KWONG XINN XIAN

MASTER OF SCIENCE

2025

EFFECT OF SERINE PROTEASE CATALYSIS AND EXPRESSION LEVEL OF SERINE PROTEASE INHIBITOR GENES IN REGULATING COAGULATION OF TACHYPLEUS AMEBOCYTE LYSATE

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KWONG XINN XIAN

Thesis Submitted in Fulfilment of the Requirement for the
Master of Science
In the Institute of Climate Adaptation and Marine Biotechnology
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2025

DEDICATION

Dedicated this thesis to:

My supervisor, Professor Noraznawati Ismail.

My beloved parents and siblings.

For all their dedication, sacrifice and endless love.

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu infulfilment of the requirement for the degree of Master of Science

EFFECT OF SERINE PROTEASE CATALYSIS AND EXPRESSION LEVEL OF SERINE PROTEASE INHIBITOR GENES IN REGULATING COAGULATION OF *TACHYPLEUS* AMEBOCYTE LYSATE

KWONG XINN XIAN

2024

Main Supervisor: Professor Dr. Noraznawati Ismail, PhD

Co-Supervisor: Dr. Fisal Ahmad

School/ Institute: Institute of Climate Adaptation and Marine Biotechnology

Horseshoe crabs as living fossils, have a unique immune system that is widely used in enzyme research. However, the effect of serine protease catalysis and its inhibitor in regulating coagulation under the induction of lipopolysaccharide is still unclear. Therefore, the current study aims to determine the effect of divalent cation and pH of buffer on the degree of gelation and proteolytic activity of trypsin-like serine protease in wild and captive of horseshoe crab blood, to compare the profiling of the TAL protein, to identify the target protein after protein profiling and to determine expression levels of serine protease inhibitor (SERPIN) genes in horseshoe crab blood during lipopolysaccharide (LPS) induction. In this study, the gel clotting method was used to determine the degree of gelation of TAL, SDS-PAGE for protein profiling, Orbital Nano LC-MS/MS for identifying target protein, and RT-PCR to determine expression levels of SERPIN genes. The current study showed that wild horseshoe crab lysate (June-1, July-1 and August-1) exhibited higher clotting levels in 1 EU/mL, 5 EU/mL, and 10 EU/mL control standard endotoxin concentration, while for the captive lysate (June-2, July-2, and August-2) showed low clotting levels in all endotoxin concentration. Among 35 different combinations of divalent cation concentration, the divalent cation of 0.15 M $MgCl_2$ + 0.15 M $CaCl_2$ had higher clotting levels for the wild batch TAL (June-1, July-1 and August-1) compared to the captive batch of TAL. In addition, the batch of TAL collected from wild horseshoe crab blood showed high proteolytic activity, and the LC-MS data of the trypsin-digested sample showed seven major proteins were detected in TAL. The expression level of SERPIN gene from July batch blood reach 5.17-fold in relative SERPIN mRNA expression with LP primer and significant increase five times compare with Jun batch which was 1 relative expression. In conclusion, wild batches of TAL in different divalent cation concentration values gave significant changes in the clotting process, which is associated with higher protein levels indicating of effect the quality of TAL decrease in captivity.

Abstrak tesis yang dikemukakan kepada Senat Universiti
Malaysia Terengganu sebagai memenuhi keperluan untuk
Ijazah Sarjana Sains

KESAN PEMANGKINAN SERINE PROTEASE DAN ARAS EKSPRESI GEN PERENCAT SERINE PROTEASE DALAM MENGAWAL PENGGUMPALAN LISAT *TACHYPLEUS* AMEOBOSIT

KWONG XINN XIAN
2024

Penyelia Utama: Professor Dr. Noraznawati Ismail, PhD

Penyelia Bersama: Dr. Fisal Ahmad

Sekolah/ Institut: Institut Adaptasi Iklim dan Bioteknologi Marin

Belangkas merupakan sejenis fosil hidup yang mempunyai sistem imun yang unik dan digunakan secara meluas di dalam kajian enzim. Walau bagaimanapun, kesan katalisis serine protease dan perencatnya dalam mengatur penggumpalan dibawah aruhan lipopolisakarida masih belum difahami. Oleh itu, kajian ini adalah untuk menentukan kesan divalen kation dan pH penampan kepada darjah pembentukan gel dan aktiviti proteolitik tripsin serine protease daripada darah belangkas liar dan terkurung, untuk membandingkan profil protein TAL, untuk pencirian protein sasaran selepas profil protein, dan untuk menentukan aras pengekspresan gen aruhan serine protease (SERPIN) dalam darah belangkas semasa aruhan lipopolisakarida (LPS). Dalam kajian ini, kaedah penggumpalan gel digunakan untuk menentukan darjah penggumpalan dalam TAL, SDS-PAGE pula digunakan untuk memprofil protein, Orbitrap Nano LC-MS/MS digunakan untuk menentukan sasaran protein dan RT-PCR digunakan untuk menentukan darjah ekspresi gen (SERPIN). Kajian ini telah menunjukkan lisat belangkas liar (Jun-1, Julai-1 dan Ogos-1) mempunyai darjah penggumpalan yang tinggi dalam aras kepekatan kawalan piawai endotoksin 1 EU/mL, 5 EU/mL dan 10 EU/mL, sementara itu, lisat belangkas terkurung (Jun-2, Julai-2 dan Ogos-2) pula menunjukkan darjah penggumpalan yang rendah dalam semua kepekatan lipopolysakarida. Sebanyak 35 kepekatan divalent kation, 0.15 M $MgCl_2$ +0.15 M $CaCl_2$ menunjukkan darjah penggumpalan yang tinggi bagi lisat belangkas liar (Jun-1, Julai-1 dan Ogos-01) berbanding dengan belangkas di dalam kurungan. Bukan itu sahaja, lisat daripada Jun-1, Julai-1 dan Ogos-1 menunjuk aktiviti protein yang tinggi dan keputusan LC-MS/MS telah menunjukkan tujuh protein utama di dalam TAL. Aras pengekspresian gen SERPIN bagi bulan Julai mencapai kadar 5.17 lebih tinggi sebanyak lima kali ganda berbanding dengan aras ekspresi bulan Jun iaitu 1 kali relatif dengan LP primer. Kesimpulannya, TAL dari belangkas liar dengan nilai kepekatan divalen kation berbeza memberi perubahan yang jelas di dalam proses penggumpalan yang berkaitan dengan aras protein tinggi. Ini menunjukkan kesan kurungan memberi penurunan dari segi kualiti TAL.

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Firstly, I would like to express my sincere gratitude to my supervisor Prof. Noraznawati binti Ismail for the continuous support of my master study, for her patience, encouragement, kindness and immense knowledge. I could not have imagined having a better supervisor for my master project. Along the journey from proposal presentation, writing proposal, progress presentation, laboratory work, until the last, thesis writing, she gave me advice, knowledge, and the encouragement to achieve the perfection for my master study. Besides, my co-supervisor, Dr. Fisal bin Haji Ahmad gave supports to me for this project. It is a pleasure to have supportive supervisors. Next, Dr. Noor Azlina Kamaruding guided me a lot to achieve my objective in my study. Here, I am appreciative and grateful to have them as my supervisors in my master project. I would also like to thank Horseshoe Crab Research Group members. They were willing to share their knowledge and provide suggestions to me. On the other hand, Dr. Ahmad Khusairi Azemi is the one of the person that I would like to thank as he had provide suggestions on my manuscript writing. Last but not least, I would like to thank my parents, Kwong Mee Chong and Nyu Mei Ching for supporting my spiritually through writing this thesis and my life in general.

APPROVALS

I certify that an Examination Committee has met on 20th September 2020 to conduct the final examination of Kwong Xinn Xian, on his Master of Science thesisentitled "**Effect Of Serine Protease Catalysis And Expression Level Of Serine Protease Inhibitor Genes In Regulating CoagulationOf *Tachypleus* Amebocyte Lysate**" in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Commiteee are as follows:

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Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotation and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.

_____

KWONG XINN XIAN

Date:

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LIST OF ABBREVIATION

$CaCl_2$	Calcium Chloride
CSE	Control Standard Endotoxin
LAL	Limulus Amebocyte Lysate
LC-MS/MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PFW	Pyrogen Free Water
rpm	Revolutions Per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SERPIN	Serine Protease Inhibitor
SDS-PAGE	Sodium Dodecyl-sulphate Polyacrylamide Gel Electrophoresis
TAL	*Tachypleus* Amebocyte Lysate

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Horseshoe crabs have many unique characteristics that had attracted researchers in the biotechnology field. The horseshoe crab is an ancient creature that presents from the upper Jurassic period and nearly unchanged for 200 million years (John et al., 2018). Today, the horseshoe crab contains four exist species which are *Carcinoscorpius rotundicauda, Tachypleus gigas*, and *T. tridentatus* which can be found in Asia, and *Limulus polyphemus* located in North America. However, the population of horseshoe crabs declined sharply when humans discovered the unique characteristic of blood. Since horseshoe crabs have been integral to the safe production of vaccines and injectable medication, the bleeding of live horseshoe crabs had led to more than a thousand deaths in the population (Maloney et al., 2018; Krisfalusi-Gannon et al., 2018). The covid-19 vaccine also increased the usage of the *Limulus* amebocyte lysate (LAL) test for clinical surgery (Gorman, 2020). Other than medication, several factors cause the horseshoe crab population to decline. *L.polyphemus* was released after hemocyte extraction but another two *Tachypleus* species were commonly used for chitin extraction, thus extraction caused deadly to horseshoe crabs (Vestbo et al., 2018). In China, horseshoe crab was also one of the foods in their meal. According to Fu et al. (2019), nearly half of northern Beibu Gulf, the coastal area consumed 10 meals per year. The farmers and fishers had a highly significant number of horseshoe crab meals.The pollution effect on seawater may affect the general health status of the horseshoe crab. The heavy metal that contained in pollutants will cause a significant negative correlation between amebocyte viability and the ratio of granule cell concentration (Kwan et al., 2018). Climate change might

harm the horseshoe crab population. The rising sea level may cause breeding habitats to decrease and be closer to the human infrastructure on the coast (Mat Zauki et al., 2019). Lastly, trash piles on the riverbank and beach had caused the habitat no longer suitable for survival and nesting (Meilana & Fang, 2020).

When the horseshoe crab was maintained in captivity its health rate was affected by multifaceted threats. The horseshoe crab maintained in captivity was more easily to being infected by disease when no water circulation in the captive (Sheikh et al., 2019). The infection of microorganisms such as *Gamma-proteobacteria*, *Rhodobacteraceae*, and *Flavobacteraceae* were increased the abundance on captive horseshoe crab shell surface and gill (Friel et al., 2020). The maintenance of horseshoe crabs in captivity will affect their health when without proper management.

The decline of the horseshoe crab population caused the Ecology Research & Development Group and IUCN Horseshoe Crab Species Survival Commission active in the conservation of horseshoe crab species (John et al., 2018). The restoration of the beach has helped the resurgence of horseshoe crabs (Mat Zauki et al., 2019). On the other hand, public awareness of conservation management must be increased to conserve and preserve all endangered species not only for horseshoe crabs (Meilana & Fang, 2020). Ecology niche modeling is a tool that helps a lot in conservation activities by predicting the response function, contribution of the environment variable, and the potential geographical range of the species (Hu et al., 2020).

The loss of a suitable living habitat had caused the horseshoe crab population to survive harder. The health of horseshoe crabs will be affected when surviving most of the time in bad environments. The biological activity in horseshoe crabs was low during unhealthy condition (Botton et al., 2022). Thus, the coagulation in horseshoe crab blood helps them to survive under pathogen infection.

The coagulation activity induced by coagulation factors in hemocytes were Factor C, Factor B, clotting enzyme, and coagulin (Kobayashi et al., 2015). There weretwo types of coagulation activation which are lipopolysaccharide (LPS) from bacteriaendotoxin and beta-glucan from fungi. The different invaders had different activatedmechanisms. For LPS, the activation of coagulation starts with Factor C after the detection of LPS (Dolejš & Vaňousová, 2015). The presence of beta-glucan activates Factor G to start the clotting effect in hemocytes. After the activation of Factor C or Factor G, the clotting activity started, and more clotting-related enzyme was secreted by the hemocyte to complete the clotting process (Das et al., 2015).

The clotting enzymes involved in coagulation activity in horseshoe crabs were classified under serine protease (Yamashita et al., 2020). When the horseshoe crab had an open wound, the serine protease such as Factor C and Factor B was secreted to form clotting to prevent hemocyte loss. The secretion of the clotting-related enzyme was caused by the expression of the immune response gene. Serine protease was important in immune response, pro-phenoloxidase activation, antimicrobial peptides, and coagulation in invertebrates (Wei et al., 2019). The expression of the serine protease and serine protease inhibitor gene in invertebrates helps the organism to regulate biological activity. After clotting was formed, the expression of serine protease inhibitor for clotting related enzyme increased to inhibit coagulation. The pro-phenoloxidase activation was used to activate the melanization of the pathogen and damage body tissue (Jeyachandran et al., 2020). For antimicrobial peptides, the big defensin, Tachyplesin and Tachycitin was released by hemocytes to help the regulation of coagulation (Wang et al., 2022). The antimicrobial peptides will inhibit the growth of bacteria in hemocyte.

Serine Protease Inhibitors (SERPIN) are needed to control the formation of clotting. The SERPIN was secreted to inhibit clotting enzyme when clotting was completely formed (Coban et al., 2022). In invertebrates, there were four different types of serine protease inhibitors were found which are Kazal, Kunitz, alpha-macroglobulin, and serpin family (Kanost, 1999; Ituarte et al., 2019). The regulation of coagulation was important to maintain the normal life of horseshoe crabs. After the digestion of the pathogen, more hemocyte was needed to replace the loss during pathogen invasion. The more nutrient was consumed for hematopoietic and replaced body tissue, then the secretion of serpin will back to normal.

In horseshoe crabs, lipopolysaccharide-induced exocytosis of granular hemocytes is a key component of innate immunity. Coagulation activity was affected by several factors. The enzyme involved in the coagulation effect was classified under serine protease. Thus, the concentration of serine protease inhibitor might affect the clotting activity. In this study, the expression level of the serine protease inhibitor gene before and after lipopolysaccharide induction will be assessed.

1.2 Problem statement

The sensitivity *Tachypleus* Amebocyte lysate reduce in captivity without a proper management especially when they are susceptible to microbial contamination. The activity of serine proteases and their inhibitor different between in wild and captive horseshoe crab blood. The clotting-related enzyme activity in captivity and wild horseshoe crab blood in regulating coagulation was still unclear. The relationship between horseshoe crab under captivity and wild collection for serine protease inhibitor gene in regulating coagulation and its protein profiling are still unknown.

1.3 Significance of the study

Enzyme is biological catalysis that plays an important role in industrial production. Many enzymes cannot be used in research and production due to time consumption, low production yield and sensitivity. In the medical field, the high sensitivity of enzymes may help a lot in the detection of endotoxin in a patient blood sample (Ma et al., 2017). The disease that enhances by bacteria can be determine in alimited of time. In this study, a wild horseshoe crab was maintained in captivity after first blood extraction. After one month of captivity, the horseshoe crab was considered as captive. This research was determined the expression level of SERPIN from wild and captive horseshoe crabs and its protein profiling after LPS induction.

1.4 Objectives

The objectives of this study are:

1. To determine the effect of divalent cation and pH of buffer on degree of gelation and proteolytic activity of trypsin-like serine protease of TAL.
2. To profile and identify TAL protein before and after LPS induction using SDS-PAGE.
3. To determine the expression level of serine protease inhibitors genes in amebocyte during multiple LPS induction.

CHAPTER 2

LITERATURE REVIEW

2.1 Horseshoe crab

The horseshoe crab is an organism that lives in marine and brackish water. The horseshoe crab was classified under Merostomata and was more closely related to Arachnidae than Crustacea (Figure 2.1). Today, four species of horseshoe crabs are distributed geographically distinctive in the world. *Limulus polyphemus* is known as an American horseshoe crab mainly found along the east coast of North America, USA and the coastline of the Yucatán Peninsula, Mexico (Smith et al., 2017). *Carcinoscropius rotundicauda*, *Tachypleus tridentatus*, and *T. gigas* are mainly distributed in Southeast Asia (Vestbo et al., 2018). The life cycle of the horseshoe crab is short compared with the mammal. The horseshoe crab was able to breed after they are 10 years old (Luo et al., 2020). Once horseshoe crabs reach breeding age, they will come to shore to make nest in a huge group. Currently, the population of horseshoe crabs is reported in declining by International Union for Conservation of Nature (IUCN) (Kwan et al., 2016; Smith et al., 2017). The sharp decrease in the horseshoe crab population is mainly caused by human activity and environmental factors. According to Smith et al. (2017), the erosion of estuarine shorelines caused by human activity resulted in a decline in the horseshoe crab population due to the sandy beach becoming an enclave. The environmental factors such as coastal change, shoreline erosion, and increasing sea level had caused the horseshoe crab to lose their suitable habitat (Smith et al., 2017). The oxygen levels, salinity, temperature beach geochemistry, and wave energy are the ecological disturbances that become more lethal to the development success of horseshoe crab eggs and larvae and reduce adult horseshoe crabs to spawn offshore (Funch et al., 2016).

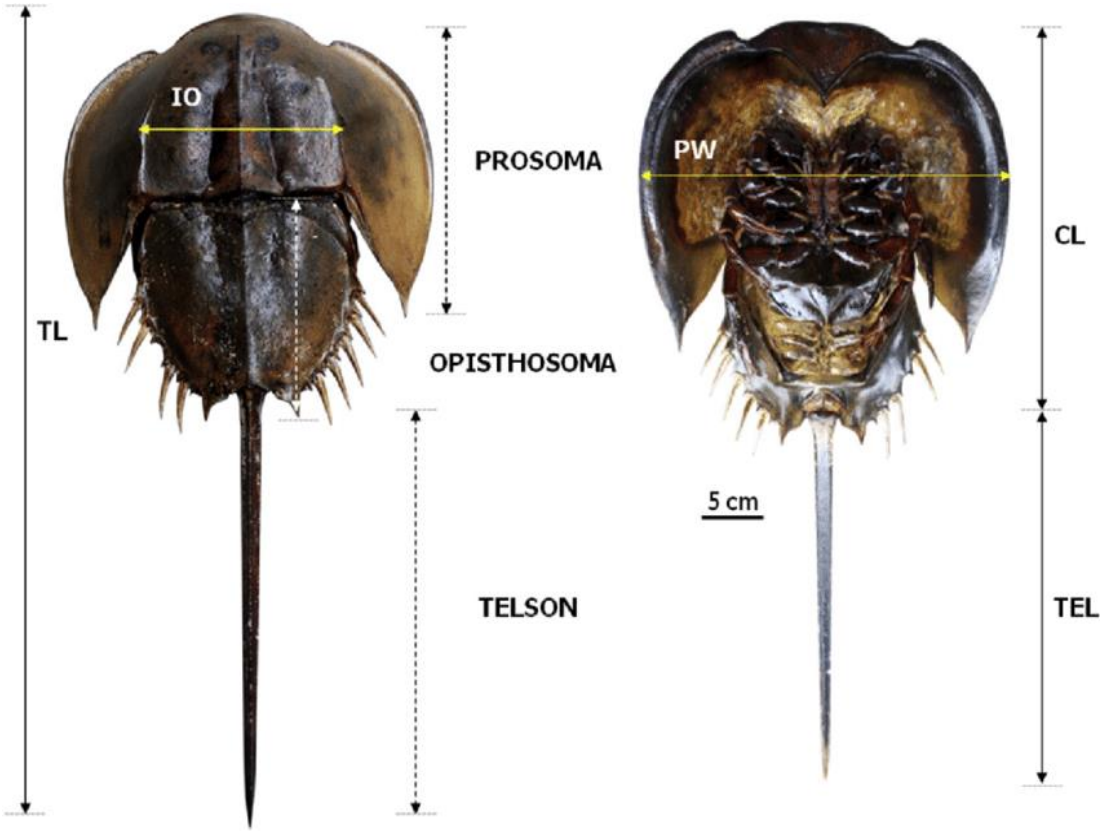

Figure 2.1: Horseshoe crab *Tachypleus* sp. CL: carapace length; IO: Intraocular distance; PW: prosomal width; TEL: telson length; TL: total length (Manca et al., 2017).

2.2 Bioactivity from horseshoe crab

The horseshoe crab was an organism that attracted researchers for bioactivity study. There were some bioactivities that found from horseshoe crab which are antimicrobial, anti-inflammatory, antitumor, and antioxidant.

2.2.1 Antimicrobial and antiviral activity

The antimicrobial activity that occurred in horseshoe crab was done by the presence of different antimicrobial peptide. The new antimicrobial peptide polyhemusin III was found from horseshoe crab *L. polyphemus* (Marggraf et al., 2018). The polyphemusin III was classified as Beta-hairpin cationic antimicrobial peptide andcan isolated from the hemocytes of horseshoe crab. Tachylesin I found in the small granules of horseshoe crab hemocyte that can bind with bacteria endotoxin also considered as antimicrobial peptide (Kushibiki et al., 2014). The presence of different types of antimicrobial peptide helps the invertebrates to fight against the bacterial infection. For antiviral activity, the tachyplesin-1 also involved in virus infection (Xie

et al., 2016). The infection of marine virus may be detected by antiviral peptide in horseshoe crab.

2.2.2 Anti-inflammatory activity

The chitosan extracted from horseshoe crab was used to prepare non-steroidal anti-inflammatory drug (Balde et al., 2020). The nonsteroidal anti-inflammatory drug was widely used in medicinal drug to reduce inflammatory activity in patients. The kynurenic acid that extracted from the horseshoe crab tail was reported to have anti-inflammatory and antioxidative (Li et al., 2021). The alkaloid was a group of compounds that can be obtained from natural product of invertebrate organisms had showed anti-inflammatory activity (Senthilkumar & Kim, 2013).

2.2.3 Antitumor

The antitumor activity was induced by the antitumor peptide. An antitumor peptide named CIGB-552 was an antitumor second-generation peptide that used in cancer therapy (Oliva Arguelles et al., 2020). It was one of the proteins derived from horseshoe crab *L. polyphemus*. On the other hand, tachyplesin-1 had a potential to become an antitumor agent (Vernen et al., 2019). The backbone cyclized tachyplesin-1 that synthesis from tachyplesin-1 showed more active in antitumor activity.

2.2.4 Antioxidant

The chitin was the second most abundant biopolymer in bio-industry. The chitosan that extracted from the horseshoe crab had showed to have antioxidant characteristic (Pati et al., 2020). The lipopolysaccharide had enhanced the antioxidant activity in invertebrates (Liu et al., 2021).

2.3 Immune system of horseshoe crab

Horseshoe crab had survived more than 200 million years, thus researchers called them living fossils. This ancient marine arthropod may survive for millions of years and may benefit from its innate immune system (Zhou et al., 2020). When a foreign body and pathogen infected horseshoe crab through an open wound, the immune system of the horsehsoe crab activated and stimulated coagulation to stop the activity of infection (Levin, 2019). This immune response is activated by a type of cell

called amebocyte or hemocyte that move freely in the hemolymph of the horseshoe crab. Hemocyte or amebocyte can be defined as a single-type cell that consists of two type of granular cell which are large and small (Figure 2.2) (Sheikh et al., 2015).

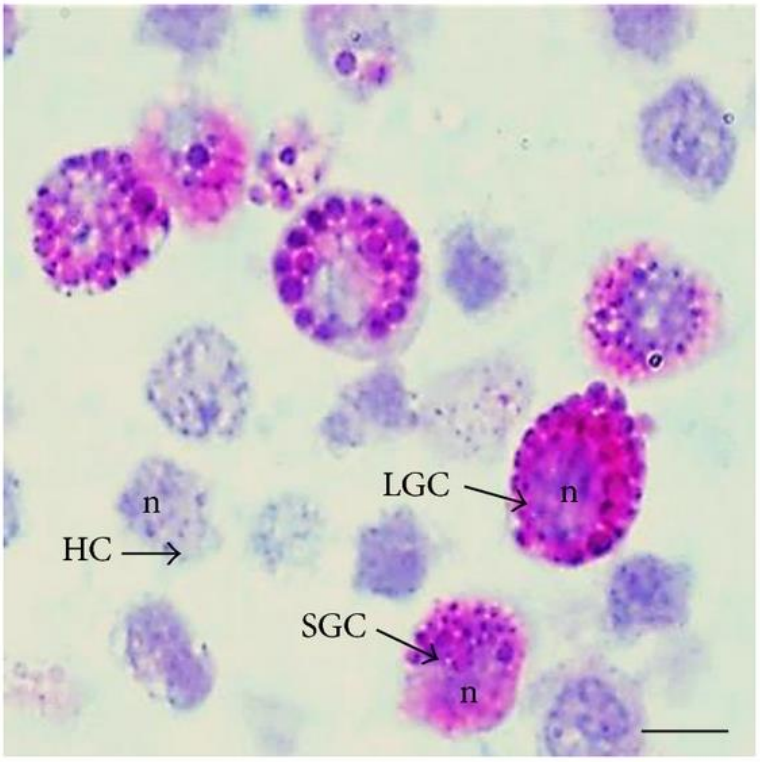

Figure 2.2: Hemocyte of invertebrate. LGC: large granular cells; SGC: small granular cells; HC: hyaline cell; n: nucleus (Senthilkumar & Kim, 2013).

In granular detected bacteria endotoxin, the clotting factor will be released. Factor B, Factor C, Factor G, and the pro-clotting enzyme are the coagulation factors found in amebocytes (Sheikh et al., 2015). The mechanism of coagulation begins with the binding of bacteria lipopolysaccharide and zymogen Factor C (Figure 2.3). For fungi, the binding of β-1,3-D-glucan with Factor G activates the coagulation process. Next, the active Factor C or Factor G will activate Factor B. Finally, blood coagulation occurred after Factor B had converted the pro-clotting enzyme to a clotting enzyme. Activation of coagulation-related protein initiates the proteolytic conversion of soluble coagulate to insoluble coagulin. The clot is subsequently stabilized by transglutaminase activity. These protein-protein cross-linking enzymes are critical interactions between coagulation and the innate immune defense as these enzymes immobilize bacteria on the clot surface. Nevertheless, the complement immune response will activate by the complement factor contained in hemolymph when microorganisms had invaded horseshoe crabs through the open wound (Sekiguchi &

Nonaka, 2015). Moreover, the prophenol oxidase in horseshoe crab blood will help in reduce the activity of microorganisms and minimize the damage caused by the invader (Wei et al., 2019). This coagulation characteristic had caused horseshoe crabblood widely used in bacteria endotoxin tests (Leskelä et al., 2020).

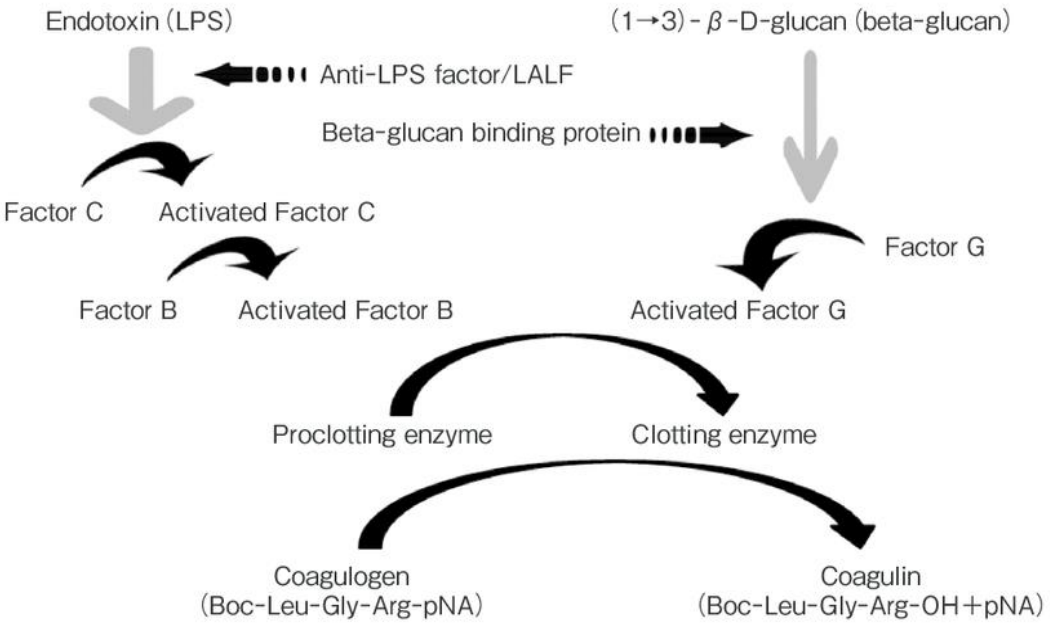

Figure 2.3: Activation of coagulation immune response in horseshoe crab (Tamura, Reich, & Nagaoka, 2016).

Horseshoe crabs have a unique immune system that helps them to survive. The horseshoe crab blood will clot when the presence of lipopolysaccharide (LPS). Lipopolysaccharide is the major component in the outer membrane of Gram-negative bacteria composed of lipid A, O antigen, and core (Giordano et al., 2020).

2.4 Serine protease and serine protease inhibitor in amebocyte

Serine protease are enzymes that cleave peptide bonds in the protein serine serves as nucleophilic amino acid at the active site and is found in both eukaryotic and prokaryotic cells. Serine proteases play an important role in physiological activities such as digestion, blood coagulation, fibrinolysis, and the immunity system (Veillard et al., 2016). The activation of trypsin-like serine proteases begins with the proteolytic activity of an inactive zymogen precursor. The activity of serine proteases depends on several factors such as temperature substrate concentration, pH, divalent cation concentration, and serine protease inhibitor (Silva et al., 2018). Serine protease

inhibitors regulated host serine protease cascade upon pathogen invasion or mechanical injury. The enzymes involved in blood coagulation were classified under the serine protease family (Maloney et al., 2018).

The expression of serine protease inhibitor (SERPIN) from SERPIN genes in amebocyte cells helps the horseshoe crab to enhance the coagulation cascade in blood. Compared to other arthropods, horseshoe carb had a large number of multi-domain serine protease inhibitors such as Kazal type serine protease inhibitor, Kunitz serine protease inhibitor, and α-macroglobulin (Blisnick et al., 2017). Three serine protease inhibitors have been characterized in *T. tridentatus,* in which *Limulus* Intracellular Coagulation Inhibitors 1,2, and 3 (LICI-1, LICI-2, and LICI-3) function as inhibition of the clotting enzymes (Gustchina Elenaand Williams, 2019). The different types of *Limulus* Intracellular Coagulation Inhibitors had different targets. For LICI-1, which was sensitive to Factor C, LICI-2 inhibited most of the clotting enzymes, and LICI-3 for Factor G. α2 macroglobulin was released to stop the activity of prophenol oxidase by secretory granules of amebocyte cells (Ponprateep et al., 2017). The serine protease inhibitor affects immunosuppression and anticoagulation, which are valuable in biotechnology development (Sheikh et al., 2021).

However, the clotting efficiency in horseshoe crab blood is affected by several factors such as pH, a divalent cation, and temperature. The clotting efficiency of horseshoe crab blood from captivity is different if compared to fresh horseshoe crab blood. The enzyme that plays important role in blood clotting was affected by an unknown reason for horseshoe crab that maintain in captivity. The concentration of amebocyte in horseshoe crabs blood effect clotting efficiency toward endotoxin. The amebocyte cell quality was located in different results in the wild and captivity in some studies (Sheikh et al., 2021).

2.5 Factor affecting serine protease catalysis

The activity of serine protease was affected by several factors, such as environmental pH, divalent cations, temperature, and inhibitors.

2.5.1 Environmental pH

The activity of serine protease like *Limulus* Factor C may affected by surrounding pH value. The protease activity increase when reaching its optimum pH

value. According to Salwan et al. (2020), 55 different types of isolated serine protease had a rise its proteolytic activity up to pH10 at 5 °C. In pH 9 condition, the serine protease will denature (Kim et al., 2018). The active site of serine protease changed and the substrate could not bind thus low biological catalysis activity occurred.

2.5.2 Divalent cation

The divalent cation play an important role in biological catalysis. The activation of serine protease by divalent cations had been proved by (Mukhtar, 2016). The calcium and magnesium ion can activate serine protease especially for cation-dependent serine protease. The conformation changes of enzymes occur when divalent cations bind to the catalytic site (Scavenius et al., 2016). The binding of divalent cations to an enzyme causes the enzyme activity to increase. On the other hand, the divalent cations not only activated the serine protease but also inhibited the serine protease. For some serine proteases, the proteolytic activity was inhibited by divalent cation. As a calcium-dependent serine protease, anthrax protective antigen was inhibited by the presence of zinc ions (Storm et al., 2018). Thus, different types of serine protease will have different divalent cation binding sites.

2.5.3 Temperature

The enzyme will denature when expose to extreme temperatures. The serine protease will unfold at high temperatures and lose its function. The unfolding of serine protease from psychrophilic marine bacteria *Vibrio* sp. was tested in high temperatures such as 37 °C, 100 °C, 200 °C and 300 °C by using a molecular dynamic simulator (Du et al., 2017). The serine protease unfolded faster at high temperatures compared to low temperatures. The stability of serine protease was depends on the interaction between amino acids such as hydrogen bonds, covalent bonding, ionic bonding, and hydrophobic interaction (Silva et al., 2018). The stability of enzymes was a big question in the biological industry. To maintain the stability of enzymes some methods have been discovered such as nanoparticle-encapsulation, polymer stabilization, crosslinking, phosphorylation, and glycosylation (Zdarta et al., 2018).

2.5.4 Anticoagulant

The coagulation activity stimulated by amebocyte may inhibit by an anticoagulant. N-ethylmaleimide and phenylmethylsulfonyl are the reagents that are

mostly used by reaserchers to study the anticoagulant effect on *Limulus* amebocyte (Romadhon et al., 2018). The anticoagulant will inhibit the activity of fibrin and reduce the coagulation effect. Some microorganism that has an anticoagulant as invade mechanism will inhibit the coagulant immune response (Strobel & Johswich, 2018). The presence of an anticoagulant in the LAL test will affect the sensitivity of amebocytes toward endotoxin.

2.5.5 Seasonal and sex different

The hemocyanin level in horseshoe crab blood was different between males and females (Owings et al., 2020). The hemocyanin level in male horseshoe crab was higher than female horseshoe crab. According to Owings et al. (2020), the hemocyanin level showed lowestin the spring season and early summer but showed highest in late summer and fall. During the summer season, the seawater temperature increase caused a decrease in oxygen affinity. The metabolism process of horseshoe crabs increases by surrounding temperature and leads to low hemocyanin concentration. From May to October, the hemocyanin level in female horseshoe crab decrease because of egg laying (Tinker-Kulberg et al., 2020). The egg-laying consumed large energy and nutrient, thus less hemocyanin was produced.

2.5.6 Heavy metal concentrations

The increased concentration of heavy metals will decrease the hemolymph composition pattern. The present of heavy metal will decrease the biological activity of horseshoe crab and caused death (Burger, 2023). The amebocyte viability and ratio of granular-spherical to granular-flattened and degranulated dendritic-like morphological states of amebocytesshow negative results in nitrite and lead heavy metal concentration (Maloney et al., 2018). The food that feeds horseshoe crabs also may affect the concentration of heavy metal in horseshoe crab blood. Different food and water quality will affect the qualityof amebocytes (Tinker-Kulberg et al., 2020).

2.6 Gene expression on immune response

In invertebrate, horseshoe crabs had a particular gene that take part in immune response activity. The lack of adaptive immune system in invertebrates, the immune response of invertebrates mostly in the form of peptide and some serine protein. The

immune response related protein such as serine proteases and its inhibitor, antimicrobial peptide, and endotoxin binding protein were expressed regarding to immune related gene (Nair et al., 2005). The expresses of serine protein for immune response such as clotting factor was important for horseshoe crab to protect itself from bacterial infection.

The serine protease and serine protease inhibitors play an important role in regulating coagulation. In horseshoe crab, there were three different serine protease inhibitor gene that help in regulating coagulation which were *Limulus* Intracellular Coagulation Inhibitor 1, 2 and 3 (LICI-1, LICI-2 and LICI-3) (Lemaite, 1997). Each inhibitors had different target, LICI-1 inhibited clotting factor C, LICI-2 for clotting enzyme and LICI-3 inhibited clotting factor G. Thus, the expression level of the serine protease gene and serine protease inhibitor gene may affect the clotting level in horseshoe crab blood.

CHAPTER 3

METHODOLOGY

3.1 Experimental design

The experimental design of the study on the effect of serine protease catalysis and expression level of serine protease inhibitor genes as shown in Figure 3.1.

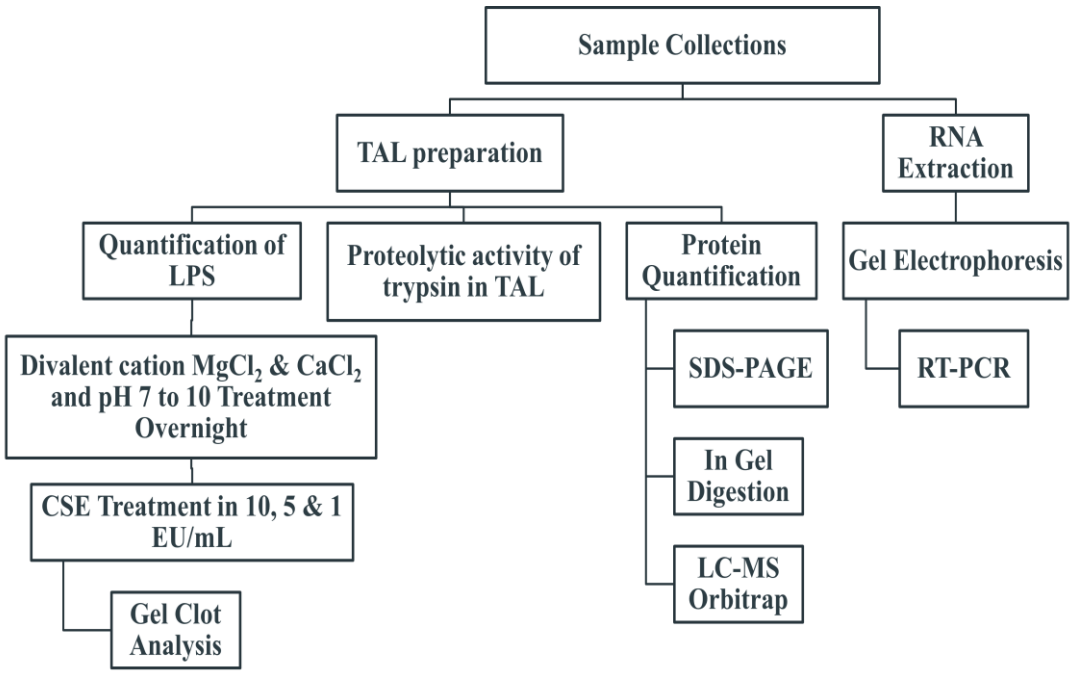

Figure 3.1: Experimental design of the study

The effect of serine protease catalysis and the expression level of serine protease inhibitor genes will be determined through a series of analysis.

3.2 Sample collection

Sample of horseshoe crabs T. gigas (Figure 2.1) were collected at Kuala Kemaman, Terengganu, Malaysia, which is located at 4º14' N 103º26' E. The samples of adult *T. gigas* were collected in three different batches of months, which are June, July and August 2020. We have designed the experiment whereby every batch will have at least 30 individuals per batch. The following months will be 30 individuals plus 30 from previous months. The frozen cockles were given hand-fed every two days and the water was replaced once per week to ensure the horseshoe crab had the same living environment. The quality of water was controlled in a suitable condition such as watertemperature, salinity, ammonium, nitrite, nitrate level, and pH value. The blood of horseshoe crabs was collected once a month. The horseshoe crabs were maintained in captivity after blood extraction. After one month of captivity, the horseshoe crabs per batch were considered as captive. The horseshoe crabs were maintained in captivity according to batch.

3.3 Blood extraction

The blood was extracted individually from the horseshoe crabs according to their body weight. The volume of blood for extraction was in the form of mL per gram. Upon collection of hemolymphs, the horseshoe crabs first were sterilized by wiping them with a piece of cotton swab in 70% ethanol. Less than 10 % body weight of horseshoe crab blood was extracted and kept in a 50 mL falcon tube. For TAL preparation, the blood was extracted following the patent described by (Noraznawati et al., 2014). The blood samples extracted from horseshoe crab were centrifuged at 750 rpm under 4 °C for 15 minutes. The centrifugation was using Centrifuge 5804 R cooler. The supernatant, the blue color solution which is plasma was transferred into a sterilized empty bottle without disturbing the pellet for future use. Next, the pellet lysate was washed by using sterilized 3 % NaCl three times with 1:3 ratios to the lysate (lysate: 3% NaCl). After that, the lysate was centrifuged at 1500 rpm for 15 minutes under 4 °C, and the supernatant was removed carefully without disturbing the pellet. Then, Pyrogen-Free water (PFW) (Associates of Cape Cod., USA) was used to wash the lysate two times.The lysate was pooled together in one flacon tube and crushed into small pieces by using a sterilized spatula. PFW was added with the ratio of 3:1 of the lysate (PFW: lysate) to dissolve the cell pellet. Next, the lysate was sonicated at

high speed at 4 °C for 15 minutes. The sonicated mixtures were incubated under 4 °C for overnight for the cell to lysis. After lysed, the lysate was centrifuged at 3000 rpm for 15 minutes at 4 °C. The supernatant was filtered by using a syringe filter and pipetted into a sterilizedvial with a rubber cap. The lysate was kept at -80 °C for overnight before being freeze-dried purpose by using a tabletop freeze dryer (Labconco, USA) under vacuum conditions.

3.4 Determination the effect of the divalent cation and pH of buffer on the degree of gelation and proteolytic activity of trypsin-like serine protease in *Tachypleus* amebocyte lysate

The determination the effect of divalent cations and pH of buffer on *Tachypleus* Amebocyte Lysate (TAL) sensitivity toward lipopolysaccharide (LPS) induction by using the gel clot method to achieve the first objective for this study. The gel clot method is a qualitative measurement in the bacterial endotoxin test.

3.4.1 Quantification of lipopolysaccharide

The qualitative of LPS was done using the gel clot method (LAL pyrotell, Cape-Cod, USA). The TAL from different batches was dissolved by using Pyrogen-Free water (PFW). After the TAL had completely dissolved, 100 µL of TAL was pipetted into pyrotube that contain 100 µL of control standard endotoxin (CSE). The CSE was prepared in 100 EU/mL, 50 EU/mL, 10 EU/mL, 5 EU/mL, 1 EU/mL, 0.5 EU/mL, 0.1 EU/mL, 0.05 EU/mL, and 0.01 EU/mL using PFW. The *Limulus* Amebocyte Lysate (LAL) stimulated with CSE was acting as positive control and PFW acted as a negative control. The pyrotubes were incubated at 37 ºC for one hour. The degree of gelation firmness will be determined following Jorgensen & Smith (1973).

3.4.2 Sensitivity of TAL

The gel clot is a qualitative method used to determine the sensitivity of TAL in this study, following method described by the manufacturer (LAL pyrotell, Cape- Cod, USA). Before being treated with divalent cations, the TAL was dissolved by using PFW according to the lysate volume before in sub-chapter 3.3. The PFW was rinsed on the inner surface of the vial to conform to the fully dissolve TAL. After the TAL had completely dissolved in PFW, the TAL was treated with divalent cation $MgCl_2$, and

$CaCl_2$ at different molarities which is 0.05 M, 0.10 M, 0.15 M, 0.20 M, and 0.25 M. The sensitivity of TAL was determined by using the gel-clot method (LAL pyrotell, Cape Cod, USA). The Control Standard Endotoxin (CSE) was prepared in 10 EU/mL, 5 EU/mL, and 1 EU/mL concentration. The 100 µLof the sample was pipetted into pyrotube and the stimulated with 100 µL of CSE at therespective concentration. LAL stimulated with CSE was prepared as positive control while LAL with PFW was a negative control. After that, all the pyrotube with test samples were vortexed for 1 minute. The samples were incubated in a water bath under37 °C for 1 hour. The degree of gelation firmness was determined following Jorgensen & Smith (1973) as Table 3.1 below:

Table 3.1 The gelation firmness in the clotting level of TAL

1:	Very weak gel with slight opacity and with some starch-like floccules adhering to the side of tubes
2:	Weak gel with slight to moderate opacity and adhesion of starch-like floccules
3:	Soft gel with moderate to considerable opacity
4:	Firm gel with considerable opacity
-ve	No visible increase in viscosity or opacity (free-flowing liquid)

3.4.3 Proteolytic activity of TAL

The determination of proteolytic activity in TAL was the second part of the first objective of this study. Azocasein had play an important role in the determination of enzyme activity especially in protease (Tamura et al., 2016). In this study, the proteolytic activity of trypsin was performed as the following method described by Tomarelli et al. (1949). The 2.5 % of azocasein (Sigma-Aldrich, USA) was reconstituted with 0.5 % of sodium bicarbonate buffer at pH 8.3. Then, 200 µL of homogenous solution of azocasein was added into a set of samples and blank. After that, sodium bicarbonate was pipetted into each set of samples at the volume 150 µL and blank for 250 µL. The 100 µL of the TAL was pipetted into the centrifuge tube. Next, the homogenous mixture of the sample and azocasein was incubated at 37 °C for 5 minutes. Trypsin was act as the positive control.

The 100 µL of trypsin, sample, and blank was pipetted into a new sterilized microcentrifuge tube and incubated in a water bath at 37 °C for 30 minutes. After incubation, 400 µL of trichloroacetic acid was pipetted into each microcentrifuge tube that contained trypsin, sample, and blank. The incubation was repeated at the same temperature for three minutes after adding trichloroacetic acid. Next, the microcentrifuge tubes with trypsin, sample, and blank were centrifuged at 8000 g at 4 °C for five minutes. After centrifuging, 100 µL of supernatant from each set of sample and blank was transferred into a new microcentrifuge tube that contained 300 µLof 0.5 M sodium hydroxide. The microcentrifuge tubes were mixed well before proceeding to the next step. The samples and blank were pipetted on a 96-well plate at volume of 250 µL. The absorbance of the samples and blank was measured by a plate reader at 440 nm wavelength. The enzyme activity of the sample will be determined by using the formula (Buarque et al., 2009).

Enzyme Activity (U/ml/min) = (A 440 nm test- A 440 nm blank)/ 0.001

3.5 Comparison of protein profile in *Tachypleus* amebocyte lysate before and after lipopolysaccharide induction

The protein profiles of TAL were compared before and after LPS induction as the second objective. To archive this objective, the SDS polyacrylamide Gel Electrophoresis (PAGE) (Bio-rad, USA) was used. Bradford Assay (Sigma-Aldrich, USA) was used to determine the concentration of protein in TAL. The protocol of Bradford assay was according to the manufacturer's guideline. The Bovine Serum Albumin (BSA) as standard was diluted by using PFW at different concentrations (0.175 mg/mL, 0.35 mg/mL, 0.7 mg/mL, and 1.4 mg/mL) to construct a standard curve. The LAL was act as control. The TAL and LAL were diluted for 2x, 5x, and 10x. After that, 5 µL of standard, LAL, and TAL were pipetted on a 96-well plate and 250 µL of Bradford reagent was slowly pipetted into the well to prevent leaky and bubble forming. The plate was then covered with aluminium foil and incubated at room temperature for 30 minutes. The absorbance of the TAL was determined at 595 nm wavelength by using a Gen5 microplate reader.

For protein profiling, the TAL will be subjected to SDS-PAGE. The method of SDS-PAGE was followed as manufacturer's protocols (Bio-Rad, CA, USA). The SDS-

PAGE gel used in protein profiling was 20% Mini-Protean TGX precast gel (Bio-rad, CA, USA, Catalog# 4561093). The protein concentration of TAL was standardized before diluted with sample buffer. The lysate was diluted with sample buffer at a ratio of 1:1. 25mM of Tris-glycine buffer with 1% SDS was act as running buffer. The sample buffer was prepared in 62.5 mM Tris-HCL with 2% of SDS, 25% of glycerol, 0.01% of bromophenol blue, and 5% of ß-mercaptoethanol. The 20 µL of lysate-sample buffer waspipetted into the well and run under 100 V for 45 minutes. Next, the gel was stained with Coomassie Brilliant Blue for at least two hours and re-staining with distilled water for 24 hours. The gels were cut and analyzed by using Orbital Nano LC-MS analysis.The samples were analyzed using Orbitrap Nano LC-MS/MS analysis and performed on a nanoflow HPLC system connected to a mass spectrometer equipped with an electrospray ionization source.

3.6 Determination of the expression levels of serine protease inhibitor genes in *Tachypleus* amebocyte lysate during multiple lipopolysaccharide induction

The RNA from horseshoe crab blood was extracted by using PureZOL RNA reagent (Bio-rad, USA) and three replicates for each sample. The method of RNA extracted was followed as the PureZOL RNA isolation reagent kit manufacturer's guideline (Bio-rad, USA, Catalog# 732-6890). One mL of PureZOL Reagent per 100 mg of the cell was added into the sample and homogenized forone minute. Then, the samples were centrifuged at 12000 g for 10 minutes at 4 °C to remove fat, polysaccharide, protein, or extracellular material and incubated for five minutes at room temperature. The Eppendorf Centrifuge MiniSpin was used for centrigation. The supernatant was then transferred into a new two mL microcentrifuge tube. After that, 200 µL of chloroform was pipetted into the sample-PureZOL mixed and shaken vigorously for 15 seconds. After incubation of 5 minutes at room temperature, the samples were centrifuged at 12000 g for 15 minutes at 4 °C. After centrifugation, the mixture was separated into three phases. It was as an upper, colorless aqueous phase, white interphase, and a lower, red organic phase. The upper phase colorless aqueous was then transferred into a new centrifuge tube. Next, 500 µLof isopropanol was pipetted into the sample for the cell to lysis and homogenized. After five minutes of incubation, the sample was centrifuged for 10 minutes at 12000 g at 4 °C.

The supernatant was discarded and the pellet was resuspended by using one mL of 75 % ethanol and vortex before centrifuging at 7500 g at 4 °C for 5 minutes. The supernatant was discarded and the pellet was dried for five minutes. The RNA was dissolved by using 30 µL of RNase-free water. The RNA was incubated at 55 °C to ensure the RNA had fully dissolved. After RNA extraction, the concentration of RNA was determined by Nano-drop UV-Vis Spectrophotometer and run gel electrophoresis with 1 % agarose gel. The transcript levels in the clot and control samples were measured by using iTaq universal STBR Green one-step kit (Bio-rad, USA) method. The first strand of cDNA was synthesized from 2 µg of RNA by primers and reverse primers. The primers were designed based on selected genes in differentially expressed proteins by manufacture (IDT DNA, USA) (Table 3.2).

The *Limulus* Intracellular Coagulation Inhibitor 1 and 2 (LICI-1 & LICI-2) can be found in *Tachypleus* sp. For internal control of qRT-PCR, the forward and reverse primersof 18S rRNA Beta actin were chosen. The master mixes of PCR for each gene were prepared according to the kit. The final volume of the qRT-PCR reaction for one tube is 20 µL. 20 µL of the qRT-PCR solution contains 0.5 µM of each forward and reverse primer, 10 µL of iTaq universal SYBR green reaction mix, 0.25 µL of iScript reverse transcriptase, 0.5 µL of RNA from each sample, and 8.25 µL of nuclease-free water. The RT-PCR reaction was performed using a 10 min 50 °C reverse transcription reaction, 1 min 95 °C denaturation, 30 sec 72 °C annealing, and the plate read. After the PCR reaction, the gel electrophoresis proceeded with 1 % of agarose gel and visualized under UV with SYBR safe.

Table 3.2 The primer set was used to determine the expression level of Serine Protease Inhibitor (SERPIN) gene.

Primer Name		Sequence (5'-3')
TICI-1	Forward	GGG TCT TGG TCA CTA TTC CTA AG
	Reverse	TTT GCG TTG GCT TCA CTA AAC
TICI-2	Forward	TGA GTC TGA CGA ACA AGT TTC C
	Reverse	CCC ATT CCG GTA GGA TGA ATA TC
LP	Forward	AAC TGG GCG TCT ATG TTG TAG
	Reverse	TGA GTG CGT TCT CTA CTT TCT C

3.7 Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). The different between the expression level of the SERPIN genes were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical significance was defined when the *p*-value was less than 0.05.

CHAPTER 4

RESULTS

4.1 Samples collection

The sample were collected at Kuala Kemaman, Terengganu. The number of collected horseshoe crab was recorded. During June 2020, there were total 37 *T. gigas* had been collected while for July 38 of *T.gigas* were collected. For August batch, 35 horseshoe crabs were collected. In the June batch, there were 21 *T. gigas* was males and 16 females. For July, there were 23 female and 15 male of *T.gigas* were collcted. Of 35 horseshoe crabs in August, there were 19 males and 16 females (Table 4.1). The horseshoe crabs were maintained in captivity after first time blood extraction.

Table 4.1 Sample collection

Months	male	Female
June	21	16
July	23	15
August	19	16
Total		110

4.2 Quantification of lipopolysaccharide induction

The quantification of lipopolysaccharide (LPS) was done by using the gel clot method of LAL assay. The sensitivity of *Tachyplues* amebocyte lysate (TAL) and

Limulus amebocyte lysate (LAL) were determined by different concentrations of controlstandard endotoxin (CSE).

In three different CSE concentrations, all samples had the highest clotting level in 10 EU/mL while the lowest in 1 EU/mL. Overall, the June-01 and August-01 of TAL hadthe highest clotting level among other batches of TAL (Figure 4.1).

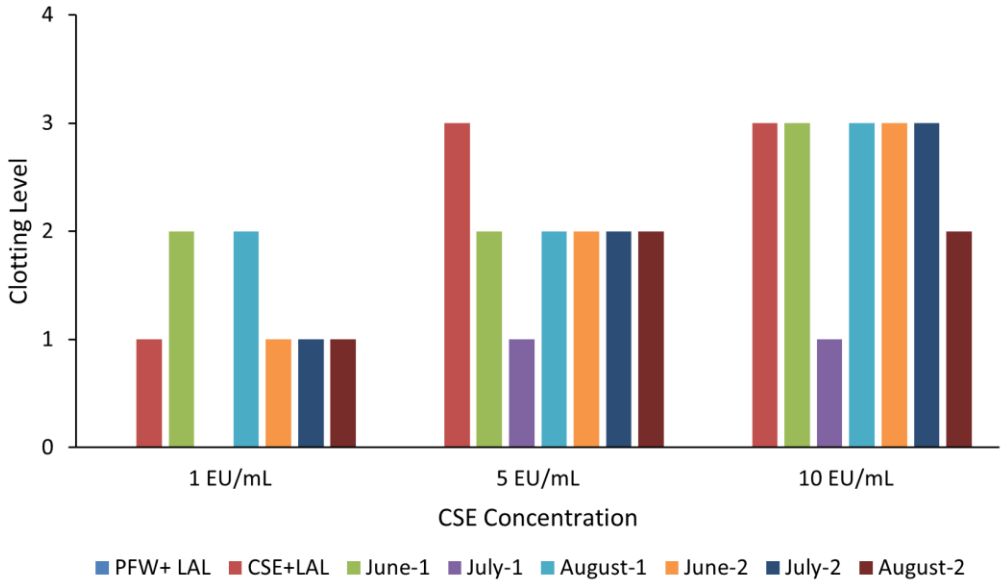

Figure 4.1: The clotting level of LAL and TAL in different concentrations of CSE; Blue: 10 EU/mL CSE; Orange: 5 EU/mL CSE and grey: 1 EU/mL CSE. CSE: control standard endotoxin.

4.3 Gel clot

The sensitivity of TAL from four different months toward CSE with different combinations of divalent cations was determined by using the gel clot method. The TAL showed a low clotting level when the CSE concentration reached 5 EU/ml. The results of sensitivity are represented in the form of clotting level (Figure 4.2).

Among 35 divalent cation concentration combinations, five different concentrations of divalent cation showed the highest clotting level among 35 different divalent cation combinations. The five concentrations are 0.05 M $MgCl_2$ +0.2 M $CaCl_2$, 0.1 M $MgCl_2$ +0.2 M $CaCl_2$, 0.15 M $MgCl_2$ +0.05 M $CaCl_2$, 0.15 M $MgCl_2$ +0.15 M $CaCl_2$ and 0.25 M $MgCl_2$ +0.05 M $CaCl_2$. TAL that was processed using from fresh blood showed the highest sensitivity toward the control standard endotoxin. The

Jun-01 means the first-time drawing blood showed level 3 clotting results in every five different concentrations of divalent cation combination. The second time drawing blood, Jun-02 showed the lowest clotting level under LPS induction (Figure 4.3). All batches of TAL showed more clotting level under 10 EU/mL LPS induction than 5 EU/mL LPS induction.

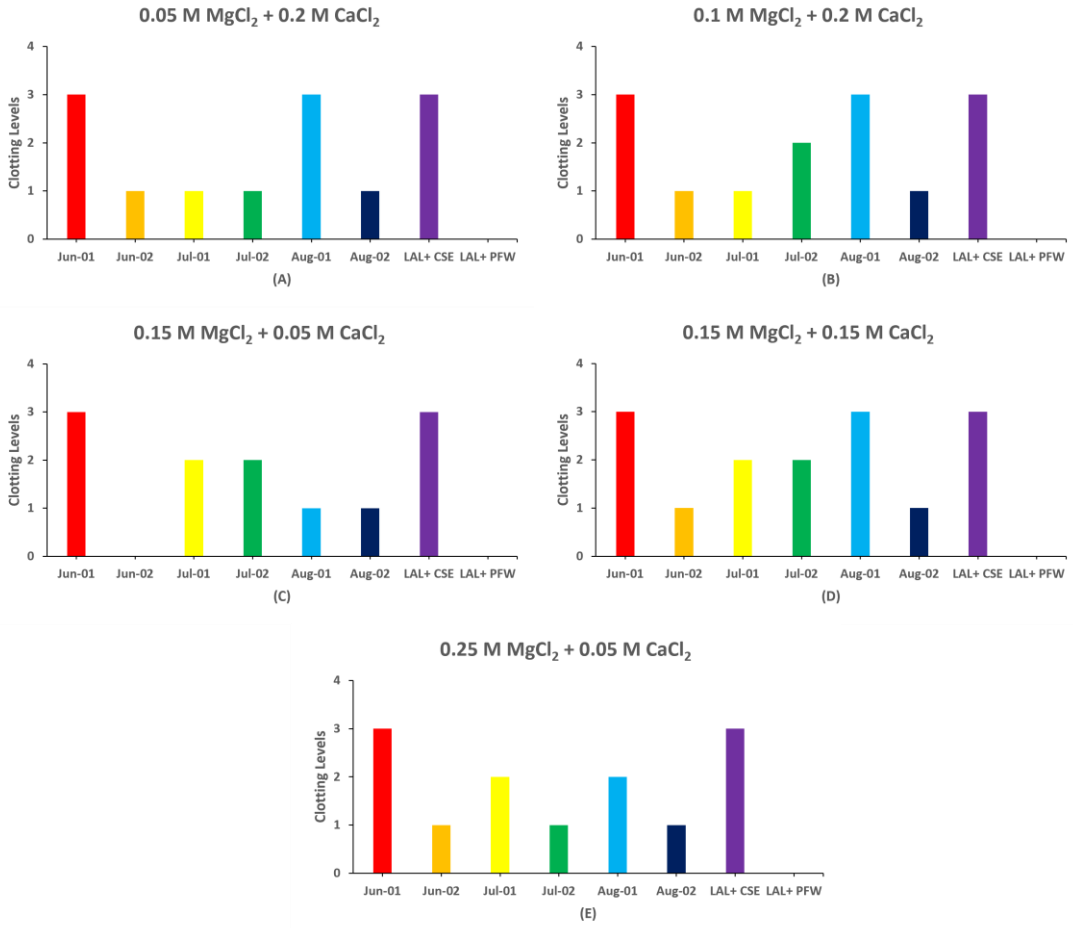

Figure 4.2: Clotting level of TAL at different concentrations of divalent cation combination in 5 EU/mL CSE concentration. June-01: June first blood draw; June-02: June second blood draw; Jul-01: July first blood draw; Jul-02: July second blood draw; Aug-01: August first blood draw; Aug-02: August second blood draw.

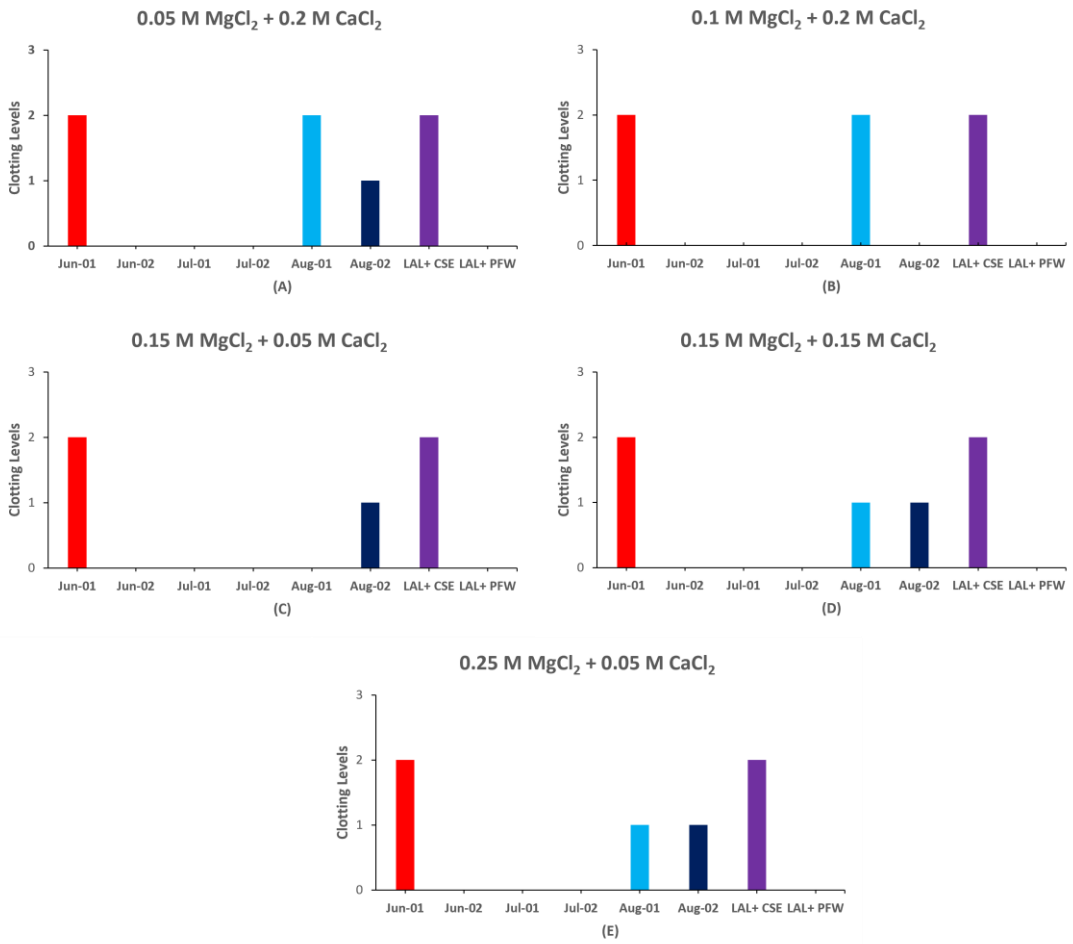

Figure 4.3: Clotting level of TAL on different concentrations of divalent cation combination in 1 EU/mL CSE concentration. Jun-01: Jun first batch; Jun-02: Jun second batch; Jul-01: July first batch; Jul-02: July second batch; Aug-01: August first batch; Aug-02: August second batch.

With the same divalent cation concentration, the Jun batch TAL had the highest clotting level compared to July and August batches. The clotting level of TAL is decreasing after the first-time blood withdrawal. In all TAL from Jun batches,the fresh bleed TAL which is Jun-01 was the highest clotting level and reached clotting level 2. The divalent cation that showed the best clotting level in all TAL batches 0.15 M $MgCl_2$ +0.15 M $MgCl_2$ combination. The next is 0.05 M $MgCl_2$ + 0.2 M $MgCl_2$ follow by 0.25 M $MgCl_2$ +0.05 M $CaCl_2$. In 6 batches of TAL, every first batch in different months showed the highest clotting level. The fresh horseshoe crab TAL has a better clotting level and is sensitive to CSE if compared to in-captivity horseshoe crab TAL. The TAL that was treated with 10 EU/mL of CSE showed more clotting level compared to 5 EU/mL.

4.3.1 Proteolytic activity of TAL

The proteolytic activity of TAL was determined by using azocasein assay.

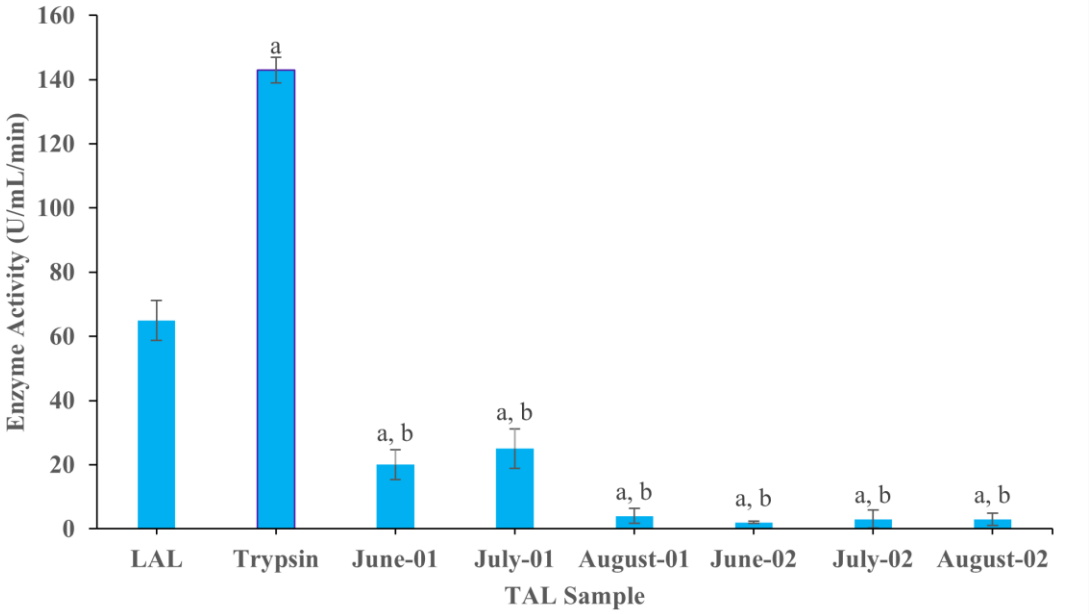

Figure 4.4: The proteolytic activity of amoebocytes from different batches. June-01: June first blood draw; June-02:June second blood draw; Jul-01: July first blood draw; Jul-02: July second blood draw;Aug-01: August first blood draw; Aug-02: August second blood draw. Data were presented as mean ± SEM (n = 3). $^{a}p < 0.05$ vs. LAL; $^{b}p < 0.05$ vs. Trypsin.

As a positive control, trypsin showed higher enzyme activity than LAL (Figure 4.4). The LAL showed the highest enzyme activity among the samples. The low proteolytic activity may result in low protein content in captivity of horseshoe crab amebocytes. The protein activity decreased when the horseshoe crab was maintained in captivity. There were no significant ($p > 0.05$) changes between the batches.

4.4 Protein profile of *Tachypleus* amebocyte lysate

The concentration of protein in TAL was determined by using the Bradford assay. Among all the batches of TAL, the June-2 batch had the highest protein concentration while June-1 reached the lowest protein concentration (Table 4.2).

Table 4.2 Protein concentration of TAL in June, July and August

Sample	Protein concentration (mg/ mL)
LAL (Control)	2.84 ± 0.11
June-1	0.67 ± 0.15
July-1	1.05 ± 0.27
August-1	1.77 ± 0.25
June-2	1.85 ± 0.14
July-2	1.61 ± 0.14
August-2	1.24 ± 0.09

The protein profile of TAL before and after LPS induction using SDS-PAGE to compare the protein in fresh and in captivity (Figure 4.5). The proteins in TAL from the different batches were profiled by using SDS-PAGE to compare the protein expression in captivity and the wild. Most of the sharp bands are present at 24 kDa size for all batches of the sample. The July-02 batch had a sharp band that was similar to August-01, however, that band was not present in another batch. As a control, LAL showed a sharp band in between 52 kDa and 76 kDa while the TAL sample would not be present. The TAL that was prepared from the first blood withdrawn from the horseshoe crab showed the sharper band in SDS-PAGE while the following batch showed faint. As result, July-02 showed more faint bands.

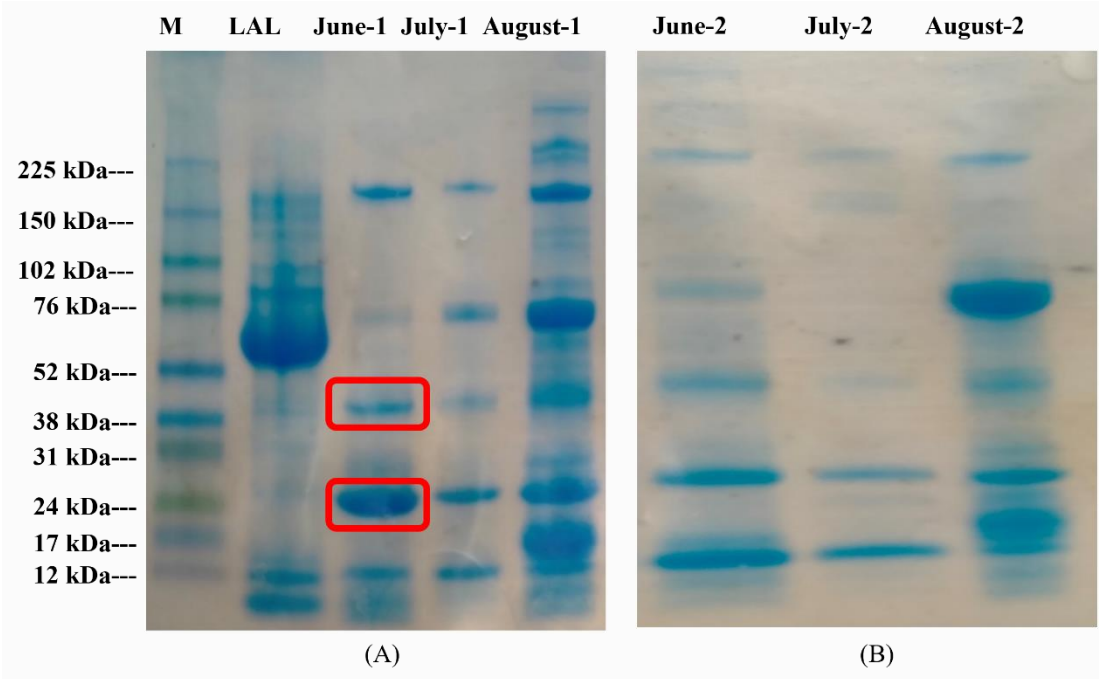

Figure 4.5: Protein profiles of TAL from different months. From left to right: Protein standard markers, LAL, June, July, and August. The LAL acted as a positive control.

M: Markers; LAL: *Limulus* amoebocyte lysate; June-1: June first blood draw; June-2: June second blood draw; July-01: July first blood draw; July-02: July second blood draw;August-01: August first blood draw; August-02: August second blood draw.

4.4.1. LC-MS sequencing

After running SDS-PAGE, the 38 kDa and 24kDa bands were cut and sent to Analytical Biochemistry Research Centre, USM for the next procedure. The 38 kDa band was present before LPS induction TAL but absent in after LPS induction TAL. The 24 kDa showed sharp band in before LPS induction but faint in after LPS induction. There were more than 20 different types of proteins that had been sequenced, however only a few proteins were related in regulating coagulation (Table 4.3).

Table 4.3 The LC-MS/MS orbitrap of TAL

	Protein ID	Protein Name	Size (Da)	Coverage (%)
1	Q27084	Tachylectin-2	28881	29
2	P00760	Cationic trypsinogen	25785	24
3	Q94JI5	Zinc finger CCCH	24982	4
4	P81475	Complement factor B-like protease	27248	4
5	Q9HQD5	Probable histidine ammonia-lysate	54073	2
6	P01024	Complement C3	187417	2
7	P12763	Alpha-2-HS glycoprotein	38419	1

4.5 Expression level of serine protease inhibitor gene

The expression level of the SERPIN gene in different months was determined using RT-PCR. The expression level of the SERPIN genes was showed different between fourmonths. By using *TICI-1* primer, the July batch horseshoe crab blood had

the highest expression among others (Figure 4.2). However, the August batch of blood showed the lowest expression level of SERPIN genes. There was no significant (p> 0.05) changed between the batch for *TICI-2* primer.

The Aug batch horseshoe crab blood showed the lowest expression level when using the *TICI-2* strand as a primer while July batch horseshoe crab blood had the highest expression level (Figure 4.7). There was significant (p< 0.05) changed between July and August for *TICI-2* primer.

The expression level of SERPIN increased continuously from Jun to July batch however decrease sharply at Aug batch for LP primer (Figure 4.8). The July batch showed the highest expression level compared to another batch. The August batch had the lowest SERPIN gene expression level. There were significant (p< 0.05) changed between July to June and July to August.

Overall, *TICI-1*, *TICI-2* and *LP* primer strands are more suitable to act as a primer in this study. The result showed a clear increase in the expression of SERPIN genes from Jun to July. The Aug had the lowest expression level of SERPIN gene in all different primer pairs.

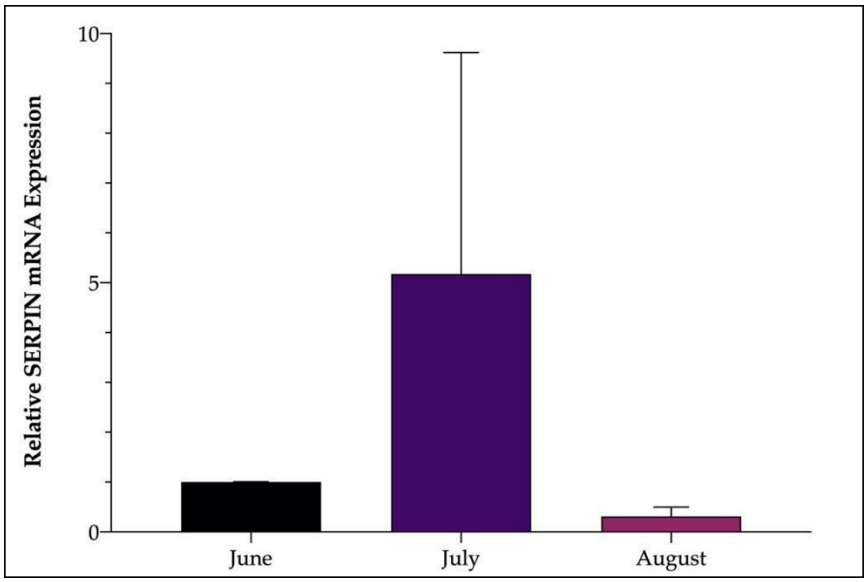

Figure 4.6: Expression level of SERPIN gene with *TICI-1* primer. Results were expressed as mean ± SEM (n=3). *p < 0.05.

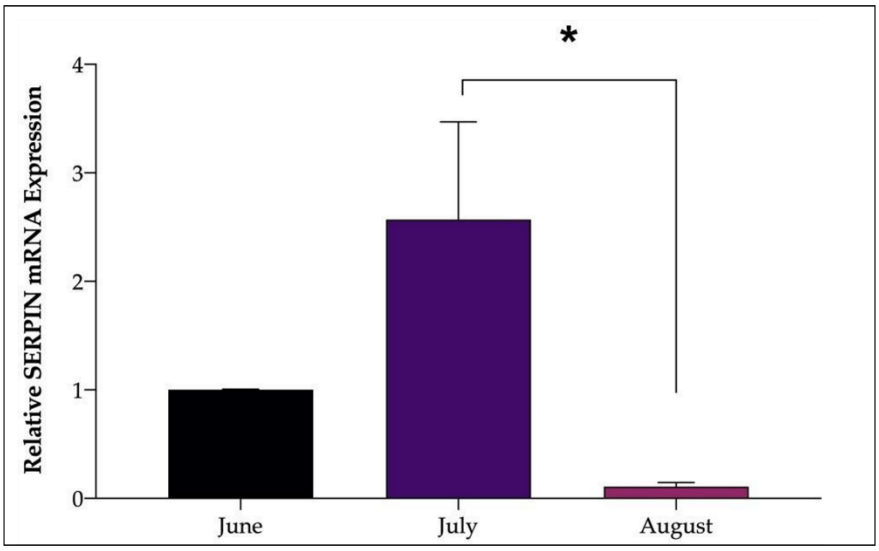

Figure 4.7: Expression level of SERPIN gene with *TICI-2* primer. Results were expressed as mean ± SEM (n=3). *p < 0.05.

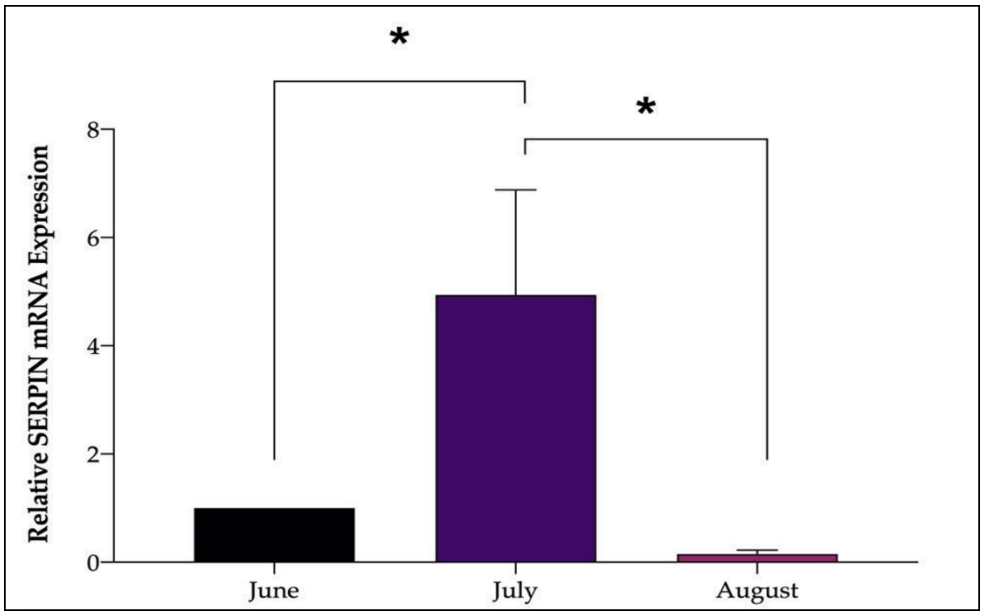

Figure 4.8: Expression level of SERPIN gene with *LP* primer. Results were expressed as mean ± SEM (n=3). *p < 0.05.

CHAPTER 5

DISCUSSION

5.1 Horseshoe crab population at Kuala Kemaman

The horseshoe crabs were collected at Kuala Kemaman and maintain in Hatchery UMT, Kuala Kemaman. The strategic location of Kuala Kemaman provided a suitable living environment for the horseshoe crab population that receives seawater inflow from the South China Sea and freshwater from the Kemaman River outflow. The occurrence of *T. gigas* was found easily during the mating season from Jun to August on the sandy beach. Kuala Kemaman also becomes a fishery landing site because of their strategic location. The collaboration with local fisheries and maintenance at a hatchery nearby had minimum the cost of sampling and traveling of the sample. A similar strategy location at Balok Beach, Pahang was done by another horseshoe crab research group on sample collection (Mat Zauki et al., 2019).

The water quality and water salinity may also affect the quality of TAL produced by captive horseshoe crabs. Thus, the environment in captivity should maintain suitable conditions for horseshoe crabs to survive. The most suitable water temperature for Asia horseshoe crab to survive was 26°C and salinity should be 34 g L^{-1} (Carmichael & Brush, 2012). The Asia species of horseshoe crab can survive in more salinity water than *L. polyphemus* (Vestbo et al., 2018). On the other hand, the diet that feeds captivity horseshoe crab also affect the regeneration of hemocyte (Kwan et al., 2014). The shellfish are the most suitable food for horseshoe crabs in the resultof 9.7 x 10^4 eggs production than none in the fish feeding group (Ying et al., 2022).

The presence of egg production proven the female horseshoe crabs were healthy and reached the protein richness limit to produce eggs.

5.2 Sensitivity of *Tachypleus* amebocyte lysate on lipopolysaccharide induction

In this study, the sensitivity of *Tachypleus* ambocyte lysate (TAL) extract from *T. gigas* toward endotoxin increase when the magnesium ion concentration is in the range of 0.1 M to 0.25 M while 0.05 M to 0.15 M for calcium ion. A different finding was done by (Tinker-Kulberg et al., 2020), by using amebocyte lysate extracted from *Limulus polyphemus.*The concentration of 10 to 25 mM of magnesium ions and 2 to 10 mM of calcium ionsincrease the sensitivity of *Limulus* lysate toward endotoxin. The sensitivity of LAL was able toclot under 0.03 EU/mL endotoxin concentration (Unger et al., 2014). The presence of a suitable concentration of $MgCl_2$ and $CaCl_2$ slightly increased the activity of zymogen toward endotoxin. The increase of Mg ion caused the increase of TAL clotting activity if compared to calcium (Harm et al., 2021). The higher concentration of Calcium ionscaused a slight decrease in TAL clotting effect. This is due to horseshoe crabs that survive in seawater environments where the Magnesium and Calcium ion ratio is 50:10 (Magnesium: Calcium). On the other hand, Calcium ions play a cofactor in many biological processes.

The hemocyanin concentrations were different between fresh from wild and in captivity. A finding was done by (Tinker-Kulberg et al., 2020), the protein contained in fresh *Limulus polyphemus* horseshoe blood decreased from 61.1 ± 9.45 mg/mL to 11.5 ± 9.45 mg/mL. The protein concentration in hemolymph needs two to six weeks to be restored to healthy rate but the restoration of the amebocyte will take up to 4 months (Tinker-Kulberg et al., 2020). The horseshoe crab that being extracted the blood for TAL preparation were not yet restored its amebocyte concentration in the blood which cause the TAL activity to decrease in the next month. The sensitivity of serine protease related to coagulation activity was determined by using the gel clot method. It had showed the serine protease that related to coagulation in the TAL were highly sensitive to lipopolysaccharide when the present of divalent cation.

5.3 Proteolytic activity of *Tachypleus* amebocyte lysate

A different proteolytic activity results with 200 U/min for hemolymph and LPS induction from whiteleg shrimp, *Litopenaeus vannamei* (Madsari et al., 2022). The concentration of protein content in hemolymph was highly correlated with the hepatosomatic index in hemolymph organisms. However, there is no other finding on the proteolytic activity of TAL between wild and captive for *Tachypleus* species. A different finding was done by using another arthropod organism such as *Galleria mellonella* showed 4 U in aseptically injured and 3 U for native (Andrejko et al., 2021). The proteolytic activity of *G. mellonella* increased when infected *Pseudomonas aeruginosa* but decrease for native state.

The binding site of serine protease in marine organism hemocyte was specific due to immune response (Söderhäll, 1983). When the horseshoe crabs were not healthy the quality of hemocytes will decrease. The horseshoe crab that maintains in captivity may bring some effect on the experiment results. According to LaDouceur et al. (2019),the infection of fungi diseases may only be found in captive animals but none in wild organisms. The pollution of heavy metal affecting living organism and may cause subhealth state to all living organisms (Nanda et al., 2019; Wu et al., 2023). The TAL was prepared from the horseshoe crab hemocyte, thus the quality of hemocyte will affect the TAL. *Tachypleus sp*. was sensitive to heavy metal and showed negative correlation in hemocyte quality (Kwan et al., 2017). The pollution of horseshoe crab habitat had cause them become unhealthy and may cause death easily when had open wound. The proteolytic activity of serine protease in TAL were showed different between wild and captivity. The protein profile of TAL can be proceeded to determine the regulatory of protein between before and after lipopolysaccharide induction.

5.4 Protein profile of *Tachypleus* amobocyte lysate

The protein profiles of TAL were done using SDS PAGE. The different protein profiling between before and after LPS induction had showed different protein regulatory after LPS induction in TAL. The August batch TAL showed more sharp band compare with Jun batch and July batch. Six sharp bandsresulted from August batch were located at 150 kDa, 76 kDa, 38 kDa, 24 kDa, 17 kDa,and 12 kDa. A similar result done by another researcher, a sharp band was located at 45 kDa (Mustopa et al., 2023). In this study, one sharp band was located between 38 kDa and 52 kDa.

The results of the LC-MS/MS analysis related to this study was discussed. The LC-MS analysis was done using *T. gigas* showed 2% of protein was related to host defense system of innate immunity and 2% was related to protein synthesis in hemocyte (Adebayo et al., 2022). Tachylectin is a type of lectin that located in hemocyte and help in the hemagglutinating effect and antimicrobial activity against many microorganisms (Adebayo et al., 2022). The tachylectin succeed located at 27 kDa and is secreted by the granular cell in the hemocyte (Bowden et al., 2020). It was specific bind to N-acetylglucosamine and N-acetylgalactosamine. A similar finding was done by Wang et al. (2021), it involved in recognition of foreign particles such as the endotoxin from bacteria. Once detect lipopolysaccharide, agglutination was processed to inhibit the activity of endotoxin. The recognition of endotoxin was startedwith the interaction of o-antigen on LPS structure (Shingate et al., 2020). The tachylectin, protease inhibitor and anti-lipopolysaccharide factor in lysate for hemolymph coagulation (Ashrafuzzaman et al., 2022). The tachylectin helps researcher to determine the sensitivity of antimicrobial action in lysate (Hou et al., 2020).

The cationic trypsinogen is a serpin inhibitor. The cationic trypsinogen was ancestral to the majority of serine protease inhibitor (SERPIN) that had to play an important role in inhibiting the serine protease activity in hemocytes and plasma (Kostin et al., 2018). A different finding from *T. tridentatus*, intracellular coagulation inhibitor (GeneBank: BAA03374.1) was located after LPS induction (Wang et al., 2021). Cationic trypsinogen is an enzyme that encodes by the PRSS1 gene (Thiel et al., 2022). The activation of trypsinogen begins with proteolysis of the peptide bond at the N-terminal end (Demcsák et al., 2019). The cationic trypsinogen (Protein ID: P00760) that was determined from TAL was recorded to function as catalytic activity and cofactor. Thepresence of a different type of trypsinogen in lysate helps the cell in biosynthesis activity (Koistinen et al., 2022).

The zinc finger CCCH domain with protein ID Q94JI5 has been sequenced in this study while a different finding was done by another researcher (Magnadóttir et al., 2021). The structure of the major zinc finger was coordinated by one or more zinc ions to stabilize the protein fold. The GATA transcription factor is a family of DNA-binding proteins that composes of zinc finger with cysteine domain that play important role in hematopoiesis for invertebrates (Li et al., 2018). The zinc finger was one ofthe most abundant proteins that especially interact with DNA, RNA and protein sequence

(Abbehausen, 2019). The FYVE zinc finger had located in a marine organism, *Hippoglossus hippoglosus* that involved in immune, gene regulatory and metabolic pathway (Magnadóttir et al., 2021). Although zinc finger is a huge protein family, however it mainly plays important role in RNA binding that can be harvestedfrom lysate (Hanhsen et al., 2022).

The α-2-HS-glycoprotein had multifunction in the hemolymph. It is a carrier protein that is synthesized by hepatocytes and found on the cell membrane. The glycoprotein was obtained after LC-MS analysis. The function of this glycoprotein may help in the activation of the immune complementary system in crustaceans (Gao et al., 2022). A different finding was done by Moriyama et al. (2022), mucin-like glycoprotein was determined at the midgut of *Plautia stali*, a sunk bug. The glycoprotein consists of two polypeptide chains which are both cleaved from a protein precursor that is encoded from a single mRNA. From the Uniport protein data bank, the α-2-HS- glycoprotein can bind with a ligand such as some lipid and thyroid hormone. The metabolism of the marine organism during injured will be activated by some other immune-related enzyme for example endocytosis induced by Alpha-2-HS-glycoprotein in the lysate. The regulatory of serine protein between before and after LPS induction in TAL was done and proceed to determine the expression level of serine protease inhibitor genes.

5.5 Expression level of serine protease inhibitor gene

After the protein profile of serine protease in TAL, the expression level of serine protease inhibitor gene was determined. In the current study, all the expressions of SERPIN genes (TICI-1, TICI-2, and LP primer) showed the highest expression in the July batch compared to the other batches. The current study showed that the expression level of SERPIN gene for July batch had significant different with Jun and August batch when TICI-2 as a primer. The TICI-1 and TICI-2 were the serine protease inhibitor that identified from the hemocyte (Ashrafuzzaman et al., 2022). The TICI-1 and TICI-2 were the inhibitor for blood coagulation in horseshoe crab that help regulating coagulation (Blisnick et al., 2017). The LP primer was design from *Limulus polyphemus* serine protease inhibitor 2.1-like with gene ID 106460682. The serine protease inhibitor *2.1 like* helpto regulate the immune response of viral infection in *Spodoptera exigua* (Li et al., 2021).

The expression level of the SERPIN gene was high when not induced with LPS. A similar finding showed the expression level of Kazal-type serine proteinase inhibitor from fresh crayfish, *Cherax quadricarinatus.* The relative expression level of Kazal-type serpin from *C. quadricarinatus* hemocyte had reached 275 (Wang et al., 2021). The serpin gene decrease sharply when the neuroendocrine-immune factor, dopamine was injected into white shrimp, *Litopenaeus vannamei* for 12 hours and back to normal level after 24 hours (Wei et al., 2019). When invertebrate organisms had an open wound and microorganisms are infected inside the body, the defense mechanism was activated. Thus, nutrition and energy were consumed more on protein synthesis such as a clotting enzyme. The increasing of immune response-related protein and decreasing the expression of its inhibitor ensure the organism to survives under microbial invasion.

The primer prepared for SERPIN expression in RT-PCR reaction used TICI-1, TICI-2, and LP DNA sequence as a template. The results of RT-PCR were related to coagulation regulation and should be higher in freshly bleed heamolymph and lower in captivity horseshoe crabs or LPS induction horseshoe crabs. This is because the TICI-1 and TICI-2 function for inhibiting the coagulation cascade (Abbas et al., 2022). The template design for LP primer was named *L. polyphemus* serine protease inhibitor 2.1 with gene ID: LOC106460682 on NCBI (Pruitt et al., 2014). The results of the expression level of SERPIN that related to the coagulation cascade were decreased when species were under LPS infection. The expression level of SERPIN for LP primer was lower than TICI-1 and TICI-2 primer because of was not specific for the clotting enzyme. The SERPIN interacts with enzymes and inhibits the spreading of hemocyte and stop the coagulation of blood (Abbas et al., 2022).

When LPS was detected by hemocyte in the horseshoe crab, the clotting mechanism induced by Factor C will clot to prevent the loss of hemocyte. In this stage, the expression level of a serine protease in hemocytes was started to increase and the expression level of serpin decreased. This is because the clotting-related enzymes were more classified as serine proteases such as Factor C. After the wound had completely sealed and entrapped the pathogen, the transglutaminase occurred in the hemocyte for preparing the complementary activity. Transglutaminase was reported which have multifunction properties that are involved in blood coagulation and immune response against bacterial infection (Maningas et al., 2013). The complementary process continued to occur after the complement-like factor was bound with the pathogen

membrane protein. There were two types of activation for complementary response in invertebrates which is through the lectin alternative pathway (Xiao et al., 2022).

The complementary system in invertebrates consists of opsonization and phagocytosis. Not like vertebrates had highly specified phagocytic cells like neutrophils, eosinophils, and macrophages, the invertebrate mainly hemocytes (Liu et al., 2020). The hemocyte-mediated phagocytosis was started when the pathogens were recognized by the phagocyte surface receptor. The phagosome was formed to enclose the foreign particle or pathogen and bind with the lysosome and finally formed the phagolysosome. After the phagolysosomes were formed, the digestive enzyme and protease were synthesized to degrade the pathogen. The expression level of serine protease increased to ensure the digestive enzymes were enough for phagocytosis. After phagocytosis, huge quantities of hemocytes were consumed, so the synthesis of new hemocytes is required after infection (Liu et al., 2021). During hematopoietic, more energy and nutrient was consumed for new hemocyte synthesis. When the level of hemocyte raised back to normal, the expression level of serine protease decreased and back to normal. The expression level of SERPIN gene increased during coagulation and decreased after clotting was formed. The expression level of SERPIN gene will be the protein issue of the effect of LPS induction.

CHAPTER 6

CONCLUSIONS

The presence of divalent cation in 0.15 M $MgCl_2$ +0.15 M $CaCl_2$ had increased the clotting activity of lysate for one clotting level. The presence of cation with suitable concentration activated the clotting enzyme that contains in amebocyte and enhanced the coagulation effect. In pH 7, the clotting level of all batches of TAL were higher than other pH buffers. When horseshoe carbs were unhealthy and stressful the results of proteolytic activity were low. The proteolytic activity of LAL showed the highest among all batches of TAL. Seven proteins that had been profile after LC- MS analysis which is tachylectin-2, cationic trypsinogen, zinc finger CCCH domain, complement factor B-like protease, probable histidine ammonia-lysate, complement C3, and alpha-2-HS glycoprotein. The expression level of three different SERPIN genes for healthy horseshoe crab was higher than captive horseshoe carb under LPS induction. The expression level of SERPIN genes showed high in fresh blood but low in captive blood. The quality of TAL was affected by environment factor,thus repeating the study by using different horseshoe crab species may provide advance result. The activity of serine protease and serine protease inhibitor in regulating coagulation was affected by environmental factor such as temperature and pH. The sample size for the study was on small scale due to the maintenance cost of captivity.

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Appendix

	Protein ID	Protein Name	Size (Da)	Coverage (%)
1	P02769	Serum albumin	69294	62
2	Q8UAA9	Beta-lactamase hydrolase-like protein	47163	1
3	Q19020	Dynein heavy chain	521584	1
4	Q92QZ2	Guanylate kinase	24847	5
5	Q21XN6	DAN-direct RNA polymerase subunit alpha	37551	1
6	Q9M2L4	Putative calcium transporting ATPase	111945	1
7	A71MR0	Alanine tRNA ligase	94550	1
8	P55068	Brevican core protein	96057	1
9	Q61361	Brevican core protein	95815	1
10	Q5XF36	Vacuole membrane protein KMS1	45848	1
11	A45UY7	50S	14760	5

1		ribosomal protein L17		
12	F4IL57	Kinesin-like protein KIN	108779	1
13	Q61329	Zinc finger homeobox protein 3	406573	1
14	O53901	Mycocerosic acid synthase-like polyketide synthase	223887	1
15	Q43432	Eukaryotic translation initiation factor 4 gamma	176651	1
16	Q03001	Dystonin	860697	0.1
17	Q26782	Proteosome subunit alpha	27526	2
18	P36107	Inositol phosphoryl ceramide synthase catalytic subunit	45193	1
19	B5RLS6	Aspartyl/glutamyl-tRNA(Asn/	54929	1

		Gln) amidotransferase subunit		
20	Q9URV2	Anaphase promoting complex subunit	165411	1
21	B6HJU6	Nonribosomal peptide synthase roqA	262820	1
22	B2IH12	Ribosomal protein L11 methyltransferase	31985	2
23	E0D7H6	Alpha-ketoglutarate-dependent dioxygenase	46007	2
24	Q7NAE5	Glutamate--tRNA ligase	57981	1
25	Q9SKQ0	Peptidyl-prolyl cis-trans isomerase CYP19-2	18464	3
26	A6UUH1	Formate-dependent phosphoribosylglycinamide	42870	1

		formyltransferase		
27	Q8EJW4	2-methyl-aconitate isomerase	41660	1
28	Q1BUU4	Phosphoribosylglycinamide formyltransferase 2	42990	1
29	Q80U40	RIMS-binding protein 2	118342	1
30	Q12150	Protein CSF1	338258	1
31	Q6LA55	UPF0648 protein C3H5.09c	311841	1
32	Q97GZ3	Protein-glutamate methylesterase	37974	2
32	Q54NX7	Uncharacterized helicase DDB_G0278827	210539	1
33	Q9A2L1	4-hydroxy-tetrahydrodipicolinate reductase	26425	5

34	Q94464	Nucleus-vacuole junction protein 2	87112	1
35	Q9SA32	Putative cyclin-B3-1	45190	3
36	Q7V8B1	Chaperone protein ClpB	95779	1
37	C1N1Y2	Translation factor GUF1 homolog, chloroplastic	77159	1