



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 conflictively "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. Alarming, 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 latissulcatus*, *Crangon crangon*, *Metapenaeus ensis*, *Marsupenaeus japonicus*, *Litopenaeus vannamei*, *Fenneropenaeus merguensis* etc.)

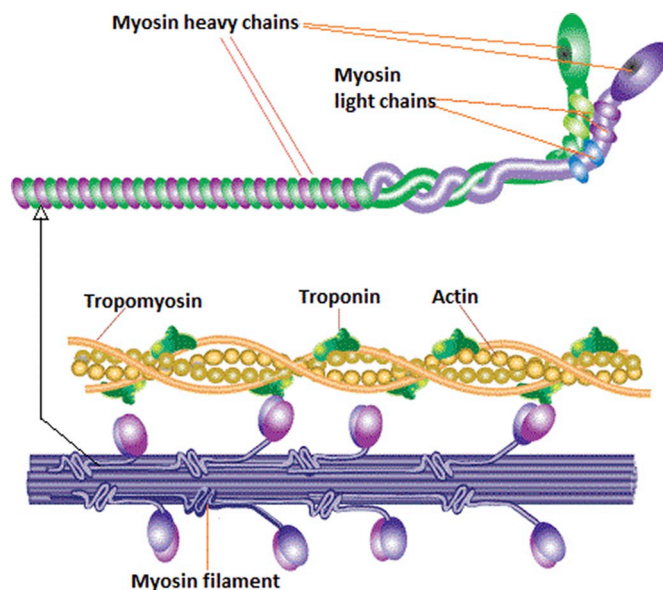


Fig. 1. Tropomyosin (major allergen) having dimeric coiled-coil structure regulates the bindings of myosin light chain, myosin heavy chain, troponin and actin (minor allergens) which helps in muscle contraction (Sigma-Aldrich, 2018).

reported in various studies. Moreover, these methodologies may be used for all prawns' species.

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 (<i>Penaeus monodon</i>)	Tropomyosin (32–39) Arginine kinase (40–42) 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	SDS-PAGE, Immunoblotting, ELISA, LC/MS, HPLC SDS-PAGE, Immunoblotting, LC/MS	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
Kuruma prawn (<i>Marsupenaeus japonicas</i>)	Tropomyosin (35)	SDS-PAGE, Immunoblotting, ELISA, CD Spectroscopy	Usui et al., 2013
King prawn (<i>Penaeus latisulcatus</i>)	Tropomyosin (32.6) Compounds having weight 36, 42, 49 kDa	SDS-PAGE, Immunoblotting, ELISA, LC/MS SDS-PAGE, Immunoblotting	Fuller et al., 2006; Sahabudin et al., 2011; Kamath et al., 2013
North-Sea prawn (<i>Crangon crangon</i>)	Tropomyosin (32–39) Actin (31) Myosin light chain (17–20) Sarcoplasmic calcium-binding protein (20–22) Arginine kinase (40) Myosin heavy chain (98) Glyceraldehyde-3-phosphate Dehydrogenase (37) Triosephosphate isomerase (27) Troponin (16.8)	SDS-PAGE, Immunoblotting, LC/MS, CD Spectroscopy SDS-PAGE, Immunoblotting, LC/MS, CD Spectroscopy	Bauermeister et al., 2011; Rahman et al., 2013
Whiteleg prawn (<i>Litopenaeus vannamei</i>)	Tropomyosin (32–39) Myosin light chain (20) Sarcoplasmic calcium-binding protein (20) Arginine kinase (40)	SDS-PAGE, Immunoblotting, ELISA, HPLC, LC/MS SDS-PAGE, 2D- SDS-PAGE, Immunoblotting, 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
Brown prawn (<i>Penaeus aztecus</i>)	Tropomyosin (36)	SDS-PAGE, Immunoblotting SDS-PAGE, Immunoblotting, ELISA, HPLC	Daul, Slattery, Reese, & Lehrer, 1994; Jeoung et al., 1997; Wild & Lehrer, 2005
Banana prawn (<i>Fenneropenaeus merguensis</i>)	Tropomyosin (34.3–37) Arginine kinase (40) Sarcoplasmic calcium-binding protein (20) Myosin heavy chain type-b (22) Hemocyanin (75) Enolase (47) Glyceraldehyde-3-phosphate dehydrogenase (30)	SDS-PAGE, Immunoblotting, LC/MS, ELISA SDS-PAGE, LC/MS, Immunoblotting	Kamath et al., 2013; Khanaruksombat, Srisomsap, Chokchaichamnankit, Punyari, & Phiriyangkul, 2014; Faisal et al., 2019
Indian prawn (<i>Penaeus indicus</i>)	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 (<i>Metapenaeus ensis</i>)	Tropomyosin (32.8)	SDS-PAGE, Immunoblotting	Leung et al., 1994; Wild & Lehrer, 2005
Sakura prawn (<i>Sergia lucens</i>)	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

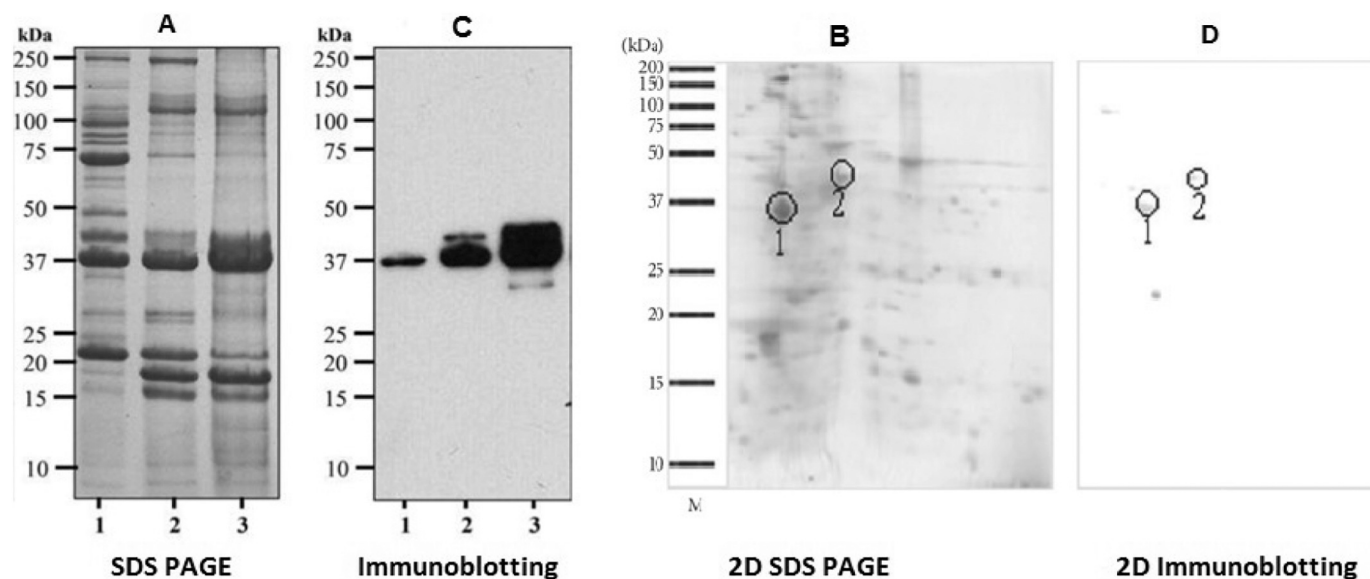


Fig. 2. Prawn protein profile in SDS-PAGE (A), Immunoblotting (B), 2D SDS-PAGE (C) and 2D Immunoblotting (D) (Kamath et al., 2014; Yadzir et al., 2015).

(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 NaHCO₃ 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 destaining 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	Gómez et al., 2015
0.01 M Tris-HCl buffer (pH 6.8) containing 2.5% SDS, 20% glycerol 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
Semi-dry immunoblot apparatus (Bio-Rad, USA)	–	Patient's serum; HRP-conjugated anti-human IgE antibody (Bethyl Laboratories, USA)	–	Usui et al., 2013
	–	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

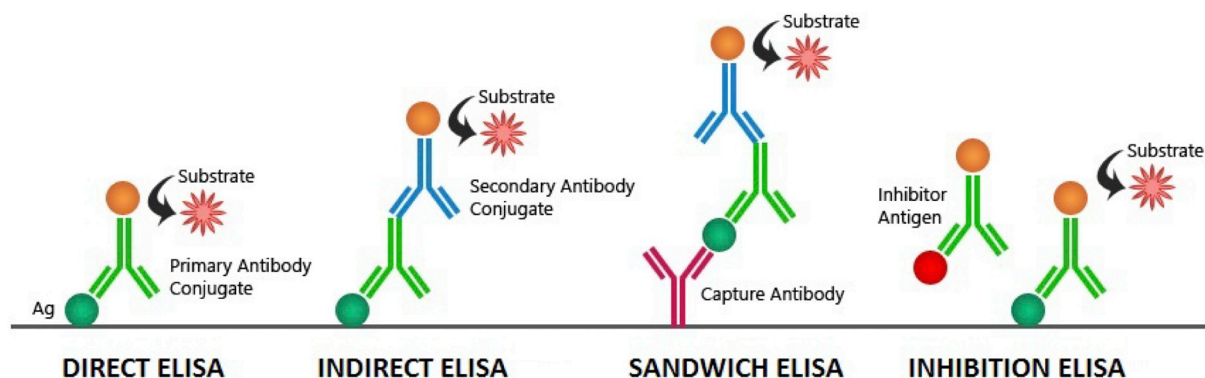


Fig. 3. Basic antigen-antibody binding principles of different types of ELISA (Boster, 2018).

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 Microton ELISA plate is coated with 100 μ L 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 μ L antisera (a primary antibody) added and incubated at 37 °C for 1.5 h. After washing 3 times with PBST, 100 μ L 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 μ L 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 μ L 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 μ L) as an inhibitor (0.02–40 μ g 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 μ L wash buffer per well, ELISA plate wells are blocked with 200 μ L ELISTOP diluent for 1 h. Exactly 100 μ L

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 μ L diluted detection antibody to all wells. After subsequent washing, 100 μ L of streptavidin-HRP is added to each well and incubated at room temperature for 30 min. After that, 100 μ L of TMB (3,3',5,5'-Tetramethylbenzidine) solution to each well, followed by 15 min incubation, 100 μ L of stop solution (2 N H₂SO₄) 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 Macrorep High Q column (Bio-Rad, Hercules, CA, USA). After that 20 μ L 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 $\times$ 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

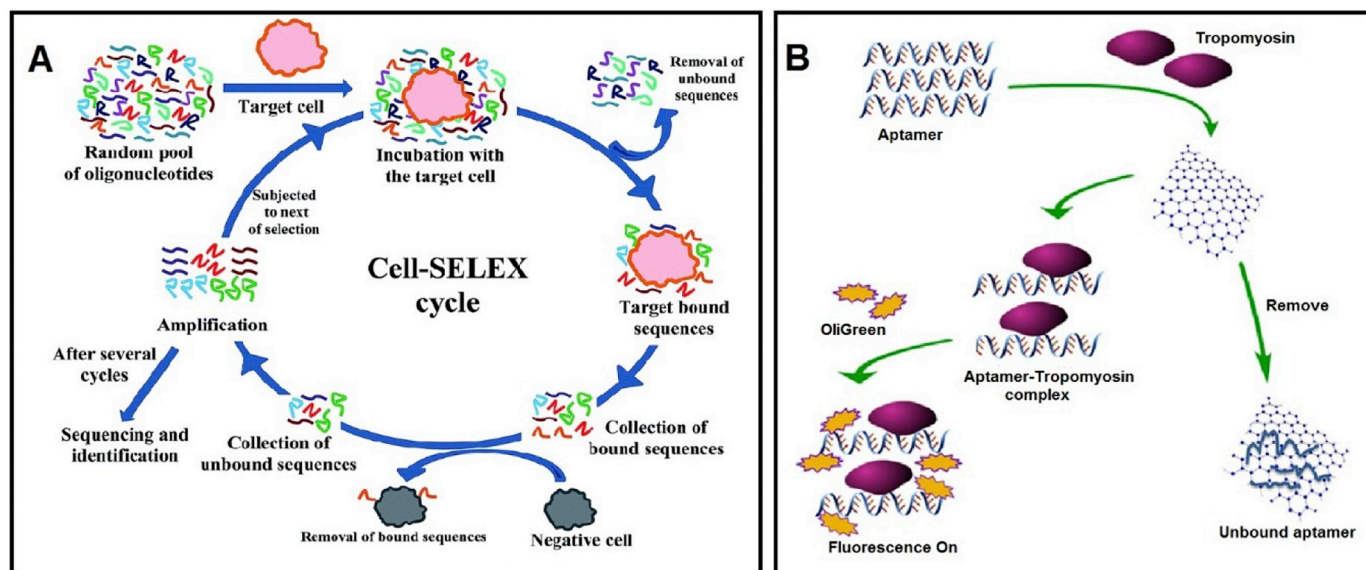


Fig. 4. Synthesis of aptamer using SELEX method (A), Detection of tropomyosin using aptamer-based technique (B) (Barman, 2015; Zhang et al., 2017).

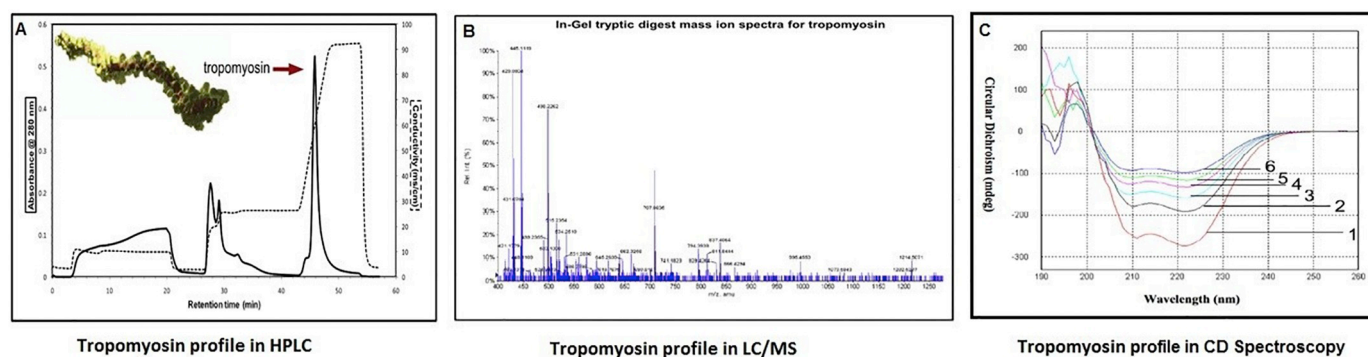


Fig. 5. Tropomyosin profile in HPLC (A), LC/MS (B) and CD Spectroscopy (C) (Kamath et al., 2013; Song et al., 2015).

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.

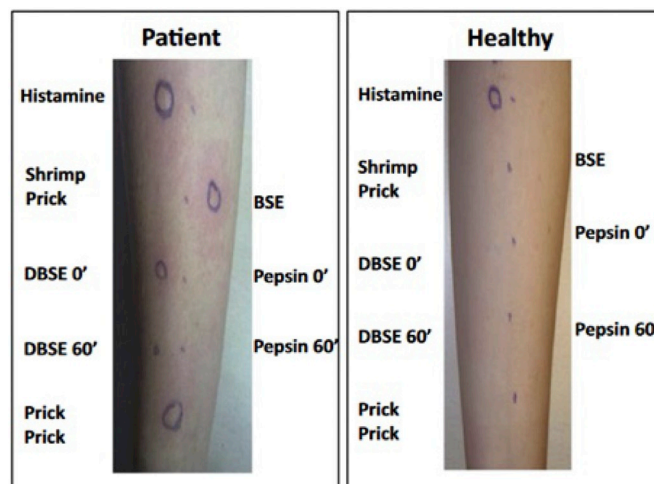


Fig. 6. Results of Skin Prick Test on the patient and healthy person (Gámez et al., 2015).

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 destaining 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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