone, as paramyosin, and the cross-reactivity of paramyosin with tropomyosin.

2. Materials and methods

2.1. Animal samples

Live or fresh specimens of the disc abalone, turban shell *Turbo cornutus*, Mediterranean mussel *Mytilus galloprovincialis*, Yesso scallop *Patinopecten yessoensis*, Japanese flying squid *Todarodes pacificus*, and common octopus *Octopus vulgaris*, were purchased at the Tokyo Central Wholesale Market. Columellar muscle was collected from disc abalone, foot muscle from turban shell and Mediterranean mussel, adductor muscle from Yesso scallop, mantle muscle from Japanese flying squid, and leg muscle from common octopus. All muscle samples were stored at -20°C until use.

2.2. Preparation of crude extracts

Each muscle sample was ground to powder in liquid nitrogen and homogenised with four volumes of 0.9 M NaCl–0.01 M phosphate buffer (pH 6.8). A part of the homogenate was centrifuged at 18,000g for 20 min, and the supernatant obtained was used as a non-heated extract. The remaining homogenate was heated in a boiling water bath for 10 min, similarly centrifuged, and the supernatant was regarded as a heated extract. Both non-heated and heated extracts were subjected to immunoblotting to examine whether the 100 kDa allergen under study is heat-stable or not.

2.3. Purification of tropomyosin

The disc abalone tropomyosin was purified from the muscle as reported in our previous paper (Motoyama et al., 2007). In brief, an acetone powder of myofibrillar proteins prepared from the muscle was extracted with 0.025 M Tris–HCl buffer (pH 8.0), containing 1 M KCl, 0.1 mM CaCl_2 and 0.1 mM dithiothreitol (DTT). The extract was subjected to salting-out with ammonium sulphate (30–60% saturation), followed by isoelectric precipitation (pH 4.6). Finally, small amounts of contaminants were removed by reverse-phase HPLC. The homogeneity of the final preparation was supported by SDS–PAGE.

2.4. Purification of a 100 kDa allergen

The muscle (5 g) of disc abalone was homogenised with 20 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.15 M NaCl and 0.5 mM cysteine. After centrifugation at 18,000g for 20 min, the supernatant obtained was regarded as a sarcoplasmic protein fraction. The residue was washed twice with the above solvent and shaken vigorously with 25 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl and 5 mM DTT at 4°C , for 14 h. Then, MgCl_2 and ATP were added to the solution until a final concentration of 1 mM for each was reached, and shaken again for 1 h. After ultracentrifugation at 117,000g for 1 h, the supernatant obtained was used as a myofibrillar protein fraction. To purify the 100 kDa allergen, the myofibrillar protein fraction was first subjected to salting-out using ammonium sulphate. The precipitate at a 10–20% saturation of ammonium sulphate was dissolved in 10 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl, 5 mM DTT, and 0.01% thimerosal, and was applied to hydroxyapatite HPLC on a Bio-Scale CHT2-I column ($0.7 \times$

5.2 cm; Bio-Rad Laboratories, Hercules, CA, USA). Elution was achieved with a linear gradient of 0.01–0.24 M phosphate buffer (pH 6.8) containing 0.9 M NaCl at a flow rate of 1 ml/min. The proteins were monitored at 280 nm with a UV detector and the eluate containing the 100 kDa allergen was collected.

2.5. Enzymatic digestion and isolation of the peptide fragments

The purified 100 kDa allergen (150 μg) was digested with 1.5 μg of lysylendopeptidase (Wako Pure Chemicals, Osaka, Japan) in 0.5 ml of 0.025 M Tris–HCl buffer (pH 8.5) containing 1 mM EDTA, 4 M urea and 0.02 M ethanolamine at 37°C , for 18 h. To isolate the peptide fragments produced, the digest was applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan), which was eluted at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0–70% in 120 min) in 0.1% trifluoroacetic acid. Peptides were monitored at 220 nm with a UV detector.

2.6. Amino acid sequencing

Amino acid sequencing was performed by the automated Edman degradation method using a Procise 492HT protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.7. Human sera

Sera were obtained from 18 crustacean-allergic patients (patients 1–18) with clinical histories of immediate hypersensitivity reactions after ingestion of crustaceans. All patients were 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 2–6 against shrimp, or both shrimp and crab. Patients 1, 2, and 12–14 were additionally shown to have CAP-RAST classes of 3–6 against molluscs. Written informed consent was obtained from each patient. In this study, sera from 19 healthy volunteers without adverse reactions after ingestion of any foods were used as controls. All sera were stored at -20°C until use.

2.8. Fluorescence ELISA and inhibition ELISA

Fluorescence ELISA was performed as reported previously (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 100 kDa allergen or the disc abalone tropomyosin solution (1 $\mu\text{g}/\text{ml}$) and reacted successively with the patient or control serum (diluted 1:200) and β -galactosidase-conjugated goat anti-human IgE antibody (0.25 $\mu\text{g}/\text{ml}$; American Qual-ex, San Clement, CA, USA). The enzyme reaction was carried out using as substrate a solution of 0.1 mg/ml 4-methylumbelliferyl- β -D-galactoside, and was stopped by addition of 0.1 M glycine–NaOH buffer (pH 10.3). Fluorescence units were measured with excitation and emission wavelengths at 367 and 453 nm, respectively. For inhibition ELISA, patient serum (diluted 1:100) was pre-incubated with an equal volume of inhibitor (100 kDa allergen or disc abalone tropomyosin) solution (0.002–20 $\mu\text{g}/\text{ml}$) at 37°C for 2 h, and then used as a primary antibody. All ELISAs (including inhibition ELISAs) were performed in triplicate, and the data obtained were expressed in mean $\pm$ SD.

2.9. SDS–PAGE

SDS–PAGE was performed on a PhastSystem apparatus (GE-Healthcare, Buckinghamshire, UK) using ready-made gels (Phast-Gel Gradient 8-25; GE-Healthcare) and ready-made buffer strips (PhastGel SDS Buffer Strips; GE-Healthcare), according to the

manufacturer's instructions. Each sample was dissolved in 0.0625 M Tris–HCl buffer (pH 7.4) containing 2% SDS, 4 M urea and 0.1 M DTT, heated at 70 °C for 10 min, and subjected to electrophoresis. Precision plus protein standards (Bio-Rad Laboratories) were run as references, along with the samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

2.10. Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as reported previously (Kobayashi et al., 2006), using a pooled serum from 10 patients (patients 1–10) or 10 healthy volunteers. Briefly, the proteins of the crude non-heated and heated extracts, firstly separated by SDS–PAGE, were electrotransferred from the gel to a polyvinylidene difluoride membrane, which was reacted successively with patient or control serum (diluted 1:200) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:5000; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The 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 1:100) was preincubated with an equal volume of inhibitor (100 kDa allergen or disc abalone tropomyosin) solution (20 µg/ml) at 37 °C for 2 h, and used as a primary antibody.

2.11. Protein determination

Protein was determined using a Protein Assay Kit (Bio-Rad Laboratories) based on the method of Bradford (1976). Bovine serum albumin was used as a standard protein.

3. Results

3.1. Purification and identification of a 100 kDa allergen in disc abalone

The 100 kDa allergen was mostly recovered in the myofibrillar protein fraction, suggesting that it is one of myofibrillar proteins. Its purification from the myofibrillar protein fraction was achieved by salting-out with ammonium sulphate (10–20% saturation), followed by hydroxyapatite HPLC on a Bio-Scale CHT2-I column. In hydroxyapatite HPLC, the 100 kDa allergen was eluted in a sym-

metrical peak at a retention time of 23.8 min (Fig. 1A). The 100 kDa allergen thus obtained was confirmed to be homogeneous, as analysed by SDS–PAGE (Fig. 1B).

Following digestion of the purified 100 kDa allergen with lysylendopeptidase, a number of peptide fragments was isolated by reverse-phase HPLC on a TSKgel ODS-120T column (data not shown). Of the isolated peptide fragments, four fragments (peptides 1–4) were randomly selected and sequenced. A database search by the BLAST algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990) revealed that the peptides 1–4 best fitted the 121–135, 147–158, 203–214 and 825–836 regions of Mediterranean mussel paramyosin (accession number AB016070 of the DDBJ/EMBL/GenBank databases), respectively (Fig. 2). The peptide 3 was completely identical with the 203–214 region of the Mediterranean mussel paramyosin, and the other peptides had only two to four alterations compared to the corresponding regions of Mediterranean mussel paramyosin. These results allowed us to conclude that the 100 kDa allergen was a paramyosin of the disc abalone.

3.2. Reactivity of patient sera to paramyosin and tropomyosin

Both paramyosin (100 kDa allergen) and tropomyosin purified from the disc abalone were evaluated for IgE reactivity by fluorescence ELISA using 18 sera from crustacean-allergic patients. Five patients (1, 2, and 12–14) had CAP-RAST classes of 3–6 against molluscs, whilst no information was available as to whether the other patients are allergic to molluscs. Nevertheless, positive reactivity to the disc abalone tropomyosin was found in as many as 16 patient sera (Fig. 3), conforming well to the current opinion that tropomyosin is a major cross-reactive allergen in crustaceans and molluscs (Chuo et al., 2000; Emoto et al., 2009; Leung et al., 1996; Motoyama et al., 2006, 2007; Reese et al., 1999; Wild & Lehrer, 2005). It was of particular interest that the same 16 patient sera also reacted to paramyosin although rather less potently than to tropomyosin. This demonstrated that paramyosin, like tropomyosin, is a major allergen of the disc abalone.

3.3. Distribution of allergenic paramyosin in molluscs

When analysed by SDS–PAGE, both 37 and 100 kDa proteins were recognised in the non-heated extracts from six species of molluscs (disc abalone, turban shell, Mediterranean mussel, Yes-

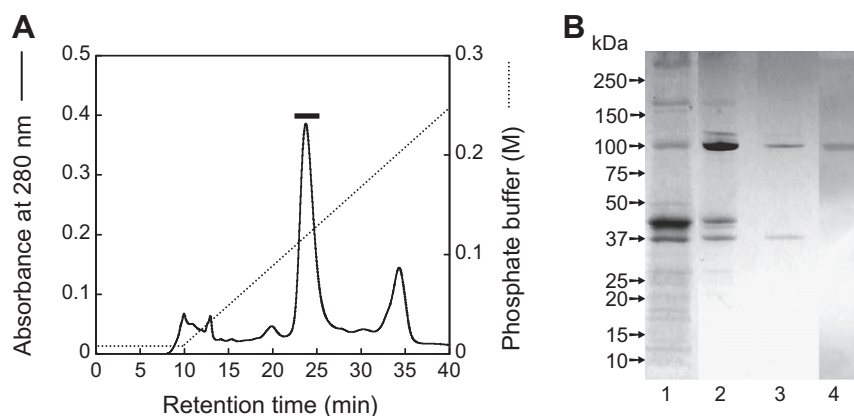


Fig. 1. Purification of a 100 kDa allergen in the disc abalone by hydroxyapatite HPLC (A) and analysis of its homogeneity by SDS–PAGE (B). (A) Sample, precipitate with a 10–20% saturation of ammonium sulphate from the myofibrillar protein fraction; column, Bio-Scale CHT2-I column (0.7 × 5.2 cm); elution, linear gradient of 0.01–0.25 M phosphate buffer (pH 6.8) containing 0.9 M NaCl; flow rate, 1 ml/min. A bar indicates the elution position of the 100 kDa allergen. (B) Samples: lane 1, sarcoplasmic protein fraction; lane 2, myofibrillar protein fraction; lane 3, precipitate with a 10–20% saturation of ammonium sulphate from the myofibrillar protein fraction; lane 4, purified 100 kDa allergen.

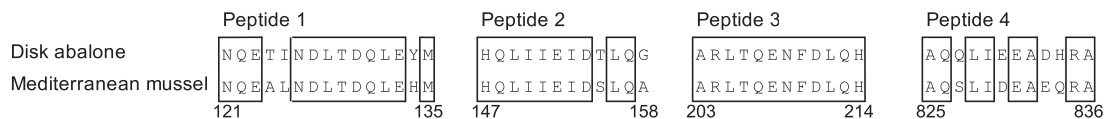


Fig. 2. Amino acid sequences of peptides 1–4 produced by digestion of the purified 100 kDa allergen with lysendopeptidase, and the corresponding regions of Mediterranean mussel paramyosin. Identical residues are boxed. The accession number (DDBJ/EMBL/GenBank) for Mediterranean mussel paramyosin is AB016070.

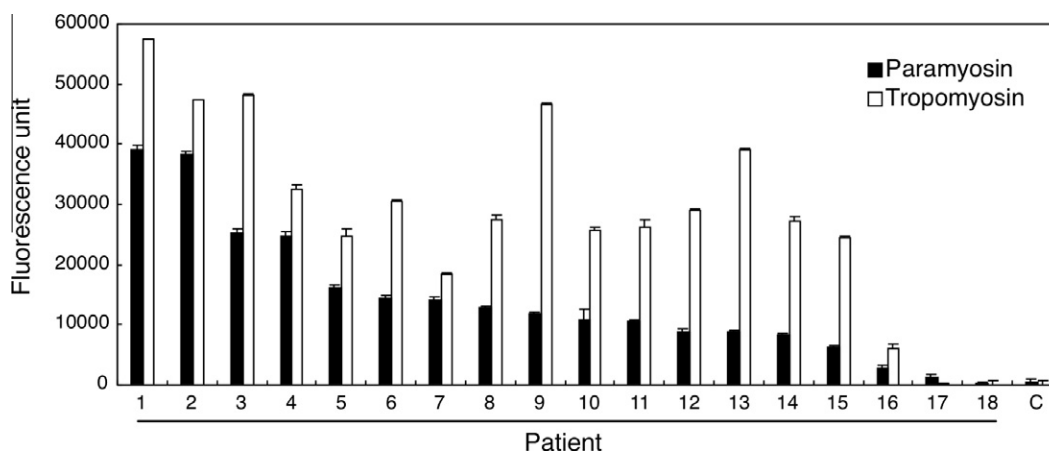


Fig. 3. Analysis of IgE reactivity of paramyosin and tropomyosin purified from the disc abalone by fluorescence ELISA using 18 patient sera. The control data (denoted by C) obtained with 19 healthy subjects were averaged and the values above mean + 2SD (fluorescence units of 1739 and 1466 for paramyosin and tropomyosin, respectively) were judged to be positive.

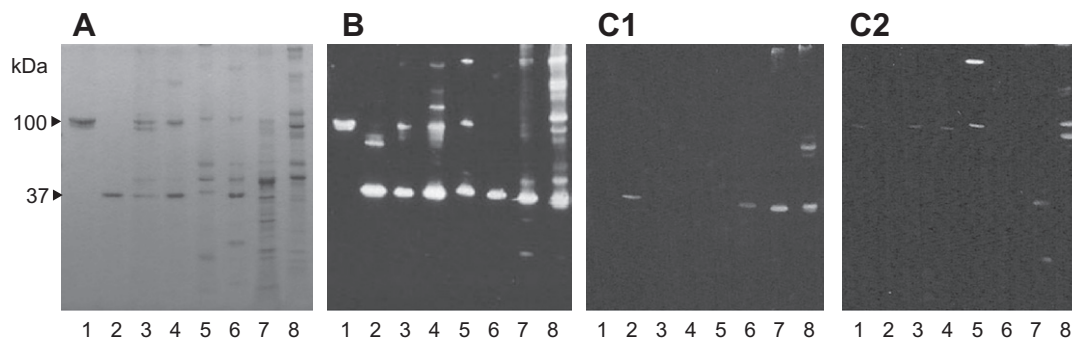


Fig. 4. Analysis of the non-heated extracts from six species of molluscs by SDS-PAGE (A), immunoblotting (B), and inhibition immunoblotting (C). Lanes: 1, disc abalone paramyosin; 2, disc abalone tropomyosin; 3, extract from disc abalone; 4, extract from turban shell; 5, extract from Mediterranean mussel; 6, extract from Yesso scallop; 7, extract from Japanese flying squid; 8, extract from common octopus. In immunoblotting, the patient serum (pooled from 10 patients; 1:200 dilution) was used as a primary antibody. In inhibition immunoblotting, the patient serum (1:100 dilution) was preincubated with an equal volume of inhibitor (disc abalone paramyosin and tropomyosin for C1 and C2, respectively) solution (20 µg/ml) prior to use as a primary antibody.

so scallop, Japanese flying squid and common octopus), although the staining intensity considerably differed from sample to sample (Fig. 4A). In immunoblotting, the control serum (pooled from 10 healthy volunteers) showed no reactivity to any proteins (data not shown) whilst the patient serum (pooled from patients 1–10) strongly reacted to the 37 kDa protein (corresponding to tropomyosin) in all the six species and weakly to the 100 kDa protein (corresponding to paramyosin) in at least four species (disc abalone, turban shell, Mediterranean mussel and common octopus) (Fig. 4B). In the case of the heated extracts, the 37 kDa protein (tropomyosin) was clearly observed in six species (Fig. 5A) and shown to be IgE-reactive as well (Fig. 5B). However, the 100 kDa protein (paramyosin) was not observed in any species, as analysed by either SDS-PAGE or immunoblotting. This suggested that paramyosin, unlike tropomyosin, becomes insoluble upon heat denaturation, being absent in the heated extracts.

3.4. IgE cross-reactivity of paramyosin with tropomyosin

In inhibition immunoblotting experiments using the disc abalone paramyosin as an inhibitor, the reactivity of the patient serum to molluscan paramyosins was completely lost, and that to molluscan tropomyosins was also lost or remarkably reduced (Fig. 4C1). On the other hand, the patient serum preincubated with the disc abalone tropomyosin showed no reactivity to both molluscan paramyosins and tropomyosins (Fig. 4C2). These results implied cross-reactivity, not only between molluscan paramyosins, but also between molluscan paramyosins and tropomyosins. Further evidence for the cross-reactivity between paramyosin and tropomyosin was obtained by the inhibition ELISA experiments. As shown in Fig. 6, the reactivity of the patient serum to either paramyosin or tropomyosin was inhibited in a dose-dependent manner by both paramyosin and tropomyosin, although much stronger inhibitory potencies were observed with tropomyosin.

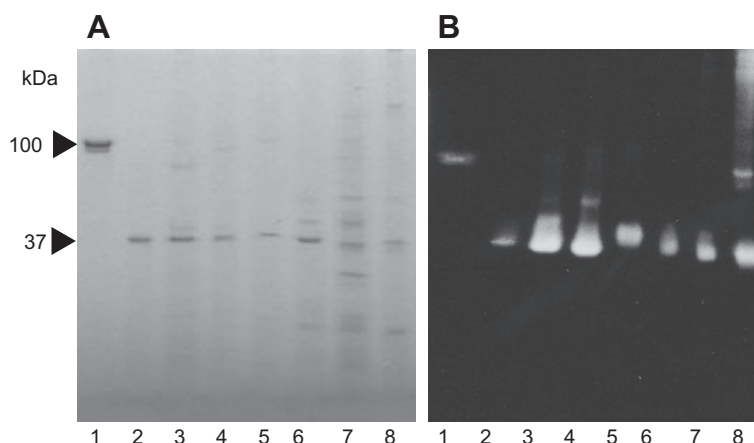


Fig. 5. Analysis of the heated extracts from six species of molluscs by SDS–PAGE (A) and immunoblotting (B) samples are the same as in Fig. 4.

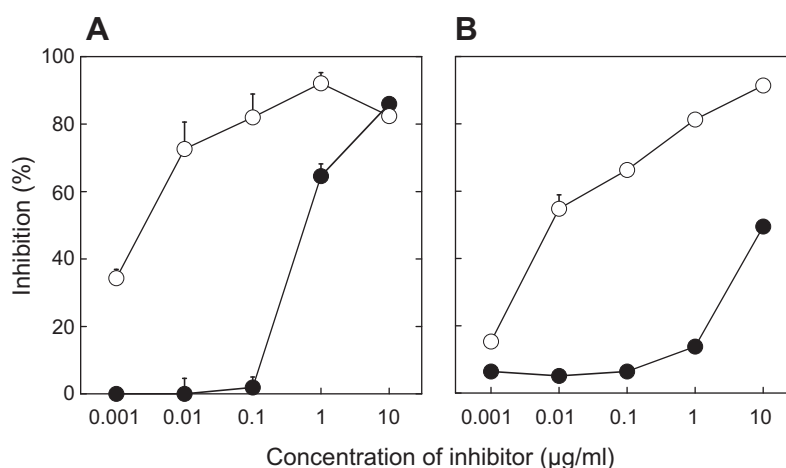


Fig. 6. Quantitative analysis of the IgE cross-reactivity between the disc abalone paramyosin and tropomyosin by inhibition ELISA. A microtiter plate was coated with 50 µl of paramyosin (A) or tropomyosin (B) solution (1 µg/ml). The patient serum (pooled from 10 patients; 1:100 dilution) was preincubated with an equal volume of the disc abalone paramyosin (●) or tropomyosin (○), and was used as a primary antibody.

4. Discussion

Tropomyosin, a 35–38 kDa myofibrillar protein, is a major allergen in molluscs as well as in crustaceans (Chuo et al., 2000; Emoto et al., 2009; Leung et al., 1996; Motoyama et al., 2006, 2007; Reese et al., 1999; Wild & Lehrer, 2005). In this study, however, a 100 kDa allergen differing from tropomyosin was purified from the muscle of the disc abalone *Haliotis discus discus* by salting-out and hydroxyapatite HPLC, and was clearly identified as paramyosin, one of the myofibrillar proteins, based on the determined partial amino acid sequence of the purified preparation. Immunoblotting and inhibition immunoblotting experiments suggested that paramyosin is not a specific allergen of the disc abalone but a cross-reactive allergen in some molluscs. Recently, arginine kinase (García-Orozco et al., 2007; Yu et al., 2003), sarcoplasmic calcium-binding protein (Ayuso et al., 2009; Shiomi et al., 2008) and myosin light chain (Ayuso et al., 2008) have been shown to be new allergens in crustaceans, whilst allergens other than tropomyosin have not been identified in molluscs. In this sense, paramyosin is the second allergen of molluscs, besides tropomyosin.

As described above, paramyosin is a cross-reactive allergen in some molluscs. Moreover, paramyosin is a major allergen in the disc abalone as demonstrated by fluorescence ELISA, in which as many as 16 of the 18 patient sera tested reacted to paramyosin, as well as to tropomyosin. This is presumably the case with other molluscan

paramyosins. Despite these circumstances, paramyosin has long been overlooked in previous studies on molluscan allergens. In relation to this, it should be noted that heating procedures were included in preparing the extracts in previous studies; the extracts were prepared from the homogenates of boiled samples (Leung et al., 1996) or by heating the homogenates of raw samples (Emoto et al., 2009; Motoyama et al., 2006). Since tropomyosin is very thermostable, it can be detected in the extracts by SDS–PAGE and immunoblotting, or can be purified from the extracts by chromatography. In contrast, paramyosin is rather unstable to heating as evidenced in this study. This thermolabile property possibly accounts for the missing of paramyosin in previous studies on molluscan allergens.

Paramyosin is an invertebrate-specific protein that forms the core of the myosin-containing thick filaments (Watabe & Hartshorne, 1990). Prior to this study, paramyosin has already been established to be one of the allergens in some invertebrates, such as house dust mites (Tsai et al., 1999, 2005) and the fish parasite *Anisakis simplex* (Pérez-Pérez et al., 2000). It is thus possible that paramyosin, like tropomyosin, is a pan-allergen in invertebrates, although it is still unknown whether crustacean paramyosin is allergenic. However, paramyosin is obviously less IgE-reactive and also less thermostable than tropomyosin, suggesting that paramyosin contributes less to the adverse reactions induced after ingestion of molluscs, especially cooked molluscs, than tropomyosin.

The most significant finding of this study is that molluscan paramyosins are cross-reactive not only with one another but also with molluscan tropomyosins. The fact that the 16 patients recognising the disc abalone tropomyosin are all sensitive to the disc abalone paramyosin can be easily understood by the cross-reactivity between paramyosin and tropomyosin. So far, tropomyosins in various species of molluscs have been elucidated for their primary structures. In contrast, no information about the primary structures of molluscan paramyosins is available, except for the Mediterranean mussel paramyosin (AB016070 of the DDBJ/EMBL/GenBank databases). At present, therefore, it is impossible to explain at the molecular level the observed cross-reactivity between molluscan paramyosins and tropomyosins.

In conclusion, the present study demonstrated that paramyosin is the second allergen of molluscs. To clarify whether paramyosin is a pan-allergen in invertebrates, it is important to examine the allergenicity of crustacean paramyosins. Furthermore, the primary structures of various molluscan paramyosins should be elucidated for a molecular understanding of the cross-reactivity, not only amongst molluscan paramyosins, but also between molluscan paramyosins and tropomyosins.

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