

Quantitative analysis of shrimp allergen in food matrices using a protein chip based on sandwich immunoassay

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Abstract Food allergy is increasingly becoming a serious concern these days. With packaged foods becoming the norm of the day, food allergy cases out of accidental consumption are becoming rampant, thereby generating great risks for the subjects involved and prompting food authorities in different countries to formulate new regulations about displaying food allergen data on food labels. Detection of food allergens is conventionally carried out by ELISA or PCR tests. These techniques are limited in that they can only detect one or few allergens at one time. Therefore, in the present study a novel sandwich protein chip assay was developed for quantitation of shrimp allergens in food matrixes. The shrimp allergen model used 3D aldehyde slides as the solid carrier, rabbit antisera as the capture reagent, and biotin-labeled monoclonal antibody as the detector reagent. Resulting antigen–antibody complexes were visualized in the presence of commercial streptavidin labeled with Cy3 to produce fluorescence for quantification. With the LOD of the protein chip being 0.054 mg tropomyosin/kg, the protein chip can quantify down to 0.096 mg tropomyosin/kg. The protein chip was not found to be sensitive to other kinds of foods but cross-reacted to some extent with allergens of some other

crustaceans. The recoveries ranged from 69.2 to 99.9%, while the intra- and inter-assay coefficients of variation were <13% and <19%, respectively. It seems that the new assay is reliable enough to detect shrimp allergens in food and food products and help minimize the instances of shrimp allergy. It is also possible to use the protein chip for simultaneous detection of other food allergens.

Keywords Allergen · Protein chip · Shrimp · Tropomyosin · Detection

Introduction

Seafood is popular around the globe not only as a delicacy but also as a highly nutritious source of animal proteins. With the application of improved processing technologies and aided by modern transportation means, seafood is now more readily available in areas that are far from where it is harvested or processed. Additionally, fish processing technologies like *surimi* have made it possible to churn out large volumes of deodorized, defatted minced marine proteins that form an indispensable raw material used in the production of a variety of imitation products such as crabmeat, crab legs, hotdogs, sausages, sauces, and flavors. Thus, consumption and subsequent exposure to some seafood allergens may not always be obvious to consumers allergic to such foods [1]. As one of the most important food allergens as illustrated by FAO, seafood occupies top spot in two out of the eight major categories of food allergens [2]. Apart from this, seafood has also been identified as a source of occupationally induced health hazards [3]. The workers engaged in the seafood processing come in direct contact with the seafood as well as the aerosols (or vapors) particularly those generated during

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cooking, can develop hypersensitivity reactions such as dermatitis, urticaria, rhinitis, conjunctivitis, asthma, and pneumonitis [4]. Previous reports have shown that about 5.9% of general public in the United States is allergic to seafood with 2.3% of them being allergic to all seafood while 2% to shellfish [5]. Though data on the prevalence of shellfish allergy is highly varied among different surveys, what is clear is that the shellfish allergy is on the rise.

Shrimps are important and highly demanded seafood. Subjects allergic to shrimp are also often allergic to other crustaceans (crabs, lobsters, etc.) and mollusks with a clinical cross-reactivity of about 50–70% due to one homologous major allergen [6], a protein, identified as tropomyosin. Tropomyosin is a heat-stable, fibrillar muscle protein with a molecular weight of about 36 kDa and an isoelectric point (PI) of 4.3. Tropomyosin reacted with the IgE in 82% of the subjects [7]. Although tropomyosin is present in all the animals, the protein from invertebrates only is recognized as a food allergen [8]. And among the invertebrates, shrimp are the most popular or sought after commodity.

Since 2006, a labeling police was formulated making it mandatory to list the eight kinds of major food allergens in food products with their common names [9]. Allergenic cross-contamination may take place between those foods that share the same production line, between seafood-based and seafood-free food products and/or inappropriately labeled food products (with traces of seafood). The urgent need, therefore, is to establish efficient and rapid detection methods for allergen control for use in food processing and for inspecting products on the shelf.

In the past few years, several methods have been established to detect allergens in food matrices [10]. Compared with other allergens, only a few methods were developed to detect seafood allergens. An enzyme-linked immunosorbent assay (ELISA) assay with limit of detection (LOD) of 4 ng/L extracts was developed by Jeoung et al. [11] to standardize shrimp tropomyosin concentrations in skin testing solutions for patients. There are some other ELISAs for tropomyosin detection in foods developed with similar LOD [12, 13]. Till now, only one quantitative ELISA kit for shellfish protein in food matrices based on the shrimp (*Penaeus latissulcatus*) tropomyosin polyclonal antibody is available in commercial market. ELISA is by far the most widely used food allergen detection technique despite its limitations of being able to detect only a few allergens in one plate at a time. There are more than 160 different kinds of allergens in foods [14], and to detect them one by one is very time-consuming and costly. Under this backdrop, an assay capable of detecting most allergens in one go is highly desirable. Protein chips, which were developed almost a decade ago, can meet this requirement [15]. Their

principle is based on the established DNA microarray technology [16] and uses covalent linking of protein ligands to the activated silica surface of microscope slides. The greatest advantage of the protein chip is that it has the potential to detect hundreds of allergens simultaneously. Some previous studies have reported the use of quantitative allergen-specific IgE detecting protein chips for drug screening [17, 18].

In this paper, we select shrimp tropomyosin as a model to study the applicability of protein chips for allergen detection in food matrices with a hope of generating some data for allergen detection in seafood using protein chips. The final aim of our work is to determine most of the major allergens in one slide.

Materials and methods

Reagents

Monoclonal antibody and rabbit polyclonal antibodies for shrimp allergen were produced in Seafood Safety Lab, Ocean University of China. Unless otherwise stated, all reagents were of analytical grade. Cy3-labeled avidin and BCA protein assay kit were purchased from Pierce (Rockford, Illinois); 3-sulfo-*N*-hydroxysuccinimide ester sodium salt, bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Missouri); 3D aldehyde slides for protein chip were purchased from CapitalBio Corporation (China, Beijing). Buffers and reagents used for protein chip were as follows: washing buffer (PBST): 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.05% Tween 20; blocking buffer: 0.01 M phosphate buffer, pH 7.4, containing 0.1% BSA (bovine serum albumin), 0.15 M NaCl; Signal scanning: Eco scan-100 produced in CapitalBio Corporation (China, Beijing).

Preparation of protein chip for spotting polyclonal antibodies

For spotting, rabbit polyclonal antibody protein extracts were dissolved in PBS containing 40% glycerol (v/v) at a concentration of 1 mg/mL and 10 μ L aliquots were plated into each well of a 384-well plate. A manual microarrayer (Whatman TY3757, UK) was used for spotting 3 nL of the protein solutions onto 3D aldehyde slides (CapitalBio Corporation, Beijing, China) at 1,500 μ m spacing. The resulting protein chips were incubated at 37 °C for 2 h and then washed with PBST for 3 times (5 min each) with shaking. After that, the slides were blocked with PBST containing 2% BSA at 4 °C overnight and washed with PBST for 3 times as before. Then, the slides were sealed in a slide box and stored at 4 °C until used.

Preparation of biotin-labeled monoclonal antibodies

For covalent conjugation with biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt, the purified monoclonal antibodies were dialyzed against 0.05 M carbonate-bicarbonate buffer (pH 9.0) overnight at 4 °C with a 3,500-molecular weight cutoff membrane and their final concentration was adjusted to 2 mg/mL according to Holmes [19]. The biotin solution with 6 mg/mL DMSO (6.5 mM) was then prepared with deionized H₂O and adding 1 part of the biotin salt solution to 100 parts of the antibody solution. The mixture was vortexed and constantly shaken at room temperature for 4 h. The reaction was terminated by adding 0.5 mL of 1 M Tris–HCl, pH 8.0. After dialyzing with PBS containing 0.1% (w/v) sodium azide for 30 h, the total protein concentration was measured and the biotinylated antishrimp tropomyosin antibody was stored at –20 °C until used.

Protein standard

Initially, a standard containing 327.68 ng/mL shrimp (*Penaeus vannamei*) tropomyosin was prepared in PBS. A series of shrimp allergen standards were prepared by serially diluting the shrimp allergen in PBS to achieve final allergen concentrations ranging from 0.32 to 327.68 ng/mL (327.68, 163.84, 81.92, 40.96, 20.48, 10.24, 5.12, 2.56, 1.28, 0.64, 0.32 ng/mL shrimp allergen). Protein concentrations were determined by Bradford method using BSA as a standard [20].

Preparation of food samples

Food samples and the processed model foods for the cross-reactivity studies were extracted at a 1:10 ratio (weight/volume) in 0.01 M PBS in a shaking water bath (NTS-4000AM, China) at 60 °C and 140 rpm for 2 h. Extracts were then centrifuged at $3,612 \times g$ (4,100 rpm) in a centrifuge (Jodan France.) at 4 °C for 30 min. Supernatants were collected and their protein contents determined by the Bradford method [20]. The clarified solutions were divided into aliquots and stored at –20 °C until analyzed. Extracts were diluted 30 times in PBS containing 1% BSA before analysis using the protein chip.

Protein chip assay

The spotted protein chips were loaded with shrimp allergen standards in twofold serial dilution of the previously described shrimp protein standards ranging from 0.32 to 327.68 ng/mL, and incubated at 37 °C for 1 h and washed 3 times with PBST. The conjugated allergens were detected by biotin-labeled monoclonal antibody conjugate in

PBS buffer (containing 1% BSA, pH 7.4) and incubated at 37 °C for 1 h. After washing the slides for 3 times with PBST, the antigen–antibody binding reaction was developed by the addition of commercial streptavidin labeled with Cy3 (diluted 1:2500 in conjugate buffer at 37 °C for 1 h). After final washing with PBST, the slides were scanned using Ecoscan100 reader. Standard curves were generated using the average of triplicate fluorescence readings against the concentration of the shrimp allergen standards.

Assay validation: specificity, accuracy, precision, limit of detection, and limit of quantitation

The cross-reactivity of shrimp tropomyosin protein chip with other crustacean species was assessed using seven other different salt- and fresh-water crustacean extracts from different locations. The concentration of the shrimp allergen in all samples was compared to the total protein concentration of their respective extracts and the relative shrimp tropomyosin contents were calculated.

The specificity of the method was assessed in cross-reactivity studies. Extracts from small yellow croaker, mackerel, wheat, milk, pork, beef, soybean, peanut, and egg were prepared as previously described. All extracts were diluted 1:30 in PBS containing 1% BSA prior to analysis with the protein chip.

The accuracy of the method was evaluated by performing recovery studies. Four different shrimp-free food samples (milk, steaming bread, hotdog, and fish *surimi*) were spiked with the shrimp allergen standard at different concentrations (2, 20, 200 µg protein/g sample) in 5 mL extraction buffer and 1 g of the respective food sample. On incubating for 15 min at RT, an additional 5 mL extraction buffer was added, and the extraction was performed as described previously. For the determination of recovery rates, the extractions were done in triplicate and the extractable proteins analyzed by the sandwich protein chip, and the mean values for the recovery were calculated.

Precision within and between assays was estimated using extracts from four different products containing shrimp (milk, steaming bread, hotdog, and fish *surimi*). The extracted samples were stored in aliquots at –20 °C and each analysis was performed with freshly thawed extract. For determination of intra-assay precision, the mean coefficients of variation (CVs) were based on eight replicates. Inter-assay precision was determined as the mean CVs on the basis of triplicate analyses on 11 different days.

The limit of detection (LOD) for the sandwich protein chip was calculated as three times to the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was taken as the lowest linear point. Additionally, the LOD in four shrimp-free

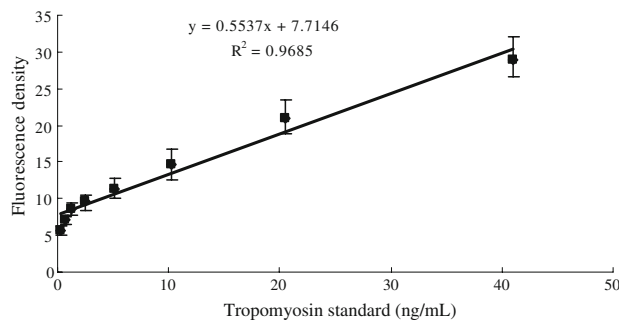


Fig. 1 Tropomyosin standard curve using eight-point linear calibration curve in protein chip. The curve shows the average values of triplicate measurements, the standard deviation in each point of the curve, and the linear regression curve and coefficient R^2

complex food matrixes (milk, steaming bread, hotdog, and fish *surimi*) was determined in the same way, on the basis of five experiments.

Cross-reactivity analyzed by 80aa segments on AllergenOnline

The identification of high identity regions of several allergens indicate similarities of IgE binding or cross-reactivity [21]. Based on AllergenOnline recommendation, every possible contiguous 80-amino acid (80aa) sequence of shrimp tropomyosin was searched from the first 1–80aa segment to the last 80aa segment and these were later compared with the database on <http://www.AllergenOnline.com> (version 9.0). Alignments having >35% identical segments are the proteins that sometimes contain similar structural motifs and thus cause cross-reactivity.

Statistical analysis

Data analysis was performed using SPSS (version 7.0). Data are shown as mean $\pm$ standard deviation (SD). Correlation and linear regression was used in this study. Differences associated with a probability of $p < 0.05$ were considered to be significant.

Results

Tropomyosin standard curve of protein chip

The tropomyosin standard curve was performed using purified tropomyosin standard with concentrations ranging from 0.32 to 327.68 ng/mL. When linear regression was applied, the effective range of the method was defined as the linear part of the curve with a correlation coefficient of ($R(2)$) > 0.95. Using this criterion, an eight-point calibration curve from 0.32 to 40.96 ng/mL (Fig. 1) was found to constitute the effective range. In order to determine the efficiency of this method, we selected three kinds of foods such as hotdogs, bread, and corn sauce which were all tropomyosin free. Results showed higher protein concentrations to cause high noise and affect the standard curve. On the contrary, with dilutions of more than 30 times, the effect on food matrices were negligible (data not shown). When using the standard curve to measure tropomyosin levels from unknown food matrices, it is therefore necessary to make a series of dilutions of the extracts. The dilutions having fluorescence density (FD) values ranging in the mid area of the linear part of the standard curve were used to calculate the tropomyosin concentrations from food samples.

Cross-reactivity of tropomyosin protein chip among different invertebrate species

Extracts of seven commonly eaten aquatic invertebrates (including crustaceans and mollusks) were tested for cross-reactivity in the tropomyosin protein chip (Table 1). Using 30 times diluted crude extracts to apply to the protein chip, three extracts gave rise to FD exceeding a concentration equivalent to 5.3 $\mu\text{g/g}$ of the tropomyosin standard, namely, the mangrove crab, greasy-back shrimp, and mantis shrimp. Mollusks such as the giant Pacific oyster, jellyfish, short-necked clam had FD corresponding to less than 0.054 $\mu\text{g/g}$. The Red swamp crayfish, the only fresh-water species tested showed slight cross-reactivity with shrimp allergen tropomyosin protein chip.

Table 1 Cross-reactivity of some foods in sandwich protein chip

Food extracts ^a	Total protein concentration (mg/mL) ^b	Equivalent shrimp tropomyosin ($\mu\text{g/g}$)
Mangrove crab (<i>Portunus sanguinolentus</i>)	2.3	7.2
Greasy-back shrimp (<i>Metapenaeus ensis</i>)	1.6	6.1
Mantis shrimp (<i>Oratosquilla oratoria</i>)	1.8	5.3
Red Swamp Crayfish (<i>Procambarus clarkii</i>)	3.1	0.3
Giant Pacific Oyster (<i>Crassostrea gigas</i>)	2.8	<0.054
Jelly fish (<i>Rhopilema esculenta</i>)	0.9	<0.054
Short-Necked Clam (<i>Moerella iridescens</i>)	1.4	<0.054

^a All food and food ingredients were extracted 1:10 (w/v) as described in “Materials and methods” and were diluted 1:30

^b Values were obtained using the Bradford protein determination method

Table 2 Cross-reactivity of some foods in sandwich protein chip

Food extracts	Total protein concentration (mg/mL) ^a	Equivalent shrimp tropomyosin (μg/g) ^b
Small yellow croaker	3.1	<0.054
Mackerel	4.5	<0.054
Wheat	2.4	<0.054
Milk	3.2	<0.054
Pork	4.3	<0.054
Beef	3.8	<0.054
Soybean	2.6	<0.054
Peanut	2.2	<0.054
Egg	6.1	<0.054

^a All food and food ingredients were extracted 1:10 (w/v) as described in “Materials and methods” and were diluted 1:30

^b Values were obtained using the Bradford protein determination method

Specificity

Two kinds of marine fish and some other common food allergens were selected to evaluate the specificity of the protein chip (Table 2). The FD equivalent to shrimp tropomyosin was measured, and the results showed that none of the food extracts exceeded FD of 0.054 μg/g.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD of the protein chip corresponded to 0.18 μg/L tropomyosin standard, equivalent to 0.054 μg/g of food, considering the sample dilution in the assay. The LOQ was 0.32 μg/L tropomyosin standard in the assay and 0.096 μg/g of tropomyosin in food. Furthermore, the LOD was assessed in four different blank food matrixes resulting in different LODs corresponding to different food matrixes. The LOD was 0.054 μg/g (milk), 0.108 μg/g (steamed bread), 0.108 μg/g (hotdog), and 0.324 μg/g (fish surimi).

Accuracy

The recovery in four kinds of blank food matrixes (milk, steamed bread, hotdog and fish surimi) were used to evaluate the accuracy of the protein chip method. All the

four matrixes were spiked with three different amounts of shrimp tropomyosin before sample extraction (Table 3). The recoveries of spiked samples ranged between 69.2 and 99.9%. The recovery differences among different tropomyosin concentration levels were within the range of the standard error.

Intra- and inter-assay precision

For assessment of the intra- and inter-assay precision of the shrimp tropomyosin protein chip, four typical food samples containing shrimp tropomyosin at different concentrations between 26 and 232 μg/g were analyzed (Table 4). The intra-assay precision, expressed as the coefficient of variation (%CV), ranged from 5 to 13%, whereas the inter-assay precision ranged from 8 to 19%.

Possible cross-reactivity analysis with AllergenOnline

Comparison of the amino acid sequences of shrimp tropomyosin with those of known allergens listed on <http://www.AllergenOnline.com> (version 9.0) revealed 45 significant matches (Table 5), including 14 crustacean species, 13 mollusks and 11 insects, if the repeated species are ignored. The 80mers identity of crustacean species was more than 96.3%, while that for mollusks was lower than 80%, and that for the insects was in between the two. The E-value of all the species was very low except for *Batillus cornutus*. But if the full alignment is compared, the identity of crustacean and mollusk allergens is lower than 65.4% except for *Batillus cornutus* (only 40AA not an intact protein).

Discussion

Food allergy is an adverse immune response to food proteins, which can lead to skin and stomach problems, and may even culminate in death. Instances of food allergy are on the rise in recent years. Seafood is recognized as one of the most important source of food allergens. Because of its nutritious value and appealing taste, seafood is consumed by more and more people. But for those allergic to seafood, strict avoidance is the only way to prevent allergic

Table 3 Recovery (%) from some possible blank food matrix with 2, 20, or 200 μg/g of shrimp tropomyosin spiked

Blank food matrix	Recovery (%) at certain amount of shrimp tropomyosin		
	2 μg/g	20 μg/g	200 μg/g
Milk	84.6 ± 9.1	88.7 ± 7.3	99.9 ± 7.4
Steaming bread	76.2 ± 14.5	77.3 ± 11.9	82.6 ± 8.2
Hot dog	77.1 ± 9.2	71.9 ± 12.5	85.1 ± 7.8
Fish surimi	69.2 ± 8.6	79.1 ± 13.6	89.3 ± 11.4

Values represent the average of three spiking experiments and are reported as mean [standard error of the mean (SEM)]

Table 4 Inter- and intra-assay variances (%CV) determined for shrimp tropomyosin protein chip methods

Food matrix	Shrimp tropomyosin ($\mu\text{g/g}$)	Intra-assay variance (%CV) $n = 8$	Inter-assay variance (%CV) $n = 11$
Fish surimi	26	13	19
Steaming bread	210	9	15
Hot dog	58	11	14
Milk	232	5	8

reactions. However, food products could be contaminated with seafood allergens via unsuitable cleaning procedures, shared instruments and production lines, or simply by accidental incorporation during production. ELISA and PCR are routinely used to detect allergens in foods. Given the existence of a large number of allergens, these techniques are not quite efficient in that they can only detect few allergens at one time. This is one of the most important factors that restricts their usage in the food industry. It is therefore necessary to develop a broad ranged, convenient, quick, and an inexpensive method to inspect the allergens in the food processing industry. In this light, protein chips could be used as they can detect a number of targets at the same time. It is reasonable that we try to establish one allergen-based protein chip and apply this method to other allergens step by step. Finally, we hope to detect most of

food allergens in one protein chip. Here we select shrimp tropomyosin as an example and try to construct a sensitive and specific protein chip method and validate its applicability with five typical food matrices.

In the present study, a novel sandwich protein chip was developed using polyclonal antibodies to shrimp tropomyosin as capture antibody and a biotin conjugate of monoclonal antibody to shrimp tropomyosin for detection. The advantage of using polyclonal antibody as a capture antibody is that it can recognize various epitopes of shrimp tropomyosin and make sure of the binding of the protein to the chip surface. After that, monoclonal antibody was used to make the detection highly specific. This versatility was considered to be favorable for designing sandwich protein chip for the purpose of detecting shrimp tropomyosin in foods. Furthermore, this technique can detect allergens in processed foods where the processes employed may cause the protein to unfold or be destroyed. The applicability of the novel protein chip in detecting shrimp allergens in different treated or processed food samples was studied in the present work.

The standard curve was linear with concentrations between 0.32 and 40.96 μg tropomyosin/L or 0.096–12.288 $\mu\text{g/g}$ food if the sample dilution in the assay is considered. Assuming that the average percentage of tropomyosin in shrimp muscles as 2% within the different species and different parts [22], the working range of the

Table 5 Some selected results about 80mers research with AllergenOnline

Hit	English name	Species	Best %ID	No. of hits > 35%	Full alignment		
					E-value	%ID	Length
1	Linnaeus	<i>Charybdis feriatus</i>	100.00	205 of 205	7.3e-068	91.70	264
2	Shrimp	<i>Metapenaeus ensis</i>	100.00	205 of 205	5.5e-077	99.60	274
3	Shrimp	<i>Marsupenaeus japonicus</i>	100.00	205 of 205	2.1e-080	100.00	284
4	Shrimp	<i>Penaeus monodon</i>	100.00	205 of 205	2.1e-080	100.00	284
5	Mantis shrimp	<i>Oratosquilla oratoria</i>	100.00	205 of 205	9.7e-079	97.90	284
6	Krill	<i>Euphausia pacifica</i>	97.50	205 of 205	5.6e-074	91.50	284
7	Midges	<i>Chironomus kiiensis</i>	96.30	205 of 205	2.2e-062	79.70	281
8	Dust mite	<i>Dermatophagoides farinae</i>	93.80	205 of 205	1.3e-066	81.70	284
9	Mite	<i>Blomia tropicalis</i>	91.20	205 of 205	4.5e-066	80.30	284
10	Mould mite	<i>Tyrophagus putrescentiae</i>	80.00	159 of 205	3e-038	69.80	199
11	Silverfish	<i>Lepisma saccharina</i>	78.70	205 of 205	9.9e-055	68.00	284
12	Snail	<i>Helix aspersa</i>	76.20	205 of 205	3.5e-052	63.70	284
13	Oyster	<i>Crassostrea gigas</i>	75.00	176 of 205	9.7e-042	65.40	228
14	Squid	<i>Todarodes pacificus</i>	75.00	205 of 205	5.3e-051	63.00	284
15	Squid	<i>Sepioteuthis lessoniana</i>	73.80	205 of 205	4.2e-051	62.70	284
16	Squid	<i>Ommastrephes bartramii</i>	73.80	205 of 205	2.4e-051	62.70	284
17	Balanus	<i>Balanus rostratus</i>	67.50	205 of 205	4.5e-047	58.70	283
18	Mussel	<i>Perna viridis</i>	66.20	178 of 205	6.5e-045	56.00	284
19	Turban	<i>Batillus cornutus</i>	40.00	6 of 205	6.8e-007	80.00	40

method (2.85–36.5 $\mu\text{g/kg}$) is robust enough to detect the allergens efficiently. In fact, if the concentration of shrimp allergen exceeds the range, it is better to dilute the samples before measurement. In order to compare the effect of food matrices, we select hotdog, bread, and corn sauce because they are typical matrices for shrimp. A kind of special shrimp hotdog is frequently consumed in China and there are breads which use shrimp as an additional nutrition. Also shrimp is often used as a kind of additives in corn soup. High concentration of food matrices also may block the binding sites of antibody or antigen. Some other proteins may also have nonspecific binding. In this assay, our protein chip shows negligible effect if we diluted the extracts 30 fold. It is useful for us to find some other ways to improve the detecting condition for further use in food processing inspection.

The specificity of the protein chip was tested by cross-reactivity experiments involving some other seafood and other typical food ingredients. With the exception of crustaceans that produce a signal equivalent to more than 0.3 mg/kg tropomyosin, no cross-reactions were observed with other samples. Even these mollusks that have been reported to cross-react with shrimp [23] did not display any cross-reaction in the assay. Other food samples such as eggs, peanuts, and beef were equally inert. Similar epitopes among crustacean allergens have been shown to be the major cause of cross-reactivity [24], and clinical evidence has proved that there is a significant cross-reaction between them [25]. Comparison of amino acid sequences by AllergenOnline showed that 80mers of all the crustacean species were more than 96.3% identical. Lesser similarity existed when the allergen was compared with the mollusks and the dust mite, whereas the remaining species did not show any similarity (>35%) with the shrimp allergen. Consistent with the known principles, our experimental data showed that crustaceans-like mangrove crab, mantis shrimp, and crayfish had a high cross-reactivity with shrimp allergen, while mollusks such as oysters showed less cross-reactivity. It seems reasonable that the dust mite has higher homology with shrimp as they belong to Arthropoda. In this study, we did not select dust mite simply because they do not form a food item. But, there are chances that these mites could potentially contaminate all kinds of foods during transportation and preservation. Therefore, it seems useful to further investigate the cross-reactivity between the dust mite and the shrimp allergen.

The LOD of the tropomyosin protein chip was found to be 0.054 $\mu\text{g/g}$ of tropomyosin in food. As mentioned earlier, the content of tropomyosin in shrimp is about 2%. Concerning the individual LOD of the food matrix surimi, it means that the protein chip we have developed can detect 16.2 mg shrimp in 1 kg surimi (which at the LOD has a tropomyosin concentration of 0.324 $\mu\text{g/g}$). So, by the time

a person allergic to shrimp consumes a kilogram of surimi, he would have consumed about 16.2 mg of tropomyosin. Although the threshold of shrimp allergen has not reported yet, it is not certain if a meager amount of 16.2 mg of tropomyosin could prove to be fatal at such low doses, especially when it is not consumed in one go. This is a testimony that the newly developed shrimp sandwich protein chip is sensitive enough to detect shrimp allergen traces in processed foods which might trigger allergic reactions in most of the sensitized consumers.

The precision of protein chip was evaluated in terms of its repeatability (intra-assay variance) and reproducibility (inter-assay variance) by spiking different levels of shrimp tropomyosin into four typical foods matrices with the tropomyosin contents ranging from 26 $\mu\text{g/g}$ (fish surimi) to 232 $\mu\text{g/g}$ (milk). The repeatability of protein chip method was found to be <13% with the reproducibility being <19%. These values are a little higher than the ELISA developed by Marianna et al. [26]. Although the precision of protein chip is a little lower than ELISAs, it is still reliable at different concentrations with assessable differences in the different samples.

According to the recommended harmonized guidelines for single laboratory validation [27], in-house validation of the shrimp sandwich protein chip was further determined. By spiking shrimp tropomyosin into four different blank matrices at different concentrations, the accuracy of the protein chip was tested by recovery experiments. For all the four food matrices, the recovery of shrimp tropomyosin was relatively low at lower concentrations and increased with increasing concentrations, a reason that could probably be attributed to poor sample homogeneity at low concentrations. The recovery of shrimp tropomyosin from milk was the highest, and reduced to about 10% for multi-component food products such as steamed bread, hotdog, and fish surimi. It is reasonable here to think that the interactions between the components of complex food may interfere with and affect the extraction procedure which may cause low recoveries. A less-than-optimal extraction procedure may partially explain the low recovery rate in some analyses. It is therefore deemed necessary to perform validation of extraction in different food matrices. For the protein chip method, the accuracy was satisfactory with variances of <16.4%. However, the trueness of the extraction as well as the method could not be determined due to unavailability of certified shrimp tropomyosin standards, and validation by an alternative analytical method was not possible either.

As discussed by Crevel et al. [28], the threshold levels of shrimp allergen is still an open question due to population aspects, differences in challenge and risk assessment protocols, and so forth. The sensitivity of the tropomyosin sandwich protein chip would be lower than 1 $\mu\text{g/g}$ in most

cases; this operative LOD is in agreement with the assay specifications considered to be necessary, in general and as such, is sensitive enough for the food industry in inspecting shrimp allergens in processed foods.

In conclusion, an effective sandwich protein chip for detecting shrimp tropomyosin in food samples was successfully developed and validated with four different typical food matrices. The shrimp tropomyosin sandwich protein chip could not only be used for raw materials and end products, but also for routine safety control inspections during food processing. Though there are a number of methods available for the detection of tropomyosin suited for food analysis, the present method has the potential of detecting most of the major food allergens simultaneously.

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