

Identification of Tropomyosins as Major Allergens in Antarctic Krill and Mantis Shrimp and Their Amino Acid Sequence Characteristics

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Abstract Tropomyosin represents a major allergen of decapod crustaceans such as shrimps and crabs, and its highly conserved amino acid sequence (>90% identity) is a molecular basis of the immunoglobulin E (IgE) cross-reactivity among decapods. At present, however, little information is available about allergens in edible crustaceans other than decapods. In this study, the major allergen in two species of edible crustaceans, Antarctic krill *Euphausia superba* and mantis shrimp *Oratosquilla oratoria* that are taxonomically distinct from decapods, was demonstrated to be tropomyosin by IgE-immunoblotting using patient sera. The cross-reactivity of the tropomyosins from both species with decapod tropomyosins was also confirmed by inhibition IgE immunoblotting. Sequences of the tropomyosins from both species were determined by complementary deoxyribonucleic acid cloning. The mantis shrimp tropomyosin has high sequence identity (>90% identity) with decapod tropomyosins, especially with fast-type tropomyosins. On the other hand, the Antarctic krill tropomyosin is characterized by diverse alterations in region 13–42, the amino acid sequence of which is highly conserved for decapod tropomyosins, and hence, it shares somewhat lower sequence identity (82.4–89.8% identity) with decapod tropomyosins than the mantis shrimp tropomyosin. Quantification by enzyme-linked immunosorbent assay revealed that Antarctic krill contains tropomyosin at almost the same level as decapods, suggesting that its

allergenicity is equivalent to decapods. However, mantis shrimp was assumed to be substantially not allergenic because of the extremely low content of tropomyosin.

Keywords Allergen · Antarctic krill · Crustacean · Mantis shrimp · Tropomyosin

Introduction

Food allergy is a representative of type I allergies that are mediated by immunoglobulin (Ig) E antibodies and is a serious problem to human health in industrialized countries (Jansen et al. 1994; Bousquet et al. 1998; Keet and Wood 2007). Sensitized subjects, who have elevated levels of IgE specific for allergic proteins (allergens), develop hypersensitive reactions, such as urticaria, asthma, diarrhea, and even life-threatening anaphylaxis, immediately after ingestion of foods containing the allergens. Of a number of allergenic foods, crustaceans are recognized as one of the most common causes of food allergy, especially in coastal countries where the consumption of crustaceans is high (Lehrer et al. 2003; Wild and Lehrer 2005). Edible crustaceans mostly belong to the order Decapoda of the class Malacostraca; hence, previous studies on crustacean allergens have focused on decapods. As a result, the major allergen has been identified as tropomyosin, a myofibrillar protein of 35–38 kDa, in common with decapods including shrimps (Shanti et al. 1993; Daul et al. 1994; Leung et al. 1994; Motoyama et al. 2007), crayfishes (Leung et al. 1998a), lobsters (Leung et al. 1998a), hermit crabs (Motoyama et al. 2007), and crabs (Leung et al. 1998b; Motoyama et al. 2007). The cross-reactivity of crustacean-allergic patients among decapods is clinically observed and

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can be understood by the high amino acid sequence identity (>90% identity) of their tropomyosins.

Besides decapods, some crustaceans are also edible although less frequently consumed than decapods and seem to be allergenic. At present, however, little information about their allergens is available. Therefore, we recently examined allergens in two species of barnacles, acorn barnacle *Balanus rostratus* and goose barnacle *Capitulum (Pollicipes) mitella*, members of the class Thecostraca that are taxonomically remote from decapods and locally consumed as a delicacy in Japan. The results showed that their major allergen is tropomyosin having cross-reactivity with decapod tropomyosins (Suma et al. 2007). It is very interesting to note that the determined amino acid sequence of the acorn barnacle tropomyosin has the highest sequence identity (76–78% identity) with tropomyosins from abalones (Japanese abalone *Haliotis diversicolor*, red abalone *Haliotis rufescens*, and tropical abalone *Haliotis asinina*) belonging to the phylum Mollusca; the sequence identity with decapod tropomyosins is only about 60%. Our results with barnacles suggest that tropomyosin is a major cross-reactive allergen not only in decapods but also in other crustaceans and that tropomyosins in crustaceans other than decapods may have unique sequence features distinguishable from decapod tropomyosins.

In view of the circumstances described above, this study was initiated to confirm whether tropomyosin is a major cross-reactive allergen in two species of edible crustaceans, Antarctic krill *Euphausia superba* and mantis shrimp *Oratosquilla oratoria*, using two species of decapods, American lobster *Homarus americanus* and black tiger shrimp *Penaeus monodon*, as references. In Japan, Antarctic krill is sometimes consumed as a substitute for shrimp, and mantis shrimp is utilized as one of the common ingredients of sushi. Both Antarctic krill and mantis shrimp are included in the class Malacostraca, which is further divided into three subclasses (Phyllocarida, Hoplocarida, and Eumalacostraca). Antarctic krill is a member of the subclass Eumalacostraca similar to decapods but belongs to the order Euphausiacea different from decapods (belonging to the order Decapoda), while mantis shrimp is a member of the order Stomatopoda of the subclass Hoplocarida. We report here the identification of tropomyosin as major allergens in Antarctic krill and mantis shrimp and their amino acid sequence characteristics compared to decapod tropomyosins.

Materials and Methods

Crustaceans

Live specimens of Antarctic krill were caught in Lat. 65°15' 67" S. and Long. 108°46'69" E. close to the Antarctic

Circle in February 2005 by the training vessel Umitaka-Maru of Tokyo University of Marine Science and Technology and immediately shelled on board the vessel. Abdominal muscle was immersed in liquid nitrogen, transported frozen to our laboratory, and kept at −80°C until use. Fresh specimens of American lobster and black tiger shrimp and live specimens of mantis shrimp were purchased at the Tokyo Central Wholesale Market. Abdominal muscle was obtained from each specimen and stored at −20°C until use. For molecular cloning experiments, part of the muscle sample of mantis shrimp was immediately frozen in liquid nitrogen and stored at −80°C until use.

Preparation of Heated Extracts

Each muscle sample was homogenized with four volumes of 0.6 M KCl in 0.01 M phosphate buffer (pH 7.0). 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 18,000×g for 20 min. The supernatant thus obtained was used as a heated extract. Protein concentrations of the heated extracts were estimated by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Purification of American Lobster Tropomyosin

Fast-type tropomyosin of American lobster was purified from the abdominal muscle as reported previously (Motoyama et al. 2007). In brief, an acetone powder prepared by the method of Greaser and Gergely (1971) was extracted with 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM CaCl₂, and 1 mM dithiothreitol. The extract was subjected successively to salting out with ammonium sulfate (50–60% saturation) and isoelectrical precipitation (pH 4.6) essentially according to the method of Cummins and Perry (1973). Finally, small amounts of impurities were removed by reverse-phase high-performance liquid chromatography HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan). The homogeneity of the final preparation was supported by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

Human Sera

Sera were donated from ten crustacean-allergic patients. The patients were all diagnosed to be allergic to crustaceans on the basis of the determined CAP-RAST classes of 2–5 against shrimp or both shrimp and crab as well as the experiences of immediate hypersensitive reactions after the ingestion of shrimp or crab. Written informed consent was obtained from each patient. In this study, sera from five

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 use.

SDS-PAGE

SDS-PAGE was performed on a PhastSystem apparatus (GE-Healthcare Bio-Sciences, Piscataway, NJ, USA) 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 GE-Healthcare Bio-Sciences. Prior to electrophoresis, each sample was dissolved in 0.0625 M phosphate buffer (pH 7.5) containing 2.5% SDS and 5% dithiothreitol and heated at 100°C for 10 min. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were run as a reference, along with samples. After running, proteins were stained with Coomassie Brilliant Blue R-250.

Immunoblotting and Inhibition Immunoblotting

Immunoblotting was performed as described in our previous paper (Motoyama 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 IgG immunoblotting to detect tropomyosin, the monoclonal antibody 5G5E1 (1 $\mu\text{g}/\text{ml}$) against American lobster tropomyosin (Lu et al. 2007) was used as a primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgM+IgG+IgA antibody (diluted 1:20,000; Cosmo Bio, Tokyo, Japan) as a secondary antibody. For the detection of IgE-binding proteins, patient serum (diluted 1:500) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:5,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) were used as primary and secondary antibodies, respectively. Antigen–antibody binding was visualized using an ECL Plus Western Blotting Detection System (GE-Healthcare Bio-Sciences) and an ECL Mini Camera (GE-Healthcare Bio-Sciences), as recommended by the manufacturer. For inhibition IgE immunoblotting, patient serum (diluted 1:250) was preincubated with an equal volume of American lobster tropomyosin solution (20 $\mu\text{g}/\text{ml}$) at 37°C for 1 h and used as a primary antibody.

Cloning Studies

Total ribonucleic acid (RNA) was extracted from 2 g of muscle sample with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and poly(A)⁺ messenger RNA (mRNA) was purified by affinity chromatography on oligo(dT)-cellulose using an mRNA Purification Kit (GE Healthcare Bio-Sciences). An aliquot (1 μg) of the purified mRNA was

converted to double-stranded complementary deoxyribonucleic acid (cDNA), followed by ligation of an AP1 adapter at both 3' and 5' ends, using the Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA). The Marathon cDNA library thus constructed was used as a template in polymerase chain reaction (PCR) reactions.

PCR amplifications were all performed using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany). At first, partial cDNA fragments were amplified using a forward primer (5'-ATCAAGAAGAAGATGCAGGCG-3') and a reverse primer (5'-TCTTCGAGCCTGTGCGACCTC-3'). Both forward and reverse primers, which were designed from the known nucleotide sequences of the crustacean tropomyosin cDNAs, were the same as employed for the cloning of decapod tropomyosin cDNAs (Motoyama et al. 2007). The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany), and four clones were analyzed for nucleotide sequences with a PRISM 310 genetic analyzer (Applied Biosystems). The remaining 3' region was amplified using the gene-specific forward primer (5'-GGAGCGTATGGATGCCCTTGA-3' for Antarctic krill or 5'-AGAAGGCCAACCAGCGCGAGGA-3' for mantis shrimp) and the AP1 primer (5'-CCATCCTAATACGACTCACTATAGGGC-3') and sequenced as described above. The 5' region was determined by sequencing of the product amplified using the gene-specific reverse primer (5'-TCAAGGGCATCCATACGCTCC-3' for Antarctic krill or 5'-GGGTGTTAGCCTTCAGCAGCTGTT-3' for mantis shrimp) and the AP1 primer.

Quantification of Tropomyosin in Crustaceans

Tropomyosin contained in the abdominal muscle of four species of crustaceans was quantified by enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody 5G5E1. ELISA was performed using a flat-bottomed polystyrene plate with 96 wells, as reported previously (Lu et al. 2007). In brief, each heated extract was diluted, coated on the plate, and immunoreacted successively with the monoclonal antibody (1 $\mu\text{g}/\text{ml}$) and horseradish peroxidase-conjugated goat anti-mouse IgM+IgG+IgA antibody (diluted 1:10,000). Enzymatic reaction was carried out using freshly prepared substrate solution (0.1% *o*-phenylenediamine and 0.03% hydrogen), and the developed color was measured at 490 nm. To make a calibration curve, various concentrations (0.03–60 ng/ml) of the American lobster tropomyosin were subjected to ELISA.

Phylogenetic Analysis

Multiple alignments of tropomyosins from various crustaceans were performed with the Clustal W program (Higgins

et al. 1994). Based on the results of multiple alignments, a phylogenetic tree was constructed using a MEGA 3.1 software (Kumar et al. 2004) by the neighbor-joining method (Saitou and Nei 1987). The robustness of topology nodes was tested by the bootstrap method with 1,000 replications.

Results and Discussion

Identification of Tropomyosins as Allergens in Antarctic Krill and Mantis Shrimp

When analyzed by SDS-PAGE, the heated extract from Antarctic krill, like those from American lobster and black tiger shrimp, produced a prominent protein band of 37 kDa at the same position as the American lobster tropomyosin used as a reference (Fig. 1a). In IgG immunoblotting, the 37-kDa protein found in the three species reacted with the monoclonal antibody 5G5E1 against the American lobster tropomyosin (Fig. 1b), demonstrating that it is tropomyosin

of each species. In the case of mantis shrimp, a 37-kDa protein assignable to tropomyosin was not recognized in the heated extract by SDS-PAGE (Fig. 1a) and only faintly by IgG immunoblotting (Fig. 1b). This suggested the much lower content of tropomyosin in mantis shrimp than in the other three species of crustaceans, as discussed below in more detail.

IgE immunoblotting, using the pooled serum from ten crustacean-allergic patients and individual five patient sera, revealed that the Antarctic krill tropomyosin is IgE-reactive irrespective the patient sera, similar to those from American lobster and black tiger shrimp (Fig. 1c). It is important to note that tropomyosin was judged to be substantially the sole allergen in Antarctic krill as well as in American lobster and black tiger shrimp. Despite the suggested low content, the mantis shrimp tropomyosin was also confirmed to be IgE reactive by the results with all patient sera, except for the patient five serum. In the heated extract from mantis shrimp, another IgE-reactive protein of 28 kDa was simultaneously detected in the experiments using the

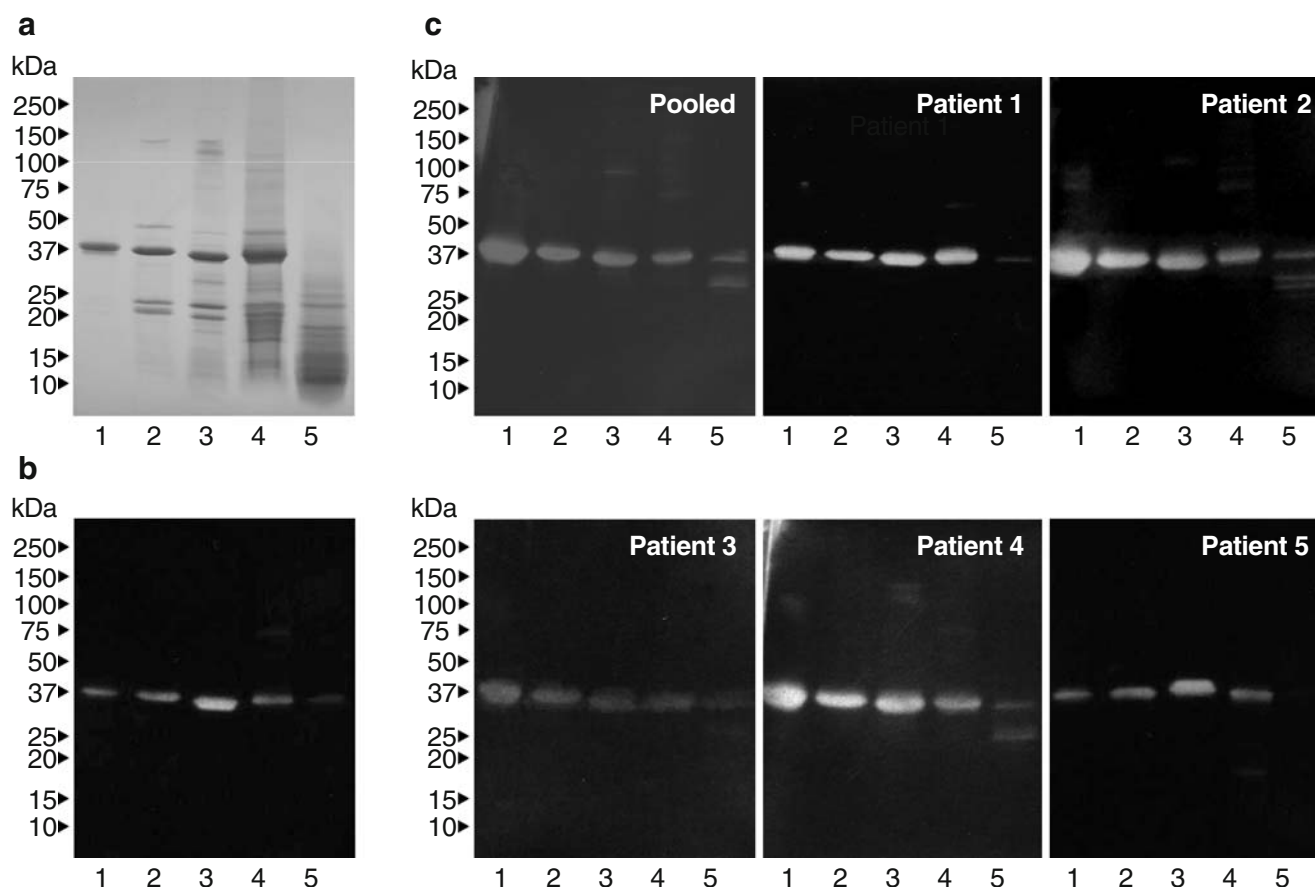


Fig. 1 Analysis of heated extracts from four species of crustaceans by SDS-PAGE (a), IgG immunoblotting using the monoclonal antibody 5G5E1 against American lobster tropomyosin (b), and IgE immunoblotting using patient sera (c). Lanes: 1, tropomyosin purified from American lobster; 2, extract from American lobster; 3, extract from

black tiger shrimp; 4, extract from Antarctic krill; 5, extract from mantis shrimp. Heated extracts (0.3 μ l each) with the following protein concentrations were subjected to SDS-PAGE: 3.6 mg/ml for American lobster, 6.6 mg/ml for black tiger shrimp, 7.5 mg/ml for Antarctic krill, and 13.7 mg/ml for mantis shrimp

pooled serum and the patient 2 and 4 sera. In inhibition IgE immunoblotting, the patient sera preadsorbed with the American lobster tropomyosin completely lost their reactivities to tropomyosins from four species of crustaceans

and the 28-kDa protein of mantis shrimp (data not shown). These results demonstrate the cross-reactivity between tropomyosins from American lobster and the other crustaceans and also suggest that the 28-kDa protein of mantis

Antarctic krill

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CTCACCTCCAGGTTTCTCAGTTTTTGTGCTTGGGTACTCACATAAAACACCGCCAAATGGACGCAATCAAGAAGAAGATGCAGGCCATG 90
                                     M D A I K K K M Q A M 11
AAAAATGGAGAAGGACTCCGCCATGGACCGTTTCTGATGCCCTGGAGGCTCAGAACAAAGGAGACCAACGCAAAAGCTGATAAGGCCGATGAT 180
K M E K D S A M D R S D A L E A Q N K E T N A K A D K A D D 41
GAGGTCCACAATCTCCAGAAGAGACTGCAGACCCCTTGAGAATGACCTCGACCAGGTCTCTGAGGCTCTGTTGAAGGCTAACACACAGTTG 270
E V H N L Q K R L Q T L E N D L D Q V S E A L L K A N T Q L 71
GTTGAGAAGGATAAGGCCCTGCAGAATGCTGAGGGTGAGGTGGCAGCACTTAACCGTCGCATCCAGCTTCTAGAAGAGGATCTGGAGCGC 360
V E K D K A L Q N A E G E V A A L N R R I Q L L E E D L E R 101
TCTGAGGAACGTTTAAACACAGCCACCACAAAGCTTGTGAAGCCTCACAAAGCTGCTGATGAGTCTGAACGTATGCGCAAGGTGTTGGAG 450
S E E R L N T A T T K L A E A S Q A A D E S E R M R K V L E 131
AACCGCTCCATGCTGGATGAGGAGCGTATGGATGCCCTTGAAGCCAACCTCAAGGAAGCCAGACTTCTGGCTGAGGAGGCCGACCGCAAA 540
N R S M L D E E R M D A L E S Q L K E A R L L A E E A D R K 161
TATGATGAGGTTGCCGTAATTAAGCATGGTTGAGGCTGACCTTGAGCTGTGCTGAGGAGAGGGCCGAGGCTGGTGAGAACAAAATGTT 630
Y D E V A R K L A M V E A D L E R A E A G E A E A G E N K I V 191
GAACTAGAGGAAGAAGCTCCGTGTTGTAGGCAACAACCTCAAATCCCTGGAAGTGTCTGAAGAAAAGGCCAACCAACGTGAGGAGGCCTAC 720
E L E E E L R V V G N N L K S L E V S E E K A N Q R E E A Y 221
AAGGAACAGATCAAGCATCTCACCCACAAGCTCAAGACGGCTGAAGCCCGTGCAGAATTGCGAGAGAGGTCCGTCCGAAACTACAGAAA 810
K E Q I K H L T H K L K T A E A R A E F A E R S V Q K L Q K 251
GAGGTTCGACAGGCTAGAAGAATGAGCTTGTCAACGAGAAGGAGAAATACAAGGCATCACTGACGAACCTTGACCAGACCTTCAACGAGCTC 900
E V D R L E D E L V N E K E K Y K G I T D E L D Q T F N E L 281
TCCGGATATTAATAATATATTTGTACCTTTACTTTATCACTCTTTAACATCATCAGCTGGATGCAATACTCTGGTTTTTACCTACCT 990
S G Y 284
GGTACCCAAGGAATTATGTTCTCTAGGAATTATGTTTTTCTGTTAGCTTTTGATTAGAATATCTAAATTCTACATCTGTGAACCAGGGTA 1080
GTTTCACCTTCATATTCATTTTA 1104
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Mantis shrimp

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CGGCCGCAAGCCCCACGTTCTTAGCTTTCTCAGTTGTTGTGCTTGGGTAACTCCCTAAAAAACCGCGCCATGGAGGCCATCAAGAAG 89
                                     M D A I K K 6
AAGATGCAGGCCATGAAGCTGGAGAAGGACAATGCCATGGAATAGGGCGGATACCTGGAAACAACAGAAACAAGGAGGCCAACACAGGGCT 179
K M Q A M K L E K D N A M D R A D T L E Q Q N K E A N N R A 36
GAGAAGGCCGAGGAGGAGGTTTACAACTTCAGAAGAGGATGCAGCAACTTGAGAAGACCTTGACCAGGTTTCAGGAACAGCTGCTGAAG 269
E K A E E E V H N L Q K R M Q Q L E N D L D Q V Q E Q L L K 66
GCTAACACCACTAAGAGAAGGACAAAGCCCTTCAGAAATGCTGAGGGTGAGGTGGCTGCCTGAACCGACGCTCCAGCTTCTTGAA 359
A N T Q L E E K D C A L N K A E G E V A L N R R I Q L L E 96
GAGGACCTGGAACGTTCTGAGGAGCGCTCAACACGGCCACCACCAAGCTCGCCGAGGCTTCCAGGCCGCCGACGAGTCCGAGCGTATG 449
E D L E R S E E R L N T A T T K L A E A S Q A A D E S E R M 126
CGCAAGGTGCTCGAGAACCCTCCCTGTCGACGAGGAGCGCATGGACGCCCTCGAGAACCAGCTGAAGGAGGCCGATTCTGGCTGAG 539
R K V L E N R S L S D E E R M D A L E N Q L K E A R F L A E 156
GAGGCAGACAGGAAATACGACGAGGTTGCCGTAAGCTGGCCATGGTTGAGGCTGACCTGGAACGTGCAGAGGAGCGCGCCGAGACTGGT 629
E A D R K Y D E V A R K L A M V E A D L E R A E E R A E T G 186
GAATCGAAGATCGTCGAGCTTGAGGAGGAGTTGAGGGTTGTTGGCAACAACCTCAAGTCTCTCGAGGTGTCCGAGGAGAAGGCCAACCCAG 719
E S K I V E L E E E L R V V G N N L K S L E V S E E K A N Q 216
CGCGAGGAGGCTTACAAAGAACAGATCAAGCAGCTTACCAACAAGTTGAAGGCGGCTGAAGCCCGTGCCGAGTTCCGCGAGAGGTCTG 809
R E E A Y K E Q I K Q L T N K A E A R A E F A E R S V 246
CAGAAGCTCCAGAAGGAGGTTCGACAGGCTCGAAGCGAACTGGTTAACGAAAGGAGAAGTACAAGTCAATTACGACGAGTTGGACCAG 899
Q K L Q K E V D R L E D E L V N E K E K Y K S I T D E L D Q 276
ACTTTCAGCGAACTTCTGGCTACTTAAACCCATAATTCTGCAAAATCTACTCTGCCATCTCTCTTCTATGCCGTCAGCTGGGCTGTAA 989
T F S E L S G Y 284
CATACTCTTTTCTAGCTTATAATTATCAGTTATTTCTGTTAACTTTTCTTCTTGTGTTGCATTTGGCCTCCAATTGAGTCTGTAAACCA 1079
AAAAAA 1085
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Fig. 2 Nucleotide sequences of cDNA encoding for Antarctic krill and mantis shrimp tropomyosin. The deduced amino acid sequences are denoted *below* the nucleotide sequences. Nucleotide and amino acid numbers are shown at the *right*. The regions corresponding to the primers used in PCR are *boxed*. Initiation and stop codons are

underlined. In the case of the mantis shrimp cDNA, the nucleotides differing from the cDNA encoding mantis shrimp *Squilla oratoria* tropomyosin (recently deposited in the DDBJ/EMBL/GenBank under the accession number of EF584510) are shown in *white* on the *black* background

shrimp may be a degraded product of tropomyosin, although it was not detected by IgG immunoblotting (Fig. 1b).

It is well known that tropomyosin is a major cross-reactive allergen in decapod crustaceans (Reese et al. 1999; Lehrer et al. 2003; Wild and Lehrer 2005). In this study, we

provided evidence that tropomyosins from two species of crustaceans, Antarctic krill and mantis shrimp that are taxonomically distinct from decapods, are allergenic and cross-reactive with those from decapods. During the preparation of this paper, essentially, the same results have

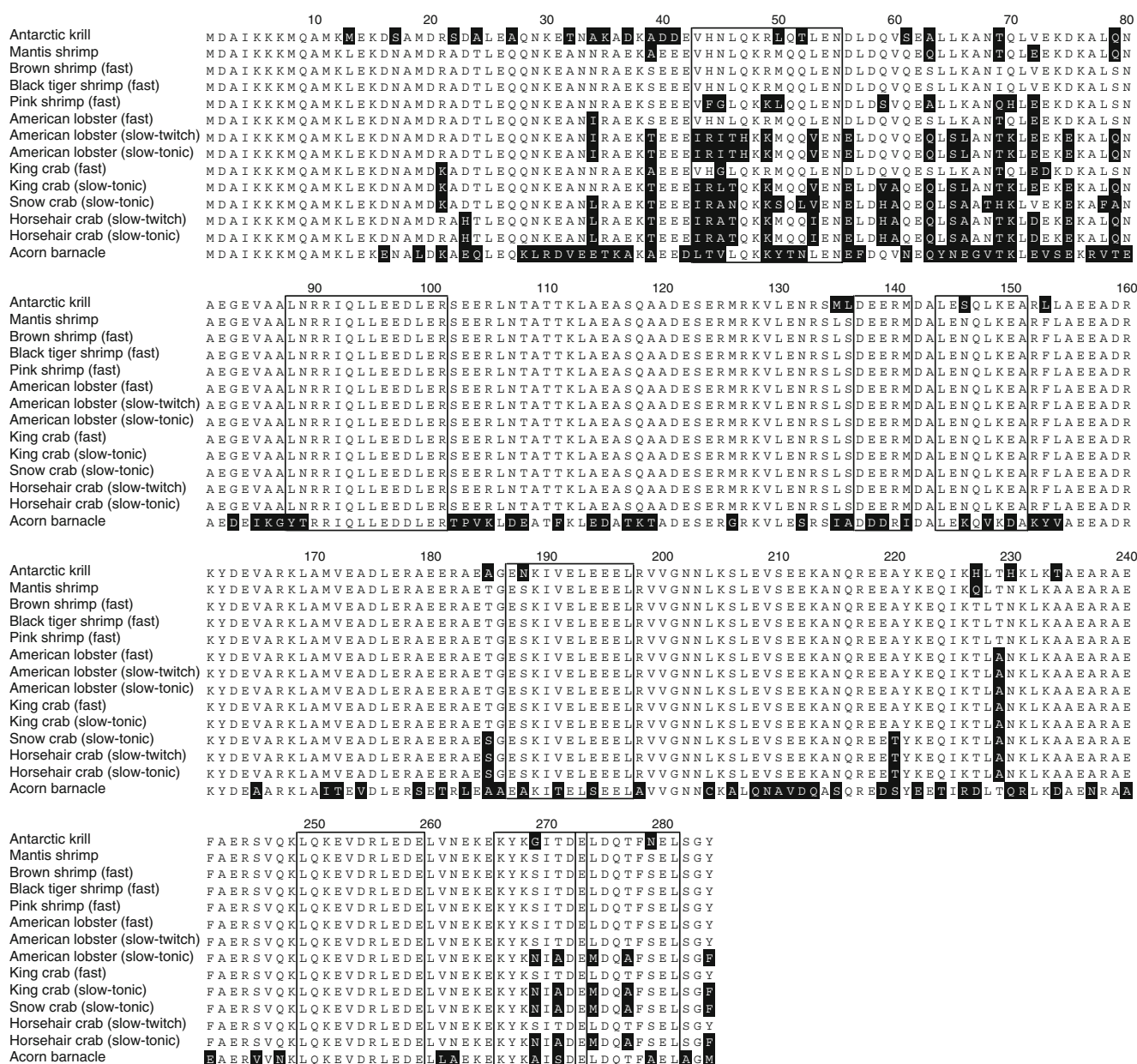


Fig. 3 Amino acid sequence alignment of crustacean tropomyosins. Tropomyosin type (fast, slow-twitch, or slow-tonic) is indicated in each parenthesis after the decapod name. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): Antarctic krill (*Euphausia superba*), AB308319; mantis shrimp (*Oratosquilla oratoria*), AB308320; brown shrimp (*Penaeus aztecus*), DQ151457; black tiger shrimp (*Penaeus monodon*), AB270629; pink shrimp (*Pandalus eous*), AB270631; American lobster (*Homarus americanus*) (fast tropomyosin), AF034954; American lobster (slow-twitch tropomyosin), AF034953; American lobster (slow-tonic tropomyosin),

AY521627; king crab (*Panulinus stimpsoni*=*Paralithodes camtschaticus*; fast tropomyosin), AB270632; king crab (slow-tonic), AB270633; snow crab (*Chionoecetes opilio*), AB270634; horsehair crab (*Erimacrus isenbekii*; slow-twitch tropomyosin), AB270635; horsehair crab (slow-tonic tropomyosin), AB270636; acorn barnacle (*Balanus rostratus*), AB269786. The residues differing from brown shrimp tropomyosin are shown in white on the black background. The regions corresponding to the IgE-binding epitopes proposed for brown shrimp tropomyosin are boxed

Table 1 Amino acid sequence identity (%) between crustacean tropomyosins

	Antarctic krill	Mantis shrimp	Brown shrimp (fast)	Black tiger shrimp (fast)	Pink shrimp (fast)	American lobster (fast)	American lobster (slow-twitch)	American lobster (slow-tonic)	King crab (fast)	King crab (slow-tonic)	Snow crab (slow-tonic)	Horsehair crab (slow-twitch)	Horsehair crab (slow-tonic)	Acom barnacle
Antarctic krill	100.0													
Mantis shrimp		90.5	89.8	89.8	88.4	89.4	85.6	84.2	89.1	83.8	82.4	84.2	83.1	57.7
Brown shrimp		100.0	97.9	97.9	96.1	98.2	95.1	93.0	98.2	92.6	89.4	93.0	91.2	60.2
(fast)			100.0	100.0	96.8	98.6	93.3	91.5	97.5	91.2	89.1	91.9	90.1	58.8
Black tiger shrimp (fast)				100.0	96.8	98.6	93.3	91.5	97.5	91.2	89.1	91.9	90.1	58.8
Pink shrimp (fast)					100.0	96.5	93.3	91.5	96.1	91.5	89.4	91.9	90.1	59.2
American lobster (fast)						100.0	94.7	93.0	98.6	91.5	89.1	92.6	90.8	60.2
American lobster (slow-twitch)							100.0	98.2	93.7	96.1	92.3	95.8	94.4	59.5
American lobster (slow-tonic)								100.0	91.9	97.9	94.0	94.4	96.1	58.8
King crab (fast)									100.0	92.3	88.7	91.9	90.8	59.9
King crab (slow-tonic)										100.0	94.7	94.7	96.5	59.2
Snow crab (slow-tonic)											100.0	94.4	96.5	58.5
Horsehair crab (slow-twitch)												100.0	98.2	59.2
Horsehair crab (slow-tonic)													100.0	58.5
Acom barnacle														100.0

been reported for Antarctic krill by Nakano et al. (2008). Recently, tropomyosin has been shown to be a major allergen in acorn and goose barnacles that are crustaceans distantly related to decapods (Suma et al. 2007). The cross-reactivity between tropomyosins from barnacles and decapods has also been demonstrated. Taken together, tropomyosin can be regarded as the major cross-reactive allergen in all crustaceans.

Nucleotide Sequences of cDNAs Encoding Antarctic Krill and Mantis Shrimp Tropomyosins

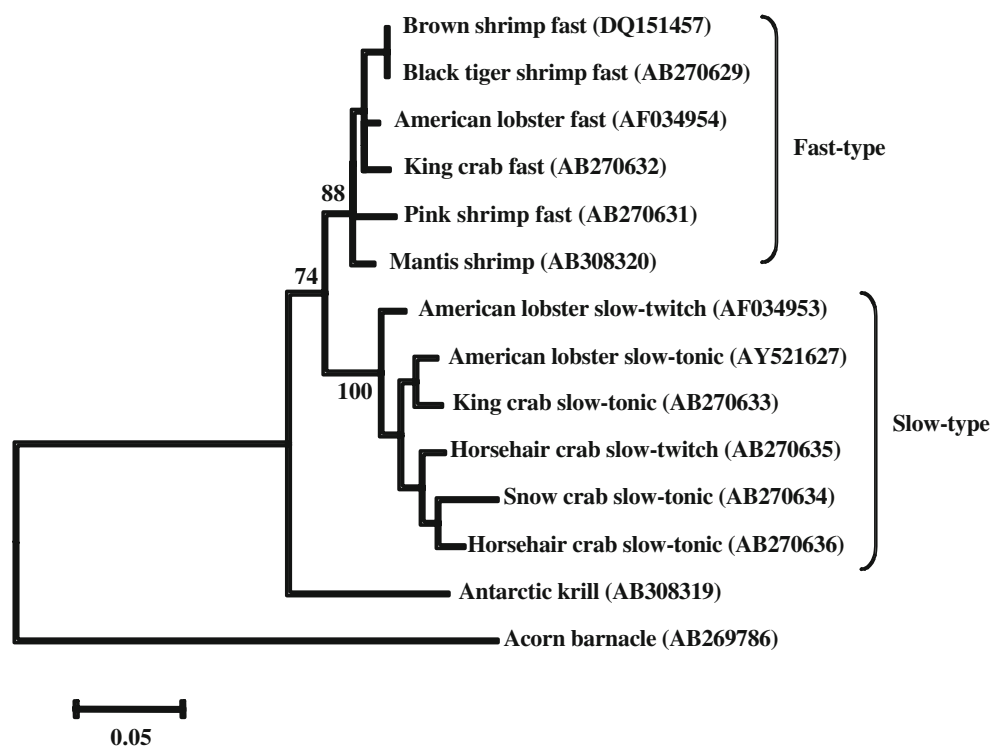
The cDNAs encoding tropomyosins from Antarctic krill and mantis shrimp were successfully cloned by PCR using the Marathon cDNA library as a template. In this study, nucleotide sequencing was performed with four clones obtained after subcloning of each PCR product. However, only one kind of cDNA was cloned from both Antarctic krill and mantis shrimp, indicating that isomeric forms of tropomyosin, if present, are trace in the abdominal muscle of both crustaceans. The elucidated nucleotide sequences of the full-length cDNAs (Fig. 2) have been deposited in the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases under the accession numbers of AB308319 for Antarctic krill tropomyosin (1,104 bp) and AB308320 for mantis shrimp tropomyosin (1,085 bp). An open reading frame of both cDNAs is composed of 852 bp coding for 284 amino acid residues, similar to the known tropomyosin cDNAs. During

the preparation of this paper, the nucleotide sequences of the full-length cDNA encoding Antarctic krill tropomyosin and the open reading frame of the cDNA encoding tropomyosin from mantis shrimp *Squilla oratoria* (probably the same species as *O. oratoria* used in this study) have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers of AB289603 and EF584510, respectively. As compared to the submitted nucleotide sequence of the Antarctic krill tropomyosin cDNA, our determined sequence has two differences in the 5' untranslated region and two differences in the open reading frame. However, there is no difference between both deduced amino acid sequences. In the case of mantis shrimp tropomyosin, our determined nucleotide sequence has as many as 11 differences compared to the submitted sequence; two of the 11 differences even lead to the replacements of amino acid residues at positions 2 (Asp for *O. oratoria* and Glu for *S. oratoria*) and 162 (Tyr for *O. oratoria* and Cys for *S. oratoria*). It is worth mentioning that Asp-2 and Tyr-162 deduced for the *O. oratoria* tropomyosin are commonly found in the known crustacean tropomyosins (refer to Fig. 3).

Amino Acid Sequences of Antarctic Krill and Mantis Shrimp Tropomyosins

The amino acid sequences of Antarctic krill and mantis shrimp tropomyosins are aligned in Fig. 3 with those from seven species of decapod crustaceans and acorn barnacle.

Fig. 4 Molecular phylogenetic tree for crustacean tropomyosins. The tree was constructed by the neighbor-joining method. Bootstrap values greater than 70% are reported on nodes of the tree. The length of branches indicates the genetic distance between the tropomyosins. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases) are shown in parentheses



Three types of tropomyosin (fast, slow-twitch, and slow-tonic) are known for decapod tropomyosins. Fast-type tropomyosins are mainly contained in the abdominal muscle of shrimps and crayfishes and both slow-twitch and slow-tonic tropomyosins in the chest protection and leg muscles of crabs including hermit crabs (Motoyama et al. 2007). As compared to the amino acid sequences of the typical fast-type tropomyosins from brown shrimp, black tiger shrimp, and American lobster, both slow-twitch and slow-tonic tropomyosins have a number of alterations (15–21 alterations) in the region 39–79, and slow-tonic tropomyosins have additional five alterations in the C-terminal region (269–284). Obviously, both Antarctic krill and mantis shrimp tropomyosins cloned in this study are devoid of the sequence features recognized for slow-type tropomyosins. Although mantis shrimp belongs to the subclass Hoplocardia differing from decapods belonging to the subclass Eumalacostraca, its tropomyosin shares as high as 96.1–98.2% sequence identities with fast-type tropomyosins and somewhat lower identities (89.4–95.1%) with slow-type tropomyosins (Table 1). In accordance with this, the molecular phylogenetic tree constructed for crustacean tropomyosins revealed that the mantis shrimp tropomyosin is classified into the fast-type tropomyosin family (Fig. 4). On the other hand, Antarctic krill, a member of the subclass Eumalacostraca, is more kin to decapods than mantis shrimp. Nevertheless, the Antarctic krill tropomyosin has diverse differences in the region 13–42, the amino acid sequence of which is highly conserved for decapod tropomyosins (Fig. 3). Therefore, it exhibits rather low sequence identities with decapod tropomyosins; the identities with fast-type and slow-type tropomyosins are 88.4–89.8% and 82.4–85.6%, respectively (Table 1). The phylogenetic tree allows us to assume that the Antarctic krill tropomyosin have diverged from both fast- and slow-type tropomyosins and evolved independently (Fig. 4).

This study showed that Antarctic krill and mantis shrimp tropomyosins are classified into the decapod tropomyosin family based on the elucidated amino acid sequences. In view of the fact that both Antarctic krill and mantis shrimp, like decapods, belong to the class Malacostraca, it is tentatively assumed that crustaceans of the class Malacostraca commonly possess tropomyosins with highly conserved amino acid sequences. On the other hand, it should be noted that the tropomyosin from acorn barnacle, a member of the class Thecostraca that is taxonomically remote from the class Malacostraca, has only about 60% sequence identity with decapod tropomyosins (refer to Table 1) but the highest identity (76–78% identity) with abalone tropomyosins (Suma et al. 2007). It is also apparent from the phylogenetic tree that the acorn barnacle tropomyosin is divergent from those of the other crustaceans

(Fig. 4). Future study is needed to clarify whether crustaceans other than members of the class Malacostraca contain tropomyosins with peculiar sequences as in the case of acorn barnacle.

Previously, eight regions (43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273, and 273–281; refer to Fig. 2) have been proposed as the major IgE-binding epitopes of the brown shrimp tropomyosin (Pen a 1; Ayuso et al. 2002a,b). These IgE-binding epitopes are completely conserved in the mantis shrimp tropomyosin, accounting for its cross-reactivity with decapod tropomyosins. In the case of Antarctic krill tropomyosin, three of the eight Pen a 1 epitopes are completely conserved, but one or two alterations are recognized in the remaining five regions. It is important to clarify whether these alterations affect the IgE-binding ability of the Antarctic krill tropomyosin. For a better understanding of the cross-reactivity with decapod tropomyosins, the numerous alterations in the region 13–42 should also be evaluated for the involvement in the IgE binding of the Antarctic krill tropomyosin.

Contents of Tropomyosin in Crustaceans

As described above, the low content of tropomyosin in mantis shrimp was qualitatively suggested by SDS-PAGE (Fig. 1a). To confirm this quantitatively, tropomyosin contents in the abdominal muscle of four crustaceans were determined by ELISA using the monoclonal antibody 5G5E1 against American lobster tropomyosin. As a result, the determined tropomyosin contents were 5.5, 6.4, 5.4, and 0.009 mg/g for American lobster, black tiger shrimp, Antarctic krill, and mantis shrimp, respectively. Since the calibration curve was made using American lobster tropomyosin, the determined values for black tiger shrimp, Antarctic krill, and mantis shrimp may have been a little underestimated or overestimated. Nevertheless, the tropomyosin content in mantis shrimp is apparently much lower than those in the other three species. It is thus assumed that mantis shrimp is much less allergenic than decapods, although its tropomyosin is cross-reactive with decapod tropomyosins. On the other hand, Antarctic krill contains tropomyosin at almost the same level as decapods, indicating that its allergenicity is equivalent to decapods. We recommend crustacean-allergic patients to avoid consumption of not only decapods but also Antarctic krill.

In conclusion, this study demonstrated that tropomyosin is a major allergen in Antarctic krill and mantis shrimp, similar to decapod crustaceans. The high sequence identity of both Antarctic krill and mantis shrimp tropomyosins with decapod tropomyosins supports the cross-reactivity between tropomyosins from both species and decapods. Quantification of tropomyosin suggests that Antarctic krill has the same potential to cause hypersensitive reactions in

crustacean-allergic patients as decapods, while mantis shrimp is much less allergenic than decapods.

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