IMPACT OF DIFFERENT PROCESSING METHODS ON IMMUNOGENICITY OF PRAWN

A thesis submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

By

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Dedicated to my beloved parents, wife and family members whose enormous encouragement enabled me to fulfil my ambitions

ABSTRACT

Prawn allergy is one of the most common seafood-borne allergies affecting more than 2% of the global population resulting in significant socio-economic problems to the society and prevention is by avoidance. Not only are some consumers affected by prawn allergy, but also the seafood industry workers (7 to 36%) related to different processing stages. The impact of different processing techniques was assessed to attenuate antigenicity of banana prawn tropomyosin (*Fenneropenaeus merguiensis)*. Frying and boiling at different temperatures, acid treatment using different acids, and storage at -20 °C up to 3 months were investigated. Untreated prawn sample was used as control. Frying significantly increased antigenicity (6 to 8 times) in temperature dependent manner, whereas the trend was fundamentally reversed with boiling. Boiling at 121 °C resulted in the lowest antigenicity (12.99 mg mL^{-1}) among all heat-treated samples, yet higher than the control (5.06 mg mL^{-1}). Freezing had initially very minor impact, although prolonged storage at -20 °C increased antigenicity slightly (2.29 mg mL^{-1}) compared to control. Antigenicity was impacted the most by reduction in pH independent on type of acid, since both acetic and HCl acids significantly reduced antigenicity of tropomyosin by ~90% compared to control. This could be considered as a new approach in processing that may potentially reduce tropomyosin derived antigenicity in prawns and prawn products.

Furthermore, changes in tropomyosin derived antigenicity of banana prawn due to high pressure processing (HPP) at 600 MPa for 5 and 10 min at various temperatures (40, 80, 120 °C) were also investigated. HPP of prawn samples at 40 and 80 °C for 5 min increased tropomyosin derived antigenicity by almost double, whereas HPP at 120 °C for 10 min decreased antigenicity by 65%, detected using enzyme linked immunosorbent assay (ELISA) kit. A significant ($P \leq 0.05$) reduction of tropomyosin antigenicity after pepsin digestion was noticeable in prawns after HPP, but not in control prawn sample. However, further digestion of the control and HPP sample with

pancreatin enzyme decreased antigenicity to ~0 mg mL^{-1}. The combination of HPP and high temperature (120 °C) in the current study can potentially reduce tropomyosin-derived antigenicity in whole prawn muscle, whereas Simulated Intestinal Fluid digestion with pancreatin enzyme may present a new prospective method to produce hypo-antigenic, enzymatically digested prawn products. Moreover, the current research implementing a new methodology using unstained gel, confirmed the actual molecular weight of banana prawn tropomyosin to be 37 kDa.

Finally, the research has explored the immunogenicity (T-helper cell-mediated immune response *in vitro*) of processed banana prawn proteins using human peripheral blood mononuclear cells (PBMCs) and then characterized these allergenic proteins using Liquid chromatography with tandem mass spectrometry (LC/MS/MS). 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 high-performance liquid chromatography (Prep-HPLC), and their antigenicity was analysed using IgG ELISA kit. Out of 39 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) showed significantly ($P < 0.05$) lower pro- and anti-inflammatory cytokine production compared to control (A10). The allergenic fractions were characterized using an LC/MS/MS, which identified 9 proteins. Among these, six proteins (tropomyosin, arginine kinase, hemocyanin, 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 tumor protein and farnesoic acid O-methyltransferase short isoform protein) identified in this study, need further investigation for their immunogenic properties.

DECLARATION

"I, Md Faisal, declare that the PhD thesis by Publication entitled "IMPACT OF DIFFERENT PROCESSING METHODS ON IMMUNOGENICITY OF PRAWN" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
2A	Shellfish allergens and impact of processing: A review	Submitted (Under review)	**International Journal of Food Science and Nutrition; SJR Q1**
2B	A review on methodologies for extraction, identification and quantification of allergenic proteins in prawns	Published	**Food Research International; SJR Q1**
3	Effects of selected processing treatments on antigenicity of banana prawn (Fenneropenaeus merguiensis) tropomyosin	Published	**International Journal of Food Science and Technology; SJR Q1**
4	Effect of simulated digestion on antigenicity of banana prawn (Fenneropenaeus merguiensis) after high pressure processing at different temperatures	Published	**Food Control; SJR Q1**
5	Immunomodulatory properties of selectively processed prawn protein fractions assessed using human peripheral blood mononuclear cells	Published	International Journal of Food Science and Technology; SJR Q1

Declaration by [candidate name]: **Signature:** [redacted] **Date:**

Md Faisal 29-08-2019

ORAL PRESENTATION

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α = alpha

β = beta

Y = gamma

˚C = degree celsius

× g = times gravitational force

µ = micro

µg = microgram

µL= microliter

AK = arginine kinase

BSA = bovine serum albumin

DBPCFC = double-blind placebo-controlled food challenge

ELISA = enzyme linked immunosorbent assay

g = gram

GLM = general linear model

h = hour

HCl= hydrochloric acid

HPLC = high performance liquid chromatography

HPP=high pressure processing

IgE = immunoglobulin E

IgG = immunoglobulin G

IL = interleukin

kDa = kilo dalton

kg = kilogram

L = litre

LC/MS/MS = liquid chromatography with tandem mass spectrometry

mg mL^{-1} = milligram per milliliter

min = minute

mL = milliliter

MLC = myosin light chain

MPa = mega pascal

MW = molecular weight

MWM = molecular weight marker

PAGE = polyacrylamide gel electrophoresis

PBMC = peripheral blood mononuclear cells

PBS = phosphate-buffered saline

pH = hydrogen ion concentration

pI = isoelectric point

Prep HPLC = preparative high performance liquid chromatography

PVDF = polyvinylidene fluoride

rpm = revolution per minute

RT = room temperature

s = second

SAS = statistical analysis software

SCP = sarcoplasmic calcium-binding protein

SDS = sodium dodecyl sulphate

SGF = simulated gastric fluid

SIF = simulated intestinal fluid

SPT = skin prick test

SSF = simulated salivary fluid

TCA = trichloroacetic acid

Th = T-helper

TM = tropomyosin

TMB = 3,3,5,5-tetramethylbenzidine

Treg = T-regulatory

UV = ultra violet

V = volts

v/v = volume per volume

w/v = weight per volume

w/w = weight per weight

Chapter 1

Introduction to the Thesis

1.1. Introduction

Food allergy is one of the main causes of food related hypersensitivity, resulting in severe anaphylaxis, gastrointestinal disorders, angioedema and atopic dermatitis (Rodgers, 2011; Wang & Sampson, 2009). It has been characterized either by IgE mediated (quick response to allergenic proteins or antigens) or non IgE mediated (delayed response) through T cell and Eosinophils (Rahaman et al 2016; Sicherer, 2016). The prevalence of food allergy has become an increasing public health burden in developed and developing countries (Tang & Mullins 2017). It has been reported as an emerging health concerns affecting 4 to 6% of kids and 1 to 3% of adults globally (Sansonetti & Medzhitov, 2009). The rate of sensitivity of food allergy varies depending on the eating habit, age and geographical distribution (Jiménez-Saiz et al 2015). More than 170 foods have been recorded as causing allergic incidences worldwide (Boyce et al., 2010). Among them, seafood has been reported as one of the most prevalent reasons of food allergies resulting in emergency hospitalization of allergic patients (Khora, 2016). The term seafood refer to specific groups of fish (having cartilages or bones) and shellfish (having exoskeleton) known as edible aquatic animals (Ruethers et al., 2018). In recent decades, trends towards balanced nutritional diet have made seafood one of the highest consumed foods worldwide (Wild and Lehrer, 2005; Sicherer et al., 2004). Due to their delicacy and high nutritional properties, seafood are widely consumed by different communities and nations globally (Ravichandran et al., 2009; Lee et al., 2012; Hoffmann, 2000) and play a significant role economically in international trade (Oosterveer 2006).

Shellfish refers to two major phyla, Arthropoda and Mollusca with an outer shell, exoskeleton. Among these edible shellfish are shrimp, prawn, lobster and crab belonging to Crustacea a subphylum of Arthropods, whereas oyster, mussel, clam, squid and snail belong to the phylum Mollusca (Ruethers et al., 2018). The top eight foods (milk, egg, crustacean-shellfish, peanut, tree nut, fish, wheat and soybean) responsible for above 90% food-related allergies are termed “The Big 8”. The

consumption of crustaceans, particularly prawns and shrimps, is the third major cause of food-related anaphylaxis in adults and children, having symptoms of asthma, respiratory disorders, long lasting gastrointestinal and dermatological disorders, laryngospasm, may even be life-threatening anaphylaxis and that persists life-long (Albrecht et al., 2009; Fuller et al., 2006; Steensma, 2003; WHO, 2001). Over 4000 prawn and shrimp species has been recorded to date, of which less than 300 have economic importance globally, and only 100 of these prawn species contribute to the principal share of world's total annual catch (De & Fransen, 2011; Chan, 1998). Banana prawn (Figure 1) is one of the commercially important and popular species due to it delicacy and nutritional properties (more than 20% of protein with essential amino acids and fatty acids in the muscle). Banana Prawn is distributed widely in South-East Asia and Australia (Held et al., 2011; Fatima et al., 2013; Nguyen et al., 2014). According to the UN Fisheries statistics, Banana prawn (*Fenneropenaeus merguiensis*) contributes about 30% of Australian total prawn production (Food and Agricultural Organization, United Nations 2012). In the current study, Banana prawn has been used as a representative sample of prawns to analyse the antigenicity and immunogenicity of unprocessed and processed prawn.

The taxonomic tree of banana prawn (https://www.cabi.org/isc/datasheet/71088) is:

Domain: Eukaryota
Kingdom: Metazoa
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Order: Decapoda
Suborder: Dendrobranchiata
Family: Penaeidae
Genus: *Fenneropenaeus*
Species: *Fenneropenaeus merguiensis*
Common or English name: Banana prawn

Figure 1: Banana Prawn (*Fenneropenaeus merguiensis*)

The prevalence of prawn allergy has been reported in more than 2% of the world's population, creating considerable socio-economic problems for the modern society (Lopata & Lehrer 2009; Lopata et al., 2010; Rahman et al., 2010; Mullins et al., 2007). In addition, an upward trend of allergic incidents caused by prawns has become an alarming issue in the food safety sector (Sicherer & Sampson, 2006).

Prawn allergy has been documented as being common in Western and Asian countries as well as Australia (Chiang et al., 2007). A study from the National Allergy Strategy Pre-budget Submission (2015–16) predicted that the proportion of Australians suffering from various kinds of allergy will rise from 20% to 26.10% by 2050. As prawn consumption rises quickly, the potential for an increased incidence of allergies will also increase at the same time (*National Allergy Strategy 2017*). In addition, in the United States, the incidence of crustacean-allergy is more than double that of peanut allergy, with most adults and children over the age of six emergency hospital visits (Liu et al., 2008; Sicherer et al., 2004). A global survey of 17,280 grownups (age group 20–44 years) in 15 different countries showed that prawn allergy prevalence was 2.3 percent (Woods et al., 2001). However, in another survey involving about fifteen thousand people, crustacean allergies in adults were nearly 5 times higher than in children (Ayuso et al., 2008). Skin Prick Test reported the maximum incidence of crustacean allergy, particularly from prawns, at 10.3 percent in Italian adults (Burney et al., 2010).

Depending on the adverse health effects mentioned above, the scientific community requires to know the presence of allergic proteins in various foods. Despite the high incidence of allergies, medications are only available for accidental consumption of prawns and avoidance is recommended to prevent prawn allergies (Jones et al., 2014).

The main allergen in prawns described in literature is tropomyosin (37 kDa), a heat-resistant water-soluble protein associated with actin filaments having a coiled α-helical structure, responsible for more than 80% prawn allergy-related incidents (Troiano, 2016; Chapman et al., 2006; Shanti et al., 1993). Tropomyosin is stated as a main allergen not only in prawns but also in other crustaceans (lobsters, krills, crabs etc.), invertebrates (house dust mites, cockroaches) and molluscs (octopus, squids, snails, clams, abalone etc.) having 90% similarity in amino acid profiles (Motoyama et al., 2007; Chapman et al., 2006). Furthermore, myosin light chain (Ayuso et al., 2008), arginine kinase (García et al., 2007), sarcoplasmic calcium-binding protein (Shiomi et al., 2008), troponin C (Bauermeister et al., 2011) and triosephosphate isomerase have also been characterized as minor allergens in prawns.

Fresh prawns are processed by various techniques before consumption to improve nutritional qualities and palatability as well as to obtain desired safety and preservation of the food (Lepski & Brockmeyer, 2013; Liu et al., 2010). In general, different processing techniques, frying, boiling, microwave roasting, grilling, steaming, and radiation may result in protein structural changes such as crosslinking between structural elements, unfolding and aggregation, as well as chemical changes (glycosylation and oxidation) of amino acid residues (Lepski & Brockmeyer, 2013; Liu et al., 2010; Yamagata & Low, 1995). Moreover, processing of prawns may abolish existing epitopes or generate new ones (formation of neoallergen) on proteins, unmask or mask epitopes in protein structures and may have direct effect on antigenicity (Taylor 2008; Lehrer et al., 2003).

In order to minimize the risk of allergic reactions to human health, several researchers have attempted to prepare hypoallergenic prawn products using various processing technologies resulting in structural modification of tropomyosin (Kamath et al., 2014; Lasekan & Nayak, 2016; Yuan et al., 2017). Researchers (Lasekan & Nayak, 2016; Rahman et al., 2010; Kamath et al., 2013 & 2014) have stated on the effect of a single heat treatment on different prawn species (king prawns, tiger and vannamei) antigenicity, but the effect of frying or boiling on prawn proteins at various temperatures have not been studied. Furthermore, studies on impact of mild acid treatment or frozen storage on prawn protein antigenicity, are lacking. Increasing demand for fresh, additive-free and prolonged shelf life of food has encouraged the improvement of high-pressure processing (HPP), a process that reduces microbial loads but maintains foods' taste, texture, colour, and dietary quality (Kaur et al., 2016; Barba et al., 2015; Briones et al., 2010). In addition, HPP has become one of the best commercial substitutions to traditional heat processing techniques for preservation of prawns, e.g. black tiger prawn (Kaur et al., 2013). However, the effect of HPP on prawn-antigenicity still remains unclear. Since different processing technologies may change the nutritional and sensory qualities, it is also necessary to study their impact on prawn antigenicity. In addition, gastrointestinal digestion of prawn proteins need further studies to understand changes in antigenicity.

Enzyme linked immunosorbent assay (ELISA) method is a widely accepted method for quick detection and quantification of antigenicity (Faisal et al., 2019), and in terms of immunogenicity studies, human peripheral blood mononuclear cells (PBMCs) have been used (Ramachandran et al., 2012). When human PBMCs are challenged with allergenic proteins, T-helper (Th) cells are stimulated to produce different cytokines (such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ etc) *in vitro*. Analysis of concentration of different cytokine profile determines the sensitivity of the allergenic protein (Wang et al., 2012; Ravkov et al., 2013). Identification and characterization of

allergenic proteins in unprocessed and processed prawns will be performed using Liquid chromatography with tandem mass spectrometry (LC/MS/MS).

1.2. Research aim and objectives

The overall aim of this research was to establish the effects of different processing methods on antigenicity and immunogenicity of allergenic prawn proteins.

The specific objectives were:

i. To investigate the effect of different processing techniques (boiling, frying, freezing, and acidification) on antigenicity of banana prawn tropomyosin.
ii. To investigate the effect of simulated digestion of banana prawn proteins after high pressure processing.
iii. To isolate and characterize the allergenic proteins using HPLC and LC/MS/MS, and investigate the immunomodulatory properties of selectively processed prawn protein fractions assessed using human PBMCs

1.3. Thesis outline

This thesis comprises of six chapters. Chapter 1 presents introduction, research aim, objectives and outline of the thesis. Chapter 2 contains the review of literature on current scientific knowledge relevant to research, the fundamental knowledge regarding allergenic prawn proteins and methodologies for isolation, identification and characterization of allergenic prawn proteins. Chapter 3 presents the effects of selected processing treatments on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) tropomyosin. Chapter 4 focuses on the effect of simulated digestion on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) after high pressure processing at different temperatures. Chapter 5 details the immunomodulatory properties of selectively processed prawn protein fractions assessed using human PBMCs. The overall conclusion is drawn from the study and the scope for future research directions are presented in chapter 6.

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Chapter 2

Review of Literature

Chapter 2 has been subdivided into chapters 2A and 2B. Chapter 2A presents a comprehensive review of current scientific knowledge on allergenic shellfish proteins, and impact of different processing methods on their antigenicity and immunogenicity. Chapter 2B focusing on globally accepted methods on extraction, isolation, identification and quantification of allergenic prawn proteins, published as a Review Article in the peer review journal.

Chapter 2A submitted Review Article

Chapter 2A presents a comprehensive review of current scientific knowledge on allergenic shellfish proteins, and impact of different processing methods on their immunogenicity. This chapter titled "**Shellfish Allergens and Impact of Processing: A Review**" by Md Faisal, Todor Vasiljevic & Osaana N. Donkor, has been submitted in the peer review journal, "International Journal of Food Science and Nutrition".

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GRADUATE RESEARCH CENTRE

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Chapter 2A

SHELLFISH ALLERGENS AND IMPACT OF PROCESSING: A REVIEW

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2A. ABSTRACT

Shellfish allergy is one of the food-borne allergies having upward prevalence all over the world. The current study discusses 19 allergenic proteins identified in different species of Crustacea and Mollusc. In addition, several undetermined proteins having allergenic sensitivity also reported with their molecular weight, need to be identified with their properties. The effect of different processing techniques implemented by several researchers and their effects on IgE reactivity has also been summarized. Several researchers stated that boiling, frying, steaming and baking increase IgE binding of shellfish allergen, whereas other reported significant reduction of IgE reactivity often create confusion to readers, may create scope for in-depth analysis. Moreover, other processing treatments (high-pressure processing, ultra-sound, gamma irradiation, chemical and enzymatic treatment) also reported in significant decrease in IgE reactivity, need to be studied for the identification of optimal range of these stated treatments.

Keywords: Crustacea; Mollusc; Shellfish; Allergy; Processing; IgE reactivity; 3D structure

2A.1. INTRODUCTION

Shellfish are well known globally as food items, these are aquatic animal with an outer shell (exoskeleton) as in oyster and other molluscs and in lobster and other crustaceans. It belongs to two major phyla, Arthropoda and Mollusca. Among the edible shellfish are shrimp, prawn, lobster and crab belonging to Crustacea a subphylum of Arthropods, whereas oyster, mussel, clam, squid and snail belong to Mollusca (Ruethers et al., 2018, Figure 2A.1). Consumption of shellfish has rapidly increased in recent years due to pleasing taste and higher nutritional values. The high intake of these shellfish and products, however, often results in adverse health impacts, termed shellfish allergy (Ravichandran et al., 2009). Shellfish allergy is an IgE mediated allergy, single to multiple symptoms mostly occur due to direct consumption, processing or inhalation (Lopata et al., 2016; Huffaker & McGhee, 2018). Moreover, sensitization due to shellfish allergy is also related with age-dependent, geographical distribution and food habits (Pascal et al., 2018). Around 170 foods are responsible for different food born allergens, among these the top eight foods, “The Big 8”, accountable for over 90% food-related allergies and shellfish is number 3 (Boyce et al., 2010; Lopata et al., 2016). Currently, shellfish allergy directly affects about 2% of people worldwide and the rate is higher among populations with greater consumption of shellfish and products (Pascal et al., 2018). The prevalence of shellfish allergy appears to be more common for Crustacean species due to higher consumption rate than Mollusc (Pascal et al., 2018; Ruethers et al., 2018).

Among the shellfish proteins, tropomyosin is known as a major allergenic protein in different species of Crustacea and Mollusca (Chapman et al., 2006; Motoyama et al., 2007; Rahman et al., 2010). However, actin, arginine kinase, myosin heavy chain, myosin light chain, troponin, sarcoplasmic calcium binding protein, hemocyanin have also been reported as minor allergens (Yang et al., 2010, Rahman et al., 2013; Guillen et al., 2014; Misnan et al., 2015). In addition to these proteins, other proteins associated with muscle, ovaries, shells and other body fragments, need to be investigated for their allergenic properties. To minimize the

antigenicity and immunogenicity of these allergenic potential proteins, several researchers (Lasekan & Nayak, 2016; Yadzir et al., 2015b; Rosmilah et al., 2012; Yu et al., 2011) have implemented different processing methods such as frying, grilling, boiling, UV-radiation, freezing, steaming, ultra-sound, microwave roasting, high pressure processing etc. The impact of these processing techniques on structural modification as well as masking-unmasking of allergenic epitopes in various proteins need to be studied more details. The aim of this review is to summarize the allergenic proteins identified in different species of shellfish and the impact of different processing methods on IgE reactivity.

2A.2. ADVERSE IMPACT OF SHELLFISH ALLERGEN

The allergic symptoms of shellfish allergy appear very quickly, usually within two hours but in some cases, these may be delayed up to six to eight hours, depending on the amount of ingestion or intensity of exposure to allergenic components (Huffaker & McGhee, 2018). Allergic symptoms include respiratory disorders, asthma, dermatological reactions involving skin irritation, itching, redness and burning of eyes, gastrointestinal disorders, rhinitis, sneezing, or in serious case even cardiac arrest which may lead to death (Jeebhay and Lopata, 2012; Lopata et al., 2010; Pascal et al., 2018). The prevalence of shellfish allergy was reported to be significantly higher than fish and peanut allergy causing in most of the emergency hospital visits for both children and adults (Liu et al., 2008; Sicherer et al., 2004). Moonesinghe et al. (2016) reported that shellfish allergy affected 5.5 % of French people, whereas 9.0% in US citizens. The highest incidence (10.30%) of shellfish allergy was stated in Italian grownups, confirmed by skin prick test (Burney et al., 2010). In the last two decades, a large number of surveys were carried out in various countries to analyse the severity of shellfish allergy. In USA more than 2% of the population is directly affected by shellfish allergy, whereas in Europe the percent is about 1.3 (Sicherer et al., 2004; Nwaru et al., 2014). On the other hand, in the Asian countries, Singapore and Philippine have been reported with more

than 5% shellfish allergy in children. In general, over 2% of global population suffer from shellfish allergy and present life-long disorder in 90% of people, (Lopata & Leher, 2009; Lopata et al., 2010; Rahman et al., 2010; Pascal et al., 2018). Moreover, the incidence of prawn allergy is also related with processing. Thomassen et al. (2016) reported more than 36% workers are affected due to handling of shellfish and inhalation of vapor during processing in the occupational environment.

2A.3. ALLERGENIC PROTEINS AND STRUCTURE

Molecular structure and prevalence of allergenic proteins based on the IgE reactivity varies from one protein to another. The three-dimensional structures of these proteins obtained from Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (www.rcsb.org) have been represented in Figure 2A.2, and the amino acid sequences (www.uniprot.org) are shown in Figure 2A.3. A brief description of different allergenic proteins identified in Crustacea and Mollusc responsible for shellfish allergy have been described below.

2A.3.1. Tropomyosin

Tropomyosin, the major allergenic protein with molecular weight between 32 to 38 kDa was first identified by Hoffman (1981) in a prawn species (Gámez et al., 2011; Pascal et al., 2015). It is accountable for above 80% of crustacean-allergic related incidents (Troiano, 2016; Chapman et al., 2006). Moreover, it is not only a crustacean allergen but also reported in Molluscs and other invertebrates (mites, cockroaches) with more than 90% homology in structure (Chapman et al., 2006; Motoyama et al., 2007). It plays an important role in muscle contraction and movement in combination with actin, myosin and troponin (Figure 2A.4).

Tropomyosin is known to be heat and high-pressure stable protein, with 284 amino acids. The reason for this stability is due to the presence of a 7-amino acids repeat sequence motif throughout the length of the protein, with the first and fourth amino acid residue being non-polar in nature, which is characteristic of coiled-coil structures (Lees & Helfman, 1991). The

highly conserved coil double α-helix forms a chain-like structure due to “head to tail” polymerization which binds firmly to the surface of actin through gestalt-binding (Pedrosa et al., 2015; Oguchi et al., 2011; Holmes & Lehman, 2008). This homodimer-coiled structure provides extra strength to maintain the secondary and tertiary structural constancy of tropomyosin during various processing (Kamath et al., 2013 & 2014).

2A.3.2. Arginine kinase

Arginine kinase (38-42 kDa) is the second identified allergen in different species of prawn, crab, lobster, crayfish and octopus (Kamath et al., 2013 & 2014; Chen et al., 2013; Bauermeister et al., 2011; Shen et al., 2012) containing 356 amino acids in the sequence. The prevalence of allergic reaction due to sensitization of arginine kinase has been reported in 67% adolescents, whereas 21 to 50% adult population have prawn allergy (Kamath et al., 2014; Yang et al., 2010). This protein is distributed in shellfish muscles in higher concentration with monomeric or dimeric structure and binds to actin protein (Uda et al., 2006; Rahman et al., 2011). Arginine kinase plays an important role as enzyme in energy metabolism, catalyzation and transportation of phosphate to regulate the concentration of ADP and ATP (Rahman et al., 2011). It is a thermo labile protein showing significant alteration in structure after heat and pH treatment (Kamath et al., 2014).

2A.3.3. Myosin

2A.3.3.1. Myosin light chain

Myosin light chain belongs to the family myosin macro-molecule complex, identified in *Litopenaeus vannamei* shrimp (Ayuso et al., 2008) and in other species such as American lobster, North Sea shrimp, crayfish and black tiger prawn (Zhang et al., 2015; Kamath et al., 2014; Bauermeister et al., 2011), as a minor allergen. The myosin macro-molecule complex consists of four myosin light chains (~18-20 kDa) and two myosin heavy chains (~200 kDa), actively involved in muscle contraction and movement through ATP hydrolyzation. Myosin light

chain with 177 amino acids, is subdivided into two groups termed regulatory light chain and essential light chain (Ruethers et al., 2018). Sensitization due to myosin light chain, has been reported to be higher in adults than in children ranging from 19 to 55% in patients with prawn allergy (Bauermeister et al., 2011; Pascal et al., 2015). Myosin light chain has shown the pH and heat stability as reported by Zhang et al. (2015).

2A.3.3.2. Myosin heavy chain

Myosin heavy chain (219 kDa) has been reported in different species of prawns (*Carangon carangon, Fenneropenaeus merguiensis*) and snails (*Helix aspersa, Theba pisana, Otala lacteal*) as a minor allergen (Martins et al., 2005; Khanaruksombat et al., 2014; Rahman et al., 2013; Pedrosa et al., 2015). The prevalence of this allergenic protein has been reported to be about 11%, however increased sensitivity in adolescents or adults has not been studied. Moreover, thermal and pH sensitivity of the protein need more studies to reveal understand of its structural modifications.

2A.3.4. Sarcoplasmic calcium-binding protein

Sarcoplasmic calcium-binding protein has been recognized as the fourth allergenic protein in different species of shrimp (*Litopenaeus vannamei, Crangon crangon*), prawn (*Penaeus monodon*), crab (*Scylla paramamosain*) and crayfish (*Procambarus clarkia*) (Shiomi et al., 2008; Ayuso et al., 2009; Bauermeister et al., 2011; Chen et al., 2013; Hu et al., 2017). The molecular weight of this minor allergen is between 20 and 24 kDa with 192 amino acid sequence (Ayuso et al., 2009; White et al., 2011). The helix-loop-helix structure of this protein consist of four domains with α and β subunits bonded to calcium ion (Ruethers et al., 2018). Sarcoplasmic calcium-binding protein has three allergenic isoforms which show over 50% sensitization in allergic patients and higher prevalence in children than adults (Ayuso et al., 2010; Kamath et al., 2017). It has been reported as a thermostable protein as well as resistant to acid and alkali (Chen et al., 2013).

2A.3.5. Troponin

Troponin is one of the minor shellfish allergens identified in several species, such as North-sea shrimp (*Crangon crangon*), Caridean shrimp (*Pandalus borealis*), Black tiger prawn (*Penaeus monodon)* and American lobster (*Homarus americanus*) with molecular weight of 20 kDa (Chao et al., 2010; Bauermeister et al., 2011; Rahman et al., 2013; Kalyanasundaram and Santiago, 2015). Troponin is formed in combination with three sub-fragments named Troponin C, I and T, of which Troponin C shows IgE reactivity (12 to 30%) in shellfish allergenic patients (Pascal et al., 2015 & 2018; Bauermeister et al., 2011). The rate of allergic prevalence in children and adults needs further investigation. The protein has 139 amino acid sequence and plays important role by regulating calcium ion concentration in conjunction with myosin light chain, actin, tropomyosin, calmodulin and parvalbumin in striated muscle (Tanaka et al., 2013; Ruethers et al., 2018; Pascal et al., 2018). The role of heat and pH treatment on the molecular structure of troponin is still not clear and needs further study.

2A.3.6. Triose phosphate isomerase

Triose phosphate isomerase is one of the minor allergens that has been reported in *Carangon carangon*, *Penaeus monodon, Octopus fangsiao and Archaeopotamobius sibiriensis* by different researchers (Bauermeister et al., 2011; Kamath et al., 2014; Lopata et al., 2016; Yang et al., 2017). The allergenic protein with dimeric structure is involved in glucose metabolism and regulate energy production (Ruethers et al., 2018). The protein contains 266 amino acid. The IgE sensitization to Triose phosphate isomerase (26-29 kDa) is reported to be lower in prevalence, ranging from 3 to 23% (Bauermeister et al., 2011; Kamath et al., 2014). Whether susceptibility of this protein is high in adults or children, is not clearly defined. To better understand the structural changes of primary and secondary structures of this protein due to heat and pH sensitivities, further investigations are needed.

2A.3.7. Hemocyanin

Hemocyanin has been detected recently as a minor allergen in different species of prawns (*Macrobrachium rosenbergii, Penaeus monodon, Fenneropenaeus merguiensis)* and crab (*Eriocheir sinensis*) (Khanaruksombat et al., 2014; Giuffrida et al., 2014; Zhang et al., 2016). Hemocyanin consist of hexamer multi-subunit structure, which transports oxygen and reacts with copper ion to form a blue-green complex (Ruethers et al., 2018). Molecular weight (74 to 75 kDa) of Hemocyanin contain 661 amino acids sequence (Lopata et al., 2016). The clinical implications in terms of allergic reaction and structural modifications due to thermal and pH changes, need further studies.

2A.3.8. Paramyosin

Paramyosin is a minor allergen identified only in mollusc species, such as Pacific oyster (*Crassostrea gigas*) and Disc abalone (*Haliotis discus discus*), still not in crustacean species (Suzuki et al., 2011 & 2014; Nugraha et al., 2018). It acts a significant role in formation of feet and mantle muscles of Turban shell and squid respectively, as it is one of the major components of white and red abductor muscle (Kantha et al., 1990). Paramyosin is pH and heat sensitive as well as water insoluble. The molecular weight is 97-100 kDa, having α-helix coil-coiled structure resembling tropomyosin, hence the term tropomyosin-A or water insoluble-tropomyosin (Ruethers et al., 2018). Due to its resemblance to tropomyosin, the characteristics of this protein needs further investigation.

2A.3.9. Other shellfish allergens

A number of proteins has been identified in different shellfish with IgE sensitivity to allergic patients. Among these allergens are Fructose 1,6 Bisphosphate aldolase (39-43 kDa), Titin fragment (70 kDa) in Black tiger prawn (*Penaeus monodon*), Actin (41 kDa), Glyceraldehyde-3-phosphate Dehydrogenase (37 kDa) in North-Sea prawn (*Crangon crangon*), Enolase (47 kDa), Glyceraldehyde-3-phosphate dehydrogenase (30 to 35 kDa), Vitellogenin (283 kDa),

14-3-3 Zeta protein (27 kDa) in Banana prawn (*Fenneropenaeus merguiensis*), α-Actinnin (100 kDa) and smooth endoplasmic reticulum Ca^{2+} ATPase (113 kDa) in Snow crab (*Chionoecetes opilio*) (Rahman et al., 2011 & 2013; Kamath et al., 2014; Khanaruksombat et al., 2014). Furthermore, Nugraha et al. (2018) reported 23 allergenic proteins in Pacific oyster (*Crassostrea gigas*) of which 12 newly identified proteins have been reported to have IgE sensitivity to allergic patients. The prevalence of these allergenic proteins, sensitivity to IgE binding and structural modification towards various processing treatments have not been studied comprehensively.

2A.3.10. Unidentified shellfish allergens

In addition to the above-mentioned shellfish allergens, various proteins have also been detected as allergens from different species of Crustacea and Mollusc based on the IgE reactivity with allergic patient's serum. Proteins with molecular weights 49 & 75 kDa in Black tiger prawn (*Penaeus monodon*); 36, 42 & 49 kDa in King prawn (*Penaeus latisulcatus*); 40, 71,& 82 kDa in common whelk (*Buccinumun datum*) and 50, 65, 75, 95 & 150 kDa in Red crab (*Charybdis feriatus*) have been reported to stimulate hypersensitivity (Sahabudin et al., 2011; Misnan et al., 2012; Kamath et al., 2014; Ruethers et al., 2018). Furthermore, Asturias et al. (2002) reported that proteins in brown garden snail with similar molecular weights show allergenic properties (*Helix aspersa*). For better understanding and greater precaution from these undetermined allergenic components, identification and characterization of these allergenic proteins are indispensable. This will contribute to the advancement of knowledge in this sector.

2A.4. CHANGES IN IMMUNOGENICITY DUE TO PROCESSING

Shellfish are processed using various processing techniques to increase palatability, prolong preservation and ensure the safety of the consumer (Lepski & Brockmeyer, 2013). Processing techniques, such as boiling, frying, grilling, steaming, baking, microwave roasting, ultra-sound,

radiation, high-pressure treatment have brought about changes in proteins and these have been studied (Lasekan & Nayak, 2016; Kamath et al., 2014; Hu & Xie, 2013), their impacts on immunogenicity (increase or decrease in allergic properties) have been summarized in Table 2A.1.

2A.5. CONTROL OF SHELLFISH ALLERGY

The diversity of Mollusc and Crustacean species creates difficulties for proper identification of allergens in foods, resulting in increasing health concerns worldwide (Lopata et al., 2016). Except emergency medication due to accidental exposure from shellfish, avoidance of shellfish is the only possible way for preventing the allergy (Pedrosa et al., 2015). Besides, proper labelling of foods regarding the presence of shellfish components may help prevent the exposure (Ruethers et al., 2018). Moreover, fast identification and analysis of allergenic proteins may create more awareness and thus control exposure. In addition, methodologies required for extraction, isolation, and analysis of shellfish allergens need to be reviewed and improved. To minimize the effect of shellfish allergy, studies have reported different processing techniques (Faisal et al., 2019; Pascal et al., 2018; Pedrosa et al., 2015). However, a complete removal of allergenic components in shellfish is yet to be achieved or prevented through drug administration (orally, sublingual, intradermal or subcutaneously) during and after the condition, will need further studies.

2A.6. CONCLUSION

Shellfish allergy is one of the IgE mediated food allergies having adverse impact on human health. Allergic proteins identified in different species of Mollusc and Crustacea by researchers has been summarized for better understanding regarding their properties and molecular structures. To date, among the allergenic proteins' tropomyosin, arginine kinase, myosin light chain and sarcoplasmic calcium-binding proteins has been characterized, as well as their

antigenicity and immunogenicity been studied in more details. Whereas, other allergenic proteins and undetermined allergenic proteins need to be identified and characterized in detail with their immunological properties. In addition, the impact of different processing treatments on allergenic proteins have not been studied in-depth and needs further investigation for advancement in knowledge on structural modifications of allergenic proteins.

2A.7. CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

2A.8. AUTHOR CONTRIBUTIONS

Md Faisal: Concept development, drafted and prepared the manuscript. Dr Osaana N. Donkor and Professor Todor Vasiljevic: Concept development, revising and editing of the manuscript.

2A.9. REFERENCES

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LEGENDS OF FIGURES

Table 2A.1: Effect of processing on immunogenicity of shellfish

Processing methods	Shellfish group	Species	Effect on immunogenicity	Reference
Boiling	Crustacea	Black tiger prawn (*Penaeus monodon*) Kuruma prawns (*Marsupenaeus japonicus*) Banana prawn (*Fenneropenaeus merguiensis*) King prawn (*Melicertus latisulcatus*) Vannamei prawn (*Litopenaeus vannamei*) Green tiger prawn (*Penaeus semisulcatus*)	Immunogenicity increased	Carnés et al., 2007 Yadzir et al., 2010 & 2015b Kamath et al., 2013 & 2014 Usui et al., 2015 Abramovitch et al., 2017
		Vannamei prawn (*Litopenaeus vannamei*)	Immunogenicity decreased	Liu et al., 2010
		Black tiger prawn (*Penaeus monodon*) Kuruma prawns (*Marsupenaeus japonicus*)	No effect on immunogenicity	Usui et al., 2013 Lasekan & Nayak, 2016
		American lobster (*Homarus* sp) Spiny lobster (*Palinurus* sp) Rock lobster (*Jasus edwardsii*) Yabby lobster (*Cherax destructor*)	Immunogenicity increased	Carnés et al., 2007 Kamath et al., 2013
		Blue swimmer crab (*Portunus pelagicus*) Mud crab (*Scylla serrata*) Sand crab (*Ovalipes australiensis*) Snow crab (*Chionocetes opilio*)	Immunogenicity increased	Abramovitch et al., 2017 Kamath et al., 2013
		White squid (*Loligo edulis*) Calamari squid (*Sepioteuthis lessoniana*)	Immunogenicity increased	Yadzir et al., 2010
	Mollusc	Carpet clam (*Paphia textile*) Asian green mussel (*Perna viridis*) Blue mussel (*Mytilus edulis*) Scallop (*Pecten fumatus*) Oyster (*Crassostrea gigas*) Sea snail (*Turbo cornutus*) Octopus (*Octopus vulgaris*)	Immunogenicity increased	Kamath et al., 2013 Yadzir et al., 2015b

		Malaysian cockle (*Anadara granosa*)	Immunogenicity decreased	Yadzir et al., 2015a
		Common whelk (*Buccinum undatum*)	No effect on immunogenicity	Lee & Park, 2004
Frying	Crustacea	Black tiger prawn (*Penaeus monodon*)	No effect on immunogenicity	Lasekan & Nayak, 2016
	Mollusc	Malaysian cockle (*Anadara granosa*)	Immunogenicity decreased	Yadzir et al., 2015a
Baking	Crustacea	Black tiger prawn (*Penaeus monodon*)	No effect on immunogenicity	Lasekan & Nayak, 2016
Steaming	Crustacea	Kuruma prawns (*Marsupenaeus japonicus*)	Immunogenicity increased	Usui et al., 2015
		Black tiger prawn (*Penaeus monodon*)	No effect on immunogenicity	Lasekan & Nayak, 2016
		Mud crab (*Scylla serrata*)	Immunogenicity decreased	Yu et al., 2011
	Mollusc	Carpet clam (*Paphia textile*)	Immunogenicity increased	Yadzir et al., 2015b
Roasting	Crustacea	Kuruma prawns (*Marsupenaeus japonicus*)	Immunogenicity increased	Usui et al., 2015
	Mollusc	Malaysian cockle (*Anadara granosa*)	Immunogenicity decreased	Yadzir et al., 2015a
		Carpet clam (*Paphia textile*)	Immunogenicity increased	Yadzir et al., 2015b
High pressure processing	Crustacea	Vannamei prawn (*Litopenaeus vannamei*)	Immunogenicity decreased	Long et al., 2015
	Mollusc	Japanese flying squid (*Todarodes pacificus*)		Jin et al., 2015
Ultra-sound treatment	Crustacea	Vannamei prawn (*Litopenaeus vannamei*)	Immunogenicity decreased	Li et al., 2006
		Mud crab (*Scylla paramamosain*)		Yu et al., 2011
Gamma irradiation	Crustacea	Brown shrimp (*Penaeus aztecus*)	Immunogenicity decreased	Byun et al., 2000
		Vannamei prawn (*Litopenaeus vannamei*)		Zhenxing et al., 2007
Protease enzymes digestion	Crustacea	Sakura shrimp (*Sergia lucens*)	Immunogenicity completely loss	Shimakura et al., 2005
		Japanese spiny lobster (*Panulirus japonicus*)		
		Tanner crab (*Chionoecetes opilio*) Horsehair crab (*Erimacrus isenbeckii*)		
		Mud crab (*Scylla paramamosain*)	Immunogenicity decreased	Yu et al., 2011
Malondialdehyde treatment	Crustacea	Greasy-back prawn (*Metapenaeus ensis*)	Immunogenicity decreased	Song et al., 2015
Glucose treatment	Mollusc	Yesso scallop (*Patinopecten yessoensis*)	Immunogenicity increased	Nakamura et al., 2005
Ribose treatment				
Maltose treatment				
Maltotrios treatment			No effect on immunogenicity	

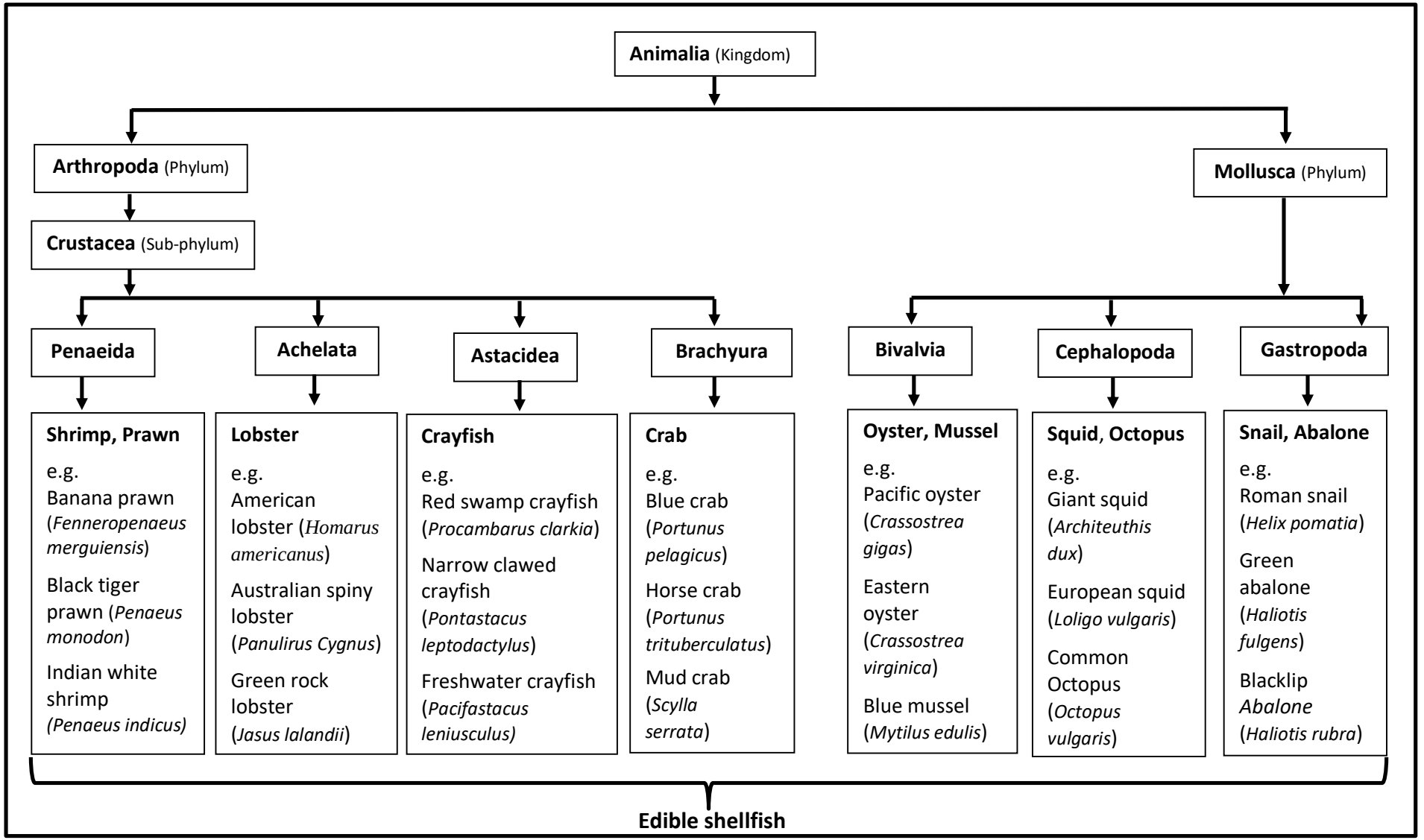

Figure 2A.1: Schematic classification of commonly consumed shellfish.

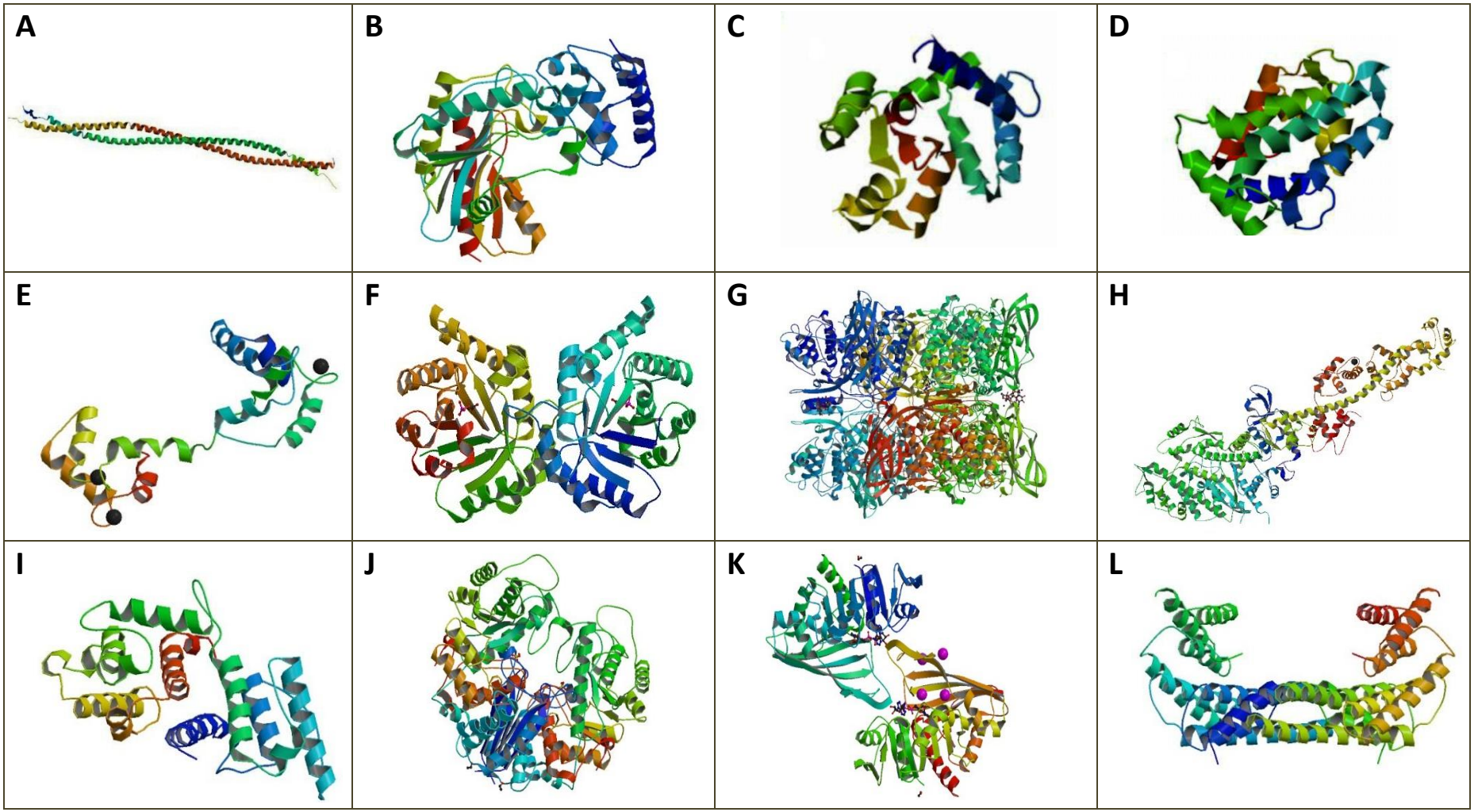

Figure 2A.2: Three-dimensional structure of shellfish allergenic proteins: A- Tropomyosin; B- Arginine Kinase; C- Myosin light chain; D- Sarcoplasmic calcium-binding protein; E- Troponin; F- Triose phosphate isomerase; G- Hemocyanin; H- Myosin heavy chain; I- Actin; J- Enolase; K- Glyceraldehyde-3-phosphate dehydrogenase; L- 14-3-3 Zeta protein.

Tropomyosin (32 kDa)

```
MDAIKKKMQA MKLEKDNAMD RADTLEQQNK EANNRAEKSE EEVHNLQKRM QQLENDLDQV QESLLKANIQ LVEKDKALSN AEGEVAALNR RIQLLEEDLE RSEERLNTAT TKLAEASQAA  120
DESERMRKVL ENRSLSDEER MDALENQLKE ARFLAEEADR KYDEVARKLA MVEADLERAE ERAETGESKI VELEEELRVV GNNLKSLEVS EEKANQREEA YKEQIKTLTN KLKAAEARAE  240
FAERSVQKLQ KEVDRLEDEL VNEKEKYKSI TDELDQTFSE LSGY (284)
```

Arginine kinase (40 kDa)

```
MADAAVIEKL EAGFKKLEAA TDCKSLLKKY LTKEVFDKLK DKKTSLGATL LDVIQSGVEN LDSGVGIYAP DAEAYTLFAP LFDPIIEDYH VGFKQTDKHP NKDFGDVNSF VNVDPEGKFV  120
ISTRVRCGRS MQGYPFNPCL TESQYKEMEA KVSSTLSSLE GELKGTYYPL TGMSKEVQQK LIDDHFLFKE GDRFLQAANA CRYWPAGRGI YHNDNKTFLV WVNEEDHLRI ISMQMGGDLG  240
QVFRRLTSAV NEIEKRIPFS HHDRLGFLTF CPTNLGTTVR ASVHIKLPKL AANREKLEEV AGKYNLQVRG TRGEHTEAEG GIYDISNKRR MGLTEFQAVK EMQDGILELI KIEKEM (356)
```

Myosin light chain (19 kDa)

```
MSRKSGSRSS SKRSKKSGGG SNVFDMFTQR QVAEFKEGFQ LMDRDKDGVI GKTDLRGTFD EIGRIATDQE LDEMLADAPA PINFTMLLNM FAERQTGESD DDDVVAKAFL AFADEEGNID  120
CDTFRHALMT WGDKFSSQEA DDALDQMDID DGGKIDVQGV IQMLTAGGGD DAAAEEA (177)
```

Sarcoplasmic calcium-binding protein (21 kDa)

```
AYSWDNRVKY VVRYMYDIDD DGFLDKNDFE CLAVRNTLIE GRGEFSAADY ANNQKIMRNL WNEIAELADF NKDGEVTVDE FKMAVQKHCQ GKKYSEFPGA FKVFIANQFK AIDVNGDGKV  120
GLDEYRLDCI TRSAFAEVKE IDDAYDKLTT EDDRKAGGLT LERYQDLYAQ FISNPNESCS ACFLFGPLKV VQ (192)
```

Troponin (15 kDa)

```
LRKAFNSFDT EGAGSINAET VGVILRMMGV KISEKNLQEV IAETDEDGSG MLEFEEFAEL AAKFLIEEDE EALKAELREA FRIYDKDCQG YITTDVLKEI LVELDPKLTP TDLDGIIEEV  120
DEDGSGTLDF DEFMEMMSG (139)
```

Triose phosphate isomerase (29 kDa)

```
MSSPRKFFVI GNWKMNVDKN RINGIVKMMN NAALDANTEA VVGCPSCYLS YAREHLTHNI GIAAQNCYKV ARGNFSGEIS PEMIKDCGCD WVILGHPERR TVFNEPDSFI AEKVAHAQEA  120
GMKIIACLCE TTDDRQKGRT EEVLFKQLTS LAAAITDWSR VVLAFEALWA SNTGVFATNQ QVQDALGLVR NWLRNNVSDK VADTTRLIYA GSVSSDNCRE MASLENLDGF LVGGASLKPD  240
IVDIINSRRS HVSELLTSLR IEEQQG (266)
```

Hemocyanin (19 kDa)

```
MRVLVVLGLI AAAAFQVVSA DVQKQKDVLY LLHRIYGDIQ DADLLATANS FDPAGGSYSD GGAAVQRLLK GLNDGRLLEQ KHWFSLFNTR HRNEALLLFD VLIHSSDWAT FVGNAAFFRQ  120
KINEGEFVYA LYVAVIHSPL TEDVVLPPLY EITPHLFTNS EVIEAAYRAK QKQTPGKFES TFTGTKKNPE QRVAYFGEDI GLNTHHVTWH MEFPFWWDDE YGHHLDRKGE NFFWVHHQLT  240
VRFDAERLSN YLDPVGELHW YKPIVDGFAP HTTYKYGGQF PARPDNVKFE DVDDVARIRD MVIVESRIRD AIAHGYIIDS HGKQIDISNE KGIDILGDVI ESSLYSPNVQ YYGALHNTAH  360
IVLGRQGDPH GKFDLPPGVL EHFETATRDP SFFRLHKYMD NIFKEHKDSL PPYTKADLEF SGVSISEVNV VGELETYFED FEYNLINAVD DAEGIPDVDI STYVPRLNHK EFTFKIDIEN  480
GGSPRLATVR IFAWPHKDNN GIEFTFDEGR WNAIELDKFW VSLAGGKNSI ERKSTESSVT VPDVPSIDTL FAKTAAGGDG LSEFASATGL PNRFLLPKGN DKGLEFDLVV AVTDGDADAA  600
VPDLHLNTKY NHYGANGVYP DKRPHGYPLD RRVPDERVFE ELPNFKHIQV KVFNHGEHIH S (661)
```

Paramyosin (97 kDa)

```
MMDYDSDIQT KVVRVNRTYN VYRGTSPSTQ NRLETVVRKE SRVINTEKQL FEAEEQRFLA RIRELEDALD GERDTRLRFE KQCAELTFQL DQMSDRLEEA SGMSSSQLDI NRKRESELVK  120
LRKDLELANA SYESTEASMR KRHQDALNDL ADQLEYMTKS KGRAEKERGQ LVAELDAFQA ANESLQKAKV RSTNKVEKEK SQLVIEIDLL QTDNESLSKS KASADAKIDS LEGSVSRLRV  240
QVDDLTRQNN DLNGLKARLT QENFDLQHQV QELDAANAGL AKAKSQLQVQ VDDLKRNLDD ESRQRQNLQV QLSALQSDYD NLNARYEEES ESASNLRAQL SRVNAEFAAL KTRYDKELLA  360
KTEEMEEMRR KLSVRIQELE DLLEQTRLRC TNLERTKSKL TAELKEVTIE LENCQIIIQD LTKRNRQLEN ENAALQKRVD ELSAENAQLR NNKHALEQEV YRLKVANAEL AEKNANLERE  480
NKNLSDQLRE ANQALKDANR LVSELTAIKA ALEAERDNLA AALRDTEDAL RDAETKLAAA QAALNQLRAE MEQRLREKDE EIDSIRKSSA RAIEELQRTL VEVETRYKTE INRIKKKYET  600
DIRELEGALD NANRANAEYL KQIKSLQHRV KDLESALEET QRQLDDCRSQ LSVSERKRIS LQQELDDCRS LLDAAERARK NAETELGETS QRLTEVQLQI TVLTNDKRRL EGDIAAMQSD  720
```

LDDALNAQRA AEERADRLAA EVNRLAGELH QEQENYKNAE SLRKQLEVEI REITVRLEEA EAFATREGKR QIAKLQARED VANLTMNKYR KAQTLIEEAE HRADNAEKNL AAVRRTRSMS VTREVTKVIK I (851)	840
Myosin heavy chain (219 kDa)	
MPGHVKKSTG PDPDPTEYLF ISREQKMKDQ TKPYDPKKSY WCPDPNEGFV ECEFQAPKGD KLVTVKLPSG ETKDFKKEQV GQVNPPKYEK CEDVSNLTFL NDPSVFYVLK SRYQAKLIYT	120
YSGLFCIAVN PYKRYPIYTN RAVKIYIGKR RNEVPPHLFA ICDGAYQNMN QERQNQSMLI TGESGAGKTE NTKKVLSYFA NVGASEKKEG ESKQNLEDQI IQTNPILEAY GNAKTTRNDN	240
SSRFGKFIRV HFAPNGKLSG ADIEVYLLEK ARVISQSPAE RGYHIFYQLM CDQIDYMKKI CCLSDDIYDY HYEAQGKVTV PSIDDKEDMQ FTHDAFDILN FSHEERDDCY KVTASVMHHG	360
NMKFKQRGRE EQAEADGTEA GEIVAKLLGV DAEELYRNFC KPKIKVGAEF VTKGMNVDQV NYNVGAMAKG LFSRVFSWLV RKCNMTLETG QTRAMFIGVL DIAGFEIFDF NGFEQICINF	480
CNEKLQQFFN HHMFVLEQEE YAKEGIVWQF VDFGMDLQAC IELFEKKMGL LSILEEESMF PKATDKTFEE KLNNNHLGKS RCFIKPKPPK AGQPENHFAI VHYAGTVSYN LTGWLEKNKD	600
PLNDTVVDQL KKASNALTVE IFADHPGQSG DGGGKGKGGK QQTGFKTVSS GYKDQLANLM KTLNATHPHF IRCIVPNEFK KPGEVDAGLI MHQLTCNGVL EGIRICQKGF PNRMPYPDFK	720
QRYNILAAKE MLEAKDDKKA ATACFERAGL DPELYRTGNT KVFFRAGVLG TLEEIRDDRI MKLVSWLQAW IRGWASRKYY SKMQKQRTAL IVMQRNIRKF KIMRSWLWYE LWIKLKPRLK	840
ATRGEEELEK LEATAVKAEE EFEKVLKVRE ELEAQNALLL AEKNELLAAV ESSKGGVSEY LDKQAKLLAQ KAELEGQLNE TLERLRKEED ARNQISNGKK KCEQEVSNLK KELEELELSV	960
QQGEQDKQTK DQQLTNLNEE ISHQEELISK VNKEKKHLQE CNQKTAEDLQ GIEDKCNNLN KVKTKLESGL DELEDTLERE KKLRAEVEKS KRKVEGDLRL TQEAVSDLER NLKELEVAAE	1080
RKEKEIAAMT AKIDDEQALV YRDQRQIKEL QARLEELEEE VEHERQARSK AEKAKNLLSR ELSELGERLD EAGGATAAQI EINKKREGEL AKVRRDIEES NLQHEAALAT LRKKHNDAVA	1200
EMSEQVDYLN KMKARTEKDK EAMKRDADDA KASMDTLARD KTTAEKTTKQ LQHQYGEICA KLDEVNRTLS DFDATKKKLA CENADLVRQL EEAENQVSQL SRVKLSLTNQ LDDTRKMCDE	1320
ESRGRATLLG KFRNLEHDIQ ALRDQLDEES DAKGDVLRML SKANAEALMW RSKYESEGVA RAEELEAARM KLAARLEEAE MQIESLNVRN LHLEKTKMRA AAELDDLHAS AERAQALASA	1440
AEKKQKNFDK IISEWKLKVD DLAAEVDASQ KECRNYSTEH FRLKAANDEN IEQLDSIRRE NKNLSDEIRD LMDQLGEGGR AYHEVQKNAR RLELEKEELQ AALEEAEAAL EQEENKVLRT	1560
QLELSQIRQE IDRRLQEKEE EFDNTRKCHQ RAIDSMQASL EVEAKGKAEA LRIKKKLESD INELEIALDH ANKANSDLHK HLRKVHDEIK DAETRVKEEQ RHASEFREQY GIAERRFNAL	1680
HGELEESRTL LEQSDRGRRH AETELNDARE QINNFTNQNT ALTASKRKLE GEMSTLQADL EEMLNEARNS EEKAKKAMLD AARLADELRS EQEHAQAQEK MRKALEITVK DLQTRLDESE	1800
SAAMKAGKKA VSNMEARIRD LESALDEEVR RHADSQKNLR KCERRIKELA FQTEEDKKNH DRMQDLVDKL QQKIKTYKRQ IEEAEEIAAL NLAKFRKTQQ ELEESEGVTI IHY (1913)	
Actin (41 kDa)	
MCDDDVSPLV VDNGSGMVKA GFAGDDAPRA VFPSIVGRPR HQGVMVGMGQ KDAYVGDEAQ SKRGILTLKY PIEHGIITNW DDMEKIWHHS FYNELRVAPE ESPVLLTEAP LNPKANREKM	120
TQIMFETFNT PAMYVAIQAV LSLYASGRTT GIVLDTGDGV THTVPIYEGY ALPHAILRLD LAGRDLTAYL MKIMTERGYS FTTTAEREIV RDIKEKLCYV ALDFESEMNV AAASSSLEKS	240
YELPDGQVIT IGNERFRCPE SLFQPSFLGM ESVGIHETVY NSIMRCDIDI RKDLFANNVL SGGTTMYPGI ADRMQKEITA LAPSTIKIKI IAPPERKYSV WIGGSILASL STFQSMWITK	360
EEYDESGPGI VHRKCF (376)	
Enolase (12 kDa)	
FTEAMRMGSE VYHHLKAVIK GRFGLDATAV GDEGGFAPNI LNNKDALTLI QESIEKAGYT GKIEIGMDVA ASEFYKGENI YDLDFKTANN DGSQKITGDQ LRDMYMEFCN EFPIVSI (117)	120
Glyceraldehyde-3-phosphate dehydrogenase (35 kDa)	
MSKIGINGFG RIGRLVLRAA LMKGAEVVAV NDPFIALDYM VYMFKYDSTH GVFKGEVKAE DGALVVNGHK IQVFNEMKPE NIPWSKAGAE YIVESTGVFT TIEKASAHFQ GGAKKVVISA	120
PSADAPMFVC GVNLEKYSKD MKVVSNASCT TNCLAPVAKV LHENFEIVEG LMTTVHAVTA TQKTVDGPSA KDWRGGRGAA QNIIPSSTGA AKAVGKVIPE LNGKLTGMAF RVPTPDVSVV	240
DLTVRLGKEC SYDDIKAAMK SAAEGPLKGV LGYTEDDVVS CDFTGDERSS IFDAKAGIQL SKTFVKVVSW YDNEFGYSHR VIDLLKHMQK ADA (333)	
14-3-3 Zeta (27 kDa)	
MSDKEEQVQR AKLAEQAERY DDMAAAMKQV TETGVELSNE ERNLLSVAYK NVVGARRSSW RVISSIEQKT EGSERKQQMA KEYREKVETE LREICQDVLG LLDMFLIPKA SNPESKVFYL	120
KMKGDYYRYL AEVATGDARA GVVDDSQKSY QEAFDIAKAE MQPTHPIRLG LALNFSVXFY EILNSPDKAC QLAKQAFDDA IAELDTLNEX XYKDXTLIMQ LLRDNLTLWT SNTQGEGDEA	240
NEGDQN (246)	

Figure 2A.3: Amino acid sequence of shellfish allergenic proteins.

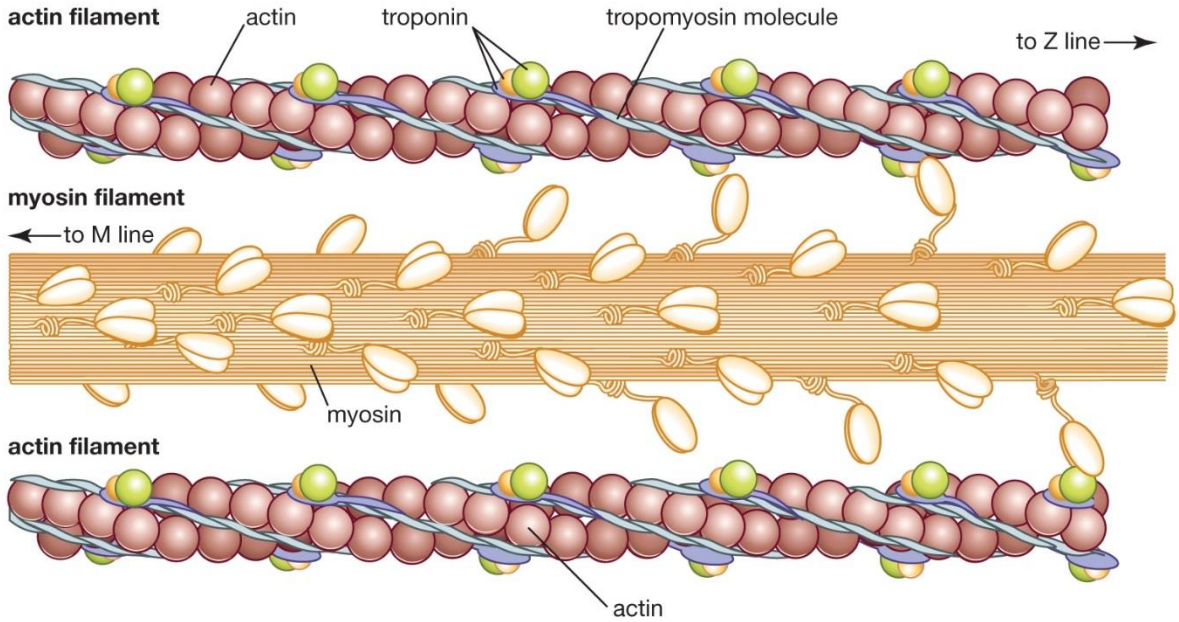

Figure 2A.4: Structure tropomyosin with actin, myosin and troponin in the muscle (Encyclopædia Britannica, Inc., 2019).

Chapter 2B published Review Article

Chapter 2B focusing on globally accepted methods for extraction, isolation, identification and quantification of allergenic prawn proteins. This chapter titled "**A review on methodologies for extraction, identification and quantification of allergenic proteins in prawns**" by Md Faisal, Todor Vasiljevic & Osaana N. Donkor, has been published in the peer review journal, "Food Research International", 121, 307-318 and included in this thesis with permission of Elsevier.

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1. PUBLICATION DETAILS (to be completed by the candidate)

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Md Faisal	85	Concept development, reviewing the relevant literature drafted and prepared manuscript	[redacted]	26-08-19
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Review

A review on methodologies for extraction, identification and quantification of allergenic proteins in prawns

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ABSTRACT

Prawn allergy is one of the most common food-borne allergies and current prevention is by avoidance. This review paper summarised different methodologies for the extraction, identification and quantification of prawn protein allergens, reported in various research studies. Following extraction, allergenic components have been analysed using well-established methodologies, such as SDS-PAGE, Immunoblotting, ELISA, CD Spectroscopy, HPLC, DBPCFC, SPT etc. Moreover, the preference towards Aptamer-based technique for allergenicity analysis has also been highlighted in this review paper. The summary of these methodologies will provide a reference platform for present and future research directions.

1. Introduction

Prevalence of food allergy in developed and developing countries becomes an increasing public health burden (Tang & Mullins, 2017). More than 170 foods all over the world are reported as the cause of these allergenic incidences (Boyce et al., 2010). Among the top 8 foods liable for over 90% of food-borne allergies, crustaceans are one causing the immense reaction in hypersensitive individuals (Fuller, Goodwin, & Morris, 2006). The term crustaceans refer to prawn, shrimp, crab, lobster, krill and crayfish, well represented in human consumption (Ruethers et al., 2018). Consumption of crustaceans especially prawn is the third important source of food-borne anaphylaxis after peanut and tree nuts in children and adults reported to affect > 2% of the global population (Lopata & Lehrer, 2009; Lopata, O'Hehir, & Lehrer, 2010; Rahman, Kamath, Lopata, & Helleur, 2010).

In 'crustacean', the terms 'shrimp' and 'prawn' are used conflictingly "even within one geographic region for the same species". Commercially, larger species are generally called 'prawns' in other English-speaking countries and 'shrimps' in the United States (Holthuis, 1980). According to famous crustacean taxonomist Tin-Yam Chan, the smaller species are termed 'shrimps', whereas the larger ones are called 'prawns' (Chan, 1998). There is no clear differentiation between the terminologies, and usage is usually interchangeable or even reversed in various countries. Therefore for the purpose of this review paper, the term 'prawn' has been used throughout the manuscript.

Prawn allergy has been reported to be very common in Asian and Western countries as well as in Australia (Chiang et al., 2007). A report by the National Allergy Strategy Pre-budget Submission 2015–16, states that around 20% of people in Australia having different types of allergy, will increase to 26.10% by 2050. Alarmingly, the rate of hospital admissions for severe allergic reactions has increased 4 fold in the last 20 years (*National Allergy Strategy,* 2017). As consumption of crustaceans rapidly rises, the potential for greater allergic incidence will increase concomitantly (*National Allergy Strategy,* 2017). Moreover, in the USA the prevalence of crustacean related allergy is more than twice that of peanut allergy, resulting in the majority of hospital emergency visits of adults and children over 6 years of age (Liu et al., 2008; Sicherer, Munoz-Furlong, & Sampson, 2004). A worldwide survey carried out in 15 different countries on 17,280 adults (age group 20–44 years) showed the prevalence of prawn allergy as 2.3% (Woods, Abramson, Bailey, & Walters, 2001). In another survey involving 14,948 individuals, however, crustacean allergies were almost 5 times higher in adults compared to children (Ayuso et al., 2008). The highest prevalence of crustacean allergy, especially from prawns, is reported in Italian adults by Skin Prick Test at 10.3% (Burney et al., 2010). Based on the above mentioned adverse health effects, the scientific community needs to understand the existence of allergenic components and their presence in different foods. Therefore the need for appropriate methods to identify allergenic components such as tropomyosin and other proteins in prawns can play an important role to raise more awareness. The main requirement and thus a problem for proper identification and quantification of allergenic components in prawns is related to the use of

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suitable extraction methods. Researchers have used different buffers of varying concentrations for the extraction processes at various centrifugal speeds to obtain maximum output (Lasekan & Nayak, 2016). After extraction, the allergenic proteins are usually visualized using established protocols of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Kamath et al., 2014). The specific IgG and IgE-binding proteins are detected using Immunoblotting (Kamath et al., 2014). For proper quantification of allergens in different prawn samples using immunological reaction-based techniques, for example, Enzyme-Linked Immunosorbent Assay (ELISA) method has been employed. Furthermore, different researchers have used various ELISA methods including Indirect, Inhibition and Sandwich ELISA to compare their IgG bindings (Abramovitch et al., 2013; Rahman, Helleur, Jeebhay, & Lopata, 2012; Song et al., 2015). Beside antibody, most contemporary aptamer-based techniques have also been introduced for recognition of prawn allergens in recent years (Tabrizi, Shamsipur, Saber, Sarkar, & Ebrahimi, 2017; Zhang, Wu, Wei, Zhang, & Mo, 2017). Separation of allergenic prawn proteins and molecular masses have been performed with various High-Performance Liquid Chromatography (HPLC) methods. Among the more frequently used are Reversed-Phase, Gel Filtration, Anion-Exchange (Kamath et al., 2014; Rahman et al., 2010) and Liquid Chromatography-Mass Spectrometry (LC/MS) (Rahman, Kamath, Gagné, Lopata, & Helleur, 2013). Moreover, the secondary structure of allergenic proteins has also been determined using Circular Dichroism (CD) Spectroscopy (Song et al., 2015). Skin Prick Test (SPT) and Double-Blind Placebo-Controlled Food Challenge (DBPCFC) have been used to measure the threshold prawn doses for patients' having prawn allergies (Gámez et al., 2015).

This review provides a collection of procedures relating to the identification and quantification of prawn allergenic proteins in detail, making it a reference platform for researchers. In addition, it will provide a clear guideline to alternative chemicals and techniques used in other methods. The overall aim of this review is to discuss comprehensively all probable methods that may be implemented in developing a potential research design to extract, identify and quantify allergenic components in prawns, as well as focus towards adaptation of newer technologies.

2. Background information on prawn allergy

2.1. Prawn allergy and allergens

The proteins in prawn can be classified into 3 groups based on their solubilities namely: sarcoplasmic, myofibrillar and stromal proteins. The myofibrillar protein is responsible for muscle contraction and made up of myosin (55–60%), actin (15–30%), tropomyosin (5%) and troponins (5%) (Sikorski, Kolokowska, & Sun, 1990). Fig. 1 shows the structure of tropomyosin, a highly conserved heat-stable myofibrillar protein (34 to 39 kDa) composed of two homodimers of α-helix coiled structure, surrounding the actin filament (Faisal, Vasiljevic, & Donkor, 2019; Holmes & Lehman, 2008; Rahman et al., 2010). Tropomyosin is liable for over 80% of all prawn-allergy related incidences (Chapman et al., 2006; Troiano, 2016). It is reported as a major allergen not only in prawns but also in other crustaceans (crabs, lobsters, krills etc.), mollusks (squids, octopus, abalone, snails, clams etc.) and invertebrates (cockroaches, house dust mites), showing 90% homology (Chapman et al., 2006; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007). However, myosin light chain (MLC), a sarcoplasmic calcium-binding protein (SCP), actin and arginine kinase (AK) shown in Fig. 1 have also been reported in few prawn species as minor allergenic compounds (Guillen, Fiandor, Del, Pedrosa, & Phillips, 2014; Misnan et al., 2015; Rahman et al., 2013; Yang et al., 2010). Table 1 summarises different methodologies used for the identification and analysis of allergenic proteins in different prawn species (*Penaeus monodon*, *Penaeus aztecus*, *Penaeus latisulcatus*, *Crangon crangon*, *Metapenaeus ensis*, *Marsupenaeus japonicas*, *Litopenaeus vannamei*, *Fenneropenaeus merguiensis* etc.) reported in various studies. Moreover, these methodologies may be used for all prawns' species.

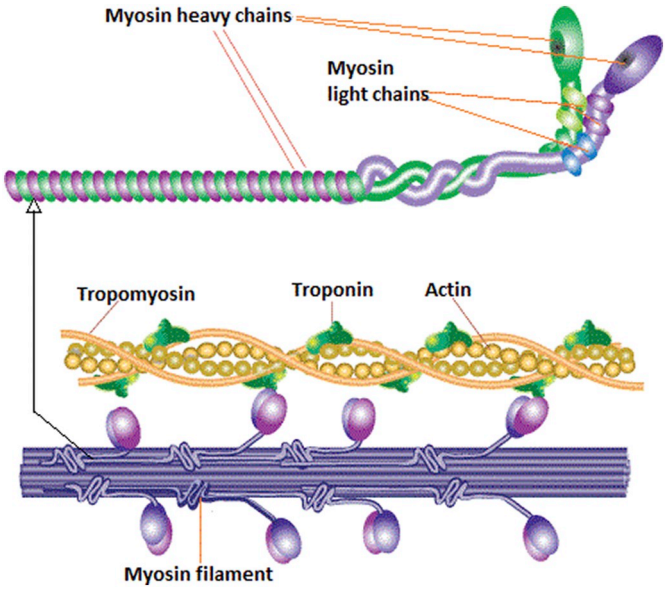

Fig. 1. Tropomyosin (major allergen) having dimeric coiled-coil structure regulates the bindings of myosin light chain, myosin heavy chain, troponin and actin (minor allergens) which helps in muscle contraction (Sigma-Aldrich, 2018).

2.2. Allergic reaction

The symptoms of allergic reaction mostly appear within 2 h (Shek et al., 2010) after ingestion of prawn or may delay up to 8 h (Villacis et al., 2006). Patients may have a localized allergic symptom relating to an organ; however, the reaction may involve multiple organs (Jeebhay, Robins, Leher, & Lopata, 2001; Maulitz, Pratt, & Schocket, 1979). People with prawn allergy frequently experience oral allergic syndrome notably itching, angioedema of the mouth, lips, and pharynx, which may appear within minutes after consumption. Also prominent in allergic people is the respiratory problem after consumption of prawn protein (Maulitz et al., 1979). The allergenicity of protein is directly related to its structure that triggers IgE antibody production (Rahman et al., 2010). Several researchers (Leung et al., 2008; Ravkov et al., 2013; Wambre, James, & Kwok, 2012; Wang et al., 2012) have reported the inducing ability of allergenic prawn proteins for the activation and proliferation of T helper (Th) cells (including Th1 and Th2 subsets) and eosinophils, as well as production of specific cytokines (such as IL-2, IL-4, IL-5, IL-10, IFN-γ etc.) in human peripheral blood mononuclear cells (PBMC). The Th1 and Th2 subsets, play a significant role in intervening the immune defense through the antibody- and cell-mediated immune response respectively, as well as their (Th1 and Th2) balance is believed as indispensable to maintain the soundness of immune response (Donkor et al., 2012; Wang et al., 2012). Moreover, allergenic proteins induce eosinophil production resulting atopic dermatitis, eosinophilic esophagitis and gastrointestinal disorders in some patients (Davis & Rothenberg, 2016; Foroughi et al., 2007; Rothenberg, 2004). Prawn allergy is long-lasting and the disorder persists during the entire life of an individual (Sicherer et al., 2004). Except for some emergency treatment following accidental exposure, there is no specific therapy available for prawn allergy and prevention is by avoidance (Ayuso et al., 2008; Fuller et al., 2006; Lieberman et al., 2010). Prevalence of prawn allergy is not only unique to consumers but also in occupational environments. Over 56.6 million employees in different countries of the world involved in numerous activities of seafood especially fish and crustaceans processing are also susceptible to life-threatening allergens

Table 1
Methodologies used for identification and analysis of allergenic proteins in different species of prawn.

Species	Identified allergen (kDa)	Method of analysis	Reference(s)
Black tiger prawn (*Penaeus monodon*)	Tropomyosin (32–39)	SDS-PAGE, Immunoblotting, ELISA, LC/MS, HPLC	Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008; Rahman et al., 2010; Sahabudin et al., 2011; Kamath et al., 2013; Kamath et al., 2014; Misnan et al., 2015
	Arginine kinase (40–42)	SDS-PAGE, Immunoblotting, LC/MS	
	Triose phosphate isomerase (26–29)		
	Titin (3000)		
	Sarcoplasmic calcium-binding protein (20 – 22)		
	Fructose 1,6 Bisphosphate aldolase (39–43)		
	Myosin light chain (19–22)		
	Compounds having weight 49 and 75 kDa		
Kuruma prawn (*Marsupenaeus japonicas*)	Tropomyosin (35)	SDS-PAGE, Immunoblotting, ELISA, CD Spectroscopy	Usui et al., 2013
King prawn (*Penaeus latisulcatus*)	Tropomyosin (32.6)	SDS-PAGE, Immunoblotting, ELISA, LC/MS	Fuller et al., 2006; Sahabudin et al., 2011; Kamath et al., 2013
	Compounds having weight 36, 42, 49 kDa	SDS-PAGE, Immunoblotting	
North-Sea prawn (*Crangon crangon*)	Tropomyosin (32–39)	SDS-PAGE, Immunoblotting, LC/MS, CD Spectroscopy	Bauermeister et al., 2011; Rahman et al., 2013
	Actin (31)		
	Myosin light chain (17–20)	SDS-PAGE, Immunoblotting, LC/MS, CD Spectroscopy	
	Sarcoplasmic calcium-binding protein (20–22)		
	Arginine kinase (40)		
	Myosin heavy chain (98)		
	Glyceraldehyde-3-phosphate Dehydrogenase (37)		
	Triosephosphate isomerase (27)		
	Troponin (16.8)		
Whiteleg prawn (*Litopenaeus vannamei*)	Tropomyosin (32–39)	SDS-PAGE, Immunoblotting, ELISA, HPLC, LC/MS	Garcia-Orozco, Aispuro-Hernandez, Yepiz-Plascencia, Calderon-de-la-Barca, & Sotelo-Mundo, 2007; Ayuso et al., 2008; Ayuso et al., 2009; Li-sha et al., 2011; Xiaoyan, Zhenxing, Hong, & Haider, 2011; Kamath et al., 2013
	Myosin light chain (20)	SDS-PAGE, 2D- SDS-PAGE, Immunoblotting, HPLC, LC/MS	
	Sarcoplasmic calcium-binding protein (20)		
	Arginine kinase (40)	SDS-PAGE, Immunoblotting	
Brown prawn (*Penaeus aztecus*)	Tropomyosin (36)	SDS-PAGE, Immunoblotting, ELISA, HPLC	Daul, Slattery, Reese, & Lehrer, 1994; Jeounga et al., 1997; Wild & Lehrer, 2005
Banana prawn (*Fenneropenaeus merguiensis*)	Tropomyosin (34.3–37)	SDS-PAGE, Immunoblotting, LC/MS, ELISA	Kamath et al., 2013; Khanaruksombat, Srisomsap, Chokchaichamnankit, Punyarit, & Phiriyangkul, 2014; Faisal et al., 2019
	Arginine kinase (40)	SDS-PAGE, LC/MS, Immunoblotting	
	Sarcoplasmic calcium-binding protein (20)		
	Myosin heavy chain type-b (22)		
	Hemocyanin (75)		
	Enolase (47)		
	Glyceraldehyde-3-phosphate dehydrogenase (30)		
Indian prawn (*Penaeus indicus*)	Tropomyosin (34)	SDS-PAGE, Immunoblotting, ELISA, HPLC	Nagpal, Pajappa, Metcalf, & Rao, 1989; Shanti, Martin, Nagal, Metcalfe, & Sabba-Rao, 1993; Wild & Lehrer, 2005
Greasy-back prawn (*Metapenaeus ensis*)	Tropomyosin (32.8)	SDS-PAGE, Immunoblotting	Leung et al., 1994; Wild & Lehrer, 2005
Sakura prawn (*Sergia lucens*)	Tropomyosin (37–38)	SDS-PAGE, Immunoblotting, ELISA, HPLC	Shimakura et al., 2005

Table 2
Occupational prawn allergy.

Causative agent	Place of contact	Symptoms/ disease (s)	Reference(s)
Prawns	Processing industry workers	Asthma, Dermatitis	Lemiere, Desjardins, Lehrer, & Malo, 1996; Rodriguez et al., 1997; Jeebhay et al., 2001; Mugica, Anibarro, & Seoane, 2003
	Researchers/ Scientists	Asthma, Contact urticaria, Dermatitis	Malo & Cartier 1993
	Fish market labors	Allergic, Contact urticaria	Taylor et al., 2000
	Caterers	Contact urticaria, Dermatitis	Goetz & Whisman, 2000
	Cafeteria employees	Asthma	Malo & Carrier, 1993; Goetz & Whisman, 2000
	Food handlers	Contact dermatitis	Musmand, Daul, & Lehrer, 1993
	Good night kissing	Fatal anaphylactic reaction	Steensma, 2003

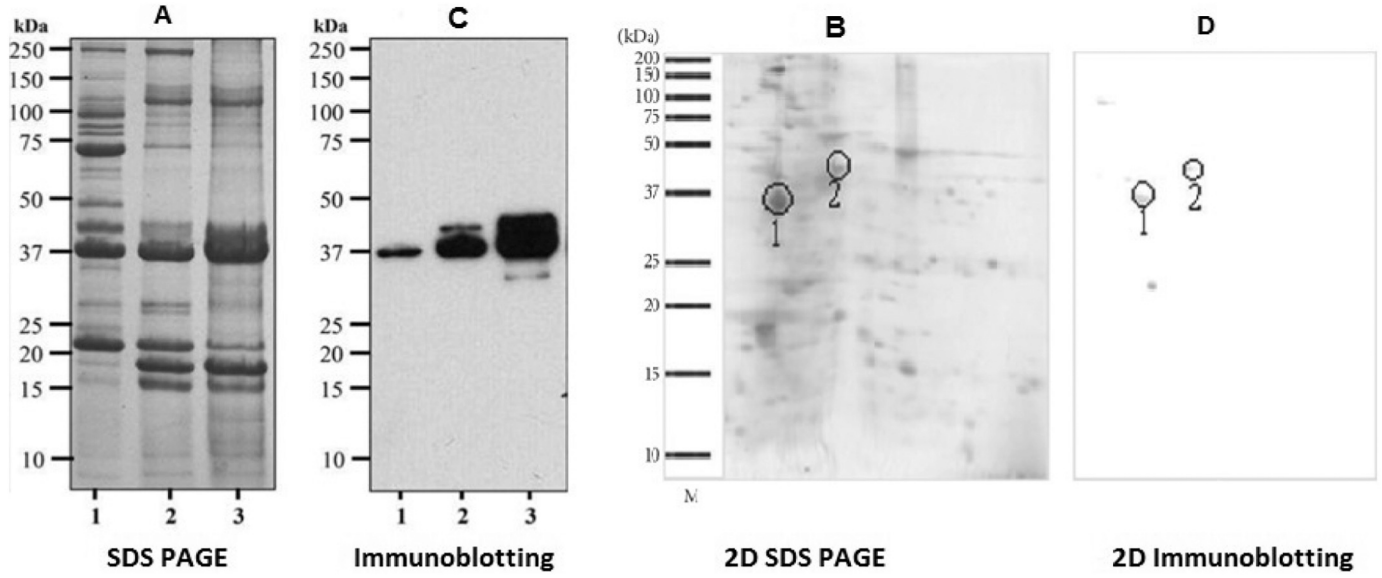

Fig. 2. Prawn protein profile in SDS-PAGE (A), Immunoblotting (B), 2D SDS-PAGE (C) and 2D Immunoblotting (D) (Kamath et al., 2014; Yadzir et al., 2015).

(FAO, 2016; Jeebhay et al., 2001). The occurrence of occupational asthma (7 to 36%) and contact dermatitis (3 to 11%) have been reported in workers related to seafood industries (Jeebhay et al., 2001). Allergic symptoms in workers related to the occupational environment due to unprotected handling of seafood and seafood products have been reported in Table 2.

3. Processing of prawns and extraction of allergenic proteins

3.1. Processing of prawn samples

Several researchers employed various processing methods to compare the changes of allergenic proteins due to processing with raw or unprocessed prawn samples. Prawn processing by frying, microwave roasting, grilling, baking, steaming and high pressure treatment after de-shelling and deveining have been discussed according to Lasekan and Nayak (2016) and Hu and Xie (2013). The oven treatment is carried out for baking at 200 °C for 4 min whereas, in frying, the sample is held in canola oil at > 200 °C for 1 min. The autoclave treatment is however held at 121 °C for 20 min and 0.14 MPa pressure. Microwave roasting used power set for 2 min at level 3 and grilling for 7 min at > 250 °C respectively. Steaming for 3 min, whereas high-pressure treatment for prawn samples is subjected to 450 MPa at 40 °C for 55 min.

Kamath et al. (2014) employed different prawn processing methods, (A): no heating of prawn sample (used as raw) and protein extraction with Phosphate Buffered Saline (PBS); (B): heating of prawn at 100 °C in PBS for 20 min; (C) heating of protein extract obtained from method (A) at 100 °C for 20 min and showed different results. Lasekan and Nayak (2016) boiled prawn in water for 5 min at 100 °C whereas, Shimakura, Tonomura, Hamada, Nagashima, and Shiomi (2005) treated prawn samples for 15 min in boiling water containing 3% NaCl to observe the changes of allergenic proteins.

3.2. Extraction of proteins from raw and processed prawns

Extraction is a crucial step for isolation and analysis of allergenic proteins. Although heat processing could interfere in protein extraction, researchers have followed the same extraction method for raw and processed prawns. The most common and widely accepted method for extraction reported by several researchers (Abramovitch et al., 2013; Gámez et al., 2015; Kamath et al., 2014; Kamath, Rahman, Komoda, & Lopata, 2013; Misnan et al., 2015; Sahabudin et al., 2011; Yadzir et al., 2012) have been described below:

Raw or processed prawn muscle is cut into smaller pieces and homogenized by utilizing an Ultra-turrax blender (IKA, Staufen, Germany) in PBS solution at 1:3 ratio, pH 7.2. After that, the protein slurry is agitated at 4 °C for 3 h and then centrifuged at 13,000 to 14,000 RPM for 15–20 min. The supernatant is carefully collected and filtered using a glass fibre filter and Millipore 0.45 μm membrane filter (Billerica, MA, USA). The resulting filtrate is preserved at −80 °C for further allergenicity investigation.

Lasekan and Nayak (2016) used 3 different buffers (A): 20 mM sodium phosphate +1 M NaCl +1% Tween 20 + 0.5 mM dithiothreitol, (B): 20 mM sodium phosphate +1 M NaCl +1% Tween 20 and (C): 20 mM sodium phosphate +1 M NaCl respectively and compared the most suitable for the extraction of prawn proteins. Extraction buffer (A) was reported to have provided the best results for protein extraction, because Tween 20 helps to improve protein interactions with solvent, whereas dithiothreitol cleaves disulfide bonds for better protein solubilisation (Lasekan & Nayak, 2016; Liu & Hsieh, 2008). Beside the above-mentioned buffers, other buffers such as 1 M KCl, 25 mM Tris-HCl (pH 8.0), PBS having protease inhibitor, 50 mM KCl, 2 mM $NaHCO_3$ buffer (pH 8.0) have also been used for the extraction of prawn proteins (Ayuso et al., 2009; Sikorski et al., 1990; Usui et al., 2013).

In order to obtain an extract rich in tropomyosin (major allergen) and other heat-stable proteins after initial extraction, an aliquot of the total protein extract is heated for 10 min in a water bath at 100 °C followed by centrifugation at 14,954 RPM for 10 min. The supernatant is designated as heat-stable protein extract and stored at −80 °C for further analysis (Lasekan & Nayak, 2016).

4. Visualization, identification, and quantification of prawn allergenic proteins

4.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Gel electrophoresis is an excellent technique for separating and identifying medium to largely sized biomolecules. The most common method to visualize protein profile is SDS-PAGE as described by several researchers (Kamath et al., 2013, 2014; Lasekan & Nayak, 2016; Lv et al., 2014; Rahman et al., 2012, 2010; Yu et al., 2011; Zhenxing, Hong, Limin, & Jamil, 2007) has been represented in Fig. 2A. Briefly, the extracted protein supernatant has been diluted with Laemmli buffer (having 5% β-mercaptoethanol) at a ratio 1:1 and boiled at 90–100 °C

for 3–5 min. To visualize the molecular weights of separated protein bands, the concentration of the separating gel played a major role in terms of resolution. It has been observed that the gel gradient of 4–20% density provided a better separation for proteins with the molecular weight range of 10 to 250 kDa. Furthermore, the intensity of protein bands was directly related to the concentration of protein loaded, hence 10–12 µL sample of 1 mg mL^{-1} was found to be appropriate for better separation on the SDS-PAGE. Separation of protein bands is attained according to the Bio-Rad recommended process at 170 V for 1 h. As a protein marker Precision Plus Protein Standard is utilized. The proteins are separated based on their molecular weight. The number of protein bands for raw prawns were around 12 to 19 whereas, for processed prawns, comparatively less number of protein bands (7 to 14) were reported on SDS-PAGE after electrophoresis by the above mentioned researchers. To visualize the separated protein bands, staining is performed with GelCode Blue Stain reagent or Coomassie brilliant blue R-250. Gel images are taken using a Tanon-4200SF (Lv et al., 2014) or Densitometer GS800 (Yadzir, Misnan, Bakhtiar, Abdullah, & Murad, 2015) or ChemiDoc (Rahaman, Vasiljevic, & Ramchandran, 2016) gel imager to enable comparison among protein bands. Nowadays, researchers are moving away from traditional staining and distaining procedure by using precast unstained gels (Faisal et al., 2019). The separated protein bands for the unstained gels are visualized through UV-activation by ChemiDoc imager within 3 to 5 min of electrophoresis (Faisal et al., 2019). Table 3 depicts the use of various buffers and gels in other studies to visualize the range of separated protein bands in prawn protein extracts.

4.2. 2-dimensional gel electrophoresis

Fig. 2B shows a 2-Dimensional Electrophoresis (2-D SDS-PAGE) separation utilizing two-step method based on different properties namely, Isoelectric Focusing (IEF) and SDS-PAGE. According to O'Farrell (1975), in the IEF mode, proteins are separated based on Isoelectric Points (pI) resulting in resolution of a more complex mixture consisting of different proteins and the relative amount of each protein determined, whereas for SDS-PAGE, proteins are separated only by their molecular weight. Specific methodologies based on 2-D SDS-PAGE are described below:

Prawn protein sample is suspended overnight in rehydration buffer (8 M Urea, 2 mM Tributylphosphine (TBP), 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 0.2% Carrier ampholytes, 0.0002% Bromophenol Blue) at room temperature and 100 µg of the sample is added to a 7 cm immobilized (pH 3–10) Bio-Rad (USA) nonlinear gradient strip (Yadzir et al., 2015). After the first step of protein separation by IEF (Bio-Rad, USA), SDS-PAGE step is performed as stated by Misnan et al. (2015) and Yadzir et al. (2015).

Liu et al. (2010) on the other hand, used a slightly different approach with 200 µg proteins separated at 8000 V for 6 h followed by equilibration steps in buffer (A): 6 M urea, 20% glycerol, 50 mM Tris-HCl, 2% SDS, 0.13 M dithiothreitol (pH 8.8) and buffer (B): 6 M urea, 20% glycerol, 50 mM Tris-HCl, 2% SDS, 2.5% iodoacetamide (pH 8.8), SDS-PAGE is done following the Bio-Rad protocol at 100 V using Criterion precast gel. Ayuso et al. (2008) however used a linear voltage slope of up to 20,000 V-hours for 17 h to separate proteins based on charge and then utilize Invitrogen Zoom Gels to perform a 2-D Gel Electrophoresis.

SDS-PAGE and 2-D SDS-PAGE methodologies are widely accepted for protein visualization, yet, there are limitations for smaller proteins (< 10 kDa) passage through the gel during electrophoretic separation. Furthermore, a comparison of relative amounts of different molecules relies on band intensities (darkness) on the gel, which sometimes shows some degree of error. Moreover, if the sample of interest is not concentrated enough, bands will be virtually invisible and difficult to measure.

4.3. Immunoblot analysis

Immunoblot analysis is a continuous process after gel electrophoresis, followed by membrane blotting and probing with antibodies. This method usually helps to immobilize proteins on membranes during repeated washing and provides higher sensitivity during antibody treatment. Extracted proteins resolved on SDS-PAGE are transferred to a 0.2 µm Polyvinylidene fluoride (PVDF) membrane or 0.45 µm nitrocellulose membrane, using a Mini Trans-Blot System at 100 V for 70 min (Bio-Rad), described by several researchers (Bauermeister et al., 2011; Rahaman et al., 2016; Song et al., 2015; Yadzir et al., 2015). Membranes are washed 2 times for 5 min each with 30 mL PBS made up of 0.1% Tween-20 (PBST) per wash. Non-fat dry milk (5% *w/v*) with PBST is used as a blocking buffer when incubated overnight at 4 °C with Immunoblots. Further incubation under constant mixing is performed with primary antibody IgG (rabbit serum) or IgE (patient's sera) for 2 h. After rinsing 5 times with PBST to remove unbound proteins, membranes are further incubated with secondary antibody (anti-IgG or anti-IgE). Antibody binding of allergenic protein is then detected using a Tanon-4200SF or ChemiDoc (Bio-Rad Laboratories) imager depicted in Fig. 2C and D (Bauermeister et al., 2011; Rahaman et al., 2016; Song et al., 2015; Yadzir et al., 2015). Beside the above-mentioned methodologies Table 4 highlights the use of different immunoblotting methods and various antibodies to detect the IgG and IgE binding of allergenic prawn proteins.

The accuracy and the better result of immunoblotting is directly related to an appropriate number of washes in-between antibody treatments. A higher number of washes often remove the target protein and provide a false negative result, whereas a lower number of washes cause darker background with false positive results. Furthermore, selection of the appropriate primary and secondary antibodies is another crucial step for immunoblotting.

Table 3
Buffers and gels used in SDS-PAGE.

Buffer	Gel used for electrophoresis	Comments	Reference(s)
Laemmli sample buffer (Bio-Rad) in the presence of 5% 2-mercaptoethanol	12% polyacrylamide separating gel and 5% stacking gel	Demonstrated approximately 23 protein bands at various molecular weights between 15 and 200 kDa for raw sample, whereas 16 to 18 protein bands for different boiled samples	Sahabudin et al., 2011; Yadzir et al., 2015
Laemmli buffer	15% running gel and 5% stacking gel	Demonstrated protein bands in between molecular weights 14.4 and 116 kDa for raw sample	Song et al., 2015
0.05 M Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 8% 2-mercaptoethanol and 0.1% bromophenol blue)	PhastGel Gradient Pre-cast 8–25%	Demonstrated protein bands molecular weight ranging between 7 and 97 kDa for raw sample	Gámez et al., 2015
0.01 M Tris–HCl buffer (pH 6.8) containing 2.5% SDS, 20% glycerin and 5% dithiothreitol			Shimakura et al., 2005
0.0625 M Phosphate buffer (pH 7.5) containing 2.5% SDS and 5% dithiothreitol			Motoyama et al., 2007

Table 4
Use of different immunoblotting methods and various antibodies to detect the IgG and IgE binding of allergenic prawn proteins.

Membrane	Immunoblotting method	Primary and secondary antibody	Comments	Reference(s)
Nitrocellulose membrane (0.45 μm)	Mini Trans-Blot System (Bio-Rad, USA)	Patient's serum; Biotinylated goat anti-human IgE (KPL, UK)	4 major IgE-binding site with molecular weight of 36, 42, 49, and 75 kDa in black tiger prawn extracts, whereas 3 major IgE-binding proteins at 36, 42, and 49 kDa in king prawn extracts. Several minor allergens at 80 and 100 kDa also observed in both species	Sahabudin et al., 2011; Misnan et al., 2015
	Surf-blot apparatus (Idea Scientific Minneapolis, USA)	Patient's serum; Alkaline phosphatase-conjugated, monoclonal mouse anti-human IgE (Southern Biotechnology Associates, USA)	–	Ayuso, Lehrer, & Reese, 2002
	Xcell II blotting apparatus (Invitrogen)	Rat anti-Tropomyosin monoclonal antibody (Abcam, UK); Rabbit anti-human IgE antibody (Dako) and goat anti-rabbit IgG-HRP conjugated antibody (Promega, USA)	IgE binding to proteins single band within the tropomyosin region (37–39 kDa) for both the raw and cooked extracts	Abramovitch et al., 2013
	Semi-dry Immunoblot apparatus (Bio-Rad, USA)	Patient's serum; Peroxidase-conjugated antihuman IgE	–	Rahman et al., 2012
Polyvinylidene fluoride (PVDF) membrane (0.2 μm)	iBlot Dry-Blotting System (Life Technologies Corporation, Carlsbad, USA)	Polyclonal prawn Tropomyosin antibody (Biorbyt Limited, UK); Horseradish peroxidase-goat anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc. USA)	Reacted with tropomyosin protein having molecular weight 38 kDa, also showed weak cross-reactivity with protein bands at approximately 40 and 45 kDa	Vang, Mæhre, Jensen, & Olsen, 2013
	Semi-dry Immunoblot apparatus (Bio-Rad, USA)	Rabbit antibody; Polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase-HRP (Promega, USA)	–	Rahman et al., 2010
		Monoclonal anti-insect tropomyosin antibody, mac-141 (Abcam, USA); Rabbit anti-mouse IgG antibody conjugated with HRP (Sigma, USA)	In raw prawn extracts, the molecular weight of these bands ranged from 31 to 36 kDa	Kamath et al., 2013, 2014
	Semi-dry transfer (Carbon plate Indian instrument)	Anti-rabbit antibody; Goat anti-rabbit IgG	–	Li-sha et al., 2011
	–	Patient's serum; HRP-conjugated anti-human IgE antibody (Bethyl Laboratories, USA)	–	Usui et al., 2013
	Semi-dry immunoblot apparatus (Bio-Rad, USA)	Patient's serum; Rabbit polyclonal anti-human IgE antibody (DAKO, USA) and goat anti-rabbit polyclonal antibody labeled with HRP (Promega, USA)	Reacted with tropomyosin having molecular weight 35 kDa	Rahman et al., 2013
	Phast-System apparatus with a PhastTransfer	Patient's serum; Peroxidase-conjugated goat anti-human IgE antibody (Cosmo Bio, Japan)	–	Shimakura et al., 2005

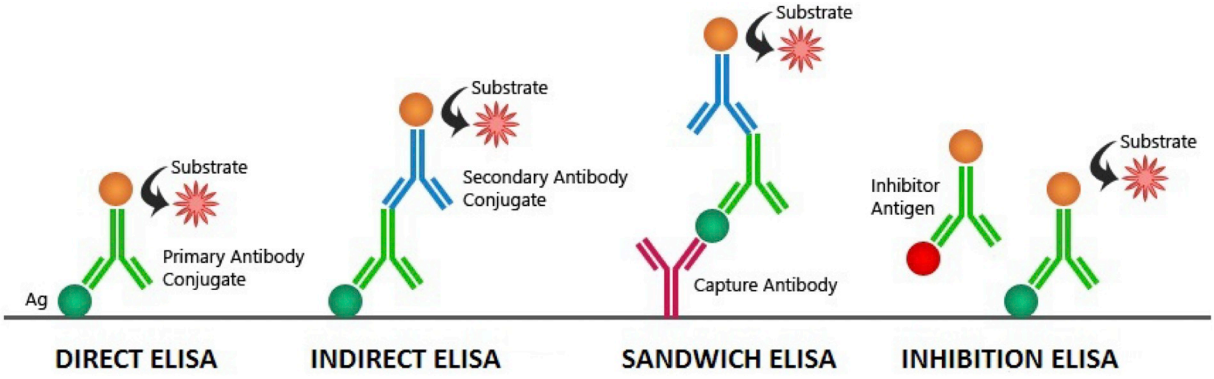

Fig. 3. Basic antigen-antibody binding principles of different types of ELISA (Boster, 2018).

4.4. ELISA

ELISA is predominantly used in the immunological reaction-based technique for allergen detection and quantification (large, small proteins and peptides). To achieve this, a calibration curve is developed based on antigen/antibody reaction of a known protein concentration. Basic antigen-antibody binding principles of different types of ELISA has been shown in Fig. 3. Boster (2018), highlights the fundamental principle of IgE or IgG binding of 4 commonly used ELISA and showed the distinctions between antigen-antibody binding processes. Shimakura et al. (2005) stated that Direct ELISA often provides false-negative results and is not suitable for prawn allergy detection. Details of other ELISA protocols namely Indirect, Inhibition and Sandwich ELISAs are described below:

4.4.1. Indirect ELISA

The IgE binding and antigenicity capability of prawn protein (tropomyosin) are assessed by indirect ELISA, using patient's sera (IgE) and rabbit polyclonal antibodies (IgG), described by Song et al. (2015). In this method, a Microlon ELISA plate is coated with 100 µL of purified tropomyosin using 50 mM sodium carbonate buffer (pH 9.6). Coated ELISA plate is incubated at 4 °C overnight to attach the antigen with IgE/IgG. After that the plate is washed 3 times using PBST and further incubated for 1.5 h at 37 °C with 1–2% bovine serum albumin (BSA) in PBST to block non-specific binding. The plate is then further washed 3 times with PBST, 100 µL antisera (a primary antibody) added and incubated at 37 °C for 1.5 h. After washing 3 times with PBST, 100 µL of secondary antibody (goat anti-human IgE or horseradish peroxidase goat anti-rabbit IgG) is added to each well and further incubated at 37 °C for 1 h. Finally, 100 µL of Tetramethylbenzidine (TMB) is added to each well after washing and incubated for 20 min at 37 °C. The antigen-antibody reaction is terminated by adding 2 to 4 M Sulphuric acid and the optical density is measured using ELISA plate reader at 450 nm wavelength (Kamath et al., 2013; Li-sha, Zhen-xing, Yi-xuan, Chen, & Hong, 2011; Usui et al., 2013). The similar method reported by Abramovitch et al. (2013) uses commercially available antibody rabbit anti-human IgE (Dako, Glostrup, Denmark) and 5% skimmed milk powder in PBST as blocking agent. The reaction is terminated in each well with 1 M HCl.

4.4.2. Inhibition ELISA

Allergenic components present in the extract as peptides are detected by Inhibition ELISA method (Lasekan & Nayak, 2016). The method uses 96-well polystyrene ELISA plates initially coated with 200 ng tropomyosin in each well and a coating buffer of sodium carbonate (15 mMol L^{-1}, pH 9.6). The plate is incubated at 4 °C for 16 h to bind the tropomyosin. After incubation plates are washed 3–5 times using TBST to remove unbound proteins and then 200 µL of blocking buffer (0.5% BSA in TBST) is used and further incubated for 2 h at 37 °C.

Another method uses a microtitre plate, with a mixture of equal volumes of human sera and digested tropomyosin (100 µL) as an inhibitor (0.02–40 µg mL^{-1}), the subsequent steps are as described for ELISA. The rate of inhibition for the reduction in IgE-binding capacity of patients' sera due to treatment with human sera is calculated using the following formula, where digested tropomyosin is used as an inhibitor:

$$\text{Inhibition rate } (\%) = (X - Y)/(X - Z) \times 100$$

Here X represents the absorbance of the patient's sera without any inhibitor. Treatment - patients' and control sera with different concentrations of inhibitors are represented as Y and Z respectively (Lasekan & Nayak, 2016; Liu et al., 2010; Shimakura et al., 2005; Song et al., 2015; Usui et al., 2013).

4.4.3. Sandwich ELISA

The Sandwich ELISA method has been reported to be more precise than the Inhibition ELISA due to its high sensitivity and less false positive results (Rahman et al., 2012). In this method, the ELISA plate is coated with a specific primary antibody to capture the target protein (tropomyosin). Unbound proteins are removed by multiple washing with PBST or manufacturer's recommended wash buffer. The enzyme-labeled secondary antibody detects the captured protein reacting with a chromogen, which helps to develop a specific color. Absorbance is measured using spectrophotometry at 450 nm (Jeounga et al., 1997; Rahman et al., 2012).

ELISA shows higher accuracy for the detection and quantification of allergenic proteins, yet in some cases high cost and short shelf-life often limit their use for long-term researches. Furthermore, a fixed number of wells (48 or 96) in commercially available ELISA plates often restricts the higher number of replications.

4.5. Interleukin cytokine assays by IgE ELISA

Due to consumption of prawn the allergenic proteins induces T helper (Th) cells (including Th1 and Th2 subsets) and eosinophils to produce specific interleukin cytokines, such as IL-2, IL-4, IL-5, IL-10, IFN-γ etc. (Ravkov et al., 2013; Wambre et al., 2012; Wang et al., 2012). Moreover, in the in-vitro system the human peripheral blood mononuclear cells (PBMC) are also stimulate with different allergenic proteins, resulting production of specific cytokines (Abramovitch, Lopata, O'Hehir, & Rolland, 2017). These cytokines can be analysed using IgE ELISA as per manufacturer's (Thermo Fisher Scientific Australia Pty Ltd.) guideline. In brief, 96 well uncoated ELISA plates (Coaster 9018 ELISA plate) are coated with captured antibody and incubated at 4 °C for overnight under continuous shaking. In the next morning, after 3 consecutive washing with 250 µL wash buffer per well, ELISA plate wells are blocked with 200 µL ELISTOP diluent for 1 h. Exactly 100 µL

of samples are added to each well and incubated at 4 °C under continuous shaking for overnight to achieve maximum sensitivity. The microwell is washed three times with washed buffer followed by addition of 100 µL diluted detection antibody to all wells. After subsequent washing, 100 µL of streptavidin-HRP is added to each well and incubated at room temperature for 30 min. After that, 100 µL of TMB (3,3′,5,5'-Tetramethylbenzidine) solution to each well, followed by 15 min incubation, 100 µL of stop solution (2 N H_2SO_4) is added to each well of ELISA plate. Finally, absorbance is measured within 10 min using ELISA plate reader (xMark microplate spectrophotometer, Bio-Rad, Tokyo, Japan) at 450 nm.

4.6. Aptamer-based technique

Aptamers are short and functional single strand nucleic acids (DNA or RNA), which are synthetically produced by a Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method, having the capacity to recognize various classes of target molecules with high affinity and specificity (Liu & Zhang, 2015; Teng et al., 2016). In recent years this technology has been precisely used for the detection of proteins (Lv, Liu, Bai, Yang, & Chen, 2015), toxins (Lu, Chen, Wang, Zheng, & Li, 2015), virus (Lai, Wang, Liou, & Lee, 2014) and other pathogens (Abbaspour, Norouz-Sarvestani, Noori, & Soltani, 2015). As a most modern concept, aptamer-based technique has been adapted for the detection of major prawn allergen tropomyosin by Tabrizi et al. (2017) and Zhang et al. (2017). Antibody-based techniques such as Immunoblotting and ELISA have been widely used with several limitations namely, instability during longer storage, dependency on animals or cell-lines for production, low specificity to target in some cases and expensive to run. Aptamers, on the other hand, use chemically synthesized DNA or RNA strands with high target binding specificity and stability in terms of storage (Teng et al., 2016; Van, 2016). Synthesis of aptamer according to the SELEX method is represented in Fig. 4A, which involves the following steps: incubation of target cell with a random pool oligonucleotide (DNA/RNA library); separation of target bound DNA/RNA sequence; amplification of target sequence by PCR; repetition of steps 1–3 for 5–15 cycles to obtain sufficient aptamers and finally, the cloning and sequencing of synthesized aptamers (Barman, 2015). On the other hand Fig. 4B shows a schematic overview of tropomyosin detection using aptamer-based technique (Zhang et al., 2017). In brief, the high affinity aptamer generated through above described SELEX method, react with prawn proteins extract containing tropomyosin. Due to specific sensitivity, aptamer binds only with tropomyosin and form aptamer-tropomyosin complex. By using graphene oxide (GO) unbound protein and aptamers are separated from aptamer-tropomyosin complex. Then the aptamer-tropomyosin complex in the supernatant is quantified using the OliGreen ssDNA reagent and the fluorescence intensities are recorded on a LS-45 Fluorescence Spectrometer (Perkin-Elmer, USA) with an excitation at 480 nm and an emission at 522 nm (Zhang et al., 2017).

5. Isolation, purification and characterization of prawn proteins and peptides by HPLC and LC/MS

To isolate, purify, determine molecular mass and characterize allergenic proteins and peptides, different separation methods namely: Reversed-phase, Gel-filtration, Anion-exchange, Hydrophobic-interaction HPLC and LC/MS are precisely used all over the world (Kamath et al., 2014; Rahman et al., 2013).

Rahman et al. (2010) and Kamath et al. (2013, 2014) used a biologic low-pressure purification system (Bio-Rad, USA) to isolate tropomyosin with the help of similar strong anion-exchange chromatographic columns. Initially by using Amikon spin filters having 3 kDa molecular weight cut-off the protein extract is exchanged into 30 mM acetate buffer, followed by loading onto a Mini Macroprep High Q column (Bio-Rad, Hercules, CA, USA). After that 20 µL of filtered sample is loaded onto the column and a mobile phase of 30 mM Tris-HCl buffer pH 6.8 is used. Elution gradient of sodium chloride (NaCl) concentration (400 to 600 mM) is performed for the separation of tropomyosin. Whereas, Ozawa, Watabe, and Ochiai (2011) used both hydrophobic interaction and anion exchange chromatography to separate prawn tropomyosin. A linear decrease in concentration of ammonium sulfate solution from 40 to 0% was performed in a hydrophobic interaction chromatography with 20 mM potassium phosphate buffer (pH 7.0). Other studies, on the other hand, used gel filtration HPLC method to determine the molecular masses of native allergens in prawns (Ishikawa, Shimakura, Nagashima, & Shoimi, 1997; Shimakura et al., 2005). In this method, TSKgel column (0.75 cm × 30 cm; Tosoh, Tokyo, Japan) is equilibrated and tropomyosin is eluted at 20 °C using 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0) maintaining 0.5 mL min^{-1} flow rate. Protein is monitored using a UV detector at 220 nm wavelength.

However, peptides with allergenic properties derived from prawn proteins have also been separated (Kamath et al., 2013, 2014). Rahman

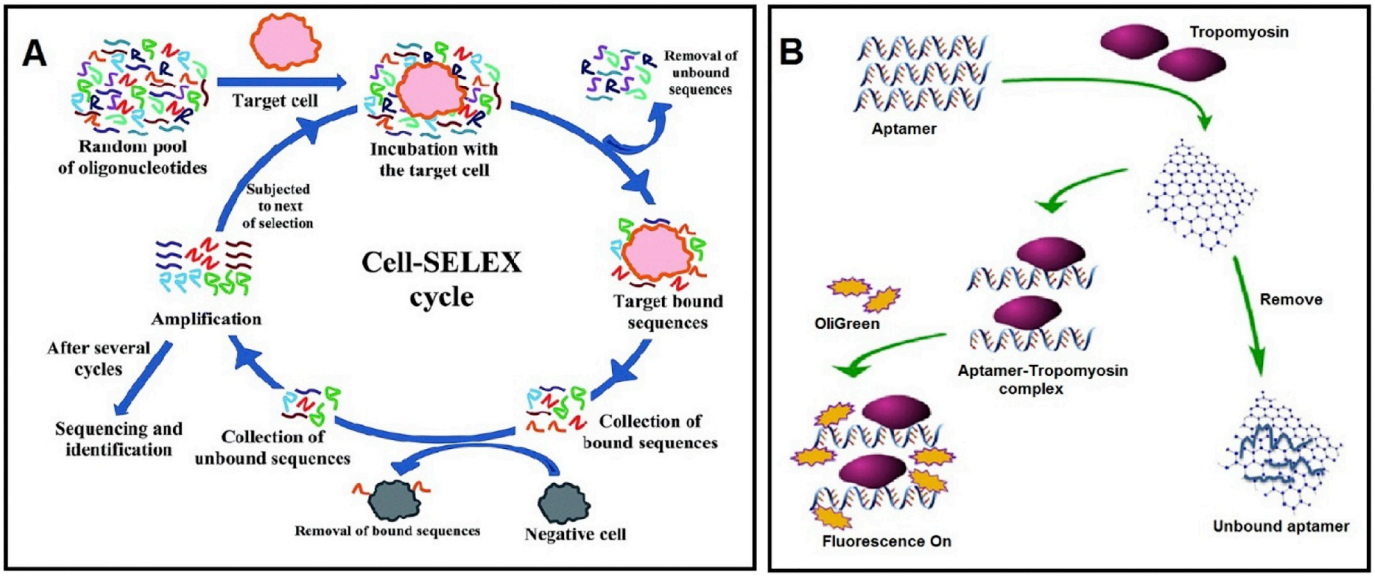

Fig. 4. Synthesis of aptamer using SELEX method (A), Detection of tropomyosin using aptamer-based technique (B) (Barman, 2015; Zhang et al., 2017).

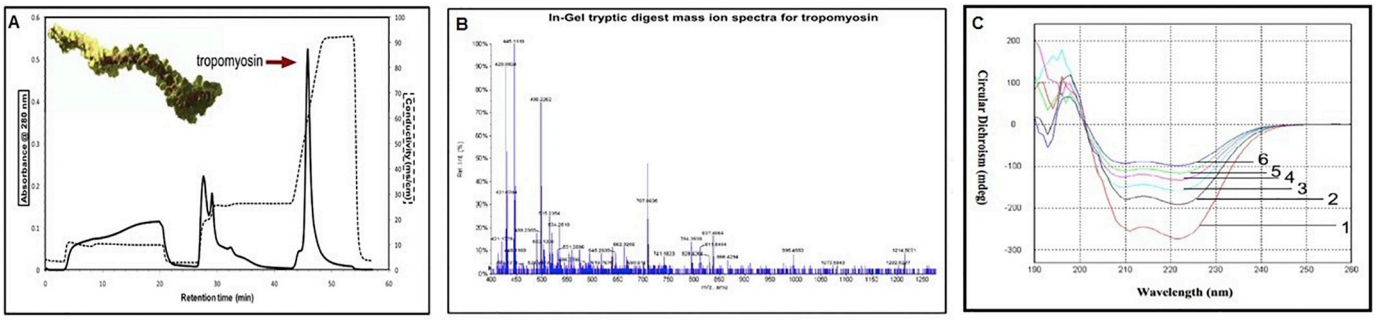

Fig. 5. Tropomyosin profile in HPLC (A), LC/MS (B) and CD Spectroscopy (C) (Kamath et al., 2013; Song et al., 2015).

et al. (2012, 2013) reported the separation of peptides of major prawn allergens at 20 °C using a Phenomenex RP Chromatography column (Kinetex C-18, 2.1 mm × 100 mm, 2.6 μM, CA, USA) on Waters Alliance 2795 HPLC system. A gradient flow of Milli-Q water with 0.1% formic acid (mobile phase A) and Acetonitrile with 0.1% formic acid mobile phase B) is used. The eluted peptides are further analysed by LC/MS method. Peptides are desolvated in electrospray ionization (ESI) mode at 250 °C with gas flow rate of 400 L h^{-1}. By using capillary and orifice cone at 3.02 kV and 40 V the ions are accelerated. Precursor ions are fragmented at low energy collision-induced dissociation using argon gas and collision energy of 13 eV. The $[M + 4H]^{4+}$ precursor ions of the unlabelled and labeled forms of the target peptide are 588.15 and 590.25 *m/z*, respectively. Finally, the processing of Data is performed using Mass Lynx 4.1 software (Kamath et al., 2013; Rahman et al., 2013, 2010). Fig. 5A and B show graphical representations of separation and elution of proteins and peptides based on polarity and molecular weight. Beside this by using the standards of different prawn allergenic proteins (for example, pure form of tropomyosin, AK, MLC, SCP etc.) and peptides on HPLC and LC/MS system, the elution time of allergenic proteins and peptides can easily be determined. This will ultimately help for the isolation, collection and purification of targeted allergenic proteins and peptides from the crude prawn protein extracts.

The efficiency of proper isolation, purification and characterizations of proteins and peptides using HPLC and LC/MS is directly related with the selection of appropriate analytical column and proper mobile phases. Moreover, the adjustment of proper flow rate and sample concentration are another crucial steps to achieve better separation of protein peaks.

6. Secondary structure analysis

The changes in the secondary structure of tropomyosin using CD Spectroscopy and reported in the literature has been shown in Fig. 5C (Song et al., 2015). A protein solution is prepared at 0.5 mg mL^{-1} in PBS (Usui et al., 2013) or 10 mM potassium phosphate buffer (Bauermeister et al., 2011; Reese et al., 2005). Far-UV CD Spectroscopy is performed using a JASCO J-815 spectropolarimeter at 185–260 nm. Scanning is performed at 50–100 nm min^{-1} with interval or response time 0.25–2.0 s and bandwidth 1–2 nm at room temperature (Lv et al., 2014; Song et al., 2015). In a similar method described by Liu et al. (2010), the CD Spectroscopy is set at a temperature range between 20 and 95 °C and a gradient of 10 °C per min or maintained at varying temperatures (25, 45 or 80) °C using a circulating water bath (Usui et al., 2013). All samples are measured 3 times, and the average results analysed with molar residue ellipticity. Final results are displayed as milli-degrees using CD analysis of Neural Networks (CDNN) 4.0 software or JASCO software which analyses the random coil proportions as well as α-helix, β-sheet and β-turn in each sample (Lv et al., 2014; Song et al., 2015; Usui et al., 2013).

7. Determination of threshold prawn doses

7.1. Double-blind placebo-controlled food challenge

The threshold prawn dose for an individual patient having prawn allergies is determined by Double-Blind Placebo-Controlled Food Challenge (DBPCFC). According to Lopata and Lehrer (2009) threshold doses range from 14 to 16 g of prawn, equivalent to 32 mg of protein. DBPCFC is performed at random in all individuals except those showing resistance to prawn allergy in the last 3 months. Challenge is considered positive in individual if similar allergy symptoms appear after 3 successive doses. A study by Gámez et al. (2015) used increasing doses of *Synuchus melantho* prawn as an active meal and milkshake pudding as placebo meal for this test. However, in a negative DBPCFC test, a total of 12 cooked prawns are subjected to individuals in an open food challenge.

Yang et al. (2010) on the other hand, employed a slightly different approach where capsules containing active prawn components are used instead of food. The capsules provided to individuals at 20 min interval, contained 500 mg lyophilized prawn or an equal amount of lactose (as placebo). The dose is gradually increased up to maximum of 8 g and individuals showing no positive reaction is subjected to open challenge at least after 48 h.

7.2. Skin Prick Test

Skin Prick Test (SPT) is performed as confirmation to allergenic response on patient's skin following the guideline of European standard (EAACI Subcommittee on Skin Tests, 1993) shown in Fig. 6.

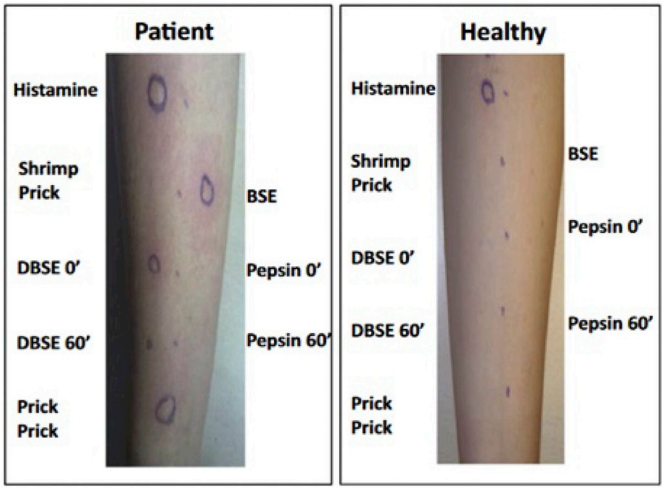

Fig. 6. Results of Skin Prick Test on the patient and healthy person (Gámez et al., 2015).

Commercial prawn extract or selected prawn species extracts liquefied in sterilized physiological saline then applied to patient's and non-allergic healthy person's skin. Saline solution (0.9%) is used as a negative control. Reactions of prawn allergy on the skin are recorded after 15 min by calculating the average diameter of duplicate wheals. To be considered as positive the wheal diameter should be at least 3 mm larger than the control measure. The SPT is performed by a nurse or another expert person (Van, Elsayed, Florvaag, Hordvik, & Endresen, 2005; Carnés et al., 2007; Yang et al., 2010; Ayuso et al., 2012; Gámez et al., 2011; Gámez et al., 2015).

8. Conclusion

For extraction of allergenic proteins from prawns, PBS with pH 7.2–7.4 is commonly used by researchers and experimentation is generally carried out at 4 °C to avoid unwanted protein denaturation. Moreover, researchers are moving away from traditional staining and distaining procedure by using precast unstained gels. With the implementation of mono and polyclonal antibodies (IgG) and Human serum (IgE), tropomyosin has been confirmed as a major allergen in prawns, but it is still not clear whether actin, MLC, AK, SCP are also allergenic in prawns. ELISA provides more accurate results in comparison to immunoblotting. It is also able to detect the presence of allergenic epitopes in peptides and low molecular weight proteins (< 10 kDa). Sandwich ELISA has been reported to be more precise in the capture of target proteins compared to other ELISA methods due to its high sensitivity and less false positive results. The identified allergens and other proteins can be isolated, purified and characterized through HPLC and LC/MS methods. Beside the above-mentioned methodologies, DBPCFC and Skin Prick Tests are worldwide accepted methodologies for determining threshold prawn doses in individuals through phenotypically visualized allergenic symptoms. Furthermore, due to greater advantages of aptamer-based technique over antibody-based techniques such as immunoblotting and ELISA, it is expected to be a most popular analytical tool in the near future for analysis of major and minor allergens in different species of prawns.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Md Faisal: Concept development, drafted and prepared the manuscript. Dr. Osaana N. Donkor and Professor Todor Vasiljevic: Concept development, revising and editing of the manuscript.

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Chapter 3

Effects of selected processing treatments on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) tropomyosin

Chapter 3 describes different processing techniques assessed to attenuate the antigenicity of banana prawn tropomyosin (*Fenneropenaeus merguiensis*). In addition, a new processing approach namely a mild acetic acid (pH 2.5) treatment and mild HCl were also used to analyse antigenicity of banana prawn. Analyses of tropomyosin antigenicity of processed prawn samples using immunoblotting and IgG ELISA kits, showed that antigenicity was dependent on processing or treatment of banana prawn. The study confirmed the actual weight of tropomyosin (37 kDa), implementing a new technology of unstained gel.

This chapter titled “Effects of selected processing treatments on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) tropomyosin” by Md Faisal, Todor Vasiljevic & Osaana N. Donkor, has been published in the peer review journal, “International Journal of Food Science & Technology” 54, 183-193, and included in this thesis with permission of Wiley.

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Effects of selected processing treatments on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) tropomyosin

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Summary Different processing techniques were assessed to attenuate the antigenicity of banana prawn tropomyosin (*Fenneropenaeus merguiensis*). Frying and boiling at different temperatures, acid treatment using different acids and storage at −20 °C up to 3 months were investigated. Untreated prawn sample was used as a control. Frying significantly increased antigenicity (6–8 times) in temperature-dependent manner, whereas the trend was fundamentally reversed with boiling. Boiling at 121 °C resulted in the lowest antigenicity (12.99 mg mL^{-1}) among all heat-treated samples, yet higher than the control (5.06 mg mL^{-1}). Freezing had initially very minor impact, although prolonged storage at −20 °C increased antigenicity slightly (2.29 mg mL^{-1}) compared with control. Antigenicity was impacted the most by a reduction in pH independent on the type of acid as both acetic and HCl acids significantly reduced antigenicity of tropomyosin by ~90% compared with control. This could be considered as a new approach to processing that may potentially reduce tropomyosin-derived antigenicity in prawns and prawn products. This study also confirmed the actual molecular weight of banana prawn tropomyosin was 37 kDa implementing a new methodology using unstained gel.

Keywords Acid treatment, banana prawn, freezing, heat treatment, prawn allergy, tropomyosin.

Introduction

Trends towards healthier dieting have made seafood as one of the most attractive foods consumed worldwide in the last few decades (Sicherer *et al*., 2004; Wild & Lehrer, 2005). Within this category, crustaceans (shrimp, crab and lobster) are one of the top eight foods that cause over 90% food-borne allergies with symptoms including respiratory disorders, laryngospasm, asthma, long-lasting gastrointestinal and dermatological symptoms, and even life-threatening anaphylaxis (WHO, 2001; Steensma, 2003; Yu *et al*., 2003). Moreover, prawn allergy in children and adults affects more than 2% of the global population, which in turn creates massive socio-economic problems to contemporary society (Mullins *et al*., 2007; Lopata *et al*., 2010; Rahman *et al*., 2010).

The main prawn protein recognised for induction of allergy is tropomyosin (TM), a muscle protein having a molecular weight of 34–39 kDa (Hoffman *et al*., 1981). Other allergenic proteins, including myosin light chain (MLC), myosin heavy chain (MHC), sarcoplasmic calcium-binding protein (SCP) and arginine kinase (AK), have also been identified recently (Ayuso *et al*., 2009; Fernandes *et al*., 2015). Tropomyosin is a heat-stable and water-soluble protein that constitutes up to ~20% of muscle protein and belongs to the family of actin filament-binding proteins. Different isoforms of this protein regulate muscle contraction through calcium sensitive interactions between actin and myosin (Hoffman *et al*., 1981; Rahman *et al*., 2010). It has been characterised as a highly conserved myofibrillar protein composed of two identical subunits, each in α-helix formation coiled around one another (Shanti *et al*., 1993).

Tropomyosin is responsible for over 80% of all prawn allergy-related incidences (Chapman *et al*., 2006; Troiano, 2016). It is termed a major allergen not only in prawns but also in other crustaceans (crabs, lobsters, krills), molluscs (squids, octopus, abalone, snails, clams) and invertebrates (cockroaches, house dust mites), showing 90% homology (Chapman *et al*., 2006; Motoyama *et al*., 2007). On the other hand, MLC, AK and SCP are also heat-resistant, but their IgE binding greatly decreases due to alteration of their

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secondary structure during processing (Ayuso *et al.*, 2009; Kamath *et al.*, 2014; Zhang *et al.*, 2015).

Banana prawn (*Fenneropenaeus merguiensis*) is one of the most popular and widely distributed prawns in the Indo-Pacific region (Held *et al.*, 2011). Prawns are processed by different methods prior to consumption to foremost achieve required level of safety and preservation and then to improve nutritional properties and palatability (Liu *et al.*, 2010; Lepski & Brockmeyer, 2013). In general, various processing methods (boiling, frying, freezing, microwave roasting, steaming, grilling, high-pressure treatment, radiation and ultrasound) result in certain structural protein modifications including unfolding, crosslinking between structural elements and aggregation, and chemical alterations such as oxidation and glycosylation of some amino acid residues (Yamagata & Low, 1995; Liu *et al.*, 2010; Lepski & Brockmeyer, 2013). In addition, processing of prawns may destroy existing epitopes or generate new ones (neoallergen formation), mask or unmask epitopes in the protein structure, which would directly affect antigenicity (Lehrer *et al.*, 2003; Taylor, 2008).

Previous studies (Rahman *et al.*, 2010; Kamath *et al.*, 2013, 2014; Lasekan & Nayak, 2016) on prawn species (tiger, vannamei and king prawns) have reported on the effect of a single temperature heat treatment on antigenicity; however, effects of sequential temperature changes in frying or in boiling have not been studied in prawns. Moreover, a study is missing that would establish an impact of frozen storage or mild acid treatment on antigenicity of prawn protein. The aim of this study therefore was to establish the impact of different processing methods and identify the most suitable processing technique to minimise tropomyosin-derived antigenicity in banana prawn.

Materials and methods

Extraction of protein from untreated prawn

Fresh banana prawns (*Fenneropenaeus merguiensis*) were supplied by a local retailer (Woolworths, Melbourne, Australia). The prawns were washed in distilled water for 2–3 min to remove external contaminants, after which the outer shells were removed and deveined using the tip of a sharp blade. The extraction of prawn protein followed an established protocol (Kamath *et al.*, 2013, 2014) with some modifications. Muscles were cut into smaller pieces and homogenised in a phosphate-buffered saline solution (pH 7.4) at 1:3 ratio using a laboratory blender (Waring 8011ES, NJ, USA) for 5 min. The slurry was agitated for 3 h at 4 °C, followed by centrifugation (Beckman Coulter Avanti J-26S XPI centrifuge, CA, USA) at 29 400× $\boldsymbol{g}$ for 15 min at 4 °C. The supernatant of the mixture was carefully separated using a micropipette and stored in a sealed labelled container at −80 °C as the control.

Sample treatment and protein extraction

Fresh prawn samples were washed with distilled water, then deshelled and deveined prior to all treatments. Prawn muscles were boiled at four different temperatures (100, 114 and 117 °C) using a Multicooker (Breville Fast Slow Pro, Sydney, NSW, Australia), while an autoclave (Atherton, Melbourne, Vic., Australia) set at 121 °C was used for boiling. In all cases, the samples were subjected to a 15-min heat treatment. For the frying process, prawn muscles were submerged in hot Sunflower seeds oil at 150, 170 or 190 °C, respectively, for 5 min each in a VersaCook™ cooker (Electronic Multi Cooker X5, Sydney, NSW, Australia). Prawn muscles were submerged in acetic acid (white vinegar) and hydrochloric acid (HCl) separately at pH 2.5 for 15, 30 or 45 min, respectively. Further treatment of fresh prawn muscles was subjected to slow freezing and then stored at −20 °C for 72 h, 1, 2 or 3 months. Furthermore, prawn proteins were also treated with a wide range citric acid-phosphate buffer (pH 2.2–7.45) to elucidate the effect of different pH levels on antigenicity. Proteins for each treatment were then extracted following the similar method described for the control sample. After extraction, protein samples were stored in sealed labelled containers at −80 °C for further analysis.

Determination of protein content

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

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE under reducing conditions was performed to visualise the total protein repertoire in the prepared extracts (Rahaman *et al.*, 2016) with slight modifications. Briefly, protein extract (2 mg mL^{-1}) was diluted 1:1 with Laemmli buffer (containing 5% of 2-mercaptoethanol) and heated for 3 min at 95 °C. Exactly 12 μg of protein was loaded onto each well in a 4–20% Mini-Protean TGX Stain-free Precast gel

(Bio-Rad Laboratories). Electrophoretic separation was achieved according to a Bio-Rad recommended process. Precision Plus Protein Unstained Standard was used as a molecular weight marker (MWM). Separated protein bands on the gel were visualised by activating protein bands for 2.5 min, and gel images and quantification were accomplished using a Chemi-Doc imager (Chemidoc MP, Bio-Rad).

Immunoblot analysis

The method used by Kamath *et al*. (2014) for immunoblot analysis was followed with some modifications. Proteins resolved by the SDS-PAGE were transferred to an activated 0.2 μm polyvinylidene fluoride (PVDF) membrane for immunoblotting to immobilise them during antibody treatment using a Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V for 7 min. The membrane was placed in a box (12 × 8.5 × 7) cm and washed 2 times in 30 mL phosphate-buffered saline containing 0.1% Tween-20 (PBST) with constant shaking at 150 r.p.m. (Ratek, Orbital mixer, Melbourne, Vic., Australia) for 5 min each. After this step, the immunoblot was incubated with a blocking buffer (5% w/v nonfat dry milk in PBST) at 4 °C for 2 h and then further incubated with an Anti-Tropomyosin antibody (MAC 141, Abcam Australia Pty Ltd, Melbourne, Vic., Australia). The Anti-Tropomyosin antibody was diluted at 1:3000 with 2.5% w/v nonfat dry milk in PBST and kept overnight at 4 °C under constant mixing at 150 r.p.m. After rinsing the membrane 5 times with PBST for 5 min per rinse, it was incubated with Goat Anti-Rat IgG H&L HRP pre-adsorbed (Abcam Australia Pty Ltd) and diluted 1:3000 in PBST for 2 h at 4 °C. IgG binding was detected using enhanced chemiluminescence (Thermo Pierce ECL Western Blotting Substrate), and images were captured by Chemi-Doc imager (Chemidoc MP, Bio-Rad).

Determination and confirmation of molecular weight of tropomyosin

To determine the actual molecular weight of tropomyosin using unstained gel, the SDS-PAGE was performed at 200 V for 45 min with untreated prawn protein extract in lanes 2–6 and precision plus protein unstained standard was used as a molecular weight marker (MWM) in lane 1 (Fig. 1). Target protein bands having molecular weight 34, 37 and 40 kDa were cut and removed from lanes 3, 4 and 5, respectively, whereas in lane 6, all the three bands (34, 37 and 40 kDa) were cut and removed. Lane 2 retained all three bands (34, 37 and 40 kDa) as a control. Figure 1 depicts the description of the methodology used for the determination of the actual molecular weight of tropomyosin.

Enzyme-linked immunosorbent assay (ELISA) kit

The presence of tropomyosin was tested in the extracted protein samples using a sandwich ELISA test kit according to the manufacturer's instructions (RIDASCREEN®FAST Crustacean, Tropomyosin, R-Biopharm, Darmstadt, Hesse, Germany). Each sample was tested separately and replicated. Briefly, 1 mL extracted protein sample was mixed with 19 mL of preheated (60 °C) allergen extraction buffer. The solution was shaken vigorously, incubated at 60 °C for 10 min in a water bath, cooled on an ice bath, and centrifuged at 2500× ***g*** (Eppendorf centrifuge 5810 R, Hamburg, Germany) for 10 min. An aliquot of 100 μL sample supernatant was transferred into each microwell pre-coated with the antibody. After 10 min of incubation at room temperature, the liquid was then poured out of the wells by turning the plate upside down on absorbent paper and tapping it vigorously three times in a row. The plate washed three times with 250 μL wash buffer. An aliquot of 100 μL conjugate solution was added to each well, shaken gently to mix, and incubated at room temperature for 10 min. The microwell was washed again three times. After that 100 μL of chromogen was added to each well, shaken gently and incubated in the dark at room temperature for 10 min to develop the blue colour. Finally, 100 μL of stop solution was added to each well to halt the reaction and absorbance reading was taken within 10 min using an ELISA plate reader (iMark microplate absorbance reader, Bio-Rad, Tokyo, Japan) at 450 nm.

Statistical analysis

Results were analysed using a general linear model (GLM) procedure of the Statistical Analysis System (SAS), version 9.2. The experiments were arranged in a randomised block design with treatments as the main factor, while the replications served as a block. The effect of different processing techniques on the antigenicity of prawn was considered to be significant at $P \leq 0.05$.

Results

SDS-PAGE protein profile in prawn samples

Control

The SDS-PAGE protein profile of banana prawn shows multiple bands between 10 and 250 kDa (Fig. 2). In case of the control, 14 protein bands were present, of which 20, 34, 37, 40, 75 and 90 kDa were prominent, with 25, 30, 45, 55, 105, 175 and 213 kDa less visible; however, one protein band over 250 kDa was also noticeable. Protein bands of molecular weights 10 and 14 kDa were not present in the control.

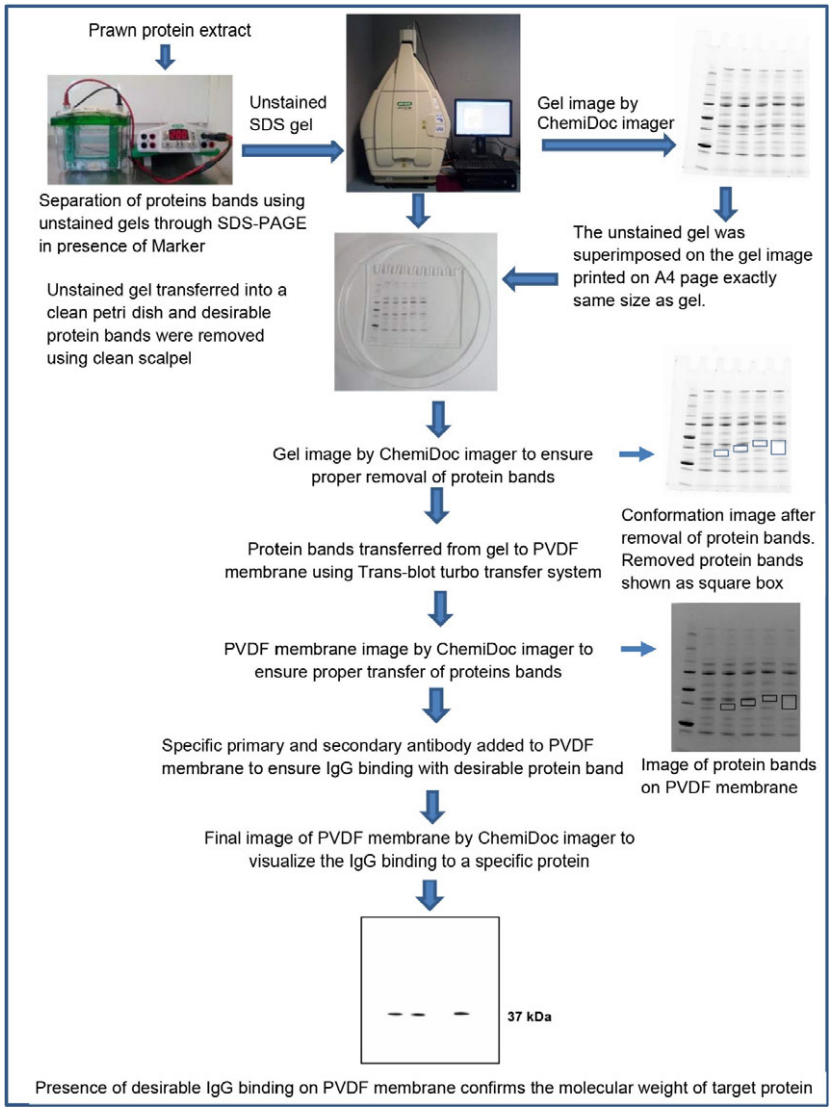

Figure 1 A flow chart for the determination of molecular weight of target protein using unstained gel. [Colour figure can be viewed at wileyonlinelibrary.com]

Heat-treated samples

For fried prawn sample extracts, protein bands did not appear sharp on the SDS-PAGE. Only seven protein bands with molecular weights 10, 14, 20, 34, 40, 42 and 75 kDa were slightly visible. The trend was similar for all three frying treatments in Fig. 2. Protein profile for boiled samples was similar to those of the fried protein extracts; however, seven of the protein bands were noted within the range of 10–250 kDa but less visible in comparison with the fried samples. Boiled prawn protein samples at 121 °C which is showing significantly lighter ($P \leq 0.05$) band intensity compared with the other bands.

Acid-treated samples

For acid-treated samples, the final pH range reached in the extracts was between 4.7 and 4.9. Acetic acid-treated samples, and mild HCl acid-treated samples are shown in Fig. 2. Prawns treated with acetic acid for 15 min separated into ten protein bands but extending treatment up to 30 or 45 min resulted in a decreased number of bands to nine in both cases. Among these protein bands, 20, 34, 40 and 75 kDa were more prominent in intensity, whereas 25, 45 and 60 kDa were less intense and 175, 213 and above 250 kDa were significantly lower in intensity compared to other bands. At all three time-points, protein bands

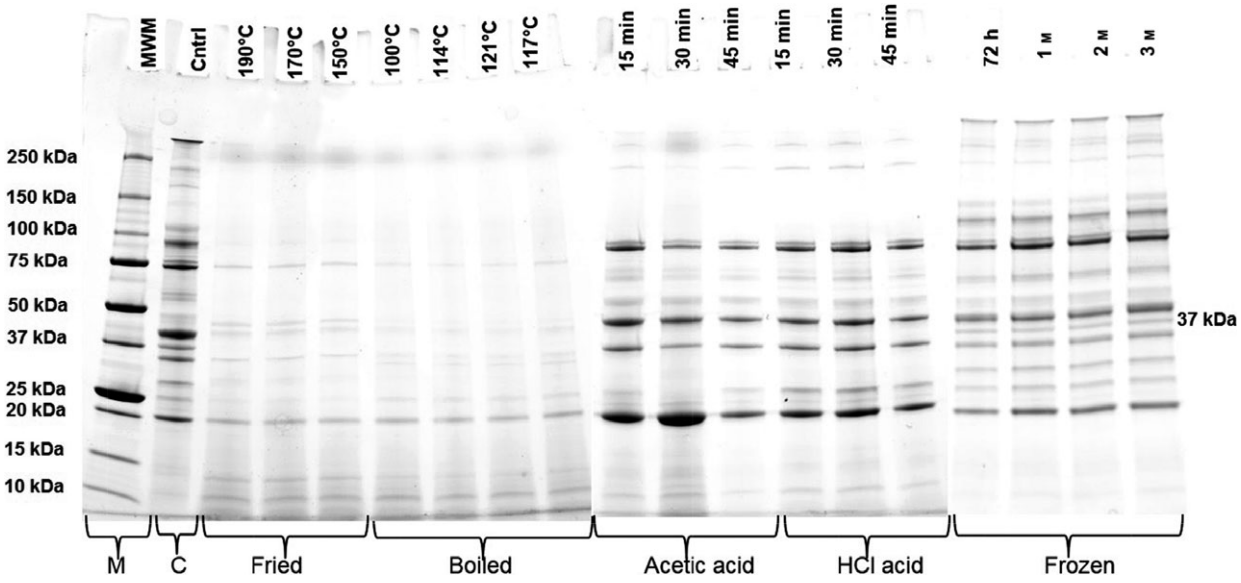

Figure 2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of control, fried, boiled, acetic acid, HCl treated and frozen prawn protein extracts.

with a molecular weight of 37 kDa were clearly absent. HCl-treated samples showed similar protein bands and intensities. On the other hand, proteins treated with citric acid-phosphate buffer (wide pH range of 2.2–7.45) (Fig. 4a) followed a similar protein profile as those obtained by treatment with acetic and HCl acids.

Frozen samples

Bands of the frozen prawn protein samples represented in Fig. 2 showed a similar pattern to the control sample although the intensity of the bands was slightly greater ($P \geq 0.05$).

Immunoblotting protein profile in control and treated prawn extracts

The monoclonal antibody binding of prawn protein profiles in the control and extracts of treated samples using immunoblotting are shown in Fig. 3. In the case of control samples, a single band was observed at 37 kDa. However, the single band intensity at 37 kDa

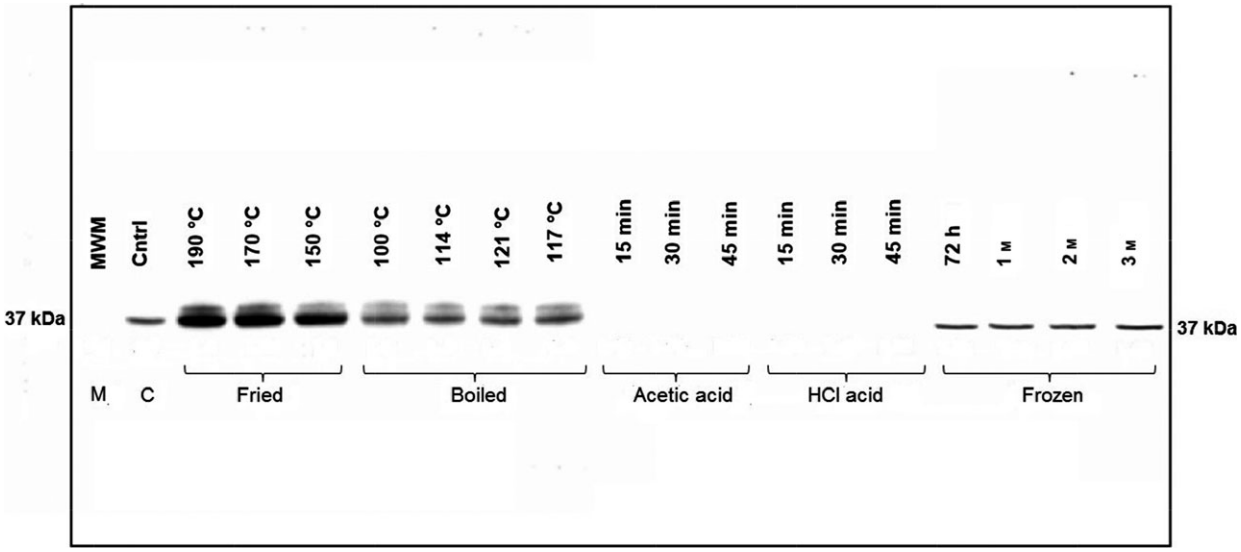

Figure 3 Immunoblotting of control, fried, boiled, acetic acid, HCl treated and frozen prawn protein extracts.

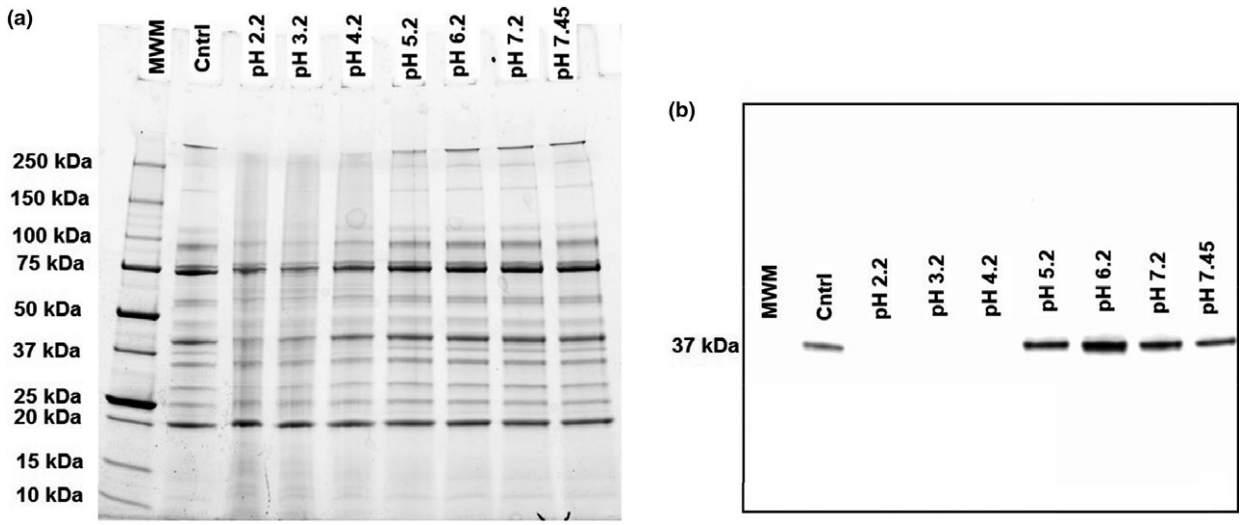

Figure 4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (a) and Immunoblotting (b) of wide pH range (2.2–7.45) buffer-treated prawn protein extracts.

for fried samples substantially increased with the rise in temperature. The temperature of boiling also impacted on antibody binding pattern since boiling at 100 and 114 °C resulted in high band intensities, whereas at 117 and 121 °C, these bands were less pronounced, yet higher than the control. After both treatments (boiling and frying), each band at 37 kDa showed a subband with slightly higher molecular weight.

No protein band was observed for samples treated with acetic and HCl acids, respectively (Fig. 3). Moreover, this study has also shown that the protein having a molecular weight (37 kDa) also disappeared on the SDS gel and PVDF membrane at pH lower than 5.0 using a wide range (pH 2.2–7.45) citric acid-phosphate buffer (Fig. 4a,b).

Frozen prawn protein extracts for the samples stored at −20 °C for 72 h show a single band (37 kDa) with a slightly increased intensity compared to the control, whereas a gradual increase in band intensity at 37 kDa for the samples stored at −20 °C for 1, 2 and 3 months, respectively, represented in Fig. 3.

Determination and confirmation of tropomyosin molecular weight in control

Unstained SDS-PAGE and immunoblotting protein profile for the determination and confirmation of actual molecular weight of tropomyosin are shown in Fig. 5a-c. After immunoblotting, single bands were

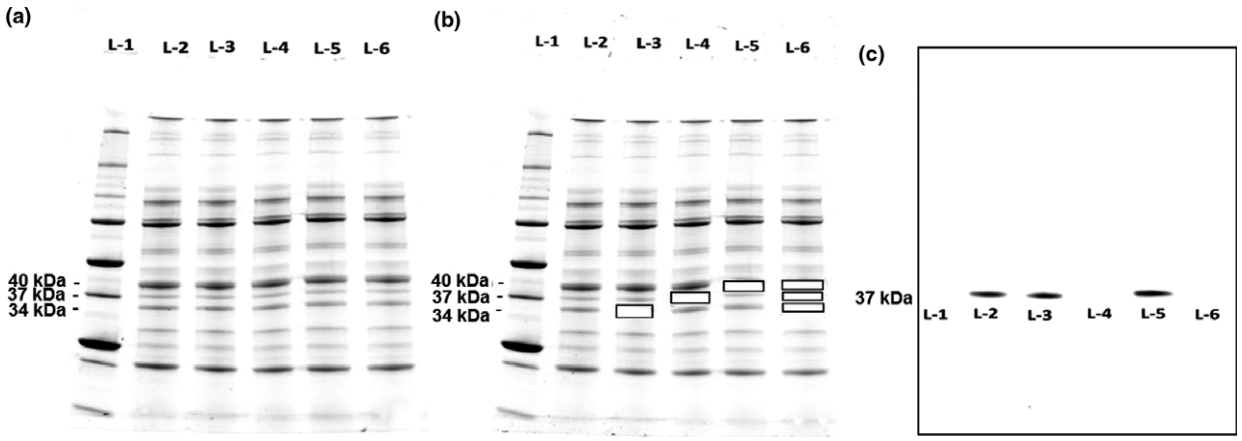

Figure 5 Confirmation of molecular weight of tropomyosin by SDS-PAGE and Immunoblotting. SDS-PAGE of untreated prawn protein extract (a); Here L-1: MWM, L-2 to L-6: untreated prawn protein extract. Protein bands cut and removed (L-3: 34 kDa, L-4: 37 kDa, L-5: 40 kDa, L-6: 34, 37 and 40 kDa) shown as square boxes on SDS-PAGE (b). Immunoblotting profile of untreated prawn protein extract (c).

Table 1 Antigenicity of treated samples assessed by ELISA

Treatments	Condition	Antigenicity (mg mL^{-1})	Treatments	Condition	Antigenicity (mg mL^{-1})	Treatments	Condition	Antigenicity (mg mL^{-1})
Control		5.06^{a}	Control	–	5.06^{a}	Control	–	5.06^{a}
Frying for	190 °C	40.93 ^{h}	Acidification by	15 min	0.43^{b}	Freezing at −20 °C	72 h	5.27^{a}
5 min	170 °C	39.40 ^{g}	acetic acid	30 min	0.44^{b}		1 month	5.84^{a}
	150 °C	31.50^{f}		45 min	0.43^{b}		2 months	5.95^{a}
Boiling for	100 °C	19.70^{e}	Acidification by	15 min	0.73^{b}		3 months	7.35^{b}
15 min	114 °C	19.35^{d}	HCl	30 min	0.67^{b}			
	117 °C	16.46^{c}		45 min	0.41^{b}			
	121 °C	12.99^{b}						

All values presented are the means for each treatment.
Superscript letters indicate significant difference within a column.

observed at 37 kDa in lanes 2, 3 and 5, whereas no band was observed in lanes 4 and 6 (Fig. 5c).

Quantification of allergen by enzyme-linked immunosorbent assay (ELISA)

Antigenicity in the control, boiled, fried, frozen and acid-treated prawn protein extracts is presented in Table 1. Frying increased antigenicity significantly ($P < 0.05$) (6–8 times) compared with the control. Similarly, boiled samples had antigenicity levels significantly ($P < 0.05$) diminished compared with fried treatments, yet higher than that of the control. On the other hand, antigenicity decreased significantly ($P < 0.05$), up to 90%, after treatment with acetic or HCl acid, compared with the control. Initial freezing had no major effect ($P > 0.05$) on antigenicity after 72 h, 1 or 2 months of frozen storage; however, the samples stored for 3 months had a significantly ($P < 0.05$) higher antigenicity compared to the control.

Discussion

Impact of processing on prawn protein profile

Prawns are usually processed by boiling, frying, freezing or pickling to increase palatability, ensure long shelf-life and improve the safety of the product (Yamagata & Low, 1995; Kumar & Basu, 2001; Liu *et al*., 2010; Lepski & Brockmeyer, 2013). However, thermal treatments have an adverse effect on structural modification of prawn proteins through denaturation, loss of primary, secondary or tertiary structures, formation of new inter or intramolecular structures, aggregation and reorganisation of disulphide bridges. These changes ultimately have either a positive or negative effect on antigenicity (Chatterjee *et al*., 2006; Oliveira *et al*., 2013).

Control

The protein bands on SDS gels for untreated prawn extracts have previously been identified as myosin light chain and sarcoplasmic calcium-binding protein 20 kDa; tropomyosin 37 kDa; arginine kinase 40 kDa; haemocyanin 75 kDa (Yu *et al*., 2003; Rahman *et al*., 2010; Kamath *et al*., 2013). In addition, other proteins having a molecular weight of 25, 50, 55, 65, 90 kDa and higher than 250 kDa were also reported (Yu *et al*., 2003; Rahman *et al*., 2010; Kamath *et al*., 2013), which instigates the need for further identification. In the control, immunoglobulin G (IgG) binds with an antigenic protein of molecular weight 37 kDa on the PVDF membrane in the current study. Previous studies, on the other hand, have detected allergenic components in different species of crustaceans with different molecular weight proteins using patients' sera (Liu *et al*., 2010; Rahman *et al*., 2010; Rosmilah *et al*., 2012; Yadzir *et al*., 2015). Kamath *et al*. (2013) used the similar monoclonal antibody for black tiger prawn proteins and found binding to a protein with a molecular weight of 37 kDa. Tropomyosin contains 284 amino acid residues, with the sequential composition of 17 different types of amino acids (www.uniprot.org/uniprot/D3XNS0). The probable binding epitope of tropomyosin with a monoclonal antibody is present at 9–19 (QAMKLEKDNAM) amino acid sequence determined in black tiger prawn (Kamath *et al*., 2013), which shows a high level of homology to banana prawn (Chakraborty *et al*., 2015).

Heat-treated prawn samples

Heat treatment during frying or boiling likely caused protein structural changes which resulted in the disappearance of most protein bands with molecular weight higher than 45 kDa (Fig. 2), whereas bands below 45 kDa were only faintly visible in the extract. These observations may be linked to fragmentation and denaturation of proteins having higher molecular weight (45–250 kDa). On the other hand, the presence of lower molecular weight protein bands such as MLC, SCP and AK indicated greater heat stability of sarcomere proteins. Fragmentation and denaturation of higher molecular weight proteins and stability of

lower molecular weight proteins exposed to heat processing have also been reported by other researchers (Oliveira *et al*., 2013; Yadzir *et al*., 2015). Proteins unfold and lose secondary and tertiary structures followed by cleavage of disulphide bonds and further formation of inter- and intramuscular interactions and rearrangements of disulphide bonds (Davis & Williams, 1998). These critical changes expose hydrophobic groups and create irreversible changes as well as aggregation in protein structures (Merril & Washart, 1998). The overall effect of prawn protein profile on SDS gel after heat exposure in this study showed similarity to various reported profiles for other prawn and crustacean species (Rahman *et al*., 2010; Kamath *et al*., 2013, 2014; Usui *et al*., 2015; Yadzir *et al*., 2015; Lasekan & Nayak, 2016).

On the other hand, immunoblotting protein profile for heat-treated samples (Fig. 3) shows that a protein at 37 kDa withstood heat treatment. This appeared due to heat stability of tropomyosin structure owing to repeating heptads of amino acid sequences along its length with the maintenance of hydrophobic core inside the structure (Brown *et al*., 2001). In addition, tropomyosin is wound tightly to the surface of actin filament through gestalt-binding interactions, which further provides rigidity (Holmes & Lehman, 2008). The IgG binding after frying has increased in a temperature-dependent manner, likely due to the breakdown of these interactions resulting in structural unfolding and ultimately unmasking of hidden epitopes of tropomyosin. In addition, tropomyosin does not contain any cysteine residues (www.uniprot.org/uniprot/D3XNS0); therefore, the formation of inter- or intradisulphide bonds is prevented, which may have prevented aggregation and thus possibly enhancing antigenicity (Albrecht *et al*., 2009; Kamath *et al*., 2013). On the other hand, boiling decreased antigenicity with the rise in temperature, which is opposite to frying, due to change in protein-solvent interactions as the water was drawn out during frying. Gestalt-binding interactions appear affected by boiling temperature and localised pressure gradients, which seem to have a counteracting effect as rise in temperature tends to minimise while the pressure favours reinforcement of these interactions. Moreover, tropomyosin contains a high number of hydrophilic amino acids, such as glutamic, lysine, arginine and aspartic, rendering it soluble in water during boiling at elevated temperature (Kunimoto *et al*., 2009; Usui *et al*., 2013). The presence of tropomyosin and its fragments during boiling of prawn has also been reported previously (Daul *et al*., 1994). The combined effect of localised pressure and solubility of tropomyosin allowed for greater interactions between tropomyosin and actin (Holmes & Lehman, 2008), likely masking epitopes and minimising antigenicity compared to frying. Lasekan & Nayak (2016) similarly reported a decline in IgE binding upon boiling at 100 and 121 °C of *Penaeus monodon* shrimp. In addition, the formation of subfragments close to 37 kDa in both boiled and fried extracts may have indicated interaction of lysine with a reducing sugar in prawn muscle through Maillard reaction (Wegrzyn & Fiocchi, 2009; Kamath *et al*., 2013; Zha *et al*., 2015).

Acid-treated prawn samples

Acetic acid- and HCl acid-treated prawn extracts rendered proteins more soluble at lower pH resulting in fewer protein bands on the SDS gel between 10 and 250 kDa compared with the control (Fig. 2). However, those that appeared had increased intensities at 20 kDa (myosin light chain and sarcoplasmic calcium-binding protein), 34, 40 (arginine kinase) and 75 kDa (haemocyanin) indicating possible accumulation of these proteins. In addition, accumulation of 20 and 40 kDa proteins after 30 min of acid treatment demonstrates further aggregation of protein due to noncovalent, especially hydrophobic, attractions and creation of hydrogen bonds (Xu *et al*., 2012).

The degree of protein disappearance and solubility for the samples treated with acetic and HCl acid (Fig. 3) is directly related to hydrophobic and electrostatic interactions. Below isoelectric point (pI), electrostatic repulsion is greater than hydrophobic attraction, ultimately enhancing the solubility of proteins (Mohan *et al*., 2007). To confirm this behaviour of acids on proteins and disappearance of 37 kDa below pH 5.0, prawn proteins were treated with a wide range (pH 2.2–7.45) citric acid-phosphate buffer (Fig. 4a,b). As the pI of 37 kDa protein is about pH 5.0, a shift in the overall electropotential of this protein below its pI was likely the cause of lack of immunoreactivity after acid treatment.

Frozen prawn samples

Freezing is widely used to retard spoilage of seafood, yet biochemical changes such as deterioration of muscle, structural changes of actomyosin complex, exposure of hydrophobic residues and ATPase enzyme activity continue during frozen storage (Inoue *et al*., 1992; Hossain *et al*., 2004; Fennema, 2008). The increased intensity of protein bands (Fig. 2) likely appeared due to the activity of an ATPase. This enzyme acts on higher molecular weight proteins during storage and gradually denatures them, producing free amino acids and peptides, which ultimately increased band intensity of lower molecular weight proteins (Fukuda, 1984; Jiang & Lee, 1985; Inoue *et al*., 1992). A similar trend in an enzymatic activity possibly exposed binding epitopes which resulted in a gradual increase immunoblotting reactivity with the

37 kDa protein (Fig. 3) during storage. Furthermore, structural changes due to the formation of larger ice-crystals during slow freezing and destruction of hydrated layers surrounding polar residues likely caused freeze-induced time-dependent denaturation of proteins (Hossain *et al.*, 2004) and exposed internal epitopes resulting in increased antibody binding.

Determination and confirmation of molecular weight of tropomyosin

The molecular weight of tropomyosin protein was confirmed (Fig. 5) using a specific antibody, which binds only to the 37 kDa protein. Yadzir *et al.* (2015) reported the molecular weight of tropomyosin as 37 kDa and determined using LC/MS, whereas other researchers have reported different molecular weights of tropomyosin ranging from 32 to 39 kDa (Table 2). We developed a method described in Fig. 1 to determine the molecular weight of banana prawn tropomyosin based on SDS-PAGE and antibodies (Anti-Tropomyosin, MAC 141; Goat Anti-Rat IgG H&L HRP pre-adsorbed). The method identified the location of the protein and corresponding molecular weight using a standard marker.

Quantification of allergen by enzyme-linked immunosorbent assay (ELISA)

The current study used sandwich ELISA quantification of antigenicity in differently processed prawn samples due to its specificity and precision (Shimakura *et al.*, 2005). Results depicting alteration in immunoreactivity of prawn antigen were clearly processing method dependent and similar to immunoblotting (Table 1). The increased intensity of IgG binding using ELISA kit was observed for frying samples in a temperature-dependent manner, whereas a reverse trend was seen for boiling. Similarly, IgG binding to the antigen for frozen samples had an upward trend. The significant decrease in antigenicity achieved by acid treatment was also likely due to partial hydrolysis of proteins, rendering epitopes unavailable. Smaller protein fragments (less than 10 kDa) likely passed through the gel during electrophoretic separation indicated by the absence of a band at 37 kDa (Fig. 3).

Conclusion

Antigenicity of prawn tropomyosin was clearly treatment dependent. Temperature and the presence of water appear important factors in governing levels of antigenicity. Elevating temperature during frying or boiling increases antigenicity by 6–8 times or 2.5–4 times, respectively. Thus, a new processing method is required to produce less antigenic prawn products. Mild acid treatment (pickling) with acetic acid may be considered as application of this approach resulted in significant reduction in, up to 90%, antigenicity. Prolonged storage (3 months or longer) of prawns at −20 °C appears to be detrimental as it increases antigenicity significantly. Considering contemporary food preparation techniques, boiling of prawns at 121 °C for 15 min appears to be more acceptable than other heat processing method, although still 2.5 times greater antigenic than raw prawn.

Table 2 Molecular weight of tropomyosin in different shrimp and prawn species

Species	Molecular weight of Tropomyosin (kDa)	References
Black tiger prawn (*Penaeus monodon*)	36	Lasekan & Nayak (2016); Sahabudin *et al.* (2011)
	34.5	Kamath *et al.* (2013)
	33	Rahman *et al.* (2010)
	38	Kamath *et al.* (2014); Wild & Lehrer (2005)
Banana prawn (*Fenneropenaeus merguiensis*)	34.3	Kamath *et al.* (2013)
Kuruma prawn (*Marsupenaeus japonicus*)	35	Usui *et al.* (2013)
King prawn (*Penaeus latisulcatus*)	32.9	Kamath *et al.* (2013)
	36	Sahabudin *et al.* (2011)
	34	Fuller *et al.* (2006)
North Sea shrimp (*Crangon crangon*)	32.8	Bauermeister *et al.* (2011)
	35	Rahman *et al.* (2013)
White leg shrimp (*Litopenaeus vannamei*)	36	Li *et al.* (2011)
Brown shrimp (*Penaeus aztecus*)	36	Wild & Lehrer (2005)
Indian prawn (*Penaeus indicus*)	34	Wild & Lehrer (2005); Shanti *et al.* (1993)
Greasy back shrimp (*Metapenaeus ensis*)	32.8	Wild & Lehrer (2005)
Sakura shrimp (*Sergia lucens*)	37–38	Shimakura *et al.* (2005)
Green tiger prawn (*Penaeus semisulcatus*)	33	Kamath *et al.* (2013)

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Conflict of interests

The authors declare that they have no conflict of interests.

Author contributions

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

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Chapter 4

Effect of simulated digestion on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) after high pressure processing at different temperatures

Chapter 4 describes the changes in tropomyosin derived antigenicity of banana prawn (*Fenneropenaeus merguiensis*) due to high-pressure processing at 600 MPa at various temperatures. In addition, the impact of digestive enzymes on tropomyosin was also analysed using simulated gastro-intestinal digestion process. Further analysis of tropomyosin antigenicity after high pressure processing and enzymatically digested prawn samples using immunoblotting and IgG ELISA kits, showed that antigenicity was dependent on processing or enzymatic digestion.

This chapter titled “Effect of simulated digestion on antigenicity of banana prawn (*Fenneropenaeus merguiensis*) after high-pressure processing at different temperatures” by Md Faisal, Roman Buckow, Todor Vasiljevic & Osaana N. Donkor, has been published in the peer review journal, “Food Control”, 104, 187-192 and included in this thesis with permission of Elsevier.

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Md Faisal	83	Concept development, research design, experiment execution, interpretation, drafting manuscript and its revision	[redacted]	23-08-19
Dr Roman Buckow	2	Research design, technical support and manuscript revision		23/8/19
Dr Osaana N. Donkor	8	Concept development, research design, support and technical expertise and manuscript revision		23/08/19
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ABSTRACT

Changes in tropomyosin derived antigenicity of banana prawn (*Fenneropenaeus merguiensis*) due to high pressure processing (HPP) at 600 MPa for 5 or 10 min at various temperatures (40, 80, 120 °C) were investigated. HPP of prawn samples at 40 and 80 °C for 5 min increased tropomyosin derived antigenicity by almost double, whereas HPP at 120 °C for 10 min decreased antigenicity by 65%, detected using ELISA kit. A significant ($P \leq 0.05$) reduction of tropomyosin antigenicity after pepsin digestion was noticeable in prawns after HPP, but not in control prawn sample. However, further digestion of the control and HPP sample with pancreatin enzyme decreased antigenicity to ~0 mg mL^{-1}. The combination of HPP and high temperature (120 °C) in the current study can potentially reduce tropomyosin-derived antigenicity in whole prawn muscle, whereas SIF digestion with pancreatin enzyme may present a new prospective method to produce hypo-antigenic, enzymatically digested prawn products.

1. Introduction

Prawn is one of the widely consumed seafood products all over the world due to its delicacy and high nutritional properties (Hoffmann, 2000; Ravichandran, Rameshkumar, & Prince, 2009). It also plays a substantial role in international seafood trade (Oosterveer, 2006) having high economic value. However, it has been declared by World Health Organization (WHO) as one of the eight major sources of food allergens due to its high antigenicity (WHO, 2001). Prawn antigenicity causes mild to severe reactions including life-threatening anaphylaxis and usually persists throughout life (Albrecht et al., 2008). The muscle protein tropomyosin has been identified as the major allergen (Steensma, 2003), although arginine kinase (García, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007), myosin light chain (Ayuso et al., 2008), sarcoplasmic calcium-binding protein (Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008), triosephosphate isomerase and troponin C (Bauermeister et al., 2011) have also been implicated and characterized as minor allergens in prawns. In spite of high allergic incidence, treatments are only available for accidental consumption of prawns and avoidance is the recommended therapy to prevent prawn allergies (Jones, Burks, & Dupont, 2014).

Growing demand for safe, fresh-tasting, additive-free and extended shelf-life of foods, have fostered the development of high pressure processing (HPP), a technology that is used to reduce microbial load but retain flavour, texture, colour and nutritional quality of many foods (Kaur, Rao, & Nema, 2016; Barba, Terefe, Buckow, Knorr, & Orlien, 2015; Briones, Perez-Won, Zamarca, Aguilera-Radic, & Tabilo-Munizaga, 2012). Studies have described several structural and biochemical changes of prawn proteins due to HPP (Bindu, Ginson, Kamalakanth, Asha, & Gopal, 2013; Büyükcan, Bozoglu, & Alpas, 2009; Joseph, Joshy, Bindu, & Kamalakanth, 2017). Moreover, HPP has become one of the best commercial alternatives to traditional heat processing methods for the preservation of prawns, e.g. black tiger prawn (Kaur, Kaushik, Rao, & Chauhan, 2013). In addition, Dang et al. (2017) stated that HPP could become a potential processing method to remove shells of shrimp as well as to prepare ready to eat shrimp. However, impact of HPP on antigenicity of prawn remains unclear.

As a novel technology HPP can change the nutritional and sensory quality suitable for human consumption (Dang et al., 2017; Barba et al., 2015), therefore its impact on antigenicity of prawn need to be studied clearly. Moreover, the changes through gastrointestinal digestion of

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HPP treated prawns has not been studied in-depth. Therefore, the aim of this study was to assess the effect of HPP at 600 MPa applied at different temperatures for 5 and 10 min, respectively, on tropomyosin derived antigenicity of banana prawn protein and its fate during gastrointestinal digestion.

2. Materials and methods

2.1. Treatment and extraction of proteins from prawn

Extraction of proteins from prawn is an important step for isolation and analysis of antigenic components. Fresh banana prawns (*Fenneropenaeus merguiensis*) were collected from a local supermarket in Australia. The prawns were washed for 2–3 min in Milli-Q water to remove external contaminants, after which the external shells were removed and deveined using the tip of a sharp blade.

HPP of whole prawn muscle was performed using a Stansted ISO-LAB FPG11501 High Pressure 3.6 L unit (Stansted Fluid Power Ltd., Stansted, Essex, UK) described elsewhere (Knoerzer, Buckow, Sanguansri, & Versteeg, 2010). 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 is 130 °C. A deionised water/propylene-glycol mixture (40% glycol) was used as the pressure-transmitting medium.

Samples were high pressure (600 MPa) treated separately for 5 and 10 min at 40, 80, or 120 °C, respectively. During high pressure treatment, the vessel was conditioned to an initial temperature, which then attained the target temperature after compression heating (Knoerzer et al., 2010). 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 or 1200 MPa min^{-1}, respectively. The temperature of the compression fluid and sample were monitored using type T thermocouple attached to the sample carrier (Knoerzer et al., 2010). All treatments were replicated on different days. The prawn muscle without any processing used as control.

The extraction of proteins from control and HPP samples were executed as described by Faisal, Vasiljevic, and Donkor (2019). In brief, prawn muscle was homogenized using a laboratory blender (Waring 8011 ES blender, NJ, USA) in phosphate buffered saline solution (pH 7.4) at 1:3 ratio for 5 min. The protein slurry was agitated for 3 h at 4 °C, trailed by centrifugation (Beckman Coulter Avanti J-26S XPI, Palo Alto, CA, USA) at 4 °C and speed of 29,400 × *g* for 15 min. The supernatant of blend (control or HPP samples) was deliberately isolated utilizing micropipette and stored in sealed containers with appropriate labelling at −80 °C until further analysis.

2.2. Determination of protein content

Determination of total protein content of each concentrate was performed by Kjeldahl method. Foss 2020 Digester Unit DS20 and Foss 2012 Distilling Unit (Hillerod, Sweden), were used for sample digestion and distillation respectively.

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The complete protein profile of extracted control and HPP samples was established by using reducing SDS-PAGE as described by Faisal et al. (2019). In brief, 2 mg mL^{-1} extract protein content from prawn sample was diluted 1:1 with 2x Laemmli buffer (containing 5% 2-mercaptoethanol) and heated at 95 °C for 3 min. Precisely 12 μg of protein was added onto each well in a 4–20% Mini-Protean TGX unstained precast gel (Bio-Rad Laboratories, Sydney, NSW, Australia). Electrophoretic separation of protein was accomplished by Bio-Rad prescribed process and Precision Plus Protein Unstained Standard was utilized as a molecular weight marker to highlight the molecular weight of separated protein bands. The protein profile on the gel was visualized through activation by Chemi-Doc imager (Chemidoc MP, Bio-Rad Laboratories).

2.4. Immunoblot analysis

Immunoblotting was performed as described by Faisal et al. (2019). Briefly, protein bands of SDS-PAGE were transferred into Polyvinylidene fluoride (PVDF) membrane utilizing the Trans-Blot Turbo Transfer System (Bio-Rad) as per manufacturer's guideline (Bio-Rad Laboratories). The membrane was blocked using 5% w/v skimmed milk in PBST followed by incubation with Anti-Tropomyosin antibody (MAC 141, Abcam Australia Pty Ltd, Melbourne, VIC, Australia) at 1:3000 dilution with 2.5% w/v skimmed milk in PBST for overnight at 4 °C under steady shaking at 150 horizontal strokes per min (Ratek, Orbital mixer, Melbourne, VIC, Australia). Following washing 5 times with PBST, the membrane was further incubated with Goat Anti-Rat Immunoglobulin-G H&L, HRP preadsorbed (Abcam Australia Pty Ltd) diluted 1:3000 in PBST for 2 h at 4 °C under constant shaking. Finally, chemiluminescence (Thermo Pierce ECL Western Blotting Substrate) was added to membrane to visualise the IgG binding using Chemi-Doc imager (Chemidoc MP, Bio-Rad Laboratories, Sydney, NSW, Australia).

2.5. Enzyme-linked immunosorbent assay (ELISA) kit

Sandwich ELISA (RIDASCREEN®FAST Crustacean, R-Biopharm, Darmstadt, Hessen, Germany) was used to measure the tropomyosin derived antigenicity in extracted protein samples. Each sample was replicated individually following manufacturer instruction. In brief, exactly 1 mL of protein extract was diluted with 19 mL of extraction buffer followed by centrifugation at 2500 × *g* (Eppendorf centrifuge 5810 R, Hamburg, Germany) for 10 min. Exactly 100 μL of sample supernatant was added into each well of antibody pre-coated microwell and incubated at room temperature for 10 min. The microwell was washed three times with 250 μL washed buffer to remove unbound proteins. An aliquot of 100 μL conjugate solution was added to each well, after gentle shaking, the plate was incubated at room temperature for 10 min. After subsequent washing, 100 μL of chromogen was added 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.

2.6. Simulated gastrointestinal digestion

Simulated gastrointestinal digestion of untreated and treated prawn samples was performed using the INFOGEST protocol as described by Minekus et al. (2014) with slight modifications. Briefly, 5 g of prawn sample was blended with 5 mL of simulated salivary fluid (SSF) electrolyte stock solution using a laboratory blender (Waring 8011 ES blender, East Windsor, NJ, USA) for 2 min. The protein slurry was further mixed with 7.5 mL of simulated gastric fluid (SGF) electrolyte stock solution, 1.25 mL pepsin stock solution [10,000 U mL^{-1} (Pepsin, Sigma, MO, USA) in SGF electrolyte stock solution] and 5 μL of $CaCl_2$ (0.3 M). The pH of the mixture was adjusted to 3.0, the volume made up to 20 mL with Milli-Q water, and digested at 37 °C in a rotary shaker (Thermo Scientific MaxQ Shaker, Marietta, OH, USA) at 100 horizontal strokes per min for 2.5 h. Afterward, 20 mL of gastric-chyme was mixed with 11 mL of simulated intestinal fluid (SIF) electrolyte stock solution, 5.0 mL of a pancreatin stock solution [100 mg mL^{-1} (Pancreatin 1X, USB, OH, USA) in SIF electrolyte stock solution], 2 mL of bile stock solution [50 mg mL^{-1} (Bile, Sigma, MO, USA) in SIF electrolyte stock solution] and 40 μL of $CaCl_2$ (0.3 M). The pH of mixture was adjusted to 7.0, and the volume made up to 40 mL with Milli-Q water, and digested at 37 °C in a rotary shaker (Thermo Scientific MaxQ Shaker) at 100 horizontal strokes per min for 2.5 h. After complete digestion Na_2CO_3

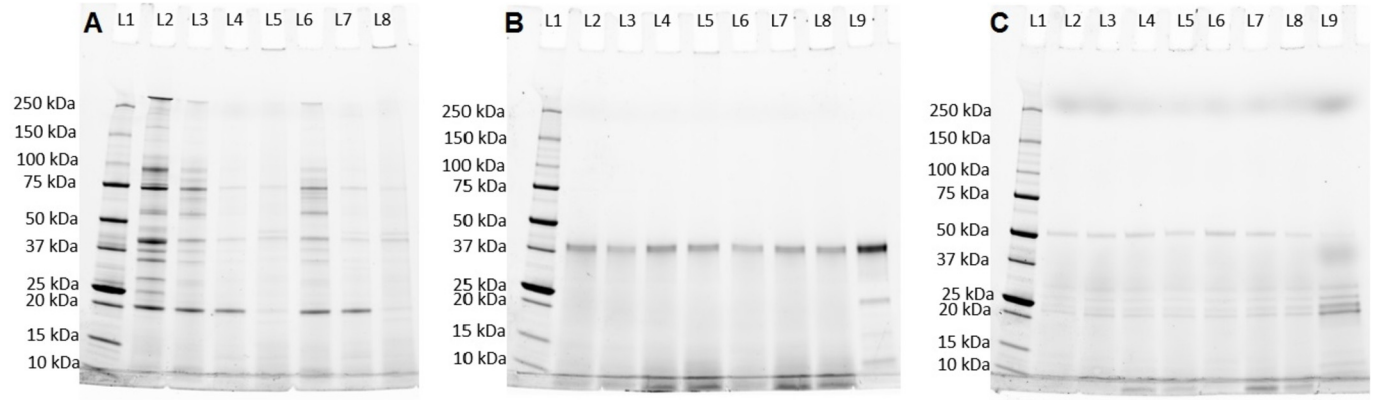

Fig. 1. SDS-PAGE of control and HPP prawn protein extracts (A), SGF (pepsin) digested protein extracts (B), and SIF (pancreatin) digested protein extracts (C): Lane 1 - standard; lane 2- control; lane 3, 4 and 5 - HPP (600 MPa) at 40, 80, and 120 °C for 5 min, respectively; lane 6, 7 and 8- HPP (600 MPa) at 40, 80, and 120 °C for 10 min, respectively, lane 9 - SGF (including pepsin (B) or pancreatin (C) enzyme) without having prawn protein extract.

(0.2 M) was added to solution to inactivate the enzymes and then stored immediately at −80 °C.

2.7. Statistical analysis

Statistical analysis of results was performed using a one way ANOVA by the Statistical Analysis System (v. 9.2). The experimental design was replicated three times. The means were compared using Tukey's Studentised Range (HDS) test. The antigenicity of HPP and enzyme digested samples was considered to be significant at $P \leq 0.05$.

3. Results and discussion

3.1. Protein profile of control and HPP prawn extracts by SDS-PAGE

The SDS-PAGE protein profile of banana prawn shows several protein bands having various molecular weights (Fig. 1A). In the control extract, 14 protein bands were observed of which molecular weights 20, 34, 37, 40, 75, 90 and over 250 kDa were more visible in intensity, whereas 25, 30, 45, 55, 105, 175 and 213 kDa were less potent (lane 2). Protein bands with similar molecular weights in untreated prawn protein extracts have also been reported in banana prawn and other prawn species in previous studies (Faisal et al., 2019; Kamath, Rahman, Komoda, & Lopata, 2013; Wu et al., 2015).

On the other hand, protein extracts of HPP treated prawn samples for 5 min (lanes 3 to 5) showed comparatively less number of protein bands compared to control (Fig. 1A). In lane 3, out of 9 visible protein bands, 20, 40 and 75 kDa bands were prominent, whereas in lanes 4 and 5, only 3 less visible protein bands were present respectively. Moreover protein extracts from the 10 min HPP treatment of prawn samples (lanes 6 to 8) showed similar protein profiles, but with lower intensity compared to the 5 min HPP treatments. In a previous study, Faisal et al. (2019), reported less number of protein bands (7 bands) in banana prawns treated at 100 °C (atmospheric pressure) and autoclaved pressure (121 °C at 0.2 MPa), respectively, for 15 min compared to the control. The probable reason of less protein bands in HPP samples was due to the combined effect of heat and high pressure resulting in disintegration of proteins into smaller molecular weight protein fragments (peptides) (less than 10 kDa). In addition, at high temperature (120 °C) proteins likely start to re-aggregate in the presence of high pressure (600 MPa) resulting in the formation of some higher molecular weight aggregates, which appeared on top of lanes 5 and 8 (Fig. 1A), whereas smaller proteins ultimately pass through the gel into the buffer (Shriver & Yang, 2011). HPP can alter the tertiary structure as well as induce denaturation of proteins by affecting the ionic, hydrogen and hydrophobic bands (Wang, Huang, Hsu, Shyu, & Yang, 2013), whereas, heat can result in changes of the secondary and tertiary structures of protein through changes in inter and intra molecular bonds (Chatterjee, Mondal, Chakraborti, Patra, & Chatterjee, 2006).

3.2. Protein profile of simulated digested prawn extracts by SDS-PAGE

Fig. 1B shows SDS-PAGE protein profiles of the control (lane 2) and HPP prawn extracts subjected to SGF digestion (lanes 3 to 8). Single protein bands of approximately 39 kDa were observed in lanes 2 to 8. The 39 kDa protein band was not from the sample but from the added enzyme (pepsin) as indicated in lane 9. Gámez et al. (2015) also reported the 39 kDa protein band on SDS-PAGE as pepsin enzyme used for SGF digestion. Moreover, protein bands above 39 kDa were completely absent (lanes 2 to 8) indicating breakdown of proteins due to pepsin enzyme activity during SGF digestion. Barrett, Woessner, & Rawlings (2012) reported that pepsin enzymes hydrolyze peptide bonds of tyrosine and phenylalanine residues. Furthermore, the intensity of aggregated proteins at the top of lanes 5 and 8 (Fig. 1B) was also reduced compared to undigested proteins (Fig. 1A) due to enzymatic digestion. In addition, the smearing of protein bands below 39 kDa to less than 10 kDa (lanes 2 to 8) resolved on SDS-PAGE can be attributed to the breakdown of proteins into peptides as well as intramolecular cross-linking, preventing linearization of protein bands with any specific molecular weight (Shriver & Yang, 2011).

Fig. 1C depicts changes in protein profiles after SIF digestion of control (lane 2) and HPP prawn samples treated with pancreatin enzymes, respectively (lanes 3 to 8), compared to undigested (Fig. 1A) and SGF digested protein profiles (Fig. 1B). On SDS-PAGE, one prominent protein band at the 50 kDa mark and 4 less intense bands in between 20 and 30 kDa can be observed in lanes 2 to 8 (Fig. 1C). These protein bands were not from the sample but from the added enzyme (pancreatin) and bile salt as indicated in lane 9. The disappearance of other protein bands (lanes 2 to 8) in Fig. 1C, were likely caused by pancreatin enzymes cleaving proteins at multiple sites including glutamic acid, lysine and arginine (Beck, 1973; Mikita & Padlan, 2007) resulting in complete hydrolysis of proteins into peptides. Smaller protein fragments from digestion, with molecular weight less than 10 kDa, passed through the gel during electrophoretic mobility (Taheri-Kafrani et al., 2009). Jin et al. (2015) treated squid at 600 MPa at 20 °C for 20 min and reported similar degradation of protein bands after SGF and SIF digestion.

3.3. Detection of antigenic tropomyosin in control and HPP prawn extracts

Detection of antigenic tropomyosin in control and HPP treated extracts was performed by immunoblotting. The binding of monoclonal antibody with prawn proteins on PVDF membrane at 37 kDa for control and HPP prawn extracts is shown in Fig. 2A. Faisal et al. (2019)

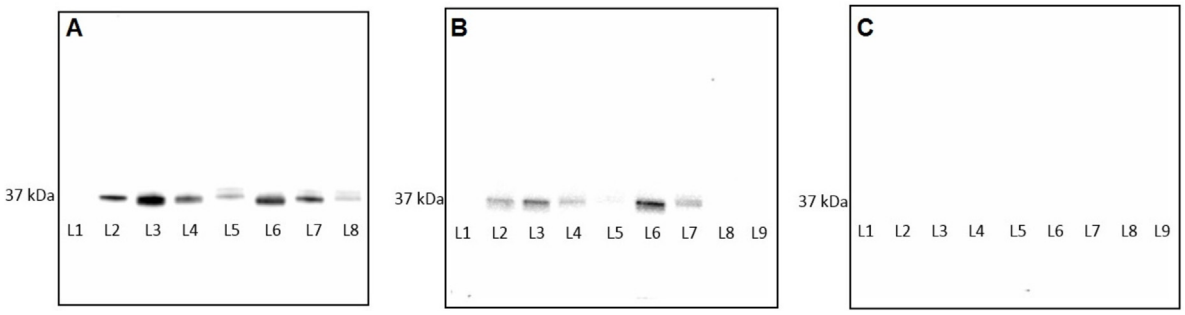

Fig. 2. Immunoblotting of control and HPP prawn protein extracts (A), SGF (pepsin) digested protein extracts (B), and SIF (pancreatin) digested protein extracts (C): Lane 1 - standard; lane 2- control; lane 3, 4 and 5 - HPP (600 MPa) at 40, 80, and 120 °C for 5 min, respectively; lane 6, 7 and 8- HPP (600 MPa) at 40, 80, and 120 °C for 10 min, respectively, lane 9 - SGF (including pepsin (B) or pancreatin (C) enzyme) without having prawn protein extract.

reported similar results for IgG binding (37 kDa as tropomyosin) in untreated banana prawn. The IgG binding for HPP prawns treated at 40 °C for 5 or 10 min, respectively (lanes 3 & 6), showed double band intensity compared to that of the control. On the other hand, HPP prawn samples treated at 120 °C for 5 or 10 min, respectively, showed less band intensity (64 and 48%, respectively) due to fewer IgG binding sites compared to that of the control (lanes 5 & 8), whereas HPP samples treated at 80 °C (5 and 10 min) resulted in similar IgG binding to the control. In a previous study, banana prawn samples were treated at 100 °C atmospheric pressure and 121 °C at 0.2 MPa for 15 min, respectively, IgG binding was reported to be 4 and 2.5 times higher respectively, compared to the control (Faisal et al., 2019). This indicates that combining HPP at 600 MPa with temperature ranging from 40 to 120 °C has a positive impact on reducing antigenicity.

Tropomyosin, having α-helix coiled structure, is twisted tightly to the surface of actin filament through gestalt-binding interactions (Faisal et al., 2019; Holmes & Lehman, 2008). HPP treatment of prawns at 600 MPa is likely to breakdown gestalt-binding interactions in tropomyosin and expose internal binding epitopes. As a result, HPP prawn samples treated at 40 °C showed significantly higher antigenicity compare to the control. Milk proteins treated at 600 MPa at 40 °C for 10 min have been shown to reveal antigenic epitopes resulting in higher antigenicity (Kleber, Maier, & Hinrichs, 2007). On the other hand, Ma, Zhou, Ledward, Yu, and Pan (2011) reported that beef muscle treated at 600 MPa for 20 min showed increasing solubility of myofibrillar proteins (actin, myosin, tropomyosin and troponin) with increasing temperature. Similarly, our study showed that at higher temperatures (80 and 120 °C), the solubility of tropomyosin increased due to breakdown of the protein structure, resulting in decreased IgG binding. Furthermore, the treatment time also plays an important role in tropomyosin solubility. For example, IgG binding was lower in samples with high pressure treated for 10 min compare to those that were treated for 5 min under similar temperature conditions.

Jin et al. (2015) reported that HPP at 600 MPa and 20 °C for 20 min, converted 53% of the α-helix of squid tropomyosin into β-sheets and random coils, resulting in substantial changes of the secondary structure and decreased antigenicity. The structural changes likely masked or destroyed binding epitopes within the protein molecule causing less IgG binding. Long, Yang, Wang, Hu, and Chen (2015) investigated a range of high pressure (100–600 MPa) treatments at various temperatures (25–75 °C) on isolated tropomyosin extract from *Litopenaeus vannamei* shrimp and reported low antigenicity for 500 MPa at 55 °C for 10 min. The main reason for the contradiction of this result with the current study is probably due to the use of different treatment conditions. On the contrary, the current study subjected whole prawn muscles to high pressure at various temperatures and found that tropomyosin behaved differently from isolated tropomyosin extracts (Gámez et al., 2015) possibly due to presence of surrounding actin, myosin and troponin molecules within the muscle.

3.4. Detection of antigenic tropomyosin in simulated digested prawn extracts

An immunoblotting method was employed to detect the presence of antigenic tropomyosin in digested samples. IgG binding observed at 37 kDa (tropomyosin) on the PVDF membrane for the pepsin digested control sample is shown in Fig. 2B (lane 2). The resistance of tropomyosin against pepsin digestion is due to the presence of its high lysine content (Li, Zhu, Zhou, & Peng, 2012; Huang et al., 2010). A similar response of tropomyosin of various crustacean species to pepsin has also been reported in several studies (Gámez et al., 2015; Wu et al., 2015). The IgG binding for pepsin digested HPP samples (lane 3) showed increased band intensity, whereas lanes 4 and 5 exhibited decreased IgG binding compared to digested control sample, due to tropomyosin solubility at higher (80 and 120 °C) temperatures. Similar IgG binding trends were observed for lanes 6 and 7, whereas lane 8 (HPP at 120 °C for 10 min) did not show any band indicating non-IgG binding. In comparison to undigested prawn extracts (Fig. 2A), digested prawn extracts (Fig. 2B) showed much lower IgG binding on the PVDF membrane. The combined effect of high pressure and temperature likely caused structural changes and partial denaturation of tropomyosin, thus accelerating pepsin digestion (Mikita & Padlan, 2007). Similar effects have been reported for autoclave treated (121 °C for 20 min) Scylla crab tropomyosin samples (Yu et al., 2011).

The pancreatin enzyme activity on control and HPP prawn samples is shown in Fig. 2C. No IgG binding for tropomyosin was observed on the PVDF membrane. Jin et al. (2015) similarly reported the absence of tropomyosin after 60 min of SIF digestion for samples treated at 600 MPa for 20 min at 20 °C. Moreover Yu et al. (2011) also reported the absence of IgG binding for tropomyosin after 120 min of SIF digestion for autoclaved treated (121 °C at 0.14 MPa for 20 min) crab sample. The authors (Yu et al., 2011) further showed that tropomyosin and its fragments were still detectable by immunoblotting after 240 min of digestion for boiled (100 °C for 20 min) sample, indicating the impact of pressure on tropomyosin degradation during SIF digestion.

3.5. Quantification of antigenicity by ELISA

The quantification of antigenicity in control and HPP prawn samples is shown in Fig. 3. HPP samples treated at 40 and 80 °C for 5 or 10 min, respectively, showed significant ($P < 0.05$) increase in antigenicity compared to the control. However, antigenicity of HPP samples treated at 120 °C for 10 min decreased significantly by 65%, similar to trends discussed in immunoblotting of HPP samples. On the other hand, the same control sample subjected to SGF digestion with pepsin enzyme showed no significant ($P > 0.05$) difference for tropomyosin antigenicity. Whereas HPP samples digested with pancreatin enzymes following pepsin digestion resulted in significant ($P < 0.05$) reduction in antigenicity similar to immunoblotting results. HPP samples treated at 120 °C for 10 min digested with pepsin enzyme showed a slight

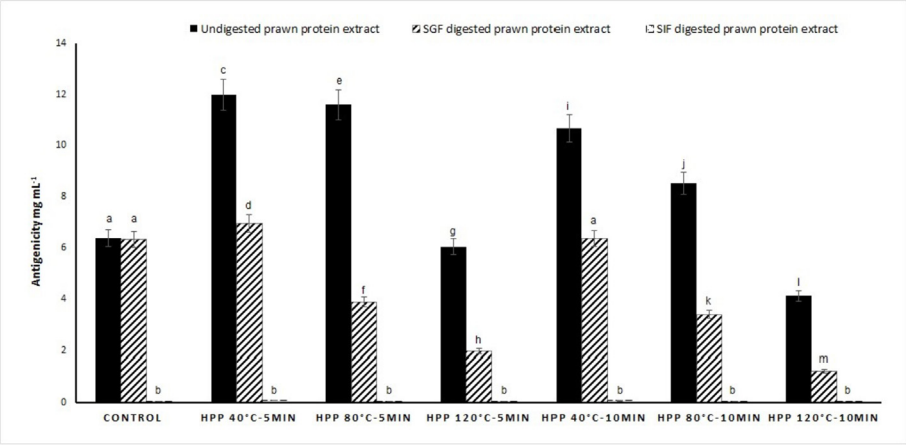

Fig. 3. Quantification of antigenicity of prawn protein extracts before and after digestion (SGF and SIF) using ELISA method for control and HPP samples processed at 600 MPa and 40, 80 and 120 °C for 5 and 10 min, respectively. (■ Undigested prawn protein extract; ▨ SGF digested prawn protein extract, ⊏ SIF digested prawn protein extract). Here the lower cases (a–m) represent significant differences ($P < 0.05$) among the treatments.

deviation compared to immunoblotting. The immunoblotting result for this sample showed complete disappearance of antigenicity, whereas the ELISA results indicated the presence of antigenicity at 1.21 mg mL^{-1}. The cause of this antigenicity is likely due to IgG binding epitopes still active in peptides resulting from enzymatic hydrolysis of proteins. Long et al. (2015) showed a declining trend of IgG binding for squid tropomyosin extract treated with 600 MPa for 10 min compared to 5 min treated samples using inhibition ELISA. The authors further reported that antigenicity of squid tropomyosin extract treated at 600 MPa decreased with increasing temperature treatment from 25 up to 75 °C.

4. Conclusion

Prawn muscles treated with 600 MPa at 40 and 80 °C for 5 or 10 min showed significant ($P < 0.05$) increased antigenicity, whereas samples treated at 600 MPa and 120 °C for 10 min decreased antigenicity by 65% compare to control. Therefore, the combination of high pressure (600 MPa) and temperature (120 °C) can potentially reduce tropomyosin-derived antigenicity in whole prawn muscle. On the other hand, prawn muscles digested in presence of pepsin enzyme showed more than 50% reduction of tropomyosin antigenicity for HPP samples, yet no significant difference for the control sample. Moreover, further digestion with pancreatin enzymes decreased antigenicity of tropomyosin up to ~0 mg mL^{-1} for control and HPP prawn samples. This potential reduction indicates digestion with pancreatin enzymes can possibly open new opportunities to produce hypo-tropomyosin-antigenic, enzymatically digested prawn protein powders. This hypo-antigenic prawn protein powders can be used as prawn seasoning in different meals, as well as flavour and taste enhancer in various biscuits, cakes, and other snack products.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Chapter 5

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

Chapter 5 describes the high-pressure processing (600 MPa) at 120 °C and mild acetic acid treatment for 15 min assessed to attenuate the antigenicity and immunogenicity of banana prawn (*Fenneropenaeus merguiensis*). The protein fractions were separated and isolated using preparative HPLC, and their antigenicity was analysed using Immunoglobulin G (IgG) ELISA kits. Immunogenicity was performed using human PBMC and further analysed using Immunoglobulin E (IgE) ELISA kits. The allergenic fractions were finally characterized using LC/MS/MS.

This chapter titled “Immunomodulatory properties of selectively processed prawn protein fractions assessed using human peripheral blood mononuclear cells” by Md Faisal, Narges Dargahi, Todor Vasiljevic & Osaana N. Donkor, has been published in the peer review journal, “International Journal of Food Science and Technology” 55, 795-804 and included in this thesis with permission of Wiley.

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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

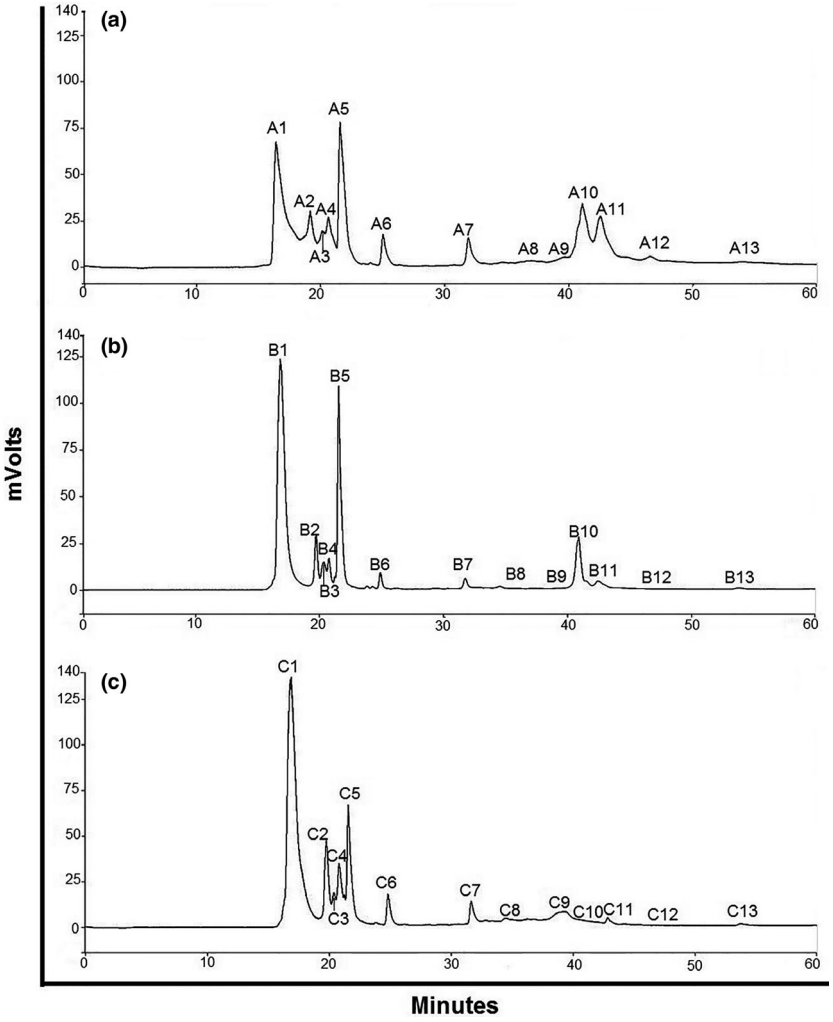

Figure 1 HPLC protein profile of untreated (a); acetic acid treated (b); and HPP at 120 °C (c) prawn protein extracts.

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).

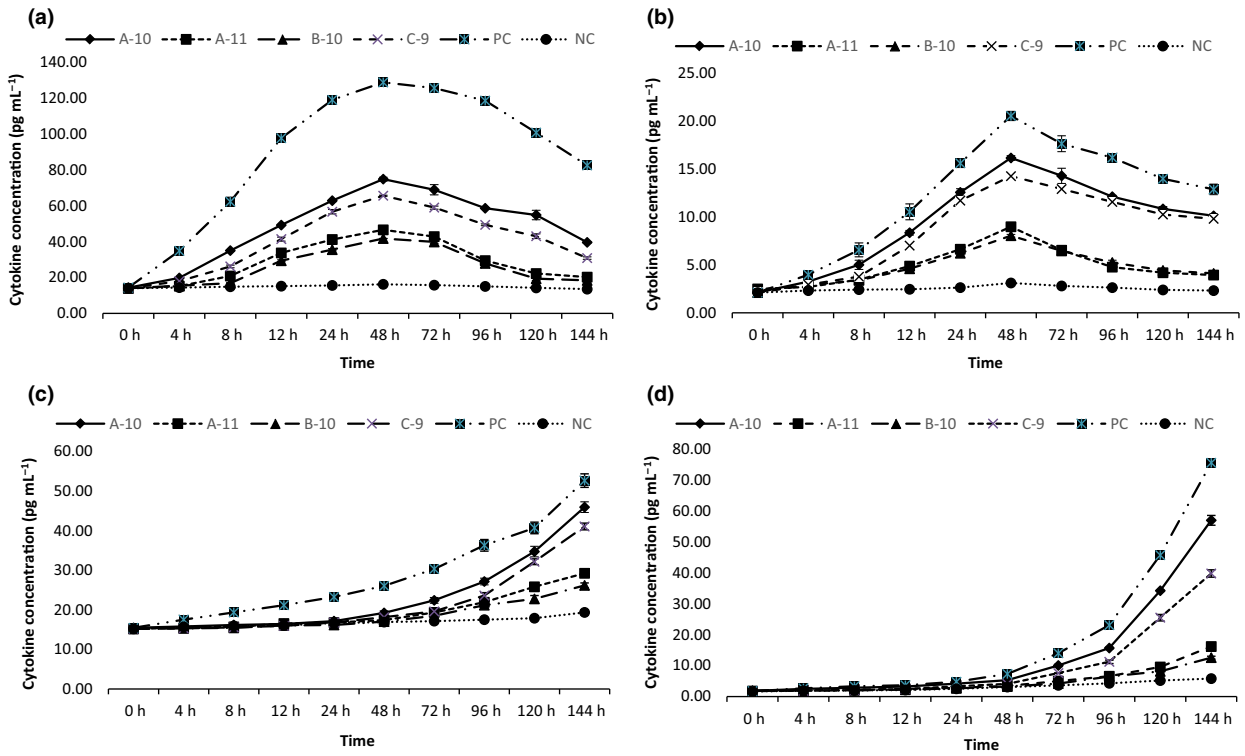

Figure 2 Changes in concentrations of cytokines in treated and untreated fractions with time – anti-inflammatory IL-10 (a) and IL-4 (b); pro-inflammatory cytokines – IFN-γ (c) and IL-17A (d). A10 and A11 from control; B10 from acetic acid treated; C9 from HPP at 120 °C treated; PC means positive control and NC means negative control. [Colour figure can be viewed at wileyonlinelibrary.com]

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 ENKKIEMELE 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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Chapter 6

Conclusions and Future Research Directions

6.1. CONCLUSIONS

Prawns are one of the top eight foods responsible for over 90% food-borne allergies. Prawns are processed using various techniques to increase palatability as well as for preservation (removal the pathogenic microorganism). The effect of various processing methods (boiling, frying, freezing, high pressure and acidification) on protein profile, antigenicity and immunogenicity were analysed in the current study.

Proteins from the muscle of unprocessed (control) and processed banana prawn were extracted using standard protocol and SDS-PAGE was used to analyse the protein profile. Unprocessed prawn showed fourteen protein bands between 10 and 250 kDa. Whereas, after frying and boiling the number of protein bands (only seven) and intensity sharply reduced due to thermal denaturation and loss of secondary and tertiary structures. Furthermore, prawn muscle treated with acetic acid and HCl also exhibited less number (nine) of protein bands between 10 and 250 kDa. The degree of protein disappearance was directly related to hydrophobic and electrostatic interactions. Prawn muscle frozen and preserved at – 20 °C showed protein profile identical to unprocessed prawn profile.

The tropomyosin antigenicity of processed prawn samples were analysed using immunoblotting and IgG ELISA kits, which showed that antigenicity was dependent on processing or treatment. Elevating frying temperature of prawn increased antigenicity 6 to 8 times, whereas increasing boiling temperature showed inverse effect. Boiling of prawn muscle at 100 °C increased antigenicity 4 times, whereas boiling at 121 °C increased antigenicity 2.5 times compared to unprocessed prawn. Results indicated that temperature and presence of water/moisture, appeared as important factors in regulating antigenicity levels. The increased antigenicity due to thermal treatment resulted in breakdown of gestalt-binding interactions of actin-tropomyosin and unfolding of secondary and tertiary structures, causing the unmasking of hidden epitopes.

Therefore, to produce less antigenic prawn products, a new processing approach namely a mild acetic acid (pH 2.5) treatment is required for a significant reduction in antigenicity by up to 90%. The current study has also revealed that antigenicity of tropomyosin was significantly reduced below the isoelectric point (pI) due to higher electrostatic repulsion than hydrophobic attraction. That ultimately enhanced the solubility of proteins resulting in significant reduction in antigenicity. Prawns are most often frozen and stored at -20 °C and no significant increase in antigenicity is observed up to two months; however, prolonged storage (more than three months) at -20 °C significantly increases prawn antigenicity.

Studies have shown that the molecular weight of tropomyosin range from 33 to 39 kDa for different species of prawns including banana prawn and thus creates confusion regarding actual molecular weight of tropomyosin. The current study on the other hand, devised and implemented a new method to determine the actual molecular weight of tropomyosin (37 kDa), using unstained SDS-PAGE technique.

Protein profile of high pressure processed (600 MPa) extracts in combination with temperature 40 °C showed less number of protein bands (nine) and at 80 and 120 °C, a substantial decrease in the number of visible protein bands (only three) occurred, compare to unprocessed prawn protein profile. In all cases samples treated for 10 min showed faint and less visible protein bands compared to 5 min treated samples. The antigenicity at 40 and 80 °C for 5 or 10 min processed protein extracts significantly increased, whereas samples treated at 600 MPa and 120 °C for 10 min showed reduced antigenicity by 65% compared to control. HPP at low temperature (40 and 80 °C) likely caused the breakdown of gestalt-binding interactions in allergic proteins and exposed hidden epitopes, resulting in increased antigenicity. Whereas at higher temperature (120 °C) in combination with HPP, solubility of allergenic protein increased which resulted in changes in binding epitopes, hence decreased antigenicity.

In addition, pepsin enzyme digested prawn muscles showed significant reduction in tropomyosin antigenicity for HPP samples by more than 50 percent. Furthermore, digestion with pancreatin enzyme has reduced tropomyosin derived antigenicity for control and HPP prawn samples to ~0 mg mL^{-1}. This significant reduction reflects that digestion with pancreatin enzymes may open new opportunities to produce hypo-tropomyosin-antigenic, enzymatically digested prawn protein powders. The hypo-antigenic prawn protein powder can be used in various meals as prawn seasoning and in various biscuits, cakes, and other snack products as a flavour and taste enhancer.

The immunogenicity of HPP (600 MPa at 120 °C for 10 min) and acetic acid (for 15 min at pH 2.5) treated prawn protein extracts were analysed *in vitro* studies using human peripheral blood mononuclear cells (PBMCs). In this regard, 39 protein fractions were isolated of which 4 showed antigenic potentials. The immunogenicity of these processed protein fractions showed significantly reduced pro- (IL-17 and IFN- γ) and anti-inflammatory (IL-4 and IL-10) cytokine production compared to control, resulting in decreased immunogenicity. These four allergenic potential protein fractions were further characterize using an LC/MS/MS, which identified 9 proteins. Among these proteins' tropomyosin is well known as major allergen in different species of prawns and shellfish, whereas arginine kinase, hemocyanin and enolase are reported as minor allergens in prawn muscle. Another two proteins (14-3-3 zeta and vitellogenin) previously identified as minor allergen in banana prawn ovaries. The remaining three proteins (translationally controlled tumour protein, beta-1,3-glucan binding protein, and farnesoic acid O-methyltransferase short isoform protein) require further investigation regarding their immunogenic properties.

6.2. FUTURE RESEARCH DIRECTION

The upward trend of prawn allergy has become increasing public health burden in developed and developing countries, yet treatment of prawn allergy is only available for accidental consumption of prawns or by avoidance. Tropomyosin and other minor allergens are important components of prawn muscle proteins and cannot be removed using biotechnological procedures. Hence, processing of prawn protein is a potential method to alter the structure before consumption to reduce antigenicity. The current research summarizes the impact of thermal, high pressure and acidification to provide in-depth knowledge on antigenicity and immunogenicity of prawn muscles. However, due to time constraint, the impact of other processing techniques, such as ultra-sound, gamma-irradiation, grilling, baking, microwave treatment etc. was not studied.

The antigenicity of prawn tropomyosin significantly increases due to boiling and frying, whereas due to acid treatment it decreased significantly. The combined effect on antigenicity of these two methods (first acidification then boiling/ frying or vice versa) are areas for future research. Moreover, the fate of high pressure processed allergenic proteins after *in simulated* digestion has been studied in the current research, yet the impact of enzymatic digestion of other processing (frying, boiling, freezing, acidification) on allergenic proteins has not been performed due to budget constraints.

The immunogenicity of acetic acid and high pressure treated protein fractions were analysed using human PBMCs. This method also can be implemented to analyse the immunogenicity of other protein fractions (isolated using preparative HPLC) of differently processed prawn protein extracts. In addition, the immunogenicity of these allergenic potential proteins performed *in vitro* using PBMCs, need to be tested on allergenic patients through skin-prick test (SPT) and Double-Blind Placebo-Controlled Food Challenge (DBPCFC) to establish their allergenic efficacy as well as provide in-depth knowledge in immunogenicity. Moreover computational based technique (*In silico* model) may be used as a primary predictor of allergens.

The current study has characterized four allergenic potential protein fractions (out of 39 protein fractions) using LC/MS/MS, detected total 9 proteins. The rest of the protein fractions also need to be analysed to map a complete profile of prawn proteins. Among the detected nine proteins six of these have been already established as allergenic proteins in prawn muscle and ovaries. The antigenicity and immunogenicity of remaining three proteins (translationally controlled tumour protein, beta-1,3-glucan binding protein, and farnesoic acid O-methyltransferase short isoform protein) need to be investigated separately to identify novel allergens.