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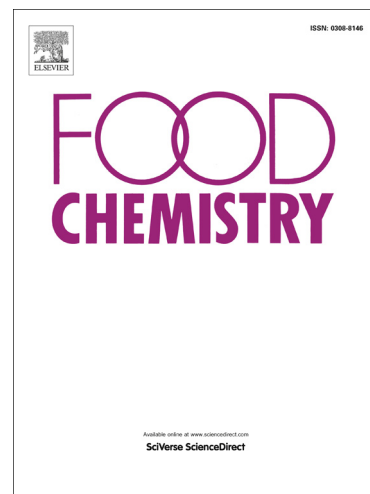
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**Development of sandwich ELISA for detection and
quantification of invertebrate major allergen tropomyosin by
a monoclonal antibody**

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ABSTRACT

Muscle protein tropomyosins of invertebrates are major allergens responsible for wide spread allergic reactions against invertebrates such as shellfish and insects. In order to develop a sandwich enzyme-linked immunoadsorbent assay (ELISA) for detection and quantification of the invertebrate pan-allergen tropomyosin, a specific monoclonal antibody (MAb), CE7B2, was produced. We have successfully established a sandwich ELISA for measuring invertebrate tropomyosin concentrations in food and food materials. The sandwich ELISA system using the MAb CE7B2 is a useful tool to detect and quantify levels of tropomyosin in food. The method is also helpful to detect mite and cockroach tropomyosins, the important indoor allergens.

Key words: Invertebrate tropomyosin; Food allergy; Monoclonal antibody; Sandwich ELISA

1. Introduction

Because of the high nutritive value, seafood including shellfish is an important food resource in the world. Shellfish such as crustaceans and mollusks are then important and common causes of food allergy (Daul & Morgan, 1993; Lopata, O'Hehir, & Lehrer, 2010). On the other hand, indoor allergies against dust mite and cockroach are now recognized as major clinical concerns (Arruda, 2005). Food and indoor allergies are commonly synonymous with type-I allergy that is mediated by immunoglobulin E (IgE) antibodies bound to mast cell. Following the reaction of allergen with specific IgE, the mast cell degranulates and releases histamine, leukotrienes and other mediators, causing hypersensitive reactions, such as urticaria, angioedema, asthma, rhinitis, vomiting, diarrhea and shock in severe cases (Daul & Morgan, 1993).

The major heat-stable allergen in shrimp was first described in 1981 (Hoffman, Day, & Miller, 1981) and later identified as tropomyosin (Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993). Studies on food allergies demonstrated that tropomyosin is an invertebrate pan-allergen and shows cross-reactivities among the given invertebrate species (Reese, Ayuso, & Lehrer, 1999).

Detection kits for shellfish tropomyosin are commercially available in Japan (Seiki, Oda, Yoshioka, Sakai, Urisu, Akiyama, et al., 2007; Shibahara, Oka, Tominaga, Ii, Umeda, Uneo, et al., 2007). However, tropomyosin is

relatively labile in enzymatic processing (Hoffman, Day, & Miller, 1981), and the tropomyosin fragmented peptides containing IgE epitope sequences remain easily in processed food. Those detection kits without IgE epitope specificities might fail to detect allergenic peptides in processed foods, such as fermented products and fish sauce. The previous studies demonstrated that an amino acid sequence around the C terminal is shared among shellfish tropomyosins and that the sequence is strongly recognized by sera of human individuals sensitive to shellfish (Emoto, Ishizaki, & Shiomi, 2009). In the present study, a MAb CE7B2 specific to the sequence was used as a capture antibody in a sandwich ELISA system to detect invertebrate tropomyosin.

Immunological reaction-based techniques have been described to identify and quantify allergens in food including enzyme-linked immunosorbent assay (ELISA) (Jeoung, Reese, Hauck, Oliver, Daul, & Lehrer, 1997). Even though some monoclonal antibodies (MAbs) had been obtained against invertebrate tropomyosin (Barletta, Butteroni, Puggioni, Iacovacci, Afferni, Tinghino, et al., 2005; Lu, Ohshima, Ushio, Hamada, & Shiomi, 2007), it is necessary to develop an MAb recognizing the tropomyosin IgE epitopes shared by invertebrate species for rapid and highly sensitive detection. Although polyclonal antibodies (PABs) are often used for the ELISA because of their low cost, PABs have some weak points; PAB is finite; each lot of antibody, even though raised against the same antigen, shows different antigen recognition ability, which would reduce the reliability of the assay.

MAbs possess a high level of selectivity for a single epitope and can be produced in unlimited amounts. We then developed a sandwich ELISA using MAb in order to provide a promising tool for the specific detection and quantification of invertebrate tropomyosins and presumably allergenic fragmented peptides in daily foods and daily life.

2. Materials and methods

2.1. Materials

The tropomyosin materials used in this study are listed in Table 1. Fresh specimens of kuruma prawn (*Marsupenaeus japonicus*), pink shrimp (*Pandalus borealis*), king crab (*Paralithodes camtschaticus*), swimming crab (*Portunus trituberculatus*), red snow crab (*Chionoecetes japonicus*), horsehair crab (*Erimacrus isenbeckii*), short-neck clam (*Raditapes philippinarum*), disk abalone (*Haliotis discus*), Sakhalin surf clam (*Pseudocardium sachalinense*), bloody cockle (*Anadara broughtonii*), Yezo scallop (*Patinopecten yessoensis*), whelk (*Neptunea polycostata*), Japanese flying squid (*Todarodes pacificus*), common octopus (*Octopus vulgaris*), sea cucumber (*Stichopus japonicus*), ascidian (*Halocynthia roretzi*), red sea bream (*Pagrus major*), American bullfrog (*Rana catesbeiana*), chicken (*Gallus gallus domesticus*) and cattle (*Bos primigenius*) were obtained from

local markets. Dust mite (*Dermatophagoides pterouyssinus*) extract and American cockroach (*Periplaneta fluiginosa*) whole body powder were obtained from Bio Stir (Tokyo, Japan).

The processed food extracts are listed in Table 2. The extracts from instant noodle soup mix of seafood style, Chinese-inspired bean-starch vermicelli soup mix (containing shellfish in materials), and Sichuan-inspired bean-starch vermicelli soup mix (showing no shellfish ingredient in the label on the package but a product through the same production line as for shellfish-containing products), shrimp cracker, octopus cracker, shrimp powder, fish sauce, kimchi (showing no shellfish ingredient in the label on the package) and kimchi sauce were obtained from Japanese local supermarkets.

All animal experiments in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Tokyo University of Marine Science and Technology and were approved by the Animal Experiment Committee in Tokyo University of Marine Science and Technology.

2.2. Preparation of tropomyosin-rich fractions and processed food extracts

The tropomyosin-rich acetone powder was prepared from each sample according to the method of Greaser and Gergely (Greaser & Gergely, 1971).

The powder was dissolved in 20 volumes of 50 mM phosphate buffer (pH 7.2), and subjected to salting-out (30-55% saturation of ammonium sulfate) to obtain myofibrillar proteins. The resulting supernatant containing tropomyosin was kept at 100°C for 30 min because tropomyosin is more stable against heat treatment compared with other myofibrillar proteins (Hoffman, Day, & Miller, 1981). After centrifugation, the resulting supernatant was desalted through a PD-10 column (GE Healthcare Japan, Tokyo, Japan) and stored at -80°C until use.

The processed food materials were separately incubated in 5 volumes of 50 mM phosphate buffer (pH 7.2) overnight at 4°C. After centrifugation at 15,000 g for 25 min, the supernatant was desalted by the PD-10 column and stored at -80°C until use.

2.3. *Synthetic peptide design*

The C-terminal region peptide sequence, NH₂-SISDELDQTFaelc-COOH, was designed based on the sequence of IgE epitope shared by some shellfish tropomyosins (American lobster, brown shrimp, mantis prawn, krill, snow crab, octopus, Japanese abalone, scallop, clam and Japanese oyster) were synthesized through the Fmoc method (Emoto, Ishizaki, & Shiomi, 2009). In this sequence, cysteine was added at the C-terminal position for the following conjugation to carrier

proteins. The designed peptide was conjugated with bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Thermo Fisher Scientific K.K., Yokohama, Japan) according to the manufacturer instructions. In this study, the KLH-conjugated peptide was used for immunization to BALB/c mice, and the BSA-conjugated peptide was used for selection of the MAbs by ELISA.

2.4. Immunization

Three individuals of female BALB/c mice (6 weeks, Clea Japan, Tokyo, Japan) were intraperitoneally immunized respectively with an emulsion of 100 μ l antigen solution containing 20 μ g of the KLH-conjugated peptide and 100 μ l TiterMax Gold (CytRx Corp., GA) on day 0 and the 14th day. The immunized BALB/c mice were given an intravenous injection the amount of 100 μ l immunogen solution 3 days (the 25th day after the first immunization) before the following cell fusion procedure.

2.5. Cell fusion and selection

The cell fusion was carried out according to the method of Kohler and Milstein (Kohler & Milstein, 1975) with slight modifications as described

previously (Lu, Oshima, Ushio, & Shiomi, 2004). The hybridoma cells were produced by fusing spleen cells from the immunized BALB/c mice with P3-X63-Ag8.U1 myeloma cells in the presence of polyethylene glycol (PEG) 1,500 (Roche Diagnostics Japan, Tokyo, Japan). The fused cells were cultured in a GIT medium (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) containing 10% BM-conditioned H1 (Roche Diagnostics Japan, Tokyo, Japan) and 1% hypoxanthine-aminopterin-thymidine (HAT) supplement (Roche Diagnostics Japan, Tokyo, Japan). Then, the positive hybridomas were screened by ELISA as described below and cloned through the limiting dilution method. The isotypes of MAbs were determined using a mouse monoclonal antibody isotyping kit (Roche Diagnostics Japan).

2.6. MAb production and purification

Ascites rich in the MAb CE7B2 was prepared by inoculating CE7B2 hybridoma cells into female BALB/c mice. The IgG antibody fractions were prepared by ammonium sulfate precipitation, followed by the protein G column purification (GE Healthcare Japan, Tokyo, Japan).

2.7. Electrophoresis and Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 10% separating gel. Proteins were visualized by staining with Coomassie brilliant blue 250R (CBB). A 37 kDa protein, which is comparable in molecular mass to the short-neck clam tropomyosin, was used as a tropomyosin marker protein. Tris-tricine SDS-PAGE was also performed with a separating gel (16.5% T, 3% C) and a stacking gel (4% T, 3% C) according to Schagger and von Jagow (Schagger & von Jagow, 1987). In this case, the gel was stained with SYPRO Ruby protein gel stain (Bio-Rad Laboratories, Inc., Tokyo, Japan). Color Marker Ultra-low range (Sigma-Aldrich Japan, Tokyo, Japan) was used as a molecular weight maker.

For Western blot analysis, the proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P^{SQ}, Merk Japan, Tokyo, Japan). The membrane was blocked overnight with Tris-buffered saline (TBS; Takara Bio Inc., Shiga, Japan) containing 5% skim milk, and incubated with MAb media or antisera in TBS containing 0.5% Tween 20 (TBST) at room temperature for 1 h, followed by reaction with Alexa Fluor 680 goat anti-mouse IgG (1:10,000 in TBST) for 1 h. Reactions were visualized with an infrared image scanner Odyssey (LI-COR, Biosciences, Lincoln, USA).

2.8. ELISA analysis

Polystyrene plates with 96 wells (Corning Japan, Tokyo, Japan) were incubated with TBS containing 1 mg/ml of the BSA-conjugated peptide or 0.2 µg/ml of myofibrillar protein samples as antigens (1: 10,000) for 1 h to select hybridoma cell lines. Each plate well was blocked with TBS containing 5% skim milk at 4°C overnight, and incubated with 100 µl of hybridoma culture supernatants at room temperature for 1 h. After washing, 100 µl of horseradish peroxidase (HRP)-rabbit anti-mouse immunoglobulin G+A+M (H+L) antibody (1: 5,000 v/v, ZYMED Laboratories, Carlsbad, USA) was added to each well and incubated for 1 h. Finally, 100 µl of freshly made substrate solution of Sigmafast OPD color former (Sigma-Aldrich Japan, Tokyo, Japan) was added and 50 µl of 1 M H₂SO₄ was added to stop the enzyme reaction. The absorbance of each well was read at 492 nm. TBS was used as a negative control.

2.9. Sandwich ELISA

Each micro plate well was coated with 100 µl of the purified MAb at 2.5 µg/ml in 50 mM carbonate buffer (pH 9.6) at 4°C overnight. The wells were washed three times with TBST, followed by incubation with 250 µl of blocking buffer (TBS containing 5% skim milk) at 37°C for 1.5 h. After washing three times with TBST, 100 µl of the purified kuruma prawn

tropomyosin, Japanese flying squid tropomyosin or food extract supernatant with different dilutions was added to each well and incubated for 1 h at 37°C. TBS was used as a negative control. After washing 3 times with TBST, 100 µl of PAb purified from an antiserum raised in rabbits against king crab tropomyosin at 8 µg/ml was added and incubated at 37°C for 1 h. After washing, 100 µl of 1:10,000 dilution of HRP-conjugated horse anti-rabbit immunoglobulin G+A+M (H+L) antibody (Thermo Fisher Scientific K.K., Yokohama, Japan) was poured and incubated at 37 °C for 1 h. Wells were washed 5 times, then added with 100 µl of Sigmafast OPD color former solution (Sigma-Aldrich Japan, Tokyo, Japan) and incubated in the dark for 30 min at room temperature. Reaction was stopped by the addition of 50 µl of 1 M H₂SO₄. The absorbance was measured at 492 nm.

The detection limit of the sandwich ELISA system for tropomyosin was calculated according to the equation of Miller and Miller (2005):

$$Ld = a + 3 \left\{ \sum (y_i - \hat{y}_i)^2 / (n - 2) \right\}^{1/2}$$

where Ld represents the value of detection limit concentration, *a* stands for the y-axis intercept deduced from line of regression of concentrations, *y_i* is the experimental values of every concentration, *ŷ_i* represents the individual values from line of regression of every concentration, and *n* is the number of samples. The mean coefficients of variation (CV) were based on three performances on three different days for determination of the inter-assay and intra-assay precision.

This sandwich ELISA was applied to determine tropomyosin levels in different processed foodstuffs. The individual results were calculated as tropomyosin equivalents in the sandwich ELISA to the total ingredient weight (Table 3). The equivalents were calculated using the standard curve for prawn tropomyosin, except that the equivalent for octopus cracker was calculated with the squid standard curve (n=3).

3. Results

3.1. Preparation of monoclonal antibody

Most of the obtained hybridomas generated against the immunogen SISDELDQTFAELC-KLH showed positive response to SISDELDQTFAELC-BSA in the first ELISA screening. However, after the second screening by ELISA and Western blot analyses using a screener blotter (WEB0921, Sanplatec corp., Osaka, Japan), only the clone CE7B2 was found positive for crustacean and molluscan tropomyosins. The subtype of the MAb CE7B2 was identified as IgG 2a with κ light chain.

3.2. Western blot analyses using the MAb

As shown in Fig. 1, the MAb CE7B2 showed specific reactivity to the invertebrate proteins with molecular mass around 37 kDa except for

American cockroach and sea cucumber, while this MAb recognized none of the vertebrate myofibrillar proteins used. The MAb CE7B2 reacted with a band of around 75 kDa in bloody cockle and bands of smaller than 20 kDa in short-neck clam, dust mite and cockroach. On the other hand, the MAb weakly reacted with a protein band of 43 kDa in American cockroach. Because sea cucumber is easy to be autodigested by its own proteases (Fu, Xue, Miao, Li, Yang, & Wang, 2005), the tropomyosin extracted from the sea cucumber was not detected in this SDS-PAGE and Western blot analyses.

Tris-tricine SDS-PAGE was performed with the short-neck clam tropomyosin as a reference molecular mass of 37 kDa. Although bands of molecular mass below 6.5 kDa were not observed in Tris-tricine SDS-PAGE (Fig. 2A), they were successfully detected by MAb CE7B2 in Western blot analyses (Fig. 2B). The MAb showed reactivity to around 37 kDa proteins of shrimp cracker, shrimp powder and octopus cracker. The MAb also reacted to the proteins of molecular mass around 17 kDa of clam; 14 kDa and 3.5 kDa of Chinese-inspired bean-starch vermicelli soup mix, Sichuan-inspired bean-starch vermicelli soup mix (showing no shellfish ingredient in the label on the package but a product through the same production lines for shellfish-containing products) and kimchi sauce. Moreover, the MAb reacted to the peptides of a molecular mass below 1 kDa.

3.3. Development of sandwich ELISA for invertebrate tropomyosins

A sandwich ELISA was developed for the detection of invertebrate tropomyosins by using the obtained MAb CE7B2 and a rabbit polyclonal antibody against king crab tropomyosin prepared previously. Standard curves for quantification of crustaceans and mollusks were generated with serial dilutions from 0.045 to 600 ng/ml of kuruma prawn tropomyosin and 0.080 to 512 ng/ml of Japanese flying squid tropomyosin, respectively (Fig. 3). The detection limit by this sandwich ELISA was 0.09 ng/ml for kuruma prawn tropomyosin and 0.64 ng/ml for Japanese flying squid tropomyosin. The CV analyses showed acceptable results of the intra- and inter-assay CVs, 1.5 to 5.1% and 1.2 to 4.2%, in the kuruma prawn tropomyosin standard curve as shown in Table 2. The CV for the Japanese flying squid tropomyosin standard curve ranged from 0.8 to 3.9% and from 0.6 to 3.2%, respectively. Further protein detections were performed for processed foods (Table 3) with the invertebrate tropomyosin sandwich ELISA. The shrimp powder showed the highest tropomyosin equivalents reaching 392 µg/g and kimchi (showing no shellfish ingredient in the label on the package) having the least value of 10 µg/g. For the cup instant soup powders, the instant noodle soup mix of seafood style took a value of 239 µg/g, followed by 106 µg/g for Chinese-inspired bean-starch vermicelli soup mix and 118 µg/g for

Sichuan-inspired bean-starch vermicelli soup mix (showing no shellfish ingredient in the label on the package but a product through the same production lines for shellfish-containing products). The levels of shrimp cracker and octopus cracker were 364 and 322 $\mu\text{g/g}$, respectively. The sauce prepared from the fish reached a value of 22 $\mu\text{g/ml}$ and the kimchi did 113 $\mu\text{g/ml}$.

4. Discussion

The MAb CE7B2 obtained in the present study reacted to proteins with a molecular mass around 37 kDa from crustaceans, mollusks and insects (Fig. 1). Previous studies reported that tropomyosins were allergic proteins with a molecular mass about 37 kDa in many invertebrate species such as shrimp (Ayuso, Lehrer, & Reese, 2002), abalone (Lopata, Zinn, & Potter, 1997), octopus (Ishikawa, Suzuki, Ishida, Nagashima, & Shiomi, 2001), dust mite and cockroach (Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002). Because tropomyosin is a major heat-stable allergen, relatively high levels of tropomyosin were detected also in processed food (Leung, Chu, Chow, Ansari, Bandea, Kwan, et al., 1994). Our MAb CE7B2 was obtained against an IgE epitope that is shared by several shellfish tropomyosins. Although some MAbs for the detection of invertebrate tropomyosins were obtained (Lu, Oshima, Ushio, & Shiomi, 2004), no MAb recognizing all invertebrate

tropomyosins has been reported. Most of the anti-tropomyosin MAbs prepared previously were found to react to only one type of crustacean, molluscan (Lu, Ohshima, Ushio, Hamada, & Shiomi, 2007; Lu, Oshima, Ushio, & Shiomi, 2004) and filarial tropomyosins (Sereda, Hartmann, Buttner, Volkmer, Hovestadt, Brattig, et al., 2010). Because these MAbs were raised against intact tropomyosins as immunogens, it is likely that they do not detect common IgE epitopes specifically. It is known that individuals sensitive to shrimp may also be allergic to other invertebrates such as crustacea, mollusk, house dust mite, cockroach and fruit fly, since the tropomyosin IgE epitope sequences are highly conserved in the invertebrates (Reese, Ayuso, & Lehrer, 1999). Our Tris-tricine SDS-PAGE results in Fig. 3 suggest that the MAb CE7B2 not only reacts to intact tropomyosin, but also recognizes the fragmented peptides with an IgE epitope sequence of SISDELDQTFAEL in the processed foods. Because this IgE epitope sequence is shared by a number of shellfish tropomyosins (Emoto, Ishizaki, & Shiomi, 2009), the MAbs which have the specificities such like the MAb CE7B2 can be used in order to check allergens in etiological foods instead of allergic patient sera.

A protein from cockroach with molecular mass around 43 kDa was detected by the MAb CE7B2 against the sequence SISDELDQTFAEL, suggesting that invertebrate tropomyosins should share similar epitope sequences of other proteins. A protein tropomodulin around 43 kDa widely

expressed in various species (Fowler, 1996) contains amino acid sequences similar to tropomyosins (Kostyukova, Hitchcock-DeGregori, & Greenfield, 2007). Although the amino acid sequences of American cockroach tropomodulin are not available in GenBank, the region (230NISDEKLEQLFAAL243) of *Drosophilidae* tropomodulin was partially similar to our target sequence SISDELDQTFAEL at the identity of approximately 65 %. This finding suggests that the MAb CE7B2 raised against the peptide SISDELDQTFAEL might react to insect tropomodulin, but the protein has not been reported as an allergen. Further studies are required to reveal whether tropomodulin is a novel allergen recognized by tropomyosin-sensitive individuals. Because the 43 kDa tropomodulin-like protein(s) has very faint immune-reactivity with the MAb CE7B2, it is likely that the 43 kDa protein(s) would not affect tropomyosin detection in the sandwich ELISA.

In this study, a sandwich ELISA method with a MAb was applied to measure invertebrate tropomyosin from the processed food extracts. The method is one of the most commonly used immunological methods for the quantitative detection of food and indoor allergens. The principal advantages of these fully quantitative assays are robustness, high sensitivity and abridgement of sample preparation procedures. Either MAbs or PAbs are used in sandwich ELISA systems. Each MAb recognizes a single epitope, while PAbs react with multi-epitopes in the target proteins. It is

widely accepted that some particular PABs may react to similar peptide structures in other related proteins, sometimes causing false positives, so that the sandwich ELISA assay has high background and limits the quantitative capability. We then used the MAb as a capture antibody given that the sandwich ELISA assay should obtain high quantitative performance to detect the intact tropomyosin and the fragmented peptides containing an IgE epitope sequence common in some invertebrates. The shellfish tropomyosin detection kits are commercially available in Japan. Two such detection kits developed against purified black tiger prawn tropomyosin have conferred the detection limit of 0.4 ng/ml (Shibahara, et al., 2007) and 0.71 ng/ml (Seiki, et al., 2007), respectively. A detection limit of the MAb 2A7H6 competitive to human IgE binding to the allergen developed previously (Lu, Ohshima, Ushio, Hamada, & Shiomi, 2007) was calculated to be 0.19 ng/ml, when evaluated using the similar sandwich ELISA developed in this work. In comparison, the present sandwich ELISA has a detection limit of 0.09 ng/ml for kuruma prawn tropomyosin, which is more sensitive than the previous system. On the other hand, the present sandwich ELISA detection limit of 0.64 ng/ml for Japanese flying squid tropomyosin confers the ability to detect mollusk and indoor pan-allergen tropomyosin. It is particularly worth mentioning that, compared to the commercially available kits, the present sandwich ELISA could detect both mollusk and dust mite samples. The CVs for the characterization of the intra- and

inter-assay variabilities suggest that the assay is highly reproducible. As described above, shellfish ingredient was not indicated in the product label of Sichuan-inspired bean-starch vermicelli soup mix. According to the determination of tropomyosin by the sandwich ELISA (Table 3) and cross-reactivity of Western blot (Fig. 2B), however, Sichuan-inspired bean-starch vermicelli soup mix showed tropomyosin level similar to Chinese-inspired bean-starch vermicelli soup mix, which contained shellfish ingredient. This result suggests that Sichuan-inspired bean-starch vermicelli soup mix was contaminated by tropomyosin in the production line which also produced shellfish-containing foods, and that the contaminated level might be allergically sufficient. Thus, the sandwich ELISA developed based on the MAb in this study is highly specific, sensitive and well suited for its application to the major food allergen tropomyosin of invertebrates.

In summary, we successfully obtained the MAb CE7B2 that reacted to tropomyosin in 16 species of invertebrates (Table 1) with high specificity and sensitivity. This MAb is considered as one of very few monoclonal antibodies against broad spectrum of invertebrate tropomyosins reported in the world. We believe that the invertebrate tropomyosin sandwich ELISA system developed in the present study could be very useful to check the quality during food processing and to ensure the safety and security of food.

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Figure legends

Fig. 1. Cross-reactivity of MAb CE7B2 to tropomyosin extracts from different crustaceans, mollusks, insects and vertebrates. (A) SDS-PAGE analysis. The electrophoretic gel was stained with CBB. (B) Western blot analysis using MAb CE7B2 raised against invertebrate tropomyosins as a primary antibody and Alex Fluor 680 goat anti-mouse IgG (H+L) as a secondary antibody.

Fig. 2. Detection of intact and fragmented invertebrate tropomyosins in different processed foods using MAb CE7B2. The short-neck clam tropomyosin was used as a molecular mass reference (37 kDa). (A) Tris-tricine SDS-PAGE analysis of processed food extracts. The electrophoretic gel was stained with SYPRO Ruby protein gel stain. (B) Western blot analysis using MAb CE7B2 against invertebrate tropomyosin as a primary antibody and Alex Fluor 680 goat anti-mouse IgG (H+L) as a secondary antibody.

Fig. 3. Standard curves of sandwich ELISA with MAb CE7B2 for kuruma prawn (●) and Japanese flying squid (○) tropomyosins. The detection limits for kuruma prawn and Japanese flying squid tropomyosins were 0.09 and 0.64 ng/ml, respectively (n=3).

Table 1

Tropomyosin materials in this study

Phylum	Class	Family	Species	Common name	
Arthropoda	Malacostraca	Penaeidae	<i>Marsupenaeus japonicus</i>	Kuruma prawn	
			<i>Pandalus borealis</i>	Pink shrimp	
		Lithodidae	<i>Paralithodes camtschaticus</i>	King crab	
	Crustacea	Atelecyclidae	<i>Erimacrus isenbeckii</i>	Horsehair crab	
		Oregoniidae	<i>Chionoecetes japonicus</i>	Red snow crab	
		Portunidae	<i>Portunus trituberculatus</i>	Swimming crab	
		Arachnida	Pyroglyphidae	<i>Dermatophagoides pterouyssinus</i>	Dust mite
	Insecta	Blattidae	<i>Periplaneta fluiginosa</i>	American cockroach	
	Mollusca	Bivalvia	Veneridae	<i>Raditapes philippinarum</i>	Short-neck clam
			Mactridae	<i>Pseudocardium sachalinense</i>	Sakhalin surf clam
Arcidae			<i>Anadara broughtonii</i>	Bloody cockle	
Gastropoda		Pectinidae	<i>Patinopecten yessoensis</i>	Yezo scallop	
		Haliotidae	<i>Haliotis discus</i>	Disk abalone	
		Buccinidae	<i>Neptunea polycostata</i>	Whelk	
		Cephalopda	Ommastrephidae	<i>Todarodes pacificus</i>	Japanese flying squid
Octopodidae			<i>Octopus vulgaris</i>	Common octopus	
Echinodermata		Holothuroidae	Holothuriidae	<i>Stichopus japonicus</i>	Sea cucumber
Chordata		Ascidia	Pyuridae	<i>Halocynthia roretzi</i>	Ascidian
	Actinopterygii	Sparidae	<i>Pagrus major</i>	Red sea bream	
	Amphibia	Ranidae	<i>Rana catesbeiana</i>	American bullfrog	
	Aves	Phasianidae	<i>Gallus gallus domesticus</i>	Chicken	
	Mammalia	Bovidae	<i>Bos primigenius</i>	Cattle	

Table 2

Coefficients of variation for intra- and inter-assay variations (n=3)

Kuruma prawn (ng/ml)	Intra-assay (% CV)	Inter-assay (% CV)
0.75	5.1	4.2
12	1.5	1.2
96	2.2	1.8
192	3.4	2.8
600	3.4	2.8
Japanese flying squid (ng/ml)		
1.28	0.8	0.6
41	3.9	3.2
164	2.4	1.9
328	1.4	1.2
512	3.1	2.2

CVs were defined as the standard deviation divided by the mean and multiplied by 100.

Table 3

Determination of tropomyosin from processed foods with the sandwich ELISA (n=3)

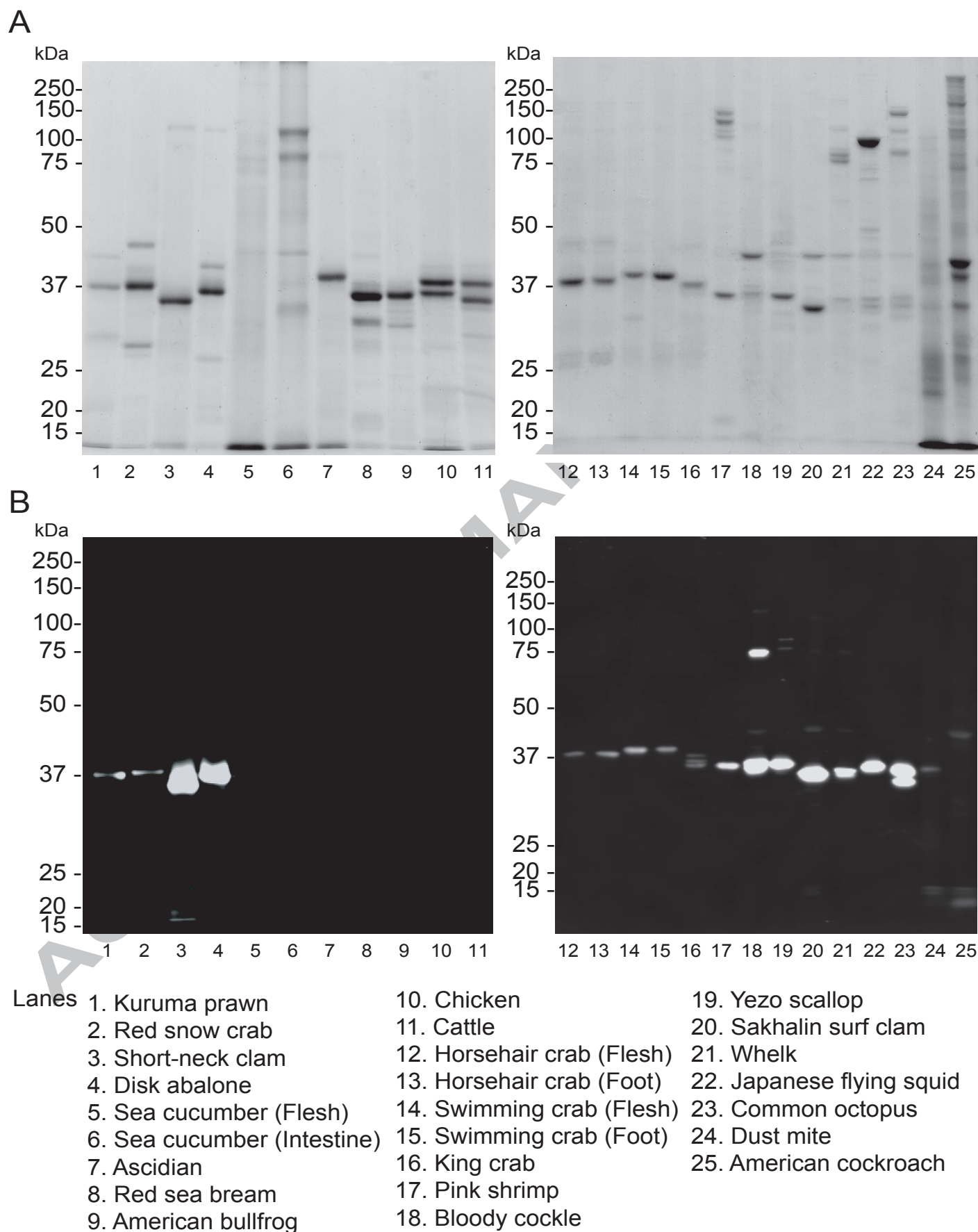
Processed foods ^a	Tropomyosin/food ^b ($\mu\text{g/g}$)
Instant noodle soup mix of seafood style	239
Chinese-inspired bean-starch vermicelli soup mix	106
Sichuan-inspired bean-starch vermicelli soup mix ^c	118
Shrimp cracker	364
Octopus cracker	322
Shrimp powder	392
Kimchi ^d	10
	($\mu\text{g/ml}$)
Fish sauce	22
Kimchi sauce	113

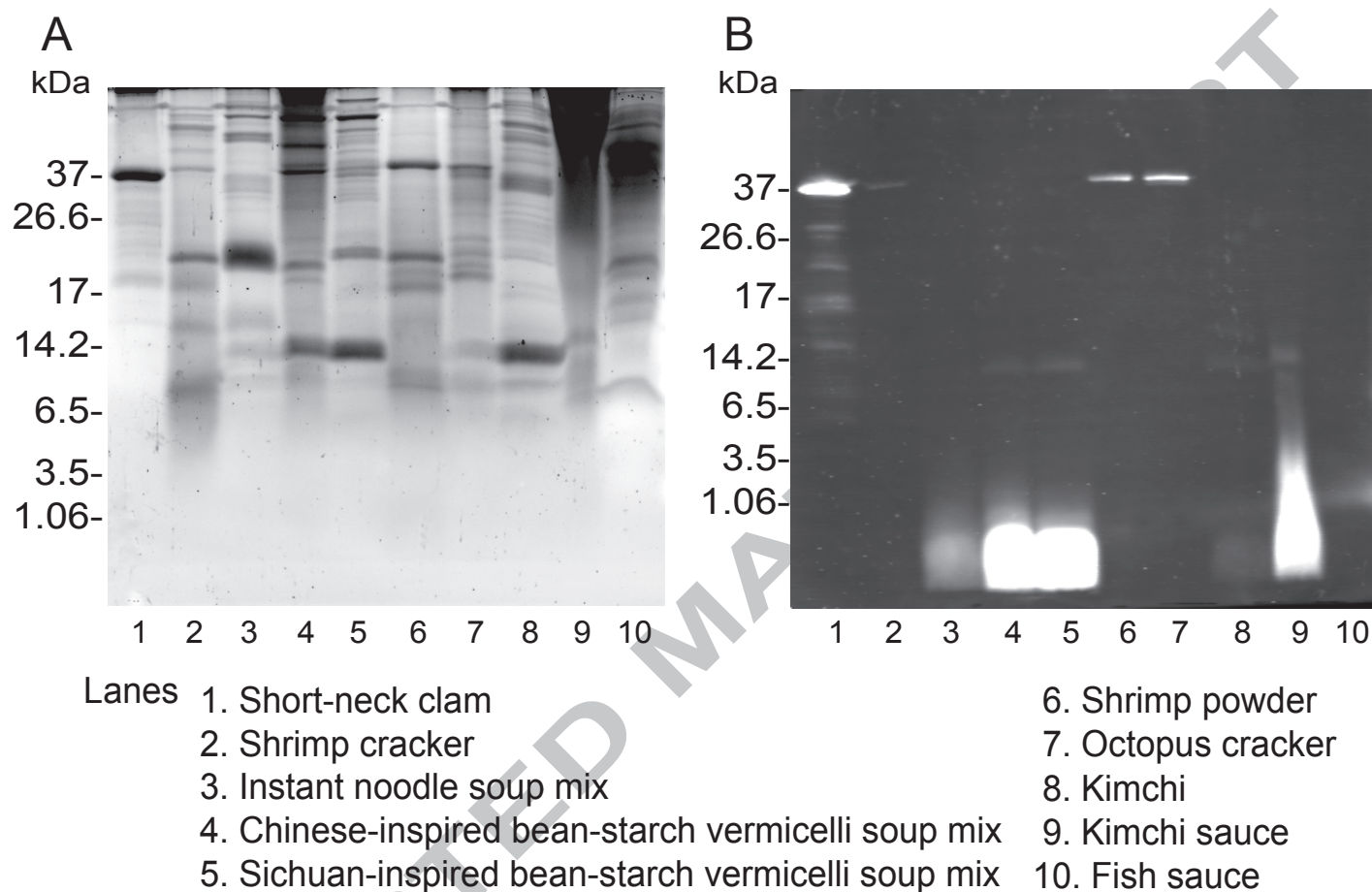
^a All tropomyosins of processed foods, except for fish sauce and kimchi sauce, were extracted as described in Materials and Methods. For fish sauce and kimchi sauce, tropomyosins were extracted from liquid mixtures.

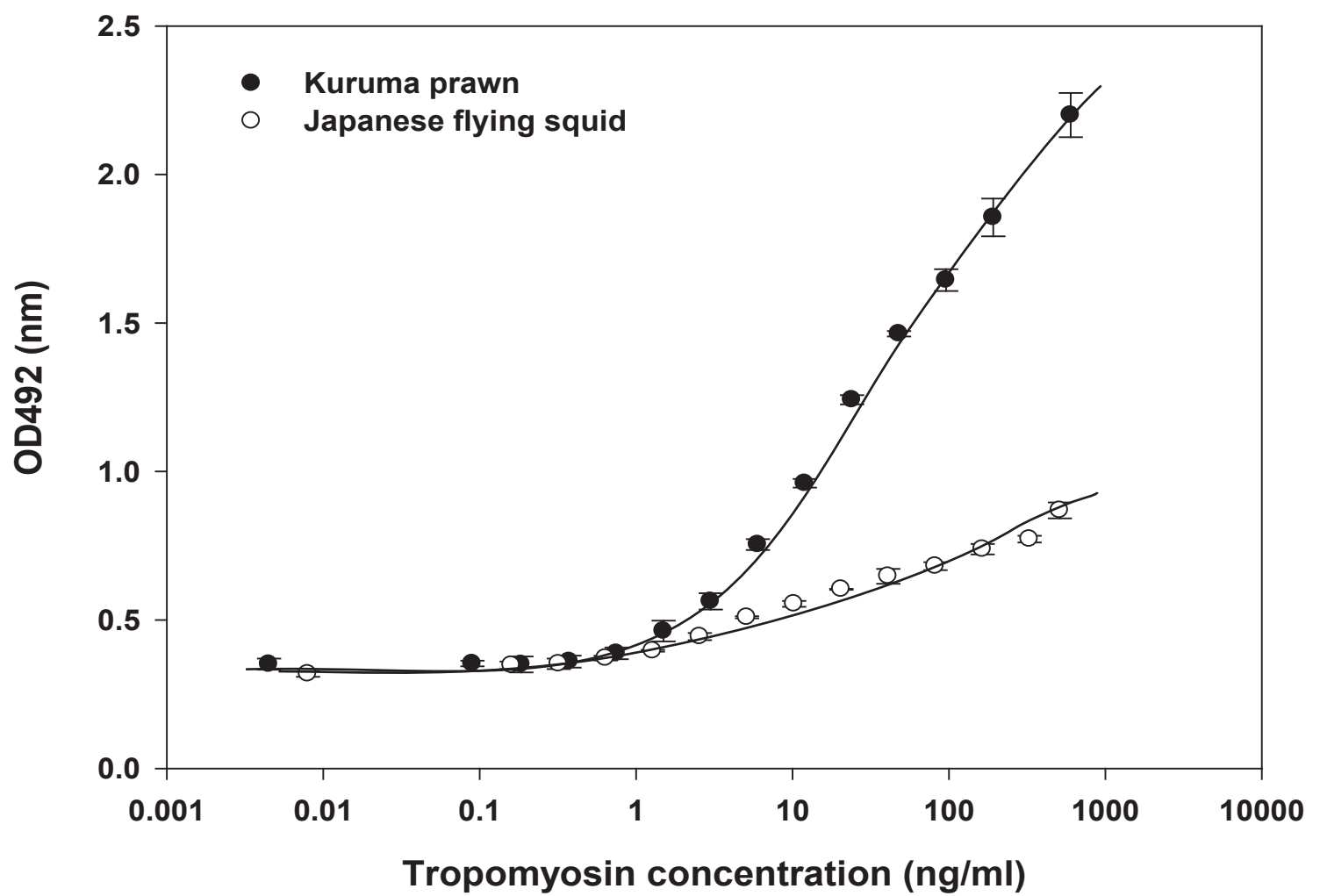
^b Tropomyosin equivalents were calculated using the standard curve for prawn tropomyosin, except that the equivalent for octopus cracker was calculated with squid standard curve (n=3).

^c Showing no shellfish ingredients but produced through the same production line for shellfish-containing products in the label on the package.

^d Showing no shellfish ingredients in the label on the package.







Highlights

- A highly sensitive monoclonal antibody (MAb) for tropomyosins was produced.
- The MAb specifically recognizes allergic tropomyosin-derived peptides.
- A sandwich ELISA based on the MAb was developed.
- The developed sandwich ELISA were successfully applied in processed foods.