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

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ABSTRACT

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

1. Introduction

Food allergy mediated by immunoglobulin E (IgE) is a public concern in industrialised countries. In sensitised subjects with high levels of specific IgE to a certain food, adverse reactions such as urticaria, asthma, diarrhoea and anaphylaxis are induced immediately after ingestion of the food; in severe cases, anaphylactic shock leads to even death. Of a number of allergenic foodstuffs, seafoods (fish, crustaceans and molluscs) are recognised as an important cause of food allergy especially in coastal countries, where their consumption is high. Previous studies showed that parvalbumin, a 12 kDa sarcoplasmic protein, is a major cross-reactive allergen in fish (Lehrer, Ayuso, & Reese, 2003; Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005; Wild & Lehrer, 2005). Besides parvalbumin, collagen has been additionally identified as a fish allergen although not major (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000). As for crustaceans and molluscs, tropomyosin, a 35–38 kDa myofibrillar protein, is a common major allergen (Chuo, Wong, & Leung, 2000; Emoto, Ishizaki, & Shiomi, 2009; Leung et al., 1996; Motoyama, Ishizaki, Nagashima, & Shiomi, 2006; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Reese, Ayuso, & Lehrer, 1999; Wild & Lehrer, 2005). IgE cross-reactivity of tropomyosin can be seen amongst crustaceans, amongst molluscs, between crustaceans and molluscs, and even between crustaceans and terrestrial arthropods, such as cockroaches and mites (Arlian, Morgan, Vyszenski-Moher, & Sharra, 2009; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002). Recently, arginine kinase (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007; Yu, Lin, Chiang, & Chow, 2003), a sarcoplasmic calcium-binding protein (Ayuso et al., 2009; Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008) and myosin light chain (Ayuso et al., 2008) have also been confirmed to be new allergens in crustaceans.

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

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

2. Materials and methods

2.1. Animal samples

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

2.2. Preparation of crude extracts

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

2.3. Purification of tropomyosin

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

2.4. Purification of a 100 kDa allergen

The muscle (5 g) of disc abalone was homogenised with 20 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.15 M NaCl and 0.5 mM cysteine. After centrifugation at 18,000*g* for 20 min, the supernatant obtained was regarded as a sarcoplasmic protein fraction. The residue was washed twice with the above solvent and shaken vigorously with 25 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl and 5 mM DTT at 4 °C, for 14 h. Then, $MgCl_2$ and ATP were added to the solution until a final concentration of 1 mM for each was reached, and shaken again for 1 h. After ultracentrifugation at 117,000*g* for 1 h, the supernatant obtained was used as a myofibrillar protein fraction. To purify the 100 kDa allergen, the myofibrillar protein fraction was first subjected to salting-out using ammonium sulphate. The precipitate at a 10–20% saturation of ammonium sulphate was dissolved in 10 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl, 5 mM DTT, and 0.01% thimerosal, and was applied to hydroxyapatite HPLC on a Bio-Scale CHT2-I column (0.7 × 5.2 cm; Bio-Rad Laboratories, Hercules, CA, USA). Elution was achieved with a linear gradient of 0.01–0.24 M phosphate buffer (pH 6.8) containing 0.9 M NaCl at a flow rate of 1 ml/min. The proteins were monitored at 280 nm with a UV detector and the eluate containing the 100 kDa allergen was collected.

2.5. Enzymatic digestion and isolation of the peptide fragments

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

2.6. Amino acid sequencing

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

2.7. Human sera

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

2.8. Fluorescence ELISA and inhibition ELISA

Fluorescence ELISA was performed as reported previously (Hamada et al., 2004). In brief, a polystyrene microtiter plate with 96 wells (Type H (black); Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of the 100 kDa allergen or the disc abalone tropomyosin solution (1 μg/ml) and reacted successively with the patient or control serum (diluted 1:200) and β-galactosidase-conjugated goat anti-human IgE antibody (0.25 μg/ml; American Qualex, San Clement, CA, USA). The enzyme reaction was carried out using as substrate a solution of 0.1 mg/ml 4-methylumbelliferyl-β-D-galactoside, and was stopped by addition of 0.1 M glycine-NaOH buffer (pH 10.3). Fluorescence units were measured with excitation and emission wavelengths at 367 and 453 nm, respectively. For inhibition ELISA, patient serum (diluted 1:100) was preincubated with an equal volume of inhibitor (100 kDa allergen or disc abalone tropomyosin) solution (0.002–20 μg/ml) at 37 °C for 2 h, and then used as a primary antibody. All ELISAs (including inhibition ELISAs) were performed in triplicate, and the data obtained were expressed in mean ± SD.

2.9. SDS–PAGE

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

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

2.10. Immunoblotting and inhibition immunoblotting

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

2.11. Protein determination

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

3. Results

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

The 100 kDa allergen was mostly recovered in the myofibrillar protein fraction, suggesting that it is one of myofibrillar proteins. Its purification from the myofibrillar protein fraction was achieved by salting-out with ammonium sulphate (10–20% saturation), followed by hydroxyapatite HPLC on a Bio-Scale CHT2-I column. In hydroxyapatite HPLC, the 100 kDa allergen was eluted in a symmetrical peak at a retention time of 23.8 min (Fig. 1A). The 100 kDa allergen thus obtained was confirmed to be homogeneous, as analysed by SDS–PAGE (Fig. 1B).

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

3.2. Reactivity of patient sera to paramyosin and tropomyosin

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

3.3. Distribution of allergenic paramyosin in molluscs

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

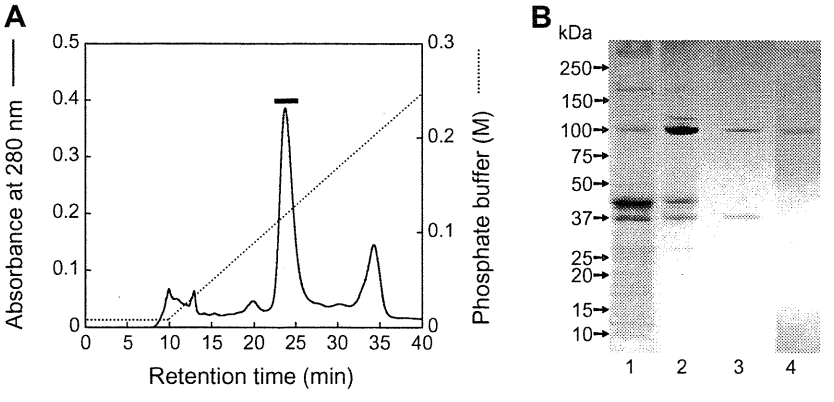

Fig. 1. Purification of a 100 kDa allergen in the disc abalone by hydroxyapatite HPLC (A) and analysis of its homogeneity by SDS–PAGE (B). (A) Sample, precipitate with a 10–20% saturation of ammonium sulphate from the myofibrillar protein fraction; column, Bio-Scale CHT2-I column (0.7 × 5.2 cm); elution,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

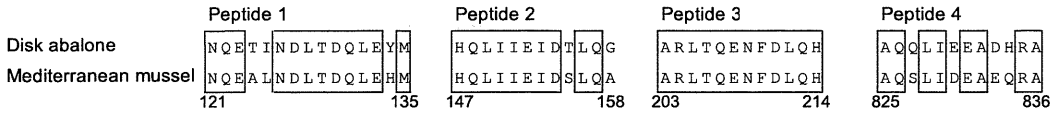

Fig. 2. Amino acid sequences of peptides 1–4 produced by digestion of the purified 100 kDa allergen with lysylendopeptidase, and the corresponding regions of Mediterranean mussel paramyosin. Identical residues are boxed. The accession number (DDBJ/EMBL/GenBank) for Mediterranean mussel paramyosin is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

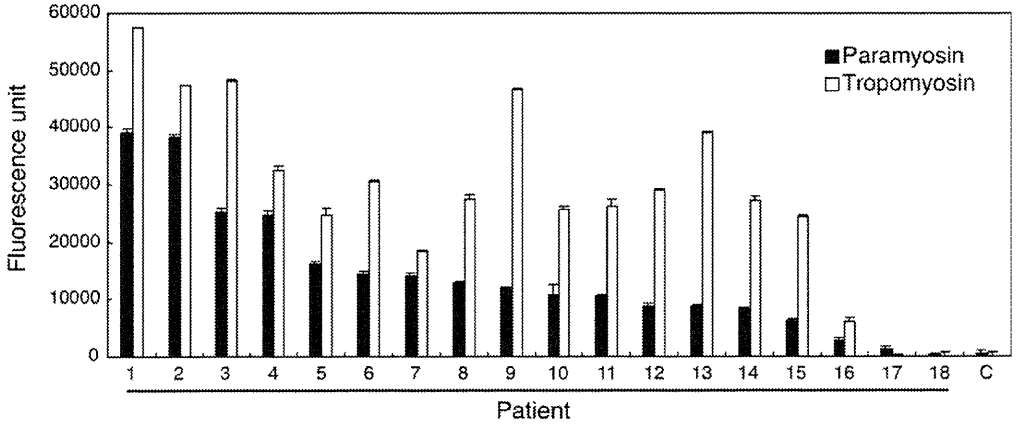

Fig. 3. Analysis of IgE reactivity of paramyosin and tropomyosin purified from the disc abalone by fluorescence ELISA using 18 patient sera. The control data (denoted by C) obtained with 19 healthy subjects were averaged and the values above mean + 2SD (fluorescence units of 1739 and 1466 for paramyosin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

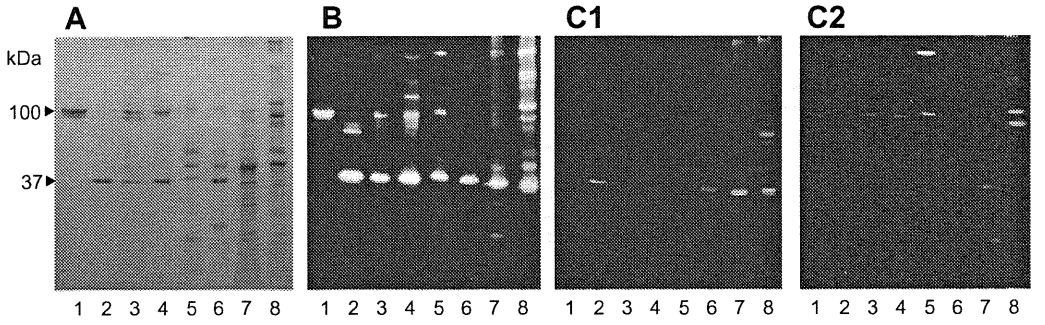

Fig. 4. Analysis of the non-heated extracts from six species of molluscs by SDS–PAGE (A), immunoblotting (B), and inhibition immunoblotting (C). Lanes: 1, disc abalone paramyosin; 2, disc abalone tropomyosin; 3, extract from disc abalone; 4, extract from turban shell; 5, extract from Mediterranean mussel;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

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

3.4. IgE cross-reactivity of paramyosin with tropomyosin

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

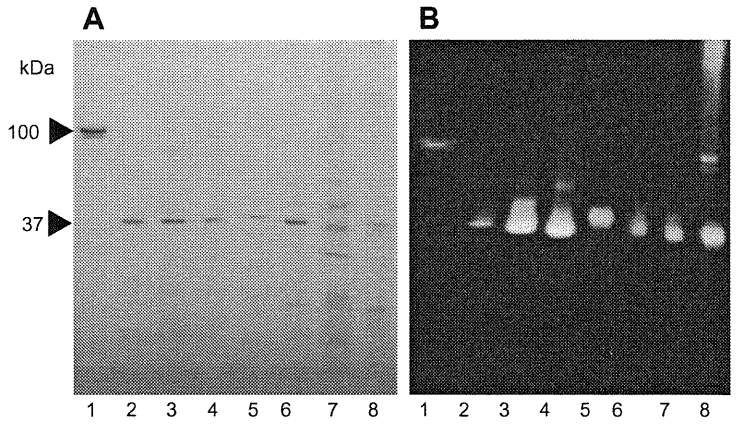

Fig. 5. Analysis of the heated extracts from six species of molluscs by SDS–PAGE (A) and immunoblotting (B) samples are the same as in Fig. 4.

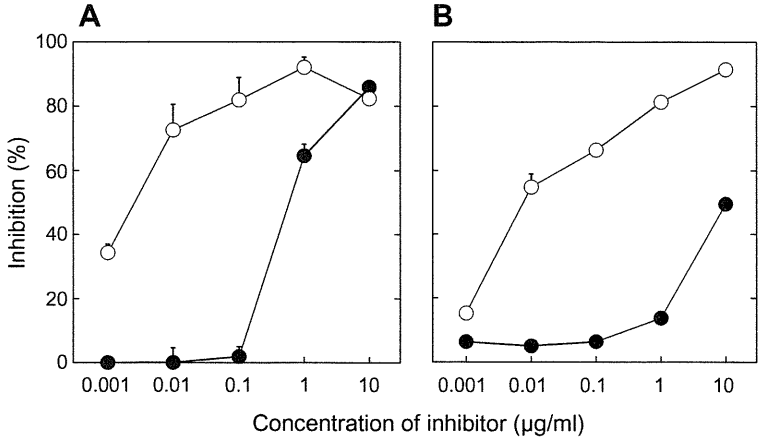

Fig. 6. Quantitative analysis of the IgE cross-reactivity between the disc abalone paramyosin and tropomyosin by inhibition ELISA. A microtiter plate was coated with 50 μl of paramyosin (A) or tropomyosin (B) solution (1 μg/ml). The patient serum (pooled from 10 patients; 1:100 dilution) was preincubated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

4. Discussion

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

As described above, paramyosin is a cross-reactive allergen in some molluscs. Moreover, paramyosin is a major allergen in the disc abalone as demonstrated by fluorescence ELISA, in which as many as 16 of the 18 patient sera tested reacted to paramyosin, as well as to tropomyosin. This is presumably the case with other molluscan paramyosins. Despite these circumstances, paramyosin has long been overlooked in previous studies on molluscan allergens. In relation to this, it should be noted that heating procedures were included in preparing the extracts in previous studies; the extracts were prepared from the homogenates of boiled samples (Leung et al., 1996) or by heating the homogenates of raw samples (Emoto et al., 2009; Motoyama et al., 2006). Since tropomyosin is very thermostable, it can be detected in the extracts by SDS–PAGE and immunoblotting, or can be purified from the extracts by chromatography. In contrast, paramyosin is rather unstable to heating as evidenced in this study. This thermolabile property possibly accounts for the missing of paramyosin in previous studies on molluscan allergens.

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

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

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

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Purification, immunological properties and molecular cloning of two allergenic parvalbumins from the crimson sea bream, *Evynnis japonica*

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ABSTRACT

Parvalbumin, a calcium-binding sarcoplasmic protein of 12 kDa, represents a major allergen of fish. Previous immunoblotting experiments suggested the presence of a 14 kDa allergen, together with parvalbumin, in sea breams. In this study, the 14 kDa allergen (PA I) and parvalbumin (PA II) were purified from the white muscle of crimson sea bream, *Evynnis japonica*, by gel filtration and reverse-phase HPLC. Amino acid sequencing of lysylendopeptidase peptide fragments demonstrated that both PA I and PA II were isoforms of parvalbumin. As analysed by ELISA, PA I showed weaker reactivities with IgG (monoclonal or polyclonal antibody) and serum IgE in fish-allergic patients than did other fish parvalbumins, including PA II. The amino acid sequences of PA I and PA II were elucidated by cDNA cloning. PA I was found to have more specific amino acid residues at several positions compared to the other fish parvalbumins.

1. Introduction

Food allergy is a representative of type I allergies, mediated by immunoglobulin E (IgE) antibodies, and is a public concern in industrialised countries since even death may occur in severe cases. Among a number of allergenic foodstuffs, fish is recognised as an important cause of food allergy, especially in coastal countries, including Japan, where their consumption is high. The major fish allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa, as demonstrated with various species of fish, such as carp (Bugajska-Schretter et al., 1999; Swoboda et al., 2002), salmon (Lindstrøm, van Dô, Hordvik, Endresen, & Elsayed, 1996), cod (Elsayed & Aas, 1971a, 1971b; Elsayed & Bennich, 1975), horse mackerel (Shiomi, Hayashi, Ishikawa, Shimakura, & Nagashima, 1998), mackerel (Hamada et al., 2003) and tuna (Shiomi, Hamada, Sekiguchi, Shimakura, & Nagashima, 1999). Besides parvalbumin, three classes of proteins, namely collagen comprised of three 100 kDa α chains (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000), aldehyde phosphate dehydrogenase of 41 kDa (Das Dores et al., 2002) and transferrin of 94 kDa (Kondo et al., 2006), have so far been identified as fish allergens, although not major ones.

It should be noted that unidentified allergens, such as a 25 kDa allergen in swordfish (Kelso, Jones, & Yunginger, 1996) and a 63 kDa allergen in codfish (Mata et al., 1994), still exist in some species of fish. Our previous immunoblotting experiments have also revealed that the red sea bream, *Pagrus major*, contains a 14 kDa allergen, together with parvalbumin (Kobayashi et al., 2006). The 14 kDa allergen was considered to be distinguishable from parvalbumin, since it did not react with the monoclonal anti-frog muscle parvalbumin antibody. Interestingly, the 14 kDa allergen (having no reactivity with the monoclonal anti-parvalbumin antibody) was also found in the crimson sea bream, *Evynnis japonica*, in our preliminary experiments, suggesting that the 14 kDa allergen seems to be commonly contained in sea breams. For a better understanding of the sea bream allergy, this study was initiated to identify the 14 kDa allergen in the crimson sea bream. As a result, the 14 kDa allergen (named PA I) was demonstrated to be a parvalbumin isoform showing less IgG and IgE reactivities than the other 12 kDa parvalbumin (named PA II) of crimson sea bream and parvalbumins of horse mackerel, *Trachurus japonicas*, and Pacific mackerel, *Scomber japonicus*. We here report the purification, immunological properties and molecular cloning of two allergenic parvalbumin isoforms, PA I and II, from the crimson sea bream.

2. Materials and methods

2.1. Fish samples

Live specimens of crimson sea bream were purchased at the Tokyo Central Wholesale Market and fresh specimens of horse mackerel and Pacific mackerel at a local retail shop. White muscle samples collected from these fish were stored at −20 °C until used. For molecular cloning experiments, the white muscle obtained from a live specimen of crimson sea bream was immediately frozen in liquid nitrogen and stored at −80 °C until used.

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2.2. Preparation of heated extract

Each muscle sample was homogenised with three volumes of 0.15 M NaCl–0.01 M phosphate buffer (pH 7.0). The homogenate was heated in a boiling water bath for 10 min, cooled to room temperature and centrifuged at 18,800*g* for 20 min. The supernatant obtained was regarded as a heated extract.

2.3. Purification procedure

Parvalbumins of horse mackerel and Pacific mackerel were individually purified from the heated extract by a combination of gel filtration and reverse-phase HPLC, as reported previously (Hamada et al., 2003; Shiomi et al., 1998). PA I and PA II of crimson sea bream were similarly purified as now described. The heated extract was subjected to gel filtration on a Sephadex G-75 column (2.5 × 100 cm; GE-Healthcare, Buckinghamshire, UK), which was eluted with 0.15 M NaCl–0.01 M phosphate buffer (pH 7.0) at a flow rate of about 35 ml h^{-1}. Fractions of 10 ml were collected, measured for absorbance at 280 nm and subjected to SDS–PAGE to identify the presence of PA I and/or PA II. The fractions containing both PA I and PA II were combined and applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh, Tokyo, Japan). The column was eluted at a flow rate of 1 ml min^{-1} with a linear gradient of acetonitrile, 0–70% (v/v) in 60 min, in 0.1% (v/v) trifluoroacetic acid. Proteins were monitored at 220 nm with a UV detector and the eluate corresponding to each peak was manually collected and analysed by SDS–PAGE.

2.4. SDS–PAGE

SDS–PAGE was performed on a PhastSystem apparatus (GE-Healthcare), using ready-made gels (PhastGel Gradient 8–25; GE-Healthcare) and ready-made buffer strips (PhastGel SDS Buffer Strips; GE-Healthcare), according to the manufacturer's instructions. Each sample was dissolved in 0.0625 M Tris–HCl buffer (pH 7.4) containing 2% (w/v) SDS, 4 M urea and 0.1 M dithiothreitol, heated at 100 °C for 10 min and subjected to electrophoresis. Precision Plus Protein Standards (10–250 kDa; Bio-Rad Laboratories, Hercules, CA, USA) were run as references, along with samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

2.5. Protein determination

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard protein.

2.6. Human sera

Sera were obtained from 12 fish-allergic patients. Written informed consent was obtained from each patient. Also, this study (using the sera) was approved by the ethics committees of Chiba University, Fujita Health University and Yokohama City University. All patients had been diagnosed to be allergic to fish at hospitals, based on the documented clinical histories of immediate hypersensitive reactions after ingestion of fish and capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) classes of 2–4 against some fish, such as salmon, mackerel and tuna. Furthermore, the patient sera were all verified to have specific IgE to fish parvalbumin by ELISA, in which both parvalbumin and collagen (purified from bigeye tuna, *Thunnus obesus* muscle) were used as antigens, as described in our previous paper (Hamada et al., 2001). In this study, a pooled serum from 10 healthy volunteers, without adverse reactions after ingestion of any foods, was used as a control.

2.7. ELISA

IgG reactivity of parvalbumins was examined by visible-light ELISA, according to the method of Ishikawa, Shimakura, Nagashima, and Shiomi (1997). In brief, a flat-bottomed polystyrene microtitre plate, with 96 wells (Type H; Sumitomo Bakelite, Tokyo, Japan), was coated with 50 μl of parvalbumin solution (0.001–10 μg ml^{-1}) and reacted successively with the monoclonal anti-frog muscle parvalbumin antibody (diluted 1:5000; Sigma–Aldrich, St. Louis, MO, USA) and peroxidase-conjugated rabbit anti-mouse IgG antibody (diluted 1:10000; Zymed Laboratories, San Francisco, CA, USA) or with the antiserum (diluted 1:5000; a gift from Mr. H. Nakajima, Maruha Co., Tokyo, Japan) raised in rabbits against Pacific mackerel parvalbumin and peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:10000; American Qualex, San Clemente, CA, USA). Enzyme reaction was carried out at room temperature for 30 min, using substrate solution, 0.1% (w/v) *o*-phenylenediamine and 0.03% (v/v) hydrogen peroxide in 0.05 M phosphate–citrate buffer (pH 5.0), and stopped by addition of 1 M sulphuric acid. The developed colour was measured at 490 nm.

IgE reactivity of parvalbumins was examined by fluorescence ELISA, as reported previously (Hamada et al., 2004). Briefly, a microtitre plate (Type H (black); Sumitomo Bakelite) was coated with 50 μl of parvalbumin solution (1 μg ml^{-1}) and reacted successively with patient or control serum (diluted 1:250) and β-galactosidase-conjugated goat anti-human IgE antibody (0.25 μg ml^{-1}; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out at 37 °C for 18 h using substrate solution, 0.1 mg ml^{-1} 4-methylumbelliferyl-β-D-galactoside-1 mM $MgCl_2$–0.01 M phosphate buffer (pH 7.0), and stopped by addition of 0.1 M glycine–NaOH buffer (pH 10.3). Fluorescence units were measured with excitation and emission wavelengths at 367 and 453 nm, respectively.

2.8. Enzymatic digestion and isolation and amino acid sequencing of peptide fragments

The purified preparations (50 μg each) of PA I and PA II were individually digested with 1 μg of lysylendopeptidase (E.C. 3.4.21.50; Wako Pure Chemicals, Osaka, Japan) in 1 ml of 0.025 M Tris–HCl buffer (pH 8.5) containing 1 mM EDTA and 2 M urea at 37 °C for 24 h. To isolate peptide fragments produced, the digest was subjected to reverse-phase HPLC on the same TSKgel ODS-120T column as described above. The column was eluted at a flow rate of 1 ml min^{-1} by a linear gradient of acetonitrile, 0–70% (v/v) in 120 min, in 0.1% (v/v) trifluoroacetic acid. Peptides were monitored at 220 nm with a UV detector. Amino acid sequencing of the isolated peptides was performed on a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA) by the MS/MS positive method. α-Cyano-4-hydroxycinnamic acid was used as a matrix.

2.9. Cloning experiments

Total RNA was extracted from 2 g of the white muscle of crimson sea bream with TRIzol reagent (Life Technologies, Rockville, MD, USA) and poly(A)+ mRNA was purified by affinity chromatography on oligo(dT)-cellulose, using a mRNA Purification Kit (GE-Healthcare). A part of the purified mRNA was converted to cDNA, followed by ligation of AP1 adapters, using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA). The Marathon cDNA library thus constructed was used as a

template in 3′ and 5′ rapid amplification cDNA ends (RACE). In combination with the AP1 adapter primer, a forward primer (PA-F with the sequence of 5′-TTGAGGAGGAGGAGCTGAAGCT-3′) and a reverse primer (PA-R with the sequence of 5′-AGCTT-CAGCTCCTCCTCCTCAA-3′), which encode the amino acid sequence 58-IEEEELKL-65 conserved in some fish parvalbumins (refer to Fig. 5, see below), were used in 3′RACE and 5′RACE, respectively. Amplification was performed using Ex Taq DNA polymerase (Takara, Otsu, Japan) under the following conditions: pre-incubation at 95 °C for 2 min; 35 cycles consisting of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; final extension at 72 °C for 7 min. The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany) and their nucleotide sequences were determined using a BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and a PRISM 310 genetic analyzer (Applied Biosystems). Based on the determined partial nucleotide sequences, 3′RACE, using a forward primer (PA I-F with the sequence of 5′-CCTTTCAAAGGACTGCAGGAT-3′ corresponding to 1-PFKGLQD-7) and AP1, was performed for PA I and 5′RACE using AP1 and a reverse primer (PA II-R with the sequence of 5′-TGCCTTAACAAGGGCAGCAAA-3′ corresponding to 102-FAALV-KA-108) for PA II. Amplified products were similarly subcloned and sequenced.

3. Results and discussion

3.1. Purification of PA I and PA II

In order to purify PA I and PA II, the heated extract from the white muscle of crimson sea bream was first chromatographed on a Sephadex G-75 column. As monitored by absorbance at 280 nm, three peaks were observed at fractions 23, 47 and 56 (Fig. 1A). Analysis by SDS–PAGE (data not shown) revealed that PA I and PA II were co-eluted in fractions 37–42. It is not strange that the fractions 37–42 have no substantial absorbance at 280 nm, since PA I is devoid of both Trp and Tyr residues, responsible for the absorbance at 280 nm, and PA II has only one Tyr residue (refer to Fig. 5, see below). When the fractions 37–42 were combined and subjected to reverse-phase HPLC on a TSKgel ODS-120T column, two prominent peaks (I and II) were recognised at retention times of 37.5 and 41.0 min (Fig. 1B). On SDS–PAGE, homogeneous PA I and PA II were found in the peaks I and II, respectively (Fig. 1C).

3.2. Identification of PA I and II as parvalbumin isoforms

Following the digestion of the purified PA I with lysylendopeptidase, peptide fragments were isolated from the digest by reverse-phase HPLC on TSKgel ODS-120T (Fig. 2A). Among them, three peptides (named peptides I-1, I-2 and I-3, in the order of elution) were randomly selected and sequenced by MALDI TOF/MS/MS, as shown in Fig. 2A. A homology search by the BLAST programme (Altschul, Gish, Miller, Myers, & Lipman, 1990) proved that the peptides I-1, I-2 and I-3 correspond to the regions 20–27, 97–107 and 45–54 of fish parvalbumins, respectively (refer to Fig. 5, see below). Thus, PA I was definitely identified as an isoform of parvalbumin. In the case of PA II, three (named peptides II-1, II-2 and II-3, in the order of elution) of the lysylendopetidase peptide fragments isolated by reverse-phase HPLC, were similarly sequenced, as shown in Fig. 2B. Obviously, the peptides II-1, II-2 and II-3, respectively, correspond to the regions 73–80, 84–96 and 55–64 of fish parvalbumins (refer to Fig. 5), supporting the idea that PA II is an isoform of parvalbumin. It should be noted that the nucleotide sequence and deduced amino acid sequence of crimson sea bream parvalbumin can be seen in the DDBJ/EMBL/GenBank databases under the accession number AB375264. From the determined amino acid sequences of the lysylendopeptidase peptide fragments, however, either PA I or PA II was judged to differ from the crimson sea bream parvalbumin so far deposited in the databases. Therefore, the parvalbumin in the databases is hereinafter called PA III.

3.3. Immunological properties of PA I and PA II

Analysis by visible-light ELISA showed that PA II had almost the same IgG reactivity with the monoclonal anti-frog muscle parvalbumin antibody as had horse mackerel and Pacific mackerel parvalbumins, while PA I was remarkably low in reactivity compared to the other parvalbumins (Fig. 3A). As for the reactivity with the antiserum raised against Pacific mackerel parvalbumin, PA I reactivity was also considerably lower than the other parvalbumins (Fig. 3B). IgE reactivity of PA I and II was assessed by fluorescence ELISA, using 12 patient sera. Although one patient serum (patient 9 serum) reacted equally to both PA I and PA II, the remaining 11 patient sera apparently showed less reactivity to PA I than to PA II; even no substantial reactivity to PA I was observed in patient 6 serum (Fig. 4A). To compare the IgE reactivity of crimson sea bream parvalbumins (PA I and PA II) with that of horse mackerel and Pacific mackerel parvalbumins, a pooled serum from 12 patients was used in ELISA, due to the scarcity of several sera. As

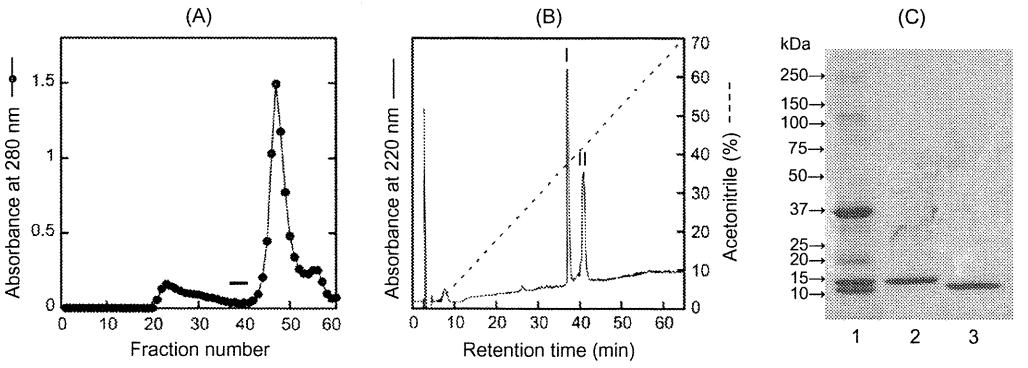

Fig. 1. Purification of PA I (14 kDa allergen) and PA II (12 kDa parvalbumin) from the crimson sea bream. (A) Gel filtration. Sample, heated extract from the white muscle of crimson sea bream; column, Sephadex G-75 column (2.5 × 100 cm); solvent, 0.15 M NaCl–0.01 M phosphate buffer (pH 7.0); volume/fraction, 10 ml. A horizontal bar indicates the fractions (fractions 37–42) containing both PA I and PA II. (B) Reverse-phase HPLC. Sample, fractions 37–42 obtained by gel filtration; column, TSKgel ODS-120T column (0.46 × 25 cm); elution, linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid; flow rate, 1 ml min^{-1}. (C) SDS–PAGE. Samples: lane 1, heated extract; lane 2, purified PA I (peak I in B); lane 3, purified PA II (peak II in B).

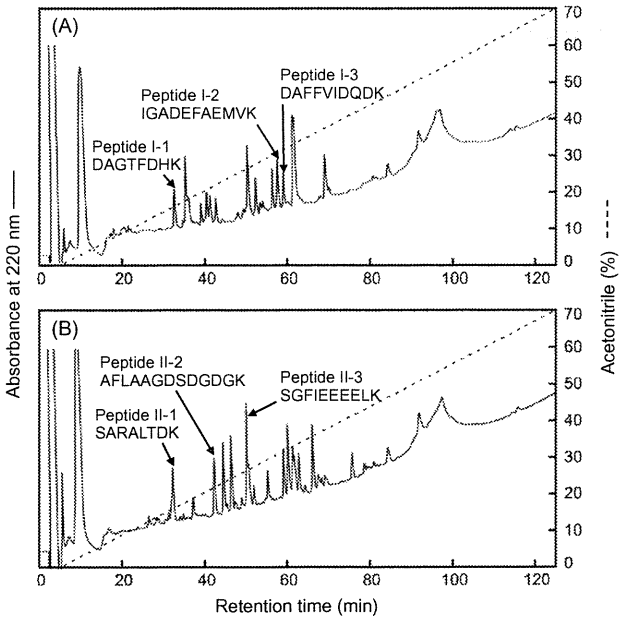

Fig. 2. Isolation of lysylendopeptidase peptide fragments from PA I (A) and PA II (B) by reverse-phase HPLC on TSKgel ODS-120T. The amino acid sequences of three peptides (I-1, I-2 and I-3) from PA I and three peptides (II-1, II-2 and II-3) from PA II, which were elucidated by MALDI TOF/MS/MS, are included in (A) and (B), respectively.

a result, PA I was apparently lower in IgE reactivity than were the other parvalbumins (Fig. 4B).

In our previous immunoblotting experiments, PA II reacted with the monoclonal anti-parvalbumin antibody but PA I did not, leading us to assume that PA I differs from parvalbumin (unpublished data). This erroneous assumption was unavoidable, in view of the fact that PA I is low in IgG reactivity, as quantitatively demonstrated by ELISA in this study. Moreover, it should be noted that the 14 kDa allergen in the red sea bream showed no IgG reactivity and was similarly assumed to differ from parvalbumin (Kobayashi et al., 2006). The 14 kDa allergen in the red sea bream is also likely to be a parvalbumin isoform with low IgG and IgE reactivities as in the case of PA I (14 kDa allergen in the crimson sea bream).

3.4. Molecular cloning of PA I and PA II

About 450 bp products were amplified by 3′RACE, using forward (PA-F) and reverse (AP1) primers. After subcloning into the pT7Blue-2 T-vector, eight clones were obtained and analysed for nucleotide sequencing. As a result, five clones were found to code for PA I, two clones for PA II and one clone for PA III. On the other hand, 5′RACE, using forward (AP1) and reverse (PA-R) primers, afforded about 250 bp products. Nucleotide sequencing of five clones obtained by subcloning showed that two clones code for PA I and three clones for PA III. On the basis of the partial nucleotide sequences, the remaining sequences of the cDNAs encoding PA I and II were analysed by 3′RACE, using forward (PA I-F) and reverse (AP1) primers, and 5′RACE, using forward (AP1) and reverse

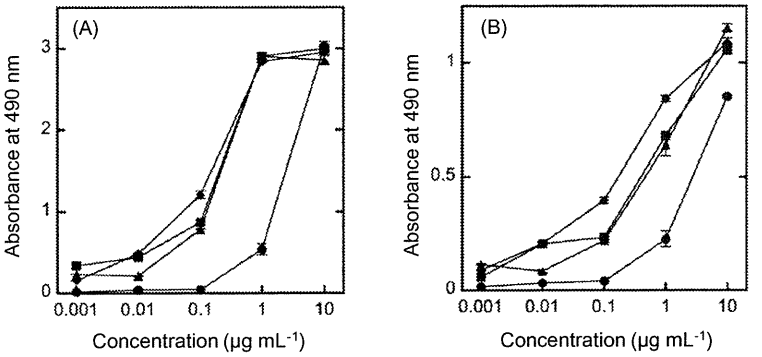

Fig. 3. Analysis of IgG reactivity of PA I (●), PA II (■), horse mackerel parvalbumin (♦) and Pacific mackerel parvalbumin (▲) by visible-light ELISA. Each datum is expressed as mean ± SD (n = 3). (A) Reactivity with the monoclonal anti-frog muscle parvalbumin antibody. (B) Reactivity with the antiserum raised against Pacific mackerel parvalbumin.

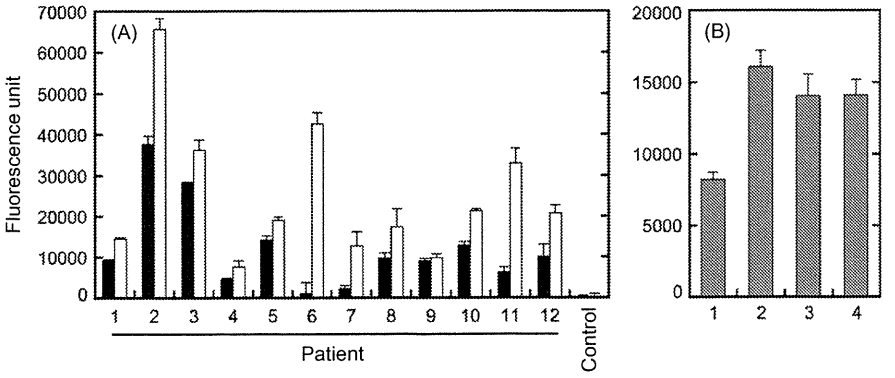

Fig. 4. Analysis of IgE reactivity of PA I, PA II, horse mackerel parvalbumin and Pacific mackerel parvalbumin by fluorescence ELISA. Each datum is expressed as mean ± SD (n = 3). (A) Reactivity of PA I (■) and II (□) with 12 patient sera. (B) Reactivity of PA I (1), PA II (2), horse mackerel parvalbumin (3) and Pacific mackerel parvalbumin (4) with a pooled serum from 12 patients.

(PA II-R) primers, respectively. Sequencing data, after subcloning, revealed that all 13 clones coded for PA I in 3′RACE and all six clones for PA II in 5′RACE. Thus, the nucleotide sequences of the full-length cDNAs (705 bp for PA I and 654 bp for PA II) encoding PA I and PA II were determined and deposited into the DDBJ/EMBL/GenBank databases under the accession numbers AB622511 and AB622512, respectively.

3.5. Amino acid sequences of PA I and PA II

An open reading frame composed of 324 bp is contained in the PA I cDNA and that of 327 bp in the PA II cDNA. The amino acid sequences of PA I (107 residues, excepting the initiating Met), and PA II (108 residues, excepting the initiating Met) are shown in Fig. 5, together with those of some fish parvalbumins, including PA III. The peptides I-1, I-2 and I-3 are all recognised in the sequence of PA I and the peptides II-1, II-2 and II-3 in the sequence of PA II, supporting the accuracy of our nucleotide sequencing.

PA I and PA II are highly homologous (72% identity) with each other and also show high identities (52–80%) with parvalbumins from various species of fish; especially high identities (75–100%) are recognised in the two calcium-binding sites (positions 51–62 and 90–101). Based on the chain length and sequence features, parvalbumins are divided into two types (α- and β-types). Both PA I and PA II are obviously β-type parvalbumins, similar to many fish parvalbumins. First, they are shorter in chain length compared to α-type parvalbumins having 109 residues or more (Moncrief, Goodman, & Kretsinger, 1990). Second, they have six residues (Ala-13, Leu-15, Cys-18, Phe-66, Gln-68 and Thr-78) characteristic of β-type parvalbumins (Goodman & Pecheré, 1977), except for the replacement of Ala-13 by Lys in PA I. PA I is a β-type parvalbumin and its molecular mass (11582.0, calculated from the amino acid sequence) is comparable to those of the other fish β-type parvalbumins. It is still unclear why PA I behaves as a larger protein (14 kDa protein) than its molecular mass in SDS–PAGE.

The IgE cross-reactivity among fish parvalbumins has been established (Hamada et al., 2004; Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005) but their common IgE-binding epitopes, responsible for the cross-reactivity, have been little understood. The linear-type IgE epitopes so far proposed for cod parvalbumin (Elsayed & Apold, 1983) and Pacific mackerel parvalbumin (Yoshida, Ichimura, & Shiomi, 2008) are considered to be not always applicable to various fish parvalbumins. Recently, the IgE reactivities of carp parvalbumin (Swoboda et al., 2007) and Pacific mackerel parvalbumin (Tomura, Ishizaki, Nagashima, & Shiomi, 2008) have been reported to be significantly reduced by depletion of Ca^{2+}, indicating the importance of conformational-type IgE epitopes for the cross-reactivity among fish parvalbumins. Although conformational IgE epitopes of the carp parvalbumin have been suggested, based on the elucidation of mimotopes (Untersmayr et al., 2006), they have not been experimentally confirmed. In the light of current knowledge, therefore, it is difficult to explain why the IgE reactivity of PA I is considerably lower than that of other fish parvalbumins. Nevertheless, it is worth mentioning that several amino acid residues, such as Lys-13, Ala-41 and Asp-45, are specifically included only in PA I, being responsible for the low IgE reactivity.

In conclusion, this study showed that the 14 kDa allergen (PA I), detected in the crimson sea bream, behaves as a larger protein in SDS–PAGE than usual parvalbumins of 12 kDa but is none other than parvalbumin. PA I is low in IgE reactivity and hence will be a useful model molecule in future study on the conformational IgE epitopes of fish parvalbumins. Such a 14 kDa allergen as PA I has already been found in the red sea bream as well (Kobayashi et al., 2006), suggesting its wide distribution in sea breams. For a better understanding of fish allergy, especially sea bream allergy,

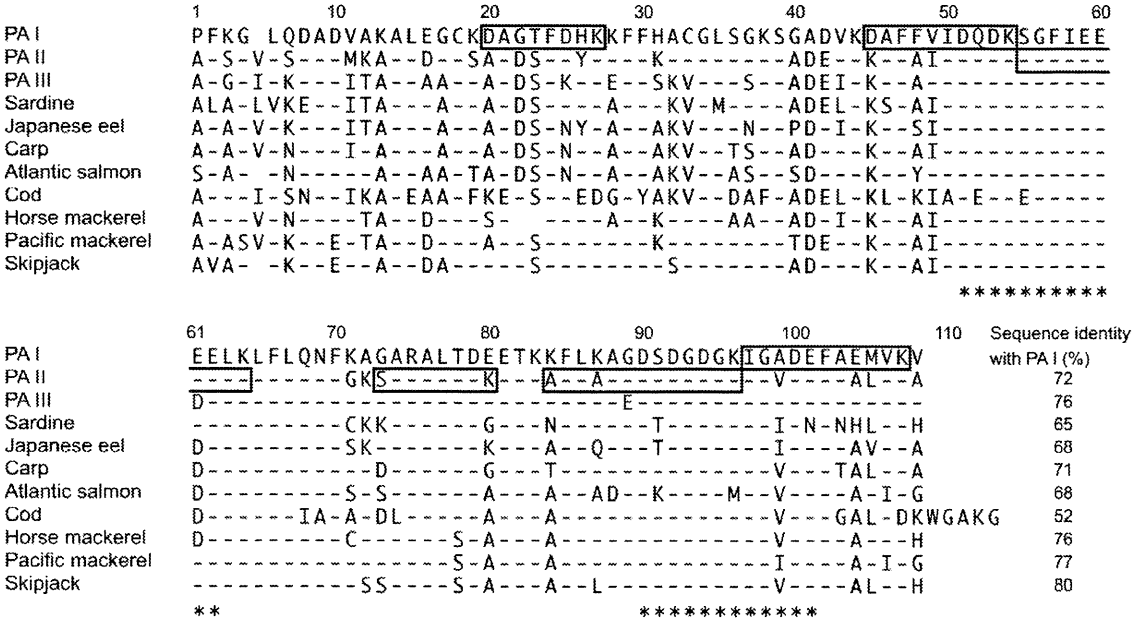

Fig. 5. Alignment of the amino acid sequences of crimson sea bream parvalbumins (PA I–III) with those of some fish parvalbumins. The residues identical with those of PA I are shown by dashes. Calcium-binding sites are indicated by asterisks under the sequence of skipjack parvalbumin. Lysylendopeptidase peptide fragments (peptides I-1, I-2 and I-3 from PA I and peptides II-1, II-2 and II-3 from PA II) are boxed. Accession numbers for parvalbumins (UniprotKB/Swiss-Prot databases for carp and cod parvalbumins and DDBJ/EMBL/GenBank databases for the other parvalbumins): PA I, AB622511; PA II, AB622512; PA III, AB375264; sardine (*Sardinops melanostictus*), AB375262; Japanese eel (*Anguilla japonica*), AB375263; carp (*Cyprinus carpio*), P02618; Atlantic salmon (*Salmo salar*), X97825; cod (*Gadus callarias*), P02622; horse mackerel (*Trachurus japonicus*), AB211364; Pacific mackerel (*Scomber japonicus*), AB091470; skipjack (*Katsuwonus pelamis*), AB375265. Note that gaps are inserted at position 5 of PA I, Atlantic salmon parvalbumin and skipjack parvalbumin and at positions 22 and 23 of horse mackerel parvalbumin.

future study is needed to identify the 14 kDa allergen in sea breams and assess its IgE reactivity.

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ARTICLE

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Differential Detection of Shrimp and Crab for Food Labeling Using Polymerase Chain Reaction

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ABSTRACT: Shrimp and crab are well-known as allergenic ingredients. According to Japanese food allergy labeling regulations, shrimp species (including prawns, crayfishes, and lobsters) and crab species must be differentially declared when ≥10 ppm (total protein) of an allergenic ingredient is present. However, the commercial ELISA tests for the detection of crustacean proteins cannot differentiate between shrimp and crab. Therefore, two methods were developed to discriminate shrimp and crab: a shrimp-PCR method with postamplification digestion and a crab-PCR method that specifically amplifies a fragment of the 16S rRNA gene. The sensitivity and specificity of both PCR methods were verified by experiments using DNA extracted from 15 shrimp species, 13 crab species, krill, mysid, mantis shrimp, other food samples (cephalopod, shellfish, and fish), incurred foods, and commercial food products. Both PCR methods could detect 5 pg of DNA extracted from target species and 50 ng of genomic DNA extracted from incurred foods containing 10 ppm (μg/g) total protein of shrimp or crab. The two PCR methods were considered to be specific enough to separately detect species belonging to shrimp and crab. Although false-positive and false-negative results were obtained from some nontarget crustacean species, the proposed PCR methods, when used in conjunction with ELISA tests, would be a useful tool for confirmation of the validity of food allergy labeling and management of processed food safety for allergic patients.

KEYWORDS: food allergy, shrimp, crab, PCR, differential detection, crustaceans

INTRODUCTION

Crustaceans are consumed in many coastal countries. In Japan, large amounts of shrimp, lobster, spiny lobster, and crab are imported from Asian countries and many other regions, and are processed as materials for commercial foods. Crustaceans are well-known allergens, and several clinical cases have been reported.[1,2] It is known that crustacean allergy generally presents as skin (urticaria, flushing, and itching) and respiratory tract symptoms (dyspnea, wheezing, and cough). Furthermore, anaphylaxis can also be induced in sensitive patients by the intake of trace amounts of crustacean.[2,3]

In many countries, it is recommended that allergenic ingredients, such as wheat, peanuts, and crustaceans, be declared on food labels to alert allergic consumers to their presence. In Japan, the Ministry of Health, Labor and Welfare (MHLW) has enforced food labeling regulations for allergenic food materials since April 2002.[4] Under this system, it was mandatory that five food items (eggs, milk, wheat, buckwheat, and peanuts) be declared on food labels, and it was recommended that 19 others, including shrimp and crab, be declared when possible. Epidemiological investigations in Japan, from 2004 to 2005, have shown that the number of patients with a crustacean allergy are large after the five food items and approximately 65% of shrimp-allergic patients cross-react with crab.[3] In other words, approximately 35% of patients allergic to shrimp have no reaction to crab, although cross-reactivity and cross-sensitization among crustaceans have been reported.[1,2] Taking these results into consideration, in 2008, the MHLW added shrimp/prawn (including crayfish and lobster) and crab as two separate groups to the list of mandatory food items to be declared.[5] Taxonomically, the species belonging to shrimp/prawn group (shrimp species, for brevity) includes those belonging to suborder Dendrobranchiata, and infraorder Caridea, Astacidea, and Achelata of suborder Pleocyemata. The species belonging to crab group (crab species) includes those belonging to infraorder Brachyura and family Lithodidae of suborder Anomura (Figure 1).

In Japanese regulation, PCR is used as a method for confirming positive ELISA screening tests and excluding false positives.[6−9] These methods are complementary and are important for accurate allergenic ingredient testing. In Japan, two commercially available ELISA kits for determining soluble crustacean protein content have already been developed as the official testing method.[10,11] The performance of both ELISA kits satisfies the validation criteria described in the official guidelines published by the Japanese government.[12] However, these ELISA kits, which target tropomyosin, cannot differentiate between shrimp and crab species due to their high amino acid homology. As Japanese labeling regulations recommend separate declaration of shrimp and crab species in processed foods, novel methods discriminating shrimp and crab species would be required to confirm the validity of the labeling.

In this study, we therefore developed two novel PCR methods for differential detection of shrimp and crab species for confirmation of the ELISA results. The sensitivity and specificity of

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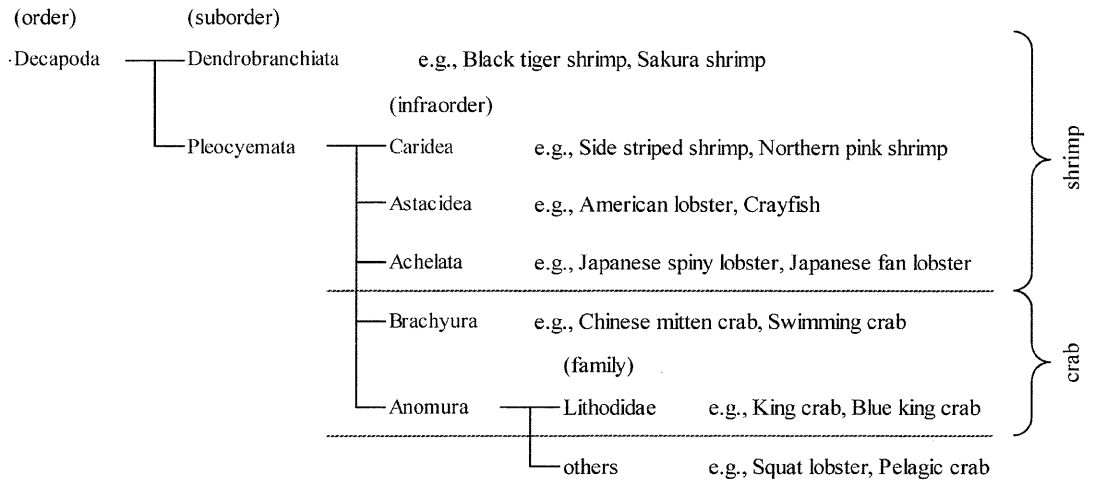

Figure 1. Target species of two PCR methods for detecting shrimp and crab species. Shrimp indicates the species which belong to the suborder Dendrobranchiata and the infraorder Caridea, Astacidea, and Achelata. Crab indicates the species which belong to the infraorder Brachyura and the family Lithoudidae.

the developed methods are examined and the application to commercially processed food analysis is also discussed.

MATERIALS AND METHODS

Samples. Fifteen shrimp species [Kuruma shrimp (*Marsupenaeus japonicus*), Whiskered velvet shrimp (*Metapenaeopsis barbata*), Black tiger shrimp (*Penaeus monodon*), Shiba shrimp (*Metapenaeus joyneri*), Sakura shrimp (*Sergia lucens*), Side striped shrimp (*Pandalopsis japonica*), Northern pink shrimp (*Pandalus eous*), Botan shrimp (*Pandalus nipponensis*), Japanese lobster (*Metanephrops japonicus*), Crayfish (*Procambarus clarkii*), American lobster (*Homarus americanus*), Japanese spiny lobster (*Panulirus japonicus*), Japanese fan lobster (*Ibacus ciliatus*), Cuba lobster (*Panulirus* spp.), and Akiami paste shrimp (*Acetes japonicus*)], thirteen crab species [Snow crab (*Chionoecetes opilio*), Red snow crab (*Chionoecetes japonicus*), Giant spider crab (*Macrocheira kaempferi*), Hair crab (*Erimacrus isenbeckii*), Dungeness crab (*Metacarcinus magister*), Deep sea red crab (*Chaceon granulatus*), Swimming crab (*Portunus trituberculatus*), Chinese mitten crab (*Eriocheir sinensis*), Spanner crab (*Ranina ranina*), King crab (*Paralithodes camtschaticus*), Blue king crab (*Paralithodes platypus*), Spiny king crab (*Paralithodes brevipes*), and Golden king crab (*Lithodes turritus*)], and three other crustaceans species that do not belong to either of the groups [Pacific krill (*Euphausia pacifica*), Mysid (*Neomysis intermedia*), and Mantis shrimp (*Oratosquilla oratoria*)] were purchased at markets in Tokyo and Chiba, Japan, or were provided by Maruha Nichiro Holdings, Inc. (Tokyo, Japan). The other samples, such as cephalopod, shellfish, fish, cereal grain, fruits, and vegetables, were purchased at local stores in Chiba.

Preparation of Incurred Foods. Six incurred foods were prepared to validate the sensitivity of each PCR detection method. Freeze-dried muscle powder of Black tiger shrimp/King crab, which was kindly provided by Maruha Nichiro Holdings, Inc., was added to materials prior to processing for a final concentration of soluble shrimp/crab protein at 10 μg/g of food sample. The muscle powder protein concentration was determined using a 2-D Quant Protein assay kit (GE Healthcare UK, Ltd., Little Chalfont, U.K.). The recipes for making each incurred sample are described below. The freeze-dried soup was made of broccoli, carrot, onion, chicken consommé, starch, and sweet cooking rice wine. Consommé, starch, sweet cooking rice wine, water, and the spiking shrimp/crab powder were stirred over medium heat until thickened. Boiled broccoli and carrot were added to the mixture, and cooled to −80 °C in a freezer, and then freeze-dried at −50 °C. The miso soup paste was made of miso, dry gluten, and dry welsh onion. Miso paste and water were thoroughly mixed with the spiking shrimp/crab powder. The mixture was packaged in a retort pouch, treated in a constant-temperature oven at 86 °C for 5 min, cooled in flowing water for 5 min, and then mixed with dry gluten and dry welsh onion. The soup powder was made of dry konjac noodles and commercial powdered flavoring, and was thoroughly mixed with the spiking shrimp powder. The dry condiment sprinkled on rice was made of fried rice cake, dried bonito, grated sesame seeds, and salt. These were mixed with the spiking crab powder and dried at 105 °C for 5 min. The rice gruel was made of rice and water. Rice and water were mixed with the spiking shrimp/crab powder and cooked in a rice cooker. The cream croquette, provided by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan), was made of milk, butter, flour, cornstarch, salt, sugar, pepper, and bread crumbs. Flour was sautéed in butter for 4 min, hot milk was added and stirred until the sauce became homogeneous, and then starch, salt, sugar, pepper, and the spiking shrimp/crab powder were added. The mixture was breaded and cooled in the freezer at −20 °C. The chicken meatball, containing shrimp/crab powder, was provided by Maruha Nichiro Holdings, Inc.[10]

Commercial Food Products. Twenty-seven commercial food products, 11 products with declaration of shrimp, 6 products with declaration of crab, 2 products with declaration of shrimp and crab, and 8 products without declaration of shrimp or crab in the list of ingredients, were purchased from local stores.

Preparation of DNA Templates. Genomic DNA was extracted from 0.2 g of sample and 2 g of homogenized incurred food or commercial food product with 2 and 20 mL of buffer G2 (Qiagen, Hilden, Germany), respectively, and purified using Genomic-tip 20/G (Qiagen) according to the manufacturer's instructions with slight modifications. DNA concentrations were determined by measuring UV absorption at 260 nm. All DNA samples were adjusted to a concentration of 20 ng/μL with TE buffer (pH 8.0) or distilled water, and used for PCR template. For the sensitivity studies, the shrimp/crab DNA samples were further diluted with 20 ng/μL of salmon testis DNA (Sigma Chemical Co., St. Louis, MO) solution. All of the DNA samples used in the specificity and sensitivity studies gave the expected PCR products with the following primer pairs: AN-5′ and AN-3′, designed to amplify a partial region of mitochondrial DNA; or CP03-F and CP03-R, designed to amplify a partial region of plant chloroplast DNA and used for the quality validation of the DNA.[13]

Primer Design. About 400 crustacean 16S rRNA gene sequences (shrimp, lobster, crab, krill, mysid, and mantis shrimp) were obtained

Table 1. Primer Sequences

detection method for	name	sequence 5′→3′ (with IUPAC mixed base codes)	blend ratio	length of the PCR product
shrimp				
F[a]	ShH12-05′-l,2	TTATATAAAGTCTRGCCTGCC	0.3	185−194 bp
R[b]	ShH 13-03′-1	GTCCCTCTAGAACATTTAAGCCTTTTC	0.1	
	ShH 13-03′-2	GTCCCTTTATACTATTTAAGCCTTTTC	0.1	
	ShH 13-03′-3	GTCCCCCCAAATTATTTAAGCCTTTTC	0.1	
crab				
F	CrH16-05′-l,2	GCGTTATTTTTTTTGAGAGTTCWTATCGTA	0.10	62 bp
	CrH16-05′-3	GCGTAATTTTTTCTGAGAGTTCTTATCATA	0.01	
	CrH16-05′-4,5	GCGTTATTTTTTTTAAGAGTACWTATCGTA	0.06	
	CrH16-05′-6	GCGTTATTTCTTTTGAGAGCTCATATCGTA	0.03	
R	CrH 11-03′	TTTAATTCAACATCGAGGTCGCAAAGT	0.2	
akiami paste shrimp				
F	AsH 11-05′	GGTTGTACAAAAAGAAAGCTGTCTCA	0.3	82 bp
R	ShH13-03′-l,2,3[c]		0.3	
mantis shrimp				
F	StH12-05′-l,2	TTGTATGAATGGTCSGACAAGAT	0.2	95 bp
R	StH12-03′-l,2	ATCGTCCCTCCATATYATTTAAGCTTTTTT	0.2	

[a] Forward primer. [b] Reverse primer. [c] A mixture of primers of equal parts of ShH13-03′-1, -2, -3.

from GenBank. In addition, 16S rRNA gene sequences of shrimp and crab species purchased for this study were determined by a direct sequencing analysis of the amplified products using the sequencing primer pairs, namely, SPP1 (forward primer 5′-CAA ATA TTG TTT CTG CCT GTT TAT C-3′ and reverse primer 5′-AAG ATT TAT AGG GTC TTA TCG TC-3′) for the upstream region and SPP2 (forward primer 5′-TTA AAG GGA CGA TAA GAC CCT ATA A-3′ and reverse primer 5′-TAG ATA GAA ACC AAC CTG GCT-3′) for the downstream region. Two sets of primer pairs based on the highly conserved sequence among the target species, ShH12-05′ and ShH13-03′ for detecting shrimp and CrH16-05′ and CrH11-03′ for detecting crab, were designed to amplify the upstream and downstream regions of the 16S rRNA gene, respectively. PCR simulations were performed with Amplify 1.0 software (Bill Engels, University of Wisconsin, Madison, WI) to predict whether PCR products of the target size would be obtained from the 16S rRNA gene sequences of crustaceans used for food in Japan, such as shrimp, lobster, crab, krill, mysid, and mantis shrimp, and some representative sequences of other crustaceans belonging to the classes Maxillopoda and Branchiopoda, which are not used for food. Two sets of primer pairs, AsH11-05′ and ShH13-03′ for detecting akiami paste shrimp and StH12-05′ and StH12-03′ for detecting mantis shrimp, were also designed in the same manner as above.

Shrimp-PCR and Akiami Paste Shrimp-PCR. For the detection of shrimp, the PCR reaction was carried out in a 25 μL reaction volume containing 0.2 mM of each dNTP, 1× buffer (PCR buffer II), 1.5 mM $MgCl_2$, 0.625 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 0.3 μM of the ShH12-05′ and ShH13-03′ primers, and 5 pg to 50 ng of template DNA. The GeneAmp PCR System 9700 (Applied Biosystems) was set at a ramping speed of 1 °C/s (9600 emulation mode). The amplifications were performed as follows: preincubation at 95 °C for 10 min; 45 cycles consisting of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; and final extension at 72 °C for 7 min. For the detection of akiami paste shrimp species, the PCR reaction was carried out as for the shrimp-PCR, with 0.3 μM of the AsH11-05′ and ShH13-03′ primers. The amplifications were performed as follows: preincubation at 95 °C for 10 min; 45 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 56 °C for 0.5 min, and extension at 72 °C for 0.5 min; and final extension at 72 °C for 7 min. The PCR products (7.5 μL) were electrophoresed on a 2% agarose gel containing 0.3 μg/mL ethidium bromide and analyzed with a ChemiDoc XRS illuminator (Bio-Rad Laboratories, Inc., Hercules, CA).

Restriction Enzyme Digestion of Shrimp-PCR Products. Seventeen microliters of shrimp-PCR product was digested with the restriction enzyme *Hae*III (Takara Bio, Inc., Shiga, Japan) in a final volume of 20 μL for 16 h at 37 °C, according to the manufacturer's instructions. The entire reaction volume was assayed on a 2% agarose gel containing 0.3 μg/mL ethidium bromide and analyzed with a ChemiDoc XRS illuminator.

Crab-PCR and Mantis Shrimp-PCR. For the detection of crab, the PCR reaction was carried out as for shrimp PCR with 0.2 μM of the CrH16-05′ and CrH11-03′ primers. The GeneAmp PCR System 9700 was run with 9600 emulation mode. The amplifications were performed as follows: preincubation at 95 °C for 10 min; 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min. For the detection of mantis shrimp species, the PCR reaction was carried out as for shrimp PCR, with 0.3 μM of the StH12-05′ and StH12-03′ primers. The amplifications were performed as follows: preincubation at 95 °C for 10 min; 34 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min. The PCR products (7.5 μL) were electrophoresed on a 3.5% agarose gel containing 0.3 μg/mL ethidium bromide and analyzed with a ChemiDoc XRS illuminator.

ELISA Procedure for Detection of Crustaceans. ELISA was performed for the determination of crustacean protein using two commercial kits: the Food Allergen Test EIA Crustacean "Nissui" (Nissui Pharmaceutical Co., Ltd.) and the crustacean kit "Maruha" (Maruha Nichiro Holdings, Inc.). One gram of the food product was extracted using 19 mL of extraction buffer. The mixture was shaken horizontally overnight at room temperature, and centrifuged at 3000*g* for 20 min after adjusting the pH to 6.0−8.0. The supernatant was filtered if necessary, diluted 20 times using each kit dilution buffer, and subjected to ELISA according to the manufacturer's instructions. Each sample extract was analyzed in duplicate (2 wells/product extraction), and the average absorbance of 2 wells was calculated.

RESULTS AND DISCUSSION

Primer Design for Shrimp-PCR and Crab-PCR. In this study, we designed PCR primers for the 16S rRNA gene of

Table 2. Predicted Specificity of Primer Pairs with PCR Simulation Software

	(A) ShH12-05′ and ShH13-03′		
crustaceans used for food	GenBank accession no.	match with 3′-end nucleotide of primers[a]	wt no.[b]
suborder Dendrobranchiata		40/40[c]	7/8[d]
e.g., *Metapenaeus affinis*	AY264904	+	5
Metapenaeus ensis	AF279810	+	5
Metapenaeus joyneri	FJ435636	+	5
Penaeus monodon	EU105471	+	/
Penaeus semisulcatus	EU024679	+	5
infraorder Caridea		20/20	15/15
e.g., *Palaemon debilis*	FM986647	+	4
Macrobrachium nipponense	FM986632	+	4
Exopalaemon modestus	EU493144	+	4
Pandalus latirostris	AB244633	+	4
Plesionika ensis	AY612883	+	4
infraorder Astacidea		21/21	7/7
e.g., *Homarus americanus*	DQ666843	+	4
Cherax tenuimanus	AF492809	+	/
Nephropsis stewarti	AY583891	+	4
Paranephrops zealandicus	EF060258	+	5
Procambarus clarkii	DQ666844	+	4
infraorder Achelata		45/45	8/8
e.g., *Palinurus delagoae*	EF546312	+	/
Palinurus mauritanicus	DQ062208	+	4
Jasus edwardsii	AF337979	+	3
Jasus lalandii	EU221225	+	/
Scyllarides latus	DQ377974	+	/
infraorder Brachyura		2/34	2/2
e.g., *Eriocheir sinensis*	AJ250642	+	4
Metacarcinus magister	AY789473	+	4
Callinectes sapidus	AJ130813	−	−
Chaceon affinis	AF100914	−	−
Telmessus cheiragonus	AB220027	−	−
infraorder Anomura		1/10	1/1
family Lithodidae			
e.g., *Lithodes aequispinus*	AF425329	−	−
Lithodes maja	AF425330	−	−
Paralithodes brevipes	AF425337	−	−
Paralithodes camtschaticus	AF425338	−	−
Paralomis granulosa	AF425339	−	−
Paralithodes sp.	AY789472	+	3
family Galatheidae			
e.g., *Munida gregaria*	EF428963	−	−
Cervimunida johni	AY351244	−	−
Pleuroncodes monodon	AY351259	−	−
family Euphausiidae		0/38	
e.g., *Euphausia longirostris*	AF281273	−	−
order Mysida		0/19	
e.g., *Mesopodopsis slabberi*	AJ966898	−	−
suborder Stomatopoda		0/15	
e.g., *Squilla mantis*	AY639936	−	−
family Balanidae		0/13	
e.g., *Semibalanus balanoides*	AY520728	−	−
order Pedunculata		0/7	
e.g., *Calantica spinosa*	AY428051	−	−

Table 2. Continued

	(B) CrH16-05′ and CrH11-03′			
crustaceans used for food		GenBank accession no.	match with 3′-end nucleotide of primers[a]	wt no.[b]
infraorder Brachyura			49/53[c]	46/53[d]
	e.g., *Atelecyclus undecimentatus*	AM946018	+	4
	Callinectes sapidus	AJ298189	+	4
	Cancer irroratus	AJ130812	+	4
	Chaceon affinis	AF100914	+	4
	Chionoecetes opilio	AY227445	+	4
	Erimacrus isenbeckii	AB197677	+	4
	Eriocheir sinensis	AJ250642	+	4
	Loxorhynchus crispatus	EU682798	+	4
	Maja brachydactyla	EU000850	−	−
	Maja squinado	DQ079723	−	−
	Portunus trituberculatus	AM410527	+	4
	Scylla serrata	AF109318	+	4
	Telmessus cheiragonus	AB220027	+	4
infraorder Anomura			12/12	12/12
family Lithodidae				
	e.g., *Lithodes aequispinus*	AF425329	+	4
	Lithodes maja	AF425330	+	4
	Paralithodes brevipes	AF425337	+	4
	Paralithodes camtschaticus	AF425338	+	4
	Paralomis granulosa	AF425339	+	4
family Galatheidae				
	e.g., *Munida gregaria*	EF428963	+	4
	Cervimunida johni	AY351244	+	4
	Pleuroncodes monodon	AY351259	+	4
suborder Dendrobranchiata			0/69	
	e.g., *Metapenaeus affinis*	AY264904	−	−
infraorder Caridea			0/49	
	e.g., *Palaemon debilis*	FM986647	−	−
infraorder Astacidea			0/35	
	e.g., *Homarus americanus*	DQ666843	−	−
infraorder Achelata			6/60	6/6
family Palinuridae				
	e.g., *Palinurus delagoae*	EF546312	−	−
	Palinurus mauritanicus	DQ062208	−	−
	Jasus edwardsii	AF337979	−	−
	Jasus lalandii	EU221225	−	−
family Scyllaridae				
	e.g., *Scyllarides herklotsii*	FJ174906	+	2
	Scyllarides latus	DQ377974	+	2
	Thenus orientalis	FJ174914	+	2
	Thenus unimaculatus	FJ174915	+	2
family Euphausiidae			0/39	
	e.g.,*Euphausia longirostris*	AF281273	−	−
order Mysida			0/19	
	e.g.,*Mesopodopsis slabberi*	AJ966898	−	−
suborder Stomatopoda			15/15	15/15
	e.g., *Squilla mantis*	AY639936	+	4
family Balanidae			0/13	
	e.g., *Semibalanus balanoides*	AY520728	−	−
order Pedunculata			0/7	
	e.g., *Calantica spinosa*	AY428051	−	−

[a] Whether the sequence of the primer's target region matches the 3′ end nucleotides of both primers in the pair is shown + (match) or − (mismatch). [b] An approximate guide to the quality of the matches and the strength of the amplifications. The larger the weight number (1 to 6), the higher the probability of amplification. [−] indicates no PCR product was predicted. [/] indicates sequence for PCR simulation was not available. [c] The number of sequences matched with the 3′ end nucleotide of the primers per the number of representative sequences that have enough length to check the nucleotides at the position corresponding to the 3′ end of the primers. [d] The number of sequences that give a PCR product of the target size per number of representative sequences that have enough length to simulate primer hybridization.

mitochondrial DNA, because multicopy genes that can be used for congeneric or conspecific classification are useful targets for constructing sensitive and specific PCR methods.[14−16] By aligning the 16S rRNA gene sequences of shrimp species, crab species, and the other crustaceans (krill, mysid, and mantis shrimp) obtained from GenBank, two sets of primer pairs for detecting shrimp (ShH12-05′ and ShH13-03′) and crab species (CrH16-05′ and CrH11-03′) were designed (Table 1). The target species for each primer pair are shown in Figure 1. Each primer was designed so that the nucleotides at the position corresponding to the 3′ end of the primer would be the same in the sequences of the target species and would differ from those of the nontarget species. In addition, the primers for detecting crab species were designed to contain a mismatch base at the second position from the 3′ end of primers in all the sequences of the target species to prevent amplification of PCR products from nontarget species.[17] Because of the diversity in 16S rRNA gene sequences within each target group and similarity between the groups to be segregated, it is difficult to design differential PCR methods for varieties of shrimp and crab species. Therefore, we employed mixed primer PCR for both shrimp and crab species to increase the sensitivity and restriction fragment length polymorphisms (RFLP) for the shrimp-PCR method to reduce the chance of false positives originating from some crab.

Specificity Analysis of Both Primer Pairs Predicted Using PCR Simulation Software. The specificity of the primer pairs was predicted with PCR simulation software. With each primer pair, PCR products of the expected sizes (≈190 bp for shrimp ShH12-05′ and ShH13-03′ and 62 bp for crab CrH16-05′ and CrH11-03′) were predicted to be produced from most of the 16S rRNA gene sequences of target species in Table 2. With the ShH12-05′ and ShH13-03′ primer pair, products of the target size were also predicted from nontarget species, such as Chinese mitten crab (*Eriocheir sinensis*) and Dungeness crab (*Metacarcinus magister*), as shown in Table 2A, and from other crustaceans such as hermit crab and some species belonging to the class Branchiopoda and the superorder Syncarida (data not shown). With the CrH16-05′ and CrH11-03′ primer pair, products of the target size were also predicted from nontarget species such as mantis shrimp (*Squilla mantis*), as shown in Table 2B, and from other crustaceans such as hermit crab (data not shown). The PCR products predicted from other crustaceans, including hermit crab, and some species not used for food would not appear to cause significant problems in developing detection methods, because those nontarget species were presumed not to be used as material for processed foods and were thus unlikely to be mixed in processed foods.

Specificity and Sensitivity of the Shrimp Detection Method (Shrimp-PCR and Akiami Paste Shrimp-PCR). The specificity and sensitivity of the proposed shrimp-PCR method combined with restriction enzyme digestion were confirmed experimentally by using food sample DNA. As shown in Figure 2, PCR products of the target size (approximately 190 bp) were amplified from 5 pg of genomic DNA of 14 target species (Figure 2A), and restriction fragments of the target size (approximately 150 bp) were also obtained by restriction enzyme digestion of these PCR products (Figure 2B), but not from the genomic DNA of akiami paste shrimp (Figure 2C). The results from shrimp-PCR of nontarget crab species and the restriction enzyme digestion of the amplified products are presented in Figure 3, panels A and B, respectively. Although a PCR product of the target size was amplified from 50 ng of the genomic DNA of some crab species, such as Red snow crab, Giant spider crab, Dungeness crab, Deep sea red crab, Swimming crab, and Chinese mitten crab (Figure 3A), a restriction fragment of approximately 150 bp was not detected from the PCR products of these crab species, except for Chinese mitten crab (Figure 3B). Thus, the restriction enzyme digestion with *Hae*III can be used for differentiating shrimp species from most of the crab species that would give false positive products in shrimp-PCR. Nonspecific products often amplified from some of the other food samples (cephalopod, shellfish, fish, cereal grain, fruits, and vegetables) were clearly different in size from the target products (data not shown).

Dried akiami paste shrimp (*Acetes japonicus*), which gave a false-negative result in shrimp-PCR, could be used in *okonomiyaki* (a Japanese pancake dish). We considered the solution of this problem to be very important in ensuring appropriate labeling. Therefore, we developed a PCR method for detecting akiami paste shrimp with the AkH11-05′ and ShH13-03′ primer pair (Table 1) for use in combination with shrimp-PCR. As the akiami paste shrimp sequence was not available from GenBank, we determined the sequence of the upstream region of 16S rRNA gene of akiami paste shrimp by direct sequencing analysis (GenBank accession number AB583753, AB583754, AB583755). Analysis of the sequence data revealed that the sequence of the forward primer (ShH12-05′ primer) binding region was different from that of Sakura shrimp (*Sergia lucens*), which belongs to the same family (Sergestidae) as akiami paste shrimp. Thus, we designed a new forward primer (AkH11-05′) for the 16S rRNA gene based on a highly conserved sequence among the akiami paste shrimp species. The proposed akiami paste shrimp-PCR method gave PCR products with a target size of 82 bp from 5 pg of genomic DNA from akiami paste shrimp and some other shrimp samples but not from 50 ng of genomic DNA from other crustaceans such as crab, krill, and mysid and other food samples (cephalopod, shellfish, fish, cereal grain, fruits, and vegetables) (data not shown).

Specificity and Sensitivity of the Crab Detection Method (Crab-PCR and Mantis Shrimp-PCR). The specificity and sensitivity of the proposed crab-PCR method was confirmed using food sample DNA. As shown in Figure 4A, a PCR product of the target size (62 bp) was amplified from 5 pg of the genomic DNA from 13 target species. As shown in Figure 4B, PCR products of the target size were amplified from 5 pg to 50 ng of mantis shrimp (squilla) genomic DNA and sporadically amplified from 50 ng of Japanese lobster and American lobster genomic DNA. Although nonspecific products were often amplified from the genomic DNA extracted from other food samples (cephalopod, shellfish, fish, cereal grain, fruits, and vegetables), all of them were different in size from the target (data not shown).

As trace mantis shrimp DNA (5 pg) also produced a product matching the target size in the crab-PCR method, commercial foods contaminated by mantis shrimp without any crab contamination could be falsely positive. Therefore, we developed the mantis shrimp-PCR method to check for the presence of mantis shrimp in a crab-PCR positive commercial food. The PCR reaction was performed for 34 cycles to detect 5 pg of mantis shrimp DNA with detection sensitivity approximately equal to that of crab-PCR. The proposed PCR method for detecting mantis shrimp with the StH12-05′ and StH12-03′ primer pair (Table 1) amplified the PCR product from 5 pg of genomic DNA from mantis shrimp but not from 50 ng of genomic DNA from other crustaceans such as shrimp and crab species (data not shown).

Specificity and Sensitivity of Shrimp and Crab Detection Methods. Both PCR methods for detecting shrimp and crab

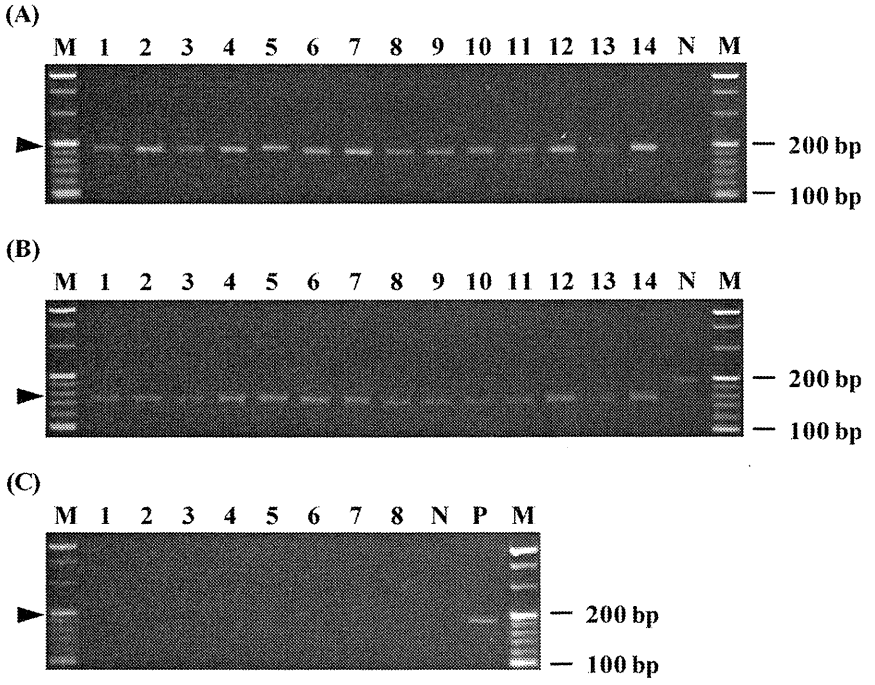

Figure 2. Specificity of the shrimp-PCR method for target species. The arrowheads indicate the expected size of PCR (A, C) and RFLP products (B). M, DNA marker (20 bp ladder). Lanes 1−14 of panels A and B, amplification of 5 pg of genomic DNA and restriction digestion of PCR products of genomic DNA, respectively, from Kuruma shrimp (1), Whiskered velvet shrimp (2), Black tiger shrimp (3), Shiba shrimp (4), Sakura shrimp (5), Side striped shrimp (6), Northern pink shrimp (7), Botan shrimp (8), Japanese lobster (9), American lobster (10), Crayfish (11), Japanese spiny lobster (12), Japanese fan lobster (13), and Cuba lobster (14). Lanes N, no template (A) and nonrestriction digestion of PCR products of Kuruma shrimp as a negative control (B). Lanes 1−8 of panel C, amplification of 50 pg (1, 2, 3, 4) and 5 pg (5, 6, 7, 8) genomic DNA from akiami paste shrimp. N, negative control (no template). P, amplification of 5 pg of Kuruma shrimp genomic DNA as a positive control.

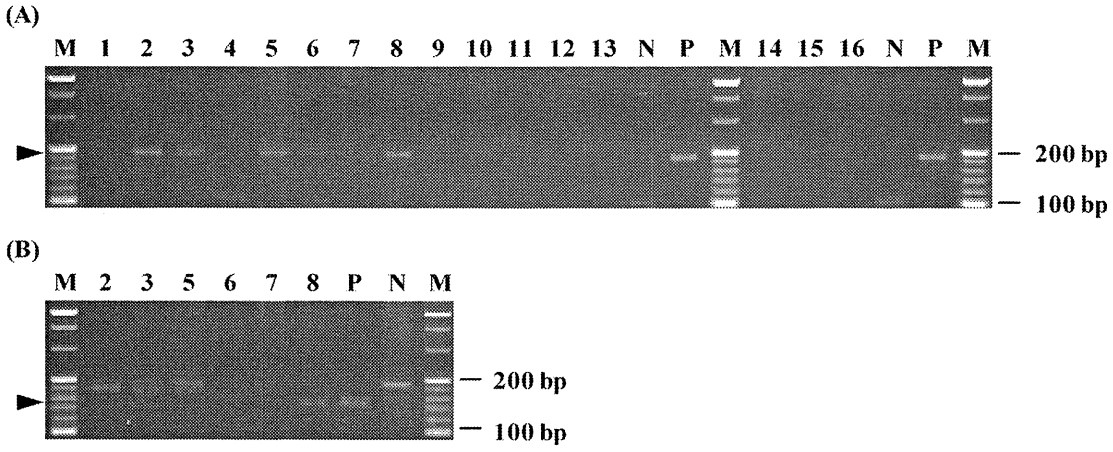

Figure 3. Specificity of the shrimp-PCR method for nontarget species. The arrowheads indicate the expected size of PCR (A) and RFLP products (B). M, DNA marker (20 bp ladder). Lanes 1−13 of panel A, amplification of 50 ng of genomic DNA from Snow crab (1), Red snow crab (2), Giant spider crab (3), Hair crab (4), Dungeness crab (5), Deep-sea red crab (6), Swimming crab (7), Chinese mitten crab (8), Spanner crab (9), King crab (10), Blue king crab (11), Spiny king crab (12), Golden king crab (13), Pacific krill (14), Mysid (15), and Mantis shrimp (16). N, negative control (no template). P, amplification of 5 pg of Kuruma shrimp genomic DNA as a positive control. Lanes 2, 3, and 5−8 of panel B, restriction digestion of PCR products of genomic DNA from crab species corresponding to lane numbers of panel A. Lanes P and N, restriction digestion (P) and nonrestriction digestion (N) of PCR product of Kuruma shrimp genomic DNA.

species gave positive results from almost all target species used for food and negative results from nontarget species, including those selected from a variety of food items. Although some false-positives and false-negatives are identified, these results show that the proposed methods can differentially detect shrimp and crab species with reasonable sensitivity and specificity. In addition, using the akiami paste shrimp- and mantis shrimp-PCR methods in combination with the shrimp- and crab-PCR methods, the specificity and accuracy of the analytical results would be improved. Sakai et al.[18] reported that marine samples such as

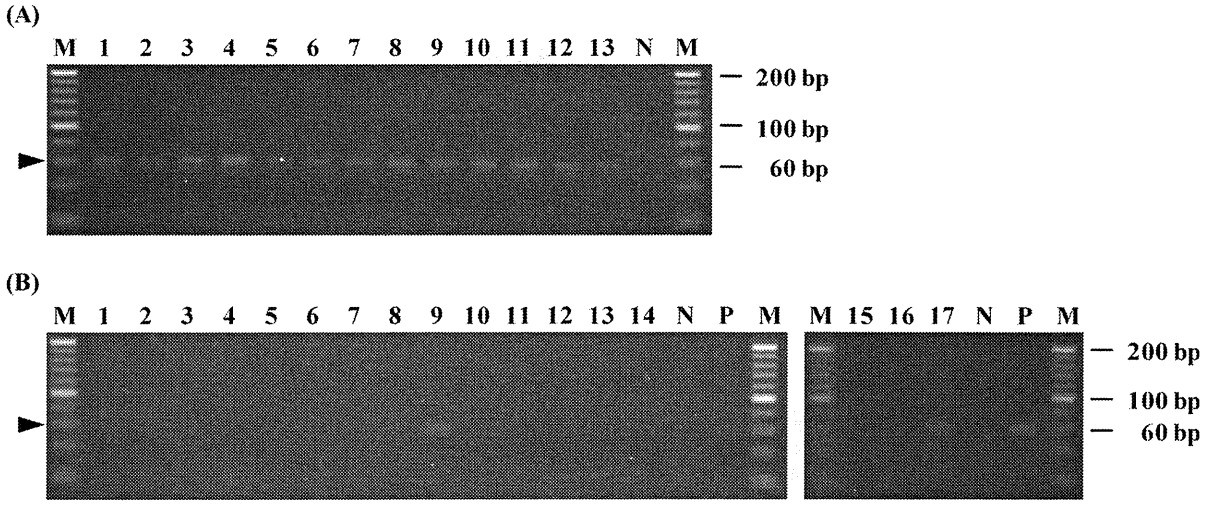

Figure 4. Specificity of the crab-PCR method. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder). Lanes 1−13 of panel A, amplification of 5 pg of genomic DNA from Snow crab (1), Red snow crab (2), Giant spider crab (3), Hair crab (4), Dungeness crab (5), Deep-sea red crab (6), Swimming crab (7), Chinese mitten crab (8), Spanner crab (9), King crab (10), Blue king crab (11), Spiny king crab (12), and Golden king crab (13). Lanes 1−17 of panel B, amplification of 50 ng of genomic DNA from Kuruma shrimp (1), Whiskered velvet shrimp (2), Black tiger shrimp (3), Shiba shrimp (4), Sakura shrimp (5), Side striped shrimp (6), Northern pink shrimp (7), Botan shrimp (8), Japanese lobster (9), American lobster (10), Crayfish (11), Japanese spiny lobster (12), Japanese fan lobster (13), Cuba lobster (14), Pacific krill (15), Mysid (16), and Mantis shrimp (17). N, negative control (no template). P, amplification of 5 pg of King crab genomic DNA as a positive control.

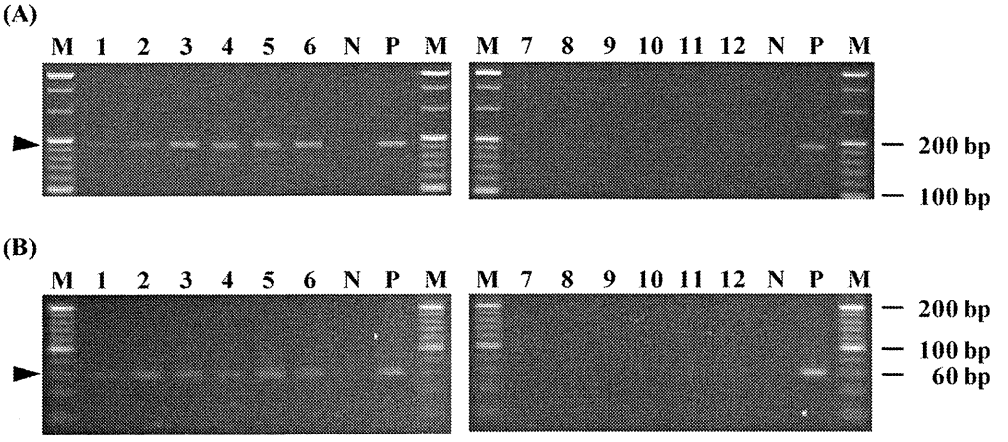

Figure 5. Sensitivity of two PCR methods (A, ShH12-05′ and ShH13-03′; B, CrH16-05′ and CrH11-03′) in incurred foods. The arrowheads indicate the expected PCR product for each primer pair. M, DNA marker (20 bp ladder). Lanes 1−6 of panel A, amplification of positive model samples, using the shrimp detection method, of 50 ng of DNA extracted from freeze-dried soup (1), paste of miso soup (2), powder of soup (3), rice gruel (4), cream croquette (5), and chicken meatball (6). Lanes 7−12 of panel A, amplification of negative model samples, using the shrimp detection method, of 50 ng of DNA extracted from freeze-dried soup (7), paste of miso soup (8), powder of soup (9), rice gruel (10), cream croquette (11), and chicken meatball (12). N, negative control (no template). P, amplification of 5 pg of Kuruma shrimp genomic DNA as a positive control. Lanes 1−6 of panel B, amplification of positive model samples, using the crab detection method, of 50 ng of DNA extracted from freeze-dried soup (1), paste of miso soup (2), sprinkling powder (3), rice gruel (4), cream croquette (5), and chicken meatball (6). Lanes 7−12 of panel B, amplification of negative model samples, using the crab detection method, of 50 ng of DNA extracted from freeze-dried soup (7), paste of miso soup (8), sprinkling powder (9), rice gruel (10), cream croquette (11), and chicken meatball (12). N, negative control (no template). P, amplification of 5 pg of King crab genomic DNA as a positive control.

laver, dried young sardine, and minced fish were frequently contaminated with crustacean. As the contamination is caused by bycatch and their feeding, those samples may be contaminated by variety of shrimp, crab, and other crustaceans including unknown falsely positive species with shrimp- or crab-PCR. Therefore, one should be careful when these commercial foods are examined and the presence of shrimp and crab species is to be comprehensively evaluated not only with the PCR and ELISA results but also the data relating to the manufacturing processes, materials, and recipes to ensure the accuracy of the list of ingredients of processed food.

Analysis of Incurred Foods. As described above, two PCR methods for shrimp and crab species have sufficiently high sensitivity to detect 5 pg of DNA from target species. Under Japanese food allergy labeling regulations, specified allergenic ingredients must be declared on the food label when 10 ppm - (μg/g or μg/mL) or more of its total protein is present in the food. Since processed commercial foods are made from many kinds of materials and processed by various methods, it is thought that the sensitivity of the detection methods could be affected by PCR inhibition, DNA degradation, and differences in DNA