

FOOD COMPOSITION AND ADDITIVES

Development and Evaluation of an Enzyme-Linked Immunosorbent Assay Using a Nonpoisonous Extraction System for the Determination of Crustacean Protein in Processed Foods

DAISUKE KOIZUMI, KAZUYA SHIROTA, and HIROSHI ODA¹

Central Research Institute, Maruha Nichiro Corp., 16-2 Wadai, Tsukuba, Ibaraki 300-4295, Japan

REIKO ADACHI, SHINOBU SAKAI, HIROSHI AKIYAMA, TOMOKO NISHIMAKI-MOGAMI, and REIKO TESHIMA²

National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Crustacean proteins are food allergens that cause severe allergic reactions in patients with food allergies; therefore, the identification of crustaceans such as shrimp, crab, and lobster as ingredients in processed food products is mandatory in Japan. We previously developed and validated an ELISA method coupled with an extraction process using the surfactant sodium dodecyl sulfate and the reductant 2-mercaptoethanol (2-ME) to quantify crustacean protein. However, 2-ME was designated as poisonous in Japan in 2008. Therefore, in this study, we developed and evaluated an ELISA method for detecting and quantifying crustacean protein that uses sodium sulfite (Na_2SO_3) in place of 2-ME for extraction. The proposed ELISA method showed high sensitivity, with an LOQ of 0.66 μg protein/g food sample. Furthermore, the proposed method showed high specificity for the Decapoda order within the subphylum Crustacea, with recoveries ranging from 83.8 to 100.8% for model processed foods, as well as high reproducibility (intra- and interassay CVs of $\leq 8.2\%$) and high correlation with our previously validated ELISA method for processed foods (correlation coefficient of 0.996). The proposed ELISA method does not require the use of poisonous reagents, provides acceptable accuracy, and is useful for the routine monitoring of food products.

Food allergies have recently become an important health problem in developed countries, with an estimated 2 to 3% or more of the worldwide population having some type of food allergy (1, 2). The effectiveness of oral, sublingual, and epicutaneous immunotherapies against food allergies has been evaluated, but no single established protocol has been shown to be both effective and safe (3, 4). Therefore, strict avoidance of the food allergen is currently the only means of preventing an adverse allergic reaction (4–6).

The labeling of allergenic foods is an important consideration for consumers with food allergies in order for them to avoid foods that might cause an allergic reaction. The regulatory framework for the labeling of allergenic foods varies among countries (7, 8). Most countries include the common allergens of milk, eggs, peanuts, wheat, crustaceans, fish, soybeans, and tree nuts on their priority allergenic foods list, as recommended by the Codex Alimentarius Commission. In Japan, the Ministry of Health, Labour, and Welfare has enforced a labeling system for allergenic food materials since April 2002 to better provide information about these foods to consumers. In this system, labeling is mandatory for seven food materials (i.e., eggs, milk, wheat, buckwheat, peanuts, shrimp, and crab) and recommended for an additional 20 food materials such as soybeans (7–11).

An allergic reaction to crustacean products can be elicited by the ingestion of even trace amounts of crustacean protein, and anaphylaxis caused by exposure to such proteins has been reported (12, 13). To protect allergic patients from exposure to hidden allergens (pre-existing allergens), we previously developed a quantitative ELISA method (14) and a qualitative lateral flow assay process (15) for the detection of crustacean protein in food products. This ELISA method (ELISA-I) was validated according to the Japanese validation protocol (16). However, the sample extraction step of ELISA-I requires the use of 2-mercaptoethanol (2-ME) as a reductant, which was designated in Japan in 2008 as being poisonous. Therefore, ELISA-I can be used only in specific facilities in which the use of poisonous compounds is permitted.

Sodium sulfite (Na_2SO_3) was recently reported to be a suitable alternative to 2-ME for ELISA-based protein detection methods (17). Because Na_2SO_3 is not designated as poisonous and is commonly used as a food additive, it is more suitable than 2-ME, especially for use in food facilities.

In this study, we developed an ELISA method (ELISA-II) in which the extraction method uses the surfactant sodium dodecyl sulfate (SDS), and the reductant Na_2SO_3 in place of 2-ME, for the determination of crustacean protein. We also evaluated the performance of ELISA-II by comparing it to ELISA-I.

Materials and Methods

Food Samples

Black tiger prawn (*Penaeus monodon*) was purchased from Integrated Aquaculture Specialist, Inc. (Cebu, Philippines).

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¹ Corresponding author's e-mail: h-oda@maruha-nichiro.co.jp

² Present address: Pharmaceuticals and Medical Devices Agency, 3-3-2 Kasumigaseki, Chiyoda-ku, Tokyo 100-0013, Japan

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Other ingredients and commercially processed foods were purchased from supermarkets in Tsukuba, Japan.

Preparation of Black Tiger Prawn Protein Standards

Freeze-dried black tiger prawn muscle powder was prepared according to a previously reported procedure (18). A 0.1 g sample of freeze-dried black tiger prawn muscle powder was added to 20 mL phosphate-buffered saline [10 mM sodium phosphate and 154 mM NaCl] containing 6 g/L SDS, 0.1 M Na₂SO₃, 10 mL/L protease inhibitor cocktail (Halt Protease Inhibitor Cocktail Kit; Thermo Fisher Scientific, Waltham, MA), and 5 mM EDTA (pH 7.4). The mixture was then extracted by shaking for 15 h at room temperature. Following extraction, the sample was centrifuged at 10 000 × 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 protein content of the obtained extract was analyzed using a 2-D Quant Kit (GE Healthcare UK Ltd, Buckinghamshire, United Kingdom).

Preparation of Model Processed Foods

Model processed foods, whose compositions were based on commercially processed foods, were prepared according to a previously reported cooking procedure (14). Specifically, the freeze-dried black tiger prawn muscle powder was spiked into several types of raw foods (described below) to a final concentration of 10–11.9 µg protein/g food sample, and then each food was cooked to prepare the processed food items containing black tiger prawn protein.

Fish meat sausages, prepared from minced fish flesh (yellow goatfish (*Mulloidichthys martinicus*), Atka mackerel (*Pleurogrammus monopterygius*), and walleye pollock (*Gadus chalcogrammus*), were thoroughly mixed with lard, sugar, salt, ice water, and the black tiger prawn spiking powder. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into 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.

Chicken meatballs were prepared from white ground chicken meat thoroughly mixed with lard, potato starch, sugar, and the spiking powder. The mixture was ground using a small cutter, and the kneaded mixture was manually placed into casings.

Tomato juice samples were prepared using commercial tomato juice thoroughly mixed with the spiking powder for 60 min at room temperature.

Freeze-dried egg soup was prepared using eggs thoroughly mixed with potato starch, milk sugar (lactose), salt, and the spiking powder. The mixture was poured into a plastic tray, frozen in a deep freezer at –80°C, and then lyophilized for 20 h at 25°C.

Preparation of Sample Solution

All samples (various crustacean samples, various molluscan samples, other food ingredients, model processed foods, and commercially processed foods (ground fish, grilled fish paste, fried ground fish cake, fermented fish, seasoning powder, seafood kimchi, spring roll, shrimp-flavored tomato sauce, deep-fried fish in batter, croquette, shrimp-flavored fried flour sticks, crab-flavored tomato sauce, dried soup, scrambled egg in

soup, lobster paste, hamburger meat, canned crab, steamed dumpling, and shrimp au gratin) were separately homogenized in an Ace AM-4 homogenizer (Nissei, Tokyo, Japan) three times for 30 s to achieve homogeneity. Sample extraction solution (19 mL) containing 6 g/L SDS and 0.1 M Na₂SO₃ (17) was added to 1 g portions of each homogenized sample, and then samples were extracted by shaking for 12 h at room temperature. Following extraction, each sample was centrifuged at 3000 × g for 20 min, and the supernatant was filtered through 5AB paper (Advantec) to obtain the extract.

Sandwich ELISA-II Procedure

Tropomyosin derived from porcine (*Sus scrofa domestica*) skeletal muscle (PTM) was added to the dilution buffer to improve the dilution linearity of this ELISA method. PTM was obtained using a previously reported purification procedure (19, 20). The monoclonal and polyclonal antibodies against black tiger prawn muscle protein described in our previous ELISA-I study (14) were also used for the new method, ELISA-II. Polystyrene 96-well microtiter plates (Thermo Fisher Scientific) were coated with 20 µg/mL monoclonal antibody in 50 mM sodium carbonate (pH 9.6) for 18 h at 4°C. After washing three times with Tris-buffered saline (TBS), the plates were blocked for 1.5 h at 25°C with TBS containing 10 g/L bovine serum albumin (BSA), 30 g/L sucrose, and 0.5 mL/L ProClin 950 (Supelco, Bellefonte, PA). Next, the blocking buffer was aspirated, and the plates were dried in an incubator for 2.5 h at 30°C, sealed in an aluminum-coated pack with a drying agent (I.D. Sheet Desiccant; I.D., Tokyo, Japan), and stored at 4°C until use. The food sample extracts were diluted 1:20 with the sample dilution buffer (TBS containing 2 g/L BSA, 30 g/L sucrose, 40 mg/L PTM, 0.5 mL/L Tween 20, and 0.5 mL/L ProClin 950). The diluted sample or a calibrator (100 µL) was added in triplicate to the coated wells, and the plates were incubated for 90 min at 25°C. After washing the plate five times with TBS containing 0.5 mL/L Tween 20 (TBS-T), horseradish peroxidase-conjugated polyclonal antibody (100 µL) was added to each well, and the plate was incubated for 30 min at 25°C. After another five washes with TBS-T, 100 µL 3,3',5,5'-tetramethylbenzidine solution (SureBlue Reserve TMB Microwell Peroxidase Substrate; KPL, Gaithersburg, MD) was added to each well as a substrate, and the plate was incubated for 20 min at 25°C. The reaction was stopped by the addition of 1 M sulfuric acid (100 µL/well). The absorbance of each well was then read using a microplate reader (SpectraMax 340PC; Molecular Devices LLC, Sunnyvale, CA) at a wavelength of 450 nm, with 650 nm as the reference wavelength. Standard curves were obtained by plotting the absorbance against the logarithm of the analyte concentration (serially diluted from 50 ng/mL).

For comparison, the samples were also subjected to our validated ELISA-I method, using 2-ME in the extraction and dilution steps, as described previously (14).

Reactivity Tests for Crustacean Protein

To examine the reactivity of the two methods toward crustacean protein, 11 crustacean samples were extracted using the sample extraction solution as described in *Preparation of Sample Solution*. The extracts were serially diluted from one to one million with sample dilution buffer and then analyzed using ELISA-I and ELISA-II. The concentration–response curves

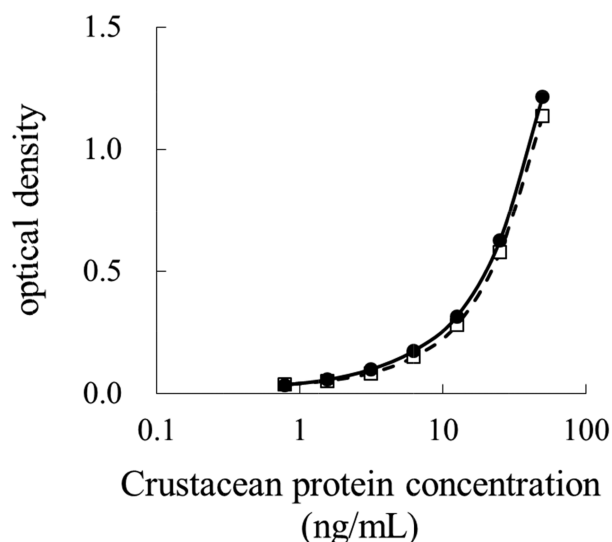


Figure 1. Crustacean protein calibration curves for ELISA-I (open squares) and ELISA-II (solid circles). ELISA-I uses 2-ME as a reductant during extraction, whereas ELISA-II uses Na_2SO_3 .

were obtained by plotting the absorbance against the logarithm of the analyte concentration, and the curves were fitted to the four-parameter logistic equation $y = \{ (A - D) / [1 + (x/C)^B] \} + D$, where A = the maximum absorbance at infinite concentration; B = the curve slope at the inflection point; C = the concentration of the analyte resulting in a 50% response (RC_{50}); and D = the minimum absorbance in the absence of analyte. The percentage reactivity values were calculated as follows: $(\text{RC}_{50} \text{ of black tiger prawn protein} / \text{RC}_{50} \text{ of target crustacean protein}) \times 100$.

Evaluation of Assay Variation, LOD, and LOQ

To determine the intra-assay precision, the mean CVs were calculated from five replicates performed on the same day. Interassay variances were calculated from experiments performed on 5 different days using model processed foods containing 10–11.9 μg black tiger prawn muscle protein/g food sample. The LOD was calculated as $3 \times$ the SD of the buffer blank mean value after 25 experiments. The LOQ was calculated as $10 \times$ the SD of the buffer blank mean values after 25 experiments.

Results and Discussion

Optimization of ELISA-II

In our previous ELISA-I study, we did not obtain satisfactory linearities of the absorbance-versus-concentration curves for

Table 1. LOD and LOQ of the two methods

Parameter	ELISA-I		ELISA-II	
	ng/mL ^a	$\mu\text{g/g}^b$	ng/mL ^a	$\mu\text{g/g}^b$
LOD	0.71	0.29	0.36	0.14
LOQ	2.25	0.90	1.64	0.66

^a Concentration of the assay solution (nanograms of protein per milliliter of assay solution).

^b Equivalent concentration in a food sample (micrograms of protein per gram of food sample).

Table 2. Reactivity of various crustacean samples using the two methods

Sample	Reactivity, %	
	ELISA-I	ELISA-II
Decapoda group		
Black tiger prawn	100.0	100.0
Northern shrimp	65.8	87.7
Japanese spiny lobster	114.3	65.2
Caribbean spiny lobster	105.6	109.9
Red king crab	38.5	50.5
Swimming crab	28.5	47.0
Other members of Crustacea		
Japanese mantis shrimp	7.6	1.8
<i>Euphausia</i>	1.2	<0.1
Opossum shrimp	0.1	<0.1
Acorn barnacle	11.3	<0.1
Goose barnacle	5.7	<0.1

extracts of the various model processed foods using sample dilution buffer without PTM. We therefore tested many different sample dilution buffers and found that adding PTM derived from *Sus scrofa domesticus*, which does not belong to the subphylum Crustacea, to the sample dilution buffer dramatically improved the linearities of the ELISA-I curves. The addition of PTM to the sample dilution buffer was necessary to obtain precise results with ELISA-I (14).

Table 3. Cross-reactivity of various molluscan samples in ELISA-II

Sample	Protein, $\mu\text{g/g}^a$
Cephalopoda class	
Common octopus	<1.0
Giant octopus	<1.0
Ocellated octopus (internal organs removed)	<1.0
Japanese common squid	<1.0
Spear squid	<1.0
Cuttlefish	<1.0
Firefly squid	<1.0
Bivalvia class	
Common scallop	<1.0
Japanese oyster	<1.0
Bloody clam	<1.0
Blue mussel	<1.0
Short-necked clam	<1.0
Common freshwater clam	<1.0
Japanese hard clam	<1.0
Sakhalin surf clam	<1.0
Gastropoda class	
Horned turban	<1.0
Japanese abalone	<1.0
Whelk	<1.0
Pond snail	<1.0

^a Micrograms of black tiger prawn muscle protein per gram of food sample.

Table 4. Reactivity of other food ingredients in ELISA-II

Sample	Protein, $\mu\text{g/g}^a$
Beef	<1.0
Pork	<1.0
Pig liver	<1.0
Chicken	<1.0
Chicken liver	<1.0
Gelatin	<1.0
Milk	<1.0
Egg	<1.0
Quail egg	<1.0
Rice	<1.0
Wheat	<1.0
Buck wheat	<1.0
Soybean	<1.0
Adzuki bean	<1.0
Coffee bean	<1.0
Banana	<1.0
Apple	<1.0
Kiwi fruit	<1.0
Orange	<1.0
Peach	<1.0
Peanut	<1.0
Walnut	<1.0
Almond	<1.0
Cashew nut	<1.0
Sesame	<1.0
Eggplant	<1.0
Pumpkin	<1.0
Onion	<1.0
Bamboo shoot	<1.0
Ginger	<1.0
Garlic	<1.0
Shiitake mushroom	<1.0
Matsutake mushroom	<1.0
Potato	<1.0
Sweet potato	<1.0
Japanese yam	<1.0
Kombu	<1.0
Sea lettuce	<1.0
Hiziki	<1.0
<i>Porphyra yezoensis</i>	<1.0
Salmon roe	<1.0
Cod roe	<1.0
Mackerel	<1.0
Salmon	<1.0
Golden threadfin bream	<1.0
White croaker	<1.0
Walleye pollock	<1.0
Pacific cod	<1.0
Cutlassfish	<1.0
Lizardfish	<1.0
Alfonsino	<1.0
Pacific whiting	<1.0
Horse mackerel	<1.0

Table 4. (continued)

Sample	Protein, $\mu\text{g/g}^a$
Sardine	<1.0
Flying fish	<1.0
Atka mackerel	<1.0

^a Micrograms of black tiger prawn muscle protein per gram of food sample.

Although we do not know why PTM improves the linearities of dilution curves, we evaluated the requirement for PTM in ELISA-II, which uses Na_2SO_3 as a reductant rather than 2-ME for extraction and dilution. We found that the addition of PTM was also necessary for obtaining satisfactory linearity with ELISA-II (data not shown). These results demonstrated that the addition of PTM to the sample dilution buffer is necessary regardless of the type of reductant used.

Calibration Curves

Figure 1 shows the calibration curves constructed for ELISA-I and ELISA-II. The two methods show similar calibration curve profiles between 0.78 and 50 ng/mL crustacean protein. In the Japanese labeling system for allergenic food materials, the enforced declaration criterion in a food sample is 10 μg crustacean protein/g food weight (9). The ELISA-II calibration curve had sufficiently high absorbance at 25 ng/mL, which is equivalent to 10 μg crustacean protein/g food sample.

LOD and LOQ

The LOD and LOQ of the two methods were determined and compared. As shown in Table 1, ELISA-II had an LOD of 0.36 ng/mL, which is equivalent to 0.14 μg crustacean protein/g food sample, and an LOQ of 1.64 ng/mL, which is equivalent to 0.66 μg crustacean protein/g food sample. These values are superior to those obtained using ELISA-I. Allergen detection methods should be capable of detecting low milligrams of protein per kilograms of food (21); thus, ELISA-II appears to detect crustacean protein with sufficient sensitivity to warrant its use as an allergen detection method.

Because ELISA-II showed an LOQ of 1.64 ng/mL, which is equivalent to 0.66 μg crustacean protein/g food sample, we set the practical LOQ to 2.5 ng/mL, which is equivalent to 1.0 μg crustacean protein/g food sample, and evaluated the reactivity and applicability of ELISA-II for use with authentic food samples. The Japanese food allergen labeling threshold, set at 10 μg protein/g food sample for all allergenic foods, is based on practical determination limits of analytical methods used to assess processed foods (9). We therefore considered that a practical LOQ of 1 μg shrimp protein/g food sample is sufficient and appropriate for allergen risk management in Japan. From a safety viewpoint, the minimum eliciting dose for allergenic food residues varies depending on the type of allergenic food and the threshold of the individual consumer with an allergy to shrimp (22). A report by the Voluntary Incidental Trace Allergen Labelling expert panel estimated a minimum eliciting dose for shrimp of 10 mg protein (22). If a consumer ate a 1 kg portion size of food containing 1 μg shrimp protein/g food sample, the intake of shrimp protein would be 1 mg shrimp

Table 5. Recovery of crustacean protein from four model processed foods

Sample	Protein, µg/g ^a	Recovery, %	
		ELISA-I	ELISA-II
Fish meat sausage	10.0	86.7	83.8
Freeze-dried egg soup	11.9	94.3	91.9
Tomato juice	10.0	93.6	91.6
Chicken meatball	10.0	87.8	100.8

^a Micrograms of black tiger prawn muscle protein per gram of food sample.

protein. Because the minimum eliciting dose of shrimp is estimated to be 10 mg of shrimp protein, this practical LOQ (1 µg shrimp protein/g food sample) is considered to provide a satisfactory safety margin for the majority of consumers with an allergy to shrimp.

Reactivity and Specificity

The reactivity and specificity of ELISA-II was tested using various crustaceans, mollusks, and other food ingredients.

As shown in Table 2, the reactivity of the Decapoda group was ≥65.2% for shrimp and lobster and 47.0–50.5% for crabs. In contrast, the reactivity of other species of Crustacea was ≤1.8%. Within the Decapoda group, ELISA-II showed lower reactivity for crab protein than for shrimp, similar to ELISA-I. Purified swimming crab tropomyosin showed high reactivity (154%) in our previous study (14), suggesting that these differences in reactivity are due to differences in tropomyosin content between shrimp and crab. As shown in Table 2, ELISA-II demonstrates higher reactivity for the Decapoda group as compared to ELISA-I, with the exception of Japanese spiny lobster. The reason for this exception is currently unknown, but Na₂SO₃ may be less effective in solubilizing the analyte protein in Japanese spiny lobster compared to the proteins of other Decapoda group species.

As shown in Tables 3 and 4, no cross-reactivity was observed for 19 types of mollusks and 57 other food ingredients. It is known that immunoglobulin E from the sera of subjects with a shrimp sensitivity reacts with both crustacean and mollusk tropomyosin (23). However, ELISA-II did not react with mollusks. Because the Japanese allergy labeling system requires that the presence of crustacean, but not mollusk, be indicated on the food label, the specificity of the proposed ELISA-II method appears promising for the monitoring and labeling of food products.

The present results suggest that ELISA-II has high specificity for the Decapoda group, which includes prawns, shrimps, lobsters, and crabs. Labeling of the presence of shrimp and crab belonging to the Decapoda order of Crustacea is mandatory on food labels, whereas the presence of other members of Crustacea is not mandatory in the Japanese labeling system (9). Therefore, ELISA-II shows sufficient specificity for conformance to the Japanese labeling system.

Recovery and Reproducibility of Model Processed Foods

To evaluate the applicability of ELISA-II for processed foods, four model processed foods containing black tiger prawn muscle protein were tested. As shown in Table 5, the recoveries of the four model processed foods ranged from 83.8 to 100.8%. The reproducibility data from the four model processed foods tested are shown in Table 6. Intra-assay CVs ranged from 2.4 to 3.1%, and interassay CVs ranged from 4.6 to 8.2%. Because there was no significant difference between the reproducibility of ELISA-I and ELISA-II in the *F*-test (*F*-value <3.7), we consider that ELISA-II shows satisfactory precision.

Application to Processed Foods

As shown in Figure 2a, we tested 34 different processed foods using ELISA-I and ELISA-II. The correlation coefficient for these results was 0.996, and the approximate linear equation was $y = 1.06x + 40.8$. Because the criterion for the Japanese food labeling system is ≥10 µg protein/g food, it was also important that ELISA-II show sufficient correlation with ELISA-I in the low-concentration region. The correlation coefficient in the <40 µg/g region for 16 different processed foods was 0.988, and the approximate linear equation was $y = 0.97x + 1.6$ (Figure 2b). Because ELISA-II showed a sufficiently high correlation coefficient with the previously validated ELISA-I (14), ELISA-II also appears to be suitable for the quantitative measurement of crustacean protein in commercial processed foods.

Of the 34 products tested, 26 products with “crustacean” included in the list of ingredients were found to contain ≥1.0 µg/g crustacean protein. Moreover, values ≥1.0 µg/g crustacean protein (but <10 µg/g) were detected in eight other products by both ELISA-I and ELISA-II, despite the lack of labeling indicating the presence of crustacean (shrimp or crab) as

Table 6. Intra- and interassay variances using model processed foods

Sample	Protein, µg/g ^a	ELISA-I		ELISA-II	
		Intra-assay CV, % ^b	Interassay CV, % ^c	Intra-assay CV, % ^b	Interassay CV, % ^c
Fish meat sausage	10.0	3.4	6.8	2.7	8.2
Freeze-dried egg soup	11.9	2.8	5.2	2.4	6.3
Tomato juice	10.0	4.3	6.4	2.6	4.6
Chicken meatball	10.0	3.0	8.2	3.1	7.0

^a Micrograms of black tiger prawn muscle protein per gram of food sample.

^b CVs were calculated from five replicates performed on the same day.

^c CVs were calculated from experiments performed on 5 different days.

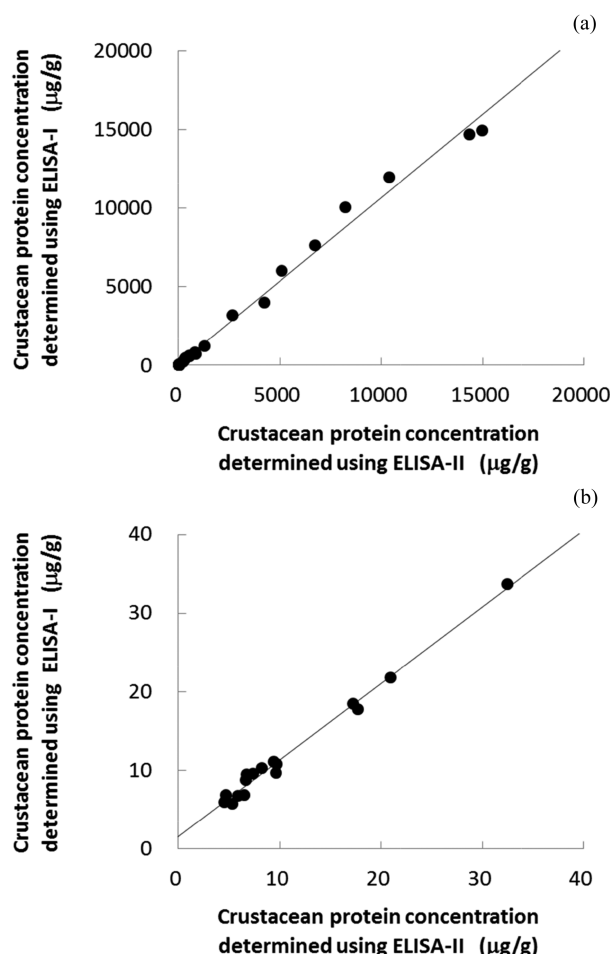


Figure 2. Analysis of various model processed and commercially processed foods using ELISA-I and ELISA-II at the (a) <15,000 µg/g region ($n = 34$; correlation coefficient, 0.996) and (b) <40 µg/g region ($n = 16$; correlation coefficient, 0.988).

an ingredient. We considered that these reactivity values were not due to a false-positive reaction in the ELISA method, but rather from contamination of the food product with crustacean protein. This contamination was most likely due to six of the eight products (three ground fish products, grilled fish paste, fried ground fish cake, and deep-fried fish in batter) being made of ground fish flesh, which often contains crustacean protein, as confirmed using a PCR method (24). Furthermore, two of the eight products (fermented fish and seafood kimchi) contained octopus, whose protein did not show cross-reactivity with ELISA (Table 3). However, octopi often eat crustaceans and, thus, their stomach will frequently contain crustaceans (25).

Conclusions

We developed and evaluated an ELISA method for detecting and quantifying crustacean protein in processed foods using an extraction system that uses Na_2SO_3 as a reductant rather than 2-ME. This change was required because 2-ME, used in our previously validated ELISA method, was designated in 2008 in Japan as being poisonous. Despite the different reductant, the proposed ELISA method using buffer containing PTM

showed high sensitivity, high specificity, sufficient recovery, high reproducibility, and high correlation with our previously validated ELISA method for the detection of crustacean protein in commercial processed foods. Because the proposed ELISA method does not require the use of poisonous reagents and provides acceptable accuracy, this method is expected to be useful for the routine monitoring of food products.

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