สมบัติและหน้าที่ขององค์ประกอบของโพรฟีนอลออกซิเดสในครัสเตเชียน

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PROPERTIES AND FUNCTIONS OF PROPHENOLOXIDASE COMPONENTS IN CRUSTACEANS

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การสังเคราะห์เมลานินเป็นกลไกการป้องกันตัวหลักที่สัตว์ไม่มีกระดูกสันหลังใช้ตอบสนองต่อเชื้อก่อโรค งานวิจัยนี้ได้ศึกษาหน้าที่ของซีรีนโปรติเนสฮอมอล็อกสองชนิดจากกุ้งกุลาดำ *Penaeus monodon* (*Pm*MasSPH1 และ *Pm*MasSPH2) จากการวิเคราะห์ลำดับกรดอะมิโน พบว่า *Pm*MasSPH1 และ *Pm*MasSPH2 มีความคล้ายกับยีน *Pl*SPH ในกุ้งเครย์ฟิช *Pacifastacus leniusculus* และมีคลิปโดเมน และซีรีนโปรติเนสไลค์โดเมนเป็นโดเมนอนุรักษ์ที่ปลาย N และปลาย C ตามลำดับ เมื่อลดการแสดงออกของยีนด้วย dsRNA *Pm*MasSPH1 และ *Pm*MasSPH2 พบว่าค่าแอคทิวิตีของเอนไซม์ฟีนอลออกซิเดสลดลง 66.5% และ 63.7% ตามลำดับ และมีปริมาณเชื้อแบคทีเรียในเลือดเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม นอกจากนี้ การลดการแสดงออกของ *Pm*MasSPH1 ยังส่งผลให้ยีนในกลุ่มเปปไทด์ต้านจุลชีพ (*Penmon*PEN3, crustin*Pm*1, และ Crus-like*Pm*) มีการแสดงออกลดลงซึ่งชี้ว่ามีกลไกเชื่อมระหว่างระบบการกระตุ้นเอนไซม์ฟีนอลออกซิเดสและการสังเคราะห์เปปไทด์ต้านจุลชีพ จากการใช้เทคนิคโคอิมมูโนพรีซิพิเทชันระหว่าง *Pm*MasSPHs และ *Pm*PPAEs พบการจับกันระว่าง *Pm*MasSPH1 and *Pm*PPAE2 นอกจากนี้ ทั้ง *Pm*MasSPH1 และ *Pm*MasSPH2 ยังสามารถจับกับเปปติโดไกลแคน (PGN) ซึ่งเป็นส่วนประกอบของผนังเซลล์แบคทีเรียแกรมบวก แสดงให้เห็นว่า *Pm*MasSPH1 และ *Pm*MasSPH2 มีความเป็นไปได้ที่จะจับกับ PGN และกระตุ้นระบบเอนไซม์ฟีนอลออกซิเดส

งานวิจัยนี้ยังได้ศึกษาหน้าที่ในการกำจัดเชื้อและการต้านจุลชีพของชิ้นส่วนที่ได้จากการตัดเอนไซม์ฟีนอลออกซิเดสในกุ้งเครย์ฟิช *P. leniusculus* ของคาสเปส 1 และโพรฟีนอลออกซิเดสแอคติเวติงเอนไซม์ (ppA) ชิ้นส่วนดังกล่าวได้แก่ *Pl*proPO-ppA ซึ่งเป็นปลายอะมิโนของ proPO หลังจากถูกตัดด้วย ppA และ *Pl*proPO-casp1 และ *Pl*proPO-casp2 ซึ่งเป็นปลายอะมิโนของ proPO หลังจากถูกตัดด้วยคาสเปส 1 จากการฉีดเชื้อควบคู่กับเปปไทด์ *Pl*proPO-ppA *Pl*proPO-casp1 and *Pl*proPO-casp2 พบว่าปริมาณเชื้อในทั้งสามกลุ่มนั้นมีปริมาณน้อยกว่ากลุ่มควบคุมซึ่งฉีดแต่เชื้ออย่างเดียว นอกจากนี้ *Pl*proPO-ppA ยังมีความสามารถในการต้านจุลชีพในหลอดทดลอง ในขณะที่ *Pl*proPO-casp1 และ *Pl*proPO-casp2 ไม่มีความสามารถดังกล่าว ในการศึกษาการตายของเชื้อพบว่าเปปไทด์ *Pl*proPO-ppA ทำให้เชื้อเกิดการจับกลุ่มกัน จากการค้นพบนี้แสดงให้เห็นถึงหน้าที่ใหม่ของ proPO ในระบบภูมิคุ้มกันของกุ้งเครย์ฟิช *P. leniusculus*

ภาควิชา ชีวเคมี
สาขาวิชา ชีวเคมี
ปีการศึกษา 2556

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
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5173884023 : MAJOR BIOCHEMISTRY
KEYWORDS: SERINE PROTEINASE HOMOLOGUES / PENAEUS MONODON / RNA INTERFERENCE / PACIFASTACUS LENIUSCULUS / PROPHENOLOXIDASE / INNATE IMMUNE SYSTEM

MITI JEARAPHUNT: PROPERTIES AND FUNCTIONS OF PROPHENOLOXIDASE COMPONENTS IN CRUSTACEANS. ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: PROF. KENNETH SöDERHäLL, Ph.D., PITI AMPARYUP, Ph.D., 90 pp.

Melanization is a major defense mechanism in invertebrate that responds to the pathogens. In this study, we investigated the function of two serine proteinase homologues (*Pm*MasSPH1 and *Pm*MasSPH2) from black tiger shrimp *Penaeus monodon*. Sequence analysis revealed that *Pm*MasSPH1 and *Pm*MasSPH2 exhibited high sequence similarity to freshwater crayfish *Pacifastacus leniusculus Pl*SPHs with the conserved clip-domain and serine proteinase-like domain at the N-terminus and C-terminus, respectively. The dsRNA-mediated gene suppression of *Pm*MasSPH1 and *Pm*MasSPH2 showed the significant decrease in hemolymph PO activity by 66.5% and 63.7%, respectively and a significant increase in the hemolymph bacterial count as compared with the control. In addition, the *Pm*MasSPH1 suppression resulted in a decrease in the expression of antimicrobial peptide genes (*Penmon*PEN3, crustin*Pm*1, and Crus-like*Pm*) suggesting the cross-talk between the proPO system and antimicrobial peptide synthesis pathway. The co-immunoprecipitation of *Pm*MasSPHs and *Pm*PPAEs indicated the protein-protein interaction between *Pm*MasSPH1 and *Pm*PPAE2. *Pm*MasSPHs exhibit binding ability to peptidoglycan (PGN), Gram-positive bacteria cell wall component, suggesting that *Pm*MasSPH1 and *Pm*MasSPH2 likely bind to PGN and activate the proPO system.

This study also investigated the cleavage of the proPO gene of freshwater crayfish *P. leniusculus* by caspases-1 and the proPO activating enzymes (ppA) and the roles of the cleaved fragments in bacterial clearance and antimicrobial activity. These fragments include PlproPO-ppA, the N-terminal part of proPO cleaved by ppA, and proPO-casp1 and proPO-casp2, the N-terminal fragments cleaved by caspase-1. The injection of the cleaved peptides along with *Escherichia coli*, *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2, showed significantly lower bacterial counts compared to the control (bacterial injection alone). *Pl*proPO-ppA displayed the antimicrobial activity in the in vitro experiment, however, proPO-casp1 and -2 did not show the sign of activity. The viability assay indicated that the viability of agglutinated *E. coli* was affected by the recombinant proPO-ppA fragment. These findings suggest a new function of proPO in the *P. leniusculus* immune response.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry	Advisor's Signature	.....
Academic Year:	2013	Co-Advisor's Signature	.....
		Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

A	absorbance
ANOVA	analysis of variance
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	base pair
cDNA	complementary DNA
CFU	colony-forming unit
clip-SPs	clip-domain serine proteinases
clip-SPHs	clip-domain serine proteinases homologues
C-terminal	carboxy-terminal
°C	degree Celsius
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dsRNA	double-stranded RNA
dTTP	deoxythymidine triphosphate
EST	expressed sequence tags
GFP	green fluorescent protein
h	hour
HET-E	incompatibility locus protein from *Podospora anserina*
HLS	hemocyte lysate supernatant
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
LRR	leucine-rich-repeat
M	molar
MAC	minimal agglutinating concentration
mg	milligram
ml	millilitre
mM	millimolar

N-terminal	amino-terminal
NACHT	Domain present NAIP, the major histocompatibility complex (MHC) class II transactivator (CIITA), HET-E and TP1
NALP	NACHT, LRR and PYD containing proteins
NAIP	Neuronal apoptosis inhibitory protein
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PGN	peptidoglycan
PI	propidium Iodide
*Pm*PPAE	*Penaeus monodon* prophenoloxidase-activating enzyme
*Pm*MasSPH	*Penaeus monodon* masquerade-like serine proteinase homologue
PO	phenoloxidase
ppA	prophenoloxidase activating enzyme
PPAE	prophenoloxidase-activating enzyme
proPO	prophenoloxidase
PYD	Pyrin domain
RNA	ribonucleic acid
RNAi	rna interference
RNase	ribonuclease
RT	reverse transcription
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope
SPH	serine proteinase homologue
TP1	telomerase-associated protein
UTR	untranslated region
WSSV	white spot syndrome virus
YH	Yellowhead
YHV	Yellowhead virus
µg	microgram
µL	microlitre

Chapter I
INTRODUCTION

1.1 General information

The aquaculture has been a high value industry over the last few decades. The industry includes fish farming, oyster farming, and especially the shrimp farming. Shrimp aquaculture has been a million dollar industry because of the high demand which lead to a high export value in many countries, including Thailand. Since 1990s, Thailand has been the world leader in black tiger shrimp farming. However, the disease problems wildly affected the shrimp production (Wyban, 2007) and drastically caused a production loss to Thai economic (Figure 1.1). The yellow head virus (YHV), white spot syndrome virus (WSSV), taura syndrome virus (TSV), and *Vibrio harveyi* have been noticed as the major pathogens that cause high mortality in shrimp aquaculture (Flegel, 2006). Recently, an emerging disease, the early mortality syndrome (EMS), was reported in 2009. The outbreak was severely acute which caused 100% mortality rate within 4-30 days post infection (Tran *et al.*, 2013). EMS caused the production loss in China before spreading to Vietnam and Malaysia followed by Thailand in 2012. From the outbreak, the shrimp production in Thailand has been drastically decreased in the past two years. To prevent the economic loss, genetic approach has been introduced to improve shrimp aquaculture and pathogen resistance.

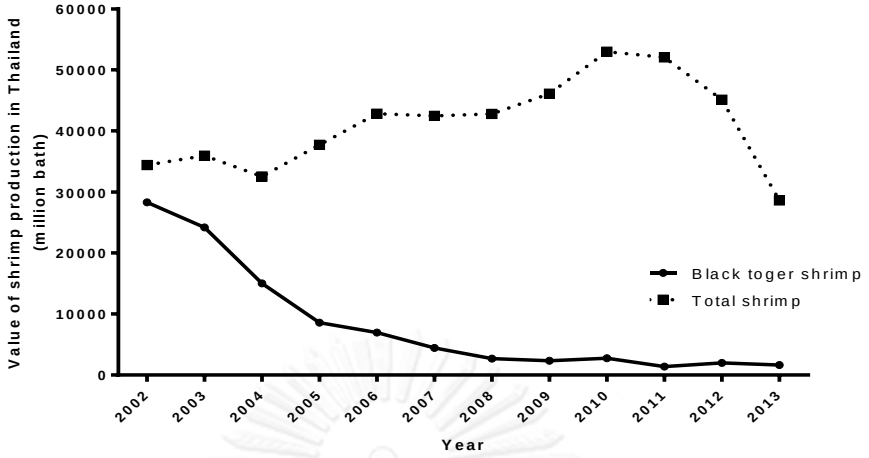

Figure 1.1 The black tiger shrimp, *Penaeus monodon*, production and the total shrimps production in Thailand in the period from 2002 to 2013. (Source: http://www.oae.go.th/oae_report/export_import/export_result.php)

1.2 Major shrimp diseases

Diseases in black tiger shrimp, *Penaeus monodon* are mainly caused by viral and bacterial pathogens. The outbreak of YHV in 1993 has become the serious problem in shrimp aquaculture (Hasson *et al.*, 1995), followed by the WSSV outbreak in 1994 to 1996 (Flegel, 1997). Recently, the new outbreak of early mortality syndrome (EMS) in 2009 has been the major cause of shrimp mortality in aquaculture (Tran *et al.*, 2013).

The white spot syndrome (WSS) is caused by an enveloped DNA virus, WSSV which is a member of family Nimaviridae. The genome of this virus contains double-stranded DNA of 292 to 305 kb. The signs of the infection include the white spots on the exoskeleton and epidermis. Shrimps infected with WSSV have high mortality rates within a few hours to a few days (Yang *et al.*, 2001, Chaivisuthangkura *et al.*, 2014).

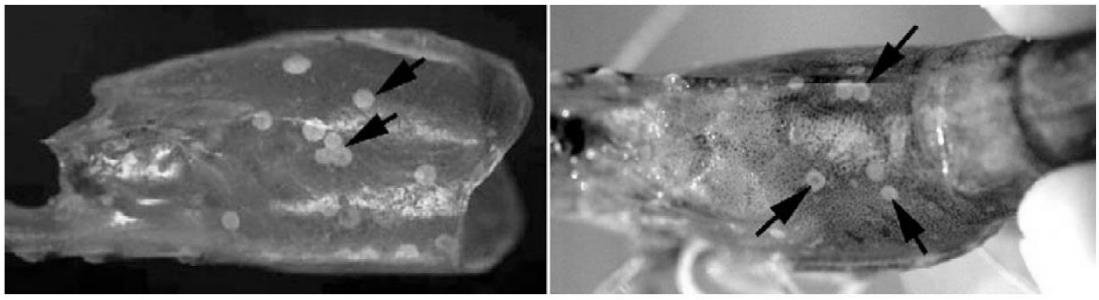

Figure 1.2 The signs of WSSV infection. The white inclusion in the cuticle (arrows) (Flegel, 2006).

The YH disease in Thailand was first reported in 1990. The disease occurs in the juvenile to sub-adult stages and causes mass mortality and production loss. The disease is caused by the YHV which is a single stranded RNA virus in the genus Okavirus, family Roniviridae. The symptoms of the YHV infection is the light yellow coloration of the dorsal cephalothorax area resulting the forming of pale or bleached appearance of hepatopancreas. The mortality rate reaches 100% within 3-5 days. There are several distinct genetic lineages (genotypes) of YHV and YHV type-1 is the type that caused major disease-related losses (Lightner, 1996, Cowley *et al.*, 1999, Chaivisuthangkura *et al.*, 2014).

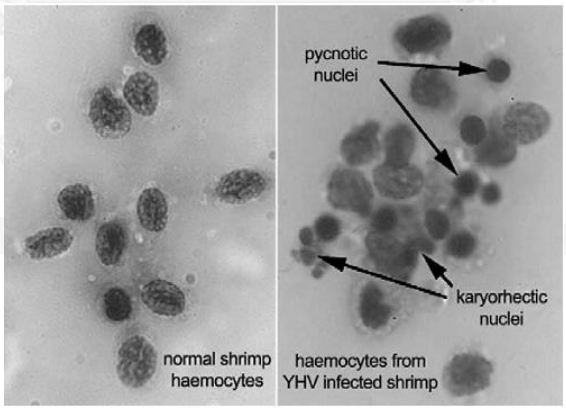

Figure 1.3 Hemocytes from YHV infected shrimp showed the disintegration of the nuclei (Flegel, 2006).

The luminescent bacterium *Vibrio harveyi* is related to the outbreak of the luminous vibriosis in hatcheries. The bacteria cause almost 100% mortality and affect in the larvae, post-larvae, juveniles, sub-adults, and adults (Nash *et al.*, 1992).

The early mortality syndrome (EMS), also known as acute hepatopancreatic necrosis syndrome (AHPNS), is a recent outbreak that causes massive production loss of shrimp aquaculture in southern China, Vietnam, Malaysia, and Thailand. The symptoms for the EMS include a pale to white hepatopancreas (HP) in the early phase of culturing in the grow-out pond because of the pigment loss in the HP R-cells. The size of HP may be reduced by 50% or more. The black spots caused by the melanin deposition from the hemocyte activity are visible in the HP. A recent study suggests that *Vibrio parahaemolyticus* is the cause of AHPNS (Tran *et al.*, 2013).

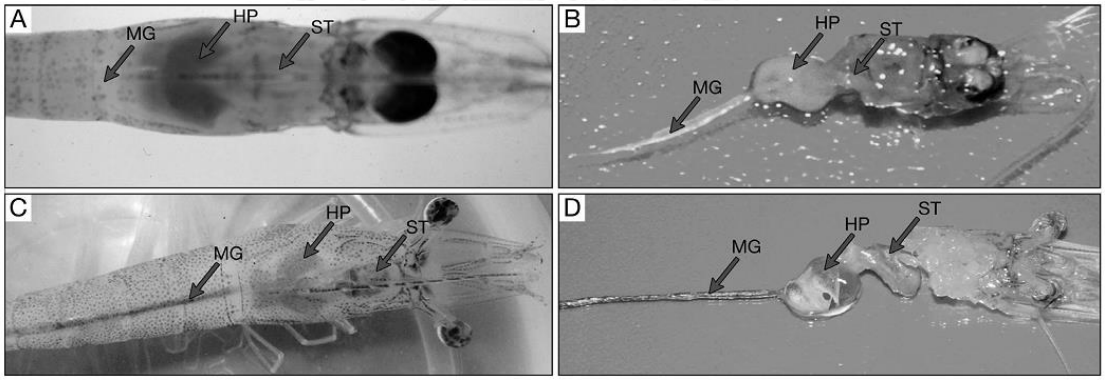

Figure 1.4 The signs of AHPNS shrimp (A, B). The pale hepatopancreas (HP) and an empty stomach (ST) and midgut (MG) compared to the normal shrimp (C,D) (Tran *et al.*, 2013)

1.3 Invertebrate immunity: innate immune system

Every living organism that are exposed to the environment need to shield themselves from harming pathogens that range from a small sized pathogens like virus, bacteria, and fungi, to larger parasites, by developing their immune systems. For this reason, they have developed a variety of the defense mechanisms, from innate immunity that is a non-specific genetically inherited immune system, to the more specific immunity called adaptive immunity (Alberts *et al.*, 2002). The adaptive immunity is mediated by antibody, which makes this system more specific to the

pathogen. Unlike the adaptive immunity, innate immunity is the pre-existing responses to invading pathogens which makes this type of response more rapid to the pathogen. Innate immune responses are found in both vertebrates and invertebrates, and are responsible for the first line of defense by recognizing the conserved structures of non-self microbial cell wall components to activate the immune cascade (Akira *et al.*, 2006) or stimulate the adaptive immune response in mammals (Hoffmann *et al.*, 1999). This response varies from the skin and mucosa as the physicochemical barrier, to the internal defense such as phagocytosis, antimicrobial peptides synthesis, and melanization.

The innate immune system is mediated by two main components; cellular and humoral responses. The cellular immune response is the type of response that blood cells are involved in, including nodule formation, phagocytosis, and encapsulation. The humoral response includes the clotting cascade, the antimicrobial peptide synthesis, and the prophenoloxidase (proPO) activating system (Kanost *et al.*, 2004, Imler and Bulet, 2005, Cerenius *et al.*, 2008).

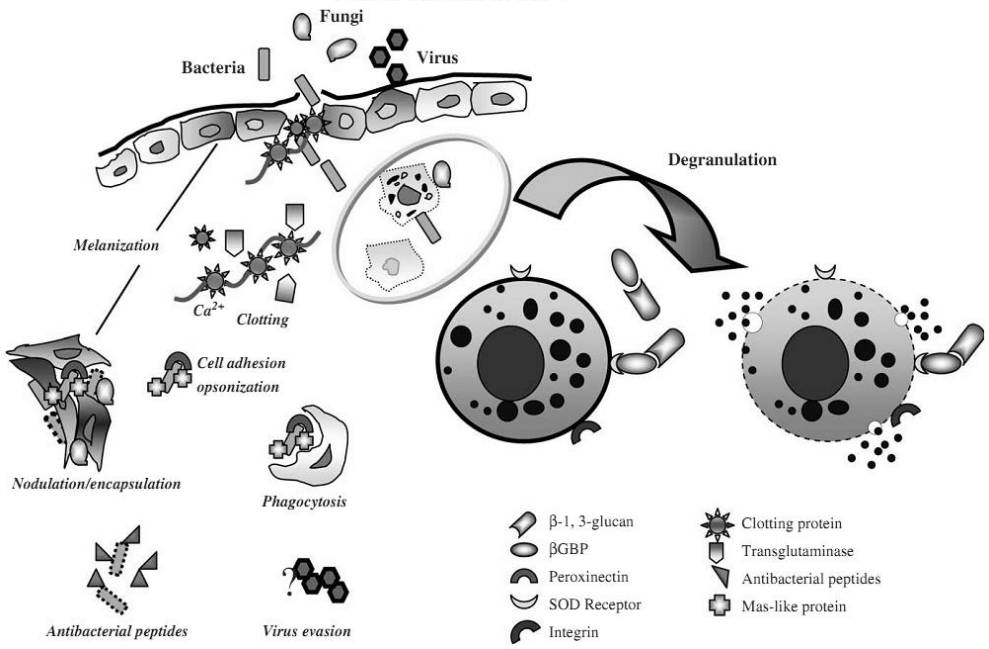

Figure 1.5 The innate immune system in crayfish or crustaceans (Jiravanichpaisal et al., 2006)

1.3.1 Cellular immune response

The cell-mediated responses are the hemocyte-mediated immune responses (Gandhe *et al.*, 2007), including nodule formation, encapsulation, and phagocytosis (Millar and Ratcliffe, 1994, Jiravanichpaisal *et al.*, 2006).

Nodulation, or nodule formation, is the aggregation of the hemocytes around the pathogens and is a response to the infection of bacteria, fungi and virus (Miller *et al.*, 1994, Trudeau *et al.*, 2001) and will later be melanized as a result of the proPO activation (Gandhe *et al.*, 2007). Encapsulation is a mechanism very similar to nodulation, but encapsulation is responsible for the larger pathogens like parasites (Götz, 1986).

Phagocytosis is the response of the hemocyte to recognize and engulf the non-self particle, i.e. bacteria or yeast. These phenomenon is common in most organisms, refers to the mechanism of the engulfment of the small pathogens by an individual hemocyte. The process was initiated by the recognition of the non-self particle followed by the uptake of the particle to destroy (Jiravanichpaisal *et al.*, 2006).

1.3.2 Humoral immune response

Humoral immune response includes the clotting system, synthesis of the antimicrobial peptides (AMPs) and melanization. The clotting system is a defence mechanism to shield the bacteria or other pathogens at the wound sites and prevent blood loss. In horseshoe crab, the clotting cascade is a result of a proteolytic cascade triggered by the pathogen associated molecular patterns (PAMPs) that bind to the receptors (Iwanaga, 2002). In insect, *Manduca sexta*, it has been demonstrated that a lectin and a serine protease homologue are involved in its clotting system (Minnick *et al.*, 1986, Finnerty *et al.*, 1999). However, in fresh water crayfish *Pacifastacus leniusculus*, the clotting occurs the cross-links of the clotting proteins in plasma by the transglutaminase enzyme (Hall *et al.*, 1999, Sritunyalucksana and Söderhäll, 2000).

1.3.2.1 Antimicrobial peptides

AMPs are conserved defense molecules that found in multicellular organisms from plants to animals. AMPs responses to the invasion of bacteria, fungi, and virus (Yasin *et al.*, 2000, Zasloff, 2002, Brown and Hancock, 2006). The peptides, which share common cationic structures, could form electrostatic and hydrophobic interactions with the outer membrane of bacteria that populated with negatively charges and disrupt the cell wall structure (Yang *et al.*, 2000, Zasloff, 2002). In penaeid shrimps, several families of AMPs have been identified, the most abundant AMPs include penaeidins, crustins, anti-lipopolysaccharide factors (ALFs), lysozymes and stylicins (Tassanakajon *et al.*, 2011, Tassanakajon *et al.*, 2013).

Penaeidins are small peptides (molecular mass approximately 5.5 to 6.6 kDa) possess anti-Gram-positive bacterial and anti-fungal activity and are expressed in hemocytes before secreted to the blood circulation as a response to the stimulants (Destoumieux *et al.*, 2000). Penaeidins structure contain a proline-rich domain (PRD) at the N-terminus and a cysteine-rich domain with six cysteine residues at C-terminus (Tassanakajon *et al.*, 2011).

Crustins are peptides containing a cysteine-rich region and a single whey acidic protein (WAP) domain (approximately 50 amino acids with conserved eight cysteine residues) at the C-terminus. Most of the shrimp crustins are Type II crustin that display anti-microbial activity against Gram-positive bacteria (Amparyup *et al.*, 2008). However, crustin*Pm*7 displays the antibacterial activity against both Gram-positive and Gram-negative bacteria including *V. harveyi* (Tassanakajon *et al.*, 2013).

ALFs are small polypeptides with exhibit the anti-Gram negative bacteria activity. They were first found in *Limulus polyphemus* hemocytes (Muta *et al.*, 1987, Wang *et al.*, 2002). Shrimp ALFs share conserved N-terminal hydrophobic and high positively charge disulfide loop region at the C-terminus (Tassanakajon and Somboonwiwat, 2011). The conserved high positive charge the disulphide loop correspond to the LPS binding (Tassanakajon *et al.*, 2013). Shrimp ALFs have been reported to have a broad antimicrobial activity against bacteria and fungi. Previous

study suggested that the ALF*Pm*s could protect shrimps from WSSV infection and prolong the shrimps survival (Tharntada *et al.*, 2009, Ponprateep *et al.*, 2012).

1.3.2.2 Melanization

Melanization is the process of melanin formation as the result of the proPO activating system that is important for most multicellular organisms, including arthropods. This system contains many different enzymes and proteins in the cascade (Figure 1.3). The main group includes the pattern recognition proteins, clip-domain serine proteinases, the proPO, and the negative regulators of the proPO cascade (Amparyup *et al.*, 2013).

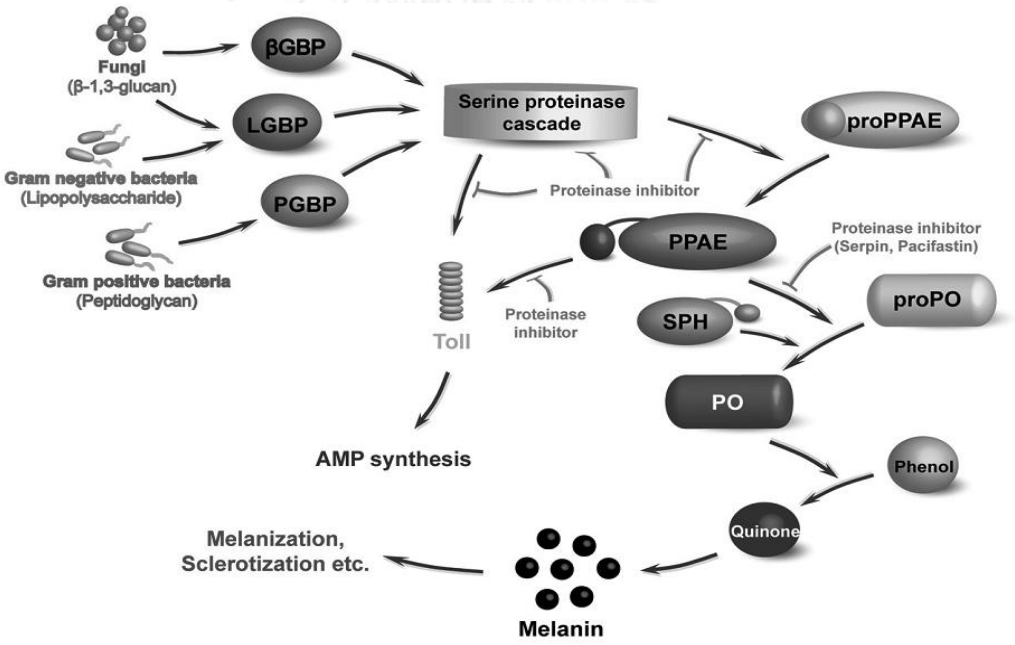

Figure 1.6 Overview of the arthropod prophenoloxidase (proPO)-activating system. (Amparyup *et al.*, 2013)

The proPO activating system is triggered by the binding of PAMPs and bind to pattern recognition receptors (PRRs). The receptors including βGBP, LGBP, and PGBP. β-Glucan-binding proteins (βGBP) are the proteins that recognise β-1,3-glucan of fungi and enhance the prophenoloxidase activity (Duvic and Söderhäll, 1990). LPS and β-1,3-glucan-binding proteins (LGBPs) could recognise the cell wall component of Gram-negative bacteria, lipopolysaccharide (LPS) as well as β-1,3-glucan of fungi (Lee *et al.*, 2000). Peptidoglycan recognition proteins (PGRPs) were found in *Drosophila* (Werner *et al.*, 2000), however, there is no report for PGRPs in crustaceans so far.

After the PAMPs are recognised by PRRs, the serine proteinase cascade is triggered which lead to the proteolytic cleavage to activate the key enzyme PO. The active PO, after the enzymatically activated by prophenoloxidase-activating enzyme (PPAE), converts monophenollic substrates into *o*-diphenolic substances (Cerenius and Söderhäll, 2004, Cerenius *et al.*, 2008, Clark and Strand, 2013). The *o*-diphenols rapidly convert into the intermediate 5,6-dihydroxyindole (DHI) which provides broad-spectrum antimicrobial activity as well as the melanin formation (Zhao *et al.*, 2007) (Figure 1.4). Melanin deposits around wounded sites or around the invading bacterial pathogens and parasites to trap and control the spreading as well as eliminating the pathogens. The prophenoloxidase activation is also associated with anti-viral activity (Ourth and Renis, 1993, Yassine *et al.*, 2012).

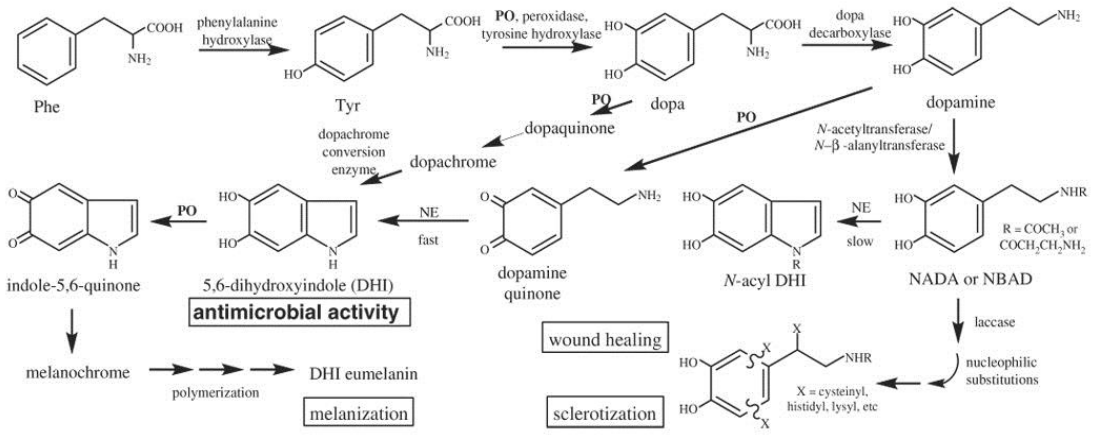

Figure 1.7 Mechanism of PO in melanization process. (Zhao *et al.*, 2007)

The melanization mediated by the proPO activating system could produce the cytotoxic compound and the over-production could cause the damages to the host cells. For this reason, the activation of proPO must be tightly regulated to prevent the over-production of the toxic compound. The melanization is controlled at multiple levels from the inhibition of SP cascades, to the melanin reaction. The proteinase inhibitors such as serpin and pacifastin have been found. Serpin was identified in insects including *D. melanogaster, A. gambiae, M. sexta*, and *T. molitor* as the inhibitors for serine proteinase in different steps of the activation (Cerenius *et al.*, 2008, Amparyup *et al.*, 2013). In crustaceans, only pacifastin has been reported as the inhibitor for PPAE, the serine proteinase at the final step of proPO activation (Liang *et al.*, 1997).

In *P. monodon*, the proPO activating system has been studied and several proteins have been reported to be involved in the system (figure 1.5). From the previous study, *Pm*LGBP has been identified as a pattern recognition protein for Gram negative bacteria and fungi (Amparyup *et al.*, 2012). *Pm*LGBP triggers the proteolytic cascade to activate the *Pm*PPAEs. *Pm*PPAEs are clip-domain serine proteinases that cleave proPOs (*Pm*ProPO1 and *Pm*ProPO2) (Sritunyalucksana *et al.*, 1999, Amparyup *et al.*, 2009) to generate the active form of POs. Two *Pm*PPAEs (*Pm*PPAE1 and *Pm*PPAE2) have been previously identified (Charoensapsri *et al.*, 2009, Charoensapsri *et al.*, 2011). The active PO will further convert phenol into quinone. In addition, *Pm*MasSPHs (*Pm*MasSPH1 and *Pm*MasSPH2) were found in *P. monodon* EST database and will be characterized for their functions in the proPO activating system in this study.

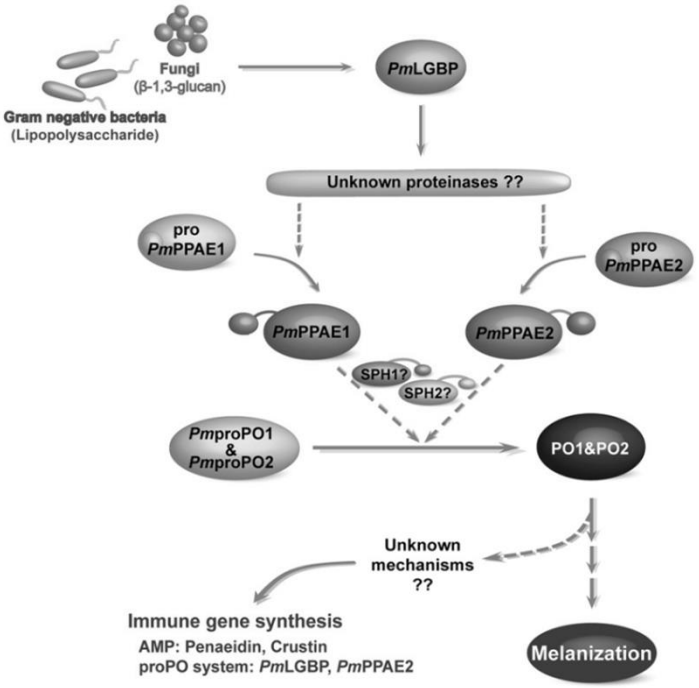

Figure 1.8 ProPO cascade in *Penaeus monodon* (Amparyup *et al.*, 2013)

1.4 The prophenoloxidase (proPO)

The key enzyme in the proPO system is the phenoloxidases (POs) which are the active form of the proPO that often produce by blood cells or hemocytes (Cerenius *et al.*, 2010). The proPO sequence was firstly reported and cloned from the freshwater crayfish *P. leniusculus* (Aspan *et al.*, 1995) and was later found in many other arthropods such as *Drosophila melanogaster* (Fujimoto *et al.*, 1995) and *M. sexta* (Hall *et al.*, 1995). In *P. monodon*, two proPO genes have been found (Figure 1.6) (Sritunyalucksana *et al.*, 1999, Amparyup *et al.*, 2009). The common structure of proPOs found in many arthropods contain two copper-binding sites without signal peptides (Figure 1.4) (Aspan *et al.*, 1995, Fujimoto *et al.*, 1995, Amparyup *et al.*, 2009, Amparyup *et al.*, 2013).

After the inactive form of proPO has been activated, active PO oxidize monophenols, such as tyrosine, to generate *o*-diphenols that will later lead to the melanin formation (Cerenius and Söderhäll, 2004, Amparyup *et al.*, 2013). Moreover,

POs also associate with cuticle sclerotization and wound healing beside the melanin formation (Zhao *et al.*, 2007).

Figure 1.9 Schematic structure of *Penaeus monodon* proPO1 and proPO2 (Amparyup et al., 2013)

1.5 Prophenoloxidase-activating enzymes (PPAEs)

The terminal proteinase in proPO activation is the PPAE. PPAEs are serine proteinase enzymes mainly containing N-terminal clip domain and trypsin-like serine proteinase domain at C-terminus that include the conserved catalytic triad (His, Asp, and Ser). Crayfish *P. leniusculus* ppA was the first PPAE to be purified and cloned (Aspán *et al.*, 1990, Wang *et al.*, 2001). Other PPAE in some arthropod species including silkworm *Bombyx mori*, tobacco hornworm *Manduca sexta*, beetle *Holotrichia diomphalia*, *Tenebrio molitor*, and shrimp *P. monodon* were also characterized (Jiang *et al.*, 1998, Lee *et al.*, 1998, Satoh *et al.*, 1999, Lee *et al.*, 2002, Charoensapsri *et al.*, 2009, Charoensapsri *et al.*, 2011). *P. monodon Pm*PPAEs are immune-responsive genes as their transcription levels increase after *V. harveyi* infection, PO activity drops and the mortality rate increases as the result of the silencing of these genes (Charoensapsri *et al.*, 2009, Charoensapsri *et al.*, 2011).

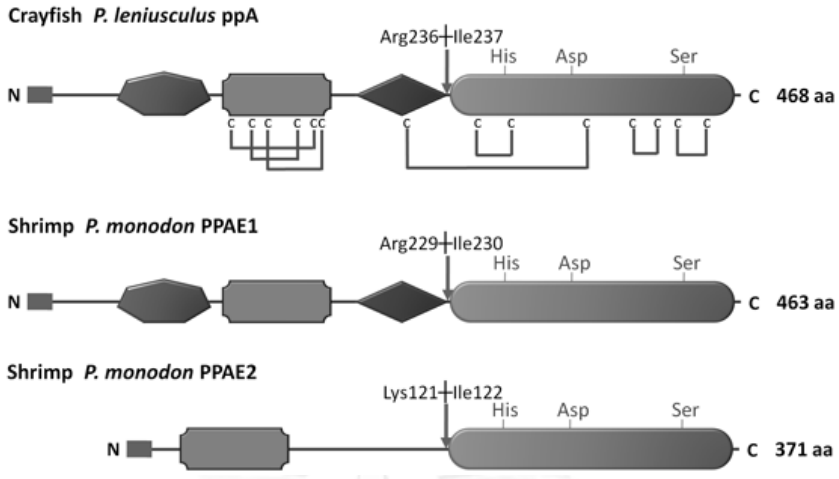

Figure 1.10 Schematic structure of crayfish *Pacifastacus leniusculus* ppA and shrimp *Penaeus monodon* PPAEs (Söderhäll et al., 2013).

1.6 Serine proteinase homologues (SPHs)

SPHs are the serine proteinase-like proteins that possess similar structure like PPAEs, containing clip-domain and serine proteinase-domain, except the Ser in catalytic triad of SPHs are replaced by Gly which results in the lack of proteolytic activity in SPHs. SPHs have been found in many arthropods such as mosquitoes *Anopheles gambiae* CLIPA8 (Yassine *et al.*, 2012), the freshwater crayfish *P. leniusculus* SPHs (Liu *et al.*, 2011), tobacco hornworm *Manduca sexta* SPHs (Yu *et al.*, 2003) the coleopteran beetle *Holotrichia diomphalia* PPAF-II (Kwon *et al.*, 2000) and mealworm *Tenebrio molitor* SPHs (Lee *et al.*, 2002). The functions of SPHs in immune system have been reported to be involved in the proPO activation including in *P. leniusculus* (Liu *et al.*, 2011). Silencing of SPHs resulting the decreasing of PO activity, *H. diomphalia* found that PPAF-II is the coactivator for PPAF-I (Kim *et al.*, 2002), and in *M. sexta* found it's SPHs promote the PO activity, and SPHs may anchor prophenoloxidase-activating proteinase1 (PAP-1) and proPO to the bacterial surface (Yu *et al.*, 2003, Gupta *et al.*, 2005). There was also a report for the function of SPH3 in *M. sexta* that involved as the antimicrobial effector (Felfoldi *et al.*, 2011).

In *P. monodon*, three *Pm*MasSPHs: *Pm*MasSPH1, *Pm*MasSPH2, and *Pm*MasSPH3, have been found from shrimp EST database (Figure 1.5). Previous study suggest that *Pm*MasSPH1, from N-terminus to C-terminus, contains single glycine-rich domain, clip domain, and serine proteinase-like domain. *Pm*MasSPH1 responses to the infection of Gram-negative bacteria *Vibrio harveyi* (Amparyup *et al.*, 2007). The further study reported that *Pm*MasSPH1 displays the opsonic activity. The function of N-terminal region glycine-rich domain and clip domain showed antimicrobial activity for Gram-positive bacteria. And the serine proteinase-like domain acts as a cell adhesive molecule and shows the binding activity to *V. harveyi,* as well as lipopolysaccharide (LPS), the Gram-negative bacterial cell wall component (Jitvaropas *et al.*, 2009). While there are various studies of SPHs in the immune system, there is nevertheless no study of these SPHs in the proPO system to date. For this reason, the investigation on their functions in this aspect is necessary.

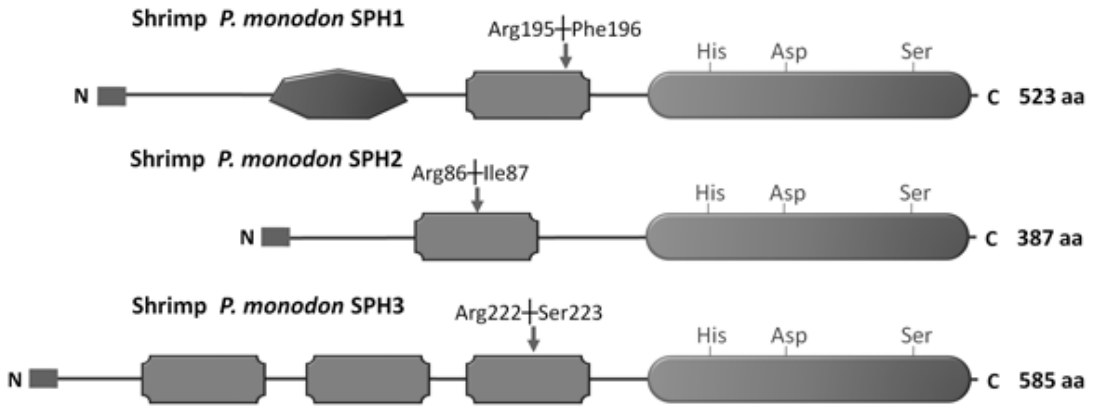

Figure 1.11 Schematic structure of *Pm*MasSPH1, 2 and 3 from shrimp *Penaeus monodon* containing the N-terminal clip-domain and the C-terminal serine proteinase-like domain

1.7 Inflammatory cytokines (interleukins) in inflammation of vertebrates

Once the pathogen invaded, immune mechanisms will be activated by the interaction of conserved PAMPs and PRRs. There are several PRRs that play roles in the self-defense manner, e.g., Toll-like receptors (TLRs) and NOD-like receptors (NLRs). NLRs are the family of soluble cytoplasmic proteins that involves in the

recognition in the presence of PAMPs. The largest NLRs subfamily is NACHT, LRR and PYD containing proteins (NALP), which could form the high molecular weight complex called inflammasome that later activates caspase-1 and processes the maturation and the secretion of inflammatory cytokines, Interleukin (IL)-1β (Martinon *et al.*, 2002, Petrilli *et al.*, 2007, Martinon *et al.*, 2009, Schroder *et al.*, 2010).

The inflammasomes are multiple protein complexes in the inflammatory process which assemble after the PRRs being triggered. The most important type of inflammasome is the NLRP3 inflammasome that could be triggered by several bacterial or fungal pathogens, some virus like influenza or parasites (Duncan *et al.*, 2009, Ichinohe *et al.*, 2009, Sollberger *et al.*, 2013). NLRP3 inflammasome consist of pyrin domains-containing protein 3 (NLRP3, also known as cryopyrin), nucleotide-binding domain (NBD), the pyrin domain (PYD) apoptosis-associated speck-like protein containing a CARD (ASC) (Walsh *et al.*, 2014) which will recruit procaspase-1 molecules causing the activation of the complexes. The complexes are then cleaved the pro-IL1.

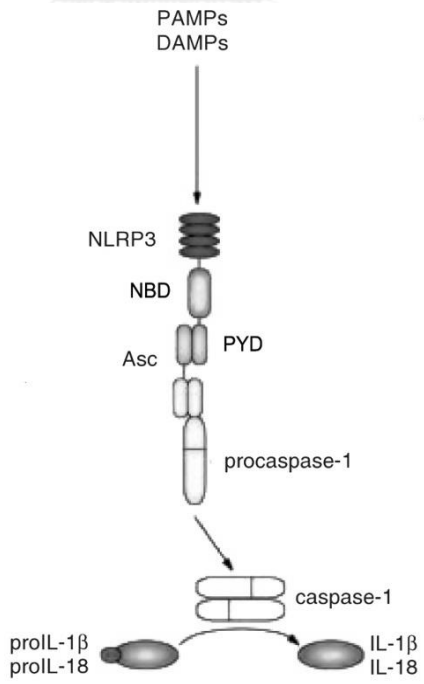

Figure 1.12 The NLRP3 inflammasome (modified from Sollberger et al. 2013)

Caspase-1, cysteine - aspartic protease that was formerly called Interleukin-1β converting enzyme (ICE), is the first caspase to be characterized and is in the cysteine proteases family (Thornberry and Molineaux, 1995, Goodsell, 2000). Unlike most caspase in the family that function as the promoter for the cleavage in the apoptosis, caspase-1 involves in the initiating the inflammation as it was found to be important in the processing and secretion of the inflammatory mediator IL-1β (Petrilli *et al.*, 2007, Franchi *et al.*, 2009).

1.8 Multifunctional proteins and their activation mechanism

1.6.1 The complement protein C3 in complement system of vertebrates

The complement system in human is the set of more than 20 plasma proteins in circular system. These proteins float in an inactive form before the first complement substance is triggered by the foreign components to induce the inflammation in response to the infection (Janeway CA Jr, 2001, Lambris *et al.*, 2008). There are three mechanisms in the complement system have been found so far, including the classical pathway, lectin pathway, and the alternative pathway. Among these pathways, complement components C3 plays role more than a single function in the immune response.

After the activation, the complement protein C3 acts as the central molecule for the effector functions including the elimination of pathogens, debris, and cellular structures (Stephan *et al.*, 2012). While the classical pathway is depend on the forming of antigen-antibody complexes and lectin pathway is depend on the binding of mannose binding lectin (MBL) before activation of the followed components, C3 in the alternative pathway could spontaneously activated in plasma at the present of foreign organism. The C3 cleavage resulting in two fragments from the single cleavage initial as C3a and C3b. C3b fragment covalently bind to the microbial surface leading to the later steps of the complement activation to finally phagocyte the cells, while C3a fragment that is released during the C3 activation appear to be the anaphylatoxins that trigger the inflammatory process (Lambris *et*

al., 2008). This information is the example of protein which containing two fragments with different functions after the activation or cleavage.

1.6.2 Hemocyanin and proPO-associated proteins in immune system of invertebrate

Hemocyanin

Hemocyanin is a plasma protein in hemolymph widely known for its role as the oxygen transporter in molluscs and arthropods. It is a metalloprotein that belongs to type 3 copper centers which is the same type as the PO and tyrosinase (Li *et al.*, 2009, Markl, 2013). In arthropods, hemocyanin contains 3 domains identified by folding motifs; domain I with five or six α-helices, domain II with four α-helices, and domain III with antiparallel β-barrel. The copper-binding site is located in the second domain (Volbeda and Hol, 1989, Hazes *et al.*, 1993, Decker *et al.*, 2007).

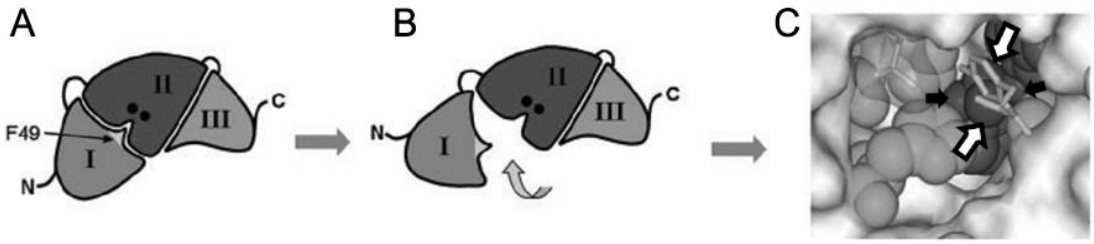

Figure 1.13 The activation of arthropods hemocyanin. The roman numerals I, II, and III indicate domain I, domain II, and domain III respectively. N and C in figure A and B indicate the N- and C-terminus. The copper center is represented by black dots in domain II of Figures A and B. Figure C displays the view into the active site after domain I has been removed, the copper atoms (dark arrows) and oxygen atoms (open arrows) are located in this figure (modified from Decker et al. 2007).

Apart from the oxygen transport, hemocyanin also possess several functions in immune response in case of the animal get infected or wounded. *P. leniusculus* hemocyanin 1 C-terminus could generate antibacterial peptide activity while hemocyanin 2 from the same organism could provide the phenoloxidase activity from the N-terminus after the proteolytic cleavage (Lee *et al.*, 2004). The C-terminal hemocyanin from *Penaeus vannamei* showed the antifungal activity (Destoumieux-Garzón *et al.*, 2001). These data provides information that hemocyanin could function other than the oxygen binding.

ProPO-associated proteins

As mentioned earlier, the proPO activating cascade is the immune response that involved the activation of proPO by PPAEs with the association with SPHs. However, several proteins in the this system exhibit the additional function beside the proPO cascade, e.g. *Pl*ppA N-terminal clip domain from crayfish shows the antimicrobial peptide activity against several tested Gram-positive bacteria (Wang *et al.*, 2001) and *Pm*MasSPH1 N-terminal clip domain displays the antimicrobial activity for Gram-positive bacteria (Jitvaropas *et al.*, 2009). Nonetheless, the activation for these functions is still unknown. For proPO, despite seeing that proPOs are similar to hemocyanin and also belongs in type 3 copper centers, the other functions apart from the PO activity have yet to be studied.

1.9 Dissertation objectives

The objectives of this study are as followed.

1. To functionally characterize shrimp *Pm*MasSPH1 and *Pm*MasSPH2

*P. monodon Pm*MasSPH1 have been identified, cloned, and characterized and have been shown that it plays roles in immune system. Here, *Pm*MasSPH1 and also *Pm*MasSPH2 were further studied for their roles in the proPO activating system *in vivo* using RNA interference technique, their involvement in bacterial clearance, and the other immune genes expression after the silencing of *Pm*MasSPHs. The function of *Pm*MasSPHs was investigated in more specific as the recombinant proteins will be produced and studied for the bacterial cell wall component binding, their ability to activate PO activity, and the protein-protein interaction with *Pm*PPAEs.

2. To investigate the immune functions of cleaved crayfish proPO

The function of the N-terminus part of the crayfish proPO was studied. The fragment of the N-terminal region, the fragment of *Pl*proPO that was cleaved by *Pl*ppA, and the two fragments from N-terminus cleaved by caspase-1 were cloned and recombinantly expressed for functional study in immune system.

This study provides further understanding of the mechanism of *Pm*MasSPH1 and *Pm*MasSPH2 in the proPO activating system and also provides new aspect for the function of proPO in the crustacean immune system.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and reagent kits

	Manufacturer
2-mercaptoethanol	Fluka
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)	Fermentas
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)	Fermentas
Agarose powder	Sekem
Rabbit anti-mouse IgG	Jackson ImmunoResearch
Ampicillin	BioBasic
Anti-His antiserum	GE Healthcare
AP Substrate Kit	Bio-Rad
Bacto agar	Difco
Bacto tryptone	Scharlau
Bacto yeast extract	Scharlau
Bovine serum albumin (BSA)	Fluka
Calcium chloride ($CaCl_2$)	MERCK
Chloramphenicol	Sigma
Coomassie brilliant blue R-250	Sigma
DNA and protein molecular weight markers	Fermentas
DNase I	Promega
E. coli strain BL21(DE3)pLysS	Novagen
E. coli strain JM109	Promega
E. coli strain Rosetta™(DE3)pLysS	Novagen
illustra RNAspin Mini Kit	GE Healthcare
ImProm-II™ Reverse Transcription System	Promega
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Fermentas
Kanamycin	BioBasic
L-3,4-dihydroxyphenylalanine (L-DOPA)	Fluka
Laminarin from *Laminaria digitata*	Sigma
Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4	Sigma
Ni-NTA Agarose	QIAGEN
pEGFP-1 vector	Clontech

pET *E. coli* T7 expression vectors	Novagen
Peptidoglycan (PGN) from *Bacillus subtilis*	InvivoGen
Pfu DNA polymerase	Promega
Propidium Iodide (PI)	Invitrogen™
Protein A–Agarose Fast Flow	Sigma
QIAprep Spin Miniprep Kit	QIAGEN
Quick Start™ Bradford Protein Assay Kit	Bio-Rad
RBC Taq DNA Polymerase	RBC Bioscience
Restriction enzymes	New England Biolabs™
SYTO®9 Green Fluorescent Nucleic Acid Stain	Invitrogen™
TA Cloning Vector	RBC Bioscience
T4 DNA Ligase	Promega
T7 RiboMAX™ Express Large Scale RNA Production System	Promega
Tryptic soy broth (TSB)	Difco

2.2 Animals

Healthy black tiger shrimps (*Penaeus monodon*), specific pathogen-free (SPF) from white spot syndrome virus (WSSV), taura syndrome virus (TSV), and yellow head virus (YHV), were obtained from local farms in southern Thailand and acclimated in laboratory's closed system with aerated 20 ppt salinity seawater at ambient temperature (28 ±4 °C) for a minimum of 3 days before the experiments. Freshwater crayfish (*P. leniusculus*) was purchased from Hjälmaren lake in Sweden and kept in laboratory's tanks with air pumps at 10°C. Only apparently healthy animals were selected for the experiments.

2.3 Sequences analysis

Nucleotide, amino acid sequences, sequence similarity and protein domains from *Pm*MasSPH1 (accession number: DQ455050) was previously described (Amparyup *et al.*, 2007). Nucleotide and amino acid sequences from *Pm*MasSPH2 (accession number: FJ620686) were analyzed by BLASTX software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for similarity searching, signal peptides and protein domains were predicted using Simple Modular Architecture Research Tool

(SMART) (http://smart.embl-heidelberg.de/). ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for sequence alignment. Phylogenetic tree was analyzed and created in Molecular Evolutionary Genetics Analysis (MEGA) version 5.2 (http://www.megasoftware.net/).

Crayfish proPO (accession number: X83494) was previously reported to contains activation site between Arg176 and Thr177 (Aspan *et al.*, 1995). The amino acid sequence was analyzed for the caspase cleavage site by PeptideCutter (http://web.expasy.org/peptide_cutter/).

2.4 Functional characterization of *Pm*MasSPH1 and *Pm*MasSPH2 genes by RNA interference

2.4.1 Preparation of *Pm*MasSPH1 and *Pm*MasSPH2 dsRNAs and shrimps *P. monodon* injection

To synthesize the dsRNA for the gene silencing, the DNA fragments were first amplified by PCR technique. Gene specific primers were designed and applied the T7 promoter sequence at 5' end, and the pair of primers were listed in table 2.1. The PCR cycle for amplification were used as followed: 94°C for 1 minute (initial denaturation stap) followed by 94°C 1 minute (denaturation step), 55°C 1 minute (annealing step), and 72°C for 1 minute (extension step). The reactions were finished after the final extension at 72°C for 10 minutes. The PCR products were visualized by agarose gel electrophoresis, and extracted from the TBE-1.5% (w/v) by NucleoSpin® Extract II kit (MACHEREY-NAGEL).

The single-stranded RNA (ssRNA), including the sense and anti-sense strand of *Pm*MasSPH1, *Pm*MasSPH2, and GFP, were separately synthesized using T7 RiboMAX™ Express RNAi System (Promega). The synthesized was done according to the manufacturer's protocol. Briefly, 1µg PCR product was used in the 20µL reaction that contain 1×RiboMAX™ Express buffer and T7 Express Enzyme Mix. The reaction was incubated at 37°C for 1 hour. The product was visualized by agarose gel electrophoresis.

After the ssRNA synthesis, the equal amount of the sense and anti-sense ssRNA was mixed and incubated at 70℃ for 10 minutes and cooled down at room temperature for 30 minutes to produce dsRNA. The template DNA was later removed by the DNase I (included in the kit) at 37℃ for 30 minutes. The dsRNA were precipitated with 1 volume of isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2) at -20℃ for 15 minutes and centrifuged at 12000×g for 20 minutes. The supernatant from the centrifugation was removed and 500µL of 70% ethanol was added to wash the pellet. After the centrifugation and removal of the supernatant, the pellet was left at room temperature and air-dry for approximately 5-10 minutes. The pellet was dissolved with nuclease-free water and was visualized by agarose gel electrophoresis and measured for the dsRNA concentration by spectrophotometry.

Shrimp injection was performed by selecting healthy shrimps, weight approximately 5 g for the gene silencing. The shrimps were intramuscularly injected with prepared 5µg/ 1g shrimp dsRNA in NaCl or 150mM NaCl for the control shrimps. The shrimps were double injected with the dsRNA at 24 hours after the first injection.

Table 2.1 List and sequences of primers used for genes silencing

Primers	Sequences (5' - 3')
*Pm*SPH1_T7F	GGATCCTAATACGACTCACTATAGGTTATAACGGACGGCGCA
*Pm*SPH1_T7R	GGATCCTAATACGACTCACTATAGGCCAGCAGGTGTCGTAGT
*Pm*SPH1_F	TTATAACGGACGGCGCAGGC
*Pm*SPH1_R	CCAGCAGGTGTCGTAGTCGAAT
SPH2iT7F	GGATCCTAATACGACTCACTATAGGGTGTGCATTAGCTGTGGCCGTC
SPH2iT7R	GGATCCTAATACGACTCACTATAGGAGCCCGAGCGCTGAATGGGTAC
SPH2iF	GTGTGCATTAGCTGTGGCCGTC
SPH2iR	AGCCCGAGCGCTGAATGGGTAC
GFPT7-F	TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA
GFPT7-R	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA
GFP-F	ATGGTGAGCAAGGGCGAGGA
GFP-R	TTACTTGTACAGCTCGTCCA

The genes silencing efficiency were determined from the genes expression in hemocyte by semi-quantitative RT-PCR. Firstly, total RNA was extracted. Hemolymph was collected from injected shrimps at 48 h post second injection for total RNA extraction by illustra RNAspin Mini Kit (GE Healthcare) following the manufacturer's protocols. Total RNA was visualized by agarose gel electrophoresis and measuring the amount of the RNA by spectrophotometer. The amount of 180 ng total RNA from each individual shrimps was subjected to the cDNA synthesis using ImProm-II™ Reverse Transcription System kit (Promega) with oligo$(dT)_{15}$ as a primer, according to the manufacturer's protocols.

Semi-quantitative RT-PCR was performed to determine gene expression and the efficiency of the gene silencing by RNAi. cDNA from each treatments were amplified with gene specific primers (Table 2.2). The PCR cycle conditions were as followed: 94 ℃ 1 minute for initialization step, 25 cycle of denaturation step, Annealing step, and elongation step are 94℃ 30 seconds 55℃ 30 seconds and 72℃ 1 minute respectively, and 72℃ 5 minutes for the final extension step. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. Band intensity in every treatments was normalized with EF1α before calculated for the relative genes expression.

Table 2.2 List and sequences of primers used for genes expression detection by semi-quantitative RT-PCR

Primers	Sequences (5' - 3')
*Pm*SPH1rtF	TACCCTCACCAGGACAGGAACG
*Pm*SPH1rtR	CTGGAAGAAAGATCCGAGCCGA
*Pm*SPH2-322-F	CAGGCGGAGAGTGTGGCATC
*Pm*SPH2-322-R	TTGTGCTCGCCCAGACGGAC
PPAE2-F	ATGCACTACCGGGTTCCCACGATC
PPAE2-R	CTAAGGTTTGAGATTCTGCACG
PEN3-F	GGTCTTCCTGGCCTCCTTCG
PEN3t-R	TTTGCATCACAACAACGTCCTA
Crus72-F	CGGCAGGTGTCCACAGATTCG
Crus72-R1	AATTGATGAGTCGAACATGCAGGCCTAT
EF1α-F	GGTGCTGGACAAGCTGAAGGC
EF1α-R	CGTTCCGGTGATCATGTTCTTGATG

2.4.2 Measuring phenoloxidase (PO) activity in the knockdown shrimps

Shrimp hemolymph PO activity was measure from dsRNA or normal saline control injected shrimps. Shrimp hemolymph was collected from shrimps at 48 hours post second dsRNA injection and measured for the protein concentration by Quick Start™ Bradford Protein Assay Kit (Bio-Rad). In each PO activity assay reaction contained the following components: 2 mg total protein from shrimp hemolymph, 10 mM Tris-HCl pH 8.0 for adjusting the volume of hemolymph to 435 µL, and 65 µL of 3 mg/mL freshly prepared L-DOPA. The reactions were incubated for 30 minutes at room temperature before added 500 µL 10% (v/v) acetic acid to stop the reaction. Total hemolymph PO activity was defined as ΔA490/ mg total protein/ minutes.

2.4.3 Analysis of bacterial count in the knockdown shrimp post *Vibrio harveyi* infection

The virulent strain *V. harveyi* 639 was cultured of in TSB at 30°C overnight as a starter for inoculation. The bacteria were cultured of at the same temperature after inoculation in 40 µL starter in 8 mL TSB, until the absorbance at 600 nm reach 0.59 - 0.60 (10^8 CFU/mL). The culture was 10 folds diluted until reach the concentration of 10^7 CFU/mL and 20µL/shrimp were injected with 20 µL of diluted culture to make 2×10^5 CFU/shrimp.

The healthy shrimps which were first injected with dsRNA for 24 hours were doubling injected with dsRNA followed by the previously prepared *V. harveyi*, simultaneously. At 6 hours after injection, hemolymph was collected from each shrimps and serially diluted to plate on LB agar plate and incubated in 30°C overnight. The number of colonies formed on the plate was multiplied by the dilution fractions before calculation for the CFU/mL and statistically analyzed.

2.5 Recombinant proteins expression

2.5.1 Construction of expression vector

*Pm*MasSPH1 and *Pm*MasSPH2 of shrimp

Primers for recombinant protein expression of mature protein from *Pm*MasSPH1 and *Pm*MasSPH2 were designed with the elimination of the signal peptides; the primers used for the construction are listed in Table 2.3. The PCR product was cloned into TA Cloning Vector and subjected to the nucleotide sequencing. The confirmed clone was sub-cloned into pET32a expression vector at the *Nco*I and *Xho*I restriction sites. The clones were then sequenced for the correct ligation and transformed into *E. coli* strain Rosetta™(DE3)pLysS expression host. *Pm*MasSPH2 SP-like domain was cloned using primer SPH2*Nco*I and SPH2*Not*I using the same protocols as mentioned above. The recombinant protein of C-terminal SP-like domain from *Pm*MasSPH1 was previously constructed (Jitvaropas *et al.*, 2009).

Table 2.3 List and sequences of primers used for *Pm*MasSPHs recombinant plasmid construction

Primers	Sequences (5' - 3')
BACSPH1-F	CATGCCATGGGCTGCTTCTTTTGGAAGGGAGAATGCA
BACSPH1-R	CGCCTCGAGCTAATGATGATGATGATGATGAATAAATCTTCCGTAGTCCCAGTCA
*Pm*SPH2-F	CATGCCATGGGCCAGAACAACCAGAACGTAAGGCT
*Pm*SPH2-R	CGCCTCGAGCTAATGATGATGATGATGATGAAATCTCACGAATTGCCTAATAAAG
SPH2*Nco*I	CATGCCATGGGCCATCATCATCATCATCATCAGGCACAGTATGCTGAG
SPH2*Not*I	ATAAGAATGCGGCCGCCTAAAATCTCACGAATTGCCTA

proPO of crayfish

Crayfish cDNA was synthesized from hemocyte total RNA and used as the following PCR template. The crayfish *Pl*proPO-ppA was amplified using specific primers, *Pl*proPO32*Eco*RI-F and *Pl*proPOppA-R. The PCR product was visualized by agarose gel electrophoresis then was extracted and digested the product with *Eco*RI and *Xho*I to ligate into pET32a expression vector at the same restriction sites. The plasmid was sequenced to confirm the nucleotides and ligation correction. *Pl*proPO-casp1 was cloned using specific primers, *Pl*proPO-F and *Pl*proPOcasp-R1, while *Pl*proPO-casp2 was cloned using specific primers, *Pl*proPO-F and *Pl*proPOcasp-R2. The following procedures were the same as *Pl*proPO-ppA mentioned above except the restriction sites, these *Pl*proPO-casp1 and *Pl*proPO-casp2 were ligated into pET28b at the *Nco*I and *Xho*I restriction sites. All three recombinant plasmids were cloned into BL21(DE3)pLysS expression host. For the recombinant protein controls, GFP gene was obtained from pEGFP-1 vector and digested with *Nco*I and *Not*I restriction enzymes. The product was visualized by agarose gel electrophoresis and the expected nucleotide size (731 bp) was cutted for the gel extraction and purification. The product was later ligated into pET32a at the same restriction sites (*Nco*I and *Not*I) and transformed into BL21(DE3)pLysS expression host for further protein expression.

Table 2.4 List and sequences of primers used for *Pl*proPO recombinant plasmid construction

Primers	Sequences (5' - 3')
*Pl*proPO32*Eco*RI-F	TTTTTTGAATTCCAGGTGACCCAGAAGTTGCTGAGGA
*Pl*proPOppA-R	CGCCTCGAGCTACCTGTTCACTTCAACCTGCATGCTT
*Pl*proPO-F	CATGCCATGGGCCATCATCATCATCATCATCAGGTGACCCAGAAGTTGCTGAGGA
*Pl*proPOcasp-R1	CGCCTCGAGCTAATCTGCCTCAAACGCGTCTCCTAAG
*Pl*proPOcasp-R2	CGCCTCGAGCTAGTCGTGACAGAATGCCAGCAGCACA

2.5.2 Protein expression

The previously constructed plasmid that transformed into the expression hosts; *E. coli* strain Rosetta™(DE3)pLysS and BL21(DE3)pLysS, were cultured in LB medium at 37°C overnight as the starter and inoculated in the new LB medium at the 1:100 ratio, the starter to the total medium. The cultures were continuously shaken at 250 r*pm* in 37°C until the A600 reach 0.6. The IPTG were added to the culture at the final concentration of 1 mM. The cultures were harvested at 6 hours after the IPTG induction by centrifugation at 7000×g for 5 minutes at 4°C. The supernatant was discarded and cell pellet were homogenized with 20mM Tris-HCl pH 8.0 before the cell disruption by the sonication. The pellet was solubilized by 8M urea.

2.5.3 Protein purification

The crude protein fractions were purified by the nickel affinity chromatography. Each fraction was separately incubated with Ni-NTA agarose before collected the flowthrough. The columns were washed and eluded the protein by increasing the concentration of imidazole in the buffer, 10mM imidazole (10mM imidazole, 50mM NaH_2PO_4, 300mM NaCl, pH 8.0), 20mM imidazole (20mM imidazole, 50mM NaH_2PO_4, 300mM NaCl, pH 8.0), 50mM imidazole (50mM imidazole, 50mM NaH_2PO_4, 300mM NaCl, pH 8.0), 100mM imidazole (100mM imidazole, 50mM NaH_2PO_4, 300mM NaCl, pH 8.0), and 250 mM imidazole (250 mM imidazole, 50mM NaH_2PO_4, 300mM NaCl, pH 8.0). Each fraction was analyzed with SDS-PAGE and the

fractions contain the purified protein were pulled together and dialyzed against 20mM Tris-HCl pH 8.0 at 4°C for 3 times to refold the peptides and to remove urea and imidazole. The purified protein was collected and kept in -20°C for further experiments.

2.5.4 SDS-PAGE and western blot analysis of the recombinant proteins

The protein fractions were analyzed with SDS-PAGE followed by the western blotting approach against the mouse anti-His antibody. The protein samples were mixed with 2×SDS loading buffer (100mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200mM β-Mercaptoethanol) to the 1× final concentration then boiled for 5 minutes. The samples were separately loaded into each well with the prestained protein ladder as a marker for the molecular mass approximating.

The protein gels were later applied in the electroblotting machine (Trans-Blot® SD Semi-Dry Transfer Cell, BioRad) to transfer the proteins onto the nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat milk in Tris Buffered Saline with Tween® 20, TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween® 20) overnight at 4°C. The milk was washed from the membrane with TBST for 5 minutes four times and probed with primary antibody, mouse anti-His antibody, at the dilution of 1:5000 in TBST. The membrane with the primary antibody was incubated for 1 hour at room temperature with mild agitation. After the primary antibody incubation, the membrane was washed with TBST for 5 minutes four times before probing with the secondary antibody at room temperature for 1 hour at the concentration of 1:10000 in TBST. The membrane was washed and detected the band by color reaction of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP/ NBT) as substrate.

2.6 Determination the protein–protein interactions of *Pm*MasSPH1 or *Pm*MasSPH2 by co-immunoprecipitation (co-IP)

The co-IP was performed to identify the protein-protein interaction ability from the paired recombinant proteins slightly modified (Isono and Schwechheimer, 2010). The recombinant proteins were paired as followed; *Pm*MasSPH1 with *Pm*PPAE1, *Pm*MasSPH1 with *Pm*PPAE2; *Pm*MasSPH2 with *Pm*PPAE1; and *Pm*MasSPH2 with *Pm*PPAE2. The 40µL of Protein A–Agarose Fast Flow was centrifuged at 2000×g for 2 minutes and the supernatant was discarded. The bead was washed twice by centrifugation with 20mM Tris-HCl pH 8.0 and supernatant was removed. After washing step, the bead was incubated with 50µg antibody for each pair of the recombinant protein (anti-*Pm*MasSPH1 for *Pm*MasSPH1 / *Pm*PPAE1 and *Pm*MasSPH1 / *Pm*PPAE2, anti-*Pm*PPAE1 for *Pm*MasSPH2 / *Pm*PPAE1, and anti-*Pm*PPAE2 for *Pm*MasSPH2 / *Pm*PPAE2) and incubated on-ice for 3 hours with mild agitation while the recombinant proteins were also separately incubated in pairs (*Pm*MasSPH1 / *Pm*PPAE1, *Pm*MasSPH1 / *Pm*PPAE2, *Pm*MasSPH2 / *Pm*PPAE1, and *Pm*MasSPH2 / *Pm*PPAE2), adjusted the volume to 1 mL with 20mM Tris-HCl pH 8.0 and incubated on ice for 3 hours.

After 3 hrs of incubation, the protein A agarose bead was centrifuged at 2000×g, the supernatant was removed and washed once with 20mM Tris-HCl pH 8.0 then incubated recombinant proteins were add to the bead. The reaction was incubated on-ice for another 3 hours with mild agitation. The incubation was centrifuged at 2000×g for 2 minutes and removed the supernatant to discard unbound recombinant proteins. The bead was washed 3 times by adding the washing buffer (50mM Tris-HCl pH 8.0, 100mM NaCl, 0.05% (v/v) Triton X-100 and 10% (v/v) glycerol) and inverting the tube several times before the centrifugation. The bead was mixed with 2x SDS loading buffer and boiled for 5 minutes. The protein bands were visualized by SDS-PAGE followed immunoblotting.

2.7 The peptidoglycan (PGN) binding assay by ELISA

To determine the PGN binding ability of *Pm*MasSPH1 and *Pm*MasSPH2, the ELISA was used in the study. The process was carried by commercial PGN in 96 well plate coating (2μg PGN per well in 50μL water). The plate was dried in 37°C incubator overnight and fixed by incubating at 60°C for 2 hours. The plate was later blocked by 200μL of 5% BSA in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) for 1 hour followed by 3 times washing with 200μL TBS. Freshly prepared recombinant proteins at various concentrations were added for 100μL in each well and incubated at 4°C overnight. The solution was discarded and the plate was washed to remove unbound protein with 200μL TBS for 3 times. After washing, 100μL of anti-His antibody, freshly prepared at concentration of 1:5000, was added in each well and incubated for 3 hours at room temperature. The plate was washed 3 times and incubated with alkaline phosphatase-conjugated anti-mouse, 1:10000 dilution in TBST, for 1 hour. After the incubation, the plate was washed twice with 200μL TBST followed by two times of 200μL water before adding the substrate (AP Substrate Kit, Bio-rad) and incubated at room temperature for 30 minutes before stopping the reaction and measuring the absorbance at A_{405}. The results were plotted by A_{405} as vertical axis against the protein concentrations as horizontal axis. To calculate the dissociation constant (K_d), a Lineweaver-Burk plot was plotted to identify the maximum binding (A_{max}) and K_d, which were calculated from the Y-intercept and slope of linear equation of

$$\frac{1}{A} = \frac{K_d}{A_{max}\,[L]} + \frac{1}{A_{max}}$$

when A was defined by absorbance at 405 nm and [L] was the protein concentration.

2.8 *In vivo* bacterial clearance

The injections of protein and bacteria were performed following the previous method (Watthanasurorot *et al.*, 2011) with slightly modifications. Briefly, wild-type strain *E. coli* was cultured in LB broth until A_{600} reach 0.5 and the bacterial cells were collected by centrifugation at 1200×g for 5 min. The pellet was resuspended in 150 mM NaCl for washing step. Centrifuged the suspension at 1200×g for 5 min, discard the supernatant. Repeated the washing step 3 times and resuspended the pellet with 150mM NaCl at 1×10^9 CFU/mL. 100µL of the bacterial suspension was mixed with 20µg recombinant protein or the 20mM Tris-HCl pH 8.0 as the control buffer, and the mixture was injected to the crayfish at the base of a walking leg. The hemolymph was bled and collected at 40 minutes and 3 hours after the injection to serial diluted with 150mM NaCl before plating on LB agar and incubate at 37°C overnight. The CFU/mL was calculated from the number of colonies form in LB agar multiplied with the dilution. The values were statistically analysed by One-Way ANOVA

2.9 *In vitro* bacterial clearance in *P. leniusculus*

To examine whether the *in vivo* bacterial clearance activity was caused by the peptides, the peptides were tested for their activity *in vitro*. The bacterial preparation for the experiment was slightly modified from previous study (Watthanasurorot *et al.*, 2011). *E. coli*, wild-type strain, was cultured in LB medium until A_{600} reached 0.5 before harvesting the cells by the centrifugation at 1200×g for 5 minutes. The cell pellet was resuspended with 150mM NaCl and centrifuged at 1200×g for 5 minutes to wash the cells. Repeated the washing process 3 times and resuspended the pellet with 150mM NaCl at 1×10^9 CFU/mL. The 100µL suspension was mixed with 20µg peptide and adjusted the volume to 1 mL, then incubated at room temperature for 1 hour with mild agitation. After the incubation, the reactions were serially diluted with 150mM NaCl. The dilutions were plated onto LB agar and incubated at 37°C overnight. The CFU/mL and the statistical analysis were calculated as described previously

2.10 Agglutination assay for the *Pl*proPOppA *in vitro*

The minimal agglutination concentration for *Pl*proPO-ppA was determined using the method from (Wu *et al.*, 2011) with slightly modification. The bacteria used in the experiment were: *Staphylococcus aureus* Cowan, *Micrococcus luteus* M III, *E. coli* D21, *A. hydrophila* B1, and *Pseudomonas aeruginosa* OT97. The overnight-cultures were harvested by the centrifugation 1200×g for 5 minutes. The supernatant was removed and the pellet was washed 3 times and resuspended in 150mM NaCl at A_{600} of 2.0 for the experiment. Each concentration of recombinant proteins were prepared by two fold serially dilution. For each reaction, 50μL recombinant protein in each dilution was mixed with 50μL bacterial suspension and incubated at room temperature for 1 hour, the incubation reactions were later observed under microscope.

2.11 The bacterial viability assay

E. coli and the protein mixtures for each treatment was prepared according to the *in vitro* bacterial clearance method described previously. *E. coli*, wild-type strain, was culture in LB medium until A_{600} reached 0.5 before harvesting the cells by the centrifugation at 1200×g for 5 minutes. The cell pellet was resuspended with 150mM NaCl and centrifuged at 1200×g for 5 minutes to wash the cells. Repeated the washing process 3 times and resuspended the pellet with 150mM NaCl at 1×10^9 CFU/mL. The 100μL suspension was mixed with 20μg peptide and adjusted the volume to 1 mL. The incubation was added with SYTO®9 (invitrogen), final concentration of 50 nM and propidium iodide (PI) to final concentration of 1μg/mL, at 5 minutes after the protein mixing and observed under the fluorescence microscope.

2.12 Scanning electron microscope analysis

E. coli and the protein mixtures for each treatment was prepared according to the *in vitro* bacterial clearance method described previously. *E. coli*, wild-type strain, was culture in LB medium until A_{600} reached 0.5 before harvesting the cells by the centrifugation at 1200×g for 5 minutes. The cell pellet was resuspended with 150mM NaCl and centrifuged at 1200×g for 5 minutes to wash the cells. Repeated the washing process 3 times and resuspended the pellet with 150mM NaCl at 1×10^9 CFU/mL. The 100µL suspension was mixed with 20µg peptide and adjusted the volume to 1 mL. After 15 and 40 minutes incubation, the cells were collected by centrifugation at 1200×g for 5 minutes. The supernatant was removed and the cell pellet was fixed with glutharaldehyde following standard procedures for SEM.

CHAPTER III
RESULTS

3.1 Functional characterization of shrimp *Pm*MasSPH1 and *Pm*MasSPH2

3.1.1 Gene characterization of *Pm*MasSPH1 and *Pm*MasSPH2 of shrimp

The proPO activating system is the major immune response in shrimp leading to the melanin formation. However, the genes that are involved in this system are poorly understood. Several studies in insects and crustaceans suggest the importance of SPHs in the proPO activating system. In this study, the SPHs were characterized to elucidate the activation mechanism of proPO in *P. monodon*. Three *Pm*MasSPHs were found in shrimp *P. monodon* EST database (http://pmonodon.biotec.or.th/) (Tassanakajon *et al.*, 2006), *Pm*MasSPH1 and *Pm*MasSPH2 were chosen for characterization in the present study. The full-length cDNA of *Pm*MasSPH1 (GenBank accession number DQ455050) has been previously analysed (Amparyup *et al.*, 2007). The cDNA consists of 1,958 bp with a 79 bp of 5' untranslated region (UTR), a 1,572 bp of open reading frame (ORF) and a 3' UTR with a poly(A) tail of 307 bp. The deduced amino acid of the protein contains 523 residues with the signal peptide for 19 amino acids at the N-terminus. The calculated molecular mass of the mature protein (504 amino acids) is 51.58 kDa with the predicted isoelectric point (p*I*) of 4.86. The putative proteolytic cleavage site was located at the beginning of the SP-like domain (between Arg250 and Ile251). The serine proteinase domain showed conserved catalytic site of His301 and Asp351, except for Ser was replaced by Gly452. The replacement suggested that the protein is a non-catalytic serine proteinase.

The cDNA sequence of *Pm*MasSPH2 (GenBank accession number FJ620686) consists of a 1,164 bp of ORF, and a 492 bp of 3' UTR with a poly(A) tail. The deduced amino acid of the protein consists of 387 residue including a signal peptide of 20 amino acid at the N-terminus. The calculated molecular mass for the mature protein (367 amino acids) is 39.37 kDa with the p*I* prediction of 7.52. The putative proteolytic cleavage site was located at the beginning of the SP-like domain

(between Ala147 and Glu148). The serine proteinase domain showed conserved catalytic site of His184 and Asp234, except for Ser was replaced by Gly336 (Figure 3.1). The replacement suggested that the *Pm*MasSPH2 is also a non-catalytic serine proteinase.

The GenBank database searching using the BlastX search program showed that *Pm*MasSPH1 exhibited 74% sequence similarity to the freshwater crayfish *P. leniusculus Pl*SPH2 (ACB41379). *Pm*MasSPH1 also showed 56% similarity to the serine proteinase-like protein from the green mud crab *S. paramamosain Sp*-SPH (ADG83846), and 44% similarity to the prophenoloxidase activating factor from *H. diomphalia Hd*PPAFII (CAC12665). The database searching also revealed that *Pm*MasSPH2 exhibited 68% sequence similarity to the freshwater crayfish *P. leniusculus Pl*SPH1 (AAX55746) and also showed 45% similarity to the *H. diomphalia Hd*PPAFII (CAC12665). The pair-wise sequence alignment of *Pm*MasSPH1 and *Pm*MasSPH2 showed the sequence similarity of 26.2%. The multiple amino acid sequence alignment between the SPHs from crustaceans and an insect (Figure 3.2) revealed the conserved clip-domain and serine proteinase-like domain with conserved catalytic triad, except Ser residues are replaced by Gly (His301, Asp351, and Gly452 for *Pm*MasSPH1 and His184, Asp234, and Gly338 for *Pm*MasSPH2). Taken together, the *Pm*MasSPH1 and *Pm*MasSPH2 were identified as *P. monodon* SPHs and have the closest sequence homology to the crayfish *P. leniusculus Pl*SPH2 and *Pl*SPH1, respectively.

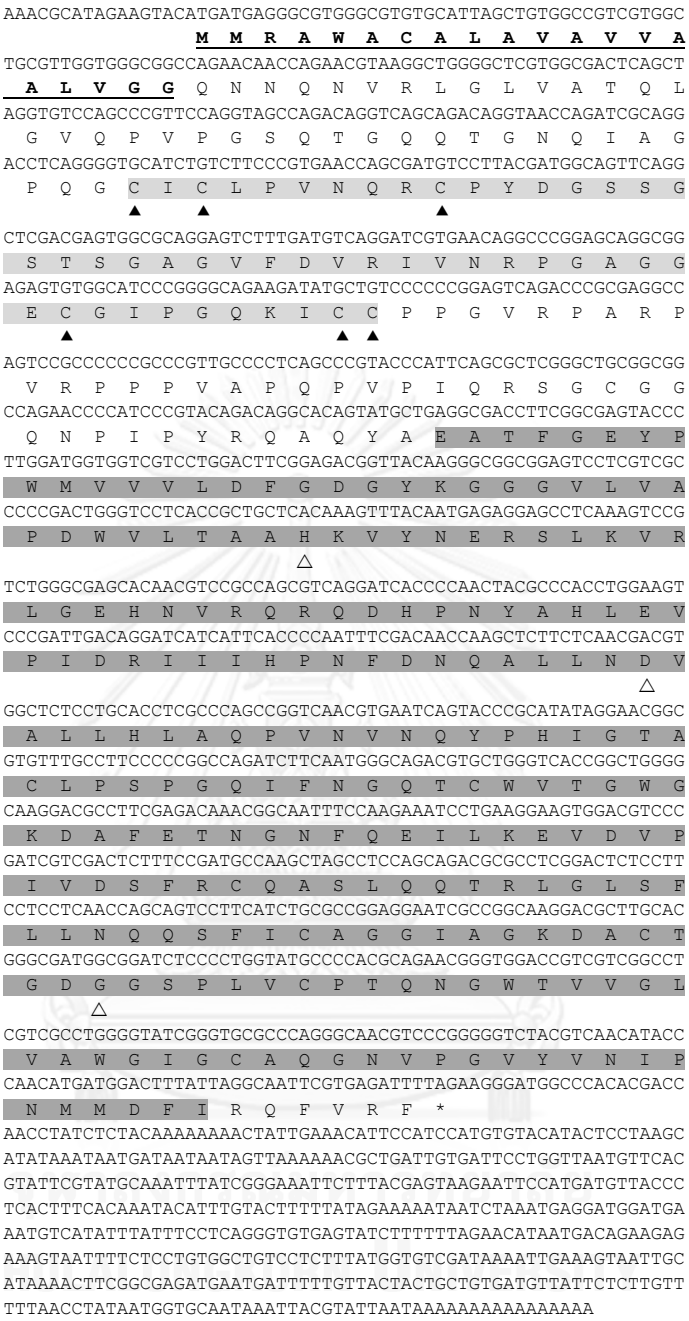

Figure 3.1 The full-length nucleotide and deduced amino acid sequences of *P. monodon* *Pm*MasSPH2. Each amino acid sequence is shown as an abbreviation under the second nucleotide of each codon. An asterisk indicates the stop codon. The predicted signal peptide is in bold and underlined. The light-grey highlight indicated the clip-domain. The black arrow heads indicate the cysteine residues in the clip-domain. The grey highlight indicates the serine proteinase-like domain. The open arrow heads indicate the sites of catalytic triad of the serine proteinase.

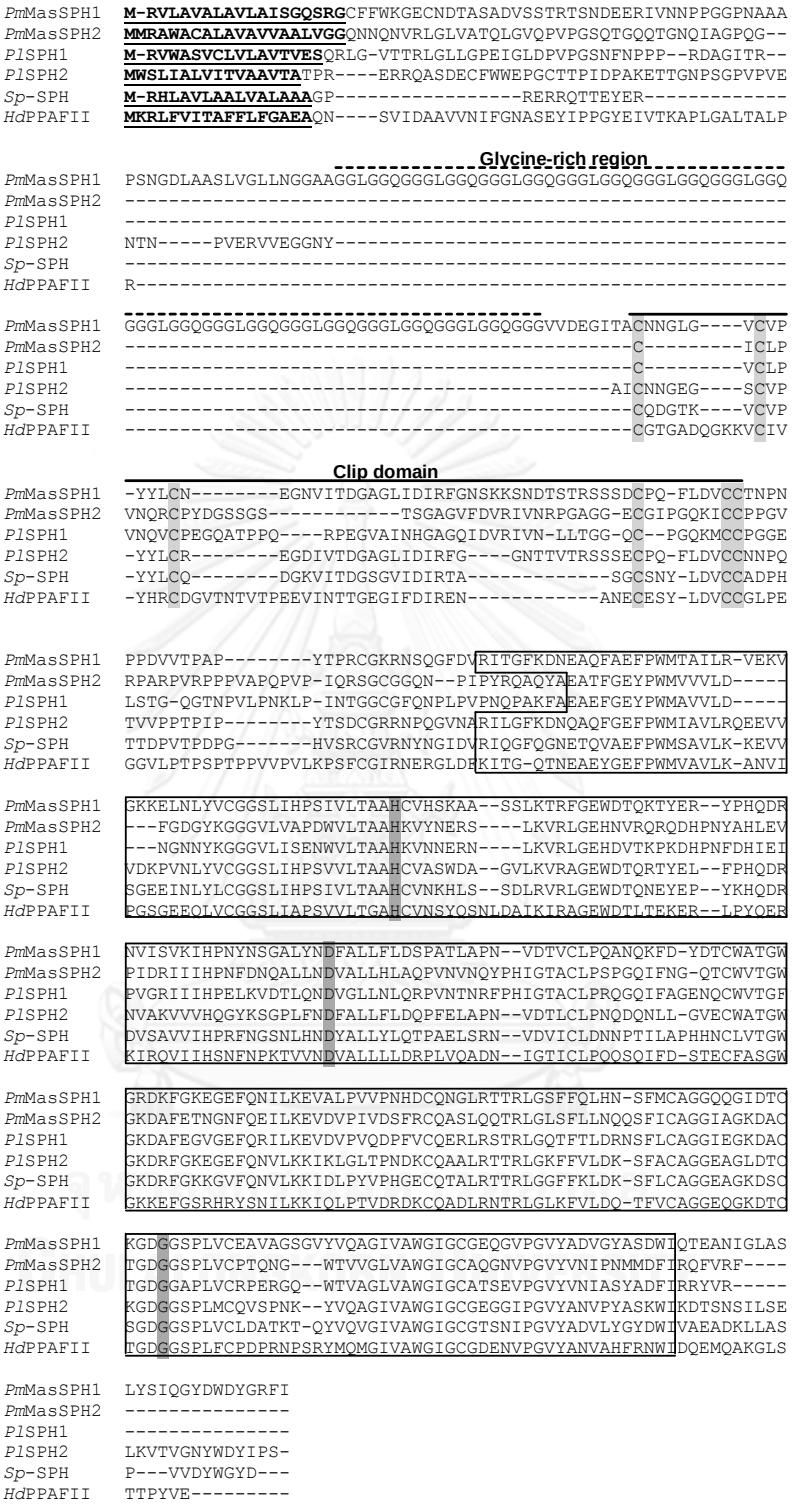

Figure 3.2 The multiple amino acid sequence alignment of *Penaeus monodon Pm*MasSPH1 (ABE03741), *Pm*MasSPH2 (ACP19560), *Pacifastacus leniusculus Pl*SPH1 (AAX55746), *Pl*SPH2 (ACB41379), *Scylla paramamosain Sp*-SPH (ADG83846), and *Holotrichia diomphalia Hd*PPAFII (CAC12665). The predicted signal peptides are in bold and underlined. The dash line indicates the

glycine-rich domain of *Pm*MasSPH1. The conserved clip-domains are indicated below the line. The light-grey highlight indicates the cysteine residues in clip-domains. The black box indicates the conserved serine proteinase-like domain with the grey highlight indicates the catalytic triads (His, Asp, and Gly residues) in the domain.

3.1.2 Functional characterization of *Pm*MasSPHs by genes silencing

3.1.2.1 The gene silencing of *Pm*MasSPH1 and *Pm*MasSPH2

In order to characterize the roles of *Pm*MasSPH1 and *Pm*MasSPH2 in the activation of proPO enzyme, the knockdown of *Pm*MasSPH1 and *Pm*MasSPH2 were performed. The dsRNA of *Pm*MasSPH1 and *Pm*MasSPH2 were synthesized and intramuscularly injected twice to the shrimps at total concentration of 10 µg/ 1 g shrimp. The dsRNA GFP (total 10 µg/ 1g shrimp) and 150 mM NaCl injections were performed along with the injection of *Pm*MasSPHs dsRNA, to serve as the control groups. At 48 h post second injection, the gene expression of *Pm*MasSPHs were determined by semi-quantitative RT-PCR technique. The gene specific primers (Table 2.1) were used to amplify the genes in each treatment and EF1α was used as the internal control for the equal amount of the cDNA template. The PCR products were visualized by agarose gel electrophoresis. The results showed the depletion of the *Pm*MasSPH1 (Figure 3.3 A) and *Pm*MasSPH2 (Figure 3.3 B) gene expression compared with the GFP dsRNA and NaCl control (Figure 3.3 A and B for *Pm*MasSPH1 and *Pm*MasSPH2, respectively). The result suggested the success of the genes silencing using dsRNA of the specific shrimp SPH genes and the dsRNA injection condition was chosen for further experiments, including the determination of PO activity in the knock-down shrimps and the involvement of *Pm*MasSPHs in the bacterial clearance.

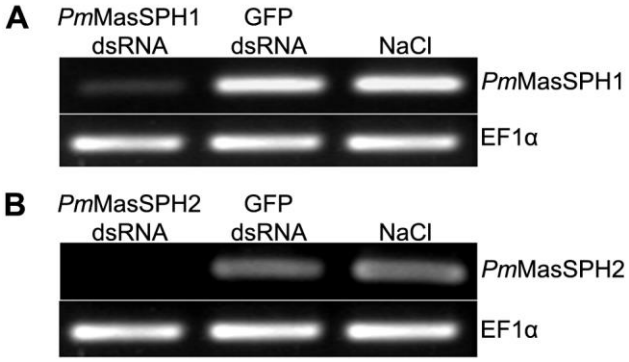

Figure 3.3 The efficiency of gene silencing of PmMasSPH1 and 2 were determined by semi-quantitative RT-PCR. Transcript level of *Pm*MasSPH1 (A) and *Pm*MasSPH2 (B) in dsRNA, GFP dsRNA, and 150 mM NaCl injected shrimp examine by RT-PCR. EF1**α** was used as an internal control. Each band represents the pooled cDNA of triplicate samples of each treatment. Each lane represents the dsRNA or NaCl injection as indicated above.

3.1.2.2 *Pm*MasSPH1 and *Pm*MasSPH2 silencing affected the hemolymph PO activity.

The expression of *Pm*MasSPH1 and *Pm*MasSPH2 was suppressed by dsRNA specific to the gene. Hemolymph PO activity was determined after *Pm*MasSPH1 and *Pm*MasSPH2 suppression to study the involvement of these *Pm*MasSPHs genes in the activation of proPO. The experiment of *Pm*MasSPH1 and *Pm*MasSPH2 was separately carried out. Shrimp hemolymph was collected from 3 treatments; *Pm*MasSPH1 or *Pm*MasSPH2 dsRNA injection, GFP dsRNA injection, and the 150mM NaCl injection. The PO activity of *Pm*MasSPH1 and *Pm*MasSPH2 knockdown shrimps was significantly decreased by 66.5% and 63.7% respectively, compared to GFP and NaCl dsRNA injected shrimps ($p < 0.05$). These results indicated that *Pm*MasSPH1 and *Pm*MasSPH2 are involved in the proPO activation.

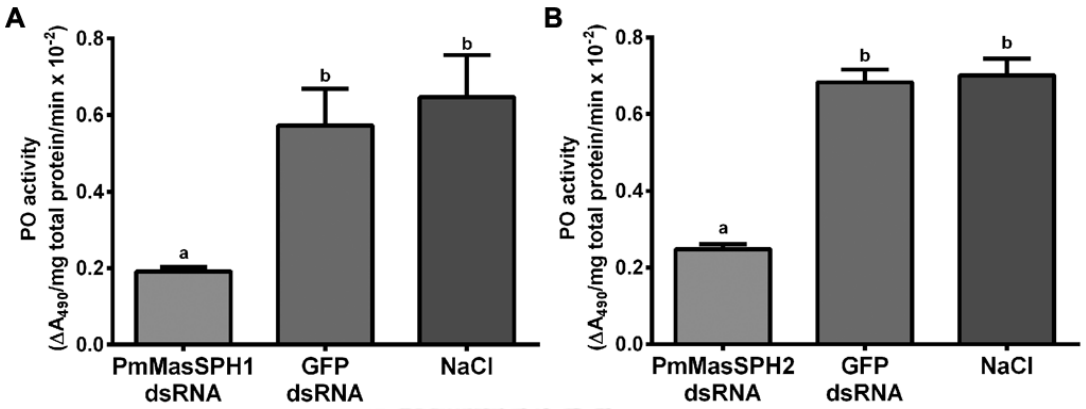

Figure 3.4 Hemolymph PO activity of the *Pm*MasSPH1 (A) and *Pm*MasSPH2 (B) silencing shrimps. Shrimps were injected with *Pm*MasSPH1 dsRNA, *Pm*MasSPH2 dsRNA. The shrimps injected with GFP dsRNA, and 150mM NaCl were served as the control group. The hemolymph was collected and measured for total hemolymph PO activity. The results from three replicated experiments are shown as mean±standard error (error bars). Means with significantly different ($p < 0.05$) are indicated by the different lower case letters (a, b) above each bar.

3.1.2.3 Bacterial number in hemolymph of the *Pm*MasSPHs knockdown shrimps

In order to investigate the effect of the *Pm*MasSPHs silencing to the hemolymph bacterial count after the bacterial injection, the knockdown shrimps were injected with *V. harveyi* at the amount of 2×10^5 CFU/shrimp along with dsRNA of *Pm*MasSPHs in the second injection. At 6 hours post injection, hemolymph from individual shrimp was collected and serially diluted to plate on LB agar and incubates at 30°C overnight. The colonies that formed on the plate were counted and statistically analysed. The CFU values from the *Pm*MasSPH1 and *Pm*MasSPH2 were compared with the values from GFP dsRNA control. The results showed the number of viable bacterial CFUs in both *Pm*MasSPH1 (11.5-fold) (Figure 3.5 A) and *Pm*MasSPH2 (7.9-fold) (Figure 3.5 B) were significantly higher compared with the GFP dsRNA control (Figure 3.5 A and B). The results suggested that both *Pm*MasSPH1 and *Pm*MasSPH2 are involved in hemolymph bacterial clearance process in *P. monodon*.

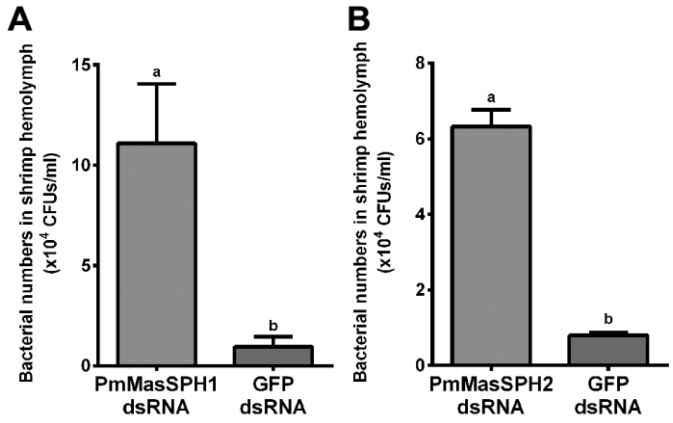

Figure 3.5 Number of viable bacteria in the hemolymph of *Pm*MasSPH1 and *Pm*MasSPH2 knockdown shrimps after the *V. harveyi* injection. Control shrimps were injected with GFP dsRNA. Each bars represented mean±SE (n=3) of the CFU/mL from each treatment. The different lower case letters (a, b) above the error bar represented the significantly difference ($p < 0.05$) between the treatment in each experiment.

3.1.2.4 Gene silencing of *Pm*MasSPH1 affected the expression of other immune genes.

The effect of gene silencing of *Pm*MasSPH1 on the expression of other immune genes was further determined by semi-quantitative RT-PCR. As shown in Figure 3.6, shrimps injected with *Pm*MasSPH1 dsRNA showed the decreasing in expression of *Pm*PPAE2 (71.4%) (Figure 3.6 B) and some antimicrobial peptide genes including *Penmon*PEN3 (54.4%) (Figure 3.6 C), crustin*Pm*1 (30.8%) (Figure 3.6 D), and Crus-like*Pm* (64.5%) (Figure 3.6 E) compared with GFP dsRNA injection.

In order to assure that the decrease in expression was not caused from non-specific gene silencing of *Pm*MasSPH1, the sequence of *Pm*MasSPH1 which was used to synthesized dsRNA in the experiment was aligned against the 4 genes that showed the sign of the decreasing expression as mentioned above. The region showed the similarity less than 20 bp in all of the alignments; *Pm*PPAE2, *Penmon*PEN3, crustin*Pm*1, and Crus-like*Pm* (Appendix). This could eliminate the possibility that the dsRNA of *Pm*MasSPH1 itself altered these immune genes, but the effect that occur was the results of *Pm*MasSPH1 silencing.

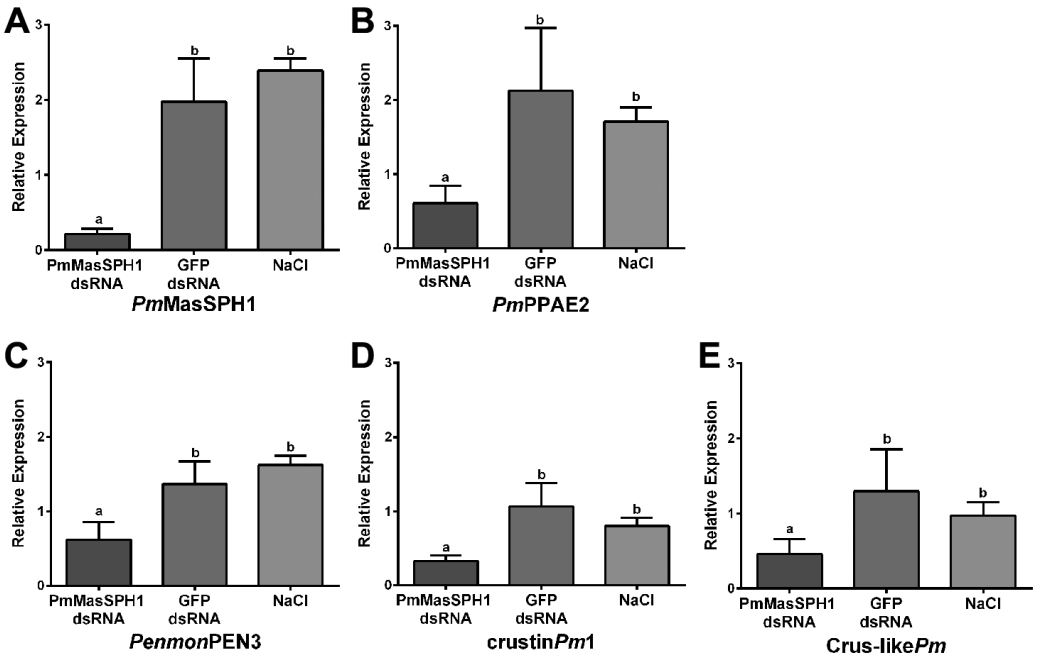

Figure 3.6 The expression pattern of *P. monodon* proPO-associated genes *Pm*MasSPH1 (A), *Pm*PPAE2 (B), and *P. monodon* antimicrobial peptides *Penmon*PEN3 (C), crustin*Pm*1 (D), and Crus-likePm (E) in the *Pm*MasSPH1 dsRNA injected shrimps. The effect of RNAi-mediated suppression of *Pm*MasSPH1 on the expression of other immune-related genes were investigated by semi-quantitative RT-PCR. cDNA of dsRNA or 150 mM NaCl injected shrimps was amplified by gene specific primers and the bands intensity were analyzed. The charts displayed the relative expression of the genes compared with EF1α, the housekeeping gene as the internal control. Each bars represented the relative expression in mean ± SE. The different lower case letters (a, b) represented the significantly different between each treatment in each chart.

3.1.3 *Pm*MasSPH1, *Pm*MasSPH2, *Pm*PPAE1 and *Pm*PPAE2 recombinant protein expression

To characterize the function of *Pm*MasSPH1 and *Pm*MasSPH2 proteins, recombinant proteins were produced in the *Escherichia coli* expression system. The recombinant proteins of *Pm*MasSPH1 and *Pm*MasSPH2 were expressed covering different domains (Figure 3.7) and were designated as r*Pm*MassPH1, rSP-*Pm*MasSPH1, r*Pm*MasSPH2, and rSP-*Pm*MasSPH2.

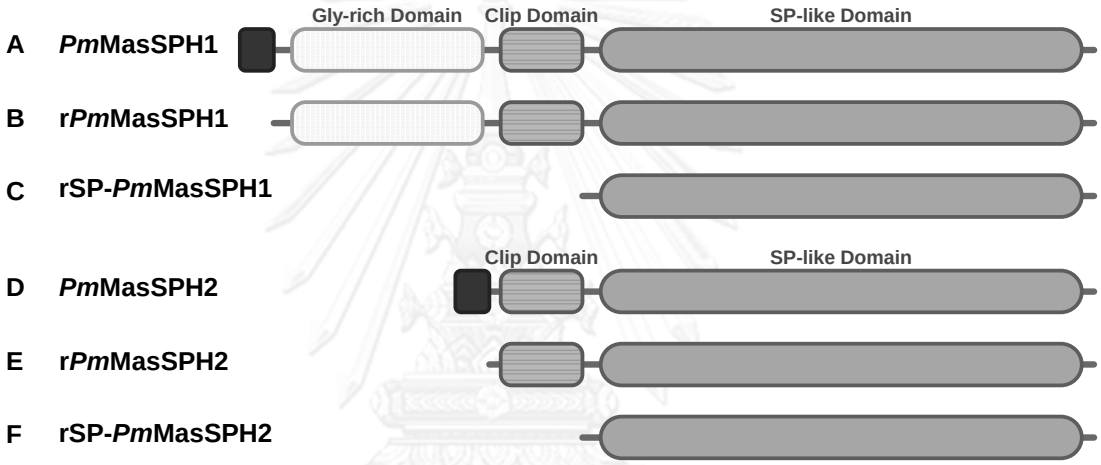

Figure 3.7 The schematic represents the constructs of recombinant proteins of *Pm*MasSPH1 (B, C) and *Pm*MasSPH2 (D, E) compared with full proteins (A, D). The black boxes represent the signal peptides. The white boxes with dots represent the glycine-rich domains. The boxes with horizontal stripes indicates the clip-domains. The grey boxes indicates the SP-like domains. The designated name for each recombinant protein are indicated on the left.

The r*Pm*MasSPH1 and r*Pm*MasSPH2 were expressed with thioredoxin (TRX) tag fused at the N-terminus, which made r*Pm*MasSPH1 contained 671 amino acids with the predicted molecular mass of 70 kDa and p*I* value of 5.79, and r*Pm*MasSPH2 contained 534 amino acids with the predicted molecular mass of approximately 57.5 kDa and pI value of 6.81. The r*Pm*MasSPH1 and r*Pm*MasSPH2 were purified by the nickel affinity chromatography and analysed by the SDS-PAGE and western blotting with the anti-His antibody as the primary antibody (Figure 3.8

and 1 and 2, respectively). The bands were at the size of approximately 70 and 58 kDa, respectively. The results indicated the purity and the specificity for further experiments.

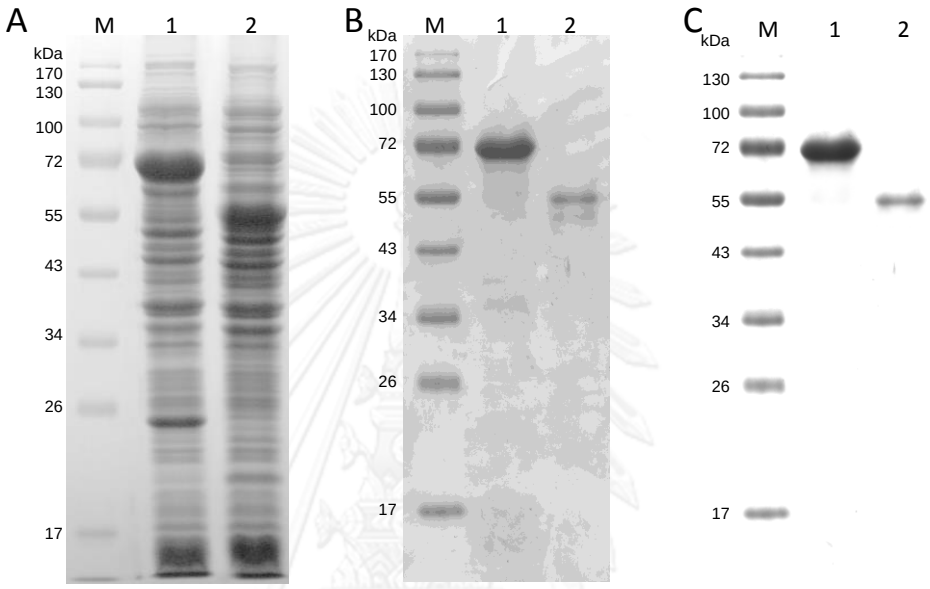

Figure 3.8 The Coomassie staining of the recombinant proteins produced in the cell lysate (A), the purified proteins (B), and immunoblotting (C) of r*Pm*MasSPH1 and r*Pm*MasSPH2. The purified proteins were analyzed by SDS-PAGE and western blotting using the anti-His antibody. Lane M, molecular weight marker with the size of the bands indicated on the left; lane 1, r*Pm*MassPH1; lane 2, r*Pm*MassPH2.

The rSP-*Pm*MasSPH1 and rSP-*Pm*MasSPH2 were expressed with His-tag fused at the C-terminus and N-terminus respectively. The predicted molecular mass of rSP-*Pm*MasSPH1 and rSP-*Pm*MasSPH2 are approximately 31 kDa and 28 kDa, respectively. The purified r*Pm*MasSPH1 and r*Pm*MasSPH2 were analysed by the SDS-PAGE and western blotting with the anti-His antibody as the primary antibody (Figure 3.9 lanes 1 and 2, respectively). The bands were the size of approximately 30 and 28 kDa, respectively. The results indicated the purity and the specificity of the proteins.

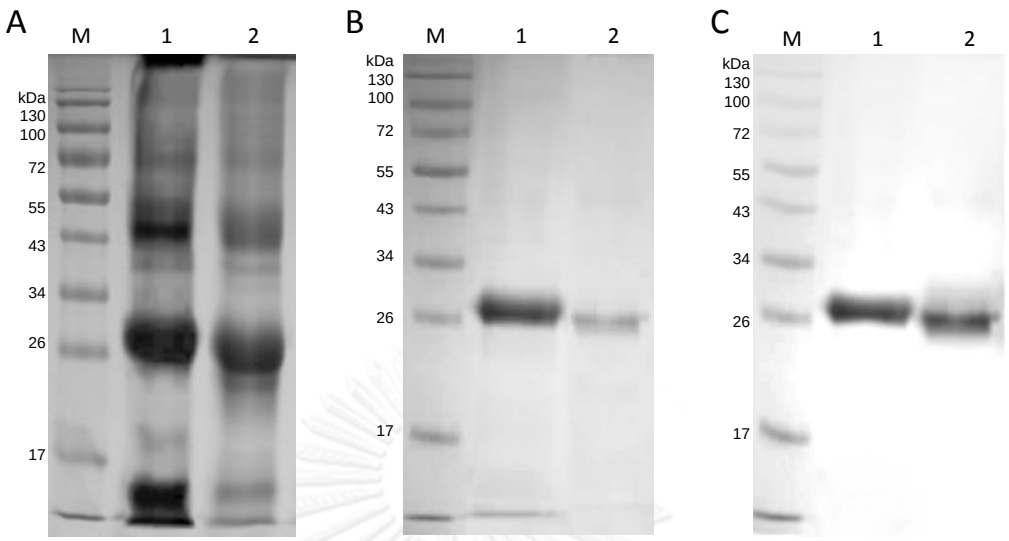

Figure 3.9 The Coomassie staining of the recombinant proteins produced in the cell lysate (A), the purified proteins (B), and immunoblotting (C) of *Pm*MasSPH1 and *Pm*MasSPH2 that were expressed in *E. coli* system. The purified proteins were analyzed by SDS-PAGE and western blotting using the anti-His antibody. Lane M, molecular weight marker with the size of the bands indicated on the left; lane 1, purified SP-*Pm*MasSPH1; lane 2, purified SP-*Pm*MasSPH2.

The recombinant proteins of SP domain of *Pm*PPAE1 (rSP-*Pm*PPAE1) (Charoensapsri *et al.*, 2009) and *Pm*PPAE2 (rSP-*Pm*PPAE2) were expressed in *E. coli* and the insoluble fraction of the proteins were subsequently purified. After SDS-PAGE analysis, the predicted molecular mass of rSP-*Pm*PPAE1 and rSP-*Pm*PPAE2 are 26.6 and 27.9 kDa, respectively. The purified recombinant proteins of rSP-*Pm*PPAE1 and rSP-*Pm*PPAE2 were confirmed by the SDS-PAGE and western blotting with the anti-His antibody as the primary antibody (Figure 3.10 lanes 1 and 2, respectively). The bands at the size of approximately 27 kDa in both recombinant proteins indicated the purity and the specificity of the proteins.

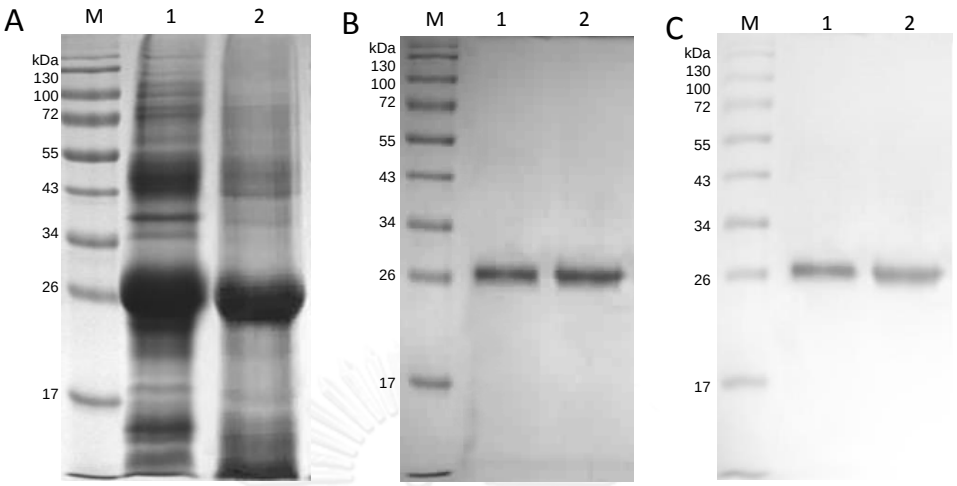

Figure 3.10 The Coomassie staining of the recombinant proteins produced in the cell lysate (A), the purified proteins (B), and immunoblotting (C) of serine proteinase-like domain of *Pm*PPAE1 and *Pm*PPAE2 using the anti-His antibody. Lane M, molecular weight marker with the size of the bands indicated on the left; lane 1, purified SP-*Pm*PPAE1; lane 2, purified SP-*Pm*PPAE2.

3.1.4 Functional characterization of *Pm*MasSPH1 and *Pm*MasSPH2 recombinant proteins

3.1.4.1 The protein-protein interaction assay by co-IP

The previous reports in insects and other crustaceans suggested the roles of SPHs as the cofactor for PPAEs, the terminal serine proteinases for the proPO activation (Cerenius *et al.*, 2008). To determine the function in *Pm*MasSPHs, the protein-protein interaction by co-IP was used to determine the interaction between *Pm*MasSPHs and *Pm*PPAEs. The protein-protein interaction experiments of *Pm*MasSPH1 and *Pm*PPAEs were carried out as followed; (A,B) *Pm*MasSPH1 and *Pm*PPAE1, (C, D) *Pm*MasSPH1 and *Pm*PPAE2 (Figure 3.11). The antiSPH1-conjugated protein A agarose beads were used. As seen in the immunoblotting, *Pm*MasSPH1 was able to specifically co-immunoprecipitate *Pm*PPAE2 (Figure 3.11 C and D, lane 2), while there was no binding observed in the detection between *Pm*MasSPH1 and *Pm*PPAE1.

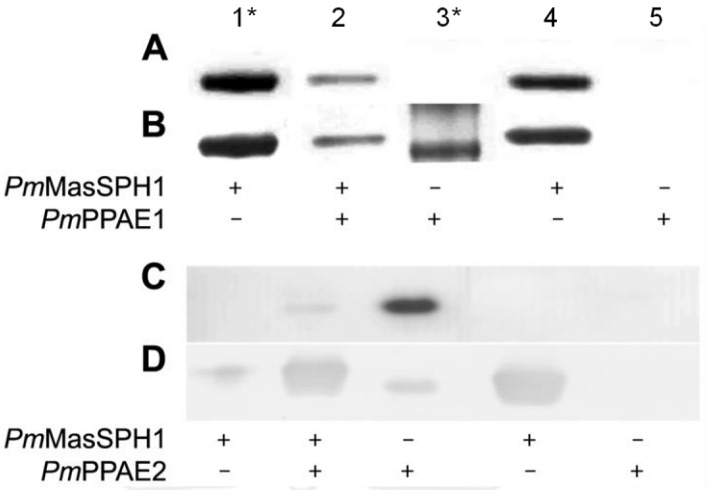

Figure 3.11 The SDS-PAGE and Western blot analysis of co-IP between *Pm*MasSPH1 and *Pm*PPAEs. The immunoblotting of co-IP between *Pm*MasSPH1 and *Pm*PPAE1 was probed with anti-*Pm*MasSPH1 antibody as primary antibody (A). The immunoblotting membrane A was striped and re-probed with anti-PPAE1 antibody (B). The immunoblotting of co-IP between *Pm*MasSPH1 and *Pm*PPAE2 was probed with anti-PPAE2 antibody as primary antibody (C). The immunoblotting membrane C was striped and re-probed with anti-*Pm*MasSPH1 antibody (D). Lanes 2, 4, and 5 represented the proteins from each co-IP reaction as indicated by + (present in the reaction) or – (not in the reaction) signs below each figure. The asterisks represent the lanes of the recombinant proteins (not in the co-IP reaction) to serve as the size indicators.

Since the unsuccessful production of the antibody specific for *Pm*MasSPH2, the anti-*Pm*MasSPH2-conjugated protein A agarose beads could not be used in the experiments. The method was substituted with the anti-*Pm*PPAE1-conjugated and anti-*Pm*PPAE2-conjugated protein A agarose beads for the *Pm*MasSPH2 and *Pm*PPAE1, and *Pm*MasSPH2 and *Pm*PPAE2 experiments, respectively. There was no binding observed in the other detection (Figure 3.12). The results suggested the recombinant SP-*Pm*MasSPH1 could bind to recombinant SP-*Pm*PPAE2 but not SP-*Pm*PPAE1. The recombinant SP-*Pm*MasSPH2 did not bind to either recombinant SP-*Pm*PPAE1 or SP-*Pm*PPAE2 *in vitro*.

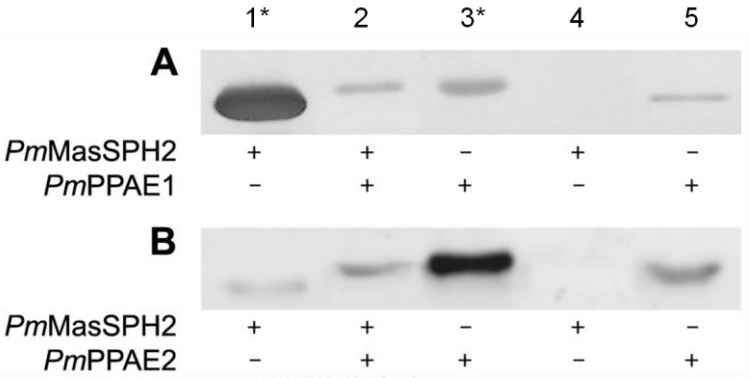

Figure 3.12 The immunoblotting of Co-IP between *Pm*MasSPH2 and *Pm*PPAE1 (A) and between *Pm*MasSPH2 and *Pm*PPAE2 (B). The immunoblotting was probed with anti-His antibody as primary antibody. Lanes 2, 4, and 5 represent the proteins from each co-IP reaction as indicated by + (present in the reaction) or – (not in the reaction) signs below each figure. The asterisks represent the lanes of the recombinant proteins (not in the co-IP reaction) to serve as the size indicators.

3.1.4.2 The binding ability of recombinant proteins to the Gram-positive bacteria cell wall component, PGN, by ELISA

One of the pathogen associated molecular patterns (PAMPs) is peptidoglycan (PGN) which is a bacterial cell wall component found mostly in Gram-positive bacteria. Unlike lipopolysaccharide (LPS) or β(1,3)-glucan, *P. monodon* *Pm*LGBP showed no binding activity to the PGN (Amparyup *et al.*, 2012). Therefore, the PGN binding proteins for the proPO activating system remain unknown. From previous study, *Pm*MasSPH1 showed the binding ability to LPS (Jitvaropas *et al.*, 2009) which provide the possibility that *Pm*MasSPH1 might plays role as the recognition protein. In this study, the recombinant protein *Pm*MasSPH1 and *Pm*MasSPH2 were investigated for the role as the recognition protein of PGN. To quantitatively measure the binding activity of *Pm*MasSPH1 and *Pm*MasSPH2 to PGN, ELISA technique was chosen for the experiment. PGN from *B. subtilis* was diluted with pathogen-free water and 2 μg PGN was coated in each well of 96-well plate. Various dilutions (0-12.5 μg/mL) of recombinant proteins were applied and the binding was assayed as described in 2.7. The results showed the binding of

recombinant *Pm*MasSPH1 and *Pm*MasSPH2 with PGN with the dissociation constant (K_d) of 6.51x10^{-8}M and 5.79x10^{-8}M respectively (Figure 3.13).

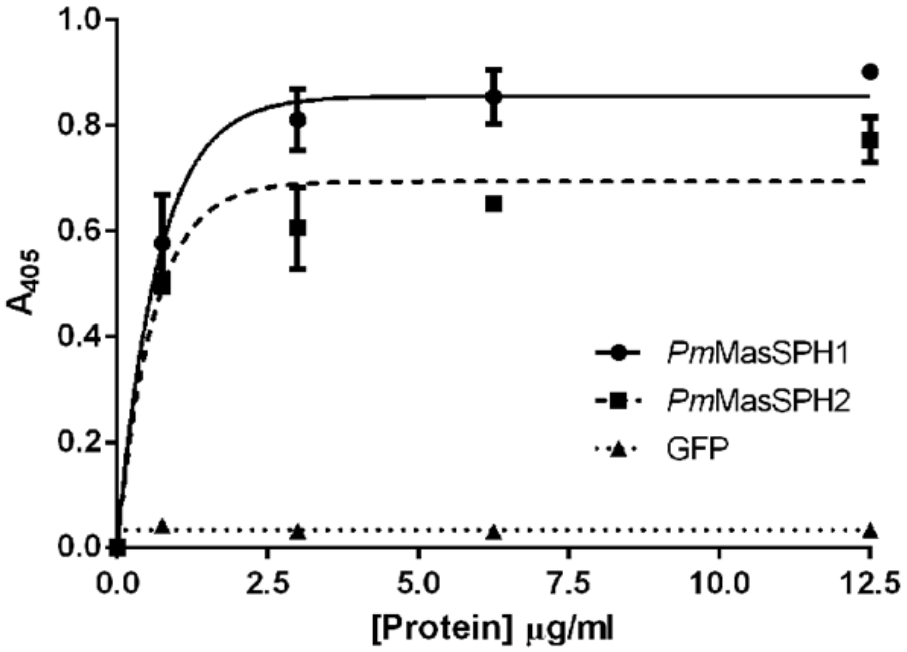

Figure 3.13 The quantitative binding of recombinant proteins *Pm*MasSPH1 or *Pm*MasSPH2 and *B. subtilis* PGN were investigated by ELISA assay. The A_{405} values from three independent replicated experiments are shown as mean±SE. The binding of *Pm*MasSPH1 and *Pm*MasSPH2 (0-12.5 µg/mL) are shown in the black line with circles and the dash line with squares, respectively. The recombinant GFP was using to serve as recombinant protein control, represented in the dot line with triangles.

3.2 Functional investigation of cleaved crayfish *Pl*proPO

3.2.1 Genes characterization of crayfish *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2

Crayfish *Pl*proPO (GenBank: CAA58471) contains a C-terminal proteinase domain and a N-terminal domain as reported in the previous study (Aspan *et al.*, 1995). The predicted cleavage site for the activation of *Pl*proPO by *Pl*ppA is located between Arg_{176} and Thr_{177}. The plasmid was constructed for protein expression. The N-terminal peptide was designated as *Pl*proPO-ppA. *Pl*proPO-ppA containes 528 bp, translated to 176 amino acids with predicted molecular mass of approximately 20 kDa and the estimated p*I* of 9.88 (Figure 3.14, black arrow head).

The first caspase-1 cleavage site in *Pl*proPO, as predicted from PeptideCutter, located between Asp_{363} and Ala_{364}. The peptide from N-terminus to the Asp_{363} was designated as *Pl*proPO-casp1. *Pl*proPO-casp1 containes 1089 nucleotides, translated to 363 amino acids with predicted molecular mass of 41.92 kDa and the estimate p*I* value of 8.55. The second caspase-1 cleavage site prediction was located between Asp_{389} and Asn_{390}. The peptide from N-terminus to the Asp_{389} was designated as *Pl*proPO-casp2. *Pl*proPO-casp2 containes 1167 nucleotides, translated to 389 amino acids with predicted molecular mass of 44.84 kDa and p*I* of 7.73 (Figure 3.14, open arrow heads).

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MQVTQKLLRRDTEMADAQKQLLYLFERPYDPINAPRADGSFLYAVAGAXTVATRFGVAPTST     62
VTVPARPDADRRLLGRAPSVPRGAVFSFFIRSHREAARDLCDVLMKTQNSTDLMQLAASVRR    124
HVNENLFIYALSFTILRKQELRGVRLPPILEVFPHKFIPMEDLTSMQVEVNRTPPTATTPLV    186

IEYGPEFANTNQKAEHRVSYWREDFGINSHHWHWHLVYPIEMNVNRDRKGELFYYMHQQMVA    248
RYDWERLSVNLNRVEKLENWRVPIPDGYFSKLTANNSGRPWGTRQDNTFIKDFRRNDAGLDF    310
IDISDMEIWRSRLMDAIHQGYMLNRNGERVPLSDNVTTGKRGIDILGDAFEADAQLSPNYLF    372

YGDLHNTGHVLLAFCHDNDNSHREEIGVMGDSATALRDPVFYRWHKFVDDIFQEYKLTQPPY    434

TMEDLSLPGVVLDKVGVVRNDQLNTLTTGWSVREFEASRGLDFNSPNPVTAHYPSRPCTLHL    496
PSPDNKQHRKPKSVTVRIYMAPKHNERGLEMGFMEQRLLWAEMDKFTQDLKPGQNQIVRASN    558
LSSITNPSGYTFRSLEAVNPANPGPPANAETNFCGCGWPEHLLLPRGKPEGMTYQLFFMLTD    620
LEKDQVDQPAGPRRCANAVSFCGILDSKFPDKRPMGFPFDRRPPPRLQDAEVTSVADYARLS    682
NMTVQDITITFLTTASRSRHDGPI                                        706
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Figure 3.14 Amino acid sequences of *Pl*proPO and its cleavage sites. The box with black arrow head represents the ppA cleavage site. The box with opened arrow heads represents the predicted caspase-1 cleavage sites.

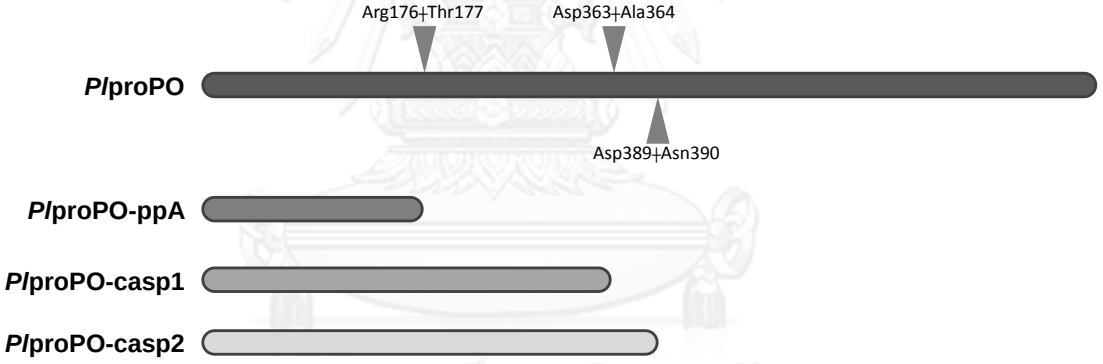

Figure 3.15 A schematic image summarized the fragments of PlproPO to be functional characterized in the study. The lines represent the fragments as labeled on the left. The arrow heads indicates the cleavage sites of each fragment.

3.2.2 Crayfish *Pl*proPO-ppA, *Pl*proPO-casp1, and *Pl*proPO-casp2 recombinant proteins expression

Recombinant plasmids of *Pl*proPO-ppA, *Pl*proPO-casp1, and *Pl*proPO-casp2 were constructed for protein expression in *E. coli* expression system. *Pl*proPO-ppA was cloned into pET32a at multiple cloning site. The recombinant plasmid was transformed into BL21(DE3)pLysS for protein expression. The recombinant protein was fused with TRX tag at the N-terminus and the total molecular mass was about 37.7 kDa with pI value of 6.80. The protein was confirmed by the immunoblotting with anti-His antibody (Figure 3.16 lane 1).

Recombinant *Pl*proPO-casp1 and *Pl*proPO-casp2 were constructed by amplifying and cloning into pET28b expression vector and transformed into BL21(DE3)pLysS for protein expression. The recombinant proteins were analyzed by SDS-PAGE and immunoblotting against anti-His antibody as primary antibody. The recombinant proteins of *Pl*proPO-casp1 and *Pl*proPO-casp2 have calculated molecular mass of 42.8 kDa and 45.9 kDa, respectively (Figure 3.16 lane 2 and 3).

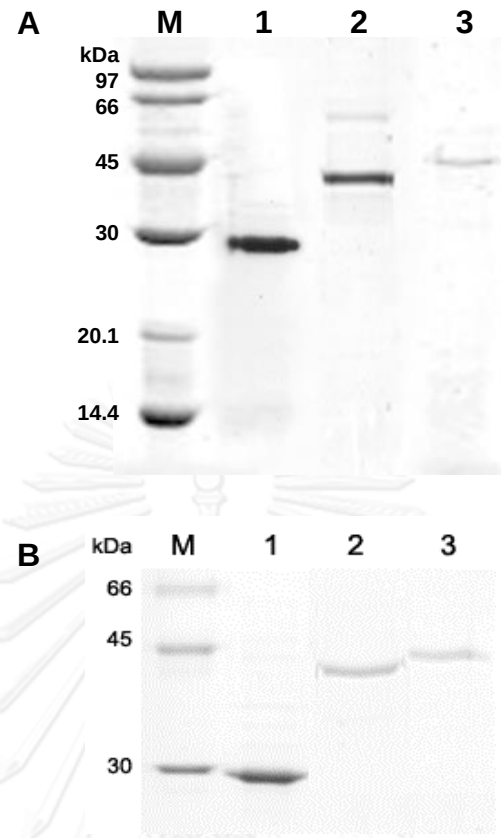

Figure 3.16 The Coomassie staining (A) and immunoblotting (B) of the *Pl*proPO-ppA, *Pl*proPO-casp1, and *Pl*proPO-casp2 recombinant proteins using anti-His antibody as primary antibody. Lane M, molecular weight marker with the size of the bands indicated on the left; lane 1, recombinant *Pl*proPO-ppA; lane 2, recombinant *Pl*proPO-casp1; lane 3, recombinant *Pl*proPO-casp2.

3.2.3 Functional characterization of *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 recombinant proteins

3.2.3.1 *In vivo* bacterial clearance of the *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 in crayfish

The non-enzymatic antimicrobial activities of *Pl*proPO fragments; *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2, were determined *in vivo* by the bacterial counting after crayfish injection of the fragments together with *E. coli*. Each crayfish was injected with 100 µL (1×10^9 CFU/mL) of *E. coli* and 20 µg of recombinant proteins or the recombinant GFP as the recombinant protein control, or 20mM Tris-

HCl pH 8.0 as the control buffer. The hemolymph of injected crayfish was bled and serially diluted to plate on the LB agar and counted for the bacterial colonies. The bacterial CFUs in 40 minutes post injection of the fragments showed lower *E. coli* number in the *Pl*proPO-ppA (83.6%), and in *Pl*proPO-casp1 treatment (72.5%) than the Tris-HCl buffer injection. However, *Pl*proPO-casp2 showed no significant decrease in *E. coli* number. At 3 hours post injection, all three treatments, *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2, showed the decrease of CFU (70.1%, 83.9%, and 75.2%, respectively) compared with the buffer-injected and GFP-injected treatment (Figure 3.17). These results suggested the activity of *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 in the bacterial clearance *in vivo*.

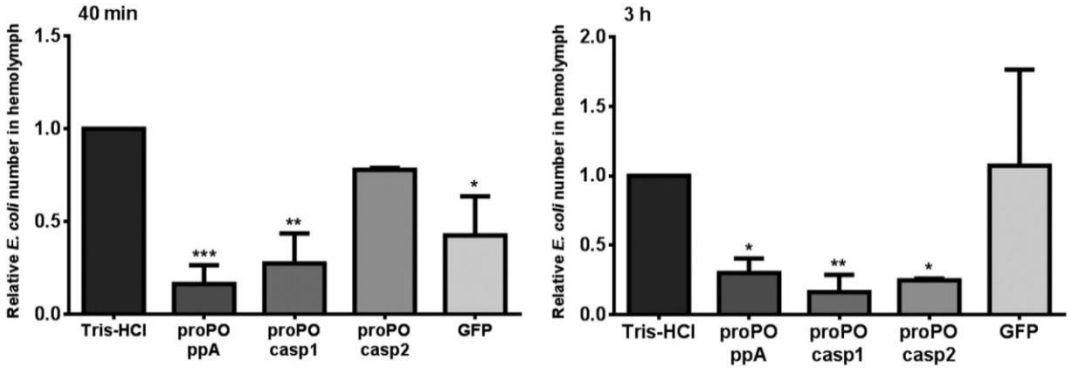

Figure 3.17 The charts display the relative *E. coli* number in the crayfish hemolymph after the injection of peptides and the bacteria. The peptides or buffer were mixed with freshly prepared *E. coli* and injected to the crayfish. The time points post injection indicated on the top of each chart, 40 minutes post injection on the left chart and 3 hours post injection on the right chart. Each bar indicates the mean (n=3) and the error bars represent standard error (SE). The values were analyzed by one-way ANOVA and the significantly difference was noted as the asterisks.

3.2.3.2 *In vitro* bacterial clearance of the *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 in crayfish

The fragments *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 showed their ability of decreasing the bacterial CFU *in vivo* and their functions were further investigated. The *in vitro* bacterial clearance assay was performed to determine whether their activity *in vivo* was caused by the fragments. *E. coli* (1×10^9 CFU/mL) and 20 µg fragments were incubated for 1 hour at room temperature with mild agitation and serially diluted to plate on LB agar. The CFUs of each treatment was calculated and analyzed. Apparently, the CFUs in the *Pl*proPO-ppA was the only treatment that CFU decreased compared to the buffer-control group (67.1%) (Figure 3.18). The result indicated the ability of the peptide to decrease the bacterial number.

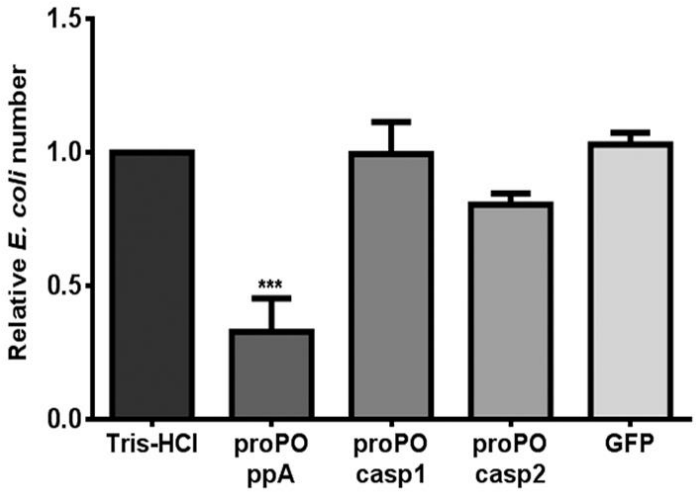

Figure 3.18 *In vitro* bacterial clearance from the *Pl*proPO fragments. The peptides were incubated together with freshly prepared *E. coli* at room temperature and were serially diluted to plate on LB agar plates. The CFUs were calculated and analyzed by one-way ANOVA. Each bar represent the mean ± SE. The asterisks indicate significant differences between treatments.

3.2.3.3 Bacterial agglutination activity of the *Pl*proPO-ppA *in vitro*

To investigate the function of *Pl*proPO-ppA in decreasing the CFU from the incubation of recombinant protein with *E. coli* (Figure 3.18), the incubation reaction including the bacteria (1×10^9 CFU/mL) and 20 µg recombinant protein was visualized under the light microscope at the same time point as the previous experiment (1 hour after mixing the bacteria and the recombinant protein). The *Pl*proPO-ppA treatment clearly displayed the sign of bacterial agglutination in contrast to all other treatments including the *Pl*proPO-casp1, *Pl*proPO-casp2, GFP-protein control and the Tris-HCl buffer control (Figure 3.19).

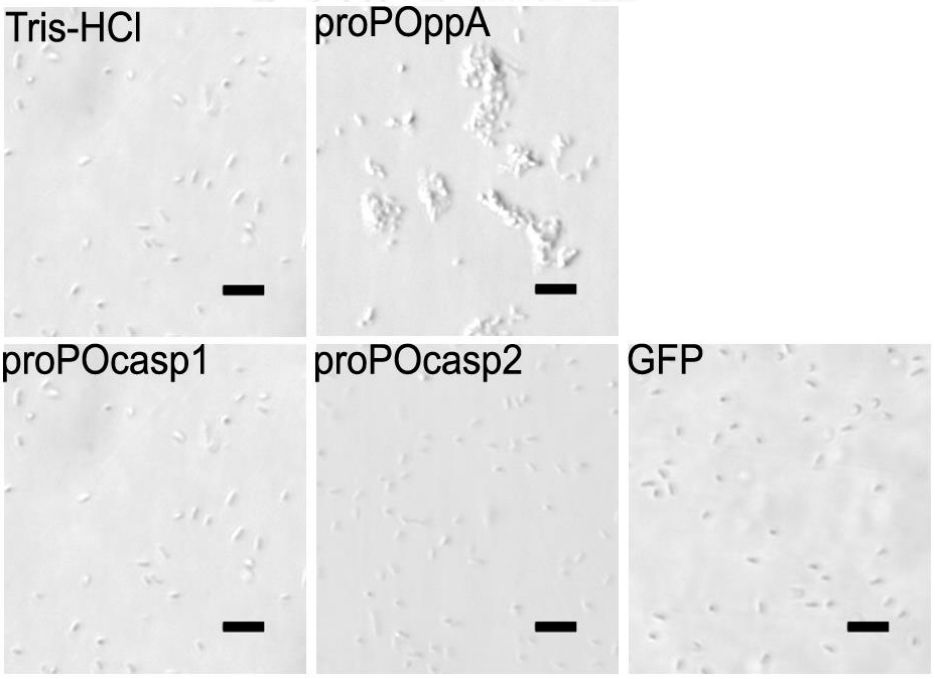

Figure 3.19 The bacterial agglutination from the *in vitro* bacterial clearance assay was observed. The *E. coli* was visualized by light microscopy after treatment with the recombinant fragments at room temperature. The treatments were indicated on the top-left. Scale bars represent 10µm.

The minimal agglutinating concentration (MAC) value for the *Pl*proPO-ppA against various bacteria as determined by incubating various concentrations of the peptide with freshly prepared bacteria. The mixtures were incubated at room temperature for 1 hour and observed under the microscope. The minimal concentration of peptide that lead to the agglutination was defined as MAC value (Table 3.1). The peptide *Pl*proPO-ppA was able to cause the agglutination in all tested bacteria, including Gram-positive *S. aureus, B. subtilis* ATCC6633, and *M. luteus*, and Gram-negative bacteria *E. coli* D21, *A. hydrophila* B1, and *P. aeruginosa* OT97.

Table 3.1 Minimal agglutinating concentration of recombinant *Pl*proPO-ppA for Gram-positive and Gram-negative bacteria

Microorganism	Minimal agglutinating concentration (μg/mL)
Gram-positive bacteria	
S. aureus	3.1
B. subtilis ATCC6633	50
M. luteus	6
Gram-negative bacteria	
E. coli D21	1.5
A. hydrophila B1	12
P. aeruginosa OT97	6

3.2.3.4 The effects of recombinant protein *Pl*proPO-ppA on bacterial viability and cell morphology

The agglutinated bacteria was stained with SYTO®9 Green Fluorescent Nucleic Acid Stain and Propidium Iodide (PI) in order to determine the viability of the bacteria. The dead bacteria could be stained with PI which appeared in red colour. The result (Figure 3.20) clearly showed the different between *Pl*proPO-ppA treatment and the buffer and GFP protein control. The agglutination that appeared only in the *Pl*proPO-ppA treatment apparently showed the red colour, indicating that cells which

clumped together are dead cells. Conversely to the *Pl*proPO-ppA treatment, the Tris-HCl buffer control treatment and GFP protein control treatment appeared the separated cells and the red-stained cells could rarely be seen.

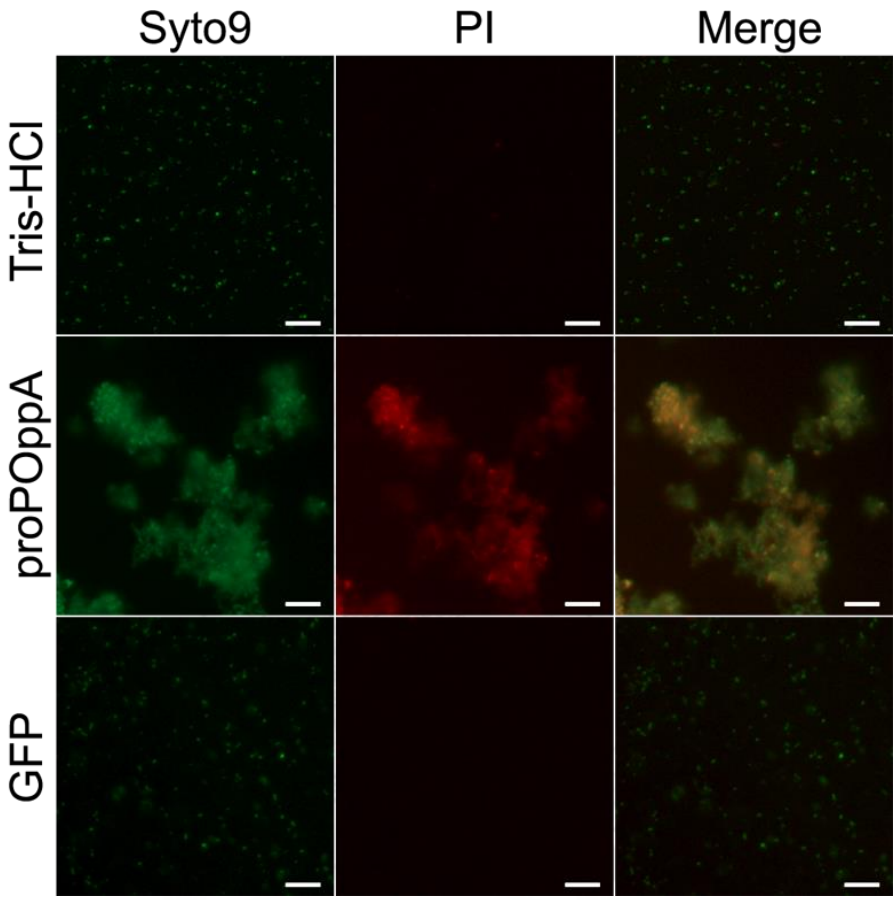

Figure 3.20 The viability staining of the *E. coli* treated with peptides or buffer. The columns indicated the Syto®9 staining, PI staining, and the merge picture of the Syto9 and PI staining, respectively. The rows indicates the treatment including Tris-HCl pH 8.0 buffer control, *Pl*proPO-ppA treatment, and GFP protein control, respectively. The scale bars represent 10µm.

The morphology of the *E. coli* visualized by SEM to determine the morphological changes that may occur and cause the cell death. The bacteria at O.D. 0.5 100 µL were mixed with 20 µg recombinant protein *Pl*proPO-ppA or GFP, and at the 15 and 40 minutes after the incubation, the samples were collected and fixed with glutharaldehyde following standard procedures for SEM. The results from 15

minutes time point showed the sign of disruption as cell wall beginning to shrink (Figure 3.21 A, white arrows) compared to the GFP recombinant protein control (Figure 3.21 B).

After 40 minutes incubation, the bacteria incubated with *Pl*proPO-ppA clearly showed the cell disruption and morphological change. The bacteria cells appeared to be flatted and distorted (Figure 3.21 C) while bacteria in the GFP control showed no sign of cell disruption (Figure 3.21 D). The results indicated the ability of *Pl*proPO-ppA in the bacterial cell disruption that may lead to bacterial clearance.

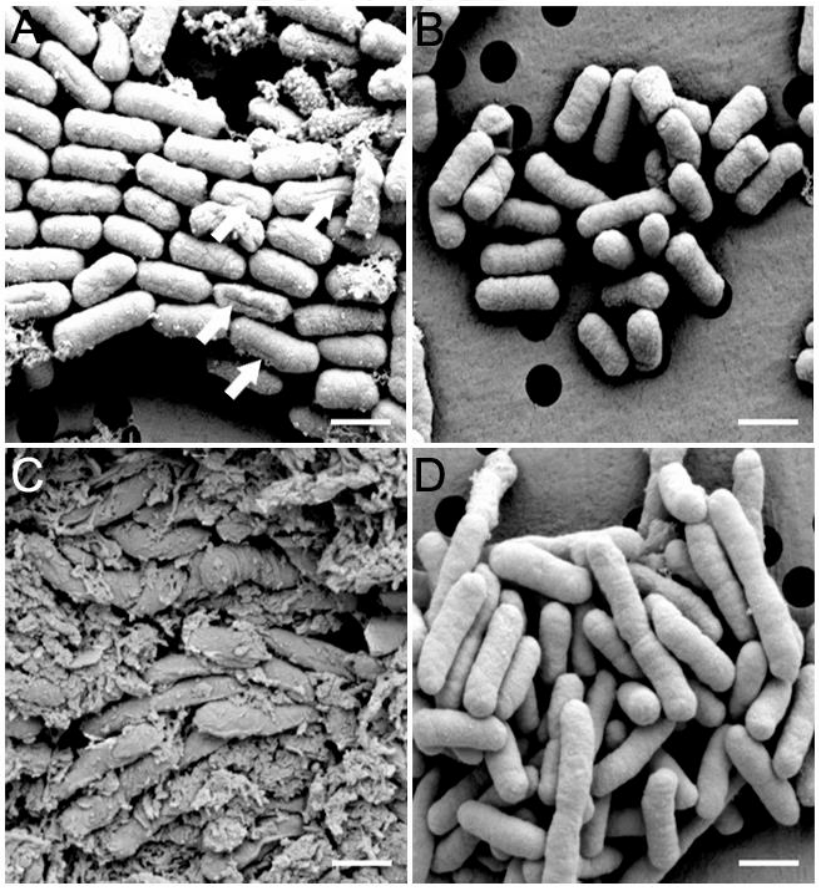

Figure 3.21 The scanning electron microscope images display the protein-bacteria incubation at different time points. A and B represent the 15 minutes post incubation while C and D show the bacterial morphology at 40 minutes after the incubation. A and C: Bacteria incubated with recombinant *Pl*proPO-ppA. B and D: Bacteria incubated with recombinant GFP. The scale bars represent 10 μm. The white arrows indicate the longitudinal lines on the *E. coli* surface in the *Pl*proPO-ppA treatment.

CHAPTER IV
DISCUSSIONS

Living organisms possess the internal self-defending mechanism called the immune system to act as a shield against the invading of various pathogens in order to maintain their biological systems to function properly. The major systems for the immunity are the adaptive immune system that provides the ability to recognize the specific pathogen which function in vertebrates, and the innate immune system that is acquired by vertebrates and invertebrates, including the crustaceans. The innate immune system consists of cellular immune responses e.g. encapsulation, phagocytosis, and nodule formation (Strand and Pech, 1995, Schmidt *et al.*, 2001) and humoral immune responses e.g. antimicrobial peptide (AMP), clotting system, and the prophenoloxidase (proPO) activation system (Lavine and Strand, 2002). The innate immune system is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) by the pattern-recognition proteins (PRP). The PAMPs are lipopolysaccharide (LPS), peptidoglycan (PGN) from bacteria and β-1,3-glucans from fungi (Nyholm and Graf, 2012). These microbial cell wall components trigger downstream immune response that will affect on the clearance of the pathogens. One of the microbial responses in crustaceans is the melanization.

Melanization is a process of the melanin synthesis. The melanin was synthesized from the hydrolysed of monophenols and *o*-diphenols to *o*-quinones which will later form the melanin (Cerenius and Söderhäll, 2004) (Cerenius *et al.*, 2008) (Amparyup *et al.*, 2013). The melanin itself helps quarantine the area of infection to prevent the spreading of the pathogens and may provide anti-fungal property (Nappi and Christensen, 2005, Yassine *et al.*, 2012). There are several by-products from the melanization reaction which are activated by an enzymatic cascade such as 5,6-dihydroxyindole (DHI) that display broad-spectrum antimicrobial activities (Zhao *et al.*, 2007).

In invertebrates, several enzymes involved in melanisation cascade including POs, PPAEs and SPHs have been functionally characterized (Cerenius *et al.*, 2008, Amparyup *et al.*, 2013). Serine proteinase (SP) and serine proteinase homologues (SPHs) are closely similar in sequence except the serine in the catalytic triad in SPs is replaced by glycine residue in SPHs. SPHs have been identified from many insects and crustaceans, for example the freshwater crayfish *Pacifastacus leniusculus Pl*SPH1 and 2, *Scylla paramamosain Sp*-SPH, *Holotrichia diomphalia Hd*PPAFII and *Manduca sexta* SPH-I, SPH-II and SPH-III. In *Penaeus monodon*, three *Pm*MasSPHs have been found in EST database (http://pmonodon.biotec.or.th/) (Tassanakajon *et al.*, 2006). In previous study, *Pm*MasSPH1 possesses the functions of opsonic activity, antimicrobial activity against gram-positive bacteria, and also possesses the binding ability to gram-negative bacterial cell wall component, LPS, and *V. harveyi* (Jitvaropas *et al.*, 2009). In this study, *Pm*MasSPH1 and *Pm*MasSPH2 were further characterized their functions in the shrimp proPO system.

*Pm*MasSPH1 shares significant similarity with *P. leniusculus Pl*SPH2, the SPH that functions in the crayfish proPO activating system (Liu *et al.*, 2011). *Pm*MasSPH2 also shares similarity with *P. leniusculus Pl*SPH1. *Pm*MasSPH1 and *Pm*MasSPH2 show high similarity to *S. paramamosain Sp*-SPH, and insect *H. diomphalia Hd*PPAFII. *Pm*MasSPH1 and *Pm*MasSPH2 also showed conserved catalytic triads to previously identified SPHs. The serine proteinase activity assay in the previous study of *Pm*MasSPH1 showed the replacement of serine caused the loss of their enzymatic activity (Jitvaropas *et al.*, 2009). The data suggested *Pm*MasSPH1 and *Pm*MasSPH2 are the serine proteinase homologues.

From the previous reports of SPHs in other organisms, *Sp*-SPH functions in the proPO activating system and also binds the gram-positive bacteria cell wall component PGN (Zhang *et al.*, 2013). *Hd*PPAFII is required in the activation of proPO in *H. diomphalia* (Lee *et al.*, 1998). The amino acid sequence alignment *Pl*SPH1, *Pl*SPH2, *Sp*-SPH and *Hd*PPAFII revealed the conserved N-terminal clip-domains and the conserved C-terminal serine proteinase-like domains in *Pm*MasSPH1 and *Pm*MasSPH2. Since *Pm*MasSPH1 and *Pm*MasSPH2 share high sequence similarity and

conserved domains which resemble the SPHs involved in the proPO system, both *Pm*MasSPH1 and *Pm*MasSPH2 may also function in the proPO activating system.

The RNAi and co-IP approaches were used in this study to identify functions of *Pm*MasSPH1 and *Pm*MasSPH2. The gene knockdown using double stranded RNA (dsRNA) in RNA interference (RNAi) technique is an effective way to investigate the gene functions. *Pm*MasSPH1 and *Pm*MasSPH2 dsRNA were injected into the shrimps to silenced the genes *in vivo,* then measured the PO activity to evaluate the involvement of the genes and proPO activating system. The dsRNA injection of these two genes was able to silence the expression of the target in hemolymph resulted in a decreasing of the PO activity by 66.5% in *Pm*MasSPH1 and by 63.7% in *Pm*MasSPH2. The decreasing of PO activity in *Pm*MasSPH1 and *Pm*MasSPH2 treatments showed the connection of these genes to the PO activation. In the previous study of *Pl*SPH1 and *Pl*SPH2 in *P. leniusculus*, which shared high similarity to *Pm*MasSPH1 and *Pm*MasSPH2, the silencing of these SPHs reduced PO activity *in vitro* (Liu *et al.*, 2011). *Sp*-SPH from *S. paramamosain* (Zhang *et al.*, 2013) also enhance PO activity as well as SPH from insect *H. diomphalia* (Kwon *et al.*, 2000) *Tenebrio molitor* SPHs (Lee *et al.*, 2002) and *Manduca sexta* SPHs (Yu *et al.*, 2003). A mosquitoe *Anopheles gambiae* CLIPA8 also showed to be involved in the melanization process (Yassine *et al.*, 2012). Thus, *Pm*MasSPH1 and *Pm*MasSPH2 function in the proPO activating system.

Not only the PO activity, but the genes expression in the knockdown shrimps were also investigated. Interestingly, the expression of *Pm*PPAE2, which is a prophenoloxidase-activating enzyme, was decreased in the *Pm*MasSPH1 dsRNA injected shrimps. Since both *Pm*MasSPH1 and *Pm*PPAE2 are the genes in proPO system, the expression change of *Pm*PPAE2 in the *Pm*MasSPH1 injected shrimps suggested that the function of *Pm*MasSPH1 might be involved with *Pm*PPAE2. To investigate the mechanism of *Pm*MasSPHs and *Pm*PPAEs in the activation of proPO, the protein-protein interaction was performed. The co-IP between *Pm*MasSPH1 and *Pm*PPAE2 could detected the bands of both proteins in the western blot membrane, suggesting that *Pm*MasSPH1 could bind to *Pm*PPAE2. Since both proteins are

essential for PO activity and *Pm*MasSPH1 interacted with *Pm*PPAE2, the possible model in this system is that the *Pm*PPAE2 interacts with *Pm*MasSPH1 and activate proPO. From the activation of proPO in some species, the process could occur effectively in the presence of another protein as the coactivator. In *H. diomphalia*, the clip-SPH, (PPAF)-II, functions as the coactivator for the PPAF-I which is the catalytic serine proteinase for the proPO in this species (Kim *et al.*, 2002). In *Bombyx mori*, it was found that the active PO forms the protein complex with various proteins in order to effectively generate melanization (Clark and Strand, 2013). On the other hand, the co-IP results between *Pm*MasSPH2 and *Pm*PPAEs showed that the band which could be detected in the western blot membrane was the *Pm*PPAEs, not *Pm*MasSPH2. This suggested the *Pm*MasSPH2 could not bind the *Pm*PPAEs and the mechanism of *Pm*MasSPH2 is still unknown. Due to the number of serine proteinases and the complexity of the proPO activating system, it is possible that *Pm*MasSPH2 interacts with other SPs in the cascade.

*Pm*MasSPH1 and *Pm*MasSPH2 also involve in *V. harveyi* clearance *in vivo* as the silencing of the genes affected the bacterial numbers in hemolymph to be 13.9-fold higher in *Pm*MasSPH1 and 7.9-fold higher in *Pm*MasSPH2 than in the control treatment. The similar function could be observed in *Pl*proPO which the silencing of this gene affect the PO activity to be decreased as well as increased the bacterial number in hemolymph (Liu *et al.*, 2007). Another report from horseshoe crab Limulus factor D, an SPH-containing protein possesses the direct antimicrobial activity (Kawabata *et al.*, 1996). The silencing of *Pm*PPAE1 and *Pm*PPAE2 also resulted in the increase of the bacterial number in hemolymph (Charoensapsri et al., 2009, Charoensapsri et al., 2011). Therefore, *Pm*MasSPHs are required in the defence system against *V. harveyi*.

In addition, besides the fact that the *Pm*MasSPH1 gene silencing affected the expression of *Pm*MasSPH1, the expression profile of several other immune genes were also changed after the silencing as well. The changes of gene expression of proPO activating system was found in *Pm*PPAE2 (28.6%), and in AMP were found in *Penmon*PEN3 (45.6%), crustin*Pm*1 (69.2%), and Crus-like*Pm* (64.5%). The plausible

explanation would be that suppression of the gene expression of *Pm*MasSPH1 affects the expression of these genes, or the similarity of *Pm*MasSPH1 dsRNA region to the effected genes caused non-specific gene silencing. Therefore, the dsRNA region in *Pm*MasSPH1 was aligned against these genes and no similarity was found between *Pm*MasSPH1 dsRNA and the effected genes in more than 20 nucleotides. The alignments confirmed that the effects were not from the non-specific suppression of dsRNA genes expression, but the depletion of *Pm*MasSPH1 expression that causes the changes. Therefore, *Pm*MasSPH1 showed the cross-talk of proPO activating system and AMP synthesis. The similar phenomenon, that a single gene did not regulate only one pathway, was found in mealworm *T. molitor* SPE which is a regulator of PO and also in the upstream of the AMP synthesis (Kan *et al.*, 2008). Another cross-talk between melanization and Toll activation was also shown in *Drosophila* that the melanin synthesis needs Toll pathway activation and Serpin27A removal (Ligoxygakis *et al.*, 2002). Also, hemolymph proteinase HP6 in *M. sexta* was shown to be able to activate both proPO-activating proteinase (proPAP1) in proPO activating system, and HP8 in the AMP synthesis parthway (An *et al.*, 2009). *M. sexta* SPH-3 was also shown that it is not responsible only in the proPO system, but also relates to the other microbial effectors (Felfoldi *et al.*, 2011).

In the previous study, *Pm*MasSPH1 has been tested for the ability to bind LPS by ELISA and found to be able to bind this Gram-negative bacteria cell wall component (Jitvaropas *et al.*, 2009). This indicates that *Pm*MasSPH1 is the multifunctional protein which involves in the activation of proPO and the pattern recognition protein. The pattern recognition protein, PmLGBP, was found to bind to LPS and β(1-3)-glucan and activate the proPO cascade (Amparyup *et al.*, 2012). While the previous study showed the binding ability of SPHs to PGN (Liu *et al.*, 2011, Zhang *et al.*, 2013). Therefore, we tested the binding of *Pm*MasSPH1 and *Pm*MasSPH2 to PGN. The result showed the binding of both *Pm*MasSPH1 and *Pm*MasSPH2 to PGN which the dissociation constant (K_d) for *Pm*MasSPH1 and *Pm*MasSPH2 are 6.51×10^{-8}M and 5.79×10^{-8}M, respectively, indicated that they can act as PGN binding proteins. The binding activity of SPHs have also been shown in *S. paramamosain* (Zhang *et al.*, 2013). In *M. sexta*, it has been found that SPH binds bacterial surface together with

PAP and proPO. The bacterial binding may function as the anchor to the enzymes as the proPO activation occurred, to limit the area of the melanization reaction to take place at the specific area and to prevent the misplacing of the cytotoxic by products (Yu *et al.*, 2003).

Prophenoloxidases are crucial for the proPO-system as they are the key enzyme that could oxidize the phenolic compound leading to melanin formation (Cerenius *et al.*, 2008, Cerenius *et al.*, 2010, Cerenius *et al.*, 2010). To date, several proPO genes have been found in many species, e.g. *M. sexta, B. mori, H. diomphalia,* shrimp *P. monodon, F. chinensis,* and crayfish *P. leniusculus* (Aspan *et al.*, 1995, Hall *et al.*, 1995, Asano and Ashida, 2001, Kim *et al.*, 2002, Amparyup *et al.*, 2009, Gao *et al.*, 2009). From the previous knowledge, the zymogen of PO, called proPO, is cleaved and the polypeptide at the C-terminus is the functioning part of the enzyme to catalyse phenol. However, the formation of the N-terminal part of the protein has not been reported. Furthermore, the components in this proPO-activating system are synthesized as pro-enzymes and stored in sub-cellular compartment until triggered. This resembles the interleukin (IL)-1β which is synthesized and stored in cytoplasm as the precursor before being released outside the cells. Since the activation of IL-1β is caused by caspase-1 (Denes *et al.*, 2012), several studies had suggested that caspase-1 and inflammasomes involved in several protein secretion (Keller *et al.*, 2008, Nickel and Rabouille, 2009, Sollberger *et al.*, 2013). Two caspase-1 cleavage sites were found in the sequence of crayfish proPO. Therefore, the fragments from caspase-1-cleavaged proPO were also studied for their functions.

Firstly, the bacterial clearance activities were tested *in vivo* and the results was clearly indicated the ability to decrease the bacterial number in crayfish hemolymph. The CFUs of *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 treatment decreased 70.1%, 83.9%, and 75.2%, respectively. These information leads to the hypothesis that these fragments could function as the AMP, and the experiment was conducted by incubation od the peptides and *E. coli in vitro*. The result showed the decrease of CFUs in *Pl*proPO-ppA treatment (67.1%). The decrease of bacterial number after the incubation indicated the bacterial clearance activity was the result

from *Pl*proPO-ppA fragment itself. On the other hand, the functions of another two fragments, *Pl*proPO-casp1 and *Pl*proPO-casp2, are still unknown but may require some other component from the crayfish. Furthermore, the incubation of the bacteria and protein was observed under light microscope to see if there were any changes of the bacteria, and the bacterial agglutination was observed. The agglutination activity was later tested against both Gram-positive and Gram-negative bacteria and the results indicated that *Pl*proPO-ppA peptide also be able to agglutinate the bacterial cell in various concentration (Table 3.1). The viability or the live-death cell assay of the agglutinated bacteria was visualized by the SYTO9 and PI stain. The agglutinated bacteria appeared the most red colour compared to the other treatment, indicated the peptide not only cause the bacterial agglutination, but may also affected the viability of the bacteria. The similar activity for a peptide generated an antimicrobial activity after being cleaved has been observed in hemocyanin where proteolytic cleavage in the plasma produces astacidin 1 (Lee *et al.*, 2004). The results also corresponded to C3 protein the multifunctional protein which plays different function after the proteolysis is C3 protein. In complement system, C3 protein is cleaved into C3a and C3b. C3b later form complex with other complement receptor to promote phagocytosis while C3a is the mediator for the inflammation (Janeway CA Jr, 2001). Another example was shown in human Eosinophil Cationic Protein (ECP) and C-terminal region of human extracellular superoxide dismutase (SOD). The antimicrobial activity of ECP displayed after ECP was incubated with bacteria and cause the bacterial agglutination. SOD also displayed antimicrobial activity against Gram-negative and Gram positive bacteria (Pasupuleti *et al.*, 2009) (Torrent *et al.*, 2012).

The bacteria were also observed the morphology changes that may cause the decrease of bacterial viability by SEM technique. The cell morphology of the *E. coli* that incubate with recombinant *Pl*proPO-ppA was clearly changed compare to the control treatment. The cells clumped together and the distortion of the cell wall was observed. The results indicating the change of bacterial morphology was the results from the peptide. The similar incident was reported in a study of funnel web spider venom that it could alter the morphology of *Shigella sp*, and the cell wall

distorted and formed the longitudinal line at the cell wall of the bacteria (Benli and Yigit, 2008). Although the specific mechanism of this fragment is still unknown, this is the first time to demonstrate the function of the N-terminus of *Pl*proPO that shows the antibacterial activity.

CHAPTER V
CONCLUSIONS

1. *Penaeus monodon Pm*MasSPH1 and *Pm*MasSPH2 were characterized for their functions in shrimp innate immune response.

    1.1 *Pm*MasSPH2 sequence was analyzed. The sequence contains 1164 bp of ORF which could be translated to a 387 amino acid protein. The calculated molecular mass for the mature protein is 39.37 kDa with the pI prediction of 7.52. The amino acid sequences alignment indicated the conserved domain of N-terminal clip-domain and C-terminal serine proteinase-like domain The catalytic triad of His, Asp and Gly suggests that *Pm*MasSPH2 is a non-catalytic proteinase homologue.

    1.2 Gene silencing of *Pm*MasSPH1 and *Pm*MasSPH2 resulted in a decrease in hemolymph PO activity, an increased in bacterial number and suppression of gene expression of *Pm*PPAE2, *Penmon*PEN3, crustin*Pm*1, and Crus-like*Pm*.

    1.3 The protein-protein interaction by co-IP suggests the binding of *Pm*MasSPH1 with *Pm*PPAE2.

    1.4 *Pm*MasSPH1 and *Pm*MasSPH2 could bind the Gram-positive bacterial cell wall component PGN.

2. *Pacifastacus leniusculus* proPO fragments including *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 were functional characterized.

    2.1 *Pl*proPO-ppA, *Pl*proPO-casp1 and *Pl*proPO-casp2 showed the bacterial clearance activity *in vivo* at 3 h. post bacterial and peptide injection. *In vitro* study showed only *Pl*proPO-ppA decreased the bacterial number in hemolymph.

    2.2 The incubation of *Pl*proPO-ppA showed the sign of bacterial agglutination. The bacterial viability assay indicated the agglutinated bacteria contained both live and dead bacteria in the cluster. The bacterial morphology in the *Pl*proPO-ppA treatment was disrupted.

    2.3 *Pl*proPO-ppA caused the agglutination against *S. aureus, B. subtilis, M. luteus, A. hydrophila,* and *P. aeruginosa*.

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APPENDIX

The sequence alignment of *Pm*MasSPH1 dsRNA

The sequence region of *Pm*MasSPH1 dsRNA, which was used to synthesized dsRNA in the experiment, was aligned against the 4 genes (*Pm*PPAE2, *Penmon*PEN3, crustin*Pm*1, and Crus-like*Pm*) that showed the sign of the decreasing expression.

1. The sequence alignment of *Pm*PPAE2 and the region in *Pm*MasSPH1 dsRNA. The asterisks indicate the similar nucleotide between the two sequences.

```
PmPPAE2              CGGCGGCGGTCACGCTCCTTGTTCTTGTGACATCTGGAGGGGCGACGAGAGATCGAAGGC
PmMasSPH1_dsRNA      -------------------------TTATAAC--------GGACGGCG-------------
                                             ** * **         ** ** **

PmPPAE2              AAGCCAGGTGCAGTGCCGGGGCACCATGCGTCCTCGTCGACTCCTGCCCCCCTGTGAAAG
PmMasSPH1_dsRNA      ----CAGG------------------------CCTCATTGAT--------------------
                        ****                         **** * **

PmPPAE2              CGCTCTTCCTGTCTCCCAACGCAGGCGACAAGCATAGGGCCCAGCAACTGATTTGTGGAA
PmMasSPH1_dsRNA      ---------------------------------ATCAGGTTTGGCAACAGC-------AA
                                                       **  **    ***** *        **

PmPPAE2              GAGAGGGCAGACGTCTTAAGGTATGTTGTGGCTCCTCAAACGTGACGCCAACGCCAAGAC
PmMasSPH1_dsRNA      GAAA---------------------------------TCTAAC--GACACCAGCACCA----
                     ** *                               ** ***  *** *** * ***

PmPPAE2              CCATAGATGTAACTCCCACCAGCAACCCCGGGGGGAACGGGAACGGACAGCTATTGCCCT
PmMasSPH1_dsRNA      -----GATCCAGTTCCGACTG-----CCCG-----------------CAGTT-----CCT
                        ***  *  *** **      ****                  *** *     ***

PmPPAE2              CAAACTGCGGACAGACCTCTAACTTGAATAAAATATTCGGTGGCGAAGCTACTGGTGTTG
PmMasSPH1_dsRNA      C------------GACGTCTG--CTGCACCAA----TC-----CGAA-------------
                     *            *** ***    ** *  **     **     ****

PmPPAE2              GCGAATTTCCTTGGATGGCTGTTTTGGGGTATAACAGTGGCTCTCTGGACTGGGAGTGTG
PmMasSPH1_dsRNA      ----------------------------------------CCCTCCGGAC-------GTG
                                                            * *** ****       ***

PmPPAE2              GAGGAGCTCTCATCAACGACCGTTACGTCCTGACAGCTGCTCACTGCGGCGATCCAGATT
PmMasSPH1_dsRNA      G------TCACGCC--CGCCCCCTACACGC---------CCCGCTGCGGCAAGAGGAACT
                     *     ** *  *  ** **  ***   *         * * ******* *     * *

PmPPAE2              TTCTGTTTGGCTCTATTCTGACTGCAATCCGTCTCGGCGAATACGACTTCTCCAAGAGCA
PmMasSPH1_dsRNA      CGCAA---GGCTTC-----GAC----GTCCGCATCACTGGAT---------TCAAGGATA
                       *     ****       ***     ****  **   * **          ****   *

PmPPAE2              AGGACTGCAACTCAGCT-GCAGACTTCTGCTTGCCCCCTGTGCAAGACTTCACGCCTGAG
PmMasSPH1_dsRNA      ACGA----GGCCCAGTTCGCGGAGTTC--------CCCTG-----GA---------TGA-
                     * **      * *** * ** ** ***        *****     **         ***

PmPPAE2              CAAGTCGTCCTCCATCCTTCCTTCAACAAGCGTGCCCCCGAGAGTGACGATATTGCGCTC
PmMasSPH1_dsRNA      CAG--------CCATCTTGC---------GCGTG-----GAGAAAGTCGGCA--------
                     **          ***** * *       *****     ****  * **  *

PmPPAE2              ATCAGACTGAACAGGAGGGTGCAACTGAACGCTGGCGTACACCCGATCTGCCTCCCTGCC
PmMasSPH1_dsRNA      ------------AGAAGG----AGCTGAACCTTTACGTGT--------------------
                                 ** ***   * ******  *  ***

PmPPAE2              GCTGGCTTAAACGTCGGCTCCTTCCTTAACGGCAGAGACGCTATCGTGATTGGCTGGGGA
PmMasSPH1_dsRNA      ----------GCGGCGGCTCCCTCATT---------------------------------
                               ** ******* ** **

PmPPAE2              CATACAGAGAGGGGCACAAATACCCAGGTGCTGCAGAAAGTCTCGCTGCCTTTCGTTGAT
PmMasSPH1_dsRNA      CAT-----------------------------------------------CCATCCATCGTT
                     ***                                               ** * * * * *

PmPPAE2              CTCGGCACCTGCAAAAGAATTCACGCAGGAGAAACACTGGTTAACGAACAGGTGTGTTTC
PmMasSPH1_dsRNA      CTCA-CAGCTGC--------TCAC----------------------------TGCGTTC-
                     ***  ** ****        ****                            ** ***

PmPPAE2              GGCGGAAGGGCGGGACAAGACTCATGCAATGGTGACTCCGGTGGCCCTCTCTTCTTGAAC
PmMasSPH1_dsRNA      -ACTCAAAGGCTG--CAAG-CTCACTCAA-------------GACCCGCT----------
                       *  ** *** *  **** ****  ***             * *** **
```

```
PmPPAE2              GCTGTCCCTGGCACCATCCTGGGCATCGTGTCGAAGGGCGGGGCGTGTGGGAGTCCCGGC
PmMasSPH1_dsRNA      ------------------------------TCGGAGAGTGGGACACCCAGAAG-----AC
                                                   *** ** * *** *     * **      *
PmPPAE2              GTGCCTGCGATCTACACCGACGTCGCCTCCTACAGGGGCTGGATCGTGCAGAATCTCAAA
PmMasSPH1_dsRNA      GTACGAGCGGT----ACC----------CTCACCAGGACAGGAACGT-------------
                     ** *  *** *    ***          *  **  ** * *** ***
PmPPAE2              CCTTAGGGCCAAAGTCGCTTATTTTTTCTACGGCCTGTGGTGTAGGCGACTTACAAAGCT
PmMasSPH1_dsRNA      CATCAGCGTGAAAATC---CATC----------------------CGAATTACAA----
                     * * ** *  *** **    **                       *** ******
PmPPAE2              TCTTTTCATTGTTGATTTATTGTTGATGTTAAATGTTGGATTTTTTGTATTATTATTTTA
PmMasSPH1_dsRNA      ------------------------------------------------------------

PmPPAE2              TTTTATTGTTCTCGAGTTTAAACAGCTACAGGAGATTAGTTGAACATGTCTTTTGTTATT
PmMasSPH1_dsRNA      CTCTGGTGCTCTCTA-------CAACGAC----------------------TTCGCTCTC
                      * *  ** **** *       ** * **                      ** * * *
PmPPAE2              CTTTTATTTCATTTATTGTTATCTGTATGATACTTGAAGTGTCCGAATGAAAGAGCTTTA
PmMasSPH1_dsRNA      CTCTTCCTT--------------------GACA--------GTCCCGCTACACTGGCCCC-
                     ** **  **                   ** *        ****   *  *   **
PmPPAE2              TATAATGTGGGCAATGAAATGCATAGAAACAACTGTTATCTTGTGATTGATGTCTTTTGA
PmMasSPH1_dsRNA      --CAACGTGGACA--------------------CCGTC-------------TGCCTCC---
                        ** **** **                 * **              ** **
PmPPAE2              TCAATTTATCGTAATCAGGCCTATCTAGTTATCTGTTTACTTATCAATTTTCAGACGTTT
PmMasSPH1_dsRNA      ---------CGCAAGCAAACC-----AGAAATTCGACTAC-------------GACACCT
                              ** ** **  **     **  **  *  ***             ***   *
PmPPAE2              TAAGCATCCATGTTTTTTTTTCTCCAGAAATGTAATCTAAATAAAAAACAGATAGTTTAA
PmMasSPH1_dsRNA      ------------------------------------------------GCTGG-----
                                                                     * * *
PmPPAE2              AAAAAAAAAAAAAAAAAAA
PmMasSPH1_dsRNA      -------------------
```

2. The sequence alignment of *Penmon*PEN3 and the region in *Pm*MasSPH1 dsRNA. The asterisks indicate the similar nucleotide between the two sequences.

```
PenmonPEN3          CCCCACCTGTAGAGGCCGAGACTCCTTGCC--CGGGTTCCTTCCTGTGTCCGCCATGCGT
PmMasSPH1_dsRNA     TTATAACGG-ACGGCGCAGGCCTCATTGATATCAGGTTTG-----GCAACAGCAAGAAAT
                        * * * *  *  *  * *** ***    * ****       *   * ** *    *

PenmonPEN3          CTCGTGGTCT----GCCTGGTC---TTCC---TGGCC----TCCTTCGCC--CTGGTCTG
PmMasSPH1_dsRNA     CTAACGACACCAGCACCAGATCCAGTTCCGACTGCCCGCAGTTCCTCGACGTCTGCTGCA
                    **   *         ** * **   ****   ** **    * * *** *  *** *

PenmonPEN3          CCAAGCCCAAGGGTACCAGGGTGGTTACACACGCCCGTTCCCCAGACCACCCTATGG-GG
PmMasSPH1_dsRNA     CCAATCCGAACCCTCCGGACGTGGTCACGCCCGCCC--CCTACACGCCCCGCTGCGGCAA
                    **** ** **   * *    ***** ** * *****   *  **  ** * **  **

PenmonPEN3          GAGGATATCATCCAGTTCC--TGTTTGCACTTCATGCCACAGGCTTAGCCCCTTACAAGC
PmMasSPH1_dsRNA     GAGGAACTCGCAAGGCTTCGACGTCCGCAT-------CACTGGATTCAAGGATAACGAGG
                    *****  **     * * *   **  ***        *** ** **      * ** **

PenmonPEN3          TCGTGCTTGCTGCAGGCAGTTAGGACGTTGTTGTGATGCAAAGCAGACATATGGTTGATG
PmMasSPH1_dsRNA     CCCAGTTCGCGG-----AGTT------CCCCTG-GATG----ACAGCCATCTTGCGCGTG
                     *  * * ** *     ****          ** ****     *** *** * *    **

PenmonPEN3          GAGAA----GACAACGAAAAACTGA--CTTCACAATGTATTAATCAGTTGTGAAGAAAGT
PmMasSPH1_dsRNA     GAGAAAGTCGGCAAGAAGGAGCTGAACCTTTAC---GTGTGCGGCGGCT-----------
                    *****    * ***  *  * ****  *** **   ** *    * * *

PenmonPEN3          GCAACCCTGATTTTGAACTGTATTTTCTAGTTCCATTTTCTTACTTTTGCTTGTGGAAAG
PmMasSPH1_dsRNA     ----CCCTCATTC--ATCCATCCATCGTTCTCACAGCTGCTCACTGC-GTTCACTCAAAG
                        **** ***   * *  *   *  * *  * **  * ** ***   * *     ****

PenmonPEN3          GATGTAGGT--ATTTGGATTTTCCAT--GAATGTATGATGAATGAAAGTGCATGTGGGAT
PmMasSPH1_dsRNA     GCTGCAAGCTCACTCAAGACCCGCTTCGGAGAGTGGGACACCCAGAAG-ACGTACGAGCG
                    * ** * *   * *         * *  **  **  **       ***  * *  * *

PenmonPEN3          GTATGTGCATACAGTCGTATTTGTC-CCAGC----AGGTCCTCGTGTATT----CACAGG
PmMasSPH1_dsRNA     GTACCCTCACCAGGACAGGAACGTCATCAGCGTGAAAATCCATCCGAATTACAACTCTGG
                    ***    **    * *      ***  ****    *  ***    * ***    * * **

PenmonPEN3          AG---------AAAGATATCGTGTTGTTTGACTTTCGTTGTAGTTATTTGTAGGTATGGGT
PmMasSPH1_dsRNA     TGCTCTCTACAACGACTTCGCTCTCCT---CTTCCTTGACAGTC--CCGCTACACTGGCC
                     *        ** **  ***   *  *   *** * *   ***     *      ***

PenmonPEN3          CTGTGTGTGGTTGGTGTTTGCATATTTCCCAAAGGACATTCGGAATTGTACTACTCTTTT
PmMasSPH1_dsRNA     CCCAACGTGGACACCGTCTGC---CTCCCGCAAGCAAACCAGAAATTCGACTAC------
                    *     ****     ** ***    * **  *** * *   * ****  *****

PenmonPEN3          ACAAATAAAATTGATATCTG----
PmMasSPH1_dsRNA     ------------GACACCTGCTGG
                               ** * ***
```

3. The sequence alignment of crustin*Pm*1 and the region in *Pm*MasSPH1 dsRNA. The asterisks indicate the similar nucleotide between the two sequences.

```
crustinPm1          CTGGAGGCGACCATGAAGGGTCTCGGAGTCATTCTGTTCTGCGTC--CTGGCGAT-----
PmMasSPH1_dsRNA     TTATAACGGACGGCGCAGG-CCTCATTGATATCAGGTTTGGCAACAGCAAGAAATCTAAC
                     *  *   ***   * ***  ***   *  **   ***  **  *  *  *  **

crustinPm1          GGCATCAGC-CCAGAGTTGGCACGGAGGTCGACCCGGAGGCTTCCCTGGTG---GAGGTA
PmMasSPH1_dsRNA     GACACCAGCACCAGATCCAGTTCCGA---CTGCCCGCAG--TTCCTCGACGTCTGCTGCA
                    * ** **** *****    *  * **   *  **** **  ****  *  *   *  * *

crustinPm1          --GACCCGGAGGCTTC---CCTGGTGGAGGTAGACCCGGAGGTAGACCCG---------G
PmMasSPH1_dsRNA     CCAATCCGAACCCTCCGGACGTGGTCACGCCCGCCCCCTACAC-GCCCCGCTGCGGCAAG
                       * *** *  ** *   * ****   *   * ***  *    * ****         *

crustinPm1          AGG--CTTCCCAAGCGTCACAGCCC----CACCCGCCTCCTGTA------GGCGCTGGT-
PmMasSPH1_dsRNA     AGGAACTCGCAAGGCTTCGACGTCCGCATCACTGGATTCAAGGATAACGAGGCCCAGTTC
                    ***  **  * * ** **   * **    ***  *  **  * *      *** * * *

crustinPm1          GCGAAACTCCAGAGAATG-----CTTTTTAC---TGCTGCGAGTCA--AGGTATGAACCC
PmMasSPH1_dsRNA     GCGGAGTTCCCCTGGATGACAGCCATCTTGCGCGTGGAGAAAGTCGGCAAGAAGGAGCTG
                    *** *  ***   * ***     * * ** *   **  *  ****   * * * ** *

crustinPm1          GAGGCACCCGTGGGCACCAAGATACTTGACTGCCCA---AAAGTCCGTGACACCTGCCCA
PmMasSPH1_dsRNA     AACCTTTACGTGTGCGGCGGCTCCCTCATTCATCCATCCATCGTTC-TCACAGCTGCTCA
                     *     **** **  *     **       ***   *  ** * * *** **** **

crustinPm1          CCGGTACGTTTTCTTGCAGT-----AGAGCAGCCAGTACCTTGCTCCAGTGACTACAA-G
PmMasSPH1_dsRNA     C---TGCGTTCACTCAAAGGCTGCAAGCTCACTCAAGACCC-GCTTCGGAGAGTGGGACA
                    *   * ****  **   **      **  **  **  ***  *** * * ** *   *

crustinPm1          TGCGGCGGCCTTGACAAGTGCTGCTTCGACAGGTGTTTGGGACAACAC--GTGTGCAAGC
PmMasSPH1_dsRNA     CCCAGAAGACGT-ACGAGCGGTACCCTCACCAG-GACAGGAACGTCATCAGCGTGAAAAT
                      * *  * * * ** ** * * *    **  * *   ** **  **   * *** **

crustinPm1          CACCTTCCTTCTATGAATTTTTTGC-CTGAAAAGGAAATTTGGAAG-----TAATTACCG
PmMasSPH1_dsRNA     C-CATCCGAATTACAACTCTGGTGCTCTCTACAACGACTTCGCTCTCCTCTTCCTTGACA
                    * * * *    **  * * *  *** **  * *   * ** *         *  **  *

crustinPm1          ATCCATGTAGAATCTGTGACTAATAAAG--------TGTTTTTCAGAGAAAAAAAAAAAA
PmMasSPH1_dsRNA     GTCC-CGCTACACTGGCCCCCAACGTGGACACCGTCTGCCTCCCGCAAGCAAACCAGAAA
                     ***  *    *   *   * **    *        **  *  *  *   ***  * ***

crustinPm1          -------AAAA----------
PmMasSPH1_dsRNA     TTCGACTACGACACCTGCTGG
                           *  *
```

4. The sequence alignment of crus-like*Pm* and the region in *Pm*MasSPH1 dsRNA. The asterisks indicate the similar nucleotide between the two sequences.

```
crus-like Pm          ------------------AGTC-CGTTCATCGCACA---GCCGAGAG-AGGAACTGTCAAG
PmMasSPH1_dsRNA       TTATAACGGACGGCGCAGGCCTCATTGATATCAGGTTTGGCAACAGCAAGAAATCTAACG
                                        * * * ** **  **     * * * ** * *** * * * *

crus-like Pm          ATGCTAAAGTTTG---TAGT----ATTATCCGTTGTCGCT--GTGGCTGTGGTAC-----
PmMasSPH1_dsRNA       ACACCAGCACCAGATCCAGTTCCGACTGCCCGCAGTTCCTCGACGTCTGCTGCACCAATC
                      *  * *      *    ***    * *  ***  **  **    * ***  * **

crus-like Pm          ---ACGCGCAGGATAAAGGCAATGCCGA----TACTCGCTTC-------CTAGGTGGAGT
PmMasSPH1_dsRNA       CGAACCCTCCGGACGTGGTCACGCCCGCCCCCTACACGCCCCGCTGCGGCAAGAGGAACT
                         ** * * ***    * **   ***     *** ***  *       * **  * * *

crus-like Pm          TGGAGTTCCTGGAGGTG-GAGTTCCTGGAGTTGG--------------AGTTCCTGGAGT
PmMasSPH1_dsRNA       CGCAAGGCTTCGACGTCCGCATCACTGGATTCAAGGATAACGAGGCCCAGTTCGCGGAGT
                       * *   * * ** **  *  *  ***** *                 *****  *****

crus-like Pm          T-----GGA-GGTGGAT-TCCTGC-CG-GGGGTTCCTGGGCATGGTGGCGTTGTTCCT--
PmMasSPH1_dsRNA       TCCCCTGGATGACAGCCATCTTGCGCGTGGAGAAAGTCGGCAAGAAGGAGCTGAACCTTT
                      *     *** *   *   ** *** ** ** *    * **** *  ** * **  ***

crus-like Pm          --GGAGGCGGTGGCCTTCTCCCTGGAGGTCAATTCGAGTGCAATTACTGCAGAACGAGGT
PmMasSPH1_dsRNA       ACGTGTGCGGCGGCTCCCTCATTCA---TCCATCC-ATCGTTCTCACAGCTGCTCACTGC
                        *   **** ***   ***  *     ** ** * *  *   * ** ** *  *   *

crus-like Pm          ACGGATACGTATGCTGCAAGCCCGGCAGGTGTCCACAGATTCGCGATACCTGCCCAGGCC
PmMasSPH1_dsRNA       GTTCACTCAAAGGCTGCAAGCTCACTCAAGACCCGC---TTCG-GAGAGTGGGACA---C
                          *  *  * ********* *        ** *   **** ** *   *  **   *

crus-like Pm          TCAGAAAGGGTGTCC-CGAT-CTGCCGTCAGGACACTGACTGCTTCGGC-------TCCG
PmMasSPH1_dsRNA       CCAGAAGACGTACGAGCGGTACCCTCACCAGGACAGGAACGTCATCAGCGTGAAAATCCA
                       *****   **     ** * *   *  *******   **  * ** **       ***

crus-like Pm          AC--AAATGCTGCTTCGACACCTGCTTGAACGACACCG-TCTGCAAACCCATCGTGGCAG
PmMasSPH1_dsRNA       TCCGAATTACAACTCTGGTGCTCTCTACAACGACTTCGCTCTCC---TCTTCCTTGACAG
                       *  ** * *  **  *   *   **  ******  ** *** *    *   * ** ***

crus-like Pm          GTTCTCAGGGATAGGCCTGCATGTTCGAC-TCATCAATTCTTTTTTATCAAAT---AAAT
PmMasSPH1_dsRNA       -TCCCGCTACACTGGCCCCCAACGTGGACACCGTCTGCCTCCCGCAAGCAAACCAGAAAT
                       * *      *  ****  **   * ***  * **           * ****    ****

crus-like Pm          GCTATAGCTGTTAATTGTTAAAAAAAAAAAAAA
PmMasSPH1_dsRNA       TCGACTAC-GACACCTGCTGG------------
                       * *   * *  *  ** *
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VITA

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