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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 α-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-γ) 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 merguiensis*) 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 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^{-1}. 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^{-1}. 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^{-1}) 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 3.5×10^7 cells mL^{-1}.

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 (3.5×10^7 cells mL^{-1}) 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, 1.66×10^6 mL^{-1}, were then co-cultured in each well with 10 μg mL^{-1} of selected prawn protein fractions in cell culture flasks and incubated at 37 °C in 5% CO_2 for 144 h. For a positive control, 1 μg 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 °C for cytokine analysis.

Cytokine assays by IgE ELISA

Concentration of different cytokines, including interleukin (IL)-4, IL-10, IL-17A and interferon (IFN)-γ, 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 °C overnight under continuous shaking. After three consecutive washes with 250 μL wash buffer per well, ELISA plate wells were blocked with 200 μL ELISTOP diluent and incubated for 1 h. Exactly, 100 μL sample/well was added and incubated at 4 °C overnight under continuous shaking to achieve maximum sensitivity. The microwells were washed three times with wash buffer followed by addition of 100 μL/well of diluted detection antibody. Streptavidin-HRP (100 μL) for detection of IL-4 and IFN-γ 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 μL of TMB solution was added to each well and incubated for 15 min at room temperature. 100 μL of stop solution (2 N H_2SO_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 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 μm, 2 cm nanoviper pepmap100 trap column with 97.5% buffer A (0.1% TFA) at a flow rate of 15 μL min^{-1}. The peptides then eluted and separated with a Thermo RSLC pepmap100, 75 μm × 50 cm, 100 Å 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 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 1900V. Peptides were selected for MSMS analysis in Full MS/dd-MS^2 (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 Triticumaestivium 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, ±10 ppm Da; peptide fragment tolerance, ±0.02 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 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 ppm and fragment tolerance at 0.5 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

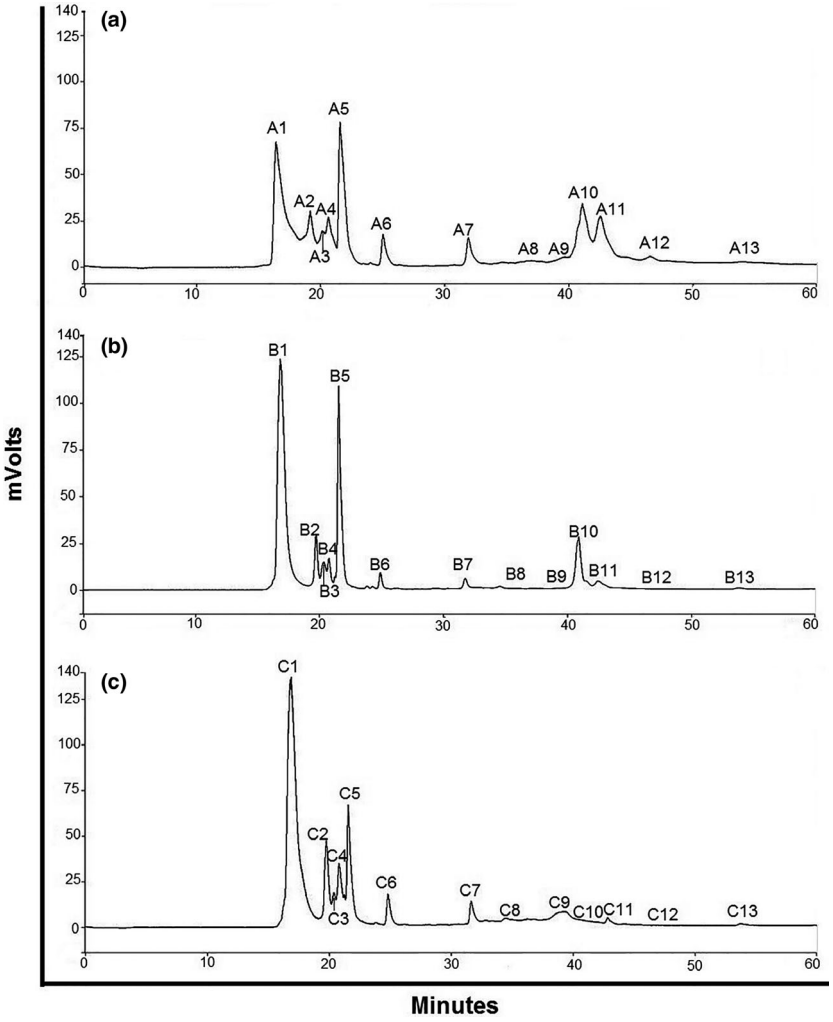

Figure 1 HPLC protein profile of untreated (a); acetic acid treated (b); and HPP at 120 °C (c) prawn protein extracts.

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 μg 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) Protein fractions with retention time (min)	Antigenicity (IgG)	Acetic acid treated for 15 min Protein fractions with retention time (min)	Antigenicity (IgG)	HPP at 120 °C for 10 min 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^{-1}
A10 (40.20–41.55)	32 μg mL^{-1}	B10 (40.20–41.50)	6 μg mL^{-1}	C10 (40.20–42.00)	–
A11 (42.10–44.00)	9 μg mL^{-1}	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 α-helix proteins into β-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).

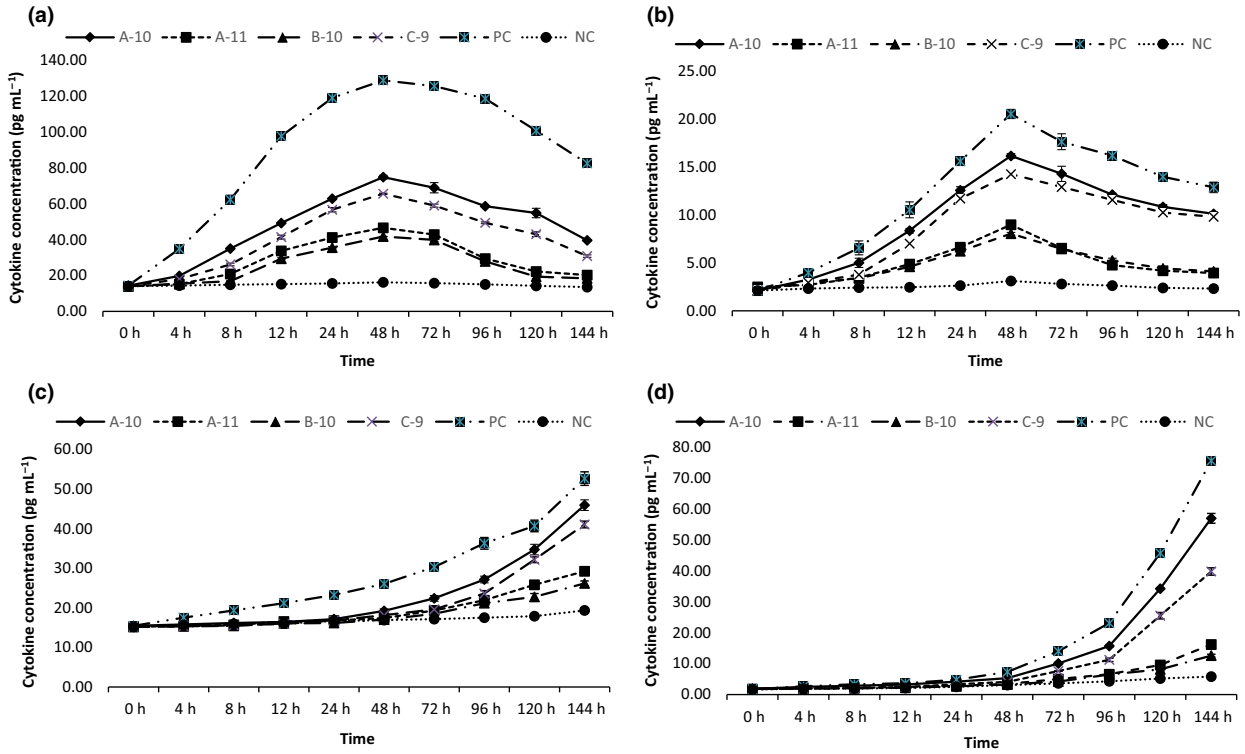

Figure 2 Changes in concentrations of cytokines in treated and untreated fractions with time – anti-inflammatory IL-10 (a) and IL-4 (b); pro-inflammatory cytokines – IFN-γ (c) and IL-17A (d). A10 and A11 from control; B10 from acetic acid treated; C9 from HPP at 120 °C treated; PC means positive control 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-γ 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=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	40.65	69.38
	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	37.00	93.66
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	11.88	66.67
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	5.00	58.33
	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	3.33	19.52
	Q07DN6	Farnesoic acid O-methyltransferase short isoform (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.62	28.82
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.52	10.71
A11 (Control)	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	35.00	88.05
	C3VUU0	Arginine kinase OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	34.58	77.25
	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	20.31	81.69
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	8.06	70.08
	Q07DN6	Farnesoic acid O-methyltransferase short isoform (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.18	30.13
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.70	32.74
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.16	19.56
B10 (Acetic acid treated)	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	27.90	67.25
	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	26.25	52.50
	C3VUU0	Arginine kinase OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	18.51	53.37
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	15.68	64.10
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	7.80	76.19
	D9I8L2	14-3-3 zeta OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	2.11	8.94
	A0A0A7D6G0	Beta-1,3-glucan-binding protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.25	5.98
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.50	3.13
C9 (HPP)	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	57.92	94.72
	C3VUU0	Arginine kinase OS=*Fenneropenaeus* merguiensis OX=71412 PE=2 SV=1	18.96	67.70
	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	15.07	54.01
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	3.90	71.79
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	2.16	30.95
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.15	29.96
	D9I8L2	14-3-3 zeta OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.84	39.43

Tropomyosin (37 KDa)

MDAIKKKMQA MKLEKDNAMD RADTLEQQNK EANNRAEKSE EEVHNLQKRM QQLENDLDQV QESLLKANIQ LVEKDKALSN AEGEVAALNR RIQLLEEDLE RSEERLNTAT TKLAEASQAA DESERMRKVL ENRSLSDEER MDALENQLKE ARFLAEEADR KYDEVARKLA MVEADLERAE ERAETGESKI VELEEELRVV GNNLKSLEVS EEKANQREEA YKEQIKTLTN KLKAAEARAE FAERSVQKLQ KEVDRLEDEL VNEKEKYKSI TDELDQTFSE LSGY (284)

Arginine kinase (40 KDa)

MADAAVIEKL EAGFKKLEAA TDCKSLLKKY LTKEVFDKLK DKKTSLGATL LDVIQSGVEN LDSGVGIYAP DAEAYTLFAP LFDPIIEDYH VGFKQTDKHP NKDFGDVNSF VNVDPEGKFV ISTRVRCGRS MQGYPFNPCL TESQYKEMEA KVSSTLSSLE GELKGTYYPL TGMSKEVQQK LIDDHFLFKE GDRFLQAANA CRYWPAGRGI YHNDNKTFLV WVNEEDHLRI ISMQMGGDLG QVFRRLTSAV NEIEKRIPFS HHDRLGFLTF CPTNLGTTVR ASVHIKLPKL AANREKLEEV AGKYNLQVRG TRGEHTEAEG GIYDISNKRR MGLTEFQAVK EMQDGILELI KIEKEM (356)

Haemocyanin (75 KDa)

MRVLVVLGLI AAAAFQVVSA DVQKQKDVLY LLHRIYGDIQ DADLLATANS FDPAGGSYSD GGAAVQRLLK GLNDGRLLEQ KHWFSLFNTR HRNEALLLFD VLIHSSDWAT FVGNAAFFRQ KINEGEFVYA LYVAVIHSPL TEDVVLPPLY EITPHLFTNS EVIEAAYRAK QKQTPGKFES TFTGTKKNPE QRVAYFGEDI GLNTHHVTWH MEFPFWWDDE YGHHLDRKGE NFFWVHHQLT VRFDAERLSN YLDPVGELHW YKPIVDGFAP HTTYKYGGQF PARPDNVKFE DVDDVARIRD MVIVESRIRD AIAHGYIIDS HGKQIDISNE KGIDILGDVI ESSLYSPNVQ YYGALHNTAH IVLGRQGDPH GKFDLPPGVL EHFETATRDP SFFRLHKYMD NIFKEHKDSL PPYTKADLEF SGVSISEVNV VGELETYFED FEYNLINAVD DAEGIPDVDI STYVPRLNHK EFTFKIDIEN GGSPRLATVR IFAWPHKDNN GIEFTFDEGR WNAIELDKFW VSLAGGKNSI ERKSTESSVT VPDVPSIDTL FAKTAAGGDG LSEFASATGL PNRFLLPKGN DKGLEFDLVV AVTDGDADAA VPDLHLNTKY NHYGANGVYP DKRPHGYPLD RRVPDERVFE ELPNFKHIQV KVFNHGEHIH S (661)

Vitellogenin (283 KDa)

MTTSKLFLVL AFVAGALAAP WTADVPRCST ECPVTGSPKL AYQPDKTYAY AYSGKSTVQL KGVDNGDTET EWTAGVDLTW ISPCDMAISF RNTKMDGARG PTAARTLERY PLVVAVVDGR VQHVCAHPED EAWAINLKKG VASAFQNSIP SLSAVSSGIT VTETDVVGKC PTKYEIETEG EKVIVVKEKN HRHCQERYPT PAALPAPWLK APLPIEESKS QCRQEIANGI YTAITCQDKN IVRPAIGIYK YVEASQYSTL RFISESSDTS AISGIPSGEL YIESLLYNHE TMKDPQLAPE LDELMKEICD KTKDTVEAEA GALVAKALHV LRRVPDTVVV ETAQKVRQGH YCSDSARLES IFLDAVAFIH ESGAVKVMVH EIENGRATGG RLALYTAALY LIPRPNIEAV KALTPLFESP RPMPSLLLAA ATMVNHYCRH TPACYEKAPV ARIAEILANR VQTHCSPSAG VEDNEVALAI FKTIGNMGVA TPAVTRAAVH CIEVEGLETS VRVAAAEAFR QANCFRPAVE KLVDIAVRPA FETEVRIASY LAAVRCAEQE HLETIIEKIS KEENTQVRGF VLGHLINIQE STCPAKENLR YLLANVVIPT DFERDFRKFS RNIDVAYHAP AFGMGAGLES NIIYAPGSFV PRAVNLKMKA DVDETHMDIA EIGARFVGIE SIIEELLGPQ GYLRTATFGK IMEDITGFAG EKGFKVMEQL KHTLRTRRSI DSSVIADFFG KLYGKSRSHT HAELFARFMG HEITYADVAE SLKGVTADTL IETFFSFFEN SLEHMKDLNL NTARTAQLSM DYSLPTIQGT PLRLRLAGTA VAGLKMEGNV NIAQILSDLG NSQTAVKFFP GLSVHATGFV GFDWFLARVG IEMQNTISSA TGAAIKIRTT ENKKIEMEYF VPEKMELLNI KAETYLVKAV GKKMTKISPS SMRDVRIQRN SCIGALEPVF GLKVCYDMNI PDVFRANALP LGEPAIAKLY VEKADPSMRG YLVTAAIKNK RGNKVIKMNV EAAGASTPRR AEMTLSYTKE EGSHIVSAKL DSSSIAAGVW TTLTNEQGHK AMETYVNFNY GQIAISRGIK LEAIAREASV GEEFQVNVFS SGTRSFPSES HIVEARFIKK TSGPEFNVDV ICRTKNALAE LFDLNIEVGA DFMKFSPKNL YPARYIPKTR IVLPVNLRKM EINAATAAWK LISYIRAGSQ SGGSREFISA LKLAKGRKDF ISVQATHTIE GTFPQNIIIK NVATAEVGRS SYKAMYDLFY HSEKMGASLE VLQAAGNEKV AHLEAIYELS GSKYCTKFLA EIPGYIQPVK VEAGIEQGAE GRYTLESAIT YGQRTVLEAS GPIMARFSSK IAKLQANIKV RAMASEPYII GANVAFGSKK QMIAMEIKGR SEAVIGLEWK MVRESSEKTT VGIVFVLPAL IENKIDAEIT DGLIHVSFNN LVLPKTSSRR RVKGFADVHI AEKKANVEFS WDADNAPEKK LVLDASLISS SANPGHAEIH GNIVIAGEPY HAKLVLTAAN LVGHMEGENG FKLILTTPSQ KTIVLGASCD IQVAGATTKV ISTVEYKNMK DRKYKYASVI AYEKLGGPFD YAVEAKVTYK QPGTAEIKLG TAVKHHWTPE EHVVAVKVEA DAPILKTPAT IEFSIHNAPN AFVGFCKIER NAPATVFEWN VQITPEGGIE AVEAGVDMKA IIEVLKIVHA AATLQEESYE TYGPHTSQYQ YRFTRPSPTS YTMQMRTPTR TMEGRAKLSP RESGIKFYPN KGKTESKYEI GYKANHEGRW GGHASKLEVR MNHPVLPKPI MAAVQYTVAE ETTKGTIELD IFPEEANKIT GTLETQRISE NAIRAEAFLT SRMLKVNPKA IITAAYAPET VAFDVVFHKT PSAAPIFAIA AKYDKTAAHN AAATFTVKME ERPVFEITAV TEPEEAATCN GIRMRAVAYA ATFGKYNVIS KMCRPAFIEV TAMRPGGAKE YIAKLGLRYP DAAEAGVYVA SGRAGETHGV AVAAVKLASP TMLKVEMAYE PQEAEAIINE MTEEFEKIAA SFTSVVMKVV EFLKQEAAAK GIQFPSSQLV NLLGVAKEEI AEIYRDIVSE AIIFDTEILG DILGSPVVSF ISRVYFGVWS EIIHLQHHIS VSLIQTIERF QEELGSISEI LMEVVMTAAR MAETGEVPAV VFDALEEIKA TKVFRIVRRE VDAILEEYPE EYEAVKHIVH NVVAILKRDV AIVRERLMEI PAVLKVIDYT MYHFHSERAF AAEAEKLVSL MLNELLFVSM ESEGNGVAVR IPLHRPLYSL TQVAQEAVPN PVTMLENLIF AYVDYIPIPV SDAIWAFYNL VPRYITDVLP PYPRTATVVG GSEILTFSGL VVRAPRSPCK VVLAAHGSHR LMMSHPQASA PAQFELKTPA ATVMIKPDFE VVVNGQPLAG SQQTIGNVRI VNTAEYIEVG CPLMKVVVAK AGEAVAVEAS GWVFGRVAGL LGPNNGEIAH DRLMPSGAAA SNPRDLVAAW QEDRQCSTPE VPRAETTVAR LIQCEALLGI RSRCNPVVHP QPFIKMCHAA HKACDAAQAY RTVCSLRGVG EVFPFGC (2586)

Beta-1,3-glucan-binding protein (227 KDa)

MSFELTTPFD AIKTLSLSAQ YSWTTSQKSA TLNITYNDKN FVLSSSLQLS TRASDITFQA RTPFSGFQHT FIEIKYDIDN REELLAFRVN ADDHRYSFVV GGFIEDKLAI FKWNLNSPFS GWTDAKFVAK IDLSSENKNL EISLEKEGDL KAIAVSGKFI GSTLDFNLQT PFRGLNNFNV FGSLNRSKRS LEMRMMNDAG HASLAGNFNS LRFNMKTPFE RAEQISWEIT KTGEGSYNAE WRRNDNYATL TIEKDGKKNS FQVNVKSEFR GWEILALTGR LDQETKQAYL SGAINEQKIT ISGSGSFGSR SNYSMKIETP YDNYRVVDIQ LDYVKRRNTM KIEASSSSSD FHLLWSRSGS GLEAHLIVPN SQQNTEISIN LTPTQGKITI TSRFEPIRDY LQEYHVNLGE TVITADHIIK LNGHEVFKME FERNASEQKG HLEIHIHVAE RHTTIHFHRE GFSKLHFLFK REVPQYGEKH FKVDITGSGA LPEKGALDIV VENTFREPAR TINARVEVDR TGARKKIKLE VTPRQSRLYV FNLEYDADLQ SPRHGDFTLS ITTPARRAAP WQNMSGNWNI ENPNDATITF TMGNVTYNAR GKLTLRESTM VLSSTNPSAE NIFLQWKFER DGNNRDYFLK LGRESKYGML KLKGTITDIA HVDIEGGFKA GPFMPNEFLF TSMWDKSNGV VTGEGTFDYG SYHGSHRLVK FERNAARKSA SFEWSATSNI PQYNSVSVSG NYDFDRKVVI FVLINADGRE SKIDVNIADI NPTRSRNTVM ISIPLLGSTF ERTELTVSHD FSHPNRKSIS AVAKFGRSQS FINAKWNRSD GFETLEGNIE AKSRFLGDFL INVRYDMSNI ADAHAEVDYS RTTTDGDKKE FKLNWTRKST DGHLENEMIF DSNFDTLSHA RAYANAEYGN NFKLLSGLDW NDKKISLTLE VRKNKVSGQL TTPFEGFETI EVDLQYKLTG KDKSVNATYQ RGDRKASLNM ELSMKGKKGG SFNVDVSTPF EVVKNLHIDG QYENKVAQIN YQRNDIQMNF SGKANIKSSK ASFDISFTPP SGQNIRIAAS YDVQDFIAGT GTEEKELASL SLEFEGNSLD FSLHGFRNDD RLYVMIHGSS SFALLKMFHL KLDSELNTEA RDGTFELTFN DFKFNVSNHF ERRENNGYYF RSKIESTLTP LPALIIGLGR EGEERIITIG YGEDKEITFS VKGKNKFLSG FSGKVDVPSL GYEGVEYEVD YSFPGDDHLQ IHVEIDLNEN GQEVEATFFL DSEGIKARLT STVLGDHSLR VRRSVSPDGF YAEAGLDDYS LKLRGGFKNE DTARGVQLEG EVFGNRFLID TLFQSEGKRY SEGKLIIHTP FPGMEKMGGL FTWSNANKKI MAHAELHLPS YITPTITGEI SLDLKKKING YVTLDVAGEE FTLKCNLAGS SISQGYTGSL EFFSPLHAVS HVVLTGDIKM QALSFLDMEV KIDAPFATHD LKLKYQLSAD KVSGEAFLES TRLYNSIQLS LNIEGLTTEN VEVDLTVNDN KINAHYTLAQ STFKFDVTTI IFNKERQFSI EAKYPSLESL EGVVAVTLEG DKHMISGSLN ISNNRIQGTL DLESDLIEGP RKLVLDVSKP SASYEQASFK IIFTSSEPHS FYLDLDLRSG LEATVKIDTP VFPKVTTTLQ VAPAIAGITI ETPKGTHKVQ VSWRQTRRMP SDWIASLELI SPLLPENYLF SVNLGSKHIM AELQTGSIKH TLEARTSVSN YGGDLSLVID TPFENINKVT LDAALNFKNN VKMDITAKFA NTLNSLRFNL DKENRKLISI VESPYIPTGM AEVEAMLTGN TNENMKMKMA LKNAEDTIAG ILNIKIKSSQ NINTNLKIIT PFKGYKKMNF GARYLKDEVT NISVFADKPL KFKADLQFGN TEDVVTTNLI VETPIEHFER IEAEMKIPLY KFAPKVMLTL PHNQHGFTAD HGSDSISQKL SAGVTVNEES YDGYYSLRTK APYELAYGYN LAHLASTRFH LRTDSSFFSV FA (2022)

Enolase (12 KDa)

FTEAMRMGSE VYHHLKAVIK GRFGLDATAV GDEGGFAPNI LNNKDALTLI QESIEKAGYT GKIEIGMDVA ASEFYKGENI YDLDFKTANN DGSQKITGDQ LRDMYMEFCN EFPIVSI (117)

Translationally controlled tumor protein (19 KDa)

MKVFKDMLTG DEMFTDTYKY EEVDDAFYMV IGKNITVTED NIELEGANPS AEEADEGTDS TSQSGVDVVI YMRLQETGFQ VKKDYLAYMK EYLKNVKAKL EGTPEASKLT SIQKPLTDLL KKFKDLQFFT GESMDPDGMV VLMDYKDIDG EERPVLYFPK YGLTEEKL (168)

Farnesoic acid O-methyltransferase short isoform (26 KDa)

RFRDIKGKTL RFQVKAAHDA HIALTSGEEE TDPMLEVFIG GWEGAASAIR FKKADDLAKV DTPDILSEEE YREFWIAFDH DVVRVGKGGE WEPFMSATVP EPFDITHYGY STGWGASGWW QFHSEMHFQT EDCLTYNFIP VYGDTFTFSV ACSNDAHLAL TSGPEETTPM YEVFIGGWEN QHSAIRLSKG EDMIKVDTPD VVCCEEDRKF YVTFKDGHIR VGYQDSDPF (229)

14-3-3 zeta (27 KDa)

MSDKEEQVQR AKLAEQAERY DDMAAAMKQV TETGVELSNE ERNLLSVAYK NVVGARRSSW RVISSIEQKT EGSERKQQMA KEYREKVETE LREICQDVLG LLDMFLIPKA SNPESKVFYL KMKGDYYRYL AEVATGDARA GVVDDSQKSY QEAFDIAKAE MQPTHPIRLG LALNFSVXFY EILNSPDKAC QLAKQAFDDA IAELDTLNEX XYKDXTLIMQ LLRDNLTLWT SNTQGEGDEA NEGDQN (246)

Figure 3 Characterisation of protein fractions by LC/MS/MS.

as a type 1 IgE-mediated allergy; hence, protein fractions A10, A11, B10 and C9 stimulated PBMCs to release Th2-type cytokines IL-4 and IL-10 (Untersmayr & Jensen-Jarolim, 2006; Barnes, 2011). However, the presence of antigens continued to stimulate the production of pro-inflammatory cytokines, which likely maintained homeostasis in the culture medium (Wang *et al.*, 2012; Gálvez, 2014). In general, fraction A10 showed significantly

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, hemocyanin 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 overies. 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.

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