JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Reliable and Sensitive Immunoassay for the Determination of Crustacean Protein in Processed Foods

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Among food allergens, crustacea such as shrimps, crabs, and lobsters are a frequent cause of adverse food reactions in allergic patients. The major allergen has been identified as a muscular protein, tropomyosin. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of crustacean protein in processed foods was developed using the sample dilution buffer that is added to porcine tropomyosin. The sandwich ELISA method was highly specific for the Decapoda group, apart from minor cross-reactivities to other crustacea and mollusks. The recovery ranged from 85 to 141%, while the intra- and interassay coefficients of variation were less than 2.8 and 8.4%, respectively.

KEYWORDS: Crustacea; food allergy; enzyme immunoassay; ELISA; tropomyosin

INTRODUCTION

In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy (*1, 2*). Burks et al. (*3*) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan, the number of patients with food allergies, especially among young children, is increasing.

To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. However, various studies have shown that severe allergenic reactions can be caused by the accidental intake of food products containing allergenic materials (*4, 5*). Therefore, sufficient information regarding potentially allergenic ingredients in food products is necessary. The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations, such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food Agriculture Organization (Codex 1998). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label eight kinds of foods that contain ingredients known to be allergens, including soybeans (FAO 1995, *6*). In Japan, the Ministry of Health, Labor, and Welfare (MLHW) has enforced a labeling system for allergenic food material since April 2002 to provide information about these foods to the allergic consumer. In this system, labeling for five food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory and is recommended for 20 other food materials, such as soybeans and shrimp. In Japan, it became clear, based on epidemiological investigations, that the number of patients with a crustacean allergy such as to shrimp or crab has increased (*7, 8*).

In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea and because the number of patients with an allergy to Crustacea has been increasing, although the crustacean allergy is still less prevalent than the peanut allergy in the food-allergic population (*9, 10*). Crustacean allergic reactions may occur due to trace amounts of the crustacean protein, and anaphylaxis to Crustacea has been reported (*11, 12*).

In the present study, we developed a reliable sandwich enzyme-linked immunosorbent assay (ELISA) method with a high sensitivity for Crustacea. We showed that this detection method could be applicable to food-processing products and that the trace amount of Crustacea contained in commercial food products can be detected using the proposed sandwich ELISA method.

MATERIALS AND METHODS

Food Samples. The black tiger prawn (*Penaeus monodon*) was purchased from Intergrated Aquaculture Specialist, Inc. (Cebu, Philippines). The common Crustacea and mollusks, namely, northern shrimp (*Pandalus borealis*), Japanese spiny lobster (*Panulirus japonicus*),

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10.1021/jf0715471 CCC: $37.00
Published on Web 10/12/2007

Caribbean spiny lobster (*Panulirus argus*), red king crab (*Paralithodes camtschatica*), swimming crab (*Portunus trituberculatus*), Japanese mantis shrimp (*Squilla oratoria*), euphausia (*Euphausia similis*), opossum shrimp (*Neomysis japonica*), acorn barnacle (*Balanus rostratus*), goose barnacle (*Pollicipes mitella*), common octopus (*Octopus vulgare*), giant octopus (*Paroctopus dofleini*), ocellated octopus (*Octopus ocellatus*), Japanese common squid (*Todardes pacificus*), spear squid (*Loligo kobiensis*), cuttlefish (*Sepia subaculeata*), common scallop (*Patinopecten yessoensis*), Japanese oyster (*Crassostrea gigas*), bloody clam (*Scapharca broughtonii*), blue mussel (*Mytilus edulis*), short-neck clam (*Tapes japonica*), common freshwater clam (*Corbicula leane*), Japanese hard clam (*Meretix lusoria*), Sakhalin surf clam (*Spisula sachalinensis*), horned turban (*Turbo cornutus*), Japanese abalone (*Haliotis discus*), and whelk (*Babylonia japonica*), and other ingredients and commercial processed foods were purchased at local stores in Japan.

Preparation of Model Processed Foods. The model processed foods were prepared according to a previously reported cooking procedure (*13*). Specifically, the freeze-dried black tiger prawn muscle powder was mixed with raw foods and then cooked to prepare the processed food items containing the shrimp protein at 10 μg/g as the final concentration. The following were prepared as the model processed foods.

Fish meat sausages were made of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and the spiking powder. Lard, salt, sugar, ice water, and the spiking powder were added to the minced fish flesh and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into the fish sausage casings. These were then heated at 121 °C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5 °C overnight.

The freeze-dried egg soup was made of egg, potato starch, milk sugar (lactose), salt, and the spiking powder. Eggs, potato starch, milk sugar, salt, and the spiking powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep freezer at −80 °C, and then lyophilized for 20 h.

The chicken meatball was made of white meat of chicken, lard, potato starch, sugar, and the spiking powder. Lard, potato starch, sugar, and the spiking powder were added to ground white meat of chicken and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into casings. These were then preserved in a deep freezer at −20 °C.

Preparation of Black Tiger Prawn Protein Standards. A 0.1 g sample of the freeze-dried black tiger muscle powder was added to 20 mL of phosphate-buffered saline [10 mM Na-phosphate, 154 mM NaCl (pH 7.4)] containing 5 g/L sodium dodecyl sulfate (SDS), 20 mL/L β-mercaptoethanol, 10 μL/mL protease inhibitor cocktail, and 10 μL/mL 0.5 M ethylenediaminetetraacetic acid (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10000*g* for 30 min and the supernatant was filtered through a 0.8 μm microfilter paper (DISMIC-25cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100 °C for 10 min. The obtained extracts were analyzed using a 2D Quant Protein Assay Kit (GE Healthcare UK Ltd. NA, England).

Purification of Black Tiger Prawn Tropomyosin (BTTM), Red King Crab, Swimming Crab, Japanese Oyster, Common Scallop, Japanese Common Squid, and Porcine Tropomyosin (PTM). The purification of the BTTM was carried out according to the methods reported by Nagpal et al. (*14*), Ishikawa et al. (*15*), and Miegel et al. (*16*), respectively. The black tiger prawn muscles (100 g) were homogenized with 200 mL of a solution containing 20 mM KCl, 1 mM $KHCO_3$, 0.1 mM $CaCl_2$, and 0.1 mM dithiothreitol (DTT). After centrifugation (3000*g* for 5 min at 4 °C), 200 mL of acetone was added to the precipitant. The suspension was filtered through cheesecloth, and the residue was then washed three times with 200 mL of acetone. Finally, the residue was allowed to dry at room temperature for 2–3 h. The dried powder was extracted overnight at room temperature with 200 mL of 25 mM Tris-HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 1 mM DTT. After filtration through cheesecloth, the residue was once more extracted with 200 mL of 1 M KCl. The extracts were combined and cooled to 4 °C. Ammonium sulfate was added to produce an approximate 30% saturation. After 2 h, the solution was centrifuged (18000*g* for 60 min at 4 °C) and ammonium sulfate was then added to the supernatant (60% saturation). After 2 h, the solution was centrifuged and the precipitant was dissolved in 20 mL of 5 mM Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$, and 0.1 mM DTT and was dialyzed overnight against 6 L of the same solution. The pH was then adjusted to 4.6 by the addition of HCl, and the tropomyosin precipitate was removed by centrifugation. The precipitate was dissolved in 25 mM Tris-HCl (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$, and 0.1 mM DTT and then chromatographed on a HiLoad Superdex 200 pg column (Φ26 mm × 600 mm; GE Healthcare UK Ltd.) equilibrated with the same buffer. Fractions of 5 mL were collected at a flow rate of 2.5 mL/min. The SDS-polyacrylamide gel electrophoresis analyses for all fractions were performed, and the fractions with the band corresponding to 37 kDa were combined (*17*). The combined fraction was then diluted with an equal volume of 0.2% trifluoroacetic acid and applied to reverse-phase high-performance liquid chromatography on a Wakosil-II 5C18 AR prep column (Φ10 mm × 250 mm; Wako Chemicals, Japan). The column was eluted at a constant flow rate of 2.5 mL/min by a gradient of acetonitrile in 0.1% trifluoroacetic acid. The tropomyosin-containing fractions were collected and lyophilized. The red king crab, swimming crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins were obtained according to the purification procedure of BTTM. Tropomyosin derived from the porcine skeletal muscle (PTM) was obtained using the purification procedure of Greaser et al. (*18*) and Bailey et al. (*19*).

Production of Monoclonal Antibodies and Rabbit Polyclonal Antibodies to BTTM. The anti-BTTM monoclonal antibodies were generated at Nippon Biotest Laboratories, Inc. (Tokyo, Japan). For the production of the monoclonal antibodies against BTTM, female BALB/c mice were immunized with the purified BTTM. Fusion of the spleen cells was performed according to the method of Kohler and Milstein (*20*). The cell culture supernatants were screened for specific anti-BTTM antibodies by a direct ELISA with purified BTTM on a solid phase. The positive hybridomas were cloned and subcloned by limiting dilution. The positive hybridoma cells were intraperitoneally administered into BALB/c mice to induce the ascite tumors. The antibody was purified from the ascite fluid using a HyperD Protein A column (Bio Sepra Inc., Marlborough, MA). The specificity of the monoclonal antibodies was demonstrated by a direct ELISA method with purified black tiger prawn, red king crab, Japanese oyster, common scallop, and Japanese common squid tropomyosins. The polyclonal antibodies were generated at Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). The rabbit antiserum against BTTM was produced by immunization of New Zealand rabbits with purified BTTM in Freund's adjuvant. Injections were repeated six times at appropriate intervals (7 days). Whole blood was collected, and the serum was separated. The polyclonal antibodies were purified from the serum using a HiTrap Protein A HP column (GE Healthcare UK, Ltd.). The polyclonal antibodies were immunoabsorbed against Japanese common squid purified tropomyosin. The immunoabsorption was performed using the Japanese common squid tropomyosin-coupled column to removed further antibodies to molluskan protein. The specificity of the absorbed polyclonal antibodies was demonstrated by direct ELISA using the various purified tropomyosins.

Preparation of Sample Solution. The samples were treated with the Ace AM-4 homogenizer (Nissei, Tokyo, Japan) a few times for 30 s for homogeneity. Nineteen milliliters of 120 mM Tris-HCl (pH 7.4) containing 1 g/L bovine serum albumin (BSA), 0.5 mL/L Tween 20, 5 g/L SDS, and 20 mL/L β-mercaptoethanol (*21*) was added to 1 g of a homogenized sample, which was then shaken for 12 h at room temperature for extraction. After the extraction, the sample was centrifuged at 3000*g* for 20 min, and the supernatant was filtered through 5AB paper (Advantec) to obtain the extract.

Procedure of the Direct ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international, Rochester, NY) were coated overnight at 4 °C with 100 μL of purified tropomyosin (0.5 μg/mL) in coating buffer (50 mmol/L sodium carbonate, pH 9.6). The plates were then washed three times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.4, containing 154 mmol/L NaCl). The plates were blocked

for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950 (Supelco, Bellefonte, PA). After the plates were washed six times with TBS containing 0.5 mL/L Tween 20 (TBS-T), diluted monoclonal antibodies or polyclonal antibodies were added to the wells and incubated at 25 °C for 1 h. After the wells were washed with TBS-T, 100 μL of horseradish peroxidase-labeled goat antimouse or antirabbit IgG serum was added to each well. After washing, 100 μL of the substrate solution containing 3,3′,5,5′-tetramethylbenzidine (SureBlue TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read on a SPECTRAmax 250 microplate reader (Molecular Devices Corp., Menlo Park, CA) at the wavelength of 450 nm.

Procedure of the Sandwich ELISA. Polystyrene 96 well microtiter plates (Nalge Nunc international) were coated with 100 μL/well monoclonal antibodies (Mab #32, 10 μg/mL; and Mab #54, 20 μg/mL in 50 mmol/L sodium carbonate, pH 9.6) for 18 h at 4 °C. After they were washed three times with TBS, the plates were blocked for 1.5 h at 25 °C with TBS containing 10 g/L BSA, 30 g/L sucrose, and 0.5 mL/L ProClin 950. After the blocking buffer had been aspirated, the plates were dried in an incubator for 2.5 h at 30 °C, sealed in an aluminum-coated pack with drying agent (I.D. Sheet Desiccant; I.D., Tokyo, Japan), and stored at 4 °C until used. The food sample extracts were diluted 1:20 with the sample dilution buffer [TBS containing 2 g/L BSA, 0.02 g/L PTM, 0.5 mL/L Tween 20, and 0.5 mL/L ProClin 950]. The diluted sample or calibrator (100 μL) was added in triplicate to the coated wells, and the plates were then incubated for 90 min at 25 °C. After the plate had been washed four times with TBS-T, horseradish peroxidase-conjugated absorbed polyclonal antibodies (100 μL) were added to each well, and the plate was then incubated for 90 min at 25 °C. After another four washes with TBS-T, 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate, KPL) as a substrate was added to each well, and the plate was incubated at 25 °C for 20 min. The reaction was stopped by the addition of 1 mol/L sulfuric acid (100 μL/well). The plate was then read using a SPECTRAmax 250 microplate reader at a wavelength of 450 nm. Standard curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration.

Eleven crustacean protein extracts and two crustacean purified tropomyosins were tested in the concentration range from 0 ng/mL to 90 μg/mL. The concentration–response curves were obtained by plotting the absorbance vs the logarithm of the analyte concentration, and the curves were fitted to a four-parameter logistic equation, $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the concentration of the analyte giving 50% responses (RC_{50}), and D is the minimum absorbance for no analyte. The reactivity values were calculated as follows: reactivity % = [RC_{50} of black tiger prawn protein (or tropomyosin)/RC_{50} of target crustacean protein (or tropomyosin)]. Seventeen molluskan sample extracts were diluted 1: 20 with the sample dilution buffer containing PTM and analyzed using the sandwich ELISA method.

Evaluation of Assay Variation. For determination of the intra-assay precision, the mean coefficients of variation (CVs) were based on 10 replicates. The interassay precision was determined as the mean CVs on the basis of triplicate analyses on 10 different days. The limit of detection (LOD) for the sandwich ELISA was calculated as three times the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean values after 25 experiments.

RESULTS

Construction of Sandwich ELISA. To evaluate the characteristics of the absorbed polyclonal antibodies and monoclonal antibodies to the BTTM, we tested the reactivity using a direct ELISA assay. We showed that the absorbed polyclonal antibody could be clearly detected for the crustacean tropomyosin but not for the molluskan tropomyosin. For the preparation of monoclonal antibodies to BTTM, nine monoclonal antibodies were obtained. Of these monoclonal antibodies, Mab #32 and #54 gave a satisfactory specificity and reactivity. In the examination of the different antibody combinations, using Mab #32 and #54 as the capture antibody and the HRP-conjugated absorbed polyclonal antibody as the detected antibody for the sandwich ELISA was found to provide the best results in terms of sensitivity and specificity to determine the total crustacean protein. However, as shown in **Figure 1**, a satisfactory dilution linearity could not be obtained when the dilution tests were performed using the tentatively constructed sandwich ELISA method and the three model processed foods. These results suggest that the food matrix could affect the dilution linearity in the tentatively constructed ELISA method. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. As shown in **Figure 2**, the dilution linearity was satisfactorily improved by the addition of the PTM to the sample dilution buffer ($r^2 = 0.996$–0.999), confirming parallelism between the calibrators and the food samples. We statistically compared the two correlation coefficients of the dilution curves obtained using a sample dilution buffer containing PTM and those obtained without PTM for the assay of the model processed foods. A statistical test between the two correlation coefficients was performed using the Z-transformation test. P values of less than 0.05 were considered statistically significant. In the case of the chicken meatball, there was a statistically significant difference between the two correlation coefficients of the dilution curve ($P = 0.036$). In the case of

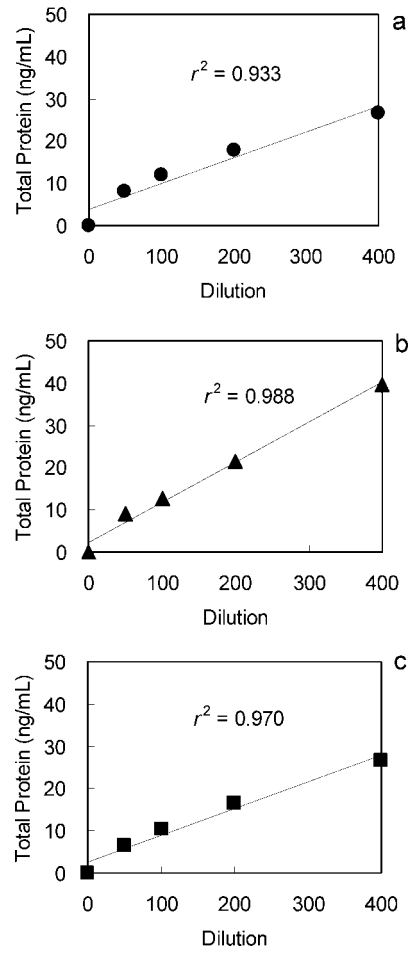

Figure 1. Linearity of dilution curves for model processed foods using the sample dilution buffer without PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

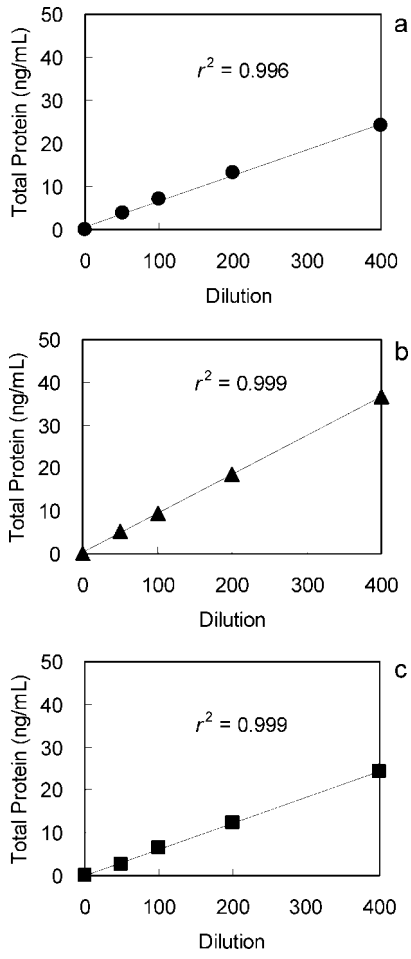

Figure 2. Linearity of dilution curves for model processed foods using the sample dilution buffer with PTM. (**a**) Fish meat sausage (solid circle); (**b**) freeze-dried egg soup (solid triangle); and (**c**) chicken meatball (solid square).

the fish meat sausage, a dilution curve using a sample dilution buffer to which was added PTM tended to show a good linearity when compared with using a tentative sample dilution buffer ($P = 0.081$). Meanwhile, there was no statistically significant difference in the freeze-dried egg soup ($P = 0.127$). These results suggested that the addition of PTM to the sample dilution buffer significantly improved the dilution linearity. Consequently, we established the sandwich ELISA method using a sample dilution buffer with PTM to minimize the food matrix effects.

Reactivity and Specificity Test. Various crustacean proteins, molluskan protein samples, and two crustacean purified tropomyosins (black tiger prawn and swimming crab) were examined to test the reactivity and specificity using the sandwich ELISA method. As shown in **Table 1**, the reactivities of the Decapoda group, which includes prawns and lobsters, are greater than 65.8%, and those of the crabs range between 28.5 and 38.5%. In contrast, the reactivities of the other Crustacea, such as the Japanese mantis shrimp, euphausia, and acorn barnacle, are less than 11.3%. The swimming crab purified tropomyosin demonstrated a reactivity of 154% as compared to the reactivity of black tiger purified tropomyosin. When all of the molluskan samples were tested, all of the levels were determined by the sandwich ELISA method to be less than 1.0 mg/kg (**Table 2**). These results suggest that the sandwich ELISA method has a specific reactivity to the Decapoda group, which includes prawns, shrimps, lobsters, and crabs.

Table 1. Reactivity Levels of Various Crustacean Samples in the Sandwich ELISA Method[a]

sample	RC_{50} (ng/mL)	reactivity (%)
Decapoda group		
black tiger prawn	9.5	
northern shrimp	14.4	65.8
Japanese spiny lobster	8.4	114.3
Caribbean spiny lobster	9.0	105.6
red king crab	24.6	38.5
swimming crab	33.4	28.5
other varieties of Crustacea		
Japanese mantis shrimp	124.4	7.6
euphausia	799.3	1.2
opossum shrimp	8060.4	0.1
acorn barnacle	83.8	11.3
goose barnacle	166.7	5.7

[a] RC_{50} is the concentration of analyte giving a 50% OD_{max} response. Reactivity % = (RC_{50} of black tiger prawn protein/RC_{50} of target crustacean protein).

Table 2. Cross-Reactivity of Various Molluskan Samples in the Sandwich ELISA Method

sample	cross-reactivity in ELISA (mg/kg)
Cephalopoda group	
common octopus	<1.0
giant octopus	<1.0
ocellated octopus	<1.0
Japanese common squid	<1.0
spear squid	<1.0
cuttlefish	<1.0
Bivalvia group	
common scallop	<1.0
Japanese oyster	<1.0
bloody clam	<1.0
blue mussel	<1.0
short-neck clam	<1.0
common freshwater clam	<1.0
Japanese hard clam	<1.0
Sakhalin surf clam	<1.0
Gastropoda group	
horned turban	<1.0
Japanese abalone	<1.0
whelk	<1.0

Limit of Detection and Limit of Quantification. The best model that describes the relationship between the absorbance and the antigen concentration is a four-parameter logistic curve (**Figure 3**). The LOD of the ELISA method determined using the standard proteins is 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and LOQ is 2.25 ng/mL, equivalent to a 0.9 mg/kg sample. Consequently, the practical determination range lies between 1.56 and 50 ng/mL. For the final evaluation of the validation data for the sandwich ELISA and its application, the LOQ for routine analysis was set to 1.0 mg/kg sample. This level was considered to give a safety margin to the majority of consumers with an allergy to peanuts (*22*).

Quantification of Crustacean Protein in Model Processed Foods Using the Sandwich ELISA. To test the applicability of the sandwich ELISA in processed foods, the crustacean protein in three model processed food samples was determined using the sandwich ELISA. As shown in **Table 3**, the mean recoveries for all three model processed food samples ranged from 85 to 141%. The precision data from the three model processed foods are shown in **Table 4**. The interassay precision across all days was 5.3, 6.2, and 8.4% CV for the three model processed foods. The intra-assay precision for the three model processed foods was 2.8, 2.3, and 2.8% CV, respectively.

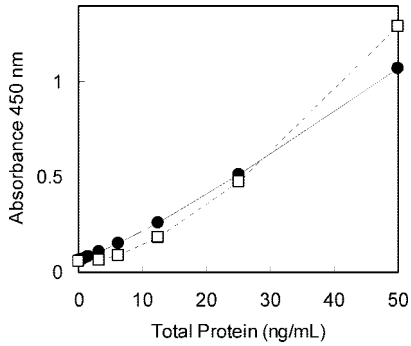

Figure 3. Representative standard curve using the shrimp protein standard in the sandwich ELISA method. The sample diluted buffer with PTM (solid circle); without PTM (open square).

Table 3. Recoveries of Crustacean Protein from Three Model Processed Foods

sample	concentration (mg/kg)	recovery (%)
fish meat sausage	25.0	96
	12.5	107
	6.3	114
	3.1	120
freeze-dried egg soup	29.8	124
	14.9	124
	7.4	125
	3.7	141
chicken meatball	25.0	97
	12.5	100
	6.3	105
	3.1	85

Table 4. Intra- and Interassay Variances in the Sandwich ELISA Method Using Three Model Processed Foods[a]

sample	concentration (mg/kg)	intra-assay	interassay
fish meat sausage	10	2.8	5.3
freeze-dried egg soup	11.9	2.3	6.2
chicken meatball	10	2.8	8.4

[a] The intra-assay variances were calculated from 10 replicates of the same extract, and the interassay variances were calculated from triplicate analysis of the same extract on 10 different days.

Application to the Commercial Food Products. Thirty-two different commercial food samples were analyzed by the sandwich ELISA method. Each commercial food was homogenized, and the extracts were obtained according to the extraction procedure described in the Materials and Methods section. As shown in **Table 5**, 15 commercial foods with a label of shrimp or crab on the ingredients list were clearly detected. In contrast, the levels in products without a label of shrimp or crab on the ingredients list were detected to be less than 1.0 mg/kg. There were no false positives from the no-declaration samples and no false negatives from the declaration samples analyzed in this study. When commercial food products containing shrimp or crab were serially diluted and assayed, each sample gave results close to linearity (r^2 = 0.993–1.000), confirming parallelism between the calibrators and the food samples. These results show that the sandwich ELISA method could appropriately determine the crustacean protein in the processed foods.

DISCUSSION

We established the sandwich ELISA method for the detection of crustacean protein that has a specific reactivity to the

Table 5. Analysis of Various Commercial Food Samples for Using the Sandwich ELISA Method

sample	declaration	substance	quantitative (mg/kg)	regression (r^2)
bean jammed	+	crab	264	0.998
seafood curry	+	shrimp	1780	0.999
beef curry	−		<1.0	
base of pilaf	+	crab	1100	1.000
cream pasta source	−		<1.0	
croquette	+	crab	404	1.000
croquette	−		<1.0	
croquette	−		<1.0	
dumpling	+	shrimp	77000	0.995
dumpling	+	crab	1040	1.000
dumpling	−		<1.0	
base of fried rice	+	shrimp	653	0.993
base of risotto	+	crab	36.7	0.998
spray-dried soup	−		<1.0	
gratin	+	shrimp	22400	0.995
gratin	−		<1.0	
snack	+	shrimp	100	0.998
cookie	−		<1.0	
Japanese rice cookie	−		<1.0	
fried food (prawn)	+	shrimp	282000	0.995
fried food (chicken)	−		<1.0	
fried food (poke)	−		<1.0	
fried food (oyster)	−		<1.0	
fried food (squid)	−		<1.0	
noodle	+	shrimp	145000	0.998
noodle	−		<1.0	
Japanese wheat noodle	−		<1.0	
steamed fish paste	+	crab	176	0.999
steamed fish paste	−		<1.0	
fried fish paste	+	shrimp	46.4	0.995
terrine	+	shrimp	1560	0.997
fish sausage	−		<1.0	

Decapoda group in Crustacea and applied this method to processed food. Jeoung et al. (*23*) already reported a determination method for tropomyosin. However, the cross-reactivity to mollusks and the application to processed foods have not yet been sufficiently clarified. Therefore, the reactivity and specificity of the sandwich ELISA method were tested using extracts from various Crustacea, mollusks, and commercial foods. In the test of all of the molluskan sample extracts, the reactivity levels were extremely low. The house dust mite was reported to cross-react with crustacean allergens (*24*). However, the monoclonal antibodies as the capture antibody do not cross-react with the house dust mite in the Western blot analysis (data not shown). These results suggest that this method would be specific to the Crustacea protein. However, the possibility of a cross-reaction with other less commonly used mollusks or other ingredients, such as crustacean extractants as seasonings, cannot be excluded and remains to be examined. It will be necessary to clarify the applicability of the present method.

The reactivities of lobster and prawn are similar to those of the black tiger prawn. Those of the crab group appear to be lower than those of the black tiger prawn. However, the purified swimming crab tropomyosin showed a high reactivity (154%). These results suggest that the variety of reactivities among the Decapoda group may be involved in the difference of the tropomyosin contents in the sample extracts.

Furthermore, we found that the addition of porcine skeletal tropomyosin to the sample dilution buffer in the sandwich ELISA method can appropriately determine the crustacean protein in processed foods without any food matrix effects.

As described in the Results section, the sample extracts of the model processed foods were serially diluted and assayed using the tentatively constructed sandwich ELISA method, and

a good linearity could not be observed ($r^2 = 0.936$–0.995). We considered that this result would be due to food matrix effects. Therefore, to improve the dilution linearity, we attempted to add the PTM to the sample dilution buffer. Consequently, the dilution linearity for model processed food was significantly improved by the addition of the PTM to the sample dilution buffer ($r^2 = 0.996$–0.999). The addition of troponin or actin failed to improve the dilution linearity (data not shown). These results suggest that tropomyosin may be involved in the food matrix effects, although the food matrix effect mechanism remains unclear. This method offers a new perspective for the determination of various proteins in processed food and is expected to be extremely useful in other protein-measuring methods using ELISA.

To evaluate the sandwich ELISA method for the determination of crustacean protein in processed foods, a recovery study and intra- and interassays were tested using model processed foods. The results of the analysis show that this method has a good accuracy and precision. The sandwich ELISA method's sensitivity was 0.71 ng/mL, corresponding to the 0.29 μg crustacean protein/g food sample weight. This result indicates that the sandwich ELISA method is suitable for detection in the presence of hidden crustacean protein in processed foods.

In conclusion, this sandwich ELISA method is shown to have an acceptable accuracy and precision and no false positive or false negative. This method has been demonstrated to be suitable for the quantitative measurement of the specific crustacean protein in processed foods without food matrix effects.

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Received for review May 28, 2007. Revised manuscript received September 3, 2007. Accepted September 18, 2007. This study was supported by the Health and Labor Sciences Research Grants for Research on Food Safety from the Ministry of Health, Labor, and Welfare of Japan.

JF0715471