โครงสร้างและหน้าที่ของเพนเลกทินและความเกี่ยวข้องกับระบบภูมิคุ้มกันในทางเดินอาหารของกุ้งกุลาดำ *Penaeus monodon*

นางสาวพชรพร อ่างทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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STRUCTURES AND FUNCTIONS OF PENLECTINS AND THEIR INVOLVEMENT IN GASTROINTESTINAL IMMUNE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*

Miss Pacharaporn Angthong

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Thesis Title	STRUCTURES AND FUNCTIONS OF PENLECTINS AND THEIR INVOLVEMENT IN GASTROINTESTINAL IMMUNE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*
By	Miss Pacharaporn Angthong
Field of Study	Biotechnology
Thesis Advisor	Professor Padermsak Jarayabhand, Ph.D.
Thesis Co-Advisor	Pikul Jiravanichpaisal, Ph.D.
	Professor Kenneth Söderhäll, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Voranop Viyakarn, Ph.D.)

.....Thesis Advisor
(Professor Padermsak Jarayabhand, Ph.D.)

.....Thesis Co-Advisor
(Pikul Jiravanichpaisal, Ph.D.)

.....Thesis Co-Advisor
(Professor Kenneth Söderhäll, Ph.D.)

.....Examiner
(Associate Professor Chanpen Chanchao, Ph.D.)

.....Examiner
(Assistant Professor Sanit Piyapattanakorn, Ph.D.)

.....External Examiner
(Sittiruk Roytrakul, Ph.D.)

พชรพร อ่างทอง : โครงสร้างและหน้าที่ของเพนเลกทินและความเกี่ยวข้องกับระบบภูมิคุ้มกันในทางเดินอาหารของกุ้งกุลาดำ *Penaeus monodon* (STRUCTURES AND FUNCTIONS OF PENLECTINS AND THEIR INVOLVEMENT IN GASTROINTESTINAL IMMUNE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. เผดิมศักดิ์ จารยะพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. พิกุล จิรวาณิชไพศาล, ศ. ดร. เคนเน็ท โซเดอร์ฮอลล์, 112 หน้า.

Tachylectin5A และ Tachylectin5B ของแมงดาทะเลญี่ปุ่นประกอบด้วยโดเมนไฟบริโนเจน (FReD) ซึ่งมีบทบาทในระบบภูมิคุ้มกัน ในการศึกษานี้ได้ทำการศึกษายีนของกุ้งกุลาดำที่มีความคล้ายกับยีน Tachylectin5 โดยให้ชื่อว่า เพนเลกทิน5-1 (PL5-1) เพนเลกทิน5-2 (PL5-2) และ เพนเลกทิน5-3 (PL5-3) ทั้งสามยีนประกอบด้วย signal peptide และโดเมนไฟบริโนเจน โดยมีตำแหน่งที่สามารถจับกับหมู่อะซิทิล (acetyl group binding site) และแคลเซียม (calcium binding site) ทั้งสามยีนมีลักษณะโครงสร้างคล้ายกับโครงสร้างของ Tachylectin5 ของแมงดาทะเลญี่ปุ่น จากการศึกษาการแสดงออกของยีน PL5-1 PL5-2 และ PL5-3 พบว่ามีการแสดงออกของยีนในเนื้อเยื่อหลายชนิด และระดับการแสดงออกของยีนในเม็ดเลือด ลำไส้ส่วนท้าย และกระเพาะอาหารสูงขึ้นเมื่อกุ้งได้รับเชื้อ *V. harveyi* หรือ *V. parahaemolyticus* 3HP (VP_{3HP}) ได้มีการผลิตโปรตีนลูกผสม PL5-1 PL5-2 และ PL5-3 ในระบบแบคทีเรีย ซึ่งโปรตีนที่ผลิตได้เป็นโปรตีนที่ไม่ละลายออกมานอกเซลล์จึงไม่สามารถนำมาใช้ศึกษาหน้าที่ของโปรตีนในระบบภูมิคุ้มกันได้ มีเพียงโปรตีน PL5-2 เท่านั้นที่สามารถแยกออกจากน้ำเลือดของกุ้งกุลาดำ และทำให้บริสุทธิ์ได้โดยใช้ affinity chromatography ดั้งนั้นจึงทำการศึกษาหน้าที่ของโปรตีน PL5-2 ในระบบภูมิคุ้มกันเท่านั้น โดยสามารถพบการแสดงออกของโปรตีน PL5-2 ในเนื้อเยื่อต่างๆ รวมทั้งน้ำเลือดของกุ้งกุลาดำ จากการศึกษาหน้าที่ของโปรตีน PL5-2 พบว่าโปรตีน PL5-2 สามารถจดจำองค์ประกอบของเซลล์แบคทีเรียกรัมบวก และแบคทีเรียกรัมลบ (ทั้งแบคทีเรียไม่ก่อให้เกิดโรค และแบคทีเรียที่ก่อให้เกิดโรค) โปรตีน PL5-2 สามารถทำให้เม็ดเลือดของมนุษย์ทั้งชนิด เอ บี และ โอ เกาะกลุ่มกันได้ และยังสามารถทำให้ทั้งแบคทีเรียกรัมลบ และแบคทีเรียกรัมบวกเกาะกลุ่มกันได้อีกด้วย ทั้งนี้อาจเป็นผลมาจากโปรตีน PL5-2 มีตำแหน่งที่สามารถจับกับหมู่อะซิทิลบนผนังเซลล์ของแบคทีเรีย ซึ่งยืนยันผลได้จากโปรตีน PL5-2 ไม่สามารถจับกับองค์ประกอบของผนังเซลล์ของแบคทีเรียได้เมื่อถูกยับยั้งด้วย GlcNAC หรือ GalNAC เพื่อให้เข้าใจหน้าที่ของยีน PL5-1 PL5-2 และ PL5-3 มากขึ้น จึงทำการยับยั้งการแสดงออกของยีนทั้งสามชนิดด้วยเทคนิค RNAi และให้กุ้งรับเชื้อ VP_{3HP} ซึ่งเป็นสาเหตุของโรคตายด่วน (AHPND) จากผลการศึกษาพบว่ากุ้งที่ถูกยับยั้งการแสดงออกยีนเพนเลกทินทั้ง 3 ยีนมีอัตราการตายสูงขึ้นหลังจากได้รับเชื้อ และกุ้งกุลาดำยังมีการเปลี่ยนแปลงพยาธิสภาพในตับรุนแรงมากขึ้น จากการที่พบโปรตีน PL5-2 ทั้งในน้ำเลือด และระบบทางเดินอาหารของกุ้งกุลาดำ อาจสามารถกล่าวได้ว่าเพนเลกทินมีหน้าที่ในการจดจำองค์ประกอบของแบคทีเรีย และสามารถยึดจับเซลล์แบคทีเรียจากนั้นจึงกำจัดออกจากร่างกาย และอาจเป็นไปได้ว่าเพนเลกทินสามารถควบคุมแบคทีเรียในลำไส้ของกุ้งได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: LECTIN / TACHYLECTIN / INNATE IMMUNITY / PENAEUS MONODON / FIBRINOGEN-RELATED PROTEIN

PACHARAPORN ANGTHONG: STRUCTURES AND FUNCTIONS OF PENLECTINS AND THEIR INVOLVEMENT IN GASTROINTESTINAL IMMUNE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: PROF. PADERMSAK JARAYABHAND, Ph.D., CO-ADVISOR: PIKUL JIRAVANICHPAISAL, Ph.D., PROF. KENNETH SöDERHäLL, Ph.D., 112 pp.

Tachylectin5A and Tachylectin5B, both contain a fibrinogen-related domain (FReD) and have been studied in japanese horseshoe crabs, *Tachypleus tridentatus* and shown to be involved in host defense. Here, we demonstrate the presence of tachylectin5- like genes in the shrimp, *P. monodon*, which we have named as *Penlectin5-1* (*PL5-1*), *Penlectin5-2* (*PL5-2*) and *Penlectin5-3* (*PL5-3*). All three lectins contain a signal peptide and a single FReD with an acetyl group and a calcium binding sites. They are structurally similar to japanese horseshoe crab tachylectin5. The *PL5-1*, *PL5-2* and *PL5-3* transcripts were expressed in various shrimp tissues in normal shrimp, and its expression was up-regulated in tissues such as hemocytes, posterior intestine or stomach following challenge with pathogenic *V. harveyi* or *V. parahaemolyticus* (3HP). The PL5-1, PL5-2 and PL5-3 proteins were expressed in a bacterial expression system and they were expressed as non-soluble proteins. Only the PL5-2 protein was possible to isolate and purify from shrimp plasma using affinity chromatography, and therefore, the PL5-2 protein was used for functional studies of this protein. The PL5-2 protein was detected in various tissues as well as in cell-free hemolymph. The biological function of the PL5-2 protein is to recognize some Gram-positive and Gram-negative bacteria regardless whether they are non-pathogenic or pathogenic. This lectin had hemagglutination activity towards all types of human erythrocytes including A, B and O type, and it had also a bacterial agglutination activity to both Gram-negative and Gram-positive bacteria. Possible binding sites of PL5-2 to bacteria could be at the N-acetyl moiety of the GlcNAc-MurNAc cell wall of the peptidoglycan, since the binding could be inhibited by GlcNAc or GalNAC. To further understand the functions and the involvements of PL5-1, PL5-2 and PL5-3 in response to AHPND in shrimp, knockdown of the PL5 genes using RNAi was performed prior to an oral administration of *Vibrio parahaemolyticus* (VP_{3HP}) causing AHPND. The results suggest that *PL5-1*, *PL5-2* or *PL5-3* silencing in shrimp challenged with VP_{3HP} showed higher increases in mortality as well as more severity of histopathological changes. The presence of PL5-2 protein in both circulating hemolymph and gastrointestinal tract, where host and microbes are usually interacting, may suggest that the physiological function of shrimp tachylectin-like proteins is to recognize and bind to invading bacteria to immobilize and entrap these microbes and subsequently clear them from circulation and the host body. It is also probably possible that the Penlectins are involved in controlling and maintaining the normal flora in the intestine.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
^{0}C	degree celcius
cDNA	complementary deoxyribonucleic acid
CFUs	colony forming units
DEPC	dithiothreitol
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRNA	double stranded ribonucleic acid
EF1-α	elongation factor 1 alpha
GFP	green fluorescence protein
IPTG	isopropyl-thiogalactoside
kDa	kilo daltan
M	molar
$MgCl_2$	magnesium chloride
mg	milligram
ml	millilitre
mM	mllimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
pI	isoelectric point
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
RNase A	ribonuclease A
RNAi	ribonucleic acid interference
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris (hydroxyl methyl) aminomethane

µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet

CHAPTER I
INTRODUCTION

1.1 Rationales

The giant tiger shrimp, *P. monodon* is one of the most economically important cultured species in Thailand. However, sustainable farming of *P. monodon* is difficult due to various factors such as the outbreak of diseases. These problems have led to a large decline in farming of *P. monodon* and this species has been replaced with *Litopenaeus vannamei*. Besides viral diseases the genus *Vibrio* is considered to be one of the most devastating pathogens among bacterial diseases in shrimp. Virulent strains of vibrios in shrimp are *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi* and *V. parahaemolyticu*s (Brock and Lightner, 1990) and these bacteria can cause mass mortalities of shrimp.

Recently, apart from vibriosis, shrimp farmers have been confronted with new or emerging shrimp diseases known as white feces disease and acute hepatopancreatic necrosis disease (AHPND). These diseases can cause significant economic losses for shrimp farmers in Thailand and other countries. Until now the causative agents are still unknown and because of this, many different factors have been proposed to be responsible for this disease. However, histopathological examination shows that the clinical sign of AHPND in both black tiger shrimp and white shrimp appear to be limited to the hepatopancreas or intestine of the gastrointestinal tract (GI tract) (Leano and Mohan, 2012). This indicates that the main target for these diseases is GI tract. To tackle and handle disease problems and to obtain a sustainable shrimp farming, understanding of their immune defenses and other factors, which will promote shrimp health is essential and urgently needed. Here a fundamental knowledge of shrimp gut immunity will be initiated since the GI tract is a significant route for pathogen entry of shrimp.

Although invertebrates lack the complex adaptive immune system in which memory is the hallmark and instead these animals rely solely on innate immunity. Howevere, their amazing diversity, abundance and evolutionary success argue that their innate immune defence is highly efficient against infections. In most cases the cuticular defenses are sufficient to protect against even quite virulent pathogens, which often only produce disease when the integument has been physically damaged. Once pathogens gain entry into the hemocoel of the host, they encounter a complex system of innate defense mechanisms involving cellular and humoral responses (Jiravanichpaisal et al., 2006).

In the absence of adaptive immunity in invertebrates, the whole defense mechanism against invading microorganisms depends on the innate immune system. Innate immunity was believed to consist largely of non-specific immune responses. Later, innate immunity has been considered to exhibit some sort of specificity as it is able to discriminate self from non-self and even between a variety of invading microorganisms (Fujita, 2002). The recognition of non-self is mediated by a series of pattern-recognition proteins (PRPs) that bind to conserved structures, so called pathogen-associated molecular patterns (PAMPs) present on the surface of microorganisms. The PRPs are now considered to be an important constituent how innate immune processes are activated. Candidates for such PAMPs are lipopolysaccharides (LPSs), peptidoglycans (PGNs), lipoteichoic acids of bacteria, glycolipids of mycobacteria, mannans of yeasts, β-1,3-glucan of fungi and double stranded RNA (dsRNA) of replicating viruses (Hoffmann et al., 1999; Soderhall and Cerenius, 1998). PRPs are conserved structures that can function as receptors or binding proteins for PAMPs and they can be localized on the surface of host cells or be secreted into the hemolymph and then they present a signal in the presence of invading pathogen in every compartment (Medzhitov and Janeway, 2002). Binding of PRPs to PAMPs initiates an activation of the host-defense system.

Lectins are proteins or glycoproteins normally without catalytic activity that can recognize and non-covalently bind to specific sugar moieties and thereby agglutinate cells by binding to cell surface glycoproteins and glycoconjugates (Lis and Sharon, 1998). Then it follows that lectins must have two binding sites for each carbohydrate

so that they can agglutinate for example bacteria. Lectins are considered as important PRPs in the innate immunity and they play significant roles in non-self-recognition and clearance of invading microorganisms, either as cell surface receptors or as soluble proteins existing in circulating fluids (Christophides et al., 2002; Yu and Kanost, 2002).

In invertebrates, lectins have been reported to be involved in various defense mechanisms such as exhibiting antimicrobial activity (Schroder et al., 2003; Sun et al., 2008), enhancing phagocytosis (Kondo et al., 1992; Mercy and Ravindranath, 1994; Sierra et al., 2005), activating the prophenoloxidase (proPO) system (Chen et al., 1995; Yu and Kanost, 2000) and inducing nodule formation or encapsulation (Koizumi et al., 1999; Ma et al., 2008).

C-type lectins are the most diverse and well studied among the lectin families in shrimp. Recently, attempts to find other lectins functioning as immune molecules have received increased attention. Tachylectins (TLs) were first cloned and characterized in Japanese horseshoe crab (*Tachypleus tridentatus*). Five types of tachylectins have been discovered and well-studied in horseshoe crab. Tachylectin1 to 4 was isolated from hemocytes and tachylectin5 from plasma. Tachylectins could exhibit bacterial agglutinating activity against Gram-positive or Gram-negative bacteria and they have hemagglutinating activity against human erythrocytes (Kawabata and Iwanaga, 1999).

Ju et al., (2009) studied the expression and function of tachylectin-related protein in amphioxus. They argue that the tachylectin transcript in gut significantly increased after being challenged with lipopolysaccharide. They also found that the recombinant protein was able to inhibit bacterial growth. In shrimp, several Tachylectin-5 (TL-5) genes have been found in *P. monodon*. It is interesting that one isoform of TL5 was mainly expressed in the hindgut and can be induced during immersion with *V. harveyi* (Soonthornchai et al., 2010).

However, the molecular mechanisms and functional involvement of lectin genes and proteins in the innate immune system of *P. monodon* are still largely unknown. Therefore, studies of the host immune responses and the role of lectins in the combat

against pathogens would contribute to the development of new and relevant approaches for disease control and sustainable shrimp farming.

In this study, the full length cDNA of lectin proteins was identified and characterized. Recombinant proteins were produced in vitro and used as an immunogens for the production of their corresponding polyclonal antibodies. Gene expression profile after bacterial infection was examined by quantitative real-time PCR. Immunohistochemistry was performed to detect the localization of interesting proteins. The recombinant proteins and the purified native lectins from shrimp plasma or tissue were used for functional studies including hemagglutinating activity, carbohydrate-binding specificity, microbial agglutination or binding assay, antimicrobial activities and RNAi were investigated.

1.2 Objectives

1. To Isolate, characterize and analyze expression of tachylectin genes and proteins involved in the immune system of *P. monodon*
2. To purify *P. monodon* tachylectin proteins from shrimp plasma for functional studies
3. To determine the biological functions of tachylectin proteins of *P. monodon*
4. To explore the effects of tachylectin gene silencing for the immunological role of the tachylectin genes

CHAPTER II
LITERATURE REVIEW

2.1 Shrimp aquaculture

Shrimp is one of the most popular seafood in the world. The production of shrimp in 2005 reached over 2.2 million metric tons (Tanticharoen et al., 2008). Shrimp farms are being created throughout the world to help meet the demand for shrimp. Farming of shrimp increased nine folds during the 1990s, and is one of the fastest growing forms of aquaculture and which now accounts for one-third of the shrimp produced globally.

Shrimp farming in Thailand is a multi-billion dollar industry, a major export product and a foreign-exchange earner. Thailand is also the world's leading exporter and the largest producer of black tiger shrimp (*Penaeus monodon*). The rapid growth of shrimp farming in Thailand has led to an economic boom, especially in the coastal provinces of the Eastern and Southern regions. Since 2002, production of *P. monodon* in Thailand has declined, and its production is replaced by the farming of the pacific white shrimp (*Litopenaeus vannamei*) (Figure 2.1).

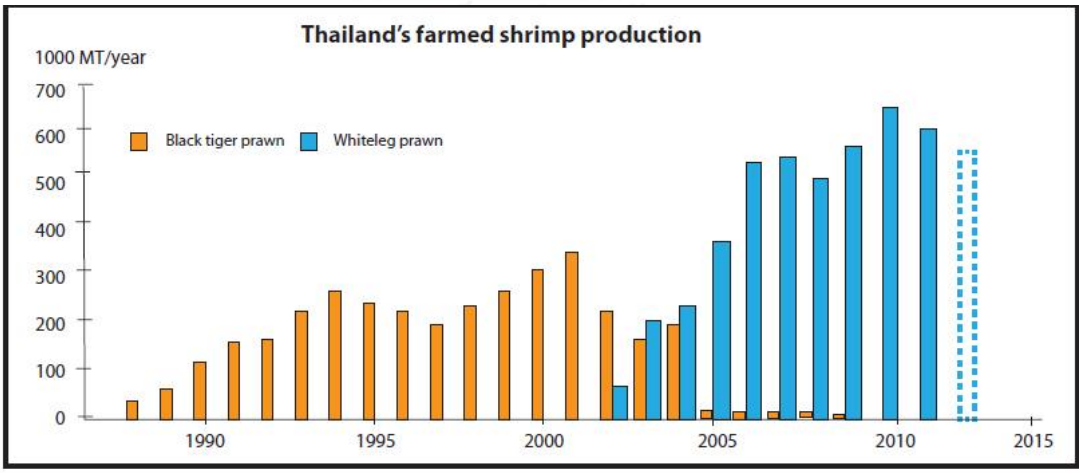

Figure 2.1 Production of *P. monodon* and *L. vannamei* in Thailand from 1990 to 2012 Source :Thai shrimp production development (2011).

The production of *P. monodon* declined due to several outbreaks of diseases and also that it has a poor domesticated broodstock. These problems have led to a large decline of farming *P. monodon* and this species has been replaced with *L. vannamei*.

Vibriosis is one major bacterial disease of shrimp and is caused by virulent strains of *Vibrio* and they belong to the species *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi* and *V. parahaemolyticus*. These bacteria outbreaks cause a mass mortality of shrimp. In late 2012, South East Asia's shrimp production has been struck by a new disease known as early mortality syndrome (EMS), or acute hepatopancreatic necrosis disease (AHPND) (Tran et al., 2013). This disease has caused a severe decrease in shrimp farming in Asia including Thailand (Figure 2.2).

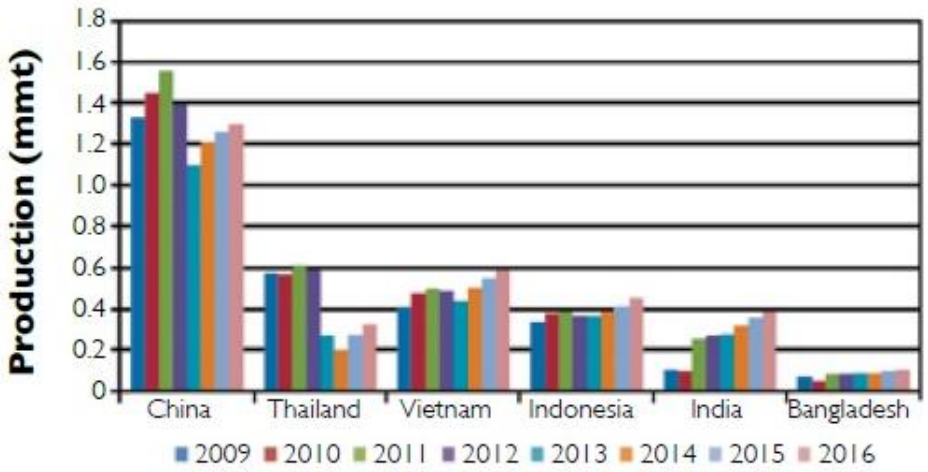

Figure 2.2 Shrimp aquaculture production in major farming nations in Asia.
Source: FAO (2009-2012) and GOAL survey (2013-2016).

2.2 Taxonomy of black tiger shrimp, *P .monodon*

The scientific name of black tiger shrimp is *Penaeus monodon* is classified as below (Bailey-Brock and Moss, 1992).

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Family: Penaeidae (Rafinesque, 1985)

Genus: Penaeus (Fabricius, 1798)

Species: *Penaeus monodon* (Fabricius, 1798)

2.3 Morphology

The shrimp body is separated into cephalothorax and abdomen (Figure 2.3). Most organs, such as gills, digestive system and heart, are located in the cephalothorax, while the muscles are concentrated in the abdomen. Appendages of the cephalothorax vary in appearance and function. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae form the jaw-like structures that are involved in food uptake. In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen.

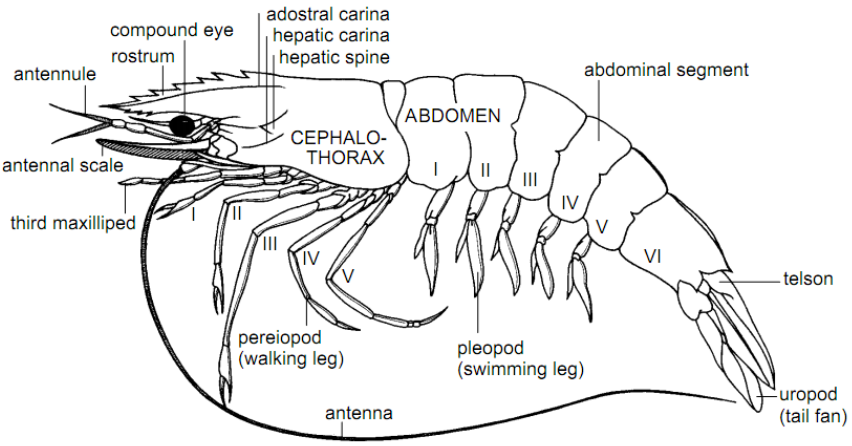

Figure 2.3 Lateral view of the external anatomy of Penaeus monodon
Source: Primavera (1990)

The internal morphology of penaeid shrimp contains circulatory, muscular, respiratory, nervous, reproductive and digestive system (Figure 2.3). Penaeids and other arthropods have an open circulatory system and the blood and the blood cells are called haemolymph and haemocytes, respectively. Crustaceans have a muscular heart that is dorsally located in the cephalothorax. The valved haemolymph vessels leave the heart and branch several times before the haemolymph arrives at the sinuses that are scattered throughout the body, where exchange of substances takes place. After passing the gills, the haemolymph returns to the heart by means of three wide non-valved openings (Bauchau, 1981). A large part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas. This digestive gland consists of diverticula of the intestine. Spaces between these hepatopancreatic tubules are haemolymph sinuses. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leaves the heart ends in the lymphoid organ, where the haemolymph is filtered. This organ is located ventro-anteriorly to the hepatopancreas. The haemocytes are produced in the haematopoietic tissue. This organ is dispersed in the cephalothorax, but is mainly present around the stomach and in the onset of the maxillipeds. Lymphoid organ and haematopoietic tissue are not shown in Figure 2.4.

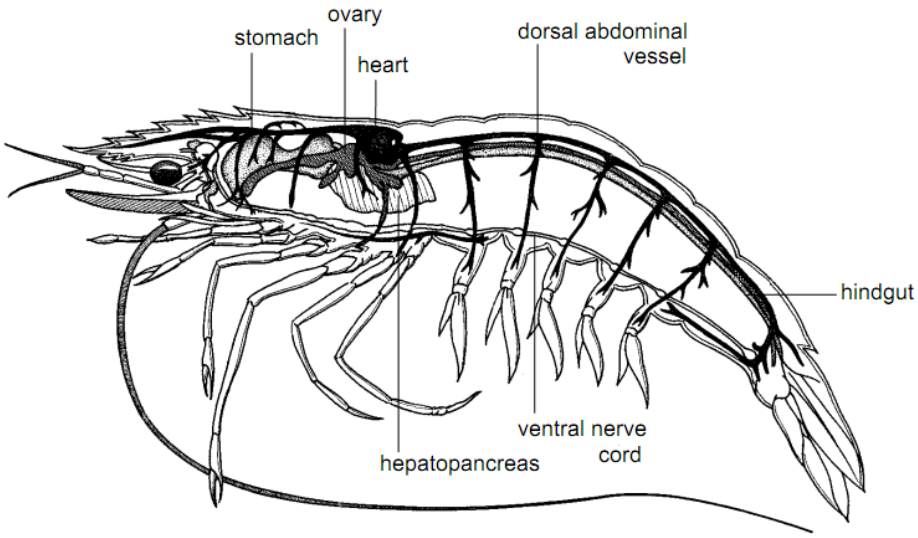

Figure 2.4 Lateral view of the internal anatomy of *Penaeus monodon*
Source: Primavera (1990)

2.4 Distribution and life cycle

The giant black tiger shrimp is widely distributed throughout the greater part of the Indo-Pacific region, ranging northwards to Japan and Taiwan, eastwards to Tahiti, southwards to Australia and westwards to Africa. Penaeid shrimp life cycle includes several distinct stages that are found in a variety of habitats (Figure 2.5). Juveniles prefer brackish shore areas and mangrove estuaries as their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities, where mating and reproduction takes place. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The eggs hatch into the first larval stage, which is the nauplius. The nauplii feed on their reserves for a few days and develop into the protozoeae. The protozoeae feed on algae, they have feathery appendages and they are elongated with a distinct cephalothorax. The early protozoea stage has a pair of protruded compound eyes and a rostrum is present in the next stage and it has a pair of uropods in the late stage of protozoea. After that, the protozoeae metamorphose into mysis. The mysis is similar to a juvenile shrimp in which segmented bodies, eyestalks and pleopods have developed. They feed on algae and zooplankton and have many of the characteristics of adult shrimp and develop into megalopas, the stage commonly called postlarvae (PL) and in this stage they look like adult shrimp and pleopods are

fully developed and functional. Larval stages inhabit plankton-rich surface water offshore, with a coastal migration as they develop.

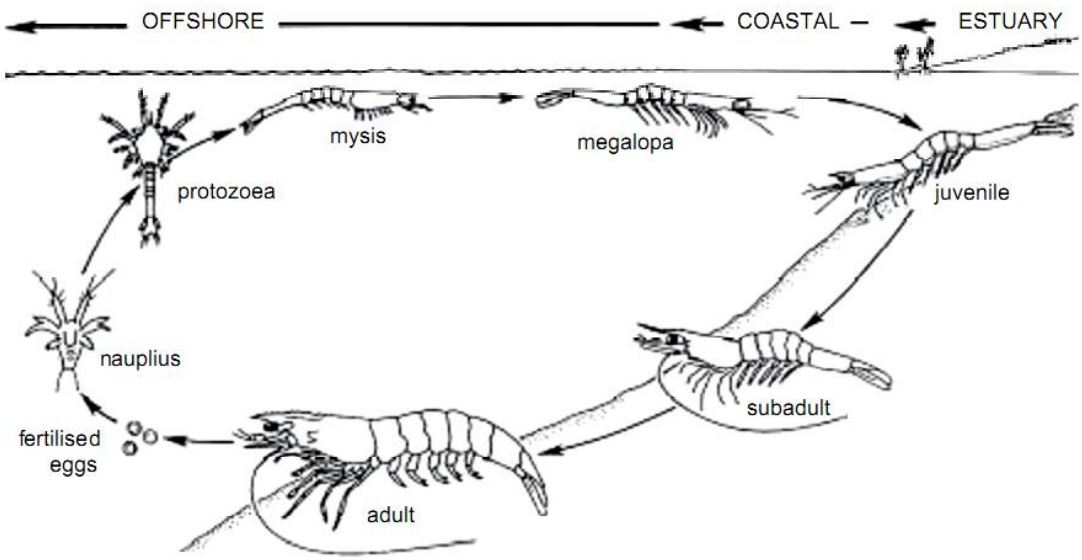

Figure 2.5 The life history of *Penaeus monodon* shrimp. Eggs hatch within 16 hours after fertilization. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The and early juvenile are called postlarvae. Transition from juvenile to takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months.
Source: Motoh (1984)

2.5 Digestive system

The digestive tract is responsible for the nutritional function. An adult decapod crustacean digestive system is divided into 3 main parts including foregut, midgut and hindgut. Generally, the foregut is composed of the mouth, oesophagus and stomach. The midgut consists of intestine, anterior midgut caeca and posterior caeca. The hindgut is composed of rectum and anus. Additionally, hepatopancreas is included in the digestive system which secretes digestive enzymes (Ceccaldi, 1989).

Moreover, digestive tract is a significant route for pathogen entry of shrimp. The pathogenic bacteria from the environment or feed can enter into the shrimp body

and colonized in the digestive system. Therefore, the immune response has a potential role to fight against the pathogen.

2.6 Major shrimp disease in Thailand

2.6.1 Luminous disease

Luminous bacterial vibriosis (LBV) is named after the luminous symptoms caused by this bacterium (Lavilla-Pitogo et al., 1990). *Vibrio harveyi* is a rod shape, gram-negative bacterium with 0.5-0.8 μm width and 1.4-2.6 μm length. Presumptive diagnosis is based on clinical signs such as blood or hepatopancreas that is streaked on a Vibrio-selective or general marine agar plate. After incubation at room temperature overnight, colonies of *V. harveyi* grow and show strong luminescence in dim light. Vibrio species live in the cultured water, in oxygen-free environments such as in the gut of aquatic animals and are sometimes found in the bottoms of shrimp ponds. Consequently, this bacteria can enter shrimp via the cuticle or sub-cuticle that also give the name shell disease or black/brown spot disease, puncture wounds so loss of limbs and cloudy musculature and the gut or hepatopancreas and/or general septicemia (Lightner, 1983). In Filipino hatcheries, the primary source of *V. harveyi* appears to be the midgut contents of female broodstock which are shed during spawning (Lavilla-Pitogo et al., 1992).

The infected shrimp show the milky white signs on the body and appendages, weakness, disoriented swimming, lethargy, loss of appetite and show black spots of melanisation on the cephalothorax (Figure 2.6). Postlarvae may display cloudy hepatopancreas and brown gills (Anderson et al., 1988; Takahashi et al., 1985). Finally, it leads to death. Histopathology of infected shrimp showed that epithelial cells of midgut and hepatopancreatic tubules are commonly detached into the lumen (Lavilla-Pitogo et al., 1990). The cuticular colonisation is the cause of necrosis of the cuticular epithelium and the formation of melanised lesions. Septic hemocytic nodules are formed in the lymphoid organ, heart, connective tissues of the gills, hepatopancreas, antennal gland, nerve cord, telson and muscle (Anderson et al., 1988; Jiravanichpaisal et al., 1994; Mohney et al., 1991). In Thailand, vibriosis in *P. monodon* has the spheroids in the lymphoid organ (Nash et al., 1992). The extracellular products (ECPs)

of *V. harveyi* are harmful to shrimp (Liu et al., 1996), and a cysteine protease is one of the major exotoxins (Liu and Lee, 1999) which appears to be important as a virulence factor (Lee et al., 1999). Extreme losses of cultured *P. monodon* in hatcheries and shrimp farms result from *V. harveyi*. These bacteria outbreaks cause mortalities of the affected shrimps up to 100%, whether they are larvae, post-larvae, juvenile, and adults (Lightner, 1983).

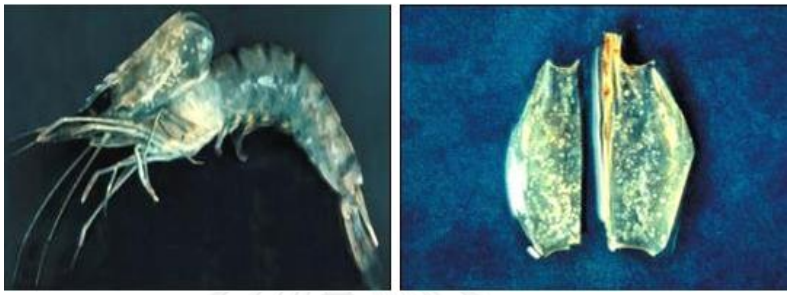

Figure 2.6 Vibriosis in shrimp. Black spots of melanization on the cephalothorax (left panel) and chronic soft shelling (right panel).
Source: http:/www.viralinfections.info/article/664934838/disease-prevention-and-treatment

2.6.2 Early mortality syndrome or Acute Hepatopancreatic Necrosis Disease (EMS or AHPND)

Recently, a newly emerging disease known as early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) caused a mass mortality of shrimp in China, Vietnam, Malaysia and Thailand. EMS/AHPND outbreaks occur within the first 30 days after stocking in a newly prepared shrimp pond and mortality can exceed 70%. The EMS/APHND is caused by a bacterial agent, which is transmitted orally, colonizes the shrimp gastrointestinal tract and produces a toxin that causes tissue destruction and dysfunction of the shrimp hepatopancreas (Tran et al., 2013). The EMS/AHPND pathogen consists of a number of unique strains of a relatively common bacterium, *V. parahaemolyticus*. This bacteria strain carries a plasmid encoded PirA and PirB which are homologus to the insecticidal Photorhandus insect-related (Pir) toxin gene (Han et al., 2015; Yang et al., 2014). Clinical signs of this

disease are slow growth, soft shells, reduced size of hepatopancreas and white hepatopancreas (Figure 2.7).

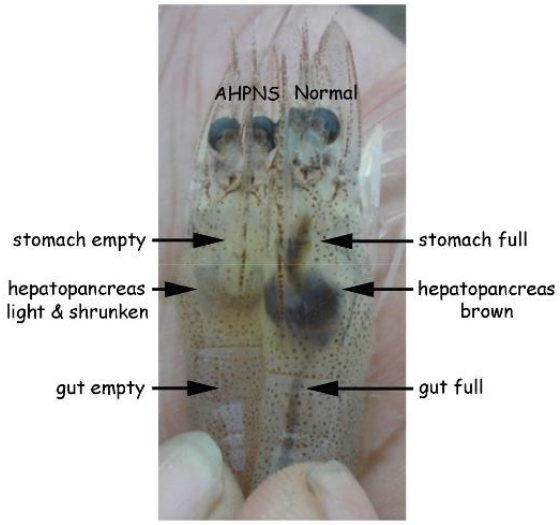

Figure 2.7 Gross signs of shrimp affected by AHPND (left) compared to normal shrimp (right).
Source: Lightner and Flegel

2.6.3 White Spot Syndrome Virus

White spot syndrome virus (WSSV) has been one of the most harmful pathogens in shrimp because of its high virulence (Flegel, 1997; Lightner, 1996), shrimp mortality can reach 100 % within 3 to 7 days of infection, they can infect all life stage of shrimp. WSSV is a double-stranded virus and they can infect various tissues including connective tissue, nervous tissue, hematopoietic tissue, muscle tissue, epidermis and gill (Takahashi et al., 1994). WSSV infected shrimp has white spots on the inner carapace, the body surface turning to red or pink (Figure 2.8).

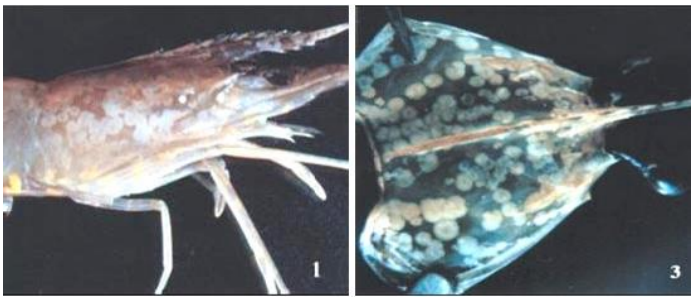

Figure 2.8 White spot syndrome virus disease. The body turning to red (left) and white spots occure on the cephalothorax (right)
Source: Lightner, 1996

2.7 Innate immune system

The immune system is commonly divided into two general systems named innate and adaptive immunity. Adaptive immunity is found only in vertebrates, which have B and T lymphocytes, which produce an infinite multitude of specific antigen receptors and antibodies through somatic gene rearrangement, whereas innate immunity is conserved in all animals throughout evolution (Murphy et al., 2008). The innate immune system uses germline-encoded receptors for recognition of common antigens on the surface of microbial pathogens. This feature distinguishes the innate immune system found in invertebrates from the adaptive immune system of the vertebrates that process a repertoire of specific antigen receptors and antibodies.

Although invertebrates lack the fine specificity of adaptive immunity that is necessary to produce immunological memory, it can distinguish self from non-self in a highly efficient defense system against microbial infections (Jiravanichpaisal et al., 2006).

The innate immune system of eukaryotes recognizes non-self through a series of pattern recognition receptors (PRRs) by binding unique microbial cell surface components, which are popularly referred to as pathogen-associated molecular patterns (PAMPs), and they seem to be shared among several groups of pathogens. Examples of PAMPs are lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acid (LTA) of Gram-positive bacteria, glycolipids of mycobacterium, β-glucans of fungi, and mannans of yeast. Recognition of PAMPs initiates various innate immune

responses, such as phagocytosis, encapsulation, lysis of the pathogen by antimicrobial peptides (AMPs), and activation of proteolytic cascade that lead to melanization as well as activation of s blood coagulation in arthropods (Figure 2.9).

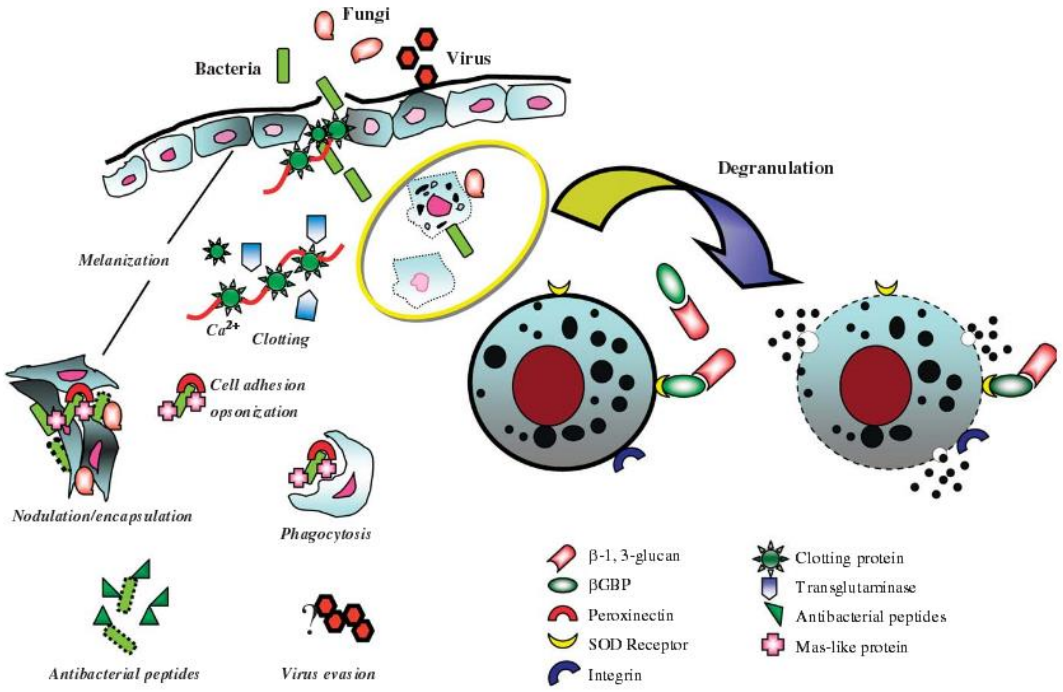

Figure 2.9 Diagrammatic view of innate immune response in crustacean
Source: Jiravanichpaisal et al. (2006)

2.8 Antimicrobial peptides

In all kingdoms, from bacteria to human, a wide variety of AMPs have been identified and characterized. Normally, AMPs contain less than 150-200 amino acid residues and have a wide variety and diversity in amino acid sequence, structure and range of activity. AMPs are active against a broad spectrum of microorganisms such as bacteria, filamentous fungi, virus and parasites (Hancock and Diamond, 2000; Pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991). Depending on their tissue distribution, AMPs ensure either a systemic or local protection of the host against pathogen.

In arthropods, a spacious activity of AMPs against bacteria and filamentous fungi has been reported in insects, horseshoe crabs, and shrimps (Bachere et al., 2004; Bulet et al., 1999; Vizioli and Salzet, 2002). In horseshoe crabs, AMPs are mainly synthesized in hemocyte and stored within cytoplasmic granules. These cells are highly sensitive to LPS which is a major outer membrane component of Gram-negative bacteria and respond by degranulating granules after stimulation by LPS. In contrast, the fat body of insects is the main site for AMP symthesis (Lemaitre et al., 1997). AMP gene transcription is induced by attacking bacteria resulting in their immediate synthesis and subsequent secretion into the blood circulation. In the last decade several AMPs have been isolated from crab, crayfish and shrimp. There are AMPs such as penaeidin, astacidin1 and 2, parasin, hipsin, antilipopolysaccharide factors (ALF), crustin and hemocyanin. Of these the penaeidin, crustin and ALF families have been studied in more detail.

2.9 Coagulation system

Coagulation is important for limiting hemolymph loss and initiating wound healing that is the first line defense of the overall invertebrate immune system. And it is quickly forming a secondary barrier to infection and immobilizing bacteria. To date, two coagulation systems have been deciphered in detail namely in crayfish and horseshoe crab. Noteworthy is that coagulation in insects is not known in any detail. In horseshoe crab, the clotting system is regulated by a proteolytic cascade that is linked with the release of antimicrobial substances (Iwanaga, 2002; Iwanaga and Lee, 2005). In another model the crayfish, the clotting system depends on a transglutaminase (TGase) dependent clotting reaction (Hall et al., 1999; Kopacek et al., 1993). In crustacean, coagulation is formed by the polymerization of a clotting protein in plasma catalyzed by a calcium ion dependent TGase, which is released from the hemocytes by foreign particle stimulation or tissue damage (Hall et al., 1999). The clotting reaction is very similar in crayfish and shrimp and TGase and clotting protein are important molecules in shrimp coagulation (Maningas et al., 2008).

TGase, a calcium ion dependent enzymes, catalyzes calcium-dependent acyl-transfer reactions between glutamine residues and lysine residues in protein substrates

in presence of calcium ion to form a solid gel at the wounding site (Wang et al., 2001). TGase gene has been cloned and localized in crayfish *Pacifastacus leniusculus* (Wang et al., 2001) and two TGase gene including STG I and STG II are characterized from black tiger shrimp (Huang et al., 2004). Another type of TGase was found to be involved in the tiger shrimp *P. monodon* (Cheng et al., 2005; Huang et al., 2004; Yeh et al., 2009).

The clotting protein, a glycoprotein, has two physiological functions including coagulation and lipid transport (Hall et al., 1995). The regulation and localization of the clotting protein are in the outer layer of stromal matrix cells of lymphoid organ (Yeh et al., 2007). Crustacean clotting proteins have been cloned and characterized from several species, and it was first cloned from the freshwater crayfish, *P. leniusculus* (Hall et al., 1999; Kopacek et al., 1993), and then was found in the tiger prawn, *P. monodon* (Yeh et al., 1999).

2.10 Melanization and Prophenoloxidase system

Melanization is an important immune component of the innate immune system of invertebrates, and is essential for defense as well as for wound healing. This mechanism provides toxic quinones e and other short-lived reaction products, these substances are also involved in formation of more long-lived products such as melanin that physically encapsulates pathogens. In addition, intermediates in the melanin pathway participate in the wound healing process by the formation of covalent links in damaged tissues resulting in sclerotization (Cerenius et al., 2008). In most invertebrates melanin synthesis is achieved by the prophenoloxidase (proPO)-system activation. The proPO cascade is initiated when microbial polysaccharides such as lipopolysaccharides, β-1,3-glucans or peptidoglycans are recognized by pattern recognition proteins and the complex formed induces activation of several serine proteinases. However, melanin synthesis must be tightly controlled because active phenoloxidase (PO) oxidizes o-diphenols into quinones that are toxic to most cells and tissues and melanin pigments are formed that also are deleterious to the host. So, the animal needs melanization inhibitors to prevent unwanted production of quinone intermediates and melanization

in places) where it is not suitable (Figure 2.6). Therefore, there are several inhibitors to the proteinase of the proPO-cascade and one group of inhibitors are the Pacifastins, and this inhibitor was first characterized in crayfish (Liang et al., 1997) and later in several shrimp species (Gollas-Galván et al., 1999; Sritunyalucksana et al., 1999; Yeh et al., 2009).

A novel 43 kDa a protein identified from the meal worm *Tenebrio molitor*, which functions as a melanization inhibition protein (MIP), and this protein can inhibit melanin formation in *T. molitor* larvae that was injected with *Candida albicans* (Zhao et al., 2005). Recently, MIP was identified from the hemolymph of crayfish, *Pacifastacus leniusculus*. Pacifastacus MIP has a similar function in *T. molitor* but has a different molecular structure. The Pacifastacus MIP is an important inhibitor of the proPO system that produced melanin in crayfish that was injected with the bacterium *Hafnia alvei* (Soderhall et al., 2009).

2.11 Pattern recognition proteins

Pathogen-associated molecular patterns (PAMPs) are present on microbial cell surface but are not present on host cells. Microorganisms have highly conserved and widely distributed signature molecules in their cell walls such as LPS or peptidoglycans of bacteria cell walls and β-1,3-glucans of fungal cell walls, which are not found on other multicellular organisms. These microbial carbohydrates can serve as discriminating molecules between self and non-self (Janeway, 1989). A number of molecules binding to PAMPs of microbes have been found in invertebrates and are called pattern recognition proteins (PRPs). PRP is localized on the surface of cells and secreted into hemolymph, ready to signal the presence of invading pathogen in every compartment. In previous studies, PRPs such as LPS binding protein (LBP), β-1,3-glucans binding protein (BGBP), peptidoglycan recognition proteins (PGRPs), and lectins have been isolated and characterized from several animals, and these pattern recognition molecules have been shown to be involved in the innate immune system in both invertebrates and vertebrates (Young Lee and Söderhäll, 2002).

The crayfish masquerade-like protein (PlMasI) was isolated from hemocytes as a pattern recognition protein that recognizes LPS of Gram-negative bacteria and β-1,3-

glucans of yeast. Besides, *PlMasI* has an opsonic and cell adhesive activity, which suggests that it plays a role as an innate immune protein (Lee and Soderhall, 2001). A recognition protein isolated from hemocytes of *P. monodon* has binding activity to LPS (Sritunyalucksana et al., 2002) and one from *L. vannamei* has binding activity to LPS and β-1,3-glucans (Cheng et al., 2005).

2.12 Lectin

Lectins/agglutinins are sugar-binding proteins or glycoproteins usually without catalytic activity. Lectins are found in all type of living organisms, both in soluble or in membrane-bound form. These proteins are crucial in the innate immune system, they have the ability to bind to specific carbohydrates expressed on different cell surface, they can bind cells and an agglutination reaction occurs. Due to that the lectins have ability to bind carbohydrates and hence agglutinate different cells such as bacteria, fungi, and yeast, it is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self-recognition reactions.

In invertebrates, lectins have various biological activities such as antimicrobial activity, cell adhesion, phagocytosis, opsonization, nodule formation and activating the proPO system. β-glucan binding proteins (BGBP) have been isolated from plasma of crayfish that can recognize β-1,3-glucan to promote the activation of the proPO-system and also to act as an opsonin to increase the phagocytic activity. Moreover, LGBP has been identified from crayfish hemocytes, they have binding activity to either LPS or β-1,3-glucans but not peptidoglycans (Lee et al., 2000). Lectins have been isolated in several shrimp including *P. japonicus* (Kondo et al., 1992), *P. californiensis* (Vargas-Albores et al., 1993; Vargus-Albores, 1995), *P. monodon* (Sritunyalucksana et al., 1999), and *L. vannamei* (Romo-Figueroa et al., 2004; Vargas-Albores et al., 1997). Sritunyalucksana et al. (2002) reported that the BGBP was constitutively expressed in hemocytes of *P. monodon* within 12 hours of the *V. harveyi*-challenge. Two other inducible immunolectins C-type lectin (Yu et al., 1999) and an LPS specific lectin (Yu and Kanost, 2000) were identified in tobacco hornworm, *Manduca sexta*, and were both found to activate the proPO system. Other interesting lectins are tachylectins (TLs) present in Japanese horseshoe crab, *Tachypleus tridentatus*, they were shown to be

involved in critical immune responses such as hemagglutination activity and antibacterial activity (Kawabata and Iwanaga, 1999).

2.13 Tachylectin

The hemolymph of horseshoe crab *Tachypleus tridentatus* contains granulated cells the socalled amoebocytes comprising 99 % of the hemocytes, and they are filled with two populations of secretory granules, named large (L)- and small (S)- granules. The granules selectively store defense molecules, such as a clotting factor, a clottable protein coagulogen, protease inhibitors, lectin, and antimicrobial peptides. The hemocyte is extremely sensitive to bacterial endotoxins such as lipopolysaccharides (LPS), and respond by degranulating the granular component after stimulation with LPS . This response is very important for host defense involving the engulfing and killing invading microbes, in addition to preventing the leakage of hemolymph. This has alos been shown to occur in crayfish (Jiravanichpaisal et al., 2006).

Five types of tachylectin (TLs), have been first identified in Japanese horseshoe crab, *Tachypleusn tridentatus* (to which the name tachylectin refers). Tachylectin-1 to 4 was isolated from hemocytes and tachylectin-5 from plasma .Tachylectins play a key role as pattern recognition molecules in the innate immune response system (Kawabata and Iwanaga, 1999).

2.13.1 Tachylectin-1 (TL-1)

Tachylectin-1 or L6 was identical to an L granule-derived protein L6 and has been purified from hemocytes (Saito et al., 1995). Tachylectin-1 is the most abundant lectin stored in L granules of hemocytes. Three types of tachylectins have been quantitated using enzyme-linked immunosorbent assay and found to be present in hemocytes at the ratio of TL-1: TL-2: TL-3 = 38:6:4 with the assumption of coagulogen as 100, which is the most abundant protein in L granules (Inamori et al., 1999). Although TL-1 shows no hemagglutinating activity, it can agglutinate sheep erythrocytes coated with LPS and this activity is inhibited by the addition of LPS. Since the S-type LPS of *Salmonella minnesota* and the R-type LPS of *Salmonella minnesota* R595 (Re) are both effective, this suggests that TL-1 recognizes an inner-core portion of LPS consisting of a disaccharide of 2-keto-3-deoxyoctonate (KDO) and lipid A.

TL-1 has antibacterial activity to Gram-negative bacteria, such as *Escherichia coli*, but not to Gram-positive bacteria (Kawabata and Iwanaga, 1999). TL-1 is a single-chain protein consisting of 221 amino acid residues with no *N*-linked sugar chain, and contains three intrachain disulfide bonds and a free Cys residue. TL-1 contains one atom of zinc but is rich in Trp (9 residues) and positively-charged amino acids (27 residues: 11 Lys, 10 Arg, and 6 His), as compared to negatively charged amino acids (16 residues: 14 Asp and 2 Glu). The isoelectric point is calculated to be 9.7. TL-1 contains six tandem repeats and each consists of 33 - 38 amino acids with 32 - 61 % internal sequence identity which is an exceptional structural feature of this protein. Based on its crystal structure, TL-1 has axis-bladed beta-propeller structure (Kawabata and Iwanaga, 1999). Interestingly, an isolectin named TL-P, which has a similar sequence to that of TL-1, has been found in the perivitel line fluid of horseshoe crab (Nagai et al., 1999). Moreover, homologues of TL-1 with unknown functions have been identified from the myxomycete *Physarum polycephalum*, named tectonins I and II, and they show 33% sequence identity to TL-1. Tectonins could recognize LPS of Gram-negative bacteria for phagocytosis, as well as have affinity for several polysaccharides for non-self recognition. Moreover, (Mali et al., 2006) have identified and characterized a tachylectin-related gene in the colonial marine hydroid, *Hydractinia echinata*, which structure was most similar to horseshoe crab TL-1. Based on RT-PCR analyzed along a developmental gradient of hydroid, the result showed that the gene was only expressed in post-metamorphic life, while no mRNA was detected during embryonic development or in planula larvae. Despite well-conserved tachylectin-like structure this *Hydractinia* tachylectin-related gene does not appear to have any function in immunity.

2.13.2 Tachylectin-2 (TL-2)

Tachylectin-2 or L10, identical to an L granule-derived protein L10, has been purified from hemocytes. TL-2 has hemagglutinating activity against human A-type erythrocytes and the activity is specifically inhibited by D-GlcNAc or GalNAc . Other monosaccharides such as D-Glc, D-Gal, D-$GlcNH_2$, D-$GalNH_2$, L-Fuc, D-Man, L-Rha, and *N*-acetylneuraminic acid have no inhibition. N-acetylallolactosamine (Galβ-6GlcNAc) shows inhibitory effect while its isomer, N-acetyllactosamine (Galβ1-

4GlcNAc) has no effect. Thus, in addition to the *N*-acetyl group, the hydroxyl group of C-4 of D-GlcNAc is important for recognition by TL-2. Ca^{2+} is not required for the hemagglutinating activity and EDTA has no effect on the activity. TL-2 also shows bacterial agglutinating activity against *Staphylococcus saprophyticus* KD. This suggests that TL-2 interacts with a precise surface component on Gram-positive bacteria such as lipoteichoic acids (LTA). Generally, LTA is composed of α-1,3-linked poly-glycerophosphate chain covalently linked to a glycolipid. Since the LTA isolated from S. saprophyticus contains α- (1-2)-linked D-GlcNAc as an additional substituent of the poly-glycerophosphate backbone (Ruhland and Fiedler, 1990). Therefore, TL-2 most likely agglutinates *S. saprophyticus* through the D-GlcNAc residues presented on the bacterial surface. TL-2 may also interact with D-GlcNAc or a specific sugar on the O-antigens of LPS (Kawabata and Iwanaga, 1999).

TL-2 is a single-chain protein consisting of 236 amino acid residues with a molecular weight of 26.7 kDa. TL-2 contains no cysteine and no *N*- and *O*-linked sugar chains. The feature of the sequence presents a five tandem repeats of 47 amino acids and sequence identity between the repeats ranges from 49-68%. TL-2 is a monomeric protein, and therefore at least two sugar binding sites must be present in the molecule for the expression of its hemagglutinating activity.

2.13.3 Tachylectin-3 (TL-3)

Tachylectin-3 (TL-3) has been purified from hemocytes as an LPS-binding lectin and exhibits hemagglutinating activity specifically against human A-type erythrocytes (Inamori et al., 1999). The hemagglutinating activity is equivalent to that of TL-2, but the activity is not inhibited by the addition of 100 mM of D-GlcNAc or D-GalNAc. However, the hemagglutinating activity of TL-3 is completely inhibited by a synthetic pentasaccharide of blood group A antigen and more strongly inhibited by S-type LPS from several Gram-negative bacteria at the ranges of 5-10 ng/ml concentrations but not by the corresponding R-type LPS, indicating the high specificity of TL-3 for *O*-antigens. TL-3 contains 123 amino acid, and consists of two repeating sequences and has partial sequence similarity with a viral neuraminidase (Inamori et al., 2001).

2.13.4 Tachylectin-4 (TL-4)

Tachylectin-4 (TL-4) has been purified from hemocytes, and contains 232 amino acids, is an oligomeric glycoprotein of 470 kDa. TL-4 has more potent hemagglutinating activity against human A-type erythrocytes than has TL-2 or TL-3. The hemagglutinating activity is inhibited more strongly by bacterial S-type LPS, but not by R-type LPS lacking *O*-antigens (Saito et al., 1997). The minimum concentration of S-type LPS from *Escherichia coli* O11:B4 required for inhibiting the agglutination of human A-type erythrocytes is 160-fold lower than that of S-type LPS from *Salmonella minnesota*. The O-antigen of *Escherichia coli* O11:B4 is built up by a unique repeating unit of a main chain containing D-Gal, D-Glc, and D-GlcNAc, and a monosaccharide side chain of colitose (3-deoxy-L-Fuc) (Knirel' Iu and Kochetkov, 1994). In this monosaccharide, colitose is the most probable candidate for a specific ligand of TL-4.

2.13.5 Tachylectin-5 (TL-5)

TL-5 has been purified from hemolymph plasma using affinity chromatography of an *N*-acetyl group immobilized resin (Gokudan et al., 1999). Two fractions, named tachylectin-5A and -5B, can be separated by step-wise elution with increasing concentration of D-GlcNAc at 25mM and 250 mM, respectively. Both TL-5A and TL-5B give a distinct band with 40 kDa on SDS-PAGE under reducing conditions but TL-5A and TL-5B form oligomers in solution with molecular masses of 210 kDa and 260 kDa, respectively, as determined by gel filtration. Both of them are composed of a number of isoproteins. TL-5A and TL5B consist of 269 and 289 amino acid residues, respectively, and identity between the two lectins is 45% and the cDNA sequence of TL-5A and TL-5B indicates that they consist of a short *N*-terminal Cys-containing segment and a C-terminal fribrinogen-like domain with the highest sequence identity (51%) to that of mammalian ficolins (Gokudan et al., 1999). TL-5, however, lack the collagenous domain found in a kind of "bouquet arrangement" of ficolin and collectins. However, electron microscopy showed that TLs-5 form two- to four-bladed propeller structures. The horseshoe crab is equipped with a unique functional homologue of vertebrate fibrinogen, coagulogen, as the target protein of the clotting

cascade but importantly this coagulogen has no seqence similarity at all with vertebrate fibrinogen (Iwanaga et al., 1998).

TL-5A and TL-5B have hemagglutinating activity against A, B, and O-types of erythrocytes, and the activity is inhibited by acetyl group-containing substances including sugars, amino acid derivatives, and acetamide. Hemagglutinating activity in plasma is found to be dependent heavily on TL-5A and TL-5B. Moreover, TL-5A and TL-5B have strong bacterial agglutinating activity against both Gram-positive and Gram-negative bacteria. Therefore, TL-5A and TL-5B must be the primary lectins that recognize invading microbes in the front line of the defense system (Kawabata and Iwanaga, 1999). Interestingly, TL-5A and TL-5B can enhance the antimicrobial activity of a horseshoe crab-derived big defensin. TL-5A was detected in heart and intestine and slightly in hepatopancreas of horseshoe crab. In contrast, TL-5B was detected only in hemocytes.

Up to date several homologoues of tachylectins have been found in slime molds (Huh et al., 1998), ancient sponge (Schroder et al., 2003), hydroid (Mali et al., 2006), carp eggs (Galliano et al., 2003), salmon (Tsoi et al., 2004), zebrafish (GenBank accession number: AAM21310), amphioxus (Ju et al., 2009a), and in mangrove horseshoe crab (Low et al., 2010).

Moreover, Tachylecin5A-like genes have been found in penaeid shrimp, *P. monodon*. It is interesting that this gene was mainly expressed in the hindgut and can be induced during immersion with *V. harveyi* (Soonthornchai et al., 2010).

CHAPTER III
RESEARCH METHODOLOGY

3.1 Characterization of the full length cDNA of Tachylectin-like lectin

3.1.1 Preparation of the 5' and 3' RACE templates

Full length cDNAs of gene homologues were characterized using a SMART RACE cDNA Amplification Kit (Clontech). Template for RACE-PCR (RACE-Ready cDNA) was prepared from messenger (m) RNA purified from total RNA extracted from intestine, using a QuickPrep *micro* mRNA Purification Kit (Amersham Phamacia Biotech). One µg of purified mRNA was combined with 2 µM of 5'-CDS primer for 5'- RACE-Ready cDNA or 3'- CDS primer A for 3' -RACE-Ready cDNA, 1 µl of 10 µM SMART II A oligonucleotide (only for 5'-RACE-Ready cDNA), and nuclease-free H2O in an amount that made the final reaction volume to 5 µl. The reaction was incubated at 70 ºC for 2 minutes and immediately cooled down on ice for 2 minutes. After that, 2 µl of 5x First-Strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM) and 1 µl of Power Script Reverse Transcriptase were added. The reaction was mixed by gently pipetting and centrifuged briefly before incubated at 42 ºC for 1.5 hours in a thermocycler. The first strand reaction product was diluted with 250 µl of TE buffer and then heated at 72 ºC for 7 minutes.

3.1.2 Rapid amplification of cDNA ends (RACE) PCR

Gene-specific primers (GSPs) were designed from their corresponding homologuos sequences in the *P. monodon* EST libraries. GSPs used in the 5' - and 3' - RACE PCRs used to amplify fragments of PL5-3 gene are summarized in Table 3.1 . The PCR amplification was carried out in 25 µl reaction mixture containing, 1X advantage 2 PCR buffer, 2X universal primer A mix (UPM), 2.5 µM GSP, 0.2 mM dNTP, 1X adventage 2 polymerase mix and 1.25 µl 5' or 3' RACE -Ready cDNA template. Two negative controls containing either UPM or GSP primer were also performed to confirm the absence of nonspecific amplified products. For 5' RACE-PCR of *PL5-3* was performed by 3 cycles of 94 ºC for 45 seconds and 72 ºC for 1.30 minutes, 3 cycles of 94 ºC for 45 seconds, 68 ºC for 45 seconds and 72 ºC for 1.30 minutes,

3 cycles of 94 ºC for 45 seconds, 66 ºC for 45 seconds and 72 ºC for 1.30 minutes, 22 cycles of 94 ºC for 45 seconds, 64 ºC for 1 minute and 72 ºC for 1.30 minutes and the final extension at 72 ºC for 7 minutes. For 3'-RACE-PCR of *PL5-3*, the PCR profiles were performed by 25 cycles of denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 1 minute and extension at 72 ºC for 3 minutes. The final extension was carried out at the 72 ºC for 7 minutes.

Table 3.1 Gene-specific primers (GSPs) used for Isolation and characterization of full length cDNA

Gene	**Primer sequence (5'-3')**
5' RACE_*PL5-3*	ACTGTTTTGGTCTGATTTTCATCTCGGA
3' RACE_*PL5-3*	CTTCAGGGGCTGGGACGACTACG

3.1.3 Agarose gel electrophoresis

Appropriate amounts of agarose and 1xTBE buffer (89 mM Tris-HCl, 8.91 mM boric acid and 2.5 mM EDTA, pH 8.0) were mixed together. Agarose was completely melted by heat and allowed to become 60 ºC before poured into the gel apparatus. The gel was left at room temperature for 30-45 minutes to completely solidify. The gel was submerged in 1xTBE buffer filled in an electrophoresis chamber and the comb was gently removed.

The products were mixed with one-fourth volume of a 10x loading dye solution (0.25% bromophenol blue and 25% ficoll in water) and loaded into the gel wells. A 100 bp DNA ladder was used as standard DNA marker. Electrophoresis was carried out at 100 volts until the tracking dye migrated about three-quarter of the gel. The gel was stained with ethidium bromide (0.5μg/ml) and destained in 1xTBE buffer. Nucleic acid products were visualized under a UV transilluminator and photographed through a red filter using a Biorad gel doc machine.

3.1.4 Elution of DNA from agarose gels

The required DNA fragment was fractionated through an agarose gel in duplicate. One was run side -by-side with a 100 bp DNA marker and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stained with ethidium bromide for 5 minutes. Positions of the DNA marker and the EtBr- stained reamplified fragment were used to align the position of the non-stained target DNA fragment.

The individual DNA bands were excised from agarose gels. DNA was extracted from the gel pieces using illustra GFX PCR DNA and gel band purification kit (GE Healthcare, UK). The extraction was carried out following the manufacturer's instruction. The gel piece was incubated with 300 µl of buffer type 3 at 60 ºC for 15 minutes with briefly vortexing every 2-3 minutes. The mixture was transferred to GFX column and centrifuged at 13,000 rpm for 30 seconds and the flow-through was discarded. Next step, 500 µl of Wash buffer type 1 was added to the GFX column and centrifuged at 13,000 rpm for 30 seconds and the flow-through was discarded again. The column was centrifuged one more time to remove trace amounts of the washing buffer. The column was then placed in a new micro-centrifuge tube and 15-30 µl of elution buffer type 4 was added to the column. The column was incubated at room temperature for 2 minutes and centrifuged at 13,000 rpm for 2 minutes. The eluted sample was stored at -20 ºC until use.

3.1.5 Ligation of the PCR product to pGEM-T easy vector

The gel-purified PCR products were cloned into the pGEM-T Easy vector (Promega). The ligation reaction was set up in a total volume of 10 µl containing approximately 50 ng of the gel-eluted PCR product, 25 ng of pGEM-T easy vector, 5 µl of 2x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% polyethylene glycol), and 3 units of T4 DNA ligase. The reaction mixture was incubated at 4 ºC overnight.

3.1.6 Transformation of the ligation product to *E .coli* host cells

3.1.6.1 Preparation of competent cells

A single colony of *E. coli* JM109 was incubated in 10 ml of LB broth (1% Bactotryptone, 0.5% Bactoyeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37 ºC overnight. The 500 µl starting culture was incubated into 50 ml of LB broth and cultured at 37 ºC with vigorous shaking to an OD600 of 0.5 to 0.8. The cells were briefly chilled on ice for 10 minutes before centrifuged at 2,700 g for 10 minutes at 4 ºC. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM CaCl2) and centrifuged as above. The supernatant was discarded and the pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and divided into 100 µl aliquots. The competent cells were stored at -80 ºC until used.

3.1.6.2 Transformation

The competent cells were thawed on ice for 5 minutes. Two to four microlitres of the ligation mixture were added and gently mixed by pipetting. The mixtures were incubated on ice for 30 minutes. The reaction tube was heat-shocked in a 42 ºC water bath for 45 seconds and then immediately placed on ice for 2 – 3 minutes. One microliter of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the tube. The samples were incubated with shaking at 37 ºC for 1.5 hours. The cultured cells were collected by centrifugation at 6,000 rpm for 1 minute at room temperature. The pellet was gently resuspended in 100 µl of SOC medium and subsequently spread onto a LB agar plate containing 50 µl/ml of amplicillin, 25 µl/ml of IPTG and 20 µl/ml of X-gal. The plates were incubated at 37 ºC overnight (Sambrook and Russell, 2001). The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

3.1.6.3 Detection of recombinant cloned by colony PCR

Recombinant clones were selected by a lacZ 'system following standard protocols (Sambrook and Russell, 2001). Only white colonies containing the inserted DNA were selected. Colony PCR was performed to identify the insert sizes of positive clones.

Colony PCR was performed in a 25 µl reaction volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X – 100, 2 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.1 µM of pUC1 (5'-TTCGGCTCGTATGTTGTGTGGA-3') and pUC2 (5'-GTGCTGCAAGGCGATTAAGTTGG-3') primers and 0.5 unit of DynazymeTM DNA Polymerase (FINNZYMES). Selected colonies were individually picked by pipette tips and mixed well in the amplification reaction. The PCR profiles was predenatured at 94 ºC for 3 minutes, followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 50 ºC for 1 minutes and extension at 72 ºC 2 minutes. The final extension was carried out at the 72 ºC for 7 minutes. The colony PCR products were electrophoretically analyzed through 1.5% agarose gel and visualized after ethidium bromide staining.

3.1.6.4 Isolation and digestion of recombinant plasmid DNA

A recombinant clone was incubated into 3 ml of LB broth (1% tryptone, 0.5% yeast extract, 0.1% NaCl) containing 50 µg/ml of amplicillin and incubated at 37 ºC with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml micro-centrifuge tube and centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded and the cell pellet was collected. After that, DNA plasmids were extracted from the *E. coli* host using illustra^{TM} plasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK). The extraction was carried out according to the manufacturer's protocol. The cell pellet was resuspended with 175 µl of the Lysis buffer type 7 containing RNaseA and thoroughly mixed by vortexing. The resuspended cells were lysed by the addition of 175 µl of the Lysis buffer type 8 and mixed gently by inverting the tube 10 times. The mixture was left for 2 minutes at room temperature. After that, 350 µl of the Lysis buffer type 9 was added to neutralize the

alkaline lysis step and mixed immediately by inverting the tube 10 times. To separate the cell debris, the mixture was centrifuged at 14,000 rpm for 15 minutes. The supernatant was transferred to the illustra plasmid mini column and centrifuged at 14,000 rpm for 1 minute. The flow-through was discarded. The column was washed with 400 µl of the Lysis buffer type 9 and centrifuged at 14,000 rpm for 1 minute. After discarding the flow-through, the column was washed again with 400 µl of the Wash buffer type 1and re-centrifuged. The flow-through was discarded. The spin tube was centrifuged for 2 minutes at 14,000 rpm to remove the residual wash buffer. The illustra plasmid mini column was placed in a new 1.5 ml micro-centrifuge tube. Plasmid DNA was eluted from the column by adding 30 µl of the elution buffer type 4. The column was left at room temperature for 2 minutes and centrifuged at 14,000 rpm for 2 minutes. The concentration of extracted plasmid DNA was measured by NanoDrop 2000c UV-Vis spectrophotometers and stored at -20 ºC.

The insert size of each recombinant plasmid was examined by digestion of the plasmid with *Eco RI*. The digestion was carried out in a 12 µl reaction mix containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 3 units *Eco RI* (Promega) and 1 µl of recombinant plasmid and incubated at 37 ºC for 4 hours or overnight before electophoretically analyzed by agarose gel electrophoresis.

3.1.6.5 DNA sequencing and data analysis

The recombinant plasmid was unidirectional sequenced using the M13 forward or M13 reverse primer on an automated DNA sequencer at Macrogen (Korea). Nucleotide sequences of 5' and 3' RACE-PCR are assembled and blasted against data in the GenBank (http://www.ncbi.nlm.nih.gov/blast) using BlastN (nucleotide similarity) and BlastX (translated protein similarity). The full length cDNA was translated to amino acid sequence by ExPASy tool (http://web.expasy.org/translate) and the amino acid sequence was analysed by SMART (http://smart.embl-heidelberg.de) to predict protein domains. The pI and molecular weight of the deduced protein were analysed using Protparam (http://www.expasy.org/tools/protparam.html).

3.1.6.6 Phylogenetic analysis and homology modeling

A phylogenetic tree was constructed using the amino acid sequences of the fibrinogen-related domain (FReD) from other organisms. Sequences alignment and molecular evolutionary analyses were conducted using MEGA6 (Tamura et al., 2013). The molecular evolution was analyzed by the Neighbor-Joining method (Saitou and Nei, 1987). Internal branch support values were from analysis of 1,000 bootstrap replicates. The evolutionary distances were computed using the Poisson correction method (Zuckercandl and Pauling, 1965).

3.2 Tissue distribution analysis of gene expression

3.2.1 Sample

Juveniles shrimp (approximately 20 g body weight, 4 month-old) were obtained from local farms in Pathumthani province. They were transported to the Center of Excellence for Marine and Biotechnology (CEMB), Chulalongkorn University and acclimated under laboratory condition for 14 days before experiments were performed. Shrimp was dissected to collect the tissue including epidermis, eye stalk, gill, heart, hemocyte, hepatopancrease, anterior intestine, posterior intestine, lymphoid organ, muscle, abdominal ganglia, pleopod, stomach, thoracic ganglia, testis, and ovary. All samples were stored at -80 ºC until use.

3.2.2 RNA extraction

Total RNA was extracted from the examined tissues using TriPure Isolation Reagent (Roche Diagnosis Indianapolis, IN, USA). A piece of tissue was immediately placed in a mortar containing liquid nitrogen and was grounded to a fine powder under frozen condition. Each sample was homogenized in 1 ml of TriPure Isolation Reagent and incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. The mixtures were subjected to chloroform extraction, 200 µl of chloroform was added then vortexed for at least 15 seconds and incubated at room temperature for 15 minutes. After centrifugation at 12,000 x g at 4 ºC for 15 minutes, the mixtures were separated into the lower phenol-chloroform

phase (red), the interphase and the upper aqueous phase (colourless). The aqueous phase containing total RNA was carefully transferred to a new micro-centrifuge tube. Total RNA was precipitated with an equal volume of isopropanol and incubated at -20 ºC for 1 hour. The samples were centrifuged at 12,000 x g at 4 ºC for 10 minutes. The supernatants were removed and the RNA pellet was washed with 1 ml of 75% ethanol followed by centrifugation at 12,000 x g at 4 ºC for 10 minutes. The ethanol was removed. The RNA pellets were air-dried for 5-10 minutes. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated H2O and was immediately used. Alternatively, the RNA pellet was kept in absolute ethanol at -80 ºC until use. The quality of extracted total RNA was examined by electrophoresis on 1.0% agarose gel.

3.2.3 DNase treatment of the extracted RNA

Chromosomal DNA contamination in RNA samples were removed by treating 15 µg of total RNA with 0.5 unit of RQ1 RNase-free DNase (Promega) at 37 ºC for 30 minutes. After incubation, the samples were purified with phenol : chloroform : isoamylalcohol (25:24:1) for 10 minutes. The sample was centrifuged at 12,000 g for 10 minutes at 4 ºC, and the upper aqueous phase was collected. The extraction process was then repeated once with chloroform : isoamylalcohol (24:1), the sample was centrifuged and the aqueous phase was collected. DNA-free RNA was precipitated with one-tenth final sample volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The mixture was incubated at -80 ºC for 30 minutes. After that, the precipitated RNA was recovered by centrifugation at 12,000 g for 15 minutes at 4 ºC. The RNA pellet was washed with 1 ml of cold 75% ethanol. Alternatively, the RNA pellet was kept in absolute ethanol at -80 ºC until use.

3.2.4 Estimation of nucleic acid concentration by spectrophotometry

Concentration of nucleic acids in the extract was determined by measuring the optical density at a wavelength of 260 nm (A260). An A260 unit of 1.0 corresponds to a concentration of 50 µg/ml double stranded DNA, 40 µg/ml single stranded RNA and 33 µg/ml oligonucleotide (Sambrook and Russell, 2001). As a result, the concentration of nucleic acid was estimated in µg/ml using the following equation:

[Nucleic acid] = A260 × dilution factor × 50 or 40 or 33

(for DNA, RNA and oligonucleotide, respectively)

Protein has a maximum absorption at 280 nm .Therefore, the purity of nucleic acid could be estimated from a ratio of OD_{260}/OD_{280}. The ratios of purified RNA were appropriately 2.0 (Sambrook et al., 2001).

3.2.5 First strand cDNA synthesis

The first strand cDNA was synthesized from 1.5 µg of DNA-free RNA using an ImPromIITM Reverse Transcription System Kit (Promega). DNA-free RNA was combined with 0.5 µg of oligo (dT15) and appropriated DEPC-treated H_2O in a final volume of 5 µl. The mixtures were incubated at 70 ºC for 5 minutes and immediately placed on ice for 5 minutes. And then the reverse transcription reaction mixture (1x reaction buffer, 2 mM $MgCl_2$, 0.8 mM dNTP Mix, 20 units Recombinant RNasin® Ribonuclease Inhibitor and 1 µl of ImProm-II™ Reverse Transcriptase) were added and gently mixed. The reaction was incubated at 25 ºC for 5 minutes and at 42 ºC for 90 minutes. The reactions were terminated by incubation at 70 ºC for 15 minutes to stop reverse transcriptase activity. Quality of the synthesized first strand cDNA was examined by spectophotometry and 1.0% agarose gel electrophoresis.

3.2.6 Tissue distribution analysis by RT-PCR

Primers were designed, based on Expressed Sequence Tag (EST) sequence of homologues of known transcripts from libraries of *P. monodon*. The pairs of primers were used to amplify *PL5-1*, *PL5-2*, and *PL5-3* (Table 3.2).

RT-PCR was performed on 25 µl reaction mixtures containing 200 ng of an appropriate first strand cDNA template derived from mRNA extracted from each dissected tissue, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% TritonX-100, 2 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of an appropriate primer pair and 1 unit of DynazymeTM DNA polymerase (FINZYMES). The reaction thermal profile of each gene is shown in Table 3.2. Five µl of the amplification products were electrophoretically analyzed though a 1.5% agarose gels. Tissue distribution of the target genes was studied in reference to that of a house- keeping gene, *Elongation*

factor-1α (*EF-1α*). The PCR profiles of *PL5-1*, *PL5-2* and *PL5-3* were performed by predenaturing at 94 ºC for 30 seconds, followed by 35 cycles of 94 ºC for 30 seconds, 60 ºC for 1 minute and 72 ºC for 3 minutes. The final extension was carried out at the 72 ºC for 7 minutes. The results were analyzed by agarose gel electrophoresis.

Table 3.2 Primer sequence and expected sizes of the PCR products for RT-PCR

Gene	Gene Bank accession No.	Primer sequence (5' to 3')	Product size (bp)
PL5-1	HC-N-N01-1087-LF,	F :GGCCTGCAGGAGATGAGAAT	386
	HC-N-N01-11852-LF	R :AATGCCGGCCTTATCATCA	
PL5-2	GlEp-N-N01-1607-LF	F :GCCAGAGCCTGAACCAGATCC	350
		R :TGTCCGTGCCAATCATACCAG	
PL5-3	HPa-N-N03-2032-LF	F :AACTACCTTTCGAGCGGCAT	493
		R: GGTTGGCGTGCAAACAGTT	

3.3 Expression of immune-related genes in *Vibrio harveyi* and AHPND challenged shrimp

3.3.1 Experimental animals

Juvenile shrimp (approximately 20 g body weight) were obtained from a commercial shrimp farm in Pathumthani province and transported to Center of Excellence for Marine and Biotechnology (CEMB), Chulalongkorn University. They were acclimated in tanks containing seawater at 16 ppt salinity with constant aeration for 2 weeks before the experiments were performed.

Adult *Artemia* were purchased from Sunday Market at Chatuchak, Thailand. They were acclimated in plastic tanks containing seawater at 30 ppt salinity with aeration overnight before used in the experiments.

3.3.2 Bacterial preparation

V. harveyi, previously isolated from wounded *P. monodon* or *V. parahaemolyticus* (3HP), isolated and identified by Centex Shrimp, Faculty of Science, Mahidol University were cultured in tryptone soya broth (TSB) supplemented with sterile 2% (W/V) NaCl and were incubated with shaking at 250 revolutions min-1 at 28^{o}C for 16 hours. After that, bacteria cells were washed twice using sterile 2% NaCl by centrifugation at 3500xg for 10 minutes at 4^{o}C. Cell pellets were resuspended in sterile 2% NaCl and then adjusted to an absorbance of 2.0 at OD 600 nm to reach approximately a concentration of 10^8 CFUs/ml.

3.3.3 Challenge test for *V .harveyi*

Shrimp were each injected intramuscularly with 100 µl of bacteria (final concentration is 10^7 cells) into the third abdominal segment. As a negative control, shrimp were injected intramuscularly with 100 µl of sterile 2% NaCl only. Four shrimp were collected at 2, 12 and 24 hours after bacteria injection. Shrimp at 2 hours without bacterial injection served as controls. To collect shrimp hemocytes, the hemolymph was bled from the ventral sinus of each shrimp using a 24 G/1/2 inch needle fitted into a 1.0 ml syringe preloaded with 500 µl of anticoagulant (10% sodium citrate, w/v). The samples were immediately centrifuged at 3,600xg at 4 ºC for 5 minutes to separate hemocytes from the plasma. The hemocyte pellets were resuspended in 1 ml of TriPure Isolation Reagent (Roche Diagnosis Indianapolis, IN, USA) homogenized and kept at -80 ºC until use. The anterior intestine and posterior intestine were dissected at the time point and they were immediately frozen in liquid nitrogen and kept at -80 ºC until used.

3.3.4 Orally challenge test for AHPND

3.3.4.1 Artemia preparation

Artemia were allowed to filter feed on *V. parahaemolyticus* (3HP) suspension for 30 minutes before they were presented to the shrimp .To estimate the bacterial load of the *Artemia* used for challenge, ten *Artemia* was homogenized in 1 ml of 2% NaCl and diluted for plate count onto TCBS agar (Oxoid).

3.3.4.2 Sample condition

Individual shrimp was placed in 5 L plastic boxes containing 1.5 L of 16 ppt salinity seawater. Each shrimp was fed with 20 *Artemia*, one Artemia was subsequently found to contain approximately 10^7 CFUs of bacteria, so each shrimp received approximately $6x10^8$ CFUs of bacteria/shrimp. Six shrimp were collected at 3, 6 and 12 hours after bacterial infection and then they were immediately frozen in liquid nitrogen and kept at -80 ºC until used. For control group, shrimp were fed with non- treated *Artemia*.

3.3.5 Total RNA extraction and the first strand cDNA synthesis

Total RNA were extracted from each dissected tissue and synthesized the first strand using the same method as described previously .

3.3.6 Quantitative real-time PCR

Expression levels of *PL5-1*, *PL5-2* and *PL5-3* were examined by quantitative Real-time PCR.

3.3.6.1 Construction of standard curve

Standard curve of each gene was constructed using the DNA segment covering the target PCR product and *EF-1α* and were amplified from primers for quantitative real-time PCR. The PCR products were cloned and Plasmid DNA was extracted and used as template for estimation of the copy number. A 10 fold -serial dilution was prepared corresponding to 10^3- 10^8 molecules/µl. The copy number of standard DNA molecules can be calculated using the following formula:

X g/µl DNA / (plasmid length in bp x 660) x 6.022 x 10^{23} =Y molecules/µl

The standard curves (correlation coefficient = 0.995-1.000 or efficiency higher than 95%) were made for each run. The standard samples were carried out in a 96 well plate and each standard point was run in duplicate.

3.3.6.2 Quantitative real-time PCR

The target transcripts and the internal control *EF- 1α* of synthesized cDNA were amplified in a reaction volume of 10 µl using 2x LightCycler® 480 SYBR Green I Master (Roche, Germany). The specific primers (Table 3.3) were used at a final concentration of 0.3 µM. The thermal profile for SYBR Green real-time PCR was 95 ºC for 10 minutes followed by 40 cycles of 95 ºC for 15 seconds, 58 ºC for 30 seconds and extension at 72 ºC for 30 seconds. Cycles for melting curve analysis were carried out at 95 ºC for 15 seconds, 65 ºC for 1 minute and at 98 ºC for continuation and cooling was 40 ºC for 30 seconds. The real-time PCR assay was carried out in a 96 well plate and each sample was run in duplicate using a LightCycler® 480 Instrument II system (Roche).

3.3.6.3 Statistical analysis

A ratio of the absolute copy number of the target gene and that of EF-1α was calculated. The relative expression levels between groups of sample were statistically tested using one - way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Significant comparisons were considered when the P value was < 0.05.

Table 3.3 Primer sequences and expected sizes of the PCR products for quantitative real-time PCR analysis

Gene	Primer sequence (5' to 3')	Product size (bp)
PL5-1	F :GCT CAA AGT CGG CAG GTA CAA	201
	R :CTC TCG TGC TCG CCT TCA TAA	
PL5-2	F :AGA CTC CTT CAC TAC CCA CGG	201
	R :TGT CCG TGC CAA TCA TAC CAG	
PL5-3	F :TGA AGT TCT CCA CCT ACG ACC A	200
	R :GCT CTT GAG GGA GTG TTT GAC G	
EF 1 alpha	F :TCCGTCTTCCCCTTCAGGACGTC	218
	R :CTTTACAGACACGTTCTTCACGTTG	

3.4 *In vitro* expression of recombinant proteins using a bacterial expression system

3.4.1 Cloning of ORF into a cloning vector (pGEM-T easy)

ORF of the desired genes were amplified from first strand cDNA derived from intestine. Primers for the amplifications were designed according to the derived full-length cDNA obtained from above and are shown in Table 3.4. Amplification products were ligated and cloned into pGEM-T easy vector. The ligation products were transformed into E. coli JM109. Plasmid DNA of the positive clone was sequenced to confirm their sequences and orientation of the inserts. The ORF-pGEM-T constructs were then used as templates for amplification of the inserts to be cloned into the expression vectors in the next step.

Table 3.4 Primer sequences used for ORF amplification of *PL5-1, PL5-2* and *PL5-3* in *P. monodon*

Gene	Primer sequence (5' - 3')
PL5-1	F :ATGGCGCTCTTGCACAAGTTCA
	R :TCAGAATGCCGGCCTTATCATC
PL5-2	F :ATGAGCCCACCGGCAATAGC
	R :CTAGAACTTTGGTTTGATCTTCATAGTC
PL5-3	F :AATGATTCGACTCGTGATCCC
	R :TTCTTTTGGATTTACTGTTTTGG

3.4.2 Cloning of recombinant expression plasmids

Primers were designed to include an appropriate restriction site before and after the start and stop codons, respectively and the signal sequences were excluded (Table 3.5). The thermal profiles were pre-denaturation at 94 ºC for 2 minutes followed by 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds and extension at 72 ºC for 2 minutes and final extension at 72 ºC for 7 minutes.

The amplified products were analyzed by agarose gel electrophoresis and the gel eluted product was digested with restriction enzyme that was specific to the introduced restriction sites. The digested products were then analyzed by agarose gel electrophoresis and eluted from the gel. The purified digested *PL5-1* and *PL5-3* product were ligated into pET32a (Invitrogen) while purified digested *PL5-2* products were ligated into pET15b (Invitrogen). The ligation products were transformed into *E. coli* JM109. The plasmid constructs were purified from the transformed *E. coli* JM109 cultures and sequenced. Plasmids of the positive clones were subsequently transformed into an expression host, *E. coli* BL21 (DE3) codon+RIPL (Stratagene).

Table 3.5 Primer sequences used for cloning of the expression plasmids of recombinant protein

Gene	Restriction site	Primer sequence (5' - 3')
PL5-1	*Bam* HI	F :TTTGGATCCACAACAGAACGAACAGATACCG
	Xho I	R :TTTCTCGAGTCATAATGCCGGCCTTATCATC
PL5-2	*Nde* I	F :TTTCATATGCTCGTGGGAAACGAAACCGTGCTC
	Bam HI	R :TTTGGATCCCTAGAACTTTGGTTTGATCTTCAT
PL5-3	*Bam* HI	F :TTGGATCCCTCCCGCCGCCCGACGCGA
	Xho I	R :TTCTCGAGCTACTGTTTTGGTCTGATTTTCA

3.4.3 Expression of recombinant proteins

A single colony of recombinant *E. coli* BL21 (DE3) codon+RIPL carrying desired recombinant plasmid was selected for expression. Cells were grown in LB medium, containing 50 µg/ml amplicillin and 50 µg/ml chloramphenicol at 37 ºC with shaking. Fifty microlitters of overnight cultured was transferred to 50 ml of LB medium containing 50 µg/ml amplicillin and 50 µg/ml chloramphenicol and further incubated to an OD_{600} of 0.4-0.6. Protein expression was induced with isopropyl-beta-D-thiogalactopyrannoside (IPTG) at a final concentration 0.5 mM. Samples were taken at 0, 1, 2, 3, 6 hours and overnight after induction with IPTG and centrifuged at 1200 x g for 1 minute. The pellets were resuspended with 1x PBS and 2x SDS-PAGE sample loading buffer before examined by 12-15% SDS-PAGE (Laemmli, 1970).

In addition, 20 ml of the IPTG induced-cultured cells at the most suitable time-intervals were taken (6 hours or overnight at 37 ºC or lower) were collected by centrifugation 5,000 rpm for 15 minutes and resuspened in 1x PBS buffer and addition lysis buffer (0.05 M Tris-HCL; pH 7.5, 0.5 M Urea, 0.05 M NaCl, 0.05 M EDTA; pH 8.0 and 1 mg/ml lysozyme). The cell walls were broken by sonication using Digital Sonifier sonicator Model 250 (BRANSON). The bacterial suspension was sonicated

2-3 times at 10-30% amplitude, pulsed on for 10 seconds and pulsed off 10 seconds in a period of 2-5 minutes. Soluble and insoluble portions were separated by centrifugation at 14,000 rpm for 30 minutes. The protein concentration of both portions was measured using a dye-binding assay (Bradford, 1972). Expression of the recombinant protein was electrophoretically analyzed by 12-15% SDS-PAGE.

3.4.4 Western blotting

The recombinant protein was analyzed in 12-15% SDS-PAGE. The electrophoresed proteins were transferred to PVDF membrane (Hybind P; GE Healthcare). The blotted membranes were washed three times with 1xTBST (0.5% Tween20) for 5 minutes, blocked with blocking buffer (5% BSA in 1xTBST) and incubated overnight at room temperature with gentle shaking. The membrane was washed three times in 1xTBST and incubated with 1:5000 dilution of an appropriate primary antibody, Mouse Anti-His (GE Healthcare) in blocking buffer (1% BSA in TBS) for 1 hour. After that, the membrane was washed three times in 1xTBST and incubated with 1:10,000 of Goat Anti-mouse-IgG-AP conjugate for 1 hour and the membrane was washed three times in 1xTBST. Visualization of immunoreactive signals was carried out by incubating the membrane in BCIP/NBT (Promega) as a substrate. The colour reaction was stopped by transferring the membrane into water.

3.4.5 Protein purification

3.4.5.1 Protein sample preparation

Protein samples were obtained from 800 ml of IPTG-induced culture prepared as above described. The cells were harvested by centrifugation at 5,000 rpm for 15 minutes. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). Samples were incubated on ice with lysis buffer for 30 minutes before they were sonicated. After that, DNA was removed by an on ice incubation with 0.03 mg/ml DNase supplemented with 1 mM $MgCl_2$. To separate the soluble or insoluble protein fractions, samples were centrifuged at 14,000 rpm for 30 minutes. Fractions containing the target expressed proteins were selected for the next purification process.

3.4.5.2 His-Tag/Ni affinity system

Recombinant PL5-2 tagged with 6 histidine residues subjected to the Ni purification system using a His GraviTrap column (GE Healthcare), containing Ni ion bound to the beads. The column was equilibrated with 10 ml of binding buffer before loading the insoluble fraction composed of the recombinant resuspended in binding buffer into the column twice. After that, the column was washed with binding buffer containing 20 mM imidazole (20 mM sidoum phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4), 5 ml of the binding buffer containing 40 mM imidazole (20 mM sodoum phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4), 5 ml of the binding buffer containing 80 mM imidazole (20 mM sodoum phosphate, 500 mM NaCl, 80 mM imidazole, pH 7.4). The recombinant protein was eluted with 6 ml of the elution buffer (20 mM sodoum phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Each fraction of the washing and eluting step were analyzed by SDS-PAGE and western blotting. The purified protein was stored at 4 ^{0}C or -20 ^{o}C for long term storage.

3.4.5.3 Polyclonal antibody production

The purified PL5 protein was concentrated and loaded into 12.5% SDS-PAGE. The specific size of protein on gel was cut and purified using Model 422 Electro-Eluter (Bio-Rad). Polyclonal antibody in rabbits against the purified proteins was made by the Faculty of Associated Medical Sciences, Chiangmai University. Western blot analysis was carried out to examine specificity and sensitivity of each antibody.

3.5 Localization of PL5-2 protein

3.5.1 Sample collection

Gills, heart, anterior intestine, posterior intestine, stomach and epidermis of juvenile *P. monodon* were separately dissected and homogenized in the sample buffer (50 mM Tris-HCl; pH 7.5, 0.15 M NaCl) supplemented with a proteinase inhibitor cocktail (EDTA free; Roche). The homogenate was centrifuged at 12,000 g

for 30 minutes at 4°C. The supernatant was transferred to a new tube. To collect shrimp hemocytes, the hemolymph was bled from the ventral sinus of each shrimp using a 24 G/1/2 inch needle fitted into a 1.0 ml syringe. The samples were immediately centrifuged at 3,600 g at 4 ºC for 5 minutes to separate hem ocytes from the plasma. The hemocytes were homogenized in sample buffer and then centrifuged at 1500 g for 15 min, and the resulting supernatant was used as hemocyte lysate (HLS).

3.5.2 Detection of PL5-2 protein in shrimp tissues by western blot analysis

Protein concentrations of the tissue extracts were determined by the dye binding method. Twenty-five micrograms of proteins were heated at 100°C for 5 min. The protein samples were loaded into 12.5% SDS-PAGE and transferred to a PVDF membrane. The localization of PL5-2 protein in various tissues was detected by western blotting as mentioned in section 3.4.4, but the first and second antibody were changed to rabbit anti PL5-2 (1 : 500) and goat anti rabbit (1 : 3,000), respectively.

3.5.3 Detection of PL5-2 protein in shrimp tissues by immunohistochemistry

Stomach, hepatopancreas, anterior intestine and posterior intestine of juvenile *P. monodon* were dissected and fixed with 4 %paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 hours at 4 ºC. The tissue samples were washed 3 times with 0.1 M phosphate buffer and washed twice with 50% ethanol. The tissue samples were stored in 70 %ethanol at 4 ºC until used.

Tissue samples were twice dehydrated through ethanol series (70%, 85%, 95% and 100% ethanol), cleared in Bioclear (Bio Optica, Milan, Italy) for 50 minutes. The paraplast was allowed to infiltrate into the tissue at 60 ºC for 1.5 hours and then embedded in paraplast. Paraplast blocks were trimmed and sectioned at 6 µm thickness and heated slides on slide warmer at 45 ºC overnight to fix. All slides were kept in -20 ºC until used.

Immunohistochemistry was performed using NovoLink Polymer Detection System (Leica microsystem, UK), the sample slides were de-paraffinized in toluene and rehydrated through ethanol series for 3 minutes twice (100%, 95%, 70% and 50% ethanol). All slides were washed in running tap water, then incubated in

Peroxidase Block for 5 minutes and washed twice in TBS buffer for 5 minutes. The section slides were incubated with Protein Block for 5 minutes and washed twice in TBS buffer for 5 minutes. After treatment, the section slides were incubated with 1:100 of anti-PL5-2 or pre-immunized for 1 hour and washed twice in TBS buffer for 5 minutes. The slides were incubated with Post Primary block for 15 minutes and washed twice in TBS buffer for 5 minutes. The slides were incubated with NovoLink Polymer (rabbit IgG primary antibody) for 15 minutes and washed twice in TBS buffer for 5 minutes. Localization of antigen was visualized using DAB working solution for 5 minutes and then the slides were rinsed in tap water. The slides were stained with hematoxylin for 5 minutes then rinsed in tap water for 5 minutes. Finally, the slides were dehydrated, mounted and observed under light microscopy.

3.6 Purification of PL5-2 protein from the *P. monodon plasma* for biological functional studies

3.6.1 Shrimp plasma preparation

The hemolymph was bled from the ventral sinus of each shrimp using a 24 G/1/2 inch needle fitted into a 1.0 ml syringe, then the hemolymph was immediately centrifuged at 3,500xg at 4 ºC for 5 minutes to separate the plasma from hemocytes. Shrimp plasma was stored at -80 °C until used.

3.6.2 Purification of PL5-2 protein

The *N*-Acetyl-D-glucosamine immobilized bead column (Sigma) was equilibrated with 10 ml of equilibration buffer (20 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0). Twenty microliter of shrimp plasma was applied to the column 5 times. The unbound plasma components were removed from the column with washing buffer (20 mM Tris-HCl, 10 mM $CaCl_2$, 500 mM NaCl, pH 8.0). Fractions of 1 ml were collected and the optical density measured at 280 nm until A280 of the effluent reached zero. The PL5-2 bound from immobilized bead was eluted with 15 mM GlcNAC in equilibration buffer. The fractions showing absorbance at 280 nm was collected and dialyzed with dialysis buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) at 4^{o}C overnight to remove

GlcNAC. Each fraction of the washing and eluting step was analyzed by SDS-PAGE electrophoresis and western blotting. The purified protein was stored at -20°C for long term storage.

3.7 Biological functions studies of PL5-2 protein

3.7.1 Hemagglutination assay

Human erythrocytes type A, B and O was withdrawn from healthy donors. Erythrocytes will be washed three times with 0.85% NaCl by centrifugation at 2500 g for 5 minutes at 4^{o}C. The 2% suspension of human erythrocytes was prepared in TBS buffer supplemented or not supplemented with $CaCl_2$ (20 mM Tris-HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5). Twenty-five microliter of purified protein of 2-fold serial dilutions in the same buffer was mixed with 25 µl of 2% suspension of human erythrocytes in a 96-well microtiter U plate. The mixtures were incubated at 37^{o}C for 1 hour and then hemagglutinating activity was observed. To determine whether calcium was required for PL5-2 protein to agglutinate human erythrocytes, each human erythrocytes prepared in TBS buffer supplemented with 10 mM $CaCl_2$ was incubated with 100 mM EDTA before added the purified protein.

3.7.2 Binding of PL5-2 protein to bacterial cells

3.7.2.1 Bacteria preparation

Gram-positive bacteria (*Bacillus megaterium* Bm11, *Micrococcus luteus* MIII, *Staphylococcus aureus* Cowan1 and *Streptococcus equi* 62), Gram- negative bacteria (*Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas aeruginosa* OT97, *Vibrio parahaemolyticus* and *Vibrio harveyi*) and other *Vibrio spp*. (*V. harveyi*, *V. parahaemolyticus*, *V. nigripulchritudo*, *V. rotiferianus*, *V. natriegens*, *V. minicus* and *V. neptunius*) were used to test the binding capacity and specificity of PL5-2. Each bacteria was separately cultured in tryptone soya broth (TSB, Oxoid) supplemented or not supplemented with sterile 2% NaCl (W/V) overnight and washed twice using sterile 0.85 or 2% NaCl by centrifugation at 3500 g for 10 minutes at 4 ^{o}C.

3.7.2.2 Bacterial binding assay

Thirty milligrams of each microorganism was incubated with 200 µl of shrimp plasma together with 10 mM $CaCl_2$ with shaking at room temperature for 1 hour. Bacterial cells were washed three times with NaCl solution by centrifugation at 3500 g for 10 min at 4 ^oC. The protein bound on the cell pellet was eluted with 4M urea in 10 mM Tris-HCl with shaking at room temperature for 15 min and centrifuged at 3500 g for 10 min at 4^oC. Eluted proteins were concentrated by acetone precipitation by incubation at -20 ^oC for 2 hours and resuspended in 4 M urea. The binding of PL5-2 protein in plasma to different microorganisms and the purified recombinant protein after binding to microorganism were examined by western blot analysis using polyclonal antibody against PL5-2 protein using the same method as described previously.

3.7.3 Bacterial agglutination assay

A. hydrophila, *V. harveyi*, *V. parahaemolyticus* (3HP) and *M. luteus* were used for agglutination assay. Each bacterial was separately cultured in TSB supplemented or not supplemented with sterile 2% (W/V) for 8 hours and washed twice using sterile 0.85 or 2% NaCl by centrifugation at 3500 g for 10 minutes at 4 ^oC. Bacterial cells were resuspended in TBS at 10^8 CFU/ml. Ten microliters of each bacteria suspension were incubated with 20 µl of purified PL5-2 protein in TBS in presence or absence of 10 mM $CaCl_2$ containing different concentrations (3.12-200 µg/ml) of purified protein. The mixture was incubated at room temperature for 1 hour and the agglutinating reaction was observed by microscopy. BSA was used as the control group.

3.7.4 Antimicrobial activity assay

A. hydrophila was used for the assay. Bacterial cells were cultured in TBS broth for 8 hours. The microorganism was washed 2 times with TBS buffer (20 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl2, pH 7.5) by centrifugation at 3500 g for 10 minutes at 4 ^oC and resuspended in TBS buffer supplemented with 10 mM $CaCl_2$ to approximately a concentration of 10^4 CFU/ml. Fifty microliters of bacteria was added to 50 µl of TBS buffer containing different concentrations (12.5-200 µg/ml) of purified

protein and 10 mM $CaCl_2$. BSA was used as the control group. The mixture was incubated at 25 ºC for 2 hours. The antimicrobial activity was determined by plate count on TSB agar plate after incubation at 28 ºC.

3.7.5 Sugar binding specificity

M .luteus, bacteria where we have found PL5-2 protein bound to the cell surface was selected and cultured in TSB broth overnight. Approximately 30 mg of bacterium was washed twice using sterile 0.85% NaCl by centrifugation at 3500 g for 10 min at 4ºC. Ten mM of mannose-6-phosphate, D-glucose, N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc), D-galactosamine hydrochloride or N-acetyl neuraminic acid was resuspended in 200 µl of purified protein and separately incubated for 30 min at room temperature then the mixtures were added together with 10 mM $CaCl_2$. The bacterial pellet was resuspended with the mixtures and incubated with rotation for 1 hour at room temperature. As a control, the bacterial pellet was incubated with 0.85% NaCl and subjected to the same treatment. Each microorganism was pelleted, and then washed 3 times with sterile 0.85% NaCl by centrifugation at 3500 g for 10 minutes at 4 ºC and resuspended in 200 µl of 0.85% NaCl. The final washed pellet was subjected to elution for 15 min by mild agitation in 4M urea in 10 mM Tris-HCl, pH 7.5. Eluted proteins were concentrated by acetone precipitation by incubation at -20 ºC for 2 hours and resuspended in 4 M urea. Then the binding of interesting protein to bacterial cell surface was detected by SDS-PAGE electrophoresis and immunoblotting using polyclonal antibody against PL5-2.

3.8 The effect of Penlectin gene silencing

3.8.1 Generation of dsRNA

A T7 promoter sequence (5'- TTATACGACTCACTATAGGG -3') was incorporated into gene specific primers for PL5-1, PL5-2 and PL5-3 at their 5' ends (PL5-1_RNAi-F/R, PL5-2_RNAi -F/R and PL5-3_RNAi -F/R) (Table 3.6) and used to amplify PCR products as the templates for dsRNA synthesis. GFP dsRNA was synthesized as a control using GFP_RNAi-F/R primer (Table 3.6).PL5-1 amplification

was performed by predenaturing at 95 ºC for 2 minutes, 5 cycles of 95 ºC for 30 seconds, 60 ºC for 45 seconds and 72 ºC for 1 minutes, 30 cycles of 95 ºC for 30 seconds, 58 ºC for 45 seconds and 72 ºC for 1 minutes and the final extension at 72 ºC for 7 minutes. PL5-2 amplification was performed by predenaturing at 95 ºC for 2 minutes, 5 cycles of 95 ºC for 30 seconds, 64 ºC for 45 seconds and 72 ºC for 1 minutes, 25 cycles of 95 ºC for 30 seconds, 62 ºC for 45 seconds and 72 ºC for 1 minutes and the final extension at 72 ºC for 7 minutes. PL5-3 amplification was performed by predenaturing at 95 ºC for 2 minutes, followed by 35 cycles of 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 1.30 minutes and the final extension at 72 ºC for 7 minutes. GFP amplification was performed by predenaturing at 95 ºC for 2 minutes, followed by 35 cycles of 95 ºC for 30 seconds, 58 ºC for 30 seconds and 72 ºC for 1.30 minutes. The final extension was carried out at the 72 ºC for 7 minutes. To generate dsRNA, 1 µg PCR product was purified by gel extraction (GE Healthcare, UK) and used as a template in vitro transcription using a MegaScript RNAi kit (Ambion, USA). The reaction mixture (nuclease-free water to 20 µl, 1 µg of a template with opposing T7 promoters flanking the transcription region, 2 µl 10X T7 reaction buffer, 2 µl ATP solution, 2 µl CTP solution, 2 µl GTP solution, 2 µl UTP solution and 2 µl T7 enzyme mix) was prepared and incubated at 37 ºC overnight. The annealed dsRNA was treated with DNase to remove DNA at 37 ºC for 15 minutes. The reaction was stopped and precipitated the dsRNA by adding 30 µl nuclease-free water and 30 µl LiCl precipitation solution and incubated at -20ºC for 30 minutes. The precipitated dsRNA was recovered by centrifugation at 12,000 g for 15 minutes at 4 ºC. The dsRNA pellet was washed twice with 1 ml of cold 70% ethanol. The concentration of dsRNA was measured by NanoDrop 2000c UV-Vis spectrophotometers and stored at -80 ºC.

3.8.2 dsRNA quantitation

One microgram of dsRNA was used for each tube. First tube, dsRNA was incubated with 0.01 µg RNaseA at 37 ºC for 30 minutes. Second tube, dsRNA was denatured at 95 ºC for 5 minutes. For the third tube, dsRNA was denatured at 95 ºC for 5 minutes before addition of 0.01 µg RNaseA and then incubated at 37 ºC for 30 minutes. dsRNA with no addition of RNaseA or heated was used as a control. The

digestion was analyzed on 1% agarose gel. The dsRNA should be resistant to RNaseA digestion.

3.8.3 dsRNA *in vivo* study

3.8.3.1 Experiment animal

P. monodon (mean body weight 2–3 g/shrimp) were obtained from Broodstock Multiplication Center, Faculty of Marine Technology, Burapha University Chanthaburi Campus, Chanthaburi, Thailand and transported to Center of Excellence for Marine and Biotechnology (CEMB), Chulalongkorn University. They were acclimated in tanks containing seawater at 15 ppt salinity with constant aeration for 2 weeks before experiments were performed.

Adult *Artemia* were purchased from Sunday Market at Chatuchak, Thailand. They were acclimated in plastic tanks containing seawater at 30 ppt salinity with aeration overnight before performed experiment.

Table 3.6 Primer sequences used for dsRNA amplification of *PL5-1*, *PL5-2* and *PL5-3* in *P. monodon*

Primer	Primer sequence (5' - 3')
PL5-1_RNAi	F :TAATACGACTCACTATAGGGGGCCTGCAGGAGATGAGAAT
	R :TAATACGACTCACTATAGGGAATGCCGGCCTTATCATCA
PL5-2_RNAi	F :TAATACGACTCACTATAGGGGCCAGAGCCTGAACCAGATCC
	R :TAATACGACTCACTATAGGGTGTCCGTGCCAATCATACCAG
PL5-3_RNAi	F :TAATACGACTCACTATAGGGAACTACCTTTCGAGCGGCAT
	R :TAATACGACTCACTATAGGGGGTTGGCGTGCAAACAGTT
GFP_RNAi	F :TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT
	R :TAATACGACTCACTATAGGGTTCTTGTACAGCTCGTCCATGC
PL5-1	F :CGGGACAACAGAACGAACAG
	R :TGCGCAATGGACAGCTGATC
PL5-2	F :CTTGCTGTTCCTCGTGACCT
	R :AAGTCCTCCTGCTCAGCGTA
PL5-3	F :TCGGCGAAGACAACGAGAAC
	R :ACGTAGATGCCGCTCGAAA

3.8.3.2 Bacterial and *Artemia* preparation

V. parahaemolyticus (3HP), was prepared as mentioned in section 3.3.2. *Artemia* were allowed to filter feed on *V. parahaemolyticus* (3HP) suspension for 15 minutes before they were presented to the shrimp. To estimate the bacterial load of the Artemia used for challenge, five Artemia was homogenized in 1 ml of 2% NaCl and diluted for plate count onto TCBS agar (Oxoid).

3.8.3.3 Orally challenge test for AHPND pathogenesis

Individual shrimp was placed in 3 L plastic box each containing 1 L of 15 ppt salinity seawater .

3.8.3.3.1 Survival rates

Shrimp were divided into five groups with 10 shrimp in each group. Each shrimp was injected with 5 µg dsRNA/g of shrimp wet body weight of *PL5-1*, *PL5-2*, *PL5*-3 or control *GFP* dsRNA dissolved in 25 µl of saline buffer (150 mM NaCl) into the third abdominal segment using 0.5 ml insulin syringes with 29-gauge needle. Control shrimp were injected with saline buffer only. The injection of dsRNA (5 µg/g of shrimp wet body weight) was repeated at 24 hours after first injection and each shrimp was fed with 5 Artemia, one Artemia was subsequently found to contain approximately 10^7 CFUs of bacteria, so each shrimp received approximately $6x10^8$ CFUs of bacteria/shrimp. The survival rates were calculated at 48 hours after second dsRNA injection. Survival and moribund shrimp were fixed with Davidson's fixative by injection into the third abdominal segment and hepatopancreas of shrimp for histopathology study. The samples were fixed for 48 hours and changed to 70% ethanol for long-term preservation.

3.8.3.3.2 Histopathology of AHPND infected shrimp

Shrimp were divided into three groups with 3 shrimp in each group and individual shrimp was placed in 3 L plastic boxes containing 1 L of 15 ppt salinity seawater. First group, each shrimp (5 µg/g of shrimp wet body weight) was injected with PL5-1, PL5-2 and PL5-3 dsRNA each, and GFP dsRNA was injected in the second group (5 µg/g of shrimp wet body weight), and saline buffer was injected in the third group. After 24 hours, a second injection of dsRNA or saline buffer was performed and shrimp were fed with Artemia as mentioned in section 3.8.3.3.1. All shrimp were fixed with Davidson's fixative by injection into the third abdominal segment and hepatopancreas of shrimp and soaked into Davidson's fixative for 48 hours then changed to 70% ethanol for long-term preservation. Tissue samples were dehydrated through ethanol series, cleared in chloroform and embedded in paraplast. Paraplast blocks were trimmed and sectioned at 5 µm thickness and tissue sections were

stained with a Mayer's hematoxylin and Eosin (H&E). The slide sections were examined under light microscopy.

CHAPTER IV
RESULTS

4.1 Isolation and characterization of the full length cDNA of *Penlectin5*

Full length sequence of PL5-3 gene was determined using Rapid Amplication of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) technique. Based on the initial partial cDNA sequence of these target genes, gene specific primers were then designed and used for RACE reactions to obtain the 5' ends of the cDNA, and the complete cDNA was assembled from multiple overlapping clones. The complete sequences were confirmed by re-amplifying the whole obtained sequences, using gene-specific primers.

The full length cDNA of *PL5-3* was 1,589 bp with an open reading frame (ORF) of 921 bp encoding a protein of 306 amino acids. The 5' and 3' UTRs were at 30 and 638 bp, respectively and the poly A additional signal (AATAAA) was located between 1,540 – 1,545 of the entire sequence (Figure 4.1). This transcript had closest similarity to Tachylectin 5B gene (*TL5B*) of japanese horseshoe crab, *Tachypleus tridentatus* (E-value = 1e-64). The deduced amino acid sequence of PL5-3 contains a putative signal peptide of 16 residues and a fibrinogen related domain (FReD) located at position 91-305 of the deduced protein sequence. The PL5-3 contains a potential N-glycosylation motif, NAT (Asp57 – Ala58 – Thr59). The calculated molecular mass of the mature PL5-3 protein is 32.6 kDa, with an estimated isoelectric point (pI) of 6.2.

The complete full length cDNA of PL5-1 and PL5-2 was previously determined (Angthong, 2010). The *PL5-1* was composed of 1,288 bp with an ORF of 939 bp encoding a protein of 312 am ino acids (Figure 4.2). PL5-1 contains a putative signal peptide of 18 residues and a FReD located at position 91 – 311 of the deduced protein. PL5-1 deduced amino acid sequence contains a N-glycosylation site NGT motif (Asp^{224} – Gly^{225} – Thr^{226}). This lectin shows 42% amino acid similarity to Tachylectin 5A (TL5A) of japanese horseshoe crab, *T. tridentatus*. The calculated molecular mass of the mature PL5-1 protein is 33.3 kDa, with an estimated isoelectric point (pI) of 5.7. The PL5-2 consists of 1,139 bp with an ORF of 768 bp corresponding to a deduced protein of 255 amino acids (Figure 4.3). PL5-2 contains a putative signal peptide of 20

residues and a FReD located at position 36 – 254 of the deduced protein. This deduced amino acid sequence contains an N-glycosylation site NET motif (Asp^{24} – Glu^{25} – Thr^{26}). The PL5-2 shares 46% amino acid similarity to TL5B. The calculated molecular mass of the mature PL5-2 protein is 27.2 kDa, with an estimated isoelectric point (pI) of 5.0.

```
1       GCTTGAGAGAAGAAGTCTTTTCAGGCAAAAATGATTCGACTCGTGATCCCCCTCATCACG
1                                       M  I  R  L  V  I  P  L  I  T
61      GCCGCTTTGGTGGCCGCCCTCCCGCCGCCCGACGCGAACGTCAACATCGAGTGGCCCGGA
11       A  A  L  V  A  A  L  P  P  P  D  A  N  V  N  I  E  W  P  G
121     GACGTTCACGTGAAAGCCCAGGGAAAGACCCTCATCGTCCTCCTCGGCGAAGACAACGAG
31       D  V  H  V  K  A  Q  G  K  T  L  I  V  L  L  G  E  D  N  E
181     AACATCAGAGTCAACATCAACGCCACCCTCATTGATGGCGACAGCGGCCTCGTCCCGCCC
51       N  I  R  V  N  I  N  A  T  L  I  D  G  D  S  G  L  V  P  P
241     ACGCCCGCGCCCACGCCCGCGCCCACGGCTCCTCCCTCTCAGCCACCGCCCGCGCCCGTC
71       T  P  A  P  T  P  A  P  T  A  P  P  S  Q  P  P  P  A  P  V
301     ATCTCGGAGAAGAACTGCCTCGACCTCAAGAACAACAACTACCTTTCGAGCGGCATCTAC
91       I  S  E  K  N  C  L  D  L  K  N  N  N  Y  L  S  S  G  I  Y
361     GTGGTGGCGCCCTACGACTGCTGCCCCGACAAGCTCGTCAGCGTCTACTGCGACATGGAC
111      V  V  A  P  Y  D  C  C  P  D  K  L  V  S  V  Y  C  D  M  D
421     ACGGACGGCGGCGGCTGGACGGTGATCCAGAGGCGCGACCAGTTCGACACGCAGCTCAAC
131      T  D  G  G  G  W  T  V  I  Q  R  R  D  Q  F  D  T  Q  L  N
481     TTCTTCAGGGGCTGGGACGACTACGTCACGGGGTTCGGCAAGCTCCGCAAGGAGTTCTGG
151      F  F  R  G  W  D  D  Y  V  T  G  F  G  K  L  R  K  E  F  W
541     CTCGGCCTCGACAACATCCACGCCTTCACGAACCAGACCAACTGCGAGATTCGCTTCGAC
171      L  G  L  D  N  I  H  A  F  T  N  Q  T  N  C  E  I  R  F  D
601     CTCGCCGACTTCGAAGGCAAGAGTCGCTGGGCGAAGTACAGTCAATTCCTGGTCAAGTCG
191      L  A  D  F  E  G  K  S  R  W  A  K  Y  S  Q  F  L  V  K  S
661     AAGGCGTCCAATTACGAGCTGAGGATAAGCGGCTACTCGGGCAACGCCGGCGACGCCATG
211      K  A  S  N  Y  E  L  R  I  S  G  Y  S  G  N  A  G  D  A  M
721     AGCTACCACAACGCCATGGTCTTCACCACCAAGGACAGTCCCGTCCACAGCGTGTGTCCG
231      S  Y  H  N  A  M  V  F  T  T  K  D  S  P  V  H  S  V  C  P
781     GAGAAGCACCAGGGGGCTTGGTGGTACAACAACTGTTTGCACGCCAACCTGAACGGACAG
251      E  K  H  Q  G  A  W  W  Y  N  N  C  L  H  A  N  L  N  G  Q
841     TACCTGGCCGGCCCTCATAACACCCTGGGCATCGGAGTGAACTGGTACGAGTGGAGGCGA
271      Y  L  A  G  P  H  N  T  L  G  I  G  V  N  W  Y  E  W  R  R
901     CTCAGGTACTCCCTCAAATTATCCGAGATGAAAATCAGACCAAAACAGTAAATCCAAAAG
291      L  R  Y  S  L  K  L  S  E  M  K  I  R  P  K  Q  *
961     AAGAAAAGGAAAGAGGAGGGAAAATCTGAACTGCTGATCACCGAGCAATCAAGAGAAATA
1021    GGGAATTGAGATCATTCAAAAAAGAAATGCGATCATTGAACGAAAATAGTTTCGGGGTCG
1081    TTGTGTAACAACGGATATAGTTAGGAGGTCATTATGTTAACATTATTCTGTTGCAGAGCT
1141    TGATCAAATGAATGATAAAAAAGAAACATCGTAAAATATCGAGAGCTATAAGAGCAAAAT
1201    GTTACGACCTCCATTATTATTCATGGAGAATCAATAATTCATAAAATGACTCTAACTACA
1261    GTAATATCATAAAGGAATATCATTACCCCGGCAGTGAATAAAAGGTATTGATCAGTAAAT
1321    GATATCAAATACATTGTTATTTATAAATGCTACTCCGTTACTGTTACTGTTCCTGATGAT
1381    AGTCGCAACACAGAATTCGATATAATAATAATGAAATGTCGAAATCCATGTATTTTATAA
1441    AATAATTATTCCTTCGGGAACGTATATGATTAATTTGATTTTGAGCATTTTCGCGTATCA
1501    AGTCGCTTACTCCTAATTTGGCTGTTCCCTTATGCATGTAATAAAGTATGGCCAATATAA
1561    AAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 4.1 Nucleotide and deduced amino acid sequences of PL5-3. The predicted ORF consists of 921 bp, corresponding to 306 amino acid residues. The putative signal peptide sequence is underlined. The FReD is in bold and italics. The NAT motif of N-linked-glycosylation site is shaded. The start and stop codons are bold and underlined and the classical polyadenylation signal sequence AATAAA is in italics.

```
1          ATCTGCAGTAGCTCCGTGTCTCTCCACTGGCAGCAGATATTAGAGAGCCGCGCGTGAACC
61         ACAGCAGAATGGCGCTCTTGCACAAGTTCATGCTGGCGAGTGCGCTCAGCATGGCCTTCG
1                    M  A  L  L  H  K  F  M  L  A  S  A  L  S  M  A  F
121        GGACAACAGAACGAACAGATACCGCGGAGCCACAGCAGCCTGGGGGATGCCACGCACTGC
18         G  T  T  E  R  T  D  T  A  E  P  Q  Q  P  G  G  C  H  A  L
181        CATCGCTCCTGCAGTCCGCGGGCTCTTCCCTAACGTGCATTGCAGCCTCCTTGATCAACT
38         P  S  L  L  Q  S  A  G  S  S  L  T  C  I  A  A  S  L  I  N
241        GCCAGGCAAACGAAAATGATCAGCTGTCCATTGCGCAAGGCCTGCGGGACACCATGTCCG
58         C  Q  A  N  E  N  D  Q  L  S  I  A  Q  G  L  R  D  T  M  S
301        AGGTGCGGCGAGCGATTGTCGACGCGGAGAGGCGGCGGCGGCGGCCAAGGCACTGCCGCG
78         E  V  R  R  A  I  V  D  A  E  R  R  R  R R P R H C R
361        ACCTGCAGCTCGACGGCGACTCCGAGTCGGGCGTCCGGCGCGTGTTCCCCTTCCGCAGTT
98         D L Q L D G D S E S G V R R V F P F R S
421        CGCCCGACGCGGCTGCGACCGTCTACTGCGACCAGGAGACCGACGGCGGCGGCTGGACGG
118        S P D A A A T V Y C D Q E T D G G G W T
481        TGTTCCAGAGGCGGCTCAAGCTCCCTGTGAGGGAGGACTTCTACCGCACGTGGGTCGAGT
138        V F Q R R L K L P V R E D F Y R T W V E
541        ACGAGCTCGGCTTCGGCCACATGGACGGCGGCGAATTCTGGCTGGGCCTCGACCTCGTGC
158        Y E L G F G H M D G G E F W L G L D L V
601        ACCGCCTGACCTCGACCGGCCTGCAGGAGATGAGAATCGACCTCACGGACTACGAGGACA
178        H R L T S T G L Q E M R I D L T D Y E D
661        ACGCCAAATGGGCCAAGTACGGGGTCTTCCACCTGGGGGACGCCAGCACCAAGTACCGGC
198        N A K W A K Y G V F H L G D A S T K Y R
721        TCAAAGTCGGCAGGTACAACGGCACAGCTGGCGATGGTTTCGGGAGTGGCAGGCACGACG
218        L K V G R Y N G T A G D G F G S G R H D
781        GCCACCCGTTTACCACGCACGATAACGACAACGACGGCGACGGTGCGAACTGTGCGGCCA
238        G H P F T T H D N D N D G D G A N C A A
841        GGTATCGCGGTGCTTGGTGGTATGACAAATGCCACATATCCAACTTGAATGGGTTTCCTT
258        R Y R G A W W Y D K C H I S N L N G F P
901        ATGAAGGCGAGCACGAGAGCTATGCGGATGGTATCGAGTGGCAACCCTGGAGAGGATATC
278        Y E G E H E S Y A D G I E W Q P W R G Y
961        ATTACTCGCTGAAAACTACAACAATGATGATAAGGCCGGCATTATGAAGCTTCTGATGTC
298        H Y S L K T T T M M I R P A L *
1021       ACTGACACACACACACACACACACATACACACACACACACACACTCACTCACTCACTCAC
1081       TCACACACATTATGTTGATATTAACTATGTCAGAAATATTTATCAGTTTACAGAAATGTA
1141       GGTGCGGATCTGGGTATTCTAAATCTAAATGCATACACGACTACAAATGTATATCTTTAC
1201       GAAATTTGTTATGAAATTTTAAATGTCTGTAGTCTGAAAATAAACATCAATAAACCCCAA
1261       AAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 4.2 Nucleotide and deduced amino acid sequences of PL5-1. The predicted ORF consists of 939 bp, corresponding to 312 amino acid residues. The putative signal peptide sequence is underlined. The FReD is in bold and italics. The NGT motif of N-linked-glycosylation site is shaded. The start and stop codons are bold and underlined and the classical polyadenylation signal sequence AATAAA is in italics (Angthong, 2010).

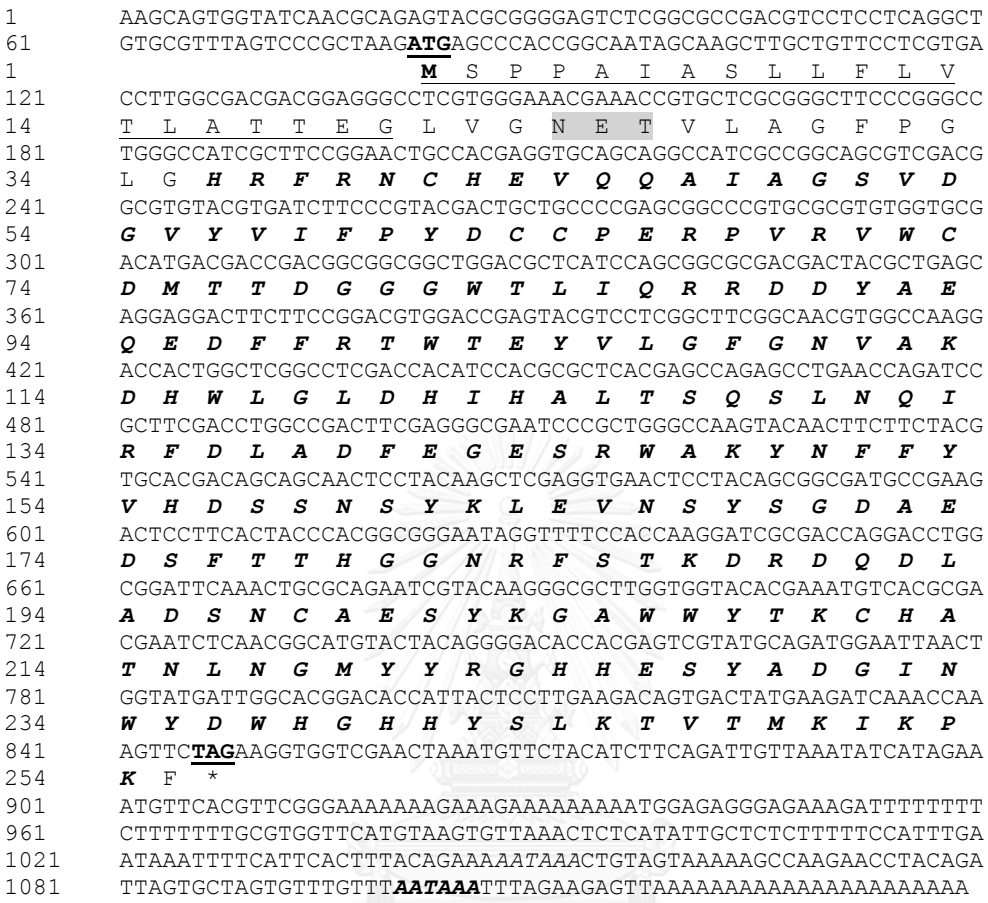

Figure 4.3 Nucleotide and deduced amino acid sequences of PL5-2. The predicted ORF consists of 768 bp, corresponding to 255 amino acid residues. The putative signal peptide sequence is underlined. The FReD is in bold and italics. The NET motif of N-linked-glycosylation site is shaded. The start and stop codons are bold and underlined and the classical polyadenylation signal sequence AATAAA is in italics (Angthong, 2010).

4.2 Sequence comparison of PL5-1, PL5-2 and PL5-3 with Japanese horseshoe crab TL5

PL5-1, PL5-2 and PL5-3 contain seven, six and seven cysteine residues, respectively. In Japanese horseshoe crab two disulphide linkages are present which can form intramolecular disulphde bonds. The respective Cys residues are also present in the PL5-1 (between Cys^{96}-Cys^{127} and Cys^{41}-Cys^{73}), PL5-2 (Cys^{255}-Cys^{268} and Cys^{198}-Cys^{211}) and PL5-3 (between Cys^{96}-Cys^{117} and Cys^{249}-Cys^{262}) (Figure 4.4, indicated by black triangles). The TL5A and TL5B FReDs comprise four residues in the Ca^{2+} binding site (Asp198, Asp200, His202, Thr204), which is providing the oxygen atoms to coordinate the calcium ion. In PL5-1 and PL5-2 only two such residues are conserved (Asp247-Asp249) and (Asp190-Asp192) to TL5A and TL5B, but PL5-3 is not conserved (Figure 4.4, marked by asterisks).

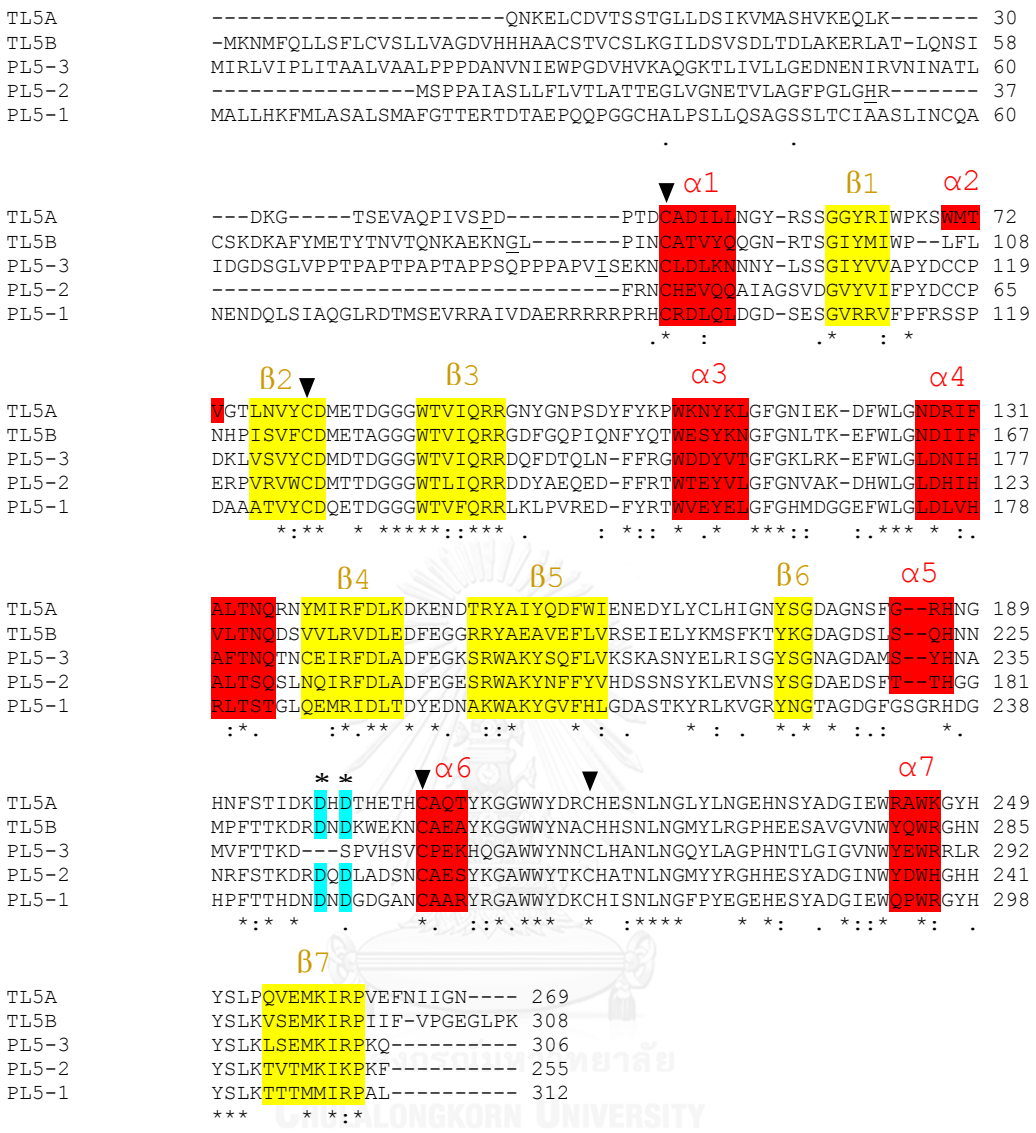

Figure 4.4 The deduced sequences of PL5-1, PL5-2 and PL5-3 are aligned with the japanease horseshoe crab TL5A and TL5B. Alpha and beta –structures are indicated by red and yellow shading, respectively. Amino acid sequences contain residues involved in Ca^{2+} binding indicated by asterisks and light blue shading, the positions of the potential intramolecular disulphide bonds in all sequences are marked with black triangle.

4.3 Phylogenetic analysis of PL5-1, PL5-2 and PL5-3

A phylogenetic tree was constructed using the neighbour-joining method. The amino acid sequences representative of FReD or tachylectin-like proteins from other organisms were used. The homology tree showed that shrimp PL5-2 and PL 5-3 are most closely related to horseshoe crab TL5B and CL5B, but they are in a different subcluster. The shrimp PL5-1, shares the highest sequence similarity (44%) with the lectin from *M. galloprovincialis,* and the similarity is much lower to the crayfish fibrinogen-like proteinsthan that of PL5-2 and PL5-3 (Figure 4.5).

4.4 Tissue distribution analysis of *PL5-1*, *PL5-2* and *PL5-3* transcripts of *P. monodon*

The tissue expression pattern of the *PL5-1*, *PL5-2* and *PL5-3* genes show that a high expression of *PL5-1* was detected in heart, anterior intestine, lymphoid organ, muscle, pleopod, thoracic ganglion and stomach (Angthong, 2010). In contrast, *PL5-2* had a high transcript level in epidermis, eye stalk, and posterior intestine, abdominal ganglion, pleopod and stomach among examined tissues (Angthong, 2010). Moreover, *PL5-3* was detected at high transcript level in anterior intestine and posterior intestine (Figure 4.6). Since *PL5-1*, *PL5-2* and *PL5-3* were differentially expressed in shrimp anterior intestine and posterior intestine and to know more specific of their expression along the intestinal tract, the expression profiles of PL5 genes were further investigated in different regions of the shrimp digestive tract including stomach, hepatopancreas, anterior part of the anterior intestine, posterior part of the anterior intestine, hindgut, anterior caecum and posterior caecum. The result shows that *PL5-1* was expressed in all tested regions of intestine and was very high in the stomach and anterior of midgut, while *PL5-2* was highly expressed in stomach, hindgut and posterior caecum (Angthong, 2010). *PL5-3* was expressed in all intestinal parts and caecum at high level, but a low transcript level was observed in stomach (Figure 4.7).

Moreover, the expression profile of *PL5-1*, *PL5-2* and *PL5-3* was also investigated in different stages of shrimp development. *PL5-2* was expressed from embryo to nauplius 3 at a low level, whereas it was expressed at significant levels from the larval stage nauplius 1 and onwards. The expression of *PL5-1* was hardly seen in

embryo, but increased significantly from nauplius 1 and onwards (Angthong, 2010). *PL5-3* was expressed from embryo to protozoea 3 and expressed at high level from mysis 1 and onwards (Figure 4.8).

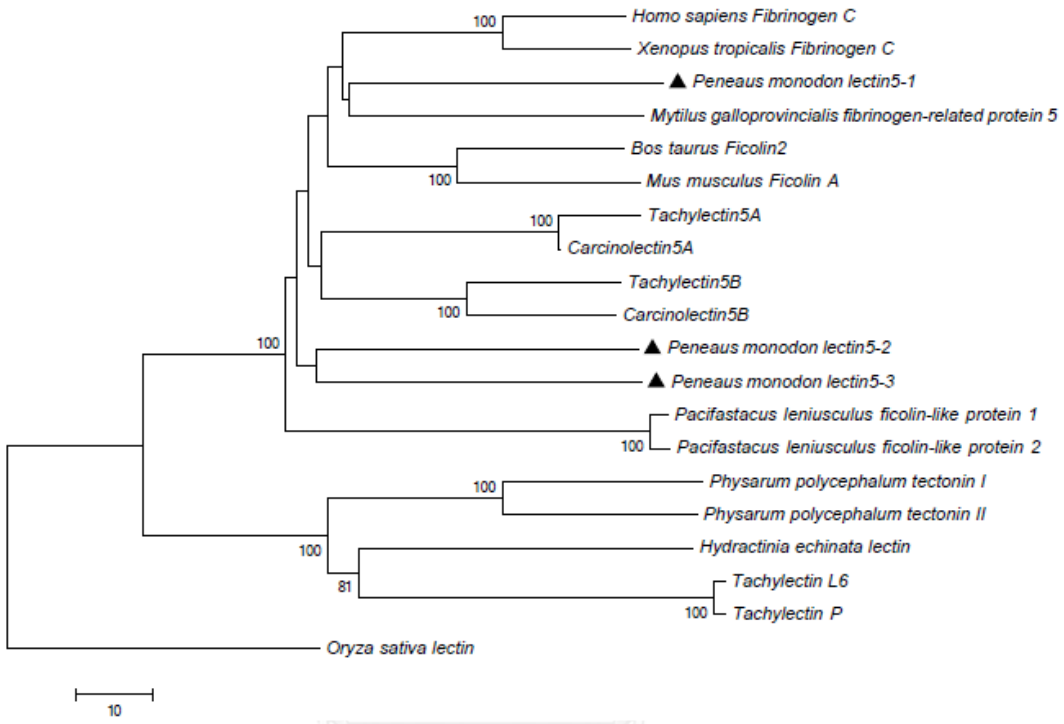

Figure 4.5 A phylogram based on multiple alignments between PL5s and other related lectin proteins: *Homo sapiens* FibrinogenC (AAH32953), *Xenopus tropicalis* FibrinogenC (NP_001072519), *Mus musculus* ficolinA (BAE53381), *Bos taurus* ficolin2 (NP_001010996), *Pacifastacus leniusculus* ficolin like proteins 1 and 2 (GU937075, GU937076), Tachylectin5B (BAA84190), Carcinolectin5B (AAY24506.1), *Mytilus galloprovincialis* fibrinogen-related protein 5 (CBM41044), Carcinolectin5A (AAY24512.1), Tachylectin5A (1JC9_A), Tachylectin L6 (AAB34596), TachylectinP (BAA88574), *Physarum polycephalum* tectoninI (AAC06200) and tectoninII (AAC06201), *Hydractinia echinata* lectin (CAI 77215) and *Oryza sativa* Japonica lectin (Group AAD27887).

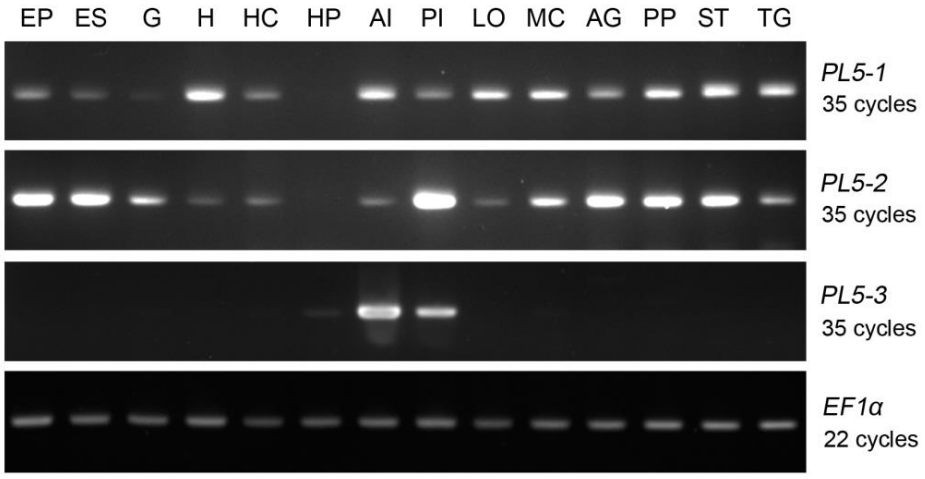

Figure 4.6 RT-PCR of *PL5-1*, *PL5-2* and *PL5-3* in different tissues including epidermis (EP), eye stalk (ES), gills (G), heart (H), hemocytes (HC), hepatopancreas (HP), anterior intestine (AI), posterior intestine (PI), lymphoid organ (LO), muscle (MC), abdominal ganglion (AG), pleopod (PP), stomach (ST), thoracic ganglion (TG) and EF1α gene served as an internal control.

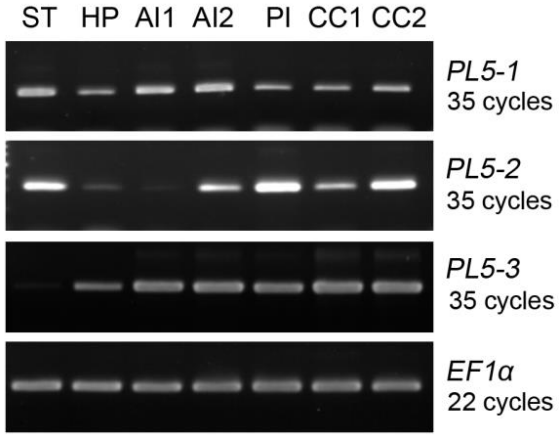

Figure 4.7 Expression profile of *PL5-1*, *PL5-2* and *PL5-3* in different parts of the shrimp digestive organ including stomach (ST), hepatopancreas (HP), anterior part of anterior intestine (AI1), posterior part of anterior intestine (AI2), posterior intestine (PI), anterior caecum (CC1) and posterior caecum (CC2) and EF1α gene served as an internal control.

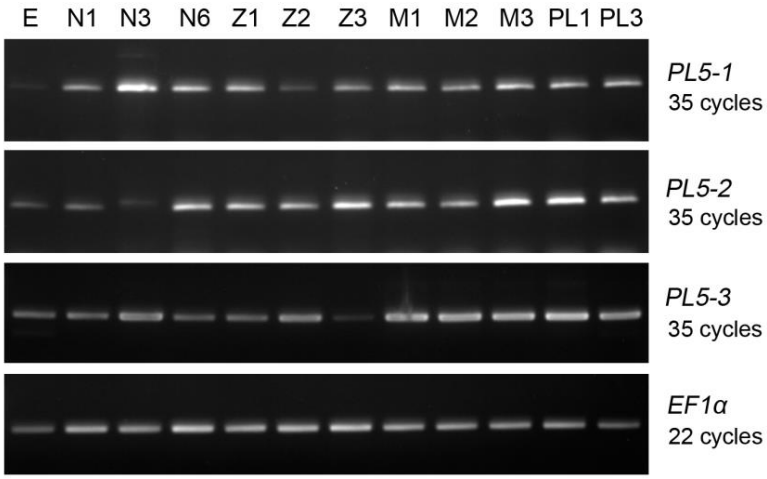

Figure 4.8 Expression of *PL5-1*, *PL5-2* and *PL5-3* in developmental embryo and larval stages of nauplius 1, nauplius 3, nauplius 6, protozoea 1, protozoea 2, protozoea 3, mysis 1, mysis 2, mysis 3, postlarva 1 and postlarva 3 and EF1α gene served as an internal control. N = nauplius, Z = protozoea, M = mysis and PL = postlarva.

4.5 *In vitro* expression of recombinant Penlectin 5 protein using the bacterial expression system

Expression of recombinant clones of PL5-1 (33.3 kDa) and PL5-3 (32.6 kDa) were selected and the expression profile of the corresponding recombinant protein was examined at 37 ºC for 0, 1, 2, 3, 4, 6 and 14 hours after IPTG induction. Due to pET 32a expression vector was used for recombinant protein expression, thioredoxin (trx) tag with a molecular weight of 18 kDa was added. Therefore, the expected size of PL5-1 and PL5-3 proteins are 51.3 kDa and 50.6 kDa, respectively.

Recombinant PL5-1 was expressed after 1 hour of 0.5 mM IPTG induction. A lower level of expressed PL5-1 was observed when the culture period was extended between 4 to 14 hours after induction (Figure 4.9A). Recombinant PL5-3 was expressed after 1 hour of IPTG induction. This recombinant protein was stably expressed throughout the cultured period (Figure 4.10A). Western blotting was also carried out to confirm the recombinant expression of target protein. The recombinant PL5-1 and

PL5-3 were recognized by the antibodies specific to the attached sequence tag (Figure 4.9B and 4.10B, respectively).

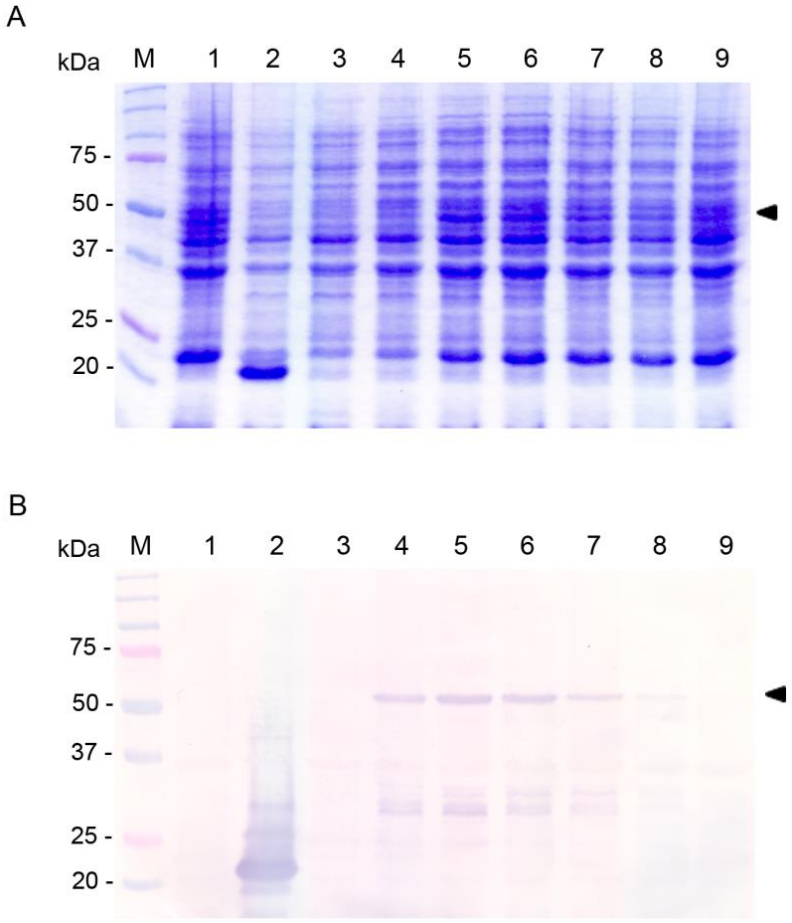

Figure 4.9 SDS-PAGE (A) and western blotting (B) showing in vitro expression of mature PL5-1 in *E. coli* BL21-CodonPlus (DE3)-RIPL at 0, 1, 2, 3, 4, 6 and 14 hours after induction with 0.5 mM IPTG (lane 3-9; A and B). *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 1; A and B) and pET-32a vector without insert in *E. coli* BL21- CodonPlus (DE3)-RIPL (lane 2; A and B) were included as the control.

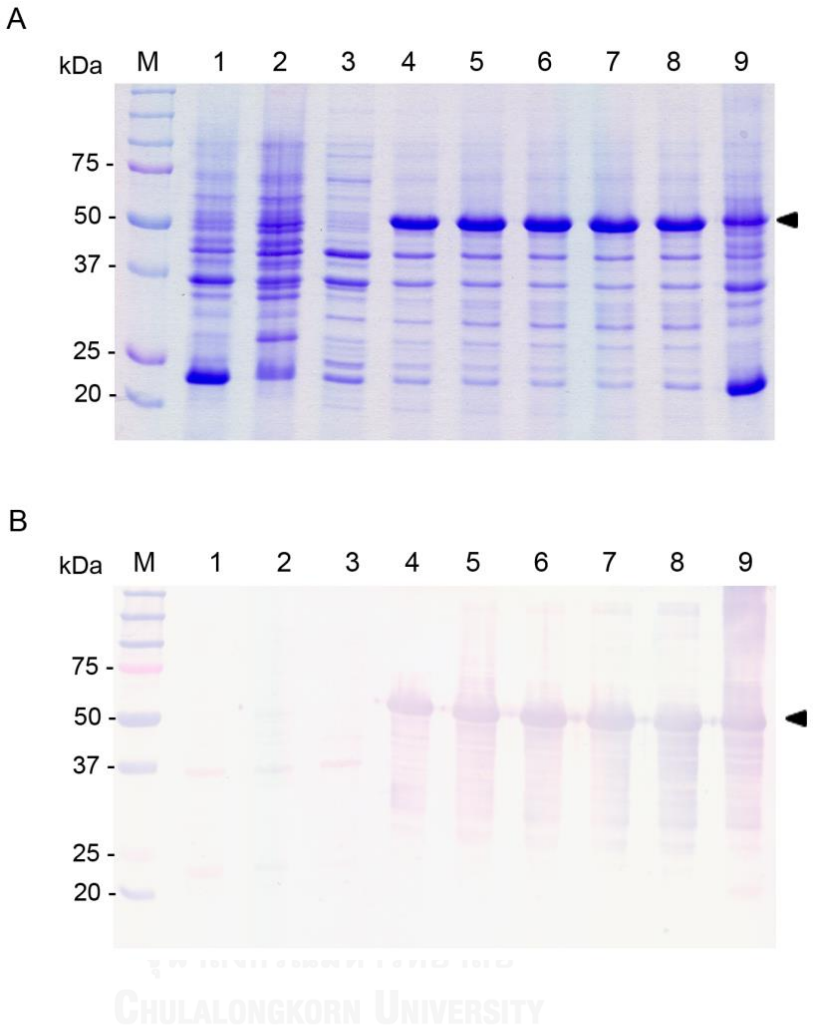

Figure 4.10 SDS-PAGE (A) and western blotting (B) showing in vitro expression of mature PL5-3 in *E. coli* BL21-CodonPlus (DE3)-RIPL at 0, 1, 2, 3, 4, 6 and 14 hours after induction with 0.5 mM IPTG (lane 3-9; A and B). *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 1; A and B) and pET-32a vector without insert in *E. coli* BL21-CodonPlus (DE3)-RIPL (lane 2; A and B) were included as the control.

The soluble and insoluble protein fraction of recombinant PL5-1 and PL5-3 were examined by SDS-PAGE. Recombinant PL5-1 and PL5-3 were cultured for 3 hours after IPTG induction at 37 ºC. Expression of recombinant PL5-1 and PL5-3 were expressed as insoluble forms (Figure 4.11). To promote the possible expression of recombinant PL5-1 and PL5-3 in a soluble form, the culture temperature was decreased to 16 ºC (Figure 4.12). However, the recombinant PL5-1 and PL5-3 were still expressed in insoluble forms and the expression level was lower than in the cultured cells at 37 ºC. Therefore, induction at 37 ºC resulted in a higher total amount of the target protein.

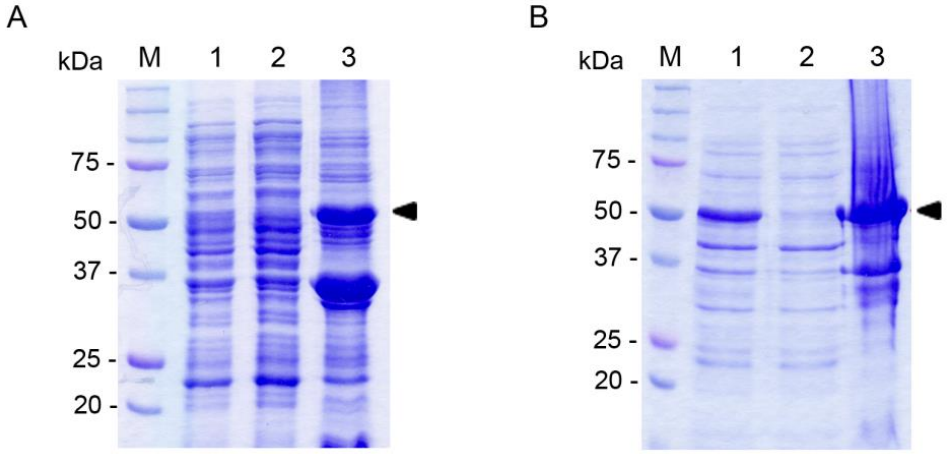

Figure 4.11 SDS-PAGE showing expression of a crude protein (lane 1), a soluble protein fraction (lane 2) and an insoluble protein fraction (lane 3) of recombinant PL5-1 (A) and PL5-3 (B) cultured cells at 37 ºC for 3 hours post 0.5 mM IPTG induction.

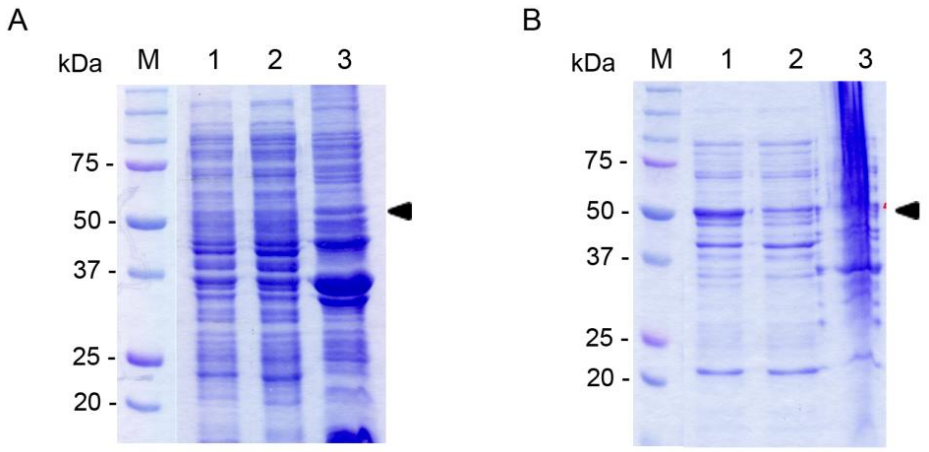

Figure 4.12 SDS-PAGE showing expression of a crude protein (lane 1), a soluble protein fraction (lane 2) and an insoluble protein fraction (lane 3) of recombinant PL5-1 (A) and PL5-3 (B) cultured cells at 16 ^{o}C for 3 hours post 0.5 mM IPTG induction.

The insoluble proteins fraction of PL5- 1 and PL5- 3 were purified under denaturing conditions using a His GraviTrap column and sent to Faculty of Associated Medical Science, Chiangmai University, for production of a polyclonal antibody made in rabbit.

The recombinant PL-1, PL5-2 and PL5-3 expressed in the bacterial expression system were all found to be inclusion proteins. However, the PL5-2 recombinant protein could be solubilized and was used to produce a polyclonal antibody.

4.6 Localization of PL5-2 protein in *P. monodon*

4.6.1 Localization of expressed proteins by western blot analysis

The protein expression profile of PL5-2 was investigated in different tissues of shrimp. PL5-2 protein and it was found to be expressed in all tested tissues including hemocytes, gill, heart, anterior intestine, posterior intestine, stomach and epidermis and also expressed in plasma at varying levels (Figure 4.13).

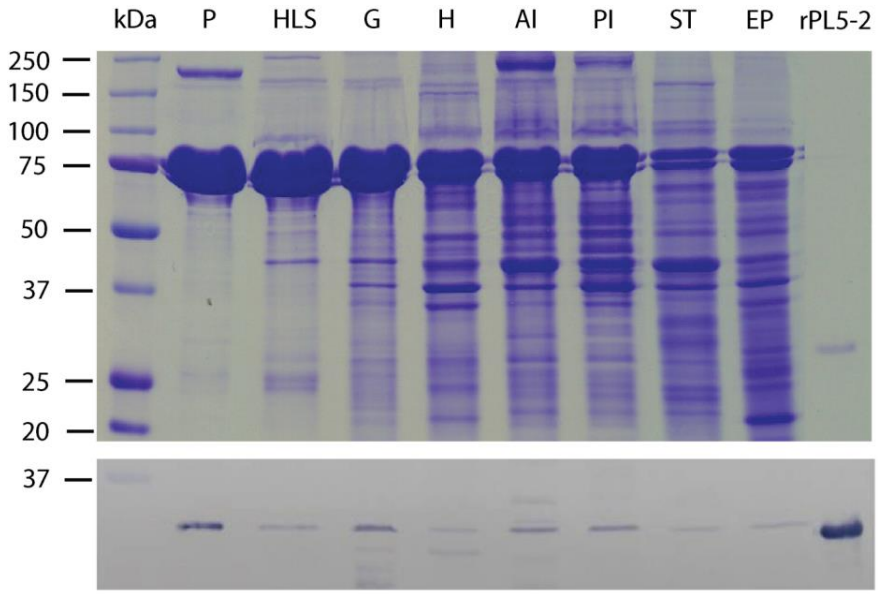

Figure 4.13 SDS-PAGE and western blot analysis showing localization of PL5-2 protein in shrimp tissues. The samples (25µg protein) from plasma (P), hemocyte lysate (HLS), gills (G), heart (H), anterior intestine (AI), posterior intestine (PI), stomach (ST), epidermis (EP) and recombinant mature PL5 (rPL5-2) served as a positive control.

4.6.2 Localization of expressed proteins by immunohistochemistry

The localization of PL5-2 was investigated in digestive tract of *P. monodon* including stomach, anterior intestine and posterior intestine. The positive immunological signal of PL5-2 was strongly detected in all examined tissues. The PL5-2 protein was located in the connective tissue of stomach and anterior intestine, and tegumental gland of the posterior intestine (Figure 4.14). Immunoreactivity was not found in the tissues when incubated with the pre-immune serum as a control.

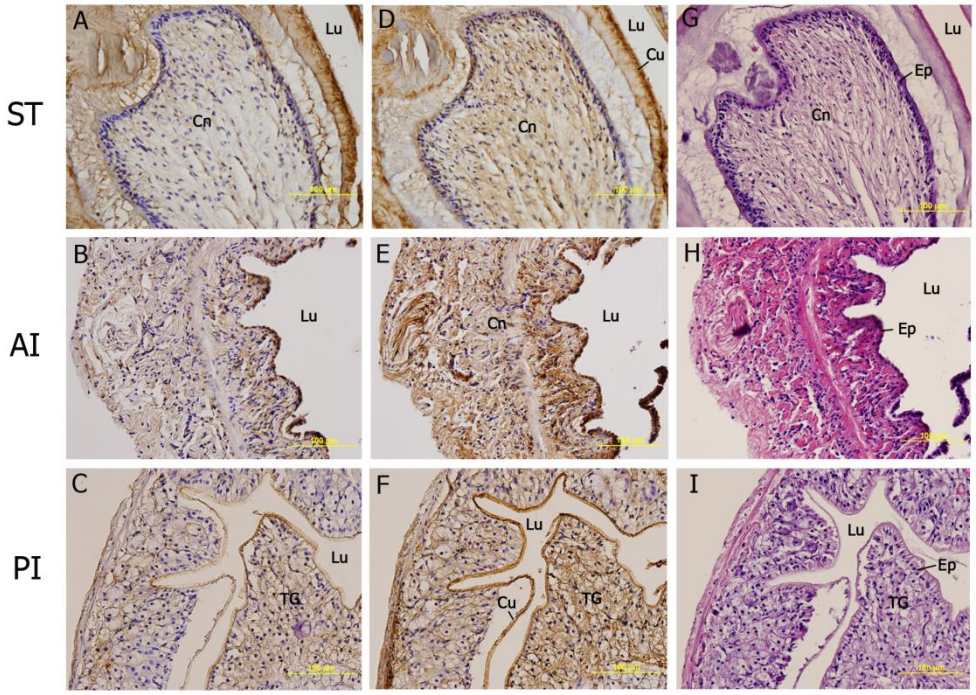

Figure 4.14 Localization of PL5-2 protein in stomach, anterior intestine and posterior intestine by immunohistochemistry. Tissue sections were incubated with pre-immune rabbit serum as a negative control (A, B and C) or the anti-PL5-2 (D, E and F). The conventional H&E staining was carried out for recognition of tissues (G, H and I). Abbreviations: stomach (ST), anterior intestine (AI), posterior intestine (PI), lumen (Lu), epithelial cell (Ep), cuticle (Cu), connective tissue (Cu) and tegmental gland (TG).

4.7 Purification of PL5-2 protein from plasma by affinity chromatography using a *N*-Acetyl-D-glucosamine affinity chromatography purification.

Since recombinant PL5-2 protein that was produced from the bacterial expression system was expressed in an insoluble form, the PL5-2 was purified from *P. monodon* plasma using *N*-acetyl-D-Glucosamine (GlcNAC) affinity chromotography. Shrimp plasma was subjected to the affinity column several times and unbound protein was repeatedly washed away from the column. The PL5-2 protein was eluted from the column by elution buffer supplemented with 15 mM GlcNAC. The fractions of purified PL5-2 protein were analysed by SDS-PAGE and western blotting to confirm that the purified protein was the PL5-2 protein using polyclonal antibody against PL5-2 (Figure 14.5).

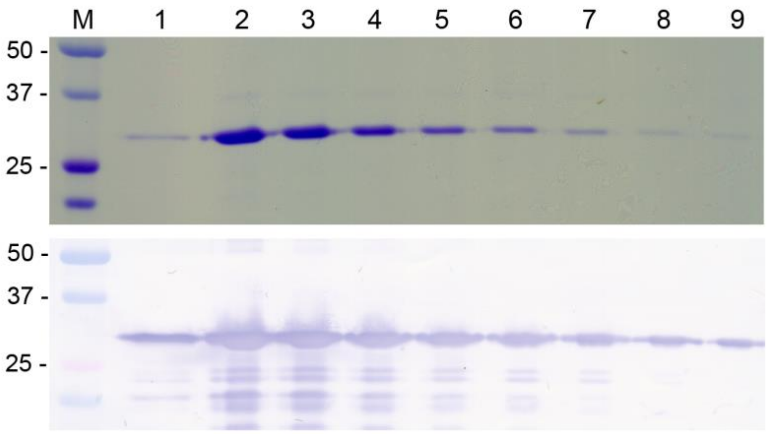

Figure 4.15 Purification of PL5-2 protein from plasma of *P. monodon*. The purified PL5-2 protein was examined by SDS-PAGE (upper panel) and western blot analysis (lower panel). Upper gel lanes 1 – 9 are eluted protein fractions stained for protein and lower gel lanes 1-9 are western blots.

4.8 Biological function of PL5-2

4.8.1 The ability of PL5-2 to bind to bacteria

Bacteria are expected to bound to PL5-2 and since *Vibrio spp*. is considered to be one of most devastating among shrimp pathogens, more strains from this genus were examined for their binding activity. The result shows that the binding of PL5-2 to *V. parahaemolyticus*1, *B. megaterium*, *S. aureus*, *S. equi*, or *V. nigripulchritudo* could not be demonstrated and PL5-2 was weakly bound to *E. coli*, *V. parahaemolyticus*2, *V. rotiferianus* and *V. natriegens* (Figure 4.16A-B). In contrast, the PL5-2 protein could strongly bind to *M. luteus* or *A. hydrophila*, as indicated by a depletion of the PL5-2 protein in the plasma after incubation plasma with each bacterium (Figure 4.16A). Moderate binding to *P. aeruginosa*, *V. haveryi*, *V. minicus* and *V. neptunius* was also observed (Figure 14.16 A-B). In addition, the binding between *V. parahaemolyticus* (3HP) and PL5-2 causing AHPND was also performed. The results showed that PL5-2 protein can bind moderately to *V. parahaemolyticus* (3HP).

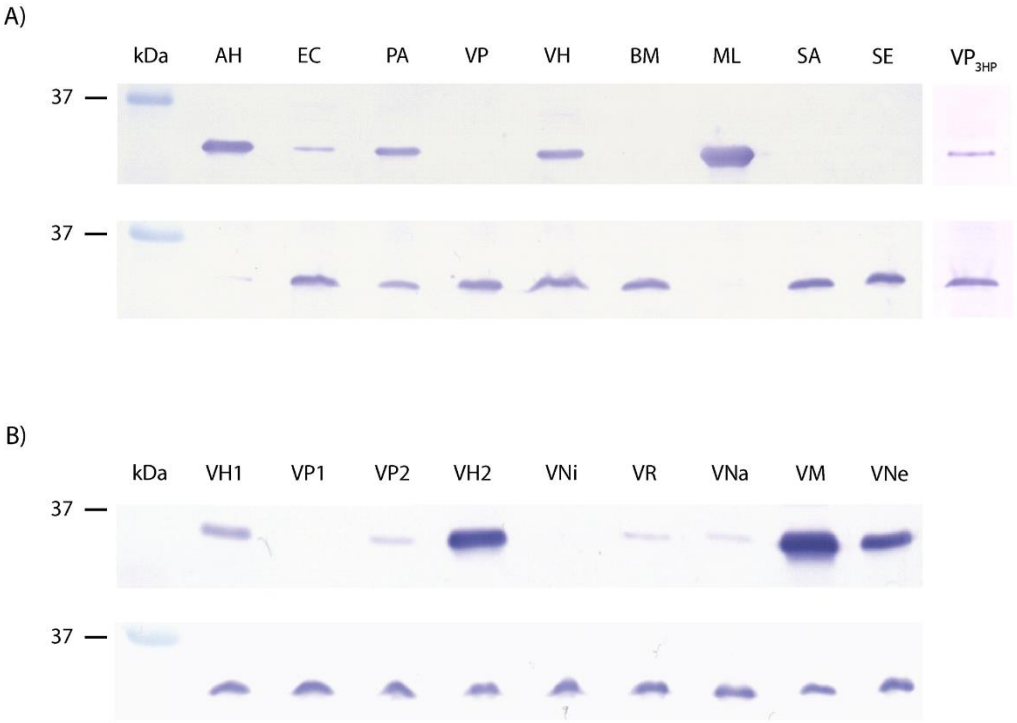

Figure 4.16 Plasma PL5-2 and its binding to different bacteria. (A) The binding of PL5-2 protein in plasma to Gram-positive or Gram-negative bacteria (Upper panel) and the amount of PL5-2 protein remaining in plasma after binding to each bacterium and this therefore represents non-bound proteins (Lower panel) as performed by western blot analysis. The bacteria included *A. hydrophila* (AH), *E. coli* (EC), *P. aeruginosa* OT97 (PA), *V. parahaemolyticus* (VP), *B. megaterium* Bm11 (BM), *M. luteus* MIII (ML), *S. aureus* Cowan1 (SA) and *S. equi* 62 (SE). (B) The binding of PL5-2 protein in plasma to different species of *Vibrio spp*. (Upper panel) and the remaining PL5 in plasma after binding to each species of *Vibrio spp*. (Lower panel). The *Vibrio spp*. include *V. harveyi* (VH1 and VH2), *V. parahaemolyticus* (VP1 and VP2), *V. nigripulchritudo* (VNi), *V. rotiferianus* (VR), *V. natriegens* (VNa), *V. minicus* (VM) and *V. neptunius* (VNe).

4.8.2 Agglutinating activity and carbohydrate binding specificity of PL5-2

A purified PL5-2 protein from shrimp plasma (Figure 4.15) was used to perform a hemagglutination assay with human erythrocytes. The PL5-2 protein agglutinated human erythrocytes type A, B and O with a minimal agglutination concentration of 9.7, 4.8 and 19.5 ng/ml, respectively (Table 4.1). Agglutination was inhibited when 100 mM EDTA was added into the solution. The purified PL5-2 protein has ability to agglutinate Gram-negative bacteria, including *A. hydrophila*, *V. harveyi* and *V. parahaemolyticus* (3HP) and also the Gram-positive bacterium, *M. luteus* (Figure 4.17). Minimal agglutinating concentrations of purified PL5-2 are shown in Table 4.2. Bacterial agglutination activity was inhibited when EDTA was added, suggesting that the agglutination activity of human erythrocytes and bacteria by purified PL5-2 was calcium dependent. GlcNAc and GalNAc were shown to effectively inhibit the binding activity of PL5-2 to *M . luteus*, at a concentration of 10 mM. No inhibition was observed when D-mannose, D-glucose, GALN-HCl or NeU5Ac was used at the same concentrations (Figure 4.18).

Table 4. 1 Hemagglutination activity of PL5-2 on human erythrocytes.

Human erythrocytes	Minnimum agglutiaction concentration (ng/ml)
A type	9.7
B type	4.8
O type	1.95

Table 4. 2 Bacterial agglutinating activity and minimal agglutinating concentration of purified PL5-2

Microorganism	Agglutinability (µg/ml)
A. hydrophila	50
V. harveyi	25
V. parahaemolyticus (3HP)	400
M. luteus	25

4.8.3 Anti-microbial activity of PL5-2

To determine whether the purified PL5-2 protein has anti-microbial activity, Gram-positive bacterium, *M. luteus* and Gram-negative bacteria, *A. hydrophila*, *V. harveyi* and *V. parahaemolyticus* were separately incubated with 50 µg/ml of purified PL5-2 protein. The results show that PL5-2 protein has anti-microbial activity against Gram-negative bacteria, *A. hydrophila*, *V. harveyi* and very weakly against *V. parahaemolyticus*, but not towards a Gram-positive bacteriium (Figure 4.19). Since purified PL5-2 protein from shrimp plasma was found to have agglutination and reduction of cell number of *A .hyrophila*, the purified PL5-2 protein was serial-diluted for determining the minimal concentration to inhibit the growth of bacteria. *A. hydrophila* was incubated with the PL5-2 protein at a concentration of 6.25-100 µg/ml. PL5-2 had significant anti-bacterial activity against *A. hydrophila* awith a minimal concentration of 25 µg/ml ($P < 0.05$) (Figure 4.20).

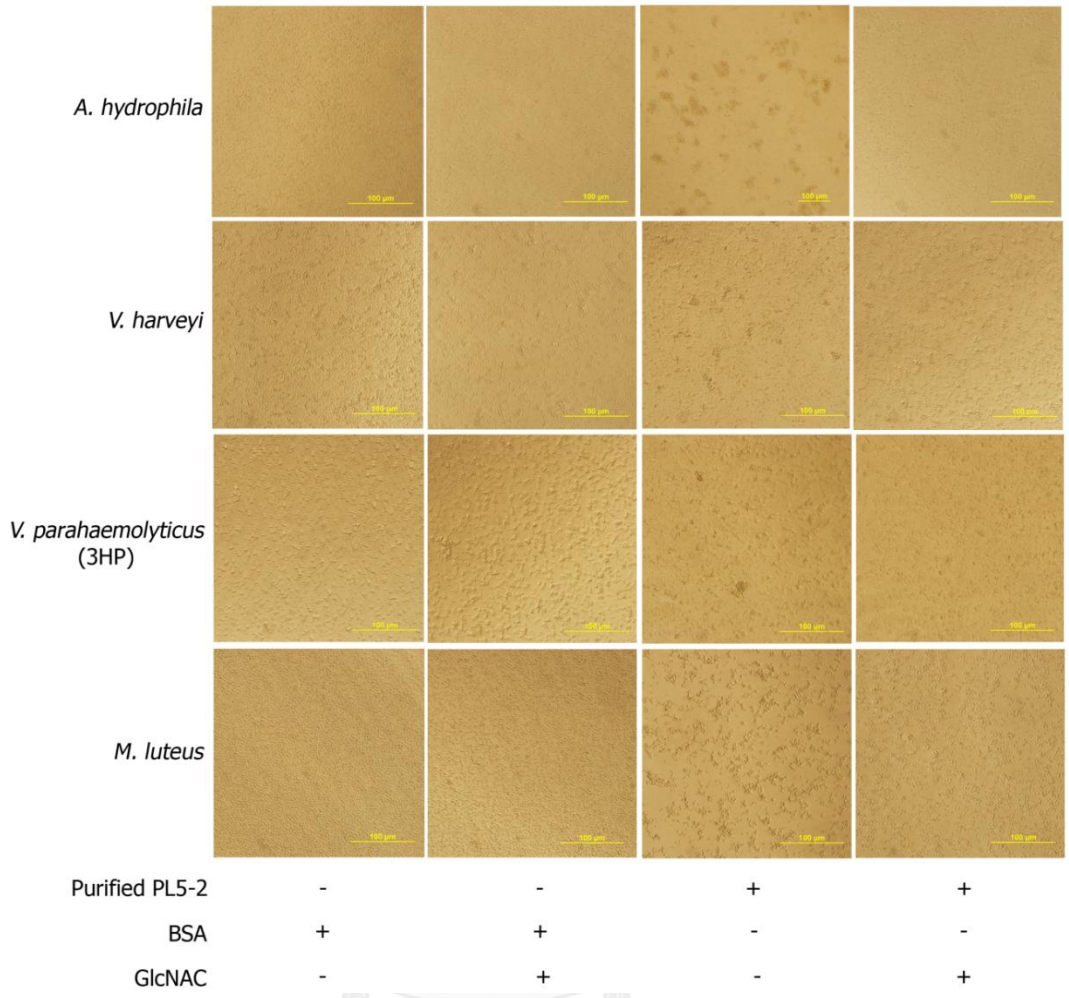

Figure 4.17 Agglutination of microorganism by the purified PL5- 2 from shrimp plasma**.**

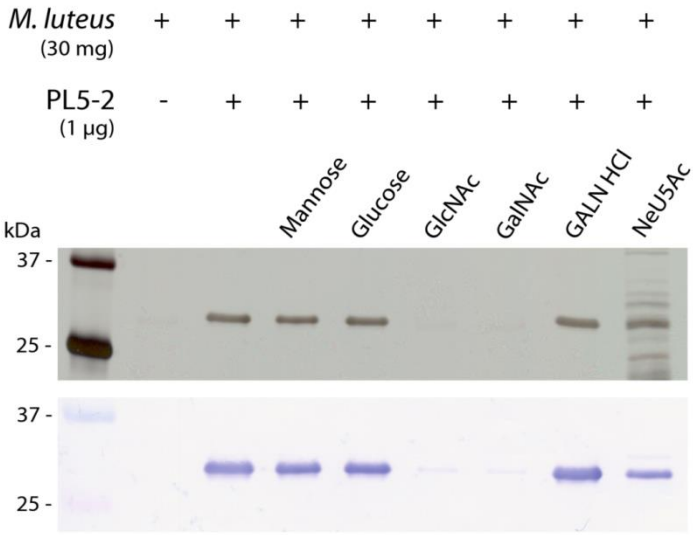

Figure 4. 18 SDS-PAGE and western blot analysis showing the inhibition of bacterial binding activity of purified PL5-2 from shrimp plasma by different sugars. Different sugars were added to inhibit the binding activity of purified PL5-2 before incubation with *M. luteus* MIII and then the bacteria were washed with NaCl solution and subsequently eluted with 4M urea in 10 mM Tris-HCl.

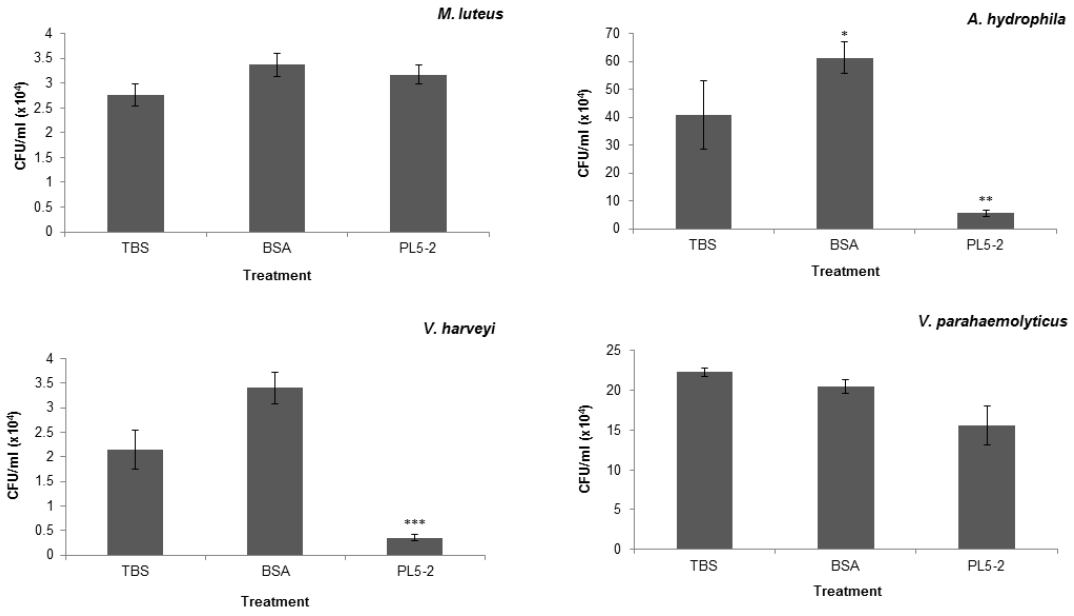

Figure 4.19 Antimicrobial activity of PL5-2 against *M. luteus*, *V. harveyi*, *A. hydrophola* and *V. parahaemolyticus* are represented as colony forming units/ml (CFUs/ml). The asterisk indicates that the expression levels are significantly different. * P <0.05, ** P<0.01, *** P<0.001 when compared to TBS buffer or BSA (*N = 3*).

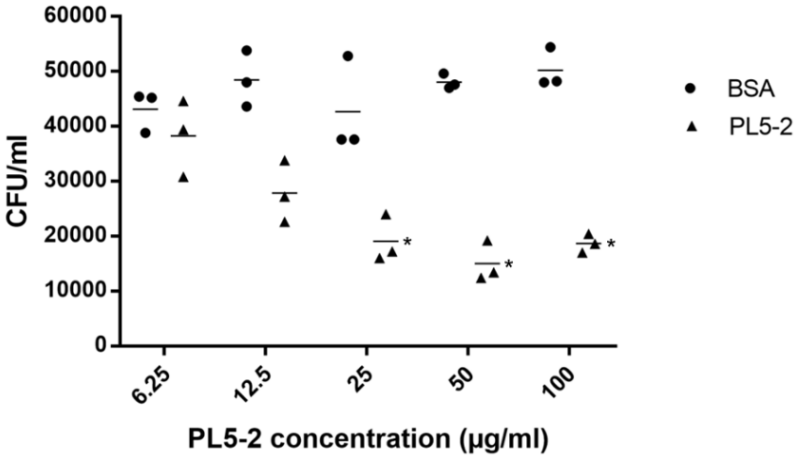

Figure 4.20 Antimicrobial activity of PL5-2 against *A. hydrophila* is shown as colony forming units (CFUs) per ml. The asterisk indicates that the expression levels are significantly different (* P <0.05)

4.9 Quantitative real-time PCR analysis of PL5-1, PL5-2 and PL5-3 in juvenile *P. monodon* after pathogenic bacteria infection

4.9.1 Expression profiles of *PL5-1* and *PL5-2* after injection with *V. harveyi* injection

The *PL5-1* transcripts in hemocytes were increased and reached a maximum at 12 h post injection with *S. aureus*, *M. luteus* or *V. harveyi* compared to those of the control (Figure 4.21A-C). However, a significant change in response to *V. harveyi* was observed ($p<0.05$, Figure 14.21C). The PL5-2 transcript level was gradually increased, but not significantly induced in the posterior intestine during injection with *S .aureus* or *M .luteus* (Figure 4.22A and B). In contrast, the expression of *PL5-2* in the posterior intestine was significantly increased at 12 h post injection with *V. harveyi* and stayed high until 24 h post injection (Figure 14.22C). This clearly shows that the transcription of *PL5-2* in posterior intestine of juvenile *P. monodon* was induced by a pathogenic bacterial infection.

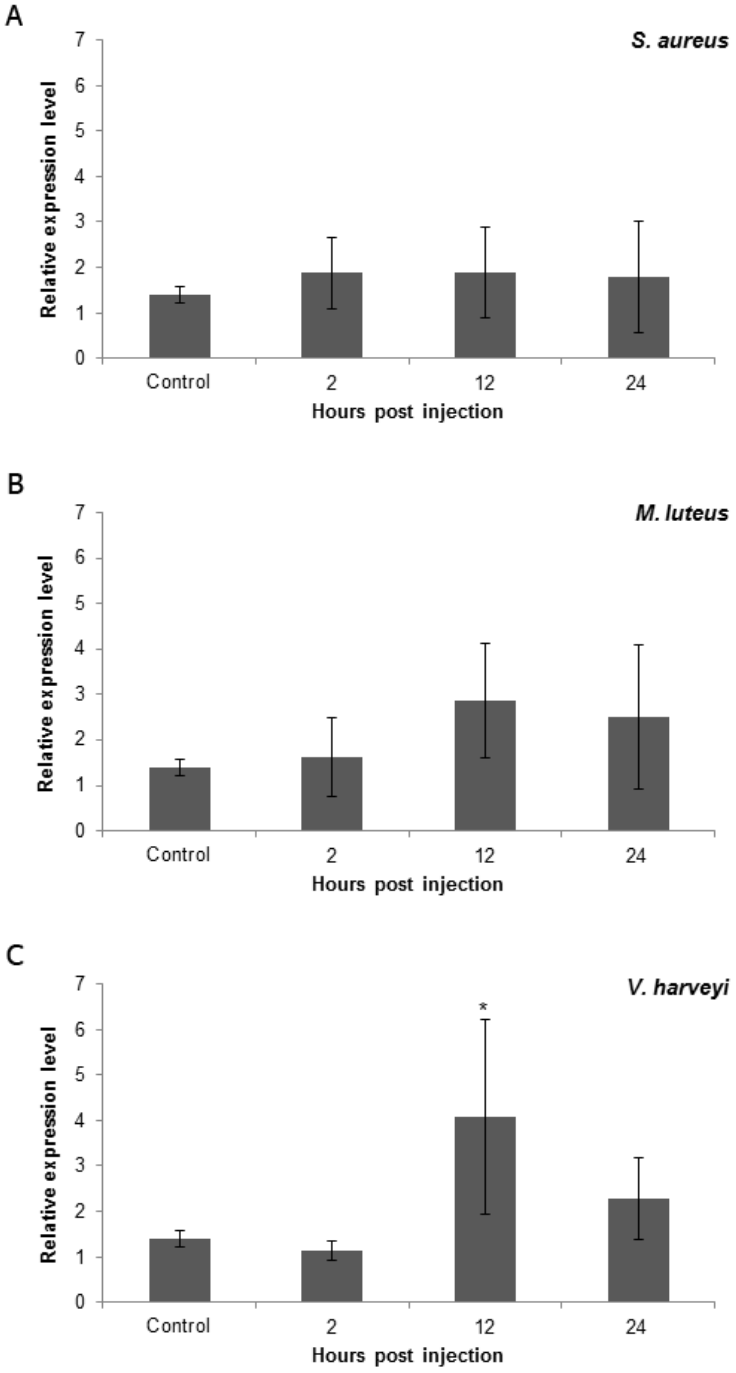

Figure 4.21 Real-time PCR analysis showing relative expression levels of *PL5-1* in hemocytes of juvenile shrimp after injection with normal saline (control group) or injected with *S. aureus* Cowan1 (A), *M. luteus* MIII (B) and *V. harveyi* (C) at 2, 12 and 24 hours. Vertical bars represent the mean ± SD (*N = 4*). The asterisk indicates that the expression levels are significantly different ($P < 0.05$).

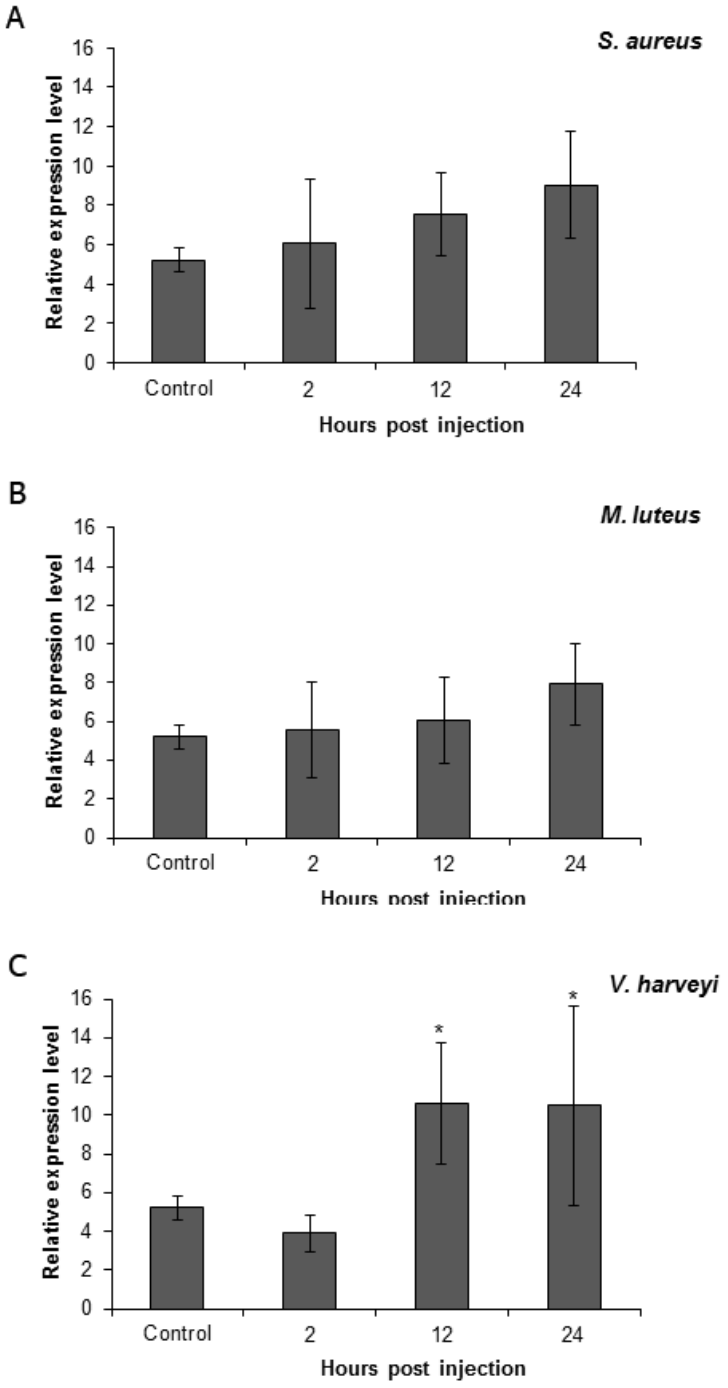

Figure 4.22 Real-time PCR analysis showing relative expression levels of *PL5-2* in posterior intestine of juvenile shrimp after injection with normal saline (control group) or injected with *S. aureus* Cowan1 (A), *M. luteus* MIII (B) and *V. harveyi* (C) at 2, 12 and 24 hours. Vertical bars represent the mean ± SD (*N = 4*). The asterisk indicates that the expression levels are significantly different ($P < 0.05$).

4.9.2 Expression profiles of *PL5-1*, *PL5-2* and *PL5-3* during AHPND causing VP_{3HP} infection

The initial step of an infection process caused by bacterial pathogens in shrimp, usually a large number of bacterial cells are observed attached to the stomach surface (Joshi et al., 2014; Soonthornchai et al., 2010). To determine the expression pattern of *PL5-1*, *PL5-2* and *PL5-3* in shrimp stomach during VP_{3HP} infection, quantitative real-time PCR was studied and the result showed that *PL5-1* and *PL5-2* were up-regulated at 6 to 12 hours post infection, whereas up-regulation of *PL5-3* was not as strong as other PL5s. (Figure 4.23). Especially for PL5-2 transcript, the expression of *PL5-2* was significantly increased at 6 hours and then decreased at 12 hours. This indicates that the transcription of *PL5-1*, *PL5-2* and *PL5-3* in *P. monodon* was induced by a pathogenic bacterial infection and that there is a significant and differente response of these isoforms to challenge with this bacterium.

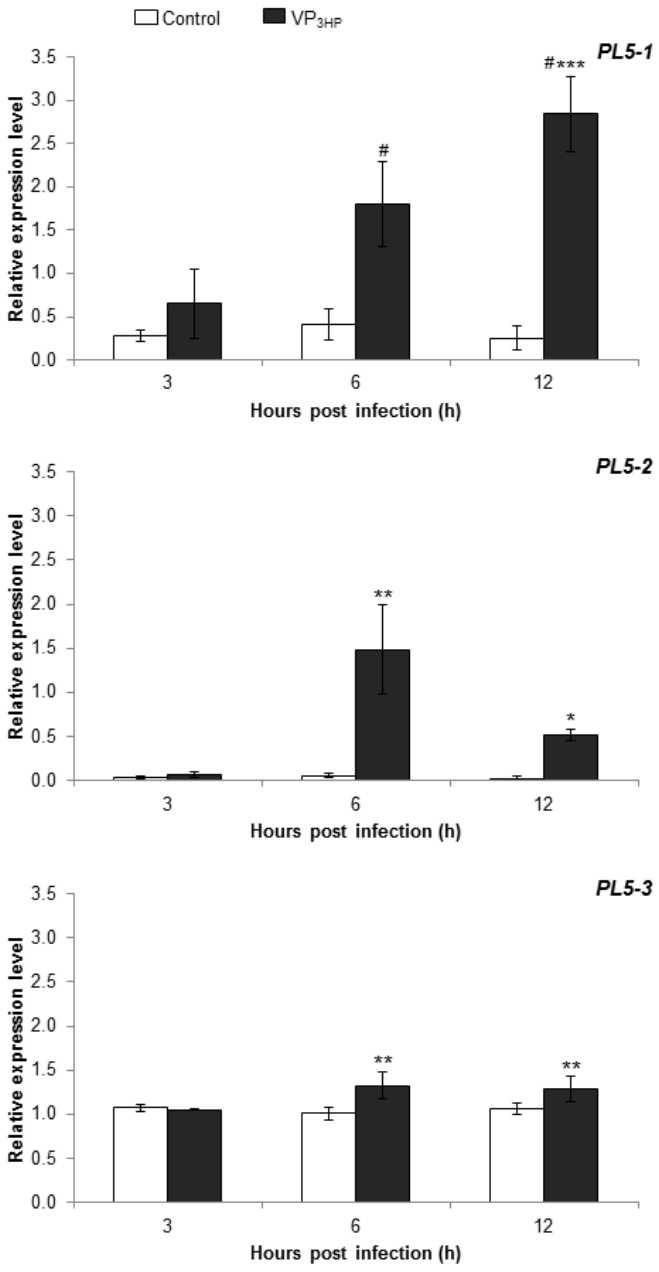

Figure 4.23 Real-time PCR analysis showing relative expression levels of *PL5-1*, *PL5-2* and *PL5-3* in stomach of juvenile shrimp after challenge with normal saline (control group) or challenged with *V. parahaemolyticus* (3HP) at 3, 6 and 12 hours. The asterisk indicates that the expression levels are significantly different. * P <0.05, ** P<0.01, *** P<0.001 when compared to AHPND at 6 hours, # P<0.001 when compared to control and AHPND at 3 hours (*N = 4*).

4.10 The effect of *in vivo* gene silencing of *PL5-1*, *PL5-2* and *PL5-3*

4.10.1 *PL5-1*, *PL5-2* and *PL5-3* dsRNA synthesis

To determine the immunological role of PL5s, *PL5-1*, *PL5-2* and *PL5-3* silencing experiments of their corresponding mRNAs were performed . The semi-quantitative RT-PCR analysis showed that the expression of *PL5-1*, *PL5-2* and *PL5-3* could be knock-down by dsRNA injection for at least 2 days after the second injections when compared with shrimp injected with *dsGFP* (Figure 4.24).

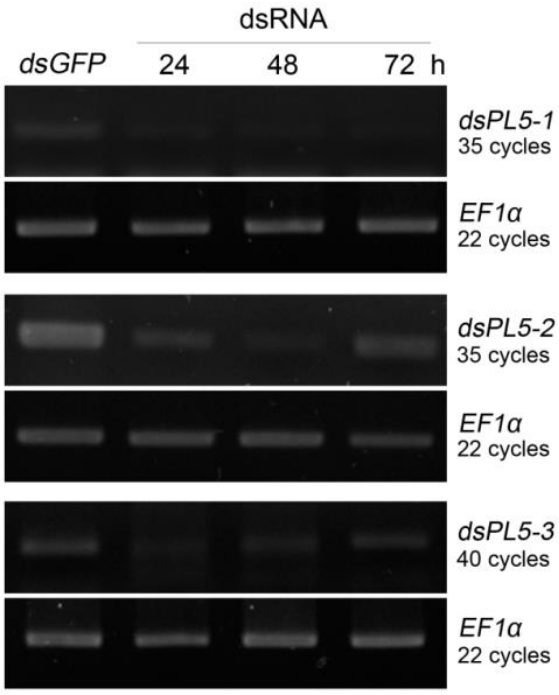

Figure 4.24 RNAi efficiency of *PL5-1*, *PL5-2* and *PL5-3*. Shrimp was injected with 5 µg of dsRNA / g shrimp which was repeated at 24 hours after the first injection and dsGFP injected animals were used as controls. The expression of *PL5-1*, *PL5-2* and *PL5-3* in stomach were analysed at 24, 48 and 72 hours after the second injection by RT-PCR and EF1α was used as an internal control.

4.10.2 Survival rate and histopathology of PL5s knocked-down shrimp

The survival rate and histopathology of shrimp were determined in the *PLs* silenced shrimp. Shrimp were injected with *PL5-1*, *PL5-2* or *PL5-3* dsRNA (N = 10) and this was repeated at 24 hours which was followed by feeding *Artemia*-VP_{3HP}. Finally, the survival rates of *PL5-1*, *PL5-2* and *PL5-3* silenced shrimp at 48 hours after exposure to VP_{3HP} was 10 %, 40 % and 10%, respectively. The survival rate of PLs silenced shrimp was lower than GFP dsRNA and normal saline injected group (80 % and 80 %, respectively) (Figure 4.25). Therefore, the reduction of *PL5-1*, *PL5-2* or *PL5-3* expression clearly effected the survival rate of shrimp receiving a VP_{3HP} infection.

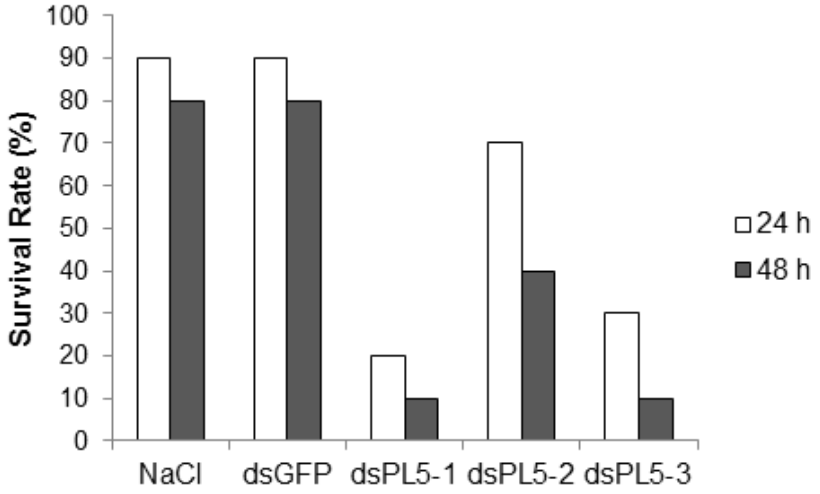

Figure 4.25 Percentage survival of shrimp at 24 and 48 hours after challenge with VP_{3HP}. Individual shrimp was injected twice with 5 µg dsRNA/g of shrimp. *dsPL5-1*, *dsPL5-2* or *dsPL5-3* before challenge. The 150 mM NaCl and dsGFP injected animals were used as control (*N = 10*).

4.10.3 Histopathological study of VP_{3HP}-challenged shrimp following PL5s silencing by dsRNA

To confirm that moribund shrimp and newly died shrimp caused by VP_{3HP} infection showed typical signs of AHPND, sagittal sections of head part of shrimp were performed and histological changes were studied. Furthermore, the severity of the histopathological changees in the hepatopancreas among the treatments were compared. Cross section of the hepatopancreas of non-infected shrimp showed that the hepatopancreas is composed of numerous hepatopancreatic tubules with intact epithelial layer (Figure 4.26A), while shrimp infected with VP3HP showed typical lesions of Acute hepatopancreatic necrosis disease of sloughed epithelial cells in the lumens surrounded by multiple layers of infiltrated hemocytes and necrotic cells an early stage of encapsulation (Figure 4.26B). All examined moribund or newly died shrimp from treatments of *PL5-1*, *PL5-2* or *PL5-3* knock-down and control groups (normal saline or *dsGFP*) which received VP_{3HP} within 24 hours, the histopathological study showed heavy infection within their hepatopancreas with numerous encapsulated tubules occupying nearly whole of the hepatopancreas organ and or the anterior part of the midgut. Almost all the tubules of the hepatopancreas were severely destroyed. Many tubule lumens contain cell debris, sloughed cells and bacterial cells (Figure 4.27A, C, E, G, I). Shrimp survivors sacrificed at 48 hours post challenge from all treatments showed a degree of infection from slight (Figure 4.27D, F), moderate (Figure 4.27B) to heavy infection (Figure 4.27H, J). Shrimp with severe infection at 48 hours post challenge were indicated by hepatopancreas shrinkage with chronic encapsulations, infected tubules walled off by infiltrated hemocytes and associated with melanin. Whereas, shrimp with slight or moderate infections showed only distorted shape of tubules (Figure 4.27F), marked infiltration of hemocytes (Figure 4.27D) or moderate necrotic foci (Figure 4.27B) in the hepatopancreas.

To determine the influence of RNAi for *PL5-1*, *PL5-2* and *PL5-3* on infection of AHPND, the histopathological changes of shrimp hepatopancreas was studied in the knock-down shrimp and control shrimp (normal saline or *dsGFP*) at 8 hours post challenge. The observation showed that all examined shrimp from *PL5-1*, *PL5-2* and *PL5-3* knock-down shrimp (*N=3*) had a severe damage of tissue throughout

the hepatopancreas with an early stage of typical AHPND lesions (Figure 4.28F, G, H), while shrimp from control groups which were infected but no silencing of the lectins ocurred , two shrimp from NaCl injected shrimp (Figure 4.28 A, B) and 3 shrimp from dsGFP injected shrimp (Figure 4.28C, D, E) showed a range of severity from relatively normal to severe infections.

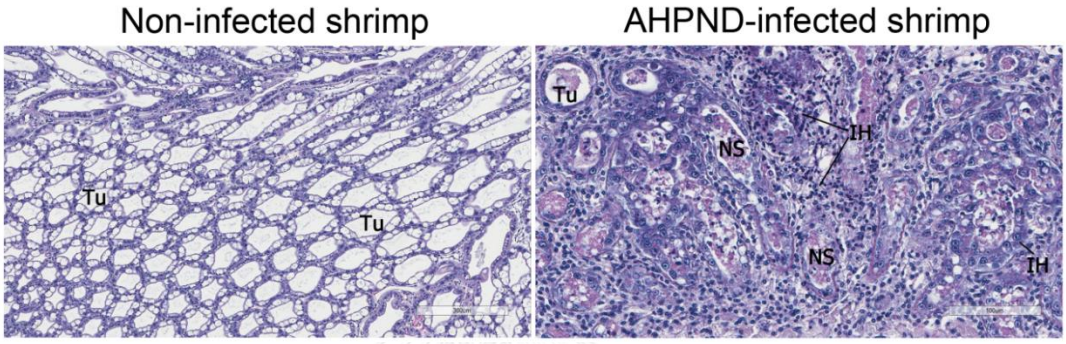

Figure 4.26 Histology analysis of representative tissues (H&E stain) showing hepatopancreas of non-infected shrimp and AHPND-infected shrimp. Abbreviation: hepatopancreas tubules (Tu), infiltrated hemocytes (IH) and necrosis sloughing of hepatopancreas tubular epithelial cells (NS).

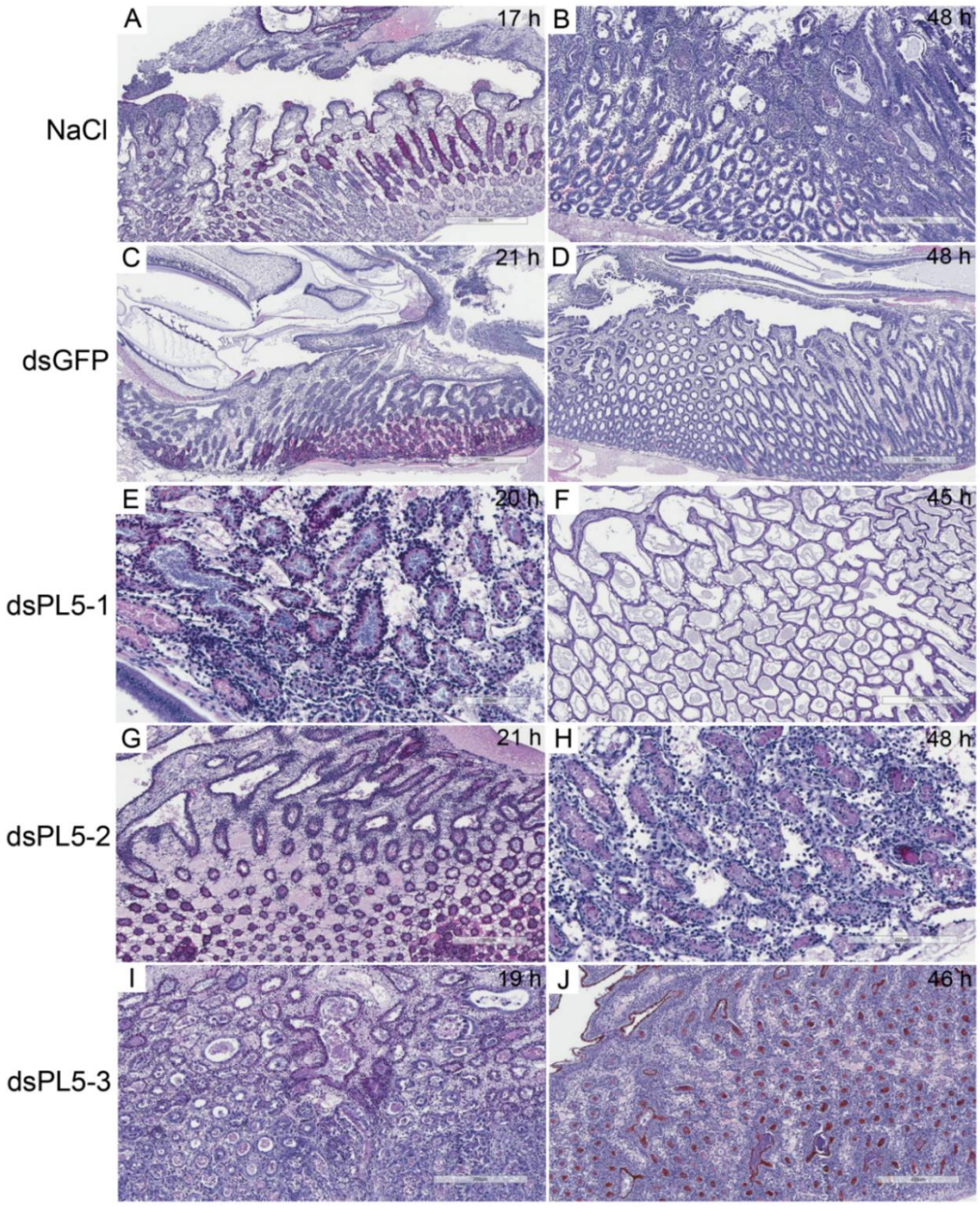

Figure 4.27 Histology analysis of representative tissues (H&E stain) showing hepatopancreas of knock-down shrimp after fed with Artemia-VP3HP. Control shrimp (NaCl injected, A and B), GFP silenced shrimp (C and D), PL5-1 silenced shrimp (E and F), PL5-2 silenced shrimp (G and H) and PL5-3 silenced shrimp (I and J).

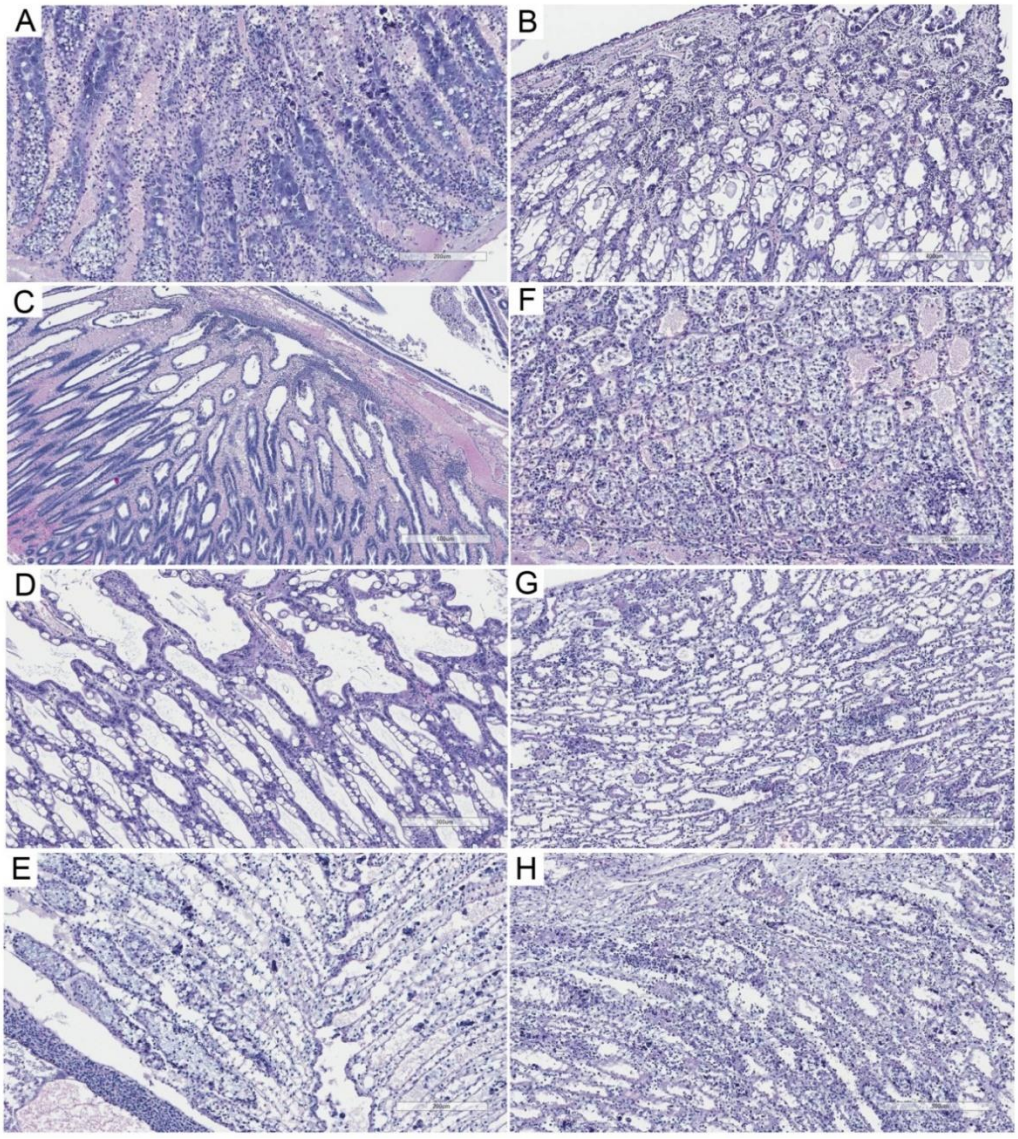

Figure 4.28 Histology analysis of representative tissues (H&E stain) showing hepatopancreas of PL5s knock-down shrimp after fed with Artemia-VP3HP at 8 h post infection. Control shrimp (NaCl injected, A and B), GFP silenced shrimp (C, D and E), PL5s silenced shrimp (F, G and H).

CHAPTER V
DISCUSSION

Shrimp is one of the most important commercial aquaculture animals in the world. However, shrimp culture has been threatened by bacterial and viral outbreaks and led to huge economic losses in the past decades due to several different diseases (Luo et al., 2007; Yang et al., 2007).

Since molecular mechanisms and functional involvement of genes and proteins related to innate immune system of *P. monodon* has received great attention, still studies of the host immune responses against pathogens would contribute to the development of relevant approaches for disease control and sustainable shrimp farming. Until now much progress at the molecular level has been reported on crustacean innate immunity focusing on proPO activating system and antimicrobial peptides (Cerenius et al., 2010; Jiravanichpaisal et al., 2006). However, much less is known of lectins as one of well-known pattern recognition proteins (PRPs). Compared to vertebrate or other arthropod lectins, the molecular features and functions of lectins in shrimp are just at the beginning of getting understood. However, studies on the role of lectins as PRPs and how they are involved in innate immunity is now receiving great attention in recent years. In earlier work since 1990, a number of natural lectins were purified and characterized by biochemical methods from hemolymph of shrimp. Subsequent work at the molecular biology level has provided structural information of many shrimp lectins. Notably almost all known shrimp lectins are member of the C-type lectin family, since Ca^{2+} are required for their agglutinating activity. Further characterization and more molecular information of other lectins might help to better understand immune defense mechanisms and functions in shrimp (Marques and Barracco, 2000). Therefore, in the present work we have made detailed molecular and functional studies of the fibrinogen related proteins Pentectin5-1 (PL5-1), Pentectin5-2 (PL5-2) and Pentectin5-3 (PL5-3) at the molecular level in shrimp and its potential role in shrimp immune responses.

5.1 Isolation and characterization of the full length cDNA of immune-related genes

The full length cDNA of *PL5-1*, *PL5-2* and *PL5-3* were successfully characterized and reported for the first time in *P. monodon*. The deduced amino acid sequence of these proteins contained the N-terminal signal peptide sequence, suggesting it is a secreted protein. The putative *N*-linked glycosylation site (N-X-S/T) was found in the deduced protein of all three genes. Alignment of PL5-1, PL5-2 and PL5-3 with Japanese horseshoe crab tachylectin 5A (TL5A), tachylectin 5B (TL5B) and other related proteins showed that the FReD of these proteins is well conserved. A phylogenetic tree was constructed based on the amino acid sequence of these shrimp FReDs and other known FReDs from vertebrates and invertebrates. The phylogenetic analysis revealed that PL5-1, PL5-2 and PL5-3 are clustered in the same large group. Although, amino acid sequences of PL5-1, PL5-2 and PL5-3 are very similar to each other, they cluster separately in the phylogenetic tree in a similar way as horseshoe crab TL5A and TL5B. This suggests that these three lectin homologous genes may have different functions.

Alignment of the deduced amino acids indicates a potential functional relationship of PL5 to TL5A. One Ca^{2+}-binding site was found in the FReD of PL5-1, PL5-2 and PL5-3 proteins and a highly conserved site for Ca^{2+} and carbohydrate binding as in Japanese horseshoe crab TL5A were also present. We also show that PL5-2 could specifically recognize GlcNAc and GalNAC, suggesting that the primary ligand-binding site is in FReD, like TL5A, and consequently is a pattern recognition receptor (Kairies et al., 2001). Although, amino acid sequences of PL5-1, PL5-2 and PL5-3 are very similar to each other, they cluster separately in the phylogenetic tree in a similar way as japanese horseshoe crab TL5A and TL5B. This may suggest that these shrimp lectin genes can have different functions.

Tachylectin and related proteins have been considered as immune effectors that recognize invading microorganisms in the first line of the defense system. Although many types of TLs have been found in horseshoe crab and other animals (Galliano et al., 2003; Huh et al., 1998; Mali et al., 2006; Romero et al., 2011; Schroder et al., 2003), so far only tachylecin5-like genes have been found in *P. monodon.* Interestingly we

found that shrimp *PmTL5B* was mainly expressed in the hindgut and it could be induced to a higher expression during immersion with *V. harveyi* (Soonthornchai et al., 2010).

Gene expression analysis and tissue distribution analysis are important and provide basic information for further analysis of functional genes. In this study, therefore, tissues distribution analysis of *PL5-1*, *PL5-2* and *PL5-3* were determined in various tissues of juvenile *P. monodon.* The expression of *PL5-1* was highly expressed in almost all examined tissues including heart, anterior intestine, lymphoid organ, muscle, pleopod, stomach and thoracic ganglion and expressed at a lower level in epidermis, eye stalk, gill, hemocyte and hepatopancreas. The *PL5-2* was expressed at the highest level among the examined organs in epidermis, eye stalk, posterior intestine with hindgut, abdominal ganglion, pleopod and stomach. On the other hand, *PL5-3* was highly expressed in anterior intestine and posterior intestine. *PL5-1*, *PL5-2* and *PL5-3* were all expressed in hemocytes but not at a high level. Although tissue distribution of *TL5A* and *TL5B* in Japanese horse-shoe crab was not studied, it is reasonable to suggest that expression of *TL5A* was high in heart while *TL5B* expression was high in hemocytes, because TL5A and TL5B were isolated from a heart and a hemocyte cDNA library, respectively (Gokudan et al., 1999). Besides these studies of the expression level of *PL5-1*, *PL5-2* and *PL5-3* in different tissues, we also focused on expression in the digestive tract since many pathogens gain entry through the mouth and then cause infection along the gastrointestinal tract or even can penetrate by themselves into the hemocoel. Surprisingly, in the gut the *PL5-2* transcript was expressed much higher in the posterior intestine than in the anterior intestine. *PL5-1* was expressed at a very high level in the anterior intestine but not in the posterior intestine. Moreover, *PL5-3* was expressed at a high level in both anterior intestine and posterior intestine. In shrimp stomach, the *PL5-2* transcript was highly expressed whereas *PL5-1* was expressed moderately and *PL5-3* was expressed at a low level. Based on phylogenetic analysis and tissue distribution results, it is possible to propose that PL5-1, PL5-2 and PL5-3 may have different roles in the immune response or they may have the same role but they are aimed at different targets.

In another species of horseshoe crab (*Carcinoscorpius rotundicauda*), CL5A and CL5B transcripts appeared to be more abundantly expressed in intestine (Zhu et al., 2006b). Another tachylectin-related homolog identified in amphioxus, *Branchiosotoma belcheri* (BbTL) which is similar to horseshoe crab TL1 has the most abundant expression in the hepatic caecum and hindgut (Ju et al., 2009b). It was noted that the epithelial layers of shrimp stomach and hindgut are lined by cuticle containing c hitin. Together with the result of the phylogenetic tree analysis, it is plausible to propose that PL5-1, PL5-3 and PL5-3 may have different roles in the immune response in shrimp or they may have the same role, but they are armed at different organs.

Penaeid larvae development is among the most complicated processes in decapod crustaceans. The crustacean larval development involves six nauplius, three zoea, and three mysis stages before reaching the postlarvae and adult stages, respectively. In a previous study, the level of proPO expression and protein was low or absent until nauplius stage four (Jiravanichpaisal et al., 2007). This was proposed to be a result of that until the larval stages starts to feed and have an intestine there is no real need for any non-self recognition system to respond to foreign molecules or pathogens, since most parasites or pathogens infect the animals through the intestine or stomach. Also the expression profile of *PL5-1*, *PL5-2* and *PL5-3* was studied in different developmental stages of shrimp. *PL5-2* was expressed at a very low level from nearly egg stage to the nauplius 3. However, from the larval stage nauplius 6 and onwards the *PL5-2* was expressed at significant levels. Similarly, the expression of *PL5-1* was hardly seen in nearly hatched eggs, but it was significantly increased from nauplius1. Moreover, *PL5-3* was expressed from embryo to protozoea 3 and expressed at high level from mysis 1 and onwards. These results suggest that *PL5-1*, *PL5-2* and *PL5-3* are highly expressed during shrimp larval development, because they are needed to be exposed and react to microorganisms from the surrounding environment. Similar result was observed with the CTRN gene, a tachylectin-like gene in *Hydractinia* and this gene was only expressed in post-metamorphic life but not in early embryos and planula larvae. This may also be important for organ development (Mali et al., 2006).

5.2 *In vitro* expression of recombinant protein and polyclonal antibody production

PL5-1, PL5-2 and PL5-3 were expressed in vitro using the bacterial expression system to produce recombinant proteins. Recombinant PL5-2 was successfully expressed using pET15b and expressed as His-tagged protein. This protein was purified and was then used to produce antibody. Recently, also PL5-1 and PL5-3 were expressed but since all PL5s were expressed as non-soluble proteins it was necessary to use the native purified proteins. By using affinity chromatography it was only possible to isolate and purify PL5-2 and this is the main reason why functional studies using a protein only was studied with this lectin.

5.3 Localization of PL5-2 protein in *P. monodon*

TLs-5 and CLs-5 from two horseshoe crabs, *T. tridentatus* and *Carcinoscorpius rotundicauda*, respectively are plasma lectins (Gokudan et al., 1999; Zhu et al., 2006b). The result of the tissue specific localization of PL5-2 shows that shrimp PL5-2 protein is present in various tissues and in the plasma. This suggests that PL5-2 is secreted from various tissues into the plasma and these molecules are important to recognize invading microbes in the first line of defense particularly in the hemolymph and in the intestine. Immunohistochemistry using the antibody agains PL5 -2 shows very similar results that the protein is found in many different tissues and they are secreted into plasma.

5.4 Biological function of purified PL5-2

Horseshoe crab tachylectins can recognize PAMPs of both Gram-positive and Gram-negative bacteria with different specificity (Saito et al., 1995; Zhu et al., 2006b). The tachylectin related protein of the sponge, *Suberites domuncula* was found to bind to LPS and to be up-regulated by challenge with LPS (Schroder et al., 2003).

Since the recombinant PL5-2 protein was expressed in an insoluble form, it was necessary to purify PL5-2 from *P. monodon* plasma using *N*-acetyl-D- Glucosamine (GlcNAC) affinity chromatography and then this purified protein could be used for

functional studies. The result shows that the purified PL5-2 protein from shrimp plasma had binding activity to various bacteria regardless whether they are pathogenic or non-pathogenic to shrimp. Although CL5A and CL5B can strongly bind to lipopolysaccharide (LPS) from *E. coli* or lipoteichoic acid (LTA) from *S. aureus* (Zhu et al., 2006a), in the present study no binding of PL5-2 to live *S. aureus* was detected. This indicates that variation in binding activity of PL5-2 may depend on the ligand structure of the PAMPs presented on the surface of the bacterial cells. We also show that the binding activity could be inhibited when GlcNAc or GalNAc was added into the samples, suggesting that PL5-2 has ability to bind to acetyl-group containing molecules present on the microbe surface.

The PL5-2 protein has hemagglutination activity, it can agglutinate all human erythrocyte types including A, B and O. Furthermore, the PL5-2 protein can agglutinate both Gram-positive and Gram-negative bacteria. Similar results were observed with TL5A and TL5B of japanese horseshoe crab, they have hemagglutinating activity against A, B, and O- types of erythrocytes. Moreover, TL5A and TL5B have strong bacterial agglutinating activity against both Gram-positive and Gram-negative bacteria. Therefore, TL5A and TL5B must be the primary lectins that recognize invading microbes (Kawabata and Iwanaga, 1999). Human erythrocyte membranes contain several sugars among which for example GlcNAC, GalNAC and NeuAc (Bladier et al., 1979), so this shows that the PL5-2 protein has a binding affinity to acetyl groups and can agglutinate erythrocytes.

PL5-2 protein has an antimicrobial activity against Gram-negative bacteria including *A. hydrophila*, *V. harveyi* and weakly against *V. parahemolyticus*, but it could not inhibit the growth of the Gram-positive bacterium, *M. luteus*. TL5A and TL5B have been shown to enhance the antimicrobial activity of a horseshoe crab-derived big defensin, which is active against both Gram-positive and Gram-negative bacteria. Purified FRFPs in the Mediterranean mussel (*Mytilus galloprovincialis*) were able to increase the hemocytic phagocytosis of fluorescent beads in vitro (Romero et al., 2011).

5.5 Expression analysis of immune-related genes by quantitative real-time PCR

Tachylectins are generally regarded as immune molecules. For example, horseshoe crab tachylectins bind to pathogen associated molecular patterns (PAMP) of Gram-positive and Gram-negative bacteria with different specificity spectra (Saito et al., 1995) . Moreover, the sponge, *Suberites domuncula*, tachylectin-related protein was found to bind to LPS and to be up-regulated by challenge with LPS (Schroder et al., 2003). The amphioxus tachylectin was significant up-regulated in the guts including the hepatic caecum and hindgut by challenge with LPS (Ju et al., 2009a). Similar results were obtained in the present study and the transcript of *PL5-2* was up-regulated by challenge with *V. harveyi* in the posterior intestine of juvenile *P. monodon*. Moreover, *PL5-1* transcript was up-regulated in hemocytes after *V. harveyi* i njection. However, the expression of *PL5-1* and *PL5-2* were not significantly increased after injection with the Gram-positive bacteria, *S. aureus* or *M. luteus* regardless of their binding ability. One reason can be that these two Gram-positive bacteria are not shrimp pathogens and then they were immediately cleared from the host within a much shorter period compared to a pathogenic bacterium such as *V. harveyi*. It means that *V. harveyi* can prolong its stay in the shrimp body using its virulence factors to fight against host immune responses. In contrast, exposure of hydroid colonies to LPS under conditions known to act ivate an immune response in this species did not result in an up-regulation of the tachylectin-related gene in hydractinia (CTRN) (Mali et al., 2006), suggesting that CTRN does not seem to be involved in innate immune responses.

Moreover, the expression profile at transcription level of *PL5-1*, *PL5-2* and *PL5-3* were investigated in shrimp infected with VP_{3HP} which is proposed to be the causative agent for AHPND. The initial step of an infection process caused by bacterial pathogens in shrimp, usually encounter that a large number of bacterial cells are attached to the stomach surface (Soonthornchai et al., 2010), therefore, stomach was the focus for this study. The results showed that *PL5-1*, *PL5-2* and *PL5-3* were up-regulated in stomach after VP_{3HP} infection, this may suggest that PL5s gene has a potential role in shrimp immunity.

5.6 The effect of *in vivo* gene silencing of *PL5-1*, *PL5-2* and *PL5-3* on resistance to VP_{3HP}

First, PL5-2 gene was silenced in shrimp and then these shrimp were fed with *Artemia* containing VP_{3HP} to determine if they were more susceptible to this bacterium. As the controls NaCl-, or *dsGFP* -injected shrimp were used. The mortality rate was 60% which shows that this lectin is of importance for the survival of this animal. When similar experiments were performed with PL5-1 and PL5-3, separately, the mortality rates were found to be much higher and nearly all (90%) shrimp died. This clearly demonstrated that these two latter lectins are of crucial importance for the resistance of the shrimp to infection with VP_{3HP}. Histopathological studies of hepatopancreas in moribund and newly dead animals showed typical signs of AHPND symptoms. Epithelial cells were sloughed into the lumen of the hepatopancreatic tubules with infiltration of hemocytes and necrotic foci around these damaged tissues (Joshi et al., 2014; Lightner et al., 2012; Tran et al., 2013). These results show that the PLs are very important for the host defense against this bacterial pathogen. The mechanism by which this occurs is most likely that these lectins prevent the bacteria from attaching to the chitin layer on the outer surface of the epithelium by binding to the acetyl groups of chitin so that VP_{3HP} with its chitin binding protein is unable to bind to the intestinal chitin. Another possible mechanism might be that these lectins bind to PAMPs on the VP_{3HP} surface and then basically agglutinate them in large clusters which will inhibit the pathogen from attaching to the host or trap the pathogen until other immune effector molecules are produced. A combination of both processes is highly likely to occur. This will prevent the bacterial pathogen from establishing an infection in shrimp.

CHAPTER VI
CONCLUSION

1. From the shrimp *P. monodon* the full length cDNAs of *PL5-1* (ORF of 939 bp which corresponds to a polypeptide of 312 amino acid), *PL5-2* (ORF of 768 bp which corresponds to a polypeptide of 255 amino acid) and *PL5-3* (ORF of 957 bp, which corresponds to a polypeptide of 318 amino acid) were characterised for the first time. PL5-1, PL5-2 and PL5-3 all contain fibrinogen domains (FReD).

2. Tissue distribution analysis shows that *PL5-1, PL5-2 and PL5-3* were expressed in almost all examined tissues of juvenile shrimp and also in the gastrointestinal tract. The three lectins were expressed at a lower level at early larval stages during shrimp development and increased from late nauplius stage and onwards.

3. A polyclonal antibody was made to the recombinant PL5-2. However, the recombinant proteins could not be used for functional studies since they were non-soluble and non-secrete proteins and as a consequence the PL5-2 protein was found to be possible to purify from plasma and was therefore used for protein functional studies.

4.The PL5-2 protein was located in all examined shrimp tissues and also in shrimp plasma.

5. The PL5-2 protein had bacteria- binding activity, hemagglutination activity, bacterial agglutination activity and antibacterial activity to live bacteria both Gram-negative and Gram-positive bacteria and the activity could be inhibited by GlcNAc or GalNAc and required calcium.

6. The expression level of both *PL5-1 and PL5-2* was significantly up-reglurated in hemocytes and posterior intestine after shrimp were injected with *V. harveyi*. The expression level of *PL5-1, PL5-2* and *PL5-3* was up-regulated in stomach after shrimp were challenged with *V. parahaemolyticus* (3HP), which is the causative agent AHPND.

7. PL5-1, PL5-2 and PL5-3 silenced gene have effected on survival rate after shrimp were exposed to pathogenic *V. parahaemolyticus* (3HP) causing AHPND. The histopathology showed higher severity changed in hepatopancreas when PL5 genes silencing, almost the tubular epithelial cells were sloughed into the hapatopancreatic lumens and hemocytes were infiltrated in the intertubular space between hepatopancreatic tubes.

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APPENDIX

VITA

Miss Pacharaporn Angthong was born on May 13, 1985 in Bangkok, Thailand. She graduated with the degree of Bachelor of Science (Fisheries Science), Faculty of Agricultural Technology from King Mongkut's Institute of Technology Lardkrabang in 2007 and the degree of Master of Science (Fisheries Science), Faculty of Agricultural Technology from King Mongkut's Institute of Technology Lardkrabang in 2010. She has studied for the Degree of Doctor of Philosophy in Biotechnology, Chulalongkorn University since 2010.

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