ALLERGENIC CHARACTERIZATION AND BIOCHEMICAL STABILITY OF ALLERGENS IN PURPLE MUD CRAB, *Scylla tranquabarica*

HASAN ALI JASIM ALSAILAWI

UNIVERSITI PENDIDIKAN SULTAN IDRIS

2020

ALLERGENIC CHARACTERIZATION AND BIOCHEMICAL STABILITY OF ALLERGENS IN PURPLE MUD CRAB, *Scylla tranquabarica*

HASAN ALI JASIM ALSAILAWI

THIS THESIS IS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(BIOLOGY)

FACULTY OF SCIENCE AND MATHEMATICS
UNIVERSITI PENDIDIKAN SULTAN IDRIS

2020

Please tick (√)
Project Paper
Masters by Research
Master by Mixed Mode
PhD

UNIVERSITI PENDIDIKAN SULTAN IDRIS
SULTAN IDRIS EDUCATION UNIVERSITY

☐
☐
☐
☐

INSTITUTE OF GRADUATE STUDIES

DECLARATION OF ORIGINAL WORK

This declaration is made on 14/7/ 2020

i. Student's Declaration:

I, HASAN ALI JASIM (P20162002429), FACULTY OF SCIENCE AND MATHEMATICS (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work Entitled ALLERGENIC CHARACTERIZATION AND BIOCHEMICAL STABILITY OF ALLERGENS OF PURPLE MUD CRAB (*Scylla tranquebarica*) is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

_____

Signature of the Student

ii. Supervisor's Declaration:

I, Prof. DR. ROSMILAH BINTI MISNAN (SUPERVISOR'S NAME) hereby certifies that the work entitled ALLERGENIC CHARACTERIZATION AND BIOCHEMICAL STABILITY OF ALLERGENS OF PURPLE MUD CRAB (*Scylla tranquebarica*) was prepared by the above named student, and was submitted to the Institute of Graduate Studies as a full fulfilment for the conferment of Doctor Of Philosophy (Biology) and the aforementioned work, to the best of my knowledge, is the said student's work.

_____ _____

Date Signature of the Supervisor

UNIVERSITI PENDIDIKAN SULTAN IDRIS
SULTAN IDRIS EDUCATION UNIVERSITY

INSTITUT PENGAJIAN SISWAZAH /
INSTITUTE OF GRADUATE STUDIES

BORANG PENGESAHAN PENYERAHAN TESIS/DISERTASI/LAPORAN KERTAS PROJEK
DECLARATION OF THESIS/DISSERTATION/PROJECT PAPER FORM

Tajuk / *Title*: ALLERGENIC CHARACTERIZATION AND BIOCHEMICAL STABILITY OF ALLERGENS OF PURPLE MUD CRAB (*Scylla tranquebarica*)

No. Matrik /*Matric No.*: P20162002429

Saya / *I* : HASAN ALI JASIM

mengaku membenarkan Tesis/Disertasi/Laporan Kertas Projek (Kedoktoran/Sarjana)* ini disimpan di Universiti Pendidikan Sultan Idris (Perpustakaan Tuanku Bainun) dengan syarat-syarat kegunaan seperti berikut:-
acknowledged that Universiti Pendidikan Sultan Idris (Tuanku Bainun Library) reserves the right as follows:-

i. Tesis/Disertasi/Laporan Kertas Projek ini adalah hak milik UPSI.
The thesis is the property of Universiti Pendidikan Sultan Idris

ii. Perpustakaan Tuanku Bainun dibenarkan membuat salinan untuk tujuan rujukan dan penyelidikan.
Tuanku Bainun Library has the right to make copies for the purpose of reference and research.

iii. Perpustakaan dibenarkan membuat salinan Tesis/Disertasi ini sebagai bahan pertukaran antara Institusi Pengajian Tinggi.
The Library has the right to make copies of the thesis for academic exchange.

ii. Sila tandakan (√) bagi pilihan kategori di bawah */ Please tick (√) from the categories below:-*

SULIT/*CONFIDENTIAL*	☐	Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub dalam Akta Rahsia Rasmi 1972. / *Contains confidential information under the Official Secret Act 1972*
TERHAD/*RESTRICTED*	☐	Mengandungi maklumat terhad yang telah ditentukan oleh organisasi/badan di mana penyelidikan ini dijalankan. / *Contains restricted information as specified by the organization where research was done*
TIDAK TERHAD / *OPEN*	☐	

_____ (Tandatangan Pelajar/ Signature)

_____ (Tandatangan Penyelia / *Signature of Supervisor*) & (Nama & Cop Rasmi / *Name & Official Stamp*)

Tarikh: _____

Catatan: Jika Tesis/Disertasi ini **SULIT** @ **TERHAD**, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh laporan ini perlu dikelaskan sebagai **SULIT** dan **TERHAD**.

Notes: If the thesis is CONFIDENTAL or RESTRICTED, please attach with the letter from the related authority/organization mentioning the period of confidentiality and reasons for the said confidentiality or restricti

ACKNOWLEDGEMENTS

Praise to Allah SWT, for giving me the chance, strength and health to accomplish this study. Without Your blessing, it is impossible for me to be at this point. Special thanks to my main supervisor, Associate Professor Dr. Rosmilah bt Misnan, for her support, guidance and advice throughout my study. Without her support and encouragement, this thesis could not have been completed. May Allah repay her kindness. I would also like to thanks to all staffs from Faculty Science and Mathematics, UPSI for their kind help on this project.

I would like also to thank my parents for their wise counsel and sympathetic ears. You are always there for me. Finally, there are my friends. We were not only able to support each other by deliberating over our problems and findings, but also happily by talking about things other than just our papers.

Thank you.

ABSTRACT

This study aims to characterize the allergenic properties and determine the biochemical stability of allergens of purple mud crab, *Scylla tranquebarica*. Raw extracts were prepared from the muscle tissues of both mixed and specific body parts. Various treated extracts were prepared from the muscles of mixed body parts. Protein profiles, allergenic proteins and cross-reactive allergens were then identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and immunoblotting inhibition test techniques, respectively, using sera from crab-allergic patients. The identification of major allergens were analyzed by mass spectrometry. This study showed that raw mixed part extract contains the most protein bands and allergenic proteins than the specific body parts and all treated extracts. Five major allergens were identified at 38, 42, 50, 63 and 73 kDa. Mass spectrometry analysis identified the 42 and 50 kDa as arginine kinase, while the 38, 63 and 73 kDa were identified as tropomyosin, actin and hemocyanin, respectively. The allergen extracts stored at -80 °C for all storage periods of 3, 6, 9 and 12 months were indicated to have the same quality as the fresh raw mixed parts extract. Immunoblotting inhibition tests showed the present of cross-reactivity among major and minor allergens of crab, with either complete or partial inhibitions. Among the specific crab parts, the female legs was found as the most allergenic parts, while the male abdomen was indicated as the least allergenic. The high pressure steamed and white vinegar treated crabs were found to have the lowest degree of stability and allergenicity among the thermal and non-thermal treated crabs, respectively. As a conclusion, the allergenicity of *S. tranquebarica* varies depending on the crab parts, processing treatments and storage conditions applied. Tropomyosin, arginine kinase, actin and hemocyanin are identified as the major and cross-reactive allergens in this crab species. Tropomyosin is indicated as the most stable allergen of *S. tranquebarica.* The implication of this study is to provide insights into local mud crab allergens to improve strategy of diagnosis, management, therapeutic and food manufacturing for crab allergic patients in this country.

PENCIRIAN ALERGENISITI DAN KESTABILAN BIOKIMIA ALERGEN KETAM LUMPUR UNGU (*Scylla tranquebarica*)

ABSTRAK

Tujuan kajian bertujuan untuk mencirikan alergen dan menentukan kestabilan biokimia alergen ketam lumpur ungu, *Scylla tranquebarica*. Ekstrak mentah disediakan daripada tisu otot kedua-dua bahagian campuran dan bahagian badan yang spesifik. Pelbagai ekstrak ketam yang dirawat disediakan daripada tisu otot campuran badan ketam. Profil protein, alergenik protein dan alergen reaktif silang kemudiannya dikenal pasti menggunakan teknik elektroforesis gel natrium dodesil sulfat-poliakrilamid (SDS-PAGE), ujian pemblotan imuno dan perencatan pemblotan imuno masing-masing, menggunakan serum daripada pesakit alahan ketam. Identifikasi alergen major kemudiannya dianalisis menggunakan spektrometri jisim. Kajian ini menunjukkan ekstrak daripada campuran ketam mentah mengandungi jalur protein dan protein alergenik yang paling banyak berbanding dengan bahagian badan spesifik dan semua ekstrak yang dirawat. Lima alergen major telah dikenal pasti pada 38, 42, 50, 63 dan 73 kDa. Analisis spektrometri jisim mengenal pasti protein 42 dan 50 kDa sebagai arginin kinase, manakala 38, 63 dan 73 kDa, masing-masing dikenal pasti sebagai tropomiosin, aktin dan hemosianin. Ekstrak alergen yang disimpan pada suhu -80 °C untuk semua tempoh penyimpanan selama 3, 6, 9 dan 12 bulan telah didapati mempunyai kualiti yang sama seperti ekstrak campuran ketam mentah segar. Ujian perencatan imuno menunjukkan kehadiran reaktiviti silang dalam kalangan alergen ketam, sama ada dengan perencatan yang lengkap atau separa. Dalam kalangan bahagian ketam spesifik, kaki ketam betina didapati bahagian yang paling alergenik, manakala abdomen ketam jantan adalah bahagian yang paling kurang alergenik. Ketam yang dikukus dengan tekanan tinggi dan ketam yang dirawat dengan cuka putih didapati, masing-masing mempunyai tahap kestabilan dan alergenisiti yang paling rendah dalam kalangan ketam yang terawat haba dan tanpa rawatan haba. Sebagai kesimpulan, alergenisiti *S. tranquebarica* berbeza-beza bergantung kepada bahagian ketam, rawatan pemprosesan dan keadaan penyimpanan yang digunakan. Tropomiosin, arginin kinase, aktin dan hemosianin dikenal pasti sebagai alergen major dan alergen reaktif silang dalam spesies ketam ini. Tropomiosin adalah alergen *S. tranquebarica* yang paling stabil. Implikasi kajian ini adalah untuk memberikan kefahaman tentang alergen ketam lumpur tempatan bagi mengukuhkan strategi diagnosis, pengurusan, rawatan dan pembuatan makanan untuk pesakit alergi ketam di negara ini.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIRC	Allergy and Immunology Research Centre
AK	Arginine kinase
CR	Cross reactivity
CUB	Combined Ultrasound and Boiling
CCE	Crab Crude Extracts
DBPCFC	Double-blind placebo-controlled oral food challenge
FAO	Food and Agriculture Organization of United Nations
HKL	Hospital Kuala Lumpur
HPS	High Pressure Steaming
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
SIF	Simulated Intestinal Fluid
TBS	Tris-buffered saline
TM	Tropomyosin
TTBS	Tween 20-tris-buffered saline
WHO	World Health Organization

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Shellfish is an aquatic shelled organism which consists of two groups; Crustaceans (Phylum Arthropods, 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 (Lee, 2016; Keenan, 1999).

Seafood plays an important role in human nutrition and health. The growing international trade in seafood species and products has added to the popularity and frequency of consumption of a variety of seafood products across many countries. The highest consumption in Europe appears to be in Iceland, where the gross per capita consumption of crustaceans and fish is about 91 kg, followed by Spain (43 kg), United Kingdom (19 kg), and Germany (13 kg), as compared to the United States of America (8 kg) and Australia (11 kg) (Ahmed et al., 2015; Food and Agriculture Organization, 2014).

Crabs are among the most important shellfish, which are classified in the Phylum Arthropoda, Subphylum Crustacean, Class Malacostraca, and Order Decapoda (Martin, Olesen, Høeg, & Høeg, 2014). Crabs belong to the Order Decapoda, which can be classified into two main groups, i.e., Brachyuran crabs and Anomuran crabs (Luque, 2015; Tsang, Chan, Ahyong, & Chu, 2011). According to Darren and Peter (1998), crabs have approximately 33 genera with approximately 165 species which are known from Indo China. Mud crabs, also known as mangrove crabs, belonged to Family Portunidae under genus *Scylla*. Four species of mud crab have been described. They are known as *Scylla serrata*, *Scylla paramamosain*, *Scylla tranquebarica*, and *Scylla olivacea* (Varadharajan & Soundarapandian, 2014; Keenan et.al, 1998). Mud crabs are highly demanded as a protein food source in Malaysia. However, the mud crab industry is still not making headway and is only carried out on a small scale by local anglers (Williner, Carvalho, & Collins, 2014).

Mud crabs, in the genus *Scylla*, inhabit brackish waters such as mangrove areas and estuaries throughout the Pacific and Indian Oceans from Tahiti, Australia, and Japan to Southern Africa (Akpaniteaku, 2014; Dai & Yang, 1991). This type of crab is an important fishery resource in Australia, Japan, Taiwan, Indonesia, and Philippines where it is also targeted for aquaculture (Azra & Ikhwanuddin, 2016; Ma et al., 2015; Watanabe et al., 1996; Watanabe & Sulistiono, 1993). In recent years, these mud crabs have been selected as one of the target species for stock enhancement programs in Japan (Azra & Ikhwanuddin, 2016). Mud crabs are important for commercial fisheries and aquaculture production throughout their distribution (Shelly & Lovatelli, 2011). Besides that, mud crabs are important in trade markets as demand for them has been reported to be increasing on a yearly basis (Bain & Mandal, 2017).

S. tranquebarica, the purple mud crabs, are a large marine portunid species and widely distributed along the Southeast China coasts and other Asian countries, such as Japan, Vietnam, India, Pakistan, Taiwan, Singapore, Indonesia, Malaysia, and the Philippines (Sun et al., 2015). In Malaysia, *S. tranquebarica* are commonly present in Sabah coastal waters where prefer mangrove forests and coastlines inundated with reduced salinity (Sharif, Kahar, Rodrigues, Ransangan, & Kian, 2016; Varadharajan & Soundarapandian, 2014; Keenan et al., 1998). *S. tranquebarica* is also declared as one of the important commercial species that are widely found in Malaysia (Fazhan, Waiho, & Ikhwanuddin, 2017; Yap, Wong, Maule, Brennan, & Lim, 2015; Keenan et al., 1998).

Both male and female crabs contribute to the local economy and are commonly consumed by local people (de Oliveira Côrtes, Zappes, & Di Beneditto,

2014). However, male and female mud crabs are difficult to differentiate. Differences between the sexes will only become more apparent as the crabs mature (Hübner, Pennings, & Zimmer, 2015; 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 ones by an increase in height for a given carapace width (Varisco & Vinuesa, 2017; Somerton, 1982). Another obvious difference between the male and the female crabs is from the external character during spawning (Somerton, 1982). Compared to the females, the males are generally larger, very dark in colour, larger in mouth, as well as broader and swollen head (Crane, 2015; Llewellyn, 2007).

However, consumption of shellfish including crabs may also cause seafood allergies (Pedrosa, Boyano-Martínez, García-Ara, & Quirce, 2015; Abramovitch et al., 2013; Lopata, O'hehir & Lehrer, 2010). The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have identified shellfish, including crabs, as one of the eight major sources of food allergens (Prester, 2016; Pedrosa et al., 2015). Allergies to crabs is one of the most common IgE-mediated food allergies and is often associated with severe reactions (Villalta et al., 2010). Signs and symptoms of crab allergies comprise of 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, Lopata & Potter, 2000; Sheffer, 1985). Exposure to crab allergens may occur through ingestion of crabs and inhalation or skin contact with crabs while processing, cooking, or working (Butt & MacDougall, 2008). Occupational asthma to crabs has also been reported in

fishermen, processing workers, shell grinders, cooks, as well as restaurant workers (Lopata & Jeebhay, 2013; Jeebhay & Lopata, 2012; Jeebhay & Cartier, 2010).

IgE-mediated hypersensitivity reactions have become important ecumenical health issues. In the United States, a survey reported that one in 50 Americans has a type of shellfish allergies, including allergy to crabs (Sockalingam, Misnan, & Yadzir, 2017; Zhang et al., 2006). The prevalence of shellfish allergy is estimated to be 0.5 to 2.5% of the general population, but higher in coastal Asian countries where shellfish constitutes a large proportion of the diet. In Malaysia, the prevalence of shellfish allergies, including crab allergy, was reported to be 44% among local patients with allergic rhinitis and asthma (Aziz et al., 2016; Shahnaz et al., 2001).

In a few studies characterising shellfish allergens, one of the most frequently recognised major allergens in both shellfish Phyla are the abundant muscle protein tropomyosin (Khora, 2016; Pedrosa et al., 2015; Rosmilah et al., 2012; Yadzir, & Murad, 2012). Tropomyosin has been demonstrated as the major allergen of numerous crab species (Pedrosa et al., 2015; Rosmilah et al., 2012; Shriver & Yang 2011; Abdul Rahman et al., 2011; Liang et al., 2008; Lehrer et al., 2003). Allergenic tropomyosin was first identified by Leung et al. (1998) in red crabs *Charybdis feriatus* (*Cha f* 1). Subsequently, tropomyosin has also been identified as the major allergens in other crab species such as Chinese mitten crabs *Eriocheir sinensis* (Liang et al., 2008), *Portunus pelagicus* (Abramovitch et al., 2013; Rosmilah et al., 2012; Huang et al., 2010), *Scylla serrata* (Liu et al., 2018; Rosmilah et al., 2015), and *Scylla paramamosain* (Han et al., 2018). Tropomyosin is considered as a pan-allergen

among invertebrates such as crustaceans, molluscs, mites, and cockroaches (Abramovitch et al., 2013; Sereda et al., 2008; Suma et al., 2007; Lehrer et al., 2003).

Besides tropomyosin, other crab allergens have also been reported, including arginine kinase, a new potential pan-allergen in *S. serrata* mud crabs (Shen et al., 2011), *S. paramamosain* (Yang et al., 2019; Han et al., 2018; Mao et al., 2013; Yu et al., 2013), and snow crabs *Chionoecetes opilio* (Abdel Rahman et al., 2011). In addition, sarcoplasmic calcium-binding protein (Hu et al., 2017; Abdel Rahman et al., 2011), troponin, α-actin, and smooth endoplasmic reticulum Ca^{2+}ATPase were also identified as allergenic proteins in crabs (Abdel Rahman et al., 2011).

The stability of allergen extracts is important for the diagnosis and treatment of allergic diseases. Several factors have been found to be important in the preservation of allergens in the extracts. Storage temperature is one of the major determinants of allergen stability in protein extracts, especially in the absence of a preservative (Jeong et al., 2013; Piboonpocanun et al., 2010). However, allergen extracts, including crabs, will be degraded and lose potency when stored over time (Jeong et al., 2013; Piboonpocanun et al., 2010). The main reason is because the allergen extracts generally contain various enzymes including proteases, which result in protein degradation and subsequently may reduce the allergenic potency of the extracts. Therefore, proper storage conditions and the addition of preservatives help to prevent protein degradation and increase the shelf life of the extracts. A study reported that the ideal storage temperature in preserving allergenicity of house dust mite and pollen extracts is at 4°C (Jeong et al., 2013). However, for shrimp extracts,

the ideal storage conditions are at -20°C for four weeks to prevent the loss of allergens (Piboonpocanun et al., 2010).

Local people commonly consume all crab parts including abdomen, claws, and legs. The meat yield, proximate composition, and fatty acid profile of two species of mud crabs, i.e., *S. serrata* and *S. tranquebarica*, were compared with respect to genders and different body parts. The results showed significant differences in biochemical composition (Sreelakshmi, 2016; Zafar, Siddiqui, & Hoque, 2004). It was reported that protein content was highest in body meat and lowest in claw meat of *S. tranquebarica*. Meanwhile, the meat from *S. serrata* and *S. tranquebarica* female crabs had significantly higher protein content than males irrespective of varied species and body parts (Sreelakshmi et al., 2016; Zafar et al., 2004; Khan, 1992).

A cross-reactivity allergy is present when the IgE antibodies against a specific allergen also recognise, bind, and induce an immune response to homolog molecules from different sources (Popescu, 2015). Cross-reactivities of tropomyosin amongst crustaceans, amongst molluscs, between crustaceans and molluscs, and between crustaceans and arthropods, such as mites and cockroaches were reported in numerous studies (Tong et al., 2018; Abramovitch et al., 2013). These are likely because of the high homology in amino acid sequence (69% to 100%) among them. Their cross-reactivities are probably due to their highly conserved IgE-binding epitopes (Tong et al., 2018). Cross-reactivities between the tropomyosin of *P. pelagicus* and *C. feriatus* (Rosmilah et al., 2012), as well as between blue swimmer crabs (*Por p* 1) and black tiger prawns (*Pen m* 1) (Abramovitch et al,. 2013), between crabs and shrimps and

scallops (Zhang et al., 2006), and between crabs and shrimps, krill, and lobsters (Nakano et al., 2008), were also frequently observed.

Seafood, including crabs could be consumed in raw or processed forms. Processed shellfish, including crabs, commonly involve several thermal treatments such as boiling, frying, or roasting (Fernandes, Pereira, Antonio, & Ferreira, 2017; Sockalingam, Misnan, & Yadzir, 2017; Ahmed, Ramaswamy, Kasapis, & Boye, 2016), or non-thermal treatments such as salting, freezing, and pickling. It was reported that processing methods could modify the allergenicity of shellfish, such as decreasing, increasing, or having no effect on the allergenicity (Fernandes et al., 2017; Ahmed et al., 2016; Abramovitch et al., 2013; Nowak-Wegrzyn et al., 2009).

Allergen stability and digestibility directly contribute to allergenicity. However, in scientific literature, the correlation between digestion stability and allergenicity is not fully clear (Utersmayra et al., 2018; Bogh & Madsen, 2016). It is increasingly acceptable that gastrointestinal digestion clearly has an influence in food allergies (Untersmayr & Jensen-Jarolim, 2008), suggesting that the digestion stability of food allergens as a criterion for assessing potential allergenicity (Moreno, 2006).

1.2 Problem Statement

Seafood plays an important role in nutrition worldwide, sustained by increasing international trade of a variety of new seafood products (Belton & Thilsted, 2014; Jeebhay et al., 2010; Lopata et al., 2007). However, consuming crabs can generate an allergic reaction mediated by IgE antibodies, which can cause a fatal effect. In fact, crabs are ranked as top seven causes of food allergic reactions in the world (Pawankar et al., 2011). Normally, harmless proteins that reacted as allergens in some hypersensitive individuals generate allergic reactions to seafood (Pawankar et al., 2011). Among portunid crabs, mud crabs are the most widely distributed and cultured group. In Malaysia, the purple mud crabs (*S. tranquebarica*) are widely consumed by the local population (Fazhan et al., 2017). However, a study on allergies to this mud crab species is unavailable in literature.

The identification of allergens is of the utmost importance because it is essential for the understanding of the specific IgE-mediated immune responses. Furthermore, their identification would aid in reliable diagnostic tests and management of patients. At present, there are numerous reports on identification of crab major allergens such as tropomyosin, arginine kinase, and actin from several crab species (Han et al., 2018; Liu et al., 2018; Rosmilah et al., 2015; Liang et al., 2008; Abramovitch et al., 2013; Rosmilah et al., 2012; Huang et al., 2010). Unfortunately, these reports are of a different species, and not *S. tranquebarica*. Currently, there has been no study on characterisation of major and minor allergens of *S. tranquebarica*.

Mud crabs are a valuable food in many countries such as Japan, China, Hong Kong, Korea, Thailand, and the USA because of their high-quality flesh, luxurious taste, and richness in protein, vitamins, and minerals (Islam, 2015). The edible flesh is in the claws, legs, and abdomens (bodies) of the crabs (Madsen, Forster, Grefenstette, Harrison, & Stern, 2017; Stewart & Reichelt, 1993). However, no reports on the characterisation of allergens from different crab parts, particularly claws, legs, and abdomens, as well as between genders have been made. The comparison of allergenic proteins between crab genders and parts is considered as an important aspect to be evaluated in allergy studies. The variations of allergenic properties in crabs' body parts and genders might play an important role in crab allergenicity.

The Skin Prick Test (SPT) is a reliable method for diagnosing IgE-mediated allergies in patients with rhino conjunctivitis, asthma, urticarial, anaphylaxis, atopic eczema, food, and drug allergies (Heinzerling et al., 2013). SPT is usually performed using crab extracts prepared from crab meat. However, to date, crab extracts that are commercially available are prepared from crab species other than *S. tranquebarica*, which might have different protein contents and allergenicity. The sources of crab parts used for extractions are not well-defined. Thus, to produce more accurate diagnostic tests for detection of specific IgE against *S. tranquebarica* allergens, allergen extracts produced from several body parts of local crabs like *S. tranquebarica* are essential to obtain accurate diagnostic results.

The practice of allergen immunotherapy is essential to the management of IgE-mediated hypersensitivities in allergic patients. The effectiveness of

immunotherapy regimens for most patients is affected by several factors, including the accuracy of skin or serum IgE testing, close correlations between test results and clinical histories, extract compositions containing the specific allergens responsible for symptomatic events, as well as the extracts qualities (Plunkett, 2016). The extraction of allergenic materials typically yields complex, heterogeneous solutions composed primarily of water-soluble proteins and carbohydrates. Several studies have examined the stability of allergens in defined mixtures representing common or potential immunotherapy vaccines. Allergen extracts are usually stored under standardised conditions to maintain their quality for years (Plunkett, 2016). However, no study has been conducted so far to determine the quality of allergen extracts of crabs, including *S. tranquebarica*, under various storage conditions. In addition, this study is very important to establish the expiration dating of the allergen extracts.

To date, only a few options are available for treatments of shellfish allergies, including crabs. Avoidance of the offending food is the only therapy recommended (Rolland, Gardner, & O'Hehir, 2009; Ayuso et al., 2008). Limitations of the diagnostic tools and treatment options make shellfish allergies more complicated to manage. Typically, patients report clinical reactions to more than one shellfish species, but whether this is a result of multiple sensitivities or IgE cross-relativities between allergens of different shellfish species is indefinite (Abramovitch et al., 2013; Lopata, Hehir, & Lehrer, 2010). Besides that, clinical cross-reactivity cannot be confirmed by only skin test positivity because of possible co-sensitisation in highly atopic individuals (Sicherer, 2001), whereas oral food challenge, which is mainly double-blind placebo-controlled oral food challenge (DBPCFC), is not easy to

perform to confirm a clinical allergy to a particular shellfish species due to various limitations (Sicherer, 2001). Cross-reactivity is also important in immunologic basis, particularly in relation to the regulation of allergic sensitisation, the risk of allergic cross-reactivity to novel food, and the identification of the cross-reactivity patterns. This is because they may reflect the patterns of clinical sensitivities (Popescu, 2015). Thus, cross-reactivity detection by using cross-inhibition assays in this research may provide more insights into the relation of crab allergen sensitisation and the clinical reactivity to the shellfish allergens.

Shellfish, including crabs, are usually subjected to some forms of food processing by either thermal, non-thermal, or both treatments. Since allergens are mainly proteins, their structure may be changed by various types of processing methods resulting in alteration of allergenicity (Chen & Phillips, 2005; Mondoulet et al., 2005). 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, Shahnaz, Zailatul, & Noormalin, 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 et al., 2015; Zailatul et al., 2012; Yu et al., 2011; Carnes et al., 2007; Martin-Garcia et al., 2007). Nevertheless, little is known about the effects of non-thermal treatments such as salting, acid, or drying on digestibility and allergenicity of crabs.

Thus, this study aimed to identify the major and minor allergens of *S. tranquebarica,* as well as determine the molecular characteristics from different body

parts and genders of this type of crab by proteomics approaches. The quality, cross-reactivity, stability, and digestibility of the allergens were also investigated experimentally. The findings from this study might contribute to the development of more efficient diagnostic kits and improve the treatment and management of patients with crab allergies in this country.

1.3 Research Objectives

The objectives of this research are as follows:

1. To identify the major and minor allergens of *Scylla tranquebarica*.
2. To evaluate the quality of allergen extracts of *S. tranquebarica* under various storage conditions.
3. To compare the allergenic properties of *S. tranquebarica* from different body parts and genders.
4. To identify the cross-reactivity characteristics between *S. tranquebarica* and common shellfish allergens.
5. To evaluate the stability and digestability of the *S. tranquebarica* allergens under various processing methods.

1.4 Research Question

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

1. What are the major and minor allergens of *Scylla tranquebarica*?
2. How the various storage conditions affect the quality of the allergen extracts of *S. tranquebarica*?
3. What are the characteristics of allergenic properties of *S. tranquebarica* from different body parts and genders?
4. What is the cross-reactivity characteristics between *S. tranquebarica* and various shellfish allergens?
5. How the various processing methods affect the stability and digestability of the allergens of *S. tranquebarica?*

1.5 Research Significant

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

The evaluation of the quality of allergen extracts under various storage conditions is important for researchers and clinicians to determine the expiration dating of the allergen extracts to be used in diagnosis or immunotherapy purposes in order to get effective and optimised results.

This study provided new data on the methods of allergen extraction from different body parts and genders of *S. tranquebarica* mud crabs, which will be useful for researchers in selecting crab parts with the highest protein components as the crab source for allergen extracts. Meanwhile, the findings will also benefit in the management of crab allergic patients by avoiding crab parts which contain more allergenic components from their diet. The information regarding the allergenicity of different crab parts and genders is also useful for the food industries in producing the least allergenic crab products by removing the most allergenic crab parts in processed foods.

Some allergens are unique markers for a specific allergen source. The value of identifying these species-specific allergens lies in being able to narrow down the primary sensitizer that causes certain reactions to just one specific source. Identifying whether the sensitisation is primary (species-specific) or a result of cross-reactivity to proteins with similar protein structures makes it easier for clinicians to judge the risks of reactions on exposure to different allergen sources (Popescu, 2015).

Meanwhile, the identification of cross-reactivities between *S. tranquebarica* and various local shellfish is vital to understand the species-specific allergens and

common allergens between shellfish. Importantly, various substances found in shellfish can trigger clinical symptoms although non-allergic in origin is similar to a true IgE-mediated allergic reaction. Because of the similarity in clinical reactions of affected individuals, it is of fundamental importance to differentiate adverse reactions from true shellfish by identification of its cross-reactivity, where the reaction is specific to the IgE reactivity of individuals. Thus, the finding of cross-reactive allergens in crabs will help to extend the relationship between crabs and other shellfish, both crustacean and mollusc groups in this country.

Besides, as shellfish including crabs are generally processed prior to consumption, the results of the effects of various processing methods on crab allergenicity will be also valuable for improving the therapeutic and management strategies towards local crab-allergic patients. Various processing treatments may be viewed as important methods of preventing allergenicity in susceptible individuals, thereby reducing treatment costs.

In general, the findings from this study will help in improving the standardisation of the diagnosis and management in crab allergies worldwide. As a result, effective and optimised management can be started, which in turn leads to improved patient health and quality of life.

1.6 Scope and Limitations of Study

This study was limited in characterising the allergenicity of one species of mud crabs, the purple crabs, i.e., *S. tranquebarica.* The crab samples were collected from only one location in Tawau, Sabah. The allergen extracts of the crabs were prepared individually from both male and female crabs, from only three crab parts which were abdomens (bodies), claws, and legs. The protein profile of the mixed crab part extracts was determined by both SDS-PAGE and 2-DE electrophoresis, while the protein profiles for the individual crab parts from processed and stored crabs were analysed solely by SDS-PAGE.

The major and minor allergens of the crabs were determined by immunoblotting and 2-DE immunoblotting tests using only 65 sera which were collected from one location, i.e., Allergy Clinic, Hospital Kuala Lumpur. For the molecular identification of crabs, only the major allergenic spot of the major allergens was identified by the mass-spectrometry analysis. Apical Scientific Sdn. Bhd., Malaysia conducted the mass spectrometry analysis.

The allergenicity comparison between different crab parts and genders, the allergen stability, as well as the quality of the allergens extracts under various storage conditions, were only evaluated experimentally based on the results of total protein contents, protein profiles, and IgE-binding profiles using only 10 to 15 selected sera. Meanwhile, for enzymatic digestion, only one serum was used. The sera were selected

based on its availability and the major allergens recognition in an immunoblotting test using the mixed body parts of *S. tranquebarica,* as mentioned above.

This study was also limited in determining the cross-reactivity patterns of *S. tranquebarica* allergens against 11 species of common local shellfish, which are green mud crabs (*S. paramamosain*), orange mud crabs (*S. olivacea*), red crabs (*Charybdis feriatus*), blue crabs (*Portunus pelagicus*), black tiger prawns (*P. monodon*), giant river prawns (*Macrobrachium rosenbergii*), pink prawns (*Penaeus latisulcatus*), squid (*Loligo edulis*), snails (*Cerithidea obtusa*), cockles (*Anadara granosa*), and clams (*Paphia textile*). The cross-reactivity was determined experimentally by an immunoblotting inhibition test. Only five selected sera were used based on their availability. In addition, the sera were chosen from patients with the history of multiple shellfish allergies.

The stability of the crab allergens was evaluated using thermal processing methods (boiling, steaming, high-pressure steaming, roasting, frying, microwave heating, and combined ultrasound boiling) and non-thermal processing methods (salting, drying, and acid hydrolysis). However, for further allergenicity test by a digestibility experiment, only three selected thermal (boiling, high-pressure steaming, and combined ultrasound boiling) and three non-thermal processing methods (dry salting, microwaved drying, and white vinegar) were tested. Meanwhile, the quality of the allergen extracts was evaluated under four storage conditions (25˚C, 4˚C, -20˚C, and -80˚C) for only up to one year period.