Table 1. Designations and Nucleotide Sequences of the Primers Used for Molecular Cloning of Crustacean Tropomyosins

designation of primer	nucleotide sequence of primer	corresponding amino acid sequence[a]
cru-f1	5′-ATCAAGAAGAAGATGCAGGCG-3′	4-IKKKMQA-10
cru-f2	5′-AGAAGGCCAACCAGCGCGAGGA-3′	212-EKANQREE-219
kuruma-f	5′-AGAAGGCTAACCAGCGCGAGGA-3′	212-EKANQREE-219
cru-r1	5′-TCTTCGAGCCTGTCGACCTC-3′	251-KEVDRLED-258
cru-r2	5′-GGATGTTAGCCTTCAGCAAGGATT-3′	62-ESLLKANIQ-70
pink-s	5′-CGTTAGAGAGAGCCTTGTCC-3′	74-KDKALSNA-81
pink-r	5′-GGTGTTGGTTAGCCTTCAGC-3′	64-LLKANTQL-71
snow-f	5′-GGAACAGATCAAGACCCTTGCC-3′	222-KEQIKTLA-229
snow-r	5′-TATGGGTAGCGGCGGACAACTGCT-3′	62-ESLLKANIQ-70
horsehair-s	5′-CCTCCTCGAGAAGCTGGATG-3′	91-RIQLLEED-98
horsehair-r	5′-CACCTCACCCTCGGCATTCT-3′	79-SNAEGEV-85
king-f1	5′-CGAGGAGGAGGTTCACGGCCTT-3′	39-AEEEVHGL-46
king-f2	5′-TGAGGAGGAGATTCGCCTTACC-3′	39-IEEEIRLT-46
king-r1	5′-TTGCGTATTCGCCTTAAGCAGGGA-3′	63-SLLKANTQ-70
king-r2	5′-CTTAGTGTTAGCCAGAGATAGCTG-3′	63-QLSLANTK-70
AP1	5′-CCATCCTAATACGACTCACTATAGGGC-3′	
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′	
AAP	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′	

[a] Refer to Figure 2 for amino acid sequences.

American lobster, for which the major allergen has previously been established to be tropomyosin (*4*), was used as a reference. Subsequently, amino acid sequences of tropomyosins from the six species of crustaceans were elucidated by a cDNA cloning technique. We report here the identification of tropomyosins as allergens in six species of decapod crustaceans and the primary structural features of their tropomyosins in comparison with the known crustacean tropomyosins.

MATERIALS AND METHODS

Crustaceans. Live specimens of kuruma prawn, pink shrimp, American lobster, and horsehair crab were purchased at the Tokyo Central Wholesale Market. Live specimens of black tiger prawn imported from Vietnam and those of king crab and snow crab caught along the coasts of Hokkaido, Japan, were kindly supplied from Nippon Suisan Kaisha. Abdominal muscle was obtained from each live specimen of black tiger prawn, kuruma prawn, pink shrimp, and American lobster and both leg muscle and chest protection muscle from king crab, snow crab, and horsehair crab. Muscle samples for extraction were stored at −20 °C until used, and those for molecular cloning experiments were immediately frozen in liquid nitrogen and stored at −80 °C until used.

Preparation of Heated Extracts. Each muscle sample was homogenized with 4 volumes of 0.6 M KCl in 0.01 M phosphate buffer (pH 7.0). Tropomyosin, a target protein in this study, is heat-stable. Therefore, the homogenate was then heated in a boiling water bath for 10 min and centrifuged at 18000*g* for 20 min to remove insoluble proteins. The supernatant thus obtained was used as heated extract. Protein concentrations of the heated extracts were estimated according to the method of Lowry et al. (*17*), using bovine serum albumin as a standard.

Purification of American Lobster Tropomyosin. An acetone powder of myofibrillar proteins was prepared from the abdominal muscle of American lobster as reported by Greaser and Gergely (*18*) and 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 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 (*19*). Finally, small amounts of impurities were removed by 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 with a linear gradient of acetonitrile (38.5−49.0% in 50 min) in 0.1% trifluoroacetic acid. The homogeneity of the final preparation was supported by SDS-PAGE. American lobster has previously been reported to contain three types of tropomyosin (fast, slow-twitch and slow-tonic) (*14*, *15*). Because the abdominal muscle of American lobster is mostly composed of fast muscle fibers (*14*), our purified preparation was judged to be fast-type tropomyosin.

Human Sera. Sera were donated from 10 crustacean-allergic patients. The patients were all diagnosed to be allergic to crustaceans on the basis of the determined capsulated hydrophilic carrier polymer−radioallergosorbent test (CAP-RAST) classes of 2−5 against shrimp or both shrimp and crab as well as on the experiences of immediate hypersensitive reactions after ingestion of crustaceans. 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 used.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed on a PhastSystem apparatus (GE-Healthcare Bio-Sciences, Piscataway, NJ) 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) were run as a reference, along with samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

Immunoblotting and Inhibition Immunoblotting. Immunoblotting was performed as described in our previous paper (*20*). 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. To detect tropomyosin, an antiserum (diluted 1:40000) raised in rabbits against king crab tropomyosin, which was a kind gift from Dr. H. Ushio of our university, was used as a primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:20000; Kirkegaard and Perry Laboratories, Gaithersburg, MD) as a secondary antibody. On the other hand, patient or control serum (diluted 1:500) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:10000; Kirkegaard and Perry Laboratories) were used as primary and secondary antibodies, respectively, to detect IgE-binding proteins. Blots were 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 μg/mL) at 37 °C for 1 h and used as a primary antibody.

Primer Design, Polymerase Chain Reaction (PCR), and Nucleotide Sequencing. Designations and nucleotide sequences of the primers used in this study are shown in **Table 1**. Both forward (cru-f1) and reverse (cru-r1) primers were designed from the known nucleotide sequences of the crustacean tropomyosin cDNAs. The nucleotide

sequence of cru-f1 is completely identical with the cDNAs encoding tropomyosins from brown shrimp (accession number of the DDBJ/EMBL/GenBank nucleotide databases: DQ151457) and red crab (AF061783) and three types of tropomyosin from American lobster (AF034954 for fast tropomyosin, AF034953 for slow-twitch tropomyosin, and AY521627 for slow-tonic tropomyosin). On the other hand, the nucleotide sequence of cru-r1 is identical with the cDNAs encoding tropomyosins from sand shrimp (U08008) and American lobster and spiny lobster (AF030063) but is different by one nucleotide from the cDNAs encoding tropomyosins from brown shrimp and red crab. Except for three primers, AP1 adapter primer (AP1), abridged universal anchor primer (AUAP), and abridged anchor primer (AAP), the other primers were designed on the basis of the determined partial nucleotide sequences. PCR amplifications were all performed using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 1 min; and 72 °C for 7 min. The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany), and at least three clones were analyzed for nucleotide sequences using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and a PRISM 310 genetic analyzer (Applied Biosystems).

Cloning Experiments for Tropomyosins of Black Tiger Prawn, Kuruma Prawn, King Crab, and Snow Crab. Total RNA was extracted from 2 g of each muscle sample with the TRIzol reagent (Invitrogen, Carlsbad, CA), and poly(A)$^+$ mRNA was purified by affinity chromatography on oligo(dT)-cellulose using an mRNA Purification Kit (GE-Healthcare Bio-Sciences). A small aliquot (1 μg) of the purified mRNA was converted to double-strand cDNA, followed by ligation of an AP1 adapter at both 3′ and 5′ ends, using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA). The Marathon cDNA library thus constructed was subjected to PCR as a template. At first, intermediate cDNA fragments were amplified using the forward primer (cru-f1) and the reverse primer (cru-r1). All amplified products were subcloned into the pT7Blue-2 T-vector and sequenced as described above. Then, the remaining 3′ region was amplified using the gene-specific forward primer (cru-f2 for black tiger prawn and snow crab, kuruma-f for kuruma prawn, king-f1 for king crab fast-type isoform or king-f2 for king crab slow-tonic-type isoform) and the AP1 primer and the remaining 5′ region using the gene-specific reverse primer (cru-r2 for black tiger prawn and kuruma prawn, king-r1 for king crab fast-type isoform, king-r2 for king crab slow-tonic-type isoform or snow-r for snow crab) and the AP1 primer. In the case of snow crab, nested PCR was additionally performed to amplify the 3′ remaining region using the gene-specific forward primer (snow-f) and the AP1 primer. All amplified products were subjected to subcloning and sequencing.

Cloning Experiments for Tropomyosins of Pink Shrimp and Horsehair Crab. Molecular cloning of pink shrimp and horsehair crab tropomyosins was simply performed by the rapid amplification of cDNA ends (RACE) method using total RNA extracted from each muscle sample, without construction of the Marathon cDNA library. For 3′RACE, first-strand cDNA was synthesized from 5 μg of total RNA using the 3′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) as described in the manufacturer's manual and used as a template. Amplification was carried out using the cru-f1 primer and the AUAP primer. 5′RACE was performed using the 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using the gene-specific reverse primer (pink-s for pink shrimp or horsehair-s for horsehair crab). The gene-specific reverse primer (pink-r for pink shrimp or horsehair-r for horsehair crab) and the AAP primer were subjected to 5′RACE reaction using the synthesized first-strand cDNA as a template. All amplified products were subcloned and sequenced.

RESULTS AND DISCUSSION

Identification of Tropomyosins as Allergens in Crustaceans. As analyzed by SDS-PAGE, a prominent protein of 37 kDa, which is comparable in molecular mass to American lobster tropomyosin used as a reference, was detected in all of the heated extracts, except for that from the chest protection muscle of king crab (**Figure 1A**). Irrespective of the species and the muscle types, the 37 kDa protein reacted with the antiserum against king crab tropomyosin (**Figure 1B**), allowing us to conclude that the 37 kDa protein is tropomyosin of each crustacean muscle. In the case of the chest protection muscle of king crab, two proteins of about 37 kDa with a considerably weaker staining intensity than tropomyosins in the other samples were observed in SDS-PAGE (**Figure 1A**). The two proteins also reacted with the antiserum against king crab tropomyosin (**Figure 1A**). Importantly, two different tropomyosins (fast and slow-tonic tropomyosins) exist only in the chest protection muscle of king crab, as described in detail below. Taken together, it is reasonable to consider that the two proteins of about 37 kDa observed in the chest protection muscle of king crab are tropomyosin isoforms. IgE-immunoblotting, using the pooled serum from 10 crustacean-allergic patients and 4 individual patient sera, showed that all tropomyosins, except for the two tropomyosin isoforms in the chest protection muscle of king crab, are IgE-reactive, regardless of the patient sera (**Figure 1C**). When the pooled control serum was used, no blots were detected in any of the heated extracts (data not shown). As for the two isoforms in the chest protection muscle of king crab, their IgE reactivity was established by the results with the pooled patient serum and the patient 2 and 4 sera. However, only one blot was observed when patient 1 and 3 sera were used, suggesting that only either one of the two isoforms is reactive with IgE in the patient 1 and 3 sera or that the two blots have appeared as one blot due to poor resolution. Apart from this problem to be clarified in future, it is important to note that tropomyosin seemed to be almost the sole IgE-reactive protein in all of the crustaceans examined, although another IgE-reactive protein of about 30 kDa was also detected in the chest muscle of king crab only when the patient 2 serum was used. In inhibition immunoblotting experiments using American lobster tropomyosin as an inhibitor, the patient sera completely lost their reactivity to tropomyosins from six species of crustaceans (only the results with the pooled patient serum are shown in **Figure 1D**), demonstrating the cross-reactivity between tropomyosins from American lobster and the other crustaceans.

Our results may be as expected from the current opinion that tropomyosin is a major and cross-reactive allergen in crustaceans (*7*, *11*, *12*). However, it should be noted that, of the six species of crustaceans used in this study, pink shrimp belonging to the suborder Pleocyemata and king crab (a kind of hermit crabs) are taxonomically distinct from the crustaceans so far studied on allergens. Therefore, this study is of value in providing experimental evidence that the current opinion is true for almost all crustaceans belonging to the order Decapoda.

Nucleotide Sequences of Crustacean Tropomyosin cDNAs. The cDNAs encoding tropomyosins from black tiger prawn, kuruma prawn, king crab, and snow crab were successfully cloned by PCR using the Marathon cDNA library as a template and those encoding tropomyosins from pink shrimp and horsehair crab by both 3′RACE and 5′RACE using total RNA. In the case of the chest protection muscle of king crab, two different cDNAs (encoding fast and slow-tonic tropomyosins as described below in more detail) were obtained; three of the five clones analyzed corresponded to the fast tropomyosin and the rest to the slow-tonic tropomyosin. On the other hand, only one kind of cDNA was cloned from the other samples. The cDNA from the leg muscle of king crab was identical with one

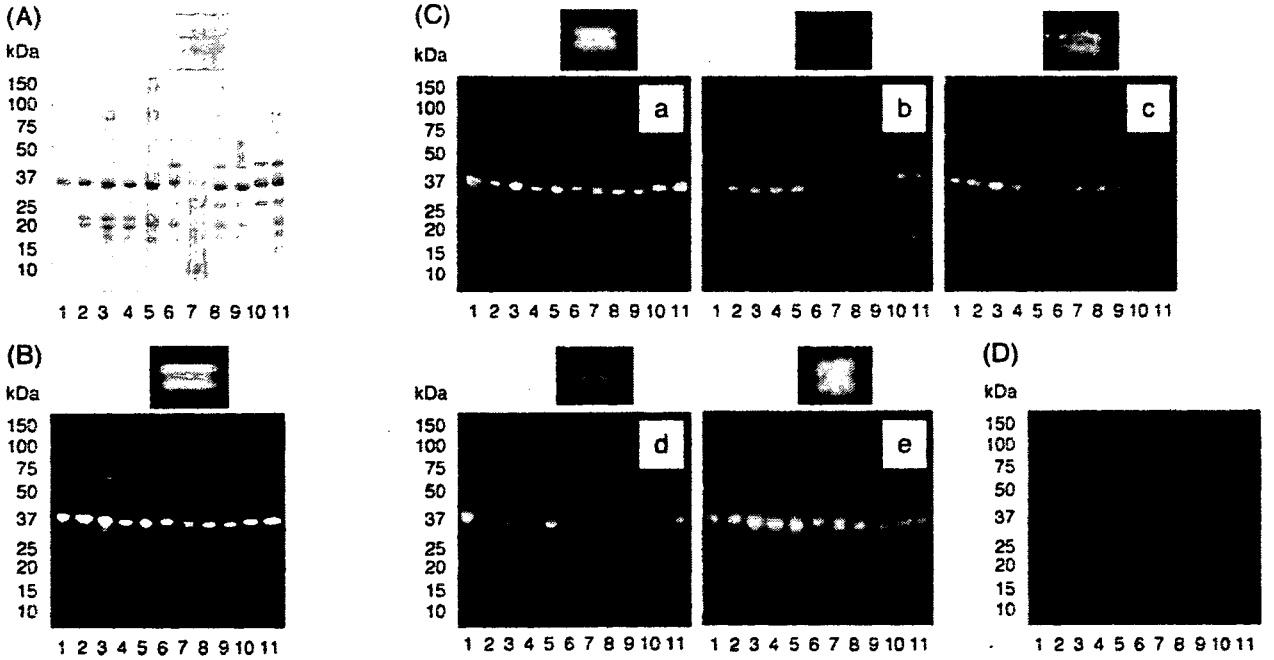

Figure 1. Analysis of heated extracts from various crustaceans by SDS-PAGE (A), IgG-immunoblotting (B), IgE-immunoblotting (C), and inhibition immunoblotting (D). Lanes: 1, tropomyosin purified from American lobster; 2, extract from American lobster; 3, extract from black tiger prawn; 4, extract from kuruma prawn; 5, extract from pink shrimp; 6, extract from king crab leg muscle; 7, extract from king crab chest protection muscle; 8, extract from snow crab leg muscle; 9, extract from snow crab chest protection muscle; 10, extract from horsehair crab leg muscle; 11, extract from horsehair crab chest protection muscle. A magnification of the 37 kDa region in lane 7 (extract from king crab chest protection muscle) is shown above each figure in A, B, and C. (A) Heated extracts (0.3 μL 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 prawn, 3.0 mg/mL for kuruma prawn, 9.8 mg/mL for pink shrimp, 3.8 mg/mL for king crab leg muscle, 7.8 mg/mL for king crab chest protection muscle, 4.2 mg/mL for snow crab leg muscle, 3.2 mg/mL for snow crab chest protection muscle, 3.2 mg/mL for horsehair crab leg muscle, and 5.6 mg/mL for horsehair crab chest protection muscle. (B) Antiserum against king crab tropomyosin was used as a primary antibody. (C) Patient sera were used as a primary antibody: a, pooled serum from 10 patients; b, patient 1; c, patient 2; d, patient 3; e, patient 4. (D) Pooled serum (diluted 1:250) from 10 patients was preincubated with an equal volume of inhibitor (American lobster tropomyosin) solution (20 μg/mL) and used as a primary antibody.

(encoding the fast tropomyosin) of the two cDNAs from the chest protection muscle. The same cDNA was found in the leg and chest protection muscles of snow crab, whereas the cDNAs from two types of muscles of horsehair crab differed from each other. These results agreed well with the data obtained in SDS-PAGE and immunoblotting showing the presence of two tropomyosin isoforms only in the chest protection muscle of king crab (**Figure 1**).

Although not shown in this paper to save space, the determined nucleotide sequences of the full-length cDNAs have been deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers AB270629 for black tiger prawn tropomyosin (1260 bp), AB270630 for kuruma prawn tropomyosin (1299 bp), AB270631 for pink shrimp tropomyosin (1050 bp), AB270632 for king crab fast tropomyosin (1493 bp), AB270633 for king crab slow-tonic tropomyosin (1559 bp), AB270634 for snow crab tropomyosin (1148 bp), AB270635 for horsehair crab slow-twitch tropomyosin (1137 bp), and AB270636 for horsehair crab slow-tonic tropomyosin (1016 bp). Irrespective of the cDNAs, an open reading frame contains 852 bp coding for 284 amino acid residues.

Amino Acid Sequences of Crustacean Tropomyosins. The amino acid sequences of eight tropomyosins from six species of crustaceans were elucidated by a cDNA cloning technique. This is the first report of the amino acid sequences of tropomyosins from shrimp (pink shrimp) belonging to the suborder Pleocyemata and hermit crab (king crab), making it possible to understand the overall features of the amino acid sequences of tropomyosins from edible crustaceans (decapod crustaceans). The known amino acid sequences of crustacean tropomyosins, including our results, are aligned in **Figure 2**. It is worth mentioning that three types of tropomyosin (fast, slow-twitch, and slow-tonic) have been identified in American lobster (*14*, *15*). As compared to the amino acid sequence of the fast tropomyosin, both slow-twitch and slow-tonic tropomyosins have as many as 15 alterations in the region 39−79 and the slow-tonic tropomyosin has 4 additional alterations in the C-terminal region. On the basis of the sequence features of the three types of tropomyosin from American lobster, the tropomyosins from the abdominal muscle of shrimps (or prawns) and spiny lobster and one tropomyosin isoform from the chest protection muscle of king crab are assignable to the fast type, that from the leg muscle of horsehair crab to the slow-twitch type, and another tropomyosin isoform from the chest protection muscle of king crab, those from the leg and chest protection muscles of snow crab, and that from the chest protection muscle of horsehair crab to the slow-tonic type. The abdominal muscle tropomyosin of pink shrimp has some differences in the region 44−72 compared to the fast-type tropomyosin but is distinguishable from the slow-type tropomyosin. Thus, we tentatively classify the pink shrimp tropomyosin into the fast-type family. In the case of red crab tropomyosin, it is apparently a member of the slow-twitch or slow-tonic type family, although its C-terminal sequence is unclear. Taken together, the relationship

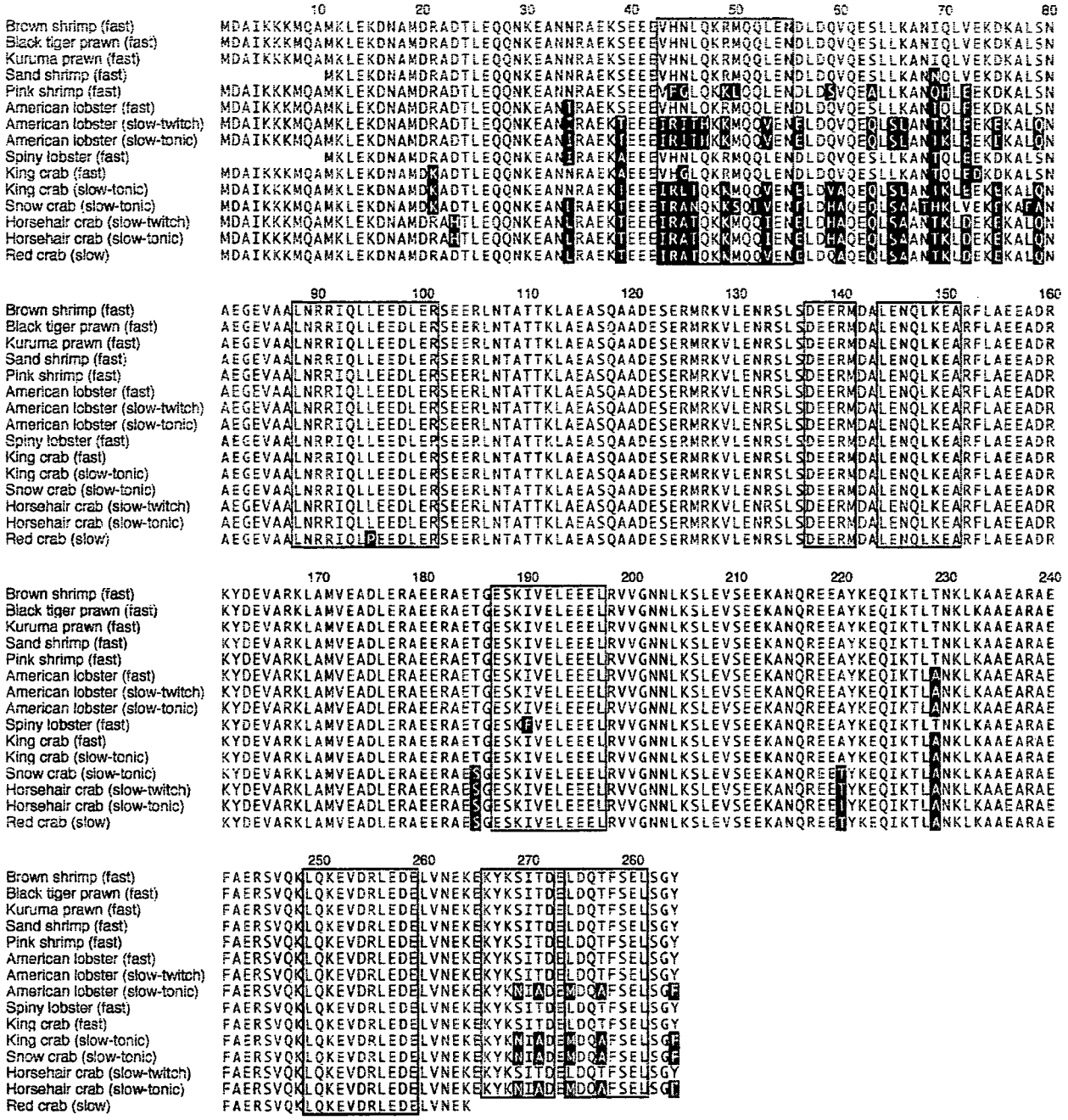

Figure 2. Amino acid sequence alignment of crustacean tropomyosins. Tropomyosin type (fast, slow-twitch, or slow-tonic) is indicated in parentheses after the crustacean name. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): brown shrimp (*Penaeus aztecus*), DQ151457; black tiger prawn (*Penaeus monodon*), AB270629; kuruma prawn (*Penaeus japonicus*), AB270630; sand shrimp (*Metapenaeus ensis*), U08008; pink shrimp (*Pandalus eous*), AB270631; American lobster (*Homarus americanus*) (fast tropomyosin), AF034954; American lobster (slow-twitch tropomyosin), AF034953; American lobster (slow-tonic tropomyosin), AY521627; spiny lobster (*Panulinus stimpsoni*), AF030063; king crab (*Paralithodes camtschaticus*) (fast tropomyosin), AB270632; king crab (slow-tonic tropomyosins), AB270633; snow crab (*Chionoecetes opilio*), AB270634; horsehair crab (*Erimacrus isenbekii*) (slow-twitch tropomyosin), AB270635; horsehair crab (slow-tonic tropomyosin), AB270636; red crab (*Charybdis feriatus*), AF061783. The residues differing from the brown shrimp tropomyosin are shown in white on the black background. The IgE-binding epitopes proposed for the brown shrimp tropomyosin are boxed.

of the major tropomyosin type in edible muscles with the taxonomical position of crustaceans is summarized as follows: fast-type tropomyosins are contained in shrimps (or prawns) and lobsters, slow-type tropomyosins in crabs, and both fast- and slow-type tropomyosins in crayfishes and hermit crabs.

As shown in **Table 2**, the amino acid sequence identities between fast tropomyosins and between slow tropomyosins are extremely high; even 100% identity is observed among the fast tropomyosins from three species of *Penacus* shrimp or prawn (brown shrimp, black tiger prawn, and kuruma prawn). The

Table 2. Amino Acid Sequence Identity between Crustacean Tropomyosins

relationship	amino acid sequence identity (%)	
	range	mean ± SD
between fast tropomyosins	96.0–100.0	98.1 ± 1.0 (n = 28)
between slow tropomyosins	92.3–98.5	96.1 ± 1.4 (n = 21)
between fast and slow tropomyosins	88.3–94.7	91.4 ± 1.4 (n = 56)

sequence identity between fast and slow tropomyosins is also considerably high. These facts seem to provide a molecular basis for the cross-reactivity among crustacean tropomyosins (*6*, *7*, *11*, *12*). More detailed discussion on the cross-reactivity among crustacean tropomyosins can be derived on the basis of the IgE-binding epitopes proposed for the brown shrimp tropomyosin (Pen a 1) (*10*, *16*). All eight epitope regions (43−55, 88−101, 137−141, 144−151, 187−197, 249−259, 266−273, and 273−281; refer to **Figure 2**) of Pen a 1 are well conserved in fast tropomyosins, with the exceptions of pink shrimp tropomyosin having four alterations in the region 43−55, spiny lobster tropomyosin having one alteration in the region 187−197, and king crab tropomyosin having one alteration in the region 43−55. This implies that the cross-reactivity among fast tropomyosins is simply realized by the common IgE-binding epitopes. In the case of slow tropomyosins, five of the eight Pen a 1 epitopes are completely or almost completely conserved. However, both slow-twitch and slow-tonic tropomyosins have as many as six to eight alterations in the region 43−55, and slow-tonic tropomyosins have additional two alterations in the two regions 266−277 and 273−281. Moreover, diverse alterations between fast and slow tropomyosins are also observed in the region 56−79, which is not identified as the Pen a 1 epitope. For a better understanding of the cross-reactivity between fast and slow tropomyosins, an examination of how the significant differences in specific regions between fast and slow tropomyosins are associated with the IgE-binding potency is needed.

In general, crustacean-allergic patients are mostly sensitive to both shrimp and crab. This is easily understood by the presence of several common IgE-binding epitopes in shrimp and crab tropomyosins. However, some crustacean-allergic patients specifically recognize only shrimp or crab. This can be realized by two possibilities. One is that both shrimp and crab have specific allergens differing from tropomyosins; for example, arginine kinase has recently been identified as a new allergen in black tiger prawn (*21*). Another possibility is derived from our finding that there are significant differences in amino acid sequences of specific regions between fast tropomyosins (mainly contained in shrimp) and slow tropomyosins (mainly contained in crab). Namely, it is possible that both shrimp-allergic and crab-allergic patients have IgE specifically binding to the regions with significant differences between fast and slow tropomyosins. In conclusion, the amino acid sequences of crustacean tropomyosins determined in this study will facilitate future molecular study to understand crustacean allergy in more detail.

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Comparative analysis of barnacle tropomyosin: Divergence from decapod tropomyosins and role as a potential allergen

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Abstract

Tropomyosin, a myofibrillar protein of 35–38 kDa, represents a major and cross-reactive allergen in decapod crustaceans. This study was initiated to clarify whether decapod-allergic patients also recognize tropomyosins of barnacles, crustaceans phylogenetically remote from decapods, which are locally consumed as a delicacy. On SDS-PAGE, a 37 kDa protein was observed in all the heated extracts prepared from two species of decapods (American lobster *Homarus americanus* and black tiger prawn *Penaeus monodon*) and two species of barnacles (acorn barnacle *Balanus rostratus* and goose barnacle *Capitulum mitella*). In immunoblotting, the 37 kDa protein was found to react with monoclonal antibodies against American lobster tropomyosin and hence identified as tropomyosin. The patient sera reacted to tropomyosins from both decapods and barnacles and the reactivity was abolished by preincubation with American lobster tropomyosin, demonstrating that barnacle tropomyosins are allergens cross-reactive with decapod tropomyosins. However, the amino acid sequence of acorn barnacle tropomyosin, deduced by cDNA cloning experiments, shares higher sequence identity with abalone tropomyosins than with decapod tropomyosins. In accordance with this, the phylogenetic tree made for tropomyosins from various animals showed that the acorn barnacle tropomyosin is evolutionally classified not into the decapod tropomyosin family but into the molluscan tropomyosin family.

Keywords: Allergen; Acorn barnacle; cDNA cloning; Cross-reactivity; Crustacea; Decapod; Goose barnacle; Tropomyosin

1. Introduction

Crustaceans are well known as a common cause of food allergy mediated by IgE antibodies. Since edible crustaceans mostly belong to the order Decapoda (including shrimps, prawns, crayfishes, lobsters, hermit crabs and crabs) of the class Malacostraca, previous studies on crustacean allergens have been performed only with decapods. As a result, the major allergen has been identified at the molecular level as tropomyosin, a myofibrillar protein of 35–38 kDa, common with the following decapods: Indian white shrimp *Penaeus indicus* (Shanti et al., 1993), brown shrimp *P. aztecus* (Daul et al., 1994), sand shrimp *Metapenaeus ensis* (Leung et al., 1994), American lobster *Homarus americanus* (Leung et al., 1998a), spiny lobster *Panulinus stimpsoni* (Leung et al., 1998a) and red crab *Charybdis feriatus* (Leung et al., 1998b).

Besides decapods, barnacles, members of the subclass Cirripedia of the class Thecostraca, are also edible crustaceans. They inhabit the rocky shores of warm seas and some species of them are consumed as a delicacy in coastal countries such as Japan and the Mediterranean countries. The subclass Cirripedia is further divided into two orders, Sessilia (acorn barnacles lacking a stalk) and Pedunculata (goose barnacles having a stalk). The acorn barnacle *Balanus rostratus* and the goose barnacle *Capitulum* (*Pollicipes*) *mitella* are the most consumed species in Japan and the goose barnacle *Pollicipes pollicipes* in the Mediterranean countries. Recently, barnacles were reported to cause IgE-mediated allergic reactions by two research groups (Moreno Escobosa et al., 2002; Marinho et al., 2006). A number of IgE-binding proteins were detected in the barnacle extract by immunoblotting experiments using sera from barnacle-allergic patients. Interestingly, one IgE-binding protein of about 37 kDa was suggested to be tropomyosin from the molecular mass. However, only one of the five barnacle-allergic patients was diagnosed to be also allergic to shrimp in one study (Marinho

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et al., 2006), while susceptibility to shrimp was unclear for the five barnacle-allergic patients in another study (Moreno Escobosa et al., 2002). It is therefore still unknown whether decapod-allergic patients recognize barnacle tropomyosin as well as decapod tropomyosin.

Tropomyosin is regarded as a pan-allergen that is implicated in IgE cross-reactivity among various invertebrates including crustaceans (decapods), mollusks, mites and cockroaches (Leung et al., 1996; Reese et al., 1999; Santos et al., 1999; Chuo et al., 2000; Ayuso et al., 2002a,b; Lehrer et al., 2003). In view of this, it is possible that barnacle tropomyosin is also cross-reactive with decapod tropomyosin, having a potential to cause adverse reactions in decapod-allergic patients. The present study was initiated to confirm this possibility by immunoblotting and immunoblotting inhibition, using two species of barnacles (acorn barnacle *B. rostratus* and goose barnacle *C. mitella*) locally consumed in Japan. Subsequently, the primary structure of acorn barnacle tropomyosin was elucidated by cDNA cloning to obtain a molecular basis for the confirmed cross-reactivity between tropomyosins from acorn barnacle and decapods.

2. Materials and methods

2.1. Crustacean samples

Live specimens of American lobster and acorn barnacle and fresh specimens of black tiger prawn *Penaeus monodon* were purchased at the Tokyo Central Wholesale Market. Specimens of goose barnacle were caught along the coasts of Noto Peninsula, Ishikawa Prefecture, and transported alive to our laboratory. Abdominal muscles collected from American lobster and black tiger prawn and muscles from acorn barnacle and goose barnacle were all stored at −20 °C until used. For cloning experiments, part of the muscle samples from acorn barnacle and goose barnacle was immediately frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Preparation of heated extract

Each muscle sample was homogenized with four volumes of 10 mM phosphate buffer (pH 7.0) containing 0.6 M KCl. Since this study was focused on tropomyosin, a thermostable protein, the homogenate was heated in a boiling water bath for 10 min to make thermolabile proteins insoluble. After centrifugation at 18,000 *g* for 20 min, the supernatant obtained was used as heated extract. Protein concentrations of the heated extracts were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.3. Purification of American lobster tropomyosin

An acetone powder of myofibrillar proteins was prepared from the deep abdominal muscle of American lobster as reported by Greaser and Gergely (1971) and 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 successively to salting-out with ammonium sulfate (50–60% saturation) and isoelectrical precipitation (pH 4.6) by the method of Cummins and Perry (1973). To remove small amounts of contaminated proteins, the tropomyosin-rich fraction thus obtained was further 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 with a linear gradient of acetonitrile (38.5–49.0% in 50 min) in 0.1% trifluoroacetic acid. The homogeneity of the final preparation was supported by SDS-PAGE. American lobster has previously been reported to contain three types of tropomyosin (fast, slow-twitch and slow-tonic) (Mykles et al., 1998; Medler et al., 2004). Since the deep abdominal muscle of American lobster is composed of fast muscle fibers (Mykles et al., 1998), our purified preparation was judged to be fast type tropomyosin.

2.4. Human sera

Sera were obtained from ten crustacean-allergic patients with clinical histories of immediate hypersensitive reactions after ingestion of crustaceans but without experiences of eating barnacles. The patients were all diagnosed to be allergic to decapod crustaceans based on the determined CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) classes of 2–5 against shrimp or both shrimp and crab. Written informed consent was obtained from each patient. In this study, sera from four healthy volunteers without adverse reactions after ingestion of any foods were pooled and used as a control. All sera were stored at −20 °C.

2.5. SDS-PAGE

SDS-PAGE was performed with a PhastSystem (GE-Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. 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 on a PhastGel Gradient 8–25 gel (GE-Healthcare Bio-Sciences). Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA) were run as a reference, along with samples. After running, proteins were visualized by staining with Coomassie Brilliant Blue R-250.

2.6. Immunoblotting

Immunoblotting was carried out as reported previously (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. To detect tropomyosin, either one of the two monoclonal antibodies (0.5 μg/mL of 2A7H6 and 1 μg/mL of 5G5E1) 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 1:5000; Kirkegaard and Perry Laboratories,

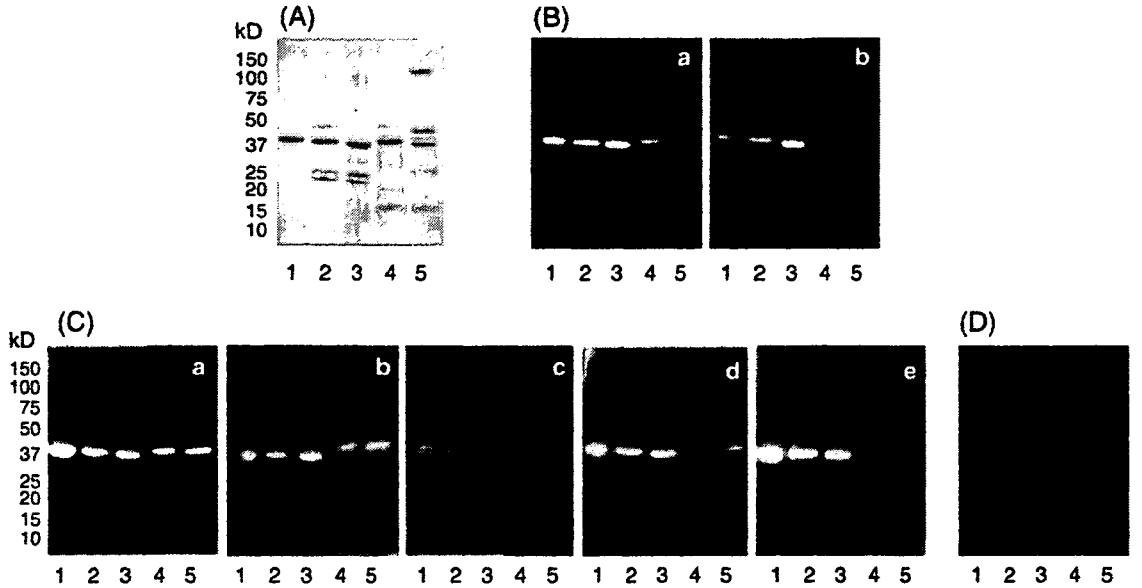

Fig. 1. Analysis of the heated extracts from decapods and barnacles by SDS-PAGE (A), immunoblotting using monoclonal antibodies against American lobster tropomyosin (B), immunoblotting using patient sera (C) and inhibition immunoblotting (D). Lanes: 1, tropomyosin purified from American lobster; 2, American lobster extract; 3, black tiger prawn extract; 4, acorn barnacle extract; 5, goose barnacle extract. (A) Heated extracts (1 μl 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 prawn, 3.6 mg/mL for acorn barnacle and 4.4 mg/mL for goose barnacle. (B) Monoclonal antibodies: a, 2A7H6; b, 5G5E1. (C) Sera: a, pooled serum from ten patients; b, patient 1; c, patient 2; d, patient 3; e. patient 4. (D) Pooled serum (diluted 1:250) from ten patients was preincubated with an equal volume of inhibitor (American lobster tropomyosin) solution (20 μg/mL) and used as a primary antibody. The same results were also obtained with the sera from patients 1–4.

Gaithersburg, MD) were used as primary and secondary antibodies, respectively. Positive bands were visualized using an ECL Plus Western Blotting Detection System (GE-Healthcare Bio-Sciences) and an ECL Mini Camera (GE-Healthcare Bio-Sciences), according to the manufacturer's instructions. For IgE-immunoblotting inhibition, patient serum (diluted 1:250) was

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ATAGCCCTTTCGAGTTTGCGGCAGCAGGATTGTGGTTCTGACAGCTCTCGTCTGCTAAAGTCACTTGTAGCTTCGACATTCCTATTTAT    89

TTTATTTTATTTGTGTGTTAGGTGAGAATAAACTAAAAATCAGCCATGGATGCCATCAAGAAGAAGATGCAGGCAATGAAATTGGAAAAG   179
                                                M  D  A  I  K  K  K  M  Q  A  M  K  L  E  K    15

GAAAATGCCTTGGATAAAGCAGAACAGTTAGAACAAAAACTTAGGGATGTTGAGGAAACCAAAGCAAAGGCAGAGGAAGATCTTACTCTT   269
 E  N  A  L  D  K  A  E  Q  L  E  Q  K  L  R  D  V  E  E  T  K  A  K  A  E  E  D  L  T  L    45

CTCCAAAAGAAATACACAAACCTCGAGAATGAGTTCGACCAAGTCAACGAACAATACAATGAAGGTGTCACCAAGCTTGAGGTCTCCGAG   359
 L  Q  K  K  Y  T  N  L  E  N  E  F  D  Q  V  N  E  Q  Y  N  E  G  V  T  K  L  E  V  S  E    75

AAACGTGTAACAGAGGCAGAAGATGAAATCAAGGGtTACACTAGGcGTATCCAACTTTTAGAAGATGACCTTGAACGTACCCCAGTCAAG   449
 K  R  V  T  E  A  E  D  E  I  K  G  Y  T  R  R  I  Q  L  L  E  D  D  L  E  R  T  P  V  K   105

TTGGACGAAGCCACCTTTAAGTTGGAAGACGCAACCAAAACAGCAGATGAGAGTGAAAGAGGACGCAAGGTACTCGAGAGCAGAAGCATT   539
 L  D  E  A  T  F  K  L  E  D  A  T  K  T  A  D  E  S  E  R  G  R  K  V  L  E  S  R  S  I   135

GCTGATGATGATAGAATTGATGCATTAGAAAAACAAGTGAAAGATGCTAAATATGTTGCTGAGGAAGCCGACCGTAAATACGATGAGGCT   629
 A  D  D  D  R  I  D  A  L  E  K  Q  V  K  D  A  K  Y  V  A  E  E  A  D  R  K  Y  D  E  A   165

GCCCGTAAACTTGCTATCACTGAAGTGGATCTTGAACGCTCCGAGACCCGATTGGAGGCTGCTGAAGCCAAAATTACCGAACTCAGTGAG   719
 A  R  K  L  A  I  T  E  V  D  L  E  R  S  E  T  R  L  E  A  A  E  A  K  I  T  E  L  S  E   195

GAGCTGGCTGTGGTTGGTAATAACTGTAAGGCCCTGCAGAACGCCGTAGACCAGGCATCTCAGAGAGAAGACAGTTACGAGGAGACTATC   809
 E  L  A  V  V  G  N  N  C  K  A  L  Q  N  A  V  D  Q  A  S  Q  R  E  D  S  Y  E  E  T  I   225

CGTGACTTGACCCAGAGACTCAAGGACGCCGAGAATCGTGCGGCGGAGGCTGAACGCGTAGTCAACAAGCTGCAGAAGGAAGTTGACAGA   899
 R  D  L  T  Q  R  L  K  D  A  E  N  R  A  A  E  A  E  R  V  V  N  K  L  Q  K  E  V  D  R   255

TTAGAAGATGAATTACTTGCTGAAAAGGAGAAGTACAAGGCGATTAGTGACGAATTGGATCAAACATTTGCTGAGTTAGCTGGCATGTGA   989
 L  E  D  E  L  L  A  E  K  E  K  Y  K  A  I  S  D  E  L  D  Q  T  F  A  E  L  A  G  M  *   284

GACATCAACTTTTGTCAGACAACAAGAAAAATACACTTCATTTACATTTCTACAAAGCCCTGTTTCGCTGTTATTGATGAT          1070
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Fig. 2. Nucleotide sequence of cDNA encoding for acorn barnacle tropomyosin. The deduced amino acid sequence is denoted below the nucleotide sequence. Nucleotide and amino acid numbers are shown at the right. The region corresponding to the primer used in 3′RACE is indicated by a straight line and those corresponding to the primers used in 5′RACE by dotted lines. An asterisk represents the stop codon (TGA).

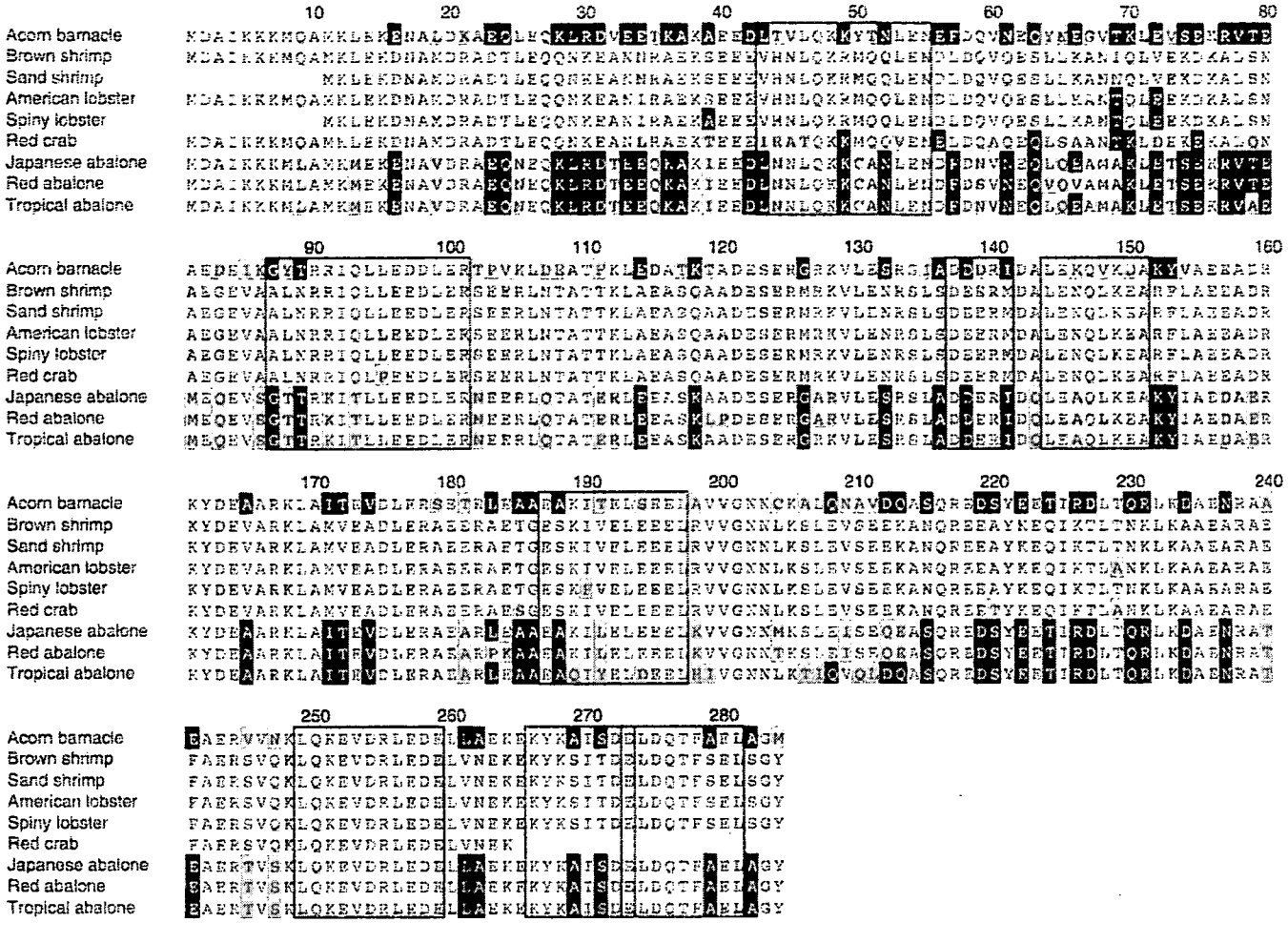

Fig. 3. Alignment of the amino acid sequence of acorn barnacle tropomyosin with those of decapod and abalone tropomyosins. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): acorn barnacle (*Balanus rostratus*), AB269786; brown shrimp (*Penaeus aztecus*), DQ151457; sand shrimp (*Metapenaeus ensis*), U08008; American lobster (*Homarus americanus*) (fast type tropomyosin), AF034954; spiny lobster (*Panulinus stimpsoni*), AF030063; red crab (*Charybdis feriatus*), AF061783; Japanese abalone (*Haliotis diversicolor*), AF216518; red abalone (*Haliotis rufescens*), X75218; tropical abalone (*Haliotis asinina*), AY320360. The residues differing from brown shrimp tropomyosin are shaded. Of the different residues from brown shrimp tropomyosin, common residues between acorn barnacle tropomyosin and other tropomyosins are shown in white on the black background. The IgE-binding epitopes proposed for brown shrimp tropomyosin are boxed.

incubated with an equal volume of American lobster tropomyosin solution (20 μg/mL) at 37 °C for 1 h and used as a primary antibody.

2.7. Cloning experiments

Total RNA was extracted from muscle sample (2 g) of either acorn barnacle or goose barnacle with the TRIzol reagent (Invitrogen, Carlsbad, CA). For 3′ rapid amplification of cDNA ends (RACE), first strand cDNA was synthesized from 5 μg of total RNA using the 3′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) as recommended by the manufacturer and used as a template. A forward primer 5′-ATCAAGAAGAA-GATGCAGGCG-3′ (corresponding to ^{4}IKKKMQA^{10}), which was designed from the conserved sequence of the known cDNAs coding for tropomyosins from brown shrimp (DDBJ/EMBL/GenBank accession number: DQ151457), American lobster (AF034954 for fast tropomyosin, AF034953 for slow-twitch tropomyosin and AY521627 for slow-tonic tropomyosin) and red crab (AF061783), was used in PCR in combination with the abridged universal amplification primer (AUAP). Amplification 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 70 °C for 1 min; and 72 °C for 7 min. After electrophoresis on a 1.2% agarose gel and extraction with a QIAquick-Gel Extraction Kit (Qiagen, Tokyo, Japan), the PCR product was determined for its nucleotide sequence using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and a PRISM 310 genetic analyzer (Applied Biosystems). 5′RACE was carried out using the 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) following the manufacturer's instructions. First strand cDNA was synthesized from 5 μg of total RNA using the gene-specific primer 5′-GCGTCCTTGAGTCTCTGGGT-3′ (corresponding to ^{229}TQRLKDA^{235}). The first 5′RACE reaction using the gene-specific primer 5′-GCGTTCTGCAGGGCCTTACA-3′ (corresponding to ^{204}CKALQNA^{210}) and the abridged anchor

primer and the second 5'RACE reaction using the gene-specific primer 5'-TAGCAAGTTTACGGGCAGCC-3' (corresponding to ^{164}EAARKLAI^{171}) and the AUAP were completed under the same conditions as adopted for 3'RACE. Nucleotide sequencing of the second PCR product was also performed as in the case of 3'RACE.

2.8. Phylogenetic analysis

Multiple alignments of tropomyosins from various animals 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 1000 replications.

3. Results

3.1. Allergens of barnacles

On SDS-PAGE, all the heated extracts from two species of decapods (American lobster and black tiger prawn) and two species of barnacles (acorn barnacle and goose barnacle) afforded a prominent protein band of about 37 kDa at almost the same position as American lobster tropomyosin (Fig. 1A). Irrespective of the species, the 37 kDa protein reacted with the monoclonal antibody 2A7H6 against American lobster tropomyosin (Fig. 1B), which recognizes both crustacean and molluscan tropomyosins (Lu et al., 2007), allowing us to conclude that the 37 kDa protein is tropomyosin of each decapod and barnacle. Very interestingly, another monoclonal antibody 5G5E1 specific only to crustacean tropomyosin (Lu et al., 2007) reacted to the 37 kDa protein in two species of decapods but showed no substantial reactivity to that in two species of barnacles (Fig. 1B). This result suggested significant differences in amino acid sequence between decapod and barnacle tropomyosins. Indeed, the elucidated amino acid sequence of acorn barnacle tropomyosin shares no high identity with decapod tropomyosins, as described below in more detail. In IgE-immunoblotting, no bands appeared when the control serum was used (data not shown), while the pooled serum from ten decapod-allergic patients and four individual patient sera were all reactive not only to tropomyosins from two species of decapods but also to those from two species of barnacles (Fig. 1C). As obviously recognized in the case of sera from patients 3 and 4, the tropomyosin bands obtained with two species of barnacles were considerably weak compared to those obtained with two species of decapods. This can be realized by assuming that the barnacle tropomyosins with significantly different amino acid sequences have a lower IgE-binding potency than the decapod tropomyosins. Besides tropomyosin, some IgE-reactive proteins, although much less potent in IgE-binding ability than tropomyosin, were also detected in the heated extracts from decapods and barnacles; for example, a 75 kDa protein in black tiger prawn reacted with the pooled

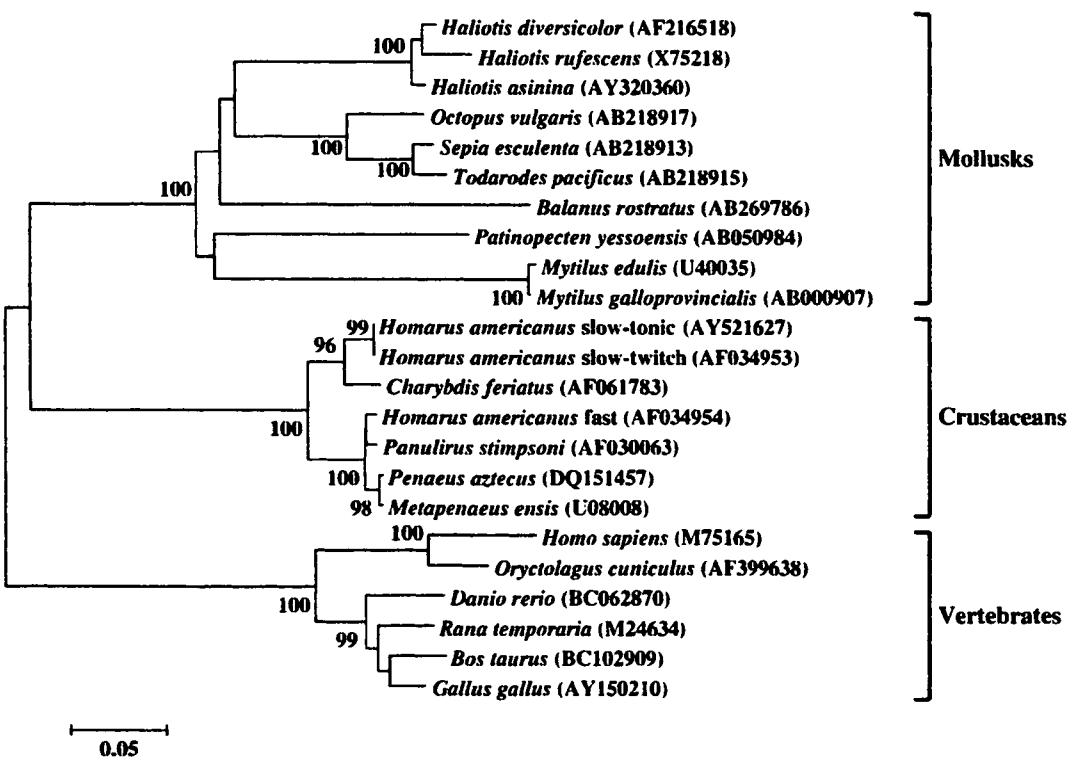

Fig. 4. Molecular phylogenetic tree for tropomyosins from various animals. The tree was constructed by the neighbor-joining method. Bootstrap values >70% are reported on nodes of the tree. The length of branches indicates the genetic distance between the tropomyosins. Accession numbers (DDBJ/EMBL/GenBank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

patient serum and a 30 kDa protein in acorn barnacle with the patient 4 serum. On the whole, however, tropomyosin was almost the sole IgE reactive protein, regardless of the crustaceans and patients. In IgE-immunoblotting inhibition, the patient sera preincubated with American lobster tropomyosin lost their reactivity to tropomyosins from both decapods and barnacles (Fig. 1D), demonstrating the cross-reactivity between decapod and barnacle tropomyosins.

3.2. Primary structure of acorn barnacle tropomyosin

In the case of goose barnacle tropomyosin, no amplified products were obtained by 3′RACE. On the other hand, a cDNA fragment encoding acorn barnacle tropomyosin was successfully amplified by 3′RACE, although the specific primer used was different by one nucleotide from the finally elucidated nucleotide sequence of the acorn barnacle tropomyosin. Based on the nucleotide sequence of the cDNA fragment, the remaining 5′ region sequence was analyzed by 5′RACE. The nucleotide sequence of the full-length cDNA (1070 bp) encoding acorn barnacle tropomyosin was thus established as shown in Fig. 2. This nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases under the accession number of AB269786.

An open reading frame of the acorn barnacle tropomyosin cDNA is composed of 852 bp coding for 284 amino acid residues from the putative initiating methionine to the putative last methionine. The deduced amino acid sequence of acorn barnacle tropomyosin is shown in Fig. 3, together with those from five species of decapod crustaceans and three species of abalones. Tropomyosins of decapod crustaceans share an extremely high sequence identity (more than 90% identity) with one another. Although acorn barnacle is a member of the Crustacea, its tropomyosin has only 58–61% sequence identities with decapod tropomyosins. Interestingly, a homology search by the BLAST program (Altschul et al., 1997) revealed that the highest sequence identity (76–78% identity) with acorn barnacle tropomyosin is displayed by abalone tropomyosins. In order to predict the evolutional position of the acorn barnacle tropomyosin, a molecular phylogenetic tree for tropomyosins was constructed. As shown in Fig. 4, crustacean (decapod), molluscan and vertebrate tropomyosins independently form a specific cluster and the acorn barnacle tropomyosin is classified into the molluscan tropomyosin family.

4. Discussion

Tropomyosin has been established to be a major and cross-reactive allergen in decapod crustaceans (Reese et al., 1999; Lehrer et al., 2003). Our immunoblotting data demonstrated that serum IgE of decapod-allergic patients reacts not only to tropomyosins from two species of decapods (American lobster and black tiger prawn) but also to those from two species of barnacles (acorn barnacle *B. rostratus* and goose barnacle *C. mitella*) locally consumed as a delicacy in Japan. IgE cross-reactivity between decapod and barnacle tropomyosins was also verified by immunoblotting inhibition using American lobster tropomyosin as an inhibitor. Based on these results, we recommend that decapod-allergic patients should avoid consumption of barnacles as well as decapods unless they are clinically diagnosed to be insensitive to barnacles.

Barnacles are not so widely consumed and hence barnacle allergies may be rare. It is, however, worth mentioning that barnacles are crustaceans but significantly distinct in appearance from decapods such as shrimps and crabs. Moreover, they are sessile crustaceans differing from decapods. Therefore, barnacles are not always correctly recognized as members of crustaceans and often misunderstood as members of sessile bivalves or snails by consumers and food servers. In other words, patients allergic to decapods but not to mollusks may mistakenly eat barnacles, resulting in the manifestation of allergic reactions. To prevent accidental incidents of fool allergy by consumption of barnacles, it is particularly important to let the public including decapod-allergic patients fully understand that barnacles are members of the Crustacea, having a potential to cause allergic reactions similar to decapods.

The significance of this study is the finding that the amino acid sequence of the acorn barnacle tropomyosin, elucidated by a cDNA cloning technique, is more homologous to those of abalone tropomyosins than to those of decapod tropomyosins. In accordance with this, the phylogenetic tree made for tropomyosins from various animals showed that the acorn barnacle tropomyosin is evolutionally classified not into the decapod tropomyosin family but into the molluscan tropomyosin family. From the viewpoint of molecular evolution, future study is needed to examine whether the sequence features observed with the acorn barnacle tropomyosin are a common feature of barnacle tropomyosins. In addition, it should be noted that such crustaceans as mantis shrimp (belonging to the order Stomatopoda of the class Malacostraca) and Antarctic krill (belonging to the order Mysidacea of the class Malacostraca) are also edible and may be allergenic. It is, therefore, interesting to elucidate the amino acid sequences of tropomyosins from these crustaceans not only for a molecular understanding on the possible cross-reactivity with decapod tropomyosins but also from the viewpoint of comparative biochemistry.

Although the sequence homology between acorn barnacle and decapod tropomyosins is not so high, the cross-reactivity between them is apparent from the immunoblotting inhibition data. This cross-reactivity can be partly realized by the fact that one (region 249–259) of the eight IgE-binding epitope regions (43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273 and 273–281; refer to Fig. 3) proposed for brown shrimp tropomyosin (Pen a 1) (Ayuso et al., 2002a,b) is completely conserved in the acorn barnacle tropomyosin. However, there are more than two alterations in the other seven regions between acorn barnacle and decapod tropomyosins. It is unclear whether these seven regions are also IgE-binding epitopes of the acorn barnacle tropomyosin. In order to understand in more detail the cross-reactivity between acorn barnacle and decapod tropomyosins, future study using synthetic peptides is needed on the IgE-binding epitopes of acorn barnacle tropomyosin. Such study will also provide helpful information on the IgE-binding epitopes of

abalone tropomyosins that are highly homologous to the acorn barnacle tropomyosin.

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Note

Specific Detection of Soybean Residues in Processed Foods by the Polymerase Chain Reaction

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A sensitive qualitative detection method for soybeans in foods by using the polymerase chain reaction (PCR) was developed. For specific detection of soybeans with high specificity, the primer pair of Gym 81/Gym 82 was designed on the gene encoding the Glycine max repetitive sequence. The trace amount of soybeans in commercial food products could be qualitatively detected by this method.

Key words: food allergy; soybean; *Glycine max* L.; detection method; polymerase chain reaction (PCR)

Food allergies have constituted an important health problem in recent years in industrialized countries, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy.[1-3] To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. Therefore, sufficient information about potentially allergenic ingredients in a food product is necessary.[4,5] In 1999, the Joint FAO/WHO Codex Alimentary Commission agreed to label eight kinds of food which contain ingredients known to be allergens, including soybeans.[6,7] In Japan, the Ministry of Health, Labor and Welfare has enforced a labeling system for allergenic food material since April 2002.[3] With this system, labeling of five food products (egg, milk, wheat, buckwheat, and peanuts) is mandatory, and is recommended for twenty other food materials such as soybeans and shrimp. Soybean labeling is particularly important, because of the almost unlimited uses of soybean, and the number of patients with an allergy to soybeans has been increasing.[8-11] In the present study, we developed a detection method for soybeans with high specificity and sensitivity using PCR. We show that the established method could be applicable for processed food products, and a trace amount of soybeans contained in a commercial food product can be detected by the proposed method.

Nine varieties of soybean (*Glycine max* L.), including seven varieties of domestic soybean (Toyokomachi, Toyomusume, Ryuhou, Tachinagaha, Enrei, Fukuyutaka and Murayutaka) and two varieties of U.S. soybean (Vinton and Navy), were collected in our laboratory. Legume relatives such as two varieties of the common bean (*Phaseolus vulgaris* cul. Tebou and *Phaseolus vulgaris* cul. Toramame), azuki bean, cowpea, broad bean, pea, lentil, lupine, chickpea and peanut, and grain samples of wheat, rye, barley, oats, corn, rice, buckwheat and rapeseed were also purchased by our laboratory. Eight kinds of nuts (almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan and walnut) were kindly provided by Morinaga Co., Ltd. Soybean-derived food materials including seasonings, lecithin, fiber and protein were collected by our laboratory, and ten kinds of commercial food products with soybeans in the list of ingredients were purchased from a local market in Japan.

The seed materials of the soybeans, other crops, food materials and commercial food products were ground with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and then the homogenous powders were used as the test samples. Wheat was used as the matrix to prepare the model mixed samples. Wheat grains were ground to a fine powder with a grinder and 0, 0.001, 0.005, 0.01, 0.1 and 100% of soybean flour (Nikka Fats & Oils, Tokyo, Japan) was then mixed according to the procedure described in a previous study with a slight modification.[12]

Genomic DNA was extracted from each plant material including soybean flour by using a silica-gel mem-

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Table 1. List of Designed Primers

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	CP 03-5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	Chloroplast DNA, sense	Plants	123 bp
	CP 03-3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	Chloroplast DNA, antisense		
B:	Gym 81	5′-TCA GCA GAT TCA AAT CTC CCA GTG A-3′	Interspersed repetitive element 1, sense	Soybean	118 bp
	Gym 82	5′-CAT CTC AAG AAG CAG AGG AAA GGA C-3′	Interspersed repetitive element 1, antisense		

A: To confirm the validity of DNA extracted from plants for the polymerase chain reaction.
B: For specific detection of soybean.

brane-type kit (DNeasy Plant Mini; Qiagen, Hilden, Germany) according to the procedure described in a previous study with some modification.[13] Genomic DNA was extracted from each soybean-derived food material and commercial food product by using an anion exchange-type kit (Genomic-tip 20/G; Qiagen, Hilden, Germany) according to the procedure described in a previous study.[14] The extracted DNA was diluted with the appropriate volume of distilled water (DW) to a final concentration of 20 ng/μl, and stored at −20 °C until needed. When the concentration of the extracted DNA was less than 20 ng/μl, an undiluted DNA extract was used for the subsequent PCR analysis. The primers were synthesized and purified in a reversed-phase column by Operon Biotechnologies (Tokyo, Japan), and then diluted with the appropriate volume of DW to a final concentration of 50 μmol/l, and stored at −20 °C until needed.

The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of the 5′ and 3′ primers, and 0.625 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), all topped up with DW. When the concentration of the extracted DNA was less than 20 ng/μl, 2.5 μl of an undiluted DNA extract was added to a PCR reaction tube. The reaction was buffered with PCR buffer II (Applied Biosystems), and amplified in a thermal cycler (PTC-220 DNA Engine DYAD; Bio-Rad Laboratories, CA, USA). The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. After PCR amplification, agarose gel electrophoresis of the PCR product was carried out according to previous studies.[12,13]

To specifically detect soybeans by using PCR, we investigated the soybean-specific genes such as the Glycine max gene for Bd 30K, the Kunitz trypsin inhibitor, beta-1,3-glucanase, cytosolic glutamine synthetase, the GB-D-II gene for proteinase inhibitor, vspA gene, the Bowman-Birk protease inhibitor, repetitive sequence (soybean interspersed repetitive element 1, SIRE-1) and satellite STR120-A.1. Based on these soybean-specific gene sequences, we designed the respective primer pair and examined PCR amplification by using the designed primer pairs and the mixed samples described later.

Consequently, the Glycine max repetitive sequence (SIRE-1) should be considered to be the most sensitive and specific for amplifying the gene sequence. Therefore, we chose a gene encoding SIRE-1 as a soybean-specific gene and designed the primer pair Gym 81/Gym 82 by referring to GeneBank Accession No. L06326. In addition, the primer pair, CP 03-5′/CP 03-3′, for the universal detection of DNA derived from plants was used to verify the extracted DNAs.[14] The primer pair, CP 03-5′/CP 03-3′, generated a 123-bp amplified fragment. The sequences of the designed oligonucleotides in this study are listed in Table 1.

As shown in Fig. 1A, a fragment (118 bp) amplified by using the primer pair Gym 81/Gym 82 was specifically detected from the soybean genomic DNA. In contrast, no amplified fragment was detected when the DNA was extracted from other legumes such as lupine and peanut, as shown in Fig. 1B. In addition, 16 kinds of other plant species (wheat, rye, barley, oats, corn, rice, buckwheat, rapeseeds, almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan and walnut) as the template DNA are shown in Fig. 1C and D. These data suggest that the soybean-genomic DNA can be specifically detected by using the Gym 81/Gym 82 primer pair.

To assess the sensitivity of the proposed PCR method, we tested the mixed wheat flour samples containing 0, 0.001%, 0.005%, 0.01%, 0.1% and 100% of the soybean flour powder. Fifty ng of the genomic DNA extracted from the mixed samples was amplified in the PCR reaction. As expected, the target sequence for the soybeans was clearly detected in the 0.001% to 100% mixed samples. This result suggested that the contaminated soybean flour in wheat flour can be at least detected at the level of 0.001% in a product without processing. To investigate the applicability of the soybean DNA detection method for commercial food products and food materials, we purchased 18 food items and tested them for the presence of soybean DNA by using the

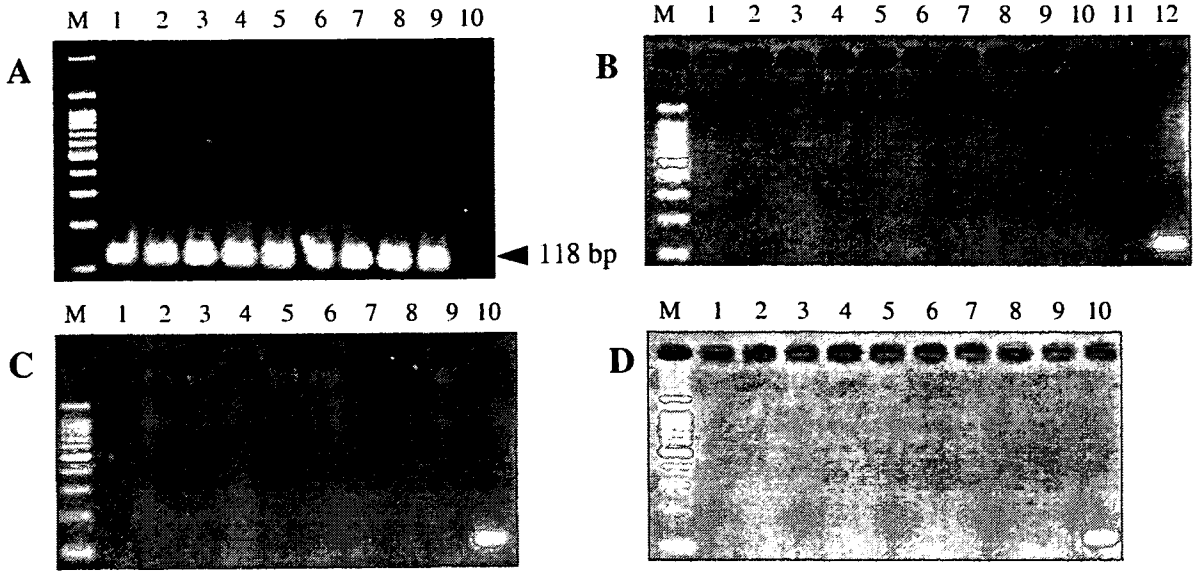

Fig. 1. Specificity of the Polymerase Chain Reaction Method Using the Primer Pair Gym 81/Gym 82 to Identify the DNA Sequence of the Gene Encoding Soybean Interspersed Repetitive Element 1.

A, Agarose gel electrophoresis of PCR products amplified from nine varieties of soybean genomic DNA [lane 1, Toyokomachi; lane 2, Toyomusume; lane 3, Ryuhou; lane 4, Tachinagaha; lane 5, Enrei; lane 6, Fukuyutaka; lane 7, Murayutaka; lane 8, Vinton; lane 9, Navy; lane 10, no template control; M, 100-bp ladder size standard]. B, Agarose gel electrophoresis of PCR products amplified from legume-relative genomic DNA [lane 1, Toramame; lane 2, Tebo; lane 3, azuki bean; lane 4, cowpea; lane 5, broad bean; lane 6, pea; lane 7, lentil; lane 8, lupine; lane 9, chickpea; lane 10, peanut; lane 11, no template control; lane 12, soybean; M, 100-bp ladder size standard]. C, Agarose gel electrophoresis of PCR products amplified from grain and rapeseed genomic DNA [lane 1, wheat; lane 2, rye; lane 3, barley; lane 4, oats; lane 5, corn; lane 6, rice; lane 7, buckwheat; lane 8, rapeseeds; lane 9, no template control; lane 10, soybean; M, 100-bp ladder size standard]. D, Agarose gel electrophoresis of PCR products amplified from nut genomic DNA [lane 1, almond; lane 2, cashew; lane 3, macadamia; lane 4, pistachio; lane 5, hazelnut; lane 6, Brazil nut; lane 7, pecan; lane 8, walnut; lane 9, no template control; lane 10, soybean; M, 100-bp ladder size standard].

proposed method. A sufficient amount (20 ng/μl) of genomic DNA for PCR was obtained from almost all the food products by using the purification method already described, except for the fried tofu and seasoning A. All the food product samples had soybean labeled on the list of ingredients. As shown in Table 2, the universal primer pair CP 03-5′/CP 03-3′ could generate a specific amplified fragment from all of the samples except for the fried tofu and seasoning A. In 16 food products except the fried tofu and seasoning A, soybean DNA was clearly detected with the PCR method by using the Gym 81/Gym 82 primer pair. Based on these results, we postulate that the absence of amplified PCR products from the fried tofu and seasoning A was probably due to the limited amount of genomic DNA in them. These results suggest that the proposed PCR method using the Gym 81/Gym 82 primer pair would be applicable for identifying soybean in processed food products, except for those that are highly processed.

In summary, a rapid PCR detection method was developed for the specific detection of soybeans. The methods reported in this study are simple, sensitive, and reliable for identifying a trace amount of soybean in processed foods. To minimize the risk of false negative or positive results, we believe that the PCR method can be used to complement the protein-based detection method.

Table 2. Investigation of Commercial Food Products (1–10) and Food Materials (11–18)

	Food item	CP 03-5′/CP 03-3′	Gym 81/Gym 82
1	Fish sausage	+	+
2	Loin roll	+	+
3	Bread	+	+
4	Soymilk drink	+	+
5	Biscuit	+	+
6	Roasted soybean tea	+	+
7	Potato chips	+	+
8	Fried tofu	−	−
9	Retort rice porridge	+	+
10	Retort sauce	+	+
11	Soy sauce	+	+
12	Miso	+	+
13	Seasoning A*	−	−
14	Seasoning B*	+	+
15	Soybean lecithin	+	+
16	Bean curd refuse powder	+	+
17	Soybean fiber	+	+
18	Soy protein	+	+

+, positive; −, negative

*Seasonings containing hydrolyzed soy protein.

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Note

Detection of Walnut Residues in Processed Foods by Polymerase Chain Reaction

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A sensitive qualitative detection method for walnut (*Juglans regia*) using polymerase chain reaction (PCR) was developed. For detection of walnuts with high specificity, the primer pair WAL-F/WAL-R was designed based on walnut matK genes. Trace amounts of walnuts in commercial food products can be qualitatively detected using this method.

Key words: allergen; walnut; pecan nut; PCR; restriction enzyme

Botanical food allergies, including allergy to walnuts (*Juglans regia*), represent a crucial and growing health problem in industrialized countries.[1–4] In Japan, it has become clear from epidemiological investigations that the number of patients with walnut allergy has increased.[5,6] The Ministry of Health of the Japanese government has enforced a labeling system for allergenic food materials since April 2002.[5] In this system, labeling of five food products (egg, milk, wheat, buckwheat, and peanuts) is mandatory, while it is recommended for 20 other food materials, including walnuts. Therefore, the development of a rapid detection method for hidden walnut residues in foods is desirable. Recently, a specific detection method for walnuts using real-time PCR equipment was reported by European researchers.[7] While the method is rapid, it also requires expensive real-time PCR instruments and specific probes. PCR methods for detecting wheat, peanuts, soybeans, and kiwifruit have already been established using conventional PCR and electrophoresis.[8–12] In the present study, we developed a detection method for walnuts with sensitivity and specificity using conventional PCR. We show that the method established can be applied to processed food products, and that even trace amounts of walnut contained in a commercial food product can easily be detected by it.

Walnuts and samples of kiwifruit, apple, yam, banana, and soybean were also purchased by our laboratory. Eight kinds of nuts (almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan nut, and walnut) were kindly provided by Morinaga Institute of Biological Science, Inc. (Yokohama, Japan).

Genomic DNA was extracted from each plant material, including walnuts, using a silica-gel membrane-type kit (DNeasy Plant Mini; Qiagen, Hilden, Germany) according to the procedure described in a previous study, with some modifications.[13] Genomic DNA was extracted from each walnut-derived food material and commercial food product using an anion exchange-type kit (Genomic-tip 20/G; Qiagen), according to the procedure described in a previous study.[10] The extracted DNA was diluted with the appropriate volume of distilled water (DW) to a final concentration of 20 ng/μl, and stored at −20 °C until needed. When the concentration of extracted DNA was less than 20 ng/μl, an undiluted DNA extract was used for the subsequent PCR analysis. The primers were synthesized and purified in a reversed-phase column by Operon Biotechnologies (Tokyo), diluted with the appropriate volume of DW to a final

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Table 1. List of Designed Primers for Detection of Walnut

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	CP 03-5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	Chloroplast DNA /sense	Plants	123 bp
	CP 03-3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	Chloroplast DNA /antisense		
B:	WAL-F	5′-GAT CTA TAT TGT TGG AAA ATG TAG C-3′	Chloroplast maturase (matK) gene/sense	Walnut	120 bp
	WAL-R	5′-GGT TAG AAT CAT TAG TGG AAA TCA G-3′	Chloroplast maturase (matK) gene/antisense		

A: For confirmation of validity of the DNA extracted from plants for polymerase chain reaction.
B: For specific detection of walnut.

concentration of 50 μmol/l, and stored at −20 °C until needed.

The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of the 5′ and 3′ primers, and 0.625 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), topped up with DW. When the concentration of the extracted DNA was less than 20 ng/μl, 2.5 μl of an undiluted DNA extract was added to a PCR reaction tube. The reaction was buffered with PCR buffer II (Applied Biosystems), and amplified in a thermal cycler (PTC-220 DNA Engine DYAD; Bio-Rad Laboratories, Hercules, CA). The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 64 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. When the CP 03-5′/CP 03-3′ primer pair was used, the annealing temperature was changed to 60 °C. After PCR amplification, agarose gel electrophoresis of the PCR product was carried out following previous studies.[13] For the design of primer pair WAL-F/WAL-R, Vector NTI Advance version 10 (Invitrogen Japan, Tokyo) was used.

To detect walnuts specifically using PCR, we investigated genes specific to walnuts. We designed primer pairs for various walnut-specific gene sequences, and examined PCR amplification using the designed primer pairs on walnuts and the other nut and food samples. We found that the walnut matK gene was the most specific for amplifying the gene sequence. Hence, we chose the gene encoding walnut matK as a walnut-specific gene, and designed primer pair WAL-F/WAL-R by referring to GeneBank accession no. AF118027. In addition, the primer pair CP 03-5′/CP 03-3′, for the universal detection of DNA derived from plants, was used to verify the extracted DNAs.[10] The primer pair CP 03-5′/CP 03-3′ generated a 123-bp amplified fragment. The sequences of the designed oligonucleotides in this study are listed in Table 1.

As shown in Fig. 1, a fragment (120 bp) amplified using primer pair WAL-F/WAL-R was specifically detected in walnut genomic DNA. In contrast, no amplified fragment was detected when the DNAs extracted from other foods, such as kiwifruit and soybean, were used, as shown in Fig. 1B. In addition, eight other nut species (almond, cashew, macadamia, pistachio, hazelnut, brazil nut, pecan nut, and walnut) were used as template DNA (Fig. 1C and D). The results suggest that only the genomic DNAs from walnuts and pecan nuts are detected using the WAL-F/WAL-R primer pair. In addition, it was confirmed that the detection limit of this PCR for both walnut and pecan nut was 0.5 pg (10 ppm equivalency) when we tested salmon genome DNA samples containing walnut DNA or pecan nut DNA (data not shown). However, according to the current Japanese article standard classification, pecan nuts are not classified as part of the walnut family, and hence, it is necessary to distinguish walnuts and pecan nuts. The matK gene, which is the target gene of this PCR, is known to exist in many edible kinds of walnut, but the gene sequence is unknown for *Juglandaceae*, *Carya illinoensis*, which is a food pecan nut. Hence, for the first time, we identified the gene sequence of matK for pecan nuts using a TA cloning technique. For insertion into the pCR®II-TOPO® vector (TOPO TA cloning® kit, Invitrogen Japan), a partial pecan nut matK gene was obtained by PCR amplification from pecan nut genome DNA with a sense primer (walnut-378F, 5′-GGA TTT CTA ACC ATC TTG TTA TCC T-3′) and an antisense primer (walnut-1295R, 5′-TCC AGA AGA TGT TAA TCG TAA ATG A-3′), under the reaction conditions described above. The 1.0-kb PCR product was cloned into pCR®II-TOPO® vector by standard procedures, and its sequence was analyzed by Hokkaido System Science (Sapporo, Japan). The pecan nut matK gene of the 148 bp fragment, including a domain amplified using the walnut PCR method, is shown in Fig. 2A.

By sequence analysis, we found that the products amplified from walnut and pecan nuts both involve the restriction enzyme site AclI, and we further determined that the products amplified from the walnut matK gene but not from the pecan nut matK gene involve a restriction enzyme site, BfaI (Fig. 2A). Therefore, to determine whether BfaI enzyme digestion discriminates between walnuts and pecan nuts, we examined digestion by the restriction enzyme with an amplification product

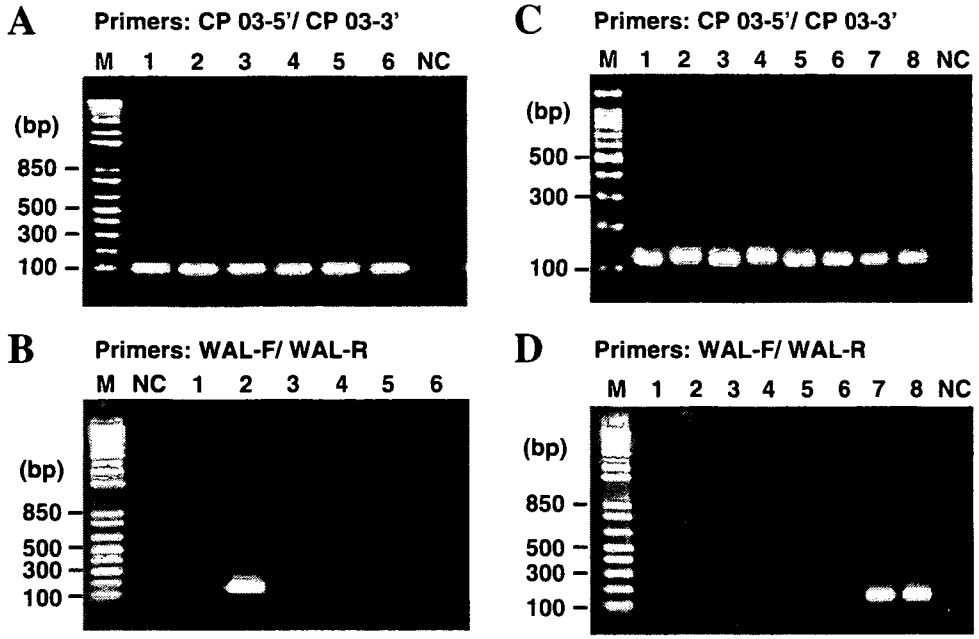

Fig. 1. Specificity of the PCR Method Using Walnut Primers (WAL-F/WAL-R).

A and B, Amplification of DNA of various plants species with primers for plants (A) or walnuts (B). Genome DNAs were obtained from kiwifruit (lane 1), walnut (lane 2), apple (lane 3), yam (lane 4), banana (lane 5), and soybean (lane 6). NC is the no-template control. Amplicons were electrophoresed in a 1.5% agarose gel. M, 100-bp ladder size standard. C and D, Amplification of DNA of various nut species with primers for plants (C) or walnuts (D). Genome DNAs were obtained from almond (lane 1), cashew (lane 2), macadamia (lane 3), pistachio (lane 4), hazelnut (lane 5), Brazil nut (lane 6), pecan nut (lane 7), and walnut (lane 8). NC is the no-template control. Amplicons were electrophoresed in a 2.5% agarose gel (plant) or a 1.5% agarose gel (walnut). M, 100-bp ladder size standard.

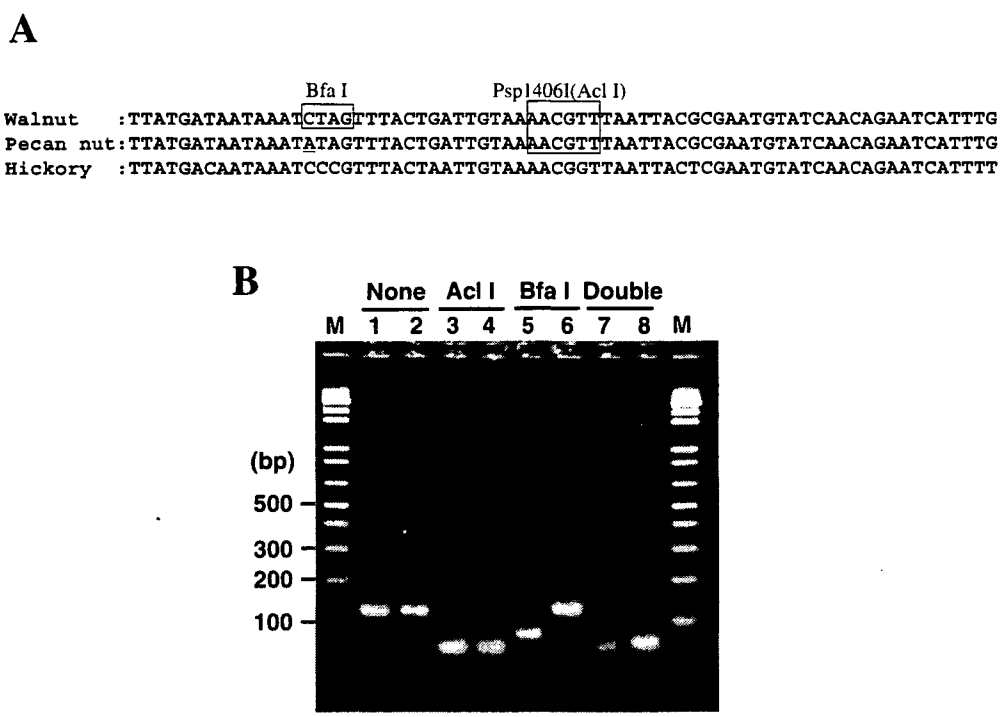

Fig. 2. Comparison of matK Genes of Walnut, Pecan Nut and Hickory (A) and Electrophoresis Analysis in 2.5% Agarose Gels of the Products Obtained with Endonuclease (B).

Primers WAL-F and WAL-R are underlined. GenBank accession nos.: walnut, AF118036, AF118027; hickory, AF118039. B, Odd numbers, walnut; even numbers, pecan nut; lanes 1 and 2, no endonuclease control; lanes 3 and 4, digestion with Acl 1; lanes 5 and 6, digestion with Bfa 1; lanes 7 and 8, digestion with Acl 1 and Bfa 1; M, 100-bp ladder size standard.