



Analytical Methods

Development and validation of a lateral flow assay for the detection of crustacean protein in processed foods

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ABSTRACT

We developed and validated a novel lateral flow assay for the detection of crustacean protein in processed foods. This assay had high sensitivity; the visual detection limit for shrimp protein extract was 25 µg/L, equivalent to 1 µg/g protein in a food sample, and results could be obtained within 20 min without sophisticated procedures or expensive equipment. Concordance between our assay and another validated quantitative enzyme-linked immunosorbent assay was 97% for commercially processed foods. This assay is rapid, simple, reliable, and highly correlated with validated enzyme-linked immunosorbent assays and is thus suitable for monitoring of food products, especially in food-processing facilities.

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1. Introduction

In recent years, food allergies have become an important health problem in developed countries; it is estimated that more than 2–3% of people have some type of food allergy (Ebisawa, 2007; Rona et al., 2007). One of the most problematic aspects of food allergies is that effective therapies have not been established. Therefore, strict avoidance of the food allergen is currently the only means of preventing an adverse allergic reaction (Sampson, 2004; Wang & Sampson, 2011). However, various studies have shown that severe allergic reactions can be caused by accidental ingestion of food products containing allergenic materials (Furlong, DeSimone, & Sicherer, 2001; Yu et al., 2006), making it necessary to develop adequate and reliable information regarding potentially allergenic ingredients in processed foods.

Labelling system for allergenic ingredients in processed foods has been discussed by international organisations such as the Codex Alimentarius Commission of the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO). In 1999, the Joint FAO/WHO Codex Alimentarius Commission Session agreed to recommend labelling of eight foods that contain ingredients known to be highly allergenic, including crustaceans (Hefle, Nordlee, & Taylor, 1996). In Japan, the Ministry of Health, Labour and Welfare (MLHW) has enforced a labelling system for allergenic food materials since

April 2002 to provide information about these foods to allergic consumers. In this system, labelling is mandatory for seven food materials, including eggs, milk, wheat, buckwheat, peanut, shrimp, and crab, and labelling for an additional 18 food materials, such as soybean, is recommended (Akiyama, Imai, & Ebisawa, 2011).

An allergic reaction to crustacean products can be elicited by ingestion of trace amounts of crustacean protein, and anaphylaxis caused by exposure to such protein has been reported (Lopata, O'Hehir, & Lehrer, 2010; Musmand, Daul, & Lehrer, 1993). To protect allergic patients from exposure to hidden allergens, we developed a quantitative enzyme-linked immunosorbent assay (ELISA) for the detection of crustacean protein (Seiki et al., 2007). The method was acceptably accurate and suitable for quantitative measurement of crustacean protein in processed foods; however, the ELISA method is relatively time-consuming and requires expensive instrumentation and well-trained people. In contrast, the lateral flow assay is simple, rapid, and can be applied to the monitoring of processed foods (Röder, Vieths, & Holzhauser, 2009).

In this study, we developed and validated a novel lateral flow assay for the detection of crustacean protein in commercially processed foods as a useful tool for monitoring food products.

2. Materials and methods

2.1. Food samples

Ingredients and commercially processed foods were purchased at local grocery stores in Tsukuba, Japan. We procured

Abbreviations: CV, coefficients of variation; mABS, milli absorbance; MS, mass spectrometry.

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commercially processed foods with and without crustacean labeling in the list of ingredients.

2.2. Preparation of shrimp protein extract and model processed foods

Shrimp protein extract was prepared from black tiger prawn (*Penaeus monodon*) (Seiki et al., 2007). Assay solutions were prepared by diluting the shrimp protein to 0.0, 12.5, 25.0, 50.0, 125.0, and 250.0 µg/L with extraction buffer containing PBS (8.1 mmol/L Na₂HPO₄, 1.47 mmol/L KH₂PO₄, 2.68 mmol/L KCl, and 137 mmol/L NaCl), 1% TritonX-100, and 0.009% NaN₃.

As a model processed food, tomato juice was prepared by adding freeze-dried black tiger prawn muscle powder at final concentrations of 0.0, 2.0, 5.0, or 10.0 µg/g; the liquid was thoroughly mixed for 60 min.

Fish-meat sausage and freeze-dried egg soup containing black tiger prawn muscle powder at final concentrations of 5.0 µg/g and 6.0 µg/g, respectively, were prepared as described (Seiki et al., 2007).

2.3. Preparation of assay solution

The samples were homogenised in an Ace AM-4 homogenizer (Nissei, Tokyo, Japan) three times for 30 s each. Extraction buffer (39 mL) was added to 1 g homogenised sample, which was then shaken using an Ace AM-4 homogenizer or VORTEX-GENIE2 (Scientific Industries Inc., New York, USA) three times for 30 s at room temperature. After extraction, the sample was centrifuged at 3,000×g for 20 min at 4 °C, and the supernatant was filtered through 5AB paper (Advantec, Tokyo, Japan).

2.4. Preparation of test strips

Test strips were prepared as described (Millipore Corporation). The monoclonal and polyclonal antibodies against black tiger prawn protein described in our previous ELISA study (Seiki et al., 2007) were used here. Monoclonal antibodies diluted in PBS (0.5 g/L) were applied to a nitrocellulose membrane in a test line, and anti-rabbit IgG antibodies diluted in PBS (2.5 g/L) were applied in a control line. The membrane was soaked in blocking solution containing 5 g/L casein and dried. Gold colloid solution (10 mL) was adjusted to pH 8.15 and 0.8 mL of 0.1 g/L polyclonal antibody was added. After 30-min incubation at room temperature, 1.2 mL of 100 g/L BSA was added. The mixture was centrifuged and the precipitate resuspended in 0.5 mL distilled water. Next, 190 µL distilled water was added to 10 µL polyclonal antibody–gold conjugate and 800 µL dilute solution; a glass fiber filter was soaked in the mixture and dried. The lateral flow devices were assembled with a mount, nitrocellulose membrane, glass fiber filter, filter paper, non-woven fabric, and cover (Fig. 1).

2.5. Evaluation of the lateral flow assay

Sample solution (2 mL) was placed in a plastic tube, and then a test strip was added for 3 s. The test strip was removed and placed on an aluminum sheet with the test line facing up for 20 min. The

signal intensity of the test line was estimated visually; when at least 1 of 3 observers considered the results positive, it was judged positive and quantitated using an Immunochromato Reader C10066 system (Hamamatsu Photonics K.K., Shizuoka, Japan). To compare the reaction intensity, sample solutions of black tiger prawn, red king crab, pollicipes, skeleton shrimp, opossum shrimp, and euphausia were diluted 1:10,000 with extraction buffer and retested. Since black tiger prawn is comprised of approximately 18.4% protein (Ministry of Education, Culture, Sports, Science and Technology, 2010), the concentration of protein contained in diluted black tiger prawn solution was equivalent to approximately 20 µg/g in a food sample. ELISA was performed as described (Seiki et al., 2007).

2.6. Evaluation of assay variation

Intra- and inter-assay precision were measured as described (Anfossi et al., 2011). Model processed foods containing black tiger prawn protein at 0.0, 2.0, 5.0, 6.0 and 10.0 µg/g were used. To determine intra-assay precision, the mean coefficients of variation (CVs) were calculated from five replicates performed on the same day. Inter-assay variances were calculated from experiments performed on five different days.

3. Results

3.1. Optimisation of the lateral flow assay

Our first approach was to select for antibody combinations showing high immunoreactivity for shrimp protein. Shrimp protein extract at 100 µg/L was not detectable with the combination of gold-conjugated monoclonal antibodies and polyclonal antibodies in the test line; in contrast, sufficient reactivity was observed for 100 µg/L shrimp protein extract with a combination of gold-conjugated polyclonal antibodies and monoclonal antibodies in the test line.

We next examined the effect of reaction time on assay sensitivity. When the reaction time was less than 15 min, detection intensity for 25 µg/L shrimp protein was very low. However, reaction times over 20 min yielded sufficient reaction intensity with 25 µg/L shrimp protein extract for visual detection.

In lateral flow assays, a false-negative reaction known as the prozone phenomenon often occurs when a sample solution contains excessive amounts of the target protein (Butch, 2000). In this study, the prozone phenomenon was observed on the control line with the concentrated crab extract (data not shown). To solve this problem, we optimised the amount of antibody in the control line and found that the concentrated crab extract does not cause the prozone phenomenon when the antibody concentration is ≥2.5 g/L.

3.2. Limit of detection for shrimp protein extract

The limit of detection (LOD) was determined for the shrimp protein extract. As shown in Table 1, reactivity was detected in samples at 12.5, 25.0, 50.0, 125.0, and 250.0 µg/L, and non-specific



Fig. 1. The lateral flow device. When test and control lines appear, the sample is positive. If test line does not appear, the sample is negative.

reaction was not detected for the 0.0 µg/L negative control. Since the ratio of positive results/total from five replicates was 5/5 for ≥ 25.0 µg/L, the visually determined detection limit of this assay was 25.0 µg/L, which is equivalent to 1 µg/g in a food sample.

3.3. Reactivity and specificity tests

The reactivity and specificity of the assay were tested using various crustaceans, molluscs, and other food ingredients. The assay was reactive for ten members of the Decapoda group and four other species of Crustacea, except acorn barnacle (Table 2). The mABS values of these reacted samples were >50.0 . The diluted solution (1:10,000) of black tiger prawn and red king crab showed reactivity, but the diluted solution (1:10,000) of four other species of Crustacea did not (data not shown). No cross-reactivity was observed in 20 different molluscan samples and 59 other food ingredients (Supplementary Tables S1 and S2).

These results show that the assay has sufficient specificity for crustacean protein testing in processed foods.

3.4. Detection limits and reproducibility for model processed foods

To evaluate the applicability of the developed assay to processed foods, model tomato juice containing black tiger prawn protein was tested. The visual LOD for model tomato juice was estimated to be 1.0 µg/g. Intra- and inter-assay variations were assessed using the model tomato juice containing black tiger prawn protein (2.0, 5.0, and 10.0 µg/g), model fish-meat sausage (5.0 µg/g) and model freeze-dried egg soup (6.0 µg/g). As shown in Table 3, the method provided satisfactory precision and sensitivity.

3.5. Application to commercial food products

We tested 104 different commercially processed foods using the assay to evaluate its applicability to processed foods. The products with crustacean included in the list of ingredients were positive, except for spring roll. Products without crustacean in the list of ingredients yielded negative results. The level of crustacean protein in these foods was analysed using the validated quantitative ELISA. Since the limit of quantification (LOQ) was 2.25 µg/L (equivalent to 0.9 µg/g in samples) (Seiki et al., 2007), the samples containing over 1.0 µg/g of shrimp protein were tentatively considered to be positive in the ELISA. As shown in Table 4, concordance between the new assay and ELISA for products containing <1.0 , 1.0–10.0, and >10.0 µg/g of crustacean protein was 100%, 88%, and 100%, respectively. Overall concordance was 97%. The ELISA detected 5.1, 1.5, and 6.4 µg/g crustacean protein in spring roll, kamaboko, and fried cake of ground fish, respectively, but the new assay did not show reactivity for these foods.

4. Discussion

We developed and validated a novel lateral flow assay for detection of crustacean protein in commercially processed foods.

Table 2

Reactivity of various crustacean samples in the lateral flow assay.

Sample	Result
<i>Decapoda group</i>	
Black tiger prawn	+
Red spotted shrimp	+
Northern shrimp	+
Spotted shrimp	+
Freshwater prawn	+
Fan lobster	+
Caribbean spiny lobster	+
Red king crab	+
Swimming crab	+
Snow crab	+
<i>Other species of Crustacea</i>	
Acorn barnacle	–
<i>Pollicipes mitella</i>	+
Skeleton shrimp	+
Opossum shrimp	+
Euphausia	+

Other lateral flow assays for the determination of food allergens have been reported (Röder et al., 2009; Wen, Borejsza-Wysocki, DeCory, & Durst, 2005). However, there has been no scientific report of a lateral flow assay to detect crustacean protein and sufficient validation of the lateral flow assay to detect food allergens, although lateral flow assays for mycotoxin are well validated (Anfossi et al., 2011). We determined the sensitivity, specificity, reproducibility, and applicability of the assay in processed foods and demonstrated that the assay is a simple and reliable method for the detection of crustacean protein in food products.

Since the major allergen of Crustacea is tropomyosin (Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993), we believed tropomyosin would be a target protein for the specific detection of crustacean protein. Detection methods for food allergens should have high specificity; however, the allergen of Mollusca is also tropomyosin, and the molluscan tropomyosin exhibits antigenic cross-reactivity to crustacean tropomyosin because of their homologous amino acid sequences (Shiomi, 2009). We previously prepared monoclonal and polyclonal antibodies with high specific reactivity to crustacean, not molluscan, protein and developed a validated ELISA method using these antibodies for detection of crustacean protein in processed foods (Sakai, Matsuda, et al., 2008; Seiki et al., 2007). In this study, we developed and validated a novel lateral flow method using the same antibodies used in the ELISA method. As expected, the lateral flow assay shows no reactivity for molluscan protein and the proteins of other major foods such as fish, meat, and crops, and the method has high specificity to crustacean protein. In the Japanese labelling system, the labelling of shrimp and crab in the Decapoda group of Crustacea is mandatory, but labelling of other groups of Crustacea, such as Mysidacea and Euphausiacea, is not (Akiyama et al., 2011). We confirmed that the new assay has high specificity to protein of the Decapoda group, similar to the ELISA method (Seiki et al., 2007). These results suggest no false-positive reactions derived from the protein of other groups

Table 1
Analysis of shrimp protein extract.^a

Sample	Assay solution (µg/L)	Equivalent concentration in a food sample (µg/g)	Result (positive/total)	mABS (average)	% CV
Shrimp protein extract	250.0	10.0	5/5	50.3	6.8
	125.0	5.0	5/5	30.8	6.4
	50.0	2.0	5/5	15.0	5.2
	25.0	1.0	5/5	11.1	7.6
	12.5	0.5	4/5	6.5	12.5
	0.0	0.0	0/5	0.5	–

^a mABS is the absorbance in the test line determined using an Immunochromato Reader. % CV is the coefficient of variation calculated from mABS.

Table 3

Intra- and inter-assay variances of the lateral flow assay using model processed foods^a.

Sample	Equivalent concentration in a food sample (μg/g)	Intra-assay		Inter-assay	
		mABS	% CV	mABS	% CV
Tomato juice	10.0	42.4	8.1	46.4	10.4
	5.0	26.0	9.4	28.5	10.8
	2.0	13.2	8.3	12.7	10.0
	0.0	0.7	—	0.9	—
Fish-meat sausage	5.0	15.8	8.8	16.3	12.6
Freeze-dried egg soup	6.0	21.6	6.9	22.0	13.5

^a To determine intra-assay precision, mean coefficients of variation (CVs) were calculated from five replicates performed on the same day. Inter-assay variances were calculated from experiments performed on five different days.

Table 4

Lateral flow assay and ELISA in processed foods.

ELISA result	Result of lateral flow assay		Concordant results/total	Concordance (%)
	—	+		
<1.0 μg/g	48	0	48/48	100
1.0–10.0 μg/g	3	23	23/26	88
>10.0 μg/g	0	30	30/30	100
Total	51	53	101/104	97

of Crustacea would occur during testing of processed foods. Thus, the new assay is suitable for the specific detection of Decapoda protein in processed foods.

Since the results of visual judgment correlated well with the value yielded by mABS (Table 1), we believed the visual judgment of three observers should be sufficient for estimating the visual detection limit in food samples. The visual detection limit for shrimp protein extract was 25 μg/L, which is equivalent to 1 μg/g protein in a food sample. Since analytical methods should detect food allergens at low concentrations (at the level of micrograms per gram of food weight) (Van Hengel, 2007), we sought to establish sufficient sensitivity for detecting food allergens in processed foods.

We prepared tomato juice as a model processed food to verify assay performance because tomato is commonly cooked with sea-food (for example, soups and ketchup) and contains polyphenolic compounds (Aherne & O'Brien, 2002) that interfere with food protein–antibody reactions in immunological assays (Bignardi et al., 2012). Our new assay has sufficient sensitivity, equivalent to 1 μg/g protein in a food sample, and sufficient reproducibility for the detection of the black tiger prawn protein in the model tomato juice.

Mass spectrometry (MS)-based methods have been adopted in the field of food testing, and some MS-based methods for the detection of food allergens have been reported (Abdel Rahman, Lopata, Randell, & Helleur, 2010; Chassaing, Nørgaard, & Hengel, 2007; Lutter, Parisod, & Weymuth, 2011). Although these methods should be highly specific and reliable, they require complex and expensive equipment, as well as technically qualified operators. Therefore, MS-based methods may be not appropriate for routine use. In contrast, our lateral flow assay is simple and requires no sophisticated procedures or expensive equipment; it is thus more suitable for routine use, especially in food-processing facilities.

Assay concordance with the validated ELISA was 97%. The ELISA showed reactivity to protein in extracts of kamaboko and fried cake of ground fish, although there was no crustacean labelling in the list of ingredients. We conclude these processed foods contain crustacean protein, because foods containing ground fish flesh often contain crustacean protein (Sakai, Adachi, et al., 2008). Since

kamaboko and fried cake of ground fish are highly processed and the extraction time in the lateral flow assay (90 s) is shorter than in the ELISA (12 h), we believe the extraction time in the lateral flow assay is too brief to detect crustacean protein in these foods. Crustacean protein was not detected by the new assay in spring roll, although the labelling declared shrimp or crab in the list of ingredients and crustacean protein was detected using the ELISA method. Since crustacean extract, which is used as seasoning in processed foods, is generally processed by protease, this difference in reactivity may be due to structural differences in the crustacean protein and differences in antibody configuration between assays. Therefore, we speculate that highly processed or enzymatically digested crustacean protein might not be detectable by the lateral flow assay in some processed foods. It will be necessary to examine the reactivity of the assay to highly processed or enzymatically digested crustacean protein.

In conclusion, we developed and validated a novel lateral flow assay for the detection of crustacean protein. Since the new assay is rapid (20 min), simple, reliable, and highly consistent with the validated ELISA, this assay is suitable for monitoring food products, especially in food-processing facilities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.10.130>.

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Supplementary data

Supplementary Table S1

Reactivity of various molluscan samples in the lateral flow assay

Sample	Result
Cephalopoda group	
Common octopus	—
Ocellated octopus (with internal organs removed)	—
Giant octopus	—
Japanese common squid	—
Spear squid	—
Cuttlefish	—
Firefly squid (with internal organs removed)	—
Bivalvia group	
Common freshwater clam	—
Short-necked clam	—
Bloody clam	—
Common scallop	—
Japanese oyster	—
Blue mussel	—
Japanese hard clam	—
Sakhalin surf clam	—
Gastropoda group	
Horned turban	—
Japanese abalone	—
Ivory shell	—
Whelk	—
Pond snail	—

Supplementary Table S2

Reactivity of other food ingredients in the lateral flow assay

Sample	Result	Sample	Result	Sample	Result
Beef	—	Peanut	—	Agar weed	—
Pork	—	Walnut	—	Sea lettuce	—
Liver of pig	—	Almond	—	Hiziki	—
Chicken	—	Cashew nuts	—	<i>Porphyra yezoensis</i>	—
Liver of chicken	—	Sesame	—	Salmon roe	—
Milk	—	Pepper	—	Cod roe	—
Gelatin	—	Wasabi	—	Mackerel	—
Egg	—	Eggplant	—	Salmon	—
Quail egg	—	Pumpkin	—	Golden threadfin bream	—
Rice	—	Onion	—	White croaker	—
Wheat	—	Bamboo shoot	—	Walleye pollock	—
Buck wheat	—	Ginger	—	Pacific cod	—
Soybean	—	Garlic	—	Cutlassfish	—
Adzuki bean	—	Shiitake mushroom	—	Lizardfish	—
Coffee bean	—	Matsutake mushroom	—	Alfonsino	—
Banana	—	Potato	—	Pacific whiting	—
Apple	—	Sweet potato	—	Horse mackerel	—
Kiwi fruit	—	Japanese yam	—	Sardine	—
Orange	—	Kombu	—	Flying fish	—
Peach	—	Wakame	—		