ลักษณะสมบัติและการวิเคราะห์หน้าที่ของยีนที่เกี่ยวข้องกับระบบโพรฟีนอลออกซิเดส จากกุ้งกุลาดำ *Penaeus monodon*

นางสาววลัยพร เจริญทรัพย์ศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF PROPHENOLOXIDASE SYSTEM-ASSOCIATED GENES FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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วลัยพร เจริญทรัพย์ศรี : ลักษณะสมบัติและการวิเคราะห์หน้าที่ของยีนที่เกี่ยวข้องกับระบบโพรฟีนอลออกซิเดสจากกุ้งกุลาดำ *Penaeus monodon* (CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF PROPHENOLOXIDASE SYSTEM-ASSOCIATED GENES FROM THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร. Kenneth Söderhäll, ดร.ปิติ อ่ำพายัพ, 114 หน้า.

ระบบโพรฟีนอลออกซิเดส (proPO system) เป็นหนึ่งในระบบภูมิคุ้มกันหลักที่ทำหน้าที่ต่อต้านการรุกรานจากเชื้อก่อโรคในสิ่งมีชีวิตที่ไม่มีกระดูกสันหลัง งานวิจัยนี้ได้พบยีนโพรฟีนอลออกซิเดสแอคติเวติงเอนไซม์ (PPAE) จำนวน 2 ชนิด (*Pm*PPAE1 และ *Pm*PPAE2) และยีนโพรฟีนอลออกซิเดสชนิดใหม่จำนวน 1 ชนิด (*Pm*proPO2) จากเซลล์เม็ดเลือดของกุ้งกุลาดำ *Penaeus monodon* จากการวิเคราะห์ลำดับกรดอะมิโนพบว่ายีน *Pm*PPAE1 และ *Pm*PPAE2 มีความคล้ายกับยีน PPAE ในกุ้ง *Pacifastacus leniusculus* และยีน PAP1 ในแมลง *Manduca sexta* 70% และ 51% ตามลำดับ ขณะที่ยีน *Pm*proPO2 มีความเหมือนกับยีน *Pm*proPO1 67% เมื่อศึกษาการแสดงออกของยีนเหล่านี้ในเนื้อเยื่อต่าง ๆ ของกุ้งกุลาดำพบว่ามีการแสดงออกมากในเซลล์เม็ดเลือด และพบว่ายีน *Pm*PPAE1 และ *Pm*proPO2 มีการแสดงออกในตัวอ่อนทุกระยะ (นอเพลียส, โพรโตซูเอีย, ไมซิส และโพสลาวา) ในขณะที่ยีน *Pm*PPAE2 และ *Pm*proPO1 มีการแสดงมากเฉพาะในช่วงปลายของการเจริญของตัวอ่อนของกุ้ง (ไมซิส และโพสลาวา)

จากการใช้เทคนิค RNA interference (RNAi) ลดการแสดงออกของยีน *Pm*PPAE1 และ *Pm*PPAE2 พบว่า dsRNA ของยีน *Pm*PPAE1 และ *Pm*PPAE2 สามารถลดการแสดงออกของยีนในเซลล์เม็ดเลือดกุ้งได้อย่างจำเพาะ และพบว่ากุ้งที่ถูกลดการแสดงออกของยีนมีค่าแอกทิวิตีของเอนไซม์ฟีนอลออกซิเดสลดต่ำลงอย่างมีนัยสำคัญ (37% และ 41% ตามลำดับ) เมื่อเปรียบเทียบกับกุ้งควบคุมที่ฉีดด้วยน้ำเกลือ นอกจากนี้ยังพบว่าภายหลังจากการฉีดด้วยเชื้อก่อโรค *Vibrio harveyi* กุ้งที่ถูกลดการแสดงออกของยีน *Pm*PPAE1 และ *Pm*PPAE2 มีอัตราการตายเพิ่มสูงขึ้นและมีจำนวนเชื้อแบคทีเรียเพิ่มมากขึ้นอย่างมีนัยสำคัญ แสดงให้เห็นว่า *Pm*PPAE1 และ *Pm*PPAE2 เป็นองค์ประกอบที่มีความสำคัญต่อระบบโพรฟีนอลออกซิเดสและยังมีบทบาทสำคัญในการต้านทานการรุกรานจากเชื้อก่อโรค *V. harveyi* เมื่อทำการลดการแสดงออกของยีน *Pm*proPO1 และ *Pm*proPO2 โดยใช้เทคนิค RNAi พบว่ากุ้งที่ถูกยับยั้งยีนมีค่าแอกทิวิตีของเอนไซม์ฟีนอลออกซิเดสลดต่ำลงอย่างมีนัยสำคัญ (75% และ 73% ตามลำดับ) เมื่อเปรียบเทียบกับกุ้งควบคุมที่ถูกฉีดด้วยน้ำเกลือ นอกจากนี้ยังพบว่ากุ้งมีอัตราการตายเพิ่มสูงขึ้นภายหลังจากการฉีดด้วยเชื้อ *V. harveyi* และมีจำนวนเชื้อแบคทีเรียในน้ำเลือดเพิ่มมากขึ้นอย่างมีนัยสำคัญ แสดงให้เห็นว่ายีน *Pm*proPO1 และ *Pm*proPO2 เป็นองค์ประกอบที่มีความสำคัญต่อระบบภูมิคุ้มกันของกุ้งในการต่อต้านการรุกรานจากเชื้อก่อโรค *V. harveyi*

จากการผลิตโปรตีนรีคอมบิแนนท์ของ *Pm*PPAE1 ในระบบการแสดงออกของ *Escherichia coli* และเซลล์แมลง พบว่าโปรตีนรีคอมบิแนนท์ที่ผลิตได้จากทั้งสองระบบไม่เสถียร จึงได้ทำการผลิตโปรตีนรีคอมบิแนนท์เฉพาะส่วนซีรีนโพรติเนสโดเมนของ *Pm*PPAE1 หลังจากนำโปรตีนรีคอมบิแนนท์ไปสร้างแอนติบอดีเพื่อติดตามการแสดงของ *Pm*PPAE1 ในน้ำเลือดของกุ้งด้วยวิธี western blot พบว่าโปรตีน *Pm*PPAE1 มีการแสดงออกเฉพาะในส่วนเม็ดเลือดของกุ้งเท่านั้น

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....วลัยพร เจริญทรัพย์ศรี.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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WALAIPORN CHAROENSAPSRI : CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF PROPHENOLOXIDASE SYSTEM-ASSOCIATED GENES FROM THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: PROF. KENNETH SÖDERHÄLL, Ph.D., PITI AMPARYUP, Ph.D., 114 pp.

The prophenoloxidase (proPO) activating system plays an essential role in the immune defence against microbial infections in many invertebrates. In the present study, two PPAE genes (designated *Pm*PPAE1 and *Pm*PPAE2) and a novel proPO (*Pm*proPO2) gene were identified from hemocytes of the black tiger shrimp, *Penaeus monodon*. Sequence analysis revealed that *Pm*PPAE1 exhibited the highest amino acid sequence similarity of 70% to a PPAE of the freshwater crayfish *Pacifastacus leniusculus*, whilst *Pm*PPAE2 showed 51% amino acid sequence similarity to the insect *Manduca sexta* PAP1. Analysis of the *Pm*proPO2 sequences showed that *Pm*proPO2 shared a 67% sequence identity to the *Pm*proPO1. Tissue expression analysis revealed that *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 mRNA transcripts were mainly expressed in hemocytes. Analysis of their larval developmental gene expression revealed that *Pm*PPAE1 and *Pm*proPO2 transcripts were expressed in all larval stages (nauplius, protozoea, mysis and post-larvae), whereas *Pm*PPAE2 and *Pm*proPO1 transcripts were mainly expressed in the late stages of larval development (mysis and post-larvae).

Double-stranded RNA(dsRNA)-mediated gene suppression of *Pm*PPAE1and *Pm*PPAE2 resulted in a significant reduction in the respective transcript levels and resulted in a significant decreased in total PO activity in the *Pm*PPAE1 (37%) and *Pm*PPAE2 (41%) knockdown shrimps as compared with saline injected group. Experimental infection of *Pm*PPAE knockdown shrimps with the highly pathogenic bacterium *Vibrio harveyi* significantly increased the cumulative mortality and the number of bacterial colonies in the silenced shrimps. These results indicate that *Pm*PPAE1 and *Pm*PPAE2 participate in the proPO system and also play an important role in the shrimp immune defence against *V. harveyi* infection. Gene silencing of *Pm*proPO1 and *Pm*proPO2 by RNA interference (RNAi) resulted in a significant decrease in the respective endogenous proPO mRNA levels in hemocytes and a reduction of total PO activity by 75 and 73%, respectively. Experimental infection of *P. monodon* with the *V. harveyi* revealed that the *Pm*proPO silenced shrimps were more susceptible to bacterial infection than the control GFP dsRNA and saline injected shrimps, suggesting that the two proPOs are important components in the shrimp immune system and play the crucial role in the defence against *V. harveyi* infection.

In addition, the mature protein of *Pm*PPAE1 was expressed in *Escherichia coli* and insect cell expression system, but the obtained recombinant proteins were not stable. The SP domain of *Pm*PPAE1 was expressed in *E. coli* system. The recombinant SP domain of *Pm*PPAE1 was used as an immunogen to generate the antibody against SP domain of *Pm*PPAE1 and western blot analysis demonstrated that the endogenous *Pm*PPAE1 was found only in hemocytes but not in cell-free plasma of *P. monodon*.

Department : .....Biochemistry..... Student's Signature Walaiporn Charoensapsri

Field of Study : ...Biochemistry..... Advisor's Signature

Academic Year : ..... 2010 ..... Co-advisor's Signature

Co-advisor's Signature Piti Amparyup.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
bp	base pair
CAC	cacodylate
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
clip-SPs	clip-domain serine proteinases
clip-SPHs	clip-domain serine proteinase homologues
C-terminal	carboxyl-terminal
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double stranded ribonucleic acid
dTTP	deoxythymidine triphosphate
EF1-α	elongation factor 1-α
EST	expressed sequence taq
GFP	green fluorescence protein
HLS	hemocyte lysate supernatant
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
M	molar
mg	milligram

ml	millilitre
mM	millimolar
MOI	multiplicity of infection
N-terminal	amino-terminal
°C	degree Celsius
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming unit
PGN	peptidoglycan
*Pm*PPAE1	*Penaeus monodon* prophenoloxidase-activating enzyme 1
*Pm*PPAE2	*Penaeus monodon* prophenoloxidase-activating enzyme 2
*Pm*proPO1	*Penaeus monodon* prophenoloxidase 1
*Pm*proPO2	*Penaeus monodon* prophenoloxidase 1
PO	phenoloxidase
PPAE	prophenoloxidase-activating enzyme
proPO	prophenoloxidase
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
r*Pm*PPAE1	recombinant *Penaeus monodon* PPAE1 protein
rSP-domain	recombinant serine proteinase domain of *Penaeus monodon* PPAE1 protein
RT	reverse transcription
SDS	sodium dodecyl sulfate
UTR	untranslated region
μg	microgram
μl	microlitre

CHAPTER I

INTRODUCTION

1.1 General introduction

The black tiger shrimp *Penaeus monodon* is one of the most economically important crustacean species in Thailand and Southeast Asian countries. In the last two decades, the shrimp industry has grown rapidly and generated billions of dollars a year in trade and employed over a million people globally. However, during the past few years, annual production of *P. monodon* farmed in Thailand was dropped owing to several relentless outbreaks of infectious diseases especially those caused by white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio harveyi* (Lightner and Redman, 1998; Tanticharoen et al., 2008). These problems have eventually lead the commercial shrimp farming to decline and resulted in a replacement of the native *P. monodon* shrimp with the Pacific white shrimp *Litopenaeus vannamei*. To prevent the spread of these disease causing pathogens and promote *P. monodon* farming to be intensive again, basic knowledge of shrimp immunity is therefore necessary to assess the new efficient strategies for microbial diseases control and further develop for sustainable shrimp aquaculture.

1.2 Shrimp larval development and life cycle

Shrimps undergo several morphologically different stages during their development (Figure 1.1). Shrimp larval development consists of six nauplius (N1-N6), three protozoea (Z1-Z3) and three mysis (M1-M3) larval stages, followed by the development of post-larvae (PL) to adult (Ronquillo et al., 2006; Türkmen, 2005). The larval development begins with a larvae hatching from the fertilized egg to the first stage known as nauplius with unsegmented spider-like body possessing three pairs of appendages. After undergoing six molting stages, nauplius metamorphoses

into the stage of protozoea. At this stage the body has feathery appendages and it is elongated with a distinct cephalothorax. The early protozoea stage has a pair of protruded compound eyes and a rostrum is present in the next stage and it has a pair of uropods in the late stage of protozoea. Three or five days later, protozoea metamorphoses into the third and final larval stage called mysis. At this stage, the morphology of the larvae is similar to a juvenile shrimp in which segmented bodies, eyestalks and pleopods have developed. Mysis lasts another three or four days, and then they metamorphose into post-larvae (PL). At the post-larval stage, they look like adult shrimps and the pleopods are fully developed and functional. All these larval metamorphosis steps are normally developed within eight or nine days (Solis, 1988). After these stages, the post-larvae will develop into juvenile and sub-adult shrimps before being develop into the mature shrimps after migrate back to the sea.

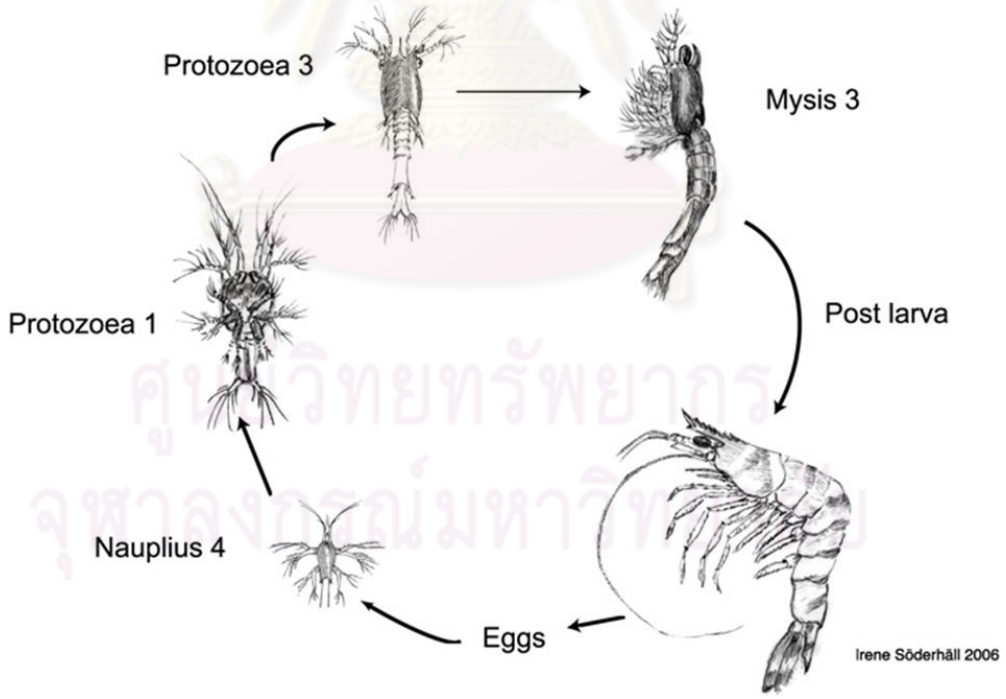

Figure 1.1 Larval development of Penaeid shrimps (Jiravanichpaisal et al., 2007).

1.3 Shrimp immune system

Like other crustaceans and invertebrates, shrimps lack an adaptive immune system; instead they solely rely on the well-developed innate immunity. Invertebrate innate immune system is composed of two main components, the cellular and humoral defence responses, and both of which are activated upon microbial infections (Gillespie et al., 1997; Jiravanichpaisal et al., 2006; Kanost et al., 2004; Lavine and Strand, 2002; Lee and Söderhäll, 2002; Schmidt et al., 2001). The cellular responses involve in the blood cells (hemocytes)-mediated immune responses such as phagocytosis, nodule formation and encapsulation (Johansson et al., 2000; Söderhäll and Cerenius, 2000), whereas humoral responses include antimicrobial peptides synthesis, the complex enzymatic cascade that regulate coagulation process, the prophenoloxidase (proPO)-activating system and the production of reactive oxygen and nitrogen intermediates (Figure 1.2) (Cerenius and Söderhäll, 2004; Destoumieux et al., 1997; Jiravanichpaisal et al., 2006; Sritunyalucksana and Söderhäll, 2000). In fact, there is an overlap between cellular and humoral defence responses due to many humoral factors affect the function of hemocytes and hemocytes are the major source of many humoral molecules (Lavine and Strand, 2002).

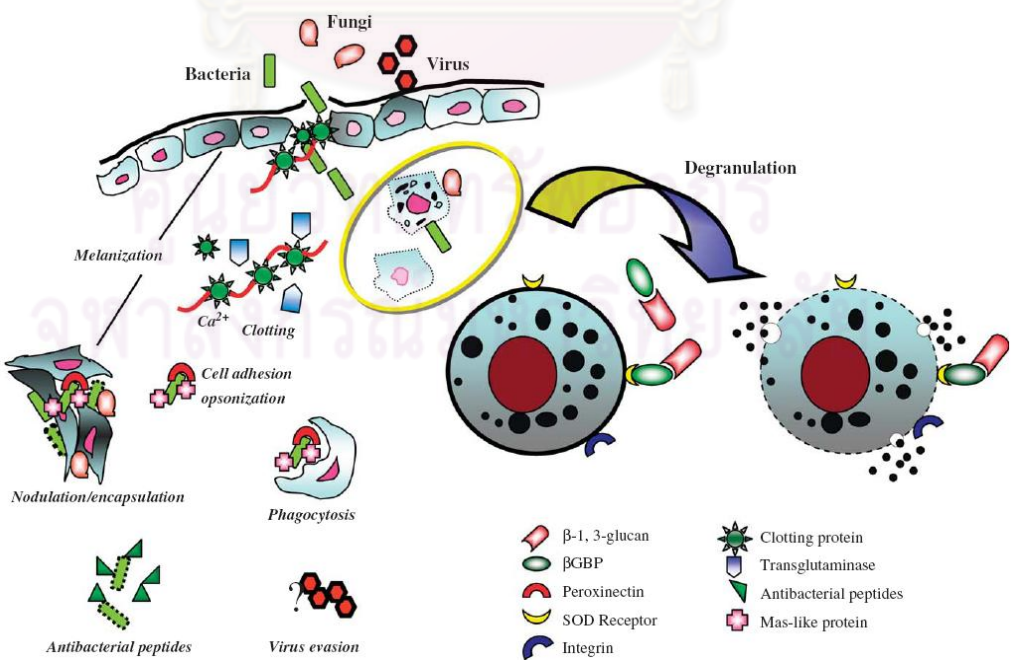

Figure 1.2 Schematic illustrations a complex system of innate defence mechanisms (Jiravanichpaisal et al., 2006).

1.3.1 Phagocytosis

Phagocytosis is an evolutionarily conserved cellular response that occurs in all metazoan phyla. Phagocytosis is considered as a primary response of hemocytes that have the capacity to recognize and destroy either small non-self particles such as bacteria, yeast and synthetic beads or senescent cells of the own organism (Lavine and Strand, 2002; Vazquez et al., 2009). It refers to the engulfment of entities by an individual cell. Phagocytosis is initiated by recognition and binding of a target particle to the phagocytic cell, followed by uptake through cytoskeleton modification and intracellular vesicular transport to phagosomes where the engulfed target is destroyed. Recognition of the target is either direct with receptor binding to the target surface or mediated via opsonization factors that mark the particle for phagocytosis (Bayne, 1990).

1.3.2 Nodule formation and encapsulation

Nodule formation and encapsulation are the multicellular immune defense against foreign invaders that too large for phagocytosis by individual hemocytes. Unlike phagocytosis, this process kills pathogens or restricts their movement and growth in the hemocoel cavity by entraped a large number of bacteria or foreign molecules within the mutilayered overlapping sheath of hemocytes and resulted in the formation of nodulation and such larger nodular aggregates will eventually be encapsulated (Lackie, 1988). This process is ultimately accompanied by blackening of the capsule due to the melanization through the activation of proPO-activating system, and finally the parasite is killed within the encapsulated capsules by several factors including the local production of cytotoxic quinones or semiquinones via the proPO activation cascade and the reactive oxygen or nitrogen intermediates (Gillespie et al., 1997; Nappi et al., 1995, 2000).

1.3.3 Antimicrobial peptide

Antimicrobial peptides (AMPs) are an integral component of the innate immune system. These peptides share certain common characteristics, such as small size, cationic character and similarities in structural patterns or motifs (Bachère et al., 2004; Jensen et al., 2006). AMPs have a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, filamentous fungi and, in some cases, viruses and protozoa (Brown and Hancock, 2006). During the past several years, several families of AMPs have been identified from the penaeid shrimp including penaeidins, crustins and anti-lipopolysaccharide factors (ALFs) (Tassanakajon et al., 2010; Bartlett et al., 2002; Destoumieux et al., 1997; Somboonwiwat et al., 2005). Penaeidins are 5.5-6.6 kDa peptides with an N-terminal proline-rich domain and a C-terminal domain containing six cysteine residues and have antimicrobial activity against Gram-positive bacteria and fungi (Destoumieux et al., 1997). Crustins are the homologues of carcinin that was firstly isolated from the shore crab *Carcinus maenas* (Brockton et al., 2007; Relf et al., 1999). Crustins are characterized as a cationic cysteine-rich 11.5 kDa AMP that contain a single whey acidic protein (WAP) domain at the C-terminus and exhibit antibacterial activity against Gram-positive bacteria (Smith et al., 2008). Anti-lipopolysaccharide factors (ALFs) are the small basic protein that originally identified from hemocyte of the horseshoe crab *Limulus polyphemus* and have the strong antibacterial effects against Gram-negative bacteria (Morita et al., 1985; Tanaka et al., 1982).

1.3.4 Coagulation system

Invertebrates have an open circulatory system. Blood coagulation is therefore essential to prevent hemolymph loss in case of injury and also help in the trapping of microbes from entering and spreading throughout the hemocoel (Iwanaga and Lee, 2005). Mechanisms of blood coagulation have been proposed based on crayfish and horseshoe crab as model organisms. In crayfish, clotting occurs through polymerization of a clotting protein in plasma and is catalyzed by a calcium ion

dependent transglutaminase (TGase) (Hall et al., 1999; Sritunyalucksana and Söderhäll, 2000; Wang et al., 2001a). In horseshoe crab, however, the clotting process is regulated by a proteolytic cascade which is activated by the microbial cell wall components (Iwanaga, 2002; Iwanaga and Lee, 2005).

1.3.5 Prophenoloxidase (proPO)-activating system

The prophenoloxidase (proPO)-activating system is known to be one of the major innate defence responses in invertebrates (Cerenius and Söderhäll, 2004; Gillespie et al., 1997; Söderhäll, 1982). Phenoloxidase (PO) is a key enzyme of the proPO cascade. PO is synthesized and maintained as an inactive precursor (zymogen) called prophenoloxidase (proPO). This precursor is activated by a limited proteolysis through the action of a serine proteinase cascade (proPO-activating cascade) upon recognition of a minute amount of microbial cell wall components such as lipopolysaccharide (LPS) and peptidoglycan (PGN) from bacteria and β-1,3-glucan from fungi (Figure 1.3) (Ashida et al., 1983; Cerenius and Söderhäll, 2004; Yoshida et al., 1986). Active PO catalyzes the oxidation of phenols to the toxic quinone precursors, which are nonspecifically cross-link neighboring molecules to form the melanin deposited at the wound site or around the pathogens (Nappi and Christensen, 2005). Quinones may also be involved in the production of cytotoxic molecules such as superoxides and hydroxyl radicals, which could help to kill the invading microorganisms (Gillespie et al., 1997; Nappi and Ottaviani, 2000).

Although PO-induced melanin synthesis is thought to be essential for host defence, it must be tightly controlled because systemic hyper-activation of the proPO system, excessive quinone formation, and inappropriate excessive melanin synthesis are also harmful to the host cells (Cerenius et al., 2008). Thus it is no surprise that proPO activation and melanization processes must be tightly regulated and modulated by various melanization regulatory molecules such as serine proteinase inhibitors (pacifastin/serpins) (De Gregorio et al., 2002; Liang et al., 1997; Ligoxygakis et al., 2002; Liu et al., 2007; Tong and Kanost, 2005; Tong et al., 2005; Zhu et al., 2003;

Zou and Jiang, 2005), PO inhibitors (Lu and Jiang, 2007), melanization inhibitors (Söderhäll et al., 2009; Zhao et al., 2005) and some other proteins (Beck and Strand, 2007; Ling et al., 2009; Rao et al., 2010).

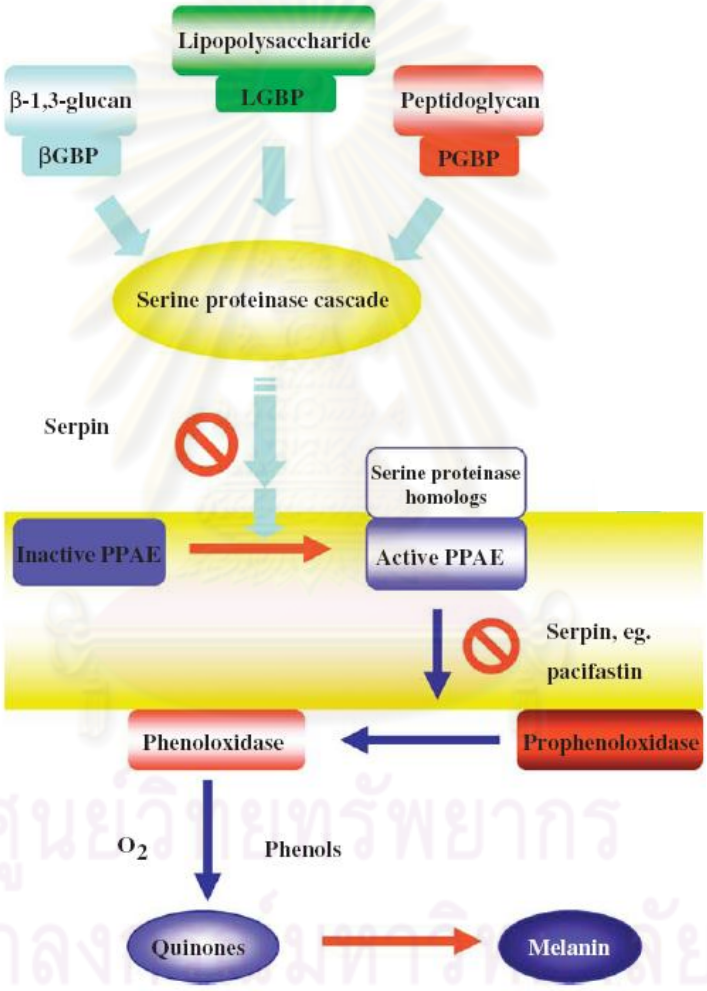

Figure 1.3 The prophenoloxidase-activating system in arthropods (Jiravanichpaisal et al., 2006).

1.4 Phenoloxidases (POs)

Phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1), a terminal enzyme of the so-called proPO system (Ashida, 1990; Söderhäll, 1982), is a bifunctional copper-containing enzyme that catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Nappi and Christensen, 2005; Sugumaran, 1996). These quinones subsequently go through the enzymatic and non-enzymatic reactions to become melanin, which is deposited at the injury site or around the intruding microorganisms (Aspán and Söderhäll, 1991; Nappi and Christensen, 2005). Due to a wide range of substrate specificity (Aso et al., 1984; Hall et al., 1995), POs may participate in multiple steps of melanin formation, wound healing, cuticle sclerotization and microbial killing (Figure 1.4) (Ashida and Brey, 1998; Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005; Sugumaran, 2002; Zhao et al., 2007).

At the beginning of the melanin biosynthetic pathway, phenylalanine is hydroxylated to tyrosine by phenylalanine hydroxylase. PO (monophenolase activity) or tyrosine hydroxylase mediates the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). DOPA is then oxidized to quinone by PO diphenolase activity. The resulting quinones are converted to melanin by a series of intermediate steps involving enzymatic and non-enzymatic reactions. In one of these enzymatic reactions, dopachrome is decarboxylated by dopachrome conversion enzyme (DCE; also known as dopachrome isomerase or dopachrome tautomerase) to form 5,6-dihydroxyindole (DHI). DHI, as well as its oxidation products, has a broad-spectrum antimicrobial activity (Zhao et al., 2007). These products spontaneously polymerize to form melanin frequently observed around wounds, pathogens, or parasites (Figure 1.4) (Sugumaran, 1996; Shelby et al., 2000; Olivares et al., 2001). A second pathway incorporating two additional enzymes contributes to the formation of melanin. The first of two additional enzymes, dopa decarboxylase (DDC), acts on the DOPA molecules formed by PO monophenolase activity. DDC decarboxylates DOPA to form dopamine, which is then acetylated to *N*-acetyldopamine (NADA) by the second enzyme, dopamine *N*-acetyltransferase. NADA undergoes polymerization and

non-enzymatic reactions lead to the formation of melanin. POs along with other enzymes, also generates dopamine quinone which rapidly cyclizes to form DHI non-enzymatically (Figure 1.4) (Saul and Sugumaran, 1988; Shelby et al., 2000).

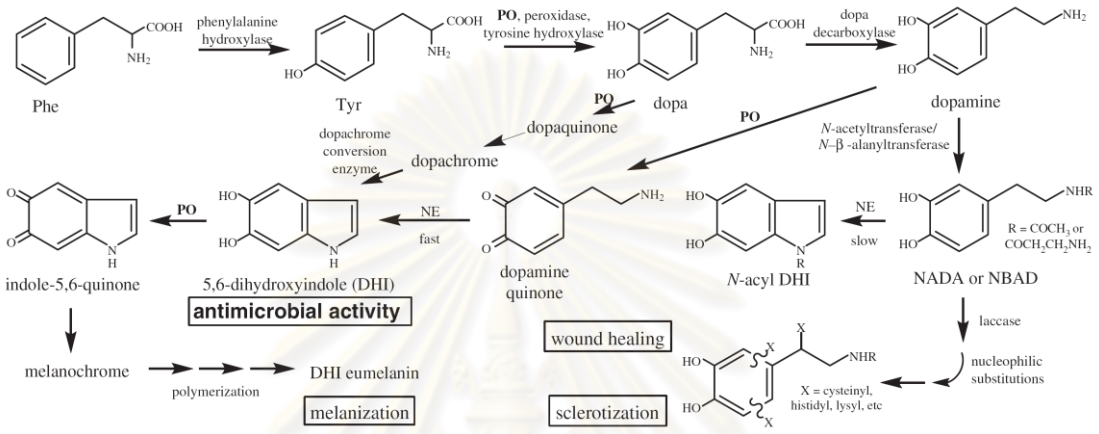

Figure 1.4 Mechanisms and physiological functions of PO-mediated reactions in insects and crustaceans. PO catalyzes the hydroxylation of tyrosine to DOPA and further oxidation of DOPA to dopaquinone. Dopaquinone can convert non-enzymatically to dopachrome, which is decarboxylated to 5,6-dihydroxyindole (DHI). DHI, as well as its oxidation products, has a broad-spectrum antimicrobial activity (Zhao et al., 2007). DOPA is also decarboxylated to form dopamine. Dopamine and DHI, after a few reaction steps involving PO, are eventually converted to melanin. In addition, dopamine can also form stable derivatives (NADA or NBAD) through acyl transfer and laccase then converts these derivatives to the oxidative intermediates for protein crosslinking during wound healing and cuticle sclerotization (Zhao et al., 2007).

The invertebrate proPO gene was first cloned from the freshwater crayfish *Pacifastacus leniusculus* (Aspán et al., 1995). Subsequently, several proPO genes have been identified and cloned from a variety of arthropods species. There are ten proPO genes in the mosquito *Aedes aegypti* (Waterhouse et al., 2007), nine in the mosquito *Anopheles gambiae* (Christophides et al., 2002), three in the fruit fly *Drosophila melanogaster* (Asada et al., 2003; Asano and Tekebuchi, 2009; Fujimoto et al., 1995; Myers et al., 2000) and the parasitoid wasp *Pimpla hypochondriaca* (Parkinson et al., 2001), two in the beetle *Holotrichia diomphalia* (Kim et al., 2002),

the silkworm *Bombyx mori* (Asano and Ashida, 2001a, 2001b; Kawabata et al., 1995) and the tobacco hornworm *Manduca sexta* (Hall et al., 1995; Jiang et al., 1997). In crustaceans, only one type of proPO gene has been reported including crayfish *P. leniusculus* (Aspán et al., 1995), penaeid shrimps *P. monodon* (Sritunyalucksana et al., 1999) and *Fenneropenaeus chinensis* (Gao et al., 2009), lobsters *Homarus gammarus* (Hauton et al., 2005), giant freshwater prawn *Macrobrachium rosenbergii* (Liu et al., 2006; Lu et al., 2006), crabs *Cancer magister* (Terwilliger and Ryan, 2006), *Scylla serrata* (Ko et al., 2007) and *Eriocheir sinensis* (Gai et al., 2008) and the water flea *Daphnia pulex* (McTaggart et al., 2009), except two proPO sequences from the *L. vannamei* (Ai et al., 2008, 2009; Lai et al., 2005; Wang et al., 2006) and *Marsupenaeus japonicus*. In animals containing several proPO genes, there are some evidences showed that these proPO genes are differentially expressed with respect to developmental stages (embryo to adult) or related to blood-feeding (Christophides et al., 2002; Li et al., 2005; Müller et al., 1999; Waterhouse et al., 2007).

Structurally, all of arthropod proPOs contain two functional copper-binding sites (copper-binding sites A and B), a proteolytic activation site and a thiol ester-like motif. All arthropod proPOs, with one exception of some proPOs from the hunting wasp, *Pimpla hypochondriaca* (Parkinson et al., 2001), lack a signal peptide for localization into the endoplasmic reticulum and the endomembrane system. POs are produced by specific hemocytes (blood cells) such as the crystal cells of the fruit fly *D. melanogaster* (Rizki et al., 1985), and oenocytoids (another type of insect blood cell) in many other insects (Shrestha and Kim, 2008) and the granular and semi-granular circulating hemocyte types in crustaceans (Johansson and Söderhäll, 1985; Perazzolo and Barracco, 1997; Söderhäll and Smith, 1983; Vargas-Albores et al., 1993).

1.5 ProPO-activating enzymes (PPAEs)

Melanization is a potent immune response mediated by phenoloxidase (PO). Biochemical studies in crustacean and large insects indicate that proPO is activated by a cascade of clip-domain serine proteinase (clip-SP) and the terminal clip-SP that carries out the proteolysis of the proPO precursor is designated as proPO-activating enzyme (PPAE) (Aspán and Söderhäll, 1991; Cerenius and Söderhäll, 2004; Gupta et al., 2005; Jiang et al., 1998, 2003a, 2003b; Satoh et al., 1999). All these PPAEs are known to consist of a trypsin-like serine proteinase domain at the C-terminus and one or two disulfide-knotted clip domains at the N-terminus (Figure 1.5) (Jiang and Kanost, 2000; Piao et al., 2005). In some insects, proPO activation also requires the catalytically inactive clip-domain serine proteinase homologues (clip-SPHs), which lack the proteolytic activity due to the replacement of the active site serine by glycine residue as cofactor to activate the proPO system (Figure 1.6) (Gupta et al., 2005; Kwon et al., 2000; Yu et al., 2003). After cleavage activation, the clip domain remains covalently attached to its catalytic or SP-like domain by an inter-chain disulfide bridge (Jiang and Kanost, 2000).

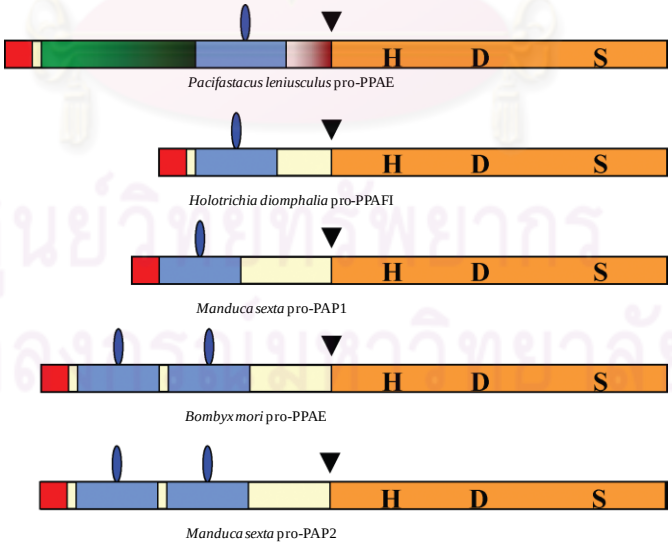

Figure 1.5 Schematic representations of the pro-form of the arthropod prophenoloxidase-activating enzymes (pro-PPAEs). The signal sequences are shown in red boxes. The blue and orange boxes indicate an N-terminal clip-domain and a C-terminal serine proteinase domain, respectively. The arrowhead indicates the proteolytic cleavage site. A glycine-rich and proline-rich region are indicated by green and brown boxes, respectively (Cerenius and Söderhäll, 2004).

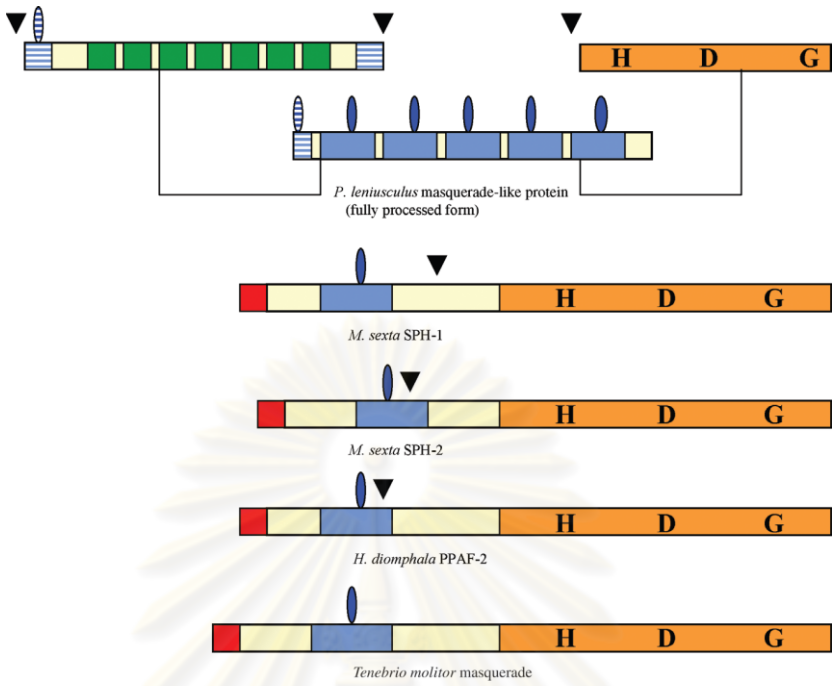

Figure 1.6 Schematic representations of the pro-form of the arthropod clip-domain serine proteinase homologues (clip-SPHs). The hydrophobic signal sequences are shown in red boxes. The blue and orange boxes indicate an N-terminal clip-domain and a C-terminal serine proteinase domain, respectively. The arrowhead indicates the proteolytic cleavage site. The green squares represent the repeated glycine-rich motifs in *Pacifastacus leniusculus* masquerade-like protein (Cerenius and Söderhäll, 2004).

Several PPAEs have been identified in many insects and a crustacean, including the tobacco hornworm *M. sexta* (Jiang et al., 1998, 2003a, 2003b), the silkworm *B. mori* (Satoh et al., 1999), the beetle *H. diomphalia* (Lee et al., 1998a, 1998b), the mealworm *Tenebrio molitor* (Kan et al., 2008) and the freshwater crayfish *P. leniusculus* (Aspán et al., 1990; Wang et al., 2001b). All PPAEs are synthesized and maintained as zymogen (pro-PPAE) and activated following either injury or infection by additional upstream specific proteolytic cleavage between the clip domain and the proteinase domain (Cerenius and Söderhäll, 2004).

In the beetle *H. diomphalia* larvae, two clip-SPs named proPO-activating factor-I (PPAF-I) and PPAF-III, and a clip-SPH named PPAF-II have been identified and biochemically characterized (Kim et al., 2002; Kwon et al., 2000; Lee et al., 1998a, 1998b). *In vitro* reconstitution experiments demonstrate that the active PPAF-I cleaves proPOs (proPO-I and proPO-II) into a smaller but inactive POs, whereas active PPAF-III cleaves the catalytically defective PPAF-II (Figure 1.7). The cleaved PPAF-II molecules form a dodecameric oligomer and is able to recruit PO molecules forming a PO·PPAF-II complex that can exhibits a strong PO activity (Piao et al., 2005).

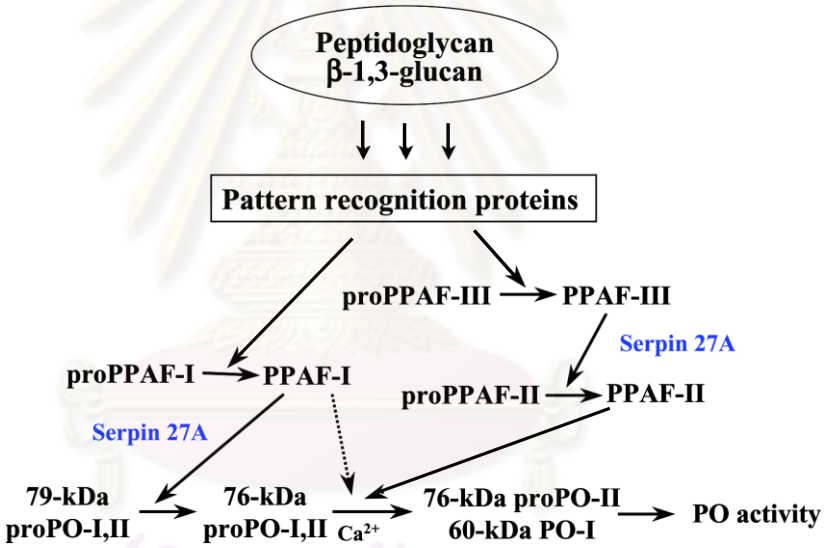

Figure 1.7 A model for the proPO system activation in the beetle *Holotrichia diomphalia* larvae. An upstream chain of pattern recognition proteins and proteinases causes the cleavage of the pro-form of prophenoloxidase-activating factor-III (proPPAF-III) into catalytically active PPAF-III. This proteinase cleaves the serine proteinase homologue proPPAF-II. The presence of cleaved PPAF-II is required for the generation of an active PO. Activation of proPOs occurs in two steps. In the first step, the proteolytic cleavage of the 79-kDa proPOs into 76-kDa proPOs is accomplished by active PPAF-I. In the second step, catalytically active PO is produced on binding to PPAF-II. The possible involvement of PPAF-I in the second step is indicated by a dashed line. Regulation of proteinases by serpin is indicated (Iwanaga and Lee, 2005).

In the mealworm *T. molitor* larvae, proPO system is triggered by sequential activation of three SPs including the modular serine proteinase (Tm-MSP), Spätzle-processing enzyme (SPE)-activating enzyme (Tm-SAE) and Spätzle-processing enzyme (Tm-SPE), which are processed sequentially whereupon, finally, active Tm-SPE cleaves Tm-proPO and a serine proteinase homologue zymogen (Tm-SPH1), leading to the formation of a stable melanization complex (Figure 1.8). This complex induces local melanin synthesis on the surface of bacteria and enables the insect host to kill the invading microbe (Kan et al., 2008; Park et al., 2007). Also, active Tm-SPE cleaves the pro-Spätzle (proSpz) into processed Spätzle (Spz) leading to the production of the antimicrobial peptide (AMP) (Figure 1.8).

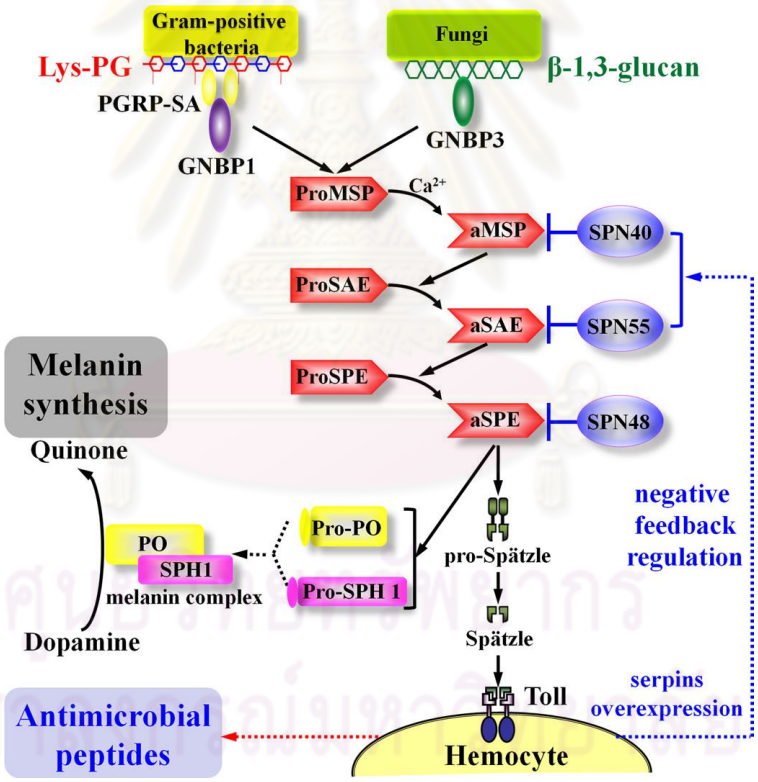

Figure 1.8 A model for the regulation of proPO activation cascade and Toll-like pathway in the mealworm *Tenebrio molitor* larvae. When presented to the host immune system, proPO system is triggered by sequential activation of three serine proteinase zymogens including the modular serine proteinase (MSP), Spätzle processing enzyme (SPE)-activating enzyme (SAE) and Spätzle processing enzyme (SPE). As a result of this three-step proteolytic cascade, proPO and SPH1 zymogen are processed and form the active melanization complex on the surface of bacteria. This complex induces local melanin synthesis. Moreover, active SPE cleaves the pro-Spätzle into processed Spätzle, leading to the production of the antimicrobial peptide. Regulation of proteinases by serpins is indicated (Jiang et al., 2009).

In *M. sexta*, three different serine proteinases with PPAE activity, PAP1, PAP2 and PAP3, were isolated and cloned (Jiang et al., 1998, 2003a, 2003b). Upon fungal or Gram-positive bacteria challenge, proHP14 becomes active in the presence of β-1,3-glucan recognition protein (Wang and Jiang, 2006). HP14 can then activate proHP21, which in turn activates proPAP2 or proPAP3 (Gorman et al., 2007; Wang and Jiang, 2007). PAP2 and PAP3 activate proPO in the presence of active SPHs (Yu et al., 2003; Wang and Jiang, 2004). Another activation pathway occurs when *M. sexta* larvae are exposed to Gram-positive or Gram-negative bacteria or fungi. Unknown serine proteinase(s) are activated, one of which then activates proHP6. Active HP6 then processes proHP8 and proPAP1 to initiate a branched pathway (An et al., 2009). Activated PAP1 activate proPO pathway in the presence of SPHs (Yu et al., 2003), while HP8 cleaves pro-spätzle to produce active Spätzle, which binds a Toll receptor to initiate a signaling pathway resulting in expression of AMPs (An et al., 2009, 2010).

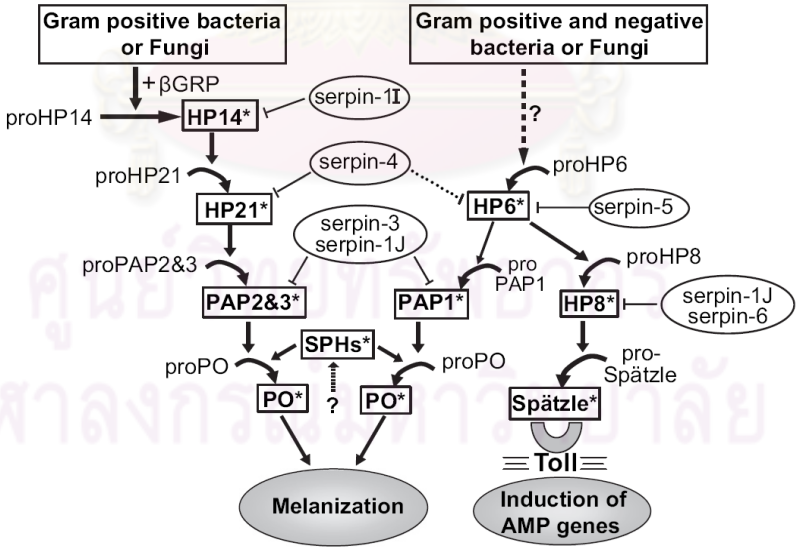

Figure 1.9 A model for the regulation of proPO activation cascade and Toll-like pathway in the tobacco hornworm *Manduca sexta*. Arrows indicate activation of downstream components or steps. Dashed arrows indicate potentially more than one step. Arrows labeled with "?" indicate steps that have not been experimentally verified. Regulation of proteinases by serpins is indicated. A dotted line represents weak inhibition of HP6 by serpin-4 (An and Kanost, 2010).

In *Drosophila*, recent genetic studies have identified two clip-SPs, MP1 and MP2/sp7/PAE1 that may act as the *Drosophila* PPAEs (Castillejo-López and Häcker, 2005; Leclerc et al., 2006; Tang et al., 2006). Overexpression of either MP1 or MP2 induces constitutive melanization and semi-lethality, while knockdown of MP1 or MP2 compromises PO activation and melanization upon microbial infection. The cleavage of proPO is blocked in the MP2 mutant flies after infection. MP1 is required for PO activation in response to both bacterial and fungal infection, whereas MP2 is more responsive to fungal infection. Acting downstream of MP2, MP1 is a good candidate to be the PPAE that directly cleaves proPO, but there is not yet direct experimental evidence for supporting this role (Tang, 2009).

In *P. monodon*, the clip-SP named *Pm*ClipSP1 and clip-SPH named *Pm*MasSPH1 have been cloned and functionally characterized (Amparyup et al., 2007, 2010; Jitvaropas et al., 2009). *Pm*ClipSP1 is not required for shrimp proPO cascade but still plays an important role in shrimp immune defence against bacterial infection (Amparyup et al., 2010). *Pm*MasSPH1 is a multifunctional immune molecule that participates in hemocyte adhesion and displays the bacterial binding and opsonic activity (Jitvaropas et al., 2009). However, the role of *Pm*MasSPH1 in shrimp proPO system is not yet to be elucidated.

1.6 Effects of the melanization on pathogen survival

Active PO produces several highly reactive and toxic compounds that are harmful to the intruding microorganisms (Cerenius et al., 2008; Christensen et al., 2005; Zhao et al., 2007). Several *in vivo* and *in vitro* report the linking between the PO activities and the pathogen survival. In *Plasmodium* susceptible mosquito strains melanization has been demonstrated to be directly involved in parasite killing, whereas in resistant host strains the parasite is not killed by melanization, but lysed by other means (Volz et al., 2006). Knockdown of the *Drosophila* melanization proteases (MP1 and MP2) strongly supported that melanization is crucial for the innate immune system against fungal infections (Tang et al., 2006). One very convincing

demonstration of the importance of PO activity for the elimination of pathogens comes from the interactions of *M. sexta* to the virulent bacterium *Photorhabdus* (Eleftherianos et al., 2007). This pathogen secretes a hydroxystilbene compound with the capacity to inhibit host PO. Bacteria with a mutated gene in the pathway for production of this inhibitor are nonvirulent, suggesting that PO activity is needed for the elimination of this bacterium (Eleftherianos et al., 2007). This conclusion is supported by the fact that knockdown of host PO will result in a much higher host susceptibility to *Photorhabdus*. Another study also carried out in *M. sexta*. When the parasitic wasp *Microplitis demolitor* lays eggs in *M. sexta* larvae, it also injects a polydnavirus (Beck and Strand, 2007). This virus produces a serine proteinase inhibitor, named Egf1.0, that blocks melanization by binding to *M. sexta* PAP3 and prevents the proPO activation. If the Egf1.0 gene became inactive, the wasp eggs become melanized and they will no longer survive in the host. The additional experiments also showed that activated PO drastically reduced both viral and parasitoid viability (Beck and Strand, 2007). *In vitro* investigations of the effects of quinones on the pathogenic organisms have also been carried out in *M. sexta* by using the isolated PO. Interestingly, dopamine and 5,6-dihydroxyindole which are produced by this enzyme exhibit a high antibacterial activity, whereas the other intermediates that produced during cuticle sclerotization were found to be less toxic to the microorganisms (Zhao et al., 2007). Thus, it is possible to suggest that the host could directly control the production of compounds with different degrees of toxicity by manipulating the levels of different melanin precursors (e.g. by regulating other enzymes such as dopa decarboxylase (DC), dopachrome conversion enzyme and peroxidase) (Christensen et al., 2005).

Furthermore, it has been reported that an active phenoloxidase preparation from *P. leniusculus* exhibited a strong antibacterial effect *in vitro* and in most cases the reduction of bacterial growth was stronger when dopamine was used as substrate as compared to L-dopa (Cerenius et al., 2010). Moreover, knockdown of the proPO transcript by double-stranded RNA (dsRNA) increased host susceptibility to a highly virulent bacterium *Aeromonas hydrophila*, suggesting the important role of phenoloxidase in defense against bacterial invasion (Liu et al., 2007). If the specific

inhibitor of the crayfish PPAE, pacifastin, is depleted by RNAi treatment this will result in higher PO activities and reduced mortality after *A. hydrophila* infections. A more detailed analysis showed that increased PO activity resulted in higher rates of phagocytosis by hemocytes, whereas depression of PO lowered the phagocytic capacity toward this bacterium (Liu et al., 2007).

1.7 Objectives of the dissertation

Several proPO system-associated genes including two different *Pm*PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) and two different *Pm*proPO genes (*Pm*proPO1 and *Pm*proPO2) have been identified from hemocytes of the black tiger shrimp *P. monodon*. The role of these gene transcripts in proPO system and shrimp immunity is yet to be clarified.

The aims of the present study are to characterize the *Pm*PPAE (*Pm*PPAE1 and *Pm*PPAE2) and *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) gene transcripts from *P. monodon* and to elucidate the tissue distribution and developmental expression profiles of these gene transcripts by semi-quantitative RT-PCR. Furthermore, the potential functions of the *Pm*PPAEs and *Pm*proPOs in shrimp immunity were elucidated by using double-stranded RNA (dsRNA)-mediated RNA interference (RNAi). The efficiency of gene knockdown was determined by semi-quantitative RT-PCR and the silenced shrimps were then assayed for PO activity and susceptibility to *Vibrio harveyi* infection. To further examine the biological function of the *Pm*PPAE1, the recombinant proteins of *Pm*PPAE1 were produced in *Escherichia coli* and insect cell expression system and further characterized for their proteinase activity and ability to activate shrimp proPO system.

This study provides the basic knowledge of shrimp proPO system and leads to better understanding of shrimp immunity that will be helpful for the control of microbial diseases and further development of sustainable shrimp aquaculture.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, laminarin from *Laminaria digitata* and the synthetic chromogenic substrates including *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were purchased from Sigma. L-3,4-dihydroxy phenylalanine (L-DOPA) was obtained from Fluka. TRI REAGENT® was purchased from Molecular Research Center. Tryptic soy broth was purchased from Difco. The dNTPs, DNA and protein molecular weight marker and isopropyl-β-D-thio galactopyranoside (IPTG) were purchased from Fermentas. Ni-NTA Agarose was purchased from Qiagen. Antibiotics including ampicillin and kanamycin were obtained from Biobasic. DNase I and *Pfu* DNA polymerase were purchased from Promega whereas *Taq* DNA polymerase was purchased from Fermentas. All of the restriction enzymes were purchased from Biolabs. The TA-cloning vector pGEM®-T Easy was purchased from Promega whilst pET28b expression vector was obtained from Novagen. The *E. coli* strain JM109 and Rosetta (DE3) pLysS was obtained from Promega and Novagen, respectively. MAX Efficiency® DH10Bac™ *E. coli* competent cells, Bluo-gal, Sf-900 II SFM, Grace's insect cell culture medium (unsupplemented), pFastBac™1 vector, Cellfectin® II Reagent and 100x Antibiotic-Antimycotic were purchased from Invitrogen Life Technologies. The insect cell line, *Spodoptera frugiperda* 9 (Sf9) cells was kindly provided by Associate Professor Dr. Sarawut Jitrapakdee from Department of Biochemistry, Faculty of Science, Mahidol University. All other common reagents were obtained from Sigma, Merck, BDH, Scharlau, Ajax and USB.

2.2 Experimental animals

Healthy black tiger shrimps, *Penaeus monodon*, were purchased from local shrimp farms in Thailand. Specific pathogen-free (SPF) *P. monodon*, which was specifically free of white spot syndrome virus (WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and *Vibrio harveyi* bacteria as determined by PCR diagnosis, were purchased from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. All shrimps were maintained in laboratory tanks with aerated seawater (20 ppt) at the ambient temperature (28 ± 4 °C) for at least 7 days prior to the experiment to allow acclimatization.

2.3 Sequence analysis of the proPO system-associated genes from *P. monodon*

The cDNA and deduced amino acid sequence of two different PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) and two different proPO genes (*Pm*proPO1 and *Pm*proPO2) from *P. monodon* were analyzed by the GENETYX 7.0 program (GENETYX corporation). Similarity searches of DNA and deduced amino acid sequence were performed using the BLASTX program (http://www.ncbi.nlm.nih.gov/blast). The putative signal peptide cleavage site and the structural protein domains were predicted by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) and simple modular architecture research tool (SMART) program (http://smart.embl-heidelberg.de/), respectively. Multiple amino acid sequence alignment was carried out using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/).

2.4 Tissue expression analysis of the proPO system-associated genes using semi-quantitative RT-PCR

2.4.1 Tissue preparation

Juvenile black tiger shrimps *P. monodon* (~ 20 g, fresh weight) were purchased from a local shrimp farm in Thailand and kept in laboratory tanks with aerated seawater (20 ppt) at the ambient temperature (28 ± 4 °C) for at least 7 days before processing. The tissues of hepatopancreas, gills, lymphoid organ, intestine and heart from three shrimps were carefully dissected on ice for subsequent total RNA extraction. Shrimp hemolymph was individually collected from the ventral sinus in an ice-cold anticoagulant solution (10% (w/v) trisodium citrate dihydrate). Hemocytes were isolated from the freshly collected hemolymph by centrifugation at 800 × g for 10 minutes at 4 °C. The hemocyte pellets from three shrimps were immediately used for total RNA isolation.

2.4.2 Total RNA isolation and DNase treatment

Total RNA were extracted from various dissected tissues of *P. monodon* using the TRI REAGENT® (Molecular Research Center). A piece of tissues (~50 - 100 mg) was homogenized in 0.2 ml of TRI REAGENT® followed by addition of 0.8 ml of TRI REAGENT® to make the final volume of 1 ml. The mixtures were centrifuged at 12000 × g for 15 minutes at 4 °C and the resulting supernatant was transferred to the new tube. Subsequently, 0.2 ml of chloroform per 1 ml of TRI REAGENT® was added to each homogenized sample. The homogenate was vortexed for 2 minutes and then chilled on ice for 30 minutes before being centrifuged at 12000 × g for 15 minutes at 4 °C to get phase separation. The upper aqueous phase which contained the isolated total RNA samples was carefully transferred to the new tube. Then, 0.5 ml of a pre-chilled isopropanol was added to the solution to precipitate the soluble RNA in each sample. The mixtures were chilled at -80 °C for 15 minutes followed by centrifugation at 12000 × g for 20 minutes at 4 °C. The RNA pellets were washed with an ice-cold 75% (v/v) ethanol, air-dried and dissolved in the appropriate volume of diethyl pyrocarbonate (DEPC)-treated water.

To eliminate the remnants of any potentially contaminating genomic DNA before being reverse transcribed into first-strand cDNA, the resulting total RNA was treated with the RQ1 RNase-free DNase I (Promega) at 37 °C for 30 minutes. The DNA-free RNA samples were then purified again with the TRI REAGENT® (Molecular Research Center) as described above. Briefly, the RNA samples were extracted with 1 ml of TRI REAGENT® and 0.2 ml of chloroform. After incubation at room temperature for 15 minutes, the mixtures were centrifuged at 12000 × g for 15 minutes at 4 °C and the aqueous phase was recovered and transferred to the new tube. The RNA sample was then precipitated by 1 volume of an ice-cold isopropanol. The mixtures were chilled for at least 15 minutes at -80 °C and the precipitated RNA was recovery by centrifugation at 12000 × g for 20 minutes at 4 °C. The RNA pellet was washed with 1 ml of an ice-cold 75% ethanol, air-dried and dissolved with an appropriate volume of DEPC-treated water.

The concentration of total RNA was spectrophotometrically determined by measuring the optical density (OD) at 260 nanometres. The quality of total RNA was observed from an optical density ratio OD_{260}:OD_{280}. Only RNAs with OD_{260}:OD_{280} greater than 1.8 (>1.8) were selected and used for further experiments.

2.4.3 First-strand cDNA synthesis

First-strand cDNAs were reverse transcribed from the poly-A tailed mRNAs using the ImProm-II™ Reverse Transcriptase System kit (Promega) following the manufacturer's instructions. Briefly, approximately 1.5 µg of DNA-free total RNA sample and 0.5 µg of oligo$(dT)_{15}$ primer were denatured at 70 °C for 5 minutes and quickly chilled on ice for 5 minutes. Reverse transcription reaction was performed in a 20 µl reaction volume containing 1x ImProm-II™ reaction buffer, 2.25 mM $MgCl_2$, 0.5 mM of each dNTP, 20 units of recombinant RNasin® ribonuclease inhibitor, 1 µl of ImProm-II™ reverse transcriptase (Promega) and the denatured total RNA. The reaction mixtures were incubated at 25 °C for 5 minutes followed by 42 °C for 90 minutes. The thermal inactivation of the reverse transcriptase was performed at 70 °C for 15 minutes. The synthesized cDNAs were stored at -80 °C until required for RT-PCR analysis.

2.4.4 Tissue distribution analysis by semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out to investigate the mRNA expression profiles of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 in different tissues of *P. monodon* including hemocytes, hepatopancreas, gills, lymphoid organ, intestine and heart. Gene-specific primers for *Pm*PPAE1 (PPAi-F and PPAi-R), *Pm*PPAE2 (*Pm*PPAE2i-F and *Pm*PPAE2i-R), *Pm*proPO1 (PO1RT-F and PO1RT-R) and *Pm*proPO2 (PO2RT-F and PO2RT-R) were designed from the full-length cDNA sequences of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2, respectively (Table 2.1). A partial fragment of the elongation factor1-α gene (EF1-α) was amplified by primer EF1α-F and EF1α-R (Table 2.1) and used as an internal control to monitor the amount of RNA/cDNA PCR template and amplification efficiency between samples. One microlitre of the first-strand cDNA was used as the PCR template in a 25 µl reaction volume containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each specific primer and 1 unit of *Taq* DNA polymerase (Fermentas). The DNA template was denatured at 94 °C for 1 minute, followed by 25 cycles of amplification (denaturing at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds). The final extension was performed at 72 °C for 5 minutes.

2.4.5 Agarose gel electrophoresis

The amplification products were analyzed through a TBE-1.5% (w/v) agarose gel electrophoresis. An appropriate percent of agarose was prepared in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA; pH 8.3) and placed into the electrophoresis chamber before submerged with appropriate amounts of 1x TBE buffer. The PCR product was then mixed with DNA loading buffer (0.25 % bromophenol blue and 25% Ficoll) and carefully loaded into each well of the agarose gel. Electrophoresis was performed at 100 volts until the bromophenol blue loading dye front moved to approximately 1 cm from the bottom of the gel. The gel was then stained with an ethidium bromide solution (2.5 µg/ml). The DNA fragments were visualized by UV-transillumination and photographed through the gel documentation

Table 2.1 Nucleotide sequence of primers used for tissue distribution and developmental expression analysis by semi-quantitative RT-PCR.

Primer	Sequence (5'– 3')
***Pm*proPO1:**	
PO1RT-F	5'- GGTCTTCCCCTCCCGCTTCG -3'
PO1RT-R	5'- GCCGCAGGTCCTTTGGCAGC -3'
***Pm*proPO2:**	
PO2RT-F	5'- GCCAAGGGGAACGGGTGATG -3'
PO2RT-R	5'- TCCCTCATGGCGGTCGAGGT -3'
***Pm*PPAE1:**	
PPAi-F	5'- CGTCTGCTTCATTGAGGGAGTG -3'
PPAi-R	5'- GTAGTAGATGGTGCCCCAGCCT -3'
***Pm*PPAE2:**	
*Pm*PPAE2i-F	5'- GCGGCGGTCACGCTCCTTGTTC -3'
*Pm*PPAE2i-R	5'- ACTCTCGGGGGCACGCTTGTTG -3'
EF1-α:	
EF1α-F	5'- GGTGCTGGACAAGCTGAAGGC -3'
EF1α-R	5'- CGTTCCGGTGATCATGTTCTTGATG -3'

2.5 Developmental expression analysis of the proPO system-associated genes using semi-quantitative RT-PCR

2.5.1 Sample preparation

P. monodon larvae from each of four different larval developmental stages including the nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15), were collected. All samples were flash-frozen in liquid nitrogen immediately and stored at -80 °C until required for RNA extraction.

2.5.2 Total RNA isolation and first-strand cDNA synthesis

Total RNA from four different larval developmental stages of whole shrimp was isolated using the TRI REAGENT® (Molecular Research Center) according to the manufacturer's instructions as described above (Section 2.4.2). The traces of genomic DNA were removed by incubated total RNA with RQ1 RNase-free DNase I (Promega) at 37 °C for 30 minutes. Total RNA was purified again using the TRI REAGENT® (Molecular Research Center) as described above. Total RNA was

spectrophotometrically quantified by measuring the optical density at 260 nanometres (OD_{260}). One microgram of the DNA free total RNA sample was reverse transcribed to first-strand cDNA using the ImProm-II™ Reverse Transcriptase System kit (Promega) and 0.5 µg of oligo$(dT)_{15}$ primer according to the manufacturer's instructions as described above. The cDNAs were stored at -80 °C until required.

2.5.3 Semi-quantitative RT-PCR analysis

The mRNA expression of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 in four different shrimp larval developmental stages including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15) stages, were analyzed by semi-quantitative RT-PCR. The gene specific primer sets for *Pm*PPAE1 (PPAi- F and PPAi-R), *Pm*PPAE2 (*Pm*PPAE2i-F and *Pm*PPAE2-R), *Pm*proPO1 (PO1RT-F and PO1RT-R) and *Pm*proPO2 (PO2RT-F and PO2RT-R) (Table 2.1) were used to amplify the mRNA transcripts in each larval stage. Shrimp EF1-α gene was included as an internal control for DNA template normalization. One microlitre of each first-strand cDNA was used as a template in a 25 µl PCR reaction volume containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each specific primer and 1 unit of *Taq* DNA polymerase (Fermentas) under the following conditions: pre-denaturation at 94 °C for 1 minute followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds. The final extension was performed at 72 °C for 5 minutes. The PCR amplicons were analyzed by a TBE-1.5% (w/v) agarose gel electrophoresis stained with ethidium bromide solution (2.5 µg/ml), visualized by UV-transillumination and photographed through the gel documentation (SYNGENE) as described above (Section 2.4.6).

2.6 Functional characterization of the proPO system-associated genes by RNA interference

2.6.1 Experimental shrimps

Shrimps used for all RNA interference (RNAi) experiments were specific pathogen-free (SPF) *P. monodon* purchased from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. Shrimps were reared in aerated seawater maintained at 20 ppt salinity and acclimatized for seven days prior to the experiments.

2.6.2 Preparation of double-stranded RNAs (dsRNAs) of the proPO system-associated genes

DNA templates for generation of double-stranded RNAs (dsRNAs) corresponding to the *Pm*PPAE1 (628 bp), *Pm*PPAE2 (667 bp), *Pm*proPO1 (280 bp) and *Pm*proPO2 (279 bp) sequences were amplified from each recombinant plasmid using gene specific primers (PPAi-F and PPAi-R for *Pm*PPAE1, *Pm*PPAE2i-F and *Pm*PPAE2i-R for *Pm*PPAE2, PO1i-F and PO1i-R for *Pm*proPO1 and PO2i-F and PO2i-R for *Pm*proPO2), designed by Primer Premier 5 program (Table 2.2). The sense and anti-sense strand DNA templates containing T7 promoter sequence at the 5' end on each different strand were then amplified by PCR using the same gene specific primers, but flanked at the 5' end by a T7 promoter recognition sequences (5'-*GGATCCTAATACGACTCACTAT AGG*-3') (Table 2.2). Two separate PCR reactions were set up, one with T7PPAi-F and PPAi-R, T7*Pm*PPAE2i-F and *Pm*PPAE2i-R, PO1iT7-F and PO1i-R and PO2iT7-F and PO2i-R (Table 2.2) for the sense strand template of *Pm*proPO1, *Pm*proPO2, *Pm*PPAE1 and *Pm*PPAE2, respectively, and the other with PPAi-F and T7PPAi-R, *Pm*PPAE2i-F and T7*Pm*PPAE2i-R, PO1i-F and PO1iT7-R and PO2i-F and PO2iT7-R (Table 2.2) for the anti-sense strand template of *Pm*proPO1, *Pm*proPO2, *Pm*PPAE1 and *Pm*PPAE2, respectively. For a negative dsRNA control, DNA template amplification was performed on the green fluorescent protein (GFP) gene. A 740-bp fragment of the GFP was amplified from the pEGFP-1 vector (Clontech) using GFPT7-F and GFP-R (Table 2.2) for the sense strand template, and GFP-F and GFPT7-R (Table 2.2) for the anti-sense strand template. The

PCR conditions consisted of an initial denaturation at 94 °C for 1 minute, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. The obtained PCR products were analyzed through a TBE-1.5% (w/v) agarose gel electrophoresis. The PCR products of the expected molecular size were excised from gels and the DNA fragments were subsequent extracted from the gel pieces using NucleoSpin® Extract II (MACHEREY-NAGEL) according to the respective manufacturer's protocol. The purified DNA fragments were analyzed through a TBE-1.5% (w/v) agarose gel electrophoresis and quantified by UV-spectrophotometer.

Sense and anti-sense single-stranded RNAs (ssRNAs) corresponded to the *Pm*proPO1, *Pm*proPO2, *Pm*PPAE1, *Pm*PPAE2 and GFP sequences were generated by *in vitro* transcription following the instructions provided for the T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega). Briefly, 1 µg of each DNA fragment was used as a template in 20 µl reaction volume containing 1x RiboMAX™ Express T7 Buffer and 2 µl of the T7 Express Enzyme Mix. Transcription reaction was carried out at 37 °C for 1 hour. The quality and quantity of the resulting ssRNA were verified by agarose gel electrophoresis and UV spectrophotometer, respectively.

To produce dsRNAs, equal amount of sense and anti-sense ssRNAs was mixed and heated at 70 °C for 10 minutes. The mixture was left at room temperature for 30 minutes to allow annealing of dsRNA. DNA template was removed by digestion with 1 µl RQ1 RNase-free DNase I (Promega) at 37 °C for 30 minutes. The potentially inhibitory or interfering components from the reaction mixtures were then removed by precipitated dsRNAs with 1 volume of isopropanol and 0.1 volumes of 3M sodium acetate (pH 5.2). The dsRNA mixtures were chilled at -20 °C for 15 minutes before being centrifuged at 12000 × g for 20 minutes at 4 °C. The dsRNA pellets were then washed with 0.5 ml of 75% (v/v) ethanol and air-dried at room temperature for 5-10 minutes before dissolved in appropriate amounts of the nuclease-free water. The formation of dsRNAs was monitored by determining the product size in TBE-1.5% (w/v) agarose gel electrophoresis based resolution. The separated dsRNAs were visualized by UV transillumination after ethidium bromide staining. The dsRNA concentration was quantified by UV spectrophotometer.

Table 2.2 Nucleotide sequence of primers used for the RNA interference (RNAi) experiments.

Primer	Sequence (5'– 3')	GenBank accession number
RNAi and RT-PCR		
PO1i-F	5'- CACCCTGAGAGCTCCCACTT -3'	AF099741
PO1i-R	5'- GAGATCCTGCAGTGTAACGT -3'	AF099741
PO1iT7-F	5'- *GGATCCTAATACGACTCACTATAGG*CACCCTGAGAGCTCCCACTT -3'	AF099741
PO1iT7-R	5'- *GGATCCTAATACGACTCACTATAGG*GAGATCCTGCAGTGTAACGT -3'	AF099741
PO2i-F	5'- CGTCTTCCTCGGCCGCTCCT -3'	FJ025814
PO2i-R	5'- CCACGGGGATACCGCTGCCA -3'	FJ025814
PO2iT7-F	5'- *GGATCCTAATACGACTCACTATAGG*CGTCTTCCTCGGCCGCTCCT -3'	FJ025814
PO2iT7-R	5'- *GGATCCTAATACGACTCACTATAGG*CCACGGGGATACCGCTGCCA -3'	FJ025814
PPAi-F	5'- CGTCTGCTTCATTGAGGGAGTG -3'	FJ595215
PPAi-R	5'- GTAGTAGATGGTGCCCCAGCCT -3'	FJ595215
T7PPAi-F	5'- *GGATCCTAATACGACTCACTATAGG*CGTCTGCTTCATTGAGGGAGTG -3'	FJ595215
T7PPAi-R	5'- *GGATCCTAATACGACTCACTATAGG*GTAGTAGATGGTGCCCCAGCCT -3'	FJ595215
*Pm*PPAE2i-F	5'- GCGGCGGTCACGCTCCTTGTTC -3'	FJ620685
*Pm*PPAE2i-R	5'- ACTCTCGGGGGCACGCTTGTTG -3'	FJ620685
T7*Pm*PPAE2i-F	5'- *GGATCCTAATACGACTCACTATAGG*GCGGCGGTCACGCTCCTTGTTC -3'	FJ620685
T7*Pm*PPAE2i-R	5'- *GGATCCTAATACGACTCACTATAGG*ACTCTCGGGGGCACGCTTGTTG -3'	FJ620685
GFP-F	5'- ATGGTGAGCAAGGGCGAGGA -3'	U55761
GFP-R	5'- TTACTTGTACAGCTCGTCCA -3'	U55761
GFPT7-F	5'- *TAATACGACTCACTATAGG*ATGGTGAGCAAGGGCGAGGA -3'	U55761
GFPT7-R	5'- *TAATACGACTCACTATAGG*TTACTTGTACAGCTCGTCCA -3'	U55761
RT-PCR		
*Pm*SP1rt-F	5'- TGAGAGCACAAATAGTGGAGGGGTA -3'	FJ620688
*Pm*SP1rt-R	5'- TGGAGGCAGGCACACAGGCAAC -3'	FJ620688
*Pm*SP2rt-F	5'- GGCGTTGGTCTTCACTGCTCTC -3'	FJ620687
*Pm*SP2rt-R	5'- CAGAACTGCCTTCCAAGGATAG -3'	FJ620687
*Pm*SPH1rt-F	5'- TACGTACTCATTGATATCAGGTTTGG -3'	DQ455050
*Pm*SPH1rt-R	5'- GCCTCGTTATCCTTGAATCCAGTGA -3'	DQ455050
*Pm*SPH2rt-F	5'- CCGTGAACCAGCGATGTCCTTA -3'	FJ620686
*Pm*SPH2rt-R	5'- GCCACACTCTCCGCCTGCTCCG -3'	FJ620686
*Pm*SPH3rt-F	5'- GCTCTTGGTGCTGCCGCTGTTG -3'	FJ620689
*Pm*SPH3rt-R	5'- CACCGTCCACGCACAGGTAATA -3'	FJ620689
EF1α-F	5'- GGTGCTGGACAAGCTGAAGGC -3'	-
EF1α-R	5'- CGTTCCGGTGATCATGTTCTTGATG -3'	-

2.6.3 *In vivo* gene silencing

For dsRNA-mediated gene silencing of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2, approximately 5 µg of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 dsRNAs dissolved in 25 µl of saline solution (150 mM NaCl) was intramuscularly injected into the third abdominal segment of the SPF shrimps (2 ± 0.2 g, fresh weight) by using a 0.5-ml insulin syringe with 29-gauge needle. Shrimps injected with an equal volume of saline solution containing 5 µg of GFP dsRNA was served as a sequence independent dsRNA control and shrimps injected with saline solution alone was included as a negative control to evaluate the effected of injection and handling. The repeat injection of dsRNAs or saline solution was carried out together with 20 µg of the lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma) and 20 µg of laminarin (β-1,3-glucan chain with some β-1,6-linked glucose units) (Sigma) at 24 hours after the first dsRNA injection. Shrimps were then reared for 48 hours after the second dsRNA injection prior to further analysis.

2.6.4 Semi-quantitative RT-PCR analysis of the knockdown shrimps

Hemolymph samples were individually withdrawn from the dsRNA-mediated knockdown shrimps at 48 hours after the second injection of either dsRNAs or saline buffer (Section 2.6.3). Total RNA was extracted from knockdown shrimp hemocytes using the NucleoSpin® RNA II (MACHEREY-NAGEL) according to the manufacturer's instructions. Contaminating genomic DNA was removed by on-column rDNase (MACHEREY-NAGEL). The RNA quantity and integrity were verified by UV spectrophotometer and TBE-1.5% (w/v) agarose gel electrophoresis. First-strand cDNA was synthesized from 180 ng of the DNA free total RNA samples using ImProm-II™ Reverse Transcriptase System kit (Promega) and oligo $(dT)_{15}$ primer according to the manufacturer's protocol. The resulted cDNAs were kept at -80 °C until required.

The efficiency of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1, and *Pm*proPO2 gene silencing was determined by semi-quantitative RT-PCR analysis using the gene specific primers PPAi-F and PPAi-R, *Pm*PPAE2i-F and *Pm*PPAE2i-R, PO1i-F and PO1i-R and PO2i-F and PO2i-R, respectively (Table 2.2). Amplification of the EF1-α gene was included as an internal control to monitor the equal loading of cDNA for analysis of the transcript levels. One microlitre of first-strand cDNA was used as a template in a 25 µl of the PCR reaction volume containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.2 mM of each specific primers and 1 unit of *Taq* DNA polymerase (Fermentas). The thermal cycling conditions consisted of 94 °C for 1 minute, followed by 25 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds and 72 °C for 5 minutes. The PCR product was analyzed by a TBE-1.5% (w/v) agarose gel electrophoresis and visualized by UV transillumination after ethidium bromide staining.

The specificity of the gene knockdown was further determined by semi-quantitative RT-PCR analysis. Specific knockdown of the *Pm*proPO1 was determined by amplification of *Pm*proPO1 silenced shrimp cDNAs with *Pm*proPO2 specific primers PO2i-F and PO2i-R (Table 2.2), whilst specific knockdown of *Pm*proPO2 was determined by amplification of *Pm*proPO2 silenced shrimp cDNAs with the *Pm*proPO1 gene specific primers PO1i-F and PO1i-R (Table 2.2). For the PPAE genes, specific gene knockdown of *Pm*PPAE1 was examined by individual amplification of the cDNA from the *Pm*PPAE1 silenced shrimp with the *Pm*PPAE2 gene specific primers *Pm*PPAE2i-F and *Pm*PPAE2i-R (Table 2.2) and specific gene knockdown of *Pm*PPAE2 was also examined by amplification of *Pm*PPAE2 silenced shrimp cDNAs with the *Pm*PPAE1 gene specific primers PPAi-F and PPAi-R (Table 2.2). The effect of gene silencing of the *Pm*PPAE1 and *Pm*PPAE2 genes on other known shrimp clip-domain serine proteinases (clip-SPs) including *Pm*ClipSP1 and *Pm*ClipSP2 and other shrimp clip-domain serine proteinase homologues (clip-SPHs) (*Pm*MasSPH1, *Pm*MasSPH2 and *Pm*MasSPH3) were verified by RT-PCR using gene-specific primers: *Pm*SP1rt-F and *Pm*SP1rt-R for *Pm*ClipSP1, *Pm*SP2rt-F and *Pm*SP2rt-R for *Pm*ClipSP2, *Pm*SPH1rt-F and *Pm*SPH1rt-R for *Pm*MasSPH1,

*Pm*SPH2rt-F and *Pm*SPH2rt-R for *Pm*MasSPH2 and *Pm*SPH3rt-F and *Pm*SPH3rt-R for *Pm*MasSPH3 (Table 2.2). A fragment of the EF1-α gene was also amplified in a separate tube to serve as an internal control for cDNA template normalization. All PCR reactions and amplification steps were carried out in a 25 µl reaction volume containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.2 mM of each specific primers, 1 unit of *Taq* DNA polymerase (Fermentas, USA) and 1 µl of each first-strand cDNA. The thermal cycling conditions consisted of 94 °C for 1 minute, followed by 25 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds and 72 °C for 5 minutes. The PCR product was analyzed by TBE-1.5% (w/v) agarose gel electrophoresis and visualized by UV transillumination after ethidium bromide staining.

2.6.5 Hemolymph phenoloxidase (PO) activity assay in the knockdown shrimps

Hemolymph was withdrawn without the use of any anti-coagulant from the ventral sinus of the randomly selected experimental silenced and control shrimps at 48 hours after the second dsRNA injection (Section 2.6.3). Total protein concentration was determined by a Quick Start™ Bradford protein assay kit (Bio-Rad). Hemolymph phenoloxidase (PO) activity was assayed by using L-3,4-dihydroxyphenylalanine (L-DOPA) (Fluka) as a substrate according to Liu et al. (Liu et al., 2007). Briefly, 2 mg of hemolymph proteins in 435 µl of 10 mM Tris-HCl buffer (pH 8.0) were incubated with 65 µl of freshly prepared L-DOPA (3 mg/ml) (Fluka) at room temperature for 30 minutes. The reaction was stopped by 500 µl of 10% (v/v) acetic acid and the remaining PO activity was spectrophotometrically monitored at 490 nanometres. The hemolymph PO activity was defined as ΔA_{490}/mg total protein/minute. Control samples were prepared using distilled water instead of shrimp hemolymph. The experiment was done in triplicate with 2-3 shrimps per group. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Duncan's test.

2.6.6 Cumulative mortality assay in the knockdown shrimps post *V. harveyi* infection

Pathogenic Gram-negative bacteria, *Vibrio harveyi* isolate 639, was revived from a glycerol stock previously stored at -80 °C using a sterile tryptic soy agar (TSA) plate. The plate was incubated at 30 °C for 18 hours. A single colony was picked and resuspended in 4 ml of tryptic soy broth (TSB) overnight at 30 °C and 40 µl was subsequently inoculated into 8 ml of TSB and cultured at 30 °C. The bacterial concentration was then established by measuring the optical density (OD) at 600 nanometres using spectrophotometer. Four different concentrations 10^6, 10^5, 10^4 and 10^3 colony forming units (CFUs) were assessed as a preliminary experiment for determining the appropriate dose for mortality assay experiments. The bacterial suspension of 2×10^5 CFUs which could kill 40-50% of the shrimps within 2-3 days was selected as the injection dose for the mortality assays.

SPF shrimps (~10 g, fresh weight) were intramuscularly injected with 25 µl of saline solution containing 20 µg of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 dsRNAs (2 µg of dsRNA per 1 g shrimp). Twenty-four hours after the first dsRNA injection, shrimps were again injected intramuscularly with 25 µl of saline solution containing dsRNAs and *V. harveyi* 639 (2×10^5 CFUs). Shrimps injected with GFP dsRNA were included as a control for non-specific dsRNA injections and shrimps injected with saline solution alone were done as an additional control group for monitoring the effect of handling and injection induced mortality. The number of dead shrimps was recorded daily for 5 days post *V. harveyi* infection. The experiment was performed in triplicate with 9-10 healthy shrimps per group. Statistical analysis of the cumulative mortality test was performed using a one-way ANOVA followed by Duncan's test.

2.6.7 Viable bacterial count in hemolymph of the knockdown shrimps post *V. harveyi* infection

Viable bacterial count was carried out according to Liu et al. (Liu et al., 2007) with some modifications. Briefly, SPF shrimps (~10 g in size) were intramuscularly injected with 20 µg of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 dsRNAs. Shrimps injected with GFP dsRNA were included as a control for non-specific dsRNA injections. Twenty-four hours after the first dsRNA injection, shrimps were re-injected with 20 µg of dsRNAs together with 2×10^5 CFUs of *V. harveyi* 639. Hemolymph samples were individually collected from the knockdown shrimp at 6 hours post *V. harveyi* injection. The collected hemolymph samples were serially diluted in phosphate buffer saline (PBS, pH 7.4) and dotted onto the LB-agar plate (10 µl/dot). The plate was incubated overnight at 30 °C and the number of bacterial cells in the knockdown shrimp hemolymph was determined by counting of *V. harveyi* CFUs. All experimental tests were conducted in triplicate with three shrimps per replicate. Statistical analysis was analyzed by one-way ANOVA followed by Duncan's test.

2.7 Recombinant protein expression of pro*Pm*PPAE1 and serine proteinase (SP) domain of *Pm*PPAE1 in *Escherichia coli* expression system

2.7.1 Construction of expression vector

The cDNA sequence encoding for the mature peptide (pro-form) and the serine proteinase (SP) domain of *Pm*PPAE1 were amplified by *Pfu* DNA polymerase (Promega) using the expression primers NcoIFull-ppA-F and NotIppA-R and NcoISP-ppA-F and NotIppA-R, respectively (Table 2.3). For convenience of cloning, an *Nco*I site and 6×histidine tag encoded nucleotides were added to the 5'-end of the NcoIFull-ppA-F and NcoISP-ppA-F primers and a *Not*I site was added to 5'-end of the NotIppA-R primer after the stop codon (Table 2.3). The *Pfu* based PCR amplification was performed at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, 72 °C for 2 minutes and the final extension was

carried out at 72 °C for 10 minutes. The PCR products of expected molecular sizes were gel purified and cloned into the cloning vector pGEM®-T Easy (Promega) before being transformed into *E. coli* JM109 competent cells by heat shock method. Individual colonies grown on LB agar plates containing 50 µg/ml ampicillin were then randomly picked and used as templates in the colony PCR screening with M13 forward and reverse primers. The PCR reaction was performed in 25 µl reaction volume containing each single colony, 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.2 mM of M13 each primer and 1 unit of *Taq* DNA polymerase (Fermentas, USA). The thermal cycling conditions were 94 °C for 3 minute, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minutes followed by 10 minutes of incubation at 72 °C. The PCR products were analyzed by 1.0% (w/v) agarose gel electrophoresis and visualized by UV transillumination after ethidium bromide staining. Recombinant plasmid DNAs were then extracted from the positive clones using a QIAprep spin miniprep kit (Qiagen) according to the manufacturer's protocols and further verified by restriction enzyme digestion and DNA sequencing (Macrogen).

To construct the expression vectors, the recombinant pGEM®-T Easy vector containing the corrected sequences of mature and SP-domain of *Pm*PPAE1 were chosen for restriction enzymes digestion with *Nco*I and *Not*I (Biolabs) and ligated into the same sites in pET28b expression vector (Novagen) before being transformed into *E. coli* JM109. Single colony was randomly picked and used as a template in a 25 µl colony PCR reaction containing 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.2 mM of specific primers for each construct and 1 unit of *Taq* DNA polymerase (Fermentas, USA). The thermal cycling conditions were 94 °C for 3 minute, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minutes followed final extension at 72 °C for 10 minutes. The PCR products were analyzed by a TBE-1.0% (w/v) agarose gel electrophoresis and visualized by UV transillumination after ethidium bromide staining. Plasmid DNA from the cultures containing the correct mature and SP domain of *Pm*PPAE1 constructs (as confirmed by colony PCR) were isolated with a QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. The

acquiring recombinant plasmids were further examined by restriction enzyme digestions and DNA sequencing (Macrogen) to verify the correct reading frame and lack of any mutations before transformed into the chemically competent *E. coli* Rosetta (DE3) pLysS for recombinant protein expression. The parental pET28b vector without any inserts was included as a negative control.

2.7.2 Recombinant protein expression

For recombinant protein expression, positive transformants screened by colony PCR reactions (as above) and negative control cells were grown overnight at 37 °C in 5 ml LB medium containing 50 µg/ml kanamycin. Subsequently, 500 µl of an overnight culture was inoculated in 500 ml fresh LB/kanamycin medium and grown at 37 °C under agitation at 250 rpm until the culture reached an OD_{600} of 0.6. The expression of the recombinant *Pm*PPAE1 (r*Pm*PPAE1) and recombinant SP domain of *Pm*PPAE1 (rSP-domain) were then induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.

Time course analysis was performed to find out the optimal induction time for recombinant protein expression. One millilitre of the bacterial cell culture was time-interval taken every hour until 5 hours post IPTG induction. The bacterial cells were harvested by centrifugation at 8000 rpm for 5 minutes at 4 °C. The bacterial cell pellets were resuspended in 1x SDS sample buffer (45 mM Tris-HCl, 10% (v/v) glycerol, 1% (w/v) SDS, 50 mM dithiothreitol and 0.01% (w/v) bromophenol blue; pH 6.8), boiled for 10 min. and the expressed proteins were analyzed by a reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting.

To determine the solubility of the expressed protein, the cultures were harvested at 4 hours (for rSP-domain) and 5 hours (for r*Pm*PPAE1) post IPTG induction and cells were centrifuged at 8000 rpm for 5 minutes at 4 °C. The bacterial cell pellets were then washed twice with 20 mM Tris-HCl buffer (pH 8.0) and resuspended in the same buffer. To determine whether native or denaturing conditions were necessary for protein purification, the cell suspensions were disrupted by

sonication for 10 minutes at 0 °C and cell lysates were then centrifuged at 11000 rpm for 20 minutes at 4 °C. Soluble and insoluble fractions were collected and subjected to SDS-PAGE analysis and the protein bands were visualized by Coomassie brilliant Blue R-250 staining.

2.7.3 Protein purification

After analysis of the protein fractions, both r*Pm*PPAE1 and rSP-domain were expressed in an insoluble form. Therefore, the pellets containing insoluble protein (inclusion bodies) were subjected to purify by a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen) under the denaturing conditions according to the manufacturer's suggestions. Briefly, inclusion bodies were washed twice in 20 mM Tris-HCl (pH 8.0) and dissolved in a denaturing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8M Urea; pH 8.0). The solutions were applied to a Ni-NTA agarose column (Qiagen), washed with a denaturing buffer containing 20 mM imidazole, and eluted by a denaturing buffer containing 250 mM imidazole. Purified proteins were resolved electrophoretically on reducing SDS-PAGE and visualized by Coomassie brilliant blue R-250 staining.

2.7.4 Protein refolding

For recombinant protein refolding, 1ml of elution fractions containing the recombinant protein were collected and pooled fractions were dialyzed against 50 mM Tris-HCl pH 8.0 overnight at 4 °C. The dialyzed protein sample was then centrifuged at 13000 rpm for 20 min at 4 °C to remove unfolded or aggregated proteins and analyzed by a reducing SDS-PAGE. The protein concentration was determined using a Quick Start™ Bradford protein assay kit (Bio-Rad) according to the manufacturer's instructions. The protein molecular weight was estimated by co-resolution with standard markers through a reducing SDS-PAGE gel.

2.7.5 Production of rabbit polyclonal antibody

For preparation of anti-SP-domain antisera, the affinity-purified recombinant rSP-domain protein was further dialyzed against the phosphate buffer saline (PBS; pH 7.4) and the obtained protein was used as an immunogen for preparing a rabbit anti-rSP-domain polyclonal antiserum by a commercial service at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

2.7.6 SDS-PAGE and western blot analysis

Protein samples were mixed with 2x SDS sample buffer (90 mM Tris-HCl, 20% (v/v) glycerol, 2% (w/v) SDS, 0.1 M dithiothreitol and 0.02% (w/v) bromophenol blue; pH 6.8) and denatured by boiling for 10 minutes. The SDS-PAGE was carried out in 1x Tris-glycine electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS; pH 8.3) at 20 mA/gel for 90 minutes. The prestained protein ladder (Fermentas) was included as the standard protein marker. Gels were stained in 0.1%(w/v) Coomassie brilliant Blue R-250 in 45%(v/v) methanol and 10%(v/v) acetic acid and then de-stained in 10%(v/v) methanol and 10%(v/v) acetic acid.

For western blot analysis, protein samples from unstained SDS-PAGE gel were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) in a transfer buffer (48 mM Tris-HCl, 39 mM glycine and 20% (v/v) methanol; pH 9.2) using Trans-Blot® SD (Bio-Rad) at 100 mA for 1 hour. The membrane was then blocked with 5% (w/v) skim milk powder in Tris buffered saline-Tween (TBST; 20 mM Tris-HCl, 150 mM NaCl and 0.05% (v/v) Tween 20; pH 7.5) at 4°C overnight followed by washing three times with the same buffer. Western blot analysis was carried out using anti-His antisera (1:3000 dilution; Amersham Biosciences) or anti-rSP-domain (1:1000 dilution) followed by incubation at 37°C for 1 hour. After washing three times with TBST buffer, the membrane was then incubated with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG) antibodies (1:5000 dilution; Jackson ImmunoResearch Laboratories, Inc.) for anti-His antisera or alkaline phosphatase-conjugated anti-rabbit IgG antibodies

(1:20000 dilution; Promega) for anti-rSP-domain at 37°C for 1 hour. After washing three times with the same buffer, antibody binding was visualized by a color reaction using 5-Bromo-4-chloro-3-indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) (Sigma) as chromogenic substrate.

2.8 Recombinant protein expression of pro*Pm*PPAE1 in insect cells using BAC-to-BAC® Baculovirus Expression System

2.8.1 Preparation of signal peptide for recombinant protein secretion

For an efficient secretion of the recombinant protein produced in insect cells using the plasmid pFastBac™1 (Invitrogen Life Technologies), the synthetic DNA fragment encoding for the signal peptide of *Manduca sexta* proPO-activating proteinase-2 (PAP-2) was generated using two olinucleotides Signal-F and Signal-R (Table 2.3). Signal-F contained the *Bam*HI restriction site fused with the sequence encoding for Met-Asn-Ile-Val-Leu-Ala-Leu-Cys-Val-Phe-Ala-Val-Ser-Ala-Ser-Phe-Ala-Ser-Gly-Gln-Ala-Met-Glu-His-Met-Glu-Phe followed by *Nco*I, *Nde*I and *EcoR*I restriction sites. Signal-R included the same restriction sites, but fused with the reverse complement sequence of the PAP-2 signal peptide. Two oligonucleotides (Signal-F and Signal-R) were annealed by incubated at 70 °C for 1 minute followed by slowly cooled to room temperature for 30 minutes. The fragments were further ligated into pGEM®-T Easy (Promega) according to the manufacturer's instruction followed by transformation into the chemically competent *E. coli* JM109 cells as described above. Colony PCR screening was then performed and the positive recombinant clone was selected followed by plasmid DNA extraction using a QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. Restriction enzyme digestion and DNA sequencing were carried out to confirm that the signal sequences were corrected. The recombinant plasmid containing the signal peptide fragment was digested with *Bam*HI and *Nco*I overnight at 37 °C. The digested products were resolved by 1.5% (w/v) agarose gel and purified using NucleoSpin® Extract II (MACHEREY-NAGEL) according to the manufacturer's instructions. The

*Bam*HI-*Nco*I signal peptide fragments were kept at -20 °C until required for fusing with the pFastBac™1.

2.8.2 Construction of recombinant transfer vector

To produce the recombinant *Pm*PPAE1 (r*Pm*PPAE1) proteins using a site-specific transposition baculovirus expression system (Bac-to-Bac, Invitrogen Life Technologies), a cDNA sequence encoding for the mature peptide of *Pm*PPAE1 with 6× histidine tag attached at the C-terminus was amplified by *Pfu* DNA polymerase (Promega) using the expression primer pairs BacPPA-F and BacPPA-R with a *Nco*I and *Xho*I sites at the 5'-end of each primer, respectively (Table 2.3). The amplification conditions consisted of an initial denaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 2 minutes and final extension at 72 °C for 10 minutes. The PCR products were separated through a TBE-1.0% (w/v) agarose gel electrophoresis and the expected DNA fragment was cut from the gel and purified by the NucleoSpin® Extract II (MACHEREY-NAGEL) following the instruction protocols. The purified DNA fragment was cloned into the pGEM®-T Easy vector (Promega) and then transformed into *E. coli* JM109 by heat shock method. The single colony grown on LB agar plates containing 50 µg/ml ampicillin were randomly selected for colony PCR screening using the *Pm*PPAE1 gene specific primers as described above. The positive clones were selected for plasmid DNA extraction using a QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was then verified by restriction enzyme digestion followed by DNA sequencing (Macrogen). The recombinant plasmid containing the correct *Pm*PPAE1 sequence was digested with *Nco*I and *Xho*I (Biolabs) and *Pm*PPAE1 DNA fragment was then gel purified using the NucleoSpin® Extract II kit (MACHEREY-NAGEL) according to the respective manufacturer's instructions.

To construct the recombinant pFastBac™1 containing PAP-2 signal peptide and the mature *Pm*PPAE1, the *Bam*HI-*Nco*I signal peptide fragment and the *Nco*I-*Xho*I mature *Pm*PPAE1 fragment were inserted into *Bam*HI-*Xho*I sites of pFastBac™1 by using a three-fragment ligation reaction. The ligation product was then transformed

into *E. coli* JM109 by heat shock method and the resulting transformants were checked by colony PCR as described above. The plasmid DNA was extracted from the positive clone using a QIAprep spin miniprep kit (Qiagen) and further verified by restriction enzyme digestion and DNA sequencing (Macrogen).

2.8.3 Preparation of recombinant bacmid DNA for BAC-to-BAC® Baculovirus Expression System

Recombinant pFastBac™1 was transformed into MAX Efficiency® DH10Bac™ *E. coli* competent cells (Invitrogen Life Technologies). *In vivo* transposition of the Signal-*Pm*PPAE1 expression cassette into bacmid DNA in *E. coli* DH10Bac™ and selection of the colonies containing recombinant bacmid was performed according to the manufacturer's instruction for BAC-to-BAC® Baculovirus Expression System (Invitrogen Life Technologies) (Figure 2.1). Briefly, 5 ng of recombinant pFastBac™1 construct was incubated with 25 µl of DH10Bac™ *E. coli* competent cells in a 2 ml round-bottom tube for 30 minutes. Transformation was performed by heat-shock method. The transformed cells were incubated at 37 °C for 4 hours with shaking. Ten-fold serial dilution of cells (10^{-1} and 10^{-2}) was then prepared and 100 µl from each dilution was plated on an LB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal and 40 µg/ml IPTG. The plates were incubated at 37 °C for 48 hours. Ten white colonies were then picked, re-streaked on the fresh LB agar plates containing the same components and further incubated overnight at 37 °C. To verify the successful of transposition, single colonies from each re-streak plate was picked for colony PCR screening using M13 forward and reverse primers. The positive clone was inoculated in a LB medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline for recombinant bacmid DNA isolation using S.N.A.P.™ MidiPrep Kit (Invitrogen Life Technologies) according to the respective manufacturer's protocols.

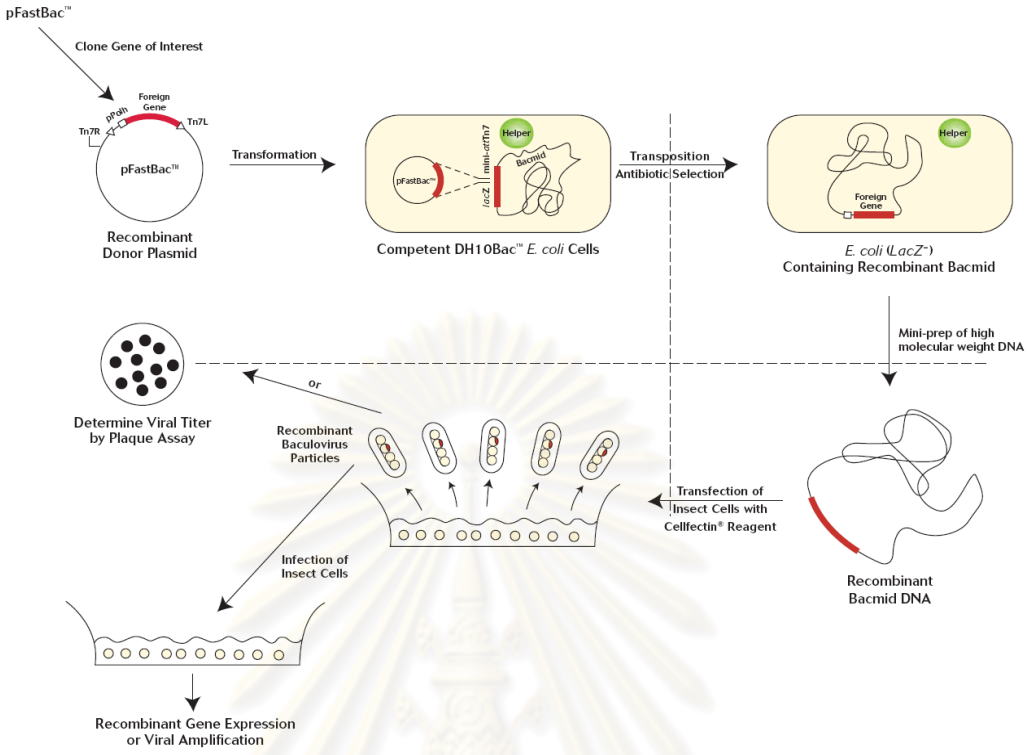

Figure 2.1 Schematic diagram illustrate the generation of recombinant baculovirus and recombinant protein expression using the Bac-to-Bac® Baculovirus Expression System.

2.8.4 Generation of *Pm*PPAE1 recombinant virus by Cellfectin Reagent-mediated transfection

Recombinant baculoviruses harboring *Pm*PPAE1 gene was generated *in vivo* using lipid-mediated transfection reaction following the instruction provided for the BAC-to-BAC® Baculovirus Expression System (Invitrogen Life Technologies) (Figure 2.1). Insect cell line *Spodoptera frugiperda* 9 (Sf9) 9×10^5 cells were seeded in to each well of the 6-well cell culture plates. The transfection reaction was prepared by incubated 1 µg of recombinant bacmid DNA dissolved in 100 µl of unsupplemented Grace's insect medium with 8 µl of Cellfectin® II Reagent (Invitrogen Life Technologies) dissolved in 100 µl of unsupplemented Grace's insect medium at room temperature for 30 minutes. The bacmid DNA:lipid complex was diluted with 0.8 ml of the antibiotic-free unsupplemented Grace's insect medium before added to the Sf9 cells followed by incubation at 27 °C for 4 hour.

Subsequently, the DNA:lipid complex was completely removed followed by addition of 2 ml of the Sf-900 II (Invitrogen Life Technologies) containing 5% fetal bovine serum (FBS; Sigma) and 1x antibiotic-antimycotic (100 units of penicillin (base), 100 µg of streptomycin (base), and 0.25 µg/ml of amphotericin B utilizing penicillin G (sodium salt), streptomycin sulfate and amphotericin B; Invitrogen Life Technologies) to the cells and the transfected cells were maintained in a 27 °C humidified incubator for 7 days or until the sign of late state of viral infection was observed.

2.8.5 Amplification of the recombinant viral stock

The P1 viral stock was obtained by collected the medium from each well at days 7 post transfection. The baculoviral stock harvested from the culture medium was filtered through a 0.45 µm filter. The P1 viral stock was used for further amplification of the virus stock. Sf9 2×10^6 cells were seeded into each well of the 6-well cell culture plate and cells were infected with an appropriate amount of P1 viral stock for 72 hours at 27 $^{\circ}$C. The medium containing P2 viral stock was then harvested and filtered through a 0.45 µm filter. Production of the high-titer P3 viral stock which used as a working viral stock was performed as described for P2 viral stock, but Sf9 cells were infected the with the P2 instead of P1 viral stock. Viral stock was kept at 4 $^{\circ}$C under light protected conditions.

2.8.6 Viral plaque assay

Viral plaque assay was performed to determine titers of the working recombinant *Pm*PPAE1 viral stocks. Sf9 cells were prepared at the cell density of 5×10^5 cells/ml in Sf-900 II SFM (Invitrogen Life Technologies) and 2 ml of cell suspensions were seeded into each well of the 6-well cell culture plate. An 8-log serial dilution (10^{-1} to 10^{-8}) of the working viral stock was prepared by diluted the P3 viral stock in an appropriate amount of the Sf-900 II SFM. One millilitre of the diluted virus (10^{-4} to 10^{-8}) and the negative control (no virus) were added into each well of the 6-well plates and cells were further incubated at room temperature for 1 hour. The medium containing viruses were then removed and cells were substituted with 2 ml of the plaquing medium (30 ml of 1.3x Sf-900 Medium and 10 ml of the melted 4%

(w/v) agarose gel). The plaquing overlay was left for hardening at room temperature for 1 hour before placing into the 27 °C humidified incubator for 10 days. Plaques were visualized by Neutral red solution staining (1 mg/ml in cell-culture grade, distilled water) and the viral titer was calculated by using the following formula and expressed as plaque forming units (pfu)/ml.

$$\text{Titer (pfu/ml)} = \text{number of plaques} \times \text{dilution factor} \times \frac{1}{\text{ml of inoculums/well}}$$

2.8.7 Optimization of the recombinant *Pm*PPAE1 production

To optimize the expression of r*Pm*PPAE1 in insect cells, Sf9 2×10^6 cells were seeded into each well of a 6-well cell culture plate and infected with the recombinant virus at a multiplicity of infection (MOI) ranging from 0.01 to 5 (0.01, 0.1. 0.5, 1, 2.5 and 5) for 3-4 days. Whole cell lysates and the culture medium were subjected to analyze by SDS-PAGE. A time course analysis of the r*Pm*PPAE1 production was then performed by infected 2×10^6 Sf9 cells with the desired MOI (MOI of 0.5). Cells and media were harvested at 24, 48, 72 and 96 hour post-infection and the proteins were resolved electrophoretically on reducing SDS-PAGE and visualized by Coomassie brilliant blue R-250 staining.

2.8.8 Recombinant protein production

To produce r*Pm*PPAE1 in insect cells, Sf9 2×10^6 cells were infected with the recombinant baculoviruses at MOI of 0.5. The infected cells were maintained at 27 °C for 96 hours before being harvested. The culture medium was aspirated and the cells collected from each well were pooled together and centrifuged at 5000 rpm for 5 minutes at 4 °C. The cell pellet was washed once with 50 mM sodium phosphate buffer containing 300 mM NaCl (pH 7.4) and resuspended with the same buffer. Cell disruption was performed by sonication for 5 minutes at 0 °C. The cell lysate was centrifuged at 11000 rpm for 5 minutes at 4 °C. The supernatant and pellet were analyzed by SDS-PAGE.

2.8.9 Recombinant protein purification

The r*Pm*PPAE1 was purified under denaturing conditions. Briefly, the infected cells were suspended in lysis buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4 and 300 mM NaCl and 8 M Urea; pH 7.4) and the insoluble material was removed by centrifugation at 11000 rpm for 20 min at 4 °C. The clear supernatant containing the r*Pm*PAPE1 was then loaded onto a HisPur Cobalt resin column (Pierce) previously equilibrated with the same buffer under the gravity flow. The flow-through fraction was collected and re-applied to the column again. The column was then washed twice with 4 ml of wash buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 300 mM NaCl, 8 M Urea and 10 mM imidazole; pH 7.4). The r*Pm*PPAE1 was eluted from the column with elution buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 300 mM NaCl, 8M Urea and 150 mM imidazole; pH 7.4). The eluted fractions were analyzed by SDS-PAGE.

Table 2.3 Nucleotide sequence of primers used for recombinant protein expression.

Primer	**Sequence (5'– 3')**
Bacterial expression system	
NcoIFull-ppA-F	5'- CATGCCATGGGC*CATCATCATCATCATCAT*GCTGTGGGTG ATGAACAGCTCTCAAG -3'
NcoISP-ppA-F	5'- CATGCCATGGGC*CATCATCATCATCATCAT*CGCATCGTGG GCGGAAAGGACGCCGA -3'
NotIppA-R	5' ATAAGAATGCGGCCGCTTAGACTGCATTATTTTTAATCC AGTC 3'
Insect cell expression system	
BacPPA-F	5'- CATGCCATGGCTGTGGGTGATGAACAGCTCTCAAG -3'
BacPPA-R	5'- CGCCTCGAGCTAATGATGATGATGATGATGGACTGCA TTATTTTTAATCCAGTC 3'
Signal-F	5'- GGATCCATGAATATCGTACTCGCTTTGTGTGTCTTCGC GGTTAGTGCAAGCTTTGCGAGTGGACAAGCCATGGAGCA TATGGAATTC -3'
Signal-R	5'- GAATTCCATATGCTCCATGGCTTGTCCACTCGCAAAGG CACTAACCGCGAAGACACACAAAGCGAGTACGATATTCA TGGATCC -3'

2.9 Functional characterization of the recombinant protein

2.9.1 Proteinase activity assay

The proteinase activity of recombinant *Pm*PPAE1 protein on the hydrolysis of synthetic chromogenic substrates was assayed in a 100 µl reaction volume of 50 mM Tris-HCl (pH 8.0) containing different amounts of recombinant protein incubated with the synthetic chromogenic substrate specific for trypsin and subtilisin (*N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide; 150 and 300 mM, respectively), chymotrypsin (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; 150 mM) and elastase (*N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide; 450 mM) at 30 °C for 20 minutes. The reaction was terminated by 50% acetic acid. The formation of *p*-nitroaniline was spectrophotometrically measured at 405 nanometres and one unit of proteinase activity was defined as ΔA_{405}/minute.

2.9.2 Phenoloxidase (PO) activity assay

To prepare the hemocyte lysate supernatant (HLS) for phenoloxidase (PO) activity assay, hemolymph was individually withdrawn from the ventral sinus cavity of the normal shrimp using the anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM trisodium citrate, 10 mM EDTA; pH 4.6) (Söderhäll and Smith, 1983). After centrifugation at 800 × g for 10 minutes at 4 °C, the hemocyte pellet was washed twice and homogenized in the cacodylate (CAC) buffer (10 mM sodium cacodylate; pH 7.0) before being centrifuged at 25000 × g for 20 minutes at 4 °C to obtain the HLS.

To examine the biological activity of the recombinant *Pm*PPAE1 protein on activation of shrimp proPO system, the HLS and the recombinant protein was preincubated with LPS (from *Escherichia coli* 0111:B4; Sigma) and laminarin (β-1,3-glucan chain with some β-1,6-linked glucose units; Sigma) at room temperature for 30 minutes. The reaction mixtures were further incubated with L-DOPA for 30 minutes at room temperature. PO activity was measured at 490 nanometres. HLS preincubated with the buffer was done as a negative control. PO activity was defined as an increase in A_{490}/mg total protein/minute.

2.10 Detection of an endogenous *Pm*PPAE1 expression in shrimp hemolymph

In an effort to investigate the expression of an endogenous *Pm*PPAE1, hemolymph was withdrawn from the ventral sinus in an anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM trisodium citrate, 10 mM EDTA; pH 4.6) (Söderhäll and Smith, 1983) and centrifuged at 800 × g for 10 min at 4 °C. The supernatant was used as cell-free plasma. The hemocyte pellet was washed twice and homogenized in 150 mM NaCl containing 2 mM EDTA followed by centrifugation at 25000 × g for 20 minutes at 4 °C. The resulting supernatant was kept on ice and used as the hemocyte lysate supernatant (HLS).

Approximately 20 µg of shrimp HLS and 100 µg of cell-free plasma protein were separated on a 15% (w/v) reducing SDS-PAGE gel and visualized by staining with Coomassie brilliant blue or identified by western blotting. For western blot analysis, separated proteins from SDS-PAGE gel were electro-transferred to the PVDF membrane (Amersham Biosciences) using Trans-Blot® SD (Bio-Rad). The membrane was blocked with 5% (w/v) non-fat dry milk in Tris Buffered Saline-Tween (TBST; 20 mM Tris-HCl, 150 mM NaCl and 0.05% (v/v) Tween 20; pH 7.5) at 4°C overnight followed by washing three times with the same buffer. Rabbit polyclonal antisera against the SP-domain of *Pm*PPAE1 (1:1000 dilution in TBST) was used as the primary antibody and membrane was further incubated at 37°C for 1 hour. After washing three times with TBST buffer, the membrane was incubated with alkaline phosphatase-conjugated to goat anti-rabbit immunoglobulin G (IgG) antibodies (1:20000 dilution in TBST; Promega) at 37°C for 1 hour. After secondary antibody incubation and washing, the alkaline phosphatase antibody-protein band complex was visualized by a color reaction using 5-Bromo-4-chloro-3-indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) (Sigma) as chromogenic substrate for alkaline phosphatase.

CHAPTER III

RESULTS

3.1 Characterization of the proPO system-associated genes from *P. monodon*

3.1.1 Characterization of *Pm*PPAE1 and *Pm*PPAE2 genes

In the black tiger shrimp *Penaeus monodon*, two putative prophenoloxidase (proPO)-activating enzymes (PPAEs) designated as *Pm*PPAE1 and *Pm*PPAE2 were identified from hemocytes of the normal shrimp by using the degenerate primer pairs designed from the conserved sequences of the PPAE genes reported in several arthropod species (Charoensapsri et al., 2009, 2011). The cDNA sequence of *Pm*PPAE1 and *Pm*PPAE2 genes were most similar to the crustacean and insect PPAEs, respectively. Consequently, these two *Pm*PPAE genes were selected for further characterization.

A full-length cDNA sequence of the putative *Pm*PPAE1 (GenBank accession number FJ595215) obtained by rapid amplification of cDNA end (RACE)-PCR, comprised of 1515 bp, including a 55 bp of 5'-untranslated region (UTR), a 1392 bp open reading frame (ORF) and a 68 bp of 3'-UTR (Figure 3.1) (Charoensapsri et al., 2009). The ORF encoded a predicted polypeptide of 463 amino acid residues with a potential signal peptide cleavage site, as identified by the online SignalP 3.0 program, located between amino acid position 18 and 19 (AA-AV). The calculated molecular mass of the mature protein (445 amino acids) was 50.54 kDa with an estimated isoelectric point (p*I*) of 7.46. The predicted protein domain of *Pm*PPAE1, as identified by SMART program, consists of an N-terminal clip domain and a C-terminal serine proteinase (SP) domain. Two potential *N*-glycosylation sites NGS and NAT were identified at the amino acid position 42 and 192, respectively, suggesting it might be a glycosylated protein.

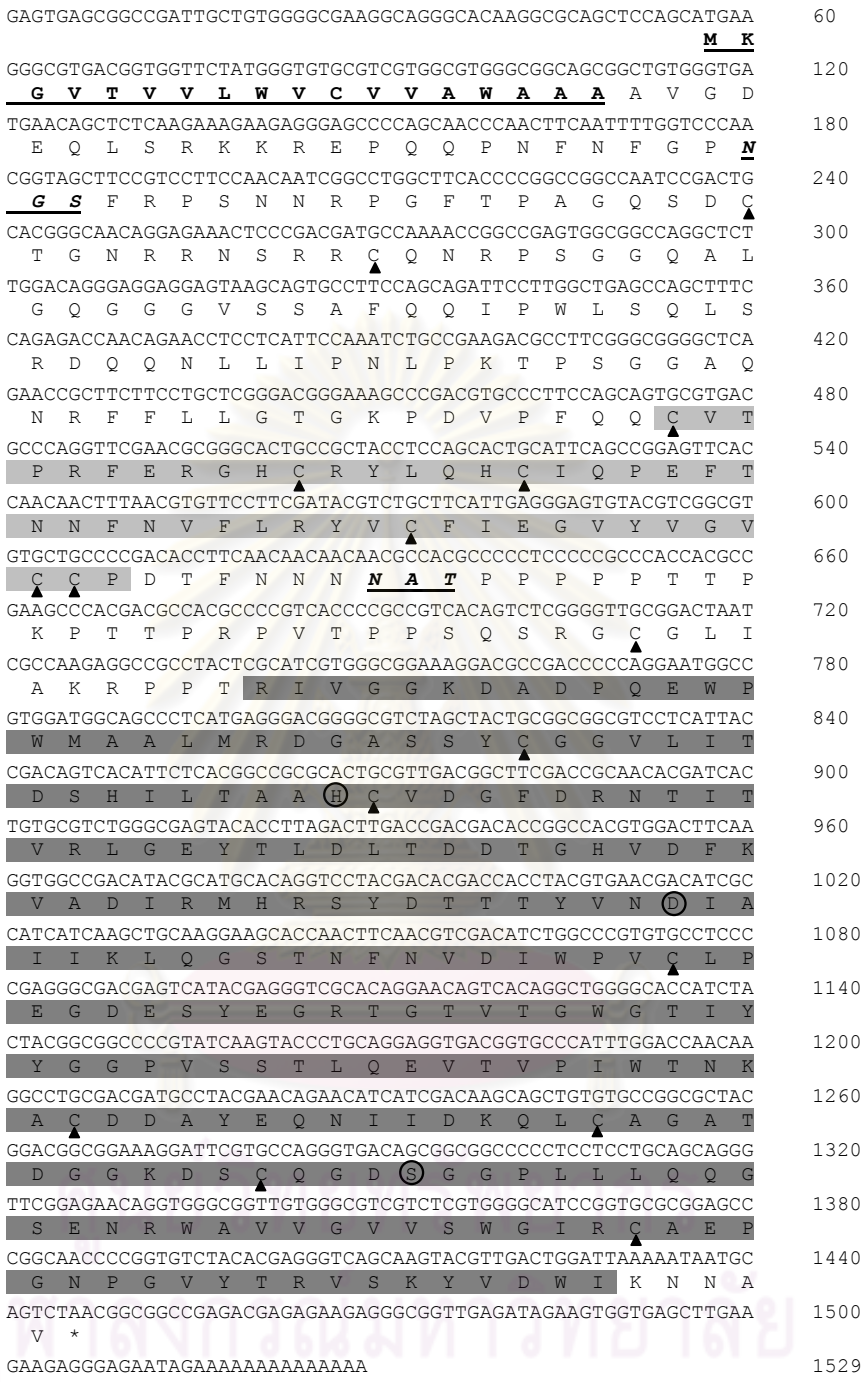

Figure 3.1 Nucleotide and deduced amino acid sequences of *P. monodon Pm*PPAE1. The deduced amino acid sequence is shown by a single abbreviation below the second nucleotide of each codon. The signal peptide is indicated with boldfaced and underlined. The clip-domain and serine proteinase domain are shown by grey and dark grey highlight, respectively. Two putative *N*-glycosylation sites are boldfaced and italicize-underlined. The catalytic residues at the active site are labeled with a circle. The cysteine residues are marked with the arrowheads.

A full-length cDNA sequence of the putative *Pm*PPAE2 (GenBank accession number FJ620685) obtained by RACE-PCR, comprised of 1557 bp, including a 29 bp of 5'- UTR, a 1116 bp open reading frame (ORF) and a 412 bp of 3'-UTR (Figure 3.2) (Charoensapsri et al., 2011). The deduced ORF of *Pm*PPAE2 encoded for a predicted protein of 371 amino acid residues with a putative 25 amino acid residues of a secretion signal peptide. The deduced mature peptide of *Pm*PPAE2 consisted of 346 amino acid residues with a calculated molecular mass of 36.52 kDa and an estimated p*I* of 7.49. SMART analysis revealed that the predicted protein domain of *Pm*PPAE2 consists of an N-terminal clip-domain and a C-terminal SP domain. One *N*-glycosylation site (NVT) was identified at the amino acid position 84 in the N-terminal part of the *Pm*PPAE2 polypeptide chain suggesting it might be a glycoprotein.

Database searching using the BlastX program revealed that *Pm*PPAE1 is most similar to the crustacean PPAE with 70% amino acid sequence similarity to the freshwater crayfish *Pacifastacus leniusculus* PPAE (AJ007668). *Pm*PPAE1 also showed the sequence similarity of 62% to the lepidopteran insects *Manduca sexta* hemocyte protease-1 (AF017663), followed by the *Bombyx mori* hemocyte protease (61% similarity; NM_001046950) and the horseshoe crab *Tachypleus tridentatus* proclotting enzyme (59% similarity; M58366), respectively. On the other hand, sequence analysis showed that *Pm*PPAE2 is more similar to the insect than those of the crustacean PPAEs with 51% amino acid sequence similarity to the proPO-activating proteinase 1 (PAP1) of the tobacco hornworm *Manduca sexta* (AF059728), followed by the *Biston betularia* proPO activating proteinase 1 (GU953224; 51% similarity) and the *Aedes aegypti* serine protease (XM_001647815; 50% similarity), respectively.

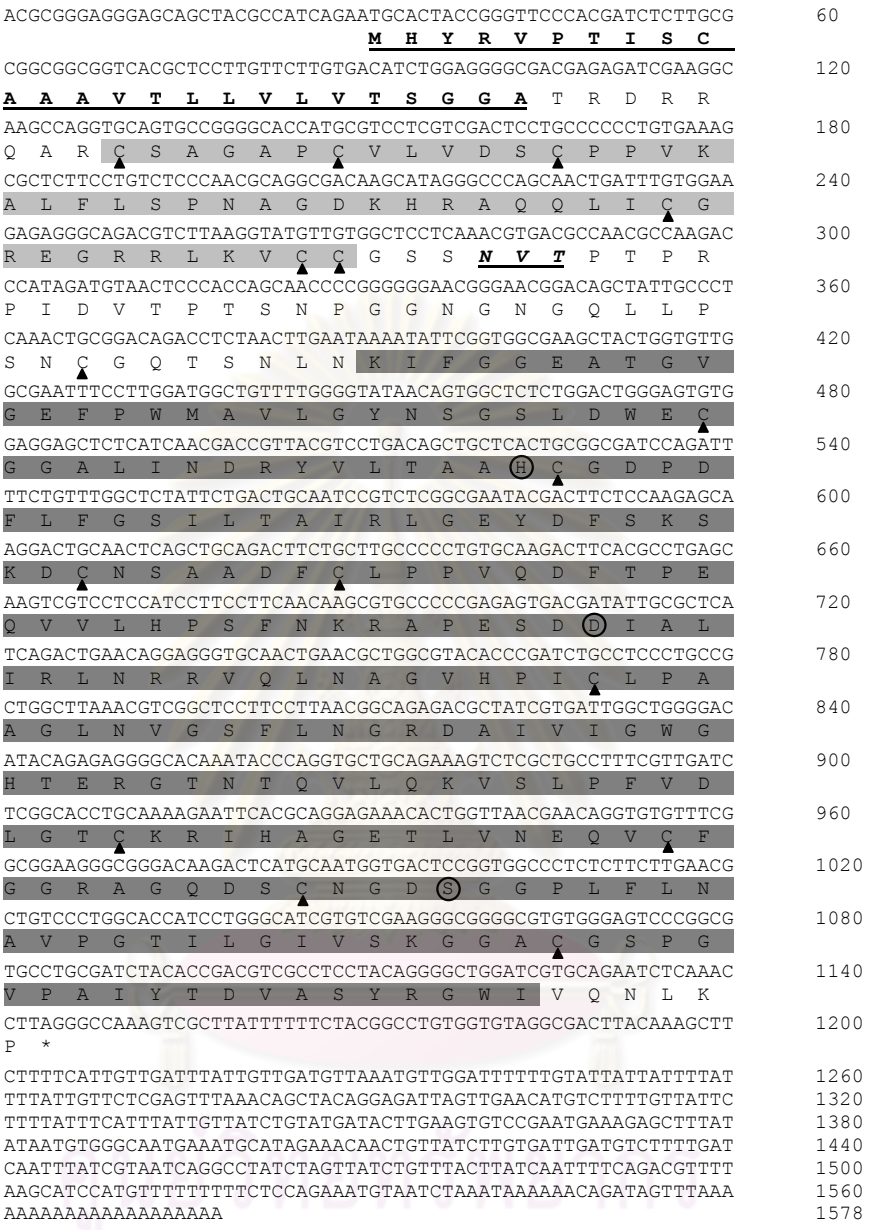

Figure 3.2 Nucleotide and deduced amino acid sequences of *P. monodon Pm*PPAE2. The deduced amino acid sequence is shown by a single abbreviation below the second nucleotide of each codon. The signal peptide is indicated with boldfaced and underlined. The clip-domain and serine proteinase domain are shown by grey and dark grey highlight, respectively. Two putative *N*-glycosylation sites are boldfaced and italicize-underlined. The catalytic residues at the active site are labeled with a circle. The cysteine residues are marked with the arrowheads.

Multiple amino acid sequence alignment of *Pm*PPAE1 and *Pm*PPAE2 with other insect and crustacean PPAEs using the ClustalW2 program indicated that both *Pm*PPAE1 and *Pm*PPAE2 shared the common structural domain features of the clip-domain serine proteinase family to those of insect and crustacean PPAEs with an N-terminal clip domain followed by a linker sequence and a C-terminal trypsin-like serine proteinase (SP) domain (Figure 3.3). Six cysteine residues that formed three disulfide bridges (Cys1-Cys5, Cys2-Cys4, Cys3-Cys6) within the N-terminal clip domain of *Pm*PPAE1 and *Pm*PPAE2 were strictly conserved and three conserved catalytic triad (His271, Asp320 and Ser413 for *Pm*PPAE1 and His165, Asp227 and Ser323 for *Pm*PPAE2) were located at the C-terminal trypsin-like serine proteinase domain (Figure 3.4). The additional N-terminal glycine-rich (glycine 13.2%) and proline-rich (proline 31.8%) domains were observed within the complete sequence of shrimp *Pm*PPAE1 and these two domains were similar to the glycine-rich (glycine 18.5%) and proline-rich (proline 31.8%) domains of the crayfish PPAE, respectively. However, *Pm*PPAE2 showed a rather different overall sequence from the *Pm*PPAE1 and crayfish PPAE due to the two additional glycine-rich and proline-rich domains were not detected. Nevertheless, *Pm*PPAE2 still has a broad similar domain structure to that of PPAEs reported in many insect and crustacean species. Taken together, the *Pm*PPAE1 was identified as a crustacean PPAE, whilst the *Pm*PPAE2 which is more similar to the insect than to those of the crustacean PPAEs was classified as a second class of the shrimp PPAE.

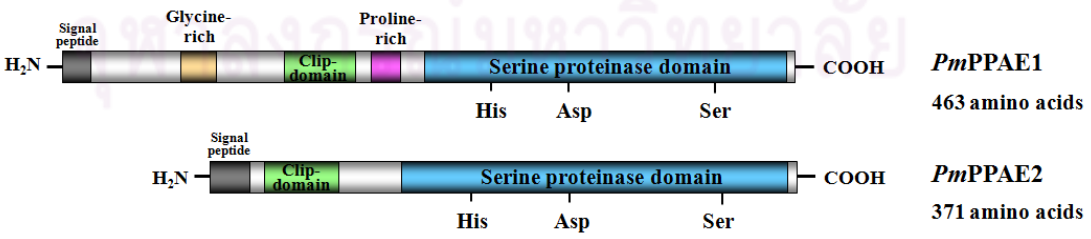

Figure 3.3 A schematic diagram representation of the domain structure of *P. monodon* *Pm*PPAE1 and *Pm*PPAE2. The clip-domain and serine proteinase domain are illustrated by green and blue boxes, respectively. Dark grey boxes indicate the putative signal peptides. Glycine-rich and proline-rich domains of the *Pm*PPAE1 are denoted by orange and pink boxes, respectively.

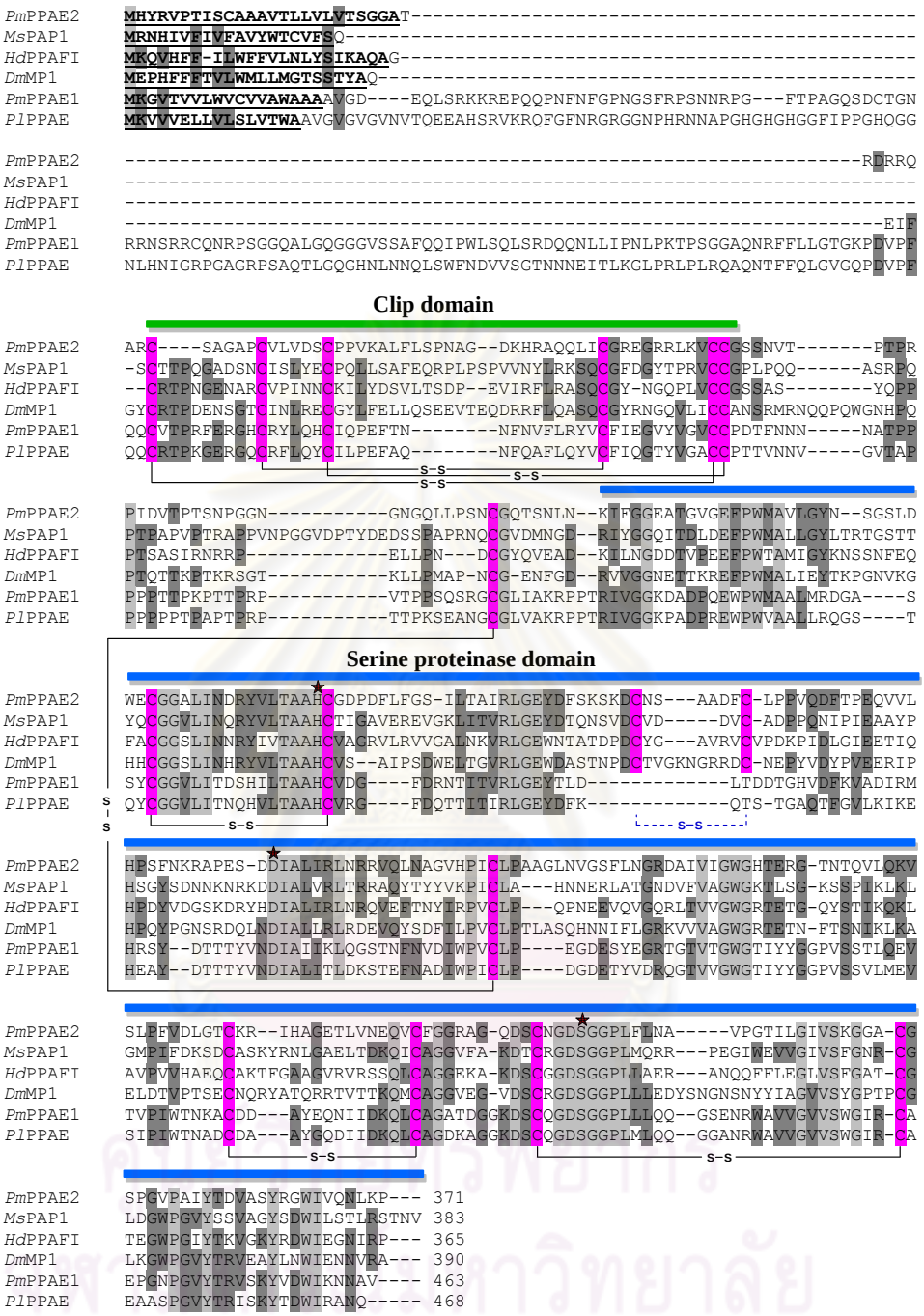

Figure 3.4 Multiple sequence alignment of *P. monodon* *Pm*PPAE1 and *Pm*PPAE2 with other arthropod PPAEs: Insects; *Manduca sexta* proPO-activating proteinase 1 (*Ms*PAP1: AF059728), *Holotrichia diomphalia* proPO-activating factor I (*Hd*PPAFI: AB013088), and *Drosophila melanogaster* Melanization protease 1 (*Dm*MP1: NM_141193); and crayfish *Pacifastacus leniusculus* proPO-activating enzyme (*Pl*PPAE: AJ007668). The signal peptides are indicated with boldfaced and underlined. The conserved cysteine residues are shown in pink highlights. The disulfide linkages are shown in black lines, whereas a blue dash line indicates the disulfide linkage that found only in the insect PPAEs and shrimp *Pm*PPAE2. Identical amino acid residues are grey shaded, whilst those conserved among three or more species are dark grey shaded. The catalytic triads are indicated by red stars.

3.1.2 Characterization of *Pm*proPO1 and *Pm*proPO2 genes

Two distinct homologous *P. monodon* proPO genes named *Pm*proPO1 and *Pm*proPO2 were found to be present in the black tiger shrimp *P. monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006). *Pm*proPO1 gene displays the same sequence and thus likely to be the same proPO gene with that previously reported by Sritunyalucksana et al. (GenBank accession number AF099741) (Sritunyalucksana et al., 1999), whereas *Pm*proPO2 gene is suggested to be a novel *P. monodon* proPO gene. Therefore, the two shrimp *Pm*proPO genes were subjected to further characterization.

The full-length cDNA sequence of the novel *Pm*proPO2 (GenBank Accession number FJ025814) which was obtained by RACE-PCR comprised of 2513 bp with a predicted 2070 bp ORF encoding for a polypeptide of 689 amino acids (Amparyup et al., 2009) (Figure 3.5). The cDNA contained a 5'-UTR of 113 bp and a 3'-UTR of 330 bp. Like other arthropod proPOs and shrimp *Pm*proPO1, no putative signal peptide cleavage site was predicted at the N-terminal part of the *Pm*proPO2, hence the predicted molecular mass and the theoretical p*I* of this protein were 79.21 kDa and 6.69, respectively (Table 3.1). Based on the sequence comparison with the putative cleavage site of *Pm*proPO1 (Sritunyalucksana et al., 1999), the proteolytic cleavage site of *Pm*proPO2 was predicted to be located between Arg45 and Leu46 and cleavage at this site generates a putative active enzyme of 74.0 kDa. Five putative *N*-glycosylation sites including NET, NET, NNS, NIS and NLS were presented in the *Pm*proPO2 sequence at the amino acid position 119, 172, 275, 375 and 432, respectively and a RGD like motif was found in the N-terminal region of the *Pm*proPO2. Structurally, the conserved copper-binding sites A and B and a thiol ester-like motif (GCGWPQHM) were also identified in the *Pm*proPO2 sequence (Figure 3.6).

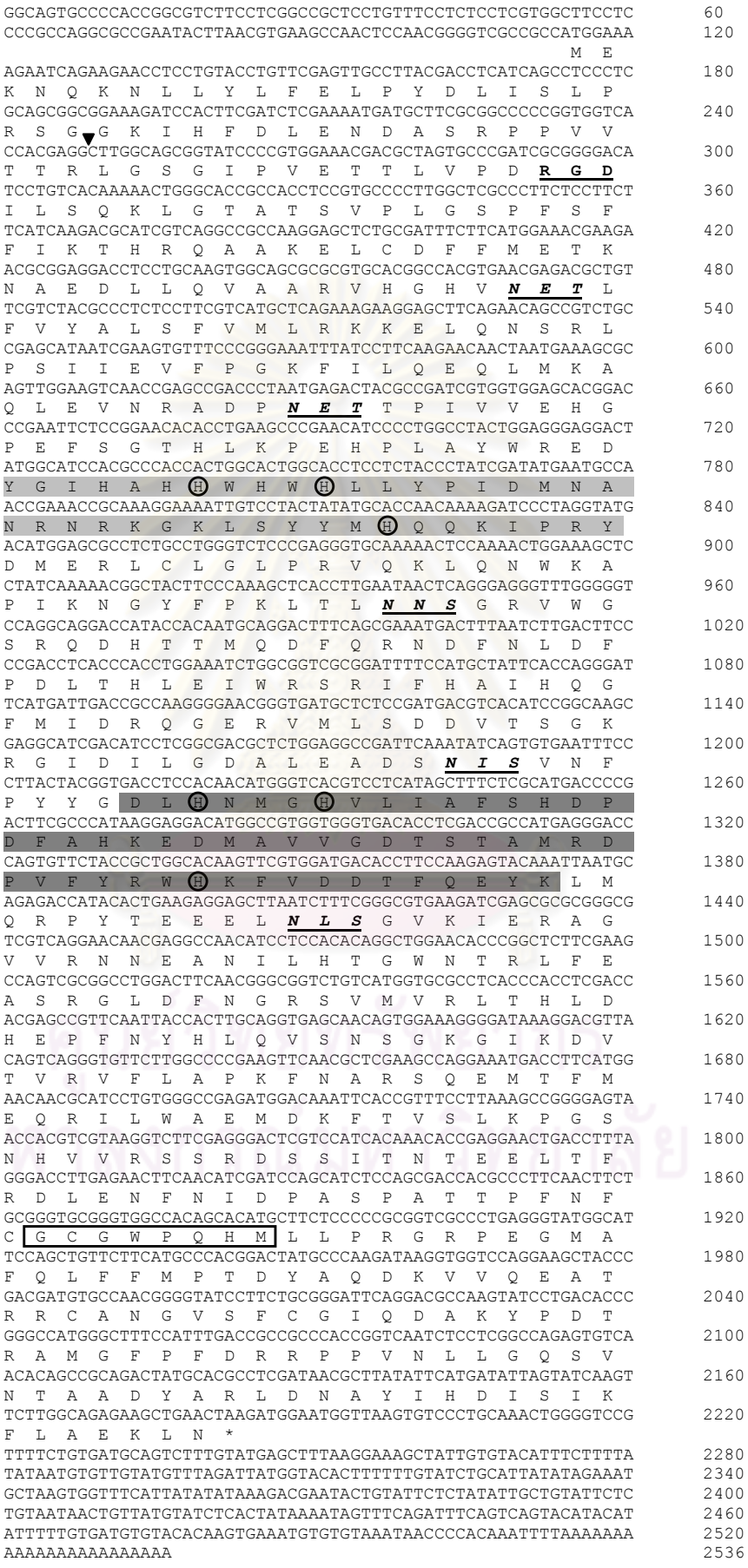
GGCAGTGCCCCACCGGCGTCTTCCTCGGCCGCTCCTGTTTCCTCTCCTCGTGGCTTCCTC 60
CCCGCCAGGCGCCGAATACTTAACGTGAAGCCAACTCCAACGGGGTCGCCGCCATGGAAA 120
M E
AGAATCAGAAGAACCTCCTGTACCTGTTCGAGTTGCCTTACGACCTCATCAGCCTCCCTC 180
K N Q K N L L Y L F E L P Y D L I S L P
GCAGCGGCGGAAAGATCCACTTCGATCTCGAAAATGATGCTTCGCGGCCCCCGGTGGTCA 240
R S G G K I H F D L E N D A S R P P V V
CCACGAGGCTTGGCAGCGGTATCCCCGTGGAAACGACGCTAGTGCCCGATCGCGGGGACA 300
T T R L G S G I P V E T T L V P D R G D
TCCTGTCACAAAAACTGGGCACCGCCACCTCCGTGCCCCTTGGCTCGCCCTTCTCCTTCT 360
I L S Q K L G T A T S V P L G S P F S F
TCATCAAGACGCATCGTCAGGCCGCCAAGGAGCTCTGCGATTTCTTCATGGAAACGAAGA 420
F I K T H R Q A A K E L C D F F M E T K
ACGCGGAGGACCTCCTGCAAGTGGCAGCGCGCGTGCACGGCCACGTGAACGAGACGCTGT 480
N A E D L L Q V A A R V H G H V N E T L
TCGTCTACGCCCTCTCCTTCGTCATGCTCAGAAAGAAGGAGCTTCAGAACAGCCGTCTGC 540
F V Y A L S F V M L R K K E L Q N S R L
CGAGCATAATCGAAGTGTTTCCCGGGAAATTTATCCTTCAAGAACAACTAATGAAAGCGC 600
P S I I E V F P G K F I L Q E Q L M K A
AGTTGGAAGTCAACCGAGCCGACCCTAATGAGACTACGCCGATCGTGGTGGAGCACGGAC 660
Q L E V N R A D P N E T T P I V V E H G
CCGAATTCTCCGGAACACACCTGAAGCCCGAACATCCCCTGGCCTACTGGAGGGAGGACT 720
P E F S G T H L K P E H P L A Y W R E D
ATGGCATCCACGCCCACCACTGGCACTGGCACCTCCTCTACCCTATCGATATGAATGCCA 780
Y G I H A H H W H W H L L Y P I D M N A
ACCGAAACCGCAAAGGAAAATTGTCCTACTATATGCACCAACAAAAGATCCCTAGGTATG 840
N R N R K G K L S Y Y M H Q Q K I P R Y
ACATGGAGCGCCTCTGCCTGGGTCTCCCGAGGGTGCAAAAACTCCAAAACTGGAAAGCTC 900
D M E R L C L G L P R V Q K L Q N W K A
CTATCAAAAACGGCTACTTCCCAAAGCTCACCTTGAATAACTCAGGGAGGGTTTGGGGGT 960
P I K N G Y F P K L T L N N S G R V W G
CCAGGCAGGACCATACCACAATGCAGGACTTTCAGCGAAATGACTTTAATCTTGACTTCC 1020
S R Q D H T T M Q D F Q R N D F N L D F
CCGACCTCACCCACCTGGAAATCTGGCGGTCGCGGATTTTCCATGCTATTCACCAGGGAT 1080
P D L T H L E I W R S R I F H A I H Q G
TCATGATTGACCGCCAAGGGGAACGGGTGATGCTCTCCGATGACGTCACATCCGGCAAGC 1140
F M I D R Q G E R V M L S D D V T S G K
GAGGCATCGACATCCTCGGCGACGCTCTGGAGGCCGATTCAAATATCAGTGTGAATTTCC 1200
R G I D I L G D A L E A D S N I S V N F
CTTACTACGGTGACCTCCACAACATGGGTCACGTCCTCATAGCTTTCTCGCATGACCCCG 1260
P Y Y G D L H N M G H V L I A F S H D P
ACTTCGCCCATAAGGAGGACATGGCCGTGGTGGGTGACACCTCGACCGCCATGAGGGACC 1320
D F A H K E D M A V V G D T S T A M R D
CAGTGTTCTACCGCTGGCACAAGTTCGTGGATGACACCTTCCAAGAGTACAAATTAATGC 1380
P V F Y R W H K F V D D T F Q E Y K L M
AGAGACCATACACTGAAGAGGAGCTTAATCTTTCGGGCGTGAAGATCGAGCGCGCGGGCG 1440
Q R P Y T E E E L N L S G V K I E R A G
TCGTCAGGAACAACGAGGCCAACATCCTCCACACAGGCTGGAACACCCGGCTCTTCGAAG 1500
V V R N N E A N I L H T G W N T R L F E
CCAGTCGCGGCCTGGACTTCAACGGGCGGTCTGTCATGGTGCGCCTCACCCACCTCGACC 1560
A S R G L D F N G R S V M V R L T H L D
ACGAGCCGTTCAATTACCACTTGCAGGTGAGCAACAGTGGAAAGGGGATAAAGGACGTTA 1620
H E P F N Y H L Q V S N S G K G I K D V
CAGTCAGGGTGTTCTTGGCCCCGAAGTTCAACGCTCGAAGCCAGGAAATGACCTTCATGG 1680
T V R V F L A P K F N A R S Q E M T F M
AACAACGCATCCTGTGGGCCGAGATGGACAAATTCACCGTTTCCTTAAAGCCGGGGAGTA 1740
E Q R I L W A E M D K F T V S L K P G S
ACCACGTCGTAAGGTCTTCGAGGGACTCGTCCATCACAAACACCGAGGAACTGACCTTTA 1800
N H V V R S S R D S S I T N T E E L T F
GGGACCTTGAGAACTTCAACATCGATCCAGCATCTCCAGCGACCACGCCCTTCAACTTCT 1860
R D L E N F N I D P A S P A T T P F N F
GCGGGTGCGGGTGGCCACAGCACATGCTTCTCCCCCGCGGTCGCCCTGAGGGTATGGCAT 1920
C G C G W P Q H M L L P R G R P E G M A
TCCAGCTGTTCTTCATGCCCACGGACTATGCCCAAGATAAGGTGGTCCAGGAAGCTACCC 1980
F Q L F F M P T D Y A Q D K V V Q E A T
GACGATGTGCCAACGGGGTATCCTTCTGCGGGATTCAGGACGCCAAGTATCCTGACACCC 2040
R R C A N G V S F C G I Q D A K Y P D T
GGGCCATGGGCTTTCCATTTGACCGCCGCCCACCGGTCAATCTCCTCGGCCAGAGTGTCA 2100
R A M G F P F D R R P P V N L L G Q S V
ACACAGCCGCAGACTATGCACGCCTCGATAACGCTTATATTCATGATATTAGTATCAAGT 2160
N T A A D Y A R L D N A Y I H D I S I K
TCTTGGCAGAGAAGCTGAACTAAGATGGAATGGTTAAGTGTCCCTGCAAACTGGGGTCCG 2220
F L A E K L N *
TTTTCTGTGATGCAGTCTTTGTATGAGCTTTAAGGAAAGCTATTGTGTACATTTCTTTTA 2280
TATAATGTGTTGTATGTTTAGATTATGGTACACTTTTTTGTATCTGCATTATATAGAAAT 2340
GCTAAGTGGTTTCATTATATATAAAGACGAATACTGTATTCTCTATATTGCTGTATTCTC 2400
TGTAATAACTGTTATGTATCTCACTATAAAATAGTTTCAGATTTCAGTCAGTACATACAT 2460
ATTTTTGTGATGTGTACACAAGTGAAATGTGTGTAAATAACCCCACAAATTTTAAAAAAA 2520
AAAAAAAAAAAAAAA 2536

Figure 3.5 Nucleotide and deduced amino acid sequences of *P. monodon Pm*proPO2. The deduced amino acid sequence is shown by a single abbreviation below the second nucleotide of each codon. The arrowhead indicates a putative cleavage site for the zymogen activation. The copper-binding sites A and B are shown by grey and dark grey highlights, respectively. The six conserved histidine residues of the two copper-binding sites are denoted by circles. The thiol ester-like motif is boxed. Five putative *N*-glycosylation sites are in boldfaced and italicize-underlined. The RGD motif is represented by boldfaced and underlined.

Table 3.1 Characteristics of *P. monodon Pm*proPO1 and *Pm*proPO2 cDNA sequences and their transcript products.

Characteristics	***Pm*proPO1**	***Pm*proPO2**
Genbank accession number	AF099741	FJ025814
Complete coding sequence	2067 bp	2070 bp
Predicted protein	688 amino acids	689 amino acids
Molecular weight	78.67 kDa	79.21 kDa
Isoelectric point (p*I*)	5.80	6.69
Predicted cleavage site	Between Arg44 and Val 45	Between Arg45 and Leu46
Putative *N*-glycosylation	Four sites [117(NET), 170(NQT), 571(NTS) and 662(NTT)]	Five sites [119(NET), 172(NET), 275(NNS), 357(NIS) and 432(NLS)]
Thiol ester-like motif	GCGWPQHM	GCGWPQHM
Copper-binding site	Copper-binding site A (3 conserved histidine residues)	Copper-binding site A (3 conserved histidine residues)
	Copper-binding site B (3 conserved histidine residues)	Copper-binding site B (3 conserved histidine residues)

Similarity searching using the BlastX program showed that *Pm*proPO2 is more similar to the proPO2 of the pacific white shrimp *Litopenaeus vannamei* (EF565469 and EU373096; 91% similarity) than that of the proPO1 from *P. monodon* (AF521948 and AF099741; 81% similarity) and proPO1 from *L. vannamei* (EU284136, EF115296 and AY723296; 81-82% similarity). Multiple amino acid sequence alignment of *Pm*proPO2 sequence with other crustacean proPO genes including *Pm*proPO1 (AF099741), proPO1 of the *L. vannamei* (*Lv*proPO1: EU284136), proPO2 of *L. vannamei* (*Lv*proPO2: EF565469), proPO of the kuruma shrimp *Marsupenaeus japonicus* (*Mj*proPO: AB073223), proPO of the freshwater crayfish *P. leniusculus* (*Pl*proPO: X83494), proPO of the european lobster *Homarus gammarus* (*Hg*proPO: AJ581662) and proPO of the mud crab *Scylla serrata* (*Ss*proPO: DQ435606) revealed that the highly conserved regions including two sites coding for copper-binding (copper-binding sites A and B) and a thiol ester-like motif were presented within the *Pm*proPO2 sequence. The six conserved histidine residues which are likely to be involved in copper-binding of the *Pm*proPO2 sequence were presented within the annotated copper-binding site A (H209, H213 and H235) and copper-binding site B (H369, H373 and H409) and a conserved thiol ester-like motif was found to be located between Gly584 to Met591 (Figure 3.7). Pair-wise alignment showed that *Pm*proPO2 exhibited 67% sequence identity to the *P. monodon* proPO1 (AF099741) and all of the copper-binding regions including copper-binding site A and copper-binding site B were also highly conserved with 67% and 72% identity, respectively.

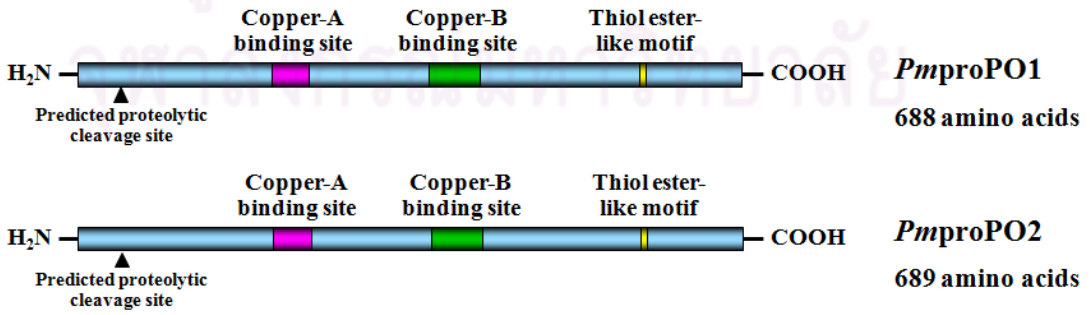

Figure 3.6 Schematic representation of *P. monodon Pm*proPO1 and *Pm*proPO2. The pink and green boxes indicate the copper-binding sites A and B, respectively. The arrowhead indicates the predicted proteolytic cleavage site. The thiol ester-like motif is shown by yellow boxes.


PmproPO2 ----------MEKNQKNLLYLFELPYDLISLPRSGGKIHFDLENDASRPPVVTTRLG 47
LvproPO2 ----------MDKSRKNLLYLFELPHDPISLPRGGGKIHFDLENDLSRPPVVATRLG 47
LvproPO1 ----------MANDQQRLLYLFELPQEPIQTPRGGGSVQFKLENDDS-PPSVATRVG 46
PmproPO1 ----------MANDQQRLLYLFELPQEDIQAPRGGGSVLFKLESDEA-PPSVATRVG 46
MjproPO ----------MNIEQQQLLYLFELPQEPLNRPRGKGSVLFVLENDDT-PPLVTTRIA 46
PlproPO MQVTQKLLRRDTEMADAQKQLLYLFERPYDPINAPRADGSFLYAVAG-ATVATRFG---- 55
HgproPO ----------MSEAQS-LLSLFEKPYSDVTTPRANESMAFELEERPVVVPRVG---- 42
SsproPO ----------MDSEQKQVLDMLQRPFR-LPTQRESHPIVFEVGTRVG---------- 36
PmproPO2 SGIPVETTLVPDRGDILSQKLGTATSVPLGSPFSFFIKTHRQAAKELCDFFMETKNAEDL 107
LvproPO2 GGVPVETTPVPDREDALPQNLGTAASVPIGSPFSFFIKSHRQAAKDLCDVFMRTKGAEDL 107
LvproPO1 L-SPSVSVPVPERKDVALQDLGTATSIPMGSAFSFFLASHRRAARDLCNVFMKTNGAEDL 105
PmproPO1 V-SPSVNLPVPERKDVTLQDLGTATSVPKGSAFSFFIASHRKAARDLCDFFMKTSGAEDL 105
MjproPO G-SPSIKLQVPERNDVALQDLGTATSIPIGSAFSFFLASHRRAAKDLCDVFMKTSGAKDL 105
PlproPO -VAPTSTVTVPARPDADRRLLGRAPSVPRGAVFSFFIRSHREAARDLCDVLMKTQNSTDL 114
HgproPO -AGP--TITLPRRPDADPSKLGTATVVPRGSAFSFFVSTHRRAAKDLCDAFMKTTGVQDL 99
SsproPO ----------GSERPPQRLTRARSIPRGTVFSIFVKSHRLAAKELCDYFMEASSVQEL 84
PmproPO2 LQVAARVHGHVNETLFVYALSFVMLRKKELQNSRLPSIIEVFPGKFILQEQLMKAQLEVN 167
LvproPO2 LQVAARVHGQVNETLFIYALSFVILRKKELRNVRLPSIIEVFPGKLIPQEQLMKAQLEVN 167
LvproPO1 MQVAARVHGKVNETLFIYAISFVILRKKELHSVRLPTMVEVFPSRFVPQEALSRAQLQVN 165
PmproPO1 MQVAARVHGKVNETLFVYAISFVILRKKELRSVRLPTMVEVFPSRFVPQEALSKAQLQIN 165
MjproPO MEVAARVHGHVNESLFIYAISFVILRKKELQSVRLPSFVEVFPSRFVPQETLAKAQIRIN 165
PlproPO MQLAASVRRHVNENLFIYALSFTILRKQELRGVRLPPILEVFPHKFIPMEDLTSMQVEVN 174
HgproPO VQFAARVKDVVNESLFIYSLSFVILHKKELRNFRLPSLVEVFPQKFVPVEHLIKSQQEAV 159
SsproPO RQRVEEVRGLVNEKLFIFALSFVIVRKPEMRHLRLPSIVEIFPCMFVPVTTVSEMEQEAR 144
Copper-A binding site
PmproPO2 RADPNETTPIVVEHGPEFSGTHLKPEHPLAYWREDYGIHAHHWHWHLLYPIDMNANRNRK 227
LvproPO2 RTDPNQSEPVVVEHGLEFSGTHLKPEHRLAYWREDYGINAHHWHWHLVYPIDMGVNRDRK 227
LvproPO1 RMDPNQSEAVIIEHGPEFSGSPVKPEHRVSYWREDYGINVHHWHWHLIYPPGMGVDRDRK 225
PmproPO1 RMDPNQTEPVIIEHGPEFSGTHLKPEHRISYWREDYGINVHHWHWHLIYPPAMGFDRDRK 225
MjproPO RMDPNQREPVIVEHGLEFSGTHLKPEHRLSYWREDYGLSVHHWHWHLIYPVGMGVDRDRK 225
PlproPO RTPPTATTPLVIEYGPEFANTNQKAEHRVSYWREDFGINSHHWHWHLVYPIEMNVNRDRK 234
HgproPO RRDENEETPIVIEHGPDFSSNTIKPEHRVAYWREDYGMNVHHWHWHLVYPIEMNLNRDRK 219
SsproPO KSTPDQ-EIVVTEYGPEFSSTHLKPEHRVAYWREDYGINSHHWHWHLVYPVDLGVMRDRK 203
PmproPO2 GKLSYYMHQQKIPRYDMERLCLGLPRVQKLQNWKAPIKNGYFPKLTLNNSGRVWGSRQDH 287
LvproPO2 GELFFYMHQQMVARYDMERLSLGLPRVQKLENWRAPIEDGYFPKLTVNNSGRAWGSRQDD 287
LvproPO1 GELFYYMHQQIIARYDMERLSLGLPRVQKLDNWRVPIEDGYFPKLTVNNSGRAWGSRQDN 285
PmproPO1 GELFYYMHQQVIARYDIERLCLGLPKVEKLDNWRIPIEDGYFPKMTVSISGRNWGSRQDN 285
MjproPO GELFYYMHQQLIARYDLERLSLGLPRVEKLDNWRVPIEDGYFPKLTISNTGRAWGTRQDN 285
PlproPO GELFYYMHQQMVARYDWERLSVNLNRVEKLENWRVPIPDGYFSKLTANNSGRPWGTRQDN 294
HgproPO GEIFFYMHQQMIARYDMERLSVGLRRVEKLENWRIPVPDGYFSKLTVNNSGRAWGTRQDN 279
SsproPO GELFFYMHQQMLARYDMDRLSVGLNRVQKLSNWRIPIPDGYFPKLTINNAGQTWGSRQDN 263
PmproPO2 TTMQDFQRNDFNLDFPDLTHLEIWRSRIFHAIHQGFMIDRQGERVMLSDDVTS-GKRGID 346
LvproPO2 TVMQDFLRNDFGLDFTDVTDLEIWRSRLFDAIHQGFMIDRAGKRVVLSDDVTS-GKRGID 346
LvproPO1 TLPKDFRRTEIG-DPVDITDLEIWRARLLGAIHQGYMVDRNGDKVPLRDDVTS-GKRGID 343
PmproPO1 TLPKDLRRRELG-EFVDITDMEIWRSRLLDAIHQGFMIDRNGDKVPLRDDVTS-GKRGID 343
MjproPO TLPKDFRRREIG-EFVDITDLEIWRSRLLDAIHQGFMVDRKGNKVPIRDDVTS-GQRGID 343
PlproPO TFIKDFRRNDAGLDFIDISDMEIWRSRLMDAIHQGYMLNRNGERVPLSDNVTT-GKRGID 353
HgproPO TFLKDFRRNDFGLQPLDITELEVWRSRLLDAIHQGYMKNPNGDTIPLSDDVTS-GKRGID 338
SsproPO SLVQDYRREDFGLLPFDVSELEQWHSRIMDAIHQGYLVDHDGNQTRLTDNVKPPEKRGID 323
Copper-B binding site
PmproPO2 ILGDALEADSNISVNFPYYGDLHNMGHVLIAFSHDPDFAHKEDMAVVGDTSTAMRDPVFY 406
LvproPO2 ILGDALEADSNLSVNSPYYGDLHNMGHVLIAFSHDPDFAHKEEMAVMGDTSTAMRDPVFY 406
LvproPO1 ILADALEADADHSVNFPYYGDLHNIGHDILAFSHDPDNAHKEEMGVVGDLGTSLRDPVFF 403
PmproPO1 ILSEALEADAELSVNFPYYGDLHNRGHDILAFSHDPDNAHKEEMGVVGDLGTSLRDPVFF 403
MjproPO ILAEALEADEDLSINYPFYGSLHNFGHDILAFSHDPDNAHKEEMGVMGDTATAMRDPVFY 403
PlproPO ILGDAFEADAQLSPNYLFYGDLHNTGHVLLAFCHDNDNSHREEIGVMGDSATALRDPVFY 413
HgproPO ILGDTLEADADLSPHYQFYGDLHNMSHVLISFSHDNDNAHKEELGVMGDPATSMRDPVFY 398
SsproPO LLGDTVEADSSISLNSLFYGDLHNMGHVVISAIHDPDYAHRENLGVMSDTATAMRDPVFY 383
PmproPO2 RWHKFVDDTFQEYKLMQRPYTEEELNLSGVKIERAGVVRNNEANILHTGWNTRLFEASRG 466
LvproPO2 RWHKFVDDTFQEYKLMQRPYTEQDLNLAGVKIERAGVVRNNEADVLHTGWNTRLFEASRG 466
LvproPO1 RLHKLVDDLFQEYKLTQPPYKEEELFLPGVRIERAGVVRDDEADVLLTGWNTREFEASRG 463
PmproPO1 RLHKLVDDLFQEYKVTQPPYTEEELFLPGVRIERAGVVRGNEADVLLTGWNTREFEASRG 463
MjproPO RWHKFVDDTFQEYKLMQRPYTEEELNFSGVKIEKVGVVRNDEADILHTGWNKRGFEASRG 463
PlproPO RWHKFVDDIFQEYKLTQPPYTMEDLSLPGVVLDKVGVVRNDQLNTLTTGWSVREFEASRG 473
HgproPO RLHKFVDDVFQAYKLTQRPYTMEDLSMPGVVVNQVSVTSKREINKLTTGWSTREFEASRG 458
SsproPO RWHKYIDDIFQEYKVIQPPYTTEELSLSSVEVVSVAVESQGQKNQLITGWSTRDFEASRG 443

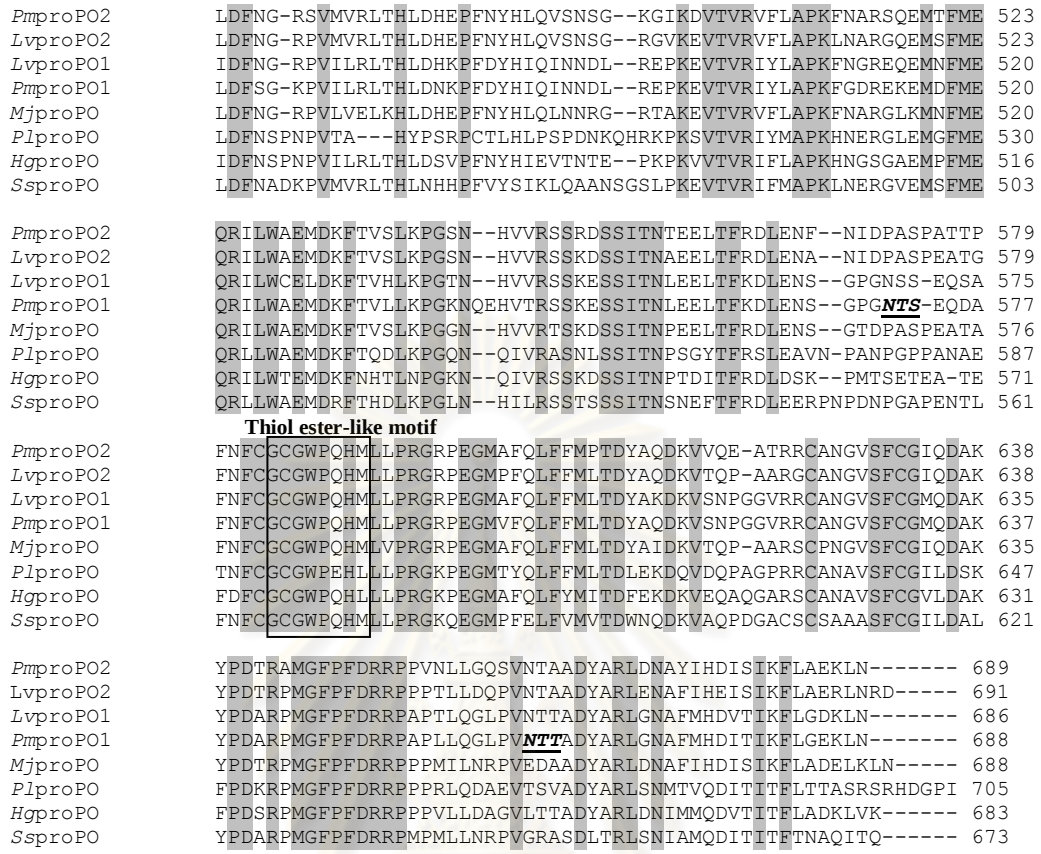

Figure 3.7 Multiple amino acid sequence alignment of *P. monodon Pm*proPO2 (AF099741) with other crustacean proPO genes including *Pm*proPO1 (FJ025814), *Litopenaeus vannamei* proPO1 (*Lv*proPO1: EU284136), *L. vannamei* proPO2 (*Lv*proPO2: EF565469), *Marsupenaeus japonicus* (*Mj*proPO: AB073223), *Pacifastacus leniusculus* (*Pl*proPO: X83494), *Homarus gammarus* (*Hg*proPO: AJ581662) and *Scylla serrata* (*Ss*proPO: DQ435606). The identical amino acid residues are shown by grey highlights. The copper-binding sites A and B and the thiol ester-like motif are boxed. The conserved histidine residues of the copper-binding sites A and B are indicated by black highlights. The putative *N*-glycosylation sites are in boldfaced and italicize-underlined. The arrowhead indicates the predicted proteolytic cleavage site for the zymogen activation. The RGD like motif of *Pm*proPO2 is represented by boldfaced.

3.2 Tissue expression analysis of the proPO system-associated genes using semi-quantitative RT-PCR

In order to examine the tissue distribution of the *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 transcripts, total RNA was extracted from different shrimp tissues, including hemocytes, hepatopancreas, gill, lymphoid organ, intestine and heart and semi-quantitative RT-PCR analysis was employed to assess the transcript expression levels by using an elongation factor 1-α (EF1-α) gene as an internal control.

The results demonstrated that *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 mRNA transcripts were mainly expressed in shrimp hemocytes and no signal was detected in all other tissues tested (Figure 3.8). However, the relative expression pattern between them was slightly different. The *Pm*PPAE1 and *Pm*proPO1 mRNA transcripts were abundantly expressed in shrimp hemocytes, whereas *Pm*PPAE2 and *Pm*proPO2 mRNA transcripts were expressed at a moderate to low level.

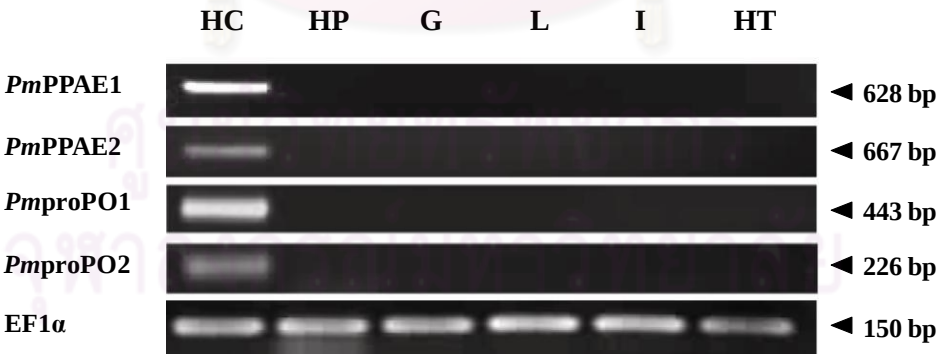

Figure 3.8 Tissue distribution analysis of the *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 transcripts by semi-quantitative RT-PCR in hemocytes (HC), hepatopancreas (HP), gills (G), lymphoid organ (L), intestine (I) and heart (HT) of *P. monodon*. The amplification reaction yielded a single amplicon of 628, 667, 443 and 226 bp of gene-specific cDNA fragments for *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2, respectively. EF1-α was used as a control housekeeping gene to standardize the amount of cDNA template in each RT-PCR.

3.3 Developmental expression analysis of the proPO system-associated genes using semi-quantitative RT-PCR

Semi-quantitative RT-PCR approach was performed to elucidate the expression level of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 transcripts during four larval developmental stages of *P. monodon*, including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15). Amplification of EF1-α was included as an internal control for cDNA template normalization.

As shown in Figure 3.9, the obtained results indicated that *Pm*PPAE1 and *Pm*proPO2 transcripts showed a broadly similar expression pattern as they were detected in all four larval developmental stages, but their relative expression level were slightly different. The *Pm*PPAE1 transcript was expressed at low level in the early stages (N3) and the expression was then increased in the later stages (Z2, M2 and PL15), whereas the expression of *Pm*proPO2 transcript remained at the same medium level in all of the four stages of larval development.

In contrast, the *Pm*PPAE2 transcript exhibited a broadly related expression pattern to the *Pm*proPO1 transcript and this expression pattern was different from those of *Pm*PPAE1 and *Pm*proPO2 transcripts in that their mRNA transcripts were not detected at the early N3 stage. Moreover, no *Pm*PPAE2 transcript was observed in Z2 stage of larval development. However, a slight increase in the transcript level of the *Pm*PPAE2 was detected when the larvae were developed to the stage of M2 and PL15, whilst the *Pm*proPO1 transcript was expressed at low level in the Z2 larval developmental stage and found to be expressed at high level in the two later stages (M2 and PL15) of larval development.

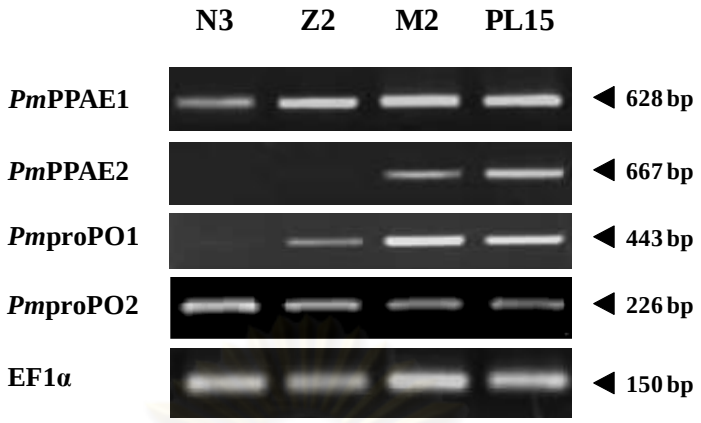

Figure 3.9 Developmental expression profile of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 transcripts by semi-quantitative RT-PCR analysis during four larval developmental stages of *P. monodon*, including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15). The amplification reaction yielded a single amplicon of 628, 667, 443 and 226 bp of gene-specific cDNA fragments for *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2, respectively. EF1-α was used as a control housekeeping gene to standardize the amount of cDNA template in each RT-PCR.

3.4 Functional characterization of the proPO system-associated genes by RNA interference

3.4.1 Determination of the gene silencing efficiency and specificity by semi-quantitative RT-PCR

3.4.1.1 The effective and specific gene silencing of the two *Pm*PPAE transcripts

To characterize the crucial roles of the two *Pm*PPAE gene products (*Pm*PPAE1 and *Pm*PPAE2) in shrimp immune system, systemic dsRNA mediated gene silencing of *Pm*PPAE1, *Pm*PPAE2, or co-silencing of both *Pm*PPAE1 and *Pm*PPAE2 was performed. Shrimps (~ 2 g) were intramuscularly injected twice with 5 µg of *Pm*PPAE1 or *Pm*PPAE2 dsRNAs, whereas shrimps injected with GFP dsRNA or saline solution alone were included as the control groups and it was showed that injection of either *Pm*PPAE1, *Pm*PPAE2 or GFP dsRNAs or injection of the saline solution alone had no effect on shrimp mortality rate during the period of 5 days post injection (data not shown).

The gene knockdown efficiency was then carried out by using semi-quantitative RT-PCR analysis and the results revealed that injection of *Pm*PPAE1 dsRNA significantly decreased the *Pm*PPAE1 transcript levels in the *Pm*PPAE1 silenced shrimp at 48 hours after the second dsRNA injection as compared to the GFP dsRNA-injected controls (Figure 3.10). The specificity of gene knockdown was further verified by examining the transcript levels of the other known clip-domain serine proteinases (clip-SPs) (*Pm*PPAE2, *Pm*ClipSP1 and *Pm*ClipSP2) and clip-domain serine proteinase homologues (clip-SPHs) (*Pm*MasSPH1, *Pm*MasSPH2 and *Pm*MasSPH3) in the same *P. monodon* samples using semi-quantitative RT-PCR and the results showed that *Pm*PPAE1 transcripts were specifically silenced by its corresponding dsRNA and no suppressive effect on the expression of other clip-SP and clip-SPH gene transcripts was observed in the *Pm*PPAE1 silenced shrimp (Figure 3.10). These results clearly demonstrated that gene silencing of *Pm*PPAE1 by using the systemic dsRNA-mediated gene silencing technique is sequence-specific.

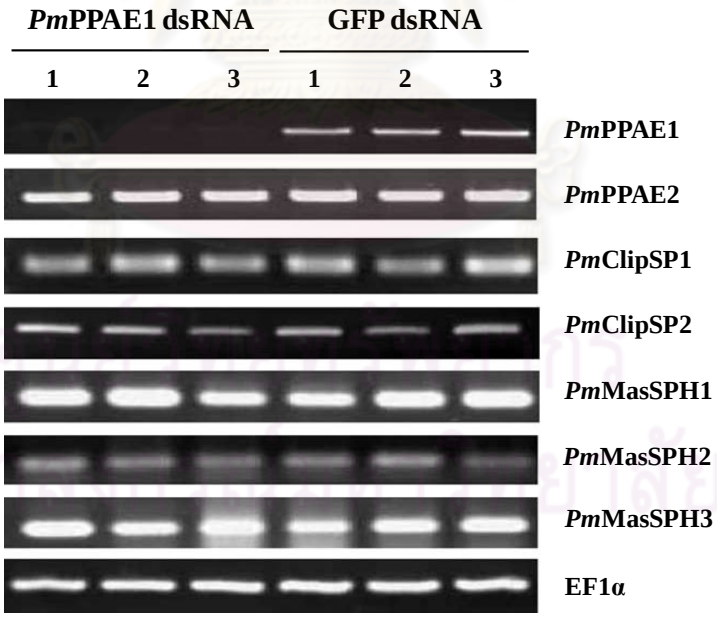

Figure 3.10 The effective and specific gene silencing of the *Pm*PPAE1 transcript levels in hemocytes of *P. monodon* at 48 hours after the second dsRNA injection. The effect of *Pm*PPAE1 dsRNA injection on the transcript expression levels of *Pm*PPAE1, and other shrimp clip-SPs (*Pm*PPAE2, *Pm*ClipSP1 and *Pm*ClipSP2) and clip-SPHs (*Pm*MasSPH1, *Pm*MasSPH2 and *Pm*MasSPH3) in *Pm*PPAE1 dsRNA- or GFP dsRNAs-injected shrimps was examined by RT-PCR using each gene-specific primer. EF1-α was used as a control housekeeping gene to standardize the amount of cDNA template in each reaction. Each lane represents cDNA from an individual shrimp.

The efficiency and specificity of gene silencing by the *Pm*PPAE2 dsRNA were also determined by semi-quantitative RT-PCR analysis and the result demonstrated that injection of *Pm*PPAE2 dsRNA significantly reduced the *Pm*PPAE2 transcript levels in the *Pm*PPAE2 knockdown shrimp at 48 hours after the second dsRNA injection and expression of the *Pm*PPAE2 was not affected by GFP dsRNA- or saline-injections (Figure 3.11). Moreover, no suppressive effect on the expression of other known clip-SPs (*Pm*PPAE1, *Pm*ClipSP1 and *Pm*ClipSP2) and clip-SPHs (*Pm*MasSPH1, *Pm*MasSPH2 and *Pm*MasSPH3) genes was observed in the *Pm*PPAE2 silenced shrimp, as compared to the GFP dsRNA- and saline-injected controls (Figure 3.11). These results clearly showed that gene silencing of *Pm*PPAE2 by using the systemic dsRNA-mediated gene silencing technique is sequence-specific.

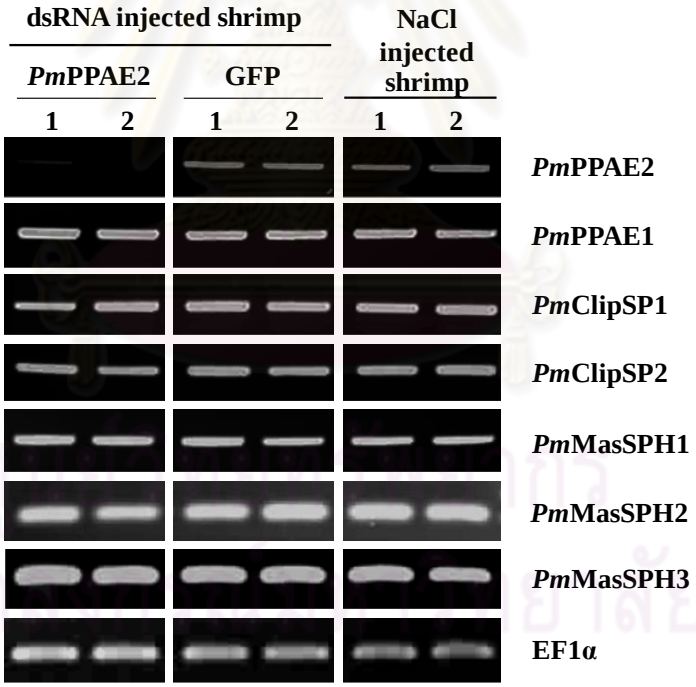

Figure 3.11 The effective and specific gene silencing of the *Pm*PPAE2 transcript levels in hemocytes of *P. monodon* at 48 hours after the second dsRNA injection. The effect of *Pm*PPAE2 dsRNA injection on the transcript expression levels of *Pm*PPAE2, and other shrimp clip-SPs (*Pm*PPAE1, *Pm*ClipSP1 and *Pm*ClipSP2) and clip-SPHs (*Pm*MasSPH1, *Pm*MasSPH2 and *Pm*MasSPH3) in *Pm*PPAE2 dsRNA-, GFP dsRNAs- or saline injected shrimps was examined by RT-PCR using each gene-specific primer. EF1-α was used as a control housekeeping gene to standardize the amount of cDNA template in each reaction. Each lane represents cDNA from an individual shrimp.

3.4.1.2 The effective and specific gene silencing of the two *Pm*proPO transcripts

In order to elucidate the essential role of the *Pm*proPO1 and *Pm*proPO2 in shrimp immune system, systemic dsRNA-mediated gene down-regulation of *Pm*proPO1, *Pm*proPO2 or both *Pm*proPO1 and *Pm*proPO2 genes was performed. Shrimps (~ 2 g, fresh weight) were intramuscularly injected twice with 5 µg of *Pm*proPO1 or *Pm*proPO2 dsRNAs. Shrimp injected with GFP dsRNA or saline solution alone were done as the control groups. Semi-quantitative RT-PCR was then performed to examine the efficiency and specificity of gene silencing in the knockdown shrimps using the gene-specific primer pairs for *Pm*proPO1 and *Pm*proPO2.

The RT-PCR results showed that the specific reduction of the *Pm*proPO1 mRNA transcript (but not *Pm*proPO2) by *Pm*proPO1 dsRNA (Figure 3.12A1) and specific reduction of *Pm*proPO2 mRNA transcript (but not *Pm*proPO1) by *Pm*proPO2 dsRNA (Figure 3.12B2) were observed in the silenced shrimps at 48 hours after the second dsRNA injection, whereas injection of GFP dsRNA or saline solution did not affected the transcription levels of either *Pm*proPO1 or *Pm*proPO2 (Figure 3.12). These results indicate that gene silencing of the two shrimp *Pm*proPO genes (*Pm*proPO1 and *Pm*proPO2) were sequence-specific and dsRNA-mediated gene down-regulation of *Pm*proPO1 and *Pm*proPO2 gene transcripts was accomplished. Although the mRNA levels of *Pm*proPO1 and *Pm*proPO2 were not entirely silenced, the levels of *Pm*proPO1 and *Pm*proPO2 mRNA transcripts were significantly suppressed.

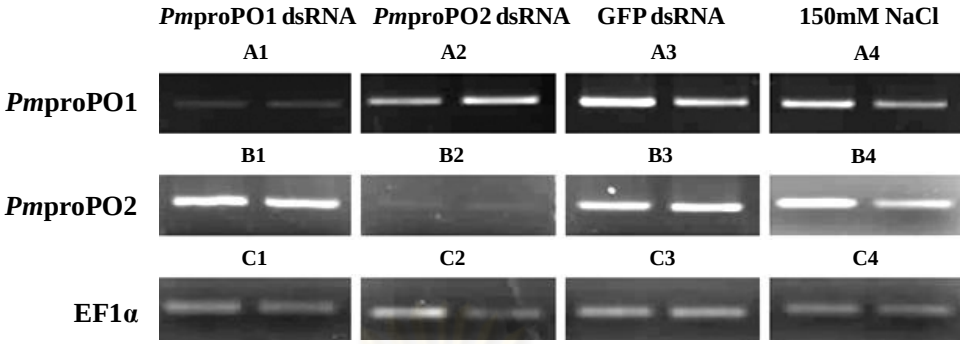

Figure 3.12 The efficiency and specificity of dsRNA-mediated gene silencing of the *Pm*proPO1 and *Pm*proPO2 in hemocytes of *P. monodon* at 48 hours after the second dsRNA injection using semi-quantitative RT-PCR. EF1-α was served as an internal control for cDNA template normalization. Each lane represents cDNA from an individual shrimp. A1-A4 represents the expression level of the *Pm*proPO1 transcript after injection of *Pm*proPO1, *Pm*proPO2 or GFP dsRNAs or saline solution alone, respectively. B1-B4 represents the expression level of the *Pm*proPO2 transcript after injection of *Pm*proPO1, *Pm*proPO2 or GFP dsRNAs or saline solution alone, respectively. C1-C4 represent the expression level of the EF1-α transcript after injection of *Pm*proPO1, *Pm*proPO2 or GFP dsRNAs or saline solution alone, respectively.

3.4.2 Determination of hemolymph phenoloxidase (PO) activity in the knockdown shrimps

3.4.2.1 Hemolymph PO activity of the *Pm*PPAE knockdown shrimps

In order to investigate the involvement of two *Pm*PPAE (*Pm*PPAE1 and *Pm*PPAE2) gene products in shrimp proPO system, hemolymph was withdrawn from the *Pm*PPAE knockdown shrimps at 48 hours after the second dsRNA injection and the hemolymph PO activity was then assayed by using L-DOPA as a substrate. Interestingly, dsRNA-mediated gene suppression of *Pm*PPAE1 and *Pm*PPAE2 transcripts significantly reduced the hemolymph PO activity in the *Pm*PPAE1 and *Pm*PPAE2 knockdown shrimps with 36.7% and 41%, respectively, whilst co-silencing of *Pm*PPAE1 and *Pm*PPAE2 transcripts resulted in non-significant difference with 41% reduction as compared to control shrimp injected with saline solution (Figure 3.13). In contrast, no significant change of hemolymph PO activity

was observed in the GFP dsRNA injected shrimp when compared to the saline-injected control ($p<0.05$, ANOVA, Duncan's test) (Figure 3.13).

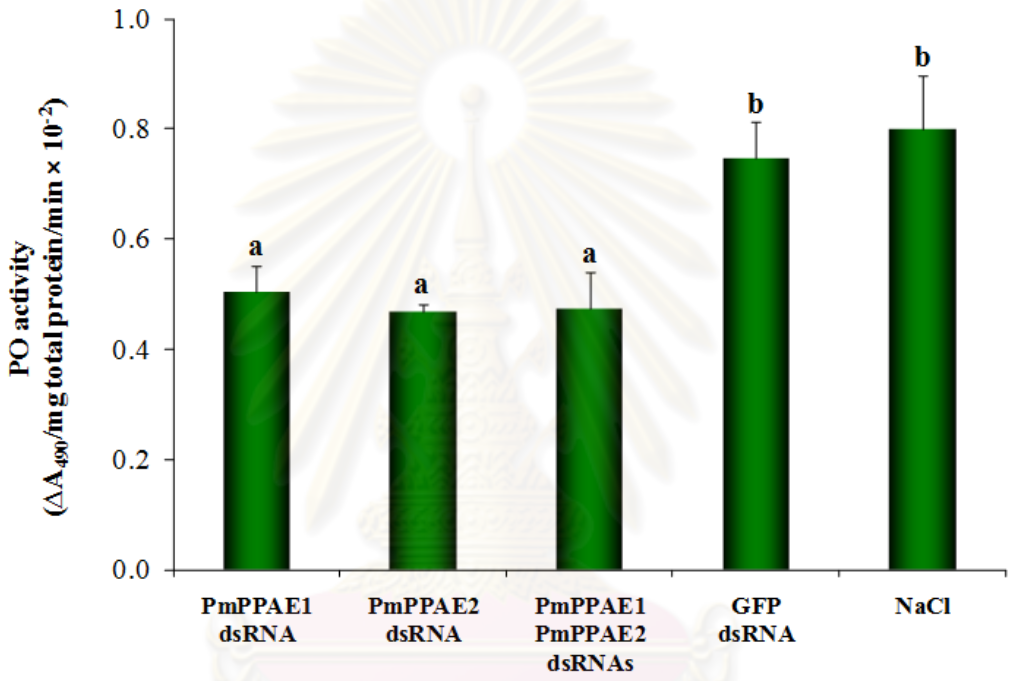

Figure 3.13 Hemolymph PO activity of the *Pm*PPAE silenced shrimps. Hemolymph was collected from the *Pm*PPAE (*Pm*PPAE1 and *Pm*PPAE2) knockdown shrimps at 48 hours after the second dsRNA injection. Control groups were shrimps injected with GFP dsRNA or saline solution. The hemolymph PO activity was measured using L-DOPA as a substrate and defined as ΔA_{490}/mg total protein/minute. The experiments were performed in triplicates and the data represents mean±standard deviation (error bars). Means with different lower case letters (above each bar) are significantly different at the $p<0.05$ level.

3.4.2.2 Hemolymph PO activity of the *Pm*proPO knockdown shrimps

To elucidate the involvement of two *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) gene products in shrimp proPO system, hemolymph was collected from the proPO silenced shrimps at 48 hours after the second dsRNA injection and subsequently subjected to assay for the hemolymph PO activity by using L-DOPA as a substrate.

As shown in Figure 3.14, dsRNA-mediated gene silencing of *Pm*proPO1 and *Pm*proPO2 transcripts significantly reduced the hemolymph PO activity in the in the *Pm*proPO1 and *Pm*proPO2 knockdown shrimps with 75% and 73%, respectively, whilst co-silencing of *Pm*proPO1 and *Pm*proPO2 transcripts efficiently reduced the hemolymph PO activity up to 88% as compared to the saline-injected control. On the other hand, no significant change in the hemolymph PO activity was observed in the GFP dsRNA injected shrimp when compared to the saline-injected control ($p<0.05$, ANOVA, Duncan's test) (Figure 3.14).

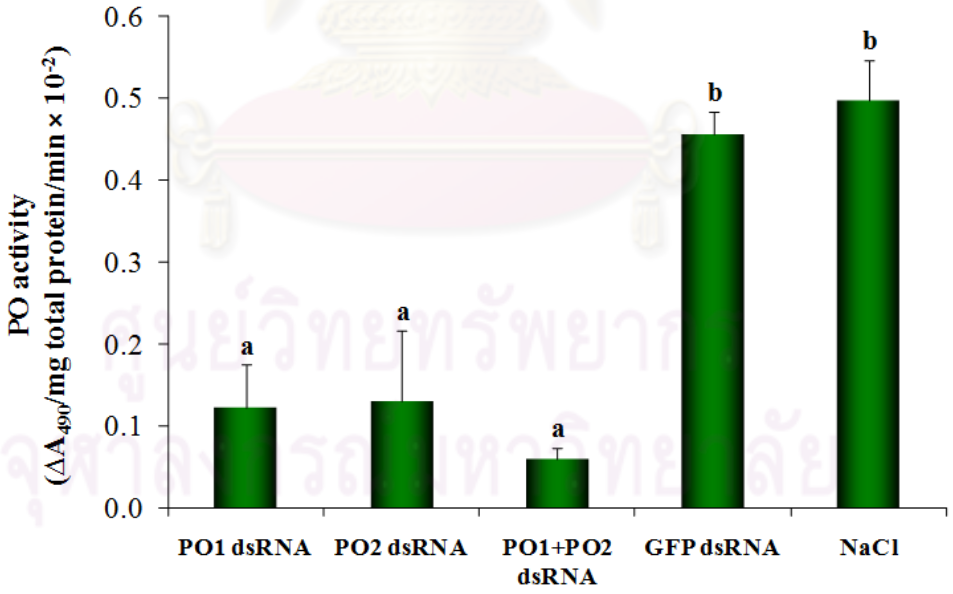

Figure 3.14 Hemolymph PO activity of the *Pm*proPO silenced shrimps. Hemolymph was collected from the *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) knockdown shrimps at 48 hours after the second dsRNA injection. Control groups were shrimps injected with GFP dsRNA or saline solution. The hemolymph PO activity was measured using L-DOPA as a substrate and defined as ΔA_{490}/mg total protein/minute. The experiments were performed in triplicates and the data represents mean±standard deviation (error bars). Means with different lower case letters (above each bar) are significantly different at the $p<0.05$ level.

3.4.3 Cumulative mortality assay in the knockdown shrimps post *Vibrio harveyi* infection

3.4.3.1 Cumulative mortality assay of the *Pm*PPAE knockdown shrimps

To elucidate the potential role of the two *Pm*PPAE (*Pm*PPAE1 and *Pm*PPAE2) gene products in the shrimp immune defense against the highly pathogenic bacterium *Vibrio harveyi* infection, the *Pm*PPAE1 and *Pm*PPAE2 silenced shrimps were challenged with the highly pathogenic bacterium *Vibrio harveyi* 639 (2×10^5 CFUs) and the mortality rate was recorded twice a day during a period of 5 days post infection.

Interestingly, the cumulative mortality of the *Pm*PPAE1 knockdown shrimp was observed at 75% within 24 hours (day 1) and then reached 100% mortality within day 3 post *V. harveyi* infection, whereas control shrimp injected with GFP dsRNA reached a total mortality of about 61.1%, which was not significantly different from the cumulative mortality of the saline injected control (55.2% mortality) (Figure 3.15).

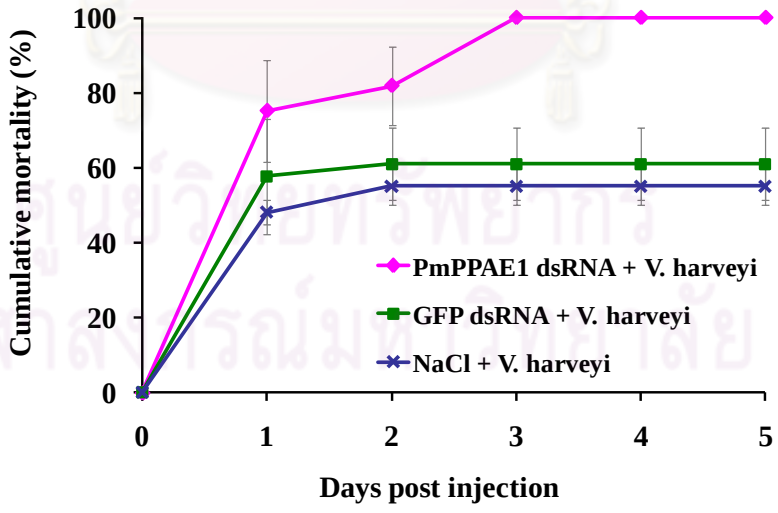

Figure 3.15 The cumulative mortality of the *Pm*PPAE1 silenced shrimps challenged with *Vibrio harveyi*. Shrimps were injected with *V. harveyi* isolated 639 (2×10^5 CFUs) following the second injection of dsRNA. Control groups were shrimps injected with GFP dsRNA or saline solution alone. Shrimp cumulative mortality was recorded twice each day for 5 days post infection. Percent mortality in each group (9–10 shrimps/group) is presented as the mean of triplicate independent experiments. The error bars indicate mean±standard deviation.

Moreover, a 77% and 83% cumulative mortality of the *Pm*PPAE2 knockdown shrimp was also observed within day 1 and 2 post *V. harveyi* infection, respectively, and the cumulative mortality remained at this level over the remainder period of 5 days post infection, which was significantly greater (3-fold) than that of the GFP dsRNA (26% mortality) or saline (28% mortality) injected controls (Figure 3.16).

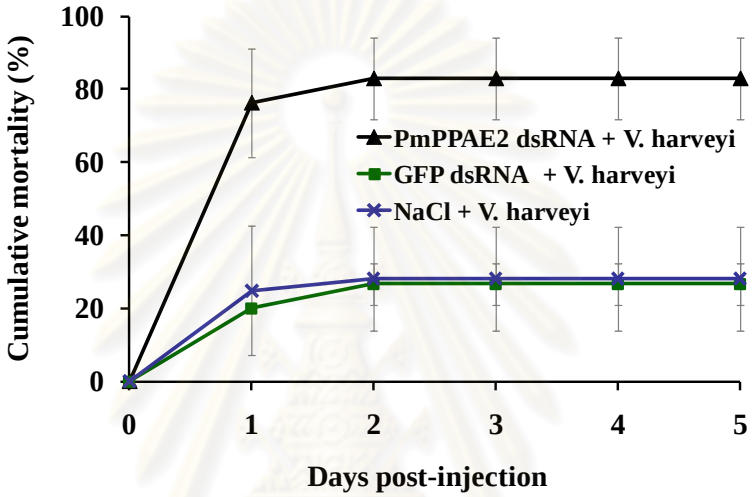

Figure 3.16 The cumulative mortality of the *Pm*PPAE2 silenced shrimps challenged with *Vibrio harveyi*. Shrimps were injected with *V. harveyi* isolated 639 (2×10^5 CFUs) following the second injection of dsRNA. Control groups were shrimps injected with GFP dsRNA or saline solution alone. Shrimp cumulative mortality was recorded twice each day for 5 days post infection. Percent mortality in each group (9–10 shrimps/group) is presented as the mean of triplicate independent experiments. The error bars indicate mean±standard deviation.

3.4.3.2 Cumulative mortality assay of the *Pm*proPO knockdown shrimps

To assess the potential role of two *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) genes in the shrimp immune defense against *V. harveyi* infection, the *Pm*proPO-knockdown shrimps were systemically challenged with the viable Gram-negative pathogenic bacterium *V. harveyi* isolate 639 (2×10^5 CFUs) and the cumulative mortality was recorded twice a day during a period of 5 days post infection. As shown in Figure 3.17, the *Pm*proPO1 silenced shrimp reached a cumulative mortality of 100% within 24 hours (day 1) post bacterial challenge, whereas the mortality of

*Pm*proPO2 silenced shrimp was 96.3% and reached 100% mortality within day 2, as compared to 61.1% and 55.2% mortality of the control groups injected with GFP dsRNA and saline solution, respectively. Furthermore, shrimp co-silenced with *Pm*proPO1 and *Pm*proPO2 dsRNAs also showed a high mortality rate of 100% within day 1 post-infection (Figure 3.17).

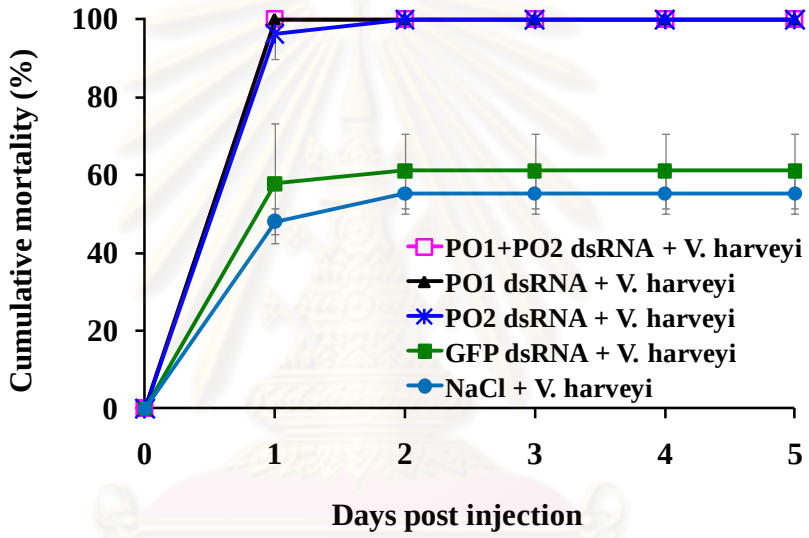

Figure 3.17 The cumulative mortality of the *Pm*proPOs silenced shrimps challenged with *Vibrio harveyi*. Shrimps were injected with *V. harveyi* isolated 639 (2×10^5 CFUs) following the second injection of dsRNA. Control groups were shrimps injected with GFP dsRNA or saline solution alone. Shrimp cumulative mortality was recorded twice each day for 5 days post infection. Percent mortality in each group (9–10 shrimps/group) is presented as the mean of triplicate independent experiments. The error bars indicated mean±standard deviation.

3.4.4 Bacterial count analysis in the knockdown shrimps post *Vibrio harveyi* infection

To investigate the potential participation of two *Pm*PPAE (*Pm*PPAE1 and *Pm*PPAE2) and two *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) gene products in the bacterial clearance mechanism, the *Pm*PPAEs or *Pm*proPOs knockdown shrimps were challenged with the pathogenic bacterium *V. harveyi* 639 (2×10^5 CFUs) and hemolymph were collected at 6 hours post challenge for bacterial colony forming units (CFUs) determination according to the modified total plate count method.

Interestingly, the results demonstrated that the number of viable bacterial CFUs was significantly increased in all of the *Pm*PPAEs and *Pm*proPOs knockdown shrimps as compared with the control shrimp injected with GFP dsRNA (Figure 3.18). A four-fold increased in the bacterial CFUs was observed in the *Pm*PPAE1 knockdown shrimp hemolymph, whilst suppression of two *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) transcripts increased the viable bacterial CFUs with a 5.7-fold higher than that of the GFP control shrimp (Figure 3.18A). Moreover, the viable bacterial cells in the *Pm*proPO co-silenced shrimp was exhibited 1.4-fold higher than that of *Pm*PPAE1 silenced shrimp (Figure 3.18A). The viable bacterial CFUs in the *Pm*PPAE2 silenced shrimp hemolymph also showed a 4.4-fold higher than that of control shrimp injected with GFP dsRNA (Figure 3.18B).

In addition, the high number of viable bacterial cells in these experiments was corresponded to the previous cumulative mortality data which also showed the high mortality rate in the *Pm*PPAE and *Pm*proPO silenced shrimps after challenged with *V. harveyi*. Taken together, these results suggests that the *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 have an essential role in protecting shrimps from the *V. harveyi* infection, or at least severe systemic infections by enhancing bacterial clearance.

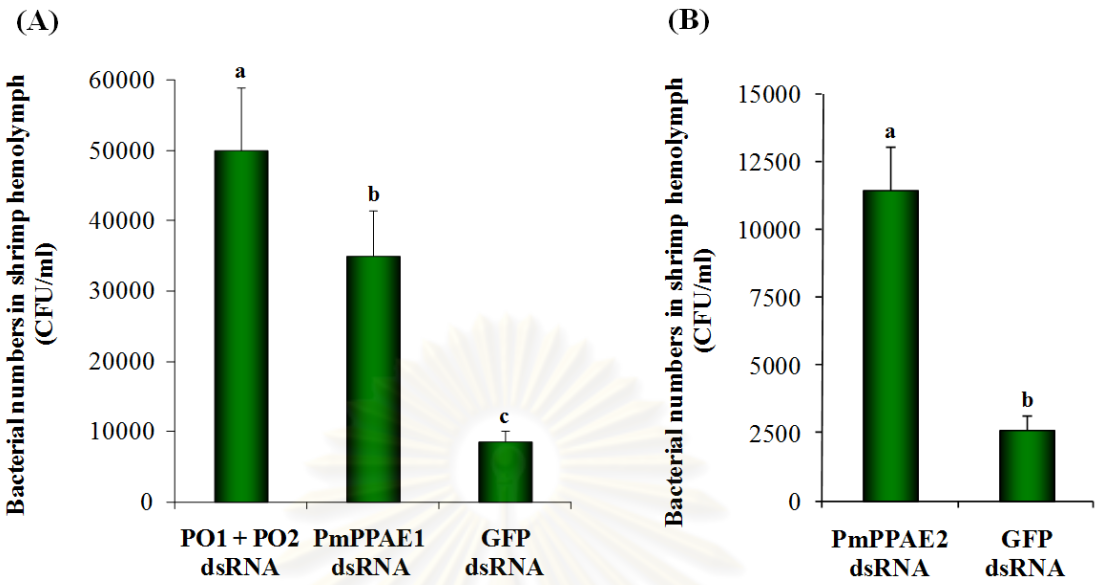

Figure 3.18 The number of viable bacterial in the hemolymph of *Pm*PPAE1 (A), *Pm*proPOs (*Pm*proPO1 and *Pm*proPO2) (A) and *Pm*PPAE2 (B) silenced shrimps at 6 hours post *Vibrio harveyi* infection. Shrimp were injected with *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) or GFP dsRNAs and then challenged with *V. harveyi* 639 (2×10^5 CFUs). A modified total plate count method was performed to determine the bacterial colony forming units (CFUs) in shrimp hemolymph. The data present mean of triplicate independent experiments. The error bars indicated mean±standard deviation. Means with different lower case letters (above each bar) are significantly different at the $p<0.05$.

3.5 Recombinant protein expression of *Pm*PPAE1 in *Escherichia coli* expression system

3.5.1 Expression of recombinant *Pm*PPAE1 (r*Pm*PPAE1)

In order to further investigate the biological function of *Pm*PPAE1, a zymogen form (without signal peptide) of *Pm*PPAE1 (pro*Pm*PPAE1) with N-terminal 6×histidine tag was recombinantly expressed in *E. coli* expression system. The expression of recombinant *Pm*PPAE1 (r*Pm*PPAE1) in *E. coli* strain Rosetta (DE3) pLysS was performed with 1 mM IPTG induction and cells were collected every hour until 5 hours post induction for SDS-PAGE analysis. As shown in Figure 3.19A, a prominent band with an apparent molecular mass of approximately 50 kDa which corresponded to the predicted molecular mass of r*Pm*PPAE1 (49.66 kDa) was

observed in the whole cell lysate at 1 hour after IPTG induction as compared to the negative control cells and the amount of r*Pm*PPAE1 protein was gradually increased as the induction time was prolonged and maximum amount of the protein expression was observed at 5 hours post IPTG induction. Western blotting using anti-His monoclonal antibody confirmed that the r*Pm*PPAE1 was successfully expressed in *E. coli* expression system (Figure 3.19B). Moreover, SDS-PAGE analysis revealed that the r*Pm*PPAE1 protein was existed in the insoluble fractions (Figure 3.19C). These results indicate that the r*Pm*PPAE1 protein was expressed as inclusion bodies in the *E. coli* Rosetta (DE3) pLysS. Consequently, only insoluble fraction was selected for subsequent purification.

3.5.2 Purification and refolding of the r*Pm*PPAE1

In order to purify the r*Pm*PPAE1 protein, inclusion body was solubilized with 8 M urea overnight at room temperature. Approximately 90% of the inclusion bodies were dissolved and only soluble r*Pm*PPAE1 was then purified by one-step Ni-NTA affinity chromatography under denaturing condition (Figure 3.20A). A major protein band of approximated molecular mass of 50 kDa, which corresponded to the predicted molecular mass of the r*Pm*PPAE1 protein, was detected in the eluted fractions as analyzed by SDS-PAGE. However, the r*Pm*PPAE1 showed low affinity binding to the Ni-NTA column due to the large amount of r*Pm*PPAE1 still remained in the flow-though fraction (Figure 3.20A). The obtained result might be suggested that the steric hindrance of the protein structure perhaps interfered 6×His tag at the N-terminus from binding with the Ni-NTA resin. To refold the r*Pm*PPAE1 protein, the elution fractions containing the recombinant r*Pm*PPAE1 protein (as analyzed by SDS-PAGE) were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0). Unfortunately, most of the purified r*Pm*PPAE1 was completely precipitated even when the pH of the Tris-HCl buffer was changing from 6.8 to 8.8 (data not shown). To solve this problem, several refolding buffer such as CAC buffer (10 mM cacodylate, 500 mM NaCl; pH 7.0) or Tris-refolding buffer (100 mM Tris-HCl, 5 mM EDTA, 15 % (v/v) glycerol, 2 mM β-mercaptoethanol; pH 8.0) were chosen for recombinant protein refolding, but none of them yields the soluble protein and the degradation products were observed even when the proteinase inhibitor (PMSF) was added into the refolding buffer (Figure

3.20B-C). Therefore, the r*Pm*PPAE1 protein produced in *E. coli* system was not sufficient for further functional analysis.

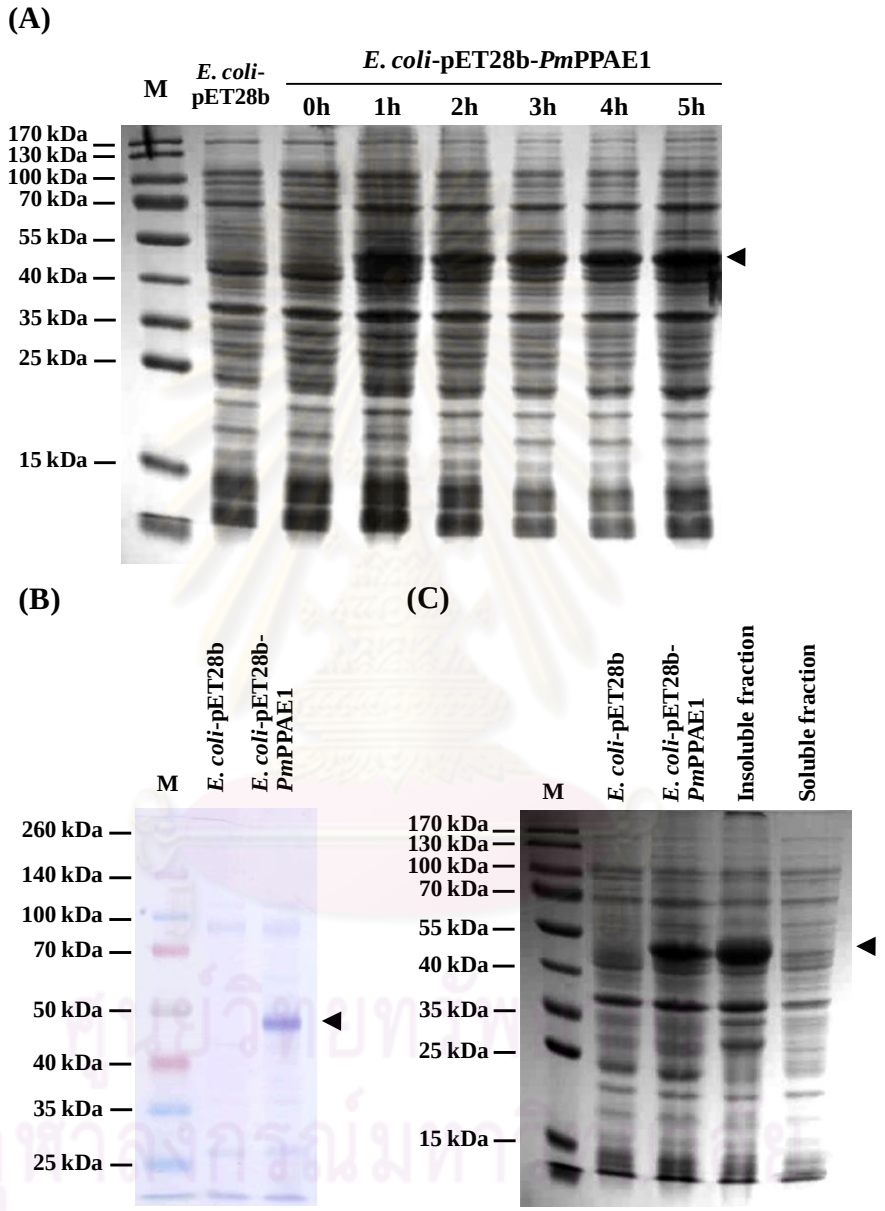

Figure 3.19 SDS-PAGE and Western blot analysis of the recombinant *Pm*PPAE1 (r*Pm*PPAE1) protein expressed in *E. coli* Rosetta (DE3) pLysS. (A) SDS-PAGE analysis of r*Pm*PPAE1 in the crude cell extracts of *E. coli* Rosetta (DE3) pLysS after induction with 1 mM IPTG for 0, 1, 2, 3, 4 and 5 hours. (B) Western blot analysis of the r*Pm*PPAE1 protein in the crude cell extracts of *E. coli* Rosetta (DE3) pLysS detected with anti-His antibody. (C) SDS-PAGE analysis of r*Pm*PPAE1 in the crude cell extract, insoluble and soluble fractions from *E. coli* Rosetta (DE3) pLysS. The crude cell extract of *E. coli* Rosetta (DE3) pLysS transformed with the parental pET28b was included as a negative control. The SDS-PAGE gels were stained with Coomassie brilliant blue. Lane M indicates the standard protein markers. The arrowheads indicate band representing the r*Pm*PPAE1 protein.

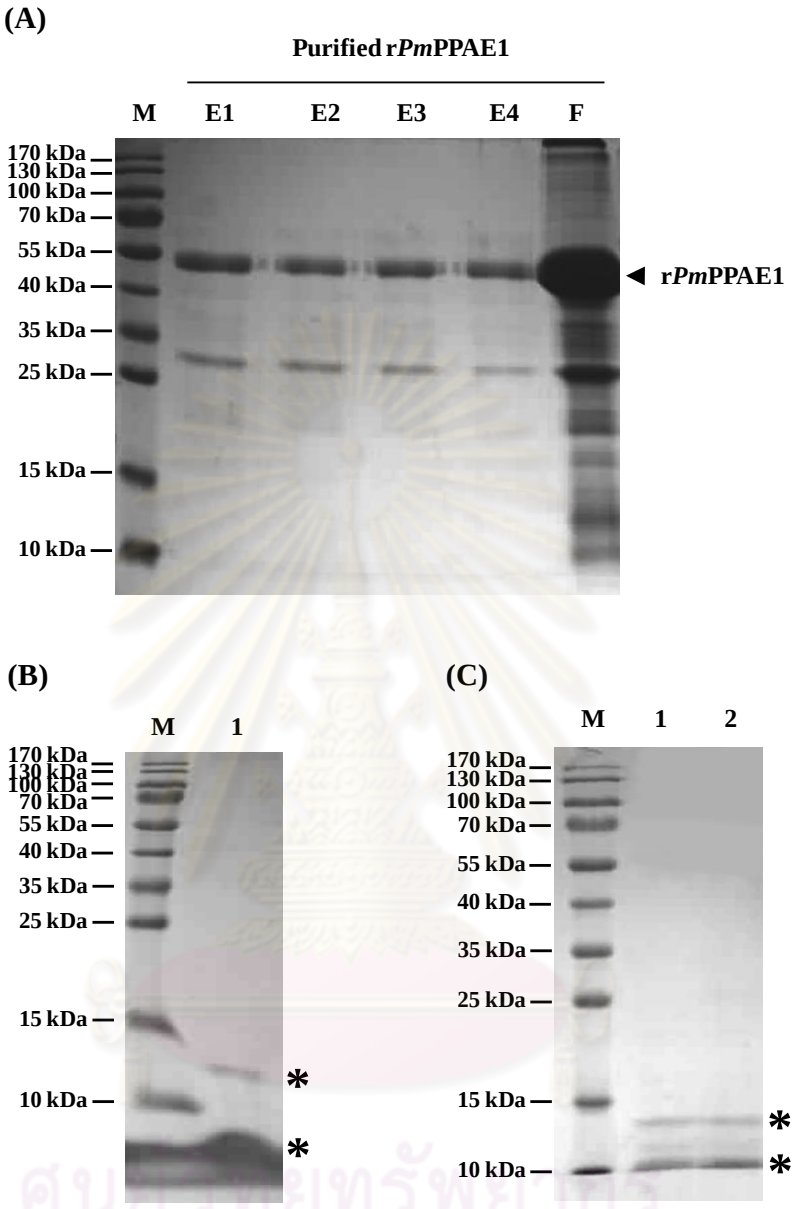

Figure 3.20 SDS-PAGE analysis of the purified recombinant *Pm*PPAE1 (r*Pm*PPAE1) protein. (A) The purified r*Pm*PPAE1 protein at different stages of purification. Lane E1-E4 indicates the first to the fourth protein fractions eluted from the Ni-NTA resin by 250 mM imidazole, respectively. Lane F indicates the flow-through fraction. (B) The purified r*Pm*PPAE1 protein after dialyzed in 10 mM CAC buffer containing 500 mM NaCl (pH 7.0). (C) The purified r*Pm*PPAE1 protein after dialyzed in 100 mM Tris-HCl buffer containing 5 mM EDTA, 15 % (v/v) glycerol and 2 mM β-mercaptoethanol (pH 8.0) with and without PMSF (Lane 1 and Lane 2, respectively). The SDS-PAGE gels were stained with Coomassie brilliant blue. Lane M indicates the standard protein markers. The arrowheads indicate band representing the r*Pm*PPAE1 protein. The asterisks represent the degradation products of the recombinant protein.

3.6 Recombinant protein expression of the serine proteinase (SP) domain of *Pm*PPAE1 in *Escherichia coli* expression system

3.6.1 Expression of recombinant SP domain of *Pm*PPAE1 (rSP-domain)

In an effort to study the function of *Pm*PPAE1 on activation of shrimp proPO system, the mature peptide of the pro-*Pm*PPAE1 protein was recombinantly expressed in *E. coli* expression system, but the r*Pm*PPAE1 was failed to renature (section 3.5). To conquer this problem, only C-terminal serine proteinase (SP) domain of the *P. monodon Pm*PPAE1 was chosen for expression in the bacterial expression system. Approximately 750 bp DNA fragment encoding for the SP domain of *Pm*PPAE1 fused with the 6×histidine tag at the N-terminus was amplified by PCR using the *Pfu* DNA polymerase and the obtained PCR product was cloned into the pET28b expression vector for production of recombinant SP-domain of *Pm*PPAE1 (rSP-domain) in the *E. coli* expression system.

To over-express the rSP-domain in bacterial expression system, the *E. coli* strain Rosetta (DE3) pLysS was transformed with the recombinant expression vector pET28b-SP-domain-*Pm*PPAE1 and cells were further induced with 1 mM IPTG for rSP-domain production. As shown in Figure 3.21A, a major protein band of approximately 27 kDa, which consistent with the estimated molecular mass of SP-domain of *Pm*PPAE1was detected only in the IPTG-inducted *E. coli* Rosetta (DE3) pLysS carrying the pET28b-SP-domain-*Pm*PPAE1, but not found in the control culture. This result suggests that the rSP-domain was successfully produced in the *E. coli* system. Moreover, SDS-PAGE analysis revealed that the rSP-domain was existed in the insoluble fractions, suggesting that rSP-domain was expressed in *E. coli* as inclusion body (Figure 3.21B).

3.6.2 Purification and functional analysis of the rSP-domain

Purification of the rSP-domain was accomplished by Ni-NTA affinity chromatography under a denaturing condition. All of the elution fractions containing the purified rSP-domain (as determined by SDS-PAGE) were pooled and dialyzed against 50 mM Tris-HCl pH 8.0. After removed the unfolded and the aggregated protein by centrifugation, coomassie brilliant blue staining following SDS-PAGE analysis indicated that the affinity-purified protein was essentially pure and intact (Figure 3.21C).

The proteinase activity of the puried rSP-domain was determined against the synthetic chromogenic substrates including *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, which were specific for trypsin/subtilisin, chymotrypsin and elastase, respectively. However, no proteinase activity was detected in all assays (data not shown). To further examine the ability of rSP-domain on activating shrimp proPO system, the rSP-domain was pre-incubated with the HLS in the presence of LPS and laminarin. PO activity was assayed by using L-DOPA as a substrate. The result showed that the rSP-domain cannot activate shrimp PO activity under the *in vitro* experimental condition as compared with the control (data not shown).

Although the rSP-domain purified from *E. coli* inclusion body was able to refold and the sufficient protein was obtained, no proteolytic activity or the ability to activate shrimp proPO system was observed. The purified rSP-domain protein was then used as an immunogen to generate the polyclonal anti-rSP-domain antisera in the immunized rabbit and western blot analysis confirmed that the polyclonal rabbit antisera reacted specifically with the rSP-domain protein (Figure 3.21D).

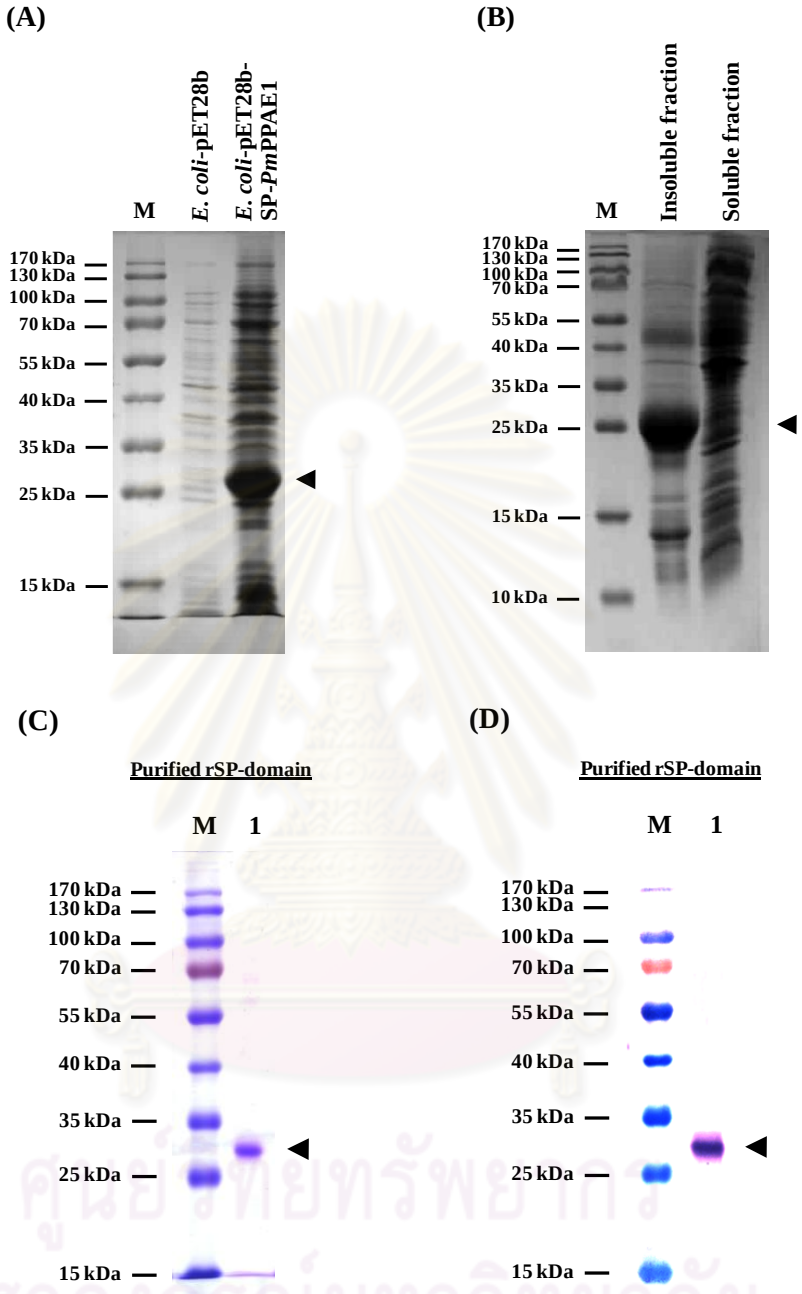

Figure 3.21 Expression and purification of the recombinant SP domain of *Pm*PPAE1 (rSP-domain) from *E. coli* Rosetta (DE3) pLysS. (A) SDS-PAGE analysis of the rSP-domain after 1 mM IPTG induction in the crude cell extracts of *E. coli* Rosetta (DE3) pLysS. The crude cell extract of *E. coli* Rosetta (DE3) pLysS transformed with the parental pET28b was included as a negative control. (B) SDS-PAGE analysis of rSP-domain in the insoluble and soluble fractions obtained from *E. coli* Rosetta (DE3) pLysS. (C) SDS-PAGE analysis of the purified rSP-domain protein. (D) Western blot analysis of the purified rSP-domain protein probing with rabbit anti-rSP-domain polyclonal antibody. The SDS-PAGE gels were stained with Coomassie brilliant blue. Lane M indicates the protein markers. The arrowhead indicates band representing the rSP-domain protein.

3.7 Production of recombinant *Pm*PPAE1 in a baculovirus infected insect cell expression system

In an attempt to produce the recombinant *Pm*PPAE1 (r*Pm*PPAE1) protein for reconstitution a branch of shrimp proPO activation cascade *in vitro*, the zymogen form (without signal peptide) of *Pm*PPAE1 protein was recombinantly expressed in insect cell system. In order to facilitate secretion and purification of recombinant protein in the baculovirus-infected insect cell expression system, *M. sexta* PAP2 signal peptide and the 6×histidine affinity tag were introduced into the N- and C-terminus of the *Pm*PPAE1 protein. The PCR-amplified cDNA fragment of *Pm*PPAE1 fused with C-terminal 6×histidine tag together with the signal peptide fragment were inserted into the transfer vector pFastBac™ 1 and sequence analysis indicated that the recombinant plasmid contained a correct open reading frame of *Pm*PPAE1 fused with signal peptide sequence and the 6×histidine tag.

To construct a recombinant baculovirus, the recombinant bacmid was generated via the *in vivo* transposition and the obtained recombinant bacmid (as confirmed by PCR approach) was transfected into the *Spodoptera frugiperda* 9 (Sf9) cells using lipid-mediated transfection method. The protein band of an apparent molecular mass of 50 kDa, which corresponded to the calculated molecular mass of the r*Pm*PPAE1 protein (51.7 kDa) was detected in the Sf9 cells at day 7 post transfection as compared to the control cells (Figure 3.22A). The baculoviral stock received from the cultures medium was then amplified and the high titer of the virus was obtained.

To express the soluble r*Pm*PPAE1 protein, Sf9 cells were infected with the recombinant baculovirus and the secreted protein was then isolated from the culture media by cobalt resin affinity chromatography. However, no protein band was observed in the conditional media suggesting that the recombinant *Pm*PPAE1 (r*Pm*PPAE1) protein did not secreted into the cell culture medium (data not shown). Nonetheless, analysis of the Sf9 cells indicated that r*Pm*PPAE1 was produced in the insoluble fraction. To optimize the r*Pm*PPAE1 production, Sf9 cells infected with the recombinant virus were harvested at 24, 48, 72 and 96 hours after viral infection. Cell

extracts were analyzed by SDS-PAGE and the results indicated that r*Pm*PPAE1 appeared to express at 48 hours post infection, but the protein level was not abundant in the insect cells. In addition, the r*Pm*PPAE1 level in the cell lysate was not increased as the infection time was prolonged and the dead cells became noticeable after 72 hours post infection as compared to the control cells infected with the recombinant virus harboring the GUS gene which became to die after 96 hours post-infection. The nature of such observation was not known, but it might be explained that the r*Pm*PPAE1 protein perhaps toxic to the cells. Therefore, the infected Sf9 cells were harvested at 72 hours post infection for recombinant protein purification under denaturing condition by a HisPur™ Cobalt resin. A protein band of approximately 50 kDa, which corresponded to the calculated molecular mass of the r*Pm*PPAE1 protein fused with the signal peptide, was detected in all of the elution fractions (Figure 3.22B). Nonetheless, the purified r*Pm*PPAE1 obtained from the insect cell was not sufficient for protein refolding and further biochemical analysis.

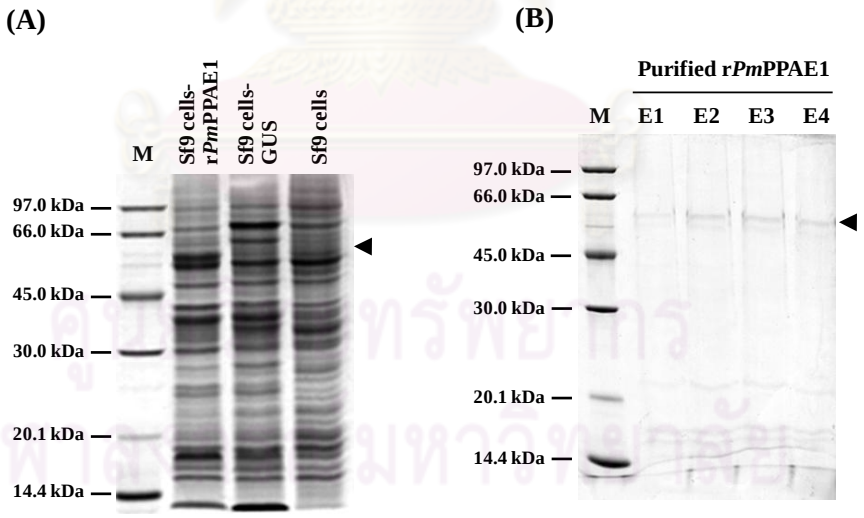

Figure 3.22 Expression analysis of the recombinant *Pm*PPAE1 (r*Pm*PPAE1) protein from insect cells expression system. (A) SDS-PAGE analysis of the r*Pm*PPAE1 produced in the insect cell lysate. Uninfected Sf9 and Sf9 cell infected with the GUS virus were used as control. (B) SDS-PAGE analysis of the affinity purified r*Pm*PPAE1. Lane E1-E4 indicated the first to the fourth protein fractions eluted from the cobalt resin by 150 mM imidazole, respectively. The SDS-PAGE gels were stained with Coomassie brilliant blue. Lane M indicates the protein markers. The arrowheads indicate band representing the r*Pm*PPAE1 protein.

3.8 Expression of an endogenous *Pm*PPAE1 in shrimp hemolymph

To detect the expression of the endogenous *Pm*PPAE1, hemolymph was collected with anticoagulant containing EDTA from the healthy shrimp. Twenty micrograms of the crude HLS and 100 µg of the cell-free plasma proteins were prepared and separated through to reducing 12.5 % (w/v) SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Western blot analysis using a polyclonal antiserum raised against the recombinant rSP-domain recognized a single protein band of approximately 49 kDa, which was corresponded to the predicted molecular weight of the putative *Pm*PPAE1, on the immunoblot of hemocytes (HLS) proteins but not in the cell-free plasma (Figure 3.23). This result suggests that the endogenous *Pm*PPAE1 was expressed only in shrimp hemocytes.

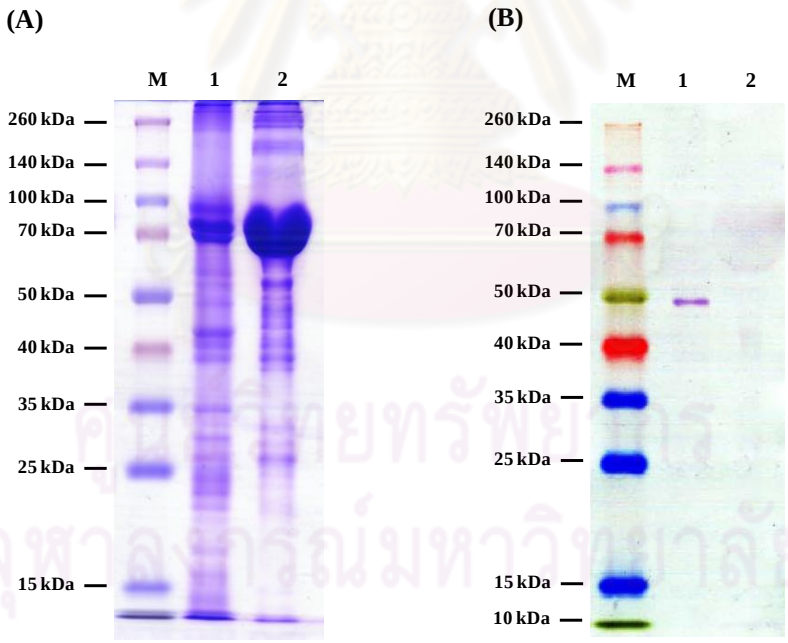

Figure 3.23 SDS-PAGE (A) and Western blot (B) analysis of the hemocyte and cell-free plasma proteins. Shrimp hemocyte lysate supernatant (HLS) (20 µg) and cell-free plasma (100 µg) proteins were separated on reducing SDS-PAGE and the proteins were stained with Coomassie brilliant blue or transferred to a PVDF membrane for Western blot analysis and probed with rabbit anti-rSP-domain polyclonal antibody. Lane 1A and 1B indicate the HLS proteins. Lane 2A and 2B indicate the cell-free plasma proteins. Lane M indicates the protein markers.

CHAPTER IV

DISCUSSIONS

Melanization through the activation of prophenoloxidase (proPO)-activating cascade is known to be one of the major defense responses that play a crucial role in invertebrate immunity (Cerenius et al., 2008; Cerenius and Söderhäll, 2004; Gillespie et al., 1997; Kanost and Gorman, 2008; Labbé and Little, 2009; Rao et al., 2010; Söderhäll, 1982; Sritunyalucksana and Söderhäll, 2000). Several proPO and proPO-activating enzyme (PPAE) genes have been reported in many arthropods (Cerenius et al., 2008, Cerenius and Söderhäll, 2004). Biochemical studies in crustacean and large insect species have established a model of proPO activation, in which proPO is cleaved after the activation cascade of clip-domain serine proteinases (clip-SPs) (An et al., 2009; Aspán and Söderhäll, 1991; Barillas-Mury, 2007; Castillejo-López and Häcker, 2005; Jang et al., 2008; Kanost and Gorman, 2008; Lee et al., 1998a, 1998b; Satoh et al., 1999; Tang et al., 2006; Wang et al., 2001b). In Penaeid shrimps, several proPO genes have been reported (Ai et al., 2008, 2009; Lai et al., 2005; Wang et al., 2006). However, the potential roles of these proPO gene products and the clip-SPs that function as a terminal proteinase (PPAE) on activation of shrimp's proPO cascade in have not yet been clarified. Moreover, the knowledge on activation and transcriptional regulation of the clip-SPs and the proPO genes in the shrimp proPO activating system is still elusive and not yet well elucidated.

In this study, two putative clip-domain serine proteinase (clip-SPs) cDNA sequences, which showed the highest sequence similarity to the arthropod proPO-activating enzymes (PPAEs) and two homologous proPO gene transcripts that showed high sequence similarity to the crustacean proPO sequences have been identified from the hemocytes of the black tiger shrimp *P. monodon* (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011).

Sequence analysis demonstrated that the first clip-SP designated as *Pm*PPAE1 is most similar to the crustacean PPAEs. *Pm*PPAE1 contains the structural domain features of the clip-domain serine proteinase family which composed of a single N-terminal clip domain and a C-terminal SP domain (Jiang and Kanost, 2000). A conserved catalytic triad of the trypsin-like SP family, which is present in all trypsin-like SPs and crucial for the formation and stabilization of the catalytic site in the three-dimensional structure of the active enzyme, was also found in the predicted mature peptide of *Pm*PPAE1 enzyme. Analysis of the *Pm*PPAE1 sequence revealed that *Pm*PPAE1 exhibited the highest amino acid sequence similarity of 70% to the PPAE of the freshwater crayfish, *Pacifastacus leniusculus*, which previously shown to be the proteinase that cleaves and activates crayfish proPO precursor without an additional protein cofactor (Aspán and Söderhäll, 1991). Moreover, an additional N-terminal glycine-rich and proline-rich domain was observed within the complete *Pm*PPAE1 sequence and these two domains were also similar to the glycine-rich and proline-rich domains of the *P. leniusculus* PPAE, respectively (Wang et al., 2001b). Based on these observations, we suggest that *P. monodon Pm*PPAE1 is a member of clip-SP family that might acts as a PPAE in shrimp proPO-activating system.

For the second clip-SP transcript (designated as *Pm*PPAE2), sequence analysis showed that *Pm*PPAE2 is more closely related to the insect PPAEs than those of the crustacean PPAEs with 51% amino acid sequence similarity to the lepidopteran insect tobacco hornworm *Manduca sexta* PAP1, a terminal PPAE that functions on cleaving and activating the proPO precursors in the requirement of the clip-domain serine proteinase homologues (clip-SPHs) (Jiang et al., 1998). The predicted amino acid sequence of *Pm*PPAE2 displays a characteristic of the clip-domain family of serine proteinase which composed of a single N-terminal clip-domain followed by a C-terminal SP domain (Jiang and Kanost, 2000). The conserved catalytic triad of the trypsin-like SP family was also presented in the predicted mature peptide of the *Pm*PPAE2 enzymes. Based on these observations, we suggest that *Pm*PPAE2 is a new member of clip-SP family that likely functions as a PPAE in shrimp proPO-activating system.

For the two shrimp proPO gene transcripts, sequence analysis revealed that the first proPO transcript (*Pm*proPO1) has the same sequence (100% identity) and thus most likely to be the same proPO gene that previously reported by Sritunyalucknana et al. (Sritunyalucksana et al., 1999), whilst the second proPO transcript homologue (*Pm*proPO2) is a novel proPO gene that exhibited a 67% amino acid sequence identity to the already reported *Pm*proPO1 (Sritunyalucksana et al., 1999). Sequence comparison also showed that all of the copper-binding regions including copper-binding sites A and B and a thiol ester-like motif are highly conserved between them.

All arthropod proPOs, with one exception of some proPOs from the hunting wasp, *Pimpla hypochondriaca* (Parkinson et al., 2001), are synthesized without a hydrophobic signal sequence. Like other arthropod proPOs, no putative signal peptide cleavage site was predicted at the N-terminal part of the deduced *Pm*proPO2 sequence. So far, analysis of most arthropod proPOs showed that cleavage of a conserved Arg - Phe linkage will results in the removal of an amino-terminal peptide containing about 50 amino acid residues from the proPO molecule (Cerenius and Söderhäll, 2004). The amino acids around this cleavage site are highly conserved among arthropod proPOs, although in most cases, it remains to be established whether an endogenous PPAE cleaves at this site. Based on the predicted proteolytic cleavage site of *Pm*proPO1 (Sritunyalucksana et al., 1999), the proteolytic cleavage site of *Pm*proPO2 was predicted to be located between Arg45 and Leu46. However, the actual cleavage site remains to be established biochemically.

Hemocyte is considered as a major tissue that synthesizes and stores the protein components of arthropod proPO system (Cerenius and Söderhäll, 2004; Johansson and Söderhäll, 1985; Perazzolo and Barracco, 1997). POs are primarily produced by specific hemocytes (blood cells) such as the crystal cells of the fruit fly *D. melanogaster* (Rizki et al., 1985), and oenocytoids (another type of insect blood cell) in many other insects (Shrestha and Kim, 2008) and the granular and semi-granular hemocyte types in crustaceans (Johansson and Söderhäll, 1985; Söderhäll and Smith, 1983). In Penaeid shrimp, enzymes of the proPO system are resided in the granular and semi-granular circulating hemocytes (Perazzolo and Barracco, 1997; Vargas-Albores et al., 1993). In the present study, tissue distribution by semi-

quantitative RT-PCR analysis revealed that *Pm*proPO1 and *Pm*proPO2 transcripts were mainly expressed in shrimp hemocytes. This is in agreement with the previously study showing that *P. leniusculus* and *P. monodon* proPO mRNAs are expressed only in hemocytes, but not in the hepatopancreas (Aspán et al., 1995; Sritunyalucksana et al., 1999). Moreover, the RT-PCR results also showed that the *Pm*PPAE1 and *Pm*PPAE2 mRNAs were mainly expressed in shrimp hemocytes, but not in other digestive tissues including the hepatopancreas and intestine. This result also resembles to the expression of the crayfish, *P. leniusculus* PPAE transcripts that detected only in the hemocytes (Wang et al., 2001b).

In many insect species for which data are available, two or more proPO genes have been reported. There are two proPO genes in the tobacco hornworm *Manduca sexta* (Hall et al., 1995; Jiang et al., 1997), the silkworm *Bombyx mori* (Asano and Ashida, 2001a, 2001b; Kawabata et al., 1995) and the beetle *Holotrichia diomphalia* (Kim et al., 2002), three in the fruit fly *Drosophila melanogaster* (Asada et al., 2003; Asano and Tekebuchi, 2009; Fujimoto et al., 1995; Myers et al., 2000) and *Pimpla hypochondriaca* (Parkinson et al., 2001), nine in the mosquito *Anopheles gambiae* (Christophides et al., 2002) and ten in the mosquito *Aedes aegypti* (Waterhouse et al., 2007). However, only one type of proPO gene has been reported in most crustacean species including crayfish *P. leniusculus* (Aspán et al., 1995), penaeid shrimps *P. monodon* (Sritunyalucksana et al., 1999) and *Fenneropenaeus chinensis* (Gao et al., 2009), lobsters *Homarus gammarus* (Hauton et al., 2005), giant freshwater prawn *Macrobrachium rosenbergii* (Liu et al., 2006; Lu et al., 2006), crabs *Cancer magister* (Terwilliger and Ryan, 2006), *Scylla serrata* (Ko et al., 2007) and *Eriocheir sinensis* (Gai et al., 2008) and the water flea *Daphnia pulex* (McTaggart et al., 2009), except two proPO sequences that reported in the two penaeid shrimps *Litopenaeus vannamei* (proPO1 and proPO2) (Ai et al., 2008, 2009; Lai et al., 2005; Wang et al., 2006) and *Marsupenaeus japonicus*. In animals with several proPO genes, there are some evidences showing that these genes are differentially expressed with respect to developmental stages (embryo to adult) or related to blood-feeding (Christophides et al., 2002; Li et al., 2005; Müller et al., 1999; Waterhouse et al., 2007). However, the

biological significance of these different expression patterns remains to be established.

Here, *P. monodon* is the third out of three reported Penaeid shrimps which have two distinct proPO genes. In order to elucidate the regulation of proPO system-associated genes during *P. monodon* larval development, semi-quantitative RT-PCR analysis was performed to determine the expression level of *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 transcripts during four larval developmental stages of *P. monodon*, including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15). The expression of *Pm*PPAE2 transcripts was not detected in the early stages of *P. monodon* larval development (N3 and Z2), but was detected at increasing levels in the later developmental stages (M2 and PL15). This contrasts to the expression of *Pm*PPAE1 transcripts that were presented in all four larval developmental stages examined, even though expressed at low levels in the N3 larvae. In the crayfish, *P. leniusculus*, proPO and PPAE transcripts were absent and detected at a low level, respectively, during the middle phase of crayfish embryo development (Zhang et al., 2010). In this study, the *Pm*proPO1 transcripts were found at very low to not detectable levels during the N3 developmental stage and gradually increased as the shrimp larval development progressed to low at the Z2 stage and high at the M2 and PL15 stages. This finding is similar to the expression of the proPO transcript previously reported by Jiravanichpaisal et al. which showed that proPO transcript was expressed of at low levels in the N4 stage and the expression was increased in later stages of *P. monodon* larval development (Jiravanichpaisal et al., 2007). In contrast to *Pm*proPO1, the mRNA expression of *Pm*proPO2 was not significantly different at fairly high levels during all four larval developmental stages examined. Comparison between the transcriptional profiles of the different two proPO and two PPAE transcripts probably suggests that *Pm*PPAE1 is likely to function as the activator of *Pm*proPO2 during the early stage, whist *Pm*PPAE2 might act as the activating proteinase of the *Pm*proPO1 in the later stages of the *P. monodon* larval development. However, the results of this study just provide a basis for developing a comprehensive understanding of structure/function relationships of the proPOs and PPAEs in shrimp proPO system.

To date, gene silencing by using double stranded RNA (dsRNA)-mediated RNA interference (RNAi) is considered to be an efficient technique that use for functional investigating of the genes of interested. The RNAi approach in the dipteran insects *Anopheles gambiae* and *D. melanogaster* established the function of the three *Anopheles* clip-SPs, CLIPB4, CLIPB8 and CLIPB14, to be involved in the proPO pathway (Paskewitz et al., 2006; Volz et al., 2005; Volz et al., 2006), and the two drosophilid clip-SPs, MP1 and MP2/sp7/PAE1, to be required for proPO activation (Castillejo-López and Häcker, 2005; Tang et al., 2006). Recently, the RNAi technique has been shown to be a powerful tool for the investigation of gene function in crustacean (Amparyup et al., 2010; Liu et al., 2007). In the freshwater crayfish, *P. leniusculus*, dsRNA-mediated gene suppression of the proPO transcripts increased host susceptibility to a highly pathogenic bacterium, *Aeromonas hydrophila* (Liu et al., 2007). If the specific inhibitor of the crayfish PPAE, pacifastin, is silenced by RNAi treatment, the higher PO activity was observed and the mortality after *A. hydrophila* infection was also reduced (Liu et al., 2007). Recently, suppression of *Pm*ClipSP1 by dsRNA-midiated RNAi in *P. monodon* demonstrated that the *Pm*ClipSP1 is not required for shrimp proPO-activation cascade, but plays an important role in the antibacterial defense against the highly pathogenic bacterium *Vibrio harveyi* (Amparyup et al., 2010). Here, the RNAi technique was applied to elucidate the role of the two PPAEs (*Pm*PPAE1 and *Pm*PPAE2) and two proPOs (*Pm*proPO1 and *Pm*proPO2) in *P. monodon* immune system.

So far, the clip-SP that functions as a proPO-activating enzyme (PPAE) in the Penaeid shrimps has not yet been identified. In this study, we report the functional characterization of the novel PPAE (*Pm*PPAE1 and *Pm*PPAE2) by using dsRNA-mediated gene silencing of the respective PPAE transcripts. Semi-quantitative RT-PCR analysis revealed that gene silencing of both *Pm*PPAE1 and *Pm*PPAE2 is sequence-specific. Interestingly, dsRNA-mediated gene suppression of *Pm*PPAE1 and *Pm*PPAE2 transcripts significantly decreased the hemolymph PO activity in the *Pm*PPAE1 (~37%) and *Pm*PPAE2 (41%) knockdown shrimps, as compared with saline injected control, suggesting that both *Pm*PPAE1 and *Pm*PPAE2 are the proteinases that function in shrimp proPO system activation. However, co-silencing of

*Pm*PPAE1 and *Pm*PPAE2 transcripts resulted in a non-significant difference with 41% reduction of PO activity as compared to control shrimp injected with saline solution. These data might suggest that both *Pm*PPAE1 and *Pm*PPAE2 participate in shrimp proPO cascade and they might be function as the activating proteinase in the same branched-pathway for activation of the shrimp proPO system. However, whether *Pm*PPAE1 or *Pm*PPAE2 are the activating proteinase that directly cleaves the *Pm*proPO1 and/or *Pm*proPO2 remains to be resolved by further investigation. Nonetheless, our data demonstrated that both *Pm*PPAE1 and *Pm*PPAE2 are required for shrimp proPO system.

The potential role of *Pm*PPAE1 and *Pm*PPAE2 in the shrimp immune defense against the bacterial infection was further investigated by systemic challenged the *Pm*PPAE1 and *Pm*PPAE2 knockdown shrimp with the highly pathogenic bacterium, *V. harveyi*. Interestingly, the *Pm*PPAE1 silenced shrimp had a cumulative mortality of 100% within day 3 post *V. harveyi* infection, as compared to the control shrimps injected with GFP dsRNA and saline solution. Moreover, knockdown of *Pm*PPAE1 strongly increased the number of viable *V. harveyi* (4-fold) in *Pm*PPAE1 silenced shrimp, as compared with GFP dsRNA injected shrimp. These results suggest that *Pm*PPAE1 functions in shrimp proPO system and also plays an essential role in a bacterial clearance pathway.

Furthermore, systemic challenge of *V. harveyi* into the *Pm*PPAE2 silenced shrimp also resulted in a significant increased in the cumulative mortality in *Pm*PPAE2 the knockdown shrimp with 77% and 83% cumulative mortality within day 1 and 2 post infection, respectively, when compared to the GFP dsRNA or saline injected controls. Moreover, the viable *V. harveyi* CFUs in the *Pm*PPAE2 silenced shrimp hemolymph also higher than that of control shrimp injected with GFP dsRNA. These results suggest that *Pm*PPAE2 participates in the proPO system in protecting shrimp from the highly pathogenic bacterium *V. harveyi* infection.

In an attempt to elucidate the involvement of the two *Pm*proPO gene products in shrimp proPO system *in vivo*, shrimps were silenced with *Pm*proPO1, *Pm*proPO2 or both *Pm*proPO1 and *Pm*proPO2 dsRNAs. Semi-quantitative RT-PCR showed that

the entire dsRNAs specifically reduced the endogenous expression of the *Pm*proPO1 and *Pm*proPO2 transcripts in the silenced-shrimp hemocytes. Although a complete silencing of the *Pm*proPO transcripts could not be achieved in the dsRNA-treated groups, the levels of *Pm*proPO1 and *Pm*proPO2 mRNA transcripts were significantly suppressed. The incomplete knockdown of the proPO transcript was also reported in the crayfish *P. leniusculus*, in which proPO dsRNA reduced the proPO transcripts about 57% (Liu et al., 2007).

Even though proPOs have long been considered to be the key enzymes in shrimp proPO-activating system, there is no direct evidence verifying its role in this system. In this study, the effect of *Pm*proPO gene knockdown on PO activity was investigated by *in vivo* RNAi experiments. The data clearly showed a significant reduction of PO activity in the *Pm*proPOs silenced shrimps, suggesting that both *Pm*proPO (*Pm*proPO1 and *Pm*proPO2) gene products are essential components of the biochemical pathway that required for the proPO-activating system in shrimps. This is the first report on the *in vivo* functional characterization of the two proPOs in shrimp immunity.

Crustacean proPO sequences are homologous with arthropod hemocyanins, and a PO-like activity in hemocyanins, the copper-containing proteins that function as arthropod oxygen transporters, have also been detected in the hemolymph of crustaceans (García-Carreño et al., 2008; Lee et al., 2004; Sellos et al., 1997). Crustacean hemocyanin, synthesized in the hepatopancreas and localized in the plasma, typically represents up to 95% of the total amount of hemolymph protein (Nagai et al., 2001). Certainly, hemocyanin is functionally converted into a PO-like enzyme by some reagents or endogenous molecules in several arthropod species (Adachi et al., 2003; Decker and Rimke, 1998; García-Carreño et al., 2008; Lee et al., 2004; Nagai et al., 2001; Pless et al., 2003). In the present investigation, knockdown of two *Pm*proPO genes by co-silencing in *P. monodon* had the strongest effect on the PO activity in the hemolymph with a 88% reduction in PO activity after LPS/PGN and laminarin (β-1,3-glucan) activation. This strongly suggests that the two *Pm*proPOs are the key enzymes contributing to the PO activity in *P. monodon* hemolymph. However, it is unclear if the remaining hemolymph PO activity (12%),

after partial co-silencing of both *Pm*proPOs is be due to the activity of hemocyanin, or other proPO isoforms, or simply the incomplete knockdown of *Pm*proPO1 and *Pm*proPO2 allowing a low level of *de novo* translation and enzyme synthesis.

The prophenoloxidase-activating system is an important defense mechanism in invertebrates against diverse pathogens (Cerenius and Söderhäll, 2004). However, the important function of proPO system in the defence against microbial infection is still highly debated (Cerenius et al., 2008). In the insects, it has been reported that the proPO reaction is not important for survival of microbial infections in *Drosophila* (Leclerc et al., 2006) and *A. gambiae* (Schnitger et al., 2007). *Drosophila* with a mutant (null) proPO-activating enzyme (PAE1) gene and *A. gambiae* with a silenced non-catalytic serine protease (CLIPA8) gene both showed depletion of PO activity, but could survive after microbial challenge, suggesting that melanization is not essential for defense against microbial infection (Leclerc et al., 2006; Schnitger et al., 2007). In contrast, the knockdown of the *Drosophila* melanization proteases (MP1 and MP2) strongly supported that melanization is crucial for the innate immune system against fungal infections (Tang et al., 2006). Furthermore, it has been shown in *P. leniusculus* that knockdown of the proPO transcripts by dsRNA increased susceptibility to *Aeromonas hydrophila* infection (Liu et al., 2007), suggesting the important role of phenoloxidase in defense against bacterial invasion. In this study, the obtained cumulative mortality results provide further evidence to support that the proPO-activating system plays an essential role in the *P. monodon* shrimp defense against the pathogenic bacterium, *V. harveyi* infection. It is noteworthy that partial silencing of either the *Pm*proPO1 or *Pm*proPO2 gene resulted in a strong PO activity reduction (73 - 75%) and a very high shrimp mortality after *V. harveyi* challenge, despite the appearance that only one type of proPO mRNA was suppressed (as shown by RT-PCR). It is possible, if not likely, that the two POs might cooperatively function in the proPO-activating system and probably acts as heteromer. In *M. sexta* and *B. mori*, two different proPOs, encoded by separate subunit genes, exist predominantly as a heterodimer in the plasma and their transcriptional expression are coordinately regulated (Asano and Ashida, 2001a, 2001b; Jiang et al., 1997; Yasuhara et al., 1995). Moreover, the obtain crystal structure data confirmed that *M. sexta*

proPO is a heterodimer which consists of 2 homologous polypeptide chains, proPO1 and proPO2 (Li et al., 2009). However, further study is required to determine the structure of the *P. monodon* proPOs whether they exist as a heteromer of two subunit types in hemolymph. In addition, the bacterial number in hemolymph of the proPO co-silenced shrimp (that is pre-injected with two *Pm*proPO dsRNAs) was shown to be increased about six-fold at 6 hours post *V. harveyi* infection. Taken together, this study clearly shown that both *Pm*proPO1 and *Pm*proPO2 are likely to be the important components of the *P. monodon* shrimp to defense against the pathogenic bacterium *V. harveyi* infection.

In the present study, the important role of two proPOs (*Pm*proPO1 and *Pm*proPO2) and two PPAEs (*Pm*PPAE1 and *Pm*PPAE2) in the *P. monodon* shrimp immune defense against systemic *V. harveyi* infections have been elucidated. In contrast to some reports in *Drosophila* (Leclerc et al., 2006) and in *A. gambiae* (Schnitger et al., 2007), that have questioned the importance of phenoloxidase in the defence against microbial infections, this study clearly demonstrated that the two proPOs and two PPAEs are the essential molecules in the shrimp innate system to defense against systemic *V. harveyi* infections. Moreover, these results are consistent with those reported in the crayfish where proPO gene knockdown resulted in an increased bacterial count of about 2.3-fold in proPO-silenced crayfish (Liu et al., 2007). Taken together, these data suggest that the genes that are involved in the proPO system of crustaceans (shrimp and crayfish) play an important role in the control of systemic bacterial infections.

Even though the proPO activation has been studied in many arthropod species, the mechanism on activation of proPO system at protein level in shrimps remains elusive. To obtain sufficient *Pm*PPAE1 protein for *in vitro* functional characterization, recombinant proteins produced from the efficient expression system appear to be highly desirable enough protein for functional analysis. In the present study, the *E. coli* expression system is the first choice that selected for production of the recombinant *Pm*PPAE1 (r*Pm*PPAE1) due to its easy to carried out and large amount of the protein will be obtained (Cabrita et al., 2004). As a result, the r*Pm*PPAE1 proteins were expressed as inclusion bodies and after purified by Ni-NTA affinity

chromatography under denaturing condition, the r*Pm*PPAE1 protein cannot renature and the degradation products were also observed. These results was similar to the recombinant proPAP2 of *M. sexta* produced from the *E. coli* system, which failed to renature and the recombinant protein contained significant amount of the degradation products and also precipitated after storage at -20 $^{\circ}$C (Ji et al., 2003).

To solve this problem, only C-terminal serine proteinase (SP) domain of the *Pm*PPAE1 was chosen for expression in the bacterial expression system. As a result, the recombinant SP domain of *Pm*PPAE1 (rSP-domain) was successfully expressed in *E. coli* system. However, *in vitro* experimental assays demonstrated that the rSP-domain lacks the proteolytic activity and the ability to activate shrimp proPO system. For the clip-domain family of serine proteinase, the clip domain remains covalently attached to its catalytic SP domain by an inter-chain disulfide bridge after proteolytic activation by cleavage between their clip and SP domains (Jiang and Kanost, 2000). In the present study, the lack of the biological activity of rSP-domain may be due to the missing of the clip-domain in the recombinant protein. However, the purified rSP-domain was further used as an immunogen to generate the rabbit polyclonal anti-rSP-domain antisera and western blot analysis using this polyclonal antiserum demonstrated that the endogenous *Pm*PPAE1 was presented only in shrimp hemocytes, but not in the cell-free plasma.

The baculovirus-infected insect cell expression system are often used to produce recombinant proteins that are not successfully expressed in *E. coli* because protein folding problem and the requirement of glycosylation or post-translational modification. In many insect species, several recombinant PPAE proteins such as *M. sexta* proPAP1 and proPAP2 were reported to be successfully expressed in the insect cell system (Wang et al., 2001c; Ji et al., 2003). In an attempt to produce the recombinant *Pm*PPAE1 (r*Pm*PPAE1) protein for reconstitution a branch of shrimp proPO activation cascade *in vitro*, the mature peptide (zymogen) of *Pm*PPAE1 protein was recombinantly expressed in the insect cell system. To facilitate secretion and purification of recombinant protein, *M. sexta* PAP2 signal peptide and the 6×histidine affinity tag were introduced into the N- and C- terminus of the *Pm*PPAE1 protein. Unfortunately, the r*Pm*PPAE1 protein did not secrete into the cell culture medium and

the r*Pm*PPAE1 protein level in the cell lysate was not increased as the infection time was prolonged and the dead cells became noticeable at day 3 post infection. Moreover, the r*Pm*PPAE1 was produced in the insoluble fraction and the purified r*Pm*PPAE1 obtained from Ni-NTA column was not sufficient for protein refolding. These results suggest that the r*Pm*PPAE1 protein perhaps toxic to the Sf9 cells, thus *Pm*PPAE1 was not successfully expressed in the insect cell expression system.

In summary, two clip-SP genes (*Pm*PPAE1 and *Pm*PPAE2) and two proPO genes (*Pm*proPO1 and *Pm*proPO2) were identified from the black tiger shrimp, *P. monodon*. Even though the *in vitro* biological activity of the recombinant mature and serine proteinase domain of *Pm*PPAE1 were not accomplished, the *in vivo* functional characterization was successfully performed and the results suggest the role of two PPAEs and two proPOs gene products in shrimp proPO system as well as an important role in the shrimp immune defense against *V. harveyi* infection. To our knowledge, this is the first time to identify and functionally characterize the components of shrimp proPO system *in vivo*. In addition, the obtained data also provides a brief outline of the expression profiles of the two *Pm*PPAE and two *Pm*proPO transcripts during several stages of *P. monodon* larval development.

CHAPTER V

CONCLUSIONS

1. Two PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) and a novel proPO (*Pm*proPO2) gene were identified from hemocytes of the black tiger shrimp *Penaeus monodon*. Sequence comparison revealed that *Pm*PPAE1 and *Pm*PPAE2 exhibited the highest amino acid sequence similarity of 70% and 51% to the freshwater crayfish *Pacifastacus leniusculus* PPAE and the tobacco hornworm *Manduca sexta* PAP1, respectively, whilst *Pm*proPO2 exhibited 67% sequence identity to the previously reported *Pm*proPO1.

2. Tissue distribution analysis showed that *Pm*PPAE1, *Pm*PPAE2, *Pm*proPO1 and *Pm*proPO2 mRNA transcripts were mainly expressed in shrimp hemocytes. Analysis of these gene transcripts during four stages of larval development revealed that *Pm*PPAE1 and *Pm*proPO2 transcripts were expressed in all stages (nauplius, protozoea, mysis and post-larvae), whereas *Pm*PPAE2 and *Pm*proPO1 transcripts were mainly expressed in the late stages (mysis and post-larvae) of larval development. These results possible suggest that *Pm*PPAE1 is likely to function as the activator of *Pm*proPO2 during the early stages, whilst *Pm*PPAE2 might acts as the activating proteinase of the *Pm*proPO1 in the late stages of *P. monodon* larval development.

3. Double-stranded RNAs (dsRNAs)-mediated gene silencing of *Pm*PPAE and *Pm*proPO transcripts resulted in a specific reduction of the respective transcript levels and significantly reduced the hemolymph PO activity in the *Pm*PPAE1 (36.7%), *Pm*PPAE2 (41%), *Pm*proPO1 (75%) and *Pm*proPO2 (73%) silenced shrimps as compared to the control group injected with saline solution. Moreover, experimental infection of *P. monodon* with the highly pathogenic bacterium *Vibrio harveyi* resulted in a significant increase in the cumulative mortality and

the bacterial number in the *Pm*PPAE and *Pm*proPO silenced shrimps. These results suggest that both *Pm*PPAEs and *Pm*proPOs are the components of proPO system and also play the crucial roles in protecting shrimp from *V. harveyi* infection.

4. The zymogen form of *Pm*PPAE1 was recombinantly expressed in *E. coli* and insect cell expression systems, but the obtained recombinant protein was not stable. Nevertheless, the recombinant serine proteinase domain of the *Pm*PPAE1 (rSP-domain) was successfully expressed in *E. coli* expression system. Moreover, the purified rSP-domain was used as an immunogen for generating the antibody against the SP domain of *Pm*PPAE1 and western blot analysis using the obtained antibody revealed that the endogenous *Pm*PPAE1 was expressed only in hemocytes but not in cell-free plasma of *P. monodon*.

5. Our data demonstrates that all of the proPO system-associated gene including two PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) and two proPO genes (*Pm*proPO1 and *Pm*proPO2) play the essential role in shrimp proPO system as well as in the shrimp immune defense against *V. harveyi* infection.

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