MOLECULAR AND ALLERGENIC CHARACTERIZATION OF TROPOMYOSIN FROM LOCAL MUD CRAB *Scylla* SPECIES

NUR FARAH HANI BINTI AZEMI

UNIVERSITI PENDIDIKAN SULTAN IDRIS

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NUR FARAH HANI BINTI AZEMI

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

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ABSTRACT

This study aims to characterize the molecular and allergenic properties of tropomyosin from local mud crab *Scylla* species. The methodology involved quantifying reference genes and tropomyosin levels in these crabs using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Additionally, recombinant tropomyosin gene and protein were created using recombinant DNA techniques. The reactivity of tropomyosin with IgE antibodies was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and anti-tropomyosin monoclonal antibody immunoblotting. Bioinformatic tools were used to predict various characteristics such as physicochemical properties, structural composition, phylogenetic relationships, IgE-epitope regions, and docking simulations of the recombinant proteins. The findings indicated that elongation factor 1 alpha (EF1α) was a suitable normalizer for qRT-PCR in the three mud crab species studied, and a combination of three reference genes per species was optimal for tropomyosin normalization. Tropomyosin was predominantly present in the abdominal muscle, with *S. tranquebarica* exhibiting the highest levels, followed by *S. olivacea* and *S. paramamosain*. A complete 855-base pair recombinant tropomyosin gene was successfully generated from a local mud crab. The induction with 1 mM IPTG at 37 °C for 4 hours resulted in 42 kDa histidine tagged-recombinant tropomyosin protein from *S. olivacea*, *S. tranquebarica* and *S. paramamosain* that was IgE-reactive to 90%, 65%, and 70% of crab-allergy patients, respectively. Analysis revealed tropomyosin as a temperature-stable coiled-coil alpha-helix protein with 11 IgE-binding sites, leading to cross-reactivity among mud crabs, other crustaceans, insects, arachnids and bacteria. Docking simulations demonstrated binding between these 11 IgE-binding epitopes and IgE antibodies, with distances ranging from 1.2 to 3.6 Angstroms. In conclusion, this study highlights the importance of comprehending the molecular and allergenic aspects of tropomyosin in local mud crab species. These findings have implications for developing diagnostic and therapeutic strategies for mud crab allergies.

PENCIRIAN MOLEKUL DAN ALERGENIK TROPOMYOSIN DARIPADA KETAM LUMPUR TEMPATAN SPESIS *Scylla*

ABSTRAK

Kajian ini bertujuan untuk mencirikan sifat molekul dan alergen tropomiosin daripada ketam lumpur tempatan spesies *Scylla*. Metodologi yang digunakan termasuk pengukuran gen rujukan dan tahap tropomiosin dalam ketam-ketam ini menggunakan *real-time quantitative reverse transcription-polymerase chain reaction* (*qRT-PCR*). Selain itu, gen dan protein tropomiosin rekombinan dicipta menggunakan teknik DNA rekombinan. Kereaktifan tropomiosin dengan antibodi IgE dinilai menggunakan elektroforesis gel poliakrilamida natrium dodesil sulfat (SDS-PAGE) dan pemblotan imuno antibodi monoklonal anti-tropomiosin. Alatan bioinformatik digunakan untuk meramal pelbagai ciri-ciri seperti sifat-sifat fizikokimia, komposisi struktur, hubungan filogenetik, rantai epitop IgE, dan simulasi penggandingan protein rekombinan. Penemuan menunjukkan bahawa faktor pemanjangan 1 alfa (EF1α) adalah penormal yang sesuai untuk qRT-PCR dalam tiga spesies ketam lumpur yang dikaji, dan kombinasi tiga gen rujukan bagi setiap spesies adalah optimum untuk normalisasi tropomiosin. Tropomiosin lebih banyak terdapat dalam otot abdomen, dengan *S. tranquebarica* menunjukkan tahap tertinggi, diikuti oleh *S. olivacea* dan *S. paramamosain*. Satu gen tropomiosin rekombinan dengan 855 pasangan bes yang lengkap telah berjaya dihasilkan dari ketam lumpur tempatan. Penginduksian dengan 1 mM IPTG pada suhu 37 °C selama 4 jam menghasilkan protein tropomiosin rekombinan dengan tag histidin 42 kDa dari *S. olivacea*, *S. tranquebarica* dan *S. paramamosain* yang reaktif terhadap IgE, masing-masing pada 90%, 65%, dan 70% pesakit alergi ketam. Analisis menunjukkan tropomiosin sebagai protein heliks alfa berpilin yang stabil suhu dengan 11 tapak pengikat IgE, yang menyebabkan reaktiviti silang di antara ketam lumpur, krustasia lain, serangga, araknida, dan bakteria. Simulasi penggandingan menunjukkan pengikatan antara 11 epitop pengikat IgE ini dan antibodi IgE, dengan jarak antara 1.2 hingga 3.6 *Angstroms*. Kesimpulannya, kajian ini menegaskan pentingnya memahami aspek molekul dan alergenik tropomiosin dalam spesies ketam lumpur tempatan. Penemuan ini mempunyai implikasi dalam pembangunan strategi diagnostik dan terapeutik untuk alergi ketam lumpur.

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α-helix	Alpha-helix
ALS	Asthma-like symptoms
APC	Antigen-presenting cell
β-actin	Beta-actin
Blast	Basic local alignment search tool
Blastn	Basic local alignment search tool nucleotide
Blastp	Basic local alignment search tool protein
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cq	Quantification cycle
DBPCFC	Double-blind placebo-controlled food challenge
ddNTPs	Dideoxy nucleoside triphosphates
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EF1α	Elongation factor 1 alpha
FAO	Food and Agriculture Organization
FcεRI	Fc epsilon receptor I
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanine cytosine
gDNA	Genomic DNA
IgE	Immunoglobulin E

IL	Interleukin
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-beta-D-thiogalactoside
mRNA	Messenger ribonucleic acid
MSC	Multiple cloning sites
NCBI	National Center for Biotechnology Information
NTC	Non-template control
OD	Optical density
OFC	Oral food challenge
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
RDT	Recombinant DNA technology
Real-time qRT-PCR	Real-time quantitative reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
S. olivacea	*Scylla olivacea*
S. paramamosain	*Scylla paramamosain*
S. serrata	*Scylla serrata*
S. tranquebarica	*Scylla tranquebarica*
S. oceanica	*Scylla oceanica*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIgE	Specific Immunoglobulin E
SPT	Skin prick test
Th	T helper
WHO	World Health Organization
18S rRNA	18S ribosomal RNA

12S rRNA	12S ribosomal RNA

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Shellfish are marine invertebrates with a shell that belong to the Phylum Mollusca (which includes clams, mussels, scallops, squids, abalones, and whelks) and the crustacean of Phylum Arthropoda (which includes crabs, prawns, lobsters, crayfishes, krills, mantis, sandhoppers, and pillbugs) (Ruethers et al., 2018). Crabs, shrimps, prawns, lobsters, and crayfishes are among the 52,000 crustacean species known worldwide, with the majority of edible species belonging to the Order Decapoda (Ghafor, 2020). With over 807,0000 tons of harvest in 2018, crab is listed as the second most commercialized decapod species after shrimp (Food and Agricultural Organization [FAO], 2020a).

The mud crab *Scylla* spp. or locally known as “Ketam Nipah” is a swimming crab found in brackish and coastal mangrove areas. Four species of mud crabs have been discovered namely *Scylla serrata* (*S. serrata*), *Scylla olivacea* (*S. olivacea*),

Scylla tranquebarica (*S. tranquebarica*), and *Scylla paramamosain* (*S. paramamosain*) (Keenan, Davie, & Mann, 1998). In Malaysia, there were *S. olivacea*, *S. tranquebarica,* and *S. paramamosain* that are commonly found in Kedah, Perak, Melaka, Terengganu, Sarawak, and Sabah (Nurul Ain, Noor Amalia, Muhammad Ali, Julian, & Seok-Kian, 2019; Haidr, Rosmilah, Som, & Alsailawi, 2018). The distribution of *S. serrata* species, on the other hand, is least reported locally based on a single study conducted in 1997, 2017, and 2020, respectively (Fazhan et al., 2020; Fazhan et al., 2017; Takeharu, 2001). Mud crab identity has been investigated in many countries including Malaysia (Ruhana & Durrah Syazwani, 2019), Bangladesh (Parvin, Islam, Hoq, & Alam, 2018; Rouf, Shahriar, Sarower, & Ahsan, 2016), Thailand (Jirapunpipat, Aungtonya, & Watanabe, 2008), Japan (Ogawa, Hamasaki, Dan, Obata, & Kitada, 2012), and India (Prasanthi & Ramesh, 2019; Devi & Joseph, 2015), resulting in different species distribution throughout the countries.

Mud crab allergy has been studied in several countries including Malaysia (Hasan, Rosmilah, Keong, & Haider, 2019; Mustafa et al., 2018; Nurul Izzah, Rosmilah, & Zailatul Hani, 2015; Rosmilah et al., 2015), China (Hu et al., 2017; Fei et al., 2016), and Australia (Crewe et al., 2016). Overall, *S. serrata, S. tranquebarica*, and *S. paramamosain* have been discovered as a cause of mud crab allergy due to the presence of several allergens while allergy to *S. olivacea* has yet not been investigated. Protein allergens present in mud crab species include tropomyosin (Hasan et al., 2019; Liu et al., 2018; Mustafa et al., 2018; Yu et al., 2011), arginine kinase (Yang et al., 2015; Mao et al., 2013; Yu et al., 2013), triosephosphate isomerase (Xia et al., 2019), sarcoplasmic calcium-binding protein (Hu et al., 2017), myosin light chain (Li et al., 2019; Abramovitch, Lopata, O'Hehir, & Rolland, 2017), filamin C (He et al., 2020), and actin (Mustafa et al., 2018), with tropomyosin is the dominant allergen being found.

Tropomyosin, a regulatory protein in crab muscle tissue, has emerged as a major allergen triggering a spectrum of allergic reactions, from rhinitis to anaphylactic shock (Lee et al., 2017; Farah Dayana, 2011). The demand for high-quality seed production in the industry has led to increased scrutiny of mud crab biology, particularly in areas such as the reproductive system (Muhd-Farouk et al., 2019). Consequently, there has been a rise in real-time qRT-PCR studies aimed at elucidating the expression patterns of functional genes during normal body development processes in mud crab species. Given its pivotal role in muscle function, tropomyosin levels significantly outweigh those of other known allergens and exhibit variation across different body parts of the crab (Kamath et al., 2013; Motoyama et al., 2007). Notably, the abdomen, cheliped, and walking legs contain edible meat, which is responsible for triggering allergic symptoms. However, despite its importance, the transcriptional level of the tropomyosin gene in these edible muscle parts remains largely unexplored, particularly in male mud crabs. This knowledge gap underscores the need for assessing tropomyosin gene expression in various tissue sources to accurately estimate the allergenicity levels in mud crab tissues (Wang et al., 2018).

Tropomyosin, recognized as a pan-allergen across various invertebrate species including crustaceans, mollusks, mites, and parasites, poses a challenge in distinguishing between allergenic and non-allergenic variants due to the lack of distinct isoform classes (Asnoussi et al., 2017; Kamath et al., 2013; Jeong et al., 2004; Ayuso et al., 2002). This variability in DNA sequences encoding tropomyosin leads to diverse protein structures, offering a unique avenue for the development of targeted immunotherapy against specific organisms (James et al., 2018; Gunning et al., 2005). To overcome the limitations associated with natural extracts in allergy diagnostics, there has been a notable shift towards recombinant allergen technology.

Recombinant allergens present a contemporary intervention providing a pure and well-defined alternative to natural extracts in diagnostic allergy tests (Smoldovskaya et al., 2016). Abundant recombinant tropomyosin proteins have been successfully produced from various crustacean species such as the crucifix crab (Cha f 1), blue swimming crab (Por p 1), and Chinese mitten crab (Eri s 1), exhibiting known molecular weights, stability, and immunoreactivity comparable to their natural counterparts (Abramovitch et al., 2013; Liang et al., 2008; Leung et al., 1998). This study seeks to bridge the existing research gap by producing recombinant tropomyosin genes and proteins from local mud crab species *like S. olivacea*, *S. tranquebarica* and *S. paramamosain*, thereby contributing to a more comprehensive understanding of shellfish allergenicity and enhancing diagnostic strategies for individuals prone to allergies.

Advances in allergen research, aided by computer technology and bioinformatic tools, have deepened our understanding of allergen properties and trimmed experimental costs (Yang et al., 2016). Analyzing amino acid sequences is pivotal in examining tropomyosin, predicting its stability and cross-reactivity across species (Asnoussi et al., 2017; Shafique et al., 2013; Chu et al., 2000; Motoyama et al., 2008). Allergens, primarily proteins, initiate allergic reactions by interacting with immune cells through various pathways (Mak & Saunders, 2006). However, the specific properties and physicochemical traits of tropomyosin from local mud crab species remain unexplored. While cross-reactivity, wherein similar allergens trigger immune responses, is evident in tropomyosin, its extent in mud crab species is uncertain (Jeong et al., 2006; Lopata, Kleine-Tebbe, & Kamath, 2016). Simulations of protein interactions can illuminate how tropomyosin interacts with IgE antibodies, providing insights into its allergenic properties. Hence, a portion of this chapter aims to

utilize bioinformatic analysis to forecast the molecular and allergenic aspects of tropomyosin from local mud crab species.

1.2 Problem statement

The problem statement revolves around the assessment of the potential of mud to cause allergic reactions, which can be determined by examining the expression levels of the tropomyosin gene. Past research has primarily focused on detecting tropomyosin, yet there is a notable gap in quantifying its levels (Wang et al., 2018; Shekhar, Kiruthika, & Ponniah, 2013). Tropomyosin, being a muscle protein, holds significance in this context. The specific issue lies in the lack of knowledge regarding the levels of tropomyosin in the edible body parts (abdomen, cheliped, and leg) of local mud crab species. The proposed research solution involves utilizing real-time qRT-PCR to measure the relative expression of tropomyosin, but a crucial requirement is the identification of a stable reference gene for normalization purposes (Bustin et al., 2009). Among the potential reference genes evaluated, including 18S rRNA, Myosin, GADPH, and EF1α, the most stable one will be selected. The overarching research objective is to determine the tropomyosin gene expression level in local mud crab species, thereby addressing the existing gap in knowledge and contributing to a better understanding of the allergenic potential associated with mud crab consumption.

The second research problem identified revolves around the introduction of recombinant tropomyosin allergen as a potential substitute for natural extracts in diagnostic tests. While natural extracts have limitations, recombinant allergens offer advantages such as control over production, consistent composition, standardized dosing, and minimized variability content (Curin, Garib, & Valenta, 2017; Larsen,

Broge, & Jacobi, 2016). However, there is a problem concerning the lack of studies focusing on recombinant tropomyosin as a replacement for natural extracts. Tropomyosin, a muscle protein, is of particular interest in this context. The specific problem lies in the absence of complete sequence information for tropomyosin gene and protein constructs from local mud crab species such as *S. olivacea*, *S. tranquebarica* and *S. paramamosain*. The proposed research solution involves cDNA cloning and subsequent expression of the recombinant tropomyosin gene in *E. coli.* The primary research objectives are to clone the recombinant tropomyosin gene and express the recombinant tropomyosin protein from local mud crab species, aiming to address the current lack of sequence information and pave the way for utilizing recombinant tropomyosin in diagnostic tests as an alternative to natural extracts.

The third research problem arises from a lack of comprehensive understanding concerning the allergenic properties of recombinant tropomyosin proteins derived from local mud crab species. Despite their designed similarity to natural proteins, there is a notable gap in knowledge regarding key aspects such as IgE-reactivity, stability, cross-reactivity, and molecular determinants, which are crucial factors in assessing allergenic potential (Nugraha et al., 2019; Hoh & Swaminathan, 2017; Smoldovskaya et al., 2016; Chen, Yang, Wei, & Tao, 2014). The identified gap underscores the limited exploration of crucial factors such as IgE-reactivity, stability, cross-reactivity, and molecular determinants associated with these proteins. To address this problem, the research solution proposes a two-fold approach involving IgE-binding tests and bioinformatic analysis. This includes investigating physiochemical properties, structural characteristics, evolutionary relationships, IgE-binding epitopes and protein-protein docking. By conducting these analyses, the research aims to elucidate the IgE-binding properties and predict the molecular and allergenic determinants of recombinant tropomyosin proteins from local mud crab species.

1.3 Research objectives

The objectives of this study are:

1. To determine the tropomyosin gene expression level in local mud crab species.

2. To determine the sequence of recombinant tropomyosin gene from local mud crab species.

3. To express the recombinant tropomyosin protein from local mud crab species.

4. To characterize the IgE-binding properties, and prediction of molecular and allergenic determinants of recombinant tropomyosin protein from local mud crab species.

1.4 Research questions

1. What is the tropomyosin gene expression level in local mud crab species?

2. What is the product of the recombinant tropomyosin gene from local mud crab species?

3. What is the product of recombinant tropomyosin protein from local mud crab species?

4. What are the IgE-binding properties of recombinant tropomyosin protein, and the predicted molecular and allergenic determinants of recombinant tropomyosin protein from local mud crab species?

1.5 Significance of research

Measurement of the level of tropomyosin expression helps to postulate the rate of allergenicity which would educate the community about the precautions of which mud crab species and which body parts are harmful. From a clinical standpoint, data can be helpful as a dietitian reference in treating allergic mud crab patients. While that, choosing the suitable real-time qRT-PCR reference gene(s) will help produce a more precise and accurate expression of tropomyosin or other genes under normal physiological conditions in local mud crab species.

The complete tropomyosin sequence obtained serves as a preliminary step and reference gene for predicting allergic tropomyosin determinants and producing recombinant tropomyosin protein in mud crab species. Furthermore, the existence of a complete tropomyosin sequence from all local mud crab species indicates the presence of nucleotide and amino acid polymorphism, which is a criterion used to distinguish the allergenicity of tropomyosin between all the local species of mud crab at the molecular level.

Purified recombinant tropomyosin is being produced for allergic-specific immunotherapy and is the ideal candidate for rational and reliable allergy diagnosis. Many clinical caused-tropomyosin can be developed as pure recombinant allergens that mimic their natural allergen's epitope properties using recombinant DNA

technology. With the existence of pure recombinant allergens, allergenic properties of individual allergens, including IgE-binding properties, can be characterized, and the fundamental mechanisms of mud crab allergy production can be understood.

Functional recombinant tropomyosin can be used as a source in desensitization treatments that only require a single specific allergen rather than several allergens. Efficient manufacturing of functional recombinant tropomyosin may also assist the development of a hypoallergenic tropomyosin vaccine specific to the species of mud crab. Furthermore, bioinformatic analysis is the easiest and quickest way to know the possible cause of allergy by comparing tropomyosin cross-reactivity with other known species. All expected allergenic properties of tropomyosin, including physicochemical properties and structure composition help, develop and generate functional recombinant tropomyosin protein. Besides, future interventions can avoid extreme allergy reactions by reducing the number of allergens for cross-reactivity as a result of amino acid homology analysis, phylogenetic tree analysis, and IgE-epitope prediction.

1.6 Scope and limitations of research

The study primarily focused on mud crab species found in specific local waters in Kedah, Terengganu, and Sabah, focusing mainly on adult male specimens to ensure consistency and control over variables. This focus enables a thorough investigation into the expression of tropomyosin across three key body parts under normal physiological conditions, aiming to enhance comprehension of its role. The decision to employ the DNA sequence of *S. serrata* for producing recombinant tropomyosin is likely influenced by its accessibility and similarity to local mud crab species. Moreover,

the inclusion of a histidine-tagged fusion protein aids in the purification and identification process during protein production. The limitation of allergenicity assessment to a small sample size of 20 sera from crab-allergic patients is attributed to practical challenges in obtaining suitable samples. Additionally, the comprehensive analysis of molecular and allergenic properties of tropomyosin, encompassing physicochemical characterization, structural prediction, epitope mapping, phylogenetic analysis, and protein-protein docking analysis, aims to offer a thorough understanding of its characteristics and interactions, demonstrating the research's methodological thoroughness and scientific objectives.