



## Original article

# Immunomodulatory properties of selectively processed prawn protein fractions assessed using human peripheral blood mononuclear cells

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**Summary** Prawn muscles were treated with acetic acid and high-pressure processing (600 MPa) separately to analyse their antigenicity and immunogenicity. The protein fractions were separated and isolated using preparative HPLC, and their antigenicity was analysed using Immunoglobulin G (IgG) ELISA kit. Out of thirty-nine protein fractions, only four (A10, A11, B10 and C9) were detected with antigenic potentials. The immunogenicity of these protein fractions was analysed using human PBMCs, and supernatants were collected at multiple times from 0 to 144 h. The treated fractions (B10 and C9) analysed using Immunoglobulin E (IgE) ELISA kit showed significantly ( $P < 0.05$ ) lower pro- and anti-inflammatory cytokine production compared with control (A10). The allergenic fractions were characterised using an LC/MS/MS, which identified nine proteins. Among these, six proteins (tropomyosin, arginine kinase, haemocyanin, enolase, vitellogenin and 14-3-3 zeta) have been established as allergenic in prawn muscle and ovaries. Other three proteins (beta-1,3-glucan-binding protein, translationally controlled tumour protein and farnesoic acid O-methyltransferase short isoform protein) identified in this study need further investigation for their immunogenic properties.

**Keywords** Antigenicity, immunogenicity, interleukin cytokine, LC/MS/MS, PBMCs, prawn allergy.

## Introduction

Prawn allergy is one of the major causes of food-borne allergies, responsible for most severe food allergy-related emergency department visits (Sicherer *et al.*, 2004; Liu *et al.*, 2008). The upward trend of prawn-induced allergic incidents has now become an alarming issue in global food safety (Sicherer & Sampson, 2006). The major allergen in prawn protein, known as tropomyosin, is responsible for over 80% prawn allergy-related incidents. It is a 37 kDa heat-stable muscle protein having an  $\alpha$ -helical structure associated with actin filaments (Troiano, 2016; Faisal *et al.*, 2019c). Beside this protein, arginine kinase, myosin light chain, actin, troponin, haemocyanin and sarcoplasmic calcium-binding protein are also known as minor allergen in prawns (Rahman *et al.*, 2013; Kamath *et al.*, 2014; Khanaruksombat *et al.*, 2014). Prawn allergy is an IgE-mediated type 1 allergy showing symptoms of severe mucocutaneous, respiratory, gastrointestinal, anaphylactic and cardiovascular (95.7%, 23.9%, 16.3%, 11.9% and 3.3%,

respectively) disorders (Sicherer, 2011; Pedrosa *et al.*, 2015). In previous studies, Ayuso *et al.* (2002) and Zheng *et al.* (2011) reported eight IgE-binding epitopes, whereas Wang *et al.* (2012) reported 17 IgE-binding epitopes on tropomyosin. As per literature, the hypersensitivity reactions due to binding of protein epitopes with IgE antibodies are termed as allergenicity, whereas binding with IgG antibodies is known as antigenicity (Verhoeckx *et al.*, 2015; Bogahawaththa *et al.*, 2017). Moreover, when allergic components stimulate the immune system of the human body involving generation of specific IgE antibodies, the resulting stimulation is known as immunogenicity (Actor, 2014).

Wang *et al.* (2012) and Ravkov *et al.* (2013) reported the ability of allergenic protein to activate and proliferate T-helper (Th) cells in human peripheral blood mononuclear cells (PBMCs). PBMCs have been extensively studied in immunological research due to the presence of highly sophisticated immune cells lending their application in *in vitro* studies. PBMCs have often been co-cultured with various immune stimulants *in vitro*, to determine their efficacy considering various parameters of immune responses, such as cytokine production

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(Ramachandran *et al.*, 2012). The Th cells, including Th1 and Th2 subsets, play important role in interfering with the immune defence through the antibody or cell-mediated immune responses. In addition, the balance between Th1 and Th2 maintained by secretion of certain types of interleukin (IL) (such as IL-2, IL-4, IL-10 and IFN- $\gamma$ ) is believed to maintain the homeostasis of immune response (Donkor *et al.*, 2012; Wang *et al.*, 2012). In addition, Th17 cells release pro-inflammatory IL-17 cytokines and may differentiate into Th1 or Treg (T regulatory) cells to regulate the balance between Th1 and Th2 cells (Korn *et al.*, 2009; Gálvez, 2014).

To minimise human health risk due to allergic reactions, several researchers endeavoured to prepare hypoallergenic prawn products through structural modification of tropomyosin using various processing technologies (Kamath *et al.*, 2014; Lasekan & Nayak, 2016; Lv *et al.*, 2017; Yuan *et al.*, 2017; Faisal *et al.*, 2019c). In previous studies, Faisal *et al.* (2019a,c) reported a significant reduction of antigenicity (IgG binding) of tropomyosin in high-pressure-processed (600 MPa) and acetic acid-treated prawn samples using immunoblotting and ELISA kits. Considering that the *in vitro* immunoassays often use crude protein extracts to measure the changes of the specific IgE sensitivity based on patient serum, the affectability and explicitness of the test are not constantly acceptable in distinguishing the actual allergenicity (Morita *et al.*, 2013; Leung *et al.*, 2014; Abramovitch *et al.*, 2017). Moreover, the crude extract contains a great deal of different types of protein matrices, which often creates difficulties to point out the role of specific proteins for IgE reactivity (Faisal *et al.*, 2019c). To resolve this problem, the use of purified allergenic proteins becomes indispensable to diagnose the specific IgE sensitivity more accurately (Morita *et al.*, 2013; Leung *et al.*, 2014). Beside this, the study on cellular immune reactivity of isolated protein fractions is limited to reveal the role of Th cells completely. Therefore, the present study aimed to examine the immunogenicity (Th cell-mediated immune response *in vitro*) of isolate protein fractions in native and processed (acetic acid and high pressure treated in combination with high temperature) banana prawn samples up to certain time using human PBMC, as well as identify and characterise the protein fractions using liquid chromatography with tandem mass spectrometry (LC/MS/MS).

## Materials and methods

### Treatment and extraction of proteins from prawn

Fresh banana prawns (*Fenneropenaeus merguensis*) were purchased from a local supermarket in Melbourne, Australia. Prawn samples were washed with distilled water, deshelled and deveined prior to all treatments.

High-pressure processing (HPP) of prawn muscle was performed using a Stansted ISO-LAB FPG11501 High Pressure 3.6 L unit (Stansted Fluid Power Ltd., Stansted, Essex, UK). The pressure vessel has a permitted initial temperature range from  $-20$  to  $110$  °C for pressures up to 800 MPa. The maximum temperature within the vessel during pressure holding was  $130$  °C. A deionised water/propylene-glycol mixture (40% glycol) was used as the pressure-transmitting medium (Knoerzer *et al.*, 2010). The processing of prawn sample with high pressure (600 MPa) at  $120$  °C for 10 min was executed as described by Faisal *et al.* (2019a). In brief during high-pressure treatment, the vessel was conditioned to an initial temperature ( $90$  °C), which then attained the target temperature after compression heating. Conditioning times for samples were short ( $<2$  min) but varied slightly depending on the applied temperature. The compression and decompression rates were set to 600 and 1200 MPa  $\text{min}^{-1}$ , respectively. The temperature of the compression fluid and sample were monitored using a type T thermocouple attached to the sample carrier. HPP treatment was replicated on different days. For acetic acid-treated samples, prawn muscles were submerged in acetic acid (commercially available white vinegar) at pH 2.5 for 15 min. The prawn muscles without any processing were used as control.

The method described by Faisal *et al.* (2019c) was implemented for the extraction of proteins from the control, HPP and acetic acid-treated samples. In brief, fresh prawn muscles were homogenised using a laboratory blender (Waring 8011ES blender, NJ, USA) in phosphate-buffered saline solution (pH 7.4) at 1:3 ratio for 5 min. The protein slurry was agitated at  $4$  °C for 3 h, followed by centrifugation (Beckman Coulter Avanti J-26S XPI centrifuge, CA, USA) at  $4$  °C and speed of 29 400 *g* for 15 min. The supernatant of the protein mixture was separated utilising micropipette and stored in properly labelled sealed containers at  $-80$  °C until further analysis.

### Determination of protein content

The total protein content of each sample was determined by the Kjeldahl method and Bradford Assay kit (Bio-Rad Laboratories, Sydney, NSW, Australia), following the manufacturer's instructions. For Kjeldahl method, a Foss 2020 Digester Unit DS20 and Foss 2012 Distilling Unit (Hillerod, Sweden) were used for sample digestion and distillation, respectively. Bovine serum albumin (BSA) was used as the protein standard for the Bradford method (Kamath *et al.*, 2013).

### Protein profiling by reversed-phase high-performance liquid chromatography (HPLC)

The proteins in control and treated (acetic acid and HPP) samples were analysed using a reversed-phase

HPLC (SHIMADZU, Prominence-i, LC-2030C, Tokyo, Japan) and a Jupiter analytical column (250 × 4.6 mm, particle size 5 µm, pore size 300 Å, connected to a security guard cartridge, wide-pore C18, 4 × 3.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of the following: (i) HPLC-grade water with 0.1% TFA (trifluoroacetic acid) and (ii) 0.1% TFA in acetonitrile with the following gradient: 5% to 90% B from 0.1 min to 50 min, then 90% B for 5 min after which it reverted to 5% B for least 5 min (total run time: 60 min) at room temperature. A 10 µL sample was injected at each run, and the flow rate was maintained at 0.2 mL min<sup>-1</sup>. Protein elution was monitored at 280 nm with UV detector.

#### Large scale protein isolation by preparative HPLC

The isolation of protein fractions in a sustainable content from different samples was conducted using a Varian HPLC system (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a C18 Jupiter preparative column (250 × 21.2 mm, particle size 5 µm, pore size 300 Å, connected to a security guard prep cartridge, C18-300A, 250 × 21.2 mm, Phenomenex). Exactly, 1 mL sample was injected in each run and the mobile phase consisted of the following: (i) HPLC-grade water with 0.1% TFA and (ii) 0.1% TFA in acetonitrile with flow rate 4.28 mL min<sup>-1</sup>. The following gradient was maintained for the separation of proteins: 5% to 90% B from 0.1 min to 50 min, then 90% B for 5 min after which it reverted to 5% B for least 5 min (total run time: 60 min) at room temperature. Proteins elution was monitored at 280 nm with a UV detector. To collect separated protein fractions, at least 25 runs/samples were performed. Total thirteen protein fractions from each sample were collected separately, and same fractions were pooled together in Falcon tubes. The eluted protein fractions in the control sample were marked as A1 to A13, whereas for acid and HPP samples marked as B1 to B13 and C1 to C13, respectively. Eluted protein fractions were frozen separately followed by freeze-drying (Dynavac FD 300 Freeze Drier, Melbourne, VIC, Australia) to concentrate the protein fractions. The freeze-dried fractions were resuspended in 2.5 mL RPMI-1640 medium to perform the following analysis.

#### Detection of allergenic protein fractions from separated samples using ELISA kit

ELISA is a widely accepted immunological based technique, used for fast detection and quantification of antigenicity (Faisal *et al.*, 2019b). Detection of allergenic protein fractions was performed using a Sandwich ELISA kit (RIDASCREEN®FAST Crustacean,

R-Biopharm, Darmstadt, Hessen, Germany). Samples were analysed following manufacturer's instructions as described by Faisal *et al.* (2019c). In brief, extracted protein fractions (250 µg mL<sup>-1</sup>) were each diluted 20-fold with extraction buffer, respectively, followed by centrifugation at 2500 g (Eppendorf centrifuge 5810 R, Hamburg, Germany) for 10 min. Exactly, 100 µL/well/sample was added into antibody pre-coated 96-microwell plate and incubated at room temperature for 10 min. The 96-microwell plate was washed three times with washed buffer followed by addition of 100 µL conjugate solution to each well and again incubated at room temperature for 10 min. After subsequent washing, 100 µL of chromogen was added/well and incubated in the dark at room temperature for 10 min. Finally, 100 µL of stop solution was added to each well and absorbance was measured within 10 min using ELISA plate reader (iMark microplate absorbance reader, Bio-Rad, Tokyo, Japan) at 450 nm.

#### Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells used in the current study have been obtained by meeting requirements of the National Health and Medical Research Council (National Health and Medical Research Council, 2007). Ethics application (ID: HRE16-058) has been approved by the Victoria University Human Research Ethics Committee. The Australian Red Cross Blood Services (Melbourne, Australia) supplied buffy coats from healthy donors. Isolation of PBMCs from buffy coat was performed using an established protocol (Donkor *et al.*, 2012; Bogahawaththa *et al.*, 2018) with slight modifications. In brief, 60 mL of buffy coat was diluted with equal amount of phosphate-buffered saline (pH 7.4) and layered gently on Ficoll-Paque Plus (GE Healthcare Pty Ltd., Silverwater, NSW, Australia). After centrifugation (Sorvall-RT7 centrifuge, DuPont, Newtown, USA) at 400 g for 25 min at 18 °C with no break, the separated layer of PBMCs was washed twice with 50 mL of RPMI-1640 immediately and centrifuged at 400 g/wash (18 °C for 10 min with break). The cell pellet was resuspended in 10 mL of RPMI-1640, and the cell concentration was calculated to be 3.5 × 10<sup>7</sup> cells mL<sup>-1</sup>.

#### Stimulation of PBMCs with isolated prawn protein fractions

Stimulation of PBMCs with the isolated nine prawn protein fractions, namely A9 to A11, control; B9 to B11, acetic acid; C9 to C11, HPP, was executed as described by Bogahawaththa *et al.* (2018) with some modifications. Freshly prepared PBMCs (3.5 × 10<sup>7</sup> cells mL<sup>-1</sup>) were resuspended in RPMI-1640

supplemented with 10% foetal bovine serum and 1% antibiotic–antimycotic solution (Sigma Aldrich Pty Ltd., Castle Hill, NSW, Australia). The cells,  $1.66 \times 10^6 \text{ mL}^{-1}$ , were then co-cultured in each well with  $10 \mu\text{g mL}^{-1}$  of selected prawn protein fractions in cell culture flasks and incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 144 h. For a positive control,  $1 \mu\text{g mL}^{-1}$  of lipopolysaccharide (LPS) from *Escherichia coli* O111: B4 (Sigma–Aldrich Pty Ltd.) was co-cultured with PBMCs, whereas unstimulated PBMCs in RPMI-1640 were used as negative control for quantifying basal cytokine production. Supernatants were collected at 0-, 4-, 8-, 12-, 24-, 48-, 72-, 96-, 120- and 144-h interval from the flasks and were stored at  $-80^\circ\text{C}$  for cytokine analysis.

#### Cytokine assays by IgE ELISA

Concentration of different cytokines, including interleukin (IL)-4, IL-10, IL-17A and interferon (IFN)- $\gamma$ , produced by stimulated PBMCs at different time periods in the presence of selected protein stimulants were quantified using enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific Australia Pty Ltd.) according to the manufacturer's guideline. In brief, ninety-six well-uncoated ELISA plates (Coaster 9018 ELISA plate) were coated with captured antibody and incubated at  $4^\circ\text{C}$  overnight under continuous shaking. After three consecutive washes with  $250 \mu\text{L}$  wash buffer per well, ELISA plate wells were blocked with  $200 \mu\text{L}$  ELISTOP diluent and incubated for 1 h. Exactly,  $100 \mu\text{L}$  sample/well was added and incubated at  $4^\circ\text{C}$  overnight under continuous shaking to achieve maximum sensitivity. The microwells were washed three times with wash buffer followed by addition of  $100 \mu\text{L}$ /well of diluted detection antibody. Streptavidin-HRP ( $100 \mu\text{L}$ ) for detection of IL-4 and IFN- $\gamma$  or avidin-HRP for IL-10 and IL-17A was added to each well and incubated at room temperature for 30 min. Following this step,  $100 \mu\text{L}$  of TMB solution was added to each well and incubated for 15 min at room temperature.  $100 \mu\text{L}$  of stop solution ( $2 \text{ N H}_2\text{SO}_4$ ) was then added to each well, and absorbance was measured within 10 min using ELISA plate reader (xMark microplate spectrophotometer, Bio-Rad, Tokyo, Japan) at  $450 \text{ nm}$ .

#### Characterisation of allergenic protein fractions

Fractions that showed significant allergenic reaction with PBMCs were analysed by a LC/MS/MS using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific). Samples were concentrated on a  $100 \mu\text{m}$ , 2 cm nanoviper pepmap100 trap column with 97.5% buffer A (0.1% TFA) at a flow rate of  $15 \mu\text{L min}^{-1}$ . The peptides then

eluted and separated with a Thermo RSLC pepmap100,  $75 \mu\text{m} \times 50 \text{ cm}$ ,  $100 \text{ \AA}$  pore size, reversed-phase nano-column with a 30 min gradient of 92.5% buffer A (0.1% formic acid) to 42.5% B (80% acetonitrile 0.1% formic acid), at a flow rate of  $250 \text{ nL min}^{-1}$ . The eluant is nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of  $1900 \text{ V}$ . Peptides were selected for MSMS analysis in Full MS/dd-MS<sup>2</sup> (TopN) mode with the following parameter settings: TopN 10, resolution 70000, MSMS AGC target 5e5, 118 ms Max IT, NCE 27, 1.8 m/z isolation window, and dynamic exclusion was set to 10 s.

Data from LC/MS/MS analysis were exported to Mascot generic file format (\*.mgf) using proteowizard 3.0.3631 (open source software, <http://proteowizard.sourceforge.net>) and searched against the Uniprot Tricumaestivium databases using the MASCOT search engine (version 2.4, Matrix Science Inc., London, UK) with all taxonomy selected. The following search parameters were used: missed cleavages, 1; peptide mass tolerance,  $\pm 10 \text{ ppm Da}$ ; peptide fragment tolerance,  $\pm 0.02 \text{ Da}$ ; peptide charge, 2+, 3+ and 4+; fixed modifications, carbamidomethyl; and variable modification, oxidation (Met). Data from LC/MS/MS run were processed using Byonic (ProteinMetrics) V 3.1-19 with no specific cleavage sites specified and a precursor and fragment mass tolerance of  $20 \text{ ppm}$ . Modifications specified were Carbamidomethyl @C fixed and Oxidation @M Variable common 1. The protein output was set to 1% FDR. Moreover, data from LC/MS/MS run were processed using Peaks studio version 8 (Bioinformatics solutions using default settings for data refinement and a parent mass tolerance of  $15 \text{ ppm}$  and fragment tolerance at  $0.5 \text{ Da}$  with a max of five peptide candidates per spectrum).

#### Statistical analysis

Statistical analysis of results was performed using the general linear model procedure of the Statistical Analysis System (SAS v.9.2) with the treatment and replications as the main factors. The effect of selected prawn protein fraction on immunogenicity of PBMCs at various times was considered significant at  $P \leq 0.05$ .

## Results and discussion

#### Profiling and isolation of prawn protein by HPLC

The HPLC protein profiles for supernatant mixture of control and treated (acetic acid and HPP) prawn samples are shown in Fig. 1(a, b and c), and retention times of eluted protein fractions are also reported in

**Figure 1** HPLC protein profile of untreated (a); acetic acid treated (b); and HPP at 120 °C (c) prawn protein extracts.

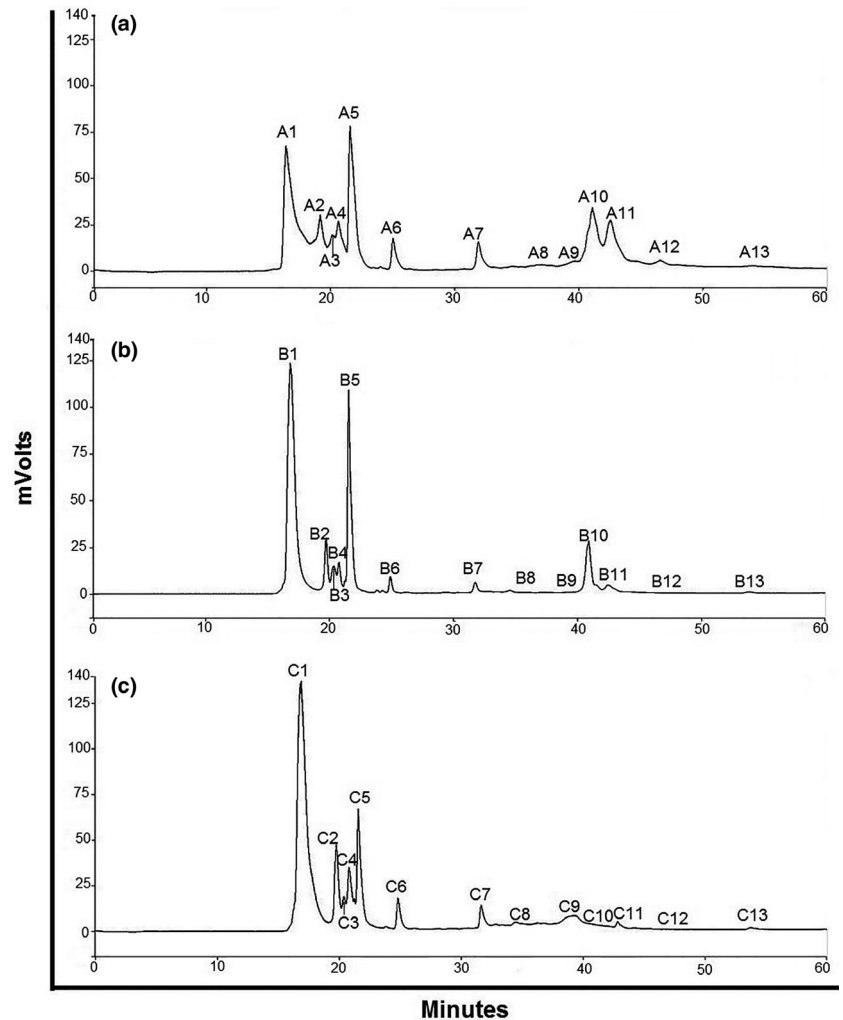


Table 1. In the control sample, two protein fractions (A10 and A11) showed significantly higher concentration than similar protein fractions (B10, B11, C10 and C11) eluted from the treated samples. However, the protein fractions C9 derived from HPP showed significantly higher concentration than A9 and B9. This was an indication that treatment had an effect on prawn proteins due to structural changes as a result of treatment of the proteins (Faisal *et al.*, 2019a,c). The combined effect of HPP and heat induced changes in primary and secondary structures through altering inter- and intramolecular bonds especially ionic, hydrogen and hydrophobic interactions (Chatterjee *et al.*, 2006; Wang *et al.*, 2013). On the other hand, Xu *et al.* (2012) reported that structural modification and aggregation of acid-treated proteins take place

due to noncovalent, especially hydrophobic, attractions and formation of hydrogen bonds.

#### Antigenicity determination in isolated protein fractions by enzyme-linked immunosorbent assay (ELISA)

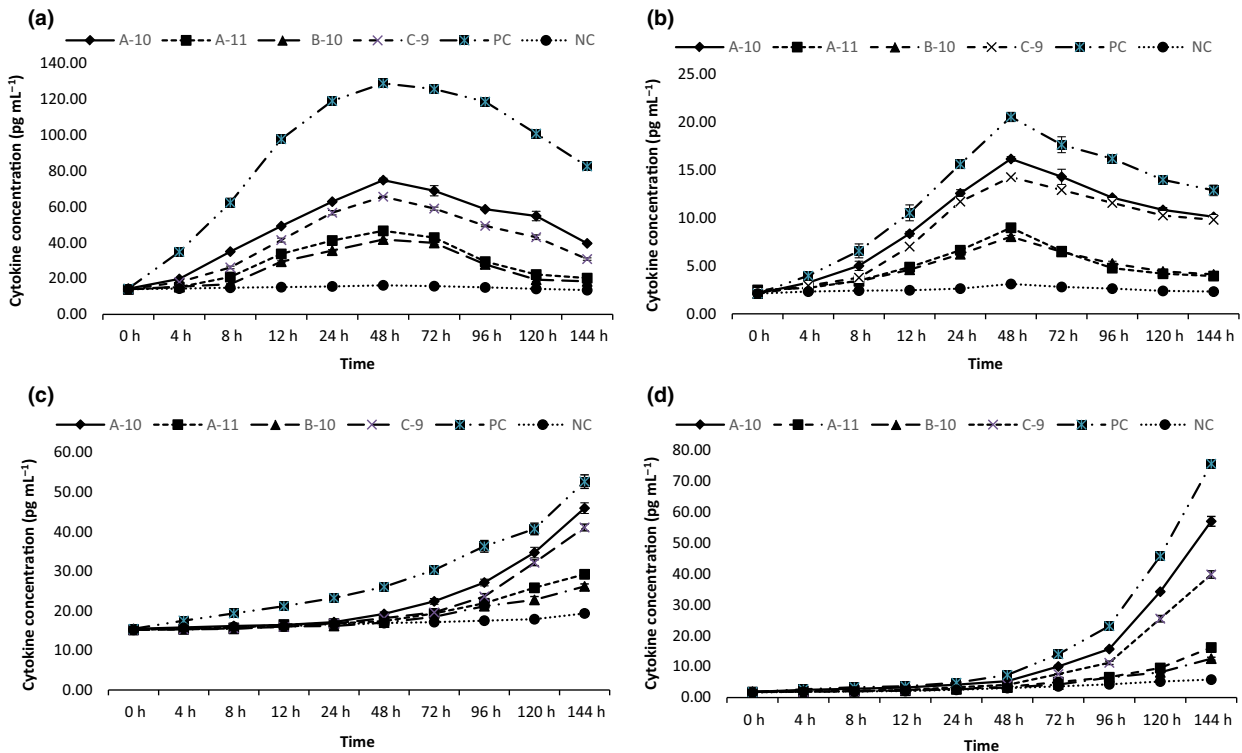
A sandwich ELISA test confirming antigenicity of protein fractions in control and treated prawn samples determined positive antigenicity in four out of thirty-nine eluted fractions, reported in Table 1. Out of the four antigens, two (A10 and A11) were from the control sample, whereas one each (B10 and C9) were from acetic acid- and HPP-treated samples, respectively. However, A10 showed the highest antigenicity ( $32 \mu\text{g mL}^{-1}$ ) followed by C9 and B10. These results indicate that antigenicity was reduced by 81.25% and

**Table 1** Antigenicity (IgG binding) analysis of eluted proteins assessed by ELISA kit

Control (Untreated)		Acetic acid treated for 15 min		HPP at 120 °C for 10 min	
Protein fractions with retention time (min)	Antigenicity (IgG)	Protein fractions with retention time (min)	Antigenicity (IgG)	Protein fractions with retention time (min)	Antigenicity (IgG)
A1 (16.00–18.00)	–	B1 (16.00–18.00)	–	C1 (16.00–18.40)	–
A2 (18.50–19.50)	–	B2 (19.40–20.00)	–	C2 (19.30–20.00)	–
A3 (19.55–20.30)	–	B3 (20.05–20.50)	–	C3 (20.10–20.40)	–
A4 (20.40–21.20)	–	B4 (20.55–21.20)	–	C4 (20.50–21.20)	–
A5 (21.30–22.50)	–	B5 (21.30–22.50)	–	C5 (21.30–22.50)	–
A6 (24.50–26.00)	–	B6 (24.55–25.30)	–	C6 (24.55–25.50)	–
A7 (31.40–32.50)	–	B7 (31.40–32.20)	–	C7 (31.40–32.30)	–
A8 (34.20–38.20)	–	B8 (34.00–38.20)	–	C8 (34.00–38.00)	–
A9 (38.50–40.00)	–	B9 (38.50–40.00)	–	C9 (38.50–40.00)	26 µg mL <sup>-1</sup>
A10 (40.20–41.55)	32 µg mL <sup>-1</sup>	B10 (40.20–41.50)	6 µg mL <sup>-1</sup>	C10 (40.20–42.00)	–
A11 (42.10–44.00)	9 µg mL <sup>-1</sup>	B11 (42.10–43.50)	–	C11 (42.40–43.50)	–
A12 (45.50–47.40)	–	B12 (45.50–47.40)	–	C12 (45.50–47.40)	–
A13 (53.20–55.10)	–	B13 (53.20–54.50)	–	C13 (53.20–54.50)	–

18.75% of C9 and B10, respectively, from a 100% of A10. Jin *et al.* (2015) stated that HPP in combination with temperature converted  $\alpha$ -helix proteins into  $\beta$ -sheets and random coils, which likely masked or destroyed the antigen-binding epitopes. Furthermore, the partial solubility of proteins due to acid treatment

likely reduced the active epitopes sites of antigens (Mohan *et al.*, 2007; Xu *et al.*, 2012) similarly observed in the current studies. Thus, processing likely masked or destroyed epitopes binding sites on antigens, resulting in less antigen–antibody binding, as previously reported (Faisal *et al.*, 2019a,c).



**Figure 2** Changes in concentrations of cytokines in treated and untreated fractions with time – anti-inflammatory IL-10 (a) and IL-4 (b); pro-inflammatory cytokines – IFN- $\gamma$  (c) and IL-17A (d). A10 and A11 from control; B10 from acetic acid treated; C9 from HPP at 120 °C treated; PC means positive control and NC means negative control.

### *In vitro* Immunogenicity analysis of selected isolated protein fractions

Antigenicity analysis of isolated protein fractions showed that potential allergenic proteins were eluted between 38.50 and 44.00 min for both control and treated samples. Based on these findings, three protein fractions from each treatment were used to challenge human PBMCs and supernatants collected at different time intervals were analysed for cytokine production (Fig. 2). Again, four protein fractions (A10, A11, B10 and C9) showed significantly ( $P < 0.05$ ) higher mean cytokine production in comparison with negative

control. On the other hand, other protein fractions tested (A9 from control; B9 and B11 from acetic acid; C10 and C11 from HPP) showed no significant ( $P > 0.05$ ) difference in cytokine concentration to negative control (data not shown). Two types of cytokines, anti-inflammatory (Fig. 2a and b) and pro-inflammatory (Fig. 2c and d), were produced in varying concentrations. The anti-inflammatory cytokine (IL-4 and IL-10) production increased in concentration with increasing time up to 48 h, whereas the pro-inflammatory cytokines (IFN- $\gamma$  and IL-17A) did not significantly increase in concentration until after 48 h up to 144 h when IL-4 and IL-10 consistently declined (Fig. 2). Prawn protein is classified

**Table 2** List of identified proteins in protein fractions by LC/MS/MS

Fractions	Protein code	Identified proteins by LC/MS/MS	Concentration of protein in fractions (%)	% of Coverage
A10 (Control)	C3VUU0	Arginine kinase OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	40.65	69.38
	D3XNS0	Tropomyosin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=4	37.00	93.66
	G3GDS2	Enolase (Fragment) OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=eno PE=4 SV=1	11.88	66.67
	Q5QD40	Translationally controlled tumour protein OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	5.00	58.33
	S5ZHH2	Haemocyanin OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=HC PE=2 SV=1	3.33	19.52
	Q07DN6	Farnesoic acid O-methyltransferase short isoform (Fragment) OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	1.62	28.82
	D2SSM3	Vitellogenin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	0.52	10.71
A11 (Control)	S5ZHH2	Haemocyanin OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=HC PE=2 SV=1	35.00	88.05
	C3VUU0	Arginine kinase OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	34.58	77.25
	D3XNS0	Tropomyosin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=4	20.31	81.69
	G3GDS2	Enolase (Fragment) OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=eno PE=4 SV=1	8.06	70.08
	Q07DN6	Farnesoic acid O-methyltransferase short isoform (Fragment) OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	1.18	30.13
	Q5QD40	Translationally controlled tumour protein OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	0.70	32.74
	D2SSM3	Vitellogenin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	0.16	19.56
B10 (Acetic acid treated)	D3XNS0	Tropomyosin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=4	27.90	67.25
	S5ZHH2	Haemocyanin OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=HC PE=2 SV=1	26.25	52.50
	C3VUU0	Arginine kinase OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	18.51	53.37
	G3GDS2	Enolase (Fragment) OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=eno PE=4 SV=1	15.68	64.10
	Q5QD40	Translationally controlled tumour protein OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	7.80	76.19
	D9I8L2	14-3-3 zeta OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	2.11	8.94
	A0A0A7D6G0	Beta-1,3-glucan-binding protein OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	1.25	5.98
C9 (HPP)	D2SSM3	Vitellogenin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	0.50	3.13
	D3XNS0	Tropomyosin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=4	57.92	94.72
	C3VUU0	Arginine kinase OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	18.96	67.70
	S5ZHH2	Haemocyanin OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=HC PE=2 SV=1	15.07	54.01
	G3GDS2	Enolase (Fragment) OS= <i>Fenneropenaeus merguensis</i> OX=71412 GN=eno PE=4 SV=1	3.90	71.79
	Q5QD40	Translationally controlled tumour protein OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	2.16	30.95
	D2SSM3	Vitellogenin OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	1.15	29.96
D9I8L2	14-3-3 zeta OS= <i>Fenneropenaeus merguensis</i> OX=71412 PE=2 SV=1	0.84	39.43	





higher cytokine production compared with fractions C9 and B10, indicative of effect of treatment on prawn proteins resulting in reduced immunogenicity. In another study, Abramovitch *et al.* (2017) also reported lower cytokine production for treated prawn muscle extract.

#### Characterisation of prawn protein fractions by LC/MS/MS

The allergenic protein fractions characterised by LC/MS/MS are listed in Table 2, and the amino acid sequences of nine identified proteins are represented in Fig. 3. In the control sample (A10, A11), the following proteins were identified: tropomyosin (37 KDa), arginine kinase (40 KDa), haemocyanin (75 KDa), enolase (12 KDa), translationally controlled tumour protein (19 KDa), vitellogenin (283 KDa) and farnesoic acid O-methyltransferase short isoform (26 KDa), as well as lesser proteins with lower molecular masses. On the other hand, with the exception of farnesoic acid O-methyltransferase short isoform (26 KDa) protein and some lesser molecular mass proteins in A10, B10 and C9 contained similar proteins in addition 14-3-3 zeta (27 KDa) identified. Khanaruksombat *et al.* (2014) identified vitellogenin and 14-3-3 zeta as minor allergens in Banana prawn ovaries and therefore are likely to be allergenic in muscle. In addition, translationally controlled tumour protein was identified in all fractions, and however, its impact on immunogenicity needs further investigation. Furthermore, in acetic acid-treated fraction B10, beta-1,3-glucan-binding (227 KDa) protein was identified (not in control and C9). Structural changes due to acid treatment (Mohan *et al.*, 2007; Faisal *et al.*, 2019a,c) and the presence of identified protein likely resulted in the lowest antigenicity and immunogenicity observed. As a consequence of treatment, LC/MS/MS identification showed the per cent of coverage (% of protein match with Database) significantly changed, compared with control. Thus, the disappearance of proteins from A10 and appearance of new proteins in B10 and C9 fractions are indications of processing effect on changes in prawn proteins; however, their impact on immunogenicity need further investigation. Tropomyosin is a major allergenic protein of Banana prawn (Faisal *et al.*, 2019a,c), whereas arginine kinase has been reported as a minor allergen in different species of prawns (Kamath *et al.*, 2013, 2014; Rahman *et al.*, 2013), and haemocyanin and enolase fragment have also been reported as minor allergens in Banana prawn muscle (Khanaruksombat *et al.*, 2014).

#### Conclusion

Processing of prawn muscle with HPP and acetic acid separately showed significant lower pro- and anti-inflammatory cytokine production resulting in decreased

immunogenicity compared to control. Characterization of treated and untreated fractions showed four common proteins (tropomyosin, arginine kinase, haemocyanin and enolase) having allergenic properties in prawn muscle. Whereas, other two proteins (vitellogenin and 14-3-3 zeta) identified are likely to have minor allergenic properties in prawn ovaries. The remaining three proteins (beta-1,3-glucan-binding protein, translationally controlled tumour protein and farnesoic acid O-methyltransferase short isoform protein) isolated from treated and untreated fractions need further studies into their immunogenic properties.

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#### Conflicts of interest

The authors declare that they have no conflicts of interest.

#### Author contributions

Md Faisal: concept development; experimental design and execution; data collection, analysis and interpretation; and manuscript writing. Narges Dargahi: experimental design and technical support. Dr Osaana N. Donkor: concept development; experimental design; technical support; and reviewing manuscript. Professor Todor Vasiljevic: concept development; technical support; and revising and editing manuscript.

#### References

- Abramovitch, J.B., Lopata, A.L., O'Hehir, R.E. & Rolland, J.M. (2017). Effect of thermal processing on T cell reactivity of shellfish allergens-Discordance with IgE reactivity. *PLoS ONE*, **12**, e0173549.
- Actor, J.K. (2014). *Introductory Immunology: Basic Concepts for Interdisciplinary Applications*. London, UK: Academic Press.
- Ayuso, R., Lehrer, S.B. & Reese, G. (2002). Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *International Archives of Allergy and Immunology*, **127**, 27–37.
- Barnes, P.J. (2011). Pathophysiology of allergic inflammation. *Immunological Reviews*, **242**, 31–50.
- Bogahawaththa, D., Chandrapala, J. & Vasiljevic, T. (2017). Modulation of milk immunogenicity by thermal processing. *International Dairy Journal*, **69**, 23–32.
- Bogahawaththa, D., Buckow, R., Chandrapala, J. & Vasiljevic, T. (2018). Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins. *Innovative Food Science & Emerging Technologies*, **47**, 301–308.
- Chatterjee, U., Mondal, G., Chakraborti, P., Patra, H.K. & Chatterjee, B.P. (2006). Changes in the allergenicity during different preparations of pomfret, hilsa, bhetki and mackerel fish as illustrated by

- enzyme-linked immunosorbent assay and immunoblotting. *International Archives of Allergy and Immunology*, **141**, 1–10.
- Donkor, O.N., Ravikumar, M., Proudfoot, O. *et al.* (2012). Cytokine profile and induction of T helper type 17 and regulatory T cells by human peripheral mononuclear cells after microbial exposure. *Clinical & Experimental Immunology*, **167**, 282–295.
- Faisal, M., Buckow, R., Vasiljevic, T. & Donkor, O.N. (2019a). Effect of simulated digestion on antigenicity of banana prawn (*Fenneropenaeus merguensis*) after high pressure processing at different temperatures. *Food Control*, **104**, 187–192.
- Faisal, M., Vasiljevic, T. & Donkor, O.N. (2019b). A review on methodologies for extraction, identification and quantification of allergenic proteins in prawns. *Food Research International*, **121**, 307–318.
- Faisal, M., Vasiljevic, T. & Donkor, O.N. (2019c). Effects of selected processing treatments on antigenicity of banana prawn (*Fenneropenaeus merguensis*) tropomyosin. *International Journal of Food Science & Technology*, **54**, 183–193.
- Gálvez, J. (2014). Role of Th17 cells in the pathogenesis of human IBD. *ISRN Inflammation*, **2014**, 928461.
- Jin, Y., Deng, Y., Qian, B., Zhang, Y., Liu, Z. & Zhao, Y. (2015). Allergic response to squid (*Todarodes pacificus*) tropomyosin. *Food and Chemical Toxicology*, **76**, 86–93.
- Kamath, S.D., Rahman, A.M.A., Komoda, T. & Lopata, A.L. (2013). Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chemistry*, **141**, 4031–4039.
- Kamath, S.D., Rahman, A.M.A., Voskamp, A. *et al.* (2014). Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: a comprehensive allergenic approach. *Molecular Nutrition & Food Research*, **58**, 1144–1155.
- Khanaruksombat, S., Srisomsap, C., Chokchaichamnankit, D., Punyari, P. & Phiriyangkul, P. (2014). Identification of a novel allergen from muscle and various organs in banana shrimp (*Fenneropenaeus merguensis*). *Annals of Allergy, Asthma & Immunology*, **113**, 301–306.
- Knoerzer, K., Buckow, R., Sanguansri, P. & Versteeg, C. (2010). Adiabatic compression heating coefficients for high-pressure processing of water, propylene-glycol and mixtures - A combined experimental and numerical approach. *Journal of Food Engineering*, **96**, 229–238.
- Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K. (2009). IL-17 and Th17 Cells. *Annual Review of Immunology*, **27**, 485–517.
- Lasekan, A.O. & Nayak, B. (2016). Effects of buffer additives and thermal processing methods on the solubility of shrimp (*Penaeus monodon*) proteins and the immunoreactivity of its major allergen. *Food Chemistry*, **200**, 146–153.
- Leung, N.Y., Wai, C.Y., Shu, S. *et al.* (2014). Current immunological and molecular biological perspectives on seafood allergy: a comprehensive review. *Clinical Reviews in Allergy & Immunology*, **46**, 180–197.
- Liu, T.H., Lin, Y.R., Yang, K.C., Chou, C.C., Chang, Y.J. & Wu, H.P. (2008). First attack of acute urticaria in pediatric emergency department. *Pediatrics & Neonatology*, **49**, 58–64.
- Lv, L., Lin, H., Li, Z., Wang, J., Ahmed, I. & Chen, H. (2017). Changes of structure and IgE binding capacity of shrimp (*Metapenaeus ensis*) tropomyosin followed by acrolein treatment. *Food and Function*, **8**, 1028–1036.
- Mohan, M., Ramachandran, D., Sankar, T.V. & Anandan, R. (2007). Influence of pH on the solubility and conformational characteristics of muscle proteins from mullet (*Mugil cephalus*). *Process Biochemistry*, **42**, 1056–1062.
- Morita, E., Chinuki, Y. & Takahashi, H. (2013). Recent advances of in vitro tests for the diagnosis of food-dependent exercise-induced anaphylaxis. *Journal of Dermatological Science*, **71**, 155–159.
- National Health and Medical Research Council, Australian Research Council, and Australian Vice-Chancellors' Committee. (2007). *National Statement on Ethical Conduct in Human Research Updated March 2014*, edited by National Health and Medical Research Council. Canberra, Australia: National Health and Medical Research Council.
- Pedrosa, M., Boyano-Martínez, T., García-Ara, C. & Quirce, S. (2015). Shellfish allergy: a comprehensive review. *Clinical Reviews in Allergy & Immunology*, **49**, 203–216.
- Rahman, A.M.A., Kamath, S.D., Gagné, S., Lopata, A.L. & Hel-leur, R. (2013). Comprehensive proteomics approach in characterizing and quantifying allergenic proteins from northern shrimp: toward better occupational asthma prevention. *Journal of Proteome Research*, **12**, 647–656.
- Ramachandran, H., Laux, J., Moldovan, I., Caspell, R., Lehmann, P.V. & Subbramanian, R.A. (2012). Optimal thawing of cryopreserved peripheral blood mononuclear cells for use in high-throughput human immune monitoring studies. *Cells*, **1**, 313–324.
- Ravkov, E.V., Pavlov, I.Y., Martins, T.B. *et al.* (2013). Identification and validation of shrimp-tropomyosin specific CD4 T cell epitopes. *Human Immunology*, **74**, 1542–1549.
- Sicherer, S.H. (2011). Epidemiology of food allergy. *Journal of Allergy and Clinical Immunology*, **127**, 594–602.
- Sicherer, S.H. & Sampson, H.A. (2006). Food allergy. *Journal of Allergy and Clinical Immunology*, **117**, S470–S475.
- Sicherer, S.H., Muñoz-Furlong, A. & Sampson, H.A. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology*, **114**, 159–165.
- Troiano, F.N. (2016). Proteomics-based characterization of food allergens and effects from food processing. PhD Thesis, The University of Foggia, Foggia, Italy. Retrieved from <https://fair.unifg.it/retrieve/handle/11369/363101/78799/Tesi%20dottorato%20Franc-esco%20N%20Troiano.pdf>.
- Untersmayr, E. & Jensen-Jarolim, E. (2006). Mechanisms of type 1 food allergy. *Pharmacology & Therapeutics*, **112**, 787–798.
- Verhoeckx, K.C., Vissers, Y.M., Baumert, J.L. *et al.* (2015). Food processing and allergenicity. *Food and Chemical Toxicology*, **80**, 223–240.
- Wang, S., Delgado, J.C., Ravkov, E. *et al.* (2012). *Penaeus monodon* tropomyosin induces CD4 T-cell proliferation in shrimp-allergic patients. *Human Immunology*, **73**, 426–431.
- Wang, C.Y., Huang, H.W., Hsu, C.P., Shyu, Y.T. & Yang, B.B. (2013). Inactivation and morphological damage of *Vibrio parahaemolyticus* treated with high hydrostatic pressure. *Food Control*, **32**, 348–353.
- Xu, Y., Xia, W., Jiang, Q. & Rao, S. (2012). Acid-induced aggregation of actomyosin from silver carp (*Hypophthalmichthys molitrix*). *Food Hydrocolloids*, **27**, 309–315.
- Yuan, F., Lv, L., Li, Z., Mi, N., Chen, H. & Lin, H. (2017). Effect of transglutaminase catalyzed glycosylation on the allergenicity and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chemistry*, **219**, 215–222.
- Zheng, L.N., Lin, H., Pawar, R., Li, Z.X. & Li, M.H. (2011). Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. *Food and Chemical Toxicology*, **49**, 2954–2960.