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Development of a sensitive sandwich-ELISA assay for reliable detection of fish residues in foods

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ABSTRACT

A new sandwich-type Enzyme-Linked Immunosorbent Assay (ELISA) method was developed based on goat IgG as capturing antibody and rabbit IgG as detecting antibody targeting soluble antigenic fish proteins in foods as detection targets. The assay has provided a relatively lower limit of quantitation (LoQ) for fish proteins with LoQ 0.5 ng/ml and appears highly sensitive. The analysis of 24 different substances, both raw and boiled, revealed no cross-reactivity above the cut-off point of the limit of quantitation. Recoveries of the SB spiked food matrices were in the range of 83–131%. Assay precision testing proved that repeatability (<5%) and reproducibility (<11%) had an acceptable level of variation. The sandwich ELISA was capable of detecting all tested commercially important fish. As a potential analytical tool, the newly developed immunoenzymatic method is suitable for detecting undeclared fish residues in real food samples available in the market, thereby will help to reduce the incidents of fish allergies.

1. Introduction

Fish allergies are considered a lifelong disease. Avoiding fish-based diets is often regarded as the only and most effective management method other than therapeutic approaches [1,2]. When it comes to convenience foods, the ingredients on display appear to fit the list. Still, there is a possibility of contact with traces of fish or their derivatives while being processed or packed with shared equipment and facilities. To guarantee the safety of consumers, several countries are obliged to label pre-packed fish and fish products as a major allergen recommended by the Codex Alimentarius Commission. The number of allergens that need to be listed on food labels varies from country to country. In the USA, fish has been declared as the Big 8 food allergen designated by the Food Allergen Labeling and Consumer Protection Act as set out in 2004 (FALCPA) by the FDA [3]. Unlike other animal based food allergies such as milk and egg allergies, fish allergy persists in young adults rather than infants [4]. Besides, countries such as the UK, Canada, Australia, New Zealand, China, Japan, and more have legally required to clearly label intentionally added fish or fish ingredients (source of fish) under the food allergy category [3].

Parvalbumin (PV) as the major allergen conserved in the finfish

species responsible for mediating IgE immune reaction [5] documented as the most suitable and popular candidate for the development of immunoassay methods due to its thermostability. However, PV content of fish can vary between individual fish species and even within the muscle part of the same fish species [6] and depending on the nature of aquaculture practices [7]. Further, the phylogenetic diversity of PV and their different degrees of cross-reactivity [4] unequivocally limiting the detection of target analyte in the food matrix. As shown by a recent study, commercially available fish ELISA kits can only identify a limited number of fish species and their products [8]. On top of that, the documented ELISA methods of fish residue detection described earlier appear to have few limitations (i.e., prolonged blocking time, prolonged enzyme-linked color reaction, undesired cross-reactivity with non-fish protein substances, inability to identify fermented fish products). These facts emphasize the requirement of sensitive and reliable assays that can be used in different food systems to detect undeclared fish residues with novel immunometric approaches.

ELISA is the most frequently used technique utilized by the food regulatory authority to test the compliance with food allergen labelling regulations, and the food industry to validate their allergen control program owing to its sensitivity, specificity, high sample throughput and

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comparatively less analytical time [9,10]. Besides, ELISA methods have the ability to directly detect the allergen/antigen itself rather than detecting a fraction of the target marker (i.e., PCR) that is not ideally the allergen/antigen itself. Though, PCR's high specificity allows as a better candidate for verifying immunological methods, its expense of needed equipment are limitations for using PCR in the food processing facility in routine analysis. Other alternative methods such as mass spectrometry for identification of PV in food products ([11]) hasn't provide quantifications and practiced personnel are necessary for handling this expensive instrumental method. To date, several in-house developed sandwich ELISA (sELISA) have been proposed for the detection of fish residues based on one particular protein (PV or 36 kDa) as detection target [12–14].

Here we chose to produce antiserum against sea bass (henceforth SB) fish (*Lates calcarifer*) targeting soluble antigenic proteins of fish species to check the possibility and suitability of a new ELISA approach to detect any traces of fish proteins using goat antiserum as capturing antibody and rabbit antiserum as the detection antibody. The method proposed by our study adhered to the general guidelines of SMPRs® published in the Journal of AOAC INTERNATIONAL, Official Methods of Analysis and Eurachem Guide with a great reliability, sensitivity and accuracy providing a useful and potential analytical tool for detecting fish residues in processed foods.

2. Materials and methods

2.1. Reagents and buffers

Reagents for the basic buffer solutions; coating buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, pH 9.6), for blocking buffer and antibody dilution buffer (0.01 M PBS; phosphate-buffered saline, 1% BSA; bovine serum albumin, 0.05% Tween 20 (v/v, pH 7.4), washing buffer (0.01 M PBS, 0.05% Tween 20 (v/v), pH 7.4), sample extraction buffer (0.01 M PBS; phosphate-buffered saline, 0.5% BSA; bovine serum albumin, 0.05% Tween 20 (v/v), 0.5% guadinine hydrochloric acid and 2% β-mercaptoethanol, pH 7.4) were purchased from Sigma-Aldrich (USA). 3,3',5,5'-Tetramethylbenzidine (TMB) was used as the chromogenic substrate purchased from Solarbio Co., Beijing.

2.2. Immunogen preparation, antiserum production and evaluation

Fresh SB extracts were produced by homogenizing 100g of the white portion of fish flesh per 500 ml of Bis-Tris buffer, supplemented with 5 mmol phenylmethylsulfonyl fluoride (PMSF) (pH 6.9) overnight. It was then centrifuged at 8000 rpm for 20 min (min) followed by filtration (0.22 μm retention particle size). Aliquots were then separated into sample tubes and stored at −20 °C until use. The protein content of the extract was determined by the bicinchoninic acid (BCA) protein assay (Bio-Rad Laboratories). Rabbit serum against SB crude proteins was produced as per the standard protocol of antibody production at Hubei Prokin Technology Co., Ltd, China. For initial antigen injection (day 0), 2–3 months old, two New Zealand rabbits were subcutaneously immunized with 50 μg of SB protein immunogen in the emulsions of Complete Freund's Adjuvant (CFA). Booster injections containing 500 μg of crude protein in Incomplete Freund's Adjuvant (IFA) were given subcutaneously (1st booster on 28 days, 2nd booster on 42nd day, and subsequent boosters at 7- day interval). Whole blood was collected on the 64th day and the serum was separated. Serum production in goat (6–7 months old) was produced at Luoyang Bai Aotong Experimental Materials Center. (Luoyang, Henan Province., China). Briefly, starting with an initial injection (day 0), containing 150 μg of crude protein emulsified with CFA was given on four different sites subcutaneously. The first booster starts with day 21 (1.5 g of antigen emulsified with IFA). Booster doses were administered in 21-day intervals for 4 Immunization cycles. By performing the indirect non-competitive ELISA method, each dilution was tested for the presence of detectable levels of antibody [15].

Overall, both 2 rabbits and goat had higher titer IgG antibody values (>50000). The antisera of 2 rabbits (pooled 1:1) and goat were separated into cryogenic vials and stored in aliquots at −80 °C until use.

2.3. Preparation of reference fish species, food/food ingredients, commercial food stuffs

Locally purchased fish and food samples were homogenized and extracted with extraction buffer (containing guadinine hydrochloric and 2-mercaptoethanol) followed by sonication for 10 min to improve the efficiency of protein extraction. All subsequent steps were similar to SB protein extraction. pH test strips were used to confirm that the food samples' pH was not strongly acidic or alkaline. Food ingredients that are commonly used as substitutes, additives or fillers of fisheries products as mentioned in Table 2 were extracted in the same manner. To prepare heated extracts, 10 ml of samples incubated in a water bath at 100 °C for 10 min. After incubation, the tubes were placed under running water for approximately 10 min, to cool down to room temperature. The tubes with heated extracts were then centrifuged at 3000×g for 5 min at room temperature. All the extracts were filter-sterilized (0.22 μm). Each of the filtrates was labelled and stored in aliquots at −20 °C for further use. The total protein content was measured by the BCA method. Filtrates were diluted in sample extraction buffer a hundred times or other appropriate dilutions ratios before being quantified by the developed assay.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), westernblot and indirect competitive ELISA

SDS-PAGE was performed following the protocols described in literature [16]. Briefly, the extracts were electrophoresed in a 1.0 mm 12% separating gel with a 5% stacking gel. The fish samples were diluted in a reducing buffer (4X) and boiled before loading them into the stacking gel. Next, 10 μl of sample or marker protein was added to each well. Coomassie Brilliant Blue R-250 (Solarbio, China) was used to stain the gels, followed by de-staining until the gel visible with clear bands. Protein bands were visualized using the ChemiDoc XRS Imaging System and Image Lab 6.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The separated SB proteins and other fish proteins from SDS-PAGE were transferred to a polyvinylidene difluoride membrane carrier (PVDF) via iBlot2 dry transfer system (Thermo Fisher Scientific Inc. Waltham, MA, USA) for 5 min at room temperature for Westernblot. The membrane was then blocked with 1% BSA for 2 h at room temperature under gentle shaking. After washing the blot three times, (15 min at each wash), it was incubated at room temperature with rabbit anti-SB detection antibody diluted 1:20000 under gentle shaking. Next, the blot was washed three times and incubated for 1 h at room temperature with 1:10000 diluted HRP-conjugated goat anti-rabbit IgG. After the final washing with PBST the results were visualized ChemiDoc XRS Imaging System and Image Lab 6.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

For indirect competitive ELISA, wells were coated with 100 μL per well of SB protein (10 μg/mL) in coating buffer and incubated overnight at 4 °C. After overnight incubation at 4 °C, the coated plates were washed three times with PBST and blocked with 1% BSA at 37 °C for 1 h (hr) 30 min. The detection antibody (diluted 1:10000) were separately mixed with increasing concentrations of other fish proteins ranging from 0 ng/ml to 500 ng/ml into individual polypropylene tubes and subsequently kept for 1 h at 37 °C for incubation. The antigen, antibody mixture was then added to the flat bottom polystyrene stripwell™ microwell plates (costar®, USA) and incubated for 1 h (100 μL/well). After washing the plates, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (cellwaylab®, China) diluted 1:6000 in PBST containing 0.5% BSA, were added to the wells (100 μL/well) and incubated for 30 min at 37 °C followed by three washes. Then, 100 μL of TMB was added to each well and allowed for incubation at 37 °C. Finally, to stop the

reaction, 2 M H_2SO_4 was added and OD was measured at 450 nm using the microplate reader (CMax Plus, Molecular Devices, LLC, San Jose, CA, USA). The concentration of analyte giving 50% response/inhibition was calculated to the following formula. % inhibition = $100 - [(\text{mean OD of inhibited well}) / (\text{mean OD of non-inhibited well}) * 100]$.

2.5. ELISA development and standard curve construction

Microwell plates were coated with 5 $\mu\text{g/mL}$ of goat antiserum in coating buffer (100 $\mu\text{L/well}$). After overnight incubation at 4 °C, the coated plates were washed three times with PBST and blocked with 1% BSA at 37 °C for 1 h (hr) 30 min. Then SB antigen as the calibrators and or samples were added (100 $\mu\text{L/well}$) and incubated 1 h, 15 min at 37 °C. Afterwards, the wells were washed three times and rabbit anti-SB serum diluted in PBST containing 0.5% BSA were added to the wells (100 $\mu\text{L/well}$) and incubated for 1 h at 37 °C. After washing the plates, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG diluted 1:6000 in PBST containing 0.5% BSA, were added to the wells (100 $\mu\text{L/well}$) and incubated for 50 min at 37 °C followed by three washes. Then, 100 μL of TMB was added to each well (100 $\mu\text{L/well}$) and allowed 9 min of incubation in the dark at 37 °C. Finally, to stop the reaction, 2 M H_2SO_4 was added and OD was measured at 450 nm using the microplate reader.

2.6. Standardization and validation of sELISA approach

2.6.1. Assay precision, limit of detection and limit of quantitation

Precision was determined by inter-plate (repeatability) and intra-plate precision (reproducibility) as calculated by the percentage of coefficient of variation (CV %). CV was calculated by dividing the pooled standard deviation (σ) of a set of measurements by the overall mean (μ) of the triplicated set of serially diluted SB protein samples in ng/ml range (100, 50, 25, 12.5, 6.25 ng/ml), which is then expressed as a percentage of variation to the mean. $\text{CV} (\%) = (\sigma / \mu) \times 100$. The intra-plate precision was evaluated by measuring the overall mean of triplicated SB proteins on four different sets of wells on a single ELISA plate. The inter-plate precision was evaluated by measuring the triplicated SB proteins assayed on four distinct ELISA plates on consecutive four-day experiments. Limit of detection (LoD) and limit of quantitation (LoQ) were calculated as the mean optical density of the buffer blank values per in triplicates ($n = 20$) plus either 3 (LoD) or 10 (LoQ) standard deviation (SD) respectively.

2.6.2. Selectivity/cross-reactivity studies

Each of the heated and non-heated food ingredients commonly used as substitutes, additives or fillers of fisheries products was analyzed in triplicates to see if the assay generates non-specific cross-reactivity for non-fish proteins. Commonly traded, commercially important edible fish species [19] of different orders was evaluated for fish species specificity.

2.6.3. Matrix studies

Five different manufactured foods were selected. Samples were homogenized and put into individual polypropylene tubes, then spiked serially diluted analytes of SB proteins (100 ng/ml, 50 ng/ml, and 25 ng/ml) by adding 100 μL of respective spiking solution per each 1g of homogenized food individually with un-spiked 0 ppb solution as a control. Adulterated food samples were kept in refrigerated conditions for 5 h, and then 10 ml of extraction buffer was added to each tube and vortexed for 5 min. Each sample was labelled and held in a horizontal shaker for 3 h, then centrifuged at $3000 \times g$ for 5 min at room temperature. Aliquots were then filtered (0.22 μm), and the samples were analyzed in triplicate. Recovery (%) = $(\text{detected protein concentration from SB ELISA} / \text{spiked SB concentration}) * 100$.

Further, the absorbance curves from the spiked food matrices (SB spiked in cuttlefish, SB spiked in shrimp) were compared with the SB

standard curve.

2.7. Undeclared fish residue detection in real food samples

Commercial foods of different categories were evaluated by the developed assay. Filtrates were diluted in sample extraction buffer for appropriate dilutions ratios before quantified by the developed assay. This assessment is intended to see if the label on the packaging is compliant with the declared fish allergy.

2.8. Statistical analysis

Data were quantified and analyzed in Microsoft Excel and GraphPad prism.

3. Results

3.1. Curve construction

An eleven-point (0.5, 1, 2.5, 5, 10, 15, 20, 30, 50, 70, 100) third-order polynomial calibration curve ($R^2 = 0.9993$) with a quantitation range from 100 ppb to 0.5 of SB proteins was constructed (Fig. 1). Values between 0 and 20 ng/ml showed linearity, $y = 0.0703x + 0.1226$, with a squared correlation coefficient $R^2 = 0.9969$ (95% CI). The absorbance values at 450 nm were displayed on the graph's y-axis with SB protein concentration on the x-axis. LoD and LoQ were calculated as 0.2 ng/ml and 0.5 ng/ml.

3.2. Validation studies

In our approach, CV% lies below 5% (<5%), which is indicative of good reproducibility within the assay (Table 1). The precision between tested plates was <11%, indicating good reproducibility between each assay. What makes different ELISA approaches reliable is the steps involved in experimenting. The optimum concentration of protein and

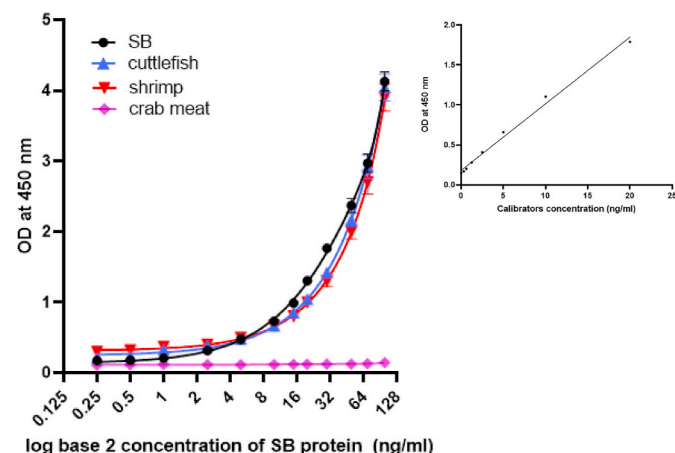


Fig. 1. SB, SB spiked cuttlefish, shrimp, and un-spiked crab meat standard curves as determined by the SB ELISA.

Abbreviations: OD; optical density. Linear curves fit with squared correlation coefficient $R^2 \geq 0.99$. SB extract was spiked into grounded muscles of fresh cuttlefish (blue) and shrimp (red) to achieve a 100 ng/ml SB standard and suitably diluted. Standard curves of appropriately diluted un-spiked crab meat (purple) extract (in extraction buffer) and calibrators (black) determined by SB ELISA. Each data point shows the average absorbance values and the standard deviations corresponding to triplicate experiments performed in three individual occasions and round to two decimal places. Error bars show the standard deviation for each set of data. Graph in the right side of the manuscript shows the linear curve of the standard calibrators in SB-sELISA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Intra- and inter-assay variances (%) for the sELISA assay.

SB protein level (ng/ml)	Intra assay		Inter assay	
	Mean $\pm$ SD	RSDr (%)	Mean $\pm$ SD	RSDr (%)
100	100.11 $\pm$ 1.47	1.47	99.3 $\pm$ 5.38	5.42
50	49.41 $\pm$ 1.08	2.19	49.35 $\pm$ 5.18	10.5
25	24.04 $\pm$ 0.25	1.04	25.12 $\pm$ 1.12	4.46
12.5	12.1 $\pm$ 0.33	2.73	12.6 $\pm$ 1.05	8.33
6.25	6.83 $\pm$ 0.06	0.89	6.22 $\pm$ 0.47	7.56

Abbreviations: SD = standard deviation. RSDr = relative standard deviation of repeatability. RSDR = relative standard deviation of reproducibility. Results are expressed as means $\pm$ SD (n = 3). All the mean values tabulated here are results of three replicate analyses and round to 2 decimal places.

antibody makes up an essential parameter for obtaining better results. Since our assay quantitation range lies between 0 and 100 ng/ml (0–100 ppb), the less-diluted samples (higher concentrations) might give false positive because large aggregates get washed off the plate. Therefore, in our assay, the extracts of common food additives, fillers, and food ingredients in the processed foods containing fish were selected for the cross-reactivity studies by making the filtrates 1% (v/v) in extraction buffer. The developed method's specificity with other mammalian, seafood and other food ingredients displayed in Table 2. Raw and heated food/food ingredients did not produce a false positive signal indicating that our anti-SB polyclonal-based sELISA approach did not cross-react with other components of non-fish substitutes, fillers or additives supporting the specificity of the developed approach.

All the fish species [19] had sufficient immunoreactive responses with the developed assay. The highest antigenic protein recovery was observed for the fish species; cod-F1, pollack-F2 and hake-F8 except for SB protein itself (Table 3). Interestingly these all are belonging to Gadiformes. These Gadiformes demonstrated above >1000 μ g/ml of the antigenic proteins from the developed SB ELISA method. Of that, the highest value was obtained for Alaska pollock (F2). This category also includes the large yellow croaker (F11), a species of fish of the Acanthuriformes order. The lowest antigenic protein recovery was observed for Pangasius (F13) fish's soluble muscle protein extract. Two Perciformes namely bluefin tuna (F5) and perch (F14) showed 523.72 $\pm$ 10.55 μ g/ml and 972.84 $\pm$ 11.42 μ g/ml respective values of antigenic fish protein content. Although, documented discussions on allergenic parvalbumin from cartilaginous fish are limited, Red stingray (F16), a

species of cartilaginous fish that habitats marine environments, showed a quantifiable recovery for the developed assay.

For a more precise evaluation process, a westernblot and indirect competitive ELISA was used to evaluate the selectivity and specificity of the detection antibody to its intended targets. A relatively high; >1000 μ g/ml (pollock, hake, large yellow croaker), medium; 1000–500 μ g/ml (tilapia, mackerel, and snakehead) and low-levels <500 μ g/ml (salmon, yellowfin sole, basa) of the soluble antigenic protein content of different fish species were evaluated in westernblot (Fig. 2) and indirect competitive ELISA (Fig. 3). The sELISA recoveries were soundly agreed with the westernblot and the concentration needed to reach 50% inhibition of the detection antibody.

Despite the level of the spiked analyte, the average recovery rates for raw pasta, oyster wine, semi-cooked shrimp balls lie above 90% (>90%). This higher recovery percentage indicates that sample matrix has minimal effect on how accurately SB proteins can be quantified with the developed assay (Table 4). Besides, raw pasta and rice-potato infants' noodles showed a relatively high recovery rate close to the known concentration of the food samples tested. Further, absorbance curves from the spiked food matrixes were compare with SB standard curve (Fig. 1). There were no significant difference observed (p < 0.05 significant level) in the means of the standard curves based on one way ANOVA test.

3.3. Detection of undeclared fish in real food samples

Almost all the pre-packaged food samples (93.75%) assayed by the developed method comply with the allergen declaration as shown in (Table 5) except for popcorn shrimp and fish oil of one manufacturer. Among thermally processed foods, lowest antigenic protein content was observed for canned tuna, which was undetectable in previous ELISA assays ([17]), possibly due to the improved extraction buffer we used in our assay. Previous studies reported that canning could significantly affect protein allergenic activity but could not abolish it [18,19]. This data certainly indicate that the developed assay can adequately quantify even a significant reduction in antigenic proteins caused by methods such as canning. The estimated antigenic protein content for salted and dried anchovy fish was 55.89 $\pm$ 2.24 μ g/ml. Fish skin and vegetable chips also gave a positive signal for the developed assay. Fish skin, however, is abundant in gelatin/collage. Therefore, it was speculated that fish muscle might not be completely removed from the skin to use as a by-product and that other sarcoplasmic muscle proteins may remain with the skin. Surimi and surimi based products estimated to have a higher antigenic protein content. Overall, all commercial food samples declared for fish allergy in the label were positive for the developed assay.

Table 2

Cross-reactivity analysis of common non-fish substitutes, fillers, additives assayed by the SB ELISA.

Ingredient	Protein concentration 1:100 dilution (μ g/ml)/(ppm)		Equivalent SB level (ng/ml) (LoQ = 0.5 ng/ml)		Ingredient	Protein concentration 1:100 dilution (μ g/ml)/(ppm)		Equivalent SB level (ng/ml) (LoQ = 0.5 ng/ml)	
	Raw	Boiled	Raw	Boiled		Raw	Boiled	Raw	Boiled
Non-fish substitutes					Additives				
Squid	77.4	63.2	<0.5	<0.5	gelatin	36.4	40.1	<0.5	<0.5
Shrimp	55	57.2	<0.5	<0.5	porcine plasma	338.7	304.1	<0.5	<0.5
Clamps	68.8	35.1	<0.5	<0.5	NFM	32	31.5	<0.5	<0.5
Crab	52.2	53.3	<0.5	<0.5	egg albumin	52.4	53.1	<0.5	<0.5
Chicken	148.4	82.1	<0.5	<0.5	garlic powder	6.5	6.6	<0.5	<0.5
Beef	82.3	84.1	<0.5	<0.5	onion powder	8.1	8.2	<0.5	<0.5
Fillers					white pepper	10.9	10.7	<0.5	<0.5
Soya	53.2	53.9	<0.5	<0.5	salt	ND	ND	<0.5	<0.5
rice flour	9.4	9.5	<0.5	<0.5	MSG	ND	ND	<0.5	<0.5
Potato starch	19.8	19.9	<0.5	<0.5	sugar	ND	ND	<0.5	<0.5
mung bean	21.4	21.4	<0.5	<0.5	corn oil	ND	ND	<0.5	<0.5
wheat flour	30.5	28.8	<0.5	<0.5	palm oil	ND	ND	<0.5	<0.5
					canola oil	ND	ND	<0.5	<0.5

Abbreviations: NFM: non-fat milk (spray dried), ND: not detected, MSG: monosodium glutamate.

All the values displayed here are means of three replicate analyses of three independent experiments and round to 2 decimal places.

Table 3

Cross-reactivity analysis of fish proteins; an assessment of antigenic protein recovery of developed SB ELISA.

Reference sample number	Common Name of reference fish sample	Fish Species	Order	Calculated soluble antigenic protein fraction from SB ELISA assay (μg/ml)
F1	Atlantic cod	<i>Gadus morhua</i>	Gadiformes	1228.77 ± 7.52
F2	Alaska pollock	<i>Theragra chalcogramma</i>	Gadiformes	1530.43 ± 12.07
F3	Atlantic mackerel	<i>Scomber scombrus</i>	Scombriformes	915.16 ± 8.77
F4	Atlantic salmon	<i>Salmo salar</i>	Salmoniformes	120.03 ± 7.85
F5	Bluefin tuna	<i>Thunnus thynnus</i>	Perciformes	523.72 ± 10.55
F6	Catfish	<i>Clarias fuscus</i>	Siluriformes	1005.61 ± 2.21
F7	Conger eel	<i>Muraenesox cinereus</i>	Anguilliformes	1235.19 ± 1.93
F8	Hake	<i>Merluccius australis</i>	Gadiformes	1424.63 ± 2.97
F9	Grass carp	<i>Ctenopharyngodon idella</i>	Cypriniformes	342.81 ± 2.03
F10	Japanese seerfish	<i>Scomberomorus niphonius</i>	Scombriformes	721.43 ± 9.11
F11	Large yellow croaker	<i>Larimichthys crocea</i>	Acanthuriformes	1127.82 ± 1.33
F12	Nile tilapia	<i>Oreochromis niloticus</i>	Cichliformes	721.78 ± 0.79
F13	Pangasius/Basa	<i>Pangasius hypophthalmus</i>	Siluriformes	180.85 ± 1.01
F14	Perch	<i>Lates niloticus</i>	Perciformes	972.84 ± 11.42
F15	Pacific pilchard	<i>Sardinops sagax</i>	Clupeiformes	805.77 ± 1.77
F16	Red stingray	<i>Dasyatis akajei</i>	Myliobatiformes	393 ± 1.33
F17	Snake head	<i>Channa asiatica</i>	Anabantiformes	523.46 ± 1.81
F18	Whiff	<i>Lepidorhombus whiffiagonis</i>	Pleuronectiformes	1015.62 ± 2.81
F19	Yellowfin sole	<i>Limanda aspera</i>	Pleuronectiformes	310.27 ± 7.79

All experimented values displayed here are results of three replicate analyses performed in three individual occasions, round to two decimal places and expressed as means ± SD (n = 3).

4. Discussion

Our study consists of two basic stages. The first is developing and validating a novel sELISA approach based on polyclonal antibodies, which has been produced in two complimentary mammalian species, followed by the second stage of evaluating undeclared fish in commercially available real food samples. In the present method, *Lates calcarifer*; a commercially important fish species around the world was selected as the source of antigen to produce antisera. For fish, the immunoreactivity of particular marker protein is highly varied among different fish species (i.e. PV). Therefore choosing a particular marker protein of single fish species as the standard for generating antibodies for immunoassays might not suitable unless a representative amount of a mixture of target proteins of different fish species use to generate antibodies ([12,13]). On the contrary, due to these variable immunogenicity of same targeted protein between fish species, one targeted protein might outperform the same target protein of another fish species in the protein mixture when generating antibodies. Thus there are no guarantee individual markers in the protein mixture will successfully become an immunogen for antibodies. In order to hinder the issue of antibody based methods for fish,

if a number of different proteins are the targets of the analysis, a higher the possibility of identifying or measuring the targeted proteins in food samples as demonstrated by our study.

As per the westernblot in our study, the detection antibodies could recognize single bands of both molecular mass of approximately 11 kDa; PV and 36 kDa proteins of selected common fish species tested. These fish proteins are known for their heat stability, species specificity and high antigenicity ([17,19–22]). Thus, both heat-stable fish species-specific 11 kDa protein and 36 kDa proteins appear to be relevant to the developed assay maximizing the target analyte detection in the food matrix. It seems, several documented fish species heated extracts, such as swordfish and gummy sharks, which do not have a PV band and have a prominent protein band of 36 kDa and species such as sturgeon, basa, rainbow trout, Atlantic salmon which have prominent PV band, but not represent a 36 kDa protein in the SDS-PAGE ([17,23]) undubitably quantifiable with our assessment.

It is an undeniable fact that due to the higher demand for food today, the food industry is increasingly focusing on creating an abundant supply of food for less money, and this is where the fillings and non-fish substitutes come in. Fillers and non-fish substitutes are additives that help to increase the weight of a low-cost raw material, thereby helping to reduce the costs of convenient foods ([24]). Twenty-four compounds in raw and boiled forms commonly used in processed fish items as non-fish substitutes, fillers, additives, and other kinds of seafood and terrestrial species like chicken and beef, were tested and did not produce non-specific cross-reactivity. This is indicative that the analyte of interest is recognized without cross-reactivity with non-fish related protein molecules by the developed sELISA approach. Several of these observations are in line with previously shown data for the less significant cross-reactivity of fish proteins with other mammalian species or other seafood species ([12–14,20] [25–30]). sELISA approach using a single mammalian species for polyclonal antibody raised against fish PV showed non-specific cross-reactivity to cephalopod samples ([13,14]). We believe that antibody generate with two complimentary mammalian species as shown by our assessment, helped to hinder this issue.

sELISA was validated with five different food matrixes. Among those manufactured food matrixes, pasta and rice-potato infants' noodles, 100% of the spiked SB proteins were recovered. However, for oyster wine, recovery appears to be relatively reduced (83%). In a previous study, the recovered spiked parvalbumin from wine by sELISA assay was low at 40% ([13]). The authors clarified the observation with fish proteins contamination due to wine clarification with gelatin. And in our study did not observe a matrix effect for oyster wine, possibly due to non-fish gelatin used in the wine clarification process. Overall, the recovery was sufficient among the matrixes suggesting that our developed assay is suitable for fish residues monitoring in processed foods.

For fish immunoassay approaches, both commercially available fish protein ELISA kits and developed ELISA methods directly targets PV in different food matrices with a LoQ in ppm or ppb range. Here we developed an approach that can detect soluble fish proteins by ppb range in raw/processed foods. Besides, the developed assay has much lower LoD/LoQ compare to commercially available fish ELISA kits and the published ELISA assays available in the literature, indicating that the immunoenzymatic method we developed claims a higher sensitivity. Due to subjective responses, it is challenging and difficult to estimate which exact quantity of fish proteins gives a clinical reaction for allergic individuals ([1]). Therefore, the absence of fish proteins in very minute amount definitely useful in particular situations when approaching more sensitive individuals.

Processing methods can cause modifications in the protein structure and these alterations often directly affect changes in the polarity and immunoreactivity of target proteins ([31]). Therefore, commercial food products we selected here were processed through a variety of processing methods such as boiling, salting, canning, drying, fermentation, frying and smoking. All the subjected thermally treated samples gave a promising immunological response for the developed ELISA. Further, a

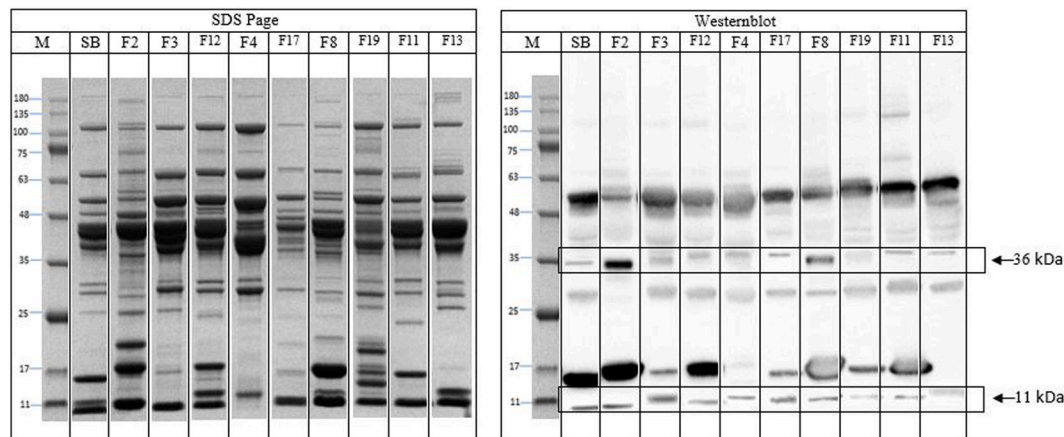


Fig. 2. SDS-PAGE of extracted fish proteins and analysis of rabbit anti-SB detection antibody specificity with other proteins using Western blotting. Rabbit anti-SB polyclonal antibodies clearly recognizes a single bands approximately at 11 kDa, 17 kDa, 25 kDa, 36 kDa and bands between 48 kDa and 63 kDa. Staining was developed for 50 s using a ChemiDoc XRS chemiluminescent imaging system. 10 μ l per lane of sample extracts loaded in to the 12% SDS-PAGE. M: molecular weight marker, F2: pollack, F3: mackerel, F12: tilapia, F4: salmon, F17: snakehead, F8: hake, F19: sole, F11: large yellow croaker, F13: basa.

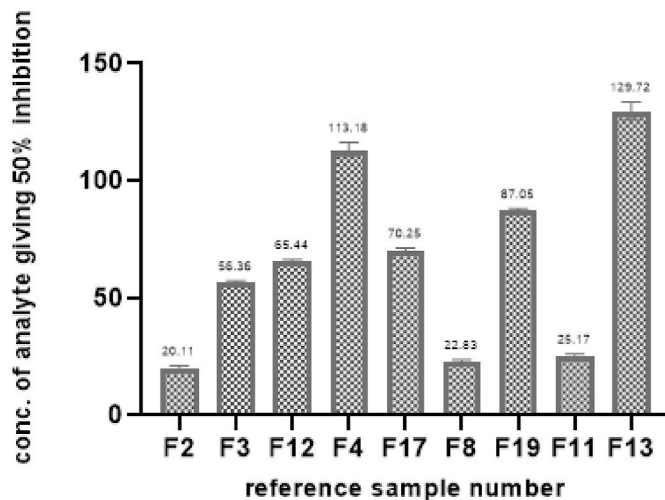


Fig. 3. Concentration of antigens giving 50% of reactivity by detection antibody.

The concentration (ng/ml) of other fish proteins [9] required to give 50% response varies between each species in a dose dependence manner. F2: pollack, F3: mackerel, F12: tilapia, F4: salmon, F17: snakehead, F8: hake, F19: sole, F11: large yellow croaker, F13: basa.

previous study based on anti-PV of SB immunogenicity demonstrated its thermostability. In addition, it could recognize most thermally treated fish species compare to other anti-fish antibodies ([17]).

Further, all the foods that were labelled as fish or fish ingredients gave a positive signal to the developed assay with the improved

extraction buffer ([32]) and complied with the allergen declaration on the label. Undeclared fish at the level of 0.43 μ g/ml– 1.29 μ g/ml were found in two commercial products (popcorn shrimp and fish oil) that might potentially pose a serious threat to fish-allergic consumers. Notably, our approach could measure fish protein content in fermented foods that could not detectable in previously developed PV based sELISA assay ([14]). During fermentation, temperature, pH, presence of salt, chemical agents could disrupt the intramolecular and intermolecular interactions causing structural alterations and denature the protein. But allergenicity isn't abolished with fermentation ([19]). Therefore it is worth mention the suitability of our approach over other assays. Overall, the sELISA approach described here is more convenient and offers manufacturers and researchers more sensitive and reliable immuno-enzymatic method to determine if their products contain undeclared fish allergenic residues to validate their allergen control program and do not have the disadvantages mentioned above.

5. Conclusion

We believe this is the first study identifying the suitability and compatibility of IgG raised against fish proteins in two different mammalian species for capture and detection antibodies to construct a novel sandwich-type ELISA approach, taking soluble antigenic proteins as detection targets. As a potential analytical tool, the developed assay is robust, specific, and precise in detecting fish proteins irrespective of the type of processing method. Since various types of complex metrics, food systems and processing conditions affect fish identification, real food samples may require different assays to ensure adequate quantification of fish residues to minimize or prevent fish allergy. Therefore, we believe that this new immuno-enzymatic method met its objective and will contribute to filling the gap in achieving that goal.

Table 4

Spike recovery (%) of manufactured food models.

Spiked level (ppb)	Canned crabmeat		Oyster wine		Semi-cooked shrimp balls		Pasta		Rice and potato infants noodles	
	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
100	96.88	9.08	90.8	9.71	98.55	13	122.62	6.45	130.79	9.15
50	95.07	5.14	86.79	5.34	92.47	3.62	104.07	2.66	117.24	3.62
25	94.85	1.22	83.21	0.25	96.2	1.08	115.26	0.45	108.42	2.41
0	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A

Abbreviations: CV = coefficient of variation, ND= Not detected, N/A = Not applicable.

All percentage values displayed here are results of three replicate analyses performed in three individual occasions and round to two decimal places.

Table 5
Detection of fish proteins in real foods.

Category	Food type/Label	Allergen declaration	Quantified fish protein level (µg/ml)
Aquatic products	seafood mixture	shrimp	ND
	hot pot mashed cuttlefish	surimi*, egg white	71.72 ± 3.05
	semi cooked cuttlefish rings	N/A	ND
	lobster balls	N/A	ND
	breaded shrimp	shrimp, soya, wheat	ND
	seafood tofu	fish	106.53 ± 2.66
	salted and dried anchovy	fish	55.89 ± 2.24
	canned tuna	fish	10.43 ± 0.81
	fish surimi	fish	85.82 ± 1.52
	fried fish cake	fish, egg, wheat, soya	192.77 ± 2.04
	seafood sticks	fish, egg, soy, wheat	118.05 ± 1.66
	Isinglass	N/A	ND
	gluten free rice pasta	N/A	ND
	fruit and nut granola	wheat	ND
Cereal products	Musli	wheat	ND
	cheerios-breakfast cereal	oat, almond, pistachio	ND
	Pasta	wheat	ND
Baby food	rice and potato infants noodles	N/A	ND
	fortified millet cereal	N/A	ND
Snacks	spinach noodles	wheat	ND
	fully fermented soda	wheat, egg, milk	ND
	biscuit	egg	ND
	egg rolls	fish, wheat, egg	1.01 ± 0.03
	fish skin and vegetable chips	fish	60.11 ± 1.72
Dips and sauces	ready to eat salmon sticks	fish	0.86 ± 0.43
	popcorn shrimp	shrimp	3.18 ± 1.80
	fish sauce	fish	4.55 ± 3.91
Beverages	manufacture 1	fish	ND
	manufacture 2	Soy	ND
	soy sauce	N/A	ND
Other edible products	oyster wine	N/A	ND
	Coffee	N/A	ND
Other edible products	fish oil manufacturer 1	N/A	ND
	manufacturer 2	N/A	0.92 ± 0.04

Abbreviations: N/A: Not available, ND: Not detected, * declared as fish surimi. All the experimented values displayed here are results of three replicate analyses performed in three individual occasions, round to two decimal places and expressed as means ± SD (n = 3).

Compliance with ethical standards

All animal experiments were performed following established guidelines and the experimental protocol in according with the Ethical Committee of Experimental Animal Care at Ocean University of China. No:SPXK20150001.

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 author.

Informed consent

N/A.

ORCID iD authorship contribution statement

Binaka Prabashini Dasanayaka: Investigation, Methodology, Data curation, Writing – original draft. **Jinlong Zhao:** Data curation, Resources. **Jiukai Zhang:** Validation, Resources. **Yuhao Huang:** Validation, Resources. **Mati Ullah Khan:** Resources. **Hong Lin:** Validation, Supervision. **Zhenxing Li:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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