



Development of ELISA Method for Detecting Crustacean Major Allergen Tropomyosin in Processed Food Samples

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Abstract

Crustacean aquatic products were recognized as allergenic food source and tropomyosin (TM) was identified as putative major allergen. At present, the most effective way to control allergenic incidence was avoidance of causative dietary source. This study reports the development of sandwich ELISA for effective detection of TM in suspected food sources by pairing two TM-specific polyclonal antibodies obtained from different species. The LOD was identified as 6.8 ng mL⁻¹, with high specificity to the TM of shrimp and crab. No cross-reactivity was observed with the TM of fish, poultry, and mammalian. The mean recoveries in food matrices were range from 87.96 to 118.77%, by both intra- and inter-assay systems with coefficients of variation of < 10% and < 25%, respectively. The sandwich ELISA can be effectively applied for detection of TM in commercial products available in the market. It facilitates the allergen labeling and management for allergenic crustacean food products by both manufacturing and regulatory agencies.

Keywords Tropomyosin · Crustacean · ELISA · Food allergen · Detection

Introduction

Food allergy affects an estimated 8% of children and 3–5% of adults worldwide (Sicherer and Sampson 2010; Gupta et al. 2011). The Food and Drug Administration (FDA) recognizes peanuts, soybeans, milk, egg, fish, crustacean shellfish, wheat, and tree nuts as the 8 most common food allergens. As one of them, crustacean aquatic products were recognized as allergenic food source with tropomyosin (TM) as its major allergen. The bad management of food allergy can reduce the quality of life for a sensitized consumer; the avoidance of diets with possible allergen content was the recommended treatment

for the prevention of allergic reaction. Adherence to avoidance of diets is challenging in the current market since the processed foods may contain trace amounts of allergens due to shared processing equipment, improper cleaning procedures, adulteration, or mislabeling of allergenic ingredients (Besler et al. 2002). To combat accidental allergen exposure, several international and national laws have been enacted by the European Food Safety Agency (EFSA), the US Food and Drug Administration (FDA), and other organizations to enforce labeling of allergenic ingredients.

Shrimp and crab are delicious high protein aquatic products that are popular because of their tasty flavor and nutrition. TM, which generally refers as the major component of the myofibril, is a heterodimer, composed of two identically coiled subunits with molecular masses of 35 to 38 kDa, has been known as the major allergens in shellfish and mollusk species, and accounts for 80% of shrimp and crab allergies (Jeoung et al. 1997; Fernandes et al. 2003). Crab tropomyosin demonstrates marked homology to the shrimp tropomyosin with remarkably high epitope sharing regions and allergic cross-reactivity (Leung et al. 1998); thus, shrimp and crab tropomyosin could be controlled in the similar approach in suspected food sources.

At present, the most effective way to deal with allergens is to detect and label allergens for consumer identification. ELISAs are the primary detection method used in food

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allergen analysis and sensitive enough to detect low concentrations of proteins in foods (Monaci and Visconti 2010; Yeung 2006). The competitive ELISA is used in certain situations when protein levels are exceptionally low or specific peptides are being targeted. When it accounts for intact allergenic proteins or larger protein fragments, sandwich enzyme-linked immunosorbent assay (sELISA) was the most commonly used detection method (Immer and Lacorn 2015).

Sandwich ELISA is currently the most reliable method employed in food industry and regulatory agency for analysis of ingredients during food technology and food safety monitoring (Poms et al. 2005). A majority of reported studies for allergen detection have showed the effective use of ELISA; consequently, less attention is given to the application to the processed food required for labeling and food safety monitoring.

Earlier studies had reported the development of an enzyme-linked immunosorbent assay for the detection of shrimp and crab allergen tropomyosin and used a monoclonal antibody system. In all of the three detection methods, limit of detection (LOD) was defined below 1 ppb (1 ng mL^{-1}) with high sensitivity which may be due to the application of the monoclonal antibodies (mAbs) in detection system (Seiki et al. 2007; Zhang et al. 2014; Lin et al. 2018). MABs possess a high level of specificity and selectivity and efficiently select a single epitope while polyclonal antibodies (pAbs) react with multiple epitopes in the target proteins. PABs were widely accepted for development of detection system over mAbs as they recognize single epitope in allergic food which may be hidden or spoiled during processing resulting in false negatives, so that the sandwich ELISA with mAbs will put consumers in more risk of food allergy issues.

Furthermore, the polyclonal rabbit anti-tropomyosin antibodies were used for both capture and detection by research groups during development assay system, wherein the biotinylated conjugate of the same antibody limits the extent and reliability of TM detection by sELISA with LOD of less than 1 ppm ($1 \mu\text{g mL}^{-1}$). It is apparently far below the methods that were used by mAbs, and it may be due to competition by antibodies for the same epitope during recognition and the labeling of antibodies causing the reduction of their sensitivity (Wang et al. 2019). In Japan, the detection criterion for ELISA in monitoring food allergen level in suspected food matrix is $10 \mu\text{g}$ food allergen protein per gram (or mL) of foods (Werner et al. 2007; Fuller et al. 2006). It was set as the threshold value to regulate commercial prepackaged foods by the regulatory agencies for labeling of allergens (Shoji et al. 2018).

Considering the fact that extraction step will cause the dilution of the sample and the 1 ppm ($1 \mu\text{g mL}^{-1}$), LOD may not satisfy the state of safe for allergen detection. It requires higher efficiency and sensitivity method to monitor trace level of allergen contamination in processed

foods. In this study, we selected two strains of TM-specific polyclonal antibody to establish a high sensitive sandwich ELISA for detection and monitoring of TM in crustacean contaminated processed foods. To avoid the risk of false negative and labeling reducing antibody sensitivity, we chose the pAbs system with non-labeled detection antibodies. Instead of labeling only detection antibody, a combined detection antibody and HRP-conjugated antibody incubation was carried out in our study to achieve the high sensitivity and short binding time. The method has high specificity and sensitivity that will be precise enough to detect tropomyosin in shrimp and related processed foods and can be effectively applicable for commercial monitoring of labeling shrimp allergens by industry for approval by regulatory agencies and consumer protection.

Materials and Methods

Materials

Mouse anti-shrimp TM IgG and rabbit anti-shrimp TM IgG antibodies were obtained from Beijing Protein Innovation. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies were obtained from ImmunoReagents. Purified shrimp TM was acquired from the laboratory of Food Safety, Ocean University of China. NFDM (non-fat dry milk) was purchased from BD Company, USA, while TMB (3,3',5,5'-tetramethylbenzidine) and single-component substrate solution was obtained from Solarbio Co., Beijing. Fresh samples of white leg shrimp (*Penaeus vannamei*), swimming crab (*Portunus trituberculatus*), short necked clam (*Ruditapes philippinarum*), large yellow croaker (*Larimichthys crocea*), bastard halibut (*Paralichthys olivaceus*), porcine, beef, chicken, and commercial processed foods were purchased at local markets in Qingdao, Shandong. All other chemicals and reagents used were pure and of analytical grade, unless otherwise stated.

Extraction and Purification of TM from Muscles

Extraction of TM

The TMs were extracted from the muscle sample following the published procedure and modified (Seiki et al. 2007). Briefly, these muscles (1 g) were homogenized with 10 mL of PBS (phosphate buffer saline) having a pH of 7.4 for 1 h at room temperature. After centrifugation at 3000 g for 20 min at room temperature, the suspension was confirmed for the ultimate tropomyosin-containing fractions.

Purification of TM

TMs from shrimp were purified by a combination of ammonium sulfate and isoelectric precipitation according to a method described previously (Bailey 1948; Lasekan and Nayak 2016), with some modification based on our lab's protocol. Firstly, the minced muscles were homogenized, centrifuged, and suspended to obtain the contents. Then, contents were washed by cold acetone to gain powders. Subsequently, precipitates were suspended and centrifuged, while the extracts were fractioned with ammonium sulfate and isoelectric precipitation. Finally, precipitations were dissolved and dialyzed overnight and then heated in a water bath at boiling temperature for 10 min to obtain TMs. TMs were stored at -20°C until used.

Production of Rabbit and Mouse Polyclonal Antibodies to TM

The polyclonal antibodies were generated at BPI Co., Ltd. (Beijing, China) for the purified shrimp TM. The rabbit antiserum against TM was produced by immunization of New Zealand rabbits with purified TM with Freund's adjuvant. Injections were repeated two times at appropriate intervals (10 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 mouse antiserum against TM was produced by immunization of Balb/c mice with purified TM with Freund's adjuvant. Injections were repeated three times at appropriate intervals (14 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.).

Evaluation of Rabbit and Mouse Polyclonal Antibodies to TM

The specificity of the polyclonal antibodies used for the development of TM detection was demonstrated by direct ELISA using the various purified TMs from different sources.

SDS-PAGE Analysis

The extracted and purified tropomyosin samples were subjected to SDS-PAGE according to the method described by Laemmli (Laemmli 1970). The samples (10 μg per well) were mixed with bromophenol blue buffer (4:1) and were heated for 7 min at 100°C . SDS-PAGE gel was prepared with 5% stacking gel and 12% running gel. Electrophoresis of the proteins was performed in the stacking gel at 80 V for 0.5 h and in the resolving gel at 120 V for 1.5 h. Coomassie Brilliant Blue R-250 (Solarbio, China) was used to stain the gels, followed by

destaining until the solution became clear. Protein bands were determined using Tanon-4200SF gel imaging and analysis software (Tanon Science and Technology Co., Ltd., Shanghai, P. R. China).

Western Blot

Samples were separated with SDS-PAGE as described earlier and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane by iBlot® 2 Gel Transfer Device (Life Technologies Co., Israel) for 7 min at room temperature. The membrane was washed with iBind solution kit. Then, it was incubated in iBind™ Western Device (Life Technologies Co., Israel) with rabbit antibodies (diluted 1:2000) or mouse antibodies (diluted 1:5000) as the primary antibody, later incubated with (HRP)-conjugated goat anti-rabbit IgG antibody (diluted 1:2000) or (HRP)-conjugated goat anti-mouse IgG antibody (diluted 1:5000) as the secondary antibody for 5 h at room temperature in total. After being washed with deionized water, the results were visualized with western blot chemiluminescent substrate. PVDF film was exposed for 10 s. Images were captured by Tanon 5200 Multi Chemiluminescent Imaging System (Tanon Science & Technology Co., Shanghai, China).

Sandwich ELISA Procedure

Polystyrene 96-well microtiter plates were coated with 100 μL well⁻¹ of polyclonal antibodies (rabbit anti-shrimp TM IgG antibodies, 1.75 $\mu\text{g mL}^{-1}$ in 50 mM sodium carbonate buffer, pH 9.6) for overnight at 4°C . Then, the plates were washed three times with PBST (PBS containing 0.05% Tween-20, pH 7.4). All consecutive washing steps performed between each operation in the tropomyosin sandwich ELISA were the same. Then, the plates were blocked for 2 h at 37°C with PBST containing 5% (0.05 g mL^{-1}) of NFDM. After washing, each well was added with 100 μL of TM (or food sample, PBS as control), followed by incubation at 37°C for 1.5 h. After washing again, 100 μL of premixed solution of mouse anti-shrimp TM IgG antibodies (diluted 1:5000) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (diluted 1:5000) was added into each well as detection antibodies and incubated at 37°C for 1 h. Followed by wash, 100 μL of TMB single-component substrate solution was added into each well and allowed for color development at 37°C in dark for 10 min. Afterwards, 50 μL of 2 M H_2SO_4 was added to each well to stop color development. Finally, the optical density at 450 nm was measured using a microplate reader (CMax Plus, Molecular Devices, LLC., San Jose, CA, USA).

Evaluation of Detection Limit, Accuracy, Precision, and Specificity of sELISA for TM Detection

The limit of detection (LOD) and quantitation (LOQ), accuracy, and precision of the developed sELISA were determined according to the Eurachem Guide (Magnusson and Örmemark 2014). LOD and LOQ were respectively calculated according to the average of 10 blank values plus 3 or 10 times the standard deviations (SD). The precision was evaluated by determining the relative SD of repeatability (RSD_r, intra-day) and reproducibility (RSD_R, inter-day) at a series of TM concentrations. Repeatability and reproducibility were evaluated by testing TM at different concentrations in 1 day ($n = 5$) or in 5 sequential days ($n = 3$), respectively. Results were calculated as follows:

$$\text{RSDr(or RSDR)}(\%) = \text{SD}/\text{mean} \times 100\%$$

The specificity of the TM specific polyclonal antibodies used for development of sELISA was determined by assessing the possible cross-reactivity TMs from swimming crab (*Portunus trituberculatus*), short necked clam (*Ruditapes philippinarum*), large yellow croaker (*Larimichthys crocea*), bastard halibut (*Paralichthys olivaceus*), porcine, cattle, and chicken proteins at 0.0625, 0.25, 1, and 4 $\mu\text{g mL}^{-1}$ concentrations.

The accuracy of the method was evaluated by performing recovery studies. Three different shrimp tropomyosin-free foods (fish meat, freeze-dried seaweed egg soup and meat sauce) were homogenized and spiked with the tropomyosin standard at levels of 5 and 10 μg of tropomyosin per gram. Then, the samples were added in a total volume of 5 mL with PBS (pH 7.4). After vortexing, the samples were incubated for 1 h in room temperature and then centrifuged at $3000\times g$ for 20 min. The suspension fractions were collected for analysis. To evaluate the recovery, extraction was performed in triplicate, and the resulting extract was analyzed by tropomyosin sandwich ELISA, and the mean value of the recovery and the standard error of the mean were calculated.

TM Detection in Commercial Food Matrices by Developed sELISA

Seventeen different commercial food samples were analyzed by the sandwich ELISA method. Commercial products are divided into two large categories according to the label of allergens, as no TM food and TM food. The TM food was further divided into sub-categories, as labeled TM food and no labeled suspect TM food. Each commercial food was homogenized, and the extracts were obtained according to the

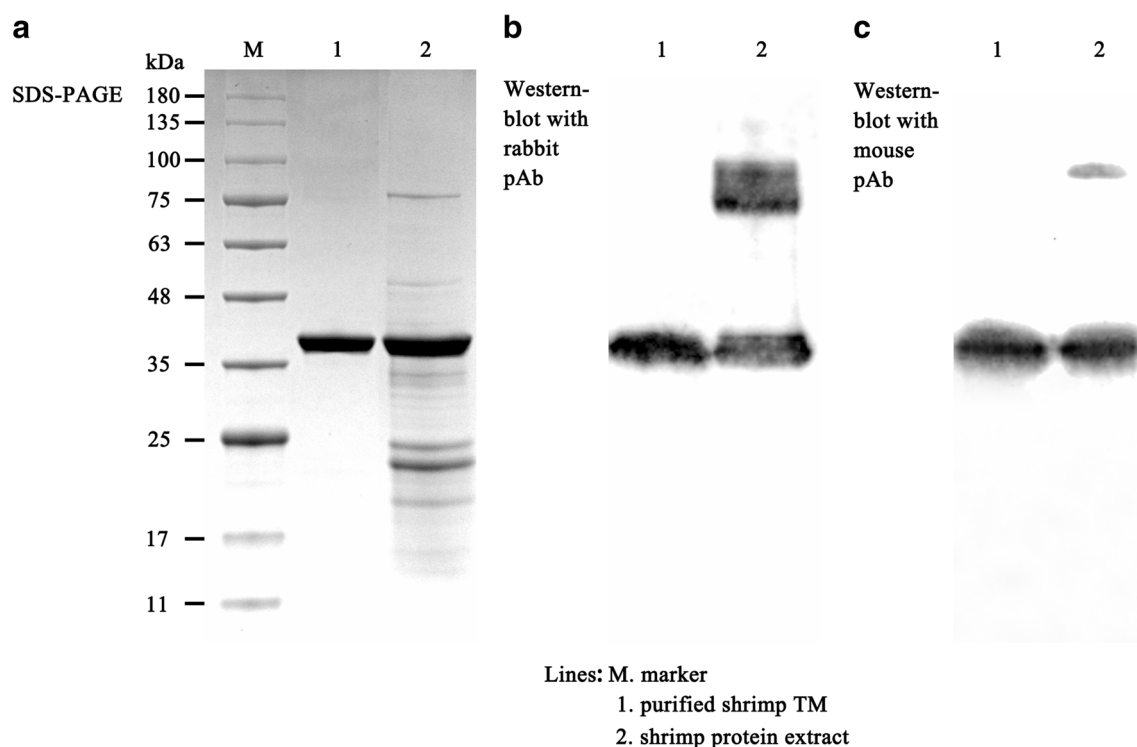


Fig. 1 Binding specificity of two rabbit and mouse anti-shrimp tropomyosin polyclonal antibody to purified tropomyosin and crude protein extraction from shrimp. M: molecular weight marker; Lane 1: purified shrimp TM (5 μg); Lane 2: shrimp protein extract (10 μg). **a** SDS-PAGE profile with 12% gel stained with Coomassie Brilliant Blue stain. **b** Western blot analysis with primary antibody as rabbit anti-shrimp

polyclonal antibody (1:2000 dilution), and the secondary antibody was HRP-conjugated goat anti-rabbit polyclonal antibody (1:2000 dilution). **c** Western blot analysis with primary antibody using mouse anti-shrimp polyclonal antibody (1:5000 dilution) and secondary antibody using HRP-conjugated goat anti-mouse polyclonal antibody (1:5000 dilution)

extraction procedure and were detected by sELISA as described in the earlier sections.

Statistical Analysis

Results were expressed as mean $\pm$ SD values wherever they were applicable considering the sample numbers used in the experiment. Statistical significance was determined using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA), and significant differences were analyzed *t* test. Statistically significant values were considered at $p < 0.05$.

Results

Shrimp Tropomyosin and Binding Ability of TM-Specific Polyclonal Antibodies

The purified shrimp tropomyosin (TM) was found to be homogenous with purity $> 90\%$. The two antibodies (rabbit and

mouse anti-shrimp TM polyclonal antibody) produced on immunization of shrimp TM were evaluated for binding ability with purified TM and crude TM. Figure 1a shows the protein profiles of purified and crude shrimp TM, and it clearly exhibited single band around 37 kDa. As shown in Fig. 1b, the rabbit pAb binds specifically with the TM band in purified TM line, but in crude TM line, the pAb binds weakly to an additional band at 75 kDa. This may be due to the minor contaminant present with purified TM during immunization. The mouse pAb, as shown in Fig. 1c, exhibit similar binding ability as observed with rabbit pAb. It also shows very weak recognition of additional 75 kDa protein in shrimp extract.

Evaluation of Possible Cross-reactivity of Antibodies to Different TMs

Various crustacean proteins were examined to test the reactivity and specificity using the two polyclonal antibodies. Figure 2a shows the protein profiles of the extraction of seven different kinds of crustacean proteins. The samples were all

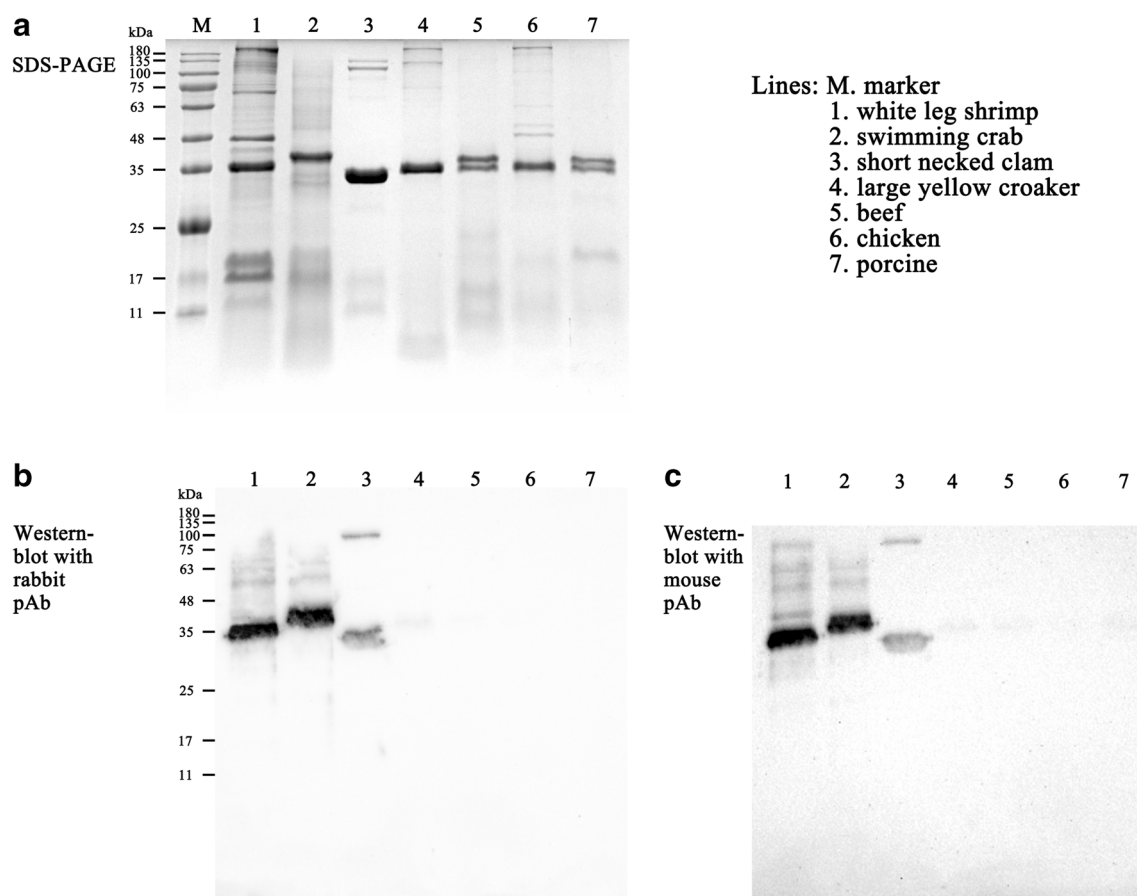


Fig. 2 Immunoreactivity and specificity of rabbit and mouse anti-shrimp tropomyosin polyclonal antibody from seven different species which are loaded at protein concentration 0.5 mg mL^{-1} . M: marker; Lane 1: white leg shrimp; Lane 2: swimming crab; Lane 3: short necked clam; Lane 4: large yellow croaker; Lane 5: beef; Lane 6: chicken; Lane 7: porcine. **a** SDS-PAGE profile of the protein extract of seven tropomyosin containing

extracts. **b** Western blot analysis, the primary antibody was rabbit anti-shrimp polyclonal antibody (1:2000 dilution), and the secondary antibody was HRP-conjugated goat anti-rabbit polyclonal antibody (1:2000 dilution). **c** Western blot analysis, primary antibody using mouse anti-shrimp polyclonal antibody (1:5000 dilution), and secondary antibody using HRP-conjugated goat anti-mouse polyclonal antibody (1:5000 dilution)

extracted with crude procedure to test the specificity of the antibodies. Due to the crude extraction, each lane covers most of the bands of this species with a dominant band at ~ 35 kDa, which represents the tropomyosin counterparts. Both shrimp and clam extracts in addition to TM had few dominant protein bands in the range of 17–100 kDa. Other extracts were found with only minor contaminating proteins. In order to ensure the identification ability of the antibodies, the western blot was employed to characterize the binding specificity of antibodies to assess the cross-reactivity to TMs of related species. As shown in Fig. 2b, the mouse anti-shrimp polyclonal antibody binds strongly to the tropomyosin components of white leg shrimp and swimming crab but weakly to the short necked clam and no binding was observed to the other TMs. Figure 2c indicates the binding ability of rabbit anti-shrimp polyclonal antibody. This showed almost equal binding to shrimp and crab with higher affinity, and a weak binding was observed with clam. None of the other TMs exhibit binding to this antibody. Both mouse and rabbit anti-shrimp TM pAbs exhibited similar binding specificities with remarkable cross-reactivity between shrimp and crab TMs. The clam showed weaker recognition with possible cross-reactivity in detection system.

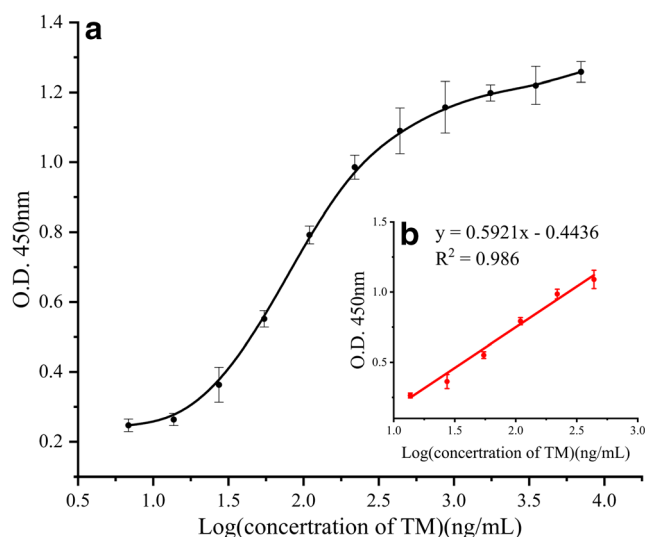


Fig. 3 A calibration curve of sELISA for detection of TM with the concentration in log 0.83 (6.8 ng mL^{-1}), log 1.14 (13.67 ng mL^{-1}), log 1.44 (27.34 ng mL^{-1}), log 1.74 (54.69 ng mL^{-1}), log 2.04 ($109.38 \text{ ng mL}^{-1}$), log 2.34 ($218.75 \text{ ng mL}^{-1}$), log 2.64 (437.5 ng mL^{-1}), log 2.94 (875 ng mL^{-1}), log 3.24 (1750 ng mL^{-1}), log 3.54 (3500 ng mL^{-1}), and log 3.84 (7000 ng mL^{-1}). **a** Standard curve of sELISA for detection of TM with the concentration in log 1.14 (13.67 ng mL^{-1}), log 1.44 (27.34 ng mL^{-1}), log 1.74 (54.69 ng mL^{-1}), log 2.04 ($109.38 \text{ ng mL}^{-1}$), log 2.34 ($218.75 \text{ ng mL}^{-1}$), and log 2.64 (437.5 ng mL^{-1}). **b** Results are expressed as means $\pm$ SD ($n=3$). Insert: linearization of data points obtained by applying log concentration to identify slope and R^2 value to calculate LOD and LOQ

Sandwich ELISA Standard Curve and Determination of LOD and LOQ

A calibration curve was obtained with TM at a concentration range from 6.8 to 7000 ng mL^{-1} . The detection of TM using two polyclonal antibodies by sELISA was plotted in Fig. 3. The curve showed typical sigmoid binding characteristics with weaker binding at low and high concentration and an exponential linear proportionate binding from 13.67 to 437.5 ng mL^{-1} concentration. The linear range of the optimized ELISA was plotted (insert to Fig. 3) taking log conversion of the data points, and proportional linear curve was observed for the detection of TM was log 1.44 (13.67) to 3.0 (437.5 ng mL^{-1}). The slope agrees with the proportional curve equation with R^2 of > 0.98 ($y = 0.5921x - 0.4436$, $R^2 = 0.986$). From the equation, the limit of detection (LOD) and limit of quantification (LOQ) were determined as 6.8 and 13.67 ng mL^{-1} , respectively.

Cross-reactivity and Specificity Test TMs by sELISA

Various crustacean protein extracts were examined to test the reactivity and specificity of developed sELISA at concentration range from 0.065 to $4 \mu\text{g mL}^{-1}$. The results are represented in Fig. 3a, the sELISA format shows high specificity and reactivity to shrimp and crab TM and there was no observed cross-reactivity of the sELISA with short necked clam (*Ruditapes philippinarum*), large yellow croaker (*Larimichthys crocea*), bastard halibut (*Paralichthys olivaceus*), porcine, beef, and chicken. The shrimp-TM specific sELISA had strong cross-reactivity and binding specificity with swimming crab (*Portunus trituberculatus*). The observed result may be due to high sequence homology and sharing of common reactive epitope of tropomyosin between shrimp and crab (Leung et al. 1998). As shown in Fig. 3b compared to shrimp, only crab TM was highly cross reactive with binding ability of 96%, whereas clam TM was binding with only 22% which was similar to negative control. The clam TM showed weaker binding with both antibodies during western blot, but it failed to detect by sELISA, indicating the detection method is applicable only for shrimp and crab TMs.

The Accuracy and Precision of the Sandwich ELISA for Detecting TM

The intra-assay and inter-assay variances were calculated from 5 replicates of the same purified TM in a range from 13.67 to 437.5 ng mL^{-1} (Table 1). Intra-assay was carried out in triplicate analysis employing same reagent and experimental conditions on 5 different days. The mean coefficients of variation were < 5 to 15% , indicating high accuracy, whereas the inter-assay was done at different experimental conditions at many time points. The results of the coefficient of variation observed

Table 1 Intra- and inter-assay variances in the developed sandwich ELISA method

TM (ng mL ⁻¹)	Intra-assay			Inter-assay ^a		
	Mean ± SD (ng mL ⁻¹)	RSDr ^b (%)	n replica	Mean ± SD (ng mL ⁻¹)	RSDR ^c (%)	n replica
13.67	14.22 ± 0.99	6.25	5	15.06 ± 3.42	20.32	5
27.34	23.43 ± 2.31	8.82	5	23.53 ± 5.73	21.77	5
54.69	54.82 ± 4.57	7.46	5	51.55 ± 10.26	17.80	5
109.38	126.82 ± 18.97	13.38	5	138.99 ± 34.23	22.02	5
218.75	259.91 ± 23.85	8.21	5	258.54 ± 68.07	23.55	5
437.5	367.16 ± 63.35	15.43	5	377.69 ± 100.32	23.76	5
	Mean	9.92		Mean	21.54	

Results are expressed as means ± SD ($n = 3$)

^a Assay was performed every 2 days for 10 sequential days

^b Relative standard deviation of repeatability for intra-assay precision

^c Relative standard deviation of reproducibility for inter-assay precision

range from 17 to 24% with slight change in precision level. The developed method appeared to be performing with less variation in standardized experimental conditions with high accuracy and reproducibility.

TM Spike and Recovery in Processed Food

To validate the applicability of the ELISA to test TM in real samples (shrimp-TM free) with matrix, recovery experiments were performed by spiking TM at 5 and 10 µg mL⁻¹ in fish (*Oreochromis* spp.) meat, freeze-dried seaweed egg soup, and meat sauce. The mean recoveries of TM from spiked in fish meat were 118% for 5 µg and 93% for 10 µg with slight variation, indicating possible matrix interference. However, with freeze-dried seaweed egg soup and meat sauce, the recovery percentage was 90 ± 2% for both 5 and 10 µg spiking of TM. The observed spiking and recovery of TM results are shown in Table 2. The reduction in the recovery was not significant and marginal, indicating no interference or cross-

reactivity of matrix compounds in the detection of TM by the developed sELISA.

Detection of TM Allergenic Residues in Processed Food

Around seventeen real food samples were procured from the market to assess the detection efficiency of the developed sELISA. The results are shown in Table 3. Based on the labeling, the samples were categorized as non-TM and TM samples. None of the control samples detected the presence of allergenic TM. Among the TM containing samples, out of the nine labeled TM ingredients, five of the samples showed positive TM with more than detection quantity. The negative results observed may be due to change in structure of TM as a result of processing methods such as fermentation, frying, and baking or may be non-shrimp or crab crustacean TM. Among the five food samples which were not labeled and suspected to contain TM, three of them were positive to TM indicating the possible cross-contamination on

Table 2 Recovery of tropomyosin from three blank food samples spiked with standard concentration of shrimp tropomyosin

Sample	TM spiked (µg mL ⁻¹ , mg g ⁻¹)	Detected concentration (µg mL ⁻¹ , µg g ⁻¹)	Recovery (%)	CV (%)
Fish meat	0	ND		3.35
	5	5.94 ± 1.01	118.77	13.86
	10	9.30 ± 0.61	93.02	5.32
Freeze-dried seaweed egg soup	0	ND		2.55
	5	4.53 ± 0.56	90.19	1.39
	10	8.86 ± 1.28	87.96	1.61
Meat sauce	0	ND		2.48
	5	4.52 ± 1.05	88.88	2.47
	10	9.09 ± 0.35	90.87	0.78

Shrimp TM was spiked at 5 and 10 µg concentration to matrix samples

All the samples were analyzed as triplicates and results were expressed as means ± SD ($n = 3$)

All the matrix samples were used at the same volume and were assayed in similar assay condition

Table 3 Analysis of various commercial real food samples for detection of the presence of TM using the developed sandwich ELISA method

Declaration	Quantitative($\mu\text{g mL}^{-1}$ *, $\mu\text{g g}^{-1}$)
None TM food	
Garlic flavored beans	ND
Sea cucumber sauce	ND
TM food	
(a) Labeled TM food	
Shrimp flavored biscuits	3.45
Puffed shrimp strips	0.21
Puffed shrimp flakes	0.15
Sea shrimp sauce	ND
Shrimp flavored beans	2.41
Crab-flavored beans	ND
Fried shrimp slices	ND
Garlic oil porridge flavored ramen	ND*
Seafood porridge flavored ramen	Positive but below the LOQ*
(b) None labled TM food	
Beef balls	0.13
(c) Suspect TM food	
Freeze-dried seaweed egg soup	ND*
Freeze-dried spinach egg soup	ND*
Salmon ham	0.38
Chicken ham	0.15
Fish meatballs	0.46

All the food samples were used at same amounts for extraction and were processed and assayed following similar experimental condition

the production line or adulteration of TM during processing methods during manufacture process. The beef ball which is a non-labeled food found contains considerable amount of TM which addresses the importance of sELISA for monitoring of the allergenic TM in processed and packed food. These results showed that the sandwich ELISA method could appropriately determine the crustacean protein in the processed foods and enables the detection of TM content in the suspected food samples.

Discussion

The present study reported the development of a quantitative detection method for shrimp and crab allergen tropomyosin using two separate polyclonal antibodies. As mentioned earlier, the sELISA reported in the literature with mAb has lower LOD around 1 ppb (Seiki et al. 2007; Zhang et al. 2014; Lin et al. 2018), with hyper predictability and the sELISA reported with single pAb had higher LOD with low specificity and predictive values. To avoid the false negative risk of monoclonal antibodies, we selected a polyclonal antibody system. The high LOD of the previous pAb's system might be due to the labeling of the detection antibodies. When the antibodies are labeled, it can

reduce the potency of antibody, therefore causing the low specificity of the method (Wang et al. 2019). So instead of using the labeled detection antibody, we used HRP-conjugated goat anti-mouse IgG antibodies that caused no influence on the detection antibody. What is more, in order to reduce the cost of the binding time, we innovatively incubated both detection and HRP-conjugated antibodies. Finally, we got low LOD and short binding time method simultaneously. Under the same polyclonal antibody system, the LOD reduced by 1 ppm to 6.8 ppb compared to the previous studies with the non-labeling of antibodies and by incubation of both of the two antibodies (Werner et al. 2007; Fuller et al. 2006).

The LOD of sELISA in this study was 6.8 ppb which is more specific and predictive with appropriate sensitivity to detect tropomyosin from shrimp and crab. The threshold level of allergen in the food has not been reported in the literature. In Japan, the threshold level of allergen in crustacean was considered as 10 ppm (Shoji et al. 2018). In addition, the threshold level for crustacean samples has not been reviewed and implemented from last 10 years as there is no report of inconvenient incidence encountered on ingestion of crustaceans. Another study with predictive threshold level shows the protein dose at which 5% of the allergic population is likely to respond with objective reactions was 1.6 mg for peanut, 1.1 mg for cow's milk, 1.5 mg for hen's egg, 7.4 mg for cashew nut, and 0.29 mg for hazelnut (Ballmer-Weber et al. 2015). Although these are no crustacean allergens involved, the milligram level of threshold can provide some references. Currently, there is no clear definition of allergen threshold from the reported literature; however, most detection methods that meet the threshold above the ppm level were considered for labeling and monitoring of allergenic contents in the foods. But considering the extraction methods for the samples containing small quantities of allergen, the LOD needs to be as low as possible for the developed detection assays.

As stated in literature, the mAb can specifically identify and bind to single epitope, and that may result in false negative when employed in sELISA for food protein detective systems according to its higher binding sensitivity (Yeung 2006). On the other hand, the same pAb can identify and bind effectively to many epitopes that may also results in some degree of false positive (Zhang et al. 2014). Compared with false positive results, the false negative is more harmful to consumers, which may cause the omission of the risk from harmful substances. In this study, the sensitivity of two pAb used was appeared to meet the threshold detection limit specified by the current researches. The cross-reactivity results showed that the sELISA method developed has high specificity to shrimp and crab tropomyosin with high sensitivity. Short necked clam had not found to be cross-reactive in the sELISA, although the two antibodies can weekly bind to the Short Necked Clam's TM in western blot analysis as shown in Figs. 2 and 4. Previous study's results showed that anti-shrimp TM

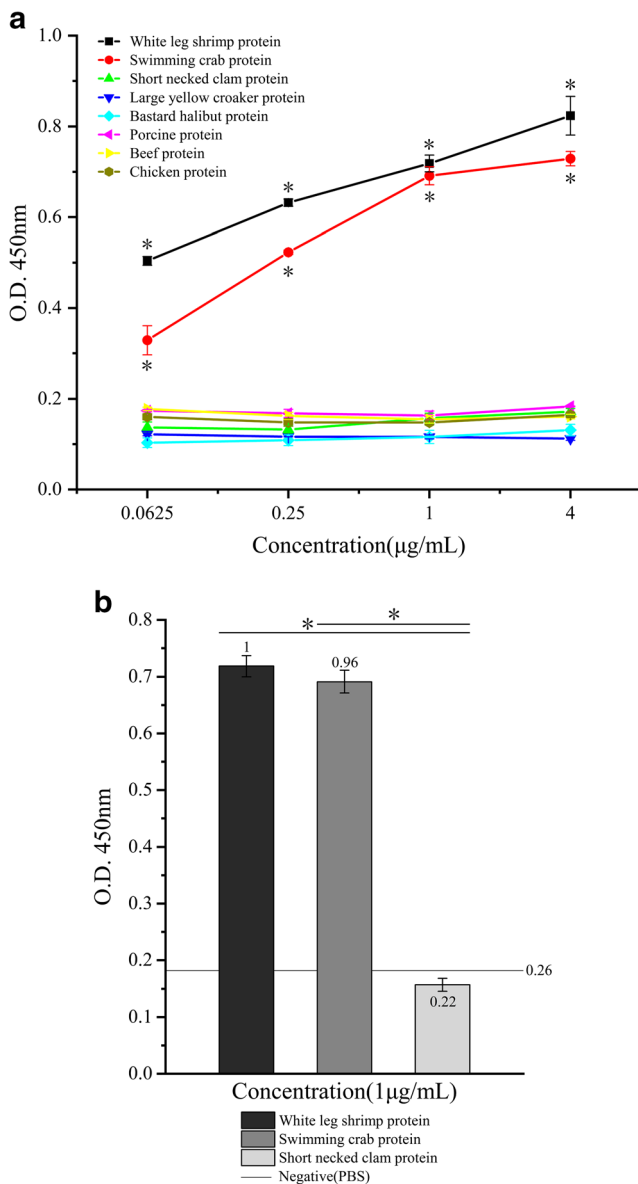


Fig. 4 Specificity of the developed sELISA, tested with TM with shrimp and other TM containing species. **a** Specificity and cross-reactivity of TMs from white leg shrimp (*Litopenaeus vannamei*), swimming crab (*Portunus trituberculatus*), short necked clam (*Ruditapes philippinarum*), large yellow croaker (*Larimichthys crocea*), bastard halibut (*Paralichthys olivaceus*), porcine, beef, chicken proteins at 0.0625, 0.25, 1, and 4 $\mu\text{g mL}^{-1}$. **b** Specificity of the developed sELISA, tested with white leg shrimp (*Litopenaeus vannamei*), swimming crab (*Portunus trituberculatus*), short necked clam (*Ruditapes philippinarum*) to predict the possible cross-reactivity and expressed as percentage value, considering binding to shrimp TM as 1. Results were expressed as means $\pm$ SD ($n = 3$). Statistically significant was considered at $p < 0.05$ signed with “asterisk”

antibodies have no cross-reactivity with common cockle (*Cerastoderma edule*). But, when it comes to sELISA, the antibodies showed little cross-reactivity with common cockle (Werner et al. 2007). We have the same point that the antibodies' ability is different from method performance. Therefore, it indicates that the method would change the identification

ability of the antibody to a certain extent. The binding epitopes of the two shrimp-specific TM antibodies employed for the sELISA detection were not the same; these pAbs developed in different host source recognize the antigen with different binding ability that allows higher sensitivity and specificity of the developed detection system.

The validation of the developed sELISA was determined according to the Eurachem Guide. The AOAC (Association of Official Analytical Chemists) has not released the standard method for performance requirements for the crustacean allergens. The matrix interference and recovery of TM on spiking were determined by three matrices (fish meat, meat sauce and soup) that contain spiked crustacean TM allergen in the practical samples. The mean recoveries of TM from spiked matrices were from 87.96 to 118.77%, indicating the method was less affected by food matrices during extraction and performance of detection assay system. Detection of antigenic proteins present in the food sample depends on protein solubility and extraction ability. Only extracted proteins were capable of being detected by the assay (Poms et al. 2004). There have been many studies on the food matrices that influence the detection of suspected antigenic proteins and minimizing the matrix effect and using suitable detergents during extraction that is compatible becomes critical factor in designing the detection assay system (Lasekan and Nayak 2016; Koizumi et al. 2018). High concentration of the denaturant and reducing agent in the extract can destroy the antigenic epitope and can alter antibody binding due to the induced structural damage. We evaluated the adaptability of the sELISA in this study (data not shown). It appeared that when the concentration of SDS and Na_2SO_3 used in protein extraction were over 0.0125% and 0.05 M, it would invalidate the method of the sELISA due to the antibody denaturation. There were very few reports on the account of extraction reliability to the detection methods. During the practical detection process, the denaturant and reducing agent will induce change to proteins in the food sample matrix. However, the high concentration of denaturant in the original extractant will have negative impact on the detection. Therefore, it is considered that the denaturant and reductant may affect the properties of antibody, which leads to inaccurate result detection. Thus, when a new extracting agent is used, it needs to be evaluated. Before we found the balance between the extraction method and detection, PBS (pH 7.4) is the better choice as it is considered to be safe and optimum for assay system.

In the present study, we used PBS (pH 7.4) as safe extractant for commercial foods as it minimized the interference in detection of crustacean allergens. The results observed were consistent and comparable with some current allergen label methods. Some surveys of commercially available food products showed the presence or absence of a precautionary warning of allergenic components that corresponds poorly with the actual presence of allergen in the product (Spanjersberg et al. 2007; Pele et al. 2007); this can lead to potential food safety problems (Spanjersberg et al. 2010; Sheth et al. 2010). A

recent study conducted by Sheth et al. (Sheth et al. 2010) in Canada showed that approximately 17% of persons with food allergies experiencing an accidental exposure attributed to products with unintentional cross-contamination during manufacturing and no precautionary statement on the label. Conversely, many products do not contain the allergen to which the precautionary warning on the label refers. The proper labeling of constituents in food sample should be a mandatory criterion to avoid accidental or unintentional problem associated with allergic reactions. The food regulatory agencies in China are planning to implement a mandatory labeling system for allergens soon. Henceforth, the reliable allergen detection methods are in need by both regulatory and manufacturing agencies for proper establishment of labeling system. Based on the analysis of the results and application study, the sELISA developed in this study could meet the need of the market and can be employed for labeling of crustacean allergen in food samples.

Conclusion

In summary, the present study successfully developed and established a sandwich ELISA method for the detection of tropomyosin with high specificity and sensitivity. The LOD was identified as 6.8 ng mL^{-1} , which was more than 100 times lower than the previous polyclonal antibody ($1 \mu\text{g mL}^{-1}$) used sELISA method. Instead of labeling only detection antibody, a combined detection antibody and HRP-conjugated antibody incubation was carried out in our study to achieve the high sensitivity and short binding time. No cross-reactivity was observed with fish, poultry, and mammalian TMs. The mean recoveries in food matrices ranged from 87.96 to 118.77%, by both intra- and inter-assay systems with coefficients of variation of $< 10\%$ and $< 25\%$, respectively. Therefore, the developed assay system can efficiently identify the shrimp and crab allergen tropomyosin in processed foods. These results suggest that our sELISA is a reliable system that can be applied to various products in the market to meet the needs of consumers to ensure the safety and security of food.

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Compliance with Ethical Standards

Conflict of Interest Zhenxing Li declares that he has no conflict of interest. Zhiwen Yu declares that he has no conflict of interest. Yaqing Wang declares that she has no conflict of interest. Siddanakoppalu N. Pramod declares that he has no conflict of interest. Lijing Zhang declares that she has no conflict of interest. Hong Lin declare that he has no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not applicable.

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