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IDENTIFICATION OF HEAT RESISTANCE PROTEINS AND ALLERGENIC CHARACTERIZTION IN MUD CRAB (*Scylla paramamosain)*

MUSTAFA NASRAT ABDULRAHEEM

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ABSTRACT

This study aimed to determine the protein profiles and allergenic characteristics in different body parts and genders of *Scylla paramamosain*. Raw and boiled extracts of a mix and six individual body parts were prepared from the respective crab muscle tissues and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using sera from 28 crab-allergic patients. The mix body part contains 24 protein fractions, while all individual body parts have almost similar protein profiles with 16 to 21 protein bands between 15 to >250 kDa. The bands above 50 kDa were recognized as heat-sensitive proteins in the boiled extracts. Therefore, the boiled extracts have fewer protein bands than the raw extracts. Both heat-sensitive and heat-resistant protein bands have capabilities to bind to IgE antibodies of the tested sera, but with dissimilar binding patterns. A heat-sensitive allergen at 95 kDa and four heat-resistant allergens at 50, 42, 38 and 36 kDa were identified as the major allergens with the frequencies of IgE-binding at 54, 50, 60, 64 and 54%, respectively. Immunoblotting of selected sera revealed various IgE-binding patterns against individual body parts. The female leg was identified as the most allergenic part than the other crab parts based on the number of IgE-binding bands, number of major allergens and IgE-binding band intensities. Only a few bands particularly the 38 kDa retain the IgE-binding capabilities in all boiled extracts. Mass spectrometry analysis identified the 38 kDa of *S. paramamosain* as tropomyosin, while the 42 kDa were identified as beta-actin. As a conclusion, this study indicated that all body parts of *S. paramamosain* have numerous heat-sensitive and heat-resistant allergenic proteins, contributing to variable allergenicity between the crab body parts. Five protein bands at 95, 50, 42, 38 and 36 kDa were identified as the major allergens of this crab. The implication is, these findings are useful for the advancement of the IgE-based diagnosis and management of crab-allergic patients.

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PENGENALPASTIAN PROTEIN TAHAN HABA DAN CIRI-CIRI ALERGENISITI DI DALAM KETAM LUMPUR (*SCYLLA PARAMAMOSAIN*)

ABSTRAK

Kajian ini bertujuan untuk menentukan profil protein dan ciri-ciri alergenisiti pada bahagian badan dan jantina yang berbeza dalam *Scylla paramamosain*. Ekstrak mentah dan rebus daripada satu campuran dan enam bahagian badan ketam disediakan daripada tisu otot ketam masing-masing dan kemudian dianalisis dengan elektroforesis gel natrium dodecil sulfat berpoliakrilamida (SDS-PAGE) dan pemblotan imuno dengan menggunakan serum daripada 28 pesakit yang alergi ketam. Campuran bahagian badan mengandungi 24 pecahan protein, manakala semua bahagian badan ketam mengandungi profil protein yang hampir sama dengan 16 hingga 21 jalur protein antara 15 hingga > 250 kDa. Kebanyakan jalur bersaiz melebihi 50 kDa dikesan sebagai protein sensitif haba dalam ekstrak rebus. Oleh itu, ekstrak rebus mempunyai kurang jalur protein berbanding ekstrak mentah. Kedua-dua protein yang sensitif haba dan tahan haba mempunyai keupayaan untuk mengikat antibodi IgE pada sera yang diuji, tetapi dengan corak mengikat yang berbeza. Satu alergen sensitif haba pada 95 kDa dan empat alergen tahan haba pada 50, 42, 38 dan 36 kDa telah dikenalpasti sebagai alergen major dengan frekuensi pengikatan-IgE masing-masing sebanyak 54, 50, 60, 64 dan 54%. Pemblotan imuno menggunakan serum terpilih mendedahkan pola pengikatan IgE yang sedikit berbeza terhadap bahagian badan ketam. Secara umumnya, kaki ketam betina dikenal pasti sebagai bahagian yang paling alergenik, berdasarkan kepada jumlah protein pengikat-IgE, jumlah alergen major dan intensiti protein pengikat-IgE. Hanya beberapa jalur terutamanya 38 kDa dapat mengekalkan keupayaan pengikatan IgE pada semua ekstrak rebus. Analisis spektrometri jisim telah mengenalpasti jalur 38 kDa sebagai tropomyosin, manakala jalur 42 kDa sebagai arginin kinase. Sebagai kesimpulan, kajian ini menunjukkan bahawa semua bahagian badan *S. paramamosain* mempunyai banyak dan pelbagai protein yang sensitif dan tahan panas yang bersifat alergenik, yang menyumbang kepada kealergian yang pelbagai di antara bahagian-bahagian badan ketam. Lima jalur protein pada 95, 50, 42, 38 dan 36 kDa telah dikenal pasti sebagai alergen major ketam ini. Implikasi kajian ini adalah, dapatan ini berguna ke arah kemajuan kaedah diagnostik berasaskan-IgE dan pengurusan pesakit alergi ketam.

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LIST OF ABBREVIATIONS

AIRC	Allergy and Immunology Research Centre
AK	Arginine kinase
CR	Cross reactivity
DBPCFC	Double-blind placebo-controlled oral food challenge
FAO	Food and Agriculture Organization of United Nations
HKL	Hospital Kuala Lumpur
IgE	Immunoglobulin E
kDa	KiloDalton
MALDI-TOF	Matrix-Assisted Laser Desorption-ionization Time of Flight
MLC	Myosin light chain
MS	Mass spectrometry
MW	Molecular weight
NIAID	National Institute of Allergy and Infectious Diseases
PBS	Phosphate buffered saline
SCP	Sarcoplasmic calcium-binding protein
SDS	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIgE	Specific IgE
SPT	Skin Prick Test
TBS	Tris-buffered saline
TM	Tropomyosin
TTBS	Tween 20-tris-buffered saline
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

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1.1 Research Background

Shellfish is an aquatic shelled organism which consists of two groups; crustaceans (phylum Arthropoda including prawns, lobsters and crabs) and molluscs (phylum Mollusca including oysters, mussels and squid (Amaral, Raposo, Morais, & Coimbra, 2018). Crab is among the most significant shellfish which contributes an important source of proteins for humans of various communities such as in China, Vietnam, Singapore, Taiwan, Hong Kong and Malaysia (Keenan, 1999).

Mud crab is highly demanded as a protein food source in many countries including Malaysia. Four species of mud crab have been described; known as *Scylla serrata, Scylla paramamosain, Scylla tranquebarica and Scylla olivacea* (Keenan, Davie, & Mann, 1998). The green mud crab (*S. paramamosain*), a large marine

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portunid species, is widely distributed along the southeast China coasts and other Asian countries, such as Japan, Vietnam, and the Philippines. *S. paramamosain* is one of the most important aquaculture animals and marine fishery resources in China because of its large market demand (Shen, 1994).

Male and female of mud crabs are difficult to differentiate. However, differences between the sexes become more apparent as the crab matured (Phelan & Grubert, 2007). For females, a prominent increase in the width of the abdomen indicates sexual maturity while for males, morphometrically mature crabs can be distinguished from morphometrically immature crabs by an increase in height for a given carapace width (Somerton, 1981). Another obvious difference between male and female is from external character during spawning (Somerton, 1981). Compared with a female, the male is generally larger, very dark in colour and has larger mouth, broader and more swollen head (Llewellyn, 2007). Both male and female crabs contribute to local economy and commonly consumed by local peoples.

However, consumption of shellfish including crab may also cause seafood allergy (Abramovitch et al., 2013). The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have identified shellfish, including crab, as one of the eight major sources of food allergens. Allergy to crab is one of the most common IgE-mediated food allergy and is often accompanied with severe reactions (Villalta et al., 2010). Signs and symptoms of crab allergy include cutaneous reactions (urticaria, angioedema, eczema), respiratory symptoms (asthma, rhinitis), gastrointestinal symptoms (diarrhoea, vomiting), and systemic reactions anaphylactic shock (Lopata & Lehrer, 2009; Liang et al., 2008;

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Lopata & Potter, 2000; Sheffer, 1985). Exposure to crab allergens might occur through ingestion, inhalation or skin contact while processing, cooking or handling crabs (Butt & Macdougall, 2008). Occupational asthma to crabs has been reported in fisherman, processing workers, shell ginders, cook as well as restaurant workers (Cartier, Malo, Ghezzo, McCants, & Lehrer, 1986; Hudson et al., 1985; Droszc, Kowalski, Piotrowska, Pawlowiez, & Pietruszewska, 1981; Gaddir, Legge, Friend, & Reid, 1980). In Malaysia, the prevalence of shellfish allergy which includes crab allergy was reported to be 44% among local patients with allergic rhinitis and asthma (Shahnaz, Balwant, & Nasaruddin, 2001).

To date, numerous studies on characterization of shellfish allergens have recognized tropomyosin, an abundant muscle protein as the major allergens in shellfish (Rosmilah, Shahnaz, Zailatul, Noormalin, & Normilah, 2012; Lopata & Lehrer, 2009), including in numerous crab species (Nugraha et al., 2018; Rosmilah et al., 2012; Shriver & Yang, 2011; Abdul Rahman, Kamath, Lopata, Robinson, & Helleur, 2011; Liang et al. 2008; Lehrer et al. 2003). Allergenic in tropomyosin was first identified by Leung et al. (1998) in red crab *Charybdis feriatus* (*Cha f* 1). Subsequently, tropomyosin has also been identified as the major allergens in chinese mitten crab *Eriocheir sinensis* (Liang et al., 2008), *Portunus pelagicus* (Rosmilah et al., 2012) and *Scylla serrata* (Rosmilah et al., 2015). Tropomyosin is considered as a pan-allergen among invertebrates such as crustaceans, mollusks, mites and cockroaches (Abramovitch et al., 2013; Sereda, Hartmann, & Lucius, 2008; Suma, Ishizaki, & Nagashima, 2007; Lehrer, Ayuso, & Reese, 2003).

Besides tropomyosin, arginine kinase has also been reported as a potential pan-allergen in mud crab *Scylla serrrata* (Shen et al., 2011), *Scylla paramamosain*

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(Mao et al., 2013; Yu et al., 2013) and snow crab *Chionoecetes opilio* (Abdel et al., 2011). In addition, sarcoplasmic calcium-binding protein, troponin, α-actin and smooth endoplasmic reticulum Ca^{2+}ATPase were also identified as allergenic proteins in crabs (Faber et al., 2017; Abdel Rahman et al., 2011; Anas et al., 2012).

1.2 Problem Statement

Seafood including crab plays an important role in nutrition worldwide, sustained by increasing international trade of a variety of new seafood products (Jeebhay & Cartier, 2010; Lopata et al., 2007). However, consuming crab can have caused allergic reactions mediated by IgE antibodies which can be fatal (Friedman et al., 2017; Visciano et al., 2016). In fact, crab was ranked as top seven of causes of food allergic reactions in the world (Pawankar, Canonica, Holgate, & Lockey, 2011). Allergic reactions to seafood are generated by normally harmless proteins that reacted as allergens in some hypersensitive individuals. Among portunid crabs, mud crabs are the most widely distributed and cultured group. However, studies on allergy to the green mud crabs, *Scylla paramamosain* are very limited, thus need more attention.

The Skin Prick Test (SPT) is a reliable method for diagnosing IgE-mediated allergies in patients with rhino conjunctivitis, asthma, urticaria, anaphylaxis, atopic eczema, food and drug allergy (Heinzerling et al., 2013). SPT is usually performed using crab extracts prepared from crab meats. However, the sources of crab parts used for extraction are not well defined. Thus, to produce more accurate diagnostic tests for detection of specific IgE against crabs including *S. paramamosain*, allergen extracts

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produced from several body parts of local crab *S. paramamosain* is essential to obtain more accurate diagnostic results.

The meat yield, proximate composition and fatty acid profile of two species of mud crabs *S. serrata* and *S. tranquebarica* were compared with respect to gender and different body parts and the results showed significant differences in biochemical composition (Sreelakshmi, Manjusha, Vartak & Venkateshwarlu, 2016; Zafar, Siddiqui, & Hoque, 2004). It was reported that total protein content estimation using the total nitrogen estimation by Kjeldahl method was highest in body meat and lowest in claw meat of *S. tranquebarica*. Meanwhile, the meat from female *S. serrata* and *S. tranquebarica* crabs had significantly higher total protein content than males irrespective of species and body parts variation (Sreelakshmi et al., 2016; Zafar et al., 2004; Khan, 1992). However, so far there is no study reported on protein profiles of *S. paramamosain*. The variations of protein content in body parts and crab gender might play an important role in allergenicity of *S. paramamosain* particularly among crab consumers, thus should also be addressed in allergenicity studies.

The main allergens causing the shellfish allergy are believed to be tropomyosin (Nugraha et al., 2018; Abdel Rahman, Kamath, Lopata, & Helleur, 2010; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007). Other than that, sarcoplasmic calcium-bnding protein (Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008), myosin light chain and arginine kinase (Garca-Orozco et al., 2007; France Sellers & Grossman, 1997), troponin C, triose phosphate isomerase, and hemocyanin (Bauermeister et al., 2011; Piboonpocanun, Jirapongsananuruk, Tipayanon, Boonchoo, & Goodman, 2011) have also been identified by proteomics, immunology

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and molecular biological approaches as important shellfish allergens. At present, there are a few reports on identification of crab allergens (Emoto, Ishizaki, & Shiomi, 2009; Ishikawa, Ishida, & Shimakura, 1998). In addition, none of these reports characterization of allergens from different crab parts particularly claws, legs and abdomens, as well as between crab genders. Currently, there has been no local study on characterization of allergenic properties of *S. paramamosain* from different crab parts and genders.

Food processing may induce allergen alterations due to epitope destruction or modifications, potentially resulting in either decreasing, enhancing or having no effect on food allergenicity (Rosmilah et al., 2012; Sathe, Teuber, & Roux, 2005). Impacts of heat treatments on the reactivity of patients' IgE antibody to numerous shellfish allergens have been widely reported (Zailatul, Rosmilah, Faizal, & Shahnaz, 2015a; Zailatul et al., 2015b; Zailatul, Rosmilah, Faizal, & Masita, 2012; Carnes et al., 2007; Martin-Garcia et al., 2007). However, comparison of protein profile and allergenic properties of different body parts and gender of *S. paramamosain* in boiled forms are not available.

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1.3 Research Objectives

Referring to the above problem statements, the general objectives of this research are as follows:

a. To determine the protein profiles of different body parts (abdomen, claws, legs) for male and female mud crab *S. paramamosain*
b. To identify the allergenic properties of different body parts (abdomen, claws, legs) for male and female mud crab *S. paramamosain*
c. To determine the effect of boiling process on allergenicity of different body parts (abdomen, claws, legs) for male and female mud crab *S. paramamosain*.
d. To identify the major allergens of *S. paramamosain* by mass-spectrometry analysis.

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1.4 Research Question

According to the research objectives, the research questions in this research are:

a. What are the protein profiles of allergen extracts from different body parts of male and female mud crab *Scylla paramamosain*?
b. What are the characteristics of allergenic proteins from different body parts of male and female mud crab *Scylla paramamosain*?
c. What are the effects of boiling on allergencity of different body parts (abdomen, claws, legs) for male and female mud crab *S. paramamosain*?
d. What are the identities of the major allergens of *S. paramamosain*?

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1.5 Research Significant

This study will provide a new data on the methods of allergen extraction from different body parts and different gender of mud crab *S. paramamosain* which will be useful for researcher in selecting the crab parts with the highest protein components as the crab source for allergen extraction. Meanwhile, the findings will also benefit in management of crab-allergic patients by avoiding crab parts which higher allergenicity from their diet. In addition, this information will be also useful for the food industries to produce crab-based food products such as crab balls, crab fillet and crab cakes with low allergenicity, by using the crab parts which have the lowest allergenicity level.

Currently, crab allergies are diagnosed using crude allergen extracts as test allergens which contain mixture of major and minor allergens. Thus, identification of crab allergens is the first step toward generating of crab allergen components, which may lead to the development of allergen panel specific for diagnosis of local crab allergy. In addition, identification of allergen is also helpful in predicting the severity of allergic reactions.

Besides, as shellfish including crab is generally processed prior consumption, the results of the effects of boiling processing methods on crab allergenicity will be valuable for improving the therapeutic and management strategies towards local crab-allergic patients.

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Meanwhile, the outcome of this study obtained from the characterization of the allergenic proteins in the different parts of *S. paramamosain* is useful in order to determine the allergen components necessary for immunotherapy. The purpose of immunotherapy is to induce immunological tolerance against the allergens (Matricardi et al., 2016; Ebo et al., 2010). Therefore, future development of hypoallergenic crab allergens will assist in the development of safer immunotherapy by preservation of the immunogenicity of the allergen but reduced its IgE binding capacity. As a result, effective and optimized management can be started, which in turn leads to improved patient health and quality of life.

1.6 Research Limitation

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This study was limited to only one species of mud crab, the green mud crab *Scylla paramamosain*. The crab samples were collected from only one location in Sungai Merbok, Sungai Petani, Kedah. The allergen extracts of the crab were prepared individually from both male and female crabs, from only three crab parts which were abdomen, claws and legs. The protein profiles of all allergen extracts were determined by SDS-PAGE, while for identification of allergenic proteins, the crab extracts were further subjected to immunoblotting experiments using 28 sera from crab-allergic patients. The sera were only collected from one population; the allergic patients in Allergy Clinic, Hospital Kuala Lumpur. This study also focuses on the investigation of the effects of only one thermal treatment, the boiling process.

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1.7 Conclusion

In summary, this chapter outlined the study background, problem statements, research objectives, research questions, significance and research limitations of this study. This chapter also provided an overview of this research in general.

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CHAPTER 2

LITERATURE REVIEW

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2.1 Shellfish

Shellfish has long been known to be a common cause of food hypersensitivity reactions (Lopata, Kleine-Tebbe, & Kamath, 2016; Kamath et al., 2014). Shellfish are generally referred to two invertebrate phyla of Athropoda which are Crustaceans, particularly prawns, lobsters and crabs, and phyla Molluscs such as oyster, snail and squid. The increasing production and consumption of shellfish have been accompanied by frequent reports of allergic reactions among consumers (Lopata et al., 2016; Chen et al., 2013).

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2.1.1 Shellfish Classification

The term shellfish is synonymous with both mollusks and crustacean, which mostly have shells or exoskeleton on their body (Lu, Ohshima, & Ushio, 2007). They are found in saltwater, terrestrials, and freshwater habitats. Shellfish comprises of all the sea animals belonging to several phyla (Amaral et al., 2018; Daul, Morgan, & Lehrer, 1993). The phylum Arthropoda consists of class crustacean with representative members as decapods such as crabs, shrimps, and lobsters, barnacles, prawns and krill. Phylum Mollusca contained class Gastropoda which comprise of abalones, periwinkles, sea slugs, conchs, limpets, snails and whelks. Class Bivalvia consists of clams, mussels, cockles, scallop and oyster, while Class Cephalopoda comprises cuttlefish, squid and octopus (Figure 2.1).

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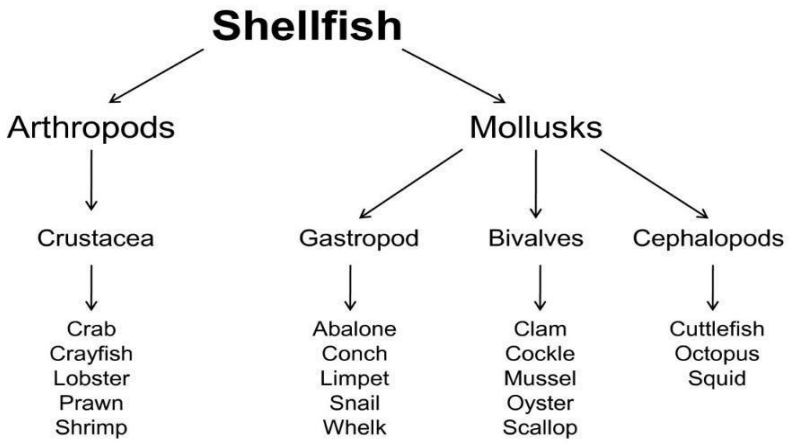


Figure 2.1. Classification of Shellfish. Adapted from Woo & Bahna, 2011.

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2.1.2 Crustaceans

The crustaceans generally live in the marine ecosystem but could be found in on land and freshwater ecosystem. There are about 50,000 to 67,000 known species of these creatures globally. However, the total number of crustaceans has been estimated to be about 10 to 100 times more than the known species (Food and Agriculture Organization, 2000). Crustaceans are a significant component of the human nutrition (Wild & Lehrer, 2005). Among crustaceans, numerous crab species such as blue crab, stone crab, Dungeness crab and mud crab are edible (FAO, 2000).

2.2 Crab

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Crabs represent a hugely diverse group including porcelain crabs, brachyuran crabs, hermit crabs and mud crab. Among crabs, mud crab is an ecologically important species of crab found in the estuaries and mangroves of Africa, Australia and Asia (FAO, 2010).

2.2.1 Mud Crab Classification, Morphology and Anatomy

Crabs are classified in the phylum Arthropoda, subphylum Crustacean, class Malacostraca, and order Decapodia (Bandral, Gupta, & Langer, 2014). Crabs include approximately 33 genera with approximately 165 species are known from Indo China. Mud crabs, also known as mangrove crabs, belong to Family Portunidae under genus

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Scylla. Four species of mud crab have been described; known as *S. serrata, S. paramamosain, S. tranquebarica* and *S. olivacea* (Keenan et al., 1998). Table 2.1 presents a full classification of the mud crab.

Table 2.1.

Classification of Mud Crab, Scylla Spp.

Phylum	**Arthropoda**
Subphylum	Crustacea
Class	Malacostraca
Subclass	Eucarida
Order	Decapoda
Suborder	Repatantia
Infraorder	Brachyura
Family	Portinuidae
Genus	*Scylla*
Species	*serrata, olivacea, paramamosain, tranquebarica*

(Adapted from King, 1995 and Keenan, 1999)

Researchers have reported that the genus *Scylla* includes several species (Keenan et al., 1998) which were classified based on their external morphology such as colour of carapace and legs, anterolateral teeth of carapace, and outer spines of cheliped and gametogenesis. Table 2.2 presents the morphological characteristics for determining the species of the adult mud crabs based on the taxonomic guide as presented by Keenan et al. (1998).

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Table 2.2.

Morphological Characteristics for Determining the Species of the Adult Mud Crab

Species	Frontal lobe spines	Cheliped	
	Height	**Carpus spines**	**Propodus spines**
S. serrata	High	Both Obvious	Obvious
S. tranquebarica	Moderate	Both Obvious	Obvious
S. paramamosain	Moderately high	Inner absent, outer reduced	Obvious
S. olivacea	Low	Inner absent, outer reduced	Reduced



S. serrata



S.olivacea



S. paramamosain



S. tranquebarica

(Adapted from Keenan et al., 1998).

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Figure 2.2 showing the anatomy of crab. In general, the crab body is protected by a tough outer shell called an exoskeleton. The head and thorax are joined together and are covered by a shell called a carapace. Crabs have four pairs of legs; one pair of swimming leg and three pairs of walking legs. Swimming legs are the last paired abdominal appendages, flattened for swimming, while the walking legs are used for movement. Juvenile and mature crabs have one pair of chelipeds which carries the large claw for protection and obtaining food. Crab also has mouth and antennae. The edible flesh are included the claws, legs and abdomen (body) of the crab (Madsen, Forster, Grefenstette, Harrison, & Stern, 2017; Leignel, Baringou, Stillman, & Thabet, 2014; Stewart & Reichelt., 1993).

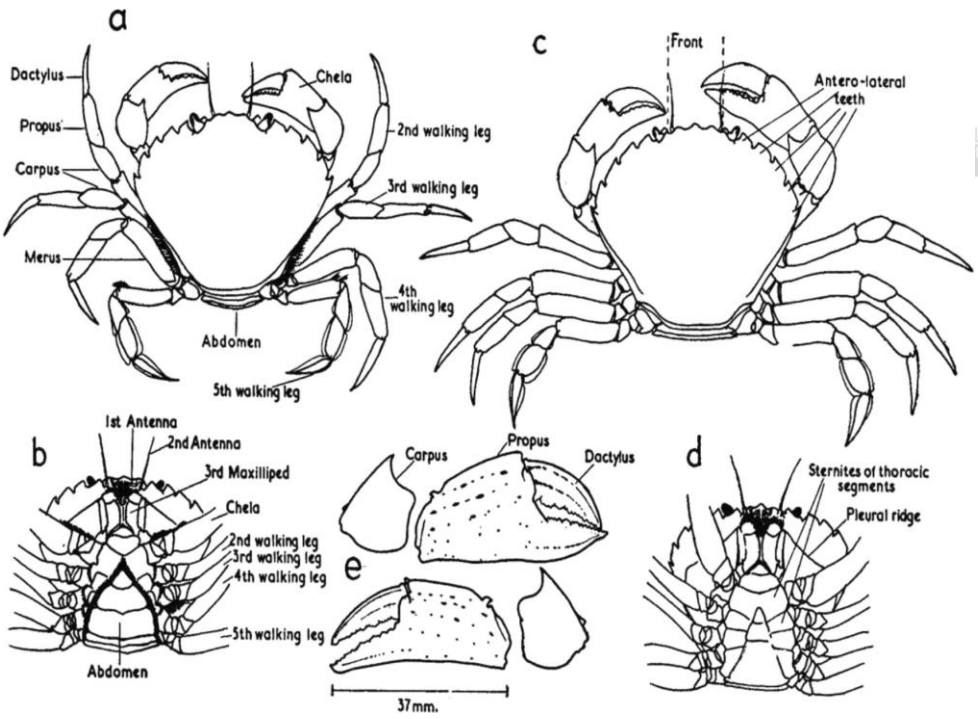


Figure 2.2. Anatomy and Morphological Criteria of Crab. A= Female Dorsal, B= Female Ventral View, C= Male Dorsal, D= Male Ventral View, E= Chelae of the Male. Adapted From Leignel Et Al. (2014).

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As juveniles, male and female mud crabs are difficult to distinguish, however the gender identity can easily determine as they mature. Male abdominal flap is narrow in shape with 3-5 fused segments, while female abdominal flap is oval and broad in shape (Keenan et al., 1998). Another difference is the claws, which are much larger in males. The male mud crabs usually grow bigger in size compared to the females (Efrizal, Arshad, Kamarudin, Saad & Amin; Keenan, 1999). Figure 2.3 shows sex differentiation between male and female mud crab.



Figure 2.3. Sexual Dimorphism of Blue Swimming Crab (*P. Pelagicus*): (A) Female-Mottle Brown (Fm-Mbr), (B) Male-Mottle Blue (M-Mbl). (C) Female-Immature (Fm-Im), (D) Female Mature (Fm-Nx), (E) Male Immature Or Mature (M-Im Pr Mr) and (F) Male-First Pleopod (M-Pl_1), Male-Second Pleopod (M-Pl_2). Adapted From Efrizal Et Al. (2015).

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2.2.2 Mud Crab Distribution

Mud crab is distributed from the Indo-West Pacific Ocean to the Red Sea along with in Australia, Taiwan, the Philippines, South Africa, Japan and the Pacific Islands and other tropical and sub-tropical regions (Hall, Motti & Kroon, 2017; Keenan et al, 1998; Stewart & Reichelt, 1993). In Asian countries, mud crab is distributed in Bangladesh, Myanmar, Sri Lanka, India, Thailand, Vietnam, The Philippine, Malaysia and Cambodia (Keenan et al., 1998). The global distribution and habitat of mud crab species are shown in Table 2.3

Table 2.3

Distribution and Habitat of Mud Crab.

Species	Distribution	Habitat
S. serrata	Indian Ocean, Red Sea, Pacific Ocean – the most widespread *Scylla* species.	Associated with mangrove forests inundated with full salinity oceanic water for the greater part of the year. Can tolerate reduced salinity.
S. paramamosain	South China Sea, Java Sea – an abundant species where it occurs	Associated with various habitats including shallow coral rubble; Shallow subtidal flats and estuarine ponds; mangrove forests.
S. olivacea	South China Sea, Indian Ocean, Pacific Ocean–moderately widespread, often associated with *S. tranquebarica.*	Associated with mangrove forests and coastlines inundated with reduced salinity seawater during the wet season.
S. tranquebarica	South China Sea, Pacific Ocean, Indian Ocean – a widespread species, often associated with *S. olivacea.*	Associated with mangrove forests and coastlines inundated with reduced salinity seawater for part of year.

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2.2.3 Mud Crab (*Scylla paramamosain)*

The distribution of *S. paramamosain* is restricted within the South China Sea and Java Sea (Le Vay, Ut, & Jones, 2001). Among the *Scylla* spp., *S. paramamosain* is one of the most abundant mud crab species inhabiting coastal and mangrove areas throughout Malaysia. The other two species are *S. olivacea* and *S. tranquebarica* (Ikhwanuddin, Bachok, Mohd Faizal, Azmie, & Abol-Munafi, 2010). The male crabs are significantly bigger and heavier, and relatively more abundant than the female, with a dominant size range of between 9.0cm and 9.9 cm with respect to the carapace width (Ikhwanuddin et al., 2010). Figure 2.4 depicts the mud crab *S. paramamosain.*



Figure 2.4. The Mud Crab *(S. Paramamosain).* Adapted From Ikhwanuddin Et Al. (2010).

The mud crab, including *S. paramamosain* are commercially important mangrove crustaceans that are extensively fished in the Indo-West Pacific regions because of their large market demand. Mud crab is a valuable food in many countries because of its high quality flesh, luxurious taste and richness in protein, vitamins and minerals (Islam, 2015). Mud crab has a thriving market in Japan, China, Hong Kong,

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Korea, Thailand and USA (Islam et al., 2017). The demand of live mud crab, soft-shelled crab and crab meat has been increasing tremendously worldwide (Islam, 2015). However, the rising popularity of crab has caused an increasing number of allergic reactions to crab. Crab including mud crab was widely reported can provoke allergic reactions in hypersensitive individuals (Woo & Bahna, 2011; Lee & Park, 2004).

2.2.4 Stages of Development in Mud Crab

The stages of development in mud crabs are similar as the other species of crabs (Figure 2.5). Mature male and female moves to the estuary and inshore areas to find mates for copulation. Mating takes place in the estuarine environment as the female remains in soft shelled condition at pubertal moulting. As the matting is completed the female migrate to the offshore for spawning and stay until hatching the eggs to first zoe. Spawning takes place in the offshore region with favorable salinity of 30 to 31 ppt. Mud crab is highly fecund and individual female can produce approximately 1 to 6 million of eggs per spawn depending on size. Hatching and larval development occurs in the offshore region and the larvae spends about 30 days as a planktonic form and complete 6 larval stages (5 zoeal stages and a megalop stage) which leads to the crab instars. The first crablet then enter into the estuarine environment and settle down on intertidal mudflats and mangrove shelters for grow out and attains maturity (Islam, 2015).

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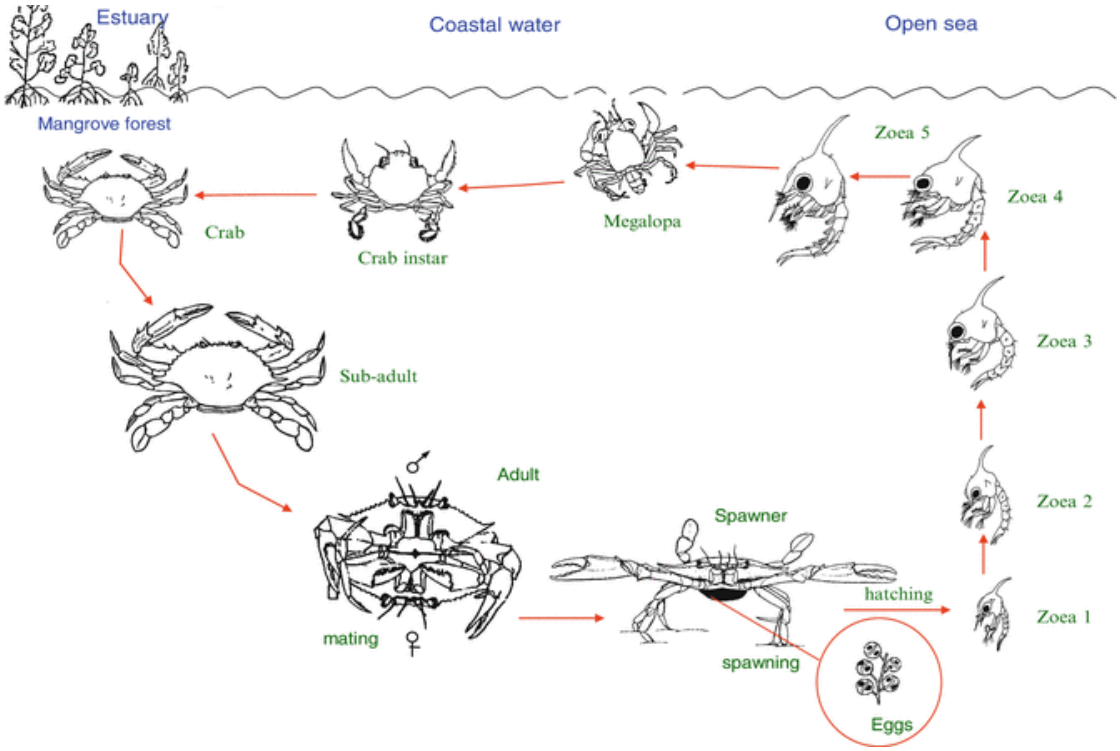


Figure 2.5. Life Cycle of Mud Crab. Adapted From Yamaguchi (1991).

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2.3 Crustaceans Allergy

Crustaceans are the major cause of shellfish allergies with severe allergic reactions (Villalta et al., 2010; Crespo & Rodriguez, 2003). Crustacean-related allergy is widely distributed due to the large availability of crustaceans. Another form of shellfish allergy (molluscan-related) is less frequently compared to crustacean allergy (Taylor, 2008). The crustacean-related allergy is IgE-mediated and classified as type I hypersensitivity reaction. The major contributors to crustacean allergy globally are the broadly consumed shrimp and prawn which serve as sources of seafood nutrition globally (Lu et al., 2007). Besides, other crustaceans including numerous species of

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crabs could also stimulating allergic reactions (Sicherer, Munoz-Furlong, & Sampson, 2004).

2.3.1 Prevalence and Epidemiology

Food allergy affects approximately 3.5 to 4.0% of the global population (Taylor, 2008). Seafood is the major cause of food allergy and food-induced anaphylaxis in adults. The commonest form of these allergies is related to shellfish allergy. About 0.5% of children suffer from shellfish allergy compared to 2.5% in adults. The general prevalence of shellfish allergy has been estimated at approximately 0.5 to 2.5% among general population, which is mainly dependent on the rate of consumption, location, and age (Moonesinghe et al., 2016; Woo & Bahna, 2011).

In a questionnaire-based international survey conducted on 17,280 adults aged 20-44 years from fifteen countries, the reported percentages of seafood-related allergies were to be 3 % in shrimp, 2.2 % in fish, and 2.3% in oyster (Woods, Abramson, Bailey, & Walters, 2001). A telephone-based survey of 14,948 individuals in the United States showed that 2-3% of the interviewed individuals are believed to be allergic to seafood, with about 2.2% being allergic to shellfish and 0.6% allergic to fish (Sicherer et al, 2004). (Crespo, Pascual, Burks, Helm, & Esteban, 1995) reported that in Spain, 6.8% among 355 children are allergic to crustacean proteins based on the skin prick test (SPT), with crustacean allergy accounting for 82.6% compared to 7.2 % of mollusk allergy. Shellfish accounted for 72.5% of the reported allergies (Khan, Orson & Ogawa, 2011).

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Asian countries have a higher prevalence of shellfish allergy compared to the western countries (Chiang, Kidon & Liew, 2007). This could be attributed to the rate of shellfish consumption where shellfish constitute a large proportion of the diet (Khora, 2016). Therefore, allergy to a particular shellfish can reflect the regional consumption of that particular species in a region (Woo & Bahna, 2011). Shek et al. (2010) reported that children of expatriates residing in Singapore (4-6 years, 0.55%; 14-16 years, 0.96%) have a higher prevalence of shellfish allergy compared to the native children (4-6 years, 1.19%; 14-16 years, 5.23%).

Few studies have investigated the natural history of shellfish allergy, and these studies point towards a long-lasting history of shellfish allergy (Wood, 2003). The prevalence of shellfish allergy is often higher than that of fish allergy in the general population. Prawn and crab are the major causes of anaphylaxis in both children and adults. Combined crustacean and mollusc allergy reportedly affects as many as 5.5% (95% CI, 4.3–7.1) of French children (5–17 years old) and 9.0% (95% CI, 6.7–11.9) of American adults (Moonesinghe et al., 2016). The prevalence of crustacean allergy alone is considerably higher, with the highest reported prevalence of 10.3% (95% CI, 7.0–14.9) determined among Italian adults who were confirmed positive by Skin Prick Test (SPT) (Burney et al., 2010).

As important crustaceans after shrimps, the prevalence of crab allergy among shellfish-allergic patients in USA was considered high, which accounted for 34.8% by (Khan et al, 2011). Similarly, (Andre, Andre, Colin, Cacaraci, & Cavagna, 1994) also reported that in France, 34% of 580 food allergy patients demonstrated IgE-mediated allergy to crabs. Meanwhile, in Malaysia, crab allergy was reported by Yadzir et al.

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(2015) and Du et al. (2015) to be 15% and 21%, respectively, both among food-allergic patients.

The frequency of shellfish allergy in certain country including Malaysia was summarized in Table 2.4.

Table 2.4

Frequency of Shellfish Allergy In Certain Country Including Malaysia

Country	Frequency	References
Taipei, Taiwan	33% adults, 88% children	Wan et al., 2010
Singapore	16.12% adults	Thong et al., 2001
	1.19% children aged from 4-6 years old	Shek et al., 2010
	5.23% teenagers aged from 14-16 years old	
Malaysia	44% asthma patients and allergic rhinitis	Shahnaz et al., 2001
Korea	7.6% children aged from 6-12 years old	Oh et al., 2004
	16.6% teenagers aged from 13-15 years old	
Japan	6.5% adults	Yoneyama & Ono, 2002
Taiwan	43% children atopic dermatitis	Huang et al., 2004
	30% adults, 15% children food allergies aged from 3-11 years old	

The inhalation, exposure or ingestion of shellfish can result in hypersensitivity reactions. The commonest source of this reaction has been attributed to their ingestion or direct contact. A double-blind placebo-controlled oral fish challenge investigation reported emesis as the most frequent sign found in patients with a positive challenge. However, the observed emesis in the patients is preceded by generalized pruritus or pharyngeal symptoms (Wild & Lehrer, 2005). Shellfish have also been implicated in

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occupational allergy, where reactions are commonly associated inhalation or a direct contact rather than ingestion. In some cases, the workers are often unable to handle raw food but can ingest it when cooked, suggesting a heat-labile allergen (Goetz & Whisman, 2000). The prevalence rate of occupational shellfish allergy has been reported at 3 to 11% (Jeebhay, Robins, Lehrer, & Lopata, 2001), including snow and king crabs which also implicated in occupational asthma (Laird & Patel, 2015).

2.3.2 Pathogenesis and Clinical Symptoms of Allergy

The immune system protects our body against pathogens and other foreign substances by producing specific antibodies or immunoglobulin from plasma cells or B-cells. Antibodies involved in allergy are Immunoglobulin E (IgE). IgE is overproduced during an allergic response. Allergic reactions involved two phases as shown in Figure 2.6. The first phase is the first exposure to an allergen, causing an allergic person becomes sensitized by producing allergen-specific IgE that binds with IgE receptors on mast cells in tissues and basophils in circulation. If the sensitized person has subsequent exposure to the same specific allergen in the second phase, this allergen will bind to the antigenic determinant site (Fab) of IgE attached to the mast cells and basophils. Binding of two or more IgE molecules to mast cells (crosslinking) is required to activate the mast cells. These activated cells result in the release of certain chemicals, such as histamine, serotonin, proteoglycans, serine protease, leukotriene and heparin, that will further bind with their receptors present in other cells and lead to inflammation, irritation, redness and other allergic symptoms. The primary function of our immune system is to defend against infection; however,

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during an allergic reaction the immune system responds against a substance that is harmless to most people (Sicherer & Sampson., 2010).

If the release of the mediators is sudden or extensive, the allergic reaction may also be sudden and severe, and anaphylaxis may occur. Allergic reactions are unique for each person. Reaction time to allergens can vary widely. Some people will have an allergic reaction immediately, for others it will take time to develop (Sicherer & Sampson., 2010).

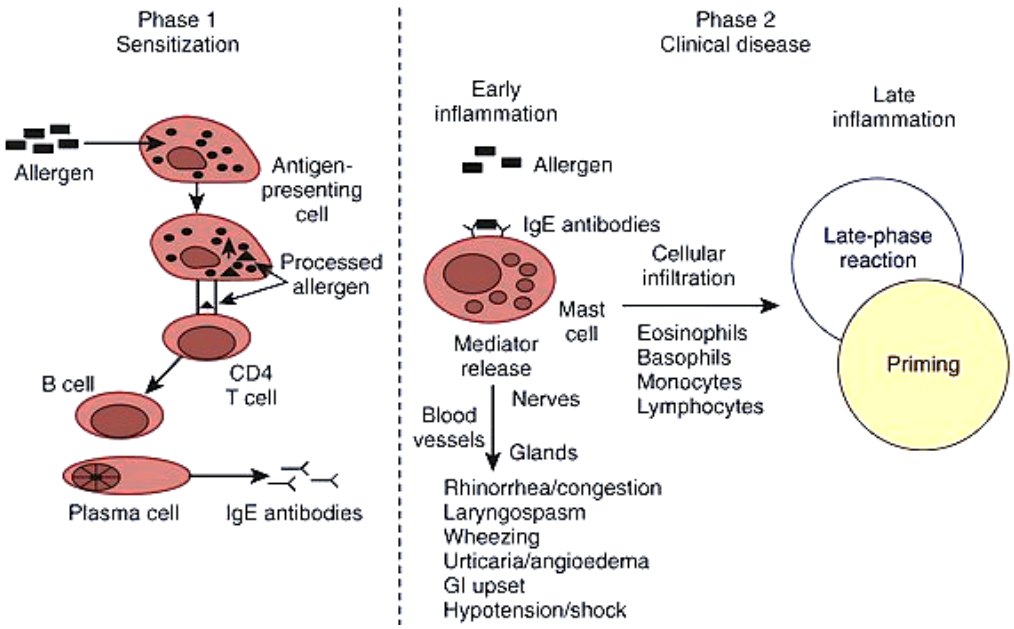


Figure 2.6. The Pathogenesis of Allergic Reactions. Adapted From Laird And Patel (2015)

Allergy to shellfish including crab can manifest in the form of mild urticaria to life-threatening anaphylaxis. Most of these reactions are IgE-mediated and may present in the form of respiratory, gastrointestinal, or cutaneous manifestations with rapid onset. In some cases, the symptoms may only be manifested within minutes of

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consuming the shellfish (Thomassen et al., 2016; Lopata, O'Hehir, & Lehrer, 2010). The clinical symptoms of shellfish allergy are presented in Table 2.4.

Table 2.5

Symptoms of Shellfish Allergic Reactions

System	Clinical Symptoms
Gastrointestinal	Nausea, Vomiting, Diarrhea, Abdominal cramping
Cutaneous	Pruritis, Dermatitis, Urticaria, Angioedema
Respiratory	Conjunctivitis, Rhinitis, Asthma, Laryngeal edema
Systemic	Anaphylactic shock, Hypotension

2.3.3 Diagnosis of Allergy

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In general, the diagnosis of allergy including crab allergy involves an investigation of the medical history of the patients, followed by diagnostic tests for *in-vivo* or *in-vitro* identification of allergic sensitization or detection of the allergen-related IgE, oral food challenge (OFC) and component-resolved diagnosis (CRD) (Matricardi et al., 2016; Yadzir et al., 2015; Muraro, Werfel, & Hoffmann-Sommergruber, 2014; Bock, Sampson, & Atkins, 1988).

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2.3.3.1 Medical History

A precise and detailed history is very important to gain information regarding the shellfish under suspicion, atopic status and clinical symptoms of the patients. An atypical or an inconsistent of clinical history usually suggests a non-atopic aetiology, such as intolerance reaction or toxicity, which cannot be regarded as an allergy (Roberts et al., 2016).

2.3.3.2 *In vivo* and *In vitro* Tests

Prick skin testing (SPT) is the main choice of *in vivo* test for patients with IgE-mediated hypersensitivity to shellfish. It is necessary to be cautious when interpreting skin test observations when the patient history is poor due to the potential cross-reacting allergens that may give false positive skin tests with unclear clinical significance (Ljerka Prester, 2105., Fernandes et al., 2003). Studies have confirmed a wheel diameter of 3 mm after SPT to be 80 to 95% predictive of a positive allergic reaction in patients with hypersensitivity to certain allergens (Jirapongsananuruk et al., 2008).

Meanwhile, the aim of most *in vitro* tests is to diagnose Type I Hypersensitivity reactions to foods or aeroallergens in patients with relative contraindications to SPT such as dermatographism, pregnancy or patients with high risk of anaphylaxis by detecting antigen-specific IgE antibodies (Wild & Lehrer, 2005). The detection of IgE antibodies to specific antigens helps in assessing

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allergies, especially food allergy. A study on food-allergic children reported 62.5% of the children specific to IgG antibodies and 22.9% specific to IgE antibodies. These antibodies were not detected in children without symptoms of food allergy (Hofman, 1995). The most common *in vitro* tests are specific IgE tests include radioallergosorbent test (RAST), enzyme-linked immunosorbent assay (ELISA), microarray, ImmunoCAP and *in-house* immunoblotting tests.

A group of researchers introduced Radioallergosorbent (RAST) in 1967 as an *in vitro* method for detecting IgE antibodies in allergic conditions. The results obtained from RAST have been positively correlated with the clinical provocation tests (Berg, Bennich, & Johansson, 1971). Furthermore, the results of specific *in vitro* reaginic antibody investigations which involve the release of histamine from excised tissue slices have been closely correlated with the results from RAST (Foucard, 1972). However, compared to SPT, RAST has been reported to be less sensitive and more expensive method for food allergy diagnosis (Sampson & Ho, 1997).

The presence of unknown allergens in food has increased the demand for sensitive, reliable and rapid allergen detection methods. One of the convenient and easy way of diagnosing food allergy in patients is the use of ELISA/EIA panels. The conventional ELISA method is mainly carried out in specialized laboratories where a considerable amount of time and sample/reagent is required. A microfluidic ELISA platform has been recently combined with a custom-designed optical sensor for the quantitative analysis of wheat gluten and *Ara h* 1. This microfluidic biosensor showed a potential sensitivity for the rapid detection of food allergens in a complex sample (Weng & Neethirajan, 2016).

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Microarray technology has also been recently deployed as a promising diagnostic test for food allergy. The first application of microarray technology for human allergic disease determination when 94 purified recombinant and natural allergen molecules were immobilized in a buffer at a concentration of 0.3 mg/mL onto a glass slide coated with a pre-activated amine (Hiller et al., 2002). Among various platforms for the *in vitro* detection of target biomolecules, microarrays provide a unique opportunity as they allow for the high-throughput screening of multiple targets simultaneously with increased sensitivity and selectivity (Jeon et al., 2018; Hiller et al., 2002). Being a new system for antibody detection, the allergen microarray has emerged as a potential way of achieving a large-scale allergen interactions profiling for simultaneous detection of IgE and IgG antibodies secreted against several allergy-inducing molecules (Ott, Baron, & Heise, 2008).

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2.3.3.3 Oral Food Challenge (OFC)

The OFC is generally performed by administering the suspected allergen source in a gradually increasing dose under medical supervision (Nowak-Wegrzyn et al., 2009). In a single-blind OFC, the patient is not aware of the food that contains the suspected allergen but the clinician is aware (Komata, Söderström, Borres, Tachimoto, & Ebisawa, 2009). In a double-blind placebo-controlled, both the clinician and patient are not aware of the presence of the challenge material, and this is the standard way of diagnosing a food allergy for both scientific and clinical purposes (Rancé et al., 2009).

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2.3.3.4Component-resolved diagnosis (CRD)

Currently, progress in laboratory diagnostics is the use of component-resolved diagnosis (CRD) which implies determination of specific IgE against purified native and recombinant allergenic molecules was developed as a new approach for diagnosing allergy. The advantage of CRD over the current methods for determining the concentration of specific IgE is that CRD can distinguish the true allergen molecules from the cross-reactive allergen molecules. CRD is used in laboratory practice as singleplex and multiplex assays. The choice of allergen for singleplex assay is based on anamnesis, clinical findings of a patient and on skin prick test results. Multiplex-microarray assays simultaneously determine multiple sIgE's against numerous allergens. Therefore, CRD enables the detection of the risk of severe symptoms and predicting the development of allergies. Thus, CRD provides a possibility for a personalized approach to the patient with allergy (Dodig & Čepelak, 2018).

2.3.4 Treatment and Management

The general approach to the management of food allergy including crustaceans such as crabs is a strict avoidance of the food source based on proven clinical reactions. It is generally advised to completely avoid all crustaceans because of the possibility of cross-reactivity between homologous allergens (Freidl et al., 2017; Sicherer, Furlong, Munoz-Furlong, Burks, & Sampson, 2001). Unfortunately, this method is not always possible due to the hidden shellfish allergens in food products and as a result, efforts

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are ongoing on new treatment approaches for food allergy such as immunotherapy (Atkins & Leung, 2007).

Allergic reactions can also be remedied with various medications. For instance, anti-histamines can provide a partial relief to the symptoms of IgE-mediated skin conditions and oral allergy syndrome. Some patients may be candidates for alternative anti-inflammatory therapy, such as troleandomycin, methotrexate, gold, hydroxychloroquine, or dapsone. In addition, the application of immunomodulator therapy, such as intravenous gamma globulin or cyclosporine, may be useful. (Sicherer & Sampson, 2010).

Efforts are currently ongoing on the development of several potential therapies for reducing symptoms of food allergic reactions. Such therapies may come in the form of immunologic manipulation of the food-allergic patient through mucosal vaccines, oral immunotherapy, peptide immunotherapy, and immunotherapy with recombinant allergens, or through food manipulation via genetic engineering (Syed, Garcia, Lyu, Bucayu, Kohli, & Ishida, 2014).

2.3.5 Crab Allergens

In general, a total of 71 proteins have been identified as food allergens so far, usually with molecular weight of 10 to 70 kDa (Velickovic, Jankulovic, & Jankov, 2005) or 5 to 100 kDa (Matsuo, Yokooji, & Taogoshi, 2015). Allergens were either identified as ‘major’ or ‘minor’ allergens depending on the prevalence of IgE-reactivity among an

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allergic population (Mueller et al., 2015). Major allergen is defined as a protein that elicits IgE binding in at least half of the sera from a group of allergic patients, while minor allergen is a protein which was detected by less than 50% of the patients' sera. At least 20 sera is sufficient for major allergen identification studies as described previously (Zailatul, Rosmilah, Faizal, Masita, & Shahnaz, 2010).

2.3.5.1 Tropomyosin (38 to 41 kDa)

To date, tropomyosin (TM) was widely reported as the most common IgE-mediated major allergens in both crustaceans and mollusks. TM was first reported in crustaceans (shrimp) by Hoffman et al. in 1981 before subsequently been demonstrated as a major allergen in various species of crabs (Rosmilah et al., 2015; Shriver & Yang, 2011; Abdel et al., 2011; Liang et al., 2008; Lehrer, Ayuso, & Reese, 2003). Several IgE-binding epitopes have been reported in TM (Motoyama et al., 2007; Leung et al., 1998).

TM is a member of the actin filament-binding protein family which has different isoforms and can be individually expressed in muscle and non-muscle tissues. In most muscles, TM acts as the regulatory switch which consists of two strands with molecular weight of 66 kDa, together with a globular protein complex called troponin which associated with the actin helix (Nevzorov & Levitsky, 2011). The calcium-dependent actin-myosin interaction is regulated by a tropomyosin-troponin complex. TM has a relative resistance to acidic conditions. TM consists of approximately 281 amino acids with monomeric molecular weight of 38 to 41 kDa

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which have been identified in the open reading frame of cloned TM in invertebrates (Liu, Liao & Charng, 2011; Liu et al., 2010; Huang et al., 2010).

TM is major shellfish allergen which accounts for the ingestion-related allergic reactions with shellfish consumption. TM has been implicated in both crustaceans and mollusks as a common allergen (Gonzalez-Fernandez Alguacil-Guillen, Cuellar, & Daschner, 2018; Suma et al., 2007; Ishikawa, et al., 1998a; 1998b; Lopata & Jeebhay 2013; Miyazawa, Fukamachi, & Inagaki, 1996), thus has been identified as a pan-allergen for cross-reactivity between invertebrates such as crustaceans, arachnids, different mollusks, and insects due to its highly conserved amino acid sequence (Wild & Lehrer, 2005).

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TM has been also identified as the major allergen in other crustaceans, mainly prawn (*Penaeus monodon, Penaeus japonicas*) and shrimp (*Pandalus eous*). Some are called *Pen a* 1 (*Penaeus aztecus*) *Pen i* 1 (*Penaeus indicus*), *Lit v* 1 (*Litopenaeus vanamei*) and *Cra c* 1 (*Crangon crangon*) (Gamez et al., 2011; Woo & Bahna, 2011; Reese, Jeoung, & Daul, 1997). Allergenic TM has also been widely reported in crabs such as red king crab (*Paralithodes camtschaticus),* snow crab (*Chionoecetes opilio),* Horsehair crab (*Erimacrus isenbeckii),* Blue swimmer crab (*Portunus pelagicus),* and Giant mud crab (*S. serrata)* (Motoyama et al., 2007). Most of these identifications were done through immunoblotting with an overall sequence homology of over 90% (Motoyama et al., 2007). Table 2.6 shows the tropomyosin that has been characterized as a crab allergens.

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Table 2.6

Summary of Tropomyosin That Has Been Characterized As Crab Allergens

Allergen	Species	Allergen identity	Size (kDa)	References
Cha f 1	*Charybdis feriatus*	Tropomyosin	34-36	Rosmilah et al., 2012 ; Yu et al., 2011; Abdel Rahman et al., 2010b
Eri s 1	*Eriocheir sinensis*	Tropomyosin	34-36	Liu et al., 2010; Liang et al., 2008
Scy s 1	*Scylla serrata*	Tropomyosin	34-36	Yu et al., 2011; Huang et al., 2010; Bugajska-Schertter et al., 2000
Por p 1	*Portunus pelagicus*	Tropomyosin	39	Wijaya et al., 2015; Jodie et al., 2013
Chio o 1	*Chionoecetes pilio*	Tropomyosin	34	Abdel Rahman et al., 2011; Gill et al., 2009; Motoyama et al., 2007

2.3.5.2 Arginine kinase (AK) (40 to 42 kDa)

Besides TM, arginine kinase (AK) has also been identified as an important major allergen in crustaceans, mainly prawn and crab (Yu et al., 2013; Liu et al., 2012; Shen et al., 2011). AK is an enzyme involved in the reversible transfer of phosphate groups between ATP and various phosphogens in invertebrates (Abdel Rahman et al., 2011). It was first identified as an allergen in prawn species including *L. vannamei, P. aztecus, F. chinensis* and *P. monodon,* using a proteomics approach (Abdel et al., 2010a; Garca-Orozco et al., 2007; Yao,Wu, Xiang, & Dong, 2005; Yu, Lin, Chiang, & Chow, 2003; France, Sellers, & Grossman, 1997). Moreover, AK has also been identified in other crab species such as *S. serrata* and *C. opilio,* with a reported patients' sera reactivity of 49% to AK (Shen et al., 2011; Abdel, et al., 2011). Additionally, AK has been identified as an allergen in other invertebrates like *Dermatophagoides farina, Plodia interpunctella,* and *Bombyx mori* (Binder et al., 2001; Liu et al., 2009; Bi & Chew, 2004). Table 2.7 shows the Arginine kinase that has been characterized as a complete shellfish allergen.

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Table 2.7

Arginine Kinase That Has Been Characterized As Shellfish Allergens.

Common name	Scientific Name	Allergen	References
Black tiger shrimp	*Penaeus monodon*	*Pen m* 2	Abdel Rahman et al., 2010
Pacific shrimp	*Litopenaeus vannamei*	*Lit v* 2	Ayuso et al., 2008
House dust mite	*Dermatophagoides pteronyssinus*	*Der p* 20	Prester, 2012
White shrimp	*Litopenaeus setiferus*	*Lit v* 2	Garcia-Orozco, 2007
Tiger shrimp	*Penaeus monodon*	*Pen m* 2	Yu et al., 2003

2.3.5.3 Sarcoplasmic Calcium-binding Protein (20 to 24 kDa)

Sarcoplasmic Calcium-binding Protein (SCP) has been reported as allergenic proteins in a number of crustacean species including black shrimp (Shiomi et al., 2008), white shrimp (Ayuso et al., 2009), North Sea prawns (Bauermeister et al., 2011), lobster marsh Red (*P. clarkii*) and lobster tight claws (*Pontastacus leptodactylus*) (Chen et al., 2013a). SCP is a protein involved in the helix-loop-helix structural (EF-hand calcium) that contains helix-loop-helix motifs to bind calcium ions (Ca^{2+}) which has a role in the buffering of calcium cytosolic in muscles. SCPs are larger proteins (20-24 kDa) that usually form dimers (White et al., 2011). Shiomi et al. 2008 was first identified SCP with allergenic characteristics in *Penaeus monodon* before being identified in *Litopenaeus vannamei* (Ayuso et al., 2009).

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2.3.5.4 Other Major Allergens

Numerous reports have identified several other potent allergens in shellfish such as heavy chain myosin, troponin, hemocyanin, actin, and amylase in several species of molluscs such as mussel and oyster (Faber et al., 2017; Taylor, 2008; Azofra & Lombardero, 2003; Martins, Peltre, da Costa Faro, Vieira Pires, & da Cruz Incio, 2004; Maeda et al., 1991; Juji et al., 1990; Koshte, Kagen, & Aalberse, 1989). However, light chain myosin was also identified as an allergen in crustaceans including shrimps *L. vannamei* and *P. monodon* (Ayuso et al., 2010; Abdel et al., 2010a; Ayuso et al., 2008) and crab (Abramovitch et al., 2013; Abdel et al., 2011). Other novel crab allergens like α-actin (42 kDa), troponin (23 kDa) and smooth endoplasmic reticulum Ca^{2+}ATPase (113 kDa) have also been identified (Abramovitch et al., 2013; Abdel et al., 2011).

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2.4 Cross-Reactivity (CR)

Cross-reactivity (CR) is an immune-mediated process of IgE antibody recognition, binding, and eliciting an immune response to homologous molecules with similar allergenic properties (Popescu, 2015). The CR of IgE tropomyosin can be clinically and experimentally observed amongst crustaceans, between crustaceans and mollusks, amongst mollusks, and between terrestrial arthropods and crustaceans (Lopata et al., 2016; Abramovitch et al., 2013; Lopata & Kamath, 2012; Reese, Ayuso, & Lehrer, 1999).

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CR is observed to frequently occur in across family of crabs, lobster, and shrimp that are phylogenetically related due to the high homology of TM structure between different crustacean species (>90% identity) (Zhang et al., 2006; Motoyama et al., 2006; Shanti et al., 1993; Reese et al., 1999; Lehrer & McCants, 1987). However, a lower degree of cross-reactivity is also observed between members of the crustacean family, such as barnacles, with amino acid sequence identity of only 61% (Suma et al., 2007; Cartier et al., 1986).

2.5 Effects of Thermal Treatments on Food Allergenicity

Food processing can alter most of the food allergenicity, either by thermal or non-thermal treatments which may results to various structural modifications including hydrolysis, cleave of peptide bonds, aggregation by non-covalent bonds and bipolar reactions, and interactions with other food molecules such as fat and carbohydrates. Thermal treatments involve dry heat such as roasting and infrared heating or moist heat such as boiling, microwave cooking, steaming and autoclaving, while the non-thermal treatments include proteolysis, fermentation, germination, enzymatic and acid treatments (Sathe et al., 2005).

In general, thermal treatment is usually applied to maintain or improve the food quality such as enhance the taste and texture and make it edible, microbiologically safe and digestible. Food may undergo many alterations during cooking process, such as water holding capacity, weight loss, colour and texture changes (Vittadini, & Barbanti, 2011), which dependent on protein denaturation and

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water loss. Moreover, the quality of foods such as meats are affected by many factors including the muscle characteristics and composition, cooking method, and cooking time and temperature (Kim, Chae, & Lee, 2001; Lee et al., 2005).

As a result of this wide range of reactions, thermal treatments will have a strong effect on allergenic proteins either by reducing (loss of epitope) or by enhancing the food allergenicity (Abdel Rahman et al., 2013). IgE-binding epitopes structure in food allergens can be either appeared as linear or conformational (Figure 2.6). Linear epitopes consist of continuous amino acid sequences, while conformational epitopes are formed by spatially adjacent amino acids that are distantly located in the primary structure of proteins (Matsuo et al., 2015). Both epitopes may responsible for allergic reactions but with different effects after subjected to heat-treatments. The conformational epitopes are destroyed due to alteration of the native protein shape by thermal treatments such as cooking, while sequential epitopes are not affected. In addition, thermal treatments may also increased the food allergenicity by generating new allergenic epitopes structure (Sampson, 2004).

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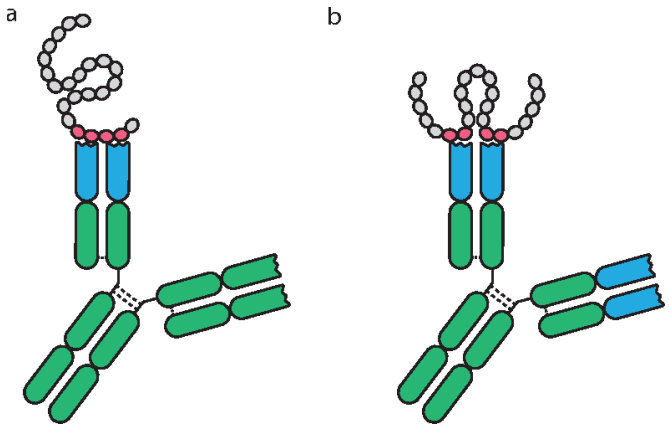


Figure 2.7. Schematic Drawing of Antibodies Binding (A) Linear Epitopes of Consecutive Amino Acid Residuse And (B) Conformational Epitopes Formed By Amino Acids Brought Togather By Protein Folding. Adapted From Forsström (2014).

Boiling is considered as the most commonly used method to cook food and are broadly used during commercial processing of foods (Dominguez, Gomez, Fonseca, & Lorenzo, 2014; Mora et al., 2011). Boiling is the rapid evaporation of liquid, which occurs when the liquid is heated to its boiling point. Heat is then transmitted to the food from the boiled water (Bayer, Koninck, Leonard, Hell, & Schulman, 2001). Thermal treatments including boiling may decrease the food allergenicity by destroying the existing epitopes on the food allergen (Latosińska & Gawdzik, 2014; Taylor, 2008), most probably because of the loss of secondary and tertiary protein structures causing it to be fragmented, loss of solubility or aggregates to form random-coil (Zailatul et al., 2015). Studies on prawn allergens have demonstrated that boiling process decreased the allerginicity of prawn by decreasing the number of allergenic bands (Zailatul et al., 2012; Latosińska & Gawdzik, 2014). Nurul et al. (2015) also reported the allergenicity of mud crab *S. serrata* was decreased after boiling treatments, suggesting that boiling treatments can be used as a tool to reduce prawn allergenicity.

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However, thermal treatments including boiling can also increase the food allergenicity by generating new epitopes, through protein aggregation and the Maillard reaction (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). Maillard reaction is the reaction between free amino groups on protein and the aldehyde or ketone groups of carbohydrate as shown in Figure 2.7, which may increase the capacity of IgE-binding in shellfish (Teodorowicz, van Neerven, & Savelkoul, 2017; Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005) as seen in the cooked extracts of black tiger prawn (Abramovitch et al., 2013; Kamath et al., 2013; Sahabudin et al., 2011), blue crab (*Portunus pelagicus*) (Abramovitch et al., 2013) and mud crab *S. serrata* (Nurul et al., 2015).

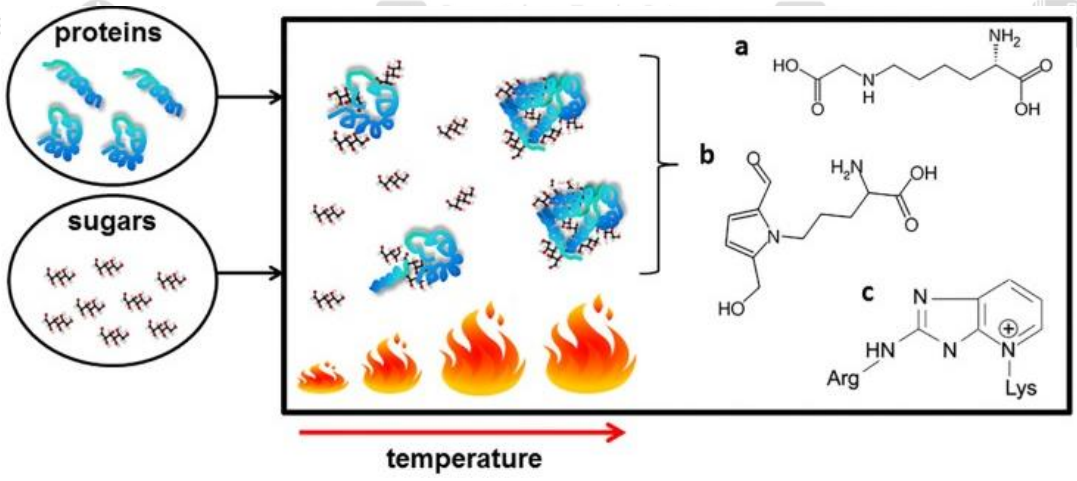


Figure 2.8. Simplified Scheme of the Formation Of Maillard Reaction Product During Food Processing. Adapted From Teodorowicz Et Al. (2017).

A study has reported a total of 42 patients had positive SPT and food challenge using cooked shrimps instead of the raw extracts (Carnés et al., 2007). Tropomyosin, the major shellfish allergen is a highly stable protein which resist to both thermal treatments and non-thermal treatments due to the highly stable alpha

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helical coils in its secondary structure such us in blue swimming crab, shellfish, mollusks (Abramovitch et al., 2013; Lopata et al., 2010; Taylor, 2008). The thermal stability of crab tropomyosin was demonstrated to be stronger in the boiled crab than raw crab. Therefore, the use of boiled tropomyosin appears to be more effective in diagnosing the seafood allergy, crab (Reese et al, 1999).

2.6 Methods of Allergen Discovery and Detection

2.6.1 Protein Profiling by Electrophoresis

Electrophoresis is a common technique that has been severally modified to enhance its resolution, quantitation, detection, and reproducibility. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) is a technique widely used to separate proteins by their molecular weights which showed appropriateness with suitable precision, accuracy, and the detection limit for assessing the protein composition of glycerinated allergen extracts of several food allergenic sources. Meanwhile, 2D electrophoresis (2-DE) is a separation technique in which proteins separation is achieved based on their isoelectric points (PI) in the first dimension and based on their molecular weight in the next dimension, it is one of the preferred techniques for the separation comparative analysis of proteins (Xu et al., 2006). In addition, the 2-DE technique of protein separation is accessible and cost-effective. It has a high resolving power and facilitates the detection of several proteins on a single gel plate (Issaq and Veenstra, 2008). With 2D-E, the basic objective is to identify new proteins and to measure their relative composition in the comparative samples. The 2-DE is a choice

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method because it can resolve large protein numbers, and can stain these proteins to enable their quantitation (Issaq and Veenstra, 2008).

2.6.2 Immunoblotting

This process is also called Western blotting. It is based on the specificity of antibodies when identifying target proteins from several unrelated protein species (Towbin et al., 1979). Immunoblotting is conventionally used to map known proteins in an electrophoretic mixture. This is a choice method for the simultaneous visualization of 1D- and 2D-separated proteins which share a common epitope related to specific post-translationally modified amino acids or to a specific functional/structural domain such as antiphosphoresidues and anti-O-linked N-acetylglucosamine antibodies (Gao et al., 2001; Buonocore et al., 1999). Immunoblotting has been widely deployed for the differential visualization of tyrosine-phosphorylation profiles in tissues and cells under varying conditions (Magi & Liberatori, 2005), as well as to study signal transduction pathways after stimulation (Soskic et al., 1999).

In proteomics, immunoblotting and 2-DE has been combined for the rapid identification and visualization of target proteins, drugs with protein adducts, (Magi et al., 1998; Magi et al., 1995), pathogen-related antigenic proteins, tumors, and allergens. Here, the patients' serum is the major antibody source (Unwin et al., 2003; Sanchez-Campillo et al., 1999). Additionally, 2D immunoblotting and mass spectrometry have been combined and used to characterize 2D electrophoretic cross-

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reactive isoforms of the same protein which for instance resulted from an alternative splicing or proteolytic cleavages (Borchert et al., 2003; Zanusso et al., 2002).

2.6.3 Mass Spectrometry

Mass spectrometry (MS) is a potential alternative to the antibody-based assays in common use due to its ability to quantitate several proteins in complex matrices with a good level of sensitivity. MALDI-TOF mass spectrometer is a leading method for the analysis of several macromolecules of biological origin (Marvin, Roberts, & Fay, 2003). It is a promising biomolecules identification method owing to its high accuracy and sensitivity combined with a wide mass range (1-300 kDa) (Sancho & Mills, 2010; Marvin et al., 2003). In allergy research, several allergens from various sources have been identified using MALDI-TOF mass spectrometer, such as crab (Rosmilah et al., 2012), squid (Yadzir et al., 2012b), mackerel (Bangping et al., 2011), wheat (Akagawa et al., 2007).

2.7 Conclusion and Research Gap

a. Production of protein extracts of S. paramamosain

The diagnosis of allergy, which is including crab allergy, is usually done using whole crude crab extracts. However, the crude crab extracts currently used for such allergy tests are prepared using only a few selected species which are not commonly consumed in Malaysia such us *S. serrata*. Thus, to enhance the accuracy and

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specificity of the diagnostic tests, the production of allergen extracts from local crabs is crucial. This study selected one of the most important mud crab species in Malaysia (*S. paramamosain*) to be investigated.

b. Determination of the protein profiles of S. paramamosain.

So far, there has been no local study on the determination of protein profiles of different body parts and gender of *S. paramamosain*. The biochemical composition and nutritional value should be known to facilitate the farming/fattening, processing, utilization, and marketing of value-added crab products for human consumption. The specific biochemical and allergenic characteristics of mud crabs with respect to differences in gender and body parts have not been studied. Hence, a study with the purpose of determining the protein profiles and subsequently allergenic profile of body and claw meats of *S. paramamosain* is essential. Therefore, in this study, the proteomic profiles of *S. paramamosain* will be investigated using SDS-PAGE technique. This study is a first step for conducting a study on the characterization of the allergenic proteins of this crab species

d. Determination of the thermal stability of allergenicity of S. paramamosain

Seafood including crabs are usually subjected to heat treatments to make it edible. So far, there has been no study on the determination of the effect of boiling on allergenicity in individual body parts and gender of *S. paramamosain*. Understanding of thermal treatments are crucial in crab allergenicity as these results would suggest the clinicians and patients the best cooking practice in decreasing crab allergenicity. Boiling is considered as the most commonly used method to cook food including

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crabs (Dominguez et al., 2014; Mora et al., 2011). Therefore, the effects of boiling on the allergenicity of *S. paramamosain* were addressed in this study.

e. Identification of the major allergens of S. paramamosain

The identification of allergens is important for the understanding of specific immune response to an allergen that would benefit in developing of reliable diagnostic tests and management of allergic patients. At present, there are a few reports on the identification of crab allergens. So far, there is no local study on identification of the allergenic proteins of S. *paramamosain.* Therefore, this study will use mass spectrometry analysis to diagnose the major and minor allergens of this species of crab.

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CHAPTER 3

METHDOLOGY

3.1 Introduction

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This chapter discussed about research design and overall research methodology. The methodology that was used in this study includes protein extraction, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting experiments.

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3.2 Research Design

Figure 3.1 shows the design of this research, while Table 3.1 indicated the details of the research design in terms of research objectives, methodology and data analysis.

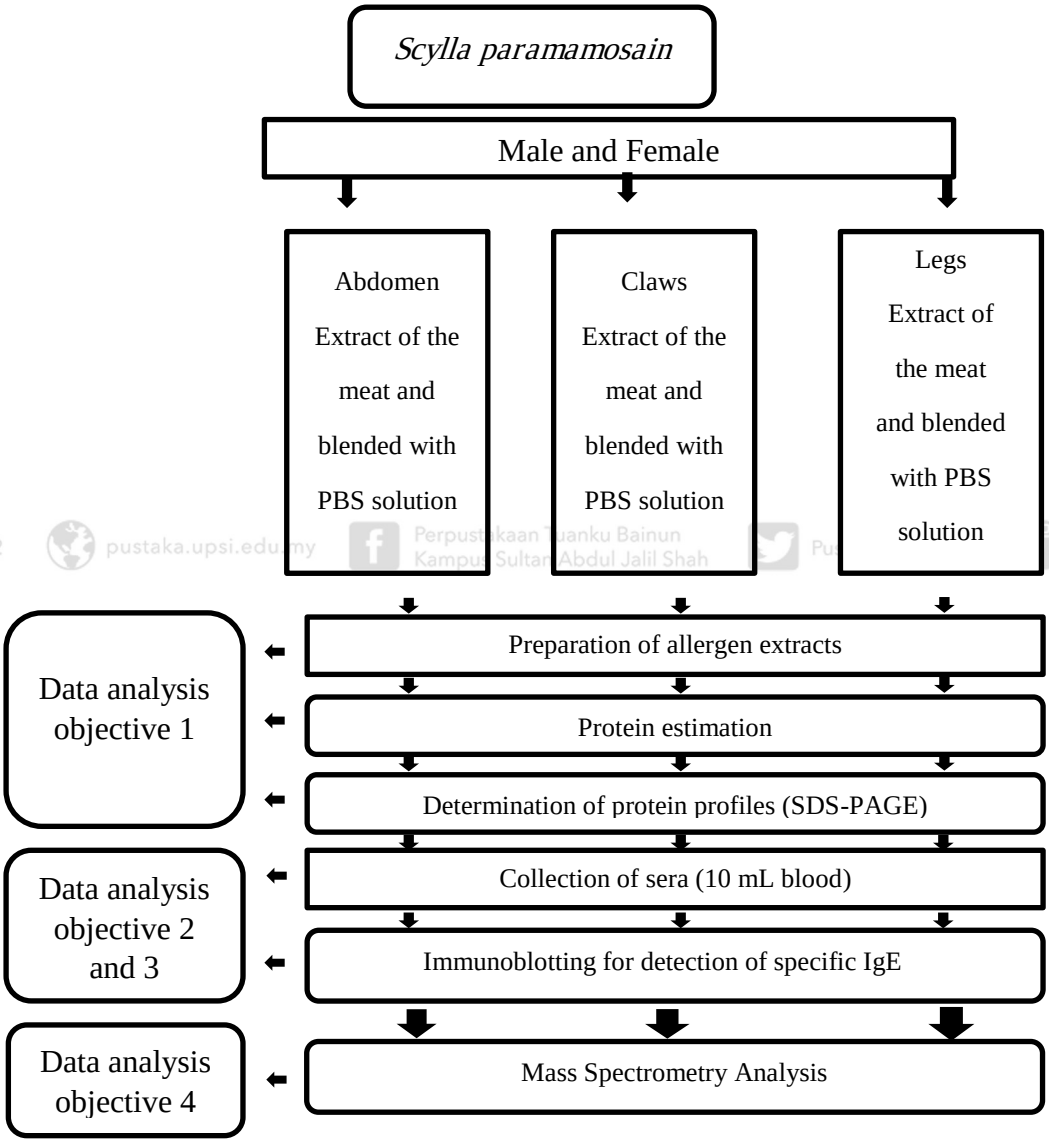


Figure 3.1. Research Design

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Table 3.1

Simplified Research Design

Objectives	Parameter	Methodology	Analysis
To determine the protein profiles of different body parts (abdomen, claws, legs) for male and female mud crab *Scylla paramamosain*	Protein profiles	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method described by Nurul et al. (2012)	Descriptive (frequency and percentage)
To identify the allergenic properties of different body parts (abdomen, claws, legs) for male and female mud crab *Scylla paramamosain*	Allergenic protein bands	Immunoblotting according to Rosmilah et al. (2012a) and Zailatul et al. (2012).	Descriptive (frequency and percentage)
To identify the effect of boiling on allergenicity of different body parts of male and female *Scylla paramamosain*	Allergenic protein bands	Immunoblotting according to Rosmilah et al. (2012a) and Zailatul et al. (2012).	Descriptive (frequency and percentage)
To identify the major allergens of *Scylla paramamosain* by mass-spectrometry analysis.	Identity of major allergens	Mass spectrometry (by using the method described by Rosmilah et al. (2013)	Descriptive (frequency and percentage)

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3.3 Methodology

3.3.1 Specimen of *Scylla paramamosain*

A total of ten samples of live adult (mature) of six males and four females of *S. paramamosain* with weight of approximately 300 gram each were collected from a local supplier from Sungai Petani, Kedah.

3.3.2 Collection of Sera

Stored sera (-20 ˚C) from 28 patients with allergy to *S. paramamosain* were used in this study. Serums from non-allergic individuals were used as negative controls. This project was approved by Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia for Ethical (Ethical Approval: NMRR-08-763-1918).

3.3.3 Sample Preparation

3.3.3.1 Crab dissection and allergen preparation

The muscle tissues from individual body parts (abdomen, all claws, and all legs) of male and female mud crab *S. paramamosain* were harvested and washed with distilled water. 5g of the muscle tissues from each body parts of both male and female were weight and stored at -20ºC until use.

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3.3.3.2 Allergen preparation

For preparation of allergen extracts, the first step was homogenization of the crab samples with phosphate-buffer saline (PBS, pH 7.2). For both male and female body parts (abdomen, claws, legs), 5g of each part was blended with 100 ml of PBS, followed by overnight extraction by using incubator shaker at 4ºC under continuous shaking (50 rpm). After that, the homogenates were centrifuged at 14,000 rpm at 4ºC for 15 min. The supernatants were filtered with filter paper, followed by filtration using sterile syringe filters 0.2 µm. Then, the crab extracts were lyophilized using a freeze dryer. The lyophilized extracts were stored at -20°C until use. Total protein content in each prepared extracts was determined using the Bio-Rad Protein Assay Kit (BioRad, USA), following the manufacturer's instructions.

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3.3.3.3 Boiling Treatment

The boiled crab extract was prepared according to the methods by Zailatul et al. (2014) with slight modifications. 10g of crab muscles were weighed. The temperature before and after boiling was measured using Thermocouple Dual Input Thermometer Type K/J. The crab samples were then boiled in PBS until the temperature inside the crab muscle reaches 100°C for approximately 10 minutes. The boiled crab samples were then subjected to protein extraction as mentioned above.

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3.3.4 Total Protein Determination

The protein concentration of each extract was determined using the Bradford Reagent Kit (Biorad, CA, USA), following the manufacturer's instructions. Bovine serum albumin (BSA) (1.4 mg/ml) was used as the protein standard. The standard protocol was performed in format of 5 ml using the dye reagent at 1x concentration. The dye was removed from 4°C storage, let it warm to ambient temperature and inverted a few times before use. Then, 2 mg/ml BSA standard was diluted with PBS to 5 concentration of protein standard as in Table 3.2. A total of 100 µl diluted BSA standard and crab extracts were use in each assay. 5 ml of 1x dye reagent was added to each samples, mix well and incubated at room temperature for 5 min. The colour intensity of the protein standards was measured using a spectrophotometer at 595 nm wavelength and a graph of absorbance against protein concentration was plotted to get a standard calibration curve.

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Table 3.2

Preparation of Standard Assay Dilution For Total Protein Assay

Tube #	Standard Volume (µl)	Source of standard	PBS volume (µl)	Final protein (µg/ml)
1	300	2 mg/ml stock	0	2,000
2	375	2 mg/ml stock	125	1,500
3	325	2 mg/ml stock	325	1,000
4	175	Tube 2	175	750
5	325	Tube 3	325	500
6	325	Tube 5	325	250
7	325	Tube 6	325	125
8 (blank)	_	_	325	0

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3.3.5 SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the protein profile in the prepared extracts by using the method described by (Rosmilah et al., 2012). Briefly, the samples were diluted with Laemmli buffer with volume: volume ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 for standardization of protein band separation in SDS-PAGE. Then, the samples and prestained molecular markers (Bio-Rad, CA) were incorporated into wells containing 12% of resolving gel followed by 5% of stacking gel using a Mini Protean 3 Apparatus (Bio-Rad, USA), and separated at 120 mA for 50 minutes. After the electrophoresis, the gel was stained using Coomassie blue R-250. Then the gel was destained in Destaining solution I and II. The molecular weight of the proteins was estimated using an imaging densitometer (Bio-Rad, CA).

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Table 3.3

Dilution Factor Used For Preparation of Sample For SDS-PAGE In The Study

Dilution factor	Diluted sample with Laemmli buffer
1:1	10 µl of sample : 10 µl of buffer
1:2	10 µl of sample: 20 µl of buffer
1:3	10 µl of sample: 30 µl of buffer
1:4	10 µl of sample: 40 µl of buffer
1:5	10 µl of sample : 50 µl of buffer
1:10	5 µl of sample : 50 µl of buffer

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3.3.6 Immunoblotting

Immunoblotting was used to identify the IgE binding components in raw mix parts extract using sera from 28 patients with crab allergy. Meanwhile immunoblotting for the individual crab parts and boiled extracts was performed using 10 selected sera which detected at least one major allergen in the raw mix parts immunoblotting.

The separated proteins of crab were electrophoretically transferred from unstained SDS-PAGE gel to a 0.45 µm pore size nitrocellulose membrane using a Mini Transblot System (BioRad, USA). The nitrocellulose blot was then cut, washed with tris-buffer saline (TBS) containing Tween 20 (TTBS) and then blocked with 5% non-fat milk in TBS. The blocked strip was then incubate overnight with individual patient's serum. The bound IgE on the strip was then detected by incubation with biotinylated goat antihuman IgE (KPL, USA) followed by incubation in streptavidin-conjugated alkaline (BioRad, USA). Finally, the alkaline phosphate Conjugate Substrate Kit (BioRad, USA) was used to detect the bound IgE.

3.3.7 Mass Spectrometry Analysis

The Coomassie-stained protein bands in SDS-PAGE gel corresponding to those recognized as the major allergens by the above sera were manually excised and subjected to destining, reduction, carbamidomethylation prior to in-gel tryptic digestion on a MassPrep workstation (Micro, UK) using modified procien Trypsin (Promega, USA). Peptide Mass Fingerprints were obtained using 5% aliquot of the

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samples, analysed on a MALDI-ToF instrument. The remaining extracts were concentrated in a speed Vac and reconstituted in 1% aqueous formic acid. This was subjected to CL-MS/MS analysis, performed on a CapLC/QToF II instrument (Micromass, UK). All fragment ion (MS/MS) spectra were search against the MSDB database using the Mascot search tool (Matrix Science).

3.4 Conclusion

In this chapter, a thorough methodology was outlined based on the research objective which includes sample selection, protein extractions, boiling treatments, total protein estimation, SDS-PAGE, immunoblotting experiments and mass spectrometry.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

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The results obtained were discussed thoroughly in this chapter. According to the research objectives that have been listed earlier in Chapter 1, findings of the results were presented and discussed.

4.2 Objective 1: To Determine the Protein Profiles of Raw and Boiled Extracts from Different Body Parts and Genders of Mud Crab *Scylla paramamosain*

Proteins play an important role for an organism as it is involved either as transporters, messengers or catalysts in human functions (Yun et al., 2009). The investigation of protein profiles can provide an overview of prawn protein characteristics in this research, which includes data and discussion of total protein concentration and protein

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profiles of raw and boiled extracts from different body parts and gender of mud crab *S. paramamosain*.

4.2.1 Total Protein Concentration of Mud Crabs

The standard curve of the Bio-Rad protein assay, using bovine serum albumin (BSA), was shown as a protein standard (BSA) in Figure 4.1, whereas the results of total protein concentration were shown in the raw and boiled treatment extracts in Table 4.1.

In general, the raw mud crab extracts have higher total protein content (17.067 to 20.334 mg/mL) than the boiled extract (6.349 to 9.814 mg/mL). The raw female leg extract showed the highest protein content of 20.334 mg/ml, while the boiled male claw extract had the lowest protein content of 6.349 mg/mL. These findings demonstrated that different crab parts and gender have varied protein content, supporting the previous findings by several researchers that reported various body parts of male and female *Scylla serrata* and *Scylla tranquebarica* had significant differences in their protein composition (Sreelakshmi et al., 2016; Zafar et al., 2004; Khan, 1992). They reported the protein content was highest in body meat and lowest in claw meat of *S. tranquebarica* than *S. serrata.* Similarly, Mia et al. (2016) also reported the cephalothorax (abdomen) of freshwater crab (*Paratelphusa lamellifrons*) contains then highest amount of protein (34.46%) followed by legs (34.23%) and cheliped (claws) which has only 32.38%. Meanwhile, the meat from female *S. serrata* and *S. tranquebarica* crabs had significantly higher protein content than males irrespective of species and body parts variation (Sreelakshmi et al., 2016; Zafar et al.,

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2004; Khan, 1992). This variations of protein content in body parts and crab gender might play an important role in allergenicity of *S. paramamosain* among crab consumers.

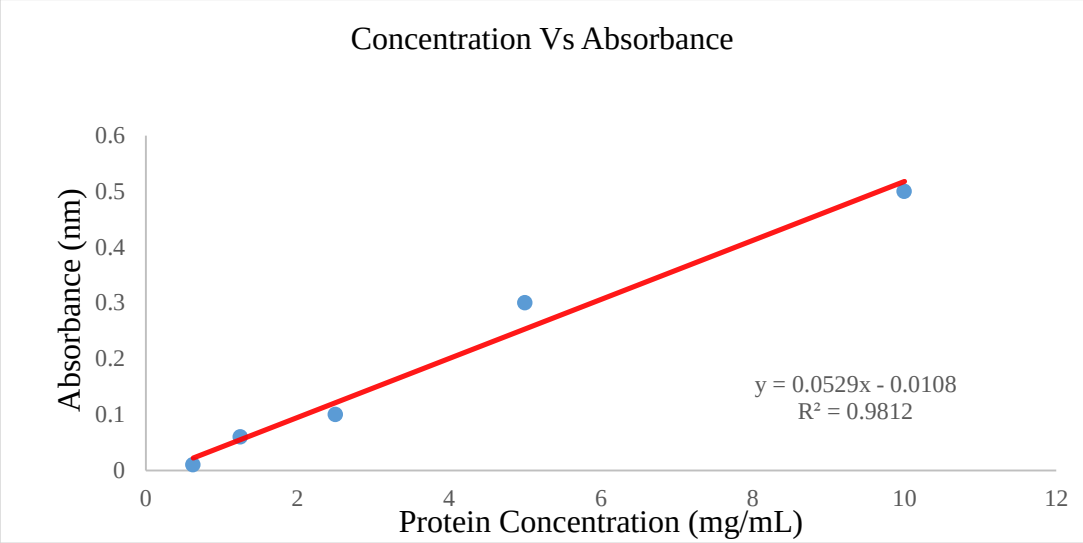


Figure 4.1. Representative Standard Curve of Bio-Rad Protein Assay, Bovine Serum Albumin (BSA). Each Experimental Point Is The Mean Of Duplicate Determinations.

Table 4.1

Total Protein Concentration (Mg/Ml) of Raw And Boiled Crab Extracts

No	Crab Parts	Protein Concentration (mg/mL)	
		Raw	Boiled
1	Mix Raw	18.577	7.660
2	Male claw	19.344	6.349
3	Male leg	17.785	8.824
4	Male abdomen	17.067	8.403
5	Female claw	19.295	9.022
6	Female abdomen	18.899	9.814
7	Female leg	20.334	8.874

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4.2.2 Protein Profiles of Crab Extracts

The protein components of raw and boiled treated extracts were separated by SDS-PAGE. Figure 4.2 and Table 4.2 displayed the comparison of protein profiles of raw mix parts and all raw individual body parts of *S. paramamosain* extracts.

Meanwhile, Figure 4.3 displayed the comparison of protein profiles of raw mix parts and male parts of S. *paramamosain* extracts in six different dilutions, while Figure 4.4 showed the comparison between the mix parts and female parts.

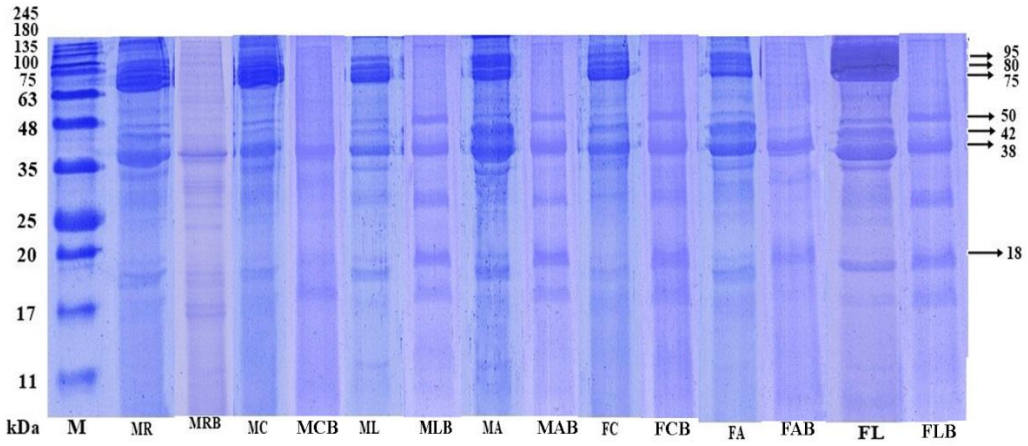


Figure 4.2. The Comparison of Protein Profiles Between Raw Extracts And Boiled Extracts From Different Genders And All Crab Parts Of Mud Crab *S. Paramamosain*. Lane MR, MRB, MC, MCB, ML, MLB, MA, MAB Are Mix Parts Raw, Mix Part Boiled, Male Claw Raw, Male Claw Boiled, Male Leg Raw, Male Leg Boiled, Male Abdomen Raw, Male Abdomen Boiled, Respectively; While FC, FCB, FA, FAB, FL, FLB Are Female Claw Raw, Female Claw Boiled, Female Abdomen Raw, Female Abdomen Boiled, Female Leg Raw And Female Leg Boiled, Respectively. M Is Molecular Weight Markers In Kilodalton (Kda).

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Table 4.2

The Comparison of Raw And Boiled Extracts of Male and Female Mud Crab Paramamosain.

No. of bands	MW (kDa)	MR	MRB	MC	MCB	ML	MLB	MA	MAB	FC	FCB	FL	FLB	FA	FAB
1	15	√	√	√	√	√	√	√	√	√	√	√	√	√	√
2	18	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3	24	√	√	√	√	√		√		√		√		√	
4	28	√	√	√		√	√	√	√	√	√	√	√	√	
5	32	√	√	√	√	√		√		√				√	√
6	35	√	√	√	√			√		√		√		√	√
7	36	√	√		√		√	√	√	√	√	√	√	√	√
8	38	√	√	√	√	√	√	√	√	√	√	√	√	√	√
9	42	√		√		√		√		√				√	
10	45	√				√		√		√		√		√	
11	50	√		√		√	√	√	√	√	√	√	√	√	
12	62	√	√	√	√	√		√		√		√		√	√
13	75	√						√		√		√		√	
14	78	√		√		√		√		√		√		√	
15	80	√		√		√				√				√	
16	86	√				√				√		√		√	
17	95	√		√		√		√		√		√		√	
18	110	√				√		√		√		√		√	
19	120	√		√		√		√		√					
20	140	√		√				√						√	
21	173	√						√		√		√			
22	190	√		√		√		√		√					
23	250	√										√		√	
24	>250	√												√	
Total band	**24**	**24**	**9**	**16**	**8**	**17**	**6**	**20**	**6**	**21**	**6**	**17**	**6**	**21**	**7**

Note: MR= mix parts raw, MRB= mix part boiled, MC= male claw raw, MCB= male claw boiled, ML= male leg raw, MLB= male leg boiled, MA= male abdomen raw, MAB= male abdomen boiled, FC= female claws raw, FCB= female claw boiled, FA= female abdomen raw, FAB= female abdomen boiled, FL= female leg raw, FLB= female leg boiled.

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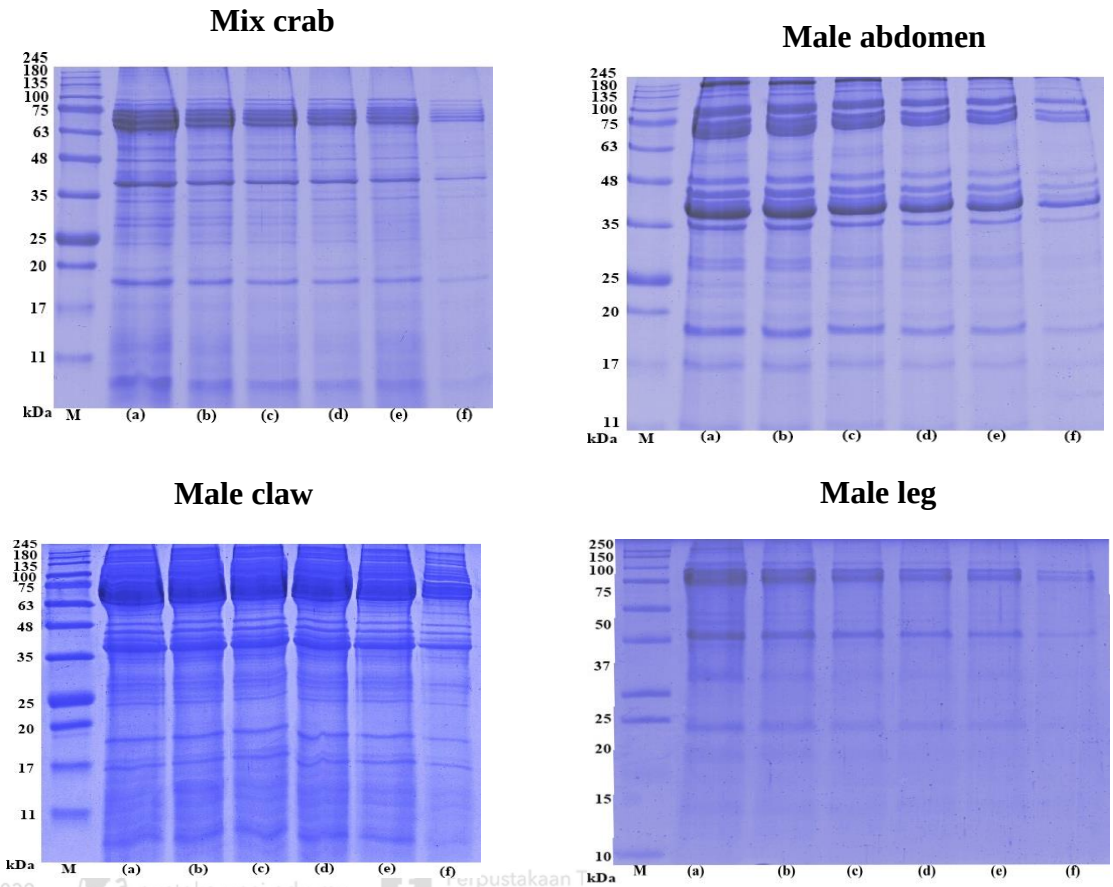


Figure 4.3. The Protein Profiles of Raw Extracts of Mix Raw Parts And Raw Male Parts of *S. Paramamosain* In Six Different Dilutions Of 1:1 (A), 1:2 (B), 1:3 (C), 1:4 (D), 1:5 (E) And 1:10 (F).

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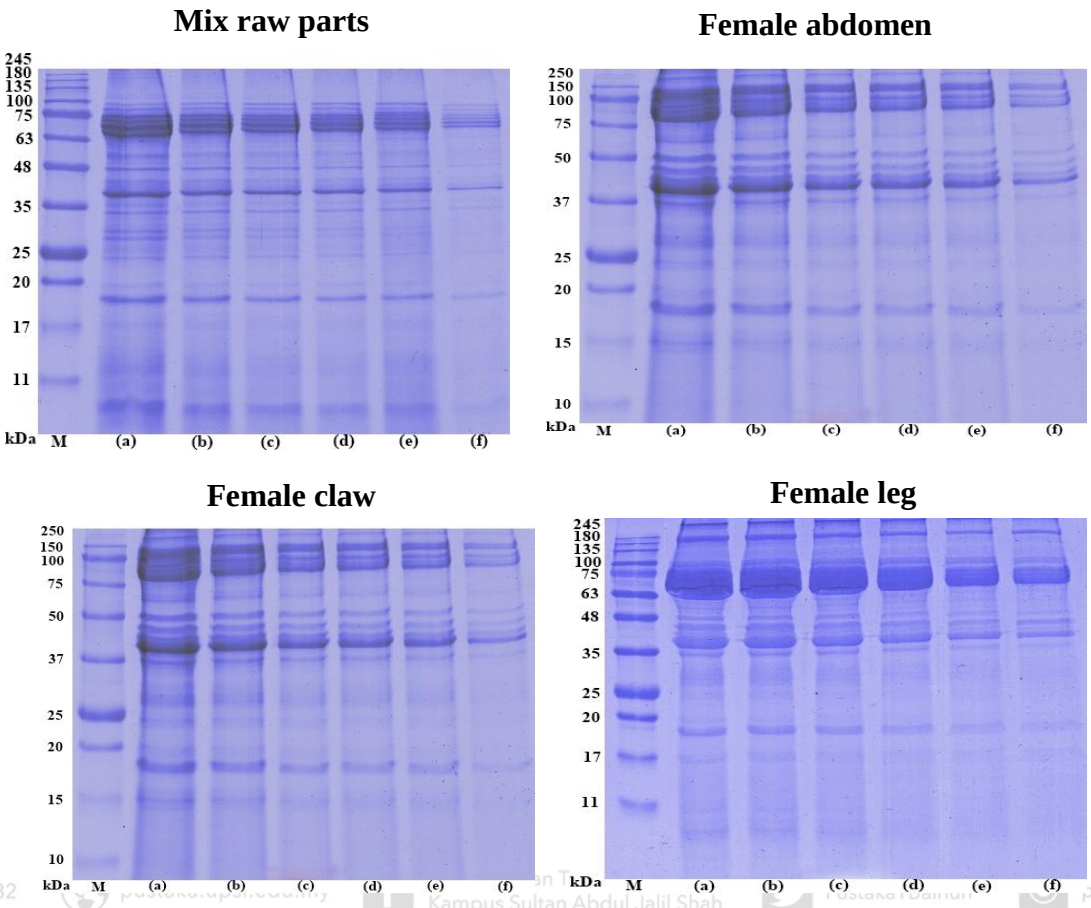


Figure 4.4. The Protein Profiles of Raw Extracts of Mix Raw Parts and Female Raw Parts of *S. Paramamosain* In Six Different Dilutions Of 1:1 (A), 1:2 (B), 1:3 (C), 1:4 (D), 1:5 (E) And 1:10 (F).

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As can be seen, the protein profiles of raw extracts from different body parts and gender of mud crab *S. paramamosain* are almost identical. All the protein bands resembles each other. Only slight differences could be seen in the intensities of the bands. The raw extract of mix crab parts revealed the highest bands of proteins; approximately 24 bands in the range of 15 to >250 kDa were fractionated. This finding is in accordance with previous study on local crab species which reported the raw extracts of blue crab *Portunus pelagicus* and red crab *Charybdis feriatus* exhibited at least 20 protein bands (Misnan et al., 2012).

Meanwhile, among the individual body parts and gender of *S. paramamosain*, at least 16 to 21 protein bands were seen. Overall, several prominent bands ranging from 140 to 75 kDa, 50 to 38 kDa and 18 kDa can be seen in all raw extracts, but with slight differences in their intensities. The male crab extracts have 16 to 21 protein bands, while the female crab extracts contained 17 to 21 protein bands. Both the female abdomen and female claws has the highest number of bands (21 bands), while the male claws has the least number of bands (16 bands). This result indicating the variation of protein profiles between the individual body parts of *S. paramamosain*, and female crab was considered has higher protein composition than the male crab. This finding is in accordance with the results of total protein contents as described above. Sreelakshmi et al. (2016) observed that the protein content of female mud crab (*Scylla serrata*) is higher than the male which also agrees with the present findings. In contrast, Kuley et al. (2008) reported that the protein content of male blue crab (*Callinectes sapidus*) is higher than the female crab. Thus, this finding supported the

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facts that a significant difference in the biochemical composition of crabs with respect to their species, gender and body parts exists.

According to SDS-PAGE analysis, both female and male mud crab extracts indicated almost homogenous protein profile patterns. The obvious bands of protein were seen at about 100, 75, 38, and 18 kDa. A similar report had shown that no significant difference was noticed in the band pattern of proteins amidst the blue crab species in terms of gender distinction (Gangar et al., 2004).

In contrast to raw mud crab extracts, boiled extracts showed different results. Figure 4.5 and Table 4.3 showed the protein profiles between raw mix parts and boiled mix parts and individual parts. Meanwhile, Figure 4.6 displayed the comparison of protein profiles of raw mix parts and all boiled male parts of S. *paramamosain* extracts in six different dilutions, while Figure 4.7 showed the comparison between the boiled mix parts and female parts.

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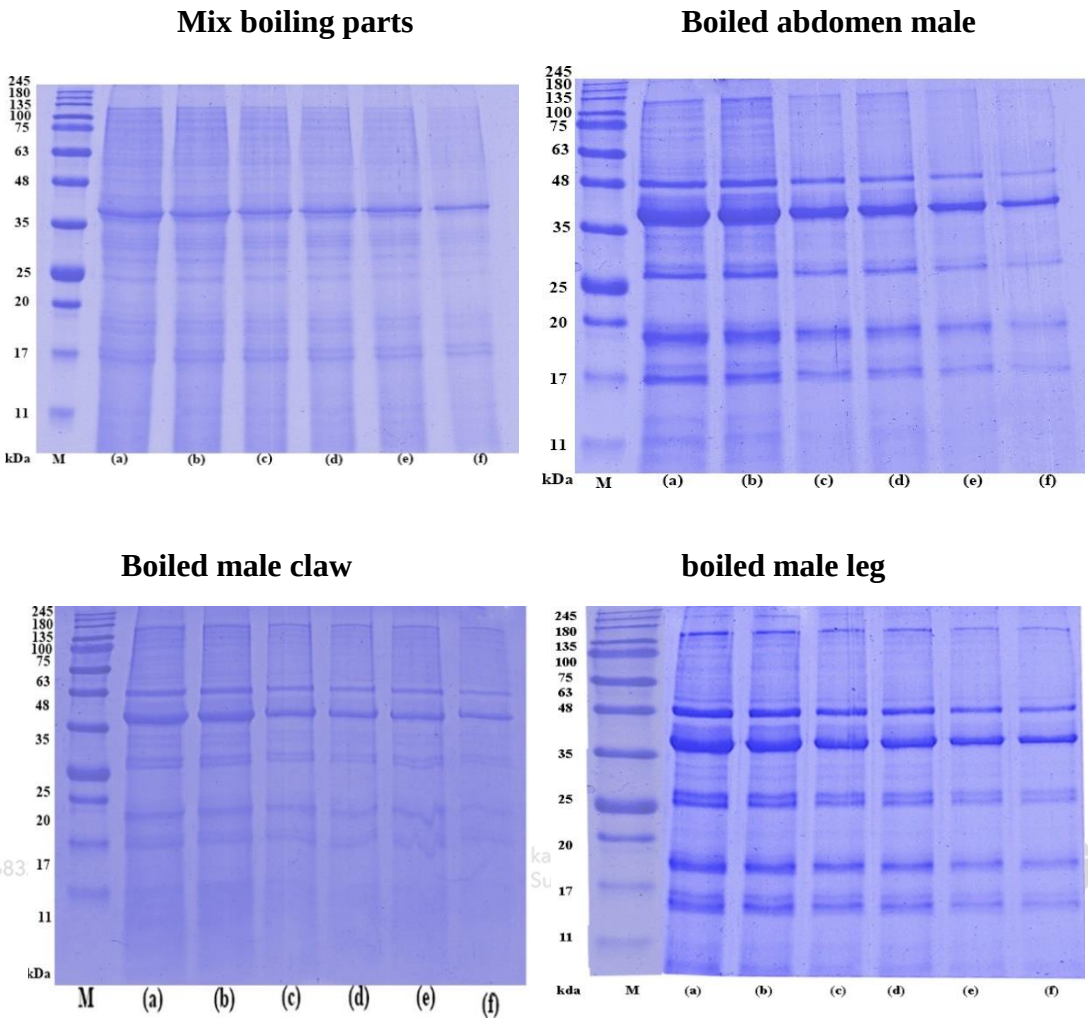


Figure 4.5. The Protein Profiles of Boiling Extracts of Mix Boiled Parts and Male Boiled Parts of *S. Paramamosain* In Six Different Dilutions of 1:1 (A), 1:2 (B), 1:3 (C), 1:4 (D), 1:5 (E) And 1:10 (F).

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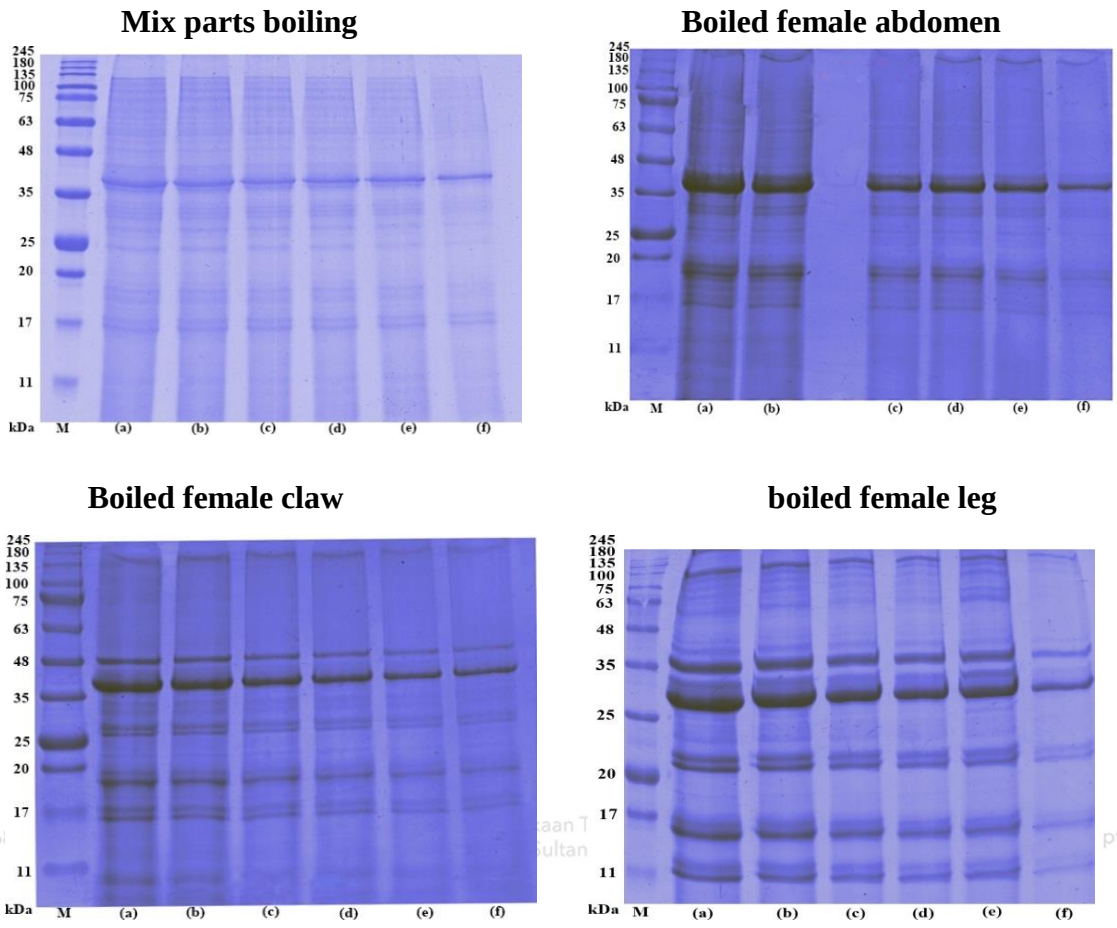


Figure 4.6. The Protein Profiles of Boiling Extracts of Boiled Mix Parts And Boiled Female Parts of *S. Paramamosain* In Six Different Dilutions of 1:1 (A), 1:2 (B), 1:3 (C), 1:4 (D), 1:5 (E) And 1:10 (F).

Mostly, the protein bands can be seen reduced when comparing to raw protein extracts. In general, a total of 10 heat-resistant protein bands between 15 to 62 kDa were detected in the boiled extracts of *S. paramamosain*, indicating that only less than half of the crab proteins are resistant to heating process, whereas the other proteins, particularly the higher molecular weight protein bands above 62 kDa are sensitive to boiling process. This finding is similar to other reports which had

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demonstrated quite a number of heat-sensitive and heat-resistant proteins are present in crustaceans including crabs (Gill et al., 2009; Samson et al., 2004).

The similarities between the raw and all the boiled extracts are that the bands of 36-38 kDa, 18 and 15 kDa remain detected in all extracts even after the heating process applied. This result shows that the 36-38 kDa protein was conserved in all the extracts regardless of the cooking process employed. It was widely reported that many food allergens are resistant to denaturation, which allows them to retain the ability to elicit an immune reaction under conditions such as heating and enzymatic degradation (Ebo & Stevens, 2001). This is seen in the case of tropomyosin (34 to 38 kDa), a highly stable major shellfish allergen, which involved in muscle contraction (Lopata & Kamath, 2012). Thus, the 38 kDa protein is most likely to be tropomyosin. However, the properties of the 18 and 15 kDa bands are rarely described in literatures, thus were remained unknown.

The band intensities of the 15, 18 and 36-38 kDa were higher compared to the raw extracts. This phenomenon has been previously reported for other shellfish allergens (Kamath et al., 2013), possibly due to the Maillard reaction. Maillard reaction is the reaction between free amino groups on protein and the aldehyde or ketone groups of sugar. This reaction has been found to increase the band intensities as well as IgE-binding capacity of certain allergens (Kamath et al., 2013).

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Meanwhile, the loss of the proteins between 42 to 250 kDa in the boiled extracts may be related to the effects of heating on proteins, indicating these proteins are heat-sensitive proteins. The disappearance of these protein bands might be due to the influences of heat on protein composition because of globular proteins degradation through heat which might destroy their tertiary structure composition, cause insolubility and change their random-coiled conformation (Peng & Gygi, 2001). Thus, distorting the structure of protein through heat processing might cause epitope elimination, unmasking or masking, modification, then decreasing, increasing or having no effect on food allergenicity (Sathe et al. 2005).

In general, among the boiled crab parts, the boiled mix body parts extract has the highest number of heat-resistant bands (9 bands) compared to the individual boiled crab part extracts, which only ranged from 6 to 8 protein bands. This is not surprising as the boiled mix parts extract was prepared from a mixture of all six body parts studied.

Among the boiled body parts, the male claw has the highest number of protein bands (8 bands), followed by female abdomen (7 bands), and whereas the other boiled crab parts revealed only six protein bands. The protein profile patterns between all boiled extracts were almost identical except for some variation of certain protein bands. For example, two bands at 28 and 62 kDa did not appeared in the male claws and female abdomen, while three bands at 32, 35 kDa and 50 kDa were detected only in the male claws and female abdomen. Meanwhile, another faint protein band at 24

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kDa was only detected in the male claws. Therefore, this study believed that the different body parts of crab have variation protein profiles without any specific pattern. However, this study supported the previous findings by Sreelakshmi et al. (2016) which showed a significant difference in the biochemical composition including protein contents of mud crabs with respect to their species, gender and body parts.

Overall, this study showed that all crab parts and genders have almost similar protein profiles in both raw and boiled forms. However, the boiling process has significantly decreased the number of protein bands in all crab parts. This finding is important because crustaceans including crabs are mostly consumed in both states (Sathe et al., 2005; Samson et al., 2004; Besler et al., 2001). Therefore, both raw and boiled forms may impact differently on crab allergenicity.

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4.3 Objective 2: To Identify the Allergenic Proteins of Raw Extracts from Different Body Parts and Gender of Mud Crab *Scylla paramamosain*

The IgE-binding proteins in raw mix *S. paramamosain* parts extract were detected by immunoblotting using 28 sera from mud-crab allergic patients. Figure 4.8 displayed the immunoblotting results, while Table 4.3 summarized the immunoblotting results analysis.

The raw extract of mix parts has 18 IgE-binding proteins between 18 to >250 kDa, indicating *S. paramamosain* has numerous allergenic proteins. This finding agrees with Nurul et al. (2015) which also detected multiple IgE-binding proteins between 23 and 250 kDa in mud crab *Scylla serrata.*

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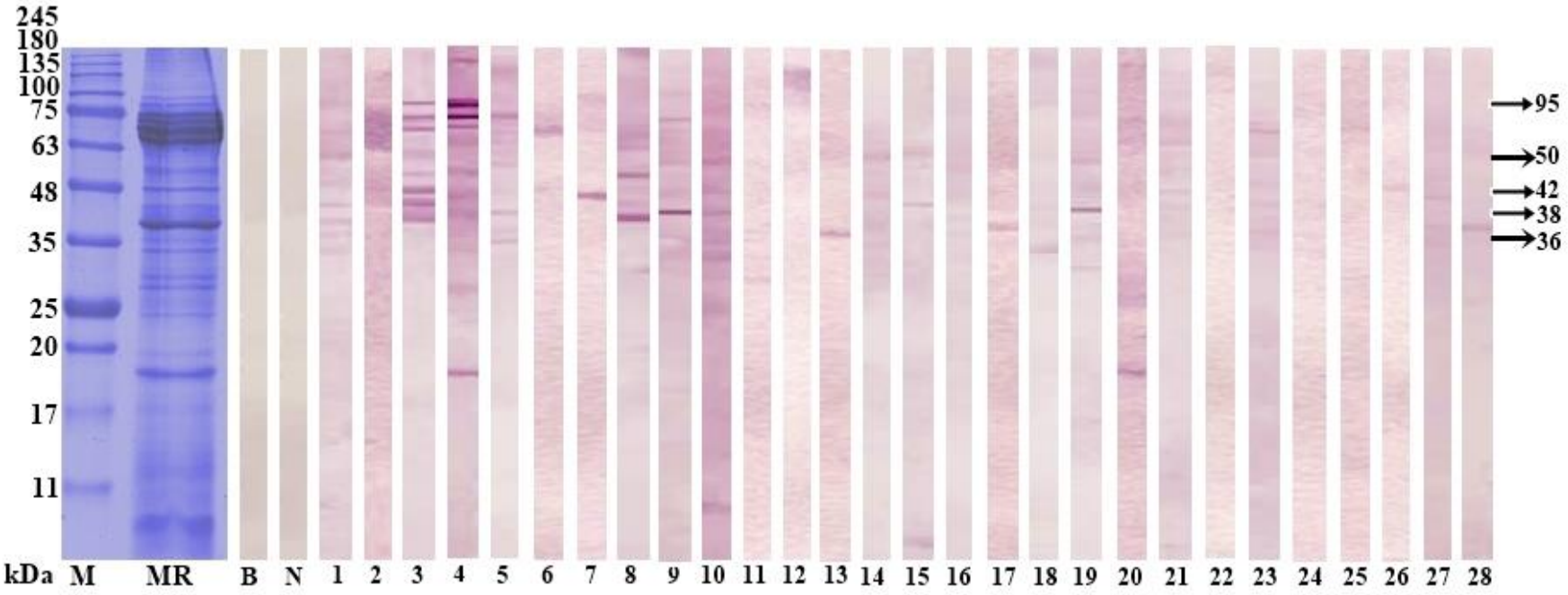


Figure 4.7. Immunoblotting Results of Raw Mix Parts of *S. Paramamosain* Using Sera From 28 Mud Crab-Allergic Patients (Lane 1 To 28). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Mix Raw; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.3

The Frequency of Ige-Binding Proteins In Immunoblotting of Raw Mix Part Extract of S. Paramamasoin Using Sera From 28 Mud Crab-Allergic Patients.

No.	Subject	>250	173	140	120	95	86	80	78	75	62	50	45	42	38	36	35	28	18	No. of bands
1	A 417/12					√					√	√	√	√	√	√		√		9
2	A 394/12					√				√	√	√		√	√	√				8
3	A 392/12					√			√	√		√	√	√	√	√				9
4	A 376/12	√		√		√				√		√	√	√	√	√			√	11
5	A 356/12	√	√			√						√		√	√	√				8
6	A 342/12					√			√	√										3
7	A 335/12					√								√	√					3
8	A 106/12	√	√			√				√	√	√		√	√	√				9
9	A 334/12					√						√		√	√	√				5
10	A 326/12	√	√			√						√	√	√	√	√				8
11	A 384/12					√											√			3
12	A 354/12			√	√															2
13	A 350/12											√				√	√			3
14	A 313/12											√		√		√	√			4
15	A 409/12											√				√	√			3
16	A 98/12													√		√				2
17	A 477/12											√		√	√	√				4
18	A 473/12					√								√				√		3
19	A 480/12					√						√		√		√				4
20	A 472/12					√			√										√	3
21	A 481/12											√								2
22	A 466/12			√																1
23	A 129/12									√				√	√	√				4
24	A 93/12							√												1
25	A 360/12									√					√					2
26	A 220/12														√					1
27	A 460/12														√					1
28	A 493/12															√				1
	N	**4**	**3**	**3**	**1**	**15**	**5**	**1**	**3**	**7**	**4**	**14**	**4**	**17**	**18**	**15**	**4**	**3**	**2**	**18**
	%	**14**	**11**	**11**	**4**	**54***	**18**	**4**	**11**	**25**	**14**	**50***	**14**	**60***	**64***	**54***	**14**	**11**	**7**	

* = Major allergen √ = IgE-binding protein N= Total number %= percentage

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Five proteins at 36, 38, 42, 50 and 95 kDa of raw mix parts of *S. paramamsain* could bind to IgE antibodies of at least half of the tested sera with the frequencies of 54, 64, 60, 50 and 54%, respectively, therefore were identified as the major allergens for *S. paramamasain.* The 36 and 38 kDa bands might be corresponded to tropomyosin, a well-known shellfish major allergen at 32 to 38 kDa (Emoto et al., 2009). To date, several crab tropomyosin including *Cha f* 1 (Leung et al., 1998), *Eri s* 1 (Liang et al., 2008), *Chi o* 1 (Abdel Rahman et al., 2010b) and *Scy s* 1 (Huang et al., 2010) have been well-characterized as major crab allergens. This protein is a regulatory protein belongs to a family of highly conserved proteins with multiple isoforms found in both muscle and non-muscle cells of all animals, with a high degree of amino acid sequence identity among different species (Lehrer et al., 2003).

This study believed that the 42 kDa major allergen bands might be identical to arginine kinase or actin as reported by other studies (Abdel Rahman et al., 2012). Arginine kinase is an enzyme which involved in cell metabolism of invertebrates for regenerating ATP during cellular activity (Chen et al., 2013; Rosmilah et al., 2012). Arginine kinase has been established as allergens in crustacean and some invertebrates (Yadzir et al., 2012; Misnan et al., 2012; Rosmilah et al., 2012; Abdel Rahman et al., 2011. Meanwhile, actin is an important contractile protein in eukaryotic cells which involved in muscle contraction and many other processes such as material transportation, cell motility, signal transduction and organelle movement (Ma, Xu, Mai, Liufu, & Chen, 2004). To date, only Abdel Rahman et al. (2011) have identified α-actin as an allergenic protein in the snow crab *Chionoecetes opiolo*.

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Meanwhile, the other major allergens of *S. paramamosain* at 50 and 95 kDa are rarely described as shellfish major allergens in literatures. However, it was interesting to note that other study on mud crab has also reported proteins of 50 and 95 kDa as IgE-binders but only as minor allergens (Nurul et al. 2015).

In addition, this study also revealed that the raw mix parts of *S. paramamosain* has numerous minor allergens with varied frequencies. However, these proteins might have only minimal or insignificant contributions on mud crab allergy, as reported by other studies (Rosmilah et al., 2016). These results supported other studies which also detected various minor allergens in crab species, but are not well-identified (Nurul et al., 2015; Misnan et al., 2012).

In general, this study revealed that individual sera exhibited a remarkable heterogeneity in detecting *S. paramamosain* allergens in the raw mix parts extract. Patient No. 4 detected the highest number of IgE-binding proteins (11 bands), while five sera (Patients No. 22, 24, 26, 27 and 28) recognized only one IgE-binding protein bands. Other study by Nurul et al. (2015) also reported similar findings in immunoblotting experiments of raw mud crab. Such IgE-reactivity variation among crab-allergic patients might be as a result of different genetic background and allergen exposures among crab allergic patients (Simpson et al., 2015), which might reflect their allergy symptom variations (Simpson et al., 2015).

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Meanwhile, to compare the IgE-binding characteristics between the raw extracts of individual body parts of *S. paramamosain,* immunoblotting experiments were conducted using the same 10 selected patient sera with sufficient quantity and having IgE-binding to at least one major allergens in immunoblotting of raw mix parts. Figure 4.9 to 4.14 represented the immunoblotting results of each raw body parts of male and female *S. paramamasain,* whereas Table 4.4 to 4.9 summarized the immunoblotting result analysis.

In this study, all 10 different sera exhibited a remarkable heterogeneity in immunoblotting of different crab part extracts. As mentioned earlier, such variation in IgE reactivity may be due to genetic variation and different exposure to allergens, which reflecting different pattern of allergen sensitization and allergy symptoms (Simpson et al., 2015).

In general, similar as the immunoblotting results of raw mix parts, immunoblotting results of raw individual crab parts and genders have also detected multiple IgE-binding proteins with various IgE-binding patterns. Among male crabs, male claws has the highest number of IgE-binding bands (19 bands), followed by male legs (15 bands) and male abdomen (14 bands). Surprisingly, among female crabs, female legs has the highest number of IgE-binding bands (20 bands), followed by female abdomen (14 bands) and female claws (13 bands). Therefore, based on the number of IgE-binding bands, the allergenicity of crab parts are in the following order: female legs > male claws > male legs > female abdomen = male abdomen > female claws. Thus, this study suggested that the female legs are the most allergenic while the female claws are the least allergenic than the other crab parts.

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Immunoblotting of male abdomen, female abdomen, female claws and female legs have identified all five major allergens of *S. paramamosain* at 36, 38, 42, 50 and 95 kDa, whereas immunoblotting of male claws and male legs have recognized only four major allergens at 36, 38, 50 and 95 kDa. Thus, according to the number of major allergens, female legs, female claws, female abdomen and male abdomen can be considered as more allergenic than other crab parts (female legs = female claws = female abdomen = male abdomen > male claws = male legs).

However, based on the IgE-binding intensity, the most prominent IgE-binding intensity was seen in immunoblotting of female legs, followed by male claws and female claws. The binding intensities between male abdomen, male legs and female abdomen are almost identical. Therefore, the allergenicity order based on the band intensities are as follows: female legs > male claws > female claws > female abdomen = male abdomen = male legs.

This finding indicated that crab parts have variable allergenicity in terms of the number of IgE-binding bands, number of major allergens and IgE-binding band intensities. Based on these three characeristics results, in this study suggested that the female legs can be regarded as the most allergenics part than the other crab parts. In general, among local consumers, crab leg is the least popular parts as it contains very little amount of meat. However, in certain countries such as Japan, the only parts that is marketed an consumed is the crab legs and claws as these parts have been claimed for their health-promoting characteristics as a valuable source of proteins and essential fatty acids (Lordan, Ross, & Stanton, 2011). Nevertheless, based on this

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findings, this study may suggest the crab-allergeic patients to avoid the consumption of female crab legs as it may have high potential to elicit allergic reactions to crabs.

However, immunoblotting as well as ELISA tests using more crab-allergic sera, supported by an extensive clinical studies among various crab-allergic patient populations are needed to confirm this finding. To the best of our knowledge, this study is the first to report the allergenicity variation between different body parts and genders of crab.

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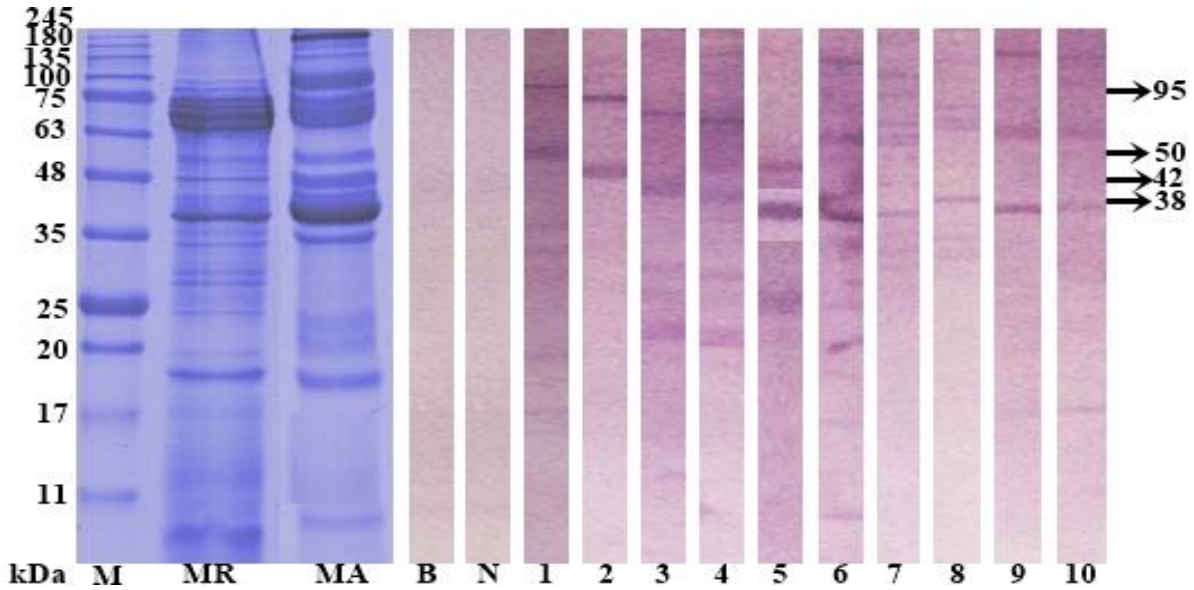


Figure 4.8. Immunoblotting Results of Raw Male Abdomen of *S. Paramamsain* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Mix Raw Sample; Lane MA Is Male Abdomen Sampel; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.4

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Raw Male Abdomen of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

No.	Subject	>250	173	140	120	110	95	86	80	78	75	62	50	45	42	38	36	35	28	24	18	15	No. of bands
1	A 417/12						√	√					√	√	√	√	√		√				8
2	A 394/12						√				√	√	√		√	√	√						7
3	A 392/12						√	√		√	√		√	√	√	√	√						7
4	A 376/12	√		√			√	√			√		√	√	√	√	√				√		10
5	A 356/12							√					√		√	√	√						5
6	A 342/12						√			√	√												3
7	A 335/12						√								√	√							3
8	A 106/12										√	√	√		√	√	√						6
9	A 334/12						√						√		√		√						5
10	A 326/12						√						√	√	√	√	√						6
	N	**1**	**0**	**1**	**0**	**0**	**8**	**5**	**0**	**2**	**5**	**2**	**8**	**5**	**9**	**9**	**8**	**0**	**2**	**0**	**1**	**0**	**14**
	%	**10**	**0**	**10**	**0**	**0**	**80**	**50**	**0**	**20**	**50**	**20**	**80**	**50**	**90**	**90**	**80**	**0**	**20**	**0**	**10**	**0**	

√ = IgE-binding protein N= Total Number % Percentage

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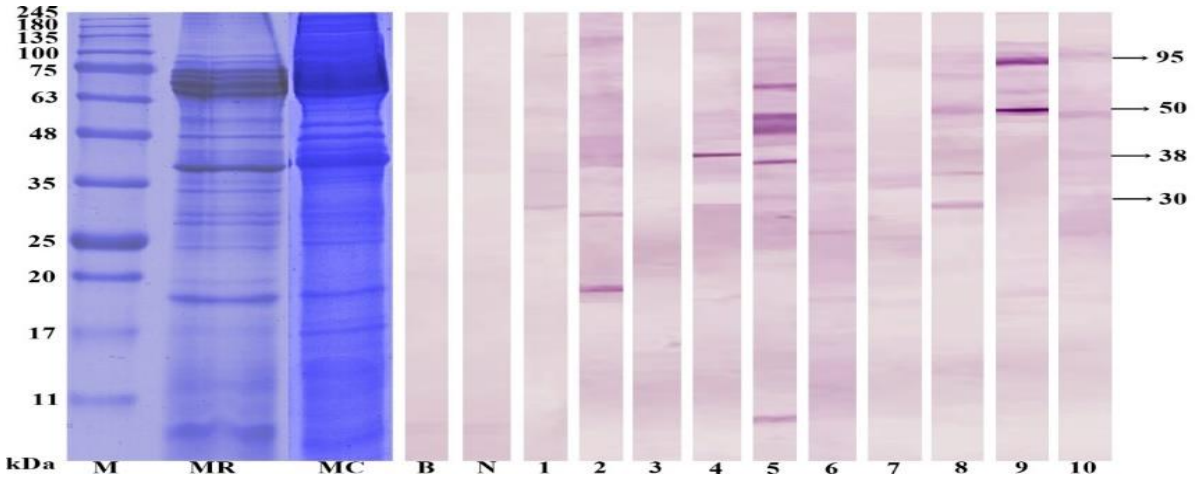


Figure 4.9. Immunoblotting Results of Raw Male Claw of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Mix Raw Sample; Lane MC Is Male Claw Sampel; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.5

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Raw Male Claw of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Male claw raw Molecular weight (kDa)																					
No.	**Subject**	**>250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12							√	√					√		√	√	√					6
2	A 394/12	√	√	√	√	√										√	√	√	√				10
3	A 392/12																			√			1
4	A 376/12																√	√		√			3
5	A 356/12	√	√					√	√				√	√		√	√					√	7
6	A 342/12															√		√					2
7	A 335/12															√		√		√			3
8	A 106/12							√	√				√			√	√	√					6
9	A 334/12						√				√					√				√			4
10	A 326/12						√					√				√		√					4
	N	**2**	**2**	**1**	**1**	**1**	**2**	**3**	**3**	**1**	**1**	**1**	**2**	**2**	**0**	**8**	**5**	**7**	**1**	**4**	**0**	**1**	**19**
	%	**20**	**20**	**10**	**10**	**10**	**20**	**30**	**30**	**10**	**10**	**10**	**20**	**20**	**0**	**80**	**50**		**10**	**40**	**0**	**10**	

√ = IgE-binding protein N= Total Number % = Percentage

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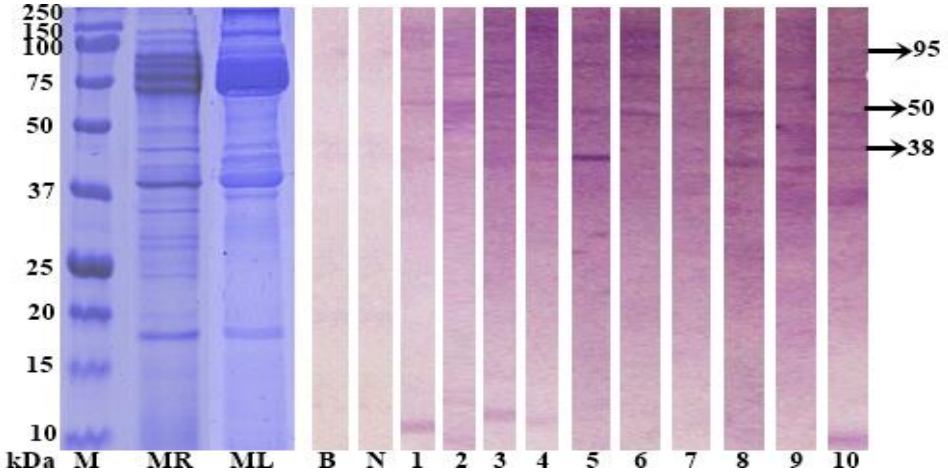


Figure 4.10. Immunoblotting Results of Raw Male Leg of *S. Paramamsain* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Mix Raw Sample; Lane ML Is Male Leg Sampel; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.6

The Frequency of Specific-Ige Binding Proteins In Immunoblotting 0f Raw Male Leg of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Male leg raw Molecular weight (kDa)																				
No.	**Subject**	**>250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12							√			√	√	√	√		√						√	7
2	A 394/12					√	√	√			√	√	√	√		√						√	8
3	A 392/12	√	√	√	√	√	√	√			√	√	√	√		√						√	12
4	A 376/12	√	√	√	√	√	√	√			√	√	√	√		√							12
5	A 356/12	√	√	√	√											√							5
6	A 342/12	√	√	√	√											√							5
7	A 335/12												√	√									2
8	A 106/12															√							2
9	A 334/12															√							1
10	A 326/12															√	√	√					3
	N	**1**	**1**	**1**	**4**	**3**	**3**	**6**	**0**	**0**	**4**	**4**	**5**	**5**		**9**	**1**	**1**	**0**	**0**	**0**	**3**	**15**
	%	**10**	**10**	**10**	**40**	**30**	**30**	**60**	**0**	**0**	**40**	**40**	**50**	**50**		**90**	**10**	**10**	**0**	**0**	**0**	**30**	

√ = IgE-binding protein N= Total Number % = Percentage

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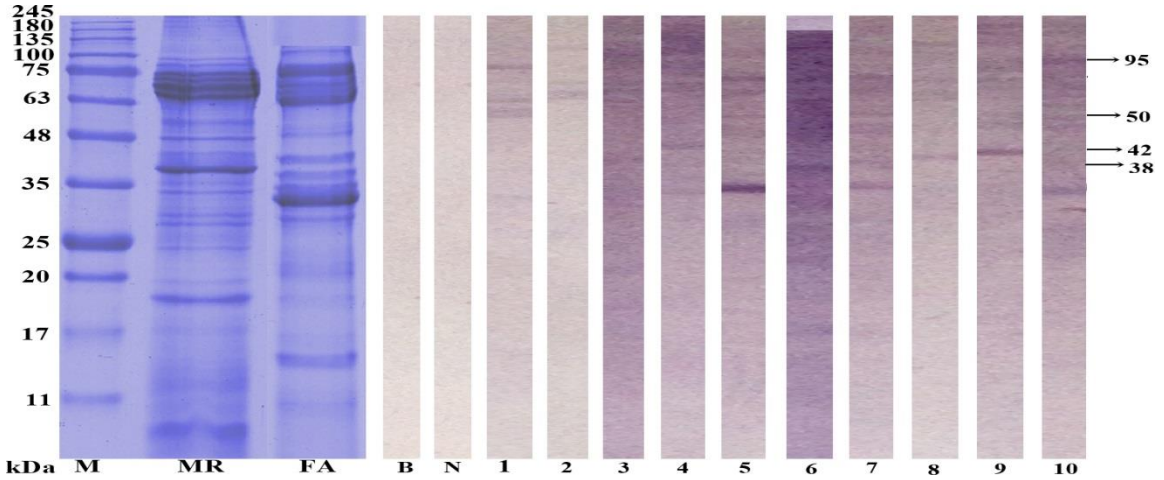


Figure 4.11. Immunoblotting Results of Raw Female Abdomen of *S. Paramamsain* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane FA Is Raw Female Abdomen Sample; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.7

The Frequency of Specific-Ige Binding Proteins In Immunoblotting 0f Raw Female Abdomen of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Female abdomen Raw																					
		Molecular weight (kDa)																					
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	**A 417/12**					√			√		√						√						**4**
2	**A 394/12**					√				√	√												**2**
3	**A 392/12**									√	√	√				√							**4**
4	**A 376/12**		√			√		√	√							√	√	√					**7**
5	**A 356/12**		√					√	√			√				√	√						**6**
6	**A 342/12**					√	√	√					√			√							**5**
7	**A 335/12**						√	√		√	√		√	√	√	√							**8**
8	**A 106/12**						√	√						√	√	√							**5**
9	**A 334/12**						√	√					√	√				√					**5**
10	**A 326/12**						√	√			√			√		√		√					**6**
	N	**0**	**2**	**0**	**0**	**4**	**5**	**7**	**3**	**3**	**5**	**2**	**3**	**4**	**2**	**7**	**3**	**3**	**0**	**0**	**0**	**0**	**14**
	%	**0**	**20**	**0**	**0**	**40**	**50**	**70**	**30**	**30**	**50**	**20**	**30**	**40**	**20**	**70**	**30**	**30**	**0**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number % = Percentage

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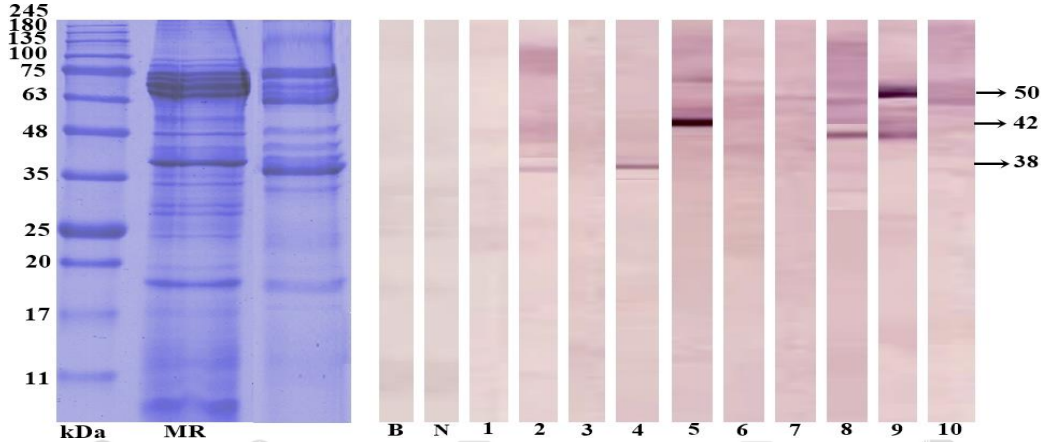


Figure 4.12. Immunoblotting Results of Raw Female Claw of *S. Paramamsain* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane FC Is Raw Female Claw Sample; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.8

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Raw Female Claw S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Female claw Raw																					
		Molecular weight (kDa)																					
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12												√			√		√					3
2	A 394/12						√	√	√	√	√	√			√	√	√						9
3	A 392/12																						0
4	A 376/12															√	√						2
5	A 356/12								√			√	√										3
6	A 342/12											√	√	√		√							4
7	A 335/12												√	√	√	√							4
8	A 106/12					√					√	√		√		√							5
9	A 334/12													√		√							3
10	A 326/12								√	√	√			√		√							5
	N	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**3**	**2**	**3**	**5**	**5**	**5**	**2**	**8**	**2**	**1**	**0**	**0**	**0**	**0**	**13**
	%	**0**	**0**	**0**	**0**	**10**	**10**	**10**	**30**	**20**	**30**	**50**	**50**	**50**	**20**	**80**	**20**	**10**	**0**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number % = Percentage

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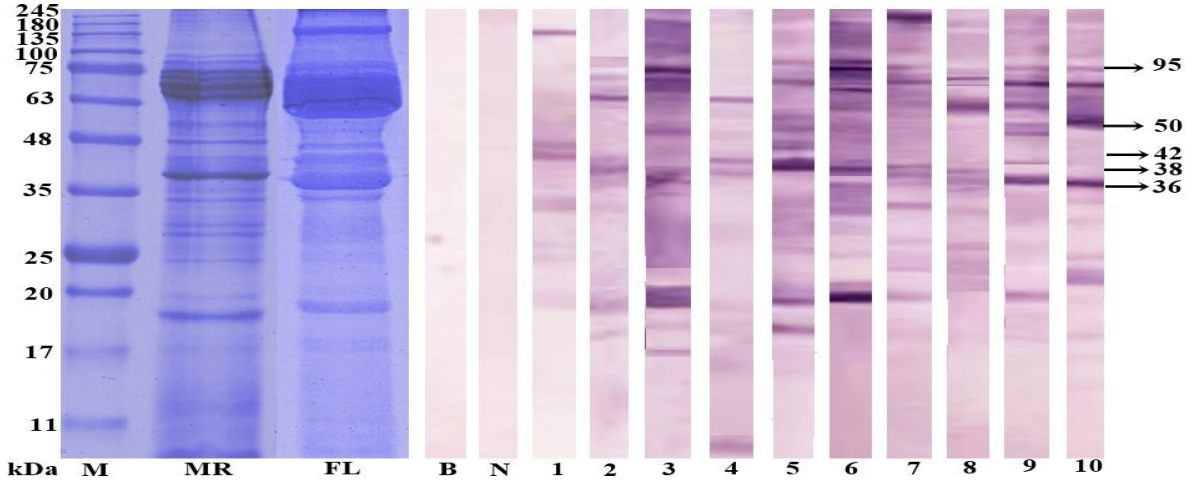


Figure 4.13. Immunoblotting Results of Raw Female Leg of *S. Paramamsain* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane FL Is Raw Female Leg Sample; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.9

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Raw Female Leg of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Female leg Raw																				
		Molecular weight (kDa)																				
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12		√	√	√			√			√		√		√	√				√	√		10
2	A 394/12					√	√	√	√		√	√	√	√	√	√	√			√	√		13
3	A 392/12			√	√	√					√	√	√	√	√	√	√			√	√	√	14
4	A 376/12								√	√	√	√	√	√	√	√	√	√		√	√		12
5	A 356/12					√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	16
6	A 342/12			√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√		18
7	A 335/12			√	√		√	√	√	√	√	√	√	√		√			√	√	√		14
8	A 106/12						√	√	√	√	√	√	√	√		√			√	√	√		12
9	A 334/12		√	√	√	√	√	√	√		√		√			√			√	√	√	√	14
10	A 326/12		√	√	√	√	√	√			√		√			√				√	√	√	12
	N	**0**	**3**	**6**	**6**	**6**	**8**	**9**	**7**	**4**	**10**	**7**	**10**	**7**	**6**	**10**	**5**	**3**	**5**	**10**	**10**	**4**	**20**
	%	**0**	**30**	**60**	**60**	**60**	**80**	**90**	**70**	**40**	**100**	**70**	**100**	**70**	**60**	**100**	**50**	**30**	**50**	**100**	**100**	**40**	

√ = IgE-binding protein N= Total Number % = Percentage

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4.4 Objective 3: To Determine the Effects of Boiling on Allergenicity of Different Body Parts and Gender of Mud Crab *Scylla paramamosain*

Figure 4.15 to 4.21 and Table 4.10 to 4.16 represented the immunoblotting results of each boiled extracts of *S. paramamasain*. This study was performed using the same 10 selected patient sera used in the immunoblotting experiments of the individual body parts as mentioned earlier. In general, immunoblotting experiments revealed that all boiled extracts had a lesser number of IgE-binding proteins than the raw extracts. Overall, a total of 13 IgE-binding proteins could retained their IgE-binding capabilities in the boiled crab extracts, thus were considered as heat-sensitive proteins, whereas the other proteins were revealed as sensitive to boiling. These results are not surprising as in SDS-PAGE results, boiled extracts were obviously had smaller number of protein bands compared to the raw extracts, most probably due to protein denaturation, as discussed earlier. These results were also consistent with previous study on mud crab *S. serrata* by Nurul et al. (2015).

Results of immunoblotting experiments for boiled extracts showed that, overall, the boiled extracts exhibited one to 13 heat-resistant IgE-binding proteins. The boiled mix parts extract has the highest number of heat-resistant IgE-binding proteins (13 bands) between 28 to >250 kDa including four major allergen bands at 36, 38, 42 and 50 kDa, detected by 90% of the tested sera. Among the individual boiled body parts, male claws has the highest number of heat-resistant bands (6 bands) at 150, 107, 95, 48, 38 and 15 kDa, followed by male legs (5 bands) at 173, 42, 38, 36 and 15 kDa, female legs (4 bands) at 107, 95, 72 and 38 kDa, and male abdomen which has only two heat-resistant bands at 50 and 38 kDa. Meanwhile, both

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boiled female abdomen and boiled female claws have only one heat resistant bands at 38 kDa. This study identified the heat-resistant proteins at 38 kDa as a stronger band with higher binding intensities in some immunoblotting results compared to the other heat-resistant bands. These findings showed that the boiled mix parts and all individual crab parts could retain their IgE-binding capabilities with at least one major allergen.

Based on the number of reacted sera in immunoblotting experiments, the boiled mix body parts elicited the highest responses with 90% of the tested sera reacted to one to four IgE-binding bands (all sera except for sera no. 10). Among the individual boiled body parts, the male leg extract elicited the highest IgE-responses, with 60% of the tested sera reacted to one to three bands (sera no. 1, 3, 4, 6, 7, and 8), followed by the boiled female leg extract which reacted to 50% of the sera (sera no. 4, 5, 8, 9 and 10) at one to three bands. Next, the boiled male abdomen extract was detected by 30% of sera (sera no. 1, 3 and 6), which reacted to only one band, either at 38 or 50 kDa. Meanwhile, for boiled male claws extract, only two (20%) of tested sera reacted (sera no. 3 and 7), which bound to four and three bands, respectively. Similarly, in female abdomen boiled extract, only 2 sera reacted to a single band at 38 kDa (sera no. 9 and 10). The crab part which has the least binding capability is the boiled female claw extract which was detected by only one sera (sera no. 2), responded to a single band at 38 kDa. Therefore, based on these results, it can be said that both boiled form of crab legs (male and female) could retained more IgE-binding capabilities compared to the other boiled crab extracts in the following order: male leg> female leg > male abdomen > male claws = female abdomen > female claws.

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In this study, all boiled extracts could conserve the capability of the 38 kDa major allergen as IgE-binder, indicating that this major allergen in all crab parts studied is unaffected by heat denaturation. Overall, the binding intensity of this band was also increased in boiled forms. Proteins that are unaffected by intense heat may penetrate the gastrointestinal tract with uninterrupted epitopes and result in allergic reactions (Shriver & Yang, 2011). This occurrence is usually observed in tropomyosin with molecular weight of 32 to 38 kDa, the heat-resistant main allergen in several invertebrates such as crabs (Rosmilah et al., 2012; Abdul Rahman et al., 2011; Liang et al., 2008; Lehrer et al., 2003; Shriver & Yang, 2011). Therefore, the 38 kDa allergens of *S. paramamosain* may be attributed to tropomyosin. Besides the 38 kDa proteins, the major allergen of 36 kDa in the boiled mix parts and male legs were also could retain its IgE-binding capability in several sera with improved intensities. Thus, this 36 kDa might be a fraction of the 38 kDa major allergen. The results obtained from this study were not different from previous reports, indicating tropomyosin was more allergenic in boiled forms (Rosmilah et al. 2012; Yadzir et al. 2010; Besler, Steinhart, & Paschke, 2001), and apparently due to the fact that boiled tropomyosin might have experienced protein-protein interactions like aggregation and Maillard reaction, hence resulted in an improved IgE binding. Consequently, boiled tropomyosin may possess higher efficiency in the diagnosis of seafood allergy (Liu et al. 2010b).

Apart from tropomyosin, the major allergen of 42 kDa was also detected in immunoblotting of two boiled extracts; the mix parts and male legs, but appeared as a weak-smeared band. This 42 kDa band is not detected as IgE-binder in the other boiled extracts, suggesting that this band was a heat-sensitive protein which could

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only retain minimal binding capacity. To date, additional shellfish major allergens at 40-42 kDa have been identified as arginine kinase (Abdel et al. 2011; Yu et al. 2003; Hendrickx, Ludikhuyze, Van den Broeck & Weemaes, 1998; Shen et al. 2011) and actin (Yadzir et al., 2016). Both allergens have been reported as heat-sensitive proteins that will be destroyed by boiling due to denaturation, thus may not detected in the immunoblotting experiments (Yadzir et al., 2016).

Similarly, the other major allergens at 50 and 95 kDa were also considered as heat-sensitive allergens, as it appeared as a weak-smeared bands in only two boiled extracts; the mix part and male abdomen. This band is rarely described as a shellfish major allergen in literatures. As mentioned earlier, the protein loss in the boiled extracts in both SDS-PAGE gel and immunoblotting tests might be due to the influences of heat on them as treatments through heating including boiling can possibly result in protein modifications including inter- or intramolecular protein crosslinking, denaturation or protein fragmentation. Alteration of protein by fragmentation caused the proteins to be fragmented to small peptides that cannot be observed on the gel as those peptide may travel through the acrylamide pores of SDS-PAGE more quickly, causing them to be eventually lost in the running buffer (Oliveira et al., 2013). Furthermore, modification of proteins through covalent bonds which includes intramolecular crosslinking can retain the band in SDS-PAGE, but with alteration of IgE-binding epitopes. Therefore, the activity of the allergen will be destroyed, decreased or possibly increased through alteration of the IgE binding epitopes.

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This study suggests that boiling were insufficient to eliminate or destroy all the mix crab parts or individual crab parts of *S. paramamosain* allergens but it can partly decrease allergenicity for patients which reacted only to the heat-sensitive allergens by reducing the IgE-binding ability. Among boiled crab parts, the boiled female claw extract can be considered as less allergenic than other boiled crab parts based on the least number of IgE-binding proteins in immunoblotting experiments. However, reduction of the number of IgE-binding proteins does not certainly mean that the patient is less allergic clinically. A claim of decreased allergic clinically can only be proven through clinical investigation with boiled extracts.

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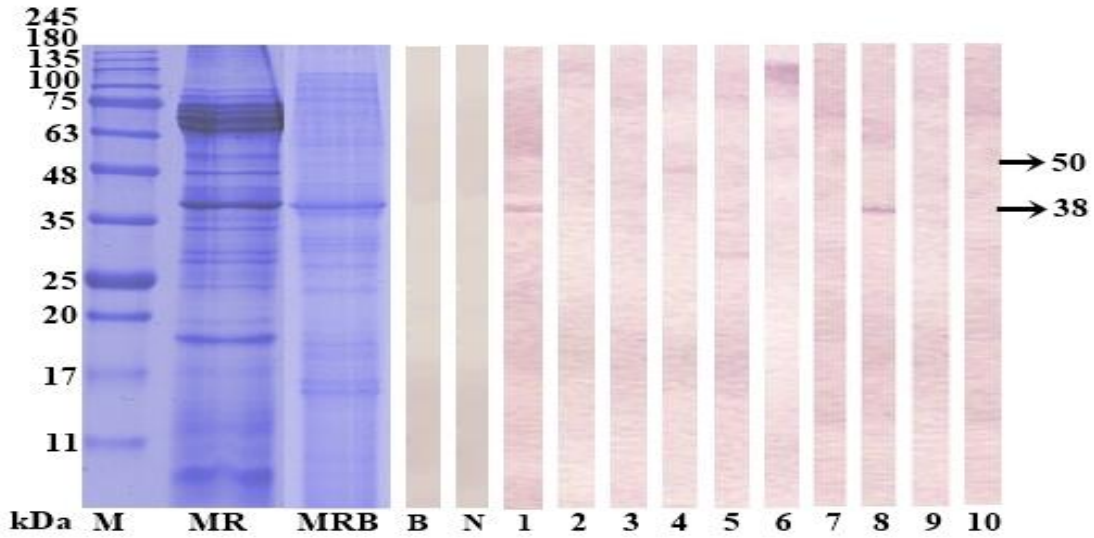


Figure 4.14. Immunoblotting Results 0f Mix Boiling Sample *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane MRB Is Boiled Mix Parts; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.10

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Mix Parts of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled mix parts																					
		Molecular weight (kDa)																					
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12											√	√			√							3
2	A 394/12			√	√	√																	3
3	A 392/12														√	√							2
4	A 376/12															√							1
5	A 356/12															√	√	√	√				4
6	A 342/12		√	√																			2
7	A 335/12	√	√	√																			3
8	A 106/12												√			√							2
9	A 334/12													√	√	√							3
10	A 326/12																						0
	N	**1**	**2**	**3**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**2**	**1**	**2**	**6**	**1**	**1**	**1**	**0**	**0**	**0**	**13**
	%	**10**	**20**	**30**	**10**	**10**	**0**	**0**	**0**	**0**	**0**	**10**	**20**	**10**	**20**	**60**	**10**	**10**	**10**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number % = Percentage

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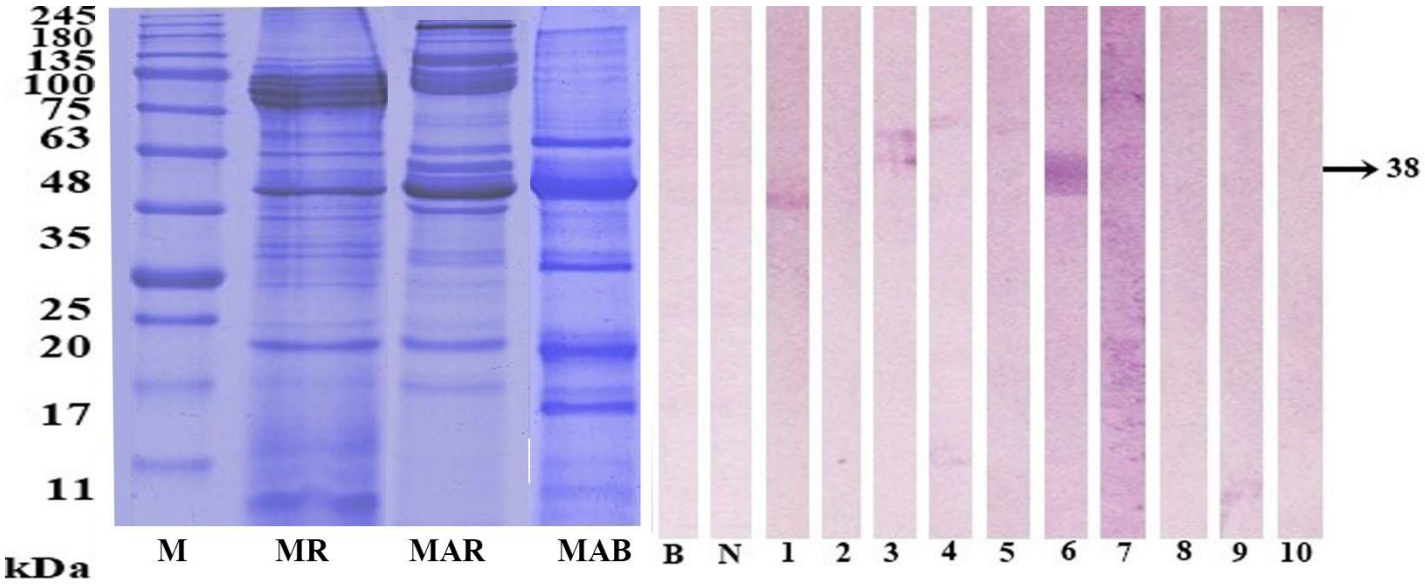


Figure 4.15. Immunoblotting Results of Boiled Male Abdomen of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane MAR Is Raw Male Abdomen; Lane MAB Is Boiled Male Abdomen; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.11

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Male Abdomen of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled male abdomen																					
		Molecular weight (kDa)																					
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12															√							1
2	A 394/12																						0
3	A 392/12												√			√							2
4	A 376/12																						0
5	A 356/12																						0
6	A 342/12															√							1
7	A 335/12																						0
8	A 106/12																						0
9	A 334/12																						0
10	A 326/12																						0
	N	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**2**
	%	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**10**	**0**	**20**	**0**	**0**	**0**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number % = Percentage

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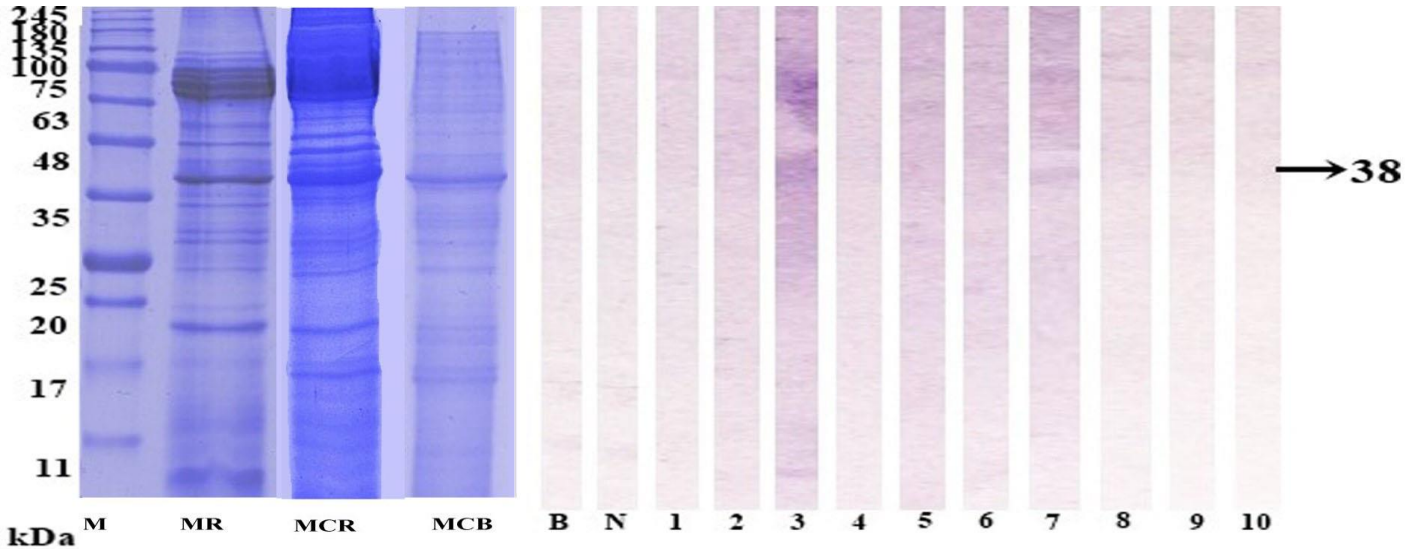


Figure 4.16. Immunoblotting Results of Boiled Male Claw of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane MCR Is Raw Male Claw; Lane MCB Is Boiled Male Claw; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.12

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Male Claw 0f S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled male claw																		
		Molecular weight (kDa)																		
No.	**Subject**	**150**	**107**	**95**	**72**	**65**	**61**	**58**	**52**	**48**	**42**	**40**	**38**	**36**	**30**	**26**	**24**	**22**	**14**	**No. of bands**
1	A 417/12																			0
2	A 394/12																			0
3	A 392/12			√						√			√						√	4
4	A 376/12																			0
5	A 356/12																			0
6	A 342/12																			0
7	A 335/12	√	√										√							3
8	A 106/12																			0
9	A 334/12																			0
10	A 326/12																			0
	N	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**1**	**6**
	%	**10**	**10**	**10**	**0**	**0**	**0**	**0**	**0**	**10**	**0**	**0**	**20**	**0**	**0**	**0**	**0**	**0**	**10**	

√ = IgE-binding protein N= Total Number % = Percentage

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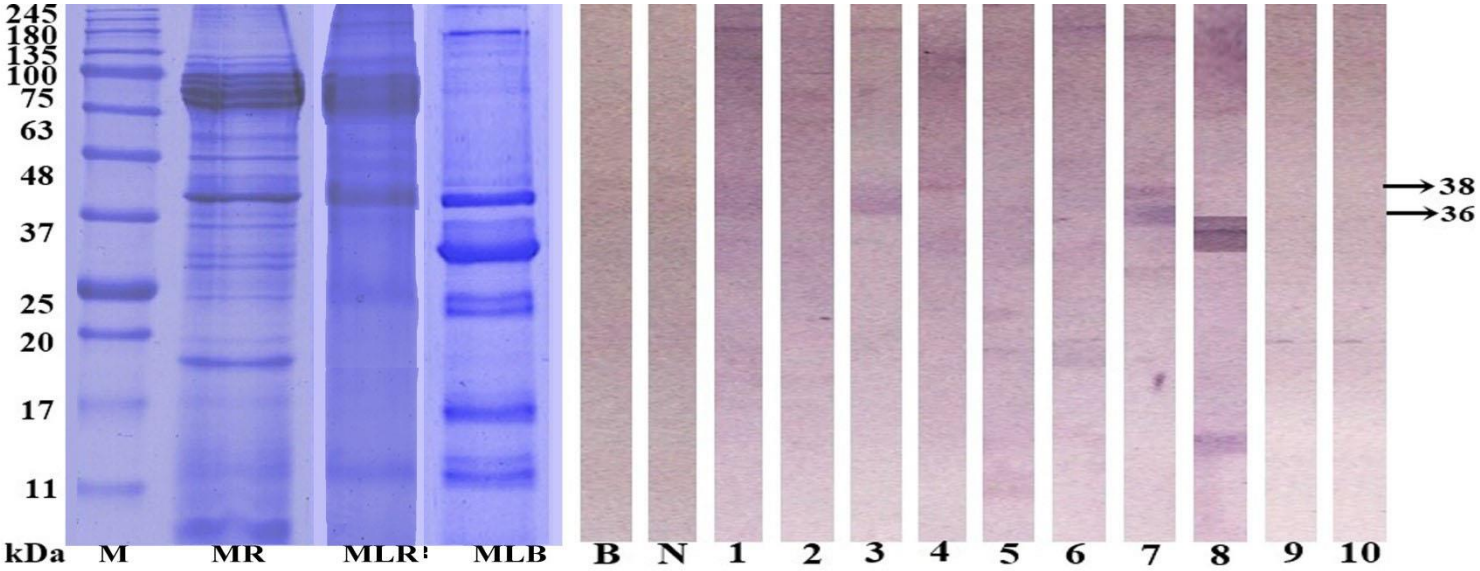


Figure 4.17. Immunoblotting Results of Boiled Male Leg of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane MLR Is Raw Male Leg; Lane MLB Is Boiled Male Leg; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.13

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Male Leg of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled male leg																					
		Molecular weight (kDa)																					
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12		√																				1
2	A 394/12																						0
3	A 392/12		√													√							2
4	A 376/12														√								1
5	A 356/12																						0
6	A 342/12		√																				1
7	A 335/12		√													√	√						3
8	A 106/12															√	√					√	3
9	A 334/12																						0
10	A 326/12																						0
	N	**0**	**6**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**3**	**2**	**0**	**0**	**0**	**0**	**1**	**5**
	%	**0**	**60**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**10**	**30**	**20**	**0**	**0**	**0**	**0**	**10**	

√ = IgE-binding protein N= Total Number % = Percentage

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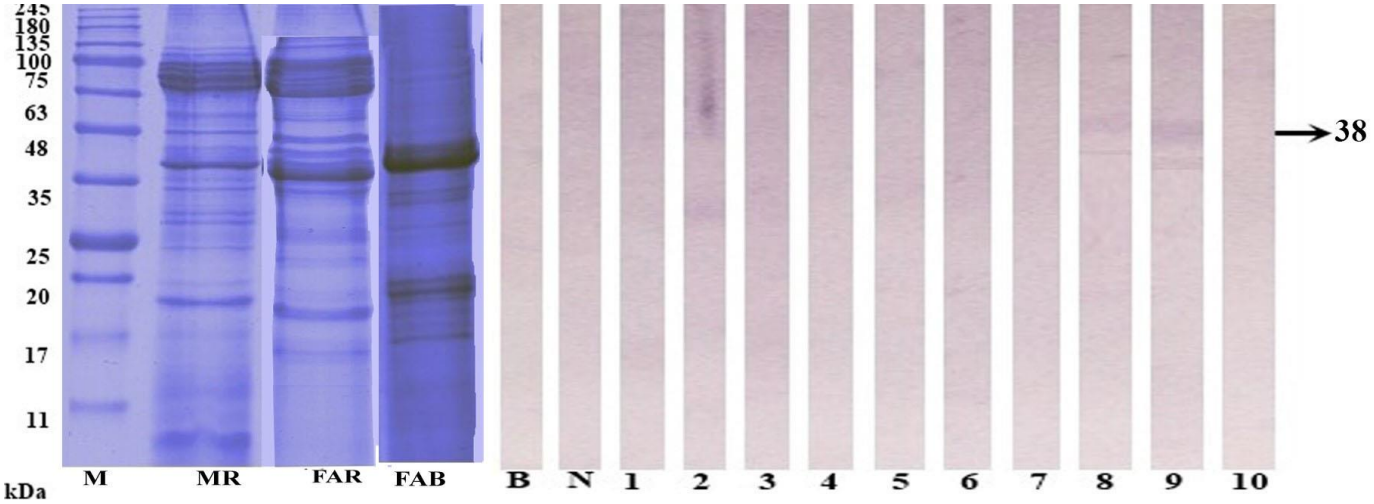


Figure 4.18. Immunoblotting Results of Boiled Female Abdomen of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane FAR Is Raw Female Abdomen; Lane FAB Is Boiled Female Abdomen; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.14

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Female Abdomen of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled female abdomen																					
		Molecular weight (kDa)																					
No.	**Subject**	**<250**	**173**	**140**	**120**	**110**	**95**	**86**	**80**	**78**	**75**	**62**	**50**	**45**	**42**	**38**	**36**	**35**	**28**	**24**	**18**	**15**	**No. of bands**
1	A 417/12																						0
2	A 394/12																						0
3	A 392/12																						0
4	A 376/12																						0
5	A 356/12																						0
6	A 342/12																						0
7	A 335/12																						0
8	A 106/12															√							1
9	A 334/12															√							1
10	A 326/12																						0
	N	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**1**
	%	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**20**	**0**	**0**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number % = Percentage

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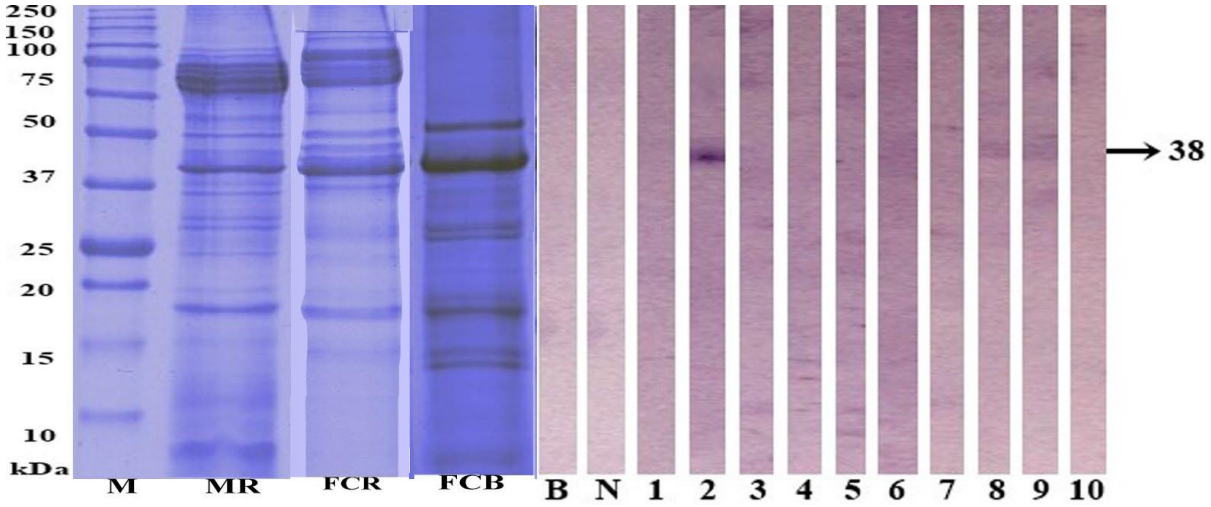


Figure 4.19 Immunoblotting Results of Boiled Female Claw of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane FCR Is Raw Female Claw; Lane FCB Is Boiled Female Claw; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.15

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Female Claw of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled female claw																		
		Molecular weight (kDa)																		
No.	**Subject**	**150**	**107**	**95**	**72**	**65**	**61**	**58**	**52**	**48**	**42**	**40**	**38**	**36**	**30**	**26**	**24**	**22**	**14**	**No. of bands**
1	A 417/12																			0
2	A 394/12												√							1
3	A 392/12																			0
4	A 376/12																			0
5	A 356/12																			0
6	A 342/12																			0
7	A 335/12																			0
8	A 106/12												√							1
9	A 334/12												√							1
10	A 326/12																			0
	N	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**1**
	%	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**30**	**0**	**0**	**0**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number % = Percentage

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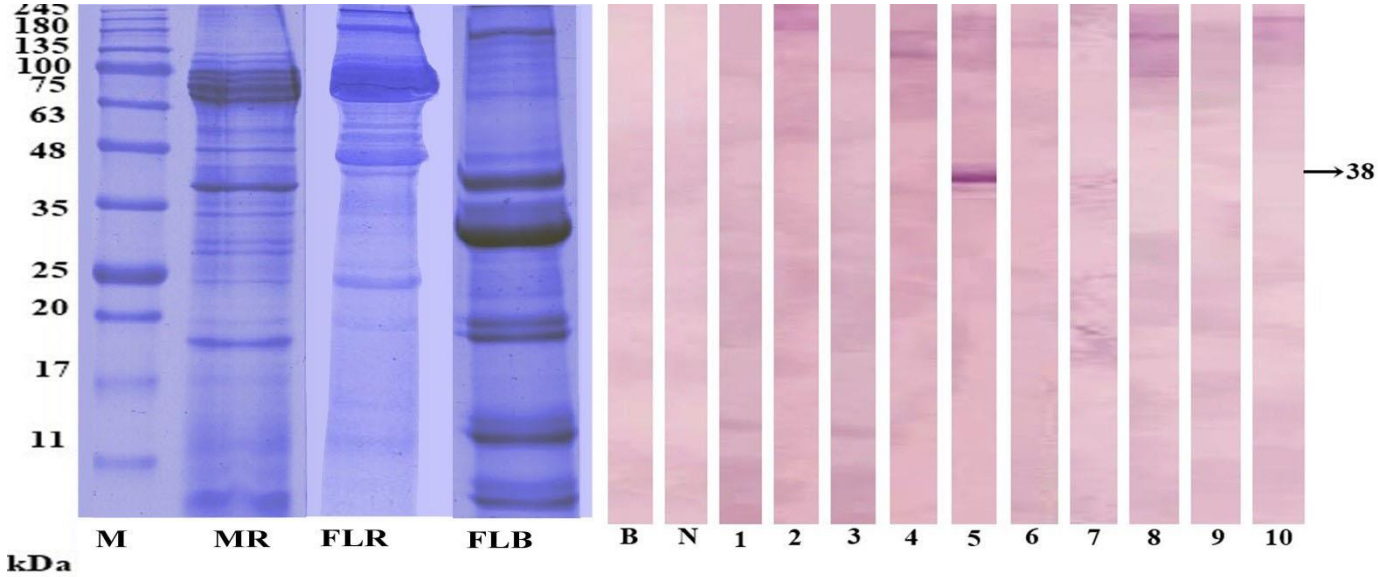


Figure 4.20. Immunoblotting Results of Boiled Female Leg of *S. Paramamsoin* Using Sera From 10 Mud Crab-Allergic Patients (Lane 1 To 10). Lane M Is Molecular Mass Markers In Kilodalton (Kda); Lane MR Is Raw Mix Parts Sample; Lane FLR Is Raw Female Leg; Lane FLB Is Boiled Female Leg; Lane B Is Blank And Lane N Is Immunoblot Using A Negative Control Serum. Arrows Indicated The Major Allergens Molecular Weight In Kda.

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Table 4.16

The Frequency of Specific-Ige Binding Proteins In Immunoblotting of Boiled Female Leg of S. Paramamosain Using Sera From 10 Crab-Allergic Patients

		Boiled female leg																		
		Molecular weight (kDa)																		
No.	**Subject**	**150**	**107**	**95**	**72**	**65**	**61**	**58**	**52**	**48**	**42**	**40**	**38**	**36**	**30**	**26**	**24**	**22**	**14**	**No. of bands**
1	A 417/12																			0
2	A 394/12																			0
3	A 392/12																			0
4	A 376/12				√															1
5	A 356/12				√								√							2
6	A 342/12																			0
7	A 335/12																			0
8	A 106/12			√	√															2
9	A 334/12		√	√																2
10	A 326/12		√	√	√															3
	N	**0**	**2**	**3**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**4**
	%	**0**	**20**	**30**	**40**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**10**	**0**	**0**	**0**	**0**	**0**	**0**	

√ = IgE-binding protein N= Total Number %= Percentage

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4.5 Objective 4: Identification of Major Allergens of *Scylla paramamosain* by Mass spectrometry

Table 4.17 summarized the mass spectrometry analysis of two major allergens of *S. paramamosain* at 38 and 42 kDa. Out of five major allergenic bands analyzed by mass spectrometry, only two major allergenic bands of 38 and 42 kDa gave significant results. No repetition was done due to financial limitation.

The 38 kDa major allergen of *S. paramamosain* showed correlation with tropomyosin (TM) from *Portunus sanguinolentus* with peptide sequence coverage of 17%. Meanwhile, the 42 kDa major allergen of *Scylla paramamosain* was found to be identical to beta-actin from *Macrophthalmus japonicus* with peptide sequence coverage 12%.

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Table 4.17

Result of Mass Spectrometry Analysis of Major Allergens of 38 and 42 Kda of Scylla Paramamosain

Spot No.	MW (kDa, pI) Observed	MW (kDa) Predicted	Protein Identification	Organism	Accession No.	Coverage of Protein sequence (%)	No of matched protein
1a	37	38, 4.71	Tropomyosin	*Portunus sanguinolentus*	A1YYV6	17	5
2a	42	42, 4.96	Beta-actin	*Macrophthalmus japonicus*	AID47198.1	12	3

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4.5.1 Tropomyosin (TM)

It is known that TM has been well-recognized as the important allergen in a wide range of shellfish species (Shen et al., 2014; Emoto et al., 2009; Martins et al., 2008; Motoyama et al., 2006; Chuo et al., 2000). Similarly, this study has also identified TM as one of the major allergens in *Scylla paramamosain*. TM with a molecular weight about 34 to 38 kDa is a group of highly conserved actin-binding proteins present in muscle and non-muscle cells and plays a central role in muscle contraction (Lopata & Kamath, 2012; Thammathongchat, Hagiwara & Sakiyama, 2010).

Table 4.18 showed the analysis of protein sequence of TM that matched with peptides from a band of *Scylla paramamosain* with molecular mass of 38 kDa. Three peptides digested from this band with the amino acid sequences of "ALQNAEGEVAALNRRIQLLEEDLE", "RSEERLAEASQAADESER" and "IVELEEER" matched with the TM sequence of *Portunus sanguinolentus* with predicted molecular mass of 37 kDa and pI of 4.71, at the respective location of 77 to 105, 113 to 125 and 189 to 197.

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Table 4.18

Protein Sequence of Tropomyosin In Database (A1YYV6; Portunus Sanguinolentus)

Size (kDa)	**No of matched peptide (n, % coverage)**	**Protein sequence of tropomyosin in database (A1YYV6; *Portunus sanguinolentus*)**	**Matched of peptide sequence (Position number)**
38	5 (17%)	**1** MDAIKKKMQA MKLEKDNAMD RADTLEQQNK EANLRAEKTE EEIRATQKKM **51** QQVENELDQA QEQLSAANTK LDEKEK**ALQN** **AEGEVAALNR** **RIQLLEEDLE** **101** **RSEER**LNTAT TK**LAEASQAA** **DESER**MRKVL ENRSLSDEER MDALENQLKE **151** ARFLAEEADR KYDEVARKLA MVEADLERAE ERAESGESK**I** **VELEEELR**VV **201** GNNLKSLEVS EEKANQREET YKEQIKTLAN KLKAAEARAE FAERSVQKLQ **251** KEVDRLEDEL VNEKEKYKSI TDELDQTFSE LSGY	ALQNAEGEVAALNRRIQL LEEDLE (77-105) RSEERLAEASQAADESER (113-125) IVELEEELR (189-197)

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4.5.2 Beta-actin

In addition to TM, beta-actin has been identified as the major allergen of *Scylla paramamosain*. The major allergenic band at 42 kDa of *Scylla paramamosain* showed high similarity to beta-actin from *Macrophthalmus japonicus* with sequence coverage of 12%, as presented in Table 4.19.

Table 4.19 showed the analysis of protein sequence of beta-actin that matched with peptides from a band of *Scylla paramamosain* with molecular mass of 42 kDa. Three peptides digested from this band with the amino acid sequences of "AGFAGDDAPRA", "VFPSIVGRPR", "SYELPDGQVIT" and "IGNER" matched with the beta-actin sequence of *Macrophthalmus japonicus* with predicted molecular mass of 40 kDa and pI of 4.96, at the respective location of 20 to 40, 240 to 250 and 251 to 255.

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Table 4.19

Protein Sequence of Beta-Actin In Database (AID47198.1; Macrophthalmus Japonicus)

Size (kDa)	**No of matched peptide (n, % coverage)**	**Protein sequence of beta-actin in database (AID47198.1; *Macrophthalmus japonicus*)**					**Matched of peptide sequence (Position number)**
42	3(12%)	**1** MCDDEVSPLV	VDNGSGMVK**A**	**GFAGDDAPRA**	**VFPSIVGRPR**	HQGVMVGMGQ	**AGFAGDDAPRA** **VFPSIVGRPR** **(20-40)**
		51 KDAYVGDEAQ	SKRGILTLKY	PIEHGIITNW	DDMEKIWHHS	FYNELRVAPE	**SYELPDGQVIT** **(240-250)**
		101 ESPVLLTEAP	LNPKANREKM	TQIMFETFNT	PAMYVAIQAV	LSLYASGRTT	**IGNER** **(251-255)**
		151 GIVLDTGDGV	THTVPIYEGY	CLPHAILRLD	LAGRDLTAYL	TKIMTERGYS	
		201 FTTTAEREIV	RDIKEKLCYV	ALDFESEMNV	AAASSSLEK**S**	**YELPDGQVIT**	
		251 **IGNER**FRCPE	SLFQPSFLGM	ESVGIHETVY	NSIMRCDIDI	RKDLFANNVL	
		301 SGGTTMYP					

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4.6 Conclusion

This study has successfully identified the major allergens as well as the IgE-binding patterns in eight protein extracts of raw and boiled *S. paramamosain*, prepared from different body parts and genders. It should be noted that these findings are new discoveries in allergenic studies as this study is the first to report the allergenicity variation between different crab parts and genders. Two major allergens of 38 and 42 kDa were successfully identified by mass spectrometry analysis as tropomyosin and arginine kinase, respectively.

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CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Introduction

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This chapter concluded the key findings of this study. All research objectives were successfully achieved. Following data analysis and a thorough discussion in Chapter 4, conclusions were made and recommendations were suggested in this chapter.

5.2 Conclusion

Analysis of total protein estimation revealed that different crab parts and gender of *S. paramamosain* have varied protein content. The protein estimation results ranges between 17.0668 mg/mL to 20.3341 mg/mL in the raw extracts. The lowest protein concentration among raw extract was seen in the raw male abdomen while the highest protein concentration was seen in the raw female leg. In contrast to raw extracts,

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boiled samples of crab showed results of protein estimation which is way lesser when compared to their respective raw parts. The result of protein estimation of boiled extracts ranges in between 6.3490 mg/mL to 9.8143 mg/mL. The lowest concentration was seen in the male claw while highest concentration was seen in the female abdomen.

The protein profiles of raw extracts from different body parts and genders of *S. paramamosain* are almost identical, except for slight differences in the bands intensities at certain molecular weights. Among the raw extracts, the mix crab parts revealed the highest bands of proteins (24 bands) between >250 to 15 kDa, whereas between the raw extracts of individual body parts, both the female abdomen and female claws have the highest number of bands (21 bands), while the male claws has the least number of bands (16 bands). However, according to SDS-PAGE analysis, both female and male mud crab extracts indicated almost homogenous protein profile patterns with four prominent bands at 100, 75, 38, and 18 kDa.

In contrast to raw crab extracts, the boiled extracts of *S. paramamosain* showed lesser bands with slightly different protein profile patterns between different body parts and genders. A total of 10 heat-resistant protein bands between 15 to 62 kDa were detected in the boiled extracts of *S. paramamosain*, whereas the other proteins, particularly the higher molecular weight protein bands above 62 kDa are sensitive to boiling process. The highly conserved bands of 36-38 kDa, 18 and 15 kDa were detected in all extracts even after the heating process applied. Among the boiled

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crab parts, the boiled mix body part has the highest number of heat-resistant bands (9 bands) followed by the male claw (8 bands), female abdomen (7 bands), and other boiled crab parts which revealed only six protein bands. The protein profile patterns between all boiled extracts were almost identical except for some variation of band intensities.

The raw extract of mix crab parts demonstarted 18 IgE-binding proteins between 18 to >250 kDa in the immunoblotting experiments, indicating *S. paramamosain* has numerous allergenic proteins. Five major allergens at 36, 38, 42, 50 and 95 kDa were identified in the raw mix parts of *S. paramamsain* with the frequencies of 54, 64, 60, 50 and 54%, respectively. Similarly, immunoblotting results of raw individual crab parts and genders have also detected multiple IgE-binding proteins with various IgE-binding patterns. Based on the number of IgE-binding bands, the allergenicity order among individual crab parts was as follows: female legs > male claws > male legs > female abdomen = male abdomen > female claws. Meanwhile, according to the number of major allergens, the allergenic order was: female legs = female claws = female abdomen = male abdomen > male claws = male legs, whereas based on the IgE-binding intensity, the most prominent IgE-binding intensity was seen in immunoblotting of female legs, followed by male claws and female claws. Thus, the allergenicity based on the binding intensities was arranged in this order: female legs > male claws > female claws > female abdomen = male abdomen = male legs. This finding indicated that in terms of the number of IgE-binding bands, number of major allergens and IgE-binding band intensities, the crab parts have variable allergenicity. Based on these three characeristics results in this

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study, the female legs can be regarded as the most allergenics part than the other crab parts.

In contrast, all boiled extracts had a lesser number of IgE-binding proteins than the raw extracts. The boiled mix parts extract has the highest number of heat-resistant IgE-binding proteins (13 bands) including four major allergen bands at 36, 38, 42 and 50 kDa. Among the individual boiled body parts, male claws has the highest number of heat-resistant bands (6 bands) including two major allergens at 95 and 38 kDa, while the female claws and female abdomen were the least allergenic, with both have only one heat resistant bands at 38 kDa. Meanwhile, according to the number of reacted sera in immunoblotting experiments, the boiled mix body parts elicited the highest responses (90%), while among the individual boiled body parts, the male leg extract elicited the highest IgE-responses (60%), followed by the boiled female leg (50%), boiled male abdomen extract (30%), male claws and female abdomen (both 20%) and female claw (10%). Thus, the allergenicity order among boiled crabs was in the following order: male leg > female leg > male abdomen > male claws = female abdomen > female claws. This finding suggests that boiling process was insufficient to eliminate or destroy all the mix crab parts or individual crab parts of *S. paramamosain* allergens but it can partly reduce allergenicity for patients who reacted only to the heat-sensitive allergens by reducing the allergenic bands.

Out of five major allergenic bands submitted for mass spectrometry analysis, two major allergens of *S. paramamosain* at 38 and 42 kDa have been successfully identified as tropomyosin and arginine kinase, respectively.

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Overall, this study concluded that all body parts and genders of *S. paramamosain* have various allergenicity in both raw and boiled forms. Five major allergens at 36, 38, 42, 50 and 95 kDa were identified as the major allergens of this crab species, and two of them, the 38 and 42 kDa were identified as tropomyosin and arginine kinase, respectively. This finding is important for clinician and patients to improve the diagnostic and management approach of crab allergenic patients in this country.

5.3 Recommendation

The findings outlined in this study indicating that all the research objectives have been achieved. The experimental data particularly the major allergens broadens the present information on mud crab allergy, offering a platform for development and improvement of diagnosis, treatment and management of crab allergic patients. However, here researcher suggested some recommendations for improvement of research approaches to fix vulnerabilities and provide better research results. Some suggestions for future research were also listed, as follows:

a. *Identification of major and minor allergens of mud crab allergic patients*

   Previous studies reported mud crab allergic patients have severe allergic reactions to crab allergens. Future studies to compare allergenic protein patterns between crab allergic patients with crab allergy and patients without crab allergy could offer an awareness into the differential IgE-binding in the

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two populations; which may lead to the development of allergen panel that was specific for crab-allergic patients.

b. *Cellular reactivity of crab allergens*

IgE reactivity of crab allergens could be confirmed by using whole blood basophil activation assay to support the immunoblotting data.

c. *Clinical food challenge study*

Food challenges such as the double blind placebo controlled food challenge tests (DBPFC) are essential for diagnosing food allergy. The result is essential to confirm the status of crab allergy and support both immunoblotting and immunoblotting inhibition data.

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d. *Production of recombinant allergen*

It is recommended that the study should be extended up to producing of recombinant protein. This recombinant protein can be used to reduce the allergic reaction on them patients in immunotherapy as it may improve safety and enhance efficacy of immunotherapy.

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