บทบาทของโปรตีนยับยั้งเมลาไนเซชันในการควบคุมระบบโพรฟีนอลออกซิเดสของกุ้งกุลาดำ *Penaeus monodon*

นายณัฐภพ หนูทวน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ROLE OF MELANIZATION INHIBITION PROTEIN IN REGULATION OF PROPHENOLOXIDASE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*

Mr. Nattaphop Noothuan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
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Thesis Title	ROLE OF MELANIZATION INHIBITION PROTEIN IN REGULATION OF PROPHENOLOXIDASE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*
By	Mr. Nattaphop Noothuan
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Professor Anchalee Tassanakajon, Ph.D.
Thesis Co-Advisor	Piti Amparyup, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Rath Pichyangkura, Ph.D.)

.....Thesis Advisor
(Professor Anchalee Tassanakajon, Ph.D.)

.....Thesis Co-Advisor
(Piti Amparyup, Ph.D.)

.....Examiner
(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

.....External Examiner
(Kallaya Sritunyalucksana-Dangtip, Ph.D.)

ณัฐภพ หนูทวน : บทบาทของโปรตีนยับยั้งเมลาไนเซชันในการควบคุมระบบโพรฟีนอลออกซิเดสของกุ้งกุลาดำ *Penaeus monodon* (ROLE OF MELANIZATION INHIBITION PROTEIN IN REGULATION OF PROPHENOLOXIDASE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ปิติ อ่ำพายัพ, 119 หน้า.

การสร้างเมลานินโดยระบบโพรฟีนอลออกซิเดส เป็นการตอบสนองทางภูมิคุ้มกันที่สำคัญในสัตว์ไม่มีกระดูกสันหลัง ระบบโพรฟีนอลออกซิเดสผลิตสารตัวกลางที่ไวต่อปฏิกิริยาและเมลานินเพื่อต่อสู้กับเชื้อจุลชีพที่บุกรุก แต่อย่างไรก็ตามระบบโพรฟีนอลออกซิเดสต้องถูกควบคุมอย่างเข้มงวดเพื่อหลีกเลี่ยงการผลิตที่มากเกินพอของสารประกอบ เช่น เมลานิน ซึ่งสามารถก่อให้เกิดอันตรายต่อเซลล์เจ้าบ้านได้ ในงานวิจัยนี้ศึกษาโปรตีนยับยั้งการสร้างเมลานินจากกุ้งกุลาดำ *Penaeus monodon* ชื่อ *Pm*MIP ซึ่งถูกค้นพบจากฐานข้อมูล *P. monodon* EST และเทคนิค Rapid Amplification of cDNA Ends (RACE) ฝั่ง 5’ และ 3’ โดย *Pm*MIP มี open reading frame ขนาด 957 คู่เบส แปลเป็นโปรตีนที่ประกอบด้วยกรดอะมิโน 318 ตัว ซึ่งทำนายว่ามีขนาด 34.75 กิโลดาลตัน และ pI 5.5 จากการวิเคราะห์ลำดับด้วย BLASTX พบว่า *Pm*MIP คล้ายกับ *Pl*MIP จากเครย์ฟิช *Pacifastacus leniusculus* 80% การวิเคราะห์ลำดับกรดอะมิโนของ *Pm*MIP แสดงถึง fibrinogen-related domain และ aspartic-rich region ยีน *Pm*MIP มีการแสดงออกมากในเนื้อเยื่อต่าง ๆ ได้แก่ เหงือก ตับ ลำไส้ อวัยวะน้ำเหลือง และกล้ามเนื้อ โดยพบว่า *Pm*MIP ในเหงือกมีการแสดงออกลดลงหลังการติดเชื้อแบคทีเรีย *Vibrio harveyi* และโปรตีน *Pm*MIP ในน้ำเลือดลดลงที่ 6 ชั่วโมง และกลับมาที่ 24 ชั่วโมงหลังการติดเชื้อ การยับยั้งการแสดงออกของยีน *Pm*MIP ด้วยการใช้อาร์เอ็นเอสายคู่ สามารถยับยั้งการแสดงออกของ *Pm*MIP ทั้งในระดับของยีนและโปรตีนได้ เป็นผลให้กิจกรรมของโพรฟีนอลออกซิเดสและกิจกรรมของโปรติเนสในเลือดเพิ่มขึ้น นอกจากนี้โปรตีนรีคอมบิแนนท์ *Pm*MIP สามารถถูกสร้างได้ในระบบ *Escherichia coli* และถูกทำให้บริสุทธิ์โดยโครมาโทกราฟีแบบจำเพาะ nickel-NTA การศึกษาหน้าที่ของโปรตีนรีคอมบิแนนท์ *Pm*MIP พบว่าโปรตีนรีคอมบิแนนท์สามารถยับยั้งกิจกรรมของโพรฟีนอลออกซิเดสในเลือดได้ จากผลดังกล่าวทั้งหมดบ่งชี้ว่า *Pm*MIP มีความเกี่ยวข้องกับระบบโพรฟีนอลออกซิเดส โดยมีบทบาทเป็นตัวควบคุมในเชิงลบ และรบกวนโปรติเนสในเลือด

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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NATTAPHOP NOOTHUAN: ROLE OF MELANIZATION INHIBITION PROTEIN IN REGULATION OF PROPHENOLOXIDASE SYSTEM OF BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: PITI AMPARYUP, Ph.D., 119 pp.

Melanization mediated by the prophenoloxidase-activating system (proPO) is an important immune response in invertebrates. The proPO system produces the reactive intermediates and melanin to fight against invading microorganism. However, the proPO system must be tightly regulated to avoid the excess production of their components such as melanin that can damage host cells. In this study, a melanization inhibition protein from black tiger shrimp *Penaeus monodon*, named *Pm*MIP, which has been previously identified from *P. monodon* EST Database and 5' and 3' Rapid Amplification of cDNA Ends (RACE), was further characterized. The open reading frame of *Pm*MIP contains 957 bp encoding a protein of 318 amino acid residues with a predicted molecular mass of 34.75 kDa and pI 5.5. The sequence analysis by BLASTX revealed that *P*mMIP exhibits 80% similarity to *Pl*MIP from crayfish *Pacifastacus leniusculus*. The amino acid sequence analysis of *Pm*MIP shows a fibrinogen-related domain and aspartic-rich region. *Pm*MIP transcript was highly expressed in various shrimp tissues such as gills, hepatopancreas, intestine, lymphoid organ, and muscle. The expression of *Pm*MIP in gill was down-regulated after *Vibrio harveyi* infection and *Pm*MIP protein in plasma was decreased at 6 h and recovered at 24 h post infection. Double-stranded RNA mediated gene silencing of *Pm*MIP could suppress both *Pm*MIP transcriptional and translational levels and resulted in an increase in hemolymph phenoloxidase activity and proteinase activity. Furthermore, the recombinant *Pm*MIP (r*Pm*MIP) protein was successfully expressed in *Escherichia coli* system and purified by nickel-NTA affinity chromatography. r*Pm*MIP was found to inhibit hemolymph PO activity. These results suggested that *Pm*MIP was involved in the proPO system by acting as a negative regulator and interfering hemolymph proteinase function.

Department:	Biochemistry	Student's Signature .....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature .....
		Co-Advisor's Signature .....
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LIST OF ABBREVIATIONS

%	percentage
℃	degree Celcius
µg	microgram
µl	microliter
µM	micromolar
A	absorbance
AHPND	acute hepatopancreatic necrosis disease
amp	ampicillin
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
C-terminus	carboxyl terminus
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double stranded deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
EF1α	elongation factor 1 alpha

EMS	early mortality syndrome
EST	expressed sequence taq
GFP	green fluorescence protein
h	hour
HL	hemolymph
HLS	hemocyte lysate supernatant
hpi	hour post infection
IgG	immunoglobulin G
IPTG	isopropyl-beta-D-thiogalatopyranoside
kana	kanamycin
kb	kilobase pair
kDa	kilodalton
Laminarin	beta-1,3-glucan
LB	Luria broth
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
ml	milliliter

mM	millimolar
Mw	molecular weight
ng	nanogram
nm	nanometer
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pI	isoelectric point
Pl	*Pacifastacus leniusculus*
Pm	*Penaeus monodon*
PO	phenoloxidase
PPAE	prophenoloxidase activating enzyme
proPO	prophenoloxidase
r	recombinant protein
RNA	ribonucleic acid
RNAi	ribonucleic acid interference

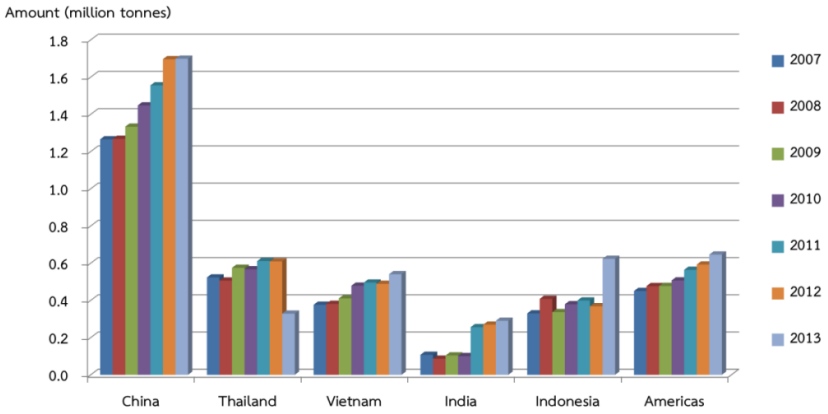
rpm	round per minute
RT	reverse transcription
s	second
SDS	sodium dodecyl sulfate
Snake	snake-like serine proteinase
SP	serine proteinase
SPH	serine proteinase homolog
ssRNA	single stranded ribonucleic acid
Tm	*Tenebrio molitor*
TSB	trybric soy broth
TSV	Taura syndrome virus
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
WSSV	white spot syndrome virus
YHV	yellow head virus

CHAPTER I

INTRODUCTION

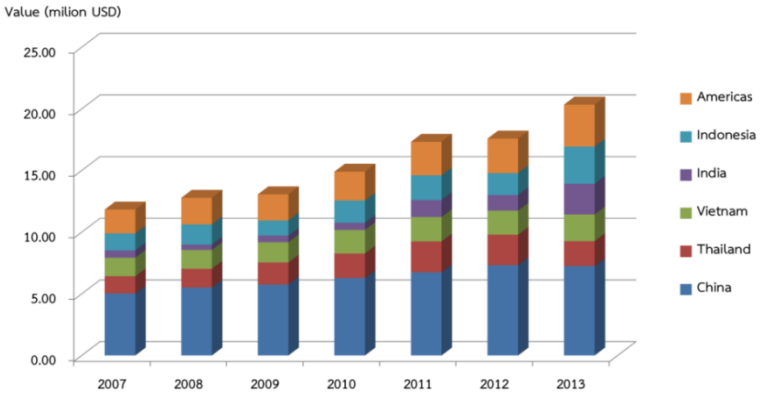
1.1 General introduction (History and Economics)

Shrimp is one of the most important commodities in the term of value. From the past, export-oriented shrimp aquaculture took off in the mid-1970s. The shrimp aquaculture industry conduced to 2.5% of total shrimp production in 1975 and gradually increased to approximately 30% in 1990s (Rönnbäck 2001). In 2003, global shrimp exports and products exceeded USD 11 billion and represented 17% of the world's total exports of seafood products (Leung and Engle 2006). Recently, cultured shrimp around 80% came from Asia with China, Thailand, Vietnam, India, and Indonesia as the major producers (Rönnbäck 2001). By the amount, China and Thailand are the leading shrimp production countries which the trend of production increased every year (source: FAO, 2015) (Figure 1.1) in corresponding with the values of shrimp production in major producing countries have grown exponentially according to FAO, 2015 (Figure 1.2).



Source: FAO, 2015.

Figure 1.1 Farm-raised shrimp production by country during 2007 – 2013.



Source: FAO, 2015.

Figure 1.2 Value of shrimp production by country during 2007 – 2013.

In Thailand, marine shrimp farming has been carrying on for over 60 years. A survey of Teinsongrusmee (1970) showed the farms were located in the province of Samut Prakarn, Samut Sakhon, Samut Songkhram, Chanthaburi, Nakhon Si Thammarat, Rayong Surat Thani, and Songkhla. The reared shrimp species comprised

banana shrimp (*Penaeus merguiensis*), school shrimp (*Metapenaeus ensis*) and a small volume of black tiger prawn (*P. monodon*) (Sutonya, 1995). In 1967, the Department of Fisheries promoted freshwater prawn (*Macrobrachium rosenbergii*) to farmer, but the supply from the freshwater prawn industry exceed demand and then the government supported the conversion of freshwater prawn to black tiger prawn, because of its large size and rapid growth rate (Singholka et al. 1980; Briggs 1994). Thailand has emerged as the world's leading farmed shrimp producer and exporter base on black tiger prawn in the early 1990s (Wyban 2007).

Since 2001, farmers of black tiger shrimp have faced Monodon Slow Growth Syndrome (MSGS), a new disease, which cause slow growth. The production led to the loss in the term of production yield and values in 2002 that was dramatically decreased to 68% by value from 2000 (Chayaburakul et al. 2004). MSGS seriously damaged the black tiger shrimp production causing the switch of the cultured species to the imported stock of white shrimp (*P. vannamei*) which shows high survival and fast growth (Chayaburakul et al. 2004; Wyban 2007). In addition, *P. vannamei* is more tolerant of higher density stocking than *P. monodon* and also resistant to Taura syndrome virus (TSV). Figure 1.3 represents the production of *P. vannamei* in 2002 great jumped to closely 200,000 metric tonnes, whereas the *P. monodon* production substantially reduced (Wyban 2007).

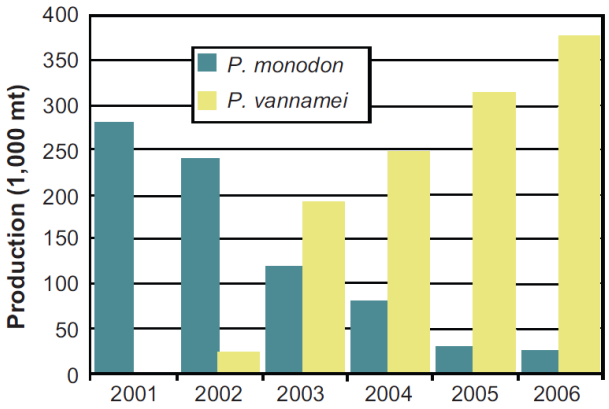
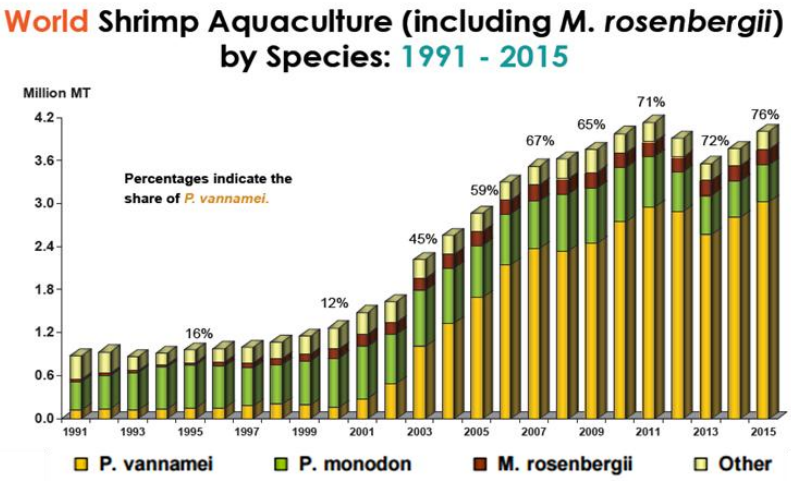


Figure 1.3 Thailand's farmed shrimp production with *P. monodon* and *P. vannamei*. (Wyban, 2007).

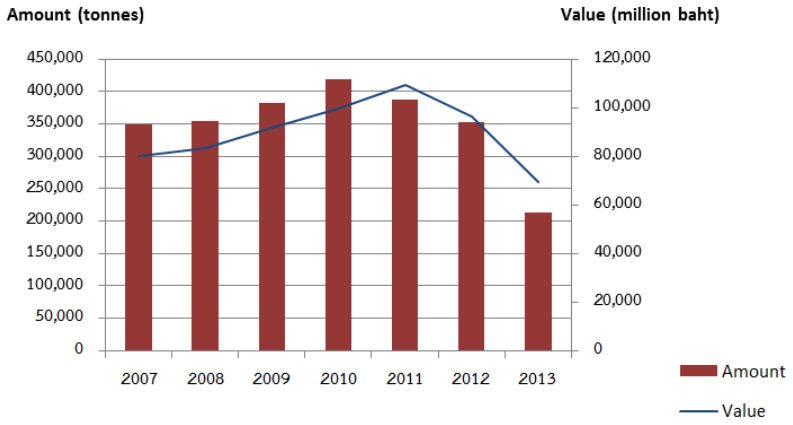
In corresponding to FAO (2013), trend of world shrimp aquaculture in Figure 1.4 illustrates the world's production of white shrimp rapidly increased while the production of black tiger shrimp gradually decreased since 2001.



Source: FAO, 2013 for 1991-2011; GOAL, 2013 for 2012-2015.

Figure 1.4 Shrimp aquaculture by species during 1991-2015.

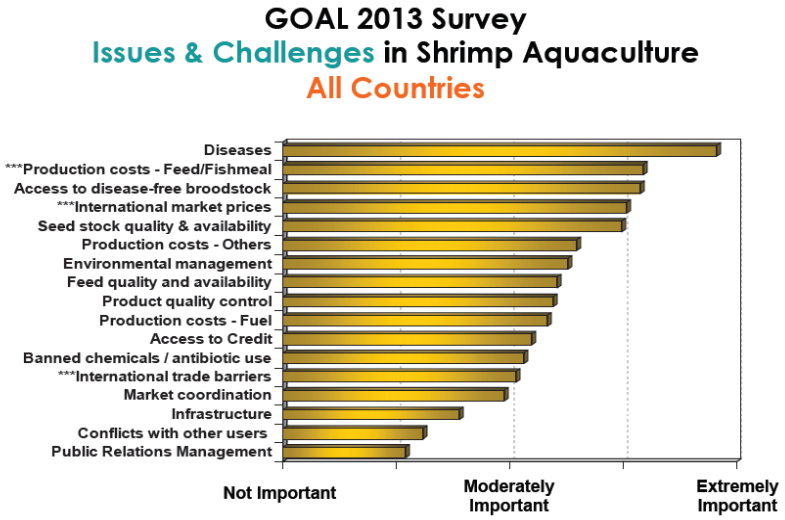
Recently, the worldwide spread of new outbreak, early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) has seriously damaged the Thailand's shrimp export which have declined from 2010 to 2013 about 7%, 16%, and 49% respectively by the production year (Figure 1.5). The disease also caused negative impact to many Asian contries (FAO, 2013) including China and Vietnam.



Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand.

Figure 1.5 Thailand's shrimp exportation during 2007-2013.

The GAA and GOAL survey the issues and challenges in shrimp aquaculture in all countries, the data presents "diseases" as a factor to be concerned, followed by production costs and access to disease-free broodstock (Figure 1.6). To prevent the spreading of the outbreaks caused by pathogens and to promote the black tiger shrimp farming, the better understanding in shrimp immunity is an essential constituent to improve the new strategy for solving all these problems.



Source: Global Aquaculture Alliance (GAA), Global Outlook for Aquaculture Leadership (GOAL), 2011.

Figure 1.6 Factors affecting shrimp aquaculture in 2013 by all countries.

1.2 Taxonomy of black tiger shrimp

Penaeus (*Penaeus*) *monodon* Fabricius, 1798

Kingdom Animalia

Phylum Arthropoda

Class Malacostraca

Order Decapoda

Superfamily Penaeoidea

Family *Penaeidae*

Genus *Penaeus*

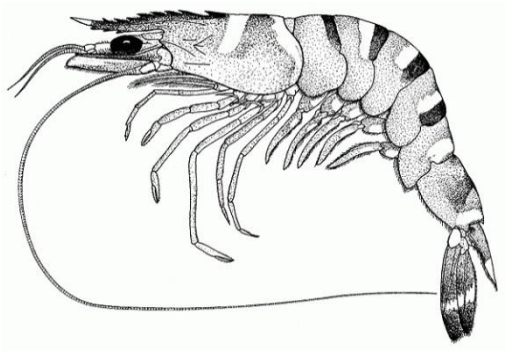


Figure 1.7 Black tiger shrimp (black and white drawing) (FAO, 1980)

FAO Names: Giant tiger prawn (En), Crevette géante tigrée (Fr), Camarón tigre gigante (Sp).

Synonymy: *Penaeus carinatus* Dana, 1852; *Penaeus tahitensis* Heller, 1662; *Penaeus semisulcatus exsulcatus* Hilgendorf, 1879; *Penaeus coeruleus* Stebbing, 1905; *Penaeus bubulus* Kubo, 1949; *Penaeus monodon monodon* Burkenroad, 1959. In older literature often confused with *P. semisulcatus*.

Local Names: Tiger prawn (S. and E. Africa), Kamba, Kamba ndogo (Swahili language, Kenya) Kalri (Pakistan), Jinga (Bombay, India), Kara chemmeen (Kerala, India), Yera (Madras, India), Bagda chingri (Calcutta, India), Ushi-ebi (Japan), Grass shrimp (Taiwan), Ghost prawn (Hong Kong), Sugpo, Jumbo tiger shrimp (Philippines), Udang windu, Udang pantjet (Indonesia), Jumbo tiger prawn, Giant tiger prawn, Black tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Kung kula-dum (Thailand), Tim sa (Vietnam).

Source Mohamed (1967); FAO (1980).

1.3 Life cycle of shrimp

There are many developmental stages in life of shrimp (Figure 1.8). After spawning, egg takes about 14-18 hours to hatch and then become the first larva stage, nauplius. The nauplius develops to protozoea, second larva stage, in 24-36 hours and this larva stage starts to eat diatom and atemaia as food source. The third larva stage is mysis that takes 3-5 day from last development. At this stage the larva begins to develop pleopods or swimmerets. After the three larva developmental stages (over 4-6 weeks), the larva develops to shrimp post larval stage. The post larva develops walking legs and can use abdominal appendages for propulsion. Juvenile is a young shrimp that is 1-2 months later in post larva. Finally, the final growth stage is adult which develop to a mature shrimp.

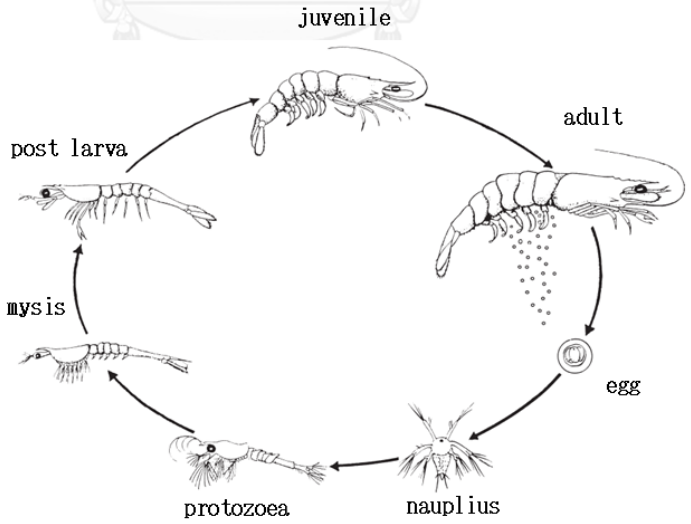


Figure 1.8 Life cycle of penaeid shrimp. (modified photo from Lee and Wickins (1992)

1.4 Anatomy of shrimp

Black tiger shrimp, has a shrimp body model including a head, five pairs of swimming legs (pleopods), five pair of walking legs (pereopods), and tail. On the head, there are many appendages such as a carapace (hard exoskeleton) enclosing the cephalothorax, a rostrum which is an extension of the carapace, as well as posterior ridge. It has six to eight dorsal teeth and two to four sig-moidal shape of ventral teeth. A telson is unarmed. *P. monodon* species is distinguished from others by the lack of an exopod (external branch) on the fifth pleopodia. (Figure 1.9)

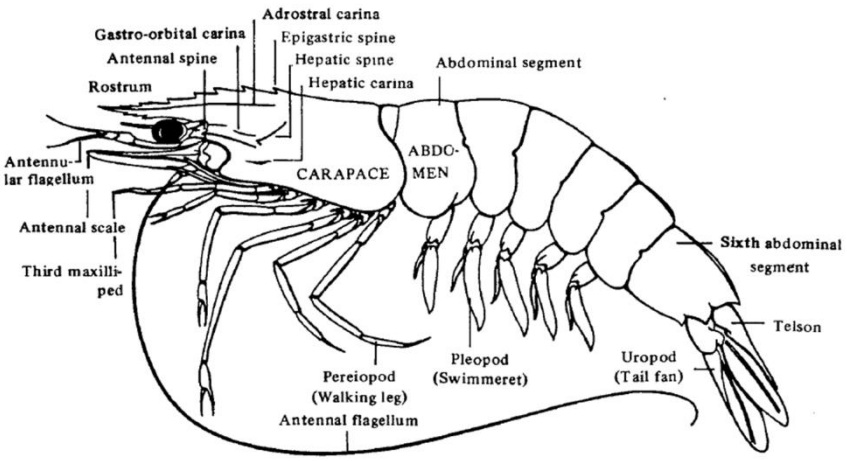


Figure 1.9 Latheral view of black tiger shrimp. (Motoh 1981)

Color of base body varies from green, brown, red, grey, or blue. On the back and tail, the colors are distinct black and white stripes and on the abdomens, the stripes alternate black/yellow or blue/yellow.

In female shrimp, there is a sperm receptacle or thelycum which locates on the last ventral thoracic segment. And the appendix masculina is an oval flap on the second pleopod which can distinguish male from female. (Dall et al. 1990)

1.5 Shrimp diseases

Diseases are the major causes of mortality in cultured shrimp and be considered as limiting factors to successful production. The disease may be caused by living agent or other influences of the environment (Johnson 1989). Infectious etiologies occur by various types of pathogens, for example, viruses, bacteria, fungus, and protozoa (Lightner 1993). A survey of GAA with disease agents in 2001 showed 60% of losses were attributed to virus and about 20% to bacteria (Flegel et al. 2008).

1.5.1 Viral diseases

Viral diseases are crucial endanger to shrimp aquaculture. There are at least 13 viral diseases in cultured penaeid shrimp (Lightner 1993; Lightner 1996). In Thailand, the concerned major viruses are white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), Taura syndrome virus (TSV), infectious hypodermal and hematopoietic virus (IHHNV) (Flegel 2006) and Laem-Singh virus (LSNV). The white spot syndrome and yellow head virus are the two major viruses which cause high mortality in shrimp.

1.5.1.1 White spot syndrome

White spot syndrome (WSD) is the most serious viral disease in shrimp aquaculture which infected shrimp showed rapid decrease in food consumption, become sluggish and have a loose cuticle (Liu et al. 2009b). Particularly, the obvious sign of WSSV infection is white spot on the carapace of shrimp (Figure 1.10 Top). The WSD exhibits 100% of cumulative mortalities within 3-10 days (Chou et al. 1995).

The causative agent of WSD is white spot syndrome virus (WSSV) which has a wide range of host among decapod crustaceans such as shrimp crab and lobster (Lo et al. 1996; Flegel 1997b). WSSV is a large circle double-stranded DNA (dsDNA) virus and assigned to genus *Whispovirus* and family *Nimaviridae* (Mayo 2002b). Virions are large (80-120 x 250-380 nm), rod-shaped to elliptical, and with a trilaminar envelope and a single tail-like appendage (Flegel 2006) (Figure 1.10 Below). The genome size of WSSV has been differently reported for different isolates and it is about 300 kbp. There are 181 open reading frames (ORFs) likely encoding functional proteins (OIE 2003).

The first occurrence of WSSV was in *P. japonicus* in Japan 1993 (Inouye et al. 1994; Nakano et al. 1994; Lightner 1999). In the same interval, the viral infection was reported in *P. japonicus*, *P. monodon* and *P. penicillatus* in Taiwan and China (Chou et al. 1995; Flegel 2006). Later on, it was reported in Thailand, Malaysia, Korea, India, Philippines, and USA (Wonteerasupaya et al. 1995; Wang et al. 1995; Kim et al. 1998;

Karunasagar and Otta 1998; Durand and Lightner 2002; Lightner 1996). Australia and Spain were also reached by the outbreak via frozen infected shrimp as fresh food for broodstock (OIE 2003). The WSSV outbreak is not affected only in Asian countries, but also almost shrimp farming in the world. The dispersions of disease are contamination of water, decomposing tissue or fecal matter, fluid from infected shrimp, and cannibal. The transmission of WSSV can occur among unrelated crustacean species. The target tissues for replication are gills, stomach and subcuticular epithelium (Flegel 2006). There are several diagnostic methods to detect the WSSV infection such as histopathology with light or electron microscopes, polymerase chain reaction (PCR), *in situ* DNA hybridization, monoclonal antibody assay by immunohistochemistry (Flegel 2006; Wongteerasupaya et al. 1996; Poulos et al. 2001).

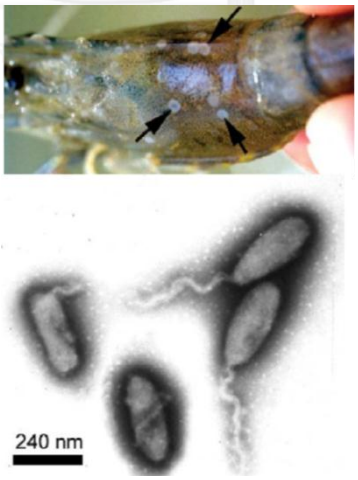


Figure 1.10 Gross sign of WSSV infection and morphology of WSSV virions. White spot on the inside of cuticle of carapace (Top). WSSV, purified virions with negative staining showing a tail-like appendage (Below). (Flegel 2006)

1.5.1.2 Yellow head disease

Yellow head disease (YHD) is one of the serious diseases affecting shrimp farming in Asia. The gross signs of YHD are yellowish cephalothorax and very pale overall body color (Lightner 1999) (Figure 1.11 Top). The disease occurs in juvenile to sub-adult stage in shrimp and is potentially lethal cause with 100% of mortality within 3-5 days (Lightner 1996). YHV infects (natural source or experimental infection) several penaeid shrimp species such *P. merguiensis, P. vannamei*, and *P. stylirostris* and other shrimps (Flegel 1997a). YHD is caused by yellow head virus (YHV) which is a rod-shaped and enveloped virion ranging about 150-200 nm in length and 40-50 nm in diameter (Figure 1.11 Below). The genome of YHV composes of single-stranded RNA (ssRNA) of positive sense with a helical nucleocapsid (Wonteerasupaya et al. 1995). Type species for YHV are genus *Okavirus* and family *Roniviridae* (Mayo 2002a). A closely related strain of YHV has been also reported in *P. monodon* from Australian shrimp farms, named Gill-associated virus (GAV) (Walker et al. 2001).

In 1990, YHV caused disease problem in Thailand at first (Limuswan 1991), but it was discovered as a new pathogen in 1992 (Boonyaratpalin et al. 1993; Chantanachookin et al. 1993; Flegel 2006). Later, the outbreaks were reported from countries in Asia. The major target tissue for replication of YHV is lymphoid organ (Khanobdee et al. 2002; Soowannayan et al. 2002). The diagnostic methods for YHD are reverse transcription-PCR (RT-PCR), histology with hematolylin and eosin (H&E)

stained gill and hemolymph smear, *in situ* hybridization, and monoclonal antibody assay by immunohistochemistry, dot blot assay and lateral flow chromatographic assay (Wongteerasupaya et al. 1997; Tang and Lightner 1999; Flegel 2006).

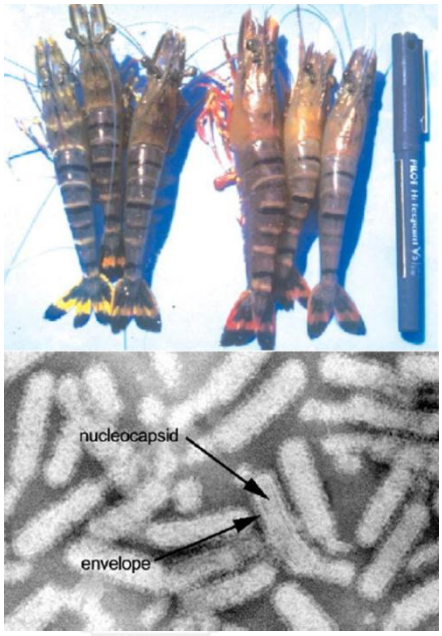


Figure 1.11 Gross sign of YHV infection and morphology of YHV virions. Shrimp on the right are bleached and yellowish cephalothorax compare to normal shrimp on the left (Chantanachookin et al. 1993) (Top). YHV, purified virions by transmission electron microscope with negative staining (Below) (Flegel 2006)

1.5.2 Bacterial diseases

1.5.2.1 Vibriosis

Vibriosis is one of major diseases in shellfish, finfish aquaculture and also all marine crustacean, including shrimps (Lightner and Lewis 1975; Adams 1991; Lightner et al. 1992). Vibriosis is caused by Gram- negative bacteria in the family *Vibrionaceae*

(Sizemore and Davis 1985). There are numerous of Vibrio species which caused vibriosis, such as *V. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. penaeicida* (Brock and Lightner 1990; Ishimaru et al. 1995) *V. damsela, V. fluvialis* and other undefined *Vibrio* species (Lightner 1996). In addition, *V. anguillarum, V. campbelli, V. nereis, V. cholera, and V. splendidus* have been reported in involvement with shrimp disease outbreaks (Chen and Hanna 1992; Lavilla-Pitogo et al. 1990; Hameed et al. 1996). *Vibrio* spp. are the chitinoclatic bacteria associated with shell disease (Cook and Lofton 1973). They may enter through the gills and the wounds in the exoskeleton or pores (Jiravanichpaisal et al. 1994) and the midgut by water, food, and sediment (Ruby et al. 1980; Jayabalan et al. 1982). Infection of Vibriosis can occur in all life stages, but more in hatcheries. *Vibrio* species are ubiquitous, widely distributed in world and are part of the natural microflora of wild and cultured shrimps (Sindermann 1990) which become opportunistic pathogens when natural defense mechanism are suppressed (Brock and Lightner 1990).

Vibrio harveyi, a luminous bacterium, is serious pathogenic bacteria causing high mortality in shrimp up to 100%. From *Vibrio harveyi* isolation showed virulent and non-virulent strains. The pathogenic mechanism has attributed to bacteriophage (Nakai et al. 1999; Nakai and Park 2002; Karunasagar et al. 2007; Vinod et al. 2006). *V. harveyi* emerges to release exotoxins (Liu et al. 1996). The first report of *V. harveyi* was found in sandbar shark (*Carcharhinus plumbeus*) in 1982 in USA (Grimes et al.

1984). In Asian countries, mass mortalities in shrimps were also reported (Sunaryanto and Mariam 1986; Baticados and Tendencia 1991).

Gross sign of Vibriosis may show lesions on the cuticle, loss of limbs, cloudy musculature, and septicemia (Lightner 1993). The lesions are brown or black and appear on the gills, appendages, or body cuticle (Sindermann 1990). In *V. parahaemolyticus or V. harveyi* infection, the rounding up and detachment of epithelial cell on the midgut trunk could be seen (Chen et al. 2000; Martin and Graves 1985). *Vibrio* infection can be diagnosed by streaking plate on Vibrio-selective media, Gram staining and observed growth in the presence of NaCl.

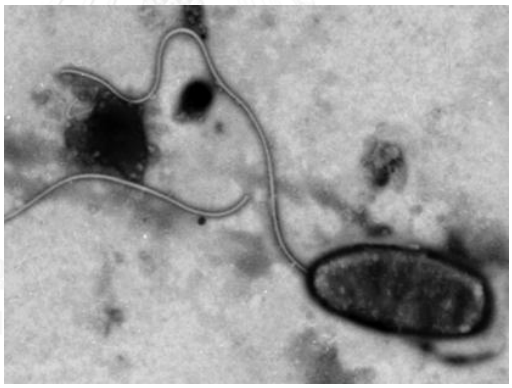


Figure 1.12 Morphology of *V. harveyi* by electron transmission microscopy. (Zhou et al. 2012)

1.5.2.2 Early mortality syndrome or acute hepatopancreatic necrosis disease

A new disease outbreak of shrimp aquaculture, early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) was first discovered in

China in 2010 and subsequently in Vietnam, Thailand, and Malaysia (FAO, 2013). This disease affects shrimp postlarvae within 20-30 days after stocking and causes up to 100% mortality (Lightner et al. 2012). The susceptible species are *P. monodon, P. vannamei*, and *P. chinensis*. A causative agent of EMS is *V. parahaemolyticus*, closely related to shrimp pathogens-luminous bacteria such as *V. harveyi* (FAO, 2013). *V. parahaemolyticus* isolates, some are virulent strain and some are not, were studied which reveals the extrachromosomal plasmid were found in virulent strain but not in non-virulent strain. The virulent strain of *V. parahaemolyticus* contains toxins-like genes in the plasmid and produces and secrets toxins that cause the EMS (Yang et al. 2014; Han et al. 2015).

The dissemination of the EMS outbreak causes the serious losses in shrimp farming in many countries. China had a loss almost 80% in shrimp culture in 2011. In Vietnam, the disease devastated a total shrimp pond area of approximately 98,000 ha since 2011 and in Malaysia, *L. vannamei* production was dropped from 70,000 to 40,000 metrics tonnes (GAA, 2012). The Thai Department of Fisheries (DOF) reported that Thai's total shrimp production in the first quarter of 2013 was 63,500 tonnes while it was 94,400 tonnes in the same period of 2012.

EMS-infected shrimp appears the signs, soft and darker shell, molting of the carapace, pale or white coloration of the hepatopancreas and becomes lethargic and anorexia (Figure 1.13). The dissection of hepatopancreas shows atrophied and whitish

with black streak epithelial cell. A secondary infection caused by opportunistic *Vibrio* bacteria affects in the sloughed masses of epithelial cell in hepatopancreas tubule lumens.

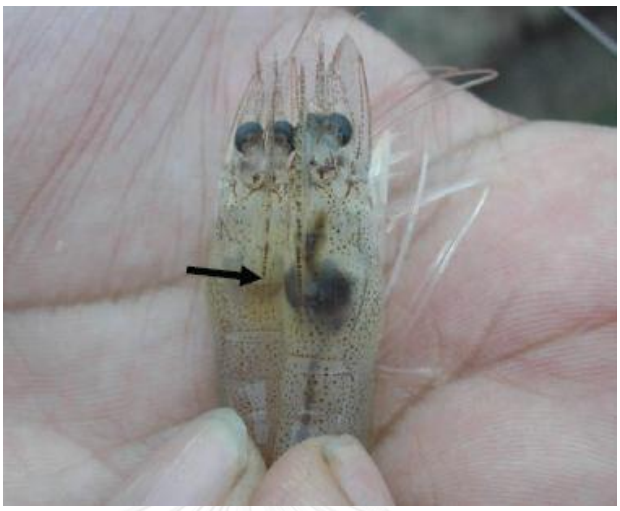


Figure 1.13 Gross sign of EMS/AHPND. Showing a pale atrophied hepatopancreas and an empty stomach and midgut (left) compare to the normal shrimp (right). (Photographed by Lightner) (FAO, 2013)

1.6 Shrimp immunity

The immunity of shrimp is absent of the adaptive immune responses, but only has the innate immune responses which play a major role in fighting pathogens invasion. The innate immunity composes of cellular immunity including phagocytosis, nodulation, and encapsulation, and humoral immunity which involves the synthesis and release of many immune proteins such as antimicrobial peptides (AMPs), proteinase inhibitors, etc (Bachère et al. 2004; Tassanakajon et al. 2013). The main immune reactions occur in hemolymph along with circulating hemocytes. The types

of hemocyte can be identified including hyaline cell (HC), semigranular hemocyte (SGH), and granular hemocyte (GC). HC has a function in phagocytosis. SCG has functions, for example encapsulation, early non-self recognition, melanization, and coagulation and also phagocytosis in some species. GH has roles in melanization, antimicrobial peptides synthesis, and cytotoxic reactions (Martin and Graves 1985; Lin and Söderhäll 2011). The hemocytes produce and store the immune molecues in the granules and release into the hemolymph upon the activation by microbial cell wall component (Sritunyalucksana and Söderhäll 2000). The pattern recognition receptors (PRRs) recognize and bind the microbial cell wall component in the term of pathogen-associated molecular pattern (PAMPs) and activate many immune responses (Yu et al. 2002; Janeway Jr and Medzhitov 2002; Wang and Wang 2013).

1.6.1 Pattern recognition receptors

The innate immune responses are activated by the invading microorganism through PRRs. The PRRs recognize pathogens by binding to PAMPs which are polysaccharides and glycoprotein on the surface of microbes, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipotechoic acid (LTA) from Gram-positive bacteria, and ß-glucan (BG) from fungi. In addition, polynucleotides, such as bacterial and viral unmethylated CpG DNA, ssRNA and dsRNA from viruses, are also the patterns (Janeway Jr and Medzhitov 2002; Tassanakajon et al. 2013; Wang and Wang 2013). Various PRRs recognize specific

PAMPs and trigger signaling pathways of the cellular and humoral immune responses. In shrimp, several PRR families have been identified, like lipopolysaccharide and ß-glucan binding proteins (LGBPs), peptidoglycan recognition proteins (PGRPs), C-type lectins (CTLs), galectins, thioester-containing proteins (TEPs), fibrinogen-related proteins (FREPs), scavenger receptors (SRs), Down syndrome cell adhesion molecules (DSCAMs), Toll like receptors (TLRs), ß-glucan binding proteins (BGBPs), serine proteinase homologs (SPHs), and trans-activation response RNA-binding proteins (TRBPs) (Wang and Wang 2013).

Many PRRs have been investigated for their recognizing properties and functional characterization. In *P. monodon*, LGBP transcript was up-regulated after *V. harveyi* challenge. Recombinant *Pm*LGBP (r*Pm*LGBP) could bind to LPS and BG and also enhance the activation of proPO system (Amparyup et al. 2012). Moreover, *L. stylirostris* LGBP transcript was induced after WSSV infections (Roux et al. 2002). Several lectins in shrimp were reported to be up-regulated after virus infections, WSSV and YHV (Ma et al. 2007; Wang et al. 2009; Junkunlo et al. 2012). The C-type lectin from *L. vannamei* showed the binding to envelope proteins of WSSV and the protection of viral infection (Zhao et al. 2009). The fibrinogen-related protein in *M. japonicus* (*Mj*FREP1) was up-regulated in bacterial and WSSV infection. r*Mj*FREP1 display agglutination activity against the Gram-positive bacteria and the FBG domain of r*Mj*FREP1 binds to PG, LPS and also VP28 of WSSV (Chai et al. 2012).

1.6.2 Clotting system

Coagulation or clot formation builds a physical barrier to prevent the loss of hemolymph and the spreading of microorganisms after the injury or infection which the rapid of blood coagulation at the injury site is necessary for survival. The coagulation in shrimp is initiated by the activation and lysis of HC, which releases components to react with plasma factors (Omori et al. 1989). The released hemocyte components, Calcium-depnedent transglutaminases (TGase), process the formation of clottable protein (CP) by catalysis of 3-(g-glutamyl) lysine crosslinks of clottable proteins, which are plasma factor, into the long chain polymers (Maningas et al. 2013).

In shrimps, CPs in hemolymph have been indentified in *P. monodon, Farfantepenaeus paulensis, M.* japonicus, and *L. vannamei*. TGase has been found in many species, *P. monodon, F. chinensis, M. japonicus,* and *L. vannamei* (Tassanakajon et al. 2013). There are at least two types of shrimp TGase (STG I and STG II) and only STG II was characterized as a hemocyte TGase involved in the coagulation (Chen et al. 2005). In silencing of TGase in *M. japonoicus* caused significantly down-regulated of AMP genes, crustin and lysozyme (Fagutao et al. 2012).

1.6.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are effectors of the innate immune response and play a crucial role to fight against invading pathogens. AMPs are small size, commonly less than 150-200 amino acid residues, and are amphipathic molecules with cationic and anion properties. The AMPs exhibit antimicrobial activity against a wide range of microorganism, such as bacteria, fungi, yeast, parasite and virus, and also anti-tumor activity (Hancock and Diamond 2000; Krepstakies et al. 2012). Many shrimp AMP families have been identified and characterized, such as penaeidins, crustins, antilipopolysaccharide factors (ALFs), lysozyme, and stylicins (Rolland et al. 2010; Tassanakajon et al. 2013). However, the difference types and/or isoforms show the different antimicrobial activity. The AMPs are mainly produced and stored in the hemocytes. Penaeidins have an unconstrained proline-rich domain (PRD) at N-terminal domain. They exhibit antifungal and anti-Gram positive bacterial activity and even immunomodulation (Tassanakajon et al. 2010; Tassanakajon et al. 2013; Li et al. 2010). Crustins are cationic peptides containing cysteine-rich regions and whey acidic protein (WAP) domain. The type II crustins and crustin*Pm*1 have the antimicrobial activity against Gram-positive. But, crustin*Pm*7 show the antimicrobial activity in both of Gram-positive and Gram-negative (Sun et al. 2010; Tassanakajon et al. 2010; Krusong et al. 2012). ALFs have two-highly conserved-cysteine residues that form disulfide loop in which highly conserved cluster of positive charged residues, in

termed as putative LPS-binding domain. The ALFs have a board antimicrobial activity against bacteria and fungi (Somboonwiwat et al. 2005; De la Vega et al. 2008). The ALFs show a binding and neutralization of LPS (De la Vega et al. 2008; Nagoshi et al. 2006; Somboonwiwat et al. 2005). Additionally, the knockdown of ALF*Pm*3 showed an increase in the cumulative mortality in WSSV-infected shrimp (Ponprateep et al. 2012). The ALF*Pm*3 could show anti-WSSV activity that yeast two-hybrid technique found a binding of WSSV proteins and neutralization of WSSV (Suraprasit et al. 2014). Lysozyme is an enzyme that cleaves the ß-1,4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in PG of bacterial cell wall and cause the cell lysis in both of Gram-positive and Gram-negative bacteria. Besides, the lysozyme could protect the WSSV infection in *L. stylirostris* (Mai and Wang 2010). Stylicins are the anionic AMP containing PRD at N-terminus and cysteine-rich domain at C-terminus. In *L. stylirostris*, *Lv*Stylisin1 exhibits poorly an antibacterial activity, but strongly in an antifungal activity (Rolland et al. 2010).

1.6.4 Prophenoloxidase system

The prophenoloxidase (proPO) activating system plays an important role in invertebrates including arthropods as a non-self-recognition system which produces reactive intermediates and melanin to fight against the pathogens. The proPO system is controlled by multistep pathway. The recognition of PAMPs by PRPs leads to the activation of serine proteinase cascade and finally results in the proteolytic cleavage

of the proPO zymogen, the key enzyme in melanin formation, to the active phenoloxidase (PO) (Söderhäll and Cerenius 1998; Cerenius and Söderhäll 2004; Cerenius et al. 2008). Biosynthesis of melanin requires both of enzymatic and non-enzymatic (spontaneous) reactions. The enzymatic step is catalyzed by PO which is considered as the rate limiting step of melanization (Figure 1.14). During proPO activation, many reactive intermediates, such as toxic quinone substrates, reactive oxygen (ROI) or nitrogen (NOI) intermediates are produced to eliminate the invading microorganism. The quinone substrates are catalyzed and polymerized into the melanin that encapsulates the pathogens. Furthermore, the intermidiates in melanin synthesis pathway also participate in the wound healing and sclerotization (Zhao et al. 2007; Cerenius et al. 2008) (Figure 1.15).

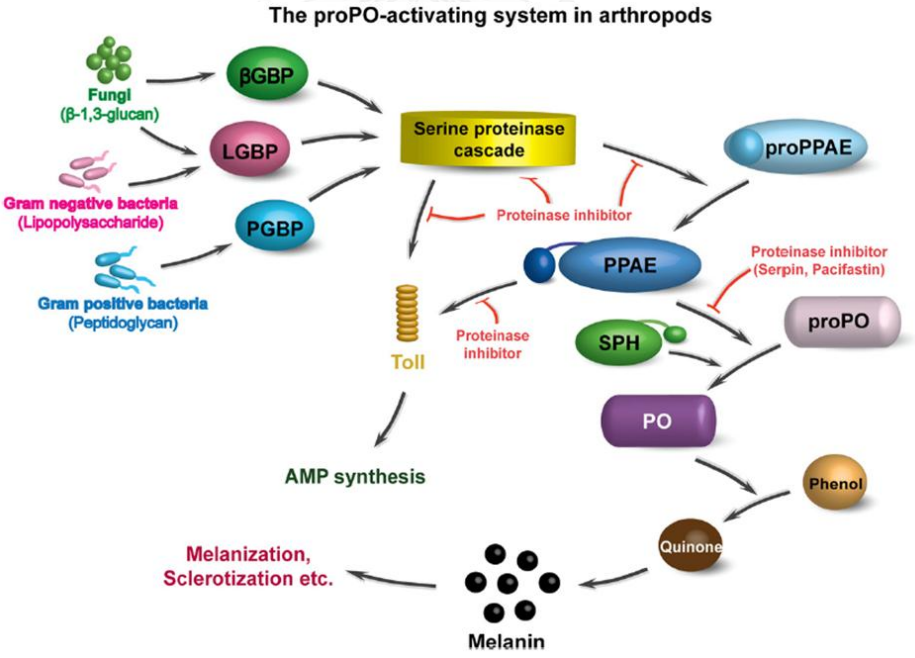


Figure 1.14 Schematic of the prophenoloxidase (proPO) system in arthropods. The PRPs recognize microbial components and trigger the SP cascade. The terminal step,

PPAE converts proPO to PO. Finally, active PO produces toxic intermediates and melanin. (Amparyup et al. 2013a)

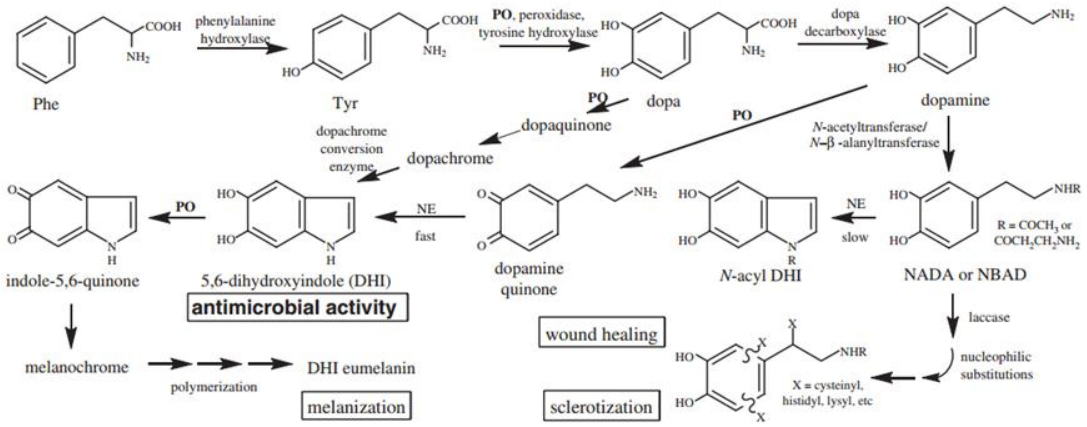


Figure 1.15 Mechanism of PO-mediated reactions in insects and crustaceans. POs and other enzymes generate 5,6-dihydroxyindole (DHI), which has a broad-spectrum antimicrobial activity, and oxidative intermediates for protein crosslinking during wound healing and cuticle sclerotization. The products of reaction spontaneously polymerize to form melanin. (Zhao et al. 2007)

The proPO system is triggered by the presence of microbial components, such as LPS, PGN, and ß-1,3-glucan and PRPs recognition, including LGBP, PGBP, and ßGBP. Many PRPs have been reported in many species, but a few PRPs in shrimp that involved in the activation of the proPO system have been found. ßGBP family contains two glucanase-like motifs that the sequence similar to the catalytic regions of microbial ß-glucanases, but lack glucanase activity and integrin-recognition motif (RGD or RGE). In crustacean, ßGBPs was characterized in crayfish *Pacifastacus leniusculus*. Crayfish ßGBP transcript is synthesized in hepatopancreas and the ßGBP

protein is released into the plasma (Duvic and Söderhäll 1990). Binding of ß-1,3-glucan and ßGBP enhances the activation of the proPO system and induces hemocyte degranulation and opsonization (Cerenius et al. 1994; Duvic and Söderhäll 1990; Thörnqvist et al. 1994). The ßGBP-HDL, similar protein to the crayfish ßGBP, was found in *L. vannamei* and *F. chinensis*. Their transcripts were found in many tissues of both species, but not in hemocytes of *L. vannamei*. However, the ßGBP-HDL proteins were detected only in plasma (Yepiz-Plascencia et al. 1998; Vargas-Albores et al. 1997). In *P. californiensis*, purified ßGBP can also activate the PO activity (Vargas-Albores et al. 1996). LGBPs have a conserve domain of glycoside hydrolase family 16 which composes of polysaccharide-binding, glucanase-, and ß-1,3-glucan-recognition motifs and two RGD motifs. Initially, LGBP was isolated from crayfish *P. leniusculus* showing a significant homology with insect GNBPs and bind to LPS or ß-1,3-glucan to activate the proPO activation (Lee et al. 2000). Several LGBPs in shrimp species have been identified, such as *P. stylirostris, L. vannamei, M. japonicus, F. chinensis and P. monodon*. LGBP mRNA was mainly expressed in hemocytes and found as an immune responsive gene (Roux et al. 2002; Cheng et al. 2005; Du et al. 2007; Liu et al. 2009a; Lin et al. 2008a; Sritunyalucksana et al. 2002). Furthermore, gene silencing of *Pm*LGBP showed a reduction of the PO activity and r*Pm*LPBP could bind to LPS and ß-1,3-glucan and activate the PO activity in the presence of LPS or ß-1,3-glucan (Amparyup et al. 2012). C-type lectins are carbohydrate-binding protein that play important role in many biological processes in both of vertebrate and

invertebrate (Vasta et al. 2007). The C-type lectin in cockroach *Blaberus discoidalis* has been reported that involve in the proPO system by the enhancement of laminarin-stimulated proPO system activation (Chen et al. 1999). In tobacco hornworm *Manduca sexta* also exhibits immulectin-1 (IML-1) and IML-2 that are PRPs for the proPO activation (Yu et al. 1999; Yu and Kanost 2004). In crustaceans, *Pc*Lec2 from *Procambarus clarkii* is participated in the proPO activation, while in *P. leniusculus* mannose-binding lectin (*Pl*-MBL) function as a regulator of the proPO system under high calcium concentration (Wang et al. 2011). In penaeid shrimp, many C-type lectins have been found, but only in *L. vannamei* was reported that *Lv*CTLD enhance the PO activity in shrimp hemolymph (Junkunlo et al. 2012).

The recognition of PRPs, then, leads to the proteolytic cascade of serine proteinase (SP). Clip-SPs have been implicated in the proPO activation cascade. The clip-SPs are produced as inactive zymogens and contain clip-domain at N-terminus and SP domain at C-terminus, which clip-domain is connected to the SP domain by a disulfide bond after the proteolytic cleavage (figure 1.16). SPs are the proteolytic enzyme and identified by the three catalytic residues (H, D, and S) that form a catalytic triad. Clip-SPs can be divided into catalytic SPs and non-catalytic SPs. The non-catalytic SPs refer to as Clip-SP homologs (Clip-SPHs) are similar sequence to the catalytic SP but a serine residue in the catalytic site is replaced by a glycine residue and that make them have no any proteolytic activity (Jiang and Kanost 2000). Several

clip-SPs that involves in the cascade have been reported. In *M. sexta*, hemolymph serine proteinase (HP) 14 and 21 are the SPs in upsteam of proPO activation (Gorman et al. 2007). In shrimp *P. monodon*, clip-SP1 and clip-SP2 were mainly expressed in hemocytes. The *Pm*ClipSP2 shows the function in the proPO system by gene silencing and activation of recombinant protein (Amparyup et al. 2013b), while *Pm*ClipSP1 plays a role in antibacterial defense but not involves in the proPO activation (Amparyup et al. 2010). In addition, *Pm*Snake, Clip-SP and similarity with HP21 about 49%, exhibits the decrease in PO activity after *Pm*Snake gene knockdown and r*Pm*Snake can activate PO activity (Ampayup et al., unpublished data). The terminal clip-SPs of the proteolytic cascades in proPO system that convert the proPO zymogen into active PO are carried out by the proPO-activating enzyme (ppA or PPAF or PPAE). PPAEs have been identified from many insects and crustaceans. In beetle *Holotrichia diomphalia*, PPAEs named PPAF-I and PPAF-III have been characterized to be the crucial components for the conversion of proPO into PO (Lee et al. 1998b; Lee et al. 1998a). Three PPAEs, PAP-1, PAP-2, and PAP-3, from *M. sexta* show their transcripts up-regulated after bacterial challenge (Jiang et al. 1998; Jiang et al. 2003a; Jiang et al. 2003b). In crustacean, crayfish PPAEs is required for producing of active PO and the rclip-domain of PPAE has an antibacterial activity (Wang et al. 2001). Two PPAEs from *P. monodon* (*Pm*PPAE1 and *Pm*PPAE2) and one PPAE from *L. vannamei* (*Lv*PPAE1) have also been found that are mainly expressed in hemocytes as immune-responsive genes in bacterial infection and involve in proPO system. Gene

knockdown of *Pm*PPAE1 and/or *Pm*PPAE2 display significantly the reduction of hemolymph PO activity. Interestingly, *Pm*PPAE1 is more closely related to the crayfish PPAE and contain glycine rich region, but *Pm*PPAE2 is similar to insect PPAEs (Jiang et al. 2010; Charoensapsri et al. 2009, 2011).

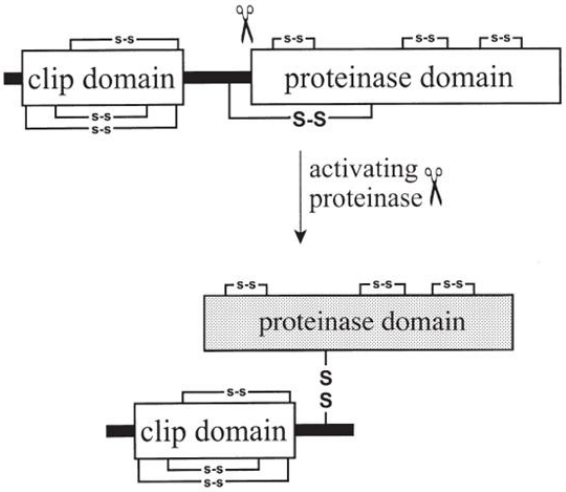


Figure 1.16 Schematic of clip domain proteinase and active clip domain proteinase. Clip domain proteinase is activated by a specific proteolysis which clip domain at N-terminus and proteinase domain at C-terminus are connected by a disulfide bond. (Jiang and Kanost 2000)

The activation of PPAEs in proPO system may require the cofactors that are Clip-SPHs to generate PO. In insect, PPAF-II from *H. diomphalia* is clip-SPH and function as a cofactor of PPAF-I and in *M. sexta*, SPH1 and SPH2 act as cofactors for PAP-2 and PAP-3 (Kim et al. 2002; Gupta et al. 2005). In *P. leniusculus*, *Pl*SPH1 and *Pl*SPH2 were reported to be involved in the PGN-induced proPO activation that may

function as cofactors in the PGN-binding complex with LGBP (Liu et al. 2011). In *P. monodon*, Three SPHs, named masquerade-like SPH1 (*Pm*MasSPH1), *Pm*MasSPH2, and *Pm*MasSPH3 have been found, but only *Pm*MasSPH1 and 2 were characterized. *Pm*MasSPH1 is a recognition protein and has antimicrobial activity against the Gram-negative bacteria *V. harveyi*. The gene silencing of *Pm*MasSPH1 and 2 significantly decreased the PO activity. The r*Pm*MasSPH1 and 2 also show the binding activity to PGN. Moreover, r*Pm*MasSPH1 is required for PPAE2 in proPO activation in the presence of PGN (Jitvaropas et al. 2009; Jearaphunt et al. 2015).

After the proteolytic cascade of SP, the PO that is a key enzyme in proPO system is converted from inactive proPO. PO is a bifunctional copper containing enzyme which possesses tyrosinase/monophenolase and catecholase/diphenolase activities. The PO catalyzed monophenols and *o*-diphenols into *o*-quinone that is precursor for melanin synthesis (Cerenius et al. 2008). PO generally contains two copper binding sites and no hydrophobic signal peptide and is produced and maintained as the inactive proPO form. Amino acid sequences of POs are similar to hemocyanins, the oxygen carrier proteins, and hemocyanins also have PO-like activity (Coates and Nairn 2014). The arthropod proPO genes was firstly found in *P. leniusculus* and following in many insects and crustaceans. In the insects, ten and nine proPO genes were reported in mosquitoes *Aedes aegypti* and *Anopheles gambiae* respectively. There are three proPOs which were found in fruit fly

Drosophila melanogaster, two in beetle *H. diomphalia*, silkworm *B. mori*, and tobacco hornworm *M. sexta*, and one in honeybee *Apis mellifera* (Amparyup et al. 2013a). The two proPOs in *B. mori* and *M. sexta* have been shown that the enzyme is composed of a heterodimer and each subunit is coded by different genes (Jiang et al. 1997; Asano and Ashida 2001a, b). But, in *D. melanogaster*, at least two of three proPOs exist as homodimers *in vivo* (Sezaki et al. 2001). In penaeid shrimp, one proPO gene was reported in *P. californiensis* and *P. semisulcatus* and two proPO genes were reported in *P. monodon*, *M. japonicus*, *L. vannamei*, and *F. chinensis* (Amparyup et al. 2013a). In *P. monodon*, the suppression of *Pm*proPO1 and *Pm*proPO2 by RNA interference (RNAi) significantly decreases in PO activity which demonstrates they are the key components in *P. monodon* proPO system (Amparyup et al. 2009). All proPOs are synthesized in cells and localized in granules, then, being release upon rupture or infection (Cerenius and Söderhäll 2004). Interestingly, a proPO (proPOb) which was found in the plasma of *M. japonicus* have been characterized that it is mainly synthesized in hepatopancreas and contains a signal peptide-like sequence at N-terminus and also show a PO activity in plasma. These characters are similar to hemocyanins but, it seems like to be proPO because the primary structure and enzymatic properties are more similar to proPOs (Masuda et al. 2012).

1.6.5 Proteinase inhibitors

Proteinase cascades play crucial roles in many biological and physiological processes, including the clotting system and proPO system, and need to be regulated to prevent excessive activation of cascades resulted in to host tissue. Proteinase inhibitors play a key role to control the pathway. Additionally, host proteinase inhibitors function in the inhibition and clearance the proteases of microorganism. Several family of serine proteinase inhibitors, such as Kazal-type serine proteinase inhibitors (KSPIs), Kunitz-type serine proteinase inhibitors (KuSPIs), serpins, alpha-2-macroglobulins (A2M) have been reported in arthropods (Kanost 1999; Tassanakajon et al. 2013). The KPIs compose of one or more Kazal domain(s) and the inhibitory activity of each KPI depends on the action of each Kazal domain which acts as a substrate analogue and competitively binds to the proteinase. In crustacean, four-domain of KPI were identified from hemocytes of *P. leniusculus* and *L. vannamei* (Johansson et al. 1994). Two types of KPIs, four- and five-domains KPIs were identified in *P. monodon* (Rimphanitchayakit and Tassanakajon 2010). The SPI*Pm*2, five-domains KPIs, was further investigate and it has the proteinase inhibitory activities, bacteriostatic and antiviral activities (Jarasrassamee et al. 2005; Somprasong et al. 2006; Donpudsa et al. 2009; Ponprateep et al. 2011). KuSPIs have been characterized from horseshoe crabs *Tachypleus tridentatus* (Nakamura et al. 1987). In shrimp, KuSPIs were identified in *Feneropenaeus chinensis* and *M. japonicus* (Kong et

al. 2013; Chen et al. 2008). A2M is a nonspecific and broad-spectrum protease inhibitor. A2M subunit contains a bait region that is susceptible to proteolytic cleavage and receptor domain. A2M inhibits the active target proteinase by a trapping mechanism, the cleavage of the bait region by proteinase leads to a conformational change that traps the proteinase in activity formed by the A2M tetramer (in vertebrate) or dimer (in invertebrate). The conformational change also leads to covalent crosslink formation between A2M thioester region and lysine side chain of proteinase which results in irreversible inhibition of the proteinase without effect of proteinase active site (Sottrup-Jensen 1989; Kanost 1999; Tassanakajon et al. 2013). A2M have been characterized in many organisms. In shrimp A2M, such as *F. paulensis*, *P. monodon*, *L. vannamei*, and *F. chinensis*, were studies and some reports showed that transcription and translation of A2M expression level is upon bacterial, viral and fungal infections (Lin et al. 2007; Lin et al. 2008b; Ma et al. 2010; Perazzolo et al. 2011; Somboonwiwat et al. 2010). Serpins act as a suicide-substrate inhibitor and contain reactive center loop (RCL) at C-terminus. The RCL consists of a scissile bond and interacts with active site of the proteinase that leads to form stable covalent complex (Kanost 1999; Gettins 2002; Tassanakajon et al. 2013). Many serpins have been identified in arthropods, including *M sexta*, *B. mori*, *T. tridentatus* and *P. leniusculus* (Kanost et al. 1989; Sasaki 1991; Liang and Soderhall 1995). Few serpins from shrimp were also characterized in *F. chinensis* and *P. monodon*. *Fc*-serpin, *Pm*SERPIN6 and *Pm*SERPIN8 are expressed upon bacterial infection, but in *Pm*SERPIN3

is constitutively expressed as a housekeeping gene (Liu et al. 2009b; Homvises et al. 2010; Somnuk et al. 2012; Wetsaphan et al. 2013).

1.7 Proteinase inhibitors and inhibitors in proPO system

There are many proteinase inhibitors and inhibitors controlling the proPO system to prevent the excess highly reactive and toxic quinone intermediate and melanin products that can damage to host cells. The melanization is regulated at multiple steps, such as SP cascade inhibition, phenoloxidase inhibition and melanization reaction inhibition. Proteinase inhibitors, such as serpins and pacifastin, have been characterized as inhibitor of proPO activation by inhibiting the SPs in cascade. In insects, serpins from *D. melanogaster*, *A. gambiae*, *M sexta*, and *T. molitor* have been identified as negative regulators of SPs cascade (Jiang et al. 1997; Zhu et al. 2003; Wang and Jiang 2004; De Gregorio et al. 2002; Gan et al. 2001; Tong and Kanost 2005; Levashina et al. 1999; Ligoxygakis et al. 2002). In crustacean, pasifastin from *P. leniusculus* has been reported as the inhibitor for *Pl*PPAE1. Pacifastin is a unique heterodimer protein with 155kDa containing a heavy chain, transferrins and a light chain, inhibitor domains (Liang et al. 1997). Only a few serpins were reported in shrimp. *Pm*SERPIN8 and *Pm*SERPIN3 from *P. monodon* inhibit the PO activity (Somnuk et al. 2012; Wetsaphan et al. 2013). Molecules that inhibit PO were identified. PO inhibitor (POI) has been characterized to be a dopa-containing 38mer peptide from housefly *Musca domestica* (Daquinag et al. 1995). In *A. gambiae*,

a protein containing several repeats of *M. domestica* POI sequence has been reported that may be involved in melanization regulation by gene silencing of POI enhanced melanization at wound sites (Shi et al. 2006). In *M. sexta*, a polypeptide homologue of housefly POI was also found and showed an inhibitory activity of PO. Moreover, a low molecular weight was found in *M. sexta* hemolymp hat strongly inhibited PO activity (Lu and Jiang 2007). In addition, the inhibitor of melanin reaction, melanization inhibiting protein (MIP), was originally identified in *T. molitor*. Injection of *Candida albicans* into *T. molitor* larvae showed the decreasing of *Tm*MIP protein in melanin-induced hemolymph. *Tm*MIP gene silencing induced the melanin synthesis and r*Tm*MIP had a inhibitory activity on melanin synthesis (Zhao et al. 2005). In crustacean, *Pl*MIP from *P. leniusculus* also showed the interference with the melanization reaction from quinone compounds to melanin and proteinase activity. *Pl*MIP was degraded when melanization induced (Söderhäll et al. 2009). In shrimp, *Pm*MIP from *P. monodon* was identified which an amino acid sequence similar to the crayfish *Pl*MIP about 80%. mRNA expression of *Pm*MIP was found mainly in many tissues, such as gill, intestine and muscle, except hemocytes and at very low levels in hepatopancreas and ovaries (Angthong et al. 2010). *Pm*MIP protein responded upon bacterial infection. However, the function of *Pm*MIP has not been studied.

1.8 Objectives

From the nucleotide sequence of *P. monodon* EST database, partial nucleotide sequence of *Pm*MIP was identified and full-length cDNA of *Pm*MIP was obtained from 3' and 5' Rapid amplification of cDNA ends (RACE) of gills cDNA (Ampayup, unpublished data).

The objectives of this thesis are to characterize *Pm*MIP from black tiger shrimp *P. monodon*. Tissue distribution of *Pm*MIP transcript was eludicated by semi-quatitative RT-PCR. Transcriptional and translational *Pm*MIP expression analysis after pathogenic bacterial *V. haveyi* challenge was carried out by quantitative real-time RT-PCR and western blot respectively. Moreover, the biological function of *Pm*MIP in proPO system was investigated by RNA interference-mediated gene silencing. The recombinant *Pm*MIP protein was produced in *Escherichia coli* system for further functional characterization.

This study provides the basic knowledge of shrimp proPO system and shrimp immune response leading to better understanding of shrimp immunity. These will be helpful for control of diseases and improvement of shrimp aquaculture.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipment and chemicals

2.1.1 Equipment

-20 °C Freezer (Whirlpool)

-80 °C Freezer (ThermoForma)

Amicon Ultra-4 concentrations (Millipore)

Automatic micropipette : P2, P10, P100, P200 and, P1000 (Gilson Medical Electrical S.A.)

Balance Satorius 1702 (Sciecntific Promotion Co.)

Biosafety LABGARD NU-813 (NuAire)

Costar® 96-well plate (Corning Incorporation)

CFX96 Touch™ real-time PCR detection system (Bio-RAD)

Dry bath incubator (Major Science)

Gel documentation (SYNGENE)

Gene Pulser (Bio-RAD)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Insulin syringes U 100 (Becton, Dickinson and Company)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Carbinets (NuAire, Inc.)

Microplate reader SpectraMax M5 (Molecular Devices)

Minicentrifuges (Costar)

Nano Drop 2000c spectrophotometer (Thermo Scientific)

Nipro disposable syringes (NISSHO)

Microcentrifuge tubes : 0.2, 0.5, and 1.5 ml (Axygen Scientific)

Nitrocellulose blotting membrane Amershem TMHybond ECL (GE healthcare)

Parafilm M® (Bemis Company)

PD-10 column (GE healthcare)

pH meter model #SA720 (Orion)

Pipette tips : 10, 200, and 1000 µl (Axygen Scientific)

Power supply Power PAC3000 (Bio-RAD)

Spectrophotometer SpectraMax M5 (Molecular Devices), DU 650 (Beckman)

Sterring hot plate (Fisher Scientific)

Touch mixer Model #232 (Fisher Scientific)

Trans-Blot SD (Bio-RAD)

Ultra sonicator (SONICS Vibracell)

Vertical electrophoresis system (Bio-RAD)

2.1.2 Chemicals, reagents, and biological substrates

100 mM dATP, dCTP, dGTP, and dTTP (Thermo Scientific)

1 kb GeneRuler DNA Ladder (Thermo scientific)

100 bp GeneRuler DNA Ladder (Thermo scientific)

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside, X-gal (Thermo Scientific)

5-bromo-4-chloro-indolyl phosphate, BCIP-T (Thermo Scientific)

Absolute ethanol (HAYMAN)

Acrylamide (GE healthcare)

Agarose (Vivantis)

Alkaline phosphatase-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories)

Alkaline phosphatase-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories)

Ammonium persulfate (USB)

Ampicillin sodium salt (Biobasic)

B9385, Boc-Val-Pro-Arg-7-amido-4-methylcoumarin hydrochloride (Sigma)

Boric acid (Merck)

Bovine serum albumin (Fluka)

Calcium choride dehydrate (Merck)

Casein enzyme hydrolysate, trypton type-I (Hi-media)

Chloroform (ACI Labscan)

Coomassie brillant blue G-250 (Research Organics)

Diethyl pyrocarbonate, DEP (Sigma)

Disodium hydrogen phosphate (Ajax Finchem)

DL-dithiothreitol (Biobasic)

Ethylenediaminetetraacetic acid, EDTA (Ajax Finchem)

Glacial acetic (Merck)

Glycerol (Ajax Finchem)

Glycine (Research Organics)

Hydrochloric acid (Merck)

Imidazole (Fluka)

Isopropanol (Burdick & Jackson)

Isopropyl ß-D-1-thiogalactopyranoside, IPTG (Thermo Scientific)

Kanamycin sulfate (Biobasic Inc.)

L-3,4-dihydroxyphenylalanine, L-dopa (Sigma)

Laminarin from Laminaria digitata (Sigma)

Lipopolysaccharide from E. coli 0111:B4 (L2630) (Sigma)

Magnesium chloride (Merck)

Methanol (Burdick & Jackson)

Mouse anti-his antiserum (GE healthcare)

N,N-dimethylformamide, DMF (Carlo Erba)

N, N, N', N'-tetramethylethylenediamine (USB)

N, N'-methylenebisacrylamide (Fluka)

Ni sepharose 6 Fast Flow (GE healthcare)

Nickle sulfate hexahydrate (Sigma)

Nitroblue tetrazolium chloride, NBT (Thermo Scientific)

pEGFP-1 vector (Clontech Laboratories)

pET28b(+) vector (Novagen)

pET43.1a(+) vector (Novagen)

Potassium chloride (Ajax Finchem)

Potassium dihydrogen phosphate (Ajax Finchem)

Prestain protein molecular weight marker, 10kDa to 200kDa (Thermo scientific)

Skim milk powder (Hi-media)

Sodium cacodylate trihydrate (Sigma)

Sodium chloride (Ajax Finchem)

Sodium citrate (Carlo Erba)

Sodium dodecyl sulfate (Vivantis)

Sodium hydrogen phosphate (Ajax Finchem)

Sodium hydroxide (Merck)

TRI REAGENT (Molecular Research Center)

Tris (Vivantis)

Tryptic soy broth (Difco)

Tween 20 (Ajax Finchem)

Unstain protein molecular weignt marker, 14.4 kDa to 116 kDa (Thermo scientific)

Urea (Research Organics)

Yeast extract powder (Hi-media)

ß-mercaptoethanol (AppliChem)

2.1.3 Kits and enzymes

DNase I (Biolabs)

*Eco*RI (Biolabs)

*Nco*I (Biolabs)

NucleoSpin® Extract II kit (MACHEREY-NAGEL)

QIAprep® Miniprep kit (QIAGEN)

*Pci*I (Biolabs)s

pGEM®-T Easy Vector Systems (Promega)

Phusion® High-Fidelity DNA Polymerase (Biolabs)

SsoFastTM EvaGreen® Supermix (Bio-RAD)

T & A Cloning vector kit (RBC Bioscience)

T7 RiboMAXTM Express RNAi System (Promega)

T4 DNA ligase (Biolabs)

Taq DNA polymerase (Thermo scientific)

Thermo ScientificTM RevertAidTM First Strand cDNA Synthesis Kit (Thermo Scientific)

2.1.4 Microorganisms

Escherichia coli strain BL21 (DE3)

Escherichia coli strain JM109

Escherichia coli strain Rosetta (DE3)

Escherichia coli strain Rosetta-gami (DE3)

Vibrio harveyi strain 639

2.1.5 Software

BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/)

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

GENETYX (Software Developmental Inc.)

NetNglyc (http://www.cbs.dtu.dk/services/NetNGlyc/)

SECentral (Scientific & Educational Software)

SMART (http://smart.embl-heidelberg.de/)

2.2 Nucleotide sequences of used primers

Table 2.1 Nucleotide sequences of primers

Primer	Nucleotide sequence	Purpose(s)
MIPT7-F	5'GGATCCTAATACGACTCACTATAGGTGTGGCAATGCTGTGGGCTG3'	RNAi
MIPT7-R	5'GGATCCTAATACGACTCACTATAGGCTCGCAGCGGACCTTCTTAG3'	RNAi
MIP-F	5'TGTGGCAATGCTGTGGGCTG3'	RNAi
MIP-R	5'CTCGCAGCGGACCTTCTTAG3'	RNAi
GFPT7-F	5'TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA3'	RNAi
GFPT7-R	5'TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA3'	RNAi
GFP-F	5'ATGGTGAGCAAGGGCGAGGA3'	RNAi
GFP-R	5'TTACTTGTACAGCTCGTCCA3'	RNAi
EF1α-F	5'GGTGCTGGACAAGCTGAAGGC3'	RNAi and real time RT-PCR
EF1α-R	5'CGTTCCGGTGATCATGTTCTTGATG3'	RNAi and real time RT-PCR
MIP_RT-F	5'TTAGCCGTAGGGCAACATTC3'	Real time RT-PCR
MIP_RT-R	5'GCACGAGGGCTTGTACATCT3'	Real time RT-PCR
MIP_NcoI-F	5'CATGCCATGGGTAACGAGCCCCTTAGCCGTAG3'	Cloning and recombinant protein expression
MIP_EcoRI His-R	5'GGGAATTCCTAATGATGATGATGATGATATCTCGGCCTGATGAGCA3'	Cloning and recombinant protein expression

Underline is T7 promoter

Italic is six residues of histidine

2.3 Sequence analysis

Nucleotide sequences were analyzed by GENETYX and BLASTX. The putative signal peptide and domain were predicted by the simple modular architecture research tool SMART version 4.0 (http://www.smart.emblheidelberg.de/). The amino acid sequence alignment were performed by ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/).

2.4 Shrimp samples

Shrimp *P. monodon* were brought from local farms in Surat Thani province and kept in aerated seawater (20 ppt) at least 7 days before performing experiments.

2.5 Total RNA extraction

Shrimp tissues (gills, hemocytes, heart, hepatopancreas, muscle, and lymphoid organ hemocytes, heart, hematopoietic tissue, gills, hepatopancreas, fore and mid gut, hind gut, lymphoid organ, and muscle) were collected from individual shrimp. For hemocytes preparation, hemolymph were collected with 10% (w/v) sodium citrate and centrifuged at 800 x g 4 °C for 10 min to separate hemocytes from plasma. Total RNA was extracted from many tissues using TRI REAGENT®. The process was described in the protocol; Samples were homogenized in 100 µl of TRI REAGENT®. Then mixture was added 900 µl of TRI REAGENT® and vortex for 2 min. Two hundred microliters of chloroform was added into the mixture, subsequent

vortex, and stored on ice for 30 min. After centrifugation at 25,000 x g 4 ℃ for 15 min, the upper phases were taken to new microcentrifuge tubes. To precipitate RNA, five hundred microliters of cold isopropanol was added and centrifuged again. Pellets were washed by 75% (v/v) cold ethanol, then air-dried for 5 min, and dissolved in DEPC-treated water. RNA purity was analyzed by 1.2% (w/v) agarose gel electrophoresis and measured the concentration at 260 nm by spectrophotometer. To remove contaminated DNA, the total RNA was treated with DNase I. The reaction contains 10 µg of total RNA in 1X DNase I buffer and 1 µl of 2 U/ul DNase I. The reaction was incubated at 37 ℃ for 30 min and then extracted total RNA again by half of reaction volume.

2.6 First strand cDNA synthesis

cDNA was synthesized from the total RNA using Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit. Briefly, the total RNA was combined with 1 µl of 100 µM oligo(dT_{18}) primer and appropriate nuclease-free water to final volume of 12 µl. Mixture was heated at 70 ℃ for 5 min and intermediately chilled on ice for 5 min. After that, the mixture was added with 8 µl of master mix, containing 4 µl of 5X reaction buffer, 2 µl of nuclease-free water, 2 µl of 10 mM dNTP mix, 0.5 µl of 20 U/µl RiboLock™ RNase Inhibitor, and 0.5 µl of 200 U/µl RevertAid™ M-MuLV Reverse Transcriptase. The reaction was incubated at 25 ℃ for 5 min, 42 ℃ for 1.30

h, and finally heated at 70 ℃ for 10 min. The total RNA and first strand cDNA were kept at -80 ℃ and -20 ℃ respectively.

2.7 Tissue distribution

Tissues from sub-adult shrimp (10-15 g) were collected for the total RNA extraction. Five hundred microliters of the total RNA from different shrimp tissues (hemocytes, heart, hematopoietic tissue, gills, hepatopancreas, fore and mid gut, hind gut, lymphoid organ, and muscle) were pooled from three individual shrimp. The pooled total RNA (1,500 ng) from each tissues were used for the first strand cDNA synthesis. The cDNA was diluted 10-fold and subjected a 1 µl into PCR reactions. Then, the expression of *Pm*MIP transcript was analyzed by semi-quantitative RT-PCR using *Pm*MIP specific primers, MIP-F/MIP-R primers (Tables 2.1). The PCR reactions consisted of 13.8 µl of PCR-water, 2.5 µl of 10X reaction buffer, 2.5 µl of 1 mM dNTPs, 2.5 µl of 2 mM of each primers, 0.2 µl of 5 U/µl of RBC *Taq* polymerase, and 1 µl of DNA template. The PCR condition was 94 ℃ for 1 min, 30 cycles of 94 ℃ for 30 sec, 55 ℃ for 30 sec and 72 ℃ for 1 min, and then a final extension at 72 ℃ 10 min. PCR products were analyzed by 1.8% agarose gel electrophoresis and visualized by UV-transillumination. For internal control, EF1α was amplified using EF1α-F/EF1α-R primers (Table 2.1). The PCR conditions were 94 ℃ for 1 min, followed by 22 cycles of 94 ℃ for 30 s, 55 ℃ for 30 sec, and 72 ℃ for 30 sec, and extension at 72 ℃ for 5 min

2.8 Gene expression and protein expression profiles after *V. harveyi* challenge

2.8.1 Preparation of *V. harveyi*

V. harveyi 639 was used in bacterial challenge experiment. Ten microliters of *V. harveyi* glycerol stock was added in 4 ml of tryptic soy broth (TSB) containing 2% (w/v) NaCl and shaken 250 rpm at 30 °C for overnight. Forty microliters of overnight culture was added into 8 ml of TSB containing 2% (w/v) NaCl and shaken 250 rpm at 30 °C for 2 - 3 h until OD_{600} reach to 0.6 (1 OD = 10^6 CFU). The cell culture was diluted 10-fold with 150 mM NaCl.

2.8.2 *V. harveyi* challenge in shrimp

Twenty microliters of diluted cell culture (2 x 10^5 CFU/shrimp) was injected into the shrimp (4-6 g) at the third segment of ventral by insulin syringes. For control, shrimp were injected with 150 mM NaCl. Then gills and hemolymph were collected at 0, 6, 24, 48, and, 72 h post injection (hpi). The gills were extracted total RNA and synthesized cDNA for gene expression analysis. The hemolymphs in anticoagulant solution, 10% (w/v) sodium citrate, were centrifuged at 800 x g at 4 °C for 10 min to remove hemocytes. Plasma were collected for protein expression analysis.

2.8.3 Detection of gene expression in *V. harveyi* challenged shrimp by real time RT-PCR

Three shrimp from each group were randomly collected. Total RNA from gills was extracted as previously described. Nine hundred nanograms of pooled total RNA from each replication was synthesized the first strand cDNA. The 1 µl of 10-fold diluted cDNA was subject into reactions following SsoFast™ EvaGreen® Supermix by using specific *Pm*MIP gene primer MIP_RT-F/MIP_RT-R (Table 2.2). The components of reaction were 5 µl of 2X SsoFast™ EvaGreen, 0.4 µl of each primer, and 3.7 µl of nuclease-free water. The cycling conditions were 95 ℃ for 8 min (1 cycle), 95 ℃ for 10 sec, 58 ℃ for 15 sec, 72 ℃ for 30 sec (39 cycles), 95 ℃ for 30 sec (1 cycle), 55 ℃ for 1 min (1 cycle), and 55 ℃ for 10 sec (1 cycle). Standard curve was performed using 5-fold diluted cDNA (1500 ng down to 2.4 ng of total RNA). Expression levels were normalized using EF1α (Table 2.2). Data were analyzed by using CFX96 Touch™ real-time PCR detection system.

2.8.4 Detection of protein expression in *V. harveyi* challenged shrimp by western blot

To determine the localization of endogenous *Pm*MIP protein in shrimp, hemolymph were collected with 10% (w/v) sodium citrate and pooled from three individual shrimp. Collected hemolymph were centrifuged at 800 x g 4 ℃ for 10 min to separate plasma from hemocytes. One hundred micograms of plasma and twenty-

five micograms of hemocyte lysate supernatant were mixed with 5X reducing SDS loading dye and heated at 100 ℃ for 10 min. Mixture were analyzed by 12.5% SDS-PAGE and western blot.

For detection of *Pm*MIP protein of *V. harveyi*-infected shrimp, fifty micograms of plasma were mixed with 5X reducing SDS loading dye and heated at 100 ℃ for 10 min. Mixture were pooled from each replication and the 50 µg of pooled mixture were subjected to 12.5% SDS-PAGE. One gel was stained with Coomassies blue and the other was performed western blot. For western blot, the gel was electro-blotted onto a nitrocellulose membrane. Then the membrane was blocked by 5% (w/v) skim milk in phosphate-buffered saline and 0.05% (v/v) Tween 20 (PBST) for 1 h. After washing in PBST for 3 x 15 min, the membrane was incubated with the rabbit anti-*Pm*MIP polyclonal antibody (1:2000 diluted in the 1% (w/v) skim milk in PBST) for 3 h and washed for 4 x 5 min. The anti-rabbit IgG alkaline phosphatase conjugated antibody from goat diluted 1:10,000 with the 1% (w/v) skim milk in PBST was incubated for 1 h and washed for 4 x 5 min. For detection, 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium (BCIP/NBT) was used as a substrate and the reaction was stopped by adding 0.02 mM ethylenediaminetetraacetic acid, EDTA.

2.9 RNA interference

2.9.1 Double-stranded RNA (dsRNA) preparation

For preparation of dsRNA, primers including T7 promoter sequence at 5' end and specific to *Pm*MIP gene, MIPT7-F/MIP-R and MIP-F/MIPT7-R (Table 2.2), were used to amplify a 434 bp region of the *Pm*MIP gene by PCR. The PCR reactions were performed as described above. The reactions were performed at 94 ℃ for 1 min, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 30 sec, and 72 ℃ for 1 min, and extension at 72 ℃ for 10 min. The PCR products were fractionated by gel extraction using Nucleospin® Gel and PCR Clean-up extraction kit. The DNA fragments were used as templates for generating sense strand and anti-sense strand by in vitro transcription using T7 RiboMAX Express RNAi System. The reaction in each strands composed 10 µl of RiboMAX express T7 2X buffer, 2 µl of enzyme mix T7 Express, and 10 µl of DNA template. Single-stranded RNAs (ssRNA) were checked by 1.2% (w/v) agarose gel electrophoresis. dsRNA was synthesized by mixing both of ssRNAs and then annealed at 70 ℃ for 10 min. The reaction was treated and incubated at 37 ℃ for 30 min with 20:1 µl of 1U/µ RQ1 RNase-Free DNase. To purify dsRNA, the DNase-treated reaction was added 0.1 volume of 3 M sodium acetate and 1 volume of cold isopropanol, and placed at -80 ℃ for 1 h. The reaction was centrifuged at 25,000 x g 4 ℃ for 15 min. Pellet was washed by cold 75% (v/v) ethanol and air-dried for 5 min. The dsRNA pellet was resuspend by nuclease-free water. dsRNA was

analyzed by 1.2% (w/v) agarose gel electrophoresis and measured the concentration at 260 nm by spectrophotometer. Control gene, the green fluorescent protein (GFP) gene was used to generate dsRNA using pEGFP-1 vector as template and GFPT7-F/GFP-R and GFP-F/GFPT7-R (Table 2.2) as specific primers.

2.9.2 dsRNA injection into shrimp

Juvenile shrimp (~3-5 g) were injected with *Pm*MIP dsRNA (2.5 μg/g shrimp) and repeated injection with *Pm*MIP dsRNA, LPS and laminarin after 24 h first dsRNA injection. The amount of LPS and laminarin were 3 μg of each component/ 1.5 g shrimp. The 25 μl of dsRNA dissolved in 150 mM NaCl was injected into the shrimp at the third segment of ventral by insulin syringes. For control, GFP dsRNA and 150 mM NaCl were used for injection.

2.9.3 Specificity of knockdown shrimp by semi-quantitative RT-PCR

RNAi silencing specificity for *Pm*MIP gene was analyzed by semi-quantitative RT-PCR using primers specific to *Pm*MIP gene, MIP-F/MIP-R (Table 2.2). At 48 h after second injection, gills were collected and then the total RNA was extract and cDNA was synthesized. The gill cDNA from each group (*Pm*MIP dsRNA, GFP dsRNA, and 150 mM NaCl) were diluted 10-fold and subjected a 1 μl into PCR reactions as previously described. The PCR profiles were performed at 94 ℃ for 1 min, followed by 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 sec, and 72 ℃ for 1 min, and extension at 72 ℃ for 10

min. The PCR products were analyzed by 1.8% (w/v) agarose gel electrophoresis. EF1α was used as the internal control.

2.9.4 Analysis of *Pm*MIP protein expression in *Pm*MIP silenced shrimp by western blot

For determination of *Pm*MIP protein in knock down shrimp, hemolymph from each group of shrimp were collected with 10% (w/v) sodium citrate. After 48 h of second injection, hemolymph were collected and centrifuged at 800 x g 4 °C for 10 min to separate plasma from hemocytes. Fifty micrograms of plasma were mixed with 5X reducing SDS loading dye and heated at 100 °C for 10 min. Mixture were subjected to 12.5% SDS-PAGE. One gel was stained with Coomassies blue and the other was performed western blot. For western blot, the gel was electro-blotted onto a nitrocellulose membrane. Then the membrane was blocked by 5% (w/v) skim milk in phosphate-buffered saline and 0.05% (v/v) Tween 20 (PBST) for 1 h. After washing in PBST for 3 x 15 min, the membrane was incubated with the rabbit anti-*Pm*MIP polyclonal antibody (1:2000 diluted in the 1% (w/v) skim milk in PBST) for 3 h and washed for 4 x 5 min. The anti-rabbit IgG alkaline phosphatase conjugated antibody from goat diluted 1:10,000 with the 1% (w/v) skim milk in PBST was incubated for 1 h and washed for 4 x 5 min. For detection, 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium (BCIP/NBT) was used as a substrate and the reaction was stopped by 0.02 mM ethylenediaminetetraacetic acid, EDTA.

2.9.5 Analysis of proPO system gene expression in *Pm*MIP silenced shrimp

For analysis of proPO system gene expression, total RNA from hemocytes was used. The hemocyte cDNA from each group (*Pm*MIP dsRNA, GFP dsRNA and 150 mM NaCl) were diluted 10-fold and subjected a 1 µl into PCR reactions. Gene expressions were examined by semi-quantitative RT-PCR using primer specific to gene in proPO system, PO1, PO2, *Pm*PPAE1, *Pm*PPAE2, *Pm*SP1, *Pm*SP2, *Pm*SPH1, *Pm*SPH2, and *Pm*Snake (Table 2.2). The amplification profiles were upon primers used. The PCR products were analyzed by 1.8% (w/v) agarose gel electrophoresis. For internal control, EF1α was amplified using EF1α-F/ EF1α-R primers (Table 2.2).

2.9.6 PO activity in knockdown shrimp hemolymph

Hemolymph from knockdown shrimp was withdrawn using 10 mM Tris-HCl pH 8.0 as anticoagulant solution. Two milligrams of total hemolymph protein was used and adjusted volume to 430 µl by 10 mM Tris-HCl pH 8.0. The concentrations of protein were measured by Bradford assay. Next, the reactions were incubated with 5 µl of 10 mg/ml LPS. After 10 min, 65 µl of 3 mg/ml substrate L-3,4-dihydroxyphenylalanine, L-DOPA, were added and then incubated for 30 min. After the time, the reactions were stopped by adding 500 µl of 10% (v/v) acetic acid. PO activity was measured the enzymatically change of L-DOPA to dopachrome at 490 nm by spectrophotometer and was reported as ΔA_{490}/mg total protein/min.

2.9.7 Proteinase activity in knockdown shrimp hemolymph

Hemolymph from knockdown shrimp was withdrawn using 10 mM Tris-HCl pH 8.0 as anticoagulant solution. Two hundred and twenty-five µg of total hemolymph protein was used and adjusted volume to 25 µl by 10 mM Tris-HCl pH 8.0. The concentrations of protein were measured by Bradford assay. Next, the reactions were added with 71 µl of proteinase buffer (100 mM Tris-HCl pH 8.0, 100 mM NaCl, and 5 mM $CaCl_2$). Then, 4 µl of 5 mM proteinase substrate B9385, Boc-Val-Pro-Arg-7-amido-4-methylcoumarin hydrochloride, were added into wells. The reactions were monitored the emission at 460 nm under excitation at 380 nm by spectrophotometer.

2.10 Cloning, production and purification of recombinant *Pm*MIP protein

2.10.1 Construction of MIP_TA vector

To ease the cloning, T & A cloning vector kit was used (Figure 2.1). *Pm*MIP gene was amplified from gill cDNA by PCR with Phusion® High-Fidelity DNA Polymerase using specific *Pm*MIP gene primer MIPNcoI-F/MIPEcoRIHis-R (Table 2.2). The 50 µl of reaction contained 10 µl of 5X buffer, 10 µl of 1 mM dNTPs, 12.5 µl of 2 mM primers, 0.5 µl of 0.02 U/µl *Phusion Taq* polymerase, 1 µl of gill cDNA, and adjusted with nuclease-free water. The PCR condition were 98 ℃ for 30 sec, 5 cycles of 98 ℃ for 10 sec, 55 ℃ for 10 sec, and 72 ℃ for 30 sec, 25 cycles of 98 ℃ for 10 sec, 60 ℃ for 10 sec, and 72 ℃ for 30 sec, and 72 ℃ for 10 min. PCR products were

attached to *Nco*I restriction site at 5’ end and six histidine residues and *Eco*RI restriction site at 3’ end. The PCR product was purified by Nucleospin® extraction kit. Next, the purified PCR product was added a poly-A tail at 3’ end followed by reaction contained 1 µl of 10X reaction buffer, 1 µl of 2 mM dATP, 7 µl of purified PCR product, and 1 µl of 5 U/µl RBC *Taq* polymerase. The reaction was incubated at 72 ℃ for 30 min. The product was purified again by Nucleospin® extraction kit. Then, the fragment was cloned into TA vector followed by the instruction, 5 µl of 1X Rapid A, 5 µl of 1X Rapid B, 2 µl of TA cloning vector, 6 µl of product, and 1 µl of 3 U/µl T4 DNA ligase. The reaction was incubated at 4 ℃ for overnight. The ligation mix was transformed into *Escherichia coli* JM109 competent cell by heat shock for recovering the recombinant plasmids. Positive clones were screened by blue white screening on Luria–Bertani (LB) agar plate supplemented with 100 µl/ml ampicillin, 20 µl/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal), 30 µl/ml isopropyl ß-D-1-thiogalactopyranoside (IPTG) and confirmed by colony PCR and DNA sequencing. The recombinant plasmid was named MIP_TA plasmid and the clone was kept as glycerol stock in -80 ℃ freezer.

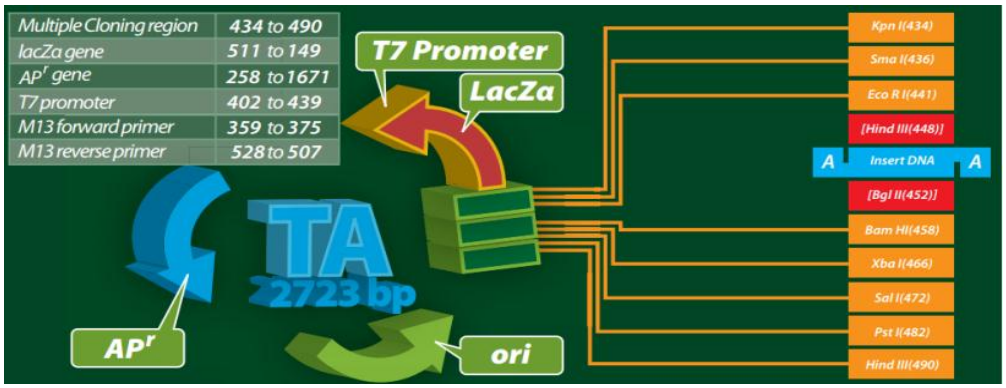


Figure 2.1 Vector map of RBC TA cloning vector

2.10.2 Construction of pET-28b_MIP and pET-43.1a_MIP

For expression of recombinant protein in *E. coli* system, pET-28b vector was used as expression vector (Figure 2.2A). *E. coli* from glycerol stock containing MIP_TA plasmid and pET-28b vector were cultured in 5 ml of LB broth for overnight and extracted for recombinant plasmids by QIAprep® Miniprep kit. The MIP_TA plasmids and pET-28b vectors were cut by *Nco*I and *Eco*RI restriction enzyme. The reaction composed of 3 µl of 10X EcoRI buffer, 1.5 µl of 10 U/µl *Nco*I, 1.5 µl of 20 U/µl *Eco*RI, 10 µl plasmids and adjusted nuclease-free water. The fragments were analysed by 1.2% (w/v) agarose gel electrophoresis, then, cut and purified by Nucleospin® extraction kit. The two fragments were ligated together by T4 DNA ligase followed of 2 µl of 10X ligation buffer, 2 µl 3 U/µl T4 DNA ligase, 2 µl of insert, and 6 µl of pET-28b vector. Then ligation reaction was incubated at 16 °C for overnight and transformed into *E. coli* JM109 competent cell by heat shock for recovering the recombinant plasmids. Positive clones on LB agar containing 50 µg/ml kanamycin

were confirmed again by colony PCR and DNA sequencing. The recombinant plasmid was named pET-28b_MIP.

pET-43.1a was also used as expression vector (Figure 2.2B), which has Nus tag to increase the solubilization of recombinant protein. The pET-43.1a has the S-tag sequence encoding the S-tag and Nus-tag sequence encoding for the Nus protein at N-terminal sequence. The vector was modified for His tag deletion and *Pci*I restriction site addition (Unpublished data). *E. coli* from glycerol stock containing MIP_TA plasmid and pET-43.1a vector were cultured in 5 ml of LB broth for overnight and extracted for recombinant plasmids by QIAprep® Miniprep kit. The MIP_TA plasmid and pET-43.1a vector were cut by *Nco*I and *Eco*RI restriction enzyme and *Pci*I and *Eco*RI restriction enzyme respectively. The reaction composed of 3 µl of 10X NEB buffer, 1.5 µl of 10 U/µl *Nco*I or *Pci*I, 1.5 µl of *Eco*RI, 10 µl plasmids and adjusted nuclease-free water. The fragments were analysed by 1.2% (w/v) agarose gel electrophoresis, then, cut and purified by Nucleospin® extraction kit. The two fragments were ligated together by T4 DNA ligase followed of 2 µl of 10X ligation buffer, 2 µl 3 U/µl T4 DNA ligase, 2 µl of insert, and 6 µl of pET-43.1a vector. Then ligation reaction was incubated at 16 °C for overnight and transformed into *E. coli* JM109 competent cell by heat shock for recovering the recombinant plasmids. Positive clones on LB agar containing 100 µg/ml ampicillin were confirmed again by

colony PCR and DNA sequencing. The recombinant plasmid was named pET-43.1a_MIP.

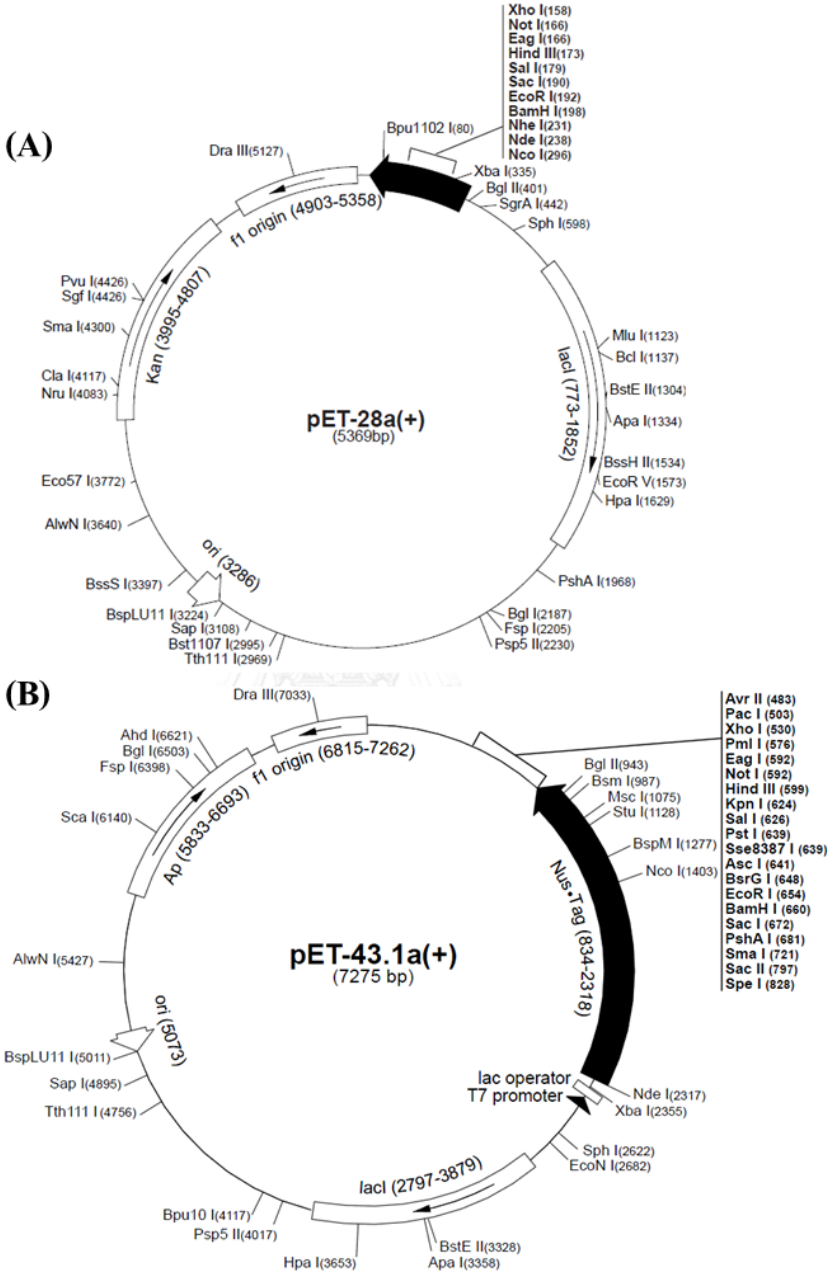


Figure 2.2 Vector map of expression vectors. (A) pET-28 vector and (B) pET-43.1 vector.

2.10.3 Expression and purification of recombinant *Pm*MIP protein

For transformation, *E. coli* stain Rosetta and BL21 were used as expression host. The pET-28b_MIP and pET-43.1a_MIP were transformed into host cells, *E. coli* Rosetta and BL21 respectively, and checked positive clones by colony PCR.

To express the recombinant *Pm*MIP protein (r*Pm*MIP), the positive clones from colony PCR were expressed in small-scale. The cells were grown in LB broth (contain 100 μg/ml ampicillin for pET-28b_MIP and 50 μg/ml kanamycin for pET-43.1a_MIP) at 37 °C in a shaking incubator (250 rpm) for overnight as starters. The starters were inoculated into LB broth ampicillin or kanamycin and shaken at 37 °C 250 rpm until the cultures reached an OD_{600} = 0.3-0.5. Then, the cultures were induced by addition of final concentration of 0.5 mM IPTG. 1 ml of culture was collected at 0, 1, 2, 3, 4, 5 and 6 h for expression level analysis and optimum time point. The cultures were centrifuged at 8,000 x g at 4 °C for 5 min. The supernatant were discharged and the pellets were mixed with 1X reducing SDS loading dye and heated for 10 min. Mixtures were subjected to 10-12.5% SDS-PAGE for coomassie blue staining and western blot. The western blot was performed by using anti-His tag monoclonal antibody as primary antibody and goat anti-mouse IgG alkaline phosphatase conjugated antibody as secondary antibody. The cultures were harvested by centrifugation at 8,000 x g at 4 °C for 5 min. The pellets were washed for twice and dissolved in 20 mM Tris-HCl pH 8.0. Then suspensions were disturb by

sonicator and centrifuged at 8,000 x g at 4 °C for 30 min. Supernatants were collected as a soluble fraction and pellets were collected as an inclusion body. The two fractions were analyzed by 10-12.5% SDS-PAGE.

To purify r*Pm*MIP-28, the pellet was dissolved in 8 M urea and added NaCl to the concentration of 300 mM NaCl. The r*Pm*MIP-28 protein was purified by Ni-NTA affinity chromatography. Column was equilibrated by equilibrated buffer (300 mM NaCl and, 50 mM $NaHPO_4$ pH 8.0 in 8 M urea) and the supernatant was subjected to the column. The column was washed by washing buffer (10, 20 and, 50 mM imidazole, 300 mM NaCl and, 50 mM $NaHPO_4$ pH 8.0 in 8 M urea) and then eluted by elution buffer (150 and 250 mM imidazole, 300 mM NaCl and 50 mM $NaHPO_4$ pH 8.0 in 8 M urea). After dialysis in 20 mM Tris-HCl pH 8.0 using 10 MWCO SnakeSkin Dialysis Tubing (Thermo Scientific, USA), the r*Pm*MIP protein was concentrated using 10 kDa Amicon Ultra-15 Centrifugal Filter (MERCK, USA). The purified r*Pm*MIP was analyzed by 12.5% SDS-PAGE and measured the concentration by Bardford assay. The purified r*Pm*MIP-28 was used for antibody production by a commercial service at Biomedical Technology Research Unit, at Chaing Mai University.

To purify r*Pm*MIP-43.1, the supernatant were added NaCl to the concentration of 1 M NaCl. The r*Pm*MIP-43.1 protein was also purified by Ni-NTA affinity chromatography. Column was equilibrated by equilibrated buffer (20 mM imidazole, 1 M NaCl and 50 mM Tris-HCl pH 8.0) and subjected the supernatant. The

column was washed by washing buffer (20, 50 and 150 mM imidazole, 300 mM NaCl and 1 M Tris-HCl pH 8.0) and then eluted by elution buffer (250 and 500 mM imidazole, 1 M NaCl and 50 mM Tris-HCl pH 8.0). After dialysis in 20 mM Tris-HCl pH 8.0 using 10 MWCO SnakeSkin Dialysis Tubing (Thermo Scientific, USA), the r*Pm*MIP protein was concentrated using 10 kDa Amicon Ultra-15 Centrifugal Filter (MERCK, USA). The purified r*Pm*MIP was analyzed by 12.5% SDS-PAGE and measured the concentration by Bardford assay. The purified r*Pm*MIP-43.1 was used for activity assay. For the control protein, which is Nus tag protein, pET43.1a plasmid was transformed into *E. coli* BL21. The same strategy was used for the expression and purification of rNUS protein with only a minor modification.

2.11 Functional characterization of recombinant *Pm*MIP protein

2.11.1 PO activity of r*Pm*MIP

Hemolymph of normal shrimp was collected from the ventral segment by insulin syringes with 10 mM Tris-HCl pH 8.0. Two hundred and fifty micrograms of total hemolymph protein was adjusted volume to 25 µl by 10 mM Tris-HCl pH 8.0 and incubated with 25 µl of r*Pm*MIP (10 µg) containing 20 mM $CaCl_2$ (For controls, rNUS and 20 mM Tris-HCl pH 8.0 were used) for 10 min and then added with 25 µl of 2 mg/ml LPS. After that the reaction was added with 25 µl of 3 mg/ml L-DOPA as a substrate. The PO activity was measured at 490 nm and defined as ΔA_{490}/mg total protein/min.

CHAPTER III

RESULTS

3.1 Sequence analysis of *Pm*MIP

The partial nucleotide sequence of *Pm*MIP was firstly identified from *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al. 2006). The full-length cDNA of *Pm*MIP was then obtained from 3' and 5' Rapid amplification of cDNA ends (RACE) of gills cDNA (Ampayup, unpublished data). The cDNA and deduced amino acid sequence of *Pm*MIP were analyzed by using the GENETYX 7.0.3 program (GENETYX Corporation). The open reading frame (ORF) of *Pm*MIP was 957 bp encoding a protein of 318 amino acids residues with the predicted molecular mass of 35.9 kDa and pI 5.5 (Figure 3.1). From BLASTX analysis, the *Pm*MIP nucleotide sequence shares 100% identity to *P. monodon Pm*MIP sequence reported previously by Angthong et al. (2010), which have been identified from intestine cDNA and exhibited 80% similarity to *Pl*MIP from crayfish *P. leniusculus*. The putative signal peptide and structural domain were predicted by the simple modular architecture research tool (SMART) version 4.0, showed fibrinogen related-domain (FReD) at C-terminus, calcium binding site and N-glycosylation position (Figures 3.1 and 3.2). The multiple sequence alignment of the deduced amino acid of *Pm*MIP with the other MIPs (*Pl*MIP from *P. leniusculus* EX571686 and *Tm*MIP from *T. molitor* AB205184.1)

revealed that *Pm*MIP shared a high similarity to *Pl*MIP (80%) but rather low similarity to *Tm*MIP (8%) (Figure 3.3).

```
AACATCGCTGGGACGGAGGCTCCTGCTGTCGATTTCTCACGAACGTCGTTCGACCAAAAT    60
                                                          M

GATCGCCATGCCCCGAATTGTGGCAATGCTGTGGGCTGTGATAGCTGTGGTGGGCGCCGC   120
  I  A  M  P  R  I  V  A  M  L  W  A  V  I  A  V  V  G  A  A

CGCCAACGAGCCCCTTAGCCGTAGGGCAACATTCCCCGCTTCCTCCAGTGAAACAAAGCA   180
  A  N  E  P  L  S  R  R  A  T  F  P  A  S  S  S  E  T  K  H

CATTGACCAGCAACTGAACGCCATCCTCAATACCCTCACGGAGAATCACAACCAGATACA   240
  I  D  Q  Q  L  N  A  I  L  N  T  L  T  E  N  H  N  Q  I  Q

GTCCTCCATTCTGGACATCATGCGCATGGTCAGACTAACCCACCGGGAAATCGCCGACCT   300
  S  S  I  L  D  I  M  R  M  V  R  L  T  H  R  E  I  A  D  L

GCGGGAGGACACTGACCTCATTTTGGGTCAGATGTACAAGCCCTCGTGCCAAGACATCGC   360
  R  E  D  T  D  L  I  L  G  Q  M  Y  K  P  S  C  Q  D  I  A

CGAGGGCCAGCCTGATCACATCGCGGGGAAGAACCAGTTCGCTACCGAGGGCGTGTACAC   420
  E  G  Q  P  D  H  I  A  G  K  N  Q  F  A  T  E  G  V  Y  T

CATTCGGCCGCCGAAGTTCAAGCCTAAGAAGGTCCGCTGCGAGCTCGGCCGGGGAGCCAT   480
  I  R  P  P  K  F  K  P  K  K  V  R  C  E  L  G  R  G  A  I

CGGATGGACGGTCATACTGGCGAGGAACGACGGGCGTGAGCCGTTTAATCGCACTTTCCA   540
  G  W  T  V  I  L  A  R  N  D  G  R  E  P  F  N  R  T  F  Q

GGATTACAAGGATGGCTTCGGAGATCCTGCGCAGGACCACTGGATCGGCCTGGACATGCT   600
  D  Y  K  D  G  F  G  D  P  A  Q  D  H  W  I  G  L  D  M  L

GCACAAGCTGACCACATGGGAGCCTCACCAACTTCGAGCAGTCATGGAGGACTTCGATGG   660
  H  K  L  T  T  W  E  P  H  Q  L  R  A  V  M  E  D  F  D  G

TTCCAAGACTTGGGTTCAGTACAACGTGTTCAGAGTGGACGGGCCTGAGGATGACTACAA   720
  S  K  T  W  V  Q  Y  N  V  F  R  V  D  G  P  E  D  D  Y  K

GCTGACGGCGGAGGAGTTCGAGGCTGACAGCGCCGCGGGAGATGGCCTCAGCATCCACAA   780
  L  T  A  E  E  F  E  A  D  S  A  A  G  D  G  L  S  I  H  N

TGGCATGAAGTTCTCCACCTACGACCATGATGATGACACCAACAGGGACGGAAACTGCGC   840
  G  M  K  F  S  T  Y  D  H  D  D  D  T  N  R  D  G  N  C  A

CAAGCTCTTTGGTGGAGGAGGTGGCTGGTGGTACAATAACTGCTACCACGTCCTCCCTAC   900
  K  L  F  G  G  G  G  G  W  W  Y  N  N  C  Y  H  V  L  P  T

CGGCACGTACCGTCACTCCGGCGGTAGCGAATACGGGGGCGTGGCCTGGTACCCTTGGAG   960
  G  T  Y  R  H  S  G  G  S  E  Y  G  G  V  A  W  Y  P  W  R

GAACGTCAAACACTCCCTCAAGAGCCTCACTCTGCTCATCAGGCCGAGATATTAGACTCC  1020
  N  V  K  H  S  L  K  S  L  T  L  L  I  R  P  R  Y  *

GGGTGACCGAGAGGCTTTGTGGCTTCCCTTGTCGACATGAGAGACAGAAACAAAGGGTAG  1080
AGTGTAAACACTAAGCAAATTTTGAAGCTCTCTAAGGCTTTTGTTTTATTTTATTTTATT  1140
GTTATTGCAAATATCGTGTGTAGGTTTGGACTGTGAGAGTATTTTTTTTCATTGTGGAAT  1200
CTATAATTGTTTATTTTTGTCATGAAGATGAATCTAATTGTGTTATGGAACATTTCTGAG  1260
CACAGTTTTGTCAAAATGGATGATTAACGTTTTTTTCAATTTTTGGCAGTGATTATTATT  1320
TTTGTCACAGTGATAAGATATTTAGCAGTGTATTCATTTATAAGGATGTCAGATTTCAAA  1380
GAGATGTGTTGGTGAACATATCGAAATGTAGTTTTAAAGAACACTCCATGTAGACTTGCT  1440
ATTAAAGACTT                                                    1451
```

Figure 3.1 Nucleotide and deduce amino acid sequences of *Pm*MIP. The predicted ORF is from the nucleotide 58-1011. The putative signal peptide is in grey shade. The

FReD is the amino acid sequence which is underlined. N-glycosylation position is in grey shade and underlined. The predicted calcium binding site is in blue box.

Figure 3.2 Illustration of FReD and putative signal peptide in *Pm*MIP. The putative signal peptide is red box and the FReD is purple box.

```
CLUSTAL 2.1 multiple sequence alignment

PlMIP           MVRGPKMAVLMVVVVATVSMVRGS----------------LAAGTNPSIARTASIPLTSA 44
PmMIP           MIAMPRIVAMLWAVIAVVG----------------------AAANEPLSRRATFPASSS 37
TmMIP           --MSPRVLLLVLGFLALSSAFEVKNLLKTLQKREVSSSLLSKSGNLNTLIRKRRDAEESA 58
                    *::  ::  .:*  .                       :.   .: *    .  *:

PlMIP           DTRQTAIESTLQTILST-MQENHNQLQSSVREVMR-----VARRTQRELTELREDTDLIL 98
PmMIP           ETKH--IDQQLNAILNT-LTENHNQIQSSILDIMR-----MVRLTHREIADLREDTDLIL 89
TmMIP           EPTSGEVISILRFFMALGWHEDKHDVLASFLEEMSDPTNNVTVIGWQRILDEQGDIKEIN 118
                :.    : . *. ::     *::::: :*. : *      :.    :.: : : * . *

PlMIP           GQMYKPSCQEVAEDLHDGTRGRTNEAIVGVYTIKPPK--YKPTQVRCELGRGAVGWTVIL 156
PmMIP           GQMYKPSCQDIAEGQPDHIAGKNQFATEGVYTIRPPK--FKPKKVRCELGRGAIGWTVIL 147
TmMIP           TIQLVIPPKPVSDGDTSYDDDDDDEDEDDDDNFLPPSTINEMKEMFEPLADLGIPGSVTA 178
                      . : :::.  .   .  :    .  .: **.   : .::   *.  .:  :*

PlMIP           SRSNGRERFNRTYREYQEGFGDPSDEYYIGNEPLHRLTTWRSHQLRVVMEDFDGQKAWVQ 216
PmMIP           ARNDGREPFNRTFQDYKDGFGDPAQDHWIGLDMLHKLTTWEPHQLRAVMEDFDGSKTWVQ 207
TmMIP           EEFNKKGELETVIYLDKDELYGKAEGFSVYNKTATVTADEVSDFVKRIKPGAFGKPSVLK 238
                 . : :  :: .    :: : . :: . : .     :   .. :: :  .  *. : ::

PlMIP           YKIFR--VAGPEDNYQLTVDEFEADSAAGDGLKIHNGMKFSTYDKDDDDNKDGNCSQLFG 274
PmMIP           YNVFR--VDGPEDDYKLTAEEFEADSAAGDGLSIHNGMKFSTYDHDDDTNRDGNCAKLFG 265
TmMIP           YDVDGNLIVDQSKVQPHLLGTLSDYYEANDDMILSGAEVIAQISQQYHQDEIPKQLAKHY 298
                *.:    : . ..        :.    *.*.: : ..  ::  .:: . :.  :    .

PlMIP           GGGGWWFTNCYHVLPTGRYRSVGGNE---YGGLAWYPWRNVKHSLKAMTLLIRTH- 326
PmMIP           GGGGWWYNNCYHVLPTGTYRHSGGSE---YGGVAWYPWRNVKHSLKSLTLLIRPRY 318
TmMIP           NNAYPLYKWRVLVNPSSYYVTRDVAVTLTYDNFDLDDKSDVEYYVYGVKKEANK-- 352
                ...   :.    * *:. *   .      *...      :*:: : .:.   .
```

Figure 3.3 Multiple amino acid sequence alignmet of *P. monodon Pm*MIP with *P. leniusculus Pl*MIP and *T. molitor Tl*MIP. The amino acid sequence of *Pm*MIP was aligned with *Pl*MIP from *P. leniusculus* (EX571686) and *Tm*MIP from *T. molitor* (AB205184.1) by ClustalW2. The yellow shade showed the same amino acids in all

species, the blue shade and the green shade showed the same amino acid, between *Pm*MIP and *Pl*MIP and *Pm*MIP and *Tm*MIP, respectively.

3.2 Tissue distribution of *Pm*MIP transcripts

The *Pm*MIP transcript was examined in shrimp tissues (hemocytes, heart, hematopoietic tissue, gills, hepatopancreas, fore and mid gut, hind gut, lymphoid organ, and muscle) by semi-quantitative RT-PCR. The results showed that *Pm*MIP transcript was highly expressed in gills, intestine, lymphoid organ, and muscle; low expressed in heart and hematopoietic tissue and fairly low in hemocytes (Figure 3.4). The presence of *Pm*MIP transcript in all examined tissues are in concordant with the work of Angthong et al. (2010). Except the hemocytes whose expression was found only in this study.

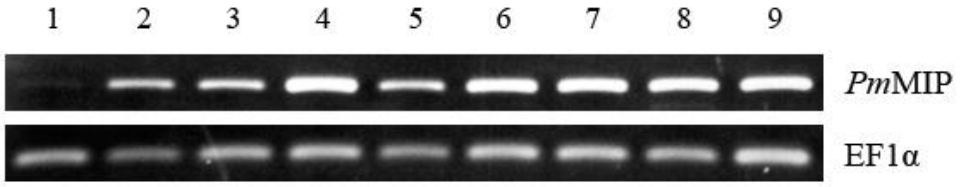


Figure 3.4 The tissue distribution of *Pm*MIP by semi-quantitative RT-PCR. Lane 1, hemocytes; Lane 2, heart; Lane 3, hematopoietic tissue; Lane 4, gills; Lane 5, hepatopancreas; Lane 6, fore and mid gut; Lane 7, hind gut; Lane 8, lymphoid organ; Lane 9, muscle. Each lane represents the pooled total RNA from three individual shrimp. Upper row, expression of *Pm*MIP. Lower row, expression of EF1α in the corresponding tissues.

3.3 Time-course analysis of *Pm*MIP gene expression after *V. harveyi* infection

*Pm*MIP is highly expressed in shrimp tissues, including gills. In this study, we examined the response of *Pm*MIP transcript to *V. harveyi* infection in gills because gills are the major site of *Pm*MIP expression. Shrimp (4-6 g) were injected with 2×10^{-5} CFU of *V. harveyi* and then gills were collected at various time points (0, 6, 24, 48, and 72 h) after injection to synthesize the first strand cDNA. The *Pm*MIP transcript level was determined by quantitative real time RT-PCR using EF1α as the internal control for normalizing the cDNA template. The result show that *Pm*MIP transcript was extremely decreased from 0.5 in 0 h to 0.07-fold at 24 h and then recovered at 48 and back to normal level at 72 h post infection (0.57- and 1.11-fold, respectively) (Figure 3.5). The result showed the *Pm*MIP mRNA expression in the gill was down-regulated after *V. harveyi* infection suggesting that *Pm*MIP was an immune responsive gene.

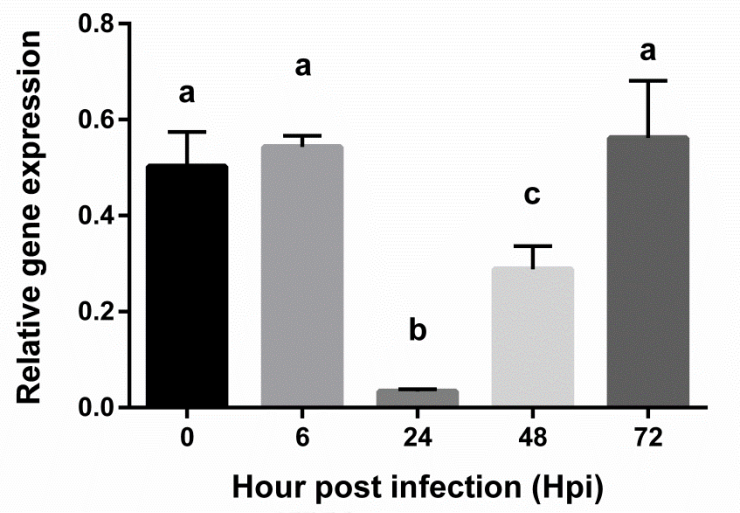


Figure 3.5 Relative expression of *Pm*MIP in gills of *V. harveyi* infected shrimp by quantitative real time RT-PCR. Relative expression levels of mRNA were calculated according to Pfaffl, 2001, using EF1α as the internal reference gene. The average relative expressions were representative of three independent repeats ± 1S.D. (error bars). Significant difference is indicated by a lowercase letter ($p<0.05$).

3.4 *Pm*MIP protein expression profile in *V. harveyi* infected *P. monodon*

It has been reported previously that *Pm*MIP transcript is expressesed in various tissues but the protein is released into the shrimp hemolymph (Angthong et al. 2010). In this study, the localization of endogenous *Pm*MIP protein in shrimp hemolymph was also determined. Hemolymph were collected and separated as plasma and hemocytes. One hundred micrograms of plasma protein and twenty-five micrograms of total protein from hemocyte lysate supernatant were analyzed by western blot using anti-*Pm*MIP antibody. A band of protein with the size

approximately 43 kDa was detected in cell-free plasma, but not in hemocytes (Figure 3.6) suggesting that *Pm*MIP protein was synthesized from different tissues and released into hemolymph.

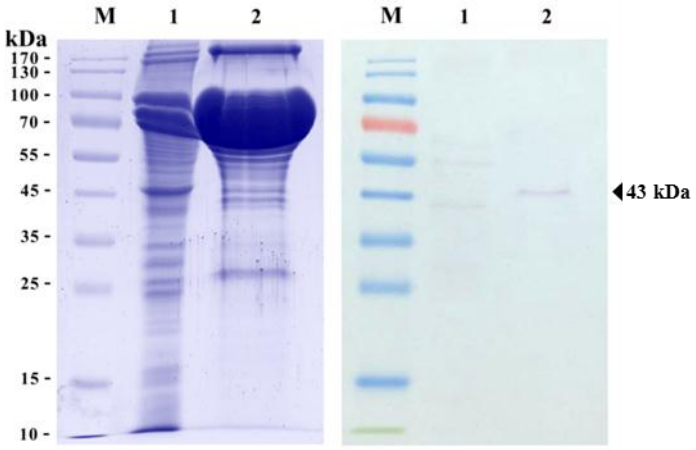


Figure 3.6 Localization of endogenous *Pm*MIP protein in shrimp hemolymph. Samples were subjected to 12.5% SDS-PAGE and performed Coomassies blue staining (Left) and western blot analysis using anti-*Pm*MIP antibody (Right). Lane M, Prestain Maker Page Ruler; Lane 1, hemocyte proteins; Lane 2, cell-free plasma proteins.

To follow the *Pm*MIP protein profile after *V. harveyi* infection, shrimp were injected with *V. harveyi* and plasma was collected at various time points (0, 6, 24, 48, and 72 h) after injection. The *Pm*MIP protein was detected by western blot using anti-*Pm*MIP antibody. The profile of *Pm*MIP protein expression showed that *Pm*MIP protein was reduced at 6 h and recovered at 24 h post injection (Figure 3.7). The result suggested that *Pm*MIP might be degraded after bacterial infection and recovered back to normal level afterward.

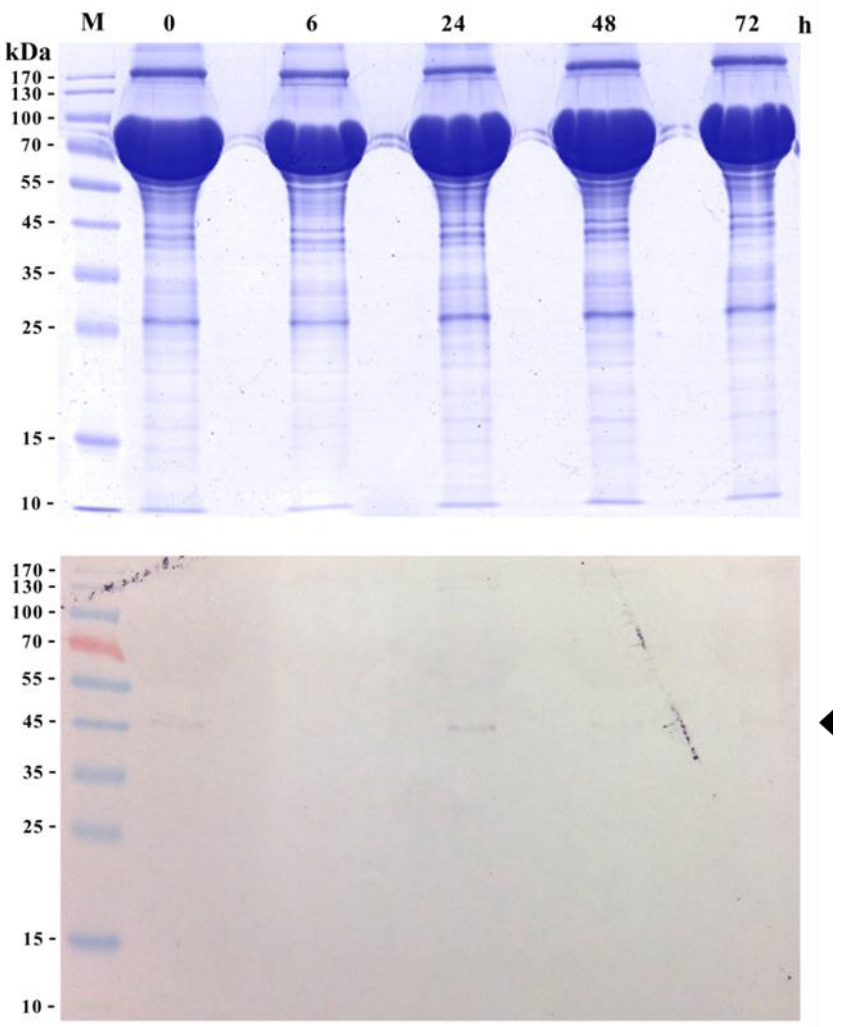


Figure 3.7 *Pm*MIP protein expression profile in cell-free plasma of *V. harveyi* infected shrimp. Samples at various time points (0, 6, 24, 48, and 72 h after injection) were subjected to 12.5% SDS-PAGE with Coomassies blue staining (B) and western blot analysis using anti-*Pm*MIP antibody (B). Lane M, Prestain Maker Page Ruler.

3.5 Silencing of *Pm*MIP by RNA interference

To study the functional of *Pm*MIP in the proPO system, gene silencing of *Pm*MIP was performed using dsRNA mediated RNA interference and assayed for PO activity.

3.5.1 Double-stranded RNA (dsRNA) preparation

The DNA fragments of *Pm*MIP gene (434 bp) and GFP gene (739 bp) were amplified using the specific primers (Table 2.1). The PCR products containing T7 promoter region at 5' end were used as templates for generating ssRNAs which are sense and anti-sense strands (Figure 3.8). Each ssRNAs was synthesized and then they were annealed to form dsRNA and treated with DNase I to remove contaminated DNA. The purified dsRNAs of *Pm*MIP and GFP were obtained with expected size of 434 and 739 bp respectively (Figure 3.9).

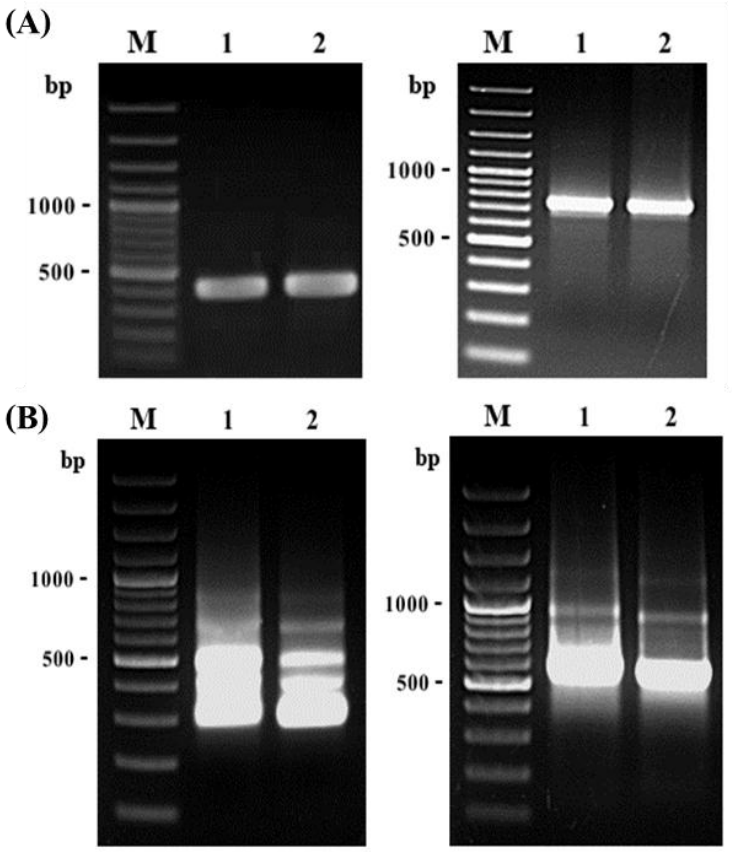


Figure 3.8 Preparation of dsDNA and ssRNA. The PCR product of *Pm*MIP (Left) and GFP (Right) was analyzed by 1.2% (w/v) agarose gel electrophoresis. (A), dsDNA amplification; (B), ssRNA synthesis. Lane M, 100 bp Marker; Lane 1, sense strand; Lane 2, anti-sense strand.

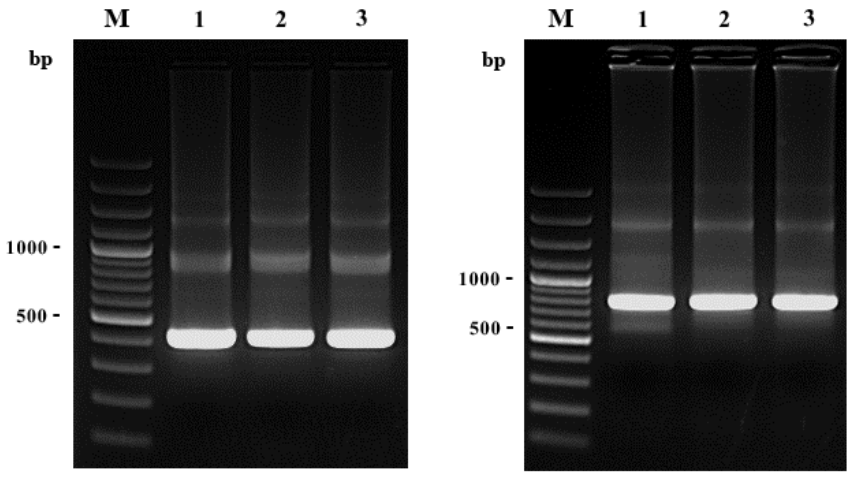


Figure 3.9 Preparation of dsRNA. The dsRNA of *Pm*MIP (left) and GFP (right) was analyzed by 1.2% (w/v) agarose gel electrophoresis. Lane M, 100 bp Marker; Lane 1, dsRNA after annealing; Lane 2, dsRNA after DNaseI treatment; Lane 3, dsRNA after precitipation

3.5.2 dsRNA-mediated supression of *Pm*MIP gene

Gene silencing of *Pm*MIP was conducted by injection of *Pm*MIP dsRNA Shrimp (3-5 g) were twice injected with 2.5 μg/g of *Pm*MIP dsRNA or controls with GFP dsRNA or NaCl. At 48 h after second injection, gills were collected and the transcripts were analyzed by semi quantitative RT-PCR. EF1α was used to normalize the amount of cDNA. The result showed that the transcript of *Pm*MIP was suppressed by *Pm*MIP dsRNA injection but not in the controls (Figure 3.10).

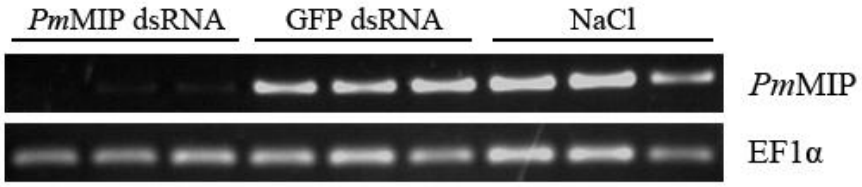


Figure 3.10 Efficiency of dsRNA-mediated *Pm*MIP knockdown by semi quantitative RT-PCR. Juvenile shrimp (3-5 g) was injected twice with 2.5 μg/g *Pm*MIP dsRNA or controls GFP dsRNA or with NaCl served as controls. EF1α was used as the internal reference gene. In shown gel, each lane repeats the results from individual shrimp.

3.5.3 Effect of suppression of *Pm*MIP gene on expression of proPO-related genes

To examine the effect of *Pm*MIP knocking down on the shrimp proPO related genes (*Pm*proPO1, *Pm*proPO2, *Pm*PPAE1, *Pm*PPAE2, *Pm*SP2, *Pm*SPH1, and *Pm*SPH2), the hemocytes of the knockdown shrimp were collected and the transcripts were analyzed by semi quantitative RT-PCR using specific primers for each gene. EF1α amplification was used as the internal control for cDNA template normalization. The results showed that supression of *Pm*MIP had no significant effect on the expression of any proPO related genes (Figure 3.11).

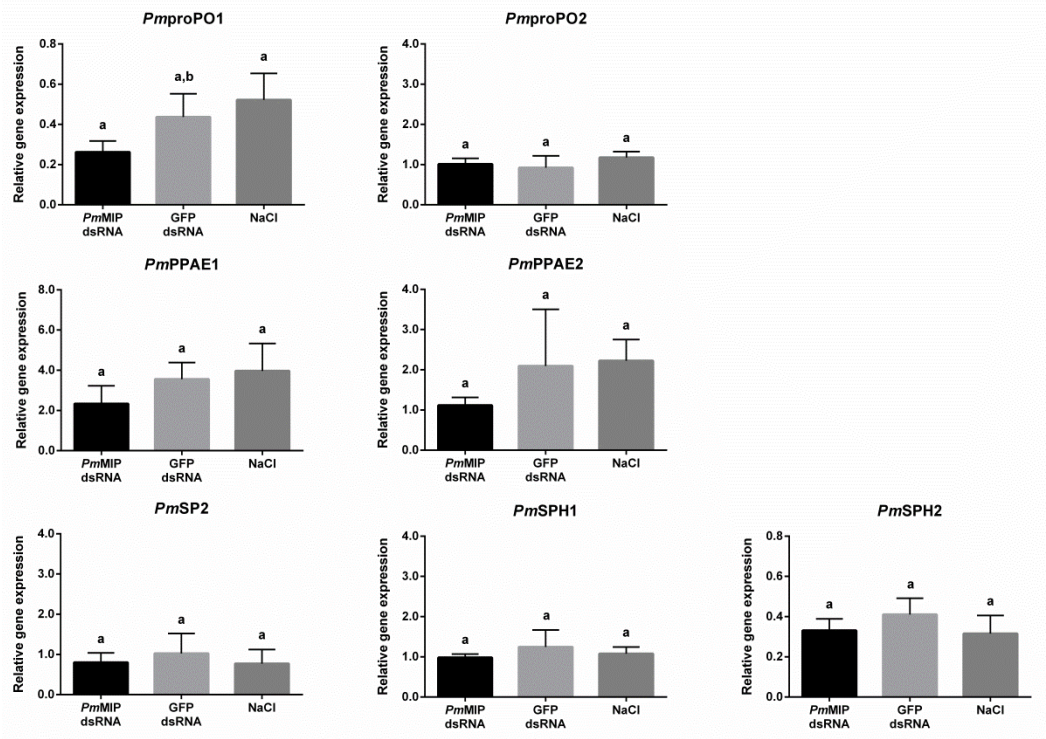


Figure 3.11 Effect of *Pm*MIP supression on the shrimp proPO related genes. The transcription levels of the genes (*Pm*proPO1, *Pm*proPO2, *Pm*PPAE1, *Pm*PPAE2, *Pm*SP2, *Pm*SPH1, and *Pm*SPH2) in *Pm*MIP dsRNA, GFP dsRNA, and NaCl injected shrimp were evaluated by semi quantitative RT-PCR using the gene specific primers for each gene. EF1α was used as an internal control to normalize the amount of cDNA template. The average relative expressions are representative of three independent repeats ± 1S.D. (error bars) Significant difference compared with control is indicated by a lowercase letter ($p<0.05$).

3.5.4 *Pm*MIP protein expression in the knockdown shrimp

To determine whether *Pm*MIP dsRNA could suppress the level of *Pm*MIP protein, the plasma protein from dsRNA-treated shrimp was analyzed by western blot using antibody against *Pm*MIP. The result showed the *Pm*MIP protein expression was decreased in *Pm*MIP dsRNA injected group as compare with the controls (Figure 3.12) suggested that *Pm*MIP dsRNA also could also suppress *Pm*MIP at the translation level.

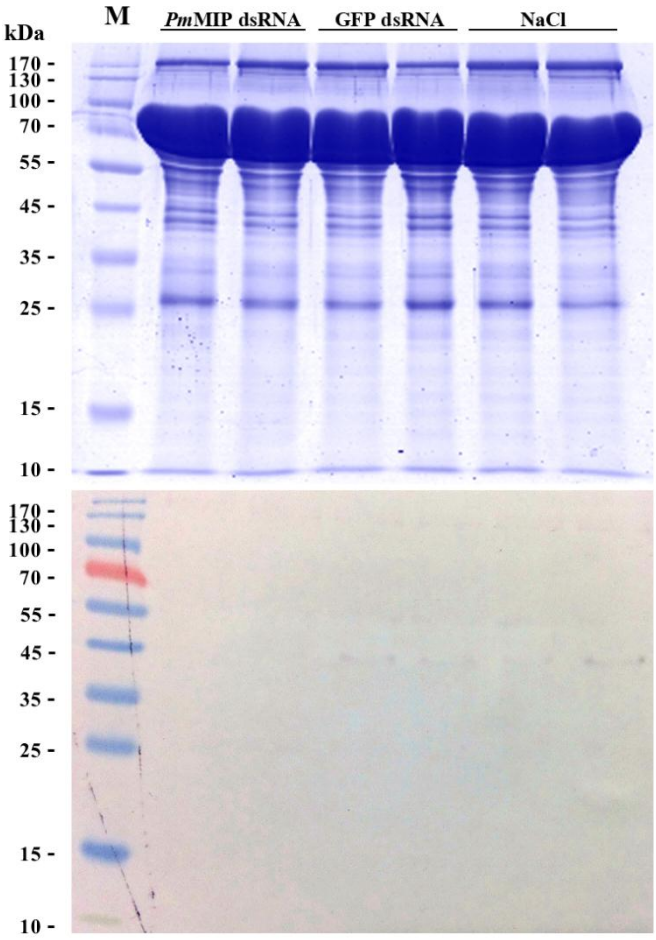


Figure 3.12 *Pm*MIP protein expression analysis in the plasma of the *Pm*MIP knockdown shrimp. Shrimp injected with GFP dsRNA or with NaCl served as controls. Samples were subjected to 12.5% SDS-PAGE and performed Coomassies blue staining

(A) and western blot analysis using anti-*Pm*MIP antibody (B). In shown gel, each lane repeats the results from individual shrimp. Lane M, Prestain Maker Page Ruler.

3.5.5 Hemolymph PO activity in *Pm*MIP knockdown shrimp

To investigate the involvement of *Pm*MIP in proPO system, the hemolymph from *Pm*MIP knockdown shrimp was subjected to a PO activity assayed by measuring the absorbance at 490 nm using L-DOPA as substrate. PO activity was defined as ΔA_{490}/mg total protein/min. The result showed that the PO activity in the *Pm*MIP silencing shrimp was increased 55% when compared with control groups (Figure 3.13). This indicates that *Pm*MIP is involved in proPO system and acts as a negatively regulator.

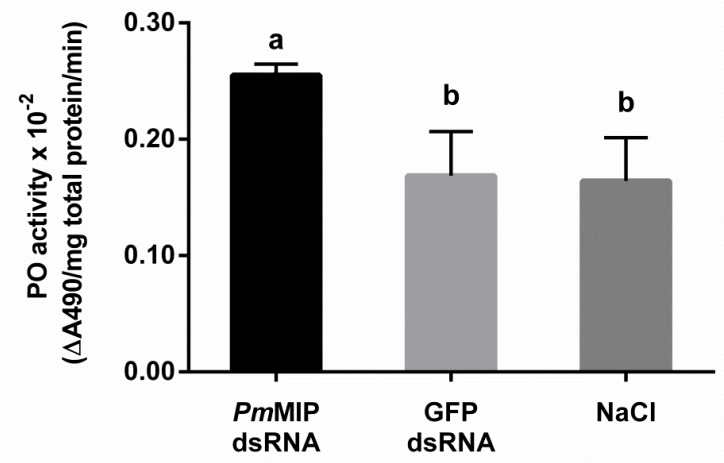


Figure 3.13 Hemolymph PO activity in *Pm*MIP knockdown shrimp. Hemolymph was collected at 48 h after the second injection of dsRNA or NaCl. The total hemolymph PO activity was defined as ΔA490/mg total protein/min. The data are shown as the mean ± 1S.D. (error bars) and are derived from three independently replicated

experiments. Means with a different lowercase letter (above each bar) are significantly different ($p<0.05$).

3.5.6 Hemolymph proteinase activity in *Pm*MIP knockdown shrimp

To further investigate the role of *Pm*MIP in melanization inhibition, we determined the proteinase activity in shrimp hemolymph proteinase activity after *Pm*MIP silencing. Hemolymph was collected from *Pm*MIP knockdown shrimp and the proteinase activity was measured by the emission at 460 nm under excitation at 380 nm using fluorescence peptide, B9385, as substrate. The proteinase activity in *Pm*MIP knockdown shrimp was significantly increased, but not in control groups (Figure 3.14). The increasing of proteinase activity suggested that *Pm*MIP might interfere the proteinase activation.

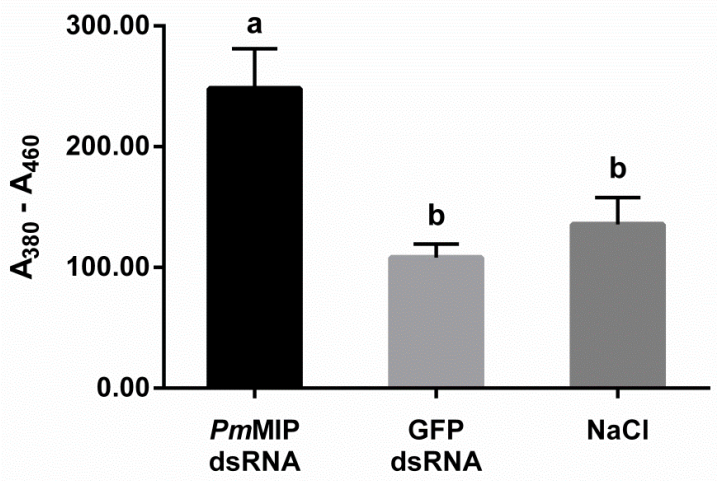


Figure 3.14 Hemolymph proteinase activity in *Pm*MIP knockdown shrimp. Hemolymph was collected at 48 h after the second injection of dsRNA or NaCl. The total hemolymph proteinase activity was measured for the fluorescence using

proteinase substrate, B9385, at emission 460 nm under excitation at 380 nm. The data are shown as the mean ± 1S.D. (error bars) and are derived from three independently replicated experiments. Means with a different lowercase letter (above each bar) are significantly different ($p<0.05$).

3.6 Recombinant *Pm*MIP protein production

To study the biological activity and produce antibody, the recombinant *Pm*MIP protein was expressed in *E. coli* system using pET vectors as expression vectors.

3.6.1 Construction of recombinant plasmids

*Pm*MIP DNA fragment with 6x His tag at C-terminus was amplified using specific primers (Table 2.1) and cloned into T & A cloning vector. The positive colony was checked by colony PCR and the sequence was confirmed by sequencing. The size of *Pm*MIP with 6x His tag was approximately 915 bp (Figure 3.12). Then the recombinant plasmid was subcloned into pET-28b vector or pET-43.1a vector. The pET-28b vector was used to produce the recombinant protein for antibody production. Whereas the pET-43.1a vector was used for increasing the solubility of recombinant protein which it has the S-tag sequence encoding the S-tag and Nus-tag sequence encoding for the Nus protein.

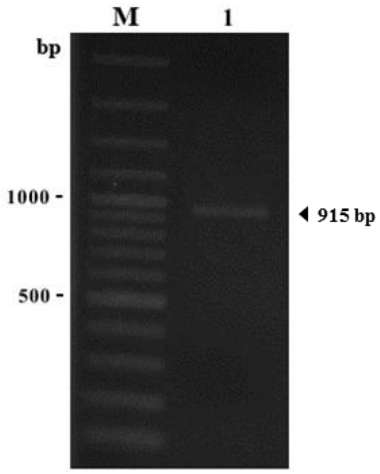


Figure 3.15 PCR product of *Pm*MIP gene amplification. PCR product was analyzed by 1.2% (w/v) agarose gel electrophoresis. M = 100 bp Marker and Lane 1 = *Pm*MIP with 6x His tag.

3.6.2 Expression and purification of recombinant protein

After the *Pm*MIP gene was subcloned into pET-28b and pET-43.1a vector. The recombinant plasmids of pET-28b_MIP and pET-43.1a_MIP were transformed into expression host *E. coli* stains rosetta and BL21 respectively. Expressions of recombinant proteins were induced by IPTG and collected at 0, 1, 2, 3, 4, 5, and 6 h after IPTG addition. The protein expressions were subjected to 12.5% SDS-PAGE and analyzed by Coomassie staining and western blot using anti-His antibody. The results showed that the recombinant proteins were expressed at various time points after IPTG induction and the highest expression of recombinant proteins was observed at 6 h after IPTG induction. The recombinant proteins with the expected molecular mass

of 34.75, 94.34, and 64.87 kDa were expressed from pET-28b_MIP in *E. coli* rosetta and pET-43.1a_MIP in *E. coli* BL21 and pET-43.1a in *E. coli* BL21 named r*Pm*MIP28, r*Pm*MIP43, and rNUS respectively (Figure 3.13).

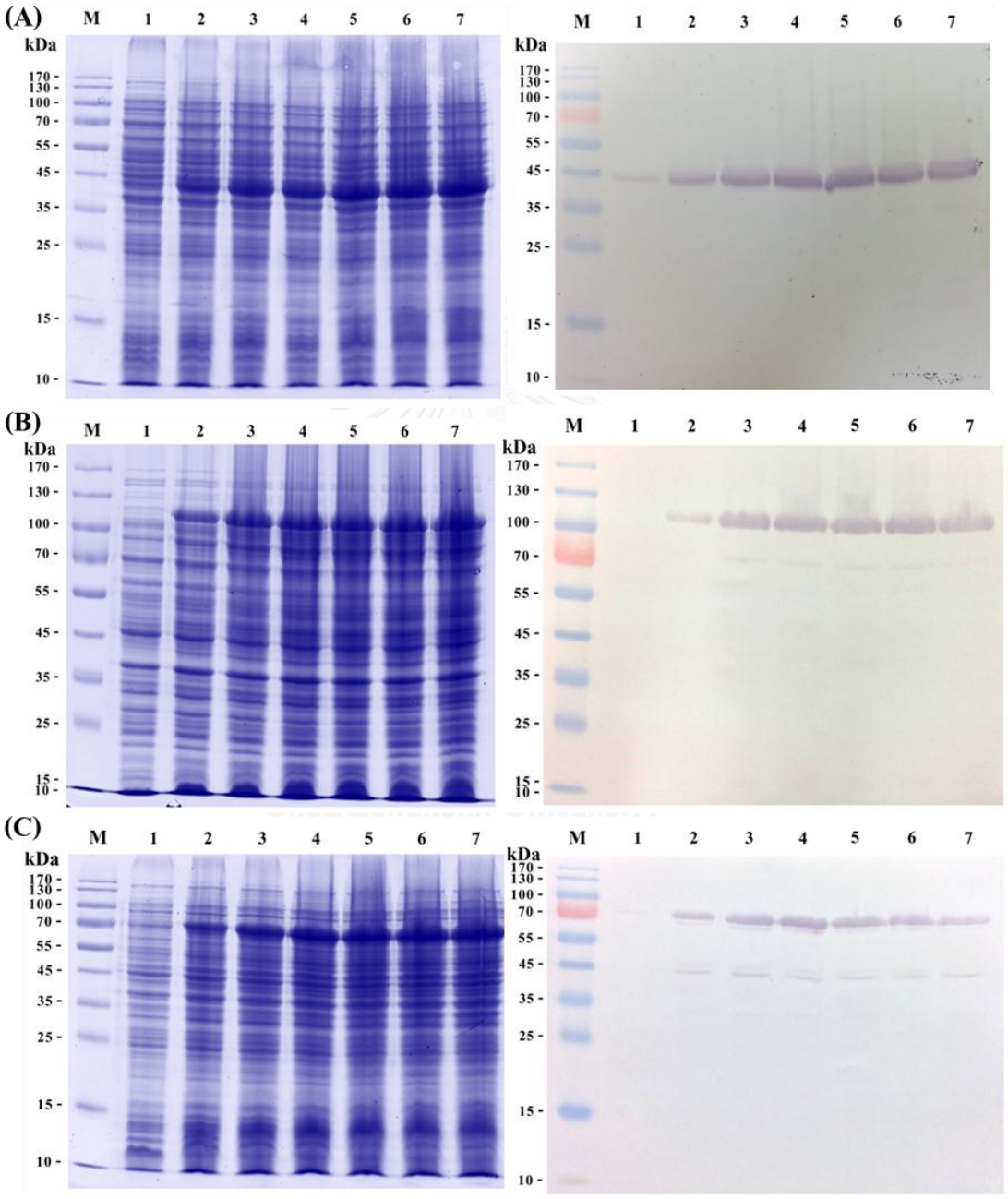


Figure 3.16 Expression of recombinant proteins after IPTG induction. The expressions were analyzed by Coomassie staining (left) and western blot using anti-His antibody

(right). (A) expression of r*Pm*MIP28 protein, (B) expression of r*Pm*MIP43 protein and (C) expression of rNUS protein. Lane M, Prestain Maker Page Ruler; Lane 1 – 7, recombinant protein expressions at 0, 1, 2, 3, 4, 5, and 6 h after IPTG induction.

To analyze the localization of recombinant protein in *E. coli*, the cultured cells were collected at 6 h after IPTG induction. Then, cell pellets were broken by sonication and separated for supernatant and pellet. The supernatant and the pellet were analyzed by 10% SDS-PAGE. The result showed that the major band of recombinant r*Pm*MIP28 protein was localized as inclusion. The major band of recombinant r*Pm*MIP43 protein was localized in both soluble fraction and inclusion. And the major band of recombinant rNUS protein was localized in soluble fraction (Figure 3.17). Using pET-43.1a as expression vector could increase the solubility of recombinant protein. The r*Pm*MIP43 were found in both fractions of soluble fraction and inclusion.

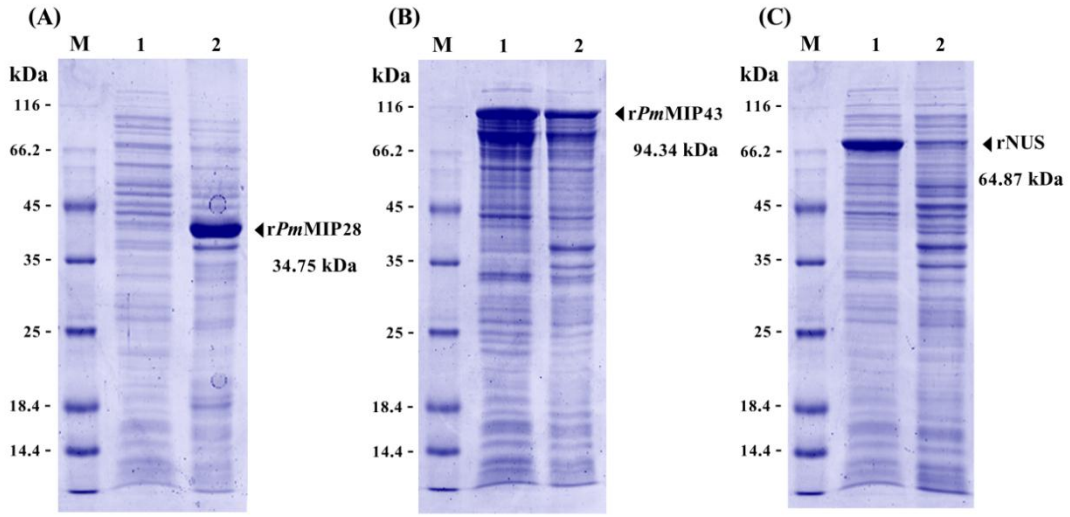


Figure 3.17 Localization of recombinant proteins in *E. coli* system. The cultured cells were collected at 6 h after IPTG induction. The supernatant and the pellet of cell lysate were analyzed by 10% SDS-PAGE. (A), expression of r*Pm*MIP28 protein; (B), expression of r*Pm*MIP43 protein; (C), expression of rNUS protein. Lane M, Unstained Maker Page Ruler; Lane 1, soluble fraction; Lane 2, inclusion bodies.

The recombinant proteins were purified by Ni-NTA affinity chromatography, dialysis, and concentration step. The purified r*Pm*MIP28 was used as immunogen and rabbit polyclonal anit-r*Pm*MIP antiserum was generated by immunization. Western blot analysis showed that the polyclonal antisera specifically reacted with the r*Pm*MIP28 (Figure 3.15). The purified recombinant proteins, r*Pm*MIP43 and rNUS, were analyzed by 10% SDS-PAGE (Figure 3.16).

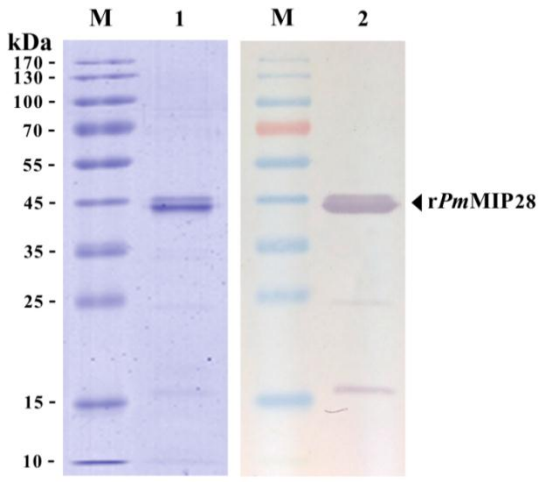


Figure 3.18 Purified r*Pm*MIP28. Sample was analyzed in 12.5% SDS-PAGE with Coomassies blue staining (left) and western blot using anti-*Pm*MIP antibody (right). Lane M, Prestain Maker Page Ruler; Lane 1-2, r*Pm*MIP28.

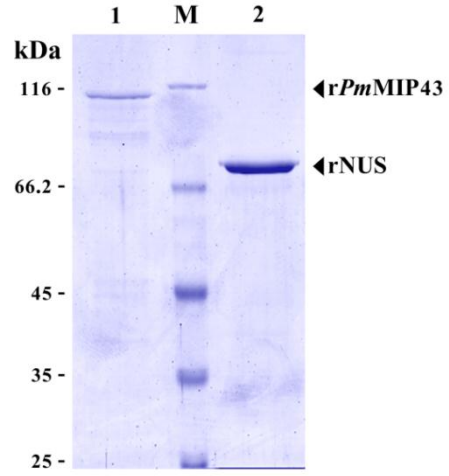


Figure 3.19 Purified r*Pm*MIP43 and rNUS. Samples were analyzed in 10% SDS-PAGE with Coomassies blue staining. Lane M, Unstain Maker Page Ruler; Lane 1, r*Pm*MIP43; Lane 2, rNUS.

3.6.3 Function of r*Pm*MIP in the inhibition of proPO system

To study the function of purified r*Pm*MIP in the proPO system, the recombinant protein, r*Pm*MIP43, was produced in *E. coli* system and purified by Ni-NTA affinity chromatography. Hemolymph from normal shrimp were collected and then incubated with r*Pm*MIP43, LPS, and substrate L-dopa. PO activity was monitored at 490 nm and defined as ΔA_{490}/mg total protein/min. The resulted showed that r*Pm*MIP43 could significantly decrease the PO activity when compare with controls, rNUS and 20 mM Tris-HCl pH 8.0 (Figure 3.20). This indicated that r*Pm*MIP might be an inhibitor in proPO system.

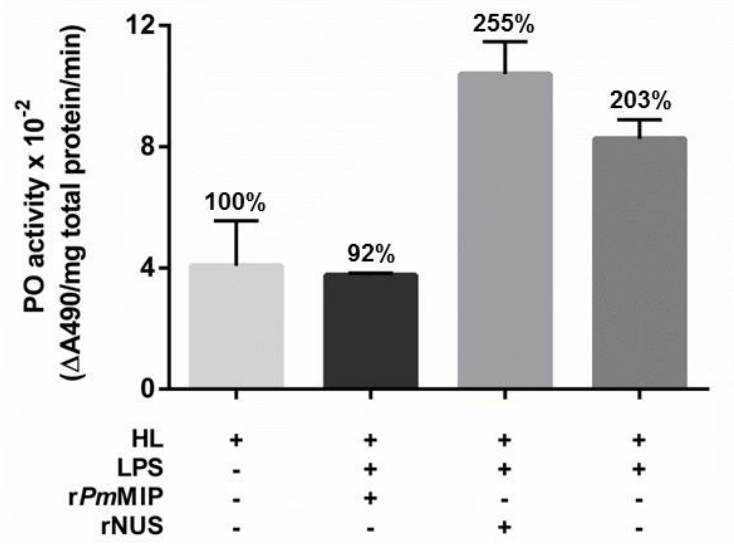


Figure 3.20 Inhibition of hemolymph PO activity by r*Pm*MIP *in vitro*. The total hemolymph (HL) was incubated with r*Pm*MIP43 and LPS. rNUS served a protein control. The data are shown as the mean ± 1S.D. (error bars) and are derived from three independently replicated experiments. The percentages of each treatment are relative to the PO activity of HL with LPS.

CHAPTER IV

DISCUSSION

Melanization is an important immune response in arthropods that leads to melanin formation and generation of toxic intermediates that can immobilize and kill invading microorganisms (Cerenius et al. 2008). Melanization is activated via the proPO sytem that is triggered though the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition proteins (PRPs), leading to the activation of SP cascade. The proPO zymogenic enzyme is converted into active PO by PPAE, the last component of SP cascade. The PO generates and catalyzes reactive compounds and melanin to eliminate and entrap invading pathogens. There are evidences that this system and its associated factors are important to host immune response. In *Drosophila*, two proteinase in melanization cascade are important to microbial infection (Tang et al. 2006). In crustacean, RNAi of proPO leads to increase of mortality after *Aeromonas hydrophila* challenge in *Pacifastacus leniusculus* which suggests that the proPO is an essential component of defense against pathogen (Liu et al. 2007). Gene silencing of two proPOs and two PPAEs in *P. monodon* show higher mortality when infection with bacteria *Vibrio harveyi*. The studies indicate that proPOs and PPAEs play important role for survival of pathogenic bacterium *Vibrio harveyi* infected shrimp (Amparyup et al. 2009; Charoensapsri et al. 2009, 2011).

These data suggest that proPO system is necessary for host defense against pathogenic infection.

Despite its important role in innate immunity, the proPO system needs to be tightly controlled because the excess reactive intermediates could damage host cells (Söderhäll and Cerenius 1998; Cerenius and Söderhäll 2004; Cerenius et al. 2008). Several proteinase inhibitors involved in proPO system have been reported in insects and crustaceans. Serpins have been characterized in insects and crustacean that involve in regulation of proPO system by inhibition of proPO activation (Cerenius et al. 2008; Jiravanichpaisal et al. 2006; Amparyup et al. 2013a). In *Drosophila*, spn27A inhibited *H. dimophalia* PPAE and mutation of spn27A caused the decrease of inhibitory activity (De Gregorio et al. 2002). Serpins from *M. sexta* have been reported that could inhibit and/or form complex with HPs and PAPs (Jiang et al. 2003b; An and Kanost 2010; Zhu et al. 2003; Tong and Kanost 2005; Wang and Jiang 2004; Zou and Jiang 2005; Christen et al. 2012). In *P. monodon*, *Pm*SERPIN8 and *Pm*SERPIN3 inhibited the PO activity but the targets are still unknown (Somnuk et al. 2012; Wetsaphan et al. 2013). Pacifastin was firstly discovered in *P. leniusculus* which is a proteinase inhibitor of crayfish PPAE (Liang et al. 1997). Moreover, the inhibitors of PO, POI and POI homologues were studied in insects (Daquinag et al. 1995; Shi et al. 2006; Lu and Jiang 2007). Recently, the negative regulators of melanization, named MIP, have been found in beetle *T. molitor* and crayfish *P. leniusculus*. They interfere

the melanin reaction from quinone compounds to melanin (Zhao et al. 2005; Söderhäll et al. 2009).

In black tiger shrimp *P. monodon*, *Pm*MIP was cloned and characterized for its mRNA expression in various shrimp tissues and in different developmental stages. Furthermore the *Pm*MIP protein was detected in hemolymph at various time points after bacterial infection (Angthong et al. 2010). In this study, we further characterize the function of *Pm*MIP in inhibition of proPO system in shrimp. The ORF of *Pm*MIP was obtained from gill cDNA by 3' and 5' RACE (Amparyup, unpublished data) which the partial nucleotide sequence of *Pm*MIP was firstly identified from *P. monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al. 2006). Sequence comparison with the previously reported *Pm*MIP sequence (Angthong et al. 2010), showed 100% identity. They obtained the sequence by 3' and 5' RACE of intestine cDNA using degenerate primers from the conserved region of crayfish *Pl*MIP. The sequence analysis showed that the ORF of *Pm*MIP consist of 957 bp encoding 318 amino acid residues. The amino acid sequences alignment of *Pm*MIP with other MIPs from crayfish *P. leniusculus* and beetle *T. molitor*, reveals that *Pm*MIP is very similar to *Pl*MIP (80%), but shares a very low similarity (8%) with *Tm*MIP. From SMART analysis, *Pm*MIP contains FReD, asp-rich region and calcium binding site which are also found in the *Pl*MIP (Söderhäll et al. 2009). The similarity of *Pl*MIP and *Pm*MIP sequence implied that *Pm*MIP may play a role in negative regulation of proPO

system. Although, the sequence of *Tm*MIP is differ from *Pl*MIP, the *Pl*MIP protein could be detected by the anti-*Tm*MIP antibody. Moreover, *Tm*MIP and *Pl*MIP have been reported to have the same function in proPO system (Söderhäll et al. 2009).

In our study, tissues distribution analysis showed that *Pm*MIP transcript was found in many tissues, such as gills, intestine, lymphoid organ, and muscle, similar to the work of Angthong et al. (2010). However, we found a very low level of *Pm*MIP mRNA expression in hemocytes whereas the previous work did not detect the transcript in this tissue which may be due to the different stages of shrimp used in the studies. In crayfish, *Pl*MIP was also found to be expressed in many tissues, except hemocyte and hematopoietic tissue (Söderhäll et al. 2009).

Time-course analysis of *Pm*MIP gene expression in gills in response to the shrimp pathogenic bacterium *V. harveyi*, revealed that *Pm*MIP transcript was significantly decreased at 24 h and recovered to normal level at 72 h post infection suggesting that *Pm*MIP gene is an immune responsive gene and down-regulated after *V. harveyi* infection. Furthermore, the endogenous *Pm*MIP protein in shrimp hemolymph was found in plasma but not in hemocytes, suggesting that *Pm*MIP protein is released from tissues into hemolymph. In beetle larva, *Tm*MIP transcript was found in fat body but *Tm*MIP protein was localized in hemolymph and plasma, not in fat body and hemocyte lysate and in crayfish, *Pl*MIP was also detected in cell-free plasma (Zhao et al. 2005; Söderhäll et al. 2009). After *V. harveyi* infection,

*Pm*MIP protein in shrimp hemolymph was decreased at 6 h and recovered at 24 h post infection in contrast to the previous research which showed that *Pm*MIP protein did not first found in plasma, but it was induced at 6 hpi, then disappeared at 24 and recovered at 48 and 72 hpi (Angthong et al. 2010). In comparison, the *Tm*MIP and *Pl*MIP proteins were found in hemolymph in normal condition which correlates with our data and *Tm*MIP protein was decreased after beetle larvae were injected with *Candida albicans* cells (Zhao et al. 2005; Söderhäll et al. 2009).

In previous study, the RNA interference (RNAi) technique has been used to elucidate the function of many genes, especially those in the proPO system. In shrimp *P. monodon*, several genes, including *Pm*LGBP, *Pm*proPO1, *Pm*proPO2, *Pm*PPAE1, *Pm*PPAE2, *Pm*ClipSP2, *Pm*MasSPH1, and *Pm*MasSPH2, were functional characterized using RNAi approach. Silencing of these genes by injection of the corresponding dsRNA resulted in the significantly decreasing of PO activity suggesting that they are involved in proPO activation (Amparyup et al. 2012; Charoensapsri et al. 2009, 2011; Amparyup et al. 2009; Amparyup et al. 2013b; Jearaphunt et al. 2015). In crayfish *P. leniusculus*, pacifastin transcript was silenced using dsRNA-mediated RNAi and the effect of gene silencing exhibited the increasing in PO activity (Liu et al. 2007). In addition, gene silencing of *Tm*MIP by RNAi treatment could increase the melanin formation (Zhao et al. 2005). Unfortunately, *Pl*MIP transcript in *P. leniusculus* could not be suppressed by RNAi technique (Söderhäll et al. 2009). Here, the

suppression of *Pm*MIP gene was successfully performed using dsRNA-corresponding *Pm*MIP and the hemolymph PO activity was increased in knockdown shrimp suggesting that *Pm*MIP is involved in proPO system and probably act as an inhibitor of the proPO pathway. Moreover, we also detected the decrease of *Pm*MIP protein in the knockdown shrimp indicating that *Pm*MIP dsRNA-mediated RNAi could suppressed both the level of *Pm*MIP transcript and the *Pm*MIP protein. In concordant reports of *T. molitor Tm*MIP and *P. monodon Pm*LGBP, the suppression of genes could decrease both the transcript and the protein expression (Zhao et al. 2005; Amparyup et al. 2012).

Interestingly, the *Pm*MIP knockdown enhanced the proteinase activity when using the commercial proteinase substrate B9375, suggested that *Pm*MIP might interfere the proteinase activity of the enzyme in the proPO system. According to Söderhäll et al. (2009), r*Pl*MIP could inhibit the proteinase activity when using the crayfish PPAE substrate S-2222. But in *T. molitor*, r*Tm*MIP could not inhibit the proteinase activity using trypsin substrate. The mechanism by which the MIP protein inhibits proteinase activity is unclear and need to be further elucidated.

To further demonstrate the role of *Pm*MIP in the inhibition of proPO system, the recombinant protein of *Pm*MIP was successfully expressed in *E. coli* system and used for in *vitro* functional characterization. The r*Pm*MIP was pre-incubated with shrimp hemolymph in the presence of LPS resulting in the reduction of PO activity.

Accordingly, r*Tm*MIP could inhibit the melanin synthesis *in vitro* in the presence of ß-1,3-glucan and r*Pl*MIP could inhibit the PO activity when pre-incubated with hemolymph prior to the LPS-PGN addition (Zhao et al. 2005; Söderhäll et al. 2009). In addition, the importance of Asp-rich region and putative calcium binding site in crayfish *Pl*MIP was investigated by deleting of the four Asp amino acids in the r*Pl*MIP and the addition of calcium in r*Pl*MIP test. The results showed the decrease of inhibitory activity in the deletion of four Asp amino acids, but the adding of calcium had no effect on the inhibitory activity of r*Pl*MIP. These suggested that the converse region of Asp-rich was involved in inhibitory activity by a different ligand (Söderhäll et al. 2009).

In conclusion, *Pm*MIP was identified from black tiger shrimp gill cDNA and found to be identical to the *Pm*MIP previously reported from the intestine cDNA and is highly similar to *Pl*MIP in crayfish. *In vivo* and *in vitro* functional characterization of *Pm*MIP was conducted and the results demonstrated that *Pm*MIP is involved in the shrimp proPO system and probably acts as a negative regulator. Moreover, the expression levels of both *Pm*MIP transcript and protein were decreased upon *V. harveyi* infection suggesting that *Pm*MIP participates in the immune response in shrimp.

CHAPTER V

CONCLUSIONS

1. A melanization inhibition protein from *P. monodon*, named *Pm*MIP, was characterized. A full-length cDNA of *Pm*MIP consists of 957 bp encoding a protein of 319 amino acid residues with a predicted molecular mass of 35.9 kDa and pI 5.5. Deduced amino acid sequence of *Pm*MIP exhibits 80% similarity to *Pl*MIP from *P. leniusculus*. *Pm*MIP composes of fibrinogen-related domain, calcium binding site and asp-rich region.

2. *Pm*MIP transcript was highly expressed in gills, hepatopancreas, intestine, lymphoid organ, and muscle and the transcript level in gills was down-regulated after *V. harveyi* challenge.

3. Endogenous *Pm*MIP protein was found in plasma, but not hemocytes. After *V. harveryi* infection, *Pm*MIP protein was decreased at 6 h and recovered at 24 h after bacterial challenge. These results suggested that *Pm*MIP was expressed in tissues and released into hemolymph upon bacterial infection.

4. Double-stranded RNA-mediated gene silencing of *Pm*MIP could suppress *Pm*MIP at both transcriptional and translational levels and resulted in an increase of hemolymph PO activity and proteinase activity.

5. The recombinant *Pm*MIP protein was successfully expressed in *Escherichia coli* and found to inhibit hemolymph PO activity *in vitro* suggesting that *Pm*MIP might play a role in proPO system by acting as a negative regulator and interfering with proteinase in hemolymph.

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APPENDIX

VITA

Mr. Nattaphop Noothuan was born on June 26, 1990 in Phichit. He graduated secondary school from Taphanhin School, Phichit. He graduated the Bachelor of Science with second-class honor from Department of Biochemistry, Faculty of Science, Chulalongkorn University. He has studied for the degree of Master of Science, program in Biochemistry and Molecular Biology, Chulalongkorn University.